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Essential Roles of Her2/erbB2 in Cardiac Development and Function

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ABSTRACT

The tyrosine kinase receptor erbB2, also known in humans as Her2, is a member of the epidermal growth factor receptor (EGFR or erbB1) family, which also includes erbB3 and erbB4. The erbBs were discovered in an avian erythroblastosis tumor virus and exhibited similarities to human EGFR (Yarden and Sliwkowski, 2001). Her2/erbB2 is highly expressed in many cancer types. Its overexpression is correlated with a poor prognosis for breast and ovarian cancer patients. ErbB receptors bind to a family of growth factors, termed neuregulins/heregulin (NRG/HRG), which comprise NRG-1, -2, -3, and -4 and include multiple isoforms. ErbB2/Her2 is an orphan receptor that does not bind ligand alone but heterodimerizes with the other erbB receptors for NRG signaling. ErbB2 is expressed in multiple neuronal and non-neuronal tissues in embryos and adult animals, including the heart. Genetic data demonstrated that erbB2 is required for normal embryonic development of neural crest-derived cranial sensory neurons. ErbB2/Her2-null mutant embryos of a trabeculation defect die before embryonic day (E) 11. To study its role at later stages of development, we generated a transgenic mouse line that specifically expresses the rat erbB2 cDNA in the heart under the control of the cardiac-specific α-myosin heavy chain promoter. When crossed into the null background, the expression of the rat erbB2 cDNA rescued the cardiac phenotype in the erbB2-null mutant mice that survive until birth but display an absence of Schwann cells and a severe loss of both motor and spinal sensory neurons. To study the role of erbB2 in the adult heart, we generated conditional mutant mice carrying a cardiac-restricted deletion of erbB2. These erbB2 conditional mutants exhibited multiple independent parameters of dilated cardiomyopathy, including chamber dilation, wall thinning, and decreased contractility. Interestingly, treatment of breast cancers over-expressing erbB2 with Herceptin (Trastuzumab), a humanized monoclonal antibody specific to the extracellular domain of erbB2, results in some patients developing cardiac dysfunction. The adverse effect is increased significantly in those patients who also receive the chemotherapeutical agent anthracycline. We found that erbB2-deficient cardiac myocytes are more susceptible to anthracycline-induced cytotoxicity. These results suggest that erbB2 signaling in the heart is essential for the prevention of dilated cardiomyopathy. These lines of mice provide models with which to elucidate the molecular and cellular mechanisms by which erbB2 signaling regulates cardiac functions. These mice also will provide important information for devising strategies to mitigate the cardiotoxic effects of Herceptin treatment, allowing for the potential expanded use of this drug to treat all cancers overexpressing erbB2.

I. Introduction

Cardiovascular diseases continue to be a major health risk and are the leading cause of death in both men and women (Michels *et al.*, 2003). Dilated cardiomyopathy (DCM) is a life-threatening heart disease characterized by cardiac enlargement and decreased contractility. Gene mutations affecting cardiac myocyte contractility (e.g., sarcomeric cytoskeletal proteins) often result in the development of DCM (Chein, 1999). In the last decade, it was discovered that the neuregulin (NRG)/erbB signaling network plays a role in the development and function of the heart, including the formation of trabeculae (Gassmann *et al.*, 1995; Lee *et al.*, 1995; Meyer and Birchmeier, 1995), angiogenesis (Russell *et al.*, 1999; Izumi *et al.*, 2002), formation of heart valves (Camenisch *et al.*, 2002), and prevention of DCM (Crone *et al.*, 2002; Ozcelik *et al.*, 2002). Her2/erbB2 has been a target for the development of therapeutic drugs. One major difficulty lies in the complexity of erbB2 signaling. It plays a role in multiple cell systems. Emerging evidence suggests that erbB2 is essential for transducing signaling elicited by multiple classes of ligands. Thus, it presents a great challenge to devise a selective treatment for a specific cell origin of disease. Comparative understanding of how erbB2 signals in cardiovascular and other cell systems will be important to the development of selective treatment of diseases involving deregulation of erbB2 function.

A. THE erbB SIGNALING NETWORK

NRGs play an important role in multiple cellular functions, including cell differentiation, migration, and survival. NRGs signal via ligand-induced dimerization between members of the epidermal growth factor receptor (EGFR) family of tyrosine kinase: namely, erbB2, erbB3, and erbB4. ErbB2 was identified as an oncogene due to its ability to induce the transformation of cultured fibroblasts (Yarden and Sliwkowski, 2001). ErbB2-overexpressing cancers usually are associated with a poor prognosis and account for about 30% of metastatic breast cancer in women.

Extensive biochemical data demonstrate that NRGs preferentially bind directly to erbB3 or erbB4 that, in turn, dimerize with other erbB receptors. The erbB2 receptor does not bind directly to any known ligands and functions primarily as a coreceptor for erbB3 and erbB4. Several biochemical studies have shown that erbB2 is the preferred dimerization partner for erbB3 and erbB4 upon NRG stimulation. Receptor dimerization results in auto- and trans-phosphorylation of intracellular domains. The phosphorylated forms of these receptors then can serve as docking sites for distinct cytoplasmic proteins involved in transducing downstream signaling cascades. The tyrosine residues that are phosphorylated upon NRG binding determine the signaling molecules recruited for a particular signaling cascade (Yarden and Sliwkowski, 2001).

Recently, two independent groups showed that the crystal structure of the erbB2 extracellular domain is a fixed, activated conformation (Cho *et al.*, 2003; Garrett *et al.*, 2003). Epidermal growth factor receptor (EGFR), erbB3, and erbB4 all undergo a ligand-dependent conformational change from an inactive to an active state. The active conformation displayed by erbB2 may help explain the potent signaling effects of this orphan receptor. Due to its active conformation, other signaling molecules can use erbB2 to rapidly transduce signals without the requirement of erbB2 ligand binding (Cho *et al.*, 2003; Garrett *et al.*, 2003). The timing, pattern, and tissue distribution of the erbB receptors, their ligand affinity, and their ability to homo- or heterodimerize underscore the diversity of the biological response resulting from distinct NRG-induced signaling pathways in target tissues.

A great deal of erbB2 signaling research has concentrated on cancer cells, with only limited studies on the signaling of NRGs in adult cardiac myocytes. The knowledge from other cell systems will provide insights into how erbB2 might signal in adult cardiac myocytes. ErbB2 has been shown to play a role in signaling elicited by interleukin (IL)-6 in transfected prostate carcinoma cells, possibly through forming complexes with the gp130 subunit of the IL-6 receptor (Qiu *et al.*, 1998). ErbB2 is transactivated by signaling of G protein-coupled receptors (Daub *et al.*, 1996). In human carcinoma cell lines, the integrin laminin receptors ($\alpha 6\beta 1$ and $\alpha 6\beta 4$) associate with erbB2 (Falcioni *et al.*, 1997). When these cells were treated with a monoclonal antibody to the $\alpha 6$ integrin, a ligand-dependent increase in erbB2 phosphorylation was seen. Cells expressing both erbB2 and $\alpha 6\beta 4$ integrin showed enhanced proliferation rates and invasiveness, suggesting that this receptor interaction might contribute to a more-malignant phenotype in carcinoma cells. In a human breast cancer cell line, known as MCF-7 cells, HRG-$\beta 1$ results in Akt activation, which is blocked by antiestrogens. HRG-$\beta 1$, bound to the erbB2/erbB3 heterodimer, in the presence of membrane estrogen receptor (ER), interacts and activates phosphatidylinositol 3-kinase (PI3-K)/Akt. Akt leads to nuclear ER-α phosphorylation, thereby altering its expression and transcriptional activity. ErbB2 has been shown to interact with the G protein-coupled growth hormone (GH) receptor in cancer cell lines (Huang *et al.*, 2003). GH, by activating extracellular signal-regulated kinases (ERKs), can modulate epidermal growth factor (EGF)-induced EGF receptor (EGFR) trafficking and signaling, suggesting mechanisms of cross-talk between the GH and EGF/erbB2 signaling system.

In the heart, G protein-coupled receptor agonists such as noradrenaline, endothelin-1, and angiotensin II are known to be involved in cardiac hypertrophy via transactivation of the EGFR (Shah and Catt, 2003). In addition, it has been shown that a serotonin (5-hydroxytryptamine, 5-HT) mouse model that expresses inactive 5HT-2B leads to embryonic and neonatal death and a specific reduction in erbB2 and lack of trabeculae in the heart. These *in vivo* data suggest that the

Gq-coupled receptor 5-HT (2B) uses the signaling pathway of tyrosine kinase receptor erbB2 for cardiac differentiation (Nebigil *et al.*, 2000). In cardiac myocytes, EGFR kinase activity is involved in protease-activated receptor (PAR)4-dependent (relative of cardiac myocyte G protein-coupled receptors) activation of the p-38 mitogen-activated protein kinase (MAPK) signaling pathway, again showing cross-talk between EGFR and G protein-coupled receptor signaling (Sabri *et al.*, 2003).

Recently, it has been shown that heparin-binding EGF-like growth factor (HB-EGF)-null mice, when viable, develop severe heart failure with grossly enlarged ventricular chambers (Iwamoto *et al.*, 2003). HB-EGF induces tyrosine phosphorylation of erbB2 and erbB4. The HB-EGF-null mice exhibited significantly lower levels of erbB2 and erbB4 phosphorylation, suggesting that HB-EGF is a cardiac survival factor partially through erbB2. Additional interactions between NRG and other signaling pathways in the heart, such as insulin-like growth factor-1 (IGF-1), have been described (Hertig *et al.*, 1999). The extent to which the NRG signaling network participates in the signal transduction of other diverse signaling pathways in the adult heart needs further investigation. Whether erbB2 associates with other G protein-coupled receptors, integrins, or gp130 in adult cardiac myocytes is unclear. It is possible that the lack of trabeculae formation in the erbB2 mutant may be due to the inactivation of a number of signaling pathways in which erbB2 functions as a central mediator.

The identification of the NRG/erbB network as potential signal integrators in response to a variety of stimuli suggests that the loss of the primary coreceptor, erbB2, either by genetic deletion (transgenic mouse models) or Herceptin treatment, cripples the coordinated cross-talk between multiple signaling pathways, resulting in loss of cellular integrity and function.

B. THE ROLE OF NRG SIGNALING IN CARDIAC DEVELOPMENT

The most-extensively studied role of NRG-mediated signaling focused on its impact on the development and maintenance of the central and peripheral nervous systems (PNS). Knockout mice provide models with which to characterize the developmental and physiological role of a gene(s) of interest. Targeted deletion of the NRG1, erbB2, or erbB4 gene results in early embryonic lethality due to cardiac developmental defects (Gassmann *et al.*, 1995; Lee *et al.*, 1995; Meyer and Birchmeier, 1995). These mouse models provided the first *in vivo* evidence of the role of the NRG/erbB signaling network for proper cardiac development. It is important to note that neither erbB2 nor erbB4 alone could compensate for the loss of the other receptor in the heart, suggesting that NRG signaling in the heart requires erbB2/erbB4 heterodimers. The embryonic lethal erbB3-null mice display a

cardiac phenotype distinct from the NRG and NRG receptor knockout mice. The loss of erbB3 expression in the cardiac cushion mesenchyme of erbB3-null mice results in thin cardiac cushions and abnormal heart valve formation (Erickson *et al.*, 1997; Camenisch *et al.*, 2002).

C. THE ROLE OF erbB2 IN DEVELOPMENT OF HYPERTROPHY AND CELL SURVIVAL

Both erbB2 and erbB4 receptors are expressed in isolated neonatal and adult cardiac myocytes (Zhao *et al.*, 1998). ErbB2/Her2 is localized to the transverse tubules (Ozcelik *et al.*, 2002) and erbB4 is localized to the calveolar microdomains within cardiac myocytes (Zhao *et al.*, 1999). *In vitro,* NRG promotes the survival of neonatal and adult cardiac myocytes and induces hypertrophic change, including changes in cell morphology, increased protein synthesis, and expression of embryonic genes (Zhao *et al.*, 1998). A soluble neuregulin-1 (NRG1, recombinant human glial growth factor 2, rhGGF2) promotes the proliferation, survival, and growth of isolated neonatal and adult cardiac myocytes. RhGGF2 provoked a two-fold increase in embryonic cardiac myocyte proliferation. RhGGF2 also promotes survival and inhibited apoptosis of serum-deprived primary cultures and induces hypertrophic growth in both neonatal and adult ventricular myocytes, which is accompanied by an increase in expression of the hypertrophic marker prepro-atrial natriuretic factor (ANF) and skeletal α-actin. NRG-1 was expressed in primary coronary microvascular endothelial cells prepared from adult rat ventricular muscle and its expression was increased by endothelin-1. This study suggested that the persistent expression of both NRG and its receptors in postnatal and adult heart poses a continuing role for the NRGs in the myocardial adaptation to physiological stress or injury. hrGGF increased protein synthesis and induced expression of ANF and sarcomeric F-actin polymerization in neonatal rat ventricular myocytes (Baliga *et al.*, 1999). NRG-1 activated P42/44 MAPK extracellular signal-regulated kinase (ERK)-2/1 and ribosomal S6 kinase (RSK)-2, both of which could be inhibited by the ERK1-antagonist PD-098059. The NRG receptors erbB2 and erbB4 are downregulated at both the message and protein levels in early stages of heart failure in animals with chronic hypertrophy secondary to aortic stenosis, suggesting a role for disabled erbB2 signaling in the transition from compensatory hypertrophy to failure (Rohrbach *et al.*, 1999).

D. erbB2 SIGNALING IN ANGIOGENESIS

A study by Russell and colleagues (1999) suggests an important role for NRG in promoting angiogenesis via activation of the erbB receptors in endothelial cells. ErbB2, erbB3, and erbB4 are all highly expressed in endothelial cells. In human umbilical vein endothelial cell (HUVEC) culture experiments, NRG

stimulation results in rapid calcium influxes and cell proliferation. *In vivo*, NRG administration led to the growth of new blood vessels in a rat corneal angiogenesis model. Antibodies directed against vascular endothelial growth factor (VEGF), in concentrations sufficient to block high levels of VEGF, had no effect on NRG-stimulated growth or tube formation of HUVEC cells. Therefore, NRG angiogenic effects appear to be independent of VEGF (Russell *et al.*, 1999). A subsequent study showed that Herceptin acts as an anti-angiogenic factor when administered to Her2-overexpressing breast tumors in mice (Izumi *et al.*, 2002). Herceptin also reduced the diameter and volume of tumor blood vessels, as compared to tumors treated with a control antibody. This expands our understanding of the antitumorigenic effects of Herceptin administration in the clinical setting.

II. Experimental Models Investigating the Essential Role of erbB2

A. THE ROLE OF erbB2 IN NEURAL AND CARDIAC DEVELOPMENT

We have investigated the developmental role of erbB2 by generating erbB2-null mice (Lee *et al.*, 1995). In this study, the mutant embryos died at embryonic day (E) 11, probably as a result of dysfunctions associated with a lack of cardiac trabeculae. Development of cranial neural crest-derived sensory ganglia was markedly affected, as was development of motor nerves. Both erbB2 and NRGs are found in the neural crest cells and migrate out of the neural tube, suggesting that both molecules act via an autocrine mechanism to play an important role in the development and differentiation of neural crest cells. Trunk neural crest cells give rise to the neuronal cells of the dorsal root ganglion, whereas cranial sensory neurons are derived from cranial neural crest cells and placodal ectoderm (D'Amico-Martel and Noden, 1983; Le Douarin and Smith, 1988).

Whole-mount immunohistochemistry with TuJ1 antibodies against neuronal tubulin revealed that the mutant embryos had no immunoreactive staining in the dorsal portion of the trigeminal ganglion or the mandibular branch of the trigeminal ganglion. The dorsal-medial portion of the trigeminal ganglion was lost in the erbB2-null embryos. There were no axonal connections between the trigeminal ganglion and the hindbrain in sections through the whole ganglion. In mutant embryos, proximal portions of the glossopharyngeal and vagus nerves were considerably smaller than control embryos, suggesting that development of the superior and jugular ganglia was severely affected. Examination of the facial motor nerves suggested that neural crest-derived neurons in the facial ganglion also were affected. Considering the evidence of erbB2 expression in neural crest cells, the results of this study suggest that a lack of erbB2 affects the normal development of cranial neural crest-derived neurons but not placode-derived

neurons at E10.5. As neural crest-derived trigeminal ganglion was markedly affected in erbB2-deficient embryos, we also investigated whether motor nerves were affected. Retrograde labeling showed that motor nerves fail to exit the hindbrain. It is likely that the development of trigeminal and facial motor nerves is secondary to a lack of the neural crest-derived portion of their respective sensory ganglia (Moody and Heaton, 1983).

B. RESCUE OF THE CARDIAC TRABECULATION IN erbB2-NULL MICE

Both erbB2 and NRGs are expressed in many non-neuronal tissues. In E9.5/10.5 embryos, erbB2 is detected in cardiac myocytes, whereas NRG is expressed in adjacent myocardium. This study suggested that erbB2 and NRG foster development of the heart via a paracrine mechanism. ErbB2 mice die of a trabeculation defect before gliogenesis in the PNS (Lawson and Biscoe, 1979) and before onset of neural muscular junction at E12 (Noakes *et al.*, 1993; Piette *et al.*, 1993). To study the role of erbB2 after E11, we genetically rescued the cardiac defect in erbB2-null mice by creating transgenic mice that expressed rat erbB2 under the control of a cardiac-specific α-myosin heavy chain (α-MHC) promoter (Morris *et al.*, 1999).

Mice expressing rat erbB2 in the heart were crossed with the erbB2-null mutants. The erbB2-null mutants that now overexpressed rat erbB2 in the heart formed cardiac trabeculae and survived to birth. However, due to the loss of innervations within the diaphragm muscle, the rescued mice were stillborn. Examination of the PNS in the rescued mutant embryos demonstrated a severe loss of both sensory and motor neurons and a complete absence of Schwann cell precursors in the peripheral nerves, demonstrating that erbB2 is required for normal development of the PNS (Morris *et al.*, 1999). The prolonged viability of these mice provided further evidence supporting the important role of the NRG/erbB signaling network in the heart.

C. CONDITIONAL HEART-RESTRICTED erbB2

The NRG/erbB receptor signaling network has been established as a key modulator/regulator of multiple developmental and physiological processes, including cardiac development and adult cardiac function. Current technology allows for the generation of mice with both temporal and tissue-specific alterations in a gene(s) of interest, referred to as conditional mutants. The conditional knockout (CKO) approach is useful to bypass early embryonic lethality, as observed in the NRG, erbB2, erbB3, and erbB4 total knockouts, as well as to define the cell-type, tissue-specific requirements of NRG signaling in the adult.

To investigate the role of erbB2 in the adult heart, we generated a line of erbB2 conditional mutant mice that lacked erbB2 in the heart and skeletal

muscle. To generate the mice, we crossed our erbB2 floxed allele mouse line to a transgenic Cre line under control of the muscle creatine kinase (MCK) promoter, a Cre line shown to promote high-efficiency recombination in atrial and ventricular lineages and postnatal skeletal muscle. A second line of heart-specific erbB2-null mice was made by breeding erbB2 floxed mice with the myosin light chain 2v (MLC2v) Cre transgenic line. The postnatal loss of erbB2 in the heart results in the progressive onset of DCM. The two independent, heart-restricted erbB2-null mouse lines display the same cardiac phenotype and differ only in the onset of DCM. Both these lines will be referred to as the erbB2 CKO mice.

The expression of erbB2 protein is significantly less in the adult hearts of erbB2 CKO mice, with no difference in the levels of the NRG receptor erbB4, compared to wild-type controls. The cardiomyopathy observed in the erbB2 CKO mice is consistent with that of dilated cardiomyopathy, including ventricular dilation, reactivation of embryonic gene expression, increase in heart:body weight ratio, and decreased cardiac contractility (Figure 1).

Transmission electron microscopy did not demonstrate alterations in cytoskeletal architecture but did reveal an increase in the number of mitochondria and vacuoles. The erbB2 CKO mutants have a significant increase in left ventricle (LV) end diastolic and end systolic dimensions (LVEDD and LVESD), decreased fractional shortening, decreased septal- and posterior-wall thickness, and decreased velocity of circumferential fiber shortening, compared to wild-type controls (Crone *et al.*, 2002). In addition, retrograde catheterization of LV of the erbB2 CKO mutants revealed marked reduction in the maximum first derivative of LV pressure (LV dP/dt max), indicative of depressed myocardial contractility. No significant changes in heart rate, LV end diastolic pressure (EDP), and tau were observed between erbB2 CKO and controls. The decrease in β-adrenergic responsiveness in the erbB2 CKO mutants is consistent with that observed in human heart failure models. However, increasing doses of the β-adrenergic agonist, dobutamine, stimulates increased contractility and relaxation in the erbB2 CKO mice, segregating the erbB2 CKO mutants from other models of DCM. This suggests that erbB2 signaling is not required for this response.

Independently, Herceptin (Trastuzumab), a humanized monoclonal antibody directed against the extracellular domain of the Her2/erbB2 receptor, was used in clinical trials to treat female patients with metastatic breast cancer. Despite its potent ability to block erbB2-mediated signaling in breast, the cardiotoxic effects of Herceptin treatment were quite severe. This observation provided an *in vivo* link between suppression of erbB2 receptor signaling and impaired cardiac function and underscores the important role of the Her2/erbB2 receptor in adult cardiac function. Ozcelik and coworkers (2002) have independently reported similar results. Their study shows that erbB2 expression is localized to the transverse tubules of the cardiac myocytes. There was no increase in apoptosis

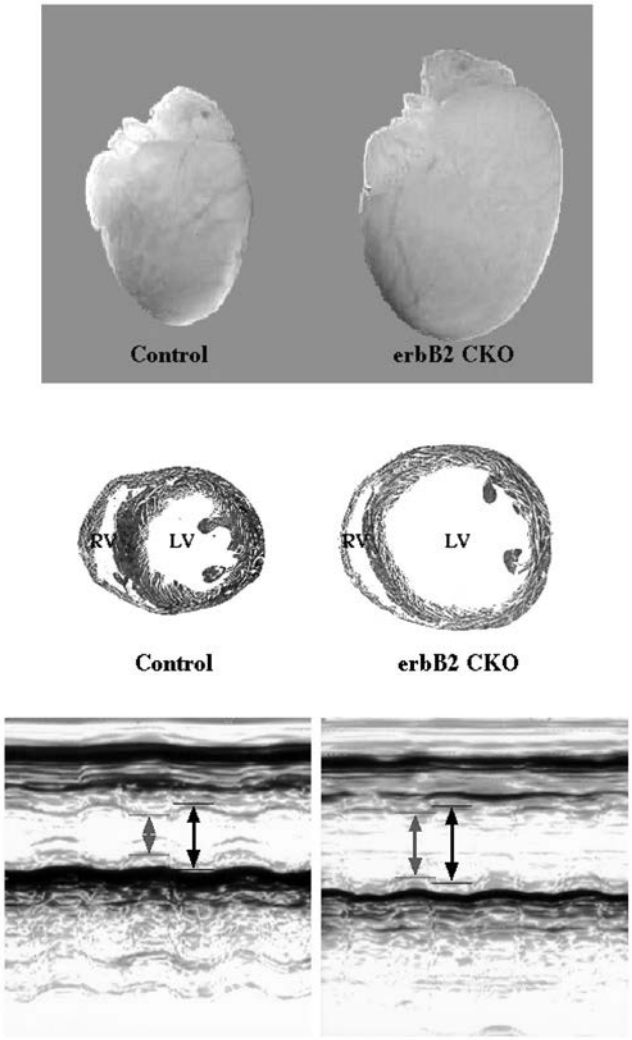

FIG. 1. ErbB2 conditional knockout (CKO) mice display multiple features of dilated cardiomyopathy. (Top panel) The heart from erbB2 CKO mutants is enlarged due to dilated left ventricle (LV), as shown in hematoxylin/eosin (H/E)-stained cross-sections of the heart (middle panel). Echocardiography (bottom panel) and hemodynamic measurements demonstrate an enlarged LV with thin walls and decreased contractility. Electron microscopy reveals intact cytostructural architecture but increased mitochondria and vacuolar structures (similar to features observed following anthracycline toxicity). ErbB2 CKO cardiomyocytes are more susceptible to anthracyline toxicity in culture. [Reprinted from Crone SA, Zhao YY, Fan L, Gu Y, Minamisawa S, Liu Y, Peterson KL, Chen J, Kahn R, Condorelli G, Ross J Jr, Chien KR, Lee KF 2002 ErbB2 is essential in the prevention of dilated cardiomyopathy. Nature Med 8(5):459–465, with permission of the Nature Publishing Group.]

rates in erbB2 CKO mutants. The researchers concluded that erbB2 is not required for cardiac myocyte survival but is needed for adult cardiac function.

Although the majority of DCM is classified as idiopathic, it is now evident that there is a strong genetic component to the disease (Chien and Olson, 2002). In clinical trials for breast cancer therapy, Herceptin was an effective single agent for abolishing erbB2-mediated signaling. However, patients receiving Herceptin either following or concurrent with anthracycline treatment experienced an increased probability of developing DCM, from 7% to 28%, respectively (Crone *et al.*, 2002). Since the majority of cancer patients have received anthracycline treatment, the effects of Herceptin treatment alone have not been fully characterized. The erbB2 CKO mice are viable and display the same cardiac dysfunction as patients treated with Herceptin. To determine whether the erbB2 CKO cardiac myocytes are venerable to anthracycline-induced cell death, as shown in patients treated with Herceptin, we treated neonatal cardiac myocytes isolated from the erbB2 CKO mice with doses of Adriamyacin (anthracycline) ranging from 0.5 to 5 mM. The erbB2 CKO mice displayed increased sensitivity to Adriamyacin-induced cytotoxicity. It is clear that the cardiotoxic effects of Herceptin are a direct consequence of the loss of erbB2 signaling in the heart.

III. Future Work

The main focus of our laboratory's work is to understand the mechanism of how inactivation of erbB2 signaling in the adult heart results in DCM. We hope to identify the signaling pathways that are absent or inactive in the erbB2 CKO DCM mouse model. The erbB2 CKO mutants provide a model that will aid in the design of therapeutic drug targets to counteract the cardiotoxic effects of Herceptin. Once the signaling pathways that are absent/altered in the erbB2 CKO mouse have been identified, Herceptin may be administered to all erbB2-positive cancers without resulting in cardiotoxicity.

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Alejandra Negro and Bhawanjit K. Brar contributed equally to this work.

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The Adrenergic Pathway and Heart Failure

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ABSTRACT

Heart failure represents the endpoint to many triggering cardiovascular pathologies. However, there are molecular and biochemical features that remain common to the failing heart, despite the varying etiologies. Principal among these is heightened activation of the sympathetic nervous system and associated enhancement of adrenergic signaling pathways via the catecholamines, norepinephrine and epinephrine. During heart failure, several hallmark alterations in the adrenergic system contribute to loss of cardiac function. To specifically study these changes in a physiologically relevant setting, we and others have utilized advances in genetically engineered mouse technology. This chapter will discuss the many transgenic and knockout mouse models that have been developed to study the adrenergic system in the normal and failing heart. These models include genetically manipulated alterations of adrenergic receptors, linked heterotrimeric G proteins, and the regulatory G protein-coupled receptor kinases (GRKs). Among the more-interesting information gained from these models is the finding that inhibition of a particular GRK — GRK2 or β adrenergic receptor kinase 1 (βARK1) — is a potential novel therapeutic strategy to improve function in the setting of heart failure. Furthermore, we will discuss recent transgenic research that proposes an important role for hypertension in the development of heart failure. Overall, genetically engineered mouse models pertaining to this critical myocardial signaling system have provided novel insight into heart function under normal conditions and during states of dysfunction and failure.

I. Heart Failure and Sympathetic Nervous System Signaling

More than 500,000 new cases of heart failure are reported each year in the United States alone, making it one of the world's most-prolific diseases. The principal function of the heart is to provide enough oxygenated blood to meet the body's metabolic demands through cardiac output. Although the heart is adaptable to many physiological conditions, various etiologies can perturb its function, leading to ventricular dysfunction and ultimately failure. The initial response of the heart to excessive stress is to enlarge morphologically to a state known as cardiac hypertrophy. Classically, cardiac hypertrophy is defined as the physiological response of the heart to an increased workload. This hypertrophy may serve as compensatory and aid in preventing progressive deterioration of cardiac function (Grossman *et al.*, 1975; Chein, 1999). However, often, the stress will

overwhelm the system, sending the hypertrophied heart into failure. As the disease progresses, the heart dilates and thins, becoming too weak to maintain adequate cardiac output. During these hypertrophic and failing processes, there is sustained heightened activation of the renin-angiotensin system and the sympathetic nervous system in an attempt by the body to maintain cardiac output and systemic blood pressure (Esler *et al*., 1997). Recent evidence suggests that the signaling pathways stimulated during the hypertrophic process, if left unchecked, participate in the pathogenesis of heart failure and may be more important in this disease process than the actual stress placed on the heart (Esposito *et al*., 2002; Rockman *et al.*, 2002).

The importance of sympathetic activity, via the catecholamines norepinephrine and epinephrine, in heart failure progression and mortality is well established (Cohn *et al.*, 1984). At a cellular level, the catecholamines act upon the heart by binding to the adrenergic receptors (ARs), which are members of the superfamily of proteins known as the G protein-coupled receptors (GPCRs) (Caron and Lefkowitz, 1993). In the heart, norepinephrine principally binds to the α_{1B}- and β_1AR, while epinephrine is a ligand for both β_1- and β_2AR (Caron and Lefkowitz, 1993). The β_1AR is the most-abundant βAR in the human heart, approaching 75% of the total number of receptors (Brodde, 1993). The βARs are coupled primarily to the heterotrimeric G protein, Gs, to stimulate adenylyl cyclase activity. This association generates intracellular cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) activation, which regulate cardiac contractility and heart rate (Bristow *et al*., 1989). β_2ARs also can couple to the G protein, Gi, which can inhibit adenylyl cyclase activity and stimulate novel mitogen-activated protein kinase (MAPK) pathways in the heart through Gα and G$\beta\gamma$ subunits (Rockman *et al.*, 2002). Alternatively, binding of norepinephrine to the α_{1B}AR elicits phospholipase C (PLC) activity via activation of the G protein, Gq, which is the principal G protein signaling pathway implicated in the hypertrophic response of the heart (Molkentin and Dorn, 2001).

Following agonist occupation of ARs, these GPCRs become substrates for regulation via G protein-coupled receptor kinases (GRKs), which phosphorylate activated receptor (Inglese *et al*., 1993). This phosphorylation facilitates binding of β-arrestins, which sterically interferes with further coupling to G proteins, thus desensitizing and uncoupling the signal. The principal GRKs involved in intracellular signaling within the heart are GRK2 (or β adrenergic receptor kinase 1, βARK1), GRK3, and GRK5, all of which have specific GPCR selectivity *in vivo* in the heart (Eckhart *et al*., 2000). As will be detailed later, GRK activity in the heart appears to play a critical role, especially in heart failure. The generalized signaling pathways, their regulation, and outcomes in the heart are depicted in Figure 1.

Importantly, in human heart failure, chronic activation of the sympathetic nervous system has adverse implications and can accelerate cardiac pathology

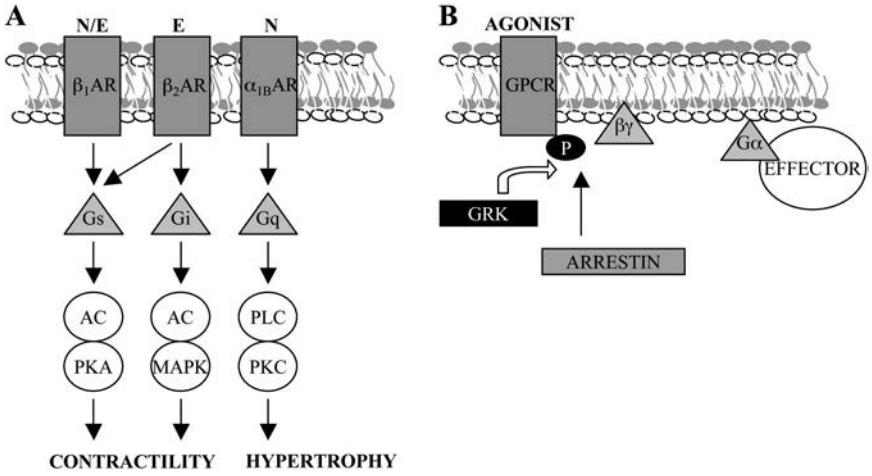

FIG. 1. (A) Schematic representation of the signaling pathways through the various adrenergic receptors (ARs) in the heart. (B) Schematic representation of the process of desensitization of G protein-coupled receptors (GPCRs). Following agonist binding to the GPCR, the associated heterotrimeric G protein splits into G$\beta\gamma$ and Gα. The Gα elicits a signal through the cell via effector molecules. In addition, the receptor is now a substrate for phosphorylation by the appropriate G protein-coupled receptor kinase (GRK). This phosphorylation facilitates arrestin binding, which sterically interferes with further G protein activation, thus desensitizing and uncoupling the signal. Abbreviations: N, norepinephrine; E, epinephrine; PKA, protein kinase A; MAPK, mitogen-activated protein kinase; PKC, protein kinase C.

(Cohn *et al*., 1984). Constant stimulation of ARs by catecholamines leads to selective β_1AR downregulation (Bristow et al., 1982,1993; Ungerer et al., 1993). However, both the β_1AR and the β_2AR are markedly uncoupled from their G proteins and effector systems (Bristow *et al.*, 1982,1989). The latter appears to be due to increased levels and GRK activity of βARK1 (Ungerer *et al*., 1993,1994). In addition, Gαi is significantly upregulated, to dampen adenylyl cyclase activation (Feldman *et al*., 1988). Overall, these molecular adrenergic changes in the failing human heart (summarized in Table I) lead to a marked attenuation of cardiac βAR signaling.

Interestingly, genetic polymorphisms in ARs have been identified and may influence individual characteristics of heart failure (Green *et al*., 1993; Podlowski *et al*., 2000; Small *et al.*, 2002). For example, individuals with an Ile164 allele for the β_2AR have significantly reduced survival (Green *et al*., 1993) and lower exercise capacity (Wagoner *et al*., 2000). In addition, polymorphisms within the β_1AR, combined with a deletion mutant found within the human α_{2c}AR gene, can act synergistically to increase the risk of heart failure in the black population (Small *et al*., 2002). These genetic studies, as well as recent heart failure drug

TABLE I
Changes in the βAR Signaling Pathway in Human Heart Failure

Molecule	Change	References
β_1ARs	↓, uncoupled	Bristow *et al.*, 1982, 1993; Ungerer *et al.*, 1993, 1994
β_2ARs	NC, uncoupled	Bristow *et al.*, 1993; Ungerer *et al.*, 1993, 1994
βARK1	↑ mRNA + activity	Ungerer *et al.*, 1993
GRK3	NC	Ungerer *et al.*, 1994
GRK5	Not studied	—
β Arrestin 1 and 2	NC	Ungerer *et al.*, 1993
Gαi	↑	Feldman *et al.*, 1988
Gαs	NC	Feldman *et al.*, 1988

[Abbreviations: AR, adrenergic receptor; GRK, G protein-coupled receptor kinase; NC, no change.]

trials, have indicated that there is still much to learn about alterations in the βAR system during cardiac failure. This includes recent studies demonstrating significant improvement in survival with βAR antagonist therapy in people with moderate and severe heart failure (Packer *et al*., 1996,2001). This is contraindicative to the short-term effects of β blockers (Epstein and Braunwald, 1966). The recent use of genetically engineered mice has provided unique experimental models for the study of cardiac adrenergic signaling alterations and the function of the normal and failing heart.

II. Mouse Models to Study the Cardiac Adrenergic System

A. LOSS AND GAIN OF AR EXPRESSION

1. The β_1AR

Gene-targeted knockout mice with disruption and ablation of the β_1AR gene generally are embryonically lethal. These mice recently have been reviewed in detail (Rohrer, 1998). Although surviving β_1AR knockout mice have normal heart rates, their response to exercise is abrogated (Rohrer *et al*., 1996). Interestingly, despite the presence of β_2ARs, there was no response to β-agonist stimulation, suggesting that β_1AR is responsible for catecholamine-induced alterations in heart rate in the mouse (Rohrer *et al*., 1996).

Using the α myosin heavy chain (αMHC) promoter to target gene expression to adult ventricular myocardium, mice that overexpress the β_1AR in the heart have been generated (Englhardt *et al*., 1999). These mice, with 5- to 15-fold overexpression compared to endogenous βAR levels, exhibit a pathology that is consistent with chronic sympathetic stimulation, with a phenotype of dilated cardiomyopathy and heart failure (Englhardt *et al*., 1999). As will be detailed, this is in striking contrast to phenotypes observed with myocardial β_2AR overexpression.

2. The β_2AR

In contrast to the β_1AR knockout mice, gene disruption of the β_2AR does not appear to significantly alter cardiac physiology (Rohrer, 1998). This suggests that, under normal conditions, the β_2AR plays no major role in murine cardiac physiology. However, the β_2AR can significantly alter cardiac physiology when overexpressed. Transgenic αMHC-β_2AR mice generated by the Lefkowitz laboratory had greater than 200-fold overexpression of endogenous βARs. These mice possessed a biochemical and physiological phenotype that mimicked maximal βAR myocardial signaling and function (Milano *et al*., 1994b). Surprisingly, even though these mice have enhanced heart rates and contractility from a young age, there is minimal pathology present, even in mice greater than 1 year of age (Koch *et al*., 2000). Furthermore, other transgenic mice with lower levels of overexpression (i.e., 30- to 50-fold) have similar characteristics (Turki *et al*., 1996). However, Liggett and colleagues (2000) have reported that a "transgene dose-response" for the β_2AR often can have delayed deleterious consequences, similar to what is observed with minimal β_1AR overexpression.

There appears to be significant Gi coupling in αMHC-β_2AR mice, as demonstrated by studies utilizing the Gi inhibitory pertussis toxin (Xiao *et al*., 1999). These data contribute to recent findings demonstrating that signaling via β_1AR and β_2AR in the heart is fundamentally different (Rockman *et al*., 2002). The overall positive effects seen with transgenic β_2AR overexpression suggest that the use of genetic engineering to replace lost βARs with the β_2AR in the failing heart may be therapeutic (Maurice *et al*., 1999). Consistent with this, we have found that cardiac β_2AR overexpression can "rescue" a mouse model of decompensated hypertrophy and heart failure due to cardiac Gαq overexpression (Dorn *et al*., 1999). However, αMHC-β_2AR mice were unable to rescue other mouse models of cardiomyopathy (Rockman *et al*., 1998b; Freeman *et al*., 2001). Moreover, β_2AR overexpression leads to functional deterioration of the heart, following induction of pressure overload (Du *et al*., 2000).

3. The α_{1B}AR

Two lines of mice have been generated using the αMHC promoter and the α_{1B}AR. These mice express either the wild-type receptor or a constitutively active mutant of the α_{1B}AR (CAMα_{1B}AR) (Akhter *et al*., 1997; Milano *et al*, 1994a). The CAMα_{1B}AR mice exhibit significant myocardial hypertrophy, suggesting that α_{1B}AR activation can induce cardiac changes independent of hemodynamic influences (Milano *et al*., 1994a). In contrast, mice overexpressing the wild-type α_{1B}AR do not develop an increase in heart size, despite displaying some biochemical characteristics of hypertrophy (Akhter *et al*., 1997). These mice, however, show a reduced tolerance to chronic α_{1B}AR stimulation, indicating that they are primed for a hypertrophic response (Iaccarino *et al*., 2001). Interestingly, the αMHC-α_{1B}AR transgenic mice have a decreased response to βAR stimulation (Akhter *et al*., 1997), which appears to be mediated via an observed increase in βARK1 expression and activity (Akhter *et al*., 1997; Iaccarino *et al*., 2001) and an activation of the sympathetic nervous system (Iaccarino *et al*., 2001). Thus, this mouse model has led to an elucidation of molecular cross-talk between the α_{1B}AR and the βAR systems in the heart. Finally, transgenic mice expressing the α_{1B}AR under the control of its isogenic promoter exhibit myocardial hypertrophy and have a surprising loss of sympathetic activity (Zusic *et al*., 2001).

B. GENETIC ALTERATION OF CARDIAC G PROTEIN EXPRESSION

1. Gαs

Transgenic mice overexpressing the stimulatory G protein Gαs in the heart have been generated and characterized (Gaudin *et al*., 1995). These mice exhibit enhanced responsiveness to catecholamines and develop cardiomyopathy as they age (Geng *et al*., 1999), in a model reminiscent of human heart failure. Interestingly, the phenotype can be rescued by chronic administration of a βAR blocker (Asai *et al*., 1999), suggesting that this phenotype, at least partially, mimics chronic sympathetic nervous system activation and enhanced βAR signaling.

2. Gαi

Targeted disruption of Gαi_2 or Gαi_3 (the major Gi subtypes in myocardium) in mice revealed that there appears to be no significant role for Gi signaling in basal cardiac function or in the response to βAR stimulation in the normal heart (Jain *et al*., 2001). In contrast to these knockout results, expression of a novel Gi-coupled receptor in the heart resulted in a large decrease in myocardial force, suggesting that defects in the Gi signaling pathway may contribute to the

development of cardiac pathology (Redfern *et al*., 1999; Baker *et al*., 2001). These results are consistent with the upregulation of Gαi, contributing to human heart failure and the uncoupling of the βAR system (Feldman *et al*., 1988).

3. Gαq

Dorn and colleagues have described transgenic mice overexpressing Gαq in the heart (D'Angelo *et al*., 1997). αMHC-Gq mice with 4-fold overexpression have cardiac hypertrophy and alterations in all of its molecular markers (D'Angelo *et al*., 1997). These animals, like the α_{1B}AR overexpressors, display abrogated αAR function. Crossbreeding the Gq mice with transgenic mice that had 200-fold overexpression of the β_2AR worsened the Gq phenotype (Dorn *et al*., 1999). However, a line of β_2AR mice with only 30-fold overexpression of the receptor rescued the cardiac hypertrophy (Dorn *et al*., 1999), suggesting that selective, controlled βAR enhancement may be beneficial. At higher levels of Gq expression in the transgenic mice, severe heart failure and early death was observed, with a component of increased myocyte apoptosis (Adams *et al*., 1998).

4. Gαq Inhibition

Due to the importance of Gq signaling in the development of cardiac hypertrophy, our laboratory set out to selectively inhibit this pathway in the heart. To achieve this, a specific peptide inhibitor consisting of the last 54 amino acids of the Gq (GqI) was developed and studied (Akhter *et al*., 1998). This GqI peptide targets the receptor-Gq interface, competitively inhibiting Gαq activation while not affecting Gs or Gi signaling (Akhter *et al*., 1998). Transgenic mice expressing the GqI peptide in the heart were shown to have attenuated responses to Gq-coupled receptor stimulation (Akhter *et al*., 1998). When these animals were subjected to an experimental model of pressure overload cardiac hypertrophy, expression of the GqI peptide in the heart significantly inhibited development of the hypertrophic phenotype (Akhter *et al*., 1998). Thus, this study identified Gq activation as the final common trigger for pressure overload hypertrophy. More recently, these mice have shown resistance to heart failure following chronic hypertrophic stimulus (Esposito *et al*., 2002), suggesting that Gq-class specific inhibition is a novel strategy to prevent ventricular dysfunction in conditions of chronic hypertrophic stress.

C. MANIPULATION OF CARDIAC GRK EXPRESSION

1. βARK1

The importance of βARK1 (GRK2) in the cardiovascular system is clearly noted by the severe cardiac malformations and embryonic death observed

following βARK1 gene ablation (Jaber *et al*., 1996). The findings suggest a possible role for βARK1 in the normal migration and differentiation of myocardial cells during heart development. Heterozygous βARK1 knockout mice with 50% less βARK1 expression and activity in myocardium have no developmental abnormalities (Rockman *et al*., 1998b). Transgenic mice that overexpress βARK1 in the heart due to the use of the αMHC promoter have an attenuated response to catecholamine stimulation with desensitized βARs (Koch *et al*., 1995). This was a significant finding, as it represents the first demonstration that βARK1 could cause the functional uncoupling of βARs *in vivo*. Furthermore, these mice demonstrate that the upregulation of βARK1 seen in human heart failure may have significance and contribute to the pathogenesis of ventricular dysfunction. Contractile responses to angiotensin II also are abrogated in αMHC-βARK1 transgenic mice, suggesting that βARK1 may be important in other receptor systems in the heart (Rockman *et al*., 1996). Interestingly, βARK1 overexpression has no effect on cardiac α_{1B}AR signaling, demonstrating GRK-GPCR selectivity *in vivo* (Eckhart *et al*., 2000).

2. Inhibition of βARK1

Since βARK1 activity is increased in heart failure and appears to play a role in uncoupling of βARs in the heart, we have studied the physiological consequences of βARK1 inhibition. To do this, a specific peptide inhibitor consisting of the last 194 amino acids of the βARK1 (βARKct) was developed and studied (Koch *et al*., 1993). The βARKct contains the $G_{\beta\gamma}$ binding domain and competes with endogenous βARK1 for $G_{\beta\gamma}$-mediated membrane translocation, a process required for βARK1 activation on activated GPCRs (Koch *et al*., 1993). When the βARKct was expressed in the hearts of transgenic mice under the control of the αMHC promoter, cardiac physiology was altered in reciprocal fashion to that seen with βARK1 overexpression (Koch *et al*., 1995). The βARKct mice have enhanced cardiac function at baseline and an augmented response to catecholamines (Koch *et al*., 1995). Importantly, using a hybrid transgenic mouse strategy where βARK1 overexpression and βARKct expression occurred *in vivo* simultaneously, we have shown that the βARKct is, indeed, inhibiting cardiac βARK1 activity (Akhter *et al*., 1999).

The phenotypes of the βARK1 and the βARKct mice are consistent with our hypothesis that this GRK plays a critical role in cardiac function and potentially in cardiac pathologies. Interestingly, heterozygous βARK1 knockout mice also have a phenotype of enhanced cardiac function (Rockman *et al*., 1998b), demonstrating that lowering βARK1 expression or its activity can have profound *in vivo* effects on cardiac contractility. Moreover, hybrid mice that express the βARKct in the heart and are heterogeneous for βARK1 gene ablation have even-greater enhancement of cardiac function (Rockman *et al*., 1998b).

In addition to heart failure, enhanced βARK1 expression and activity has been shown to be indicative of several models of cardiac hypertrophy (Koch *et al.*, 2000). Enhanced βARK1 activity in the hypertrophied heart has been shown to be responsible for the loss of βAR inotropic reserve seen in this pathological condition (Choi *et al.*, 1997). To study the inhibition of βARK1 during hypertrophy, we used a novel transgenic mouse model. The βARKct was targeted to the heart using the cardiac ankyrin repeat protein (CARP) promoter, which turns off during adulthood. However CARP belongs to a family of fetal genes, such as atrial natiuretic factor (ANF), that can be reactivated in adult ventricular myocardium by stress. CARP-βARKct mice lose βARKct expression after 3 weeks of life and adult mice do not have enhanced contractility (Manning *et al*., 2000). However, following induction of pressure overload hypertrophy, expression of the βARKct is seen once again in the myocardium, resulting in improved βAR responsiveness and cardiac function (Manning *et al*., 2000).

3. Cardiac Transgenic Studies with GRK3 and GRK5

Following the profound effects seen with βARK1 manipulation in the hearts of transgenic mice, we studied the physiological consequences of GRK3 and GRK5 overexpression. These two GRKs are found normally in the heart but their overall role in cardiac signaling is not well understood, although GRK5 has been found to be upregulated in some animal models of heart failure (Ping *et al*., 1997; Vinge *et al*., 2001). Unlike βARK1, both GRK3 and GRK5 homozygous knockout mice are viable with no overt cardiac phenotype (Wess, 2000). Overexpression of these GRKs in the heart has, however, led to unexpected and interesting results that have uncovered novel aspects of GRK regulation *in vivo* in the heart. GRK3 (also known as βARK2) previously was thought to be an isozyme of βARK1, since it is highly homologous and appeared to have the same *in vitro* GPCR activity (Benovic *et al*., 1991; Freedman *et al*., 1995). However, when αMHC-GRK3 mice were generated and studied, there were no signaling alterations in the cardiac βAR system (Iaccarino *et al*., 1998a). This was the first demonstration *in vivo* that GRK3 was different from βARK1, with a unique GPCR specificity profile. Further investigation revealed that thrombin signaling in the heart was uncoupled in these mice, demonstrating that the thrombin receptors are *in vivo* substrates for GRK3 (Iaccarino *et al*., 1998a). The difference in these GRKs may lie in the $G_{\beta\gamma}$ binding regions, which is the area between GRK3 and βARK1 that is the most divergent (Muller *et al*., 1997). Thus, there may be selective GPCR-mediated translocation of these GRKs. In hybrid transgenic mice with different GRKs overexpressed along with the α_{1B}AR, it was found that GRK3 is also the primary kinase for desensitization of this AR in the heart (Eckhart *et al*., 2000).

GRK5, which is the second-highest expressing GRK in the heart, is not regulated by $G_{\beta\gamma}$ and thus would be expected to have different receptor substrates in the heart. However, like βARK1 overexpressing mice, αMHC-GRK5 transgenic mice had severely blunted βAR inotropic responses *in vivo* in the heart, demonstrating that this GRK also could desensitize cardiac βARs (Rockman *et al*., 1996). These mice exhibited GPCR substrate selectivity, compared to βARK1, as responses to angiotensin II were not altered, whereas this Gq-coupled receptor system was desensitized in mice overexpressing βARK1 (Rockman *et al*., 1996). GRK5 also has some activity against cardiac α_{1B}ARs, again demonstrating a difference with βARK1 (Eckhart *et al.*, 2000). The overall significance of the findings that GRK5 may be altered in heart failure is not clear at this time but obviously could have important implications.

III. βARK1 Inhibition and Rescue of Murine Models of Heart Failure

One interesting area where this research has led us is to investigate whether inhibition of βARK1 activity could be a novel therapeutic strategy for improving function of the failing heart. Over the last few years, this has become possible to study in the mouse, as murine models of cardiomyopathy have been described, many of which have important manifestations of the human condition. These models have been the result of a specific gene deletion in the mouse or cardiac-specific overexpression of a heart failure-inducing transgene. Powerful information can be generated by cross-breeding αMHC-βARKct mice and various heart failure models to test the hypothesis that $G_{\beta\gamma}$-βARK1 inhibition could be beneficial. Simply studying the cardiac phenotype of these novel hybrid mice could give an answer and provide information on the role of βARK1 and GRK activity in the pathogenesis of the various heart failure etiologies, specific for the different models. We have studied six different heart failure models with βARKct mice and, for the most part, have seen overwhelming rescue. Table II summarizes our findings over the last 3–4 years using this novel genetic approach; some of the more-important findings are detailed in the next paragraph.

The first murine heart failure model rescued by any genetic manipulation was done by us with βARKct animals (Rockman *et al*., 1998a). The model of heart failure was due to the gene knockout of the muscle LIM protein (MLP), a cytoarchitectural protein and conserved regulator of myogenic differentiation (Rockman *et al.*, 1998a). The improvements made by the βARKct in this model of dilated cardiomyopathy ($MLP^{-/-}$) included restoring cardiac chamber dilation, increasing basal contractility, and enhancing βAR function (Table II). A second model of heart failure with different characteristics of disease and rescue was due to cardiac-targeted overexpression of calsequestrin (CSQ), a high-capacity calcium-binding protein (Harding *et al*., 2001). These mice have much more-severe disease than the $MLP^{-/-}$ animals and experience early mortality, as

TABLE II
Murine Models of Heart Failure and Rescue Status with Cardiac βARKct Expression

Murine model	Phenotypic change with βARKct	Reference
$MLP^{-/-}$ knockout	Functional rescue with restored βAR responsiveness	Rockman *et al.*, 1998a
Transgenic cardiac CSQ overexpression	Functional rescue and cardiac dimensions with improved survival	Harding *et al.*, 2001
Transgenic cardiac expression of a mutant myosin heavy chain (HCM)	Functional rescue, prevention of hypertrophy, and dimensions and improved exercise tolerance	Freeman *et al.*, 2001
Transgenic cardiac overexpression of MCP-1	Prevention of hypertrophy	Khouri *et al.*, 2002
Transgenic cardiac overexpression of dominant-negative mutant of CREB ($CREB_{A133}$)	Restoration of βAR signaling, no functional or mortality rescue	Eckhart *et al.*, 2002a
Transgenic cardiac overexpression of Gαq	No rescue	Dorn *et al.*, 1999

[Abbreviations: $MLP^{-/-}$, homozygous knockout of muscle LIM protein; CSQ, calsequestrin; MCP-1, monocyte chemotactic protein-1; CREB, cyclic AMP-responsive element binding protein.]

all mice are dead within 20 weeks of life. Thus, in this study, we were able to carry out a survival test. βARKct expression nearly doubled the life-span of these heart-failure mice (Harding *et al.*, 2001). In addition to survival, the βARKct prevented excessive ventricular deterioration and improved cardiac function (Harding *et al.*, 2001). Furthermore, combination of β-blocker therapy (metoprolol) and βARKct expression was synergistic in improving survival (Harding *et al.*, 2001). These data are particularly interesting, given the clinical promise of β-blocker therapy in human heart failure (Packer *et al.*, 1996,2001). Studies from our laboratory have also shown that chronic βAR blockade by carvedilol decreases the expression of βARK1 in the heart and reduces cardiac GRK activity (Iaccarino *et al.*, 1998b).

As detailed in Table II, cardiac βARKct expression also has rescued other models of heart failure, including one with cardiac-targeted overexpression of a mutant form of the αMHC gene (HCM) that is associated with human hypertrophic cardiomyopathy (Freeman *et al.*, 2001). Interestingly, expression of the βARKct in the Gαq mice had no effect on the Gq phenotype, unlike the β_2AR (Dorn *et al.*, 1999). In the Gq model of decompensated cardiac hypertrophy,

βARK1 is not upregulated, suggesting that the βARKct is acting specifically to inhibit GRKs. However, the exact mechanism of the βARKct may involve sequestration of $G_{\beta\gamma}$ from other signaling pathways, such as those involved in the activation of phosphoinositide-3 kinase (PI3K) (Naga Prasad *et al*., 2000,2001) and $I_{K,Ach}$ channels (Clapham and Neer, 1997). The contribution of these other potential $G_{\beta\gamma}$ effects to the salutary effects of βARKct in heart failure remains to be determined. Finally, the therapeutic benefit of the βARKct may involve enhanced signaling through other GPCRs such as angiotensin II receptors.

IV. Hypertension, the Adrenergic Pathway, and Heart Failure

The American Heart Association suggests that the presence of hypertension or high blood pressure in a patient doubles that person's risk for developing heart failure. In essential hypertension, elevated blood pressure has been associated with increased sympathetic output (Mark, 1990), suggesting that the catecholamines and associated adrenergic pathways may be involved in this pathology. It also implicates hypertension as a potential primary component in the development of heart failure. Indeed, studies have shown that agents that reduce blood pressure, no matter what the mechanism, all appear to eventually reverse hypertrophy (Testa *et al*., 1996; Anker *et al*., 1997).

We previously discussed the importance of Gq signaling in cardiac hypertropy and heart failure. As was detailed, transgenic mice that express the GqI peptide inhibitor of Gq can prevent the development of hypertrophy and heart failure in a pressure-overload model (Akhter *et al*., 1998; Espositio *et al*., 2002). To study the potential contribution of the vascular system and its associated alterations in blood pressure to this hypertrophic response, we developed a line of trangsenic mice that express GqI in vascular smooth muscle cells under the control of the SM22α promoter (Keys *et al*., 2002). Following chronic Gq agonist administration, we observed an attenuation of mean arterial blood pressure and an inhibition of cardiac hypertrophy in the transgenic mice with vascular GqI expression (Keys *et al*., 2002). In contrast — and somewhat unexpectedly — when the GqI was expressed in the heart, neither hypertension nor hypertrophy was inhibited (Keys *et al*., 2002). These findings suggest that, during hypertension, the vascular system is the principal determinant of cardiac hypertrophy, rather than direct stimulation of the heart itself.

Interestingly, impairment of the vascular βAR system has been shown in human and animal models of hypertension (Feldman, 1990; Brodde and Michel, 1992). More specifically, elevations in βARK1 expression have been found in lymphocytes of hypertensive patients (Gros *et al*., 1997,1999). Recently, we generated transgenic mice that express βARK1 in the vascular smooth muscle, again using the SM22α promoter (Eckhart *et al*., 2002b). These mice display attenuated vascular βAR signaling, an increase in mean blood pressure, and

develop cardiac hypertrophy (Eckhart *et al.*, 2002b). This again implicates the adrenergic system in hypertrophy – and, in particular, cardiovascular βARK1 activity — which also proposes a link between hypertension and heart failure.

IV. Conclusion

The development of transgenic mouse models has provided a broader understanding of the physiological impact of individual proteins during heart failure. Overall, through our efforts detailed herein and those from other laboratories around the world, there are currently at least 75 genetically altered mouse models available to study the role of particular signaling systems in the heart (Chu *et al.*, 2002). This review has focused on the adrenergic signaling pathway under normal conditions and during heart failure. It is evident that transgenic mice have given us insight into the role of adrenergic system in the heart that otherwise would not have been possible. In the future, we hope that this knowledge may yield novel therapeutic interventions for the treatment of cardiac disease. In fact, adenoviral-mediated delivery of the βARKct and β_2AR to larger animal models of heart failure has resulted in beneficial effects (White *et al.*, 2000; Shah *et al.*, 2001; Tevaearai *et al.*, 2002), suggesting that gene-therapy strategies may, indeed, target these AR abnormalities in heart failure in the coming years and offer new hope to patients suffering from this disease of epidemic proportions.

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Effects of Thyroid Hormone on the Cardiovascular System

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ABSTRACT

Increased or reduced action of thyroid hormone on certain molecular pathways in the heart and vasculature causes relevant cardiovascular derangements. It is well established that overt hyperthyroidism induces a hyperdynamic cardiovascular state (high cardiac output with low systemic vascular resistance), which is associated with a faster heart rate, enhanced left ventricular (LV) systolic and diastolic function, and increased prevalence of supraventricular tachyarrhythmias — namely, atrial fibrillation — whereas overt hypothyroidism is characterized by the opposite changes. However, whether changes in cardiac performance associated with overt thyroid dysfunction are due mainly to alterations of myocardial contractility or to loading conditions remains unclear. Extensive evidence indicates that the cardiovascular system responds to the minimal but persistent changes in circulating thyroid hormone levels, which are typical of individuals with subclinical thyroid dysfunction. Subclinical hyperthyroidism is associated with increased heart rate, atrial arrhythmias, increased LV mass, impaired ventricular relaxation, reduced exercise performance, and increased risk of cardiovascular mortality. Subclinical hypothyroidism is associated with impaired LV diastolic function and subtle systolic dysfunction and an enhanced risk for atherosclerosis and myocardial infarction. Because all cardiovascular abnormalities are reversed by restoration of euthyroidism ("subclinical hypothyroidism") or blunted by β-blockade and L-thyroxine (L-T4) dose tailoring ("subclinical hyperthyroidism"), timely treatment is advisable in an attempt to avoid adverse cardiovascular effects. Interestingly, some data indicate that patients with acute and chronic cardiovascular disorders and those undergoing cardiac surgery may have altered peripheral thyroid hormone metabolism that, in turn, may contribute to altered cardiac function. Preliminary clinical investigations suggest that administration of thyroid hormone or its analogue 3,5-diiodothyropropionic acid greatly benefits these patients, highlighting the potential role of thyroid hormone treatment in patients with acute and chronic cardiovascular disease.

I. Introduction

Thyroid hormone has relevant effects on the cardiovascular system (Klein and Ojamaa, 2001). Many symptoms and signs recognized in patients with overt hyperthyroidism and hypothyroidism are due to the increased or reduced action of thyroid hormone on the heart and the vascular system, respectively, and the related hemodynamic derangements (Table I). In recent decades, it has emerged

TABLE I
Hemodynamics and Cardiac Function in Overt Thyroid Dysfunction

Parameter	Normal values	Hyperthyroidism	Hypothyroidism
Blood volume (% of normal value)	100	105.5	84.5
Heart rate (bpm)	72–84	88–130	60–80
Cardiac output (L/min)	4.0–6.0	$>$ 7.0	$<$ 4.5
Systemic vascular resistance (dyn·sec/cm^{-5})	1500–1700	700–1200	2100–2700
Left ventricular ejection fraction (%)	$>$ 50	$>$ 65	$\leq$ 60
Isovolumic relaxation time (msec)	60–80	25–40	$>$ 80

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that subclinical thyroid dysfunction may affect the cardiovascular system, which may increase cardiovascular risk. It is becoming increasingly apparent that acute and chronic cardiovascular disease may alter thyroid hormone metabolism and contribute to cardiovascular impairment. This chapter will provide an overview of the basic mechanisms underlying the effects of thyroid hormone on the cardiovascular system and their clinical correlates, then address the potential benefit of thyroid hormone treatment in patients with cardiovascular disorders.

II. Cellular Effects of Thyroid Hormone on the Cardiovascular System

Most of the molecular and cellular mechanisms responsible for the cardiovascular effects of thyroid hormone have been clarified. As shown in Figure 1, thyroid hormone may exert both genomic and nongenomic effects on cardiac myocytes. The genomic effects of thyroid hormone are mediated by the transcriptional activation or repression of specific target genes that encode both structural and functional proteins (Dillmann, 1990). This process begins with the entry of triiodothyronine (T3), the biologically active thyroid hormone, into the cardiomyocyte through specific transport proteins located within the cell membrane (Everts *et al.*, 1996). To date, there is no clear evidence of a biologically relevant conversion of thyroxine (T4) to T3 in cardiomyocytes (Everts *et al.*, 1996). Once in the cardiomyocyte, T3 enters the nucleus and interacts with

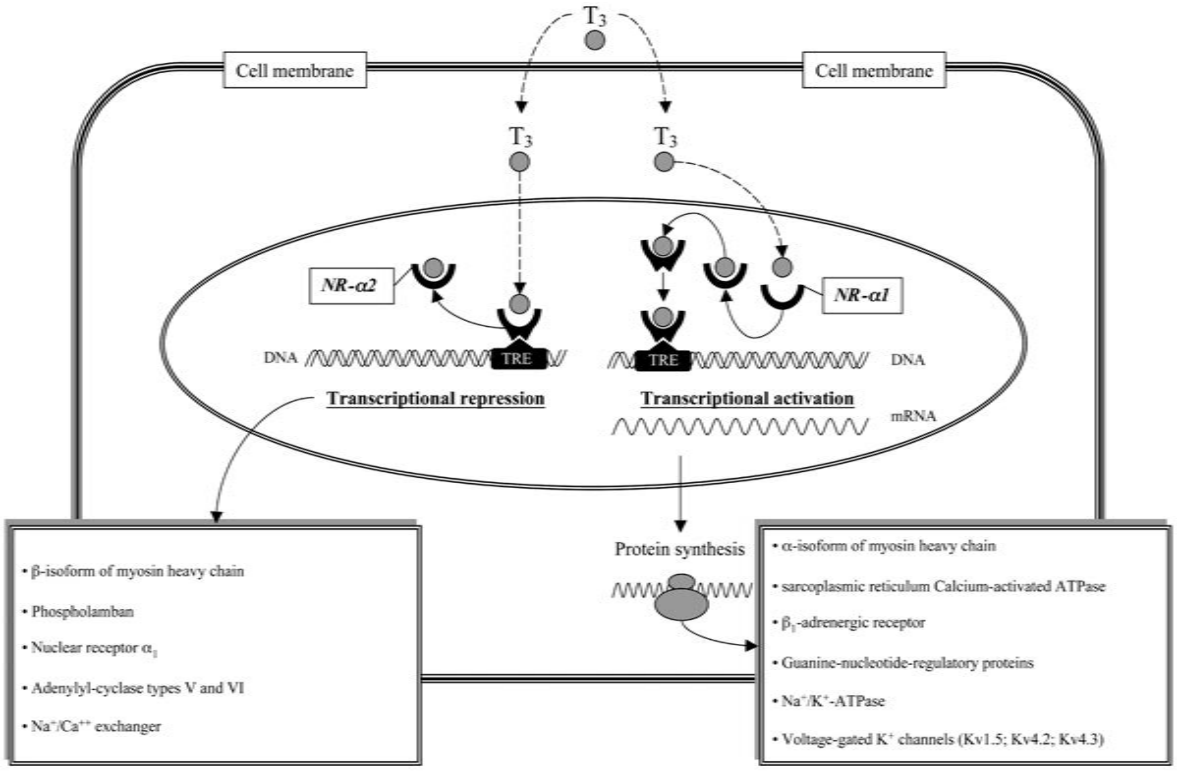

FIG. 1. Genomic effects of thyroid hormone (T3) on cardiomyocytes. NR, triiodothyronine nuclear receptor; TRE, thyroid hormone responsive element (see text for details).

specific transcriptional activators (nuclear receptor α1) or repressors (nuclear receptor α2). Occupancy of these receptors by T3, in combination with recruited cofactors, allows the thyroid hormone-receptor complex to bind (nuclear receptor α-1) or release (nuclear receptor α-2) specific sequences of DNA (thyroid-responsive elements) that, in turn, by acting as cis- or trans-regulators, modify the rate of transcription of specific target genes (Brent, 1994).

Among various proteins whose expression is modulated at transcriptional level (Figure 1), the most-extensively characterized are myosin heavy chains (Morkin, 1993; Ojamaa *et al.*, 1996b) and the sarcoplasmic reticulum protein involved in the regulation of intracellular calcium handling, namely, calcium-activated ATPase and its inhibitory cofactor, phospholamban (Dillmann, 1990; Kiss *et al.*, 1994). Many studies *in vitro* and in experimental rats incontrovertibly show that thyroid hormone upregulates the expression of the α-isoform of the myosin heavy chain in cardiomyocytes, while it downregulates the β-isoform (Morkin, 1993; Ojamaa *et al.* 1996b). There is some evidence that this regulation also occurs in humans (Ladenson *et al.*, 1992). However, the magnitude of this phenomenon is certainly less pronounced than in rodents — a finding that fuels uncertainties about the functional correlate of this molecular effect. In humans, the β-isoform of the myosin heavy chain is more prevalent than the α-isoform (Magner *et al.*, 1988; Ladenson *et al.*, 1992) and the ratio is only marginally modified by thyroid hormone (Ladenson *et al.*, 1992).

In contrast, several lines of evidence suggest that some abnormalities of cardiac function in patients with thyroid dysfunction directly reflect the effects of thyroid hormone on calcium-activated ATPase and phospholamban, which are involved primarily in the regulation of systodiastolic calcium concentrations in cardiomyocytes (Dillmann, 1990; Kiss *et al.*, 1994). Sarcoplasmic reticulum calcium-activated ATPase is responsible for the rate of calcium reuptake into the lumen of the sarcoplasmic reticulum during diastole that, in turn, is a major determinant of the velocity of myocardial relaxation after contraction (Dillmann, 1990; Kiss *et al.*, 1994). However, the performance of sarcoplasmic reticulum calcium-activated ATPase is influenced by the level of expression of phospholamban: the higher the phospholamban expression, the lower the sarcoplasmic reticulum calcium-activated ATPase activity (Kiss *et al.*, 1994). In this regard, it has been extensively demonstrated that thyroid hormone upregulates expression of the sarcoplasmic reticulum calcium-activated ATPase and downregulates expression of phospholamban, thereby enhancing myocardial relaxation (Dillmann, 1990; Kiss *et al.*, 1994). Indeed, the improved calcium reuptake during diastole may favorably affect myocardial contractility. In fact, the greater reduction in cytoplasmatic concentration of calcium at end-diastole increases the magnitude of the systolic transient of calcium that, in turn, augments its availability for activation of tropo-myosin units. In fact, in phospholamban-deficient mice, cardiac contractility was found to be increased, with no further

increase after thyroid hormone treatment (Kiss *et al.*, 1998). This finding strongly supports the key role of sarcoplasmic reticulum proteins and their effects on intracellular calcium handling in thyroid hormone-mediated changes in systo-diastolic cardiac function in patients with thyroid dysfunction. In this context, it is important to recognize that thyroid hormone also modifies the expression of other ion channels, such as Na^+/K^+-activated ATPase, Na^+/Ca^{++} exchanger, and some voltage-gated K^+ channels (Kv1.5, Kv4.2, Kv4.3), thereby coordinating the electrochemical and mechanical responses of the myocardium (Gick *et al.*, 1990; Ojamaa *et al.*, 1999).

In addition to these genomic effects, thyroid hormone produces changes in cardiac inotropism and chronotropism more rapidly than would be expected from regulation of gene expression, which usually take minutes to hours to be phenotypically and functionally appreciable. This calls into question the involvement of nongenomic mechanisms (Davis and Davis, 1993; Walker *et al.*, 1994). Some evidence indicates that thyroid hormone promotes the acute phosphorylation of phospholamban and that this action attenuates the inhibitory effect of phospholamban on sarcoplasmic reticulum calcium-activated ATPase (Ojamaa *et al.*, 2002). Interestingly, the fact that this process is mediated at least in part by the activation of intracellular kinase pathways involved in signal transduction of the adrenergic stimulus (Ojamaa *et al.*, 2002) may help to explain functional analogies between the cardiovascular effects of thyroid hormone and those promoted by the adrenergic system (Levey and Klein, 1990). Indeed, although most of the cardiovascular manifestations associated with hyperthyroidism and hypothyroidism mimic a condition of increased and reduced adrenergic activity, respectively, the sensitivity of the cardiovascular system to adrenergic stimulation does not seem to be substantially altered in these conditions (Hoit *et al.*, 1997; Ojamaa *et al.*, 2000).

Thyroid hormone also exerts an important effect on the vascular system. It acutely reduces peripheral vascular resistance by promoting relaxation in vascular smooth-muscle cells (Klemperer *et al.*, 1995; Ojamaa *et al.*, 1996a; Park *et al.*, 1997). A single study has reported that chronic thyroid hormone excess exerts profound effects on vascular reactivity by improving both endothelium-dependent and -independent mechanisms (Napoli *et al.*, 2001).

III. Overt Hyperthyroidism

Palpitation is one of the most-common symptoms associated with overt hyperthyroidism (Nordyke *et al.*, 1988). Continuous, ambulatory, 24-hour electrocardiogram (ECG) monitoring characteristically demonstrates that heart rate is constantly increased during the day and exaggerated in response to exercise, although its circadian rhythm usually is preserved (von Olshausen *et al.*, 1989; Cacciatori *et al.*, 1996). Analysis of heart rate variability reveals sympatho-vagal

unbalancing with a relative increase in sympathetic tone (Cacciatori *et al.*, 1996). In this respect, although β-adrenergic blockade usually attenuates tachycardia in patients with overt hyperthyroidism, heart rates remain slightly higher, in comparison with euthyroid controls. This supports the notion that thyroid hormone is able to directly affect sinus node firing (Sun *et al.*, 2001).

About 5–10% of overt hyperthyroid patients have atrial fibrillation, which may be the presenting problem (Sawin *et al.*, 1994; Auer *et al.*, 2001). Atrial fibrillation usually reverts to sinus rhythm with achievement of euthyroidism (Nakazawa *et al.*, 1982), the reversion rate decreasing with age and duration of arrhythmia (Nakazawa *et al.*, 1982; Nordyke *et al.*, 1988). Therefore, achievement of the euthyroid state should be the primary treatment strategy of atrial fibrillation associated with overt hyperthyroidism. β-adrenergic blockade may be used effectively to control the ventricular rate. In all instances, electrical or pharmacological cardioversion should be an option only after achievement of euthyroidism. In this context, it remains controversial whether overt hyperthyroid patients with atrial fibrillation should receive anticoagulant therapy. In general, the risk of bleeding with anticoagulant therapy always should be weighed against the risk of systemic embolization (Gilligan *et al.*, 1996) and the decision made on a case-by-case basis. In general, it would be appropriate to administer anticoagulant agents to older patients with known or suspected heart disease or with atrial fibrillation of longer duration (Petersen and Hansen, 1988; Gilligan *et al.*, 1996).

Systolic arterial pressure is almost invariably increased and diastolic arterial pressure decreased in subjects with overt hyperthyroidism, so that pulse pressure is characteristically wider and mean arterial pressure is only marginally decreased (Graettinger *et al.*, 1959; Theilen and Wilson, 1967; DeGroot and Leonard, 1970). These hemodynamic changes are associated with a remarkable increase in cardiac output and a notable reduction in peripheral vascular resistance, thereby resulting in the classic hyperdynamic cardiovascular state (Graettinger *et al.*, 1959; Theilen and Wilson, 1967; DeGroot and Leonard, 1970).

The main determinants of increased at-rest left ventricular (LV) performance in patients with overt hyperthyroidism have not been definitively established (Biondi *et al.*, 2002a). The high cardiac output state results from a remarkably faster heart rate and a slightly augmented stroke volume that, in turn, is associated with normal or marginally enlarged LV end-diastolic size and normal or marginally decreased LV end-systolic size (Biondi *et al.*, 2002a). As a result, LV ejection fraction, an index of overall systolic chamber function, characteristically is increased in overt hyperthyroid subjects (Biondi *et al.*, 2002a). Whether this process is sustained mainly by a true enhancement of myocardial contractility or by the interaction of hemodynamic factors is unclear (Biondi *et al.*, 2002a). Studies by Merillon and colleagues (1981) and by Feldman and coworkers (1986) are emblematic of the controversy surrounding this argument.

Merillon and coworkers (1981) assessed LV function in seven overt thyrotoxic subjects by cardiac catheterization, compared with 11 euthyroid controls atrially paced at a near-identical heart rate. They found no differences between the two groups in such parameters of contractile performance as LV ejection fraction, rate of rise of LV pressure as a proportion of the total pressure, velocity of circumferential fiber shortening, and ratio of LV end-systolic pressure to end-systolic volume. Conversely, it was noted that atrial pacing, but not hyperthyroidism, was accompanied by a marked reduction in both end-diastolic volume and pressure and by a significant increase in systemic vascular resistance and mean aortic pressure. As expected, cardiac performance was not increased in atrially paced subjects. Although atrial pacing and hyperthyroidism are not strictly comparable (acute vs. chronic condition), the authors concluded that there was no realistic increase in the true level of myocardial contractility independent of changes in heart rate and preload in human hyperthyroidism.

Feldman and colleagues (1986) studied LV function in 11 hyperthyroid patients by means of echocardiography and in 11 age-matched normal subjects. They found no differences between the two groups in LV end-diastolic diameter or in end-systolic meridional wall stress. Differently, the rate-corrected mean velocity of circumferential fiber shortening (a measure of LV function claimed to be independent of preload and heart rate) was much higher in hyperthyroid patients. As a result, when LV end-systolic wall stress was related to the rate-corrected velocity of fiber shortening, the values of hyperthyroid patients were above the mean regression line for normal subjects, reflecting the presence of an increased contractile state. The authors, however, did not consider that the normal end-diastolic dimension, despite the augmented heart rate in their patients, corresponded to an effective increase in preload, given the inverse relationship between the two variables. Furthermore, the noninvasive method of assessing myocardial contractility by relating the rate-corrected circumferential fiber-shortening velocity to meridional LV end-systolic wall stress may overestimate myocardial performance in pathophysiological states characterized by simultaneous increases in preload and heart rate.

Based on these remarks, it may be assumed that the notably faster heart rate in hyperthyroid patients masks the actual increase in cardiac preload, leading to an underestimation of the extent to which the Frank-Starling mechanism contributes to improving cardiac performance (Biondi *et al.*, 2002a). Indeed, there is evidence that cardiac preload is increased in overt hyperthyroidism. Some studies have demonstrated clearly that blood volume is enlarged in patients with overt hyperthyroidism (Gibson and Harris, 1939; Anthonisen *et al.*, 1960). Other studies showed that the renin-angiotensin-aldosterone system is activated in hyperthyroid patients (Resnick and Laragh, 1982). In addition, thyroid hormone has been shown to upregulate erythropoietin secretion and, in turn, red blood cell mass, which may also contribute to the increase in total blood volume (Gibson

and Harris, 1939; Graettinger *et al.*, 1959; Klein and Levey, 1984). Moreover, the analysis of LV diastolic function in different investigations has almost invariably revealed an increase in indices of early LV filling and a faster LV relaxation independent of the effect of heart rate (Lewis *et al.*, 1979; Friedman *et al.*, 1982; Mintz *et al.*, 1991; Kahaly *et al.*, 1999). This pattern of diastolic function is consistent with a greater venous return and enhanced ventricular suction, suggesting that the improvement in diastolic function would allow the increased venous return to be accommodated without relevant changes in filling pressure. This interpretation is supported by the observation of comparable values of LV end-diastolic volume and pressure in hyperthyroid patients and normal subjects (Merillon *et al.*, 1981).

It is, therefore, conceivable that the hyperthyroid heart increases its performance through the advantageous modulation of hemodynamic loads, rather than through recourse to its inotropic reserve (Biondi *et al.*, 2002a). In this regard, it is important to underline that the mechanisms that utilize myocardial contractility involve an ever and necessarily greater myocardial metabolic demand. On the contrary, recourse to the hemodynamic loads to improve cardiac performance is an energetically favorable mechanism. Indeed, recourse to the latter mechanism optimizes the cardiac mechanic-energetic utilization in a condition such as hyperthyroidism, in which a clear increase of myocardial energetic consumption is already present (Bengel *et al.*, 2000).

The complicated and delicate interaction between the different factors determining cardiac function in the patient with overt hyperthyroidism may explain, at least in part, two clinical observations. The first, and more frequent, is reduced exercise tolerance (Kahaly *et al.*, 1998,1999); the second – less-frequently encountered than in the past — is development of congestive heart failure (CHF) (Magner *et al.*, 1988). On the one hand, reduced exercise tolerance of hyperthyroid patients may result from their reduced cardiovascular reserve (Kahaly *et al.*, 1998,1999). In fact, the mechanisms used by the cardiovascular system during exercise are almost largely used at rest. On the other hand, CHF may be precipitated by the occurrence of atrial fibrillation. The loss of atrial contribution to ventricular filling and the reduced diastolic time may severely compromise diastolic dynamics, increasing end-diastolic pressure and promoting systemic congestion. Occasionally, CHF may develop as a result of the so-called "rate-related cardiomyopathy" (Magner *et al.*, 1988).

IV. Subclinical Hyperthyroidism

Subclinical hyperthyroidism is characterized by subnormal or suppressed thyrotropin (TSH) serum levels in the presence of circulating thyroid hormones in the normal range for the general population (Biondi *et al.*, 2002c). It may be due to an intrinsic pathology of the thyroid gland (endogenous subclinical

hyperthyroidism) but, more often, is the consequence of suppressive or replacement L-thyroxine (L-T4) therapy (exogenous subclinical hyperthyroidism) (Biondi *et al.*, 2002c). Exogenous subclinical hyperthyroidism is the condition more-frequently seen in clinical practice (Biondi *et al.*, 2002c).

Over past decades, a number of studies have investigated the effects of subclinical hyperthyroidism on the heart, showing that this condition may be associated with important abnormalities of cardiac structure and function (Biondi *et al.*, 2002c). The more-consistent abnormalities found in patients with subclinical hyperthyroidism are increased heart rate and prevalence of supraventricular arrhythmias and enhanced LV mass (Biondi *et al.*, 1993,1994,1996,1999b,2000; Fazio *et al.*, 1995; Ching *et al.*, 1996; Shapiro *et al.*, 1997; Mercuro *et al.*, 2000). The latter feature often is associated with slightly enhanced systolic function and almost always with impaired diastolic function due to slowed myocardial relaxation (Biondi *et al.*, 1993,1994,1996,1999b,2000; Fazio *et al.*, 1995; Ching *et al.*, 1996; Shapiro *et al.*, 1997; Mercuro *et al.*, 2000). The increase in LV mass is due to increased wall thickness without changes of cavity dimension ("concentric remodeling"). It rarely corresponds to an actual LV hypertrophy and is related to the duration of subclinical hyperthyroidism rather than to circulating thyroid hormone levels. The mechanism responsible for the increase in LV mass has not been completely clarified. In general, it is assumed to develop in response to a chronic hemodynamic overload due to the mild hyperkinetic cardiovascular state (Klein, 1988; Fazio *et al.*, 1995). In fact, as happens with classic models of overload-induced cardiac hypertrophy, the increase of LV mass observed in patients with subclinical hyperthyroidism is associated with slowed myocardial relaxation and impaired ventricular filling. It has been suggested that this diastolic dysfunction results from altered intracellular calcium handling due to reduced expression of sarcoplasmic reticulum calcium ATPase and/or increased expression of phospholamban, with the consequent delayed sarcoplasmic reuptake of calcium. However, this view would contrast with the well-known effects of thyroid hormone on such genes (Dillmann, 1990; Kiss *et al.*, 1994). Therefore, it may be hypothesized that, in the long-term, the effects of chronically increased cardiac workload on calcium metabolism would prevail on those promoted by thyroid hormone. Be that as it may, abnormalities of LV morphology and function promptly normalize with achievement of euthyroidism and are effectively attenuated by β-blocking drugs (Biondi *et al.*, 1994,1995,1999b, 2000). This supports the concept that cardiac involvement in subclinical hyperthyroidism is reversible and mostly determined by functional mechanisms.

Importantly, compelling evidence exists that subclinical hyperthyroidism is associated with increased cardiovascular mortality (Parle *et al.*, 2001). Although the mechanism responsible for this association remains obscure, several factors may contribute to this phenomenon (Biondi *et al.*, 2002b). There are indications that patients with subclinical hyperthyroidism, particularly the elderly, have an

increased risk of atrial fibrillation (Sawin *et al.*, 1994; Auer *et al.*, 2001), which may enhance the incidence of thromboembolic events (Petersen and Hansen, 1988; Ladenson, 1993). It has been shown that the increase of LV mass *per se*, even in the absence of a clear LV hypertrophy, and of heart rate are associated with an increased risk of sudden death (Haider *et al.*, 1998; Greenland *et al.*, 1999). Moreover, it is well-recognized that diastolic dysfunction may precede development of more-severe LV dysfunction. Indeed, especially in the elderly, it may precipitate cardiac decompensation and CHF. Therefore, the current opinion is to avoid or correct subclinical hyperthyroidism in all patients affected with benign thyroid disease (Biondi *et al.*, 2002c). On the contrary, when subclinical hyperthyroidism is a therapeutic indication (e.g., in patients with differentiated thyroid cancer), L-T4 should be administered at the lowest dose sufficient to achieve a stable TSH suppression, eventually associated with chronic β-blockade (Biondi *et al.*, 2002c). Contextually, subclinical hyperthyroidism should be suspected in all subjects with a history of atrial fibrillation, especially in the elderly and in those with underlying cardiac disease who complain of worsening of angina pectoris or cardiac decompensation.

V. Overt Hypothyroidism

The cardiovascular effects of thyroid hormone deficiency are opposite to those caused by thyroid hormone excess. However, the clinical presentation of overt hypothyroidism is not obvious and most patients have few symptoms and signs (Klein and Ojamaa, 2000). Bradycardia and systemic hypertension, with narrow pulse pressure and slightly increased mean arterial pressure, and some degree of exercise impairment are the most-common findings in patients with overt hypothyroidism (McAllister *et al.*, 1995; Klein and Ojamaa, 2000).

Many patients with overt hypothyroidism have abnormal standard ECG, including QT interval lengthening and flattening or inversion of the T wave (Fredlund and Olsson, 1983; Klein and Ojamaa, 2000), which reflects the prolonged cardiac action potential (Ojamaa *et al.*, 1999). In addition, overt hypothyroid patients are more prone to ventricular arrhythmias, particularly in the presence of an underlying ischemic heart disease, due to increased electrical dispersion in the myocardium (Fredlund and Olsson, 1983; Klein and Ojamaa, 2000).

The prevalence of systemic hypertension is nearly three-fold higher in patients with overt hyperthyroidism than in euthyroid subjects. In addition, in patients with systemic hypertension, overt hypothyroidism is associated with higher blood pressure (Endo *et al.*, 1979; Saito *et al.*, 1983; Streeten *et al.*, 1988; Klein, 1989; Fletcher and Weetman, 1998; Fommei and Iervasi, 2002). Two factors contribute to systemic hypertension in overt hypothyroidism. The first, and certainly the most-widely recognized, is the remarkable increase in periph-

eral vascular resistance (Klein and Ojamaa, 2000). The second, and more-recently documented, is the increase in arterial stiffness, which likely results from myxedema of the arterial wall (Dernellis and Panaretou, 2002; Obuobie *et al.*, 2002). In general, systemic hypertension associated with overt hypothyroidism is poorly controlled by conventional treatments, whereas it promptly improves with achievement of euthyroidism (Dernellis and Panaretou, 2002). This finding would encourage the routine assessment of thyroid function in all patients with preexisting systemic hypertension that becomes resistant to pharmacological treatment.

The most-consistent cardiac abnormality recognized in patients with overt hypothyroidism is impairment of LV diastolic function, which is characterized by slowed myocardial relaxation and impaired early ventricular filling (Crowley *et al.*, 1977; Wieshammer *et al.*, 1989). LV systolic function usually is only marginally subnormal, as demonstrated by slightly reduced values of ejection fraction and stroke volume (Crowley *et al.*, 1977; Wieshammer *et al.*, 1989). On the one hand, the reduced cardiac preload, in combination with bradycardia and slightly depressed myocardial contractility, accounts for a subnormal cardiac output in overt hypothyroidism (Crowley *et al.*, 1977; Wieshammer *et al.*, 1989). On the other hand, the lower cardiac performance and the abnormalities in peripheral and proximal vascular function may contribute to the poor exercise tolerance in overt hypothyroidism (McAllister *et al.*, 1995).

Occasionally, cardiac function may be further compromised by the development of pericardial effusion, which occurs with severe, long-standing overt hypothyroidism (Ladenson *et al.*, 1992). In addition, overt hypothyroidism may be associated with some increase in LV mass. However, as shown by necropsy and ultrasound investigations, the increase in LV mass does not correspond to myocardial hypertrophy *sensu strictu* but rather to interstitial myxedema (Aber, 1964). By increasing wall stiffness, cardiac myxedema may further compromise LV mechanics, contributing to reduced cardiac output.

In this context, it is important to recall that although overt hypothyroidism is associated with a lower myocardial oxygen demand, myocardial mechanical work efficiency is worse than in euthyroid controls and improves with achievement of euthyroidism (Bengel *et al.*, 2000). The increase in peripheral vascular resistance and arterial stiffness in overt hypothyroidism contributes to increased cardiac afterload, one of the major factors determining myocardial oxygen consumption (Bengel *et al.*, 2000). The disproportionate increase in myocardial oxygen uptake with respect to the level of cardiac performance may, therefore, explain at least in part why overt hypothyroidism may precipitate or worsen angina in patients with suspected or known ischemic heart disease (Keating *et al.*, 1960) and why some of these patients have an improvement in anginal symptoms after thyroid hormone replacement is initiated.

Overt hypothyroidism may be particularly hazardous in the elderly, independent of the presence of underlying cardiovascular disease. Aging is accompanied by the development of cardiac hypertrophy and interstitial fibrosis, which may be responsible *per se* for diastolic dysfunction and reduced cardiovascular performance. Therefore, the onset of overt hypothyroidism in this vulnerable population occasionally may precipitate cardiac decompensation and CHF. In a single study, it was estimated that approximately 30–50% of elderly subjects with heart failure have normal values of ejection fraction (diastolic heart failure) (McDermott *et al.*, 1995). Noteworthy, diastolic heart failure was strongly associated with hypothyroidism and was more prevalent in women (McDermott *et al.*, 1995). Therefore, thyroid function should be routinely assessed in older patients with newly diagnosed or worsening heart failure.

VI. Subclinical Hypothyroidism

Subclinical hypothyroidism (SH), defined by elevated serum TSH level in the presence of normal levels of free thyroid hormones, is common in the adult population, especially among women above 60 years of age (Tunbridge *et al.*, 1977; Canaris *et al.*, 2000). Up to two thirds of patients have serum TSH between 5–10 mU/L and thyroid autoantibodies (Tunbridge *et al.*, 1977; Canaris *et al.*, 2000). Almost half of these individuals may progress to overt thyroid failure (Vanderpump *et al.*, 1995; Huber *et al.*, 2002).

Striking evidence indicates that elevated TSH levels in SH patients do not reflect pituitary compensation to maintain euthyroidism but mild tissue hypothyroidism *sensu strictu* (Andersen *et al.*, 2002). Several changes in metabolic and organ function indexes have been reported in most clinical investigations of patients with persistent SH (Biondi *et al.*, 2002c). Deviation from normality progressively increases with serum TSH level ("dosage effect" phenomenon) (Lekakis *et al.*, 1997; Bindels *et al.*, 1999; Faber *et al.*, 2002).

In general, resting heart rate and blood pressure are normal in SH subjects (Biondi *et al.*, 1999a; Monzani *et al.*, 2001; Di Bello *et al.*, 2002; Vitale *et al.*, 2002). However, significant hypofunctional abnormalities in the parasympathetic nervous system and an increased prevalence of systemic hypertension have been reported in patients with SH (Kahaly, 2000; Luboshitzky *et al.*, 2002).

The most-consistent cardiac abnormality recognized in SH patients is LV diastolic dysfunction, characterized by slowed myocardial relaxation and impaired early ventricular filling, both at rest and with exercise (Biondi *et al.*, 1999a; Monzani *et al.*, 2001; Di Bello *et al.*, 2002; Vitale *et al.*, 2002; Brenta *et al.*, 2003). Often, this is associated with a variable impairment in LV systolic function at rest (Biondi *et al.*, 1999a; Monzani *et al.*, 2001; Di Bello *et al.*, 2002; Vitale *et al.*, 2002), which becomes more consistent on exercise (Foldes *et al.*, 1987; Kahaly, 2000; Brenta *et al.*, 2003). In a comprehensive study of exercise

capacity, it was reported that SH is associated with impairment of several exercise-related cardiopulmonary responses, resulting in some degree of exercise impairment (Kahaly, 2000).

Recently, a strong association between SH and atherosclerotic cardiovascular disease, independent of the traditional risk factors (i.e., hypercholesterolemia, hypertension, smoking, diabetes mellitus), was noted in a large cross-sectional survey of postmenopausal women (the Rotterdam Study) (Hak *et al.*, 2000). Although the mechanism responsible for this association remains to be clarified, compelling evidence indicates that SH is associated with an atherogenic lipid profile, characterized by increased circulating levels of total and low-density cholesterol and increased levels of oxidized low-density lipoproteins (Duntas, 2002; Duntas *et al.*, 2002). It has been reported that SH may affect the hemostatic profile, thereby promoting a hypercoagulable state (Müller *et al.*, 2001). Moreover, a reduced endothelium-dependent, flow-mediated vasodilation, which is an early marker of atherosclerosis, has been reported in patients with SH (Lekakis *et al.*, 1997).

Importantly, when SH patients are treated with L-T4, most metabolic and cardiovascular abnormalities improve or even normalize (Danese *et al.*, 2000; Biondi *et al.*, 2002c). However, whether patients with SH should be treated remains the subject of disagreement (Chu and Crapo, 2001; McDermott and Ridgway, 2001).

VII. Thyroid Function in Cardiovascular Disorders

The relationship between thyroid status and the cardiovascular system is not unidirectional. A body of data indicates that acute and chronic cardiovascular disorders may alter the metabolism of thyroid hormone.

Within 4 hours after acute uncomplicated myocardial infarction, circulating T3 and T4 levels are reduced by about 20% and 40%, respectively (Franklyn *et al.*, 1984). In patients with chronic heart failure, serum thyroid hormone concentration decreases and reverse-T3 increases, the magnitude of changes paralleling the degree of functional impairment as assessed by the New York Heart Association classification (Hamilton *et al.*, 1990). In patients undergoing cardiopulmonary or coronary-aortic bypass surgery, circulating T3 levels fall significantly in the postoperative period (Holland *et al.*, 1991; Klemperer *et al.*, 1995; Bettendorf *et al.*, 1997).

In this regard, several lines of evidence suggest that the altered thyroid status in patients with cardiovascular disorders could modify cardiac gene expression and contribute to impaired cardiac function (Klein and Ojamaa, 1998). In animals, a low serum concentration of T3 induced by caloric restriction was associated with impaired cardiac contractility and altered gene expression similar to that seen in experimental hypothyroidism, which normalized with replacement

doses of T3 (Katzeff *et al.*, 1997). Decreased expression of thyroid hormone nuclear receptor α-1 associated with increased expression of the thyroid hormone nuclear receptor α-2, typical of tissue hypothyroidism, recently was reported in myocardial biopsy from patients with chronic heart failure (Kinugawa *et al.*, 2001). Of note, this abnormal pattern of thyroid hormone receptor expression was correlated with the reduced myocardial expression of the α-isoform of myosin heavy chain and the increased expression of the β-isoform of myosin heavy chain and of the atrial natriuretic peptide, thereby implicating tissue thyroid status in the development of the failing heart phenotype (Kinugawa *et al.*, 2001). In 23 patients with advanced chronic heart failure, a single intravenous dose of T3 elicited a significant hemodynamic improvement by reducing peripheral vascular resistance and increasing cardiac output (Hamilton *et al.*, 1998). Treatment with low-dose L-T4 daily for 12 weeks greatly benefited 20 patients with chronic heart failure by reducing peripheral vascular resistance, increasing cardiac output, and improving exercise performance (Moruzzi *et al.*, 1996). Moreover, in patients undergoing cardiopulmonary or coronary artery bypass surgery, intravenous treatment with T3 immediately before and/or after surgery significantly improved postoperative cardiac function and reduced somewhat the extent of surgical mortality (Klemperer *et al.*, 1995; Cimochowski *et al.*, 1997; Mullis-Janson *et al.*, 1999; Bettendorf *et al.*, 2000). In no instance did thyroid hormone treatment deteriorate cardiac function or induce myocardial ischemia rhythm disturbances or other untoward effects (Klemperer *et al.*, 1995; Moruzzi *et al.*, 1996; Cimochowski *et al.*, 1997; Hamilton *et al.*, 1998; Mullis-Janson *et al.*, 1999; Bettendorf *et al.*, 2000).

In line with these preliminary observations, studies with experimental models of LV dysfunction and preliminary clinical investigation of patients with chronic heart failure reported that the thyroid hormone analog 3,5-diiodothyropropionic acid, whose biological activity profile is similar to that of thyroid hormone except for effects on heart rate, elicited relevant hemodynamic improvements by reducing systemic vascular resistance and improving both systolic and diastolic LV function. This was accompanied by an increase in cardiac output and improved lipid profile (Morkin *et al.*, 2002).

VIII. Conclusion

Thyroid dysfunction causes remarkable cardiovascular derangements. Because signs and symptoms referable to the cardiovascular system may be the only manifestations of overt thyroid dysfunction and because persistent subclinical thyroid dysfunction may notably increase the cardiovascular risk, thyroid status should be systematically investigated in all patients with newly diagnosed or worsening cardiovascular disease, especially the elderly. In addition, patients with acute or chronic cardiovascular disorders have abnormalities in peripheral

thyroid hormone metabolism that may alter cardiac function. The finding that administration of thyroid hormone or its analog 3,5-diiodothyropropionic acid may greatly benefit these patients should foster further investigation in this area.

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Functional Significance of Tie2 Signaling in the Adult Vasculature

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ABSTRACT

Abundant data now demonstrate that the growth of new blood vessels, termed angiogenesis, plays both pathological and beneficial roles in human disease. Based on these data, a tremendous effort has been undertaken to understand the molecular mechanisms that drive blood vessel growth in adult tissues. Tie2 recently was identified as a receptor tyrosine kinase expressed principally on vascular endothelium. Disrupting Tie2 function in mice resulted in embryonic lethality with defects in embryonic vasculature, suggesting a role in blood vessel maturation and maintenance. Based on these studies, we undertook a series of studies to probe the function of Tie2 in adult vasculature that will form the focus of this chapter. Consistent with a role in blood vessel growth in adult vasculature, Tie2 was upregulated and activated in the endothelium of rat ovary and in healing rat skin wounds, both areas of active angiogenesis. Moreover, Tie2 was upregulated in the endothelium of vascular "hot spots" in human breast cancer specimens. Surprisingly, Tie2 also was expressed and activated in the endothelium of all normal rat tissues examined, suggesting a role in maintenance of adult vasculature. To determine the functional role of Tie2 in tumor vasculature, a soluble Tie2 extracellular domain (ExTek) was designed that blocked the activation of Tie2 by its activating ligand, angiopoietin 1 (Ang1). Administration of recombinant ExTek protein or an ExTek adenovirus inhibited tumor growth and metastasis in rodent tumor models, demonstrating a functional role for Tie2 in pathological angiogenesis in adult tissues. To begin to understand the endothelial signaling pathways and cellular responses that mediate Tie2 function, we identified signaling molecules that are recruited to the activated, autophosphorylated Tie2 kinase domain. Two of these molecules, SHP2 and GRB2, are part of the pathway upstream of mitogen-activated protein kinase (MAPK) activation, a pathway that may be responsible for morphogenetic effects of Tie2 on endothelial cells. Another signaling molecule, p85, is responsible for recruitment of phosphatidylinositol 3 kinase (PI3-K) and activation of the Akt/PI3-K pathway. Akt/PI3-K has emerged as a critical pathway downstream of Tie2 that is necessary for cell survival effects as well as for chemotaxis, activation of endothelial nitric oxide synthase, and perhaps for anti-inflammatory effects of Tie2 activation. Taken together,

these studies and many others demonstrate that the Tie2 pathway has important functions in adult tissues, in both quiescent vasculature and during angiogenesis, and help to validate the Tie2 pathway as a therapeutic target.

I. Introduction

Angiogenesis is the complex and dynamic process by which blood vessels are assembled and remodeled to form a functional vasculature. A large body of evidence has shown that angiogenesis is a crucial event for normal embryonic development. In adulthood, the vasculature is notably quiescent but angiogenesis is required for normal female reproductive function and likely is required for wound healing. Importantly, however, angiogenesis contributes to the pathogenesis of a number of diseases, including cancer, arthritis, obesity, atherosclerosis, and two common causes of blindness, diabetic retinopathy and macular degeneration (Folkman, 2001). Conversely, other diseases (e.g., coronary artery disease, peripheral vascular disease) are characterized by failure of the compensatory angiogenic response. Involvement of angiogenesis, or failure of angiogenesis, in these important diseases has led to a tremendous effort on the part of academic and industry researchers to define the molecular mechanisms that drive blood vessel assembly and remodeling.

Of the molecular mechanisms identified to date, activation of endothelial receptor tyrosine kinases (RTKs) by polypeptide growth factors appears to play a pivotal role in blood vessel growth and differentiation (Yancopoulos *et al.*, 2000; Conway *et al.*, 2001). Indeed, growth factors such as fibroblast growth factor, hepatocyte growth factor, platelet-derived growth factor, and the ephrins have been shown to modulate angiogenesis, suggesting that signaling by multiple RTKs is required for the proper assembly of blood vessels. Despite the role of these factors in angiogenesis, the expression of their cognate RTKs outside of the vasculature has dampened enthusiasm for these pathways as therapeutic targets. Importantly, RTKs for two families of angiogenic growth factors — the vascular endothelial growth factor family (VEGF) and the angiopoietin (Ang) family — are expressed predominantly on vascular endothelial cells, making them attractive targets for both pro- and anti-angiogenic therapy (Yancopoulos *et al.*, 2000; Conway *et al.*, 2001; Jones *et al.*, 2001a; Loughna and Sato, 2001). This review will focus on work from our laboratory to elucidate the role of the Ang RTK, Tie2, in adult vasculature. The elegant and important work from our colleagues is cited where it has provided context for our studies and where it has confirmed or extended our work.

II. Function of Ang/Tie2 Signaling in the Embryonic Vasculature

Tie2 originally was described as the second member of an orphan RTK subfamily expressed predominantly in the embryonic endothelium (Dumont *et*

al., 1992; Iwama *et al.*, 1993; Maisonpierre *et al.*, 1993; Runting *et al.*, 1993; Sato *et al.*, 1993; Schnurch and Risau, 1993; Ziegler *et al.*, 1993). Tie1, the original member of the subfamily, and Tie2 share an identical domain structure, with an unusual N-terminal ligand-binding domain, a single transmembrane domain, and an intracellular tyrosine kinase domain (Figure 1). Tie2 was found to be highly conserved across vertebrate species, predicting the importance of its biological function. In fact, the domain structure of Tie2 is highly conserved from zebrafish to human, with the greatest amino acid homology occurring in the kinase domain (Figure 2) (Lyons *et al.*, 1998).

Consistent with its expression pattern, disrupting the function of Tie2 in transgenic mice resulted in early embryonic lethality secondary to vascular abnormalities (Dumont *et al.*, 1994; Sato *et al.*, 1995). Grossly, the patterning of large vessels such as the dorsal aorta was normal. However, Tie2-/- embryos failed to develop the normal hierarchy of vascular elements, suggestive of a failure of vascular branching and differentiation. Ultrastructurally, vessels of

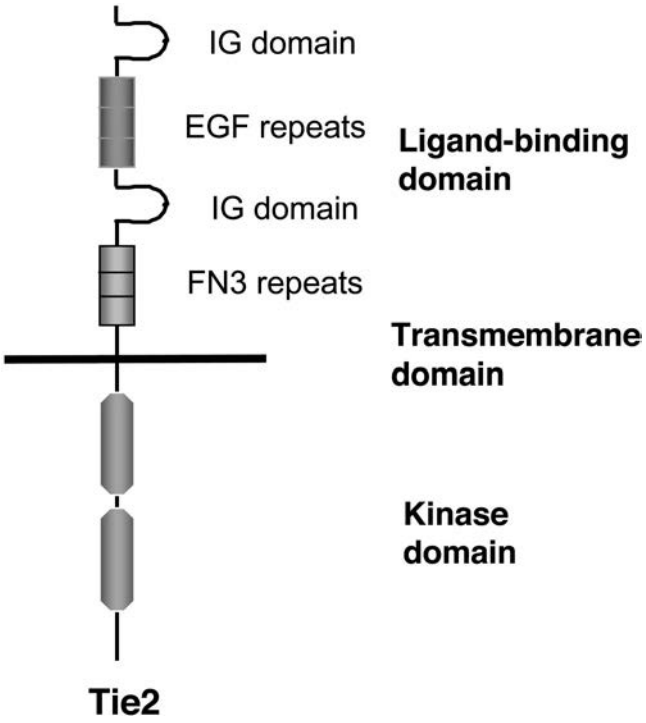

FIG. 1. Tie2 is a receptor tyrosine kinase with a unique extracellular ligand-binding domain comprised of immunoglobulin (IG) domains, epithelial growth factor (EGF) repeats, and fibronectin-like 3 (FN3) repeats.

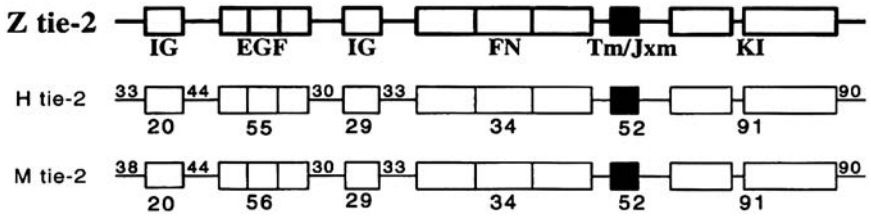

FIG. 2. Tie2 is highly conserved among vertebrate species from zebrafish to human, with the highest homology in the kinase domain.

Tie2-/- embryos showed a decreased number of endothelial cells and decreased contact between endothelial cells and the underlying perivascular cells (pericytes and smooth muscle cells), suggesting a role in the maturation and stabilization of the embryonic vasculature.

Tie2 was deorphanized with the discovery of the angiopoietins, Ang1 and Ang 2 (Davis *et al.*, 1996; Maisonpierre *et al.*, 1997). As is the case with other RTKs, Ang1 binding stimulated autophosphorylation of the kinase domain of Tie2. However, unlike activation of most growth factor RTKs, Ang1 activation of Tie2 did not stimulate mitogenesis, suggesting a novel role in endothelial biology. In contrast to Ang1, Ang2 did not stimulate Tie2 autophosphorylation but instead blocked Ang1-mediated Tie2 activation and endothelial migration, suggesting that Ang2 was a naturally occurring inhibitor of Tie2 activation (Maisonpierre *et al.*, 1997; Witzenbichler *et al.*, 1998; Teichert-Kuliszewska *et al.*, 2001). Consistent with this finding, mice lacking functional Ang1 expression and mice overexpressing Ang2 both displayed a phenotype similar to Tie2-/- mice (Suri *et al.*, 1996; Maisonpierre *et al.*, 1997). Recently, however, it has been demonstrated that, under some circumstances, Ang2 can stimulate Tie2, suggesting that the action of Ang2 as a Tie2 agonist or antagonist is context dependent (Kim *et al.*, 2000b; Teichert-Kuliszewska *et al.*, 2001; Gale *et al.*, 2002). Two other Tie2 ligands have been identified that have disparate effects on Tie2 activation (Valenzuela *et al.*, 1999). Demonstrating their selectivity, none of the Tie2 ligands identified to date bind to or activate the closely related Tie1 RTK. Taken together, these findings indicate that the Ang/Tie2 pathway is a finely regulated one that plays crucial roles in the remodeling and maturation/stabilization of the embryonic vasculature. Based on these studies, we hypothesized that Tie2 signaling would have important roles in the normally quiescent adult vasculature.

III. Role of Tie2 in the Normal Adult Vasculature

To begin to explore the role of Tie2 signaling in adult vasculature, it was first necessary to determine the pattern of Tie2 protein expression (Wong *et al.*,

1997). As anticipated from studies in mouse embryos, immunohistochemical analysis demonstrated that Tie2 was expressed in adult rat tissues undergoing angiogenesis. For example, during ovarian folliculogenesis, Tie2 was expressed in the neovessels of the developing corpus luteum. Interestingly, Maisonpierre and colleagues found that Ang1 and Ang2 also were expressed in the corpus luteum, with Ang2 localizing to the leading edge of proliferating vessels and Ang1 localizing diffusely behind the leading edge (Maisonpierre *et al.*, 1997). Based on this pattern of expression, it was suggested that Ang2-mediated inhibition of Tie2 activation serves to "destabilize" the vessel, to make it responsive to other angiogenic growth factors such as VEGF. Subsequently, Ang1-mediated activation of Tie2 would trigger remodeling and stabilization of the neovasculature.

Similar to the ovary, Tie2 was expressed on the endothelium of neovessels in skin wounds. By western analysis and RNase protection assay, Tie2 was expressed in normal skin, upregulated during active wound angiogenesis, and downregulated as newly formed vessels regressed. As an indicator of Tie2 activation, Tie2 phosphorylation could be detected in skin wounds at all stages of the healing process. Somewhat surprisingly, however, Tie2 also was expressed and phosphorylated in quiescent vasculature of normal skin. Taken together, the expression pattern in the ovary and in healing skin wounds suggested that, as in embryonic vasculature, the Tie2 pathway played a role in the development and remodeling of neovessels in adult vasculature.

The expression and apparent activation of Tie2 in the normally quiescent skin vasculature prompted a broader survey of Tie2 in normal adult tissues. By immunohistochemistry, Tie2 was found to be expressed in endothelium of all adult tissues examined and in most, if not all, blood vessels, including arteries, veins, and capillaries. Studies in transgenic mice expressing marker transgenes driven by the Tie2 promoter have confirmed the broad expression of Tie2 in adult vasculature (Schlaeger *et al.*, 1997; Motoike *et al.*, 2000). In fact, Tie2-cre transgenic mice now serve as a powerful tool to examine the role of more-broadly expressed genes, specifically in endothelium (Kisanuki *et al.*, 2001; Theis *et al.*, 2001; Forde *et al.*, 2002; Takahashi *et al.*, 2003; Vicent *et al.*, 2003). In addition to its broad endothelial expression pattern, Tie2 phosphorylation was detected in every normal adult tissue examined, strongly suggesting a role for Tie2 in the maintenance of quiescent adult vasculature.

Indeed, more-recent studies in transgenic and knockout (KO) mice suggest a critical role for Tie2 in later stages of embryonic vascular development and in adult vasculature. Conditional expression of Tie2 in endothelium of mice homozygous for a Tie2 null allele partially rescued the embryonic lethality of the Tie2 null phenotype (Jones *et al.*, 2001b). Unfortunately, embryos were viable only until embryonic day (E) 15.5 and demonstrated signs of necrosis and vascular hemorrhage, suggesting that this approach did not allow a full rescue of

the Tie2 null phenotype. However, turning off Tie2 expression with doxycycline in "rescued" animals dramatically increased the number of apoptotic endothelial cells, compared to doxycycline-treated, wild-type littermates. In support of these findings, mice chimeric for wild-type and doubly homozygous Tie2 and Tie1 null alleles lack mutant endothelial cells in the vasculature from E15.5 into adulthood (Puri *et al.*, 1999). In contrast, endothelial cells doubly heterozygous for Tie2 and Tie1 null alleles could be found in the embryonic vasculature but were completely absent in the adult vasculature. These findings demonstrate the importance of Tie2 signaling in the later stages of embryonic development and strongly suggest a role for Tie 2 in the adult vasculature. However, further studies using conditional null alleles or perhaps more-effective "rescue" strategies are required to fully assess the role of Tie2 in quiescent adult vasculature.

IV. Role of Tie2 in Pathological Angiogenesis

The expression and activation of Tie2 during angiogenesis in normal adult vasculature suggested a role in pathological angiogenesis. To begin to understand the role of Tie2 in pathological neovascularization, Tie2 expression was assessed in a large number of human breast cancer tumor specimens (Peters *et al.*, 1998). Consistent with results in adult rat vasculature, Tie2 was expressed in the vascular endothelium in both normal breast tissue and in breast tumors. However, the proportion of Tie2-positive tumor microvessels was increased in tumors, compared to normal breast tissue. Moreover, Tie2 expression was concentrated in "vascular hot spots" at the leading edge of invasive tumors. Subsequently, expression of Tie2 in human breast tumors has been confirmed and Tie2 expression has been shown in a number of other human tumors, including ovarian tumors, hepatocellular tumors, and glioblastoma (Stratmann *et al.*, 1998; Hayes *et al.*, 2000; Hata *et al.*, 2002; Tanaka *et al.*, 2002).

The expression of Tie2 in tumor vasculature suggested a role for Tie2 in tumor angiogenesis and in other settings where pathological angiogenesis contributes to disease progression. However, elucidating the functional significance of Tie2 expression in pathological angiogenesis required development of a specific inhibitor of Tie2 activity (Lin *et al.*, 1997,1998a). Since angiopoietins bind with high affinity to the Tie2 extracellular domain, we reasoned that a soluble, recombinant Tie2 extracellular domain should be an efficient inhibitor of angiopoietin-mediated Tie2 activity (Figure 3A). To explore this idea further, a fusion protein consisting of the full-length Tie2 extracellular domain with a 6-His tag (ExTek.6His) was constructed in a baculovirus expression vector and produced in insect cells. Purified ExTek.6His inhibited Ang1 and Ang2 binding to immobilized Tie2 very potently, with IC_{50}s of 110 nM and 159 nM, respectively (Figure 3B). ExTek.6His also inhibited Ang1-mediated Tie2 phosphorylation and cell survival but had no effect on proliferation of a rat mammary tumor cell

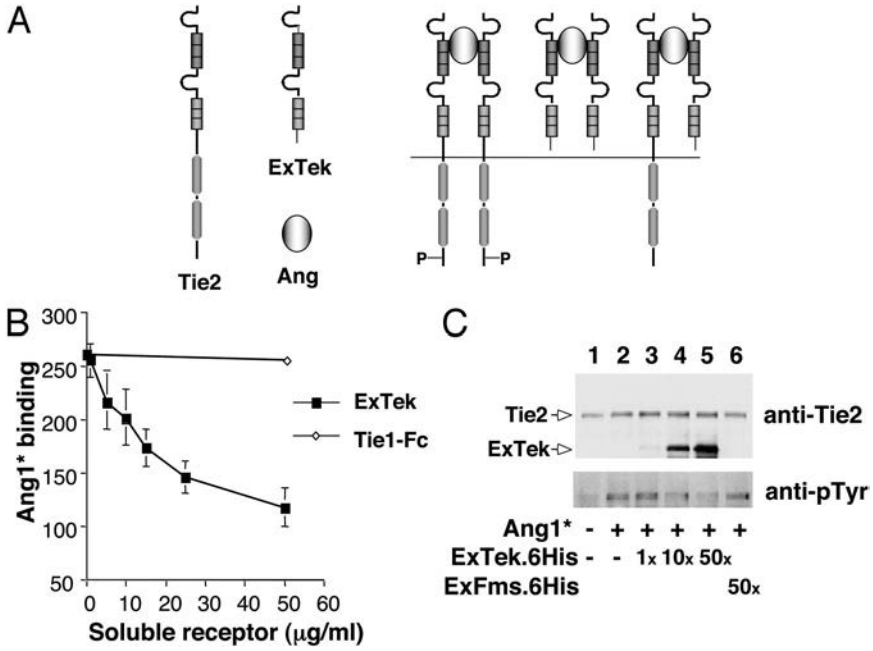

FIG. 3. ExTek is a potent inhibitor of Tie2 activation. (A) ExTek has two potential mechanisms of action. ExTek could simply sequester angiopoietin (Ang) and prevent binding to Tie2. Alternatively, ExTek could have a dominant-negative effect by binding both Ang and Tie2, to prevent proper oligomerization and activation of Tie2. (B) ExTek inhibited Ang1* binding to immobilized Tie2 extracellular domain. Binding of Ang1* (800 ng/ml) to immobilized Tie2-Fc was done in the presence of increasing ExTek concentrations. The Tie1-Fc was used as a control to demonstrate the specificity of the binding inhibition. (C) ExTek inhibited Ang1-stimulated Tie2 autophosphorylation. Serum-starved EAhy926 cells were stimulated with Ang1* (150 ng/ml) plus the indicated amount of ExTek protein or control protein, ExFms (extracellular domain of c-fms). Tie2 and Tie2 phosphorylation levels then were detected by Tie2 immunoprecipitation and western blotting with anti-Tie2 and antiphosphotyrosine antibodies, respectively.

line. These results suggested that, as is the case for other RTKs, ligand-mediated dimerization was required for activation of Tie2 and that ExTek should be an effective inhibitor of Tie2 activation *in vivo*.

As an initial attempt to explore the role of Tie2 in tumor angiogenesis, a single dose of recombinant ExTek was applied directly to rat mammary tumors grown in a transparent cutaneous window chamber (Lin *et al.*, 1997). In this setting, ExTek-treated tumors grew more slowly (i.e., more than 75% reduction in tumor volume) and were less vascular (i.e., 40% reduction in tumor vessel length density) than tumors treated with a control protein. Confirming these results, systemic administration of ExTek using an adenoviral vector inhibited

the growth and metastasis of two different murine tumors, a mammary carcinoma and a melanoma (Lin *et al.*, 1998b). In particular, ExTek adenovirus markedly reduced the number and size of lung metastases when co-injected intravenously with tumor cells or when administered in a model of spontaneous metastatic tumor development. Interestingly, corneal angiogenesis induced by tumor cell-conditioned medium was inhibited by recombinant ExTek, despite the presence of VEGF. Moreover, mammary tumor growth in the tumor window model was significantly inhibited by either recombinant ExTek or a recombinant VEGFR2 extracellular domain (Lin *et al.*, 1997,1998a). These results suggest that Ang/Tie2 and VEGF pathways are both pivotal ones that are either independent or interdependent for tumor angiogenesis. Other studies independently employing different versions of the Tie2 extracellular domain have shown similar effects in different tumor models (Siemeister *et al.*, 1999; Stratmann *et al.*, 2001; Tanaka *et al.*, 2002). Taken together, these findings indicate that Tie2 signaling plays an important role in the development of the tumor vasculature and suggest that this pathway may be involved in the pathogenesis of other angiogenic diseases.

In light of these studies, it is surprising that Ang1 is not upregulated in most tumors. In fact, overexpression of Ang1 in tumor models has resulted in decreased tumor growth, an effect possibly related to Ang1-mediated stabilization of the tumor vasculature, making it resistant to angiogenic stimuli (Hayes *et al.*, 2000; Shim *et al.*, 2001,2002; Hawighorst *et al.*, 2002; Stoeltzing *et al.*, 2002,2003). These findings suggest that the inhibition of tumor angiogenesis by ExTek may not be due to a blockade of Ang1-mediated Tie2 activation. Rather, a number of studies have shown that Ang2 is upregulated in the tumor vasculature (Stratmann *et al.*, 1998; Tanaka *et al.*, 1999; Koga *et al.*, 2001). Consistent with an active role in tumor angiogenesis, Ang2 is localized in the endothelium of proliferating neovessels at the interface between tumor and normal tissue (Holash *et al.*, 1999; Zagzag *et al.*, 1999). Based on these findings, it is possible that ExTek might function to inhibit some positive angiogenic function of Ang2 in tumor vasculature.

Consistent with this notion, recent studies have demonstrated that, in cultured endothelial cells, with longer exposures times or at higher concentrations, Ang2 can act as a Tie2 agonist (or perhaps a partial agonist) (Kim *et al.*, 2000a; Teichert-Kuliszewska *et al.*, 2001). In addition, mice carrying a homozygous Ang2 null allele have profound defects in the growth and patterning of lymphatic vasculature and a failure of remodeling and regression of the hyaloid vasculature that surrounds the neonatal lens (Gale *et al.*, 2002). When the Ang2 gene was replaced with Ang1, the lymphatic defects, but not the vascular remodeling defects, were rescued, suggesting that Ang2 functions as a Tie2 antagonist in blood vasculature but as a Tie2 agonist in developing lymph vasculature. These findings suggest that the context dependence of Ang2 may be vascular bed specific. Whereas Ang2 normally is expressed in arterial smooth

muscle, it appears to be expressed in endothelium only under conditions of vascular remodeling. Arterial expression of Ang2 suggests an as-yet-unknown role in arterial endothelial maintenance that may be related to its agonist effects during lymphatic remodeling. Interestingly, VEGFR3, a receptor that normally is expressed predominantly in adult lymphatic vasculature, was found to be upregulated in vascular endothelium of breast tumors (Valtola *et al.*, 1999). It is tempting to speculate that at least some tumor vessels may take on a more-lymphatic phenotype and thus respond to Ang2 as an agonist instead of an antagonist. Clearly, further work will be required to fully understand the biology of the Ang/Tie2 pathway in tumor vasculature.

The efficacy of ExTek in tumor models suggested a role for Tie2 in other angiogenic diseases. Similar to the tumor vasculature, Tie2 and Ang2 are upregulated in human retina in eyes from patients with ischemic retinal disorders (Takagi *et al.*, 2003). In a mouse model of ischemic retinopathy, Ang2 expression was markedly upregulated in the ischemic retina and closely associated with retinal neovasculature (Hackett *et al.*, 2000). In this setting, either local delivery of a recombinant, soluble Tie2-Fc by intravitreal injection or systemic delivery of ExTek by intramuscular injection of AdExTek inhibited retinal neovascularization (Hangai *et al.*, 2001; Takagi *et al.*, 2003). Similar results were obtained with AdExTek in a murine model of laser-induced choroidal neovascularization (Hangai *et al.*, 2001). These findings, together with results using tumor models, establish a role for the Tie2 pathway in pathological angiogenesis and suggest that modulation of the Tie2 pathway could have broad application in the myriad of angiogenesis-dependent diseases.

V. Tie2 Activation and Signal Transduction

Clarification of the role of Tie2 in normal vasculature and in pathological angiogenesis will require a deeper understanding of the molecular and cellular mechanisms of Tie2 activation and signal transduction. It is now generally accepted that RTK activation and signaling are initiated by ligand-mediated receptor dimerization (Schlessinger, 2000). Receptor dimerization results in cross-phosphorylation of each member of the dimer pair on specific tyrosine residues. Receptor cross-phosphorylation has a dual effect on receptor function: first, to further enhance the receptor's kinase activity and second, to provide binding sites for signaling molecules possessing phosphotyrosine binding domains (i.e., SH2 and PTB domains) (Pawson, 2002). The complement of signaling molecules that is recruited to any given RTK dictates the cellular responses that follow receptor activation and thereby the biological activity of the pathway.

With this background, it was apparent that determining sites of receptor phosphorylation on Tie2 and the signaling molecules that associate with these

sites would yield insights into the molecular and cellular functions of Tie2. To begin to identify signaling molecules that associate with Tie2, we constructed a recombinant Tie2 kinase domain fused to glutathione-S-transferase (GST) at the N-terminus and to a heart muscle kinase phosphorylation site (RRASV) at the C-terminus (Huang *et al.*, 1995). This fusion protein, termed GTEKH, could be purified efficiently on a glutathione sepharose column and ^{32}P-labeled to high specific activity. Importantly, GTEKH was heavily tyrosine phosphorylated after purification, while a mutated, kinase-inactive GTEK remained unphosphorylated, strongly suggesting that GTEKH phosphorylation was due to autophosphorylation. Using radiolabeled, autophosphorylated GTEKH as an affinity reagent to probe an *E. coli* expression library yielded two src homology 2 (SH2) domain-containing proteins, the adaptor protein GRB2 and the protein tyrosine phosphatase SHP2. Confirming the findings in cloning experiments, endogenous GRB2 and SHP2 from endothelial lysates associated with wild-type GTEK immobilized on glutathione agarose beads. Since phosphotyrosine residues in the C-tail of other RTKs are common sites of interaction with downstream signaling molecules, three tyrosine residues in the C-terminal tail of Tie2 were mutated to phenylalanine (Y1101F, Y1107F, and Y1112F) (Figure 4). The Y1101F mutation markedly reduced the association of GRB2, while the SHP2 association remained intact. Conversely, the Y1112F mutation markedly decreased association of SHP2 but left GRB2 association intact. Similarly, synthetic phosphopeptides encompassing Y1101 and Y1112 specifically blocked association of GTEK with GRB2 and SHP2, respectively.

These findings indicated that GRB2 and SHP2 associate with autophosphorylated Tie2 at distinct tyrosine residues and strongly suggested a role for these signaling molecules in Tie2-mediated cellular responses and biological activity. Abundant evidence has shown that both GRB2 and SHP2 play important roles in

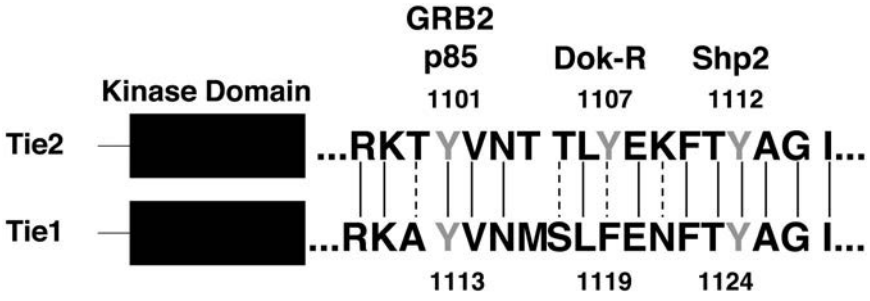

FIG. 4. Aligned peptide sequences of the C-tail tyrosine residues of Tie2 and Tie1. Some of the proteins known to associate with Tie2 are listed above their putative sites of association. Note that tyrosine 1113 of Tie1 conforms to the consensus sequence for binding p85. Note also that the Dok-R binding site on Tie2, Y1107, is not present on Tie1.

the activation of the ras–mitogen-activated protein kinase (MAPK) pathway, one known to influence multiple cellular responses. Indeed, Ang1 has been shown to transiently enhance MAPK activation in cultured endothelial cells and in rat aortic ring explants, while MAPK inhibition attenuated Ang1-mediated angiogenesis in rat aortic ring explants (Fujikawa *et al.*, 1999; Kim *et al.*, 2002; Zhu *et al.*, 2002). However, in cell culture, inhibition of MAPK activation had no effect on Ang1-mediated endothelial cell survival or migration (Fujikawa *et al.*, 1999). These findings suggest that MAPK activation downstream of Tie2 during angiogenesis may be more important for endothelial differentiation (capillary morphogenesis) than for endothelial recruitment and maintenance (Figure 5).

In addition to its positive role in MAPK activation, SHP2 may negatively regulate RTK signaling by dephosphorylating autophosphorylation sites. SHP2 phosphatase activity is enhanced by occupancy of its SH2 domains, so it is possible that recruitment of SHP2 to activated Tie2 could enhance SHP2 phosphatase activity and thereby limit phosphorylation of the activated receptor (Pluskey *et al.*, 1995). Consistent with this possibility, mutation of the SHP2 binding site (Y1112F) on Tie2 results in enhanced autophosphorylation and

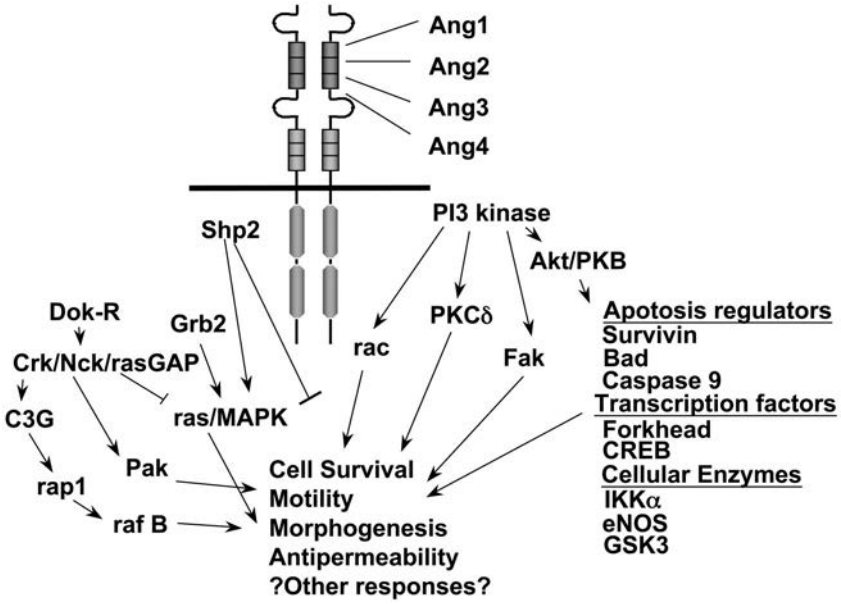

FIG. 5. Signaling pathways downstream of Tie2 activation and the cellular responses they may promote. Abbreviations: PI3, phosphatidylinositol 3; PKB, protein kinase B; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; CREB, cyclic adenosine monophosphate response binding protein; IKK, I kappa B kinase complex; eNOS, endothelial nitric oxide synthase; GSK, glycogen synthase kinase.

increased activation of downstream signaling pathways (Kontos *et al.*, 1998; Jones *et al.*, 2003). Recently, the structure of the Tie2 kinase domain has revealed that Y1112 is not solvent exposed but rather the phenyl ring occupies a hydrophobic pocket and the hydroxyl group forms hydrogen bonds with residues in the C-terminal lobe of the Tie2 kinase (Shewchuk *et al.*, 2000). This positioning of Y1112 directs the C-terminal tail of Tie2 towards the substrate-binding site, suggesting an autoinhibitory function. Supporting this model, a 16-residue C-tail truncation of Tie2 that includes Y1112 but spares Y1107 and Y1101 enhances the *in vitro* activity of the Tie2 kinase domain and yields a receptor with enhanced baseline and ligand-mediated autophosphorylation and signaling (Niu *et al.*, 2002). Taken together, these data indicate that Y1112 may participate in two levels of negative regulation: first, to maintain Tie2 in its inactive state by obscuring the kinase active site and second, to attenuate signaling by the activated Tie2 by recruiting SHP2. These findings increase our understanding of the molecular mechanisms of Tie2 activation and may provide insights into the development of therapeutic agents for the modulation of Tie2 activity.

Because the expression cloning approach used to identify SHP2 and GRB2 had limited ability to detect low-affinity interactions, we used a Tie2 kinase bait in the yeast two-hybrid system to screen a human fetal heart library. One of the clones identified in this screen encoded the C-terminal SH2 domain of the p85 subunit of phosphatidylinositol 3 kinase (PI3-K) (Kontos *et al.*, 1998). Like GRB2, mutating Y1101 markedly reduced binding of p85 to Tie2 in yeast-interaction assays and in GST pull-down assays. Because Tie2 ligands were unavailable at the time, we constructed a chimeric receptor consisting of the extracellular domain of c-fms (CSF-1 receptor) and the kinase domain of Tie2 that could be activated using recombinant CSF-1. Mirroring *in vitro* experiments, ligand stimulation of the wild-type Tie2 kinase resulted in activation of PI3-K, as measured by accumulation of membrane-bound 3-polyinositides (predominantly PI 3,4 bisphosphate). Mutation of Y1101 to phenylalanine abrogated this effect. Further confirming the functional significance of the interaction of Tie2 with p85, CSF-1 stimulation resulted in plasma membrane translocation and activation of Akt, a serine/threonine kinase known to be a downstream effector of PI3-K. Consistent with Akt being downstream of PI3-K, CSF-1-mediated Akt activation was blocked by mutation of Y1101 and by the selective PI3-K inhibitor, wortmannin.

With discovery of the Tie2 ligands, it subsequently was shown by a number of investigators that Ang1- and, at least under certain circumstances, Ang2-mediated stimulation of endogenous Tie2 in endothelial cells resulted in the activation of Akt in a PI3-K-dependent manner (Jones *et al.*, 1999; Kim *et al.*, 2000a; Papapetropoulos *et al.*, 2000). Moreover, these studies demonstrated that activation of the PI3-K/Akt pathway was required for Ang1-mediated cell

survival and chemotaxis. As mentioned previously, these responses were not inhibited by a selective MAPK inhibitor (Fujikawa *et al.*, 1999). A more-recent study has shown that the adaptor protein, Dok-R, is recruited to activated Tie2 by association of its protein tyrosine binding (PTB) domain with Y1107 (Jones *et al.*, 2003). Dok-R subsequently is phosphorylated on multiple tyrosine residues, resulting in recruitment of additional signaling molecules with putative roles in a number of cellular responses, including cell migration. Predictably, recruitment and phosphorylation of Dok-R were attenuated by mutation of Y1107, as was the chemotactic response to Ang1. Surprisingly, however, association and phosphorylation of Dok-R also were attenuated by mutation of Y1101, by mutation of the Dok-R PH domain, or by treatment of cells with a PI3-K inhibitor. These findings suggested that the signaling cross-talk between the PI3-K pathway and the Dok-R pathway is required for an optimal chemotactic response downstream of Tie2. Other recent studies have shown that Tie2-mediated activation of the PI3-K/Akt pathway is required for endothelial nitric oxide synthase (eNOS) activation, focal adhesion kinase activation, and protease secretion, all of which may contribute importantly to Tie2 function during angiogenesis (Kim *et al.*, 2000c; Babaei *et al.*, 2003). Supporting this idea, eNOS KO mice had a markedly reduced angiogenic response to Ang1 in the Matrigel plug assay (Babaei *et al.*, 2003). Based on these studies, it is likely that PI3-K activation and PI3-K-dependent cellular responses are pivotal for the function of Tie2 in the maintenance and in the angiogenic response of the adult vasculature.

Like Tie2, the closely related Tie1 receptor is required for normal embryonic vascular development. We recently have shown that activation of a c-fms/Tie1 receptor also stimulates the PI3-K/Akt survival pathway, suggesting a similar role for Tie1 in maintenance of the adult vasculature (Kontos *et al.*, 2002). Consistent with this possibility, in chimeric WT/Tie1 KO mice, Tie1 null endothelial cells contribute normally to the vasculature until E10.5 but are increasingly underrepresented after E12.5 and into adulthood (Puri *et al.*, 1995). These studies indicate that the Tie1 and Tie2 pathways have both overlapping and nonoverlapping functions. Although a ligand for Tie1 has yet to be identified, our study using a cfms/Tie1 chimeric receptor suggests that Tie1 may be activated by ligand-mediated dimerization. However, another recent study suggests that Tie1 may be activated via a heterotypic interaction with Tie2 (Marron *et al.*, 2000). Clarification of the mechanism by which Tie1 is activated will be important to fully understand the relationship and function of these two crucial endothelial signaling pathways.

VI. Therapeutic Modulation of Tie2 Activation

The studies described above implicate Tie2 signaling in vascular maintenance and in angiogenesis in adult tissues and suggest that therapeutic modula-

tion of Tie2 activation may be beneficial. However, important questions remain unanswered. Perhaps the most-pressing unanswered question is the mechanism underlying the differential effects of Ang1 and Ang2 on Tie2 activation. Recent data have demonstrated that the C-terminal fibrinogen domain is required for Tie2 binding of both ligands, suggesting that differential oligomerization of Ang1 vs. Ang2 might be responsible for the observed functional differences (Procopio *et al.*, 1999; Davis *et al.*, 2003). However, in endothelial cells (ECs), dimers of either Ang1 or Ang2 fail to activate Tie2, whereas tetramers of Ang1 activate Tie2 but tetramers of Ang2 not only fail to activate Tie2 but inhibit binding of Ang1 tetramers (Davis *et al.*, 2003). Based on these findings, it was suggested that a subtle difference in the binding of Ang1 vs, Ang2 fibrinogen domains might be responsible for their contrasting activities on ECs. However, another group has shown that the binding sites for Ang1 and Ang2 on Tie2 are grossly indistinguishable (Fiedler *et al.*, 2003). In view of these data, it may be more likely that differences between Ang1 and Ang2 are due to expression of an Ang2 coreceptor in endothelial cells. The expression of such a coreceptor, or lack thereof, might explain the different responses of lymphatic vs. blood vascular endothelium and perhaps some of the apparently disparate effects of Ang1 and ExTek on pathological angiogenesis.

Based a broad range of data, we must continue to explore the therapeutic potential of modulating the activity of the Tie2 pathway. From our studies and those of many others, it appears that therapeutic manipulation of angiogenesis will likely be an important clinical application of Tie2 biology. Most of the data suggest that activation of Tie2 plays an important role in blood vessel assembly and that inhibiting the Tie2 pathway will inhibit pathological angiogenesis. Although the therapeutic utility of soluble receptors has been shown for other ligand/receptor systems, further proof of concept of Tie2 inhibition for antiangiogenesis would be greatly facilitated by the advent of potent and selective small molecule inhibitors of the Tie2 kinase. Such inhibitors would be powerful tools to explore the role of Tie2 in quiescent adult vasculature.

In addition to antiangiogenesis, recent data suggest that enhancing Tie2 activation can enhance angiogenesis in normal tissues and improve collateral development and blood flow to ischemic tissues. For example, transgenic overexpression of Ang1 in the skin increased dermal vascularity and exogenous delivery of Ang1 alone or in combination with VEGF improved collateral development and perfusion to ischemic skeletal muscle (Shyu *et al.*, 1998; Suri *et al.*, 1998; Chae *et al.*, 2000). In addition to its pro-angiogenic effects, Tie2 activation attenuates the action of pro-inflammatory mediators and decreases vascular leak, suggesting that Tie2 activation may have other applications in vascular disease, such as plaque stabilization in atherosclerosis and limiting cerebral edema after ischemic stroke (Thurston *et al.*, 1999; Gamble *et al.*, 2000; Kim *et al.*, 2001). Indeed, recent studies have shown that Ang1 is protective in

rodent models of pulmonary hypertension and allograft atherosclerosis (Nykanen *et al.,* 2003; Zhao *et al.,* 2003). Additionally, since VEGF enhances expression of inflammatory mediators and increases vascular permeability, coadministration of Ang1 with VEGF could represent an improved approach to proangiogenesis. Continued improvement of gene transfer vectors for delivery of endogenous Tie2 ligands or continued development of recombinant Tie2 ligands that are more homogeneous and easier to produce could yield effective therapeutic agents for ischemic and other vascular disorders. Alternative strategies to enhance Tie2 activation — such as direct kinase activators or inhibition of protein tyrosine phosphatases such as SHP2 and others that negatively regulate Tie2 — may be effective future therapeutic tools.

Although a tempting therapeutic target, modulation of Tie2 signaling in the adult vasculature should be approached with caution. Data presented earlier indicate that Tie2 likely plays a role in maintenance of the adult vasculature, perhaps mediating endothelial survival. Our studies with ExTek, however, suggest that at least short-term inhibition of Tie2 signaling will be well tolerated. Improved approaches for conditional KOs of Tie2 or the development of potent and selective Tie2 inhibitors will be necessary to determine the effects of chronic blockade of the Tie2 pathway in adult vasculature. It is possible that enhanced Tie2 signaling may have adverse consequences in the adult vasculature. We and others have shown that mutations in Tie2 that cause inherited venous malformations enhance ligand-independent and ligand-dependent Tie2 kinase activity (Vikkula *et al.,* 1996; Calvert *et al.,* 1999). Despite these findings, chronic expression of Ang1 in murine skin enhanced dermal vascularity without apparent adverse effects. In addition, studies in animal models of ischemia indicate that short-term enhancement of Tie2 signaling is sufficient to improve collateral circulation without evidence of untoward effects. Taken together, these data strongly suggest that, although caution is warranted, therapeutic modulation of the Tie2 pathway to inhibit or enhance blood vessel growth and remodeling will have a favorable therapeutic index.

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Angiostatin and Anti-angiogenic Therapy in Human Disease

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ABSTRACT

Many diseases have abnormal quality and/or quantity of vascularization as a characteristic feature. Cancer cells elicit the growth of new capillaries during neovascularization in a process termed angiogenesis. In diabetics, pathologic angiogenesis in various tissues is a clinical feature of many common complications. Therefore, the diabetic cancer patient warrants special consideration and extra care in the design of anti-angiogenic treatments without adverse side effects. Some treatment regimens that look promising *in vitro*, in animal models, or in early clinical trials may be contra-indicated for diabetics. This chapter will review the common complications of diabetes, with emphasis on the angiogenic pathology. Recent research related to the mechanism of action and basis for specificity of the anti-angiogenic peptide, angiostatin, will be the focus. The aim is to shed light on areas in which more research is needed with respect to angiostatin and other anti-angiogenic agents and the microenvironmental conditions that affect their activities, in order to develop improved therapeutic strategies for diabetic cancer patients.

I. Introduction

Normal tissue function relies on an adequate supply of nutrients and oxygen from pre-existing blood vessels. It is well established that tumor growth depends on development of a new vascular supply, a process known as angiogenesis (Folkman, 1971). The discovery of factors that mediate this process has significantly increased our understanding of many normal and pathological circumstances. For example, angiogenesis is desirable in wound healing and recovery from cardiac ischemia but undesirable in the pathogenesis of psoriasis (Oates, 2002) or when metastastic tumor tissue develops a blood supply, allowing it to grow and spread (Weidner *et al.*, 1991,1993; Macchiarini *et al.*, 1992). The quest has been to isolate naturally occurring agents or develop new ones that aid in the regulation of this process. Judah Folkman initially developed the hypothesis that naturally occurring agents existed that inhibited angiogenesis. The first two, discovered in his laboratory, were angiostatin (O'Reilly *et al.*, 1994a) and endostatin (O'Reilly *et al.*, 1997).

As tumors grow in size, they become hypoxic and acidotic and elaborate several growth factors to stimulate local blood vessels to sprout branches.

Endothelial cells then begin proliferating towards the tumor and form tubular structures that become blood vessel branches. These new blood vessel branches differ from normal blood vessels. Some agents can target them while not harming intact, mature vasculature elsewhere in the body. For example, newly formed blood vessels are "leaky" or exceptionally permeable (Dvorak *et al.*, 1988). They also are tortuous, with blind ends and incomplete drainage or backflow (Secomb *et al.*, 1993; Kimura *et al.*, 1996) and therefore contain a mixture of arterial and venous blood (Kallinowski *et al.*, 1988; Vaupel, 1997; Vaupel *et al.*, 1989,1998). This makes them poor at removing catabolites, including carbon dioxide (CO_2) (Boyer and Tannock, 1992).

Angiostatin is endogenously produced in the tumor stroma. Therefore, circulating levels can control metastatic cell proliferation until a primary tumor is removed. Before the discovery of angiostatin, it was believed that when cancer recurred after surgical removal of a primary tumor, it was because the surgeon had left some tumor cells behind. It is now generally accepted that micrometastases already have seeded elsewhere in the body but were inhibited from proliferating by endogenous production of angiostatin or other inhibitors from the primary tumor. These micrometastases then came under permissive growth conditions when the primary tumor was removed. The discovery of angiostatin elicited great excitement because it seemed likely that exogenous administration after primary tumor removal could prevent metastatic growth. However, one problem is the short half-life of this peptide, leading to a need for continuous administration. Identifying receptors for these molecules was deemed a high priority, so that alternate molecules could be developed that have the same targets and are more stable, more effective, and easier to manufacture and administer.

Angiostatin is found naturally in significant amounts in the circulation of patients with primary tumors (Canfield *et al.*, 1986; Cao *et al.*, 2000; OSI Pharmaceuticals *et al.*, 2001). When a primary tumor is removed, metastases may experience lower circulating levels of endogenous inhibitors. Local blood vessels respond to the malignant cells' elaboration of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). Local blood vessels then respond by sprouting branches to feed the metastases. At this time, small micrometastases may grow beyond the 2-mm size, which is functionally dormant, and become a threat to the patient when rapid growth causes local damage. Angiostatin can maintain metastases in a dormant state in laboratory animals when administered exogenously (O'Reilly *et al.*, 1994a,b,1997). Research on the mechanism of action of angiostatin delineates some parameters affecting its activity that will require evaluation, in order to design treatments that yield the most-effective combinations of agents. More-complicated issues are whether these treatments are applicable in patients having a variety of vascular pathologies. Diabetic patients have other body compartments that feature abnormal vascularization. Understanding how these anti-angiogenic agents work and the conditions in the

various compartments will enable predictions about possible side effects, permitting further drug development. Many agents are in clinical trials (Table I), with alarmingly little known about their mechanisms of action and potential side effects.

II. Stages of Tumor Angiogenesis

Angiogenesis begins when a fibrin clot forms on the adventitial surface of an existing blood vessel (Muthukkaruppan and Auerback, 1970; van Hinsbergh *et al.*, 2001), followed by sprouting of new capillaries. The initial phase begins with increased vascular permeability and local degradation of the vessel wall (i.e., extracellular matrix (ECM) or basement membrane). The endothelial cells enter the tumor stroma, migrate toward a stimulus such as VEGF or FGF, and proliferate behind the leading edge. At this time, the cells may be most vulnerable to agents that interfere with their proliferation, since they lack protection from other cell types (Egginton *et al.*, 2000). The next step in vessel formation is recruitment of pericytes, followed by smooth muscle cells.

III. On/Off Switches for Angiogenesis: a Delicate Balance

Angiostatin is a fragment of a larger protein, plasminogen, with an activity distinct from the parent protein. It may be cleaved from the original precursor protein by enzymes under various conditions; normally, in cases where a pro-angiogenic stimulus first has promoted blood vessel development (such as during wound healing), which must then be inhibited when the desired level of vascularity has been achieved. Angiostatin has a short half-life of about 15 minutes (Gonzalez-Gronow *et al.*, 1990; DeMoraes *et al.*, 2001; Fortier *et al.*, 2001). Consistent with these results, exogenous administration of angiostatin at doses ranging from 15–300 mg/m^2/day showed rapid clearance of this protein and no side effects in a phase I clinical trial performed at Thomas Jefferson University (DeMoraes *et al.*, 2001; Fortier *et al.*, 2001). Based on animal studies, however, if administered continuously in addition to endostatin, metastasis may be discouraged from growth for prolonged lengths of time (Yokoyama *et al.*, 2000). Combination therapies with two new agents are necessarily slow to be tested in humans. Each compound has to be tested individually first in patients and, if they are only effective together, the potential efficacy may not be discernable in early trials. Recent combinations that have been studied in animal models include angiostatin with chemotherapy (te Velde *et al.*, 2002) and angiostatin with radiation (Mauceri *et al.*, 1998). Basic research is needed to understand mechanisms and predict which combinations will be most effective, so that trials can be done in the correct context, the right tumor types, and thus in patients that will benefit the most. Furthermore, deleterious side effects should

TABLE I
Antiangiogenic Agents

Angiogenesis inhibitors	Target	Trial phase	References
ABX EGF, Cl-1033, PKI-166, EGF vaccine, EKB-569, GW2016, ICR-62, EMD 55900, CP358, PD153035, AG1478	EGFR	I	Bier *et al.*, 1995; Schober *et al.*, 1995
IMC-C225 (Erbitux), ZD1839 (Iressa), OSI-774	EGFR	II/III	Robert *et al.*, 2001; Albanell *et al.*, 2002; Baselga *et al.*, 2002; Herbst *et al.*, 2002c; Ranson *et al.*, 2002
Erlotinib (tarceva)	EGFR	III	OSI Pharmaceuticals *et al.*, 2001
Angiostatin	ATP synthase on endothelial cells; intracellular pH regulation; angiomotin; annexin II	I	Moser *et al.*, 1999, 2001; Troyanovsky *et al.*, 2001; Wahl *et al.*, 2001, 2002a; Wahl and Grant, 2002
Arrestin, endostatin	$\alpha_1\beta_1$ on endothelial cells	No, I/II	Colorado *et al.*, 2000; Mundhenke *et al.*, 2001; Eder *et al.*, 2002; Herbst *et al.*, 2002a,b; Sudhakar *et al.*, 2003; Thomas *et al.*, 2003
BAY 12-9566 & w/fluorouracil or doxorubicin	Metalloproteinase inhibitor-2, -3, -9 (multiple target)	I	Heath *et al.*, 2001
Bevacizumab (avastin)	VEGF antagonist	II, III	Chen *et al.*, 2001
Carboxyamidotrizole, and w/paclitaxel	Inhibits endothelial cells calcium influx	I	Kohn *et al.*, 2001
Canstatin, EMD121974, S-24, vitaxin	$\alpha_v\beta_3$ integrin antagonist	I,I,I,I/II	Gutheil *et al.*, 2000; Eder *et al.*, 2002; Herbst *et al.*, 2002b; Thomas *et al.*, 2003
Dimethylxanthenone acetic acid	Unclear, induces TNF-α and nitric oxide I	No	Galbraith *et al.*, 2002
IM862	Activates NK cells	I	Tulpule *et al.*, 2000
Interleukin-12, interleukin-2	Induces CD8+ T-cell receptor & $\alpha\beta$+T cells	I	Brivio *et al.*, 2002
NM-3	VEGF inhibitor	I	Reimer *et al.*, 2002
HuMV833 PTK787, ZK22584	VEGF receptor antagonists	I	Jayson *et al.*, 2002; Turetschek *et al.*, 2002

RhuMab, Angiozyme (ribozyme)	VEGF receptor antagonists	II	Sandberg *et al.*, 2000; Gordon *et al.*, 2001
IMC-1C11	VEGFR-2 antagonists	I	Posey *et al.*, 2003
Neovastat, Marimastat, Prinomastat, BMS-275291, COL-3, MM1270	Matrix metalloproteinase inhibitors	I/II/III	Levitt *et al.*, 2001; Rudek *et al.*, 2001; Batist *et al.*, 2002; Shepherd *et al.*, 2002; Reber *et al.*, 2003
SU101, SU6668, SU11248	PDGFR, VEGFR, bFGF (multiple targets) endothelial cells/pericytes	I	Eckhardt *et al.*, 1999; Whatmore *et al.*, 2002; Bergers *et al.*, 2003
SU5416, with Paclitaxel, w/Gemcitabine & Cisplatin, and w/Irinotecan & Cisplatin and w/radiation	VEGFR-2 antagonists	II,III	Stopeck *et al.*, 2002
Razoxane	Topoisomerase II inhibitor	II	Braybrooke *et al.*, 2000
Squalamine lactate	Sodium/proton antiporter isoform III	I	Akhter *et al.*, 1999; Bhargava *et al.*, 2001
Tecogalan	Inhibits bFGF binding	I	Eckhardt *et al.*, 1996
Temozolomide & PEG interferon α2b	Unknown	I	Agarwala and Kirkwood, 2003
Tetrathiomolybdate	Anticopper agent	I,II	Brewer *et al.*, 2000; Redman *et al.*, 2003
TNP-470	Inhibits endothelial cell proliferation	I	Logothetis *et al.*, 2001
Thalidomide, CC-5013 (immunomodulatory derivative of thalidomide), and with taxotere	Unknown	I	Baidas *et al.*, 2000; Figg *et al.*, 2001; Short *et al.*, 2001; Daliani *et al.*, 2002; Escudier *et al.*, 2002; Gutheil and Finucane, 2002
Tumstatin	$\alpha_V\beta_3$ integrin antagonist	No	Sudhakar *et al.*, 2003
2-methoxyestradiol	Hypoxia inducible factor (HIF1α)	I	Lakhani *et al.*, 2003
VEGF trap	Decoy soluble VEGFR	I	Herbst *et al.*, 2002c

[Abbreviations: EGFR, epidermal growth factor receptor; ATP, adenosine triphosphate; VEGF, vascular endothelial growth factor; TNF, tumor necrosis factor; NK, natural killer; VEGFR, VEGF receptor; PDGFR, platelet-derived growth factor receptor; bFGF, bovine fibroblast growth factor.]

be predictable and avoidable if comparison between compartmental microenvironment and tumor microenvironment are made and the effects of these determinants on the drug activity are understood.

IV. Mechanisms of Action of Angiostatin

One major theme that has emerged from research done thus far on antiangiogenic compounds is that the extent of cell attachment to a matrix and the nature of the matrix are critical determinants of the compound's activity. This is a complex issue because tumor stroma is comprised of a variety of proteins, often in abnormal relative concentrations. Extensive literature indicates that, in addition to tumor cell modulation of stroma composition, stromal components impact tumor cells. This two-directional signaling is part of the malignant phenotype and often is regulated at the post-transcriptional level. This pioneering work, recently reviewed by Roskelley and Bissell (2002), has delineated the importance of tumor stroma in tumor cell behavior. Recent work in the field of angiogenesis has shown that many of the same microenvironmental factors are critical to the endothelial cell response to the tumor microenvironment and can determine the phenotype of these cells and the outcome of exposure to various stimuli (e.g., administration of exogenous angiostatin) (Wahl and Grant, 2002).

Angiostatin is an internal fragment of plasminogen and may contain either the first three (K1–3) or four (K1–4) kringle domains. A similar activity has been reported for kringle 5 (K-5) of plasminogen (Liu *et al.*, 2000; Gonzalez-Gronow *et al.*, 2003). Three receptors on endothelial cells have been reported for angiostatin to date. Adenosine triphosphate (ATP) synthase was the first receptor identified by Moser and colleagues (1999). The presence of this typically mitochondrial enzyme on the endothelial cell surface was somewhat surprising. Binding of angiostatin to surface-associated ATP synthase since has been confirmed by other research groups (Wahl and Grant, 2002; Arakaki *et al.*, 2003). Additional reports have confirmed the unexpected finding that many of the enzymes and components of the mitochondrial electron transport chain and ATP synthesis generating mechanisms are located on the plasma membrane of endothelial cells (Yegutikin *et al.*, 2002; Arakaki *et al.*, 2003). Two other potential target receptors of angiostatin that have been reported are angiomotin (Troyanovsky *et al.*, 2001) and integrin $\alpha_v\beta_3$ (Tarui *et al.*, 2001). The distribution of these receptors on the surface of endothelial cells may be located at sites so proximal to one another that angiostatin may be able to interact with more than one simultaneously. Alternatively, multiple targets could be implicated in binding to the different kringles of angiostatin as a function of receptor and peptide presentation.

Parameters affecting angiostatin's interaction with the ATP synthase receptor have not been completely elucidated but recent work indicates that the

synthase may be situated in a caveolar compartment (Moser *et al.*, 2001) and generates ATP on the cell surface (T.L. Moser, D.J. Cheek, J.A. Roy, T.A. Ashley, M.D. Goodman, A.E. Paradis, B. Li, D.J. Kenan, and S.V. Pizzo, unpublished results). It has been reported that one of angiostatin's receptors is proximal to focal adhesion kinase (FAK) and that binding catalyzes FAK phosphorylation in the absence of integrin clustering that usually is triggered by cell attachment to substrate (Claesson-Welsh *et al.*, 1998). This implies that FAK phosphorylation occurs under aberrant circumstances (i.e., when endothelial cells are not tightly attached to their normal substratum). In the usual scenario, attachment to a substrate such as fibronectin causes integrins to form tetramers. FAK is phosphorylated and organizes, allowing cytoskeletal elements to form structurally organized arrays to carry out cell spreading (Figures 1 and 2). In the tumor microenvironment, several parameters differ. Matrix composition includes collagen, laminin, and many other proteins (Canfield *et al.*, 1986; Baatout and Cheta, 1996; Grant and Kleinman, 1997; Aoudjit and Vuori, 2001). The pH and the oxygen levels are low and the degree of attachment is compromised because cells are rapidly dividing and moving into areas requiring a blood supply. This means that different integrin isoforms may be activated to differing extents

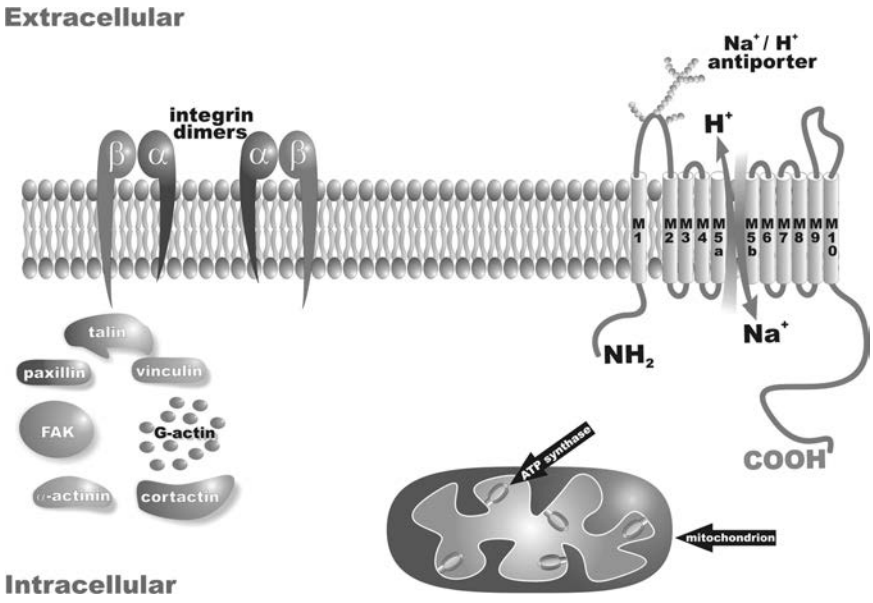

FIG. 1. Endothelial cell membrane in an unattached cell at normal pH ($\approx$ 7.3). The integrin subunit are in $\alpha\beta$ dimers, the sodium proton antiporter (NHE) is minimally active, focal adhesion kinase (FAK) is unphosphorylated, cytoskeletal elements are unassembled, and adenosine triphosphate (ATP) synthase is active primarily within the mitochondria.

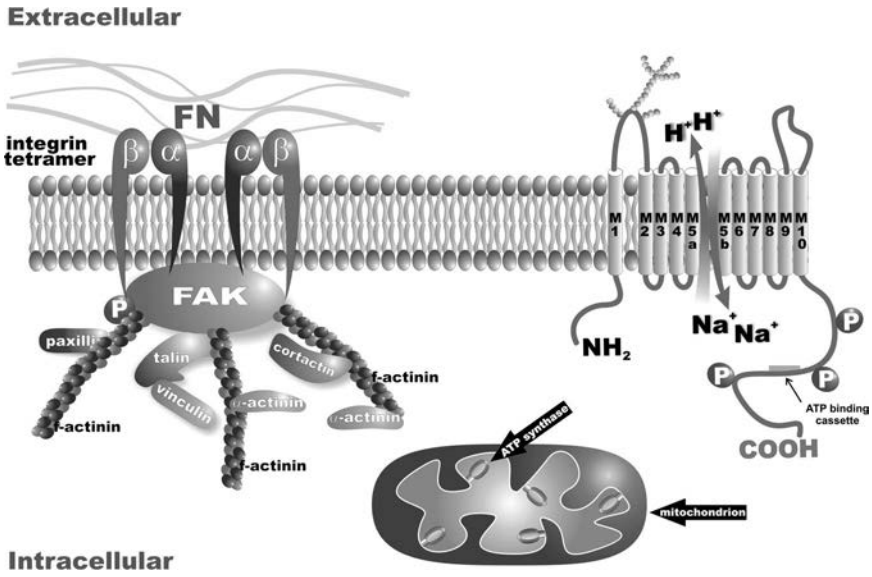

FIG. 2. Endothelial cell attached to fibronectin at normal pH (≈ 7.3). Integrins are clustered into tetramers in response to attachment to fibronectin, FAK is phosphorylated, NHE is more active, cytoskeletal elements are assembled to promote cell attachment and spreading, and ATP synthase remains in mitochondria.

(Schwartz *et al.*, 1991b; Agius *et al.*, 1996; Coopman *et al.*, 1996; Erdreich-Epstein *et al.*, 2000). Thus, the distribution of other molecules on the cell surface and their activities will be affected. When extracellular pH is low, typically the sodium/proton antiporter, isoform 1 (NHE-1), is activated to maintain intracellular pH in the viable range. This occurs as a function of attachment, phosphorylation, ATP binding, and the presence of growth factors in fibroblasts, where it has been studied in the most detail (Schwartz *et al.*, 1991a; McSwine *et al.*, 1994). It has been reported that migrating cells have NHE-1 clustering at the leading edge of lamellopodia in work done using fibroblasts (Grinstein *et al.*, 1993) and melanoma cells (Akasaka *et al.*, 1995) and that they co-localize in partially attached and moving cells with FAK (Schwartz *et al.*, 1991a).

We recently demonstrated that when coupled with extracellular acidification, angiostatin caused a precipitous decline in cytosolic intracellular pH (Wahl *et al.*, 2001,2002a; Wahl and Grant, 2002). Low extracellular pH could affect receptor levels, receptor distribution, angiostatin binding, and angiostatin conformation. ATP synthase distribution on the endothelial cell surface was reported to be more focal at low pH (Wahl and Grant, 2002). Receptor binding interactions with angiostatin as a function of extracellular pH currently are being studied in our laboratories. At normal extracellular pH, both ATP synthase and NHE-1 would

be less active, whether or not cells are attached. However, in the tumor microenvironment (Figures 3 and 4), there is less attachment to a variety of substrate proteins, a lack of integrin clustering, and aberrant FAK phosphorylation. Because of the low extracellular tumor pH, NHE-1 is activated and ATP synthase is activated and organized focally (Wahl and Grant, 2002). When angiostatin enters this scenario, it could bind to ATP synthase, which could, in turn, disrupt FAK, ATP synthesis, and the function of NHE-1. A model for how angiostatin binding affects endothelial cells in a tumor microenvironment — based on recent research, the literature, and conjecture — is shown in Figure 4.

V. Other Anti-angiogenic Agents

Other anti-angiogenic agents have been discovered or developed. In some cases, the receptors have been identified (Table I). However, little is known about how their signals are transduced, the basis for their selectivity, or their degree of

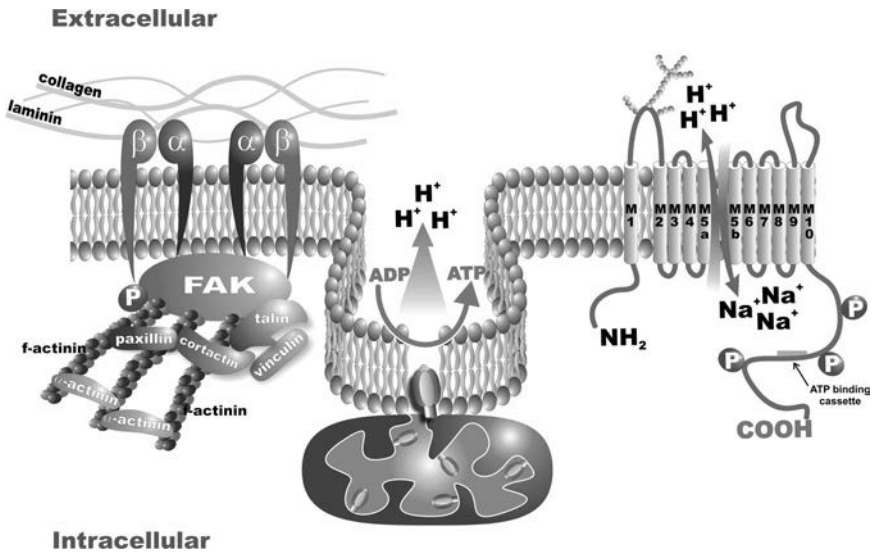

FIG. 3. Endothelial cell in a tumor microenvironment with a low and variable pH. Cells are partially attached to various matrix proteins, including collagen and laminin, different integrin isoforms cluster in tetramers, and ATP synthase appears in caveolae situated near FAK. This ATP synthase now generates ATP on the plasma membrane. FAK is phosphorylated. Extracellular pH is lower (6.7 on average) and migration of endothelial cells is required; therefore, NHE is very active, highly phosphorylated, and has ATP bound on the cytoplasmic tail. NHE molecules congregate at the leading edge of lamellopodia to generate a sodium gradient to bring about cell movement. Signals are transduced to cytoskeleton, which catalyzes a rearrangement of cytoskeletal elements. Cells then proliferate and can differentiate into tubular structures.

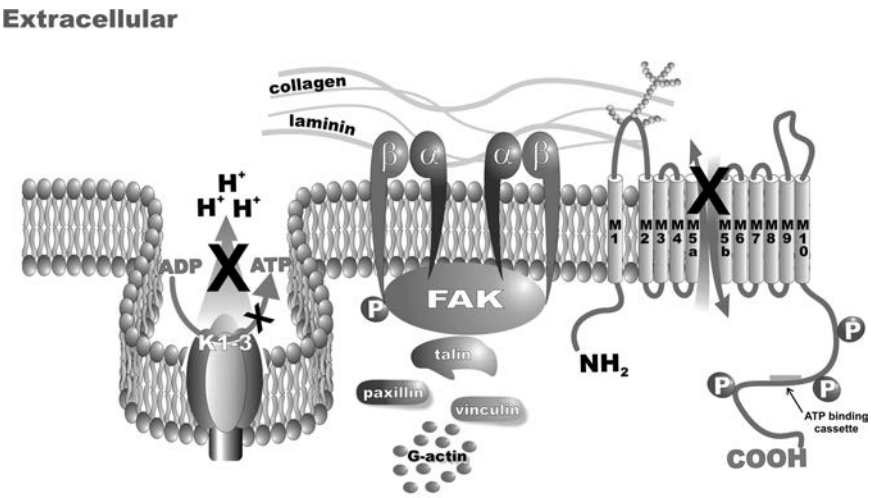

FIG. 4. Proliferating endothelial cells in the tumor microenvironment in the presence of angiostatin. Angiostatin binds to the catalytic subunit of ATP synthase. ATP synthesis ceases and inhibits the synthesis of extracellular ATP. Rapid proliferation loosens attachment of the cells, integrin tetramers dissociate into dimers, and NHE molecules disaggregate. FAK is phosphorylated (as in Figure 2) but under conditions where attachment is not being maintained. NHE is inhibited by lack of attachment, dephosphorylations, and loss of ATP. Intracellular pH decreases below 6.5 and metabolism ceases. The cell has received conflicting signals, which trigger a signal transduction cascade ending in apoptosis.

selectivity. Features of the tumor microenvironment that have been shown to be critical for demonstrating angiostatin activity are delineated below, emphasizing parameters that should be evaluated for other anti-angiogenic agents that may be useful to determine in which clinical scenario(s) each is likely to be most effective.

Some characteristic features of the tumor microenvironment may be shared by other improperly vascularized areas. This means that anti-angiogenic therapies could cause complications for cancer patients with diabetes mellitus. Drugs for treating tumors may have undesirable side effects in other tissues in diabetics to a greater extent than in nondiabetics. Some of these features have been described but have not been characterized fully in terms of application of anti-angiogenic agents. Differences between vasculature arising in different locations, in endothelial cells from the various tumor types, and in different ECM microenvironments are also areas where further basic research is warranted.

Some major parameters that need to be examined for each anti-angiogenic agent under consideration for clinical use are described in Section VI.

VI. Features of the Tumor Microenvironment: Potential Mediators of the Response of Tumor Stromal Endothelial Cells to Anti-angiogenic Drugs

A. VEGF & FGF

VEGF and FGF are two growth factors secreted by metastatic or primary tumor cells to encourage new vessel branch development. These growth factors are used as markers for vessel growth. In the first phase I clinical trial of angiostatin at Thomas Jefferson University, the protocol was designed to administer daily doses of intravenous (IV) angiostatin for 2-week intervals, separated by 1-week interruptions. Both VEGF and FGF rebounded rapidly in the circulation just 1 day after daily dosing stopped after each dosing interval (DeMoraes *et al.*, 2001). This made it evident that daily dosing without interruption would be necessary to maintain the effects of angiostatin. It indicated that agents with a slower clearance time would probably be more practical clinically. Table I lists some anti-angiogenic agents currently in clinical trial. If these are expressed in excess and/or at inappropriate times, hypervascularization could occur.

B. ENDOGENOUS INHIBITORS

Other endogenous inhibitors of angiogenesis have been reported, including angiogenin (Fett *et al.*, 1985), endostatin (O'Reilly *et al.*, 1997), thrombospondin-1 (Nicosia and Tuszynski, 1994), interferons (Dinney *et al.*, 1998), platelet factor-4 (Nicosia *et al.*, 1994), and 16 kDa prolactin (Hanahan and Folkman, 1996). Numerous other inhibitors, both naturally occurring and synthetic, are listed in Table I. Their relative abundance can play a role in the overall picture in various body compartments. Angiostatin has proven to be one of the most potent. *In vitro*, angiostatin is relatively specific for tumor endothelial cells and inhibits their proliferation (Moser *et al.*, 1999), migration (Moser *et al.*, 1999), tube formation (Moser *et al.*, 1999; Wahl *et al.*, 2002a), vessel network formation in the embryonic body model (Eriksson *et al.*, 2003), and formation of sprouts from mouse aortic rings (Hajitou *et al.*, 2002). Exceptions to this specificity include a published study on smooth muscle cells (Walter and Sane, 1999) and one on neutrophils (Benelli *et al.*, 2002). *In vivo*, however, angiostatin inhibits primary tumor metastasis in mice (O'Reilly *et al.*, 1994b), vascular proliferation in the chick chorioallantoic membrane (CAM) assay (Gately *et al.*, 1996), and neovascularization in the corneal pocket assay (White *et al.*, 2003).

Angiostatin is found in normal human plasma at a concentration of $\approx$ 6–12 nM (Soff *et al.*, 1999). The concentration of angiostatin in the urine of cancer

patients is significantly higher than in other patients (Cao *et al.*, 2000). Elevated levels of angiostatin have been observed in the ascites fluid from ovarian cancer patients as well (Yokoyama *et al.*, 2000). More recently, angiostatin was found to be elevated during impaired production of nitric oxide in coronary angiogenesis (Matsunaga *et al.*, 2002) and in the bronchoalveolar lavage fluids from patients with acute respiratory distress syndrome (ARDS) (Lucas *et al.*, 1998). These findings may indicate that angiostatin functions not only as a tumor-specific inhibitor but also as a regulator of angiogenesis in other scenarios.

C. PERICYTES & SMOOTH MUSCLE CELLS

Pericytes and smooth muscle cells are recruited to surround the endothelial cell tubes in developing blood vessels, so reduced numbers of them could affect the levels of various stimulators and inhibitors to which endothelial cells are exposed. Platelet-derived growth factor (PDGF) plays a major role in pericyte recruitment. When vessels initially form, their vulnerability may be maximal in part because these other cell types are not there to provide a mechanical barrier. A recent study indicates that retinal capillary coverage by pericytes was crucial for survival of endothelial cells in retina subjected to hypoxic conditions, particularly under the stress conditions of diabetes (Hammes *et al.*, 2002). Pericytes also secrete similar growth factors and thus modulate the ECM (Allt and Lawrenson, 2001). Smooth muscle cell proliferation also is inhibited by angiostatin (Walter and Sane, 1999).

D. INTRACELLULAR & EXTRACELLULAR pH

Both intracellular and extracellular pH affect angiostatin activity. The average extracellular pH (5.6–7.6) in tumors is lower and more variable than in normal tissue (7.2–7.6), yet tumors have a normal average intracellular pH (Yamagata and Tannock, 1996). It has been reported that angiostatin profoundly affects intracellular pH in endothelial cells (Wahl and Grant, 2002; Wahl *et al.*, 2002a). This effect is manifested only at low extracellular pH. This implicates pH-regulating transporters that are activated at low extracellular pH as a potential class of targets for anti-angiogenic agents in areas of tumors where extracellular pH is low. These potentially include NHE and the H^+-linked monocarboxylate transporter (MCT).

E. ATP SYNTHASE RECEPTOR LEVELS

Modulation of angiostatin's receptor levels may be a determinant of angiostatin activity. Research has shown that ATP synthase distribution can be altered as a function of ECM composition and extracellular pH. When cells are allowed to attach to fibronectin, histochemical assays using a primary antibody

directed against the β subunit of ATP synthase showed no detectable enzyme on the cell surface. However, when cells were plated on Matrigel to simulate the tumor stroma, the enzyme was revealed on the cell surface (Wahl and Grant, 2002). When cells at normal and low extracellular pH were compared, the distribution at low pH was more punctate. This was likely related to the organization of focal adhesion plaques and how they assemble for migration (Wahl and Grant, 2002). Other microenvironmental parameters have not been evaluated in terms of this receptor and these factors have not been evaluated for other anti-angiogenic agents.

F. HYPOXIA

Hypoxia often — but not always — goes hand in hand with acidity, although there is some disparity in spatial distribution (Vaupel *et al.*, 1989,1998; Brizel *et al.*, 1996; Gullege and Dewhirst, 1996; Helmlinger *et al.*, 1997). These two features have not been teased apart experimentally to determine the relative importance of each and the degree of overlap. Most studies thus far are either conducted at low pH or done under hypoxic conditions but not both. Future studies with new anti-angiogenic agents, such as 2-methoxyestradiol (2ME2) or Panzem ® (Entremed, Inc.), a hypoxia-inducible factor (HIF)-1 α inhibitor (Mabjeesh *et al.*, 2003), will help determine the extent to which targeting this receptor will be useful in a variety of tumor types. There is evidence that hypoxia is a feature of diabetic complications such as renal disease (Ries *et al.*, 2003), poor circulation in lower extremities (Fife *et al.*, 2002), and retinopathy (Drasdo *et al.*, 2002).

G. BICARBONATE STATUS

Bicarbonate (HCO_3^-) is a parameter that has not been considered in most *in vitro* work but should receive more attention. In the tumor microenvironment, catabolite removal by inefficient and abnormal blood vessels is poor, resulting in CO_2 buildup (Newell *et al.*, 1993; Helminger *et al.*, 2002). Both these aspects contribute to the low pH environment. In some work at low pH, the pH is lowered by altering sodium bicarbonate concentration (Chu and Dewey, 1988; Chu *et al.*, 1990; Wahl *et al.*, 1996; Owen *et al.*, 1997). In this type of experiment, the buffering capacity of the medium will be low, causing more potential extracellular pH fluctuation. Furthermore, this approach has two experimental variables, pH and bicarbonate, without distinction as to which is causing the effects on cells in the tumor microenvironment. Research studies have been performed where bicarbonate is maintained at 26 mM and the incubator CO_2 is raised to 17% from 5%, to create acidic and tumor-like conditions (Wahl and Grant, 2002; Wahl *et al.*, 2002a,b). This is an appealing model, since physiologic bicarbonate is 26 mM and the tumor is high in CO_2, which is a likely cause of elevated carbonic

anhydrase levels in tumors (Beasley *et al.*, 2001; Giatromanolaki *et al.*, 2001; Koukourakis *et al.*, 2001; Olive *et al.*, 2001; Bui *et al.*, 2003; Swinson *et al.*, 2003). It has been proposed that carbonic anhydrase inhibitors could relax pericytes, cause vasodilation, and enhance blood supply to the retina by increasing intracellular pH and decreasing extracellular pH (Reber *et al.*, 2003). It would be even more physiologic to study tumor cell behavior in a hypoxic chamber inside a 17% CO_2 incubator, to further mimic the part of the tumor microenvironment most likely to promote new blood vessel branch growth. Evaluation of low extracellular pH, low oxygen, and the combination in terms of therapeutic responses to agents and phenotype of cells in such environments should help characterize responses in the tumor microenvironment more completely.

H. LACTIC ACIDOSIS

Another reason, other than high CO_2 and poor perfusion, for the acidic microenvironment in tumors is excessive production of lactic acid (Vaupel *et al.*, 1989; Walenta *et al.*, 1997; Brizel *et al.*, 2001; Wahl *et al.*, 2002b). One indicator of the contribution of lactic acidosis is measuring levels of the transporter used to remove it from the cell. This transporter, MCT, is elevated in human melanoma (Wahl *et al.*, 2002b) and may be elevated in other tumors of neural crest origin, including brain tumors. MCT levels are elevated in the retina of diabetics, indicative of elevated lactic acid in that compartment (Knott *et al.*, 1999). Intracellular and extracellular pH levels, functionality of other transporters used to regulate intracellular pH, and degree to which MCT is elevated may be critical to understanding the degree to which a pH regulation inhibitor can affect a certain cell type and to which angiostatin can affect intracellular pH in endothelial cells in the low pH tumor microenvironment. Intracellular and extracellular pH measurements in normal and pathologic body compartments aside from the tumor microenvironment will help address the relative importance of these parameters. In addition, a relationship between areas of hypoxia and acidity is likely, although the relative importance of each has not been addressed in any one study.

I. GLUCOSE, HYPERGLYCEMIA & ACIDIFICATION

Oral glucose is used clinically to lower tumor pH before thermoradiotherapy (Thistlethwaite *et al.*, 1987; Leeper *et al.*, 1994,1998; Engin *et al.*, 1995). The mechanism is via increased glycolysis in the tumor microenvironment, producing lactic acid (Burd *et al.*, 2001). In diabetes, glucose overload may occur, so the patient's tissues already may be more acidic. This could lead to potentiation of angiostatin's activity in the body, since its activity is greatest when pH is low (Wahl and Grant, 2002; Wahl *et al.*, 2002a). Other enzymes relating to glycolysis may impact this axis, including lactate dehydrogenase (Koslowski *et al.*, 2002),

glut-1 receptor levels (Thews *et al.*, 2003), and hexokinase levels (Wachsberger *et al.*, 2002). In the diabetic, it is believed that complications arise to a greater extent in patients with the most poorly managed glucose levels (DCCT Research Group, 1993), although this is still a matter of considerable debate (Craighead, 1994). Individual variations in glycation (non-enzymatic glycosylation) of proteins are not only affected by degree of management but also by other unknown parameters. Facchiano and coworkers (2002) have reported that FGF (isoform 2) is more glycosylated in diabetic mice and is less chemotactic and pro-angiogenic as a result.

J. MATRIX

Matrix composition could be used to distinguish physiologic compartments, if qualitative and/or quantitative differences can be characterized in nondiabetics and diabetics with and without cancer. With respect to angiostatin, research indicates that the vulnerability of endothelial cells in the tumor microenvironment stems in part from the fact that when they are rapidly proliferating, their attachments are compromised. Furthermore, substrates they come in contact with in the tumor microenvironment differ, leading to different integrin isoform expression, with subsequent differences in signal transduction. This would contribute to the explanation of why mature vessels appear to be unaffected by angiostatin. It also would explain the targeting of endostatin to integrins (Sudhakar *et al.*, 2003), which then would be less apt to be engaged in attachment. Migration of tumor cells is often along the ECM of the basal lamina. When mice are injected with a melanoma cell line selected for metastatic seeding in the lung, the cells migrate specifically and secondary lung tumors are formed. When they are inhibited from binding fibronectin or laminin, over 90% of the cells fail to reach the lungs (Humphries *et al.*, 1986). In order to enter a blood vessel, tumor cells lyse the collagenous matrix by secreting various plasminogen activators. Antibodies directed against plasminogen activators have been shown to inhibit metastasis (Ossowski and Reich, 1983). The urinary-type plasminogen activation complex (u-PA) is one of the major regulators of ECM remodeling. u-PA converts plasminogen to plasmin, which degrades matrix and indirectly activates other metalloproteinases (Vassalli *et al.*, 1991). Enriched levels of u-PA and its receptor (u-PAR) are found on the leading edge of migrating cells. Plasminogen activator inhibitor type 1 (PAI-1), a u-PA antagonist, mediates cell adhesion and spreading by forming a bridge between the cell surface and the matrix directly regulating adhesion (Planus *et al.*, 1997). u-PA expression has been correlated with angiogenesis and poor prognosis (Kaneko *et al.*, 2003).

Matrix metalloproteinases (MMP) are enzymes that digest/degrade matrix proteins, enabling metastasizing cells to migrate. Matrix metalloproteinase inhibitors (MMPI) exist in a balance and can be offset in pathologic conditions

(Spranger *et al.*, 2000). Another factor warranting further study is the composition of matrix in various locations, which will affect the degree of enzyme activity needed to impact upon the matrix. A third consideration is that enzymatic alteration of matrix composition may affect where tumor cells disseminate and may cause digestion of other pathologic matrices in nearby or distant areas. This issue may require consideration in glomerulosclerosis and other complications of diabetes in which basement membrane thickening is a feature. Finally, MMP/MMPI balance may affect wound healing, an important consideration for patients that may need surgery during their course of treatment (Lockhart *et al.*, 2003).

K. TUMOR VESSEL ARCHITECTURE

Tumor blood vessel branches are inefficient and tortuous. In tumor vessels, backflow often occurs, mixing arterial and venous blood, and vessels leak (Secomb *et al.*, 1993; McDonald and Baluk, 2002). Some of these characteristics are potentially exploitable by anti-angiogenic agents and antivascular agents, again, hopefully, with some specificity for the uniquely abnormal features of these vessels. More studies are needed to characterize vasculature in different tumor types, as a function of different body locations of metastasis, and the degree of resemblance to other vasculature in normal and pathologic instances that can coexist in patients.

VII. Models Used to Study Angiogenesis in Various Tissues

A. *IN VITRO* MODELS

Many cell types are used to study angiogenesis from macrovascular vessels, microvascular vessels, various locations in the body, and from various animal species. Although little information is available about the differences between these various cell types, these differences are extremely important if research findings are to be translated into practical, predictive information. Models include the tube differentiation assay (Kubota *et al.*, 1988; Grant *et al.*, 1990,1995; Grant and Kleinman, 1997), the migration/scratch assay, and the rat aortic ring assay (Nicosia and Ottinetti, 1990; Grant and Kleinman, 1997). For angiostatin, data have been confirmed in all three of these models (Wahl *et al.*, 2002a). However, for other agents, one system may demonstrate activity more than another, which will lead to elucidation of what models may be most useful for which agents or diseases. Physiologic models are critical to getting meaningful results.

B. ANIMAL MODELS

Several animal models are available to study angiogenesis, including the corneal pocket (Muthukkaruppan and Auerback, 1970; O'Reilly *et al.*, 1994b; Sood *et al.*, 1999), the chick embryo (Risau and Lemmon, 1988; O'Reilly *et al.*, 1994b; Eriksson *et al.*, 2003), implantation of human cells into a mouse or a rat (xenografts) in various locations (Danielsen and Rofstad, 2000; Shan *et al.*, 2003), and the angioreactor model (Guedez *et al.*, 2003). Which are best to model which tumor types and clinical scenarios? Considerations include the fact that blood vessels in a human xenograft usually are a combination of human and animal vessels. Tumors often are placed in animals in locations other than the ones where they naturally occur. Model evaluation is critical to determining which one may be most physiologically meaningful. A knowledge of the pathogenesis of the particular type of cancer, the degree of vascularity it possesses, and the microenvironment of the location into which it will be grafted are all critical considerations.

C. DESIGN OF CLINICAL TRIALS: TIMING IS EVERYTHING

In the angiostatin phase I clinical trial at Thomas Jefferson University, wound healing was normal in a patient needing acute unexpected surgery while on angiostatin (DeMoraes *et al.*, 2001). Other anti-angiogenic agents may differ, however, so this must be considered, preferably prior to introduction of agents in the clinic. Another consideration in trials is that the typical measure of efficacy — tumor growth delay assessments — may not be perfectly applicable to anti-angiogenic drugs. Some are initially slow to show responses (D'Amato *et al.*, 1994). Others may cause necrosis followed by swelling, which is mostly inflammatory cells and fluid. Thus, even though a large part of the tumor mass is killed, the initial effect is an apparent increase in tumor size (Kaban *et al.*, 1999). Most traditional therapies are directed at achieving maximum results during a brief window of opportunity, while most anti-angiogenic therapies will need to be administered chronically and indefinitely to prevent blood vessel branches from sprouting (Kaban *et al.*, 1999). Therefore, animal model experiments will have to be designed to take these differences into account.

The parameters described above are emerging as important for cancer pathogenesis and treatment outcome, yet most are not considered during experimental work on anti-angiogenic drugs. Conversely, when pro-angiogenic drugs are administered for other conditions, patients with neoplastic disease may have counter-indications. Some of these drugs are listed in Table II.

VIII. Diabetes

Some sequelae of diabetes mellitus include impaired wound healing, characterized by both spontaneous and wound-induced ulcerations, particularly of the

TABLE II
Pro-angiogenic Agents

Drug	Target	Approved or potential use	References
Regranex (becaplurmin)	Unknown	Diabetic foot ulcers (Ortho-McNeil Pharmaceuticals)	Fruhstorfer, 2000
SIKVAV	Unknown	Cardiovascular ischemia prevention	Grant and Zukowska, 2000
Angiopoietin I	Tie-2 on endothelial cells	Cardiovascular ischemia prevention	Cascone *et al.*, 2003; Fiedler *et al.*, 2003
Angiopoietin II	Tie-2 on endothelial cells	Cardiovascular ischemia prevention	Fiedler *et al.*, 2003; Zhang *et al.*, 2003
Neuropeptide Y	NPY2	Wound healing	Grant and Zukowska, 2000; Ekstrand *et al.*, 2003; Lee *et al.*, 2003

lower limbs, and retinopathy. Both these conditions are consequences of insufficient perfusion. If limbs become acidotic and hypoxic, why don't they invoke the growth of new and normal blood vessels? Do they produce compounds that antagonize angiogenesis (e.g., angiostatin, endostatin) in higher amounts than those that promote it (e.g., VEGF, FGF) initially, then not turn off those signals at the right time? If so, exogenous administration of either type of compound could make things worse. With retinopathy, hypervascularization occurs at later stages, where there could be a positive impact from anti-angiogenic therapy. All potential side effects should be evaluated in diabetics, whether or not they have complications, since the eventual complication rate is high. Those that are known to have a vascular component are discussed in the following sections.

A. RETINOPATHY

Retinopathy, followed by macular degeneration, develops in 50–98% of diabetics within 15 years of diagnosis and is the most-devastating complication. It is characterized by basement membrane thickening, pericyte degeneration, microaneurysm formation, and focal capillary closure and acellularity, with subsequent abnormal proliferation of endothelial cells. Although laser treatment can prevent blindness in most cases, vitreous cavity bleeding and retinal detachment are problematic and preventative therapies would be more desirable (Danis *et al.*, 2001).

Among those issues being investigated are the effects of chronic intracellular hyperglycemia and abnormal glycation (Kaban *et al.*, 1999; Noma *et al.*, 2002; Verstappen *et al.*, 2003), disturbances in the polyol pathway (Funatsu *et al.*, 2003), and protein glycation (Funatsu *et al.*, 2002). All sugars, including D-glucose and D-galactose, can initiate protein glycation. They produce stable adducts that accumulate on long-lived biological molecules such as hemoglobin and proteins of the extracellular matrix (e.g., collagen). The rate of this reaction increases with glucose concentration. When basement membranes were glycated heavily *in vitro*, increased proliferation of endothelial cells and decreased proliferation of pericytes was reported (Kalfa *et al.*, 1995; Beltramo *et al.*, 2003). Synergistic effects of diabetes and hypertension have been reported in an animal model and increased number of caveolae in endothelial cells and pericytes, basement membrane thickening, and decreased cell-cell contact (Hillman *et al.*, 2001). As the most metabolically active tissue in the body, the retina has a high demand for oxygen. In diabetes, capillaries supplying the retina may become clogged, resulting in oxygen deprivation (Barinaga, 1995). The retina then produces VEGF but the vessels don't form in a proper or orderly manner. A similar process happens in premature infants. If this abnormal development could be stopped or re-directed, the blindness that often ensues could be preventable. One successful approach to therapy for retinopathy is photocoagulation. After this therapy was administered to diabetic retinas, endogenous angiostatin release was detected, which likely contributes to the success of the treatment (Spranger *et al.*, 2000). Some studies have linked high production of VEGF and low production of endostatin with severity of diabetic retinopathy (Noma *et al.*, 2002), diminished benefits from surgical intervention to treat cataracts (Funatsu *et al.*, 2003), and increased risk of macular edema (Funatsu *et al.*, 2002). Inflammatory mechanisms may influence retinal neovascularisation. Induction of cyclooxygenase-2 inhibitors has been shown to inhibit it by antagonizing prostaglandin E2 (Sennlaub *et al.*, 2003).

B. ULCERATIONS AND DRY GANGRENE

Ulceration and dry gangrene occur in diabetes due to insufficiency of circulation to the lower limbs. What is the mechanism by which this hypovascularity develops? Is there a lack of pro-angiogenic cues or responses to them or counterbalancing anti-angiogenic factors? Recently, Regranex (Ortho MacNeil Pharmaceuticals, Raritan, NJ) was approved for use in combating circulation impairment in the lower extremities. It is important to keep in mind that if a person had an undiagnosed cancer at the time of treatment, his/her malignant condition could be made worse as a result of treatment.

C. OTHER COMPLICATIONS

Other complications in which the role of angiogenesis is unknown include glomerulosclerosis, necrotizing papillitis, focal demyelination, and peripheral neuropathy. Peripheral neuropathy is also a side effect of some chemotherapeutic drugs (Verstappen *et al.*, 2003); thus, alternatives to these should be prescribed for diabetic cancer patients. Neuropathy may be related to the polyol pathway, in which aldose reductase and sorbitol dehydrogenase are the two key enzymes that handle excess glucose by converting it to sorbitol (Oates, 2002). Sorbitol toxicity has been implicated in neuron, kidney, and retinal damage. It may act by creating an osmotic gradient leading to swelling or have direct toxicities (Craighead, 1994). Diabetics have impaired wound healing, increased rejection of transplanted organs, and impaired formation of coronary collaterals, all of which have the lack of angiogenesis in common.

IX. Understanding the Basis for Selectivity Is the Key to Prudent Trial Design

Anti-angiogenic therapies for cancer patients are most desirable if they don't inhibit wound healing. Conversely, development of pro-angiogenic drugs for diabetics that work well in the retina and wounds but don't stimulate tumor growth is needed. Combination therapies that aren't antagonistic can be developed only if there is detailed analysis of the microenvironment and cell types in the different physiologic and pathologic compartments of the patient, so the desirable selectivity can be achieved and undesirable side effects can be avoided. The key is selectivity as a function of cell type, microenvironment, and for the various pathologies in which abnormal vascularity is a feature.

Much remains to be learned about the mechanisms for selectivity of anti-angiogenic agents in the tumor microenvironment and other locations. There is evidence that the tumor microenvironment is acidotic, hypoxic, and pro-angiogenic, so it could follow that pH or oxygen elevation could enhance effects in desired locations for pro-angiogenesis. Could impairment of intracellular pH regulation be causative in the etiology of the reduced perfusion in the limbs of diabetics? If it is compromised, how? There may be abnormalities in pH regulation in the diabetic retina or the ulcerative wound. If so, could counteractive drugs be developed that stimulate ion transport and encourage angiogenesis? Another aspect of drug potency is pH-dependent activity. Many features must be taken into account to understand the interactions that will lead to a positive, negative, or no effect for each agent.

X. Conclusions

In summary, concepts are emerging from research reported on the mechanism of action and basis for specificity of angiostatin. The data indicate that the

many new anti-angiogenic agents that have been developed and tested need to be characterized in terms of microenvironmental influences that affect their activity and other undesirable actions in other types of tissue, in patients with and without secondary pathologies. This chapter delineates some of activity parameters for this class of agents that are needed to enable effective translation to the clinic, with predictive information regarding harmful side effects, so that patients at risk can be handled accordingly.

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Balancing Contractility and Energy Production: The Role of Myocyte Enhancer Factor 2 (MEF2) in Cardiac Hypertrophy

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ABSTRACT

Cardiac hypertrophy — that is, enlargement of the heart resulting from increased myocyte size — is observed with many forms of human heart disease. It may arise secondary to an insult, such as infarct or chronic hypertension, or may occur as a consequence of a genetic defect, such as in hypertrophic cardiomyopathy. Traditionally, it has been widely believed that hypertrophy occurred as an adaptive response to normalize increased wall stress due to disease. Recently, however, it has been observed that while hypertrophy initially appears to improve the function of the heart following insult, over time, it frequently leads to a decompensated state, characterized by fibrosis and chamber dilation, resulting in overt heart failure. Hypertrophy also occurs during fetal development, immediately after birth, and in trained athletes; however, it does not lead to decompensation in these states. Experiments over the last 15 years have implicated similar signaling pathways in both pathological and physiological hypertrophic responses. Recently, important differences have been demonstrated that might hold the key to the development of effective new treatments for human diseases. This chapter focuses on how these hypertrophic responses differ from one another phenotypically and discusses how inefficient or impaired energy metabolism in the heart may contribute to the development of pathological responses. We also discuss recent evidence that the myocyte enhancer factor 2 (MEF2) transcription factor family, which previously has been shown to be important in cardiac development and hypertrophy, may have a role in regulation of cardiac energy metabolism.

I. Cardiac Hypertrophy

The term "cardiac hypertrophy" refers to enlargement of the heart due primarily to an increase in the size of individual cardiomyocytes. The term often is used to refer to a pathological response to an experimental insult or to the introduction of a transgene. However, it is important to differentiate pathological hypertrophy from physiological hypertrophy, in which cardiac enlargement occurs without significant clinical consequences. It is also important to differentiate between early stages of pathological hypertrophy, during which changes in cardiac structure compensate for increased stress on the heart, and later stages, when the heart becomes decompensated, resulting in cardiac failure and increased morbidity and mortality.

A. PHYSIOLOGICAL HYPERTROPHY

After birth, the heart continues to grow for a limited period to adapt to increased workload. In particular, the left side of the heart undergoes hypertrophy, while the right side actually gets slightly smaller due to changes in blood pressure throughout the heart that occur soon after birth. The first few breaths after birth fill the lungs with air, decreasing afterload on the right side of the heart and increasing pulmonary vascular blood flow. This increases blood flow to the left side of the heart, increasing preload. Mean arterial blood pressure rises throughout the newborn's body and increases the mechanical afterload on the heart. It is the increased workload on the heart, specifically on the left side, that leads to increased heart size. The changes in cardiac size after birth result from both hypertrophy and hyperplasia of cardiomyocytes but as cardiomyocytes terminally differentiate during the first few weeks of life, further increases in heart size throughout life are due primarily to hypertrophy (Oparil *et al.*, 1984).

Cardiac hypertrophy also occurs following (typically long-term) exercise training. The heart increases in size and mass following resistance training such as weight lifting, although there is some debate as to whether true hypertrophy occurs in this scenario. Normalizing the increased heart weight to the increased body weight of the athletes appears to greatly reduce the increased ratio typically observed in true hypertrophy (Oakley, 2001; Haykowsky *et al.*, 2002). Nevertheless, resistance athletes frequently demonstrate some degree of concentric hypertrophy (increased wall thickness with normal chamber dimensions). In the case of isotonic training (e.g., distance running), cardiac hypertrophy is much more obvious and often presents as an eccentric hypertrophy (increased wall thickness with increased chamber dilation) (Oakley, 2001).

Hypertrophy in athletes is frequently of sufficient degree to cause alterations in electrocardiograms, although these changes do not seem to correlate with increased susceptibility to disease (Oakley, 2001). Indeed, cardiac hypertrophy in athletes appears to be beneficial, since stroke volume is increased at the same time that resting heart rate is decreased, resulting in more-efficient pumping of blood (Scharhag *et al.*, 2002). Despite news media reports of college or professional athletes dropping dead of heart attacks or arrhythmias during practice, sudden cardiac death is extremely rare among elite athletes and occurs less frequently than in the general population (Futterman and Myerburg, 1998). Recent studies have concluded that the vast majority of cases of sudden death in athletes occur as a result of previously undiagnosed genetic predisposition or defect that becomes exacerbated during exercise (Futterman and Myerburg, 1998; Basso *et al.*, 1999). Furthermore, the typical sequelae of pathologic hypertrophy (i.e., increased fibrosis and cardiac decompensation) usually do not follow physiological cardiac hypertrophic growth, indicating that this form of hypertrophy is truly beneficial to the individual.

B. PATHOLOGICAL HYPERTROPHY

Pathological forms of cardiac hypertrophy frequently occur following acute events, such as myocardial infarction, or accompanying chronic insults such as hypertension. In these examples, hypertrophy is thought to be an attempt to relieve increased transmural wall stress in the heart by thickening the wall, thereby exploiting the law of Laplace (Morisco *et al.*, 2003). Various inherited genetic disorders also result in cardiac hypertrophy as either a primary or secondary endpoint. For example, hypertrophic cardiomyopathy is an inherited disease that can result from a wide variety of genetic lesions, including mutations in contractile proteins such as β-myosin heavy chain that may directly result in cardiac hypertrophy (Arad *et al.*, 2002). Alternatively, hypertrophy may represent a secondary response to a distal lesion, such as occurs in pheochromocytoma, a tumor that uncontrollably releases epinephrine and norepinephrine to continually activate adrenergic pathways in the heart (Prichard *et al.*, 1991). Pathologic hypertrophy thus typically represents an attempt by the heart to alleviate a stress or a response to inappropriate alteration of prohypertrophic signaling pathways.

Pathologic hypertrophy essentially can be divided into two subcategories, representing two different stages of the pathologic process.

1. Compensation

The early stage of pathologic cardiac hypertrophy is termed "compensation" because, in response to a stress, the heart walls thicken in an attempt to compensate for the increased stress. This enlargement is due to increased cardiomyocyte size as well as to increased deposition of collagens and other extracellular matrix components in a process called fibrosis, which may account for a significant proportion of the size increase. By increasing cardiac wall volume, fibrosis helps alleviate transmural stress. However, stiffness of the cardiac muscle increases and compliance decreases as fibrosis progresses (Jalil *et al.*, 1989). Over time, fibrosis actually can impair normal cardiac function (see following section on decompensation). Since fibrosis does not typically occur during physiologic hypertrophy, it is a logical therapeutic target, especially since significant fibrosis is usually a harbinger of a shift to decompensation.

A classic example of compensation occurs in response to hypertension. Increased afterload due to increased mean arterial pressure activates stretch receptors in cardiomyocytes. For example, β-integrin in the sarcolemmal membrane is coupled via its cytoplasmic tail to a complex of proteins in the Z-disc, including α-actinin and talin (Sadoshima and Izumo, 1997). Through a process that is not yet completely understood, cell stretching stimulates this complex and leads to activation of downstream signaling pathways, including the Ras/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase/AKT/

glycogen synthase kinase (PI-3K/AKT/GSK3)-β/calcineurin cascades (Sadoshima and Izumo, 1997). Activation of components of both of these pathways has been linked to cardiac hypertrophy in experimental animals. For example, transgenic mice overexpressing activated forms of the MAPKs mitogen extracellular signal-regulated kinase (MEK)1 and MEK5 present with concentric and eccentric hypertrophy, respectively (Bueno *et al.*, 2000; Nicol *et al.*, 2001). Interestingly, however, mice overexpressing MEK1 appear to model a physiological form of hypertrophy, with no signs of disease at 12 months of age, despite hypertrophy, whereas MEK5-overexpressing mice quickly proceed to failure. Overexpression of a constitutively active form of calcineurin also leads to dramatic hypertrophy, eventually leading to decompensation and failure (Molkentin *et al.*, 1998). Conversely, overexpression of constitutively active GSK-3β, which counteracts the actions of calcineurin by phosphorylating and inactivating downstream targets of calcineurin (the nuclear factor of activated T cell (NFAT) transcription factors), blunts the hypertrophic response to a variety of stimuli, including systemic isoproterenol administration and aortic banding (Antos *et al.*, 2002).

Many other factors and pathways are involved in activation of the hypertrophic program in the heart, including neurohumoral factors (e.g., angiotensin II, adrenergic stimulation) and paracrine influences and interactions between cardiomyocytes and other cell types found in the heart (e.g., fibroblasts, endothelial and vascular smooth muscle cells). Comprehensive coverage of these pathways, however, is beyond the scope of this review. The reader is directed to several recent reviews (Sadoshima and Izumo, 1997; Frey and Olson, 2003).

2. Decompensation

Compensated cardiac hypertrophy frequently devolves into the latter stage of hypertrophy, decompensation. As mentioned earlier, significant cardiac fibrosis typically precedes decompensation and likely plays a causative role. As collagen fibrils are deposited in the cardiac interstitium, cardiac function becomes progressively impaired. Eventually, the heart is unable to pump enough blood to meet the body's demands and patients go into overt cardiac failure. Although various treatments are available to heart failure patients that attempt to preserve cardiac function, mortality remains high.

Although fibrosis can reduce stroke volume and ejection fraction, the ramifications may be more serious for diastolic, rather than systolic, function. Many heart failure patients actually have preserved systolic function but increased wall stiffness due to fibrosis results in reduced diastolic filling, with the net result that less blood is available to be pumped to the body during each beat (Kitzman, 2000). Nonetheless, it is clear that increased fibrosis is a significant problem in patients with cardiac hypertrophy. Several factors have been identi-

fied that can stimulate fibrosis in animal models, such as transforming growth factor-beta1 (TGF-β1); however, the signaling cascade(s) leading to fibrosis remain unclear (Kuwahara *et al.*, 2002). Fibrosis does not typically occur during exercise-induced hypertrophy. At the same time, many of the same hypertrophic signaling pathways appear to be involved in both physiological and pathological responses. For example, recent studies with transgenic mice expressing inhibitors of calcineurin showed attenuation of cardiac hypertrophy in response to not only exercise but also to aortic banding (pressure overload model), isoproterenol (β-adrenergic activation), and transgenic calcineurin overexpression (De Windt *et al.*, 2001; Rothermel *et al.*, 2001). Together, these data indicate a role for calcineurin in both physiological and pathological cardiac hypertrophy. Since similar genetic pathways may be activated in both forms of hypertrophy, examining how the metabolism of the exercised heart contrasts with that of the failing heart may shed light on the phenomena of fibrosis and hypertrophy in general and lead to improved therapies.

II. Energy Metabolism in Hypertrophy

In contrast to most other tissues that rely on glucose oxidation for energy, cardiomyocytes depend primarily on the mitochondrial oxidation of fatty acids for fuel, deriving up to 60% or more of the cell's energy budget from this source (van der Vusse *et al.*, 2000). Fatty acids are a richer energy source than glucose: complete oxidation of a six-carbon fatty acid yields 44 molecules of adenosine triphosphate (ATP), compared to 38 from glucose oxidation. However, fatty acid oxidation is completely dependent on oxygen, which is critically important in the context of the extremely high ATP demands of the cardiomyocyte, while glucose oxidation has a lower oxygen requirement. During short-term exertion or ischemia, when oxygen supply may be severely limited, glycolysis can become more important than oxidation for meeting the cell's energy requirements. However, anaerobic glycolysis provides only two molecules of ATP per glucose molecule, since glucose is metabolized to lactate instead of to acetyl-CoA, which enters the Krebs cycle and drives oxidative phosphorylation (Carvajal and Moreno-Sanchez, 2003). A switch to glucose as a preferred substrate, with a concomitant reduction in fatty acid oxidation, also occurs during cardiac hypertrophy and failure (see below). The heart can metabolize a variety of other energy sources (e.g., ketone bodies, lactate), when present in sufficient quantities. The contribution of various energy-producing pathways to net ATP synthesis can be affected by various metabolites and intermediaries found in cardiomyocytes, including malonyl-CoA and L-carnitine (Carvajal and Moreno-Sanchez, 2003). The energy-producing machinery of the heart thus is highly tuned to meet the specific and significant demands of individual myocytes. Stresses acting on these

processes that may affect the efficiency of energy production may, therefore, have serious consequences for the heart as a whole.

It is difficult to determine whether changes in myocardial metabolism necessarily precede pathologic hypertrophy or simply whether metabolic changes occur only after the hypertrophic cascade has begun. The reality is likely that both scenarios occur to some degree in human disease: metabolic changes may occur as a result of insult or genetic defect/predisposition, leading to hypertrophy, which, in turn, imposes its own metabolic consequences on cardiomyocyte function and exacerbates the situation. The specific genotype of the individual would be expected to influence not only whether pathologic hypertrophy occurs in response to a particular stress but also how quickly the disease progresses and in what form it manifests (e.g., concentric vs. eccentric hypertrophy, left-sided vs. right-sided failure). The interplay between genotype and phenotype is highly complex, with each side of the equation influencing the other. Therefore, it may be unlikely that a one-size-fits-all therapy will be found, although continued basic research will highlight promising avenues, while revealing dead ends.

It is certainly true that defects in metabolism can result in hypertrophy and related cardiomyopathies such as dilated cardiomyopathy, in which cardiac chambers become dilated and the ventricular walls thinned, resembling decompensation and resulting in heart failure. Many cardiac diseases result from primary or secondary mitochondrial defects. For example, both dilated and hypertrophic cardiomyopathy have been associated with point mutations in mitochondrial DNA coding for tRNAs and metabolic genes such as cytochrome b and cytochrome c oxidase (Marin-Garcia and Goldenthal, 2002; Antonicka *et al.*, 2003). Release of cytochrome c from mitochondria activates caspase-9-mediated apoptosis (Green and Reed, 1998). Increased cardiomyocyte apoptosis can cause dilated cardiomyopathy and heart failure (Wencker *et al.*, 2003; Yamamoto *et al.*, 2003). In some instances, defects in mitochondria are secondary to other phenomena, yet still evoke cardiac hypertrophy. Recently, it was shown that deletion of the intermediate filament protein desmin results in extensive derangement of mitochondria in cardiac and skeletal muscles of desmin-null mice (Milner *et al.*, 2000). This loss precedes development of cardiac hypertrophy, which eventually devolves into ventricular dilation and cardiac failure (Milner *et al.*, 1999). It would appear, then, that alteration of normal mitochondrial structure and/or function may lead to hypertrophy and cardiac failure.

Conversely, recent studies indicate that energy metabolism in the heart changes during pathologic hypertrophy. It has been well documented that during cardiac hypertrophy and failure, the contribution to energy production by glycolysis is augmented significantly (Allard *et al.*, 1994; Leong *et al.*, 2002). At the same time, energy production by fatty acid oxidation significantly decreases as glucose becomes the favored fuel (Allard *et al.*, 1994; Sack *et al.*, 1996). This

shift may represent an adaptive response whose purpose is to preserve cardiac function under increased demands, since glucose oxidation requires less oxygen consumption than fatty acid oxidation. Evidence for this comes from reports that heart failure patients given medications that inhibit fatty acid oxidation and favor glucose oxidation have improved prognoses with reduced mortality (Eichhorn *et al.*, 1994; Wallhaus *et al.*, 2001).

The mechanism by which this switch in substrate utilization occurs may involve regulation of transcription and mobilization of the major glucose transporters in the heart, GLUT1 and GLUT4. GLUT1 is responsible for basal glucose import into cardiomyocytes, while GLUT4, although present at higher levels than GLUT1, is responsible for insulin-mediated glucose import (Young *et al.*, 1999). In response to increased energy demand by the heart — for example, during ischemia — there is increased recruitment of both transporters to the sarcolemmal membrane, although the response is greater for GLUT4 (Young *et al.*, 1997). Results in skeletal muscle also suggest that expression of GLUT4 may increase with exercise (Langfort *et al.*, 2003). In cardiac hypertrophy, GLUT1 levels rise, while GLUT4 expression is reduced, which may account for the insulin-independent increase in glucose uptake noted in hypertrophy (Montessuit and Thorburn, 1999; Liao *et al.*, 2002). In overt cardiac failure, however, GLUT1 levels decrease, suggesting the possibility that the transition from compensated to decompensated hypertrophy may involve significant loss of ATP-generating capability by glucose oxidation (Razeghi *et al.*, 2002). This concept is supported by the report that cardiac-specific overexpression of GLUT1 was able to prevent conversion of hypertrophy to heart failure by preserving glucose import and glycolysis following ascending aortic constriction (Liao *et al.*, 2002). It is also intriguing that cardiac-specific deletion of GLUT4 in mice results in cardiac hypertrophy but with preserved contractile function and no fibrosis, similar to compensated hypertrophy (Abel *et al.*, 1999). This deletion may mimic the natural decrease in GLUT4 expression that accompanies hypertrophy and suggests that a decrease in energy production capability may precede disease (Liao *et al.*, 2002).

Ultimately, it may be loss of energy stores, either due to decreased synthesis or increased consumption of high-energy compounds like ATP, which may be the critical factor in the shift from compensated to decompensated hypertrophy. Jung and Dietze reported in a survey of ^{31}P nuclear magnetic resonance (NMR) studies in humans that the phosphocreatine/ATP ratio, a measure of energy stores in the heart, was reduced in patients with aortic stenosis or mitral regurgitation (Jung and Dietze, 1999). In contrast, the hearts of elite cyclists showed no change in this ratio, suggesting that fundamental differences exist between cardiac metabolism in physiological and pathological hypertrophy. Recent studies in a pig model of myocardial infarction to produce cardiac remodeling and failure also demonstrate loss of energy stores, with the loss proportional to the severity

of the disease (Liu *et al.*, 2001; Ye *et al.*, 2001). Another recent study has identified myocardial fatty acid metabolism as an independent predictor of left ventricular mass in heart disease arising as a consequence of hypertension, although it is unclear whether changes in fatty acid metabolism also predict cardiovascular morbidity and mortality (de las Fuentes *et al.*, 2003). Together, these findings reveal that energy metabolism changes significantly in the hypertrophied heart but also suggest that the changes that occur during physiological vs. pathological hypertrophy are different. A recent paper has advanced an intriguing hypothesis of inefficient ATP utilization underlying hypertrophic cardiomyopathy and provides an interesting model for how both contractile and noncontractile protein mutations can lead to ATP loss (Ashrafian *et al.*, 2003). These cited studies suggest that defects in ATP generation or usage, resulting in decreased energy stores, actually may underlie pathologic hypertrophic scenarios in general, regardless of the exact cause. Potential regulatory pathways involved in this process will be discussed later. Unfortunately, while significant resources have been devoted to analyzing pathologic changes in the heart, much less work has been done to identify metabolic changes occurring during physiological hypertrophy. Because it is not clear exactly how these two phenotypes differ, further research is needed in this area.

Another issue requiring further examination is the mechanism by which changes in metabolism can lead to hypertrophy. One argument suggests that altered metabolism results in less-efficient production of ATP by the various bioenergetic pathways in the heart. The net result is that more fuel is required to produce each mole of ATP and thus more fuel is required to do a given amount of work. If fuel is limiting, work output consequently will decrease. Since the definition of heart failure is "the inability of the heart to pump sufficient blood to meet the body's needs," if inefficiency is high enough, work output can decrease enough to put the heart into failure. Any added stresses, such as increased afterload due to hypertension or valve stenosis, or genetic defects that reduce work output (e.g., contractile protein defects) would exacerbate the consequences of the underlying inefficiency.

Indirect evidence to support this hypothesis has been found in studies of the adenosine monophosphate-activated protein kinase (AMPK). AMPK is activated in response to an elevation in the ratio of AMP:ATP. As muscle shifts from a sedentary to active state, increased metabolism will raise the turnover rate of ATP, resulting in an increased pool of AMP and a decrease in ATP levels, thereby activating AMPK. AMPK activation increases the activity of numerous downstream pathways involved in energy production. Therefore, AMPK behaves as a bioenergetic sensor and feedback apparatus, becoming activated when energy stores are low and, in turn, activating energy replenishment programs, including fatty acid oxidation and glycolysis (Hopkins *et al.*, 2003). As one would expect, AMPK becomes activated in the heart during exercise, with the

degree of activation proportional to the degree of work performed (Coven *et al.*, 2003). However, it also has been reported that AMPK is activated during pressure-overload hypertrophy (Tian *et al.*, 2001). This activation suggests that energy stores were being depleted in the hypertrophied heart, in agreement with the report noted earlier in which ^{31}P NMR revealed energy depletion (Jung and Dietze, 1999). It must be noted that, at this time, it is unclear whether inefficiency of ATP synthesis or work performance, or even some other cause, is behind the energy depletion observed in pressure-overload hypertrophy.

Another possibility for how altered energy metabolism leads to cardiac hypertrophy involves generation of reactive oxygen species (ROS), such as superoxide and hydroxyl radicals, and hydrogen peroxide. Exactly how ROS cause hypertrophy is unclear but numerous studies have implicated ROS production in the hypertrophic process. During the progression of hypertrophy to failure in a guinea pig model, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, an important source of ROS, is activated, with increased expression of various subunits of the enzyme and increased production of superoxide radicals, a ROS that can lead to production of more-toxic species (Li *et al.*, 2002). Recently, it was shown that the transgenic calcineurin mouse model of hypertrophy produces increased levels of superoxide (Sayen *et al.*, 2003). Electron spin trap experiments have demonstrated increased generation of hydroxyl radicals arising from superoxide radicals and hydrogen peroxide in failing canine hearts (Ide *et al.*, 2000). Furthermore, the degree of cardiac dysfunction correlated positively with levels of ROS generation.

ROS may arise from numerous sources within cardiomyocytes as well as within other cell types found in the heart. Normal oxidative metabolism produces ROS due to leakage out of the electron transport chain. Numerous antioxidant systems exist within the cell to prevent damage (Czubryt *et al.*, 1996). If ROS synthesis becomes highly elevated or antioxidant levels reduced, ROS may become toxic by interacting with and inactivating or destroying almost any molecule in the cell. However, it is unclear what sources may produce sufficient quantities of ROS to affect cardiac metabolism in otherwise-healthy tissue. One theory suggests that some ROS are generated during normal fatty acid oxidation, when some metabolites are only partially oxidized to form radical species. As fatty acid oxidation increases to meet increased workload, ROS are generated in greater numbers. If the workload elevation is chronic, over time, the antioxidant defenses of the cell are neutralized and ROS builds to toxic levels. Another possibility is that increased workload over time may increase necrosis of cardiomyocytes. As cells die, monocytes and macrophages move into the myocardium, both of which produce high levels of ROS during respiratory bursts (Czubryt *et al.*, 1996). The exact site of ROS production may be unimportant, since some species (e.g., hydrogen peroxide) freely cross cell membranes, allowing migration of ROS into cardiomyocytes and resulting in damage.

Finally, it has been reported that inhibition of oxidative phosphorylation in $Ant1^{tm2Mgr}$ (-/-) knockout mice results in increased production of hydrogen peroxide, which can contribute to the formation of more-toxic ROS (Esposito *et al.*, 1999). This is especially important when considering the inhibition of oxidative phosphorylation that occurs when oxygen is limited, such as during ischemia. This may be highly relevant in some forms of hypertrophy such as idiopathic dilated cardiomyopathy, where tissue perfusion and oxygen delivery can be reduced in the cardiac wall (van den Heuvel *et al.*, 2000).

III. Myocyte Enhancer Factor 2 (MEF2), Hypertrophy, and Energy Metabolism

Our laboratory has focused on genetic signaling pathways involved in the process of cardiac hypertrophy. Of particular interest has been the MEF2 family of transcription factors, which consists of four members (A, B, C, and D) (Black and Olson, 1998). MEF2 transactivates expression of numerous hypertrophic marker genes. MEF2 activity measured in a transgenic mouse expressing a MEF2-responsive reporter is increased during hypertrophy due to overexpression of activated calmodulin-dependent protein kinase IV (CaMKIV) (Passier *et al.*, 2000). MEF2 activity is attenuated by the class II histone deacetylases (HDACs), which bind specifically to MEF2 and block transactivation (Lu *et al.*, 2000a,b; McKinsey *et al.*, 2000a,b). The class II HDACs include HDAC4, -5, -7, and -9. This repression is relieved by phosphorylation of HDACs by calcium/CaMK and other kinases, which phosphorylate HDACs on two conserved serine residues. This phosphorylation inhibits HDAC binding to MEF2 and creates a target binding site for the chaperone protein 14-3-3, which then binds to the HDAC and escorts it out of the nucleus (McKinsey *et al.*, 2000b). This mechanism may explain, at least in part, the hypertrophy observed in CaMKIV-overexpressing mice. Release of HDACs is accompanied by association of the histone acetyltransferase p300 with MEF2 and consequent activation of MEF2 target genes (Eckner *et al.*, 1996; Sartorelli *et al.*, 1997; Slepak *et al.*, 2001).

Since MEF2 appears to play a role in the hypertrophic response, and since class II HDACs repress MEF2 activity, we sought to determine whether a mutant HDAC that constitutively binds MEF2 could repress the hypertrophic program. We used human HDAC5 in which serines 259 and 498 were mutated to alanines (HDAC5-S259/498A) to prevent phosphorylation. This construct successfully blocked the hypertrophic response of cardiomyocytes to prohypertrophic agents such as phenylephrine (Zhang *et al.*, 2002). However, initial attempts to create transgenic mice expressing this construct in the heart were unsuccessful, since only mosaic animals were obtained at birth that did not transmit the transgene to their offspring, leading us to suspect that the transgene may be lethal in the embryo. We therefore incorporated a tet-off system to allow heart-specific

conditional induction of the transgene in mice. In the presence of the tetracycline analog doxycycline, the transgene is silent but becomes fully activated within a few days after withdrawal of the drug (Yu *et al.*, 1996). Besides blocking hypertrophy, it was hoped that gene expression changes in these animals might indicate the existence of as-yet-unidentified MEF2 target genes.

Although several markers of hypertrophy appear to be downregulated initially, several days after transgene induction, these markers (including atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), and cardiac ankyrin repeat protein (CARP)) become highly upregulated. This is possibly due to induction of a hypertrophic signaling cascade that is independent of MEF2. In fact, female transgenic mice develop a dilated cardiomyopathy resembling end-stage heart failure within 30 days of transgene activation, characterized by chamber dilation and ventricular wall thinning (M. Czubryt and E. Olson, unpublished data). However, the model was particularly interesting because male mice, in contrast to the females, died within 8 to 10 days after withdrawal of doxycycline, exhibiting signs of acute heart failure, including bradycardia and lethargy. Examination of the hearts of male mice revealed myocyte loss and damaged mitochondria in the remaining cells, although there was no change in cardiac mass (Czubryt *et al.*, 2003). A gene chip analysis of these animals revealed significant downregulation of numerous enzymes involved in fatty acid oxidation, including the transcriptional coactivator peroxisome proliferator-activated receptor (PPAR) gamma coactivator-1α (PGC-1α).

PGC-1α coactivates the PPAR class of transcription factors, which are related to the nuclear hormone receptor superfamily (Lehman and Kelly, 2002). PPARγ regulates the expression of several key enzymes involved in fatty acid oxidation. The expression of at least two of these enzymes is coactivated by PGC-1α (Vega *et al.*, 2000). Our mice expressing the HDAC5 mutant transgene showed a dramatic downregulation of PGC-1α expression, as well as the two known coactivated targets of PGC-1α, and several other enzymes that may, in fact, be additional targets of PGC-1α (Czubryt *et al.*, 2003). Expression of PPARγ itself was not affected in these animals.

Examination of the PGC-1α promoter revealed the existence of two putative MEF2 binding sites, which we confirmed did, in fact, bind MEF2 specifically and conferred MEF2 and HDAC5 regulation to a luciferase reporter under control of the PGC-1α promoter (Czubryt *et al.*, 2003). Chromatin immunoprecipitation analysis revealed significant deacetylation of the more distal of the two MEF2 sites, in response to transfection of neonatal cardiomyocytes with an adenovirus expressing the HDAC5 mutant. It therefore appears that PGC-1α is a *bona fide* target of MEF2 and HDAC signaling. A recent study by Handschin *et al.* (2003) suggests that calcineurin may play a role in regulation of PGC-1α expression and reports that PGC-1α itself is required for activation of the PGC-1α promoter by MEF2. In contrast to our findings, this report did not observe activation of the

promoter by MEF2 alone. However, this may be due to differences in the cell types chosen for reporter assays, since the cultured muscle cells used in this study may be deficient in endogenous PGC-1α (Michael *et al.*, 2001). This discrepancy also may be due to the fact that our study used a 3-kb promoter fragment containing two MEF2 sites, compared to the 2-kb promoter fragment used in Handschin's study that contained only one MEF2 binding site, which may alter sensitivity of the promoter to MEF2 activation.

Our results suggest that MEF2 may play a much-wider role in cardiac metabolism than previously suspected, including regulation of fatty acid oxidation in the heart and maintenance of mitochondrial function. In support of this hypothesis, our laboratory recently reported that deletion of MEF2A in mice results in derangement of mitochondrial structure, significant loss of mitochondria accompanied by reduced cytochrome c oxidase activity, cardiac dilation, and activation of a fetal gene program reminiscent of that activated in heart failure (Naya *et al.*, 2002). A recent report suggests that PGC-1α may have a wider role to play than initially believed, since PGC-1α coactivated the expression of several MEF2 target genes involved in oxidative slow fiber formation in skeletal muscle (Lin *et al.*, 2002). One of the MEF2 targets coactivated in this study, myoglobin, may be considered a bioenergetic protein but the other target, troponin I slow, regulates contractile activity. In light of the variety of genes regulated by MEF2 that are coactivated by PGC-1α, it is intriguing to consider the possibility that PGC-1α and MEF2, together, may be involved in the regulation of both bioenergetic and hypertrophic contractile genes under MEF2 transactivational control.

When the heart undergoes a sustained increase in workload, many changes occur in the milieu of the cardiomyocyte, including ATP depletion and increased calcium levels (Fralix *et al.*, 1991). In light of a potential role for MEF2 in regulating expression not only of contractile proteins but also fatty acid oxidation enzymes, a model can be envisioned in which MEF2 and PGC-1α coordinately regulate the heart's response to increased workload (Figure 1). The decrease in ATP levels and rise in intracellular calcium concentration activates the two major pathways of this model by activating AMPK and CaMK, respectively. Activation of these pathways eventually results in stimulation of MEF2 transactivation and increased synthesis of PGC-1α. By regulating contractile protein and metabolic enzyme gene transcription with the same transcription factor, both the capability to do more work by increasing myofiber number and the capability to supply the energy necessary to perform that work can be up- and downregulated proportionally to one another. An accumulating body of evidence supports many aspects of this model.

With sustained work, the AMP:ATP ratio increases, as energy supplies are exhausted faster than they can be replenished, eventually triggering energy-generation programs through activation of AMPK. Recently, it has been shown

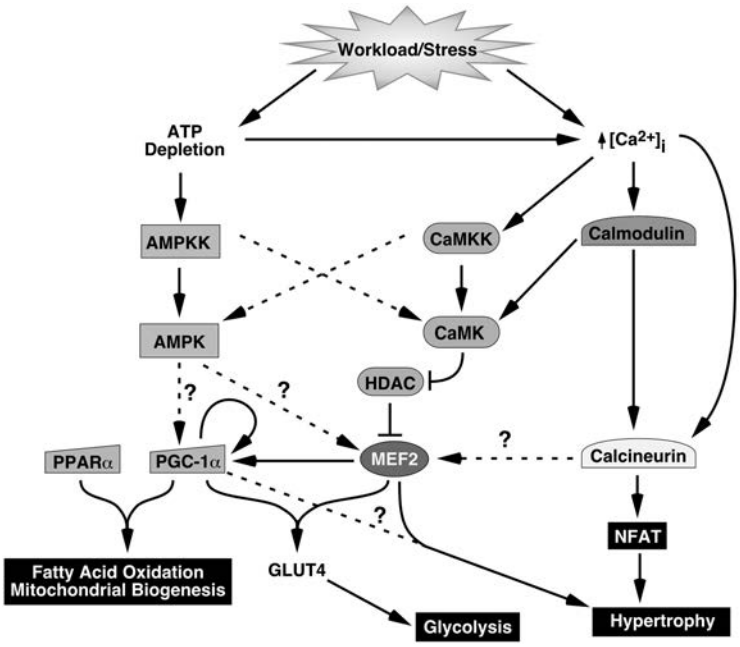

FIG. 1. Myocyte enhancer factor 2 (MEF2) participates in transducing adenosine triphosphate (ATP) and calcium signals to mediate hypertrophic gene expression. In response to increased workload or stress (e.g., inefficient coupling of excitation and contraction, inefficient contraction due to genetic defect), ATP levels fall and intracellular calcium concentration rises. The rise in intracellular calcium may be exacerbated when ATP levels are low due to reduced activity of calcium pumps in the sarcolemma or sarcoplasmic reticulum, resulting in reduced calcium sequestration during relaxation. Increased adenosine monophosphate (AMP):ATP ratio activates AMP-activated protein kinase (AMPK) kinase, or AMPK directly, while at the same time, increased calcium concentration activates calcium/calmodulin-dependent protein kinase (CaMK) kinase and calmodulin, which, in turn, activate CaM kinases. Activated CaMKs activate MEF2-regulated transcription by phosphorylating histone deacetylases (HDACs), which repress MEF2 transactivation, resulting in HDAC release from MEF2 and export of HDACs from the nucleus by 14-3-3. Activated AMPK increases transcription of peroxisome proliferator-activated receptor (PPAR) gamma coactivator-1α (PGC-1α) and MEF2A and MEF2D via an unknown mechanism. PGC-1α also coactivates its own expression, ostensibly mediated by MEF2. PGC-1α and PPARγ cooperate to drive expression of fatty acid oxidation enzymes and promote mitochondrial biogenesis. PGC-1α and MEF2 cooperate to drive expression of GLUT4, thereby increasing glycolysis and/or glucose oxidation by augmenting glucose import, and may participate in driving expression of genes involved in hypertrophy such as contractile proteins. Increased intracellular calcium concentration and activation of calmodulin also activate the protein phosphatase calcineurin, which dephosphorylates and activates the nuclear factor of activated T cell (NFAT) transcription factor family that is involved in driving the hypertrophic program. Calcineurin may activate MEF2 directly but the mechanism of this process has not been elucidated. Numerous other signaling pathways are expected to interact with components of this model — for example, the phosphatidylinositol 3-kinase/glycogen synthase kinase-3β (PI-3K/GSK-3β) pathway — since GSK-3β regulates NFAT transcriptional activity.

that activation of AMPK results in increased expression of PGC-1α, while a dominant-negative AMPK mutant blocked an increase in PGC-1α expression in response to ATP depletion (Terada *et al.*, 2002; Zong *et al.*, 2002; Irrcher *et al.*, 2003). At the same time, the expression of GLUT4, the primary insulin-sensitive glucose transporter in the heart and a direct transcriptional target of PGC-1α and MEF2 (Michael *et al.*, 2001), is increased in response to increased intracellular calcium or in response to the AMPK agonist AICAR (5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside) (Ojuka *et al.*, 2002b). Increased GLUT4 expression may be at least partially responsible for providing the glucose necessary to drive increased glycolysis during short-term energy demands such as exercise. In the same study, it was noted that the increase in intracellular calcium or treatment with AICAR also induced expression of MEF2A and MEF2D. Together, these findings support our model in which MEF2 and PGC-1α (as well as their potential downstream targets) act downstream in a pathway originating with energy status sensing by AMPK.

The activation by increased intracellular calcium is of particular note for three reasons. First, increased rate of contraction of cardiac muscle results in increased intracellular calcium concentration, likely due to decreased efficiency of calcium sequestration following the action potential or simply due to decreased time for sequestration as heart rate climbs (Braveny, 2002). Second, calcium activates the CaMKs, which have been demonstrated to drive MEF2 transcriptional activity by phosphorylating HDACs to regulate their subcellular distribution (McKinsey *et al.*, 2000b; Zhao *et al.*, 2001). Third, calcium also activates the protein phosphatase calcineurin, which independently drives the hypertrophic response in the heart (Molkentin *et al.*, 1998). It also has been observed that periodic or sustained increases in intracellular calcium in muscle cells result in increased mitochondrial biogenesis (Ojuka *et al.*, 2002a,2003). This finding is supportive of our model, since overexpression of PGC-1α has been shown to be sufficient for mitochondrial biogenesis (Lehman *et al.*, 2000). Calcium may act through MEF2 to activate expression of PGC-1α, resulting in the reported observation. It should be noted that cross-talk may occur between these energy- and calcium-responsive pathways, since AMPK kinase (AMPKK) can activate CaMK, while CaMKI kinase (CaMKIK) can activate AMPK through phosphorylation events, although the physiological relevance of this observation is unknown (Hawley *et al.*, 1995).

MEF2 and PGC-1α are, therefore, in an ideal position to respond to both calcium and energy depletion signals to drive the response to exercise, increasing transcription of both fatty acid oxidation enzymes and possibly contractile genes. However, it is still unclear exactly how AMPK may activate MEF2 or what all of the downstream targets of this pathway may be. It is also unclear whether there are differences in how this pathway may respond to periodic increased workload, as in exercise-induced physiologic hypertrophy, compared to sustained increased

workload as in pathologic forms of hypertrophy. Indeed, the periodicity of stress on the heart in exercise, compared to the chronic stress of disease, may prove to be the single most-important factor in the differential responses of physiologic vs. pathologic hypertrophy. MEF2 is strongly activated during pathologic hypertrophy (Passier *et al.*, 2000) but may be only weakly activated by exercise (H. Wu and E. Olson, unpublished data). At the same time, PGC-1α expression is upregulated in exercise-induced hypertrophy but is downregulated in pathologic forms of hypertrophy (Baar *et al.*, 2002; Lehman and Kelly, 2002; Garnier *et al.*, 2003). PGC-1α expression is also upregulated in skeletal muscle following exercise (Baar *et al.*, 2002). This disjunction between MEF2 activity and PGC-1α expression suggests that there are as-yet-unidentified factors involved in this model that may modulate precise control over transcription of genes encoding contractile proteins or bioenergetic enzymes. An uncoupling of MEF2 and PGC-1α pathways may be a critical feature of pathologic hypertrophy.

One possible model for disease progression is as follows: during exercise or other short-term stress, activation of AMPK and CaMK increases activity of MEF2, which then drives PGC-1α expression. Together, these two factors increase transcription of the GLUT4 gene; individually, PGC-1α coactivates PPARγ to increase transcription of genes encoding enzymes involved in fatty acid oxidation, while MEF2 (possibly with the involvement of PGC-1α and likely in conjunction with calcineurin activation) drives contractile gene expression. With chronic stress, an unknown factor inhibits transcription of PGC-1α, while MEF2 activity remains constant or may even increase, particularly if calcineurin is activated by elevated intracellular calcium. One possibility for inhibition of PGC-1α expression is specific recruitment of HDACs to the PGC-1α promoter. The net result is downregulation of fatty acid oxidation enzymes and GLUT4, while contractile protein expression is maintained, resulting in hypertrophy. GLUT1 levels increase to stimulate glucose import, supporting increased glycolysis and glucose oxidation and providing energy to maintain a compensated state. If the stress is prolonged sufficiently or if there are underlying genetic defects resulting in inefficient energy production or use, the heart shifts from compensation to decompensation. Elevated ROS production or myocyte dropout may accelerate this process. Fibrosis may occur as a result of physical stresses on the heart — for example, from increased wall tension, increased afterload (e.g., hypertension), or increased preload (e.g., reduced systolic ejection). Therapeutic intervention at a variety of points along this pathway may provide relief for cardiac patients, as some therapies already are proving, such as glycolysis-promoting agents, antihypertensives, and positive inotropes. As our understanding of the contributions of various pathways to the etiology of hypertrophy and heart failure improves, novel treatments will present themselves.

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Phosphorylation of RNA Polymerase II in Cardiac Hypertrophy: Cell Enlargement Signals Converge on Cyclin T/Cdk9

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ABSTRACT

Cardiac myocyte enlargement is the eponymous characteristic of cardiac hypertrophy, regardless of the instigating signal. Such triggers include biomechanical stress (e.g., work load, compensation for ischemic damage), sarcomeric protein mutations, cytoskeletal protein mutations, abnormal energetics, G protein-coupled receptors for ligands (including angiotensin II and endothelin-1), or their signal transducers within cells. In turn, increased myocyte size reflects increased RNA and protein content per cell as responses to these stimuli. In eukaryotic cells, the large subunit of RNA polymerase II (RNAPII) becomes extensively phosphorylated in its serine-rich C-terminal domain (CTD) during the transition from transcript initiation to transcript elongation — that is, "escape" of RNAPII from the promoter-proximal region into the open reading frame. Although this process is believed to be crucial to productive synthesis of mRNA and is known to be governed by two atypical cyclin-dependent kinases, Cdk7 and Cdk9, surprisingly little is understood of how regulatory pathways within cells intersect these RNAPII-directed protein kinases. Investigations of the CTD kinase module in cardiac hypertrophy provide a tentative initial map of a molecular circuit controlling cell size through regulated phosphorylation of RNAPII.

I. Overview

In pathophysiological terms, and in weighing potential nodal control points for therapeutic interventions, cardiac hypertrophy is usefully viewed as a problem in signal transduction coupled to transcriptional control (Sadoshima and Izumo, 1997; Hunter and Chien, 1999; Molkentin and Dorn, 2001; Akazawa and Komuro, 2003; Frey and Olson, 2003; Olson and Schneider, 2003). Hypertrophy is the normal mode of cardiac growth after birth but also the form of growth the heart exhibits when subjected to biomechanical stresses such as pressure overload (MacLellan and Schneider, 2000; Pasumarthi and Field, 2002). Hemodynamic stress upon the heart can be thought of as extrinsic if resistance to ventricular ejection is increased, such as in hypertension, obstructive valvular disease, or congenital narrowing of the aorta. By contrast, intrinsic hemodynamic

stress occurs because of diminished pump function, with myocardial infarction the most-prevalent example, or with cardiomyopathic mutations. As the result of increased load, a number of physiological and molecular changes ensue within the myocardium, resulting in the aggregate "hypertrophic phenotype." Teleologically, at least some elements of this response may be salutary, at least in the short run. For instance, concentric hypertrophic growth (ventricular wall thickening) likely serves to ameliorate the abnormal increase in wall stress, per a basic physical principle, the law of Laplace. Morever, alterations in specific genes' expression during hypertrophy are likely to encompass some adaptive or cytoprotective components that benefit cardiomyocyte metabolism, contractility, or survival. However, the adverse aspects of this transcriptional program have received increasing attention and can include the induction of overtly pro-apoptotic genes that are sufficient, through myocyte death, to trigger organ-level dysfunction (Yussman *et al.*, 2002). Coupling wall stress to cardiac hypertrophic growth and, often, to eventual heart failure involves an intricate web of signal transduction cascades and transcriptional regulators, both in cardiomyocytes themselves and autocrine/paracrine interactions with cardiac fibroblasts and other types of heart cells (Sadoshima and Izumo, 1997; Molkentin and Dorn, 2001; Akazawa and Komuro, 2003; Frey and Olson, 2003; Olson and Schneider, 2003).

How does the heart "know" that it should work harder? Ideas regarding some sort of mechanical sensor, which *a priori* should exist, have been proposed for decades. However, no responsible molecule has been identified conclusively for initiating mechanical signal in the heart. Among the divergent classes of molecules suggested as serving this role are proteins that couple the extraceullar matrix to the cytoskeleton (e.g., integrin-binding proteins, focal adhesion kinase). A muscle LIM protein (MLP)/telethonin (T-cap) complex in the Z-band of the sarcomere may be important in cardiomyocyte stretch sensing (Knoll *et al.*, 2002). One recent study suggests that cell stretch causes a calcium-permeable, growth factor-regulated channel of the transient receptor potential family to translocate to the sarcolemma; overexpression of this channel causes cardiomyopathy in mice (Iwata *et al.*, 2003). For some cells, stretch-activated nuclear calcium channels mediate the transcriptional response to changes in cell shape (Itano *et al.*, 2003). It is not known if this is true for the heart. While the most-proximal stem in mechanotransduction is still the least defined, functionally important downstream signals generated because of load on the heart are many and varied.

As in applying Koch's postulates of causality to other biological problems, dissecting the cascades for cardiac hypertrophy entails finding mediators that ultimately fulfill the following experimental critieria: 1) they are induced or more active after load; 2) they are sufficient to promote one or more components of hypertrophy when supplied exogenously or expressed as a gain-of-function mutation; and 3) they can be shown to be necessary for one or more components

of hypertrophy when their repression or function is blocked. Perhaps the best-posed proximal mediators for cardiac hypertrophy are heterotrimeric G proteins (Adams and Brown, 2001). G protein-coupled receptors of relevance to cardiac hypertrophy include those for the cardiac agonists angiotensin II, endothelin-1 (ET-1), and α_1-adrenergic ligands, each of which has trophic effects on cardiac myocytes apart from peripheral actions such as vasoconstriction. The G proteins implicated in cardiac hypertrophy include $G_{\alpha q}$, G_{11}, $G_{\alpha s}$, and $G_{\alpha i}$, with the case being especially strong for $G_{\alpha q}$ and G_{11}, acting redundantly. Other components of this signal transduction pathway include but are not limited to the mitogen-activated protein kinase (MAPK) family, protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3-K), Akt, calmodulin-dependent protein kinases, and the calmodulin-dependent phosphatase calcineurin (Molkentin *et al.*, 1998; Bueno *et al.*, 2000; Zhang *et al.*, 2000; Chen *et al.*, 2001; Molkentin and Dorn, 2001; Crackower *et al.*, 2002; Minamino *et al.*, 2002; Shioi *et al.*, 2002; Olson and Schneider, 2003). Of these, we will return to $G_{\alpha q}$ and calcineurin subsequently.

A central event for signal transduction in hypertrophy is the modification of ubiquitous and cardiac-enriched transcription factors. The former group includes cyclic AMP (cAMP)-responsive element binding protein/activating transcription factor (CREB/ATF), nuclear factor of activated T cells (NFAT), class II histone deacetylases, and histone acetylases (p300, CREB-binding protein), whereas the latter includes serum response factor (SRF), myocyte enhancer factor-2 (MEF2), homeodomain protein Nkx2.5, and zinc finger protein GATA4, with proteins of both classes serving as coactivators. Transcriptional adaptors that promote the assembly of multifactor complexes are themselves the target of signaling cascades that control key functional properties, such as histone acetylase activity or nuclear localization. Among other targets, these alterations turn on the set of genes known as immediate-early transcription factors (e.g., Fos, Myc, Jun, Egr-1). Certain of the newly synthesized, repressed, and modified proteins influence upstream components of the hypertrophic pathway, activating their activators, activating parallel modules, or providing a negative-feedback loop. Relationships among the pathways for hypertrophy are labyrinthine but no more so than, say, for the cell cycle in proliferating cells. Like "transformation" in cancer biology, which is more-instructively viewed in terms of its discrete elements (e.g., loss of replicative senescence, escape from apoptosis, tumor angiogenesis, metastasis), the hypertrophic phenotype is an aggregate of modular responses — including plasticity of cardiac gene expression (activation of a largely fetal-like gene program), production of growth factors and cytokines, apoptosis, and fibrosis — of which myocyte enlargement is the pathognomic one.

The physical enlargement of cardiomyocytes occurs through a global increase in RNA and protein content per cell. Knowledge concerning the mechanisms for hypertrophic growth is surprisingly scant, compared to the wealth of

new insights concerning transcriptional plasticity (Olson and Schneider, 2003). In this review, we consider recent discoveries that implicate the phosphorylation of RNA polymerase II (RNAPII) in cardiac hypertrophy at the sites that are crucial for transcription elongation (Sano *et al.*, 2002; Sano and Schneider, 2003). What kinases are responsible? How do hypertrophic cascades couple to the kinases? What is the evidence that changes in the RNAPII-directed protein kinase circuit are causative with respect to growth? Is such growth adaptive, phenotypically neutral, or adverse?

II. The RNAPII Phosphorylation-Dephosphorylation Cycle

RNA transcription — which genes are expressed and at what levels — is integral not only to the definition of cell identity during lineage decisions for embryogenesis but also to the cell's adaptation to the environment in diverse pathophysiological states. Hence, transcription is the executor of both "nature" and "nurture." mRNA synthesis directly couples the DNA genetic code to the cell- and organ-level phenotypes that are manifested as the consequence of protein expression: genome, transcriptome, proteome, and phenome. Yet, RNA transcription encompasses three phases — initiation, elongation, and termination — of which just the first has been studied widely (Orphanides and Reinberg, 2002). Transcription initiation begins with the formation of a preinitiation complex that brings together many general transcription factors that bind DNA and one another, including the general transcription factors TFIIA, TFIIB, TFIIC, TFIID, TFIIE, TFIIF, and the subunits of RNAPII. The carboxy-terminal domain (CTD) of the largest subunit of RNAPII contains an evolutionally conserved, 52-copy repeat of a 7-amino acid motif (YSPTSPS).

RNAPII is recruited to promoters in its hypophosphorylated form (RNAPIIa) and subsequently phosphorylated at Ser2 and Ser5 of the heptapeptide repeat, in the transition from initiation to elongation (Komarnitsky *et al.*, 2000; Lin *et al.*, 2002; Orphanides and Reinberg, 2002; Pokholok *et al.*, 2002; Shim *et al.*, 2002; Shilatifard *et al.*, 2003). Importantly, the state of phosphorylation of the CTD is critical to transcription initiation, elongation, and mRNA processing, rather than merely denoting this change of state. Two kinases responsible for phosphorylation of the CTD are cyclin-dependent kinase 7 (Cdk7) (Roy *et al.*, 1994) and Cdk9 (Zhu *et al.*, 1997). Cdk7 phosphorylates Ser5 of the heptapeptide repeat between initiation and promoter clearance, whereas Cdk9 phosphorylates the CTD at Ser2, allowing RNAPII to overcome proximal promoter pausing and migrate into the open reading frame (Orphanides and Reinberg, 2002). The names of these two kinases allude to cyclins as their principal binding partners: cyclin H for Cdk7 (Makela *et al.*, 1994) and cyclin T or K for Cdk9 (Peng *et al.*, 1998a,b). The cyclin H/Cdk7 complex has a third component with the more-than-usually memorable name, ménage-a-trois (MAT1) (Devault *et al.*, 1995;

Tassan *et al.*, 1995a). In carrying out its role as a CTD kinase, this trimeric complex is part of the larger general transcription factor TFIIH (Serizawa *et al.*, 1995; Shiekhattar *et al.*, 1995). Cyclin H/Cdk7 also functions as a Cdk-activating kinase (CAK), regulating different phases of the cell cycle by activating other Cdks (Fisher and Morgan, 1994; Harper and Elledge, 1998). The presence of MAT1 in the complex is thought to shift the substrate preference towards the RNAPII CTD (Rossignol *et al.*, 1997; Yankulov and Bentley, 1997).

The cyclin T/Cdk9 heterodimer is known as positive transcription elongation factor-b (P-TEFb) (Marshall and Price, 1992,1995; Conaway and Conaway, 1999; Price, 2000). The cyclin T family is made up of three isoforms — cyclin T1, cyclin T2a, and cyclin T2b — which are known to be functionally distinct in some settings. More distantly related, cyclin K also activates Cdk9. Among the known specificities, the best-studied is Cdk9's recruitment to the human immunodeficiency virus (HIV) genome exclusively by cyclin T1, via the HIV Tat protein and a 59-base transactivation-responsive region (TAR) in the 5′ untranslated region (UTR) of nascent HIV transcripts (Wimmer *et al.*, 1999). Several transcription factors are reported to bind cyclin T2 preferentially (Simone *et al.*, 2002a,b). Negative regulators of transcript elongation include negative elongation factor (NELF) and DRB sensitivity-inducing factor (DSIF) (Wada *et al.*, 2000; Yamaguchi *et al.*, 2002). To inhibit positive transcription elongation, DSIF binds to RNAPII directly and NELF binds to nascent RNA transcripts and RNAPII/DSIF (Yamaguchi *et al.*, 2002). The ability of Cdk9 to promote elongation occurs in part by impairing the association of RNAPII with these key inhibitors. Thus, CTD phosphorylation by Cdk9 enables RNAPII to migrate into the open reading frame and differing elongation factors are bound to the polymerase, depending on position.

Dephosphorylation of the CTD takes place after transcription termination, through the action of a specific TFIIF-associated CTD phosphatase, FCP1 (Cho *et al.*, 1999; Kobor *et al.*, 1999; Kobor and Greenblatt, 2002; Lin *et al.*, 2002; Kamada *et al.*, 2003). FCP1 may preferentially dephosphorylate Ser2 of the CTD (Hausmann and Shuman, 2002) (cf. Lin *et al.*, 2002), whereas a new family of small CTD phosphatases preferentially dephosphorylates the CTD at Ser5 (Yeo *et al.*, 2003). By catalyzing this dephosphorylation after transcription termination, FCP1 prepares RNAPII for another round of transcription. FCP1 likely has an additional role in transcript elongation beyond its function as a CTD phosphatase (Kobor and Greenblatt, 2002; Mandal *et al.*, 2002). Both functions are regulated through its phosphorylation by protein kinases that are yet to be identified (Friedl *et al.*, 2003).

The RNAPII CTD is also the substrate for a third Cdk complex, cyclin C/Cdk8. In this case, however, the kinase acts as a negative regulator of transcription, by phosphorylating the CTD before RNAPII is recruited to promoters (Tassan *et al.*, 1995b; Hengartner *et al.*, 1998; Akoulitchev *et al.*, 2000).

Hyperphosphorylated RNAPII cannot be recruited to promoters; thus, Cdk8 prevents transcription from commencing. Cdk8 also phosphorylates cyclin H, repressing the CTD kinase activity of TFIIH and disrupting its ability to activate transcription (Akoulitchev *et al.*, 2000).

This phosphorylation-dephosphorylation cycle — in its relation to the cardiac growth signals we studied — is illustrated, in abbreviated form, in Figure 1 (upper panel).

III. Cdk7 and Cdk9 Are Targets of the Signaling Cascades for Biomechanical Stress

Bridging the two ideas described earlier — increased RNA content per cell and the established mechanism of transcription elongation — might Cdk7 and Cdk9 be important in cardiac hypertrophy? For this to be true, Cdk7, Cdk9, or both would first need to be expressed and active in the heart. Indeed, both kinases are expressed and active in embryonic myocardium, with function decreasing dramatically from the embryo stage to the adult, as measured by immune complex kinase assays and the phosphorylation of endogenous RNAPII (Sano *et al.*, 2002) (Figure 1a, left). This downregulation was readily explained by the downregulation of both Cdks and the two corresponding cyclins as the heart matures. The net result was a fall in the fraction of phosphorylated RNAPII in myocardium, from 30% in embryos to just 4% in normal adults.

Conversely, both Cdk7 and Cdk9 were activated during chronic cardiac hypertrophy *in vivo*, provoked by means of biomechanical stress (partial aortic constriction) and by two of its genetically best-proven downstream mediators, $G_{\alpha q}$ (Adams *et al.*, 1998; Wettschureck *et al.*, 2001) and calcineurin (Molkentin *et al.*, 1998; Rothermel *et al.*, 2001). In contrast, acute mechanical stress caused activation only of Cdk9, not Cdk7 (Figure 1a, right). The same was true for isolated neonatal rat cardiomyocytes challenged with ET-1 (Sano *et al.*, 2002). Hence, the principal difference observed was between long-term and short-term growth signals, not between hypertrophy of the intact heart and its surrogate in cultured cells.

The preference for Cdk9 activation as a ubiquitous or at least highly generalizable response to hypertrophic cues prompted the hypothesis that Cdk9 might be the more important of these two CTD kinases in cardiac hypertrophy. Furthermore, Cdk9 has the important technical and conceptual advantages inherent to being an immediate-early response and, hence, is perhaps more likely to be direct. Using catalytically inactive dominant-negative (dn) forms of both kinases, it was demonstrated that dn Cdk9 was sufficient to block ET-1-induced hypertrophy in cultured cardiomyocytes, whereas dn Cdk7 was not (Sano *et al.*, 2002) (Figure 1b). Under these conditions, at least, Cdk9 was essential for myocyte growth. As other critieria to support this interpretation, similar results

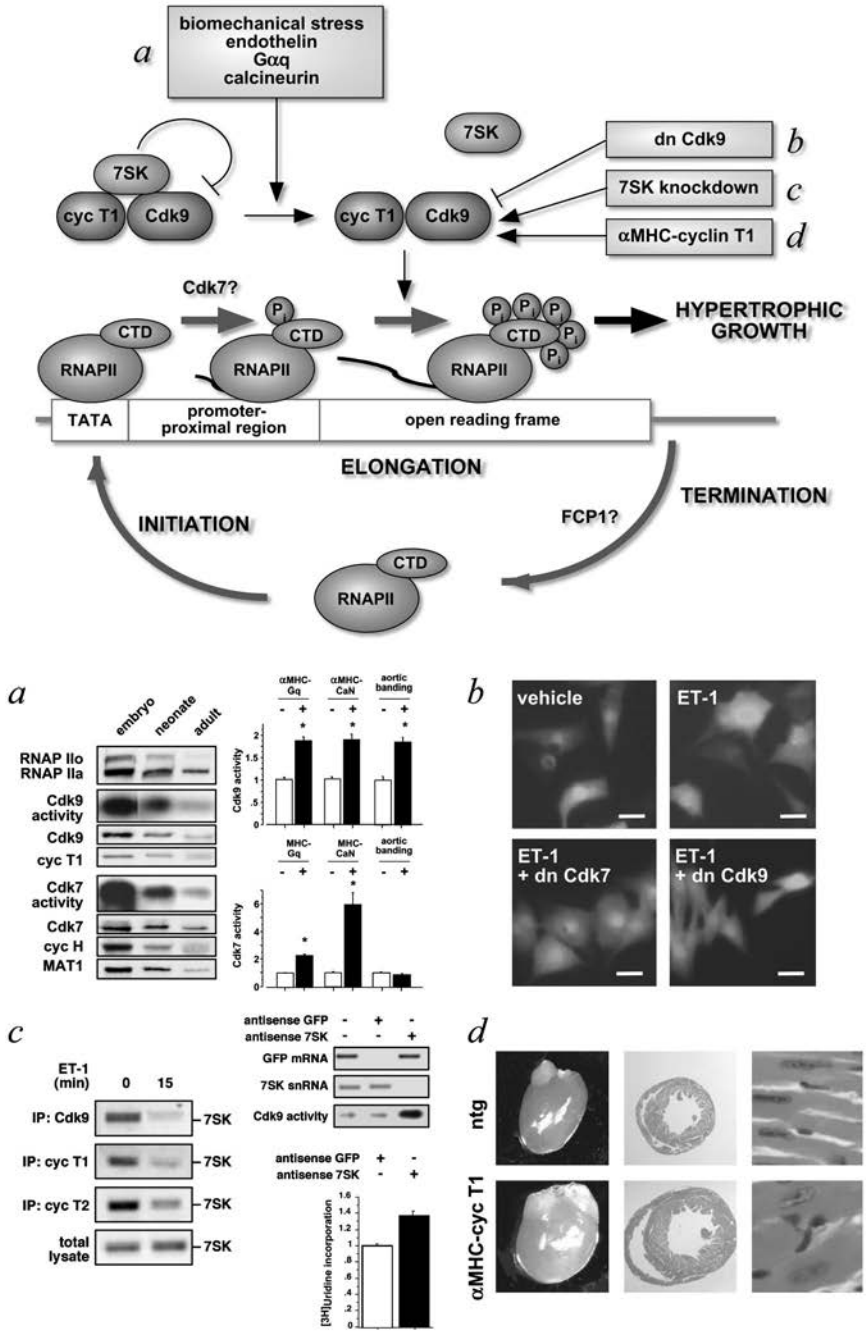
were obtained using 5,6-dichloro-1-D-ribofuranosylbenzimidazole, a pharmacological inhibitor of transcript elongation that is active preferentially against Cdk9. Furthermore, the phosphorylation of RNAPII triggered by ET-1 occurred at Ser2 of the heptapeptide repeat, the site preferred by Cdk9 (Sano *et al.*, 2002).

IV. Trophic Signals Dissociate an Endogenous Inhibitor from Cdk9

A quandary remained: how was Cdk9 becoming activated in these four pathobiological settings — ET-1, $G_{\alpha q}$, calcineurin, and biomechanical stress — and was a unitary mechanism possibly shared across this series of interrelating signals? A more-specific mystery was that many common and expected mechanisms were easily excluded. Unlike the easy and intuitively satisfying explanation for downregulation of CTD kinase activity with age (the observed downregulation of all four proteins, the two kinases and their two cyclins), no analogous change was occurring as an early response to trophic signals to account for the rapid reinduction of Cdk9 activity: neither levels of Cdk9, levels of the T cyclins, nuclear localization, nor physical assembly of cyclin T/Cdk9 heterodimers increased (Sano *et al.*, 2002).

In 2001, two groups independently identified an astounding endogenous inhibitor of the cyclin T/Cdk9 complex, namely "7SK," a small nuclear RNA (snRNA) of no prior established function (Nguyen *et al.*, 2001; Yang *et al.*, 2001). Based on this breakthrough, the presence of the inhibitor in physical association with cardiac P-TEFb was ascertained directly, using immune complex reverse transcription-polymerase chain reaction (RT-PCR) and sequencing the recovered cDNA (Sano *et al.*, 1999). Binding was confirmed independently using biotinylated RNA complementary to 7SK snRNA for affinity purification, then Western blotting for the co-recovered proteins. In all the hypertrophic models we studied, acute and chronic alike, the activation of Cdk9 occurred through dissociation of this 7SK transcript (Sano *et al.*, 2002) (Figure 1c).

The next question asked was, if signals for cardiac hypertrophy can activate Cdk9, can activating Cdk9 suffice to cause hypertrophy? As pivotal evidence supporting a causal role of the endogenous inhibitor, antisense 7SK was sufficient to increase Cdk9 activity and RNA production (Sano *et al.*, 2002) (Figure 1c). Thus, pharmacological, physiological, and genetic instigators of hypertrophy each removed an inhibitor from Cdk9, whereas removing the inhibitor, by suppressing its expression, was enough to activate Cdk9 and elicit spontaneous growth. Whereas this antisense study ablating 7SK snRNA answered the question conclusively for cultured cells, a corresponding answer for mouse myocardium was sought by forcibly expressing cyclin T1 at the level normal for embryonic or neonatal myocardium. To accomplish this, transgenic mice were engineered using the cardiomyocyte-specific α-myosin heavy chain (MHC) promoter to overexpress cyclin T1 in the heart. Cdk9 activation and concentric


a
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d
Cdk7?
CTD
RNAPII
HYPERTROPHIC GROWTH
TATA
promoter-proximal region
open reading frame
ELONGATION
TERMINATION
FCP1?
INITIATION
embryo
neonate
adult
RNAP IIo
RNAP IIa
Cdk9 activity
cyc T1
Cdk7 activity
Cdk7
cyc H
MAT1
αMHC-Gq
αMHC-CaN
aortic banding
MHC-Gq
MHC-CaN
vehicle
ET-1
ET-1 + dn Cdk7
ET-1 + dn Cdk9
ET-1 (min)
0
15
IP: Cdk9
IP: cyc T1
IP: cyc T2
total lysate
antisense GFP
antisense 7SK
GFP mRNA
7SK snRNA
[3H]uridine incorporation
ntg
αMHC-cyc T1

cardiac hypertrophy resulted, in a dose-dependent fashion, evidenced by an increase in the heart-weight-to-body-weight ratio and in myocyte size (Sano *et al.*, 2002) (Figure 1d).

V. Cdk9 as a Therapeutic Target

The published findings summarized earlier point to Cdk9 as a potentially druggable target for intervention in the future. The logic for this is simple and builds plausibly on the knowledge that increased cardiac mass, in population-based studies of heart disease, is a potent and independent risk factor for cardiac demise (reviewed in Sano and Schneider, 2002). If cardiac hypertrophy is ultimately harmful to the organism, beyond some given duration or threshold for growth, perhaps blocking the activity of Cdk9, at least partially, might be beneficial in the treatment of heart disease, by reducing cardiomyocyte growth. Yet, this extrapolation rests on several untested assumptions.

First, if Cdk9 is to be a therapeutic target in humans and not just in mice, then it must be demonstrably germane to human heart disease. In preliminary studies of human heart failure, we recently confirmed the predicted increase in Cdk9 and Cdk7 activity (M. Sano and M.D. Schneider, unpublished results).

Second, even if this is the case, does increasing Cdk9 activity confer increased risk of developing heart failure? Cyclin T1 transgenic mice, our model of Cdk9 activation, appear grossly normal, apart from heart size, under unstressed conditions. Increasingly, however, there is recognition that manipulations of the genome can be incompletely informative or even misleading, if the "phenome" is studied merely as a basal state. This was true, for example, in our prior studies of a proximal MAPK kinase kinase, MEKK1. With little or no findings from the germline deletion by itself, a critical role for MEKK1 was unmasked when tested in the context of a Gq gain-of-function: deleting MEKK1 prevented $G_{\alpha q}$-induced growth (Minamino *et al.*, 2002). Like $G_{\alpha q}$ at the dose studied, cyclin T1 was well tolerated as a gain-of-function mutation, expressed at the level normal in earlier stages of life, each transgene resulting separately in

FIG. 1 *(facing page).* Activation and function of the positive transcription elongation factor-b (P-TEFb) (cyclin T/Cdk9) in cardiac muscle hypertrophy. (Upper panel) Schematic representation of the RNA polymerase II (RNAPII) phosphorylation-dephosphorylation cycle and its modulation by physiological, pharmacological, and genetic triggers of hypertrophy. Upstream signals (a) and direct manipulations of Cdk9 activity (b-d) correspond to data illustrated in the lower panels. [Reproduced and modified from Sano M, Abdellatif M, Oh H, Xie M, Bagella L, Giordano A, Michael LH, DeMayo FJ, Schneider MD 2002 Activation and function of cyclin T-Cdk9 (positive transcription elongation factor-b) in cardiac muscle-cell hypertrophy. Nature Med 8:1310–1317, with permission of the Nature Publishing Group.]

mild concentric hypertrophy without cell death, fibrosis, or overt dysfunction. However, crossing cyclin T1 with $G_{\alpha q}$ transgenic mice — creating the double-transgenic model — increased Cdk9 activity more so than either alone, increased the heart-weight-to-body-weight ratio more than either of the single transgenics, and induced apoptosis, with the functional sequelae of heart failure and early demise (M. Sano and M.D. Schneider, unpublished data). Completely blocking the increase of Cdk9 function in cardiomyocytes with dnCdk9 prevented Gq-induced growth, yet was tolerated poorly under stress. The same exacerbation and mortality were true, using mechanical load as the hypertrophic signal, in concert with dnCdk9 or cyclin T1. This suggests that Cdk9 is an essential protein at some intermediate level of activity and that graded, titratable inhibition, whether genetic or drug based, would more likely be of benefit than complete disruption of this signaling module.

VI. Unanswered Questions and Future Prospects

In summary, we have shown that Cdk7 and Cdk9 both become activated by chronic mechanical load or genetic triggers of hypertrophy, using the G protein $G_{\alpha q}$ and the calcium-dependent phosphatase calcineurin. Each of these in transgenic mice reproduces and may be essential for the hypertrophic phenotype (Sano *et al.*, 2002). In acute load or cell-culture models of cardiac hypertrophy, only Cdk9 activity increased. Across this gamut of hypertrophic triggers (hemodynamic, genetic, and pharmacological), a unifying property was activation of Cdk9 not by increased assembly with its functional partner cyclin T but, rather, through dissociation of a remarkable endogenous inhibitor, the noncoding 7SK snRNA. Cdk9 function was sufficient to compel hypertrophic growth in culture (shown by an antisense knockdown of 7SK snRNA), obligatory for hypertrophy in culture (shown by dnCdk9), and sufficient for hypertrophy in mouse myocardium (shown by forced expression of its activator cyclin T1). Findings that implicate Cdk9 as a potential therapeutic target in cardiac disease also include the abnormally increased activity of this kinase in human heart failure and the catastrophic interaction between cyclin T1 and other hypertrophic cues in transgenic mouse models.

P-TEFb — the cyclin T/Cdk9 heterodimer — lies at an intriguing interface between the forefront of fundamental studies into general transcription factors, on the one hand, and disease-driven studies of pathobiological growth, on the other. Tantalizing insights are emerging but many questions surrounding its structure, activity, and function remain to be answered.

Consider, first, its endogenous inhibitor, the 7SK snRNA. Recent findings implicate a novel protein, ménage a quatre (MAQ1), as recruited to the N-terminal homology region of cyclin T via this noncoding transcript, a relationship that resembles (and competes with) the binding of cyclin T1 by the HIV Tat

protein and HIV TAR RNA. Thus, it has been proposed that lentivirus has subverted the regulation of P-TEFb by the MAQ1–7SK protein-RNA complex (Michels *et al.*, 2003). However, the participation of MAQ1 in growth signaling *in vivo* and its specific function even in cell culture are still mysteries. Futhermore, even though the 7SK-MAQ1 complex was found to inhibit P-TEFb function, it is unknown how signals release this complex from P-TEFb and which component of the complex is their primary target.

Several cyclins besides T1 partner with Cdk9, including cyclins T2a and T2b, the alternatively spliced products of a separate gene. Like cyclin T1, the T2 cyclins are found in the cardiac P-TEFb/7SK RNA complex but, unlike cyclin T1, both are upregulated highly in the adult heart, compared to myocardium of the embryo or neonate (Sano *et al.*, 2002). Aside from Tat binding just to cyclin T1, functional distinctions among these isoforms are largely conjectures.

Taking a step back from the partnering cyclins, Cdk9 has been shown to associate with other proteins in the cell, such as the molecular chaperones heat shock protein 70 and chaperone complex Hsp90/cyclin-dependent complex 37 (O'Keeffe *et al.*, 2000). Whereas activation of Cdk9 in *acute* hypertrophic models was traced, thus far, only to the dissociation of 7SK RNA, the *chronic* hypertrophic models were found to cause not only decreased binding of the inhibitor but also increased expression of the chaperone proteins and increased assembly of cyclin T/Cdk9 (M. Sano and M.D. Schneider, unpublished data). Is this pathway needed for normal activation of Cdk9? Conversely, are Cdk9 activation and its resulting impact on transcription essential targets of these chaperones?

To date, we have learned more of Cdk9 than of Cdk7 in the coupling of hypertrophic signals to RNAPII phosphorylation. However, it should not be overlooked that all chronic hypertrophic signals (long-term load, $G_{\alpha q}$, and calcineurin) also activated this second of the CTD kinases. Experimentallly, the barriers to studying Cdk7 activation in cardiac hypertrophy are formidable, as no *in vitro* model has been identified in which its activation is perturbed and activation of Cdk7 *in vivo* has, thus far, always been concomitant with activation of Cdk9 (Sano *et al.*, 2002). What, then, is the role, if any, of Cdk7? If consequential, how is its activity regulated by the growth signals that also impinge on Cdk9? Conversely, for RNAPII to be competent for recruitment into the preinitiation complex, it must be in the dephosphorylated form. Although critical to the cycling of RNAPII, nothing is presently known about the CTD phosphatases in cardiac hypertrophy.

Although the results discussed here are interpreted in the broadest possible biochemical context — namely, the function of CTD phosphorylation in transcription elongation generically — it can be envisioned that some genes might be regulated more than others by this mode of transcriptional control. As yet, nothing is known of specific genes' susceptibility to regulation by hypertrophic

signals at this step. Such an understanding of CTD kinases in cardiac hypertrophy is likely to require both flavors of scientific "chips" — genome-wide microarray comparisons of gene expression and, more mechanistically, chromatin immunoprecipitation. The latter would be indispensable to pinpoint promoter escape and identify the protein-protein associations that occur with the elongating form of RNAPII provoked by cardiac growth signals.

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Cardiomyocyte Calcium and Calcium/Calmodulin-dependent Protein Kinase II: Friends or Foes?

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ABSTRACT

Calcium (Ca^{2+}) is a critical second messenger in cell signaling. Elevated intracellular Ca^{2+} can activate numerous Ca^{2+}-regulated enzymes. These enzymes have different subcellular localizations and may respond to distinct modes of Ca^{2+} mobilization. In cardiac muscle, Ca^{2+} plays a central role in regulating contractility, gene expression, hypertrophy, and apoptosis. Many cellular responses to Ca^{2+} signals are mediated by Ca^{2+}/calmodulin-dependent enzymes, among which is the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). Putative substrates for CaMKII include proteins involved in regulating Ca^{2+} storage and release, transcription factors, and ion channels. The major isoform of CaMKII in the heart is CaMKIIδ. Two cardiac splice variants, CaMKIIδ_B and δ_C, differ in whether they contain a nuclear localization sequence. Our laboratory has examined the hypothesis that the nuclear δ_B and the cytoplasmic δ_C isoforms respond to different Ca^{2+} stimuli and have distinct effects on hypertrophic cardiac growth and Ca^{2+} handling. We have shown that pressure overload-induced hypertrophy differentially affects the nuclear δ_B and the cytoplasmic δ_C isoforms of CaMKII. Additionally, using isolated myocytes and transgenic mouse models, we demonstrated that the nuclear CaMKIIδ_B isoform plays a key role in cardiac gene expression associated with cardiac hypertrophy. The cytoplasmic CaMKIIδ_C isoform phosphorylates substrates involved in Ca^{2+} handling. Dysregulation of intracellular Ca^{2+} and resulting changes in excitation-contraction coupling characterize heart failure and can be induced by *in vivo* overexpression of CaMKIIδ_C and phosphorylation of its substrates. The differential location of CaMKII isoforms and their relative activation by physiological vs. pathological stimuli may provide a paradigm for exploring and elucidating how Ca^{2+}/CaMKII pathways can serve as both friends and foes in the heart.

I. Intracellular Ca^{2+} Regulation

As a second messenger, Ca^{2+} regulates acute physiological functions, including contraction of cardiac, skeletal, and smooth muscle and release of hormones and neurotransmitters. Ca^{2+} also regulates more-chronic cellular responses, including cell proliferation and cell survival. Dysregulation of intracellular Ca^{2+} homeostasis can lead not only to loss of normal physiological control mechanisms but also to pathological changes in cell growth.

Intracellular cytosolic Ca^{2+} concentrations are regulated carefully to remain at $\approx$ 100 nM under resting conditions. This occurs even in the face of mM levels of Ca^{2+} in the extracellular space and high Ca^{2+} in intracellular organelles such

as the endoplasmic reticulum (ER). Acute increases in Ca^{2+} are needed to elicit physiological responses and are achieved through the actions of hormones or neurotransmitters on cell-surface receptors. In smooth muscle and secretory glands, ligands such as norepinephrine, acetylcholine, endothelin-1, and angiotensin II stimulate G protein-coupled receptors (GPCR) coupled to Gq to activate phospholipase C (PLC), catalyze phosphatidylinositol biphosphate (PIP_2) breakdown, and generate inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 mobilizes Ca^{2+} from the ER/sarcoplasmic reticulum (SR), transiently increasing Ca^{2+}. In skeletal muscle, nicotinic receptor activation by acetylcholine depolarizes the membrane potential to induce Ca^{2+} release from the SR. In cardiac muscle, Ca^{2+} transients occur with every heart beat. The amplitude of these transients is increased via stimulation by norepinephrine of β-adrenergic receptors coupled to Gs, which activate adenylate cyclase to increase cyclic adenosine monophospate (cAMP). Subsequent activation of protein kinase A (PKA) phosphorylates Ca^{2+} regulatory proteins such as voltage-dependent Ca^{2+} channels and phospholamban (PLB), leading to increased Ca^{2+} influx and releasable SR Ca^{2+}.

II. Ca^{2+}-regulated Enzymes and Ca^{2+}/Calmodulin-dependent Protein Kinase

Once neurotransmitters or hormones elevate Ca^{2+}, any of a number of Ca^{2+}-regulated enzymes — including protein kinases, protein phosphatases, phospholipases, nitric oxide synthases, cysteine protease calpains, and endonucleases — can be activated. For some of these, Ca^{2+} induces activation by binding to calmodulin (CaM), an intracellular Ca^{2+} sensor. Three Ca^{2+}/calmodulin-dependent enzymes have significant roles in cardiac function: Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase or CaMK), protein phosphatase 2B (calcineurin), and myosin light chain kinase (MLCK). In contrast to CaMK and calcineurin, which have broad substrate specificities, MLCK is a dedicated enzyme that phosphorylates only the regulatory light chain of myosin II, modulating Ca^{2+} sensitivity of myofilaments and cardiac contractility (Sweeney *et al.*, 1993) and sarcomere organization during cardiac hypertrophy (Aoki *et al.*, 2000). This review will consider the role of Ca^{2+}-dependent enzymes in regulating gene expression, Ca^{2+} handling, and apoptosis, focusing primarily on CaMK.

CaMKs are ubiquitous mediators of Ca^{2+} signaling (Braun and Schulman, 1995). This multifunctional serine/threonine family, consisting of CaMKI, -II, and -IV, has an extremely wide tissue distribution and is represented to varying degrees in all eukaryotic systems examined. Studies carried out over the past decade demonstrate that CaMKs can phosphorylate multiple substrates and regulate numerous cellular functions. CaMKI and CaMKIV are monomeric

enzymes that are activated by phosphorylation through an upstream kinase (CaMK kinase) (Lee and Edelman, 1994; Tokumitsu *et al.*, 1995). These isoforms are expressed at very low levels in the heart (Edman and Schulman, 1994; Colomer *et al.*, 2003). In contrast, CaMKII, the major cardiac isoform, is a multimer of 6–12 subunits encoded by four separate genes: α, β, γ, and δ (Braun and Schulman, 1995). Binding of Ca^{2+}/CaM to CaMKII leads to its activation and subsequent autophosphorylation, rendering it autonomous (i.e., active) in the absence of Ca^{2+}/CaM (Braun and Schulman, 1995). Several laboratories have demonstrated that the δ subunit of CaMKII predominates in the heart and that distinct splice variants of CaMKIIδ, characterized by the presence of a second variable domain, exist (Edman and Schulman, 1994; Baltas *et al.*, 1995; Mayer *et al.*, 1995). Of particular interest, the δ_B subunit contains an 11-amino acid nuclear localization signal (NLS) that localizes it to the nucleus. In contrast, the δ_C isoform lacks the NLS and localizes to the cytoplasm (Edman and Schulman, 1994; Srinivasan *et al.*, 1994; Ramirez *et al.*, 1997). Heteromultimers comprised predominantly of δ_B subunits (CaMKIIδ_B) localize to the nucleus, while those with δ_C (CaMKIIδ_C) localize to the cytoplasm (Srinivasan *et al.*, 1994).

III. Role of Ca^{2+} Signaling in Gene Expression and Cardiac Hypertrophic Growth

Ca^{2+} is a well-established regulator of transcriptional changes in gene expression (Hardingham and Bading, 1998). Changes in intracellular Ca^{2+} also have been suggested to mediate cardiac hypertrophic responses (for a review, see Frey *et al.*, 2000). Modulations in Ca^{2+} levels would need to be transmitted to the nucleus to affect transcriptional regulation of genes associated with cardiac hypertrophy. The nuclear isoform of CaMKII (CaMKIIδ_B) would, therefore, be the isoform predicted to play a predominant role in Ca^{2+}-mediated transcriptional gene regulation. Both Ca^{2+} and CaM can translocate into the nucleus and could activate nuclear localized CaMKII (Heist and Schulman, 1998). There also is evidence for independent regulation of nuclear Ca^{2+} via IP_3 receptors localized in the cell nucleus (Malviya and Rogue, 1998). CaMK and calcineurin have been shown to play critical and often synergistic roles in transcriptional regulation in cardiomyocytes (Passier *et al.*, 2000) (Figure 1). There is growing evidence that the amplitude, frequency, source, and subcellular localization (spatial and/or temporal modes) of Ca^{2+} signals are determinants of distinct transcriptional responses (Berridge, 1997; Dolmetsch *et al.*, 1997), allowing regulation of diverse cellular processes in response to the same second messenger (Ca^{2+}). In this regard, it may be important to note that CaMK is activated by high and transient Ca^{2+} spikes and its activity is dependent on Ca^{2+} spike frequency

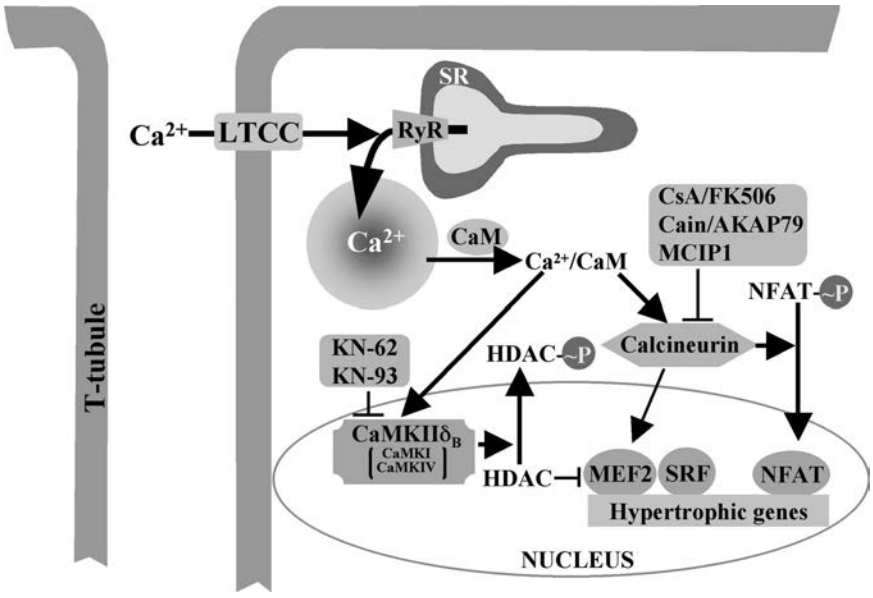

FIG. 1. Calcium (Ca^{2+}) signaling in cardiac gene expression and hypertrophic growth. Ca^{2+} binds to calmodulin (CaM), which, in turn, activates Ca^{2+}/calmodulin-dependent protein kinase (CaMK) or calcineurin. CaMK can phosphorylate histone deacetylase (HDAC) and dissociate it from myocyte enhancer factor 2 (MEF2) and/or serum response factor (SRF), thereby relieving HDAC inhibition of MEF2 and/or SRF transcriptional activity. Calcineurin can dephosphorylate the nuclear factor of activated T cells (NFAT), which enables it to translocate to the nucleus to induce hypertrophic gene expression. Calcineurin also can activate MEF2 through a post-translational mechanism. Inhibition of calcineurin by cyclosporin A (CsA)/FK506, Cain/AKAP79, or modulatory calcineurin-interacting protein (MCIP)1 blocks calcineurin signaling pathways, while inhibition of CaMK by KN-62 or KN-93 blocks CaMK signaling pathways.

(Hudmon and Schulman, 2002), whereas calcineurin responds to low, sustained Ca^{2+} plateaus (Dolmetsch *et al.*, 1997).

A. CAMK SIGNALING IN GENE EXPRESSION AND CARDIAC HYPERTROPHY

1. The Effect of CaMK on Gene Transcription

CaMK has been suggested to regulate gene expression via activation of several different transcription factors, including activation protein 1 (AP-1) (Ho *et al.*, 1996), CAAT-enhancer binding protein (C/EBP) (Wegner *et al.*, 1992), activating transcription factor (ATF-1) (Sun *et al.*, 1996), serum response factor (SRF) (Misra *et al.*, 1994), cAMP-response element binding protein (CREB)

(Sheng *et al.*, 1991), and myocyte enhancer factor 2 (MEF2) (Passier *et al.*, 2000). CaMKII and CaMKIV have been shown to activate CREB by phosphorylation of Ser133 (Matthews *et al.*, 1994). Surprisingly, CREB phosphorylation does not appear to be altered in transgenic mice expressing CaMKIIδ_B (Zhang *et al.*, 2002) or CaMKIV (Passier *et al.*, 2000), both of which localize to the nucleus. Therefore, phosphorylated CREB cannot account for the long-term changes in cardiac function in these mice. Another transcription factor, MEF2, is upregulated during cardiac hypertrophy (Kolodziejczyk *et al.*, 1999; Lu *et al.*, 2000) and has been suggested to act as a common endpoint for hypertrophic signaling pathways in the myocardium (Kolodziejczyk *et al.*, 1999; Lu *et al.*, 2000). Studies using transgenic overexpression of CaMKIV demonstrate that MEF2 is a downstream target for CaMKIV (Passier *et al.*, 2000). The mechanism by which CaMK signaling activates MEF2 *in vivo* remains to be determined but recent studies have suggested that MEF2 interacts with class II histone deacetylases (HDACs) and other repressors that normally limit expression of MEF2-dependent genes (Figure 1). CaMKI and CaMKIV activate MEF2 by dissociating HDACs and other repressors (Lu *et al.*, 2000). Most recently, SRF has been shown to be activated by CaMKIV in a similar manner (i.e., by dissociating HDACs) (Davis *et al.*, 2003). Both MEF2 and SRF activation have been demonstrated to occur in response to activation of the noncardiac CaMKI and IV isoforms. However, the ability of CaMKII to regulate HDAC — and thereby activate MEF2 and SRF — has not been explored and the selectivity of the nuclear vs. cytoplasmic CaMKIIδ isoforms is undetermined. Our preliminary studies indicate that the nuclear CaMKIIδ_B overcomes HDAC5-mediated repression of MEF2 activity, while the cytoplasmic CaMKIIδ_C does not (T. Zhang and J.H. Brown, unpublished data). These findings are consistent with our earlier work implicating CaMKIIδ_B (vs. δ_C) in control of gene expression (Ramirez *et al.*, 1997).

2. The Role of CaMK in Hypertrophic Growth

Studies from a variety of *in vivo* preparations — including hypertensive rat hearts, coronary artery-ligated rabbit hearts, and transverse aortic-constricted (TAC) mouse hearts — have demonstrated increased CaMKII expression and activity in hypertrophied myocardium (see Table I for a summary) (Currie and Smith, 1999; Boknik *et al.*, 2001; Hagemann *et al.*, 2001; Zhang *et al.*, 2003). Studies using isolated cardiomyocytes and CaMK inhibitors KN-62 or KN-93 also suggested that CaMKII was involved in cardiomyocyte hypertrophy induced by agonists such as endothelin-1, leukemia inhibitory factor (LIF), and phenylephrine (Sei *et al.*, 1991; Ramirez *et al.*, 1997; Kato *et al.*, 2000; Zhu *et al.*, 2000). Several transgenic mouse models subsequently confirmed a role for CaMK in activation of the hypertrophic gene program and development of

hypertrophy (Table I). Transgenic mice overexpressing calmodulin were generated nearly 10 years ago and shown to develop severe cardiac hypertrophy (Gruver *et al.*, 1993). This subsequently was demonstrated to be associated with an increase in the autonomous activity of CaMKII *in vivo* (Colomer and Means, 2000). Pronounced hypertrophy also develops in transgenic mice that overexpress CaMKIV (Passier *et al.*, 2000). This is associated with specific changes in gene expression. However, CaMKIV knockout (KO) mice still are able to develop hypertrophy after TAC (Colomer *et al.*, 2003), presumably because CaMKIV is not one of the major CaMK isoforms present in the heart (Edman and Schulman, 1994; Colomer *et al.*, 2003).

Since hypertrophic growth is associated with a specific program of altered gene expression, and CaMKII is implicated in this response, we hypothesized that CaMKII isoforms expressed in the nucleus would selectively regulate hypertrophic transcriptional responses. In support of this, we reported that transient expression of the nuclear δ_B isoform of CaMKII in neonatal rat ventricular myocytes induced expression of the atrial natriuretic factor (ANF) gene, an established indicator of cardiomyocyte hypertrophy, as indicated by enhanced transcriptional activation of an ANF-luciferase reporter gene and increased ANF protein (Ramirez *et al.*, 1997). The nuclear localization signal of

TABLE I
Summary of Animal Models Showing Ca^{2+}/Calmodulin-dependent Protein Kinase (CaMK) Involvement in Cardiac Hypertrophy

Animal model	Phenotype and effects	References
Hypertensive rat models	Cardiac hypertrophy and increased CaMKII expression	Hagemann *et al.*, 2001
Spontaneously hypertensive rats	Cardiac hypertrophy and increased CaMKII activity	Boknik *et al.*, 2001
Coronary artery ligation rabbit	Cardiac hypertrophy and increased CaMKII activity	Currie *et al.*, 1999
Transverse aortic constricted mice	Cardiac hypertrophy and increased CaMKII expression and activity	Colomer *et al.*, 2003; Zhang *et al.*, 2003
Calmodulin TG mice	Severe cardiac hypertrophy and increased CaMKII activity	Gruver *et al.*, 1993; Colomer *et al.*, 2000
CaMKIV TG mice	Cardiac hypertrophy through MEF2 activation	Passier *et al.*, 2000
CaMKIIδ_B TG mice	Cardiac hypertrophy and dilated cardiomyopathy	Zhang *et al.*, 2002

Abbreviations: TG, transgenic; MEF, myocyte enhancer factor.

CaMKIIδ_B was shown to be required for this response, as transient expression of CaMKIIδ_C did not result in enhanced ANF expression. Indeed, CaMKII heteromultimers formed predominantly of δ_C subunits were excluded from the nucleus and failed to induce ANF expression (Ramirez *et al.*, 1997). These findings are consistent with the observation that constitutively active CaMKI and CaMKIV, which enter the nucleus, induce a hypertrophic response in cardiomyocytes *in vitro* (Passier *et al.*, 2000). We recently reported that hypertrophic growth occurs in transgenic mice that overexpress the CaMKIIδ_B isoform, which is highly concentrated in cardiomyocyte nuclei (Zhang *et al.*, 2002).

B. CALCINEURIN SIGNALING IN CARDIAC HYPERTROPHY

1. Calcineurin and Transcriptional Regulation

Ca^{2+}/calmodulin-dependent protein phosphatase 2B (PP2B) or calcineurin is a serine/threonine protein phosphatase that is activated by sustained elevations in intracellular Ca^{2+}. The sufficiency of calcineurin to promote cardiac hypertrophy has been demonstrated *in vitro* and *in vivo* (Molkentin *et al.*, 1998; De Windt *et al.*, 2000). *In vitro*, adenoviral expression of calcineurin is sufficient to induce hypertrophy in neonatal rat ventricular myocytes (De Windt *et al.*, 2000). *In vivo*, cardiac-specific overexpression of an activated truncation mutant of calcineurin leads to profound hypertrophy that rapidly progresses to dilated heart failure by 2–3 months of age (Molkentin *et al.*, 1998). In cardiomyocytes, calcineurin dephosphorylates the nuclear factor of activated T cells (NFAT) transcription factor, which then translocates to the nucleus and interacts with the cardiac-restricted zinc finger transcription factor GATA4 to activate the gene expression of B-type natriuretic factor, a marker for cardiac hypertrophy and heart failure (Molkentin *et al.*, 1998). Another line of evidence for NFAT regulating cardiac transcriptional responses downstream of calcineurin is that transgenic (TG) mice overexpressing a constitutively nuclear NFATc4, one of the NFAT family members, develop profound hypertrophy within 2–3 months of age, whereas overexpression of full-length NFATc4 does not produce detectable hypertrophy (Molkentin *et al.*, 1998). The transcription factor MEF2 also is involved in calcineurin signals through a post-translational mechanism (Blaeser *et al.*, 2000). Two other transcription factors, NF-κB and Elk-1, can be regulated by calcineurin (Meyer *et al.*, 1997; Tian and Karin, 1999).

2. Calcineurin Involvement in Hypertrophic Responses

A calcineurin inhibitor cyclosporin A (CsA) has been shown to attenuate the hypertrophic response induced by phenylephrine, angiotensin II, and endothelin-1 in cardiomyocytes (Molkentin *et al.*, 1998; Zhu *et al.*, 2000), suggesting that calcineurin is activated in response to these hypertrophic agonists. Increased

calcineurin activity, as well as increased calcineurin mRNA and protein expression, subsequently has been demonstrated in cardiomyocytes stimulated with phenylephrine, angiotensin II, and serum (Taigen *et al.*, 2000). Myriad studies have demonstrated that calcineurin is activated in pressure overload- and exercise-induced hypertrophy (for a review, see Molkentin, 2000). However, others have reported either no change or decreases in cardiac calcineurin activity (for a review, see Molkentin, 2000). Disparate findings also are evident in studies using systemic administration of calcineurin inhibitors, CsA, or FK506 to evaluate the role of calcineurin in cardiac hypertrophy (Olson and Williams, 2000). More-specific inhibitors of calcineurin might provide less-equivocal information. For example, Cain/Cabin-1 or A-kinase anchoring protein 79 (AKAP79) inhibited calcineurin activity and have been shown to attenuate phenylephrine and angiotensin II-induced cardiomyocyte hypertrophy *in vitro* (Taigen *et al.*, 2000) and reduced catecholamine infusion or pressure overload-induced hypertrophy *in vivo* (De Windt *et al.*, 2001). In addition, the response to hypertrophic stimuli is impaired in calcineurin-deficient mice and calcineurin dominant-negative TG mice (for a review, see Wilkins and Molkentin, 2002). Similarly, cardiac-specific overexpression of modulatory calcineurin-interacting protein 1 (MCIP1) prevents the hypertrophic response to calcineurin overexpression, isoproterenol infusion, exercise, and pressure overload (for a review, see Wilkins and Molkentin, 2002). However, studies with MCIP1 also indicate differential effects, depending on the nature of the hypertrophic stimulus. Thus, in MCIP1-null mice, the hypertrophic response to activated calcineurin overexpression was exacerbated, whereas the response to pressure overload or chronic adrenergic stimulation was blunted (Vega *et al.*, 2003).

IV. Cardiac Ca^{2+} Transients and Physiological Ca^{2+} Regulation

In the heart, intracellular Ca^{2+} transients are regulated in a beat-to-beat manner (70 beats/minute in human, $>$ 400 beats/minute in mice), acting as transducers of excitation-contraction coupling (E-C coupling). During the cardiac action potential, Ca^{2+} enters the cell through voltage-dependent Ca^{2+} channels (L-type Ca^{2+} channel) and subsequently binds and activates the ryanodine receptor (RyR) on the SR to trigger further Ca^{2+} release (Figure 2A). This process, termed Ca^{2+}-induced Ca^{2+} release (CICR), serves to amplify and coordinate the Ca^{2+} signal. The elemental event in Ca^{2+} release from the SR is the Ca^{2+} spark. Ca^{2+} sparks occur at low frequency in diastole and in a synchronized manner, which leads to large Ca^{2+} transients and coordinated contractions during systole. To allow for muscle relaxation between contractions, cytosolic Ca^{2+} must be decreased quickly. This is accomplished by the Na^{+}/Ca^{2+} exchanger (NCX), which removes Ca^{2+} to the extracellular space, and by the SR Ca^{2+}ATPase (SERCA), which mediates Ca^{2+} uptake into the SR.

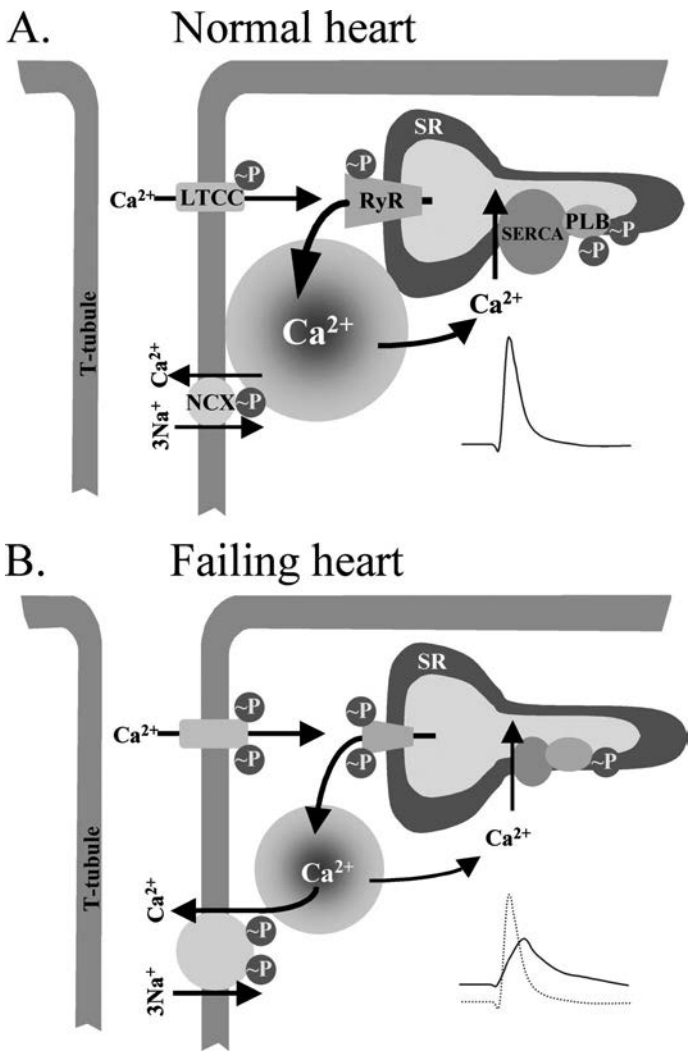

FIG. 2. Ca^{2+} regulation in ventricular myocytes. (A) Normal physiological Ca^{2+} regulation. Ca^{2+} entering through the L-type Ca^{2+} channel (LTCC) triggers Ca^{2+} release through ryanodine-receptor (RyR). Ca^{2+} reuptake into the sarcoplasmic reticulum (SR) is mediated by the SR Ca^{2+} ATPase (SERCA), which is negatively regulated by phospholamban (PLB). The Na^{+}/Ca^{2+} exchanger (NCX) can serve to remove Ca^{2+} from the cytosol. Phosphorylations (shown as ~P) of the LTCC, RyR, NCX, and PLB regulate their functions. A normal Ca^{2+} transient is illustrated by the solid line. (B) Pathophysiological changes associated with heart failure, including altered expression levels of Ca^{2+} regulatory proteins (as indicated by difference in size compared to (A)) and altered phosphorylation state (indicated by the number of ~ P). The Ca^{2+} transient (solid line) is smaller and slower in failing heart (compared to normal, shown as broken line in (B)).

Two types of Ca^{2+} channels (L and T) contribute to Ca^{2+} influx. The L-type Ca^{2+} channel (LTCC) predominates in the ventricle. Ca^{2+} current through LTCC (I_{CaL}) is the most-important trigger of Ca^{2+} release. This current is regulated by voltage and cytosolic Ca^{2+} (Bers and Perez-Reyes, 1999). Stimuli that increase cAMP and activate PKA clearly enhance single-channel activity as well as whole-cell Ca^{2+} currents in cardiomyocytes (Bers, 2002). This contributes significantly to the positive inotropic effects of the β-adrenergic receptor pathway.

The RyR (RyR2 in heart) channel is a tetrameric structure comprised of four monomeric subunits, each of $\approx$ 565,000 Daltons. Ca^{2+} binds to and activates the RyR to release Ca^{2+} from the SR. SR Ca^{2+} release also is regulated by SR Ca^{2+} content, with decreases in SR Ca^{2+} content facilitating closure of RyR (cessation of Ca^{2+} release) and increases in SR Ca^{2+} content increasing the Ca^{2+} sensitivity of RyR to open (Bers, 2002). The RyR has been shown to form a macromolecular complex with PKA, protein phosphatases PP1 and PP2A, FK-506 binding protein (FKBP 12.6), mAKAP, and sorcin (Meyers *et al.*, 1995; Marx *et al.*, 2000). Recently, we showed that CaMKII also associates with the RyR (Zhang *et al.*, 2003). RyR activity is regulated by its phosphorylation state, the best-described mechanism being RyR phosphorylation by PKA, which results in dissociation of FKBP12.6 from the channels and increased channel open probability (Marx *et al.*, 2000).

Ca^{2+} uptake into the SR is mediated by SERCA. SERCA plays an important role in the declining phase of the Ca^{2+} transient. The activity of SERCA2a, the isoform expressed in the heart, is regulated by intracellular Ca^{2+} concentration and PLB. PLB is an endogenous inhibitor of SERCA that is regulated by phosphorylation. PLB is phosphorylated at Ser16 by PKA (Simmerman *et al.*, 1986), which decreases its ability to inhibit SERCA activity. Phosphorylation of PLB by PKA thus accelerates Ca^{2+} uptake, contributing to the more-rapid decline of Ca^{2+} transients and contractions induced by β-adrenergic receptor stimulation (lusitropic effect). PLB is also phosphorylated at Thr 17 (Simmerman *et al.*, 1986), a site for CaMKII. The effects of this phosphorylation are more controversial.

The Na^+/Ca^{2+} exchanger, NCX1, is highly expressed in the heart. Under physiological conditions, the major role of NCX is to extrude Ca^{2+}, although NCX can act in the reversed mode (Ca^{2+} influx mode), which occurs in the presence of high intracellular Na^+ and high membrane voltage (Nuss and Houser, 1992). Hilgemann and colleagues have demonstrated that PIP_2 is a positive regulator of NCX and suggested that this is the primary mechanism for ATP-dependent NCX activation (Hilgemann and Ball, 1996). Stimuli coupled to Gq and phospholipase C activation could affect the local cellular PIP_2 level. This might contribute to the regulation of NCX activity, a hypothesis that experimentally requires validation. Although regulation of NCX activity by kinases is not

established, it has been suggested that β-adrenergic agonists enhance NCX activity through PKA (Perchenet *et al.*, 2000) and that α-adrenergic agonists and phorbol ester enhance NCX activity through PKC (Iwamoto *et al.*, 1996).

V. Physiological Role of CaMK in Cardiomyocyte Ca^{2+} Handling

CaMKII has been implicated in the modulation of several key proteins involved in acute regulation of ventricular myocyte Ca^{2+} homeostasis. Published studies demonstrate that CaMKII can phosphorylate RyR (Witcher *et al.*, 1991; Hain *et al.*, 1995), SERCA (Xu *et al.*, 1993; Toyofuku *et al.*, 1994), PLB (Le Peuch *et al.*, 1979; Simmerman *et al.*, 1986), and the LTCC or an associated regulatory protein (Dzhura *et al.*, 2000). Thus, CaMKII has the potential to significantly affect acute Ca^{2+} regulation and E-C coupling in cardiomyocytes (Figure 3).

Studies using CaMKII inhibitors in isolated cardiomyocytes suggest that CaMKII is the mediator of Ca^{2+} current (I_{Ca}) facilitation (Anderson *et al.*, 1994; Xiao *et al.*, 1994; Yuan and Bers, 1994), although the site of CaMKII action is

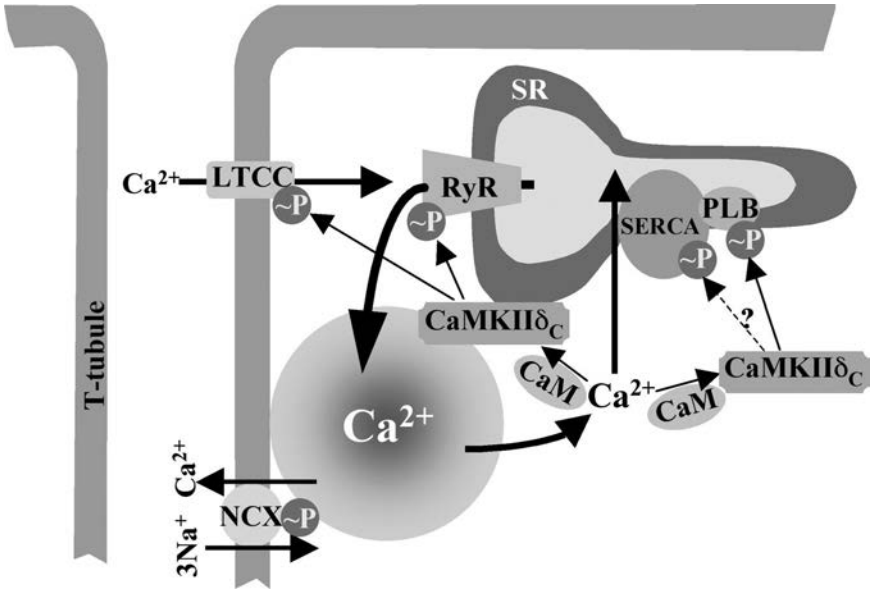

FIG. 3. Regulation of cardiomyocyte Ca^{2+} homeostasis by CaMKII. CaMKII can phosphorylate the RyR2 at Ser2809 (and others), SERCA2a at Ser38, PLB at Thr17, and the LTCC or an associated regulatory protein (the actual site is unknown). Most of these phosphorylations have functional consequences, indicating that CaMKII could profoundly affect Ca^{2+} handling in cardiomyocytes.

not clear. It is likely that CaMKII phosphorylates the L-type Ca^{2+} channel complex or an associated regulatory protein, since CaMKII colocalizes with LTCC in myocytes (Xiao *et al.*, 1994) and CaMKII can activate LTCC when it is applied to the cytoplasmic face of excised cell membrane patches (Dzhura *et al.*, 2000). It has been reported that CaMKII is involved in the development of early after-depolarizations (EADs) and arrhythmias as a consequence of LTCC activation *in vitro* and *in vivo* (Anderson *et al.*, 1998; Wu *et al.*, 1999,2002).

CaMKII has been reported to phosphorylate the same site (Ser2809) as PKA on the RyR2 (Witcher *et al.*, 1991; Marx *et al.*, 2000; Rodriguez *et al.*, 2003). However, the functional consequence of CaMKII-mediated phosphorylation is not yet clear. RyR2 phosphorylation by CaMKII has been suggested to alter RyR2 gating properties. While some studies indicate that CaMKII increases RyR2 open probability (Witcher *et al.*, 1991; Hain *et al.*, 1995), others find that CaMKII decreases RyR2 open probability (Lokuta *et al.*, 1995). To assess the intrinsic effect of CaMKII on E-C coupling, Bers' laboratory used voltage-clamped ventricular myocytes and demonstrated that, for a given SR Ca^{2+} load and I_{Ca} trigger, inhibition of CaMKII by KN-93 diminished the Ca^{2+}-dependent increase in SR Ca^{2+} release (relative to total SR) (Li *et al.*, 1997). This finding supports the hypothesis that activation of CaMKII by Ca^{2+} transients phosphorylates RyR2 and enhances the efficacy of E-C coupling in cardiomyocytes. Whether CaMKII phosphorylation of RyR2 can dissociate FKBP12.6, as observed for PKA, is still not clear. An important recent finding from Colyer's laboratory is that CaMKII appears to phosphorylate at least four sites, in addition to Ser2809, on RyR2 *in vitro* (Rodriguez *et al.*, 2003). Whether this occurs *in vivo* and how this alters E-C coupling have not been established.

As previously discussed, phosphorylation of PLB plays a major role in regulating cardiac SERCA activity. PLB can be phosphorylated by CaMKII at Thr17 (Le Peuch *et al.*, 1979; Simmerman *et al.*, 1986), a site adjacent to the PKA phosphorylation site (Ser16). Phosphorylation of PLB relieves its inhibitory effects on SERCA activity, thereby accelerating Ca^{2+} transport. The functional role of dual-site PLB phosphorylation has been examined extensively over the past decade. Several studies have suggested that PLB phosphorylation at Ser16, the PKA site, is a prerequisite for PLB phosphorylation at Thr17 *in vivo* (Luo *et al.*, 1998) and is sufficient to mediate the maximal contractile effects of β-adrenergic stimulation (Chu *et al.*, 2000). However, emerging evidence has shown that Thr17 phosphorylation of PLB by CaMKII can occur independently of Ser16 phosphorylation. First, it has been demonstrated in genetically targeted mice that mutation of Ser16 in PLB (S16A) does not prevent Thr17 phosphorylation (Chu *et al.*, 2000). Second, it has been reported that electrical pacing of rat ventricular myocytes increases CaMKII-dependent phosphorylation of PLB at Thr17 in a frequency-dependent manner, without altering Ser16 phosphorylation (Hagemann *et al.*, 2000). Third, it has been reported that an increase in Thr17

phosphorylation can be produced by an increase in intracellular Ca^{2+} (with a simultaneous inhibition of phosphatases or acidosis) in the absence of β-adrenoceptor stimulation (Mundina-Weilenmann *et al.*, 1996; Vittone *et al.*, 1998). Finally, in CaMKIIδ_C TG mice, PLB phosphorylation at the CaMKII site was increased without increased phosphorylation of the PKA site (Zhang *et al.*, 2003). These observations all indicate that PLB phosphorylation by CaMKII can occur independently of and serve a function distinct from that of PLB phosphorylation by PKA under physiological or pathophysiological conditions. Of particular interest, recent studies using PLB site-specific mutant mice have shown that both PLB phosphorylation sites are involved in the mechanical recovery after ischemia, with Thr17 appearing to play the major role (Said *et al.*, 2003). In addition, it recently was reported that targeted inhibition of CaMKII in cardiac longitudinal SR transgenic mice causes selective decreases in PLB phosphorylation at Thr17 but not at Ser16 and results in cardiac dysfunction (Ji *et al.*, 2003).

Frequency-dependent acceleration of relaxation (FDAR) is an important intrinsic physiological mechanism that contributes to faster relaxation and diastolic filling as heart rate increases. Published studies have suggested that FDAR is mediated by CaMKII rather than PKA (Bassani *et al.*, 1995) and might be due to enhanced SR Ca^{2+} uptake secondary to CaMKII-mediated PLB phosphorylation (Schouten, 1990). However, while PLB would appear to be a likely target for CaMKII effects on FDAR, this response is still prominent in PLB KO mice. Moreover, it can be inhibited by the CaMKII inhibitor, KN-93 (DeSantiago *et al.*, 2002). Thus, targets of CaMKII other than PLB must contribute to FDAR.

The question of whether SERCA function is regulated directly by CaMKII phosphorylation remains controversial. Some investigators have shown that CaMKII can directly phosphorylate cardiac SERCA (SERCA2a) on Ser38, resulting in increases in its enzymatic activity and therefore the maximal velocity (V_{max}) of Ca^{2+} transport (Xu *et al.*, 1993; Toyofuku *et al.*, 1994). However, studies from other groups present contradictory findings (Odermatt *et al.*, 1996; Reddy *et al.*, 1996). One study failed to observe phosphorylation of SERCA2a by CaMKII (Reddy *et al.*, 1996) and another observed CaMKII-mediated phosphorylation but did not observe stimulation of SERCA2a activity (Odermatt *et al.*, 1996). Thus, the physiological role of SERCA2a phosphorylation by CaMKII remains unclear.

VI. Pathophysiological Changes of Ca^{2+} in the Heart

A variety of stresses are imposed upon the heart by pathophysiological conditions, including high blood pressure, ischemia, infarction, and virus infection. Since cardiomyocytes are terminally differentiated and have lost their ability to proliferate, they adapt by increasing cell size, resulting in myocyte hypertrophy and cardiac enlargement. Although this is a compensatory response,

it also serves as an independent risk factor for development of heart failure, which is characterized by the heart's inability to generate sufficient force for blood ejection. The failing heart exhibits not only structural changes but also marked alterations in Ca^{2+} regulatory protein expression and phosphorylation. Ca^{2+} transients in ventricular myocytes isolated from failing hearts are generally of reduced amplitude and characterized by slower times to peak and to decline. The diastolic level of Ca^{2+} is higher in failing vs. normal heart. Changes in expression levels and activity of several Ca^{2+} regulatory proteins are thought to underlie the dysregulation of Ca^{2+} transients in heart failure (Figure 2B). Diminished Ca^{2+} transients are major determinants of the decreased cellular contractile function. Indeed, Ca^{2+} dysregulation, with resultant changes in E-C coupling, has been suggested as a causal mechanism of heart failure. Ca^{2+} dysregulation may contribute to cardiomyocyte apoptosis (e.g., via mitochondrial Ca^{2+} overloading or calpain activation), leading to progressive loss of cardiomyocytes. Myocyte cell death is not compensated by myocyte cell replacement and, accordingly, myocyte loss is considered to contribute to the development of heart failure.

Furthermore, less Ca^{2+} release is induced by a given Ca^{2+} current in heart failure, a phenomenon referred to as decreased E-C coupling gain (Gomez *et al.*, 2001). Decreased RyR expression, observed in numerous models of heart failure, could contribute to this. However, as described earlier, RyR function is also highly regulated by its phosphorylation. RyR hyperphosphorylation is observed in heart failure (Marks, 2000; Marx *et al.*, 2000). RyR hyperphosphorylation results in the dissociation of FKBP12.6 from RyR, enhancing Ca^{2+} leakage during diastole and thereby decreasing the pool of Ca^{2+} available for release during the contractile cycle (Marks, 2000; Reiken *et al.*, 2003a). Therefore, RyR hyperphosphorylation has been suggested to contribute to the decrease in E-C coupling gain in heart failure through the decrease in SR Ca^{2+} loading. The maladaptive effects of RyR hyperphosphorylation may be one reason that chronic β blockade improves cardiac contractility and prolongs survival in patients with heart failure. Indeed, recent data indicate that β blockade normalizes the phosphorylation status of RyR, resulting in RyR stabilization and decreased Ca^{2+} leakage from the SR in both animal models and failing human heart (Doi *et al.*, 2002; Reiken *et al.*, 2003b). In addition to this hypothesized mechanism, spatial remodeling of T tubules (decreased number of T tubules) and/or the dyad (i.e., distance between LTCC and RyR) have been postulated as mechanisms for the decreased E-C coupling gain (Gomez *et al.*, 2001).

No changes in peak Ca^{2+} currents are observed in most studies of heart failure (Benitah *et al.*, 2002), although channel density has been suggested to be reduced (He *et al.*, 2001). Increased open probability of LTCC, perhaps as a result of increased basal phosphorylation, might compensate for the decreased channel density (Schroder *et al.*, 1998; X. Chen *et al.*, 2002). The functional

contribution of changes in Ca^{2+} currents to heart failure requires further investigation.

SR Ca^{2+} uptake is decreased in many animal models of heart failure. This defect, like increased diastolic Ca^{2+} leak through phosphorylated RyR, would diminish the releasable Ca^{2+} pool and thus contribute to impaired contractile function. Decreases in SR Ca^{2+} uptake also contribute to the prolonged Ca^{2+} transients observed in cardiomyocytes from failing heart. A mechanistic basis for the decreased Ca^{2+} uptake is diminished SERCA protein expression, observed in some, but not all, experimental models of heart failure (Movsesian *et al.*, 1994; Schwinger *et al.*, 1995). In support of a pathophysiological role for decreased SERCA expression, recent evidence indicates that adenoviral SERCA gene delivery improves cellular contractile function and Ca^{2+} transients in human heart failure (Del Monte *et al.*, 2002).

PLB generally is not decreased to the same extent as SERCA in heart failure; thus, there is a lower ratio of SERCA/PLB and enhanced SERCA inhibition in heart failure (Houser *et al.*, 2000). PLB phosphorylation also appears to be decreased (Huang *et al.*, 1999; Houser *et al.*, 2000), providing another mechanism for enhanced SERCA inhibition in heart failure. Interestingly, recent evidence indicates that a mutation in PLB can, by itself, lead to human heart failure (Schmitt *et al.*, 2003). Development of PLB inhibitors therefore has been touted as a promising therapeutic strategy to improve SR Ca^{2+} content and other cellular defects in heart failure (Chien *et al.*, 2003).

The role of NCX in removing cytosolic Ca^{2+} gains importance in the face of decreased SR Ca^{2+} uptake in heart failure. In many, but not all, cases (for a review, see Sipido *et al.*, 2002), NCX is upregulated in heart failure. This is thought to be a compensatory response to prevent Ca^{2+} overloading. Recently, it has been reported that NCX is hyperphosphorylated by PKA, resulting in increased activity of the exchanger in failing heart (Wei *et al.*, 2003). While increased NCX activity initially would be adaptive, excessive or sustained NCX activation could further contribute to decreased SR Ca^{2+} content by removing cytosolic Ca^{2+}, diminishing systolic Ca^{2+} transients and contractile function.

VII. Pathophysiological Role of CaMK in the Heart

A. ROLE OF CAMK IN THE DEVELOPMENT OF HEART FAILURE

Changes in CaMKII have been associated with development of heart failure. It has been reported that CaMKII activity is increased $\approx$ 3-fold and that CaMKII expression is increased $\approx$ 2-fold in human failing hearts with dilated cardiomyopathy (Hoch *et al.*, 1999; Kirchhefer *et al.*, 1999). Conversely, in a rat heart failure model induced by myocardial infarction (Netticadan *et al.*, 2000) and in

a canine model of heart failure produced by intracoronary microembolization (Mishra *et al.*, 2003), CaMKII activity and expression are reduced. We and others observed that, in the TAC-induced hypertrophy mouse model, there is not only acute CaMKII activation but also an increase in CaMKIIδ_C expression that is sustained for at least 1 week (Colomer *et al.*, 2003; Zhang *et al.*, 2003). Upregulation and activation of CaMKII have been correlated with changes in cardiac function (cardiac index and ejection fraction) in patients (Hoch *et al.*, 1999; Kirchhefer *et al.*, 1999). Sustained CaMKII activation could play a causal role in the development of heart failure. However, whether changes in CaMKII are causal or secondary to the development of cardiac diseases remains to be established. Nonetheless, the findings clearly indicate that regulatory changes in CaMKII expression occur in association with cardiac pathology.

B. EFFECTS OF CAMKIIδ_C OVEREXPRESSION ON CA^{2+} HANDLING IN TRANSGENIC MICE

As was discussed, CaMKII plays important roles in Ca^{2+} cycling and E-C coupling in cardiomyocytes *in vitro*, although the extent to which this can occur *in vivo* has not been addressed. Accordingly, our laboratory generated TG mice that expressed the cytoplasmic CaMKIIδ_C isoform in the heart and examined phosphorylation of Ca^{2+} regulatory proteins and concomitant changes in cellular Ca^{2+} regulation. These mice developed a dilated cardiomyopathy characterized by a significant decrease in cardiac function and premature death (Zhang *et al.*, 2003). The time course for development of functional impairment and extent of lethality were related to the gene dosage for CaMKII expression in several TG founder lines (Zhang *et al.*, 2003). Further studies exploring the mechanisms by which CaMKII expression induced these phenotypic changes examined changes in the phosphorylation state of Ca^{2+} regulatory proteins in cardiac homogenates and alterations in Ca^{2+}-handling properties in adult cardiomyocytes isolated from the TG mice (Maier *et al.*, 2003; Zhang *et al.*, 2003).

We hypothesized that the initial effects of CaMKIIδ_C on Ca^{2+} handling would be adaptive responses that serve to increase contractile function. Our finding of a rapid increase in CaMKII activation in response to pressure overload induced by TAC (Zhang *et al.*, 2003) is consistent with CaMKII serving such an adaptive role. Enhanced PLB phosphorylation, which would be expected to increase SR Ca^{2+} transport and SR Ca^{2+} stores, was evidenced by the increased PLB phosphorylation at Thr17 site in CaMKIIδ_C TG mouse hearts vs. wild type (WT) (Zhang *et al.*, 2003). We also demonstrated that RyR2 phosphorylation was increased in CaMKIIδ_C TG vs. WT mouse hearts. This was established both by back phosphorylation (Zhang *et al.*, 2003) and through use of a phospho-specific antibody for Ser2809 that showed a significant increase in phospho-RyR2 in TG vs. WT hearts (T. Zhang and J.H. Brown, unpublished data).

As discussed earlier, hyperphosphorylation of RyR2 by PKA is suggested to play a major role in the etiology of heart failure (Marx *et al.*, 2000). We hypothesized that this also might occur with CaMKII-mediated changes in RyR2 phosphorylation and function. The functional consequences of RyR2 phosphorylation were assessed in myocytes isolated from the CaMKIIδ_C TG mice (Maier *et al.*, 2003). Diastolic Ca^{2+} spark frequency was increased in TG myocytes and the sparks were of increased width and prolonged duration. The overall diastolic SR Ca^{2+} leakage was 4.3-fold higher in TG vs. WT cardiomyocytes. Importantly, SR Ca^{2+} release during twitch (relative to the total SR Ca^{2+} pool) was found to be increased in TG vs. WT, despite lower SR Ca^{2+} load and diastolic intracellular Ca^{2+}. Another important finding was that RyR2 phosphorylation and Ca^{2+} spark frequency were increased at early stages, prior to development of failure (assessed by echocardiography), in CaMKIIδ_C TG mouse hearts. We also reported that the increased Ca^{2+} spark frequency in isolated TG myocytes was normalized by acute CaMKII inhibition and that CaMKIIδ was associated with the RyR2 in immunoprecipitation studies (Zhang *et al.*, 2003). Taken together, these data demonstrate that CaMKII mediates RyR2 phosphorylation *in vivo*, that this results in increases in SR Ca^{2+} spark frequency, and that these precede (and could therefore be causal in) the development of failure (Zhang *et al.*, 2003).

Other changes in Ca^{2+} handling were observed in CaMKIIδ_C TG mice, including direct effects of overexpressed CaMKIIδ_C on peak I_{Ca} and FDAR (Maier *et al.*, 2003). Peak I_{Ca} was slightly increased in TG vs. WT and was acutely reversed by CaMKII inhibition. However, CaMKII-dependent I_{Ca} facilitation still was present in TG mice, suggesting that CaMKII activation in the TG mouse hearts was not sufficient to saturate I_{Ca} facilitation. FDAR was only slightly (albeit significantly) enhanced in TG mice (Maier *et al.*, 2003), implying that CaMKII is involved in FDAR but that endogenous CaMKII levels in WT mice may not be rate limiting. Changes in SERCA (decreased) and NCX (increased) expression and function observed in CaMKII TG mice are presumably secondary to heart failure development in these mice, since there is no evidence that CaMKII can directly downregulate SERCA2a or upregulate NCX function. Similar changes in SERCA2a and NCX function are seen in numerous heart failure models.

VIII. Ca^{2+} and Apoptosis

A. IMPORTANCE OF APOPTOSIS IN ISCHEMIA/REPERFUSION INJURY AND THE DEVELOPMENT OF HEART FAILURE

Apoptosis or programmed cell death plays an important role in development and has been implicated in diseases, including cancer, autoimmune diseases, and

degenerative disorders. Apoptosis is widely recognized as a factor contributing to heart failure. Over the last decade, it has been shown that apoptotic cell death is associated with myocardial infarction, ischemia/reperfusion, and cardiomyopathy (Gottleib *et al.*, 1994; Olivetti *et al.*, 1997). The development of apoptosis is highly regulated in a step-by-step fashion by many signaling events, providing the potential for multiple sites of therapeutic intervention. There are two relatively distinct pathways in apoptosis: the intrinsic (mitochondrial) pathway and the extrinsic (death receptor) pathway. Both eventually converge on caspase-3, a key component of the apoptotic machinery.

In response to ischemia/reperfusion, both apoptosis and necrosis are observed and the contribution of each to cardiomyocyte cell death is controversial. However, it has been reported that caspase inhibitors, which block apoptosis, protect against ischemia/reperfusion damage (Yaoita *et al.*, 1998). A mitochondrial permeability transition pore inhibitor has been shown to be protective against ischemia/reperfusion in ventricular myocytes and isolated hearts (Griffiths and Halestrap, 1993; Murata *et al.*, 2001). These findings implicate apoptosis in the pathological response to ischemia/reperfusion. Interestingly, while the border zone of infarction shows apoptotic cell death, infarct zones in the myocardium show necrosis, raising the possibility that cell death starts by way of the more-tidy apoptotic pathway but ultimately regresses to one with necrotic features (Yaoita *et al.*, 1998).

Myocyte loss by apoptosis is recognized as a major contributing factor in the transition from compensated (adaptive) hypertrophy to heart failure (maladaptation). Conditional KO of gp130, a receptor through which cytokines regulate cardiomyocyte survival, has been reported to result in massive induction of myocyte apoptosis and rapid onset of dilated cardiomyopathy in response to aortic banding (Hirota *et al.*, 1999). Direct induction of apoptosis by conditional caspase activation also induces heart failure in transgenic mice (Wencker *et al.*, 2003). Activation of the Gαq signaling pathway, when sustained or exacerbated, induces heart failure with apoptosis (Adams *et al.*, 1998,2000). The peripartum cardiomyopathy seen in Gαq transgenic mice is markedly attenuated by caspase inhibition (Hayakawa *et al.*, 2003).

B. CA^{2+}, PERMEABILITY TRANSITION PORE, AND CARDIOPROTECTION

Many experimental models of cardiomyocyte apoptosis — including ischemia/reperfusion and sustained Gαq signaling — are associated with mitochondrial changes due to the opening of the mitochondrial permeability transition pore (PT pore) (Halestrap *et al.*, 1997; Adams *et al.*, 2000). The PT pore is considered to be a megachannel with permeability to ions and solutes up to $\approx$ 1500 daltons. Although not all of the molecules comprising the PT pore are

known, the adenine nucleotide translocator (ANT) and voltage-dependent anion channel (VDAC) are among them. Long-lasting PT pore opening has been reported to dissipate the mitochondrial membrane potential and decrease ATP production (Kroemer and Reed, 2000). Cytochrome *c* is released through the PT pore and acts, along with other cytosolic factors, to promote caspase-9 activation. Subsequent caspase-3 activation mediates DNA cleavage into nucleosomal fragments. PT pore opening has been reported to be regulated by several factors. Increases in Ca^{2+}, reactive oxygen species (ROS), and proapoptotic Bcl-2 family proteins (e.g., Bad, Bax, tBid) induce PT pore opening, while antiapoptotic Bcl-2 family proteins such as Bcl-2 and Bcl-xl inhibit the PT pore (for a review, see Kroemer and Reed, 2000).

The involvement of Ca^{2+} in regulating mitochondrial function has been appreciated for many years. Mitochondria take up cytosolic Ca^{2+} via a Ca^{2+} uniporter. Recent studies show that mitochondrial Ca^{2+} changes in a beat-to-beat manner in cardiomyocytes (Robert *et al.*, 2001). Furthermore, a close relationship between the SR and mitochondria in cardiomyocytes is illustrated by studies demonstrating that Ca^{2+} derived from SR (via Ca^{2+}-induced Ca^{2+} release mechanism) is efficiently transmitted to the mitochondria (Szalai *et al.*, 2000). A more-recent focus has been on the role of mitochondrial Ca^{2+} in PT pore opening (Crompton, 1999; Murata *et al.*, 2001). Experiments using isolated mitochondria or permeabilized cells clearly demonstrate that high concentration of Ca^{2+} can induce PT pore opening (Crompton, 1999). Our unpublished observations demonstrate that ionomycin, a Ca^{2+} ionophore, induces PT pore opening (assessed by loss of mitochondrial membrane potential) and induces DNA laddering in neonatal rat ventricular myocytes. We also have observed that cytosolic Ca^{2+} dysregulation and resultant Ca^{2+} overloading directly induce PT pore opening and apoptosis in cardiomyocytes subject to sustained stimulation of Gαq signaling pathways, while chelation of Ca^{2+} with EGTA (ethylene glycol-bis(beta-amino ethyl ether)-N, N, N′, N′-tetraacetic acid) inhibits these responses (S. Miyamoto and J. H. Brown, unpublished data).

Another mitochondrial channel that may be involved in regulating mitochondrial Ca^{2+} concentration and PT pore function is the mitochondrial ATP-sensitive potassium channel (mitoK_{ATP}). MitoK_{ATP} has attracted considerable interest since Liu and coworkers (1998) reported that diazoxide selectively opens these channels and confers cardiac protection against subsequent ischemia. Although not all of the operative cardioprotective mechanisms have been elucidated, it has been shown that diazoxide treatment inhibits mitochondrial Ca^{2+} overloading (Murata *et al.*, 2001). Three mechanisms by which mitoK_{ATP} channel opening could lead to protection have been suggested. One is that K^+ influx reduces the driving force for Ca^{2+} by mitochondrial membrane depolarization (Holmuhamedov *et al.*, 1999). Second, opening the mitoK_{ATP} channel induces moderate production of ROS, which contributes to cardioprotection by

stimulating protein kinases, in particular, PKC (Forbes *et al.*, 2001). Third, it has been proposed that treatment with diazoxide results in release of mitochondrial Ca^{2+} by inducing flickering PT pore opening (low-conductance state) (Katoh *et al.*, 2002).

Recently, another Ca^{2+}-dependent pathway has been suggested to be responsible for cytochrome *c* release during ischemia/reperfusion (M. Chen *et al.*, 2001,2002). Calpain, a Ca^{2+}-dependent cysteine protease, is activated by the rapid influx of Ca^{2+} stimulated by reperfusion and cleaves Bid, a proapoptotic BH3-only Bcl-2 family member. The product of the cleavage, tBid, induces mitochondrial dysfunction. Calpain has been recognized as an important mediator of necrotic cell death through proteolysis; thus, these results suggest possible cross-talk between apoptotic and necrotic pathways.

C. CAMK INVOLVEMENT IN APOPTOSIS

There is limited but compelling evidence that CaMKII can mediate signal transduction in apoptosis. Selective inhibitors of CaMKII significantly inhibit apoptotic responses induced by tumor necrosis factor alpha (TNFα), ultraviolet irradiation, and the natural toxin microcystin (Wright *et al.*, 1997; Fladmark *et al.*, 2002). In addition, overexpression of active forms of CaMKII can induce apoptosis (Fladmark *et al.*, 2002). CaMKII has been shown to regulate expression and phosphorylation of c-FLIP (cellular Fas-associated death domain-like interleukin-1β-converting enzyme inhibitory protein), thus modulating Fas-mediated signaling (Yang *et al.*, 2003). Our recent collaborative studies demonstrated that apoptosis induced by β1-adrenergic receptor stimulation in adult mouse cardiomyocytes occurs through activation of CaMKII rather than PKA (Zhu *et al.*, 2003). We reported that CaMKII inhibition protected cardiomyocytes against apoptosis and that expression of the cytoplasmic CaMKIIδ_C (but not the nuclear CaMKIIδ_B) enhanced the apoptotic response (Zhu *et al.*, 2003). Further investigation is needed to elucidate the downstream targets of CaMKII in apoptotic signaling pathways. However, it is intriguing to consider that chronic activation or increased expression of CaMKIIδ_C not only contributes to the altered Ca^{2+} handling and contractile dysfunction associated with heart failure but also serves as a mediator of apoptotic loss of cardiomyocytes.

IX. Summary

Changes in intracellular Ca^{2+} underlie multiple cardiomyocyte responses and are critical to the fundamental function of the heart as a pump. Studies carried out over the past decade demonstrate that CaMK is a ubiquitous transducer of Ca^{2+} signals. Thus, the possible role of CaMKII in cardiac function has generated considerable interest. The discovery of distinct CaMKIIδ splice

variants that localize to the cytoplasm vs. the nucleus suggests that there could be precise spatial regulation of CaMKII activation and function in cardiomyocytes. Whether the cardiac CaMKII isoforms are differentially regulated by Ca^{2+} mobilized in response to particular stimuli or arising from distinct sources remains to be determined. Data presented herein suggest that the isoforms may play distinct roles in transcriptional regulation, Ca^{2+} homeostasis, and control of apoptosis. Information on dynamic changes in CaMKII isoform activation during the cardiac contraction cycle and on more-chronic changes in CaMKII activation and expression in adaptive and maladaptive phases of cardiac diseases will be necessary to gain further insight into physiological and pathological functions of CaMKII. It is abundantly clear, however, that there are multiple avenues for manipulating cardiac Ca^{2+}/CaMKII signaling pathways that hold considerable therapeutic potential.

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Hypertension and Obesity

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ABSTRACT

Obesity is a common problem in much of the western world today in that is linked directly with several disease processes, notably, hypertension. It is becoming clear that the adipocyte is not merely an inert organ for storage of energy but that it also secretes a host of factors that interact with each other and may result in elevated blood pressure. Of particular importance is the putative role of leptin in the causation of hypertension via an activation of the sympathetic nervous system and a direct effect on the kidneys, resulting in increased sodium reabsorption leading to hypertension. Obesity *per se* may have structural effects on the kidneys that may perpetuate hypertension, leading to an increased incidence of end-stage renal disease that results in further hypertension. Adipose tissue may elaborate angiotensin from its own local renin-angiotensin system. The distribution of body fat is considered important in the genesis of the obesity-hypertension syndrome, with a predominantly central distribution being particularly ominous. Weight loss is the cornerstone in the management of the obesity-hypertension syndrome. It may be achieved with diet, exercise, medications, and a combination of these measures. Anti-obesity medications that are currently undergoing clinical trials may play a promising role in the management of obesity and may also result in lowering of blood pressure. Antihypertensives are considered important components in the holistic approach to the management of this complex problem.

I. Introduction

Obesity is rapidly turning into an "epidemic" afflicting much of the industrialized world, resulting in a prohibitive health and economic burden on society (Mark *et al.*, 1999a; Hall *et al.*, 2001,2002; Sowers, 2001). A predominantly "central" or "visceral" obesity pattern triggers a myriad of cardiovascular, renal, metabolic, prothrombotic, and inflammatory responses that are essentially maladaptive, some of which constitute the "cardio-metabolic syndrome" (to be defined). These responses, individually and interdependently, lead to a substantial increase in cardiovascular disease (CVD) morbidity and mortality, including

hypertension, coronary heart disease (CHD), congestive heart failure (CHF), sudden cardiac death (SCD), stroke, and end-stage renal disease (ESRD) (Garrison *et al.*, 1987; Mark *et al.*, 1999a; Zhang and Reisin, 2000; Hall *et al.*, 2001,2002, Sowers, 2001). This review will highlight the causative role that adipose tissue may play in the genesis of hypertension, or what recently has been referred to as "obesity hypertension." Epidemiological studies have revealed a strong relation between obesity and hypertension. Data from the National Health and Nutrition Examination Survey (NHANES) (Figure 1) show a remarkable and linear relationship between rise in body mass index (BMI) and systolic, diastolic, and pulse pressures in the American population. In regression models corrected for age-related rise in blood pressure, a BMI gain of 1.7 kg/m^2 in men and 1.25 kg/m^2 in women or an increase in waist circumference of 4.5 cm for men and 2.5 cm for women corresponds to an increase in systolic blood pressure of 1 mmHg (Kissebah and Krakower, 1994). Obesity by itself possibly accounts for 78% and 65% of essential hypertension in men and women, respectively, according to data from the Framingham Cohort (Kannel *et al.*, 1993). Animal experiments and human studies have confirmed this causation and given insight into the mechanisms involved (Reisen *et al.*, 1978; Alexander *et al.*, 1979; Reaven, 1993; Carrol

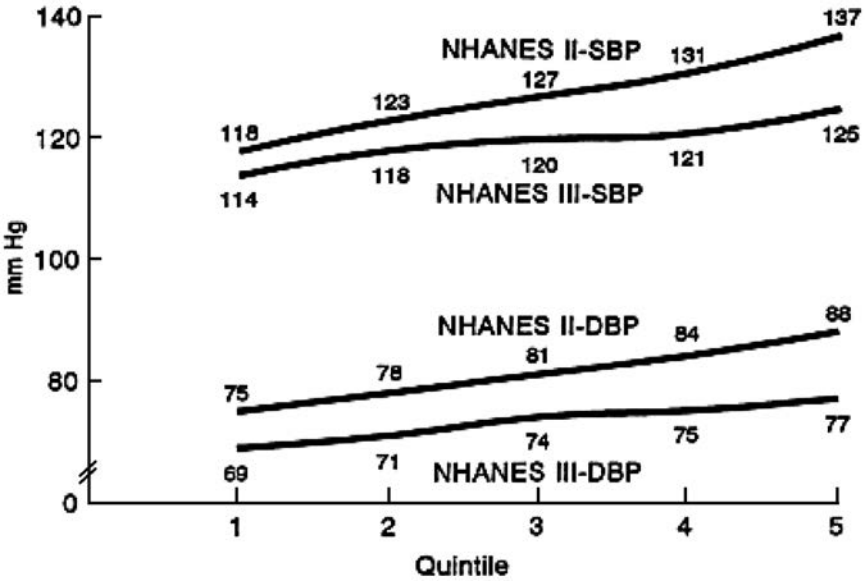

FIG. 1. Line graph shows age-adjusted systolic blood pressure (SBP) and diastolic blood pressure (DBP) from the National Health and Nutrition Examination Surveys (NHANES) II and III by NHANES III quintile of body mass index. [Source: Centers for Disease Control and Prevention, National Centers for Health Statistics.]

et al., 1995; Van Vleit *et al.*, 1995; Hall *et al.*, 1996; Hall, 1997; Rocchini *et al.*, 1999; Antic *et al.*, 2000, Dobrian *et al.*, 2000; Weyer *et al.*, 2000; Rocchini, 2002). Hyperinsulinemia, hyperleptinemia, hypercortisolemia, renal dysfunction, altered vascular structure and function, enhanced sympathetic and renin-angiotensin system activity, and blunted natriuretic peptide activity stand out as major contributory mechanisms to "obesity hypertension" (Tuck *et al.*, 1981; Rocchini *et al.*, 1989a; Hall *et al.*, 1993a,1996,2001,2002; Kissebah and Krakower, 1994; Cignolini *et al.*, 1995; Mark *et al.*, 1999a; Mansuo *et al.*, 2000; Zhang and Reisin, 2000; Engeli and Sharma, 2001,2002).

II. Epidemiology

The prevalence of obesity is rapidly increasing in developing as well as industrialized countries (Mark *et al.*, 1999a; Hall *et al.*, 2001,2002; Sowers, 2001). Up to 61% of Americans are either overweight or obese, according to data from the 1999 census. Economic costs attributable to obesity in the United States in 2000 were estimated at $117 billion (Garrison *et al.*, 1987; Flegal *et al.*, 1998; Zhang and Reisin, 2000; Sowers, 2001). Increasing obesity is common to all developed societies, particularly among children. The Nurses' Health Study involving 80,000 women revealed that a 5-kg weight gain after age 18 was associated with a 60% higher relative risk of developing hypertension (Huang *et al.*, 1998), compared to those women who gained 2 kg or less. Those who gained 10 kg or more increased their risk by 2.2-fold. Similar increases have been observed in other populations (Wilsgaard *et al.*, 2000) and in children (He *et al.*, 2000).

Cardio-metabolic syndrome prevalence has been estimated at 22% of the U.S. population. It increases with age and varies according to ethnicity. Mexican-Americans have the highest age-adjusted prevalence (31.9%) (Sowers, 2001). Among African-Americans and Mexican-Americans, the prevalence is nearly twice as high in women than in men (57% and 26% higher prevalence, respectively) (Kissebah and Krakower, 1994; Cignolini *et al.*, 1995; Hall *et al.*, 2001,2002).

III. Definitions

A. BODY MASS INDEX

Overweight is defined as a BMI $>$ 25. (BMI is body weight in kilograms divided by the height in meters, squared, expressed as wt (in kg)/height (m^2).) Obesity is defined as a BMI $>$ 30; morbid obesity is a BMI $>$ 35 (Sowers, 2001).

B. CENTRAL OBESITY

The distribution of weight gain is of crucial importance. A predominantly "central" pattern of weight gain, as measured by a waist-to-hip ratio, is considered more ominous from cardiovascular and glycemic standpoints because it confers a far higher risk than that expected by BMI measurements. Central obesity is often referred to as abdominal, upper-body, male-type, android, or visceral obesity vs. female-type or gynoid obesity, where there is preferential fat accumulation in the gluteal and femoral distribution (Kissebah and Krakower, 1994; Hall, 1997; Hall *et al.*, 2001,2002; Rocchini *et al.*, 2002). Abdominal obesity is diagnosed clinically by a waist-to-hip ratio that is > 0.95 in men and $>$ 0.85 in women. Although clinical measures are considered acceptable in the diagnosis of true central obesity, better imaging techniques such as dual-energy X-ray absorptiometry (DEXA) or an abdominal computed tomography (CT) scan probably are required for accurate description. The pattern of obesity is important. For example, athletes such as football players often have BMIs higher than 35, making them morbidly obese by definition. A closer look may reveal a total body fat lower than 8%, quite different than the figures for those who are truly obese. Therefore, obesity defined solely by BMI may be an imperfect approximation. Clinicians must always be aware of this possible fallacy, further emphasizing the importance of the pattern of fat distribution.

C. CARDIO-METABOLIC SYNDROME

The presence of an interesting constellation of findings variously referred to as the cardio-metabolic syndrome, the metabolic syndrome, the deadly quartet, insulin resistance syndrome, and syndrome X is now an established predictor for premature and often severe CVD.

The metabolic syndrome is defined by guidelines from the National Cholesterol Education Program (Adult Treatment Panel (ATP) III). These guidelines suggest that the clinical identification of the metabolic syndrome be based upon the presence of any three of the following: 1) abdominal obesity, defined as a waist circumference in men $>$ 102 cm (40 inches) and in women $>$ 88 cm (35 inches) (it was noted by ATPIII that some men with lower waist circumference (i.e., 94–102 cm) may develop insulin resistance due to genetic factors); 2) triglycerides $\geq$ 150 mg/dL (1.7 mmol/L); 3) high-density lipoprotein (HDL) cholesterol $<$ 40 mg/dL (1 mmol/L) in men and $<$ 50 mg/dL (1.3 mmol/L) in women; 4) blood pressure $\geq$130/$\geq$85 mmHg; or 5) fasting glucose $\geq$ 110 mg/dL (6.1 mmol/L) (National Institutes of Health, 1998).

The World Health Organization (WHO) has proposed a different definition for the metabolic syndrome. A diagnosis of the metabolic syndrome required the presence of either hyperinsulinemia or a fasting plasma glucose $\geq$ 110 mg/dl (6.1 mmol/L) and an additional two characteristics from among the following: 1)

abdominal obesity, defined as a waist-to-hip ratio > 0.90, a BMI ≥ 30 kg/m^2, or a waist girth ≥ 94 cm (37 inches); 2) dyslipidemia, defined as serum triglyceride ≥ 150 mg/dL (1.7 mmol/L) or HDL cholesterol < 35 mg/dL (0.9 mmol/L); or 3) blood pressure $\geq 140/90$ mmHg or the administration of antihypertensive drugs.

IV. Alterations in Renal Function and Structure, Leading to Obesity-related Hypertension

A. RENAL HEMODYNAMIC CHANGES

A high-fat diet consistently results in enhanced sodium reabsorption by the kidneys (Figure 2). Interestingly, weight loss in the same individual promotes urinary sodium excretion (Figure 3), demonstrating a direct link between BMI and sodium retention (Tuck *et al.*, 1981; Lucas *et al.*, 1985; Reaven and Hoffman, 1987; Landsberg and Krieger, 1989; Rocchini *et al.*, 1989a; Tuck, 1992; Reisin *et al.*, 1993; Baron *et al.*, 1995; Kassab *et al.*, 1995; Hall *et al.*, 1996,1999a; Sugarman *et al.*, 1997; Bloomfield *et al.*, 2000; Stern *et al.*, 2001; U.S. Renal Data System, 2001). Obesity is associated with activation of the renin-angiotensin system (RAS) (Tuck, 1992; Zhang and Reisin, 2000; Hall *et al.*, 2001,2002), increased sympathetic nervous system (SNS) activity (Landsberg and Krieger, 1989; Carrol *et al.*, 1995; Mark *et al.*, 1999a; Weyer *et al.*,

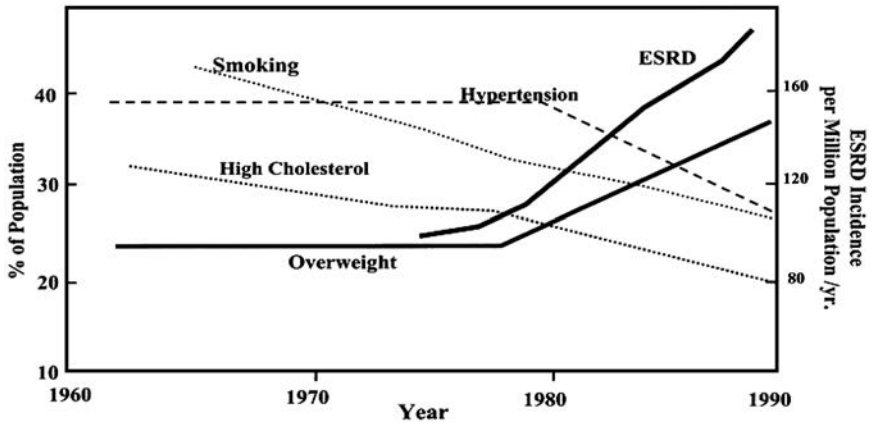

FIG. 2. Estimated prevalence of cardiovascular risk factors, as assessed by NHANES I and II and NHANES II incidence of end-stage renal disease (ESRD) reported by the United States Renal Data Systems Surveys. [Reprinted with permission from Hall JE, Brands MW, Dixon WN, Smith MJ Jr 1993 Obesity-induced hypertension: renal function and systemic hemodynamics. Hypertension 22:292–299. Copyright Lippincott Williams & Wilkins.]

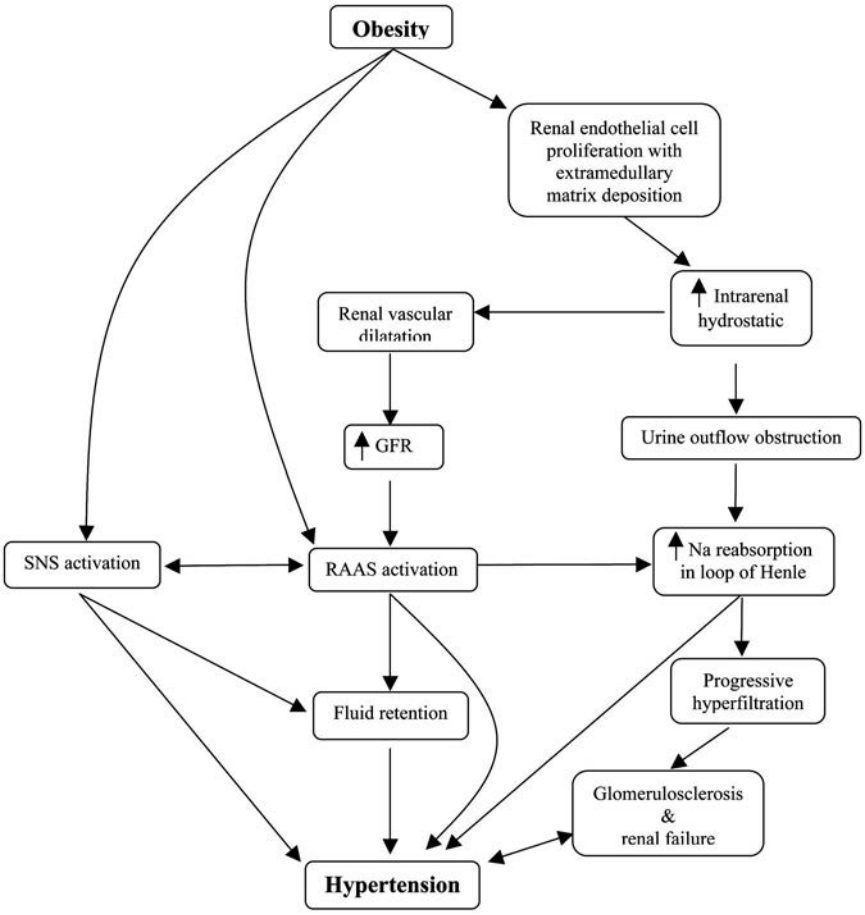

FIG. 3. Mechanisms responsible for progressive renal failure in obesity hypertension. Structural and functional alterations in the kidneys collaborate, resulting in activation of the sympathetic nervous system (SNS) and the renin-angiotensin aldosterone system (RAAS), leading to fluid retention. Once ESRD develops, it perpetuates hypertension. Abbreviations: GFR, glomerular filtration rate; Na, sodium. [Reprinted with permission from Rocchini AP, Key J, Bordie D, *et al.* 1989 The effect of weight loss on the sensitivity of blood pressure to sodium in obese adolescents. N Engl J Med 321:580–585. Copyright 1989 The Massachusetts Medical Society.]

2000; Abate *et al.*, 2001; Hall *et al.*, 2001), and hyperinsulinemia (Reaven, 1993; Weyer *et al.*, 2000), all of which may contribute to sodium reabsorption and associated fluid retention and may therefore contribute to renal obesity hypertension (Reisen *et al.*, 1993; Burt *et al.*, 1995; Hall *et al.*, 1999b; Engeli and Sharma, 2001; Stern *et al.*, 2001). A compensatory lowered renal vascular

resistance, elevated kidney plasma flow, increased glomerular filtration rate, and the higher blood pressure associated with obesity are important in overcoming increased sodium reabsorption. Neurohumoral factors like angiotensin II (AII), sympathetic system, and cytokines — either individually or synergistically — are involved in these compensatory mechanisms. In obese hypertensives, an inappropriately small natriuretic response to a saline load at mean arterial or intraglomerular pressure is seen and is often referred to as impaired pressure natriuresis (Figure 3) (Kassab *et al.*, 1995; Hall *et al.*, 1996; Hall, 1997; Engeli and Sharma, 2001). These adaptive changes increase glomerular wall stress and, especially in the presence of other risk factors such as hyperlipidemia and hyperglycemia, may provoke glomerulosclerosis, proteinuria, microalbuminuria, and loss of nephron function in the "obese" kidney, even before structural microscopic changes are evident. The combination of these potentially nephrotoxic mechanisms, including hyperfiltration and hypertension, may initiate and, indeed, perpetuate renal damage. The early glomerular hyperfiltration in obesity is often as great as that observed in uncontrolled type I diabetes.

B. RENAL STRUCTURAL CHANGES

Altered intrarenal physical forces are believed to play a role in sodium retention, mainly by slowing down the tubular flow rate (Weisinger *et al.*, 1974; Reisen *et al.*, 1984; Wesson *et al.*, 1985; Arnold *et al.*, 1994; Hall, 1994; Alonso-Galicia *et al.*, 1995; Bruzzi *et al.*, 1997; Sugarman *et al.*, 1997; Hall *et al.*, 1998; Bloomfield *et al.*, 2000; Henegar *et al.*, 2001; Kambham *et al.*, 2001; Okosun *et al.*, 2001; Sundquist *et al.*, 2001). Observations from obese animals and humans have shown an increase in renal weight attributable to endothelial cell proliferation and intrarenal lipid and hyalouronate deposition in the matrix and inner medulla. These depositions in the tightly encapsulated kidney lead to distortion of the intrarenal mechanical forces. The interstitial fluid hydrostatic pressure is elevated to 19 mmHg in obese dogs, compared to 9–10 mmHg in lean dogs. This increase in pressure and volume causes parenchymal prolapse and urine outflow obstruction, resulting in slow intrarenal flow and increased sodium reabsorption. Of particular importance is the increased sodium reabsorption in the loop of Henle, which leads to a feedback-mediated renal vascular dilation, elevation of glomular filtration rate (GFR), and stimulation of the renin-angiotensin aldosterone system (RAAS), despite volume expansion overcoming the increased tubular reabsorption and maintaining sodium balance. However, persistent glomerular hyperfiltration — in combination with glucose intolerance, hyperlipidemia, and hypertension — results ultimately in glomerulosclerosis and renal failure.

The structural changes occurring in the human kidney as a consequence of obesity have been demonstrated in a large retrospective human study that

incorporated 6800 renal biopsies. Obesity was associated with a peculiar obesity-related glomerulopathy, defined as focal segmental glomerulosclerosis associated with enlargement of the glomerulus itself. The mean BMI in these patients was 41.7 kg/m^2. Compared with the idiopathic variety, obesity-associated focal segmental glomerulosclerosis had lower rates of nephrotic syndrome, fewer lesions of segmental sclerosis, and a greater glomerular size. The disease course in this population was dictated largely by the presence of co-morbid conditions such as hypertension and dyslipidemia. A recent study of the German WHO MONICA (Monitoring of Trends and Determinants in Cardiovascular Disease) Augsberg study population found a steady increase in the presence of microalbuminuria, an early marker of renal vascular damage with quintiles of waist-to-hip ratio. In this study, the odds ratio for the development of microalbuminuria for individuals with central obesity was not very different from that for patients with hypertension. Thus, over a span of time, obesity by itself may perpetuate hypertension by these structural damages.

The parallel increase in the prevalence of obesity and ESRD (Figure 4), in addition to the close association between obesity and type II diabetes and hypertension that are the two major risks of ESRD, has led to speculation that obesity may account for at least half of ESRD in the United States.

In summary, persistent obesity causes renal injury and functional nephron loss, contributing to elevated blood pressure, which in turn leads to further renal injury, thereby setting off a vicious circle of events leading to further elevated BP and renal injury. Interestingly enough, it is difficult to dissociate the cause from the effect in this circle, since the overall burden of obesity may be strongly time dependent (Figure 4).

V. Role of SNS, Biochemical, Neurochemical, and Hormonal Mediators

A. SYMPATHETIC NERVOUS SYSTEM

Several mechanisms and mediators have been postulated as causative in the genesis of adrenergic overactivity in the obese (Hall *et al.*, 1993b; Grassi *et al.*, 1995,1998; Rumantir *et al.*, 1999; Esler, 2000; Mansuo *et al.*, 2000; Weyer *et al.*, 2000). Notable are elevated insulin levels with insulin resistance; activation of renal afferent nerves that, in turn, are stimulated by increased intrarenal pressures, leading to the subsequent activation of renal mechanoreceptors; plasma free fatty acids (FFAs); angiotensin II; elevated leptin levels; potentiation of central chemoreceptor sensitivity; and impaired baroreflex sensitivity.

There are several reasons to believe that SNS activation may contribute significantly to the obesity-hypertension syndrome. Obese subjects have elevated sympathetic activity, measured both directly and indirectly. A diet that results in obesity has been found to increase baseline plasma norepinephrine levels, to

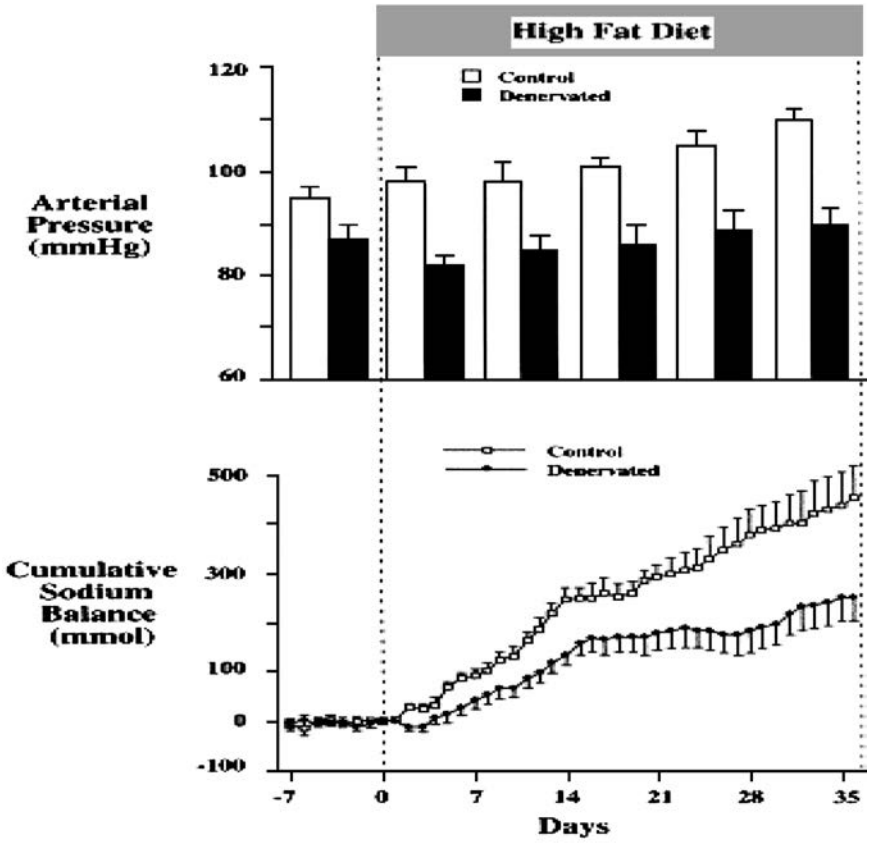

FIG. 4. Effects of 5 weeks of a high-fat diet on mean arterial pressure and cumulative sodium balance in dogs with innervated kidneys (control) and bilaterally denervated kidneys (denervated). [Reprinted with permission from Kassab S, Kato T, Wilkins C, Chen R, Hall JE, Granger JP 1995 Renal denervation attenuates the sodium retention and hypertension associated with obesity. Hypertension 25:893–897.]

amplify the SNS response to stimuli such as handgrip and upright posture, and to increase catecholamine turnover in peripheral tissues. In addition to elevated renal sympathetic activity, muscle sympathetic activity is elevated in obese hypertensive subjects, when measured with microneurographic methods (Grassi *et al.*, 1998). Weight loss, on the other hand, reduces sympathetic activity, which further supports this hypothesis. Interestingly, Pima Indians do not demonstrate a markedly increased sympathetic tone when obese and also do not get hypertensive to the same degree as other racial groups. Also, medications that abolish or diminish the central sympathetic drive cause a greater blood pressure reduc-

tion in obese as compared to lean subjects (Hildebrandt *et al.*, 1993; Licata *et al.*, 1994a; Wofford *et al.*, 2001). Indeed, in dogs fed a high-calorie diet, a combined blockade of α and β receptors lowered blood pressure more significantly in obese than in lean dogs. Clonidine, which blocks central $\alpha 2$ receptors and thereby markedly reduces the central sympathetic drive, blunts hypertension in dogs fed a high-calorie diet. Similar results have been observed in humans. To further support the hypothesis, sympathetic denervation of the kidneys has a significant negative effect on renal sodium retention and thereby on obesity hypertension. In dogs fed a high-fat diet, renal nerves appear crucially important for sodium retention and hypertension. In dogs with denervated kidneys, sodium retention was markedly attenuated, leading to a lower blood pressure. Therefore, it appears that renal nerves play a pivotal role in salt retention, impaired pressure natriuresis, and hypertension.

B. INSULIN RESISTANCE AND HYPERINSULINEMIA

Hyperinsulinemia was considered a key factor governing obesity-induced hypertension in the recent past. It is well known that obese subjects have markedly elevated insulin levels, which are required to maintain glucose and fatty acid metabolism, and often are resistant to the actions of insulin on peripheral tissues (DeFronzo *et al.*, 1975; Rocchini *et al.*, 1990,1996; Hall *et al.*, 1993b; Bjorntorp and Rosmond, 2000). This resistance to insulin is not universal to all tissues; some tissues retain their sensitivity to its effects, referred to as selective insulin resistance. Studies in the acute setting suggest that insulin may contribute to sodium retention and sympathetic activity and therefore to hypertension (Rocchini *et al.*, 1987,1989b). However, on further investigation, elevated insulin levels in both acute and chronic settings have not been found to affect salt excretion, sympathetic activity, and blood pressure in humans and dogs (Hall *et al.*, 1986,1995; Hildebrandt *et al.*, 1999a). In fact, infusions to raise insulin levels to match those found in the obese actually lower blood pressure, suggesting a dominant depressor effect for insulin. Even in obese dogs resistant to the metabolic effects of insulin, infusions did not have a pressor effect. A central nervous system (CNS) infusion of insulin did not demonstrate a change in blood pressure in the dog.

In rats, however, hyperinsulinemia does elevate blood pressure. The underlying mechanisms are probably mediated by a complex interaction between insulin, the RAAS, and thromboxane (TXA2) activity. This hypothesis was further tested when blockade of RAAS or TXA2 abolished the blood pressure rise (Brands *et al.*, 1997; Keen *et al.*, 1997; Sechi, 1999). It therefore appears that the initial enthusiasm generated by rodent studies in hyperinsulinemia as causative for hypertension in the obese may not be extrapolated to humans.

However, some researchers believe that insulin resistance plays a part in blood pressure regulation. This association, although controversial, is based upon the following hypotheses. There is evidence that insulin-mediated vasodilatation is impaired in an insulin-resistant state, which may lead to hypertension (Laasko *et al.*, 1990). In healthy subjects, insulin infusions stimulate endothelin-1 and nitric oxide (NO) activity (Cardillo *et al.*, 2000). In subjects who have developed hypertension, reduced insulin-mediated vasodilatation is associated with reduced endothelial NO production. Also, insulin increases renal sodium reabsorption (Gupta *et al.*, 1992). Obese individuals tend to have glomerular hyperfiltration and these increased filtration rates correlate well with fasting insulin levels (Ribstein *et al.*, 1995). A class of drugs called thiazolidinediones that are insulin sensitizers has been shown to have antihypertensive properties in rats and humans. It has been demonstrated that troglitazone significantly reduced both systolic and diastolic blood pressures, as assessed by 24-hour blood pressure monitoring in normotensive, insulin-resistant human subjects associated with improved insulin sensitivity and glucose tolerance (Nolan *et al.*, 1994).

C. RESISTIN

Resistin is a newly discovered signaling protein that probably plays an important role in the development of insulin resistance and is thought to be the missing link between obesity and diabetes. The role of resistin in hypertension is being researched. A recent study from China reported that resistin gene polymorphism is an independent factor associated with systolic and diastolic blood pressures in type 2 diabetics. Diabetics with the GG genotype were found to have a higher prevalence of hypertension in this population (Tan *et al.*, 2003). Other reports in Caucasian and Japanese populations found no association between resistin gene polymorphism and type 2 diabetes but the single nucleotide polymorphism (SNP) in the promoter region was a significant predictor of the insulin-sensitivity index. It was therefore hypothesized that noncoding SNPs in the resistin gene might influence insulin sensitivity in interaction with obesity.

D. FREE FATTY ACIDS

High FFA levels are thought to raise blood pressure by increasing sympathetic activity or enhancing the sympathetic vascular responses (Stepniakowski *et al.*, 1995; Grekin *et al.*, 1997). Subjects with visceral obesity deliver a FFA load to the liver that, in turn, activates hepatic afferent pathways that may lead to sympathetic activation and contribute to insulin resistance (Bergman *et al.*, 2001). FFA levels in obese subjects are approximately 2-fold higher than in lean subjects. Also, an acute rise in FFA levels induced by intralipid infusion increases vascular reactivity to α-adrenergic agonists. They may also enhance reflex vasoconstrictor responses in the peripheral circulation. Infusions of oleic

acid into the portal of systemic circulation in rats increased their blood pressure and heart rates. These effects were abolished by adrenergic blockade. Interestingly, however, portal vein infusion caused a greater blood pressure rise than systemic infusions, emphasizing the potential role of afferent pathways in the liver. In humans, high rates of intralipid and heparin infusion caused a small (i.e., 4 mmHg) increase in mean blood pressure (Steinberg *et al.*, 1997). On the other hand, long-term effects of FFAs on blood pressure are less clear and warrant further investigation (Hildebrandt *et al.*, 1999b). In dogs, infusion of long-chain fatty acids had no effect on blood pressure, in contrast to rats that demonstrated a rise in blood pressure and heart rate. Neither cerebral (via vertebral artery) nor systemic infusion of long-chain fatty acids in dogs has a significant effect on arterial pressure, renal function, or hemodynamic responses. Therefore, the relationship between elevated FFA levels and hypertension currently is tenuous at best and merits further investigation.

E. LEPTIN

Leptin is a 167-amino acid peptide that has been the subject of intense research and discussion in recent years due to its possible role in the obesity-hypertension syndrome. Figure 5 schematically demonstrates the possible mechanisms via which leptin may exert its pressor effects (Zhjang *et al.*, 1994; Pellymounter *et al.*, 1995; Lee *et al.*, 1996; Haynes *et al.*, 1997,1998a; Casto *et al.*, 1998; Flier, 1998; Lu *et al.*, 1998; Onions *et al.*, 1998; Shek *et al.*, 1998; Hall *et al.*, 1999b; Mark *et al.*, 1999b; Lembo *et al.*, 2000; Rahmouni *et al.*, 2001; Carlyle *et al.*, 2002). It is secreted by white adipocytes (Maffei *et al.*, 1995; Considine, 2001). Leptin levels correlate well with the body adipose tissue stores. Levels of 5–15 μg/ml are noted in lean individuals and are typically elevated in most obese subjects (Considine *et al.*, 1996). It crosses the blood-brain barrier to the CNS via a saturable, transport-mediated endocytosis, where it binds to its receptors (Ob-R) in the lateral and medial regions of the hypothalamus (Golden *et al.*, 1997). The Ob-Rb is a full-length receptor with a transmembrane domain and a long intracellular carboxyl-terminal tail. The Ob-Ra, Ob-Rc, and Ob-Rd are prematurely terminated receptor proteins with short intracellular tails. The Ob-Re lacks the transmembrane domain and thus may function as a soluble receptor to bind and inactivate the circulating leptin. This binding of leptin to its receptor triggers a series of reactions and neuropeptide pathways that serve to regulate energy balance by reducing appetite and increasing energy expenditure via stimulation of the adrenergic system (McAuley *et al.*, 1993; Thornton *et al.*, 1994; Fan *et al.*, 1997; Huszar *et al.*, 1997; Haynes *et al.*, 1999). Evidence for the fact that leptin acts as a feedback inhibitor of food intake and regulates body weight comes from genetic studies in mice and humans. Mice that lack the ability to synthesize leptin (i.e., ob/ob mice) or that have mutations of the leptin receptor

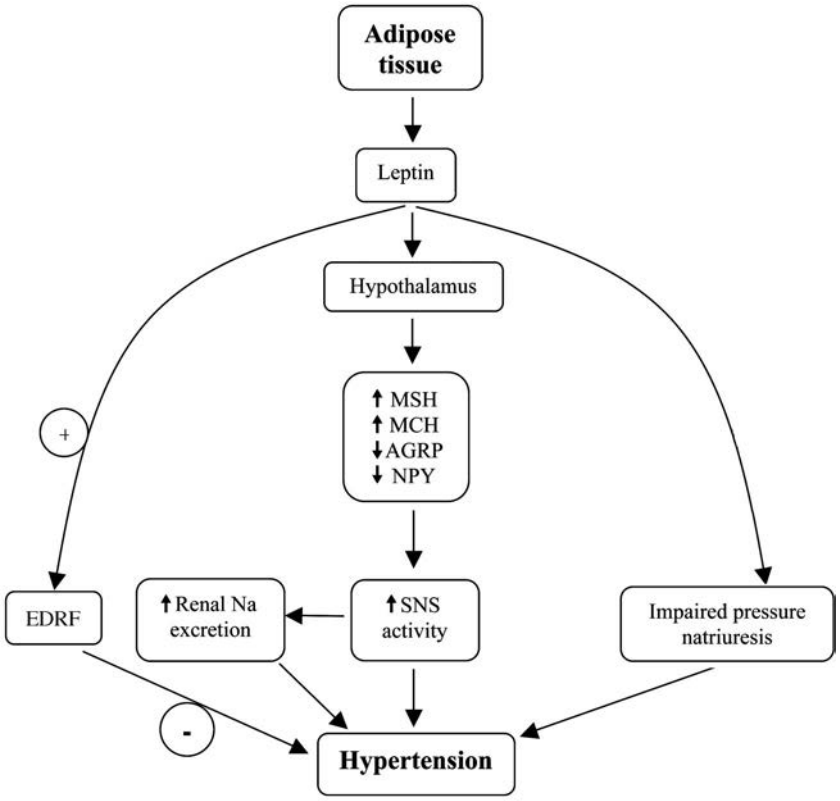

FIG. 5. A summary of the interactions through which leptin is thought to contribute to hypertension. Abbreviations: α-MSH, α-melanocyte-stimulating hormone; MCH, melanin-concentrating hormone; AGRP, agouti-related peptide; NPY, neuropeptide Y; EDRF, endothelium-derived relaxing factor; SNS, sympathetic nervous system; Na, sodium.

(i.e., db/db mice) develop extreme obesity. Mutations of the leptin gene in humans result in extreme obesity but are very rare and do not contribute significantly to the obesity epidemic.

Several rodent studies have indicated that intravenous (IV) and intracerebroventricular (ICV) infusion of leptin increases sympathetic activity in kidneys, adrenals, and brown adipose tissue (BAT) after 2–3 hours of infusion (Figure 6) (Shek *et al.*, 1999; Aizawa-Abe *et al.*, 2000). Leptin infusion in rodents causes mild acute blood pressure elevation, whereas long-term infusions cause a more-significant and sustained rise (Haynes *et al.*, 1998b; Mark *et al.*, 1999b). Failure of blood pressure to rise significantly in the acute scenario may be explained by a concomitant stimulation of NO synthesis, which, in turn, coun-

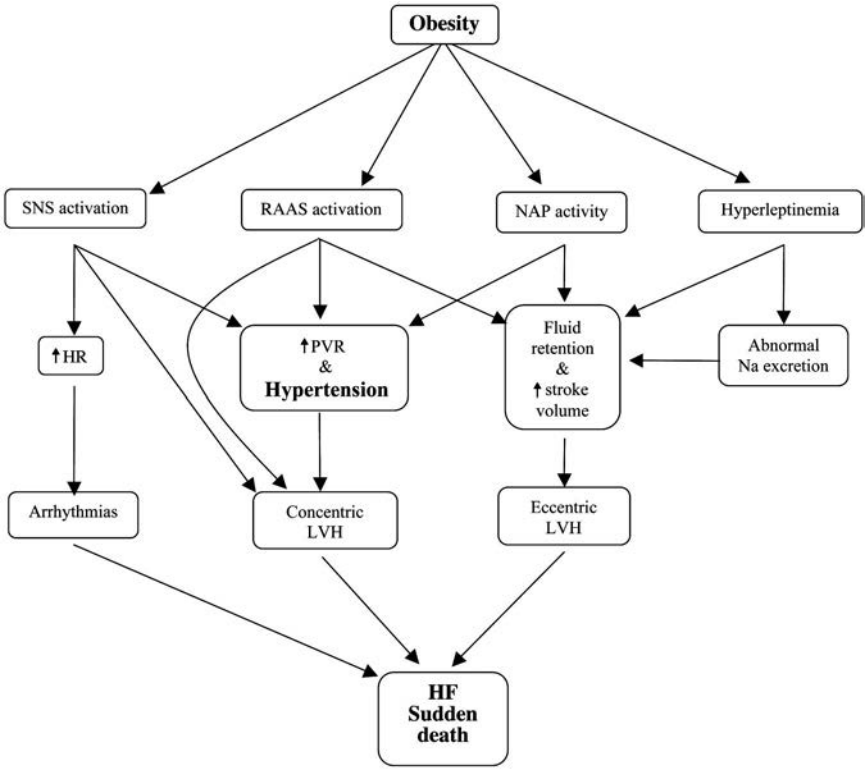

FIG. 6. A summary of the mechanisms by which obesity may lead to excessive cardiovascular morbidity and mortality. Abbreviations: SNS, sympathetic nervous system; RAAS, renin-angiotensin aldosterone system; NAP, natriuretic peptide; HR, heart rate; PVR, peripheral vascular resistance; Na, sodium; LVH, left ventricular hypertrophy; HF, heart failure; ↑, increase.

teracts its pressor effects (Hirose *et al.*, 1998; Suter *et al.*, 1998; Frubeck, 1999; Lembo *et al.*, 2000; Kuo *et al.*, 2001). An alternative explanation is that leptin's sympathetic effect on peripheral vasoconstriction is probably not potent enough to produce significant blood pressure elevation, while the main leptin pressor effect is via the SNS, causing avid renal salt retention and hence hypertension. The rise in blood pressure with leptin infusion is slow in onset and occurs despite a reduction in food intake that accompanies it that would tend to reduce blood pressure. Moreover, the hypertensive effects of leptin are amplified when NO synthesis is impaired experimentally. Conceivably, NO production in the obese is impaired due to endothelial dysfunction.

Transgenic mice that overexpress leptin have elevated blood pressure levels comparable to those produced by leptin infusions. Importantly these chronic

blood pressure elevations are abolished completely by α and β-adrenergic blockade (Shek *et al.,* 1999; El-Haschimi *et al.,* 2000). Also, obese mice that are leptin deficient and obese rats with a leptin receptor mutation usually do not have elevated blood pressure, compared with lean control mice. This suggests that hyperleptinemia with normally functioning leptin receptors is crucial for leptin-induced hypertension (VanHeek *et al.,* 1997; Ishizuka *et al.,* 1998). The role of minor differences in the production and sensitivity to leptin towards contributing to obesity is less well understood.

Women typically have higher leptin levels than men (Flier, 1998) but these differences may not be accountable by fat mass alone. The effect of ethnicity on leptin levels is even less well understood. Blacks may have higher leptin concentrations than whites, even when adjusted for fat volume. Another important concept that has gained ground is the presence of selective leptin resistance (El-Haschimi *et al.,* 2000). Obese humans continue to overeat, despite elevated circulating leptin levels, indicating a failure of feedback mechanisms on satiety. Yet they develop hypertension, indicating that the effect of leptin on the sympathetic system is intact. As attractive as this hypothesis may sound, it has been tested only in rodents and not in humans (VanHeek *et al.,* 1997). The complexity of the relationship between adiposity, leptin, and long-term blood pressure control is further illustrated by the fact that lower body obesity causes greater increases in leptin than visceral obesity, even though visceral obesity is more closely associated with hypertension.

VI. Role of Neuropeptide Y, Melanocortin-4 Receptors, and Other Neurochemicals

Activation of leptin receptors in the arcuate nucleus initiates a cascade of downstream events. These include inhibition of neurons containing neuropeptide Y (NPY), an orexigenic peptide, and stimulation of neurons containing pro-opiomelanocortin (POMC), the precursor of α-melanocyte-stimulating hormone (MSH), an anorexigenic peptide. NPY is a potent and effective orexigenic agent upon acute and chronic administration. Chronic ICV infusion of NPY in rats produces an obesity syndrome. It has been postulated that endogenous NPY may have an important role in basal and starvation-induced food intake. However the receptor subtype that mediates the orexigenic effects of NPY is unknown. The negative effect of leptin on NPY formation in the hypothalamus is thought to be the primary mediator causing appetite suppression. NPY injections into the thalamus reproduce all features of hypoleptinemia (e.g., obesity, excessive eating behavior, decreased BAT heat production) (Mountjoy *et al.,* 1994; Boston *et al.,* 1997; Huszar *et al.,* 1997; Seeley *et al.,* 1997; Haynes *et al.,* 1998a; Vaisse *et al.,* 1998; Yeo *et al.,* 1998). The ob/ob phenotype in ob/ob mice appears to be mediated, in part, by increased NPY because ob/ob mice in which NPY

expression has been knocked out (NPY^{-}/NPY^{-}) are substantially less obese than ob/ob mice with normal NPY expression. However, obesity in these mice is severe, even in the absence of NPY expression, and they respond normally to the satiety effects of leptin, indicating that leptin must act on other targets to induce satiety. The administration of NPY into the vagal nucleus of tractus solitarius or caudal ventrolateral medulla causes a reduction in blood pressure (Schorr *et al.*, 1998). Since hyperleptinemia causes a reduction in brain NPY levels, it is possible that the hypertensive effects of leptin may be mediated by a reduction in NPY levels. At present, there is little evidence regarding the putative role of reduced central levels of NPY in mediating the effects of leptin on sympathetic activity and arterial pressure.

The POMC pathway may interact with leptin to stimulate sympathetic activity and regulate energy balance. MC4-R may play an important role in feeding mechanisms. While the central administration of MC4-R agonists decreases feeding, MC4-R deletion induces obesity in rats. α-MSH, which is derived from the breakdown of POMC, may be the endogenous ligand for MC4-R. POMC expression in the arcuate nucleus may be increased by leptin, an effect that could be part of a feedback pathway for control of appetite and sympathetic activity. Increasing leptin, associated with obesity, would stimulate arcuate POMC expression and α-MSH, which would then act elsewhere in the hypothalamus on MC4-R-expressing neurons, causing decreased food intake and increased sympathetic activity. Other factors that may influence the MC4-R pathway include agouti-related peptide (AgRP), which acts as an antagonist on this receptor. Yellow agouti mice with ectopic overexpression of agouti peptide are obese and hyperleptinemic owing to antagonism of the hypothalamic melanocortin system by the excess agouti protein. Treating rodents with an MC4-R antagonist completely abolishes the increased renal sympathetic activity associated with short-term ICV leptin infusion in rats. Surprisingly, MC4-R blockade does not prevent leptin-induced stimulation of sympathetic activity in BAT. This suggests that the thermogenic effects of leptin in BAT are not mediated through MC4-R, whereas the short-term effect of leptin to enhance renal sympathetic activity appears to depend on intact MC4-R. These paradoxical effects of MC4-R blockade on BAT and renal sympathetic activity suggest that leptin may activate the SNS through complex central pathways.

The orexins and melanin-concentrating hormone (MCH), which regulate appetite and energy homeostasis, are recently discovered mediators (Sakurai, 1999; Shirasaka *et al.*, 1999). Short-term experiments involving ICV injections of orexins increase renal sympathetic activity, heart rate, and arterial pressure in rats and thereby raise blood pressure. Control mechanisms for orexin release are opposite to those of leptin in that orexin expression usually increases with starvation and decreases with increased energy intake. Therefore, orexin levels are reduced in situations when leptin is increased (e.g., obesity) and thus

probably do not mediate the hypertensive effects of leptin. However, the importance of orexins and other neurochemical pathways in the hypothalamus in modulating the chronic effects of leptin on sympathetic activity, thermogenesis, and arterial pressure is largely unexplored.

VII. Role of Adipose Tissue and Systemic RAAS

A. SYSTEMIC RAAS

The RAAS seems to be activated in obesity, despite a state of volume expansion and sodium retention. Elevated serum aldosterone levels have been reported in the obese (Granger *et al.*, 1994; Schorr *et al.*, 1998; Engeli *et al.*, 2001,2002) and it is postulated that a yet-unidentified factor (possibly a fatty acid) derived from adipose tissue causes the release of a hepatic factor that, in turn, steps up aldosterone synthesis (Goodfriend *et al.*, 1998,1999). Reports in the literature suggest that a relationship between plasma angiotensin (Ang II) levels, plasma renin activity (PRA), and plasma angiotensin-converting enzyme (ACE) with BMI exists in humans (Licata *et al.*, 1994b; Cooper *et al.*, 1997,1998). Sharma and colleagues reported a relationship between plasma leptin and Ang II levels and leptin and PRA. Since plasma leptin levels correlate with adipose tissue mass (Engeli *et al.*, 2000), it is fair to speculate that adipose tissue may directly contribute to circulating Ang II. Sharma and coworkers also noted that a positive family history of hypertension predicts a relationship between plasma Ang II and blood pressure, suggesting a role for genetic factors in Ang II-related blood pressure response (Schorr *et al.*, 1998).

The effect of weight loss on RAAS also has been investigated. On a short-term scale, Tuck and Sowers studied the effect of weight loss on RAAS and blood pressure in 25 obese patients placed on a 12-week weight-reducing diet (Tuck *et al*., 1981). Sodium intake was either medium or low; PRA, aldosterone, and mean arterial pressure (MAP) were significantly reduced. The reduction in PRA but not in aldosterone correlated with weight loss in both sodium-intake groups. MAP fell significantly and to the same degree in both groups, correlating with weight loss throughout the study and with PRA from weeks 4–12. These results suggest that weight loss is accompanied by reductions in PRA and aldosterone and that PRA reductions may contribute to the decline in blood pressure (Bloem *et al.*, 1995; Umemura *et al.*, 1997; Uckaya *et al.*, 1999; Engeli and Sharma, 2002).

In a nutshell, despite evidence in favor of a stimulated RAAS in obesity, the role of genetic factors continues to be investigated. There is little information on the long-term effect of weight loss on the RAAS; larger studies are needed to investigate the short-term effect.

B. ADIPOSE TISSUE RAAS

It is becoming increasingly evident that adipose tissue possesses a local RAAS that plays an important role in adipose tissue function. Recent studies suggest that adipose tissue angiotensinogen mRNA expression is higher in abdominal than subcutaneous adipose tissue and therefore may be associated with increased cardiovascular risk. Fat cells possess the capability to synthesize all components of the RAAS. They apparently also express Ang II receptors, which make them a target for the paracrine-produced Ang II (Giacchetti *et al.*, 1999). That local Ang II may be a growth factor for fat cells is suggested by experiments in angiotensinogen-knockout (KO) mice that exhibit low blood pressure, have hypotrophic fat cells with reduced fatty acid synthase activity, and demonstrate abnormal, diet-induced weight gain (Frederich *et al.*, 1995; Engeli *et al.*, 2000). It has been suggested from studies in these animals that adipose tissue RAAS may govern blood pressure. Massiera and coworkers expressed the angiotensinogen (AGT) gene in adipose tissue of AGT-KO mice, creating a transgenic-KO mouse model in which production of AGT is limited to adipose tissue. In these animals, systemic levels of AGT increased to 20% of wild-type levels, demonstrating that AGT produced in fat cells can enter the circulation (Massiera *et al.*, 2001). Blood pressure and sodium homeostasis were restored in the transgenic animals, lending further credence to the theory that an increased fat cell mass may result in higher circulating AGT levels, a finding confirmed in obese individuals.

Additional evidence supports the role of adipose tissue in causation of hypertension. A transgenic mouse that overexpresses the cortisol-forming enzyme 11β-hydroxysteroid dehydrogenase (HSD) 1 develops obesity hypertension and all features of the metabolic syndrome (Masuzaki *et al.*, 2001). This model was found to overexpress angiotensin in adipose tissue and elevated serum angiotensin levels. Obese hypertensive women have been found to overexpress genes encoding renin, angiotensin-converting enzyme (ACE), and Ang II type 1 receptor in subcutaneous abdominal fat cells (Gorzelniak *et al.*, 2002). Despite the fact that expression of the AGT gene is lower in the obese, the high Ang II levels in circulation may be derived from the huge fat cell mass, resulting in hypertension. It recently has been reported that mutations in the DD phenotype of the ACE gene may be associated with obesity hypertension in Caucasian men.

VIII. Role of the Natriuretic Peptide System

The natriuretic peptide system consists of the atrial natriuretic peptide (ANP), the brain natriuretic peptide (BNP), and the C-type natriuretic peptide (CNP), each encoded by a separate gene (Rosenzweig and Seidnan, 1991; Maack, 1992; Maoz *et al.*, 1992; Nakao *et al.*, 1992; Sarzani *et al.*, 1993,1996;

Dessi-Fulgheri *et al.*, 1998). They are synthesized predominantly in the heart, brain, and kidneys. They work via specific receptors, namely, NPr-A, NPr-B, and NPr-C (Nakao *et al.*, 1992; Levin *et al.*, 1998). The natriuretic peptides have salutary effects on plasma volume, renal sodium handling, and blood pressure and cause a reduction in sympathetic tone in the peripheral vasculature. They dampen baroreceptor function and lower the activation threshold of vagal efferents, suppressing reflex tachycardia and vasoconstriction resulting from decreased extracellular volume. Transgenic mice that overexpress the ANP gene have lower blood pressure, while animals with inactivated ANP gene are prone to develop salt-sensitive hypertension (Melo *et al.*, 1998,2000). Therefore, the natriuretic peptides have a protective role on the development of hypertension due to their natriuretic and vasodilator effects as well as due to their inhibitory effect on the SNS and the RAAS.

It has been noted that the NPr-C receptor is overexpressed in adipose tissue in the obese. This may adversely affect the systemic activity and actions of the natriuretic peptides, leading to sodium retention and hypertension. It has been reported that ANP plasma levels are lower in the obese hypertensive than in obese normotensive subjects (Dessi-Fulgheri *et al.*, 1997). These obese hypertensive subjects also demonstrate a stronger response to exogenously administered ANP, as measured by blood pressure reduction, natriuresis, and increases in urine cyclic guanylate monophosphate (cGMP) excretion (Dessi-Fulgheri *et al.*, 1999). These effects are pronounced after these subjects have been on a low-calorie diet for a span of time, rather than at baseline. The ANP response to salt load was found to be suppressed in the obese, compared to a potent ANP elevation in the lean. Concomitantly, PRA and aldosterone were suppressed in the obese when saline challenged. Obese rats that were subjected to 40% weight loss by caloric restriction demonstrated a significant increase in circulating ANP levels as well as a strong decrease in PRA (Crandall *et al.*, 1989). A recent study reported an association between a promoter variant at position -55 in the NPr-C gene, a higher blood pressure, and lower ANP plasma levels in obese hypertensive subjects (Sarzani *et al.*, 1999). Further studies are needed to understand the role of this finding re: its possible contribution to blood pressure elevation.

IX. Cardiovascular System Changes

A. VASCULAR ADAPTATIONS

An alteration in ions at the cellular and molecular level appears to be important in regulating vascular smooth muscle tone. These may be deregulated in the obese, resulting in abnormal vascular responsiveness (Messerli *et al.*, 1981; Frohlich *et al.*, 1983; Assmann and Schulte, 1998). Insulin works as a vasodilator by inhibiting voltage-gated Ca^{2+} influx. It also stimulates glucose transport and

phosphorylation of glucose to glucose-6-phosphate, which further activates Ca^{2+} ATPase transcription and increases cellular Ca^{2+} efflux. These actions result in a net decrease in intracellular Ca^{2+} and, therefore, decreased vascular resistance. These effects are blunted in obesity due to insulin resistance, leading to increased vascular resistance (Terazi, 1983; Licata *et al.*, 1990; Rocchini *et al.*, 1992; Rochstroph *et al.*, 1992; Schmieder and Messerli, 1993; Weir *et al.*, 1998; Zemel, 1998).

Resnick and colleagues (1997) discovered that increased abdominal visceral fat, decreased intracellular magnesium, and advanced age were closely associated with reduced aortic distensibility. In this study, magnetic resonance imaging (MRI) was utilized to evaluate the aorta of normal and hypertensive subjects.

Jacobs and Sowers studied the effect of weight reduction on peripheral vascular resistance (PVR), reflected by forearm vascular resistance and MAP in obese persons. Weight loss resulted in a clearly significant decrease in PVR and MAP (Jacobs *et al.*, 1993).

B. CARDIAC ADAPTATIONS

Obesity is a major cause of type I diabetes, hypertension, and hyperlipidemia. These disorders individually and synergistically increase the cardiovascular risk, as shown in the Prospective Cardiovascular Munster (PROCAM) study (Assmann and Schulte, 1988).

Prolonged hypertension in lean subjects tends to produce a concentric left ventricular hypertrophy (LVH), whereas the predominant pattern of cardiac hypertrophy noted in obese hypertensives is eccentric. With concentric hypertrophy, cardiac dilatation is a late and terminal event (Frohlich *et al.*, 1992; Simon *et al.*, 1994). Heart failure is more common in the obese subject, even when corrected for the presence of hypertension (Drenick *et al.*, 1980). The presence of both obesity and hypertension in a patient results in a mixed pattern of cardiac hypertrophy, caused by an elevation in both cardiac preload and afterload (Messerli *et al.*, 1983). Obesity results in increased preload due to an expanded vascular volume, while the high afterload can be accounted for by the presence of hypertension and SNS activation. After adjustment for established risk factors, the risk of heart failure increases by 5% in men and 7% in women for each BMI increment of 1 (Kenchaiah *et al.*, 2002). Compared with subjects having a BMI below the obese range, obese subjects have double the risk of heart failure, after adjustment for co-morbid risk factors. Autopsy data from the Mayo Clinic reveal that the average cardiac mass is 467 g in obese hypertensive subjects, compared with 367 g in obese individuals without heart disease and 272 g in nonobese hypertensive subjects (Smith and Willius, 1933). Since LVH itself is a major risk factor for sudden death and death due to progressive cardiac decompensation, it may partially explain the increased incidence of cardiovas-

cular morbidity and mortality in the obese (Lip *et al.*, 1994). Mononuclear cell infiltration in and around the sinoatrial node, with fat deposition all along the conduction system, is present in the myocardium of obese individuals (Bharati and Lev, 1995). Lipomatous hypertrophy of the interatrial septum also has been noted in obesity (Basa *et al.*, 1994). All these changes make the myocardium in the obese hypertensive an ideal substrate for cardiac arrhythmia and sudden death (Duflou *et al.*, 1995).

X. Proinflammatory and Prothrombotic Changes

A. ADIPOSE TISSUE IS A SOURCE OF INFLAMMATORY CYTOKINES AND HORMONES

Adipose tissue in general and central adipose tissue in particular is recognized as a rich milieu and source of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and C-reactive protein (CRP), and plasminogen activator inhibitor (PAI-1). As such, obesity has been suggested to be a low-grade inflammatory condition increasingly important in the causation and progression of hypertension and atherosclerosis (Festa *et al.*, 2000; Das, 2001). It is thought that the central adipocyte synthesizes TNF-α, which, in turn, stimulates IL-6, considered a major regulator in the production of acute-phase reactants such as CRP, PAI-1, and fibrinogen from the hepatocyte.

It is possible that the relationship between obesity and vascular disease may depend in part on the increased production and release of these inflammatory mediators from adipose tissue. A direct cause-and-effect relationship, however, has not been clearly established. It is not known, for example, whether long-term treatment with aspirin or other nonsteroidal anti-inflammatory drugs reduces the level of inflammatory cytokines and CVD in obese patients.

B. HYPERCOAGULATION AND INCREASED RISK OF THROMBOSIS IN OBESITY

Increased risk for thrombosis may contribute to a higher incidence of heart disease and stroke in obesity. Obesity is associated with polycythemia. Epidemiologic evidence exists that hypercoagulation and impaired fibrinolysis activity are related to BMI or waist-to-hip ratio. For example, obese subjects have higher levels of factor VII antigen, fibrinogen, plasminogen, and PAI-1 activity, all of which have been suggested to increase the risk for CVD (Alessai *et al.*, 2000; Chu *et al.*, 2001; Rissanen *et al.*, 2001). The increased flux of FFAs in obesity may promote thrombosis by increasing protein C, PAI-1, and/or platelet aggregation. Adipose tissue production of leptin or inflammatory mediators have been suggested as important factors causing increased thrombosis (Konstantinides *et*

al., 2001) but these aspects of the relationship between obesity and CVD are poorly understood and require additional study. Other mechanisms for platelet activation in the obese recently have been elucidated, at least in part. Davi and coworkers (2002) reported that a visceral or android pattern of obesity is associated with lipid peroxidation and persistent platelet activation, both of which appear partially reversible with weight loss. An association has been proposed between a circulating soluble CD40 ligand (CD40L) and platelet activation. CD40L is a trimeric, transmembrane protein of the TNF family that is inactive in resting platelets but is rapidly activated and expressed on the platelet surface when the platelet is stimulated. Therefore, increased levels of circulating CD40L represent an index of platelet activation. Desideri and Ferri (2003) addressed the relationship between central obesity and circulating levels of soluble CD40L and the effect of weight loss on this measure of platelet activation in a prospective intervention cohort study. It was found that baseline levels of circulating CD40L and 8-iso-PGF2α levels (a marker of lipid peroxidation) were significantly higher in obese vs. nonobese subjects. BMI levels correlated well with levels of CD40L and 8-iso-PGF2α. After a 16-week intervention period of caloric restriction and weight loss, levels of these markers went down, corresponding with a commensurate lowering of the BMI in the obese subjects.

XI. Management

A. PHYSICAL EXERCISE

Increased physical activity is perhaps the most-widely prescribed and the most-poorly complied with of all weight-reduction strategies. It requires considerable time, thought, and effort from the patient and the therapist. Physical activity is essential for long-term weight control. It appears to be the best predictor of maintenance of weight loss. Even in the absence of weight loss, increased physical activity is associated with other desirable outcomes, such as improved cardiovascular health and perhaps reduction in insulin resistance. Studies suggest that, in overweight and obese persons, physical activity is independently associated with the redistribution of adiposity away from the abdomen. It is important not to set inappropriately ambitious physical activity goals, since they increase the likelihood that the exercise program will be abandoned. High levels of aerobic physical activity at a fitness center are not needed to obtain health benefits. Several studies have compared the impact of lifestyle activity vs. physical exercise and demonstrated that they result in similar health benefits (e.g., control of hypertension and body fat) (Andersen *et al.*, 1999). Based on these and other observations, it is clearly inappropriate to suggest to patients that the only acceptable physical activity included in their

weight-loss program would be vigorous in nature. Although some may choose this, those who don't should be encouraged to start with low-impact, moderate-intensity physical activity (i.e., walking) of short duration, with a goal of accumulating at least 30 minutes of physical activity on most days of the week. A recent report suggests that physical activity participation for 60 minutes per day will impart increased benefits and is more likely to help maintain weight loss.

B. CALORIC RESTRICTION

Caloric restriction seems the most-important component of obesity management. A daily caloric reduction of 500–1000 cal produces a weight loss of 0.45–0.90 kg/week. Individually planned diets that incorporate all essential dietary components should be made available to everyone entering a weight-loss program. Many types of diets — including high-protein, low-carbohydrate, and low-fat — have been promoted. The individual macronutrient component of diet is controversial and has not been determined (Jackic *et al.*, 2001). All low-calorie diets produce weight loss and macronutrient composition seems less important than the number of calories cut (Freedman *et al.*, 2001).

C. BEHAVIORAL MODIFICATION

Behavioral therapy is a very important component of any weight-loss program. It helps patients develop the skills they need to identify and modify eating and activity behaviors. It also helps remodel thinking patterns that undermine weight-control measures (Wadden *et al.*, 2000). Important components of behavioral therapy are self weight monitoring, measuring intake by keeping a food log, and physical activity. Other strategies are identification of stimuli that trigger unusual, irregular food intake or binging on food and problem solving, which identifies problems and proposes solutions. Behavioral strategies encompass the active and supportive involvement of the social and family fabric that surrounds the patient. Stress and time management are also essential to a comprehensive weight-loss program. Patients who appear depressed and have poor self-image should receive appropriate referral to psychotherapists, support groups in the community, and, if deemed necessary, a psychiatrist. Professional help from dieticians, nurse educators, and exercise physiologists should be readily available (Boutelle and Kirschenbaum, 1998).

D. PHARMACOTHERAPY

Because antihypertensive drug treatment in obesity hypertension is not yet based on evidence from large, randomized, controlled trials that have specifically addressed this population, it remains empirical. No definitive guidelines have been framed; therefore, only suggestions can be made based upon assumed

pathophysiologic mechanisms and clinical experience. For example, since β-blockers can result in weight gain and impair glucose tolerance, their use in uncomplicated obesity and hypertension cannot be recommended as routine first-line therapy (Sharma *et al.*, 2001). This recommendation cannot extend across the board to patients admitted to critical care units with acute coronary syndromes where β-blockers are not only essential but also may be lifesaving. They may be used to manage patients with hypertension uncontrolled with multiple other agents. The importance of adrenergic blockade in the management of this syndrome cannot be dismissed (Wofford *et al.*, 2001). It was shown by Masuo *et al.* (2001a,b) that subjects who were resistant to weight loss-induced blood pressure reductions possessed a highly active SNS and would potentially benefit from adrenergic blockade. Calcium channel blockade cannot be recommended in obese hypertensive patients, since some small studies have cast doubt on their efficacy. No definitive recommendations can be made until data from large, randomized, controlled trials are gathered (Schmieder *et al.*, 1993; Masuo *et al.*, 2001a,b).

ACE inhibitors and angiotensin-receptor blockers (ARBs) may be considered preferred agents for several reasons. First, they are metabolically neutral, except for the risk of hyperkalemia, which can be managed effectively with careful monitoring. Second, there is evidence from the Heart Outcomes Prevention Evaluation (HOPE) and Captopril Prevention Project (CAPPP) trials (Hansson *et al.*, 1999; Yusuf *et al.*, 2000) that ACE inhibitors may prevent or retard the development of type 2 diabetes. Third, this class of agents may be the most effective in preventing the onset and progression of proteinuria that are responsible for the inexorable progression to obesity-hypertension-related ESRD. Finally, this class of agents may be the most effective to prevent and manage eccentric cardiac hypertrophy and thus progression to heart failure. Low-dose ACE inhibitors may be more effective than thiazides (Reisin *et al.*, 1997; Yusuf *et al.*, 2000; Wofford *et al.*, 2001). Therefore, it may be reasonable to recommend ACE inhibitors and ARBs as first-line therapy for obesity-related hypertension. Thiazide diuretics may be an effective class of agents for this group of patients, since they directly address the volume-expanded state of the obesity-hypertension syndrome (Reisen *et al.*, 1997) and may prevent cardiac dilatation by preload reduction. Caution must be exercised with use in this class of patients, primarily for the risk of exacerbating dysglycemia and erectile dysfunction, which are quite prevalent in this population. Excessive diuresis may result in a state of volume contraction, triggering further activation of the SNS and the RAAS, resulting in avid renal salt and fluid retention, with exacerbation of hypertension. Indeed, a combination of ACE inhibitors and low-dose thiazides may be more effective than either strategy alone in this population. Having said that, it is important to note that there is little evidence to support these hypotheses.

Weight loss — achieved by exercise, behavioral modification, pharmaceutical agents, or a combination — is another important and effective means of reversing insulin resistance, elevated leptin levels, decreased cardiac output, and activated SNS (Emdin *et al.*, 2001; Itoh *et al.*, 2001; Nakano *et al.*, 2001). Despite significant initial success, long-term and sustained weight loss is difficult to achieve with diet and exercise alone.

Some recently developed pharmaceutical agents (e.g., orlistat, sibutramine) have shown good promise in achieving significant and sustained weight loss in the range of 5–10%. In a recent meta-analysis that pooled data from trials using orlistat (30% of participating subjects were hypertensive), significant blood pressure reductions were noted after 1 year in subjects who lost 5% or more weight. The blood pressure reductions were on the order of 7 mmHg systolic and 5.5 mmHg diastolic, comparable to those achieved with antihypertensives used as single agents (Zavoral, 1998). Other cardiovascular risk factors improved significantly in subjects who took orlistat for more than 2 years (Rossner *et al.*, 2000). Since orlistat has no negative effect on the cardiovascular risk profile, it can be safely recommended for treating the obese patient with hypertension.

Sibutramine, another anti-obesity medication, acts by inhibiting serotonin and norepinephrine uptake. In doing so, it enhances satiety and increases energy utilization (Hansen *et al.*, 1999). A recent trial called STORM (Sibutramine in Obesity Reduction and Management) revealed significant and sustained weight loss comparable to that achieved with orlistat. Improvement in metabolic parameters such as blood glucose and lipid levels was also noted (Hansen *et al.*, 2001). However, small increases in blood pressure and heart rate were noted and no net blood pressure reductions could be achieved commensurate with the degree of weight loss. In another trial with sibutramine, those who maintained weight at 1 year had no significant changes in blood pressure and heart rate, when compared to baseline (McMahon *et al.*, 2000). Therefore, sibutramine cannot be recommended in the hypertensive-obese subject. If it must be used, careful blood pressure monitoring is warranted with use of effective antihypertensive medications.

Novel weight-loss medications have been reported recently. A randomized control trial conducted with zonisamide (an anti-epileptic agent with dose-dependent biphasic dopaminergic and serotonergic activity) resulted in significantly greater weight loss and reductions in blood pressure, compared to lifestyle intervention alone in the extended phase of the trial. The drug was found to be safe, with fair compliance (Gadde *et al.*, 2003). Therapy with a recombinant variant of ciliary neurotrophic factor (rhvCNTF) resulted in significant weight loss. This agent that was developed for the management of amyotrophic lateral sclerosis (a type of motor neuron disease) did not prevent disease progression but resulted in weight loss. It is thought to exert its effect by binding to the CNTF receptor and activating leptin-like intracellular signaling pathways (janus kinases

and signal transducers and activators of transcription 3) in hypothalamic nuclei, thereby regulating food intake and body weight (Ettinger *et al.*, 2003). Despite significant weight loss, no significant effect on blood pressure was noted. The drug must be administered subcutaneously. Other agents such as SR141716 (Rimonabant) are in phase 3 trials. Rimonabant acts by blocking a cannabinoid receptor in the CNS that, when activated, stimulates hunger.

E. ROLE OF BARIATRIC SURGERY

The National Institutes of Health criteria for obesity surgery eligibility are a well-informed and motivated patient with a BMI $>$ 40 or a patient with less-severe obesity (i.e., BMI $>$ 35) with high-risk, co-morbid conditions (e.g., type 2 diabetes, cardiopulmonary problems). Trials such as the Swedish Obesity Study (SOS) found that weight-reduction surgery in the morbidly obese with significant co-morbidities can result in improved metabolic parameters and blood pressure. Surgery is an option for patients in whom all conservative measures, including pharmaceutical agents, have failed and in those who cannot tolerate drugs due to their prohibitive side effects. These procedures entail considerable operative risk and pose ethical and scientific questions, so cannot yet be routinely recommended (Sjostrom *et al.*, 2001; Torgerson and Sjostrom, 2001; Laimer *et al.*, 2002; Nabro *et al.*, 2002).

XII. Summary

This review has discussed some of the mechanisms involved in the causal relation between obesity and hypertension. Obesity causes a constellation of maladaptive disorders that individually and synergistically contribute to hypertension among other cardiovascular morbidity. Well-designed, population-based studies are needed to assess the individual contribution of each of these disorders to the development of hypertension. Control of obesity may eliminate 48% of the hypertension in whites and 28% in blacks. We hope that this chapter will help scientists formulate a thorough understanding of obesity hypertension and form the basis for more research in this field, which greatly impacts on human life.

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Obesity, Insulin Resistance, and Cardiovascular Disease

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ABSTRACT

The ability of insulin to stimulate glucose disposal varies more than six-fold in apparently healthy individuals. The one third of the population that is most insulin resistant is at greatly increased risk to develop cardiovascular disease (CVD), type 2 diabetes, hypertension, stroke, nonalcoholic fatty liver disease, polycystic ovary disease, and certain forms of cancer. Between 25–35% of the variability in insulin action is related to being overweight. The importance of the adverse effects of excess adiposity is apparent in light of the evidence that more than half of the adult population in the United States is classified as being overweight/obese, as defined by a body mass index greater than 25.0 kg/m^2. The current epidemic of overweight/obesity is most-likely related to a combination of increased caloric intake and decreased energy expenditure. In either instance, the fact that CVD risk is increased as individuals gain weight emphasizes the gravity of the health care dilemma posed by the explosive increase in the prevalence of overweight/obesity in the population at large. Given the enormity of the problem, it is necessary to differentiate between the CVD risk related to obesity *per se*, as distinct from the fact that the prevalence of insulin resistance and compensatory hyperinsulinemia are increased in overweight/obese individuals. Although the majority of individuals in the general population that can be considered insulin resistant are also overweight/obese, not all overweight/obese persons are insulin resistant. Furthermore, the cluster of abnormalities associated with insulin resistance – namely, glucose intolerance, hyperinsulinemia, dyslipidemia, and elevated plasma C-reactive protein concentrations — is limited to the subset of overweight/obese individuals that are also insulin resistant. Of greater clinical relevance is the fact that significant improvement in these metabolic abnormalities following weight loss is seen only in the subset of overweight/obese individuals that are also insulin resistant. In view of the large number of overweight/obese subjects at potential risk to be insulin resistant/hyperinsulinemic (and at increased CVD risk), and the difficulty in achieving weight loss, it seems essential to identify those overweight/obese individuals who are also insulin resistant and will benefit the most from weight loss, then target this population for the most-intensive efforts to bring about weight loss.

I. Introduction

Introduction of a specific immunoassay for quantifying plasma insulin concentrations (Yalow and Berson, 1960) had an enormous impact on understanding the relationship between obesity, insulin resistance, plasma insulin concentrations, type 2 diabetes, and cardiovascular disease (CVD). In that publication, Yalow and Berson pointed out that plasma immunoreactive insulin concentrations following an oral glucose challenge were as high, if not higher, in

patients with what was then called "maturity-onset diabetes" than in a population with normal glucose tolerance. This observation led them to speculate that the tissues of patients with maturity-onset diabetes did not respond normally to insulin: in more-contemporary terms, they were insulin resistant. Shortly thereafter, papers began to appear that initiated a controversy concerning the relationship between obesity, insulin resistance, and hyperinsulinemia that continues to this day. Karam and colleagues, using a somewhat different insulin immunoassay (Karam *et al.*, 1965), argued that plasma insulin concentrations were elevated in response to a glucose challenge only in patients with maturity-onset diabetes who were also obese. Based on these findings, they concluded that it was obesity, not maturity-onset diabetes, which was associated with insulin resistance. The suggestion by Karam and colleagues that obesity was associated with insulin resistance was consistent with the results of Rabinowitz and Zierler (1962), who had used the perfused forearm technique to provide direct evidence that insulin-stimulated glucose disposal was impaired in obese individuals. Although the observation that obesity was associated with insulin resistance did not necessarily mean that obesity was the sole cause of the defect in insulin action, the results of these and somewhat-similar studies have led to a commonly expressed view that insulin resistance, and its metabolic consequences, are simple manifestations of obesity.

An alternative view, and one that has been the focus of our research group for approximately 35 years, is that obesity is only one of several factors that modulate insulin action and by no means does insulin resistance equal obesity. The remainder of this chapter will summarize our efforts to address the relationship between obesity, insulin resistance, and CVD.

II. Relationship Between Obesity, Plasma Insulin Concentration, and Type 2 Diabetes

Our research group has published several papers that addressed the relationship between obesity, plasma insulin concentration, and type 2 diabetes. The results of these studies have been quite consistent in showing that plasma insulin concentrations in patients with type 2 diabetes are more closely associated with degree of hyperglycemia than with obesity. This section will discuss the results of three publications relevant to this issue.

Although the paper by Karam and coworkers (1965) was interpreted as showing that plasma insulin concentrations were high only in patients with type 2 diabetes who were also obese, the researchers did not control for the fact that the nonobese patients were significantly more hyperglycemic. Therefore, we (Reaven and Miller, 1968) determined the plasma glucose and insulin responses to oral glucose in 125 individuals, divided into four groups on the basis of progressive degrees of hyperglycemia: 1) normoglycemic; 2) impaired glucose

tolerance; 3) mild diabetes (hyperglycemia); and 4) severe diabetes (hyperglycemia). Each of these four groups was further subdivided on the basis of degree of obesity into those who were less than 10% overweight, 10–30% overweight, or 30–50% overweight. The total integrated insulin responses of these experimental groups to an oral glucose challenge are shown in Figure 1, the legend of which defines the glucose levels of the four groups. It can be seen from these data that the total plasma integrated insulin response was somewhat higher than normal in the group with impaired tolerance, comparable to normal in patients classified as having mild diabetes, and somewhat lower in those with severe diabetes. These data showed that the insulin response to an oral glucose challenge varied considerably as a function of degree of hyperglycemia. Furthermore, when individuals were matched for plasma glucose concentration, differences in weight had very little effect on insulin response. Thus, the results of Karam and associates were essentially self-fulfilling in that they compared nonobese patients with relatively severe hyperglycemia to obese patients with lower levels of plasma glucose concentration. We would have come to the same confounded conclusion if we had compared plasma insulin concentrations without matching for both weight *and* degree of hyperglycemia.

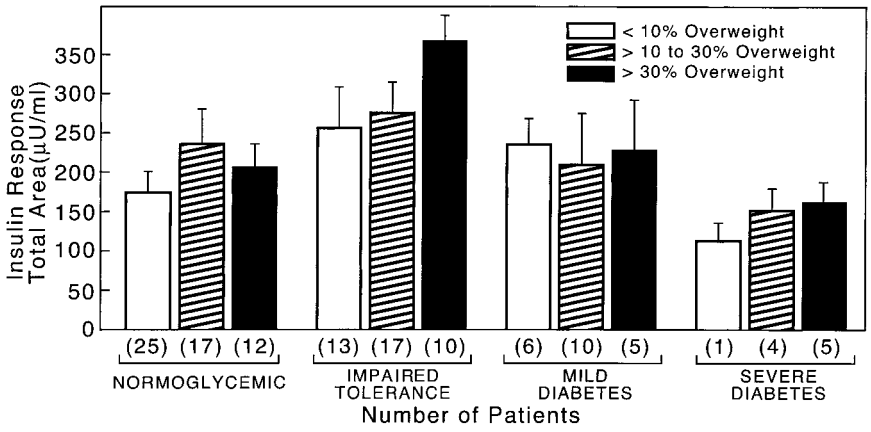

FIG. 1. Total integrated plasma insulin response to a 75-g oral glucose challenge in four groups of individuals differing in their degree of glucose tolerance. Within each glucose tolerance group, the individuals were subdivided further on the basis of their degree of obesity. Mean ± fasting plasma glucose concentrations were 115 ± 4 and 217 ± 13 in the patients with "mild" and "severe" diabetes, respectively. The mean ± plasma glucose concentrations 120 minutes after oral glucose challenge were 98 ± 3, 140 ± 6, 221 ± 15, and 411 ± 22 in the normoglycemic, impaired tolerance, "mild," and "severe" diabetes groups, respectively. [Reprinted with permission from Reaven GM, Miller R 1968 Study of the relationship between glucose and insulin responses to an oral glucose load in man. Diabetes 17:560–569.]

Two other studies subsequently were performed in which the relationship between plasma insulin concentration and obesity was compared in patients with type 2 diabetes, always making sure to match subjects for obesity as well as degree of hyperglycemia. In the first study (Hollenbeck *et al.*, 1984), we were able to demonstrate that both resistance to insulin-mediated glucose disposal and the plasma insulin response to an oral glucose challenge were comparable when obese and nonobese patients with type 2 diabetes were matched for degree of hyperglycemia. Finally, when questions were raised as to the specificity in earlier studies of the measurements of plasma insulin concentrations, we determined specific plasma insulin concentrations throughout the day in response to meals in both obese and nonobese individuals with varying degrees of glucose tolerance (Reaven *et al.*, 1993). The results once again demonstrated that elevated plasma insulin concentrations in patients with glucose intolerance and/or type 2 diabetes varied as a function of degree of hyperglycemia, not body weight.

III. Obesity and Insulin Resistance

The observation that the hyperinsulinemia associated with glucose intolerance was not a simple function of obesity strongly suggested that this would also be true of the relationship between obesity and insulin resistance. This issue was first addressed in a study of 36 nondiabetic volunteers whose mean relative body weight (determined by Metropolitan Life Tables) was 1.21 (Olefsky *et al.*, 1974). Insulin-mediated glucose disposal was quantified by the insulin suppression test, in which the steady-state plasma glucose (SSPG) concentration is determined at the end of a 180-minute infusion of insulin and glucose, with simultaneous suppression of endogenous insulin secretion (Shen *et al.*, 1970; Greenfield *et al.*, 1981; Pei *et al.*, 1994). Under these conditions, the steady-state plasma insulin (SSPI) concentration at the end of the 180-minute infusion in all individuals is similar both in kind (exogenous) and amount. Since the amount of glucose infused is also similar in all subjects, the SSPG concentration is a direct measure of how effective insulin was in disposing of the infused glucose load: the higher the SSPG concentration, the more insulin resistant the individual. The results of this study indicated that the correlation coefficient (r-value) between SSPG concentration and relative body weight was 0.46. In other words, differences in adiposity accounted for approximately 25% of the variability in insulin-mediated glucose disposal in these overweight/obese individuals.

Although a relatively crude estimate of adiposity was used in this initial study, the results strongly suggested that being overweight increased the likelihood that an individual would be insulin resistant, although that the relationship was relatively modest in magnitude. Indeed, in a subsequent collaborative study performed in nondiabetic volunteers of both Pima Indian and European ancestry, the magnitude of the relationship between several estimates of adiposity and

insulin-mediated glucose disposal was quite similar to our earlier findings (Bogardus *et al.*, 1985). Parenthetically, it is important to note that, in the same study, we found that differences in physical fitness, as quantified by measurement of maximal aerobic capacity, were as powerful as variations in adiposity in modulation of insulin action. Finally, we recently completed a study of 314 healthy, nondidabetic, normotensive volunteers and the relationship between insulin resistance (SSPG) and body mass index (BMI) (Figure 2) (Abbasi *et al.*, 2002). These data indicate that there is a statistically significant correlation between SSPG and BMI but, again, it can be seen that differences in BMI accounted for approximately 25% of the variability in insulin-mediated glucose disposal in this large population of healthy volunteers. Based upon these results, as well as evidence from several other studies published over the last 25 years, we believe it reasonable to conclude that obesity, along with physical inactivity, can account for approximately 50% of the variability in insulin-mediated glucose disposal in healthy, nondiabetic, normotensive individuals.

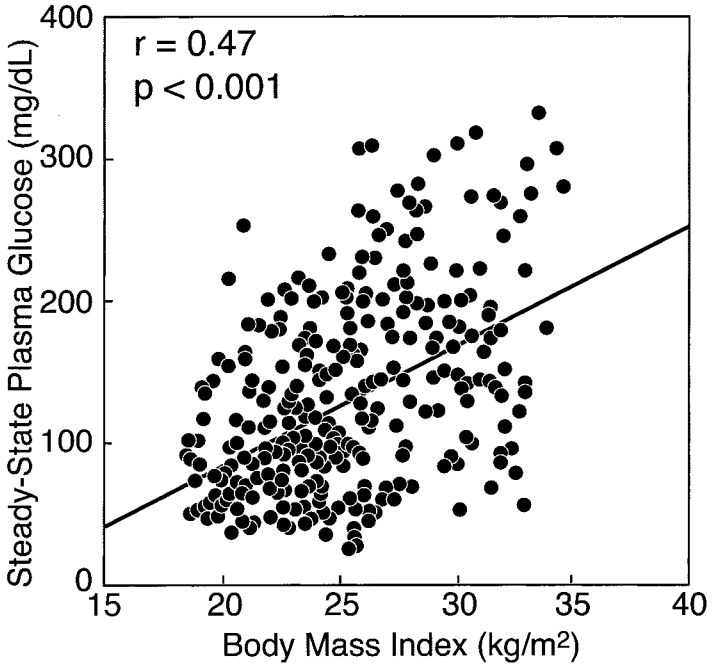

FIG. 2. Relationship between body mass index (BMI) and steady-state plasma glucose (SSPG) concentration in 314 healthy volunteers. [Reprinted with permission from Abbasi F, Brown BWB, Lamendola C, McLaughlin T, Reaven GM 2002 Relationship between obesity, insulin resistance, and coronary heart disease risk. J Am Coll Cardiol 40:937–944.]

IV. Obesity, Insulin Resistance, and CVD Risk

It can be seen from inspection of the data in Figure 2 that SSPG values varied several-fold in those individuals classified as being obese (BMI $>$ 30.0 kg/m^2). The obvious question that must be asked is whether CVD risk factors were similar in all the obese individuals in this study, or varied as a function of degree of insulin resistance? One way to approach this question is to calculate the correlation coefficients between each of the two variables, BMI and SSPG, and a number of CVD risk factors. The results, displayed in Table I, indicate that although almost all of the relationships depicted between the CVD risk factors measured and both BMI and SSPG were statistically significant, with differences in the magnitude of the correlation coefficients (r-values). Thus, BMI was more closely associated with age, systolic blood pressure (SBP), and concentrations of total and low-density lipoprotein cholesterol (LDL-C). In contrast, SSPG was more closely associated with diastolic blood pressure (DBP), triglyceride (TG), lower concentrations of high-density lipoprotein cholesterol (HDL-C), and higher plasma glucose and insulin responses to an oral glucose load. When these

TABLE I
Correlation Coefficients (r-values) Between Cardiovascular Disease Risk Factors, Body Mass Index (BMI), and Steady-state Plasma Glucose (SSPG)

	r Values	
Risk factors	BMI	SSPG
Age	0.19*	0.11**
SBP	0.29	0.16*
DBP	0.14*	0.20
Total cholesterol	0.35	0.24
TG	0.44	0.51
LDL-C	0.36	0.22
HDL-C	−0.38	−0.41
Glucose response	0.31	0.57
Insulin response	0.38	0.63

All r-values were statistically significant at the $p < 0.001$ level, with the exception of * $p < 0.05$ and ** $p = 0.16$. Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol. Glucose and insulin responses are the total integrated area for the 180 minutes following a 75-g oral glucose challenge. [Reprinted with permission from Abbasi F, Brown BWB, Lamendola C, McLaughlin T, Reaven GM 2002 Relationship between obesity, insulin resistance, and coronary heart disease risk. J Am Coll Cardiol 40:937–944.]

same relationships were evaluated by multiple regression analysis, the results were quite similar. Thus, BMI was an independent predictor of SBP and total and LDL-C concentrations, whereas SSPG was an independent predictor of DBP and glucose response. Finally, although both BMI and SSPG were independent predictors of TG, HDL-C, and insulin concentrations, the magnitude of the relationship was much greater in the case of SSPG. Perhaps the relationship between BMI, SSPG, and CVD risk factors can be best understood by inspection of the data in Figures 3–5. To create these figures, we relied upon the results of a previous prospective study (Yip *et al.*, 1998) that showed that 15% of apparently healthy individuals whose SSPG concentrations were in the upper tertile of the population at large developed manifest CVD over the approximately

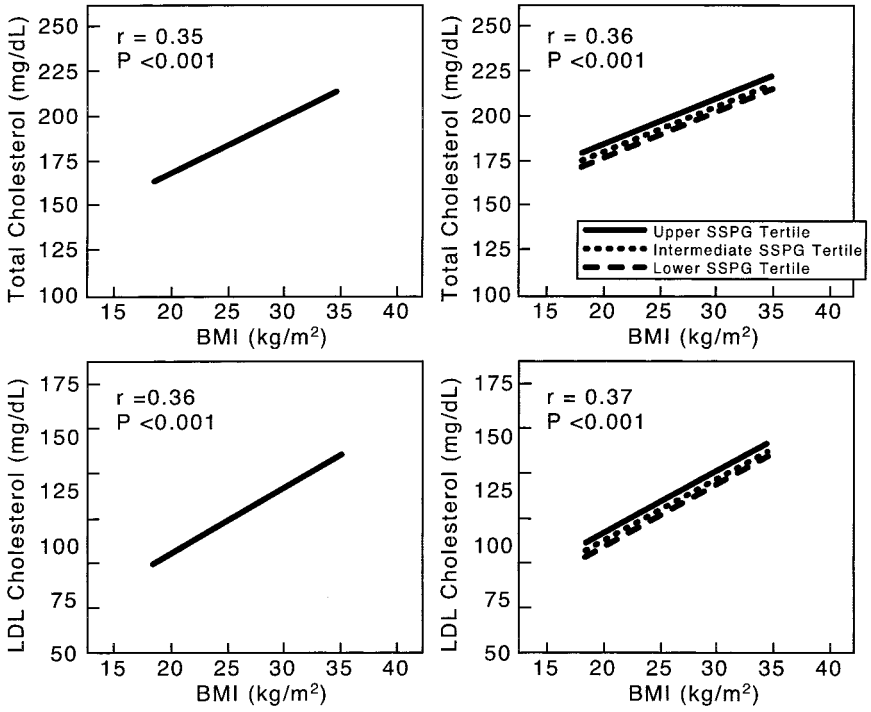

FIG. 3. The best-fit line describing the relationship between BMI and plasma concentrations of total cholesterol (upper panels) and low-density lipoprotein cholesterol (LDL-C) (lower panels) in 314 healthy volunteers. In each case, the panels on the left describe the relationship in the entire population, whereas the panels on the right portray the relationship when the group is divided into tertiles on the basis of their degree of insulin resistance (SSPG concentration). The r-values in the left panels are the correlation coefficients for the group as a whole, whereas the values on the right indicate the correlation coefficients when the relationships between BMI and the metabolic variables are adjusted for differences in degree of insulin resistance.

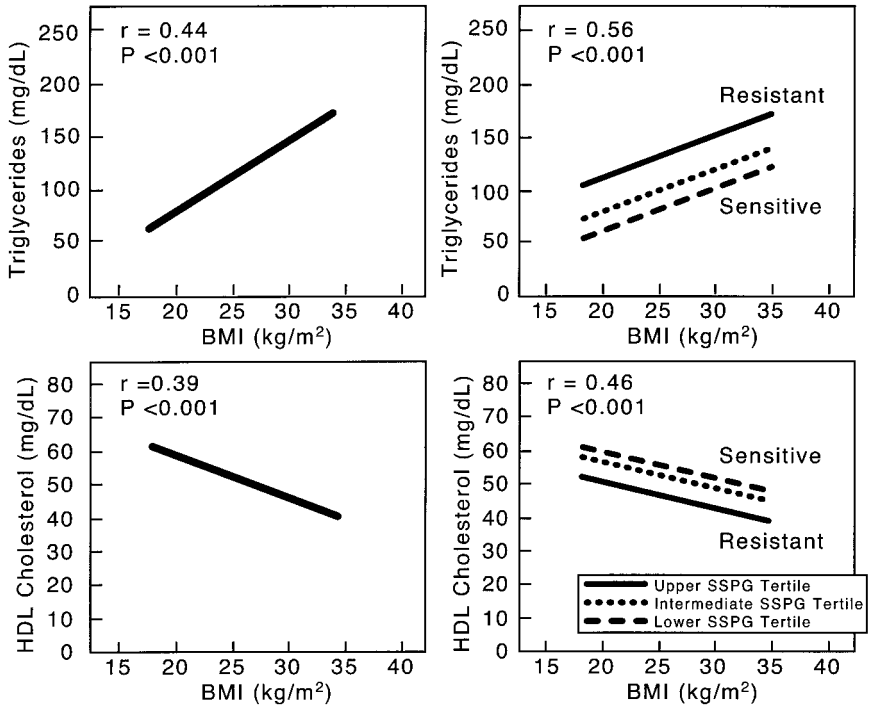

FIG. 4. The best-fit line describing the relationship between BMI and the concentrations of plasma triglyceride (TG) (upper panels) and low-density lipoprotein cholesterol (HDL-C) (lower panels) in 314 healthy volunteers. In each case, the panels on the left describe the relationship in the entire population, whereas the panels on the right portray the relationship when the group is divided into tertiles on the basis of their degree of insulin resistance (SSPG concentration). The r-values in the left panels are the correlation coefficients for the group as a whole, whereas the values on the right indicate the correlation coefficients when the relationships between BMI and the metabolic variables are adjusted for differences in degree of insulin resistance.

5-year period of observation. In contrast, none of those in the lower SSPG tertile displayed any evidence of CVD during this period. A subsequent prospective study (Facchini *et al.*, 2001) confirmed the differences between the CVD risk of the upper and lower SSPG tertiles as well as pointed out that adverse clinical outcomes of those in the upper SSPG tertile were not limited to CVD. Based upon these findings, we operationally defined individuals whose SSPG concentrations fell within the upper tertile of the population at large as being insulin resistant (IR) and those with SSPG concentrations in the lowest SSPG tertile as being insulin sensitive (IS).

We then calculated the best-fit line for describing the relationship between BMI and the CVD variables in question in the entire population as well as

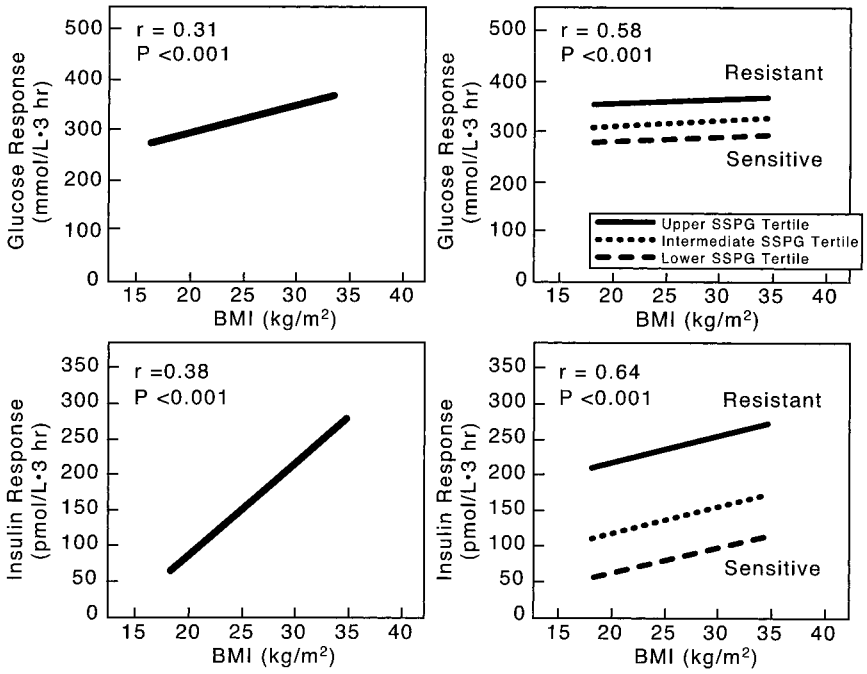

FIG. 5. The best-fit line describing the relationship between BMI and the plasma glucose (upper panels) and insulin responses to a 75-g oral glucose challenge (lower panels) in 314 healthy volunteers. In each case, the panels on the left describe the relationship between the entire population, whereas the panels on the right portray the relationship when the group is divided into tertiles on the basis of their degree of insulin resistance (SSPG concentration). The r-values in the left panels are the correlation coefficients for the group as a whole, whereas the values on the right indicate the correlation coefficients when the relationships between BMI and the metabolic variables are adjusted for differences in degree of insulin resistance.

separately for individuals in the IR, IS, and intermediate SSPG tertiles. This approach was used to create the relationships shown in Figures 3–5 between BMI, SSPG, and CVD risk factor. In each figure, the two left panels illustrate the relationship between BMI and the specific CVD risk factor for the entire population, whereas the right panels display the best-fit line between BMI and the CVD risk factor for the individuals in the three SSPG tertiles. If we focus on Figure 3, it is apparent from these data that the relationship between BMI and total and LDL-C concentrations seen in the entire group (left panels) does not vary as a function of SSPG tertile (right panels). In other words, at any given BMI, the total and LDL-C concentration were relatively similar in IR and IS individuals.

In contrast, the data in Figures 4 and 5 dramatically illustrate that the relationship between BMI and plasma TG and HDL-C concentrations (Figure 4) and plasma glucose and insulin concentrations in response to the oral glucose challenge (Figure 5) vary substantially as a function of insulin-mediated glucose disposal rates. More specifically, at any give BMI, the plasma TG concentrations are much higher and the HDL-C concentrations are much lower in the IR as compared to the IS tertile. Similarly, plasma glucose and insulin concentrations following an oral glucose challenge are two to three times higher in IR as compared to IS individuals.

Based upon these results, it is obvious that not all overweight/obese individuals are insulin resistant and that although insulin resistance and the CVD risk factors measured tend to increase as individuals get heavier, it is those overweight/obese individuals who are also insulin resistant that are at greatest CVD risk.

V. Does Weight Loss Decrease CVD Risk?

The studies to be discussed in this section fall into two categories. To begin with, it is necessary to address the issue of whether or not insulin resistance and/or compensatory hyperinsulinemia have any effect on the ability of overweight/obese individuals to lose weight. We will then discuss the improvement in CVD risk factors associated with weight loss.

A. RELATIONSHIP BETWEEN INSULIN RESISTANCE AND ABILITY TO LOSE WEIGHT

There appears to be considerable popular acceptance of the view, aggressively trumpeted in several popular diet books, that all overweight/obese individuals are insulin resistant and that it is their insulin resistance that prevents them from losing weight. Put more bluntly, this view states that the daylong hyperinsulinemia that permits insulin-resistant individuals to maintain normal, or near-normal, glucose tolerance prevents effective weight loss in overweight/obese individuals. In our first effort to address this issue (McLaughlin *et al.*, 1999), we performed SSPG concentrations in a series of obese individuals in apparently good health. They were then placed on a calorie-restricted diet and followed for 2 months.

The relationship between their degree of insulin resistance (SSPG concentration) and daylong insulin response to standard test meals before the period of weight loss is shown in Figure 6. It can be seen from this figure that the concentrations of both SSPG (two top panels) and plasma insulin (two lower panels) varied widely in the volunteers in this study. However, it is apparent from the data in the top two panels that there was absolutely no correlation between

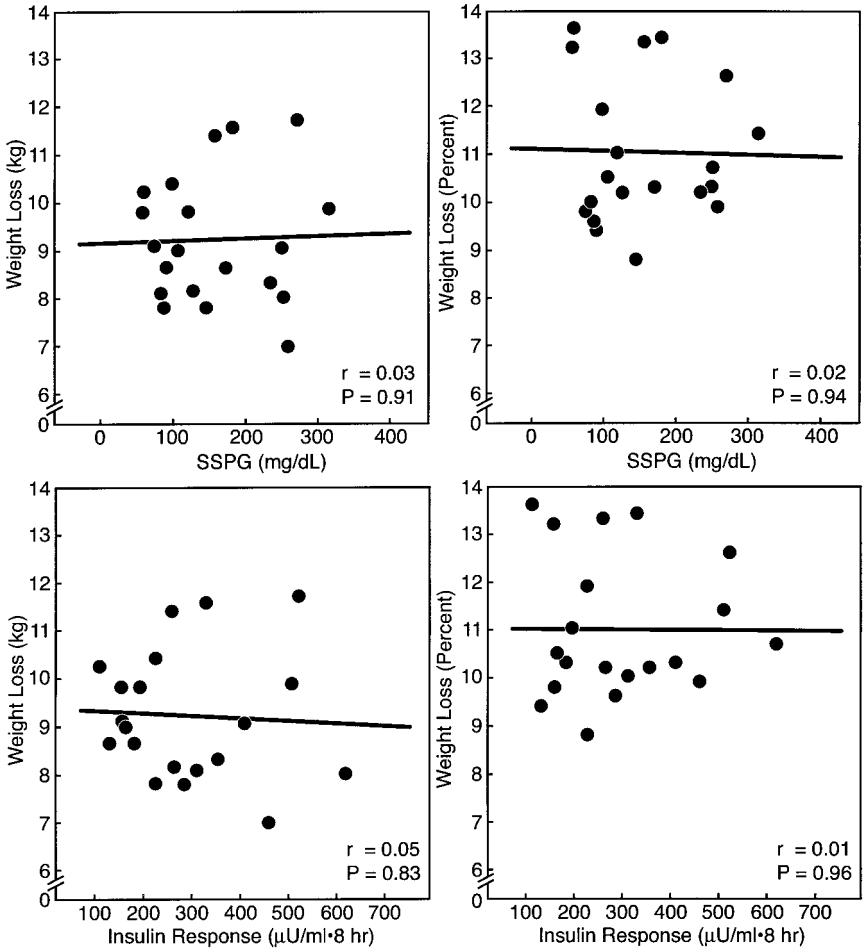

FIG. 6. The relationship between weight loss in response to 2 months of a calorie-restricted diet and the baseline SSPG concentration (upper panels) and daylong integrated plasma insulin response to breakfast and lunch (lower panels). [Reprinted with permission from McLaughlin T, Abbasi F, Carantoni M, Schaaf P, Reaven GM 1999 Differences in insulin resistance do not predict weight loss in response to hypocaloric diets in healthy obese women. J Clin Endocrinol Metab 84:578–581. Copyright The Endocrine Society.]

baseline SSPG concentration and weight loss. The data in the two lower panels indicate that there was also no relationship between baseline daylong insulin concentrations and ability to lose weight. To express it more succinctly, the obese individuals in this study lost weight as effectively, or ineffectively, irrespective of their degree of insulin resistance or daylong hyperinsulinemia.

Although the results displayed in Figure 6 provided evidence that the ability of obese subjects to lose weight in response to calorie-restricted diets was independent of differences in SSPG and daylong insulin concentrations, this conclusion could be criticized on two counts. In the first place, the period of weight loss was only 2 months long. Because obesity usually results from slow but cumulative weight gain, the clinical significance of the study could be questioned. Second, our weight-loss protocol was highly controlled, relying primarily on a liquid nutritional formula rather than "real" food. Thus, while the experimental approach may have been useful in attempting to eliminate the possible confounding effect of varied caloric intake between subjects, it could be argued that it had little relevance to the manner in which weight-loss programs usually are carried out. Thus, a second study was initiated to extend our earlier findings and the protocol was amended to the address the two potential problems just outlined (McLaughlin *et al.*, 2001). Specifically, the period of weight loss was extended to 4 months and the diet intervention was limited to nutritional advice, with all foods prepared by the subjects at home. In addition, to maximize potential for weight loss, the dietary advice was supplemented by administration of the appetite suppressant sibutramine.

The BMI of the volunteers enrolled in this study ranged between 30.0 and 36.0 kg/m^2. They were subdivided at baseline into IR (SSPG = 219 $\pm$ 7 mg/dL) and IS (SSPG = 69 $\pm$ 6 mg/dL) subgroups. The baseline weight of the two groups was similar (87 $\pm$ 2 kg vs. 84 $\pm$ 2 kg) and there was no difference in the amount of weight loss after 4 months of a calorie-restricted diet in the IR and IS subgroups (8.6 $\pm$ 1.3 kg vs. 7.9 $\pm$ 1.4 kg).

Thus, doubling the weight-loss period and having the participants prepare all their own food did not change the overall conclusion that differences in baseline values of insulin-mediated glucose disposal and circulating insulin concentrations have little, if any, effect on the ability of an individual to lose weight.

B. EFFECT OF WEIGHT LOSS ON CVD RISK FACTORS

Although differences in insulin-mediated glucose disposal do not affect the ability of obese individuals to lose weight in response to a calorie-restricted diet, they certainly modify the benefits associated with weight loss. For example, Figure 7 contains measurements of body weight and SSPG concentrations before and after weight loss in 40 obese women, divided into IR and IS subgroups (McLaughlin *et al.*, 2002). The two groups were not different in terms of age, weight, BMI, or waist circumference at baseline. By selection, the SSPG concentrations were higher in the IR group (228 $\pm$ 8 vs. 76 $\pm$ 5 mg/dL). The results in Figure 7 (left panel) again show that differences in insulin resistance did not affect weight loss. Weight decreased by a comparable amount (8.7 $\pm$ 0.9 vs. 8.4 $\pm$ 0.8 kg) and to a similar final value in both groups. SSPG concentration

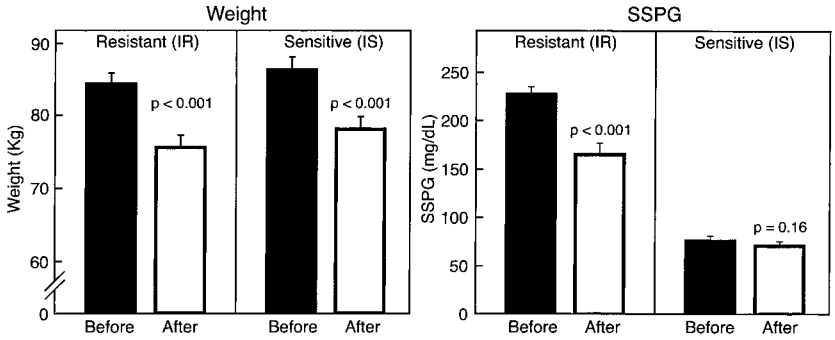

FIG. 7. Weight (left panels) and SSPG concentrations (right panels) before and after 4 months of a calorie-restricted diet in equally obese individuals, subdivided into insulin-resistant (IR) and insulin-sensitive (IS) subgroups on the basis of their SSPG concentrations at baseline. [Reprinted with permission from McLaughlin T, Abbasi F, Lamendola C, Liang L, Reaven G, Schaaf P, Reaven P 2002 Differentiation between obesity and insulin resistance in the association with C-reactive protein. Circulation 106:2908–2912.]

decreased significantly (right panel) to 165 ± 12 mg/dl ($p < 0.001$) with weight loss in the IR group, although this value was still greater than the post-weight loss value in the IS group (75 ± 5 mg/dl, $p < 0.001$). SSPG concentration did not change significantly in the IS group with weight loss.

Daylong plasma glucose and insulin concentrations for both groups, before and after weight loss, are shown in Figure 8. Daylong plasma glucose concentrations (top panel) were significantly higher at baseline in the IR group than in the IS group ($p = 0.005$). In addition, plasma glucose concentrations declined significantly ($p < 0.001$) with weight loss in the IR group. The daylong response was no longer significantly higher than in the IS group ($p = 0.29$). In contrast, daylong plasma glucose responses were essentially the same in the IS group, before and after weight loss.

The IR group had significantly greater daylong plasma insulin concentrations (Figure 8, lower panel) than the IS group at baseline ($p < 0.005$). Furthermore, although daylong integrated insulin concentrations were significantly lower following weight loss ($p = 0.01$) in the IR group, it should be emphasized that the post-weight loss daylong insulin response in this group was still elevated, as compared with the post-weight loss daylong insulin response in the IS group ($p > 0.001$). Finally, daylong insulin concentration curves were similar before and after weight loss in the IS group.

In addition to the higher daylong plasma glucose and insulin concentration, we had shown in an earlier study that insulin-resistant women had higher plasma TG and lower HDL-C concentrations as well as higher plasma concentrations of plasminogen activator inhibitor-1 (Abbasi *et al.*, 1999). Given this cluster of

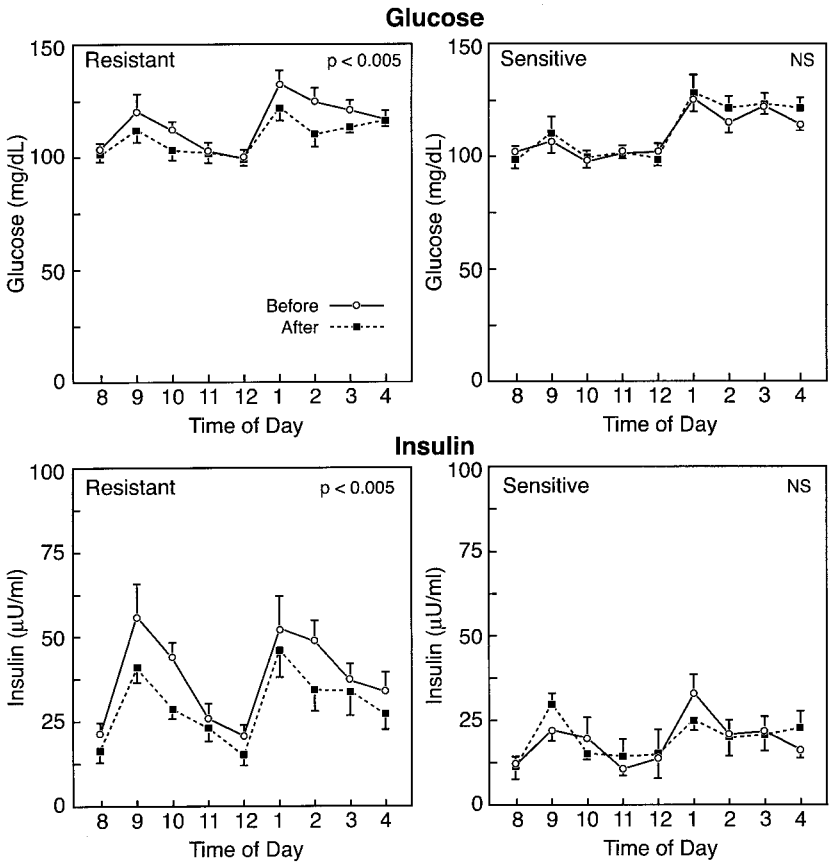

FIG. 8. Daylong plasma glucose (upper panels) and insulin responses (lower panels) before and after weight loss in obese individuals subdivided into IR and IS subgroups on the basis of their SSPG concentrations at baseline. [Reprinted with permission from McLaughlin T, Abbasi F, Lamendola C, Liang L, Reaven G, Schaaf P, Reaven P 2002 Differentiation between obesity and insulin resistance in the association with C-reactive protein. Circulation 106:2908–2912.]

abnormalities known to increase CVD risk, we also compared concentrations of high-sensitivity C-reactive protein (CRP) in the IR and IS groups of obese women. These results are seen in Figure 9, which presents CRP concentrations of the two groups, before and after weight loss. Baseline CRP concentrations were significantly higher in the IR group than in the IS group (0.39 ± 0.08 vs. 0.12 ± 0.03 mg/dL, $p = 0.001$). Furthermore, whereas plasma CRP concentrations were significantly lower after weight loss in the IR group ($p = 0.04$), there was no decline in CRP concentrations in the IS group. Despite the fall in CRP concentrations with weight loss in the IR group, the post-weight loss CRP

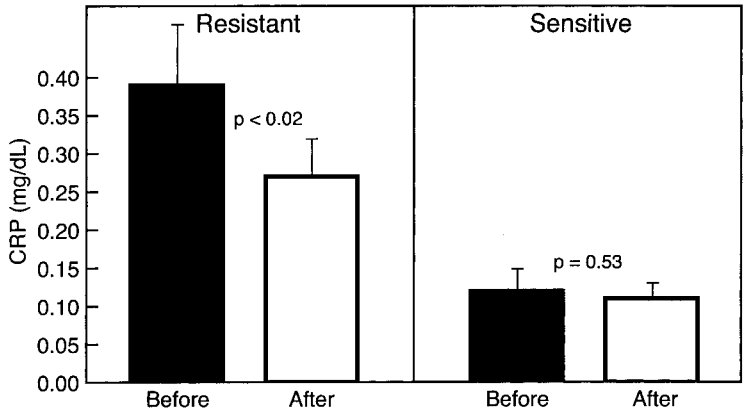

FIG. 9. C-reactive protein (CRP) concentrations before and after weight loss in obese individuals divided into IR and IS subgroups on the basis of their SSPG concentrations at baseline. [Reprinted with permission from McLaughlin T, Abbasi F, Lamendola C, Liang L, Reaven G, Schaaf P, Reaven P 2002 Differentiation between obesity and insulin resistance in the association with C-reactive protein. Circulation 106:2908–2912.]

concentrations were still higher in the IR group than in the IS group (0.27 ± 0.05 vs. 0.11 ± 0.02, $p = 0.008$).

VI. Conclusions

Recent reports (Kuczmarski *et al.*, 1997) indicate that more than 50% of the U.S. population is overweight (BMI > 25.0 kg/m^2), with approximately 20% designated as obese (BMI > 30 kg/m^2). The disease-related implications of this epidemic have received an enormous amount of publicity in the popular media but public awareness of the untoward effects of excess weight has not led to an effective approach to dealing with the dilemma. The gravity of the problem is accentuated in light of the report that only about half of the physicians polled provided weight-loss counseling (Galuska *et al.*, 1999) and that pharmacological treatment of weight loss is not being used appropriately in overweight individuals (Khan *et al.*, 2001).

Given the importance of excess adiposity as increasing risk of CVD, type 2 diabetes, and hypertension (West and Kalbfleisch, 1971; Havlik *et al.*, 1983; Rimm *et al.*, 1995), and the combination of an increase in the prevalence of overweight/obesity and a health care system unprepared to deal with this situation, it is essential that considerable thought be given as to how to best address this dilemma. In this context, it must be emphasized that CVD, type 2 diabetes, and hypertension are characterized by resistance to insulin-mediated

glucose disposal (Reaven, 1988,2001) and that insulin resistance, as well as the compensatory hyperinsulinemia associated with insulin resistance, have been shown to be independent predictors of all three clinical syndromes (Yip *et al.*, 1998; Zavaroni *et al.*, 1999; Facchini *et al.*, 2001). It has been apparent for many years that overweight/obese individuals tend to be insulin resistant and become more insulin sensitive with weight loss (Olefsky *et al.*, 1974). In light of these observations, it seems reasonable to suggest that insulin resistance is the link between overweight/obesity and the adverse clinical syndromes related to excess adiposity. Based on this fundamental premise, our research group has performed a series of studies over the past several years in an effort to provide insights into the nature of this relationship. The evidence summarized in this review has shown that the more overweight an individual is, the more likely he/she will be insulin resistant and at increased risk to develop all the abnormalities associated with this defect in insulin action. However, we do not believe that all overweight/obese individuals are insulin resistant, any more than all IR individuals are overweight/obese. More importantly, there is compelling evidence that CVD risk factors are present to a significantly greater degree in the subset of overweight/obese individuals that are also insulin resistant. Not surprisingly, we have also demonstrated that an improvement in CVD risk factors with weight loss occurs to a significantly greater degree in those overweight/obese individuals who were also insulin resistant at baseline. In view of the ineffectiveness of current clinical approaches to weight loss, it seems necessary to recognize that not *all* overweight/obese individuals are at equal risk to develop CVD and that it is clinically useful to identify those at highest risk. If this is done, intense efforts at weight control can be brought to bear on those who not only need it the most but also have the most to gain by losing weight.

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Leptin and the Cardiovascular System

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ABSTRACT

Obesity is associated with increased cardiovascular morbidity and mortality, in part through development of hypertension. Recent observations suggest that the cardiovascular actions of leptin may help explain the link between excess fat mass and cardiovascular diseases. Leptin is an adipocyte-derived hormone that acts in the central nervous system to promote weight loss by decreasing food intake and increasing metabolic rate. Leptin causes a significant increase in overall sympathetic nervous activity, which appears to be due to direct hypothalamic effects and is mediated by neuropeptide systems such as the melanocortin system and corticotropin-releasing hormone. Renal sympathoactivation to leptin is preserved in the presence of obesity, despite resistance to the metabolic effects of leptin. Such selective leptin resistance, in the context of circulating hyperleptinemia, could predispose to obesity-related hypertension. Some *in vitro* studies have suggested that leptin may have peripheral actions such as endothelium-mediated vasodilation that might oppose sympathetically induced vasoconstriction. However, we and others have shown that leptin does not have direct vasodilator effects *in vivo*. The fact that chronic leptin administration or overexpression of leptin produces hypertension supports the concept that the hemodynamic actions of leptin are due predominantly to sympathetic activation. Exploration of the sites and mechanisms of leptin resistance should provide novel therapeutic strategies for obesity, insulin resistance, and hypertension.

I. Introduction

Obesity has become one of the most-serious health problems in industrialized societies. Weight gain is associated with a high risk of developing cardiovascular and metabolic diseases such as coronary heart disease, hypertension, diabetes, and dyslipidemia. Epidemiological studies have documented a close relationship between body mass index (BMI) and cardiovascular events (Hubert *et al*., 1983; Kushner, 1993; Kopelman, 2000). The association between body weight and blood pressure has been found even in normotensive subjects with normal BMI (Stamler *et al*., 1978; Mikhail and Tuck, 2000). Subsequently, clinical studies have demonstrated that weight loss induced by low-calorie diet or gastric bypass reduces arterial pressure and corrects diabetes and other comorbidities associated with obesity (Carson *et al*., 1994; Richards *et al*., 1996). Several experimental models of obesity-induced hypertension have been devel-

oped. Different species — including the dog, rabbit, rat, and mouse — develop obesity associated with an increase in blood pressure when fed a high-fat diet (Kaufman *et al*., 1991; Mills *et al*., 1993; Montani *et al*., 2002). Some genetic models of obesity (e.g., the Zucker fatty rat, agouti obese mouse) are also used as models of obesity hypertension (Kurtz *et al*., 1989; Mark *et al*., 1999).

Until recently adipose tissue was considered exclusively as a body energy store without other function. However, the realization that adipocytes can produce many hormones — including leptin, adiponectin, resistin, atrial natriuretic peptide, and angiotensinogen — have led to a view that this tissue is an endocrine secretory organ (Bradley *et al*., 2001). Hormones produced by the adipose tissue may act locally to affect adipocyte growth and differentiation or may be released into the circulation to act elsewhere. Among the different hormones released by adipose tissue, leptin has probably drawn the most attention in recent years.

II. Leptin

Classical lesion studies established the importance of the hypothalamus in control of energy homeostasis (for a review, see Elmquist *et al*., 1999). It was then postulated that to control body fat stores, the brain must receive afferent input in proportion to the current level of body fat. Coleman (1973) was the first to demonstrate the existence of a circulating factor that plays a major role in the regulation of body weight. Using parabiosis methodology, he found that obesity in the ob/ob mouse was due to lack of this factor, whereas obesity in the db/db mouse was caused by reduced sensitivity to the factor. The much-later cloning of the ob gene revealed the identity of the adiposity factor, which was termed "leptin" (Zhang *et al*., 1994). This hormone is a 167-amino acid protein expressed mainly by adipocytes and released in the blood in proportion to the size of adipose tissue, which is consistent with leptin's role as a signal of the energy stores (Figure 1). Leptin gene expression and secretion are increased by overfeeding, high-fat diet, insulin, glucocorticoids, endotoxin, and cytokines and decreased by fasting, testosterone, thyroid hormone, and exposure to cold (Coleman and Hermann, 1999; Fried *et al*., 2000). Leptin gene expression also is modulated by the sympathetic nervous system. For example, we found that blockade of norepinephrine synthesis with alpha-methyl-para-tyrosine or pharmacologic sympathectomy with 6-hydroxydopamine increases leptin mRNA as well as plasma leptin (Sivitz *et al*., 1999). These observations suggest that the sympathetic nervous system tonically inhibits leptin gene expression through a negative-feedback loop to adipose tissue.

In lean subjects, leptin circulates at low levels (i.e., 5–15 ng/ml) as a free form and attached to binding proteins (Sinha *et al*., 1996). Plasma leptin is transported to the central nervous system (CNS) by a saturable, unidirectional

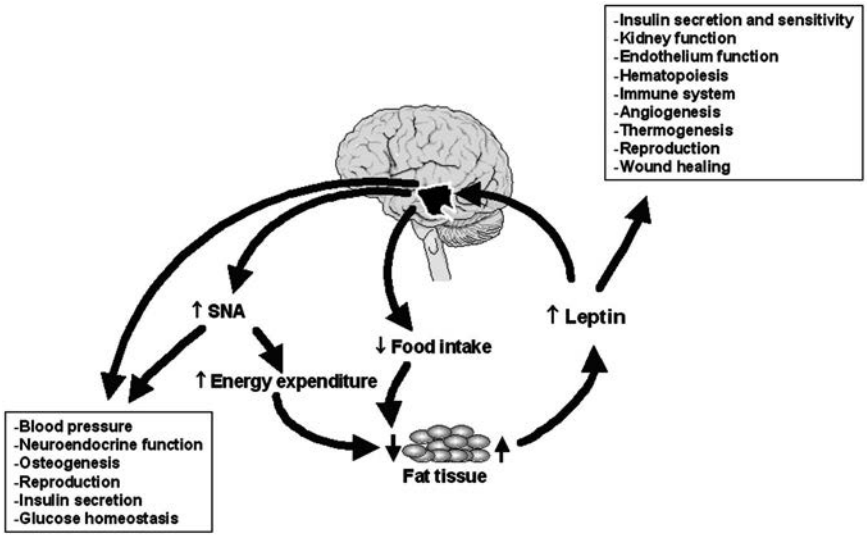

FIG. 1. Role of leptin in the regulation of body weight and other functions. Leptin is secreted by adipocytes and circulates in the blood in a concentration proportional to fat mass content. Action of leptin on its receptor present in the hypothalamus inhibits food intake and increases energy expenditure through stimulation of sympathetic nerve activity (SNA). Leptin modulates different other functions by direct peripheral action in various tissues or through the modulation of SNA.

system (Banks *et al*., 1996), involving binding of leptin to the short form of the leptin receptor located at the endothelium of the vasculature and the epithelium of choroid plexus (Bjorbaek *et al*., 1998a). Some evidence suggests that leptin also may be produced in the brain (Wiesner *et al*., 1999); however, the physiological role of leptin produced locally in the brain and its autocrine and/or paracrine effects remain unknown. Leptin action in the CNS promotes weight loss by decreasing food intake and increasing energy expenditure (Figure 1). The severe obesity and hyperphagia caused by the absence of leptin in rodents and humans demonstrate the importance of this hormone for the control of body weight and food intake (Friedman and Halaas, 1998). Although the main role of leptin is as an adipostat, more-recent studies have shown that leptin has a broad range of effects in different tissues (Wauters *et al*., 2000; Margetic *et al*., 2002). Leptin affects these different functions either by direct action in peripheral tissues or through its action in the CNS (Figure 1).

III. Leptin Receptor and Mechanisms of Signaling

The leptin receptor is a protein with a single transmembrane domain belonging to the cytokine superfamily. Six different alternatively spliced iso-

forms of this receptor have been identified (designated Ob-Ra to Ob-Rf) (Tartaglia, 1997). Five isoforms (Ob-Ra to Ob-Rd and Ob-Rf) share an identical extracellular domain but differ in the length of their intracellular domain. Ob-Re, which lacks both the transmembrane and intracellular domains, is a soluble form of the receptor. The Ob-Rb form that encodes the full receptor, including the long intracellular domain, appears to mediate most of the biological effects of leptin (Chen *et al*., 1996; Tartaglia, 1997). As predicted by parabiosis studies, the db/db mouse lacks the Ob-Rb form of the receptor, resulting from premature stop codon at the 3′-end of the Ob-Rb transcript (Chen *et al*., 1996; Chua *et al*., 1996). In contrast, the obese Zucker rat lacks all forms of the leptin receptor, caused by an amino acid substitution (glutamine → proline) in the extracellular domain common to all known isoforms of the leptin receptor (Iida *et al*., 1996; Phillips *et al*., 1996).

The janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway was the first signaling mechanism associated with the leptin receptor (Vaisse *et al*., 1996). Activation of this signaling pathway in the hypothalamus is initiated upon the conformational changes in the leptin receptor triggered by leptin binding. Intracellular JAK proteins (JAK2 and possibly JAK1) that are associated with the binding motifs located in the proximal region of the leptin receptor then are activated by transphosphorylation. Activated JAK proteins, in turn, phosphorylate tyrosine residues of the receptor, providing docking sites for STAT proteins, which become tyrosine-phosphorylated by JAK. Phosphorylated STAT molecules dimerize and translocate to the nucleus to modulate the transcription of target genes (Figure 2). While *in vitro* studies have shown that leptin receptors signal through different STAT molecules, *in vivo* studies have demonstrated that, in the hypothalamus, this occurs specifically through activation of STAT3 (Bates *et al*., 2003). Leptin's effects on the newly identified suppressors of the cytokine signaling family (SOCS) represent a negative-feedback mechanism for this pathway (Bjorbaek *et al*., 1998b; Emilsson *et al*., 1999). SOCS proteins negatively regulate the JAK/STAT pathway, either by directly blocking JAKs or through inhibition of further STAT phosphorylation.

More recently, several other intracellular signaling mechanisms have been associated with the leptin receptor. By engaging JAK2, the leptin receptor is able to stimulate insulin receptor substrate (IRS)-2, which, in turn, activates phosphoinositol-3 kinase (PI_3-K) through an association to its regulatory subunit (Niswender *et al*., 2001). This pathway appears to be involved not only in the modulation of neuronal firing rate via the activation of the potassium-adenosine triphosphate (ATP) channels (Niswender and Schwartz, 2003) but also in the modulation of gene transcription through a phosphodiesterase 3-cyclic adenosine monophosphate (cAMP) mechanism (Zhao *et al*., 2002). The leptin receptor also was found to be able to activate the extracellular factor-regulated kinases

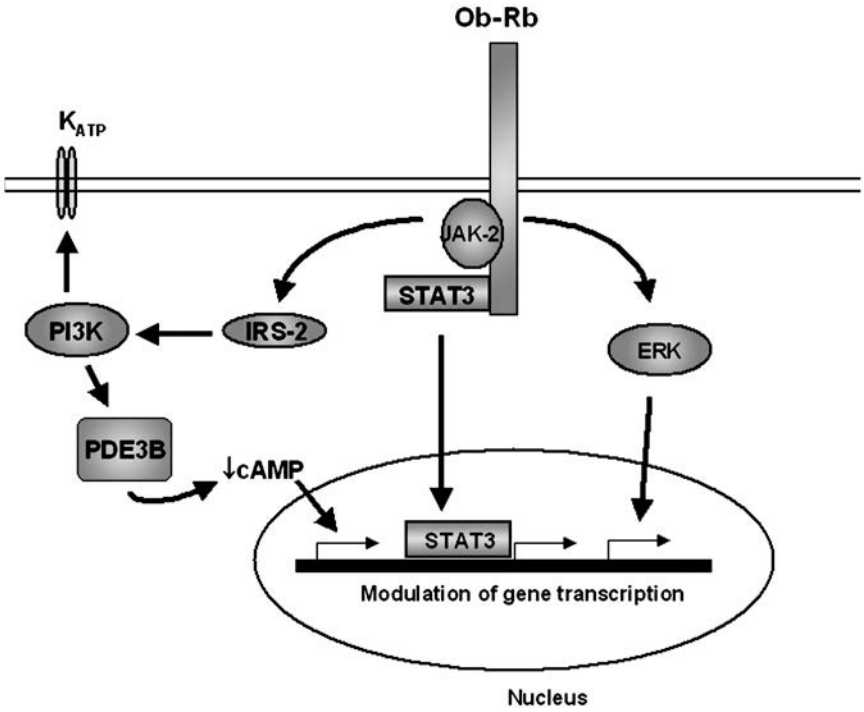

FIG. 2. Molecular mechanisms involved in leptin receptor (Ob-Rb) signaling in the hypothalamus. Leptin modulates gene transcription via activation of signal transducer and activator of transcription (STAT) proteins, phosphoinositol 3 kinase (PI3-K, via the phosphodiesterase 3B (PDE3B)), and a member of the mitogen-activated protein kinase, extracellular factor-regulated kinase (ERK). The PI3-K pathway also appears to be involved in the modulation of neuronal firing rate via the activation of the potassium-adenosine triphosphate channels (K_{ATP}).

(ERKs), a member of the mitogen-activated protein kinase (MAPK) (Bjorbaek *et al*., 2001; Bates *et al*., 2003), by different mechanisms, including direct phosphorylation by JAK2. This ERK pathway appears to be involved in leptin-induced immediate early-response gene expression, c-fos.

IV. Leptin and the Sympathetic Nervous System

A. SYMPATHETIC EFFECTS OF LEPTIN

Consistent with its role in the regulation of energy expenditure, leptin was found to increase norepinephrine turnover in brown adipose tissue (BAT) (Collins *et al*., 1996), suggesting activation of sympathetic outflow to this tissue.

Using multifiber recording of regional sympathetic nerve activity (SNA), we evaluated the effects of leptin on the sympathetic outflow to different beds (Haynes *et al*., 1997). As expected, we found that intravenous administration of leptin in anesthetized Sprague-Dawley rats caused a significant and dose-dependent increase in SNA to BAT (Figure 3). Unexpectedly, leptin caused sympathoactivation to other beds not usually considered thermogenic, such as the kidney, hindlimb, and adrenal. Satoh *et al*. (1999) investigated the effect of leptin on circulating catecholamines and found that leptin administration caused a significant and dose-dependent increase in plasma concentration of norepinephrine and epinephrine.

We have shown that the leptin-induced regional increases in SNA respond nonuniformly to baroreflex activation and hypothermia (Hausberg *et al*., 2002a,b). Leptin-induced increases in renal SNA can be suppressed by baroreflex activation, suggesting that the increase in renal SNA subserves circulatory functions. In contrast, leptin-induced BAT sympathoactivation was not prevented by baroreflex activation, suggesting the recruitment of sympathetic fibers that serve thermogenic or metabolic, rather than circulatory, functions (Hausberg *et al*., 2002a). The effect of leptin on regional SNA response to hypothermia differs

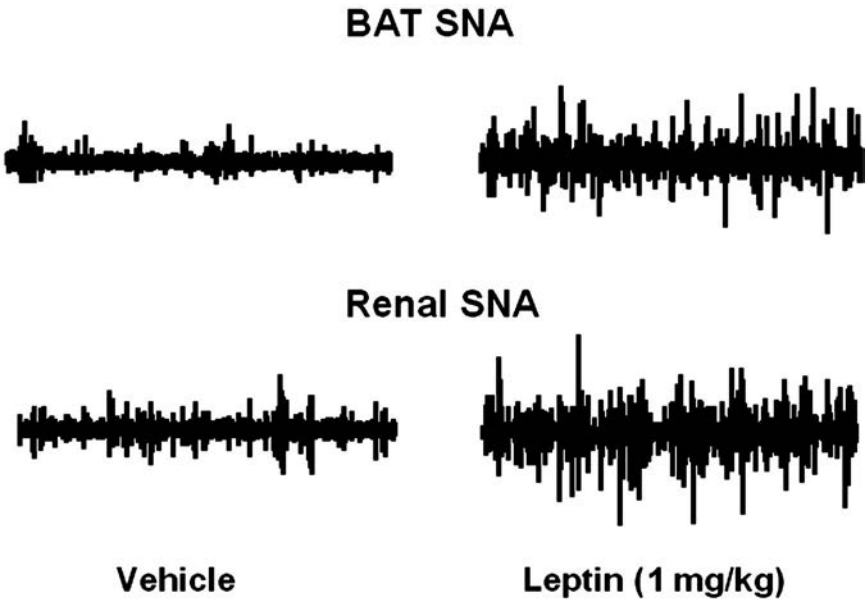

FIG. 3. Effects of intravenous administration of leptin (1 mg/kg), as compared to vehicle, on SNA to brown adipose tissue (BAT) and kidney in Sprague-Dawley rats. Leptin caused significant increases in both renal and BAT SNA.

between sympathetic fibers that serve circulatory or thermogenic functions. Leptin, at a low dose that does not alter baseline SNA, acutely enhances sympathetic outflow to BAT in response to hypothermia in lean rats. This effect is specific for thermogenic SNA because leptin does not affect the response of renal SNA to hypothermia (Hausberg *et al*., 2002b).

Although some reports have shown that leptin could increase SNA through stimulation of peripheral afferent nerves (Niijima *et al*., 1998; Tanida *et al*., 2000), our data support the concept that sympathoactivation to leptin is due to the action of this hormone in the CNS. First, leptin-induced sympathoexcitation remains apparent after transection of the sympathetic nerves distal to the recording site and disappears after ganglion blockade with intravenous chlorisondamide (Haynes *et al*., 1997). These findings indicate that the increase in SNA resulted from efferent sympathetic nerves rather than afferent nerves. Second, direct administration of leptin to the third cerebral ventricle, at subsystemic doses, increases SNA (Haynes, 2000) and dose-dependently increases plasma catecholamines (Satoh *et al*., 1999). Third, sympathoactivation to intravenous leptin can be abolished completely by selective lesioning of the hypothalamic arcuate nucleus (Haynes, 2000). The arcuate nucleus of the hypothalamus is also considered the major site of leptin action to control body weight and food intake (Schwartz *et al*., 2000).

In humans, there is no direct evidence for a role of leptin in the regulation of the sympathetic nervous system because results from leptin infusions in humans are lacking. In nonhuman primates, however, leptin has been shown to activate the sympathetic nervous system, as assessed by an increase in circulating norepinephrine levels after a single cerebroventricular administration of leptin (Tang-Christensen *et al*., 1999). Indirect evidence suggests that leptin may be important for control of SNA in humans. A positive and significant correlation between muscle SNA and plasma leptin concentration has been reported in healthy, nondiabetic men (Snitker *et al*., 1997). Also, serum leptin levels are a strong positive determinant of resting metabolic rate, which is under sympathetic control, suggesting that action of leptin on SNA is a determinant of energy expenditure in humans. In addition, Jeon *et al*. (2003) have shown that the correlation between leptin and resting metabolic rate is lost in patients with a disrupted sympathetic nervous system caused by spinal cord injury. These patients had also a lower resting metabolic rate. Together, these findings strongly support the concept that leptin influences energy expenditure through the sympathetic nervous system in humans.

B. SYMPATHETIC EFFECTS OF LEPTIN IN OBESITY

Several lines of evidence suggest that obesity is associated with activation of the sympathetic nervous system. Plasma and urinary catecholamines are in-

creased in obese humans as well as in obese animal models (Sowers *et al*., 1982; Young and Landsberg, 1982; Rocchini *et al*., 1989). Using direct measurement with microneurography, several groups have shown increased SNA to skeletal muscle in obese subjects, as compared to lean individuals (Vollenweider *et al*., 1994; Grassi *et al*., 1995). Norepinephrine spillover techniques have demonstrated that human obesity is associated with increased SNA to a key organ of the cardiovascular homeostasis, the kidney (Vaz *et al*., 1997). Elevated renal SNA is also reported in animal models of obesity, including rats on high-fat diet (Iwashita *et al*., 2002). These findings demonstrate that enhanced SNA is a common feature of obesity, which would play a major role in obesity-induced hypertension and cardiovascular disease (Hall *et al*., 2000). However, the mechanisms responsible for increased SNA in obesity remain unknown.

Obesity is known to be associated with circulating hyperleptinemia, reflecting resistance to leptin because, despite high circulating levels of leptin, such subjects remain obese (Considine *et al*., 1996). Under these circumstances, in order for leptin to have a role in obesity-related hypertension, one must postulate that any leptin resistance is selective, with preservation of sympathetic responsiveness. Indeed, we have demonstrated that in some animal models, including agouti mice and diet-induced obesity, leptin resistance is selective, with sparing of the effects of leptin on renal SNA. For example, in agouti mice, the anorexic and weight-reducing effects of leptin were less in the obese mice, compared to lean littermates. However, the increase in renal SNA in response to leptin was identical in both lean and obese mice (Correia *et al*., 2002; Rahmouni *et al*., 2002). Eikelis *et al*. (2003) recently have shown the existence of a strong correlation between leptin plasma concentration and renal SNA across a broad range of leptin values in men of widely differing adiposity. This indicates that leptin may be the main cause of sympathoactivation associated with obesity in both animal models and humans (Figure 4).

C. MECHANISMS OF LEPTIN-INDUCED SYMPATHOACTIVATION

The receptor-mediated sympathoexcitatory effect of leptin is supported by the absence of SNA response to leptin in obese Zucker rats (Haynes *et al*., 1997). However, it was not clear which form of the leptin receptor was involved, since Zucker rats lack all forms of the leptin receptor. We recently demonstrated an absence of renal SNA response to leptin in db/db mice that indicates that the effects of leptin on sympathetic outflow are mediated by the long-form Ob-Rb of the leptin receptor (Rahmouni *et al*., 2003b).

As mentioned earlier, the leptin receptor has divergent signaling capacities and modulates the activity of different intracellular enzymes. Although STAT signaling was thought to be the main pathway that mediates the leptin action in the hypothalamus, PI_3-K has been found to play a pivotal role in the effect of

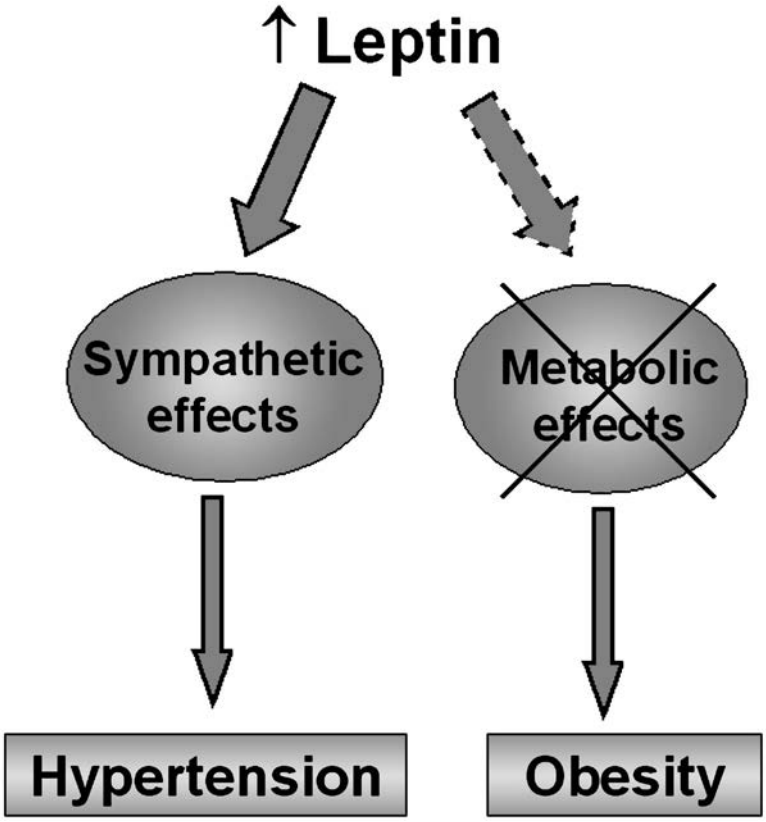

FIG. 4. Concept of selective leptin resistance: there is resistance to the appetite- and weight-reducing actions of leptin but preservation of the sympathetic actions. This phenomenon might explain in part how hyperleptinemia could be accompanied by obesity (partial loss of appetite and metabolic actions of leptin) but still contribute to sympathetic overactivity and hypertension because of preservation of the sympathetic actions of leptin to some organs involved in blood pressure regulation (e.g., kidney).

leptin on food intake (Niswender and Schwartz, 2003). Our group has demonstrated that PI_3-K plays a major role in the transduction of leptin-induced changes in renal sympathetic outflow. We compared renal sympathoactivation to leptin before and after intracerebral administration of PI_3-K inhibitors. Both LY294002 and wortmannin markedly attenuated the increase in renal SNA induced by leptin, without affecting sympathoactivation to stimulation of the melanocortin system (Rahmouni *et al.*, 2003a). The role of PI_3-K and other pathways in leptin-induced sympathoactivation to different other beds — including BAT, hindlimb, and adrenal gland — remains unknown. Leptin likely controls sym-

pathetic nerve activity in a tissue-specific manner, for several reasons. First, activation of arterial baroreceptors and hypothermia modulate differentially leptin-induced sympathoactivation to the kidney and BAT (Hausberg *et al*., 2002a,b). Second, in diet-induced obese mice, lumbar SNA responses to leptin are attenuated, as compared to lean mice, whereas leptin-induced increases in renal SNA occur with the same time course and magnitude in both diet-induced obese and lean mice (K. Rahmouni, D.A. Morgan, A.L. Mark, W.G. Haynes, unpublished data).

Several hypothalamic neuropeptides, monoamines, and other transmitter substances have been identified as candidate mediators of leptin action in the hypothalamus. These include melanocortins, neuropeptide Y (NPY), corticotropin-releasing hormone (CRH), melanin-concentrating hormone, and cocaine- and amphetamine-regulated transcript (CART) (Schwartz *et al*., 2000). Therefore, leptin could cause regional sympathoactivation through stimulation of different neuropeptides. In the neural melanocortin system, alpha-melanocyte stimulating hormone (α-MSH) is derived from pro-opiomelanocortin (POMC) and acts mainly on melanocortin-4 receptors (MC-4R). Both renal and lumbar sympathoactivation to leptin seem mediated by the melanocortin system because blockade of melanocortin receptors with SH9119 (Haynes *et al*., 1999) or agouti protein (Dunbar and Lu, 1999) inhibits the renal and lumbar SNA response to leptin. However, SHU9119 does not block leptin-induced sympathoactivation to BAT (Haynes *et al*., 1999). Using a MC-4R knockout mouse, we recently confirmed that the renal SNA response to leptin is mediated by MC-4R. Indeed, we have shown a gene dose effect, with MC4-R heterozygotes having 50% of the normal response to leptin and homozygote knockouts having no renal SNA response to leptin (Rahmouni *et al*., 2003b). The interrelationship between leptin and the melanocortin system appears to be more complex than first thought because absence of the leptin receptor in db/db mice attenuates the renal SNA response to stimulation of the MC-3/4R with MTII (Rahmouni *et al*., 2003b). This was not expected because the melanocortin system was considered downstream to leptin. Although the mechanisms of this attenuated SNA response to MTII in db/db mice remain unknown, one possibility relates to the increased expression of the agouti-related protein in these mice (Korner *et al*., 2001), which is known to at least partially block the melanocortin receptors in the brain.

The increase in BAT SNA seems to depend on neuropeptides other than the melanocortin system. Given that intracerebral CRH increases SNA to BAT, we investigated the role of this system in leptin-induced sympathoactivation to this tissue. Our results show that a CRH receptor antagonist blocked leptin-induced sympathoactivation to BAT but not to the kidney (Correia *et al*., 2001a). In summary, leptin appears to causes regional sympathoactivation via different neuropeptide pathways, with melanocortins mediating renal sympathoactivation and CRF mediating BAT SNA to leptin.

V. Leptin and Blood Pressure

A. PRESSOR EFFECTS OF LEPTIN

Leptin-induced activation of SNA to organs such as the kidney was the first indication of the potential role of this hormone in regulation of blood pressure. The sympathetic nervous system is an important component in the control of renal function (DiBona and Kopp, 1997). Long-term renal sympathetic stimulation by leptin would be expected to raise arterial pressure by causing vasoconstriction and by increasing renal tubular sodium reabsorption. Dunbar *et al*. (1997) have shown that the sympathoactivation to leptin is followed by a slow but progressive increase in mean arterial pressure. Shek *et al*. (1998) demonstrated that intravenous infusion of leptin at a dose that increased plasma leptin from 1 to 94 ng/ml for 12 days increased arterial pressure and heart rate, despite a decrease in food intake that would be expected to decrease arterial pressure. Leptin-induced increases in arterial pressure probably are due to a central neural action of this hormone because intracerebroventricular administration of leptin mimics the effects of systemic administration (Correia *et al*., 2001b). The substantial dose-dependent increase in heart rate, as well as the greater response to air-jet stress observed in leptin-treated rats, supports central activation of the sympathetic nervous system (Correia *et al*., 2001b). Finally, blockade of the adrenergic system inhibits the pressor response to leptin (Carlyle *et al*., 2002).

Further evidence for the pressor effects of leptin derives from studies of transgenic mice overexpressing leptin in the liver (Aizawa-Abe *et al*., 2000). These mice had 10-fold increases in plasma leptin and decreased body weight. Despite the decreased body weight, the transgenic mice overexpressing leptin had significantly higher arterial pressure than nontransgenic littermates. The transgenic mice also had increased urinary excretion of norepinephrine, a marker of sympathetic nervous system activity. The increase in arterial pressure was normalized after alpha-adrenergic or ganglionic blockade, again demonstrating the importance of the sympathetic nervous system in the pressor effects of leptin.

We evaluated arterial pressure in obese, leptin-deficient ob/ob mice and their wild-type, lean controls (Mark *et al*., 1999). Despite body weights nearly twice as high as their lean controls, the leptin-deficient ob/ob mice had lower arterial pressure. Aizawa-Abe *et al*. (2002) subsequently reported that administration of leptin to ob/ob mice (so-called leptin reconstitution) increased systolic blood pressure by as much as 25 mmHg, despite decreases in food intake and body weight. These findings demonstrate that leptin contributes physiologically to the regulation of arterial pressure.

In contrast to leptin-deficient ob/ob mice, agouti yellow obese mice have elevated arterial pressure, despite the fact that they have milder obesity than ob/ob mice (Mark *et al*., 1999). Obesity induced by a high-fat diet is also

associated with an increase in arterial pressure (Mills *et al*., 1993; Iwashita *et al*., 2002). The presence of high circulating levels of leptin associated with the selectivity in leptin resistance (i.e., preserved ability of leptin to increase renal SNA) (Figure 4) could explain the hypertension in these different models of obesity. Other mechanisms may contribute to the development of obesity-related hypertension. For example, *in vitro* studies have shown that leptin causes oxidative stress in cultured endothelial cells by increasing the generation of reactive oxygen species (ROS) (Bouloumie *et al*, 1999; Yamagishi *et al*., 2001). Leptin also has been shown to stimulate the secretion of proinflammatory cytokines (e.g., tumor necrosis factor-alpha, interleukin-6) that are known to promote hypertension (Loffreda *et al*., 1998).

As has been described for renal SNA, the melanocortin system appears to mediate the effect of leptin on blood pressure. First, pharmacological activation of melanocortin receptors for 14 days caused significant increases in arterial pressure, despite decreases in food intake and body weight (Kuo *et al*., 2003). Second, inhibition of melanocortin receptors blocks the increase in blood pressure induced by leptin (Dunbar and Lu, 1999).

In order to investigate whether the increase in arterial pressure induced by leptin is due to enhanced salt sensitivity, we studied the effects of a high-salt diet on the pressor responses of intracerebroventricular administration of leptin. The increase in arterial pressure was similar in leptin-treated rats fed a low- or high-salt diet, indicating that leptin-dependent mechanisms in the CNS do not alter arterial pressure sensitivity to salt (Correia *et al*., 2001b).

In humans, several studies have shown that plasma leptin is related to blood pressure in both normotensive and hypertensive subjects (Hirose *et al*., 1998; Schorr *et al*, 1998). A positive correlation has been observed between longitudinal changes in leptin and the arterial pressure (Itoh *et al.*, 2002). Farooqi *et al*. (1999) have reported that replacement leptin therapy in a child with congenital leptin deficiency for 1 year caused a drastic decrease in body weight (16 kg). This weight loss would be expected to lower arterial pressure substantially but the arterial pressure did not change. These observations are consistent with a pressor action of leptin offsetting the depressor action of weight loss.

B. DEPRESSOR EFFECTS OF LEPTIN

Recently, several studies have suggested that leptin may have direct vascular effects that tend to decrease arterial pressure. The vascular endothelium is an important component in the control of arterial pressure homeostasis (Contreras *et al*., 2000). Endothelial cells release several vasoactive factors, of which nitric oxide (NO) is perhaps the most important with potent vasodilator action. Functionally competent leptin receptors are present on endothelial cells (Sierra-Honigmann *et al*., 1998) and leptin administration in rat causes a dose-dependent

increase in NO metabolite concentrations. In one study in anesthetized rats, infusion of leptin during inhibition of NO synthesis increased arterial pressure (Fruhbeck, 1999). Leptin also decreased arterial pressure after suppression of sympathetic influence using ganglionic blockade (Fruhbeck, 1999) or chemical symapathectomy (Lembo *et al*., 2000). Furthermore, *in vitro* studies have shown that leptin evokes an endothelium-dependent relaxation of arterial rings (Kimura *et al*., 2000; Lembo *et al*., 2000). Therefore, it has been argued that these vasodilator effects of leptin might oppose its neurogenic pressor action.

In contrast to these reports, Gardiner *et al*. (1999) found no evidence for a vasodilator action of leptin in conscious rats. These authors showed that leptin does not change blood flow in different beds, including renal, mesenteric, and abdominal arteries. The presence of the NO synthase inhibitor, L-NAME, failed to unmask any pressor effect of leptin (Gardiner *et al*., 1999). Similarly, we found that leptin does not have substantial direct or indirect vasodilator effects *in vivo*. Indeed, leptin, at a concentration sufficient to increase sympathetic nerve outflow, did not change arterial pressure or blood flow measured from the mesenteric, lower aortic, and renal arteries (Mitchell *et al*., 2001). Blockade of the adrenergic system or NO synthase did not reveal any pressor effect of leptin (Mitchell *et al*., 2001). Furthermore, leptin did not alter the sympathetically mediated vasomotor response in hindlimb or kidney to stimulation of the splanchnic sympathetic nerve trunk (Jalali *et al*., 2001). Kuo *et al*. (2001) found that blockade of NO synthesis augmented the heart rate and renal vascular and glomerular responses to leptin but did not substantially augment the pressor response to leptin. Thus, the role of NO in blood pressure responses to leptin remains controversial but consistent negative results of studies in conscious animals argue against a meaningful stimulation of NO generation.

VI. Renal Effects of Leptin

Besides the indirect action of leptin on renal function via sympathoactivation, leptin could exert a direct effect on the kidney. In the rat, leptin receptor expression has been found in the inner zone of the medulla and pyramid, associated with the vascular structures, tubules, and ducts (Serradeil-Le Gal *et al*., 1997). Several investigators have shown that acute administration of leptin in anesthetized or conscious normotensive lean rats produces significant increases in sodium excretion and urine volume without significant effects on renal blood flow, glomerular filtration rate, or potassium excretion (Jackson and Li, 1997; Villarreal *et al*., 1998; Beltowski *et al*., 2002). As expected, these saliuretic effects of leptin were blunted in the Zucker rat (Villarreal *et al*., 2000) but also in high-fat-diet, obese rats (Beltowski *et al*., 2002). Surprisingly, spontaneously hypertensive rats also were refractory to the diuretic and natriuretic effects of leptin, due perhaps to enhanced renal SNA, because renal denervation restored

the saliuretic response of these rats to leptin (Villarreal *et al*., 2000). A subsequent study by Shek *et al*. (1998) suggested that the natriuretic action of leptin is not operative at physiological levels because a chronic increase in leptin concentrations within the physiological range in rats did not produce natriuresis, despite an increase in arterial pressure. This study (Shek *et al*., 1998) provided no support for a significant natriuretic action of leptin and instead suggested that leptin actually may produce a modest antinatriuretic effect (probably due to the activation of the renal SNA) that opposes pressure natriuresis.

However, leptin appears to have an important role in obesity-induced renal damage such as glomerular hyperfiltration and increased urinary albumin loss. In glomerular endothelial cells, leptin stimulates cellular proliferation, transforming growth factor-beta$_1$ (TGF-β_1) synthesis, and type IV collagen production. Conversely, in mesangial cells, leptin upregulates synthesis of the TGF-β type II receptor, but not TGF-β_1, and stimulates glucose transport and type I collagen production (Wolf *et al*., 1999). Chronic leptin treatment induces glumerosclerosis and proteinuria in normal rats (Wolf *et al*., 1999). Therefore, leptin may be of relevance to the development of glomerular pathology associated with obesity.

VII. Leptin and Other Cardiovascular Risks

Longitudinal and cross-sectional studies have shown an association between serum leptin concentrations and various cardiovascular risks, including stroke (Soderberg *et al*., 1999a), chronic heart failure (Leyva *et al*., 1998; Schulze *et al*., 2003), acute myocardial infarction (Soderberg *et al*., 1999b), coronary heart disease (Wallace *et al*., 2001), and left cardiac hypertrophy (Paolisso *et al*., 1999). Most of these studies have statistically corrected for differences in body adiposity, though such statistical techniques can be inadequate when there are large differences between groups in confounding variables. Further studies, including animal experimentation, are needed to strengthen and clarify the link between leptin and cardiovascular disease (CVD). The mechanisms by which leptin could contribute to these complications remains unknown. Although the action of leptin in the CNS and the activation of the sympathetic nervous system could play a major role in CVD, the presence of the leptin receptor in the heart suggests that leptin could modulate cardiac function directly (Wold *et al*., 2002). For example, Nickola *et al*. (2000) have shown a direct effect of leptin on cardiomyocyte contraction that may contribute to altered myocardial function. Interestingly, the cardiac contractile response to leptin was blunted in the spontaneously hypertensive rat (Wold *et al*., 2002). An effect of leptin on the adenylate cyclase system, the main effector of beta-adrenergic receptors, was demonstrated in a cardiac cell line (Illiano *et al*., 2002).

VIII. Conclusion

The epidemic of obesity has led to an unwelcome epidemic of CVD, including hypertension. The mechanisms of obesity-related CVD are not fully understood but the discovery of leptin and its effects on the sympathetic nervous system may provide a partial explanation. Leptin has diverse cardiovascular actions, although sympathoactivation is probably the most important. The concept of selective leptin resistance may explain how leptin could contribute to obesity-related hypertension, despite loss of its metabolic effects. Increasing knowledge of the mechanisms and sites of leptin resistance should provide new insights into the pathophysiology of obesity and its treatment.

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The Use of Animal Models to Dissect the Biology of Leptin

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ABSTRACT

Our understanding of the effects of leptin have stemmed mainly from animal studies, which continue to leave important clues of its roles in physiology, metabolism, neuroscience, and cell signaling. Since its discovery, leptin has been linked to various pathways, either directly at its primary site of action in the hypothalamus, or indirectly via downstream effector pathways such as in adipocytes and muscle. Leptin's importance is exemplified by the lack of redundant backup mechanisms, since leptin-deficient mice are obese, diabetic, and sterile. Investigations uncovering the pleiotropic actions of leptin were unfolded mainly from rodent models. Thus, this chapter focuses on these studies and, more specifically, on those findings recently brought forward by transgenic mice overexpressing leptin. The vast amount of biology that has been ascribed to leptin encompasses effects on food intake, insulin sensitivity, adiposity, thermogenesis, reproduction, immunity, and bone regulation. Mechanisms underlying leptin's action revolve essentially around neural pathways but also encompass to a lesser extent peripheral mechanisms. The roles of leptin along these axes are reviewed, with particular emphasis on pathways and phenotypes generated by transgenic hyperleptinemia. An evolutionary significance of hyperleptinemia in association with development of leptin resistance is suggested as a protective armament against some of the detrimental effects caused by hyperleptinemia in transgenic mice overexpressing leptin.

I. Introduction

The discovery of the adipocyte-derived hormone leptin (Zhang *et al.*, 1994) opened a new field of investigation, heightened interest in dissecting pathways regulating energy homeostasis, and a fresh viewpoint on treating obesity and associated disorders such as diabetes, syndrome X, polycystic ovary syndrome, and hypertension. It has long been known that obesity exacerbates these conditions; thus, the discovery of leptin replaced the interest brought earlier by adipsin (Flier *et al.*, 1987) and tumor necrosis factor (TNF) alpha (Hotamisligil *et al.*, 1993), by providing a new entry point to delineate mechanisms of energy homeostasis. Preparation of recombinant leptin allowed its exogenous effects to be tested on normal and leptin-deficient *ob/ob* mice (Campfield *et al.*, 1995; Halaas *et al.*, 1995; Pelleymounter *et al.*, 1995; Weigle *et al.*, 1995). The major outcomes from these early studies showed a significant decrease in food intake,

increase in energy expenditure, and selective attenuation of the adipose fat mass. Another major effect of leptin was on the reproductive axis (Chehab *et al.*, 1996), which is directly linked to an organism's nutritional status. It was shown that leptin treatment of *ob/ob* mice could rescue their lifelong sterility, while, in normal mice, it could suppress fasting-induced anestrous (Ahima *et al.*, 1996) and stimulate the onset of puberty (Ahima *et al.*, 1997; Chehab *et al.*, 1997; Yura *et al.*, 2000). Other functions of leptin were associated with immunity (Lord *et al.*, 1998) and a central regulation of bone formation (Ducy *et al.*, 2000; Takeda *et al.*, 2002). The failure of exogenous leptin treatment to attenuate the obesity in other animal models of obesity — such as the *db/db* mouse, the agouti yellow mouse, and the melanocortin 4 receptor (MC4-R) null mouse (Huszar *et al.*, 1997), which all are associated with elevated leptin levels — gave rise to the concept of leptin resistance and hyperleptinemia, a condition reflected in most forms of human obesity (Considine *et al.*, 1996). All these findings suggested that leptin has multiple roles beyond its body weight-thinning effects, as initially suggested by its name (Flier, 1998). Thus, to study leptin's systemic and chronic effects and to delineate mechanisms of action, transgenic animal models of leptin overexpression are warranted. This review is aimed mainly at pinpointing the most-relevant aspects of leptin biology that have and could emerge from overexpressing leptin in the mouse.

II. Animal Models of Leptin Overexpression

To date, there have been reports of four transgenic mouse models overexpressing leptin. One model resulted from leptin overexpression at very high levels under control of the liver-specific albumin promoter (Ogawa *et al.*, 1999). Two additional models used the fatty acid binding protein aP2 promoter to direct expression of leptin specifically to adipocytes, resulting in, in one case, moderately elevated leptin levels (Qiu *et al.*, 2001b) and in the other, very low leptin levels (Ioffe *et al.*, 1998). A fourth model directed leptin expression to the osteoblast using the alpha-1 collagen promoter (Takeda *et al.*, 2002). Although the low aP2-expressing line and the osteoblast line did not significantly alter circulating levels of leptin, they addressed specific questions regarding the sensitivity of *ob/ob* mice to small amounts of leptin and local effects on bone formation, respectively. Overexpression of leptin in a rat model also was generated using an adenoviral vector (Chen *et al.*, 1996). This review mainly addresses studies that emerged from the high and moderately elevated leptin-overexpressing lines (referred herein as LepTg) and the rat model, all of which investigated metabolic and broader effects of leptin.

III. Food Intake

Exogenous administration of leptin to *ob/ob* mice resulted in dose-dependent reductions in food intake (Pelleymounter *et al.*, 1995), with the greatest inhibition

occurring during the early phases of leptin treatment. This demonstrated the exquisite sensitivity of *ob/ob* mice to leptin. In normal mice, large leptin doses were required to result in a 35% reduction of food intake, which eventually stabilized to match the food intake of nonleptin-treated normal mice (Halaas *et al.*, 1995). These studies demonstrate that normal mice respond to leptin treatment and adapt to its anorectic effects. In LepTg mice overexpressing 12-fold and 8-fold leptin, reductions in food intake were 25% and 15%, respectively (Ogawa *et al.*, 1999; Qiu *et al.*, 2001b). This once again demonstrated the dose-dependent effect but, interestingly, the lack of adaptation to anorectic effects of leptin. Adult rats infected with an adenoviral leptin construct resulted in a 10-fold hyperleptinemia and a 30–50% reduction in food intake (Chen *et al.*, 1998). These decreases in food intake are likely to represent near-maximal levels of tolerance that a mouse can sustain without suffering devastating physiological consequences of a caloric deficit. The implications of this functional hyperleptinemia (as opposed to hyperleptinemia associated with leptin resistance) suggests that while there may be a saturable transport of leptin to the hypothalamus (Banks *et al.*, 1996), normal circulating endogenous leptin levels are far below the threshold required to achieve a transport saturation. Because the 12-fold overexpressing LepTg line resulted in a larger decrease in food intake than the 8-fold overexpressing line, it is reasonable to assume that an 8-fold leptin overexpression still does not result in transport saturation. Mechanisms leading to chronic reductions of food intake in response to functional hyperleptinemia are likely to involve a continuous downregulation of the orexigenic pathway involving neuropeptide Y (NPY) neurons and activation of the anorectic pathway mediated by pro-opiomelanocortin (POMC) neurons and the melanocortin 4 receptor (MC4R) (Cowley *et al.*, 2001). Thus, steady-state reductions of food intake in normal mice suggest that pharmacological manipulations of MC4R could result in long-term reductions in food intake in individuals who might be responsive to leptin. Overall, the anorectic effects of leptin in an organism susceptible to hyperleptinemia appear to be dose related. While they are more pronounced when exogenously administered for short periods, they last longer when chronically secreted from a transgene. Interestingly, experiments by Coleman and Hummel (1969) involving the parabiotic union of *db/db* mice (overexpressing leptin) with normal mice led to wasting and death of the normal parabiont. In contrast, LepTg mice, while continuously responding to transgenic hyperleptinemia, do not suffer from energy deprivation or a wasting phenotype, suggesting that sensitivity to hyperleptinemia is not detrimental to energy homeostasis. It is to be noted that leptin-mediated anorexia is not associated with the detrimental effects of fasting, which unlike leptin treatment (Wang *et al.*, 1999), result in elevated circulating free fatty acids (FFAs), showing an unusual role for leptin in lipolysis.

IV. Regulation of Adiposity

The characteristic and most-prominent feature of exogenous leptin treatment by any means was a decrease in body weight, which was accounted for almost entirely by a reduced adipose tissue mass. In LepTg mice, the extent of this effect correlated with transgenic leptin levels and varied from a small fat mass (Qiu *et al.*, 2001b) to a virtual absence (Chen *et al.*, 1996; Ogawa *et al.*, 1999), reflecting quantitative effects of leptin on the fat mass. When adipose tissue could be recovered, histological analysis revealed that, compared to normal mice, LepTg adipocytes were 4-fold smaller and were either depleted of triacylglycerol stores or contained minute lipid droplets (Qiu *et al.*, 2001b; Ogus *et al.*, 2003). Whether these small cells represent preadipocytes, adipocytes, or a mixture of both remains to be determined. However, mRNA expression of the fatty acid binding protein aP2, which is expressed only in mature adipocytes, failed to reveal any quantitative differences between normal and leptin transgenic mice. This suggests that the majority of these cells consist of mature adipocytes, albeit without visible intracellular lipids. Recovery and growth of these adipocytes *in vitro* in a medium rich in glucose, insulin, and insulin-like growth factor-1 (IGF-1), which are all significantly decreased in LepTg mice, revealed a rapid accumulation of triacylglycerols, suggesting a heightened sensitivity to lipid accumulation (Ogus *et al.*, 2003).

The histological makeup of the adipose tissue in the leptin-overexpressing mice is not unique to leptin (Figure 1) but rather characteristic of lean mice with small fat masses, such as fasted mice, transgenic mice overexpressing a dominant-positive form of sterol regulatory element-binding protein-1c (SREBP1c) (Shimomura *et al.*, 1998), forkhead box subclass C member 2 (FOXC2) (Cederberg *et al.*, 2001), or knockout (KO) mice for either the type II regulatory subunit of protein kinase A (Cummings *et al.*, 1996), protein tyrosine phosphatase 1B (Elchebly *et al.*, 1999; Klaman *et al.*, 2000), or the lipid-coating protein perilipin (Martinez-Botas *et al.*, 2000; Tansey *et al.*, 2001). Thus, the convergence of different pathways to a common phenotype – namely, the depletion of triacylglycerols from adipocytes — demonstrates the various effectors that impact the adipose mass. The question of whether these molecules belong to a common pathway orchestrated by leptin remains to be answered.

In nonadipose tissues (e.g., liver, muscle), leptin prevents the lipotoxicity associated with triacylglycerol buildup (Lee *et al.*, 2001) by activating 5′-adenosine monophosphate (AMP)-activated protein kinase (AMPK), which stimulates fatty acid oxidation via inhibition of acetyl coenzyme A carboxylase. Leptin's action on stimulating AMPK activity is mediated chiefly via the central nervous system (CNS), with a moderate peripheral action (Minokoshi *et al.*, 2002). Since triacylglycerol buildup in muscle is associated with insulin resistance, it is conceivable that leptin treatment

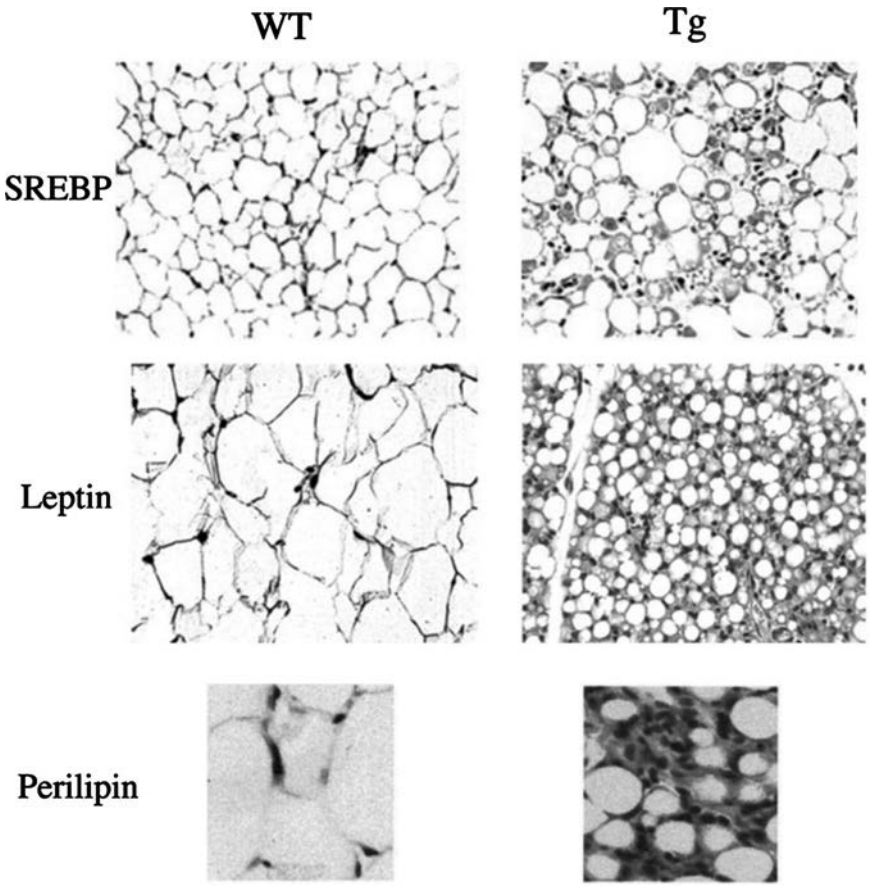

FIG. 1. Histology of white adipose tissue, stained with hematoxylin/eosin (H/E) from normal and lipodystrophic mice overexpressing either sterol regulatory element-binding protein-1c (SREBP-1c), leptin, or homozygous for a targeted deletion in perilipin. The extent of lipid accumulation and adipocyte sizes of the transgenic (Tg) mice are greatly reduced, compared to those of wild-type (WT) mice. [Reprinted with permission from Shimomura S, Hammer RE, Richardson JA, Ikemoto S, Bashmakov Y, Goldstein JL, Brown MS 1998 Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose tissue: model for congenital generalized lipodystrophy. Genes Dev 12:3182–3194; Qiu J, Ogus S, Lu R, Chehab FF 2001 Transgenic mice overexpressing leptin accumulate adipose mass at an older, but not younger, age. Endocrinology 142:348–358. Copyright The Endocrine Society; Tamsey JT, Sztalryd C, Gruia-Gray J, Roush DL, Zee JV, Gavrilova O, Reitman ML, Deng CX, Li C, Kimmel AR, Londos C 2001 Perilipin ablation results in a lean mouse with aberrant adipocyte lipolysis, enhanced leptin production and resistance to diet induced obesity. Proc Natl Acad Sci USA 98:6494–6499. Copyright 2001 National Academy of Sciences, U.S.A.]

might alleviate this effect by stimulating intramuscular lipolysis, thereby improving the diabetes phenotype.

V. Adaptation to Cold and Diet-induced Thermogenesis

Brown adipose tissue (BAT) is greatly affected in LepTg mice. However, in contrast to white adipose tissue, brown adipocytes appeared entirely devoid of triacylglycerol stores, at least as seen by light microscopic analysis (Qiu *et al.*, 2001b), suggesting a dysfunctional hypothalamic-BAT axis. A prominent characteristic of rodents is their adaptation to cold temperatures, a mechanism that ensures their survival in nature. The underlying mechanism for adaptive thermogenesis involves the uncoupling of oxidative phosphorylation to generate heat via increased expression and activity of mitochondrial uncoupling protein 1 (UCP-1) in BAT. Moreover, UCP-1 expression and activity can be stimulated via diet-induced thermogenesis, converting excess calories into heat (Himms-Hagen, 1984) and protecting the animal against obesity to some extent. Leptin's role in thermogenesis is evidenced by the cold intolerance of leptin-deficient and leptin receptor-deficient obese mice and, paradoxically, by leptin-overexpressing lean mice. Indeed, LepTg mice, unlike normal mice, were sensitive to a cold challenge and quickly succumbed to hypothermia if not rescued from the cold. This effect was independent of UCP-1 expression in BAT, since restoration of triacylglycerols in adipose tissue via high-fat diet feeding resulted in normal cold tolerance of the leptin transgenic mice (Qiu *et al.*, 2001b). Therefore, the absence of triacylglycerols in BAT failed to provide substrates for the ability of UCP-1 to uncouple oxidative phosphorylation. On the other end of the spectrum, obese mice deficient in either leptin or leptin signaling are also cold intolerant, despite the massive accumulation of triacylglycerols in BAT (Trayhurn and James, 1978; Trayhurn, 1979). Therefore, leptin plays a primordial role in regulating the hypothalamic-BAT axis. Any interference with its regulation, whether manifested in an excessively lean or obese state, is reflected by a perturbation of thermogenesis. This effect appears to be quantitative and subject to threshold levels of triacylglycerols. For example, unlike LepTg mice, perilipin KO mice could adapt to the cold (Figure 2), despite their reduced fat mass and significantly decreased intracellular triacylglycerols. However, when further depleted of their residual intracellular triacylglycerols by fasting, they could not withstand a cold challenge (Martinez-Botas *et al.*, 2000). Lipoatrophy of the perilipin KO mice is not as severe as that of LepTg mice, which quickly succumb to a cold challenge in the fed state. Thus, regulatory thermogenesis requires the presence of leptin and of a critical intracellular level of triacylglycerols in adipocytes, to elicit the induction of UCP-1 expression and activity, thereby ensuring survival of the animal at low temperatures.

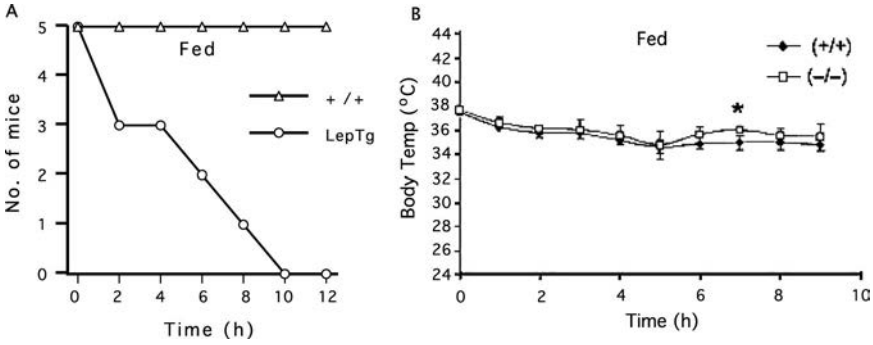

FIG. 2. Differential effects of thermogenesis in lipodystrophy in *ad libitum*-fed LepTg and perilipin knockout (KO) mice. (A) Number of LepTg mice (○) that did not tolerate the cold, resulting in a significant drop of their body temperature to 17.4°C–25°C, compared to the stable body temperature of normal mice (△). (B) Perilipin KO (−/−) were insensitive to the cold and adjusted appropriately, similar to WT controls (+/+). [(B) Reprinted with permission from Martinez-Botas J, Anderson JB, Tessier D, Lapillonne A, Chang BH, Quast MJ, Gorenstein D, Chen KH, Chan L 2000 Absence of perilipin results in leanness and reverse obesity in Lepr (*db/db*) mice. Nature Genet 26:474–479. Copyright Nature Publishing Group.]

The mechanism by which leptin induces UCP-1 is mainly through the sympathetic nervous system (SNS) (Collins *et al.*, 1996), since central administrations of leptin resulted in increased UCP-1 expression. In addition, the MC4R, a downstream target of leptin, appears to mediate this effect because the leptin-mediated induction of UCP-1 expression could be blocked with SHU9119, a potent antagonist of alpha-melanocyte stimulating hormone (α-MSH) (Satoh *et al.*, 1998). Since α-MSH acts as an agonist at the MC4R, one would expect that MC4R KO mice would not tolerate the cold. However, this is not the case. Adult — and therefore obese — MC4R null animals have normal body temperature and tolerate cold well (Ste Marie *et al.*, 2000), suggesting that additional α-MSH-mediated mechanisms are involved in the SNS response to cold.

VI. Glucose Homeostasis and Insulin Sensitivity

A hallmark of leptin treatment of *ob/ob* mice was their improved insulin sensitivity and amelioration of their hyperglycemia independently of effects on food intake (Halaas *et al.*, 1995). Thus, the link between insulin sensitivity, glucose metabolism, and leptin became the focus of intense investigation. Although circulating glucose levels in LepTg mice ranged from normal (Ogawa *et al.*, 1999) to low (Qiu *et al.*, 2001b), plasma levels of insulin, FFAs, and triglycerides were depressed in both transgenic lines. The antidiabetic properties of leptin also were demonstrated in lipoatrophic diabetic mice treated with leptin,

either exogenously, as with the aP2-nSREBP1c transgenic mice (Shimomura *et al.*, 1998), or by crossing the LepTg mice with the A-ZIP-F1 KO mice (Ebihara *et al.*, 2001) that are lipoatrophic and diabetic (Moitra *et al.*, 1998). Although the mechanisms underlying this leptin-mediated improvement in insulin sensitivity remain to be determined, it is likely that the steatosis associated with lipodystrophy could be ameliorated by leptin. This makes it as a prime candidate for the treatment of lipodystrophies such as in the Berardinelli-Seip syndrome (Berardinelli, 1954; Seip and Trygstad, 1963; Savage and O'Rahilly, 2002).

VII. Reproduction

While the role of leptin in energy homeostasis was the center of investigation, due mainly to the morbid obesity of leptin-deficient *ob/ob* mice, their infertility had not caught up with the excitement of leptin's discovery, even though it had been investigated in parabiosis experiments (Lane, 1959) prior to those by Coleman (Coleman and Hummel, 1969). The finding that leptin rescued the sterility of *ob/ob* mice (Chehab *et al.*, 1996) and advanced the puberty of normal mice (Ahima *et al.*, 1997; Chehab *et al.*, 1997; Yura *et al.*, 2000) carved a place for it in reproductive biology.

It is often assumed — erroneously — that obesity is always associated with hypogonadism and infertility. While this may be correct for the Prader-Willi syndrome (Hamilton *et al.*, 1972) and the rare occurrences of leptin and leptin receptor mutations (Montague *et al.*, 1997; Clement *et al.*, 1998), only 60% of obese patients with the Bardet-Biedl syndrome exhibit hypogonadism (Jones, 1988). Association studies between nonsyndromic obesity and hypogonadism remain scarce and worthy of investigation.

In animal models, obesity caused by leptin or leptin receptor mutations results in infertility, whereas KO mice at the MC4-R (which is downstream of leptin) are obese and fertile. So what is the role of leptin in reproduction and what is its mechanism of action on the reproductive system? Exogenous leptin treatment of normal mice advanced vaginal opening (Figure 3) by a few days (Ahima *et al.*, 1997; Chehab *et al.*, 1997). However, in *ad libitum*-fed rats, leptin did not significantly alter the normal course of puberty, although it was able to partially reverse the delay in sexual maturation caused by reduced feeding (Cheung *et al.*, 1997). Studies have shown that intracerebroventricular (ICV) injections of leptin induce prepubertal secretion of luteinizing hormone (LH) (Dearth *et al.*, 2000), whereas leptin antibodies inhibit the leptin-mediated stimulation of LH release (Carro *et al.*, 1997). Transgenic mice oversecreting 12-fold excess leptin showed early vaginal opening (Yura *et al.*, 2000) but those with 8-fold overexpression did not (F.F. Chehab, unpublished observations). Thus, for leptin to significantly impact the onset of puberty and the firing of the reproductive system, elevated leptin levels analogous to the prepubertal leptin

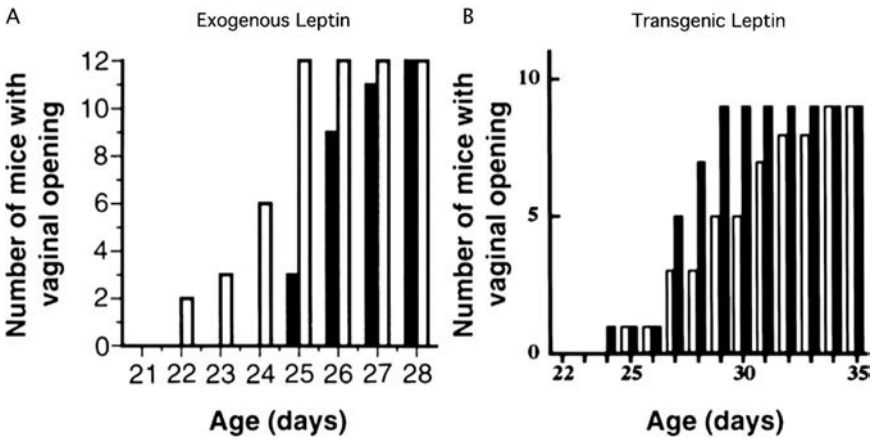

FIG. 3. Stimulation of puberty by exogenous leptin treatment or transgenic hyperleptinemia. Leptin treatment of prepubertal normal mice resulted in earlier vaginal opening (A), similar to LepTg mice (B), demonstrating a stimulatory effect on the reproductive system. [(A) Reprinted with permission from Chehab FF, Mounzih K, Lu R, Lim ME 1997 Early onset of reproductive function in normal female mice treated with leptin. Science 275:88–90. Copyright The American Association for the Advancement of Science; (B) from Yura S, Ogawa Y, Sagawa N, Masuzaki H, Itoh H, Ebihara K, Aizawa-Abe M, Fujii S, Nakao K 2000 Accelerated puberty and late-onset hypothalamic hypogonadism in female transgenic skinny mice overexpressing leptin. J Clin Invest 105:749–755.]

spike found in mice (Ahima *et al.*, 1998) and rats (Nagatani *et al.*, 2000) may be required. In the former case, this preceded the appearance of estradiol but coincided with that of insulin (Figure 4), suggesting an indirect stimulation of estradiol secretion and a concomitant action with insulin. Furthermore, the presence of a transgene overexpressing small amounts of leptin onto the *ob/ob* background rescued only the infertility of the phenotype, without affecting obesity (Ioffe *et al.*, 1998), demonstrating the exquisite sensitivity of the reproductive system to leptin. Similarly, human studies have shown that leptin-deficient patients who fail to enter puberty naturally progress through puberty with leptin treatment (Farooqi *et al.*, 1999). Paradoxically, in rhesus monkeys, associations between prepubertal leptin rise and the onset of puberty were lacking in one study (Plant and Durrant, 1997) but were evident in another using a different leptin-sampling paradigm (Suter *et al.*, 2000). Nevertheless, exogenous leptin treatment of rhesus monkeys did not appear to elicit precocious puberty, which could be attributed to the fact that the monkeys had developed leptin antibodies (Barker-Gibb *et al.*, 2002).

The role of leptin as a conveyor of energy stores to the reproductive system is logical. While studies have concentrated on the fact that leptin may perform this function single-handedly, the complexity of the mammalian system and the

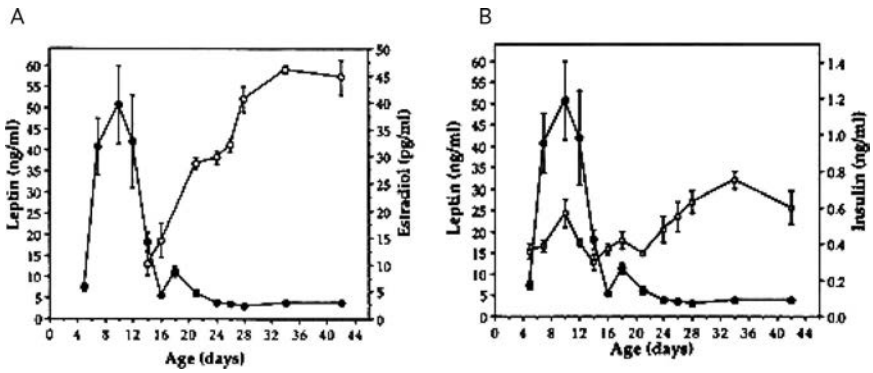

FIG. 4. Circulatory levels of leptin, estradiol, and insulin in neonatal female mice. (A) The leptin spike at 10 days of age preceding that of estradiol is consistent with a stimulatory role of leptin on the reproductive axis around the time of puberty. (B) The coincident rise in leptin and insulin at 10 days of age suggests that leptin and insulin may act together to stimulate the reproductive system. [Reprinted with permission from Ahima RS, Prakabakaran D, Flier JS 1998 Postnatal leptin surge and regulation of circadian rhythm of leptin by feeding. J Clin Invest 101:1020–1027.]

redundancy of supportive mechanisms ensuring the survival of the reproductive system — and therefore of the species — suggest that leptin might recruit other factors (e.g., insulin, IGF-1) to stimulate the release of gonadotropin-releasing hormone (GnRH) and open the gate of the reproductive system. Variations in the expression of leptin's cofactors at critical times during the progression to puberty may be detrimental to the reproductive system. For example, disruption of insulin signaling in the brain has revealed disturbances of the reproductive system (Burks *et al.*, 2000). ICV injections of IGF-1 have been known for some time to induce early puberty (Hiney *et al.*, 1991,1996). Thus, the actions of leptin, insulin, and IGF-1 in the brain could constitute a driving force aimed at the stimulation and firing of the reproductive system.

A mechanism of action of leptin on the reproductive system remains elusive. However, two critical components of the leptin energy homeostasis pathway have been ruled out as playing a role in reproduction and, in fact, have dissociated the obesity from the sterility phenotypes. As previously stated, MC4R KO mice are obese but fertile, ruling out that the MC4R and its downstream targets are critical for reproduction. Thus, bifurcation from the leptin-energy homeostasis pathway could occur at the MC4-R or upstream from it. Furthermore, the role of orexigenic NPY appears to be central for the leptin-reproductive pathway. NPY inhibits reproductive function in male and female rats (Clark *et al.*, 1985). Its overexpression in obese-sterile, leptin-deficient *ob/ob* mice is reduced by leptin treatment (Stephens *et al.*, 1995), which is accompanied by a rescuing of the sterility phenotype (Chehab *et al.*, 1996).

NPY's inhibitory effect on reproductive function appears to be mediated by the Y4 receptor, since *ob/ob* mice homozygous for an Y4 receptor deletion remain obese but are fertile (Sainsbury *et al.*, 2002). The exclusion of a major leptin signaling event in the hypothalamus stemmed from the finding that mutation of the critical tyrosine 1138 phosphorylation site in the long form of the leptin receptor, largely responsible for signal transducer and activator of transcription (STAT)-3 activation, resulted in an obese but fertile mouse (Bates *et al.*, 2003). Thus, the pathway elicited by tyrosine 1138 phosphorylation is not critical for reproduction. These observations suggest that the leptin-reproductive pathway bifurcates from the leptin-energy homeostasis pathway prior to the MC4-R and may involve a separate signaling pathway at other leptin receptor sites. The complexity of this pathway is demonstrated by the fact that modifier genes rescued the sterility phenotype of leptin-deficient mice, demonstrating the multifactorial nature of the leptin-reproductive pathway (Ewart-Toland *et al.*, 1999; Qiu *et al.*, 2001a).

VIII. Leptin and the Melanocortin System

It has been demonstrated that a major downstream target of leptin-binding neurons is a downregulation of NPY neurons and a stimulation of MC4R neurons, which act to stimulate and inhibit food intake, respectively (Cowley *et al.*, 2001). While this topic has been extensively reviewed, the issue of adult-onset obesity, as in agouti yellow obese mice (^{y}A), has received little attention. The molecular defect in ^{y}A mice has been elucidated and consists of a blockade at the MC4R by the agouti protein, which normally is expressed only in melanocytes, but becomes ubiquitously expressed in the agouti mouse. As a result, it competes with α-MSH binding at the MC4R (Bultman *et al.*, 1992; Miller *et al.*, 1993; Lu *et al.*, 1994). These studies led to the discovery of agouti-related protein (AgRP) (Ollmann *et al.*, 1997), which is the endogenous competitor of α-MSH. AgRP and α-MSH act together to regulate energy homeostasis (Ebihara *et al.*, 1999). Interestingly, AgRP overexpression in transgenic mice leads to obesity (Ollman *et al.*, 1997), whereas its deletion by gene targeting did not result in any obvious phenotype, even in the absence of NPY (Qian *et al.*, 2002). This suggests that other factors may regulate energy homeostasis via the MC4R, either in the presence or absence of AgRP and NPY.

The issue of adult-onset obesity is central and has been neglected. For example, in the ^{y}A mouse, despite the ubiquitous expression of agouti protein and its antagonism of melanocyte signaling and pigmentation from an early age, it is not clear why antagonism at the MC4R is delayed and not evident from a young age. To address whether leptin sensitivity is reduced from a young age in agouti mice, we crossed LepTg mice with ^{y}A mice and generated double heterozygotes for ^{y}A and the leptin transgene. The resulting mice were largely devoid of

adipose mass, similar to the transgenic mice, but showed the characteristic agouti color of ^{y}A mice (Figure 5). The statistical differences in body weight, reflecting the lean phenotype of the double heterozygotes, did not last beyond 6 weeks in females and 8 weeks in males (Figure 5), when obesity started to become evident and the lean phenotype caused by transgenic hyperleptinemia started to wane. The color coat of the mutant mice demonstrated expression of the agouti protein from a young age; studies have shown that the MC4R also is expressed from a young age (Mountjoy and Wild, 1998). Then why is the obesity associated with the lack of neuronal MC4R signaling not manifested from a young age, as in *ob/ob* and *db/db* mice, but manifested at a later age, as in ^{y}A and MC4R KO mice? One would have to speculate that neuronal projections downstream of the MC4R conceivably either do not develop or fail to be activated until a later age. Thus, these animal models will be valuable in elucidating pathways downstream of MC4R neurons that may develop to counteract leptin sensitivity.

IX. Leptin and the Sympathetic Nervous System

The binding of leptin to its receptor in the arcuate nucleus eventually is reflected by the adrenergic stimulation of lipolysis (Collins *et al.*, 1996) on adipocytes via beta3 adrenergic receptors (β3AR). Deletion of the β3AR by gene targeting did not yield to overt obesity, due to an upregulation and activation of β1AR and β2ARs (Susulic *et al.*, 1995). In addition, deletion of β1, -2, and -3

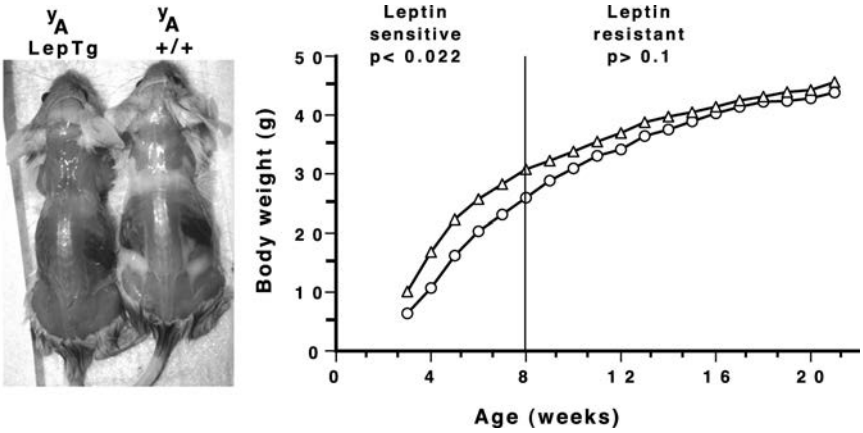

FIG. 5. Sensitivity and resistance of agouti yellow (^{y}A) mice to transgenic leptin. The photograph shows the lack of subcutaneous adipose mass in 6-week-old agouti LepTg males (^{y}A LepTg), compared to yellow agouti (^{y}A +/+) controls without transgenic hyperleptinemia. The growth curve shows the biphasic states of the ^{y}A/LepTg mice (○) between 3–8 weeks of age, when their body weights are significantly less than those of ^{y}A +/+ mice (△).

ARs yielded mice that were slightly obese on a chow diet but grossly obese on a high-fat diet (Bachman *et al.*, 2002). This suggested that βARs are necessary to counteract diet-induced obesity (DIO). We asked whether the leptin-mediated stimulation of the SNS acting on adipocytes would be attenuated by the absence of β3AR on adipocytes. This effect would be most noticeable in animals with no or little fat (e.g., LepTg mice). Thus, generation of LepTg mice with no β3AR resulted in a lean phenotype similar to that of LepTg mice with β3AR, as judged by their growth curves (Ke *et al*., 2003) (Figure 6). This infers a normal response of adipocytes to leptin-mediated SNS activation of lipolysis. Therefore, leptin-mediated activation of the SNS is indifferent to the absence of β3AR, which are chiefly found on adipocytes.

Another axis that has been investigated is the renal axis, with possible connections to hypertension. Leptin treatment caused a dose-dependent increase in sympathetic nerve activity to the kidney (Haynes *et al.*, 1997). This increase could be blocked by the MC4-R antagonist SHU9119, thereby involving the melanocortin system in the sympathoexcitatory effects of leptin on the kidney (Haynes *et al.*, 1999). LepTg mice consistently had elevated blood pressure, whereas the low blood pressure characteristic of *ob/ob* mice was reversed with leptin treatment (Aizawa-Abe *et al.*, 2000). However, ICV administrations of SHU9119 increased food intake in the LepTg mice but did not affect their blood pressure, suggesting that the leptin-mediated effect on blood pressure involves a

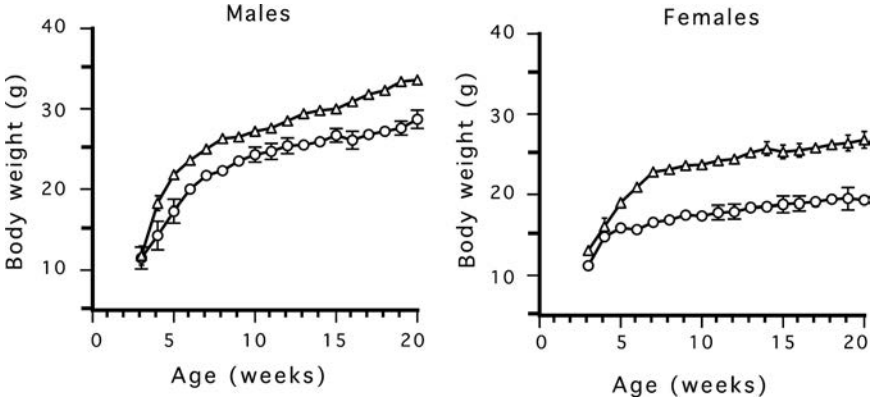

FIG. 6. Transgenic hyperleptinemia exerts its effects independently of beta3-adrenergic receptors (β3ARs). Growth curves of β3AR KO mice (βARKO) with (○) and without (△) transgenic hyperleptinemia. The body weights of the LepTg mice on a null β3AR background remain smaller than those of βARKO mice without hyperleptinemia, demonstrating the lack of specificity of the leptin-mediated response to β3ARs. [Reprinted from Ke Y, Qiu J, Ogus S, Shen WJ, Kraemer PB, Chehab FF 2003 Overexpression of leptin in transgenic mice leads to decreased basal lipolysis, PKA activity and perilipin levels. BBRC 312:1165–1170.]

different pathway than the melanocortin system. Thus, to reconcile the differences between the SHU9119 blockade of the leptin-mediated sympathetic activation of the kidney and its lack of effect on the blood pressure of the LepTg mice, one would have to assume that the two pathways are centrally divergent at the MC4-R.

X. Leptin and the Bone Axis

Increased weight bearing caused by obesity results in increased bone remodeling and decreased bone removal. This observation is significant in obese, postmenopausal women, who show diminished rates of hip fractures caused by osteoporosis. Thus, a mechanism linking obesity and osteoporosis would be of great interest in the treatment of osteoporosis in nonobese women. The observation that ICV leptin treatment of normal and leptin-deficient *ob/ob* mice resulted in bone loss led to the hypothesis that leptin participates in the modulation of bone formation via its action on the hypothalamus, acting as an inhibitor of this process (Ducy *et al.*, 2000) independently of its anorexigenic function (Takeda *et al.*, 2002). Based on an increased bone mass in leptin-deficient mice, we asked whether an excess of leptin, as found in the LepTg mice, would result in decreased bone mass. Histomorphometric studies showed that bone formation was not affected by transgenic hyperleptinemia (F.F. Chehab and M. Horowitz, unpublished observations), despite a modest and significant decrease in bone weight, which could be attributed to the smaller weight and lean phenotype of the LepTg mice. Thus, leptin deficiency strengthens bone formation, while its abundance in organisms that respond to hyperleptinemia does not appear to cause a detrimental effect on bone remodeling.

XI. Resistance to Transgenic Hyperleptinemia

It is clear from the phenotype of LepTg mice that they chronically respond to transgenic hyperleptinemia, as evidenced by reductions in food intake and fat mass, two parameters that are characteristic of a leptin response. We found that even though the LepTg mice continue to secrete transgenic leptin throughout their life, there are at least three situations that make them resistant to transgenic hyperleptinemia: aging, a high-fat diet, and genetics (Figure 7).

When maintained on a C57BL/6J/DBA heterogeneous genetic background, the LepTg mice exhibit a lean phenotype characterized by very little fat mass, which gradually builds up to approximate that of normal mice around 33–36 weeks of age (Qiu *et al.*, 2001b). On a pure C57BL/6J genetic background, the LepTg mice remain lean for a longer period of time than on a mixed genetic background but eventually also gain large amounts of fat that remain, however, significantly less than those of nontransgenic littermates. Our observation that the LepTg mice fail to remain lean over time, an effect more pronounced in males than females, is puzzling and interesting. It has been reported that aging results

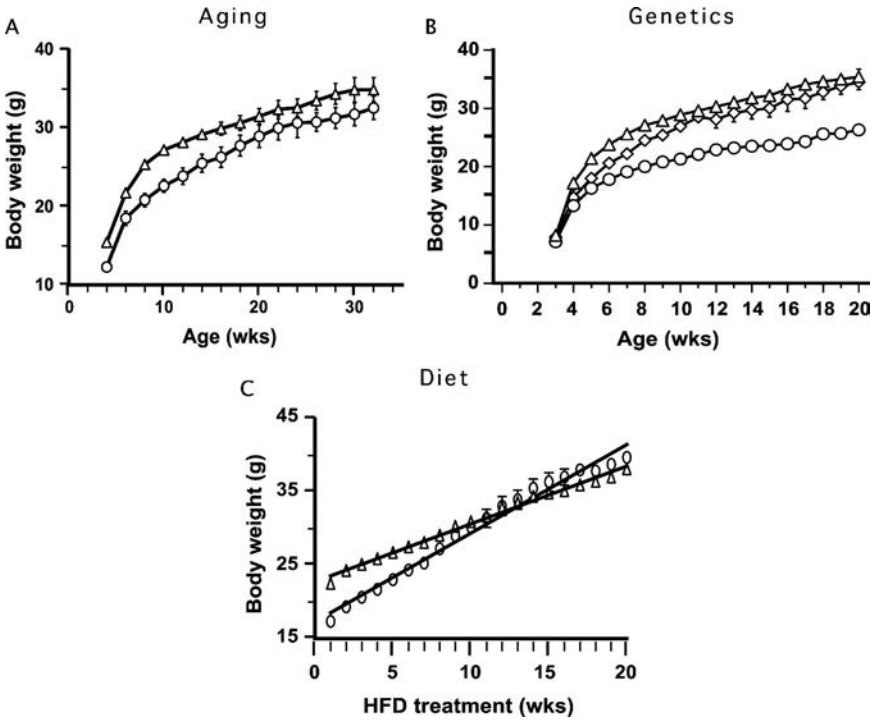

FIG. 7. Resistance to transgenic hyperleptinemia as a result of aging, genetic background, and diet-induced obesity (DIO). (A) At a young age, the body weights of LepTg mice on a mixed genetic background (○) are lower than those of normal mice (△), reflecting lower adiposity, but then start to approach the body weights of normal mice at a later age. (B) Genetic resistance to transgenic hyperleptinemia in LepTg males bred on a mixed genetic background (◇) is demonstrated by their normal body weight, compared to WT (△) and LepTg (○) mice on the same genetic background. (C) Body weights of high-fat diet-fed C57BL/6J normal (△) and LepTg (○) mice show the precipitous DIO of LepTg mice. [(A) Reprinted with permission from Qiu J, Ogus S, Lu R, Chehab FF 2001 Transgenic mice overexpressing leptin accumulate adipose mass at an older, but not younger, age. Endocrinology 142:348–358. Copyright The Endocrine Society; (C) Modified with permission from Ogus S, Ke Y, Qiu J, Wang B, Chehab FF 2003 Hyperleptinemia precipitates diet-induced obesity in transgenic mice overexpressing leptin. Endocrinology 144:2865–2869. Copyright The Endocrine Society.]

in a leptin resistance state (Scarpace *et al.*, 2000a,b). Thus, it would make sense to assume the same for the LepTg mice. Whether the effects of functional transgenic hyperleptinemia become blunted with aging or whether a mechanism that supercedes the transgenic leptin effect becomes established with age remains to be determined. However, our studies tend to favor the latter hypothesis because transgenic mice that have accumulated adipose mass with age remain sensitive to peripheral administrations of large doses of leptin (Qiu *et al.*, 2001b).

An unexpected finding underlying the loss of effect associated with functional transgenic hyperleptinemia was a high-fat diet. Since the young LepTg mice are sensitive to transgenic hyperleptinemia — as indicated by a reduction in food intake and adipose tissue mass — we reasoned that they actually would be resistant or show at least an attenuated obesity on a high-fat diet treatment. This was clearly not the case when they were fed a diet consisting of 42% milk fat, as they manifested a more-precipitous obesity than that of normal mice fed the same diet (Ogus *et al.*, 2003). This interesting finding raises a number of issues as to the cause of their resistance to transgenic hyperleptinemia. In the absence of significant differences in high-fat food intake, one should look for differences in energy expenditure, metabolism, or diet composition. It is possible that the levels of hyperleptinemia were not sufficient to counteract the rapid buildup of adipose mass, indicating that the ratio of leptin to fat accumulation would be the culprit of the resulting phenotype. Moreover, the composition of the high-fat diet, which consisted of 61% saturated and 39% unsaturated fats, may be critical. Thus, feeding the LepTg mice different amounts and types of fat might provide clues about the interactions between their hyperleptinemia and their diet, possibly extending the resulting observations to the broader problem of hyperleptinemia in DIO in animals and humans.

Another interesting finding in our LepTg mice is the fact that some on the mixed genetic background exhibit a transgenic hyperleptinemia that is not associated with a lean phenotype, even from a young age (Figure 7). When backcrossed to the C57BL/6J genetic background for 6–10 generations, this effect is lost, pointing to a powerful effect of modifier genes from the C57BL/6J and DBA/2J genetic backgrounds. Backcrossing the LepTg mice to the DBA/2J background revealed that, at the N5 generation, the LepTg mice remain lean, similar to C57BL/6J LepTg mice. Thus, genetic leptin resistance must have arisen from a polygenic interaction of the two genomes. Furthermore, crosses of genetically resistant LepTg mice did not result in recapitulation of the phenotype according to Mendel's laws for single-gene disorders. Thus, it is unlikely that dominant or recessive genes accounted for this early leptin resistance phenotype. Instead, it suggests that modifier genes had accumulated in these mice but that genetic crosses allowed their segregation. Although these findings reinforce the effect of genetics on the phenotype of leptin resistance, along with the data mentioned earlier, they pinpoint what has commonly been suspected: that complex genetics account for the common DIO phenotype.

XII. Perspectives on Mechanisms of Leptin Resistance

Leptin resistance refers to a state of elevated leptin levels without a lean phenotype but, paradoxically, with an obese state. It has been suggested that the hypothalamus may be insensitive to elevated leptin levels but would, on the

contrary, sense low leptin levels, such as in fasting states. This would activate a survival response that would conserve energy resources by shutting down, for example, the reproductive system. Remarkably, the response to fasting can be rescued by leptin injections, demonstrating that leptin mediates the starvation-induced response (Ahima *et al.*, 1996).

What distinguishes LepTg mice with elevated leptin levels and a clear response to hyperleptinemia from hyperleptinemia in common obesity remains for the most part unknown. It is unlikely, but still not completely ruled out, that the primary defect could lie in the saturation transport of leptin to the hypothalamus. There appears to be enough leeway to allow large quantities of leptin (such as in LepTg mice) to elicit a physiological response (Ogawa *et al.*, 1999; Qiu *et al.*, 2001b). The culprit may, however, be in the diet, which could block leptin's access to the hypothalamus or its central action. It has been shown that prolonged, high-fat diet feeding of normal mice fails and reduces STAT-3 activation in the hypothalamus following peripheral and central administrations of leptin, respectively (El-Haschimi *et al.*, 2000). The contribution of the diet and its mechanisms of action to the establishment of a leptin-resistant state in LepTg mice will be invaluable in deciphering pathways that result in dysfunctional hyperleptinemia. One possibility is that components of a high-fat diet might alter the permeability of leptin to the brain and reduce leptin signaling in the hypothalamus.

XIII. Evolutionary Significance of Hyperleptinemia

The possibility that resistance to hyperleptinemia might have evolved as a protective mechanism to ensure the survival of an organism is a worthwhile hypothesis. As previously noted, LepTg mice are cold sensitive and succumb rapidly to a low temperature. Should a similar situation arise in wild habitats, such mice would be quickly eliminated by natural selection, unless they are capable of developing leptin resistance. This is particularly significant for hibernating animals, which stock up on energy stores prior to hibernation, increasing their adiposity and leptin levels. Should they fail to develop leptin resistance, the process of adipose mass deposition would fail. Another example is pregnancy. We have shown that leptin resistance is established in midpregnancy in normal mice (Mounzih *et al.*, 1998) as a mechanism to increase energy intake to build up enough adipose tissue in anticipation of the large energy requirements of lactation. If the mother fails to build up adipose tissue, lactation and survival of the pups would be seriously jeopardized. Therefore, the need to develop a resistance to hyperleptinemia is teologically warranted and would protect the organism against the detrimental effects of functional hyperleptinemia to ensure its survival. Thus, although leptin resistance in pregnancy makes sense and is essential, it occasionally extends beyond gestation, resulting in postpartum

weight retention. This again demonstrates a close interaction between the reproductive system, leptin signaling, and obesity.

XIV. Concluding Remarks

Much remains to be learned about the biology of leptin and its direct and indirect effects on various systems. The use of leptin-deficient, leptin-resistant, and leptin-overexpressing mice has demonstrated the detrimental consequences of leptin resistance and sensitivity, albeit in different ways. Animal models will continue to unravel the biology of leptin and are destined to unlock leptin resistance and provide a treatment for obesity. However, timed leptin resistance, as in pregnancy, is essential and would need to be maintained to ensure the survival of the next generation.

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Insulin and Leptin as Adiposity Signals

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ABSTRACT

There is now considerable consensus that the adipocyte hormone leptin and the pancreatic hormone insulin are important regulators of food intake and energy balance. Leptin and insulin fulfill many of the requirements to be putative adiposity signals to the brain. Plasma leptin and insulin levels are positively correlated with body weight and with adipose mass in particular. Furthermore, both leptin and insulin enter the brain from the plasma. The brain expresses both insulin and leptin receptors in areas important in the control of food intake and energy balance. Consistent with their roles as adiposity signals, exogenous leptin and insulin both reduce food intake when administered locally into the brain in a number of species under different experimental paradigms. Additionally, central administration of insulin antibodies increases food intake and body weight. Recent studies have demonstrated that both insulin and leptin have additive effects when administered simultaneously. Finally, we recently have demonstrated that leptin and insulin share downstream neuropeptide signaling pathways. Hence, insulin and leptin provide important negative feedback signals to the central nervous system, proportional to peripheral energy stores and coupled with catabolic circuits.

I. Overview

When maintained on an *ad libitum* diet, most animals — including humans — are able to precisely match caloric intake with caloric expenditure, resulting in relatively stable energy stores as adipose tissue (Kennedy, 1953; Keesey, 1986). Growing emphasis has been placed on the role of the central nervous system (CNS) in controlling this precision of energy homeostasis. However, to balance the energy equation, the brain must be able to receive several kinds of input from the periphery. Some of these messages should provide information about the status of peripheral energy stores in the form of the adipose mass.

Compelling evidence implicates at least two peripheral hormones as providing key afferent information to the CNS concerning the amount and distribution of body fat. Leptin, a recently described peptide hormone secreted from adipocytes in proportion to fat mass (and especially to subcutaneous fat mass) (Masuzaki *et al.*, 1995; Dua *et al.*, 1996; Montague *et al.*, 1997) has received tremendous attention in recent years. Considerable evidence suggests that leptin acts as one of the body's adiposity signals (Zhang *et al.*, 1994; Matson *et al.*,

1996; Friedman, 1997; Buchanan *et al.*, 1998; Woods *et al.*, 1998; Schwartz *et al.*, 2000). Leptin levels in the blood are correlated with body fat. Administration of exogenous leptin reduces food intake and increases energy expenditure. Furthermore, when energy balance is suddenly changed (for example, if an individual is fasted for a day), plasma leptin levels decrease far more than body adiposity in the short term (Woods *et al.*, 1997; Buchanan *et al.*, 1998). Hence, although much has been written about leptin as an adiposity signal, it is not ideal in and of itself, suggesting that at least one additional signal must exist. The logical candidate is the pancreatic hormone, insulin.

Plasma insulin levels are directly correlated with adiposity (Woods *et al.*, 1998). They correlate better with visceral than subcutaneous fat. Moreover, when energy balance changes, plasma insulin follows these changes faithfully. Hence, leptin and insulin together provide information to the brain not only about the size of the fat mass but also about its distribution and important recent changes of metabolic status. The effort of many laboratories, including part of our own, is concentrated on elucidating how insulin and leptin interact with central neural systems controlling energy homeostasis.

II. Insulin as an Adiposity Signal

Considerable evidence suggests that insulin is a key peripheral regulator of food intake and body adiposity. After Kennedy (1953) hypothesized that fat stores produce a hormone that acts as a negative feedback control for adiposity, one early suggestion was that this signal was insulin (Baskin *et al.*, 1987; Woods *et al.*, 1996). Data supporting this possibility have been collected over the past three decades and include studies using several species and techniques (Woods *et al.*, 2003). To summarize that literature, levels of plasma insulin correlate directly with body weight and with body adiposity in particular (Bagdade *et al.*, 1967; Polonsky *et al.*, 1988a,b). Obese animals and humans have higher basal insulin levels and secrete more insulin in response to a meal than do lean individuals (Bagdade *et al.*, 1967; Woods *et al.*, 1974). Plasma insulin levels also reflect more-acute changes in energy status. Insulin increases during meals and any other condition of positive energy balance and decreases during fasting and other periods of negative energy balance. The major stimulant of insulin secretion is an increase of local glucose levels in the pancreas. The degree of glucose-stimulated insulin secretion is a direct function of body fat (Bagdade *et al.*, 1967; Woods *et al.*, 1974; Polonsky *et al.*, 1988a,b). The increment of insulin in response to glucose lasts only as long as glucose is elevated. Then, insulin is rapidly cleared from the blood, since it has a half-life of only 2 to 3 minutes. Obese animals and humans are said to be insulin resistant because more insulin is required to maintain a normal level of blood glucose. This is important because

insulin resistance and visceral fat correlate with levels of insulin, type 2 diabetes mellitus, and obesity.

The second line of evidence consistent with insulin being a prime candidate for an adiposity signal is the finding that insulin better maps acute changes of energy metabolism and adiposity than its adipocyte cousin leptin. Insulin secretion faithfully tracks changes of energy balance on the order of minutes to hours, as opposed to days, and these changes are always in direct proportion to the size of the adipose mass. Furthermore, insulin plays a key role in the regulation of glucose and lipid utilization and storage. Without sufficient insulin, most tissues cannot take up much glucose, so glucose accumulates in the blood. At the same time, adipocytes cannot take up and store fat. When insulin resistance occurs in obesity, and more insulin is secreted to regulate glucose, the excess insulin causes increased accumulation of fat in adipocytes. Hence, disruptions of insulin sensitivity are associated with both obesity and diabetes. Many reviews of these phenomena have been written (Schwartz *et al.*, 1992; Porte *et al.*, 1998; Woods *et al.*, 1998,2003).

Additional support for the hypothesis that insulin acts as an adiposity signal includes the fact that insulin receptors and insulin receptor mRNA are found in CNS regions involved in the regulation of food intake and body weight (Schwartz *et al.*, 1992; Campfield *et al.*, 1996; Matson *et al.*, 1996; Woods *et al.*, 2003). In particular, insulin receptors in the arcuate nucleus of the hypothalamus (ARC) are prime candidates for translators of an adiposity signal. Third ventricular (i3vt) administration of insulin decreases expression of the anabolic effector peptide, neuropeptide Y (NPY) in the ARC (Schwartz *et al.*, 1992; Sipols *et al.*, 1995). ARC NPY fibers project to the paraventricular nucleus (PVN) of the hypothalamus. Administration of insulin into the i3vt of the brain also causes increased expression of corticotropin (ACTH)-releasing hormone mRNA in the PVN (Sipols *et al.*, 1995; Schwartz *et al.*, 1996b). These results demonstrate that the insulin signal is tied to changes in food intake associated with hypothalamic neuropeptides. The mediation of insulin's catabolic effects is detailed below.

One requirement of a humoral adiposity signal is that it should gain access to the brain. Consistent with this, insulin has been found to enter the brain via a saturable transport process that moves it from plasma into brain interstitial fluid (Hachiya *et al.*, 1988). The rate of entry of insulin to CNS is well mapped to normal fluctuations of plasma insulin levels, although higher levels exceed the saturation point (Baura *et al.*, 1993; Woods *et al.*, 2003). With very high plasma levels, the entry of insulin to brain remains relatively constant. This is important for regulation of body weight because some types of obesity are known to be associated with disruptions of the insulin transport system. A review of this is found in Woods *et al.* (2003).

Finally, and importantly, administration of exogenous insulin into the brain reduces food intake and increases energy expenditure, consistent with its being

an adiposity signal. Repeated administration of insulin in small doses, or as a continuous infusion, elicits decreased food intake and increased energy expenditure after several hours and lasts for the duration of the treatment (Woods *et al.*, 1974,1996; Air *et al.*, 2002a). Administration of insulin into the brain also potentiates the anorexic effects of peripherally administered cholecystokinin (CCK) (Figlewicz *et al.*, 1995; Riedy *et al.*, 1995). This suggests that insulin modulates the body's response to short-term signals that terminate meals. Importantly, administration of insulin peripherally, in amounts that do not cause hypoglycemia, decreases food intake (Nicolaidis and Rowland, 1976; Vanderweele *et al.*, 1982; McGowan *et al.*, 1990; Woods *et al.*, 2003). In agreement with these data, administration of antibodies to insulin into the brain increases food intake and body weight (Strubbe and Mein, 1977; McGowan *et al.*, 1992). It is important to note that there is no evidence that alterations in food intake after administration of insulin, either systemic or central, are secondary to aversive consequences (Chavez *et al.*, 1995). That is, administration of exogenous insulin into the brain does not appear to make animals ill. Collectively, these data suggest that insulin provides a signal of adiposity to the CNS and that the signal is capable of altering food intake, based on the state of energy balance.

In summary, insulin, aside from its important peripheral action for the metabolism of fuels, satisfies all of the criteria for consideration as a general adiposity signal. Indeed, Woods and colleagues (Porte *et al.*, 1998; Woods *et al.*, 1998) have suggested that the administration of insulin does not simply act to change food intake *per se* but rather helps determine the level of fat that will be maintained and defended by the animal. That is, levels of circulating insulin help modulate the long-term level of fat stored in the body in any particular environment. It is unclear at present whether, in the obese insulin-resistant state, there are parallel changes of central insulin-sensitive systems. Niswender and Schwartz (2003) published an excellent recent review of insulin as an adiposity signal.

III. Leptin as an Adiposity Signal

First described in 1994 (Zhang *et al.*, 1994), leptin has proven to be a key metabolic protein that has actions throughout the body. Leptin quickly overshadowed insulin as the best-known adiposity hormone because it is secreted from adipocytes themselves. However, unlike insulin, leptin can be given systemically with few adverse side effects such as hypoglycemia. Analogous to what occurs with insulin, plasma leptin levels are correlated directly with adiposity. Likewise, circulating leptin is transported into the brain via a saturable process (Banks *et al.*, 1996; Schwartz *et al.*, 1996a). Leptin receptors exist in many brain areas, including the ARC (Banks *et al.*, 1996; Schwartz *et al.*, 1996b). Within the brain, leptin has many actions, including reducing food intake and increasing energy

expenditure. This is supported further by the finding that prolonged administration causes loss of body fat and body weight. A current hypothesis is that leptin, being secreted in proportion to total body adiposity, conveys the overall nutritional status to the brain (Ahima *et al.*, 2000). Low leptin levels are considered to indicate depleted fat stores and reduce or turn off functions that require adequate energy stores to be successful (e.g., reproduction). During weight loss, plasma leptin decreases (Boden *et al.*, 1996; Havel *et al.*, 1996; Ahren *et al.*, 1997; Keim *et al.*, 1998); analogously, weight gain is associated with an increase in leptin secretion (Seeley *et al.*, 1996; Ahren *et al.*, 1997).

The importance of leptin as an adiposity signal to the brain is supported further by the phenotype of animals that either do not synthesize it (*ob/ob* mice that have a mutation in the leptin gene) (Zhang *et al.*, 1994) or that have genetic mutations that compromise functioning of the leptin receptor (*db/db* mice and fatty Zucker *fa/fa* rats) (Chua *et al.*, 1996). These animals are characterized by hyperphagia and extreme obesity. Administering small amounts of leptin into the brains of *ob/ob* mice reverses this syndrome (see reviews in Tartaglia *et al.*, 1995 and Woods *et al.*, 1998).

The leptin receptor, a member of the cytokine receptor family, exists in many natural forms in the brain and rest of the body. The leptin receptor can be considered to have two functional parts. The extracellular domain recognizes and interacts with extracellular leptin and is identical for all known leptin receptors. When appropriately activated, the intracellular domain causes certain cellular events to be initiated. Variations in the length of the intracellular domain determine the type of action leptin exerts on a cell (Tartaglia *et al.*, 1995). All intracellular forms of the leptin receptor activate the Janus kinases (JAK) (JAK-signal transducer and activator of transcription (STAT) pathway of tyrosine protein kinases). The form found in the hypothalamus, including the ARC, is the longest one, termed OB-Rb, and has the capacity to activate several intracellular signaling pathways besides JAK-STAT. This form also activates transcription factors (Niswender and Schwartz, 2003).

Leptin signaling (actually, reduced leptin signaling) has been hypothesized to regulate many vital systems when animals are severely hypocaloric and have low body fat. Leptin is secreted from adipocytes in direct proportion to the amount of stored fat, especially the amount of subcutaneous fat. Leptin secretion rate and leptin mRNA expression are two to three times higher in subcutaneous than visceral fat, in part due to the larger adipocytes relative to visceral fat (Dua *et al.*, 1996; Montague *et al.*, 1997; Bray and Popkin, 1998; Samaras *et al.*, 1998). However, the actual stimulus is related more to the metabolic activity of the fat cell than to fat storage, such that dissociations can occur between stored fat and leptin release, particularly during a fast. Nonetheless, under normal conditions, plasma leptin levels are a reliable and rather stable indicator of body fat, since leptin's half-life is about 45 minutes.

Soon after the discovery of leptin, it was reported that when mice are administered exogenous leptin into the brain, they reduce their food intake and body weight (Campfield *et al.*, 1995). Our laboratory replicated this phenomenon in rats (Seeley *et al.*, 1996) and found that leptin exerts this effect without causing signs of illness (Thiele *et al.*, 1997). We also mapped the location of OB-Rb in the brain and determined where leptin administration elicits c-Fos expression (Thiele *et al.*, 1997; van Dijk *et al.*, 1999). We also found that leptin acts in the ARC to reduce NPY synthesis and to stimulate pro-opiomelanocortin (POMC) synthesis (Seeley *et al.*, 1997; Hagan *et al.*, 1999; van Dijk *et al.*, 1999). We proposed a model based upon the opposing actions of anabolic neuropeptides such as NPY and agouti-related peptide (AgRP) and catabolic neuropeptides such as alpha-melanocyte stimulating hormone (α-MSH) (Schwartz *et al.*, 1997). We also reported that the ability of leptin to reduce food intake critically depends upon "downstream" stimulation of activity at melanocortin-4 (MC-4) receptors, since the administration of mixed MC3/4 antagonists or specific MC-4 antagonists attenuates leptin's ability to reduce food intake and induce fos activity in the PVN (Seeley *et al.*, 1997). Like others, we found that the administration of MC-4 agonists reduces food intake and body weight (Thiele *et al.*, 1998; Benoit *et al.*, 2000; McMinn *et al.*, 2000).

IV. Similarities and Differences Among Adiposity Signals

It is worth considering why there should be more than one adiposity signal to the brain. The simple answer is that redundancy is the rule rather than the exception in the control of energy homeostasis. Nonetheless, insulin reflects different fat stores, genders, and risk factors for developing type 2 diabetes mellitus and various cardiovascular problems than does leptin. That is, whereas the levels of insulin and leptin both signal the degree of adiposity to the brain, each has important other functions throughout the body. Insulin is a major controller of the levels and utilization of glucose throughout most of the body. Low circulating leptin and the resultant decrease of leptin signaling have been hypothesized to regulate many vital systems when animals are severely hypocaloric and have low body fat.

There are other fundamental differences. For one, the secretion of insulin is adjusted in response to every acute change of metabolism. Its levels increase during meals or when glucose is elevated for some other reason and decrease during stress and exercise. The half-life of insulin in the blood (2–3 minutes) is consistent with its role as a minute-to-minute indicator of ongoing metabolism; all of its fluctuations are directly proportional to total body fat. Leptin is secreted from adipocytes in direct proportion to ongoing metabolic activity of the fat cell. Plasma leptin levels are, therefore, a reliable and rather stable indicator of body fat. Hence, insulin levels reflect the interaction of ongoing metabolic processes

and body adiposity, whereas leptin levels reflect the activity of adipose cells more directly.

Another difference is that insulin secretion reflects the amount of visceral white adipose tissue, whereas leptin secretion reflects total fat mass, especially subcutaneous fat (Masuzaki *et al.*, 1995; Dua *et al.*, 1996; Montague *et al.*, 1997). This is potentially quite important with regard to the message conveyed to the brain, since visceral fat carries a greater risk for the metabolic complications associated with obesity than does subcutaneous fat. Elevated visceral fat is associated with an increased incidence of insulin resistance, type 2 diabetes mellitus, hypertension, cardiovascular disease, and certain cancers. Hence, while circulating levels of leptin and insulin each convey specific information as to the distribution of fat, the combination of the two conveys information about the total fat mass of the body.

Regardless of the differences, leptin and insulin both provide important afferent information to the brain. Even though they are not made in the brain, the brain nonetheless contains specific receptors for both. Each is transported from the blood, through the blood-brain barrier, by a receptor-mediated mechanism (Porte *et al.*, 1998; Woods *et al.*, 1998,2003). Therefore, biologically active leptin and insulin are delivered into the brain interstitial fluid, where they can interact with receptors on neurons. The ARC contains receptors for each in particularly high concentrations (Banks *et al.*, 1996; Schwartz *et al.*, 1996b; Woods *et al.*, 2003) and each signal has important shared mediators in the hypothalamus. We have found that when insulin and leptin are administered into the brain in combination, they initially interfere with each other's action such that the net catabolic effect is less than the sum of the individual effects. However, after 4 hours, the two peptides are simply additive (Air *et al.*, 2002b). There are several reviews of this literature (Porte *et al.*, 1998; Woods *et al.*, 1998). A diagram of stimulation of the ARC by leptin and insulin is depicted in Figure 1.

V. Central Effector Systems

Signals indicating adiposity, as well as those indicating ongoing metabolic processes and what is being eaten and processed in the gut, converge on the CNS. Within the CNS, in order to regulate food intake and body weight effectively, these signals need to interact in meaningful ways and to engage neurochemical systems that influence energy intake and energy expenditure. The best known of these CNS systems are in the ventral hypothalamus. They can be roughly divided into those whose activity reduces body fat (catabolic effector systems) and those whose activity increases body fat (anabolic effector systems). Anabolic effectors elicit increased food intake, decreased energy expenditure, and consequently increased stored energy in the form of adipose tissue. They are hypothesized to

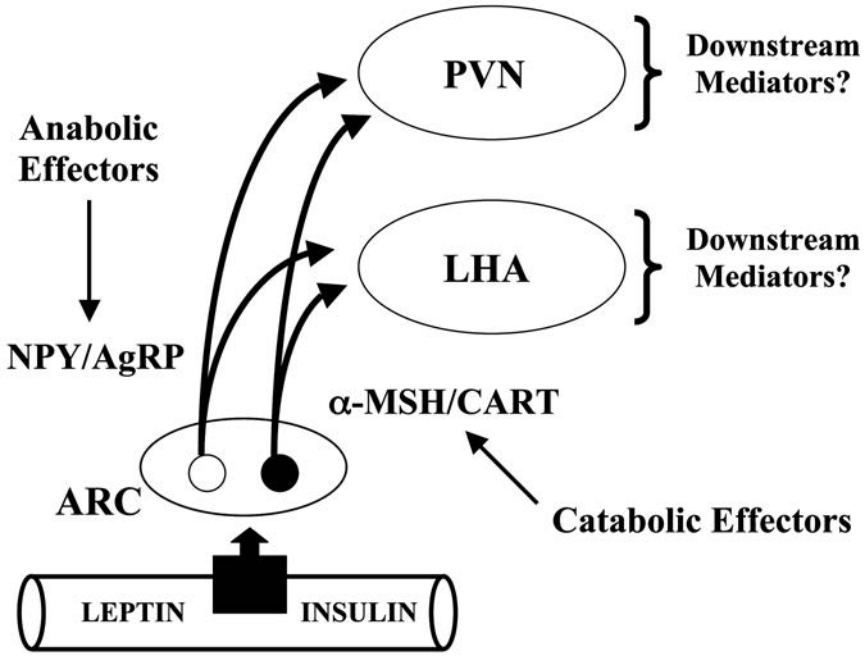

FIG. 1. A diagram representing stimulation of the arcuate nucleus of the hypothalamus (ARC) by leptin and insulin. A neuroanatomical schematic of the hypothalamic melanocortin system control of food intake. Abbreviations: PVN, paraventricular nucleus; LHA, lateral hypothalamic area; NPY, neuropeptide Y; AgRP, agouti-related protein; MSH, melanocyte-stimulating hormone; CART, cocaine- and amphetamine-regulated transcript.

become more active when energy stores are low, as indicated by reduced levels of insulin and leptin (i.e., when the body is in negative energy balance). Catabolic effectors do just the opposite. Activated by positive energy balance, they decrease food intake, increase energy expenditure, and result in decreased adipose tissue mass. A critical aspect of this negative-feedback model is that hormones responsive to the level of adiposity inhibit anabolic pathways, while activating catabolic pathways. It is the balance between these two pathways that ultimately determines the animal's ingestive behavior and defended level of adiposity.

The catabolic and anabolic effector systems are in actuality a series of discrete neurotransmitter systems and axonal pathways in the brain. Many of the key details of this overall schema have emerged in the last few years. Although receptors for leptin and insulin are located throughout the CNS, both are concentrated in the ARC in the ventral hypothalamus. Hence, ARC neurons are

sensitive to these hormones and consequently to the amount of adipose tissue in the body.

A. ANABOLIC EFFECTOR SYSTEMS

The best-described anabolic effector peptide in the brain is NPY. Although NPY mRNA and peptide are distributed widely throughout the CNS, NPY-containing cell bodies in the ARC are especially important in the control of energy homeostasis (Schwartz *et al.*, 1992). Although these ARC NPYergic neurons directly influence several areas of the brain, major projections are to the nearby PVN and the lateral hypothalamic area (LHA). ARC NPYergic neurons respond to negative energy balance (e.g., food deprivation) by synthesizing more NPY mRNA and consequently release more NPY in the PVN (Kalra *et al.*, 1991; Schwartz *et al.*, 1992) and presumably the LHA as well. Importantly, animals in negative energy balance have low levels of adiposity hormones, resulting in elevated NPY mRNA in the ARC. Local replacement of either insulin or leptin in the vicinity of the ARC normalizes the elevated NPY mRNA in the ARC of fasted animals (Schwartz *et al.*, 1992; Sipols *et al.*, 1992). Hence, the activity of these ARC NPY neurons is under the direct influence of at least two adiposity signals.

Consistent with its being an anabolic effector peptide, administration of exogenous NPY into the PVN or into the adjacent i3vt elicits a rapid and robust increase in food intake (Clark *et al.*, 1984; Sahu and Kalra, 1993; Stanley *et al.*, 1993; Seeley *et al.*, 1995) and decrease in energy expenditure (Billington *et al.*, 1994). Repeated administration of NPY produces uncompensated increases in food intake, body weight, and adiposity. Local administration into the ARC of compounds that result in less NPY being synthesized result in reduced food intake and body weight (Akabayashi *et al.*, 1994). Hence, NPY meets all of the criteria of an anabolic effector peptide.

B. CATABOLIC EFFECTOR SYSTEMS

The catabolic counterpart of the ARC NPY system also appears to reside within the ARC. Considerable evidence implicates the ARC melanocortin system as an important catabolic effector system. Melanocortins are a family of peptides that include ACTH and α-MSH. The precursor molecule for ARC melanocortins is POMC. In addition to several other important neuropeptides, POMC encodes α-MSH, a transmitter that functions as an agonist at several classes of melanocortin receptors within the hypothalamus (especially within the PVN and LHA) (Cone, 1999,2000). When administered into the i3vt, α-MSH and other melanocortin receptor agonists (including the synthetic drug MTII) reduce food intake and body weight, whereas administration of synthetic melanocortin receptor antagonists (e.g., SHU-9119) increases food intake and body weight (Tsujii and

Bray, 1989; Fan *et al.*, 1997; Seeley *et al.*, 1997; Thiele *et al.*, 1998). POMC gene expression is reduced in negative energy balance (Schwartz *et al.*, 1997) and increased in positive energy balance (Hagan *et al.*, 1999).

Consistent with the hypothesis that the melanocortin system is important in mediating the effects of leptin, leptin receptors are found on POMC neurons, leptin stimulates POMC mRNA, and a melanocortin receptor antagonist blocks the effect of leptin to reduce food intake (Seeley *et al.*, 1997). Analogously, insulin receptors are found on POMC neurons, insulin stimulates POMC mRNA, and SHU 9119 attenuates insulin's anorexic action (Benoit *et al.*, 2002). All of this evidence points to the endogenous POMC/α-MSH/melanocortin receptor hypothalamic system as being a key catabolic effector pathway capable of eliciting robust effects on food intake and body weight that mediates the effect of adiposity signals in the CNS.

A compelling body of evidence implicates the hypothalamic melanocortin system as one of the central effectors controlling food intake and energy balance. The evidence for this is multifold. First, consider the phenotype of the agouti mouse. This yellow mouse has an autosomally transmitted trait resulting from a mutation of the agouti gene that results in ectopic expression of the agouti protein (Bultman *et al.*, 1992). In melanocytes, this inappropriate expression results in continuous antagonism of α-MSH signaling. The resulting phenotype is a yellow coat. However, the agouti mouse is obese as well as yellow. This observation led to the hypothesis that ectopic agouti protein antagonizes central melanocortin receptors involved in food intake. Indeed, though melanocortins have been known to influence food intake since the 1980s, it was only during the last decade that their receptors were cloned and localized to the hypothalamus. As predicted, agouti is an antagonist of these receptors (Lu *et al.*, 1994). Importantly, an endogenous AgRP subsequently was found to be produced almost exclusively in the ARC and to project to the PVN and LHA (Schwartz *et al.*, 1997).

Consistent with the hypothesis that the melanocortin system mediates the effects of adipose hormones is the finding that expression of melanocortin gene products is regulated by energy balance. During periods of negative energy balance (and, consequently, low adipose hormones), expression of AgRP mRNA is increased, while expression of POMC is decreased (Mizuno *et al.*, 1999). During positive energy balance (and high levels of adipose hormones), on the other hand, expression of POMC mRNA is increased and AgRP is decreased. Furthermore, as previously discussed, POMC-containing neurons also have receptors for leptin (Cheung *et al.*, 1997; Mountjoy and Wong, 1997; Seeley *et al.*, 1997). All these findings suggest that the hypothalamic melanocortin system is a likely central target of adipose signals and a mediator of their effects on food intake.

The brain expresses two types of melanocortin receptors, MC3R and MC4R (Cone, 1999,2000). Distribution of MC3R is limited to areas of the hypothala-

mus, while MC4R are located throughout the brain. Most data suggest that MC4R are most critical for melanocortin involvement in food intake. Indeed, an important piece of evidence linking melanocortins to food intake also supports the hypothesis that MC4R is the critical receptor. Mice with targeted deletion of the MC4R gene are phenotypically similar to the yellow (agouti) mouse (Huszar *et al.*, 1997). While not yellow, they exhibit profound obesity as well as hyperinsulinemia. As predicted, nonselective MC3/4R agonists (e.g., MTII) do not reduce food intake in MC4R-/- mice (Marsh *et al.*, 1999). While the issue is complicated by findings that MC3R-/- mice have increased body fat but not increased food intake (Chen and Marsh, 2000), such findings nonetheless support the hypothesis that the melanocortin system plays an important role in the control of energy balance.

Additional evidence supporting a role for melanocortins in the control of food intake comes from experimental administrations of both naturally occurring and synthetic peptides. i3vt administration of α-MSH decreases food intake (Tsujii and Bray, 1989; McMinn *et al.*, 2000), as does i3vt administration of synthetic agonists, including MTII and Ro27–3225 (Fan *et al.*, 1997; Thiele *et al.*, 1998; Benoit *et al.*, 2000). Conversely, administration of melanocortin receptor antagonists, such as AgRP or the synthetic agonist SHU-9119, elicits long-lasting increase in food intake (Fan *et al.*, 1997; Ollmann *et al.*, 1997; Rossi *et al.*, 1998; Hagan *et al.*, 1999,2000). While these data are consistent with the hypothesized role of the melanocortin system in the control of food intake, the presence of both an endogenous agonist and antagonist of the same receptor makes this system a prime candidate for the translation of negative-feedback signals from adipose tissue.

Figure 1 depicts a neuroanatomical schematic of the hypothalamic melanocortin system control of food intake. POMC neurons in the ARC express receptors for adiposity signals (e.g., leptin). These hormones convey an adiposity signal to the brain, which is in part received by ARC POMC neurons. To mediate the anorexic effects of leptin and insulin, these neurons, in turn, release α-MSH. Leptin and insulin also function to reduce NPY and AgRP expression. Figure 1 also depicts the most-characterized projection sites of these ARC POMC neurons, the PVN and the LHA, where the MC3/4R thought to be most important in the control of food intake and body weight are localized. However, relatively little experimental attention has been given to either 1) the downstream projections of these PVN neurons or 2) MC3/4R in other areas of the brain that might play important roles in the control of ingestion.

Importantly, the melanocortin system projects to multiple areas in the brain. Because food intake and body weight regulation are likely controlled by several CNS processes, the possibility remains that melanocortin peptides might alter food intake and body weight in multiple ways. For example, POMC-expressing neurons and the densest expression of MC4R actually are found in the caudal

brainstem (Kishi *et al.*, 2003). Additionally, MC4R are expressed in the infralimbic and insular cortices, LHA, bed nucleus of the stria terminalis, lateral parabrachial nucleus, nucleus of the solitary tract, hippocampus, amygdala, and the dorsal motor nucleus of the vagus (Lindblom *et al.*, 2002; Zhou *et al.*, 2002; Alvaro *et al.*, 2003; Kishi *et al.*, 2003). Thus, the melanocortin system may play important roles in the detection of interoceptive state signals (e.g., hunger and satiety), taste processing, or even reward or learning about ingested foods (Saper *et al.*, 2002).

VI. Synergy and Interactions of the Systems

Leptin and insulin fill distinct niches in the endocrine system. Although leptin has been implicated in several systemic processes (e.g., angiogenesis) (Schwartz *et al.*, 1996a), the primary role of leptin appears to be as a negative-feedback adiposity signal that acts in the brain to suppress food intake and net catabolic effector peptides (Porte *et al.*, 1998; Woods *et al.*, 1998,2003). Consistent with this, animals lacking leptin or functional leptin receptors are grossly obese. Insulin, in contrast, has a primary action in the periphery to regulate blood glucose and stimulate glucose uptake by most tissues. Analogous to leptin, however, deficits in insulin signaling are associated with hyperphagia in humans. Animals that lack normal insulin signaling in the brain are also obese.

The potential for redundancy between leptin and insulin has been highlighted by several recent studies in which leptin and insulin have been found to share intracellular and neuronal signaling pathways. While the melanocortin system has long been thought to mediate the central actions of leptin, recent studies in which insulin significantly stimulated POMC expression in fasted rats and insulin-induced hypophagia was blocked by a nonspecific melanocortin receptor antagonist (Fan *et al.*, 1997; Ollmann *et al.*, 1997; Seeley *et al.*, 1997; Rossi *et al.*, 1998; Hagan *et al.*, 1999,2000) strongly support a role for the melanocortin system in the regulation of energy balance by insulin as well. Furthermore, phosphatidylinositol-3-OH kinase (PI3-K), an enzyme that is an intracellular mediator of insulin signaling, appears to play a crucial role in the leptin-induced anorexia signal transduction pathway (Niswender and Schwartz, 2003). While these data are consistent with the concept that leptin and insulin share such pathways, they also suggest that, over time, this redundancy dissipates and their pathways diverge.

VII. Additional Complexity of the Leptin/Insulin-Melanocortin System

Syndecans are a family of highly abundant cell-surface heparan sulfate proteoglycans (HSPGs) (proteins with covalently attached, highly acidic sugar chains) that are unique in their ability to bind extracellular peptides such as

hormones and growth factors. They act as coreceptors by modulating interactions of peptide ligands with their activity-generating receptors. In mammals, the syndecans are comprised of four transmembrane HSPGs and members of the glypican family of glycerophosphoinositol-linked HSPGs. Together, they account for nearly all HSPGs unbiquitously expressed at cell surfaces (Bernfield *et al.*, 1999; Park *et al.*, 2000). Syndecan family members are found on virtually every cell type (Bernfield *et al.*, 1999) but are differentially expressed in a tissue-specific manner. Syndecan-1 is found predominately on epithelial cells, while syndecan-3 is found primarily on neural crest-derived cells and neurons. They are induced during development and injury and in response to a wide spectrum of physiological stimuli (Lauri *et al.*, 1998; Bernfield *et al.*, 1999; Hsueh and Sheng, 1999).

During the course of studies on the function of syndecan-1, Reizes and colleagues (2001) discovered that the syndecans play an important role in the regulation of food intake and body weight. In those studies, they induced overexpression of syndecan-1 in mice. For reasons that still are not clear, this did not result in ubiquitous expression but rather yielded high levels of syndecan-1 expression in specific areas of the brain where it would normally not be found. In particular, the syndecan-1 transgene was highly expressed in hypothalamus. Syndecan-1 transgenic mice have severe maturity-onset obesity and type II diabetes. The phenotype of this obesity closely resembles that of previously characterized mice with disruptions of the melanocortin signaling pathway, including the agouti yellow, AgRP overexpressers, and MC4R knockout mice (Huszar *et al.*, 1997; Ollmann *et al.*, 1997). Additionally, syndecan-1 was found to potentiate the obesity of the yellow (Ay/a) mice and to potentiate the activity of AgRP and agouti signaling protein in cell-culture preparations. Finally, it was discovered that syndecan-1 promoted obesity only when it was expressed at the cell surface. Mice that constitutively shed syndecan-1 because the membrane-binding region of the gene had been deleted had a complete reversal of the obese phenotype (Reizes *et al.*, 2001). These findings are consistent with the hypothesis that the syndecan-1 transgene acts as a coreceptor for AgRP on MC3R- and MC4R-containing neurons. This, in turn, led to the hypothesis that syndecan-3, normally expressed in hypothalamic tissues, is a coreceptor for endogenous AgRP antagonism.

VIII. Dysregulation of Energy Homeostasis

Obesity is increasing and has been declared an epidemic. The epidemiological data are quite compelling and have been summarized in several reviews (Bray and Popkin, 1998; Samaras *et al.*, 1998). Additionally, studies in animals provide strong experimental evidence that increasing dietary fat accelerates the development of obesity. Across numerous experiments, diets, and species, the

conclusion that increased consumption of high-fat (HF) diets leads to increased body fat is inescapable. There are many reviews of this literature (Hill *et al.*, 1992; Warwick and Schiffman, 1992; Warwick, 1996; Golay and Bobbioni, 1997; West and York, 1998) and several valuable points have emerged from them. Perhaps most importantly, strong genetic influences dictate whether or not a given individual will be prone or resistant to becoming obese when exposed to a HF diet (Levin and Routh, 1996; West, 1996; Leibel *et al.*, 1997; Levin *et al.*, 1997; Reed *et al.*, 1997; West and York, 1998). As Bray and Popkin point out, a HF diet can be viewed as the environmental agent that acts on a susceptible host animal to produce the noninfectious disease, obesity (Bray *et al.*, 1990).

Experiments in which animals were rendered obese, then placed on a low(er)-fat diet, have been somewhat equivocal, with some reporting loss of body weight (Hill *et al.*, 1992) and others no weight reduction (Faust *et al.*, 1978; Rolls *et al.*, 1980; Harris *et al.*, 1986; Uhley and Jen, 1989; Hill *et al.*, 1992). One important parameter is evidently the age at which the obesity is initially induced. Younger rats (as well as older rats made obese by maintenance on a HF diet for longer intervals) may increase their number of adipocytes (Lemonnier, 1972; Faust *et al.*, 1978; Hill *et al.*, 1992). When subsequently placed on a low-fat (LF) diet, such animals tend not to lose weight (or body fat). However, if the number of adipocytes does not increase, obese animals placed on a LF diet lose weight to the level of rats never made obese at all (Hill *et al.*, 1992). Without assessing fat cell number, we have observed that adult rats given a HF diet and held at an obese weight for a prolonged interval lose weight to control levels when returned to a LF diet. One conclusion that has been reached from this literature is that it is easier to induce obesity in a lean individual with a HF diet than it is to induce leanness in an obese individual with a LF diet (Hill *et al.*, 1992; Bray and Popkin, 1998).

Importantly, the consequences of obesity, including dietary-induced obesity, are well documented and include type II diabetes and insulin insensitivity. Furthermore, some detrimental effects of dietary fat are not limited to obese individuals. For example, we recently demonstrated that while dietary-induced obesity decreases central sensitivity to the anorexic effects of central insulin administration, increased dietary fat, in the absence of frank obesity, attenuates the potency of central insulin to reduce food intake and body weight.

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Leptin Receptor Signaling and the Regulation of Mammalian Physiology

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ABSTRACT

While the hormone leptin and its receptor were discovered relatively recently, a great deal is already known about the molecular details of leptin receptor (LR) signaling and physiologic regulation. While multiple alternatively spliced LR isoforms exist, only the long (LRb) form associates with the Janus kinase 2 (Jak2) tyrosine kinase to mediate intracellular signaling. LRb initiates signaling via three major mechanisms: 1) Tyr_{985} of LRb recruits SH2-containing tyrosine phosphatase (SHP-2); 2) Tyr_{1138} of LRb recruits signal transducer and activator of transcription 3 (STAT3); and 3) tyrosine phosphorylation sites on the receptor-associated Jak2 likely recruit numerous undefined signaling proteins. The $Tyr_{985} \rightarrow$ SHP-2 pathway is a major regulator of extracellular signal-regulated kinase (ERK) activation during leptin signaling in cultured cells, while the $Tyr_{1138} \rightarrow$ STAT3 pathway induces the feedback inhibitor, suppressor of cytokine signaling 3 (SOCS3), as well as important positive effectors of leptin action. The Jak2-dependent activation of the insulin receptor substrate (IRS) protein $\rightarrow$ phosphatidylinositol 3-kinase (PI3′-K) pathway appears to regulate membrane potential in LRb-expressing neurons and contributes to the regulation of feeding. The $Tyr_{1138} \rightarrow$ STAT3 pathway mediates transcriptional regulation of the hypothalamic melanocortin pathway *in vivo*. This pathway is required for the regulation of appetite and energy expenditure by leptin. Interestingly, the $Tyr_{1138} \rightarrow$ STAT3 pathway does not strongly regulate neuropeptide Y (NPY) and thus is not required for the control of reproduction and growth. Thus, other as-yet-undefined leptin receptor signals are central to these and perhaps other aspects of leptin action.

I. Background

A. THE *ob* and *db* LOCI

Although leptin was identified only recently (1994), the history of leptin began several decades ago with the description of the *obese (ob)* and *diabetes (db)* mouse models that spontaneously and independently arose in the mouse breeding program at the Jackson Laboratories (Flier, 1995; Friedman and Halaas, 1998). In the homozygous state *(ob/ob* or *db/db),* mutations at these loci cause hyperphagia and decreased metabolic rate (resulting in morbid obesity), predisposition to diabetes, and endocrine disturbances. While genetic studies mapped

these two genes to separate loci, their relatedness was suggested early on by an intriguing set of parabiosis studies by Douglas Coleman in which the circulatory systems of pairs of wild-type, *ob/ob*, and *db/db* mice were joined. Parabiosis of *ob/ob* animals with either wild-type or *db/db* mice abrogated the phenotype of *ob/ob* mice, suggesting that the *ob* gene product (missing in *ob/ob* mice) was a circulating factor (a hormone) present in wild-type and *db/db* mice. In contrast, neither wild-type nor *ob/ob* mice could alter the phenotype of *db/db* mice to which they were joined, suggesting that the *db/db* mouse was insensitive to this factor. Coleman theorized that the receptor for the *ob* factor was mutated in the *db/db* mouse.

The predictions of these classic experiments were confirmed when the *ob* gene was positionally cloned in a large mouse genetics study. The gene encodes a predicted secreted protein, termed leptin (Greek: leptos = thin) (Flier, 1995; Friedman and Halaas, 1998). Soon after the cloning of leptin, it was demonstrated that leptin is synthesized and secreted from adipocytes and that treatment of *ob/ob* mice with leptin reversed their phenotype (Halaas *et al.*, 1995). Treatment of *db/db* mice with leptin was ineffective, confirming their insensitivity to this factor.

Following the cloning of leptin, the leptin receptor (LR) was cloned quickly by biochemical techniques employing labeled leptin (Tartaglia *et al.*, 1995). Soon thereafter, the original *db* mutation from Jackson Laboratories and several other independently arising *db* mutations were shown to lie within the leptin receptor gene *(lepr)* (Chua *et al.*, 1996).

B. LEPTIN COMMUNICATES THE STATUS OF BODY ENERGY STORES TO THE CENTRAL NERVOUS SYSTEM

Adipose cells are the primary source of leptin. Leptin production by fat cells in culture is stimulated by glucocorticoids and indicators of acute nutritional influx such as insulin and inhibited by the counter-regulatory hormones and their intracellular signaling mediators (MacDougald *et al.*, 1995; Rentsch and Chiesi, 1996; Slieker *et al.*, 1996). This regulation of leptin production by insulin (feeding → increased insulin → increased leptin) and counter-regulatory hormones (fasting → increased counter-regulatory hormones → decreased leptin) suggests that leptin might serve as an indicator of energy balance (i.e., that increased energy stores yield increased leptin levels). Indeed, circulating leptin levels strongly correlate with body adiposity and changes in acute nutritional status (Frederich *et al.*, 1995; Maffei *et al.*, 1995; Considine *et al.*, 1996).

The stimulation of leptin production by glucocorticoids appears counterintuitive in this light, however, since circulating glucocorticoids are important mediators of the stress and starvation responses. Indeed, while plentiful data suggest that insulin is a critical mediator of increased leptin production *in vivo*,

the diurnal pattern of leptin levels and numerous other data argue against an important role for circulating glucocorticoids in the stimulation of leptin production (Licinio *et al.*, 1997). In contrast, adipocyte-produced autocrine- or paracrine-acting glucocorticoids may be involved, since the production of glucocorticoids by adipocytes increases with the accumulation of triglycerides. Mouse models with increased glucocorticoid production specifically in adipocytes have elevated leptin levels (Bornstein *et al.*, 1997).

Circulating leptin levels thus reflect the status of body energy reserves. Leptin regulates energy balance throughout the body by controlling processes involved in energy intake and utilization. Leptin regulates energy expenditure via the reproductive, growth, autonomic, and other axes of the neuroendocrine system as well as by the immune system (Ahima *et al.*, 1996; Lord *et al.*, 1998) (Figure 1). Thus, in times of inadequate energy stores (e.g., starvation), leptin

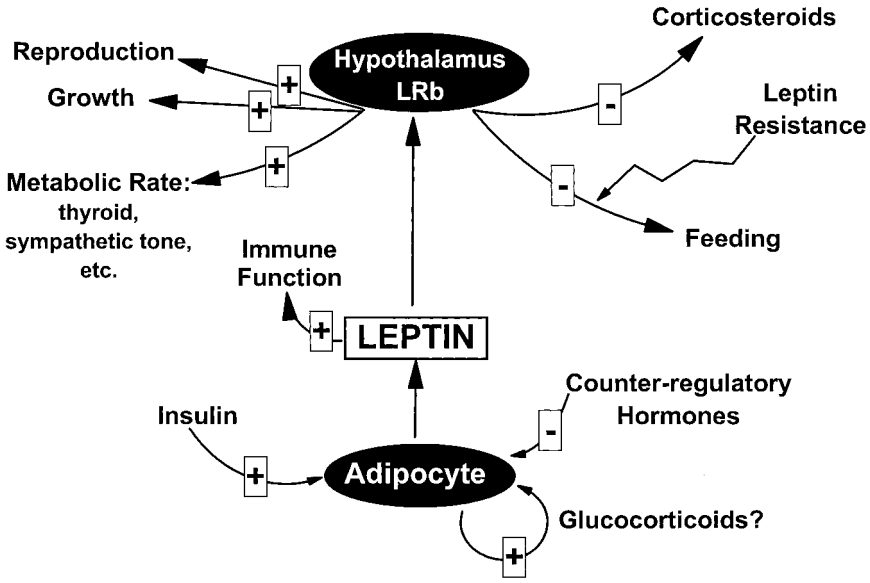

FIG. 1. Leptin signals the status of body energy stores to the hypothalamus and neuroendocrine system. Leptin production in adipocytes is regulated by indicators of acute nutritional status, such as insulin and counter-regulatory hormones, as well as by fat content (perhaps signaled via the autocrine/paracrine production of glucocorticoids). Leptin heightens immune responsiveness by direct effects upon the immune system and regulates feeding and neuroendocrine function by activating the long (LRb) form of its receptor in the brain, most notably in the hypothalamus. Adequacy of leptin levels (reflecting adequate nutrition) suppresses feeding and the hypothalamic drive to produce glucocorticoids as well as permits the expenditure of energy by the reproductive, growth, and thyroid axes. Leptin resistance in common obesity appears to most strongly impact the ability of leptin to inhibit feeding.

levels fall; these low leptin levels enhance appetite and decrease energy utilization. Conversely, with adequate energy stores, high leptin levels decrease the drive to eat and permit utilization of energy by the above-mentioned systems.

Lack of the leptin signal in mice (and humans) genetically null for leptin *(ob/ob* mice) or the leptin receptor (LR) *(db/db* mice) results in obesity secondary to increased feeding and decreased energy utilization (Montague *et al.*, 1997; Clement *et al.*, 1998; Elmquist *et al.*, 1998; Friedman and Halaas, 1998). These animals also display a phenotype reminiscent of the neuroendocrine starvation response, including hypothyroidism, decreased growth, and infertility, in addition to decreased immune function. Indeed, exogenous leptin replacement during food restriction restores each of these functions as well as decreases appetite (Ahima *et al.*, 1996).

C. LEPTIN RECEPTORS AND SITES OF LEPTIN ACTION

Multiple LR isoforms exist; all are products of a single *lepr* gene and result from alternative mRNA splicing and/or proteolytic processing (Chua *et al.*, 1997; Tartaglia, 1997). LR isoforms fall into three classes: secreted, short, and long. The secreted forms are alternative splice products (e.g., the murine LRe) or proteolytic cleavage products of membrane-bound LR forms: These contain only extracellular leptin-binding domains and complex with circulating leptin, perhaps regulating free leptin concentrations (Ge *et al.*, 2002). Short- (e.g., LRa) and long-form (LRb) receptors contain identical extracellular and transmembrane domains as well as the same first 29 intracellular amino acids and diverge in sequence secondary to alternative splicing of 3′ exons. LRb is highly conserved among species and possesses an intracellular domain of approximately 300 residues (Chua *et al.*, 1997; Tartaglia, 1997).

While the function of the short LR forms remains unclear, LRb is critical for leptin action. Indeed, the originally described *db/db* mice lack only LRb (as a consequence of a mutation that results in mis-splicing of the LRb message) but exhibit a phenotype indistinguishable from that of leptin-deficient *ob/ob* animals and of db^{3J}/db^{3J} mice (which are deficient in all LR isoforms) (Chua *et al.*, 1997; Tartaglia, 1997; Friedman and Halaas, 1998).

Much of leptin's action is attributable to effects in the central nervous system (CNS), especially in the basomedial hypothalamus, the site of highest LRb expression (Elmquist *et al.*, 1998,1999). Here, leptin acts on neurons that regulate levels of circulating hormones (e.g., thyroid hormone, sex steroids, growth hormone) (Elmquist *et al.*, 1999; Inui, 1999). Leptin action on these hypothalamic neurons also regulates the activity of the autonomic nervous system, although direct leptin action on brainstem LRb-expressing neurons may play a role in this and other leptin actions (Elmquist *et al.*, 1997; Grill *et al.*, 2002). Leptin action on the immune system appears to result from direct action on

LRb-expressing T cells (Lord *et al.*, 1998). Leptin also may regulate glucose homeostasis (independently of effects on adiposity). Leptin regulates glycemia at least partly via the CNS but also may directly regulate some metabolic tissues (Liu *et al.*, 1988; Kieffer *et al.*, 1997; Kulkarni *et al.*, 1997; Burcelin *et al.*, 1999).

D. LEPTIN RESISTANCE AND OBESITY

Over one quarter of adult Americans are obese. The incidence of obesity continues to rise in the United States as well as in other industrialized nations. Obesity is a major risk factor for type 2 diabetes, cardiovascular disease, and some forms of cancer (Roth, 1998). Since administration of leptin to rodents decreases food intake and increases energy expenditure, resulting in loss of fat mass, leptin initially was hailed as a potential cure for obesity (Tartaglia, 1997; Elmquist *et al.*, 1998,1999; Friedman and Halaas, 1998; Inui, 1999).

With the exception of rare human patients with genetic leptin deficiency, however, circulating leptin levels correlate well with body mass index (BMI) and total body fat mass. Hence, obese individuals have elevated circulating leptin levels but this abundant leptin fails to mediate weight loss, suggesting that most human obesity represents a form of leptin resistance. Indeed, although therapy with exogenous leptin does augment weight loss, the effects of leptin are modest at the doses that have been tested. A number of potential mechanisms have been postulated to underlie leptin resistance, including defects in leptin access into the brain, in LRb signaling, or in pathways/neurons that mediate downstream leptin action. We predict that defects in LRb signaling or downstream effectors will underlie leptin resistance, since leptin may gain direct access (without need for transport across the blood-brain barrier) to neurons in the basomedial hypothalamus that are important for the regulation of energy balance.

II. LR Signaling Systems

A. LEPTIN SIGNALING AT THE OUTSET

My graduate training in the laboratory of Morris White, Ph.D. at the Joslin Diabetes Center focused on the role(s) played by insulin receptor substrate-1 (IRS-1) in cellular insulin signaling. After defending my thesis, I returned to medical school to complete the clinical component of my training. I realized that I preferred to focus on research in the long term, as opposed to continuing with clinical practice. Thus, it was in the late winter and early spring of 1997 that I found myself finishing my medical education and thinking about a new scientific path to follow to establish my independence. Possessing a background in basic tyrosine kinase signaling, I wished to play to this strength as well as to continue working in a direction relevant to diabetes and the control of metabolism. It was

clear that switching away from insulin signaling and IRS-l would facilitate my transition to independence. Although I considered for a time studying signaling by cytokines in immune function, the initial cloning of the leptin receptor just had been reported to great fanfare (Tartaglia *et al.*, 1995). LRb was clearly a type I cytokine receptor that mediated cell signaling via a member of the Jak family of tyrosine kinases. The obese and diabetic phenotype of mice deficient in leptin or LRb made it clear that it was a metabolically relevant target. While the hubbub surrounding leptin action was huge (and remains high), it was clear that most investigators had priorities other than studying the intricate details of LR signaling and cell biology, although this area would likely prove to be interesting and important.

The homology of LRb with other type I cytokine receptors (e.g., the gp 130 subunit of the interleukin (IL)-6 receptor family) suggests a number of signaling functions that were tested quickly by a number of groups: leptin binding to LRb activates Jak kinases and mediates cytokine receptor-like signals, including those mediated by the latent transcription factor, signal transducer and activator of transcription 3 (STAT3) (Baumann *et al.*, 1996; Tartaglia, 1997; White *et al.*, 1997). A specific tyrosine residue (Tyr_{1138}) in the intracellular domain of LRb mediates the activation of STAT3.

We felt that LRb was likely to mediate other important signals as well and set out to identify them. In general, tyrosine kinase-based signaling is mediated by the recruitment of signaling molecules to tyrosine-phosphorylated motifs on the receptor and/or tyrosine kinase (Koch *et al.*, 1991) — in this case, on the intracellular domain of LRb and its associated Jak kinase(s). This recruitment of downstream signaling proteins generally is mediated by specialized phosphotyrosine binding domains (e.g., src homology 2 (SH2) domains) in the signaling proteins. These SH2 domains generally require the presence of phosphotyrosine in the target protein for high-affinity binding but also recognize surrounding amino acid residues. Thus, each tyrosine phosphorylation site on a receptor recruits specific SH2 domain-containing proteins, based upon the amino acid motif in which the phosphotyrosine residue is located. In this manner, the residues Met-Pro-Gln (MPQ) following Tyr_{1138} of LRb dictate binding of STAT3 when Tyr_{1138} becomes phosphorylated (Baumann *et al.*, 1996; Tartaglia, 1997; White *et al.*, 1997).

Understanding the signals mediated by LRb requires identifying the tyrosine residues within the LRb/Jak kinase complex that are phosphorylated during signaling and subsequently determining the signaling proteins that interact with these sites. While the identification of LRb phosphorylation sites should be relatively straightforward, identifying phosphorylation sites on the associated Jak kinase is more complicated, given that it requires identifying the Jak kinase isoform(s) utilized by LRb (Ihle, 1995) as well as sorting through a greater number of tyrosine phosphorylation sites (Feng *et al.*, 1997). Furthermore, in

order to understand the failure of short LR isoforms to compensate for the lack of LRb in *db/db* animals, it is critical to define their ability to mediate Jak-dependent signaling.

In order to avoid potential artifacts introduced by the endogenous (mostly short-form) LR isoforms expressed in most cells, we employ a chimeric receptor (ELR) that contains the extracellular ligand-binding domain of the erythropoeitin (Epo) receptor fused to the transmembrane and intracellular domains of LR forms (Banks *et al.*, 2000). The EpoR is expressed on few cells or cell lines, so ELR permits us to use Epo to stimulate signaling via various LR intracellular domains without triggering endogenous receptors.

B. JAK KINASE SELECTIVITY AND UTILIZATION BY LR ISOFORMS

There are four known members of the Jak family: Jak1–3 and Tyk2. Jak1, Jak2, and Tyk2 are widely expressed, whereas Jak3 is found only in cells of the hematopoeitic/immune systems (Ihle, 1995). We expressed ELR in 32D myeloid progenitor cells and assessed its ability to mediate the activation of the commonly expressed Jak kinases, using the endogenous (Jak-promiscuous) IL-3 receptor as a positive control for the activation of each kinase. This analysis demonstrated that the intracellular domain of ELR activated only Jak2, not Jak1 or Tyk2 (Kloek *et al.*, 2002). Furthermore, while disruption of Jak2 expression in fibrosarcoma cells abrogated ELR signaling, disruption of Jak1 or Tyk2 had no effect. Thus, Jak2 represents the unique Jak kinase involved in signaling by the intracellular domain of LRb.

All functional cytokine receptors contain a proline-rich "Box 1" motif that is required for Jak kinase interaction and activation. Additional less-conserved sequences (sometimes referred to as "Box 2") COOH-terminal to Box 1 are also important for Jak kinase interactions and likely function in Jak kinase isoform selectivity (Ihle and Kerr, 1995; Taga and Kishimoto, 1997). Our deletion analysis demonstrated that, in addition to Box 1 sequences, intracellular residues 31–36 of LRb (i.e., immediately downstream of the alternative splice junction following amino acid 29) are required for Jak2 activation (Kloek *et al.*, 2002). Interestingly, we observed that high-level overexpression of Jak kinases by transient transfection could decrease the stringency of the requirement for residues 31–36, consistent with the ability of others to observe (relatively weak) LRb-Jak1 and LRa-Jak2 signaling under transient transfection conditions (Bjorbaek *et al.*, 1997). These results suggests a model in which Box 1 is absolutely required for all cytokine receptor-Jak kinase interactions, with the more-flexible role (at least under experimental conditions) for residues homologous to intracellular amino acids 31–36 of LRb in determining the specificity of the Jak kinase that interacts with a particular receptor. Indeed, homology between the Box 2 regions of LRb and other Jak2-associated cytokine receptors suggests that

a loosely conserved E/N-X_{0-2}E/N-X_{0-2}L/I motif mediates Jak2 association (Kloek *et al.*, 2002).

This motif is absent from all described short LR isoforms, explaining the inability of these molecules to mediate leptin action in *db/db* animals (Tartaglia, 1997; White *et al.*, 1997; Kloek *et al.*, 2002). The lack of Jak2 signaling by short LR isoforms suggests we cannot rule out, on the basis of the failure of these receptors to transmit any leptin signal, the possibility that Jak2 may be capable of mediating some physiologic leptin actions in the absence of LRb tyrosine phosphorylation sites.

C. LRb TYROSINE PHOSPHORYLATION

There are three conserved tyrosine residues on the intracellular domain of LRb: Tyr_{985}, Tyr_{1077}, and Tyr_{1138} (Tartaglia, 1997; White *et al.*, 1997; Banks *et al.*, 2000). In the human long-form LR, two additional intracellular tyrosine residues exist, albeit within motifs that do not appear to be particularly hydrophilic and accessible to kinases (Tartaglia *et al.*, 1995). In order to determine whether any of these sites might be candidates for phosphorylation, we generated a bacterial fusion protein containing all of the tyrosine residues of the intracellular domain of the human long-form LR (except the Tyr_{1138} homologue) and subjected it to phosphorylation with recombinant Jak2. Analysis of the resulting phosphorylated glutathione-S-transferase (GST)-LR demonstrated tyrosine phosphorylation of the Tyr_{985} homologue but not of any other sites, suggesting that Tyr_{985} and Tyr_{1138} might be the unique sites of LRb tyrosine phosphorylation (M.G. Myers, Jr., unpublished observations).

In order to examine the phosphorylation of tyrosine residues on the intracellular tail of LRb in intact cells, we mutated the three LRb tyrosines individually and in combination and examined the phosphorylation of the mutant receptors during signaling in 293 cells (Banks *et al.*, 2000). This analysis confirmed that Tyr_{985} and Tyr_{1138} are phosphorylated during receptor signaling but Tyr_{1077} is not phosphorylated and does not contribute to signaling. Thus, three primary intracellular signaling pathways emanate from LRb (Figure 2): those originating directly from Jak2 tyrosine phosphorylation sites, from Tyr_{985} of LRb, and from Tyr_{1138} of LRb.

D. SIGNALING VIA TYR_{985} MEDIATES SHP-2 RECRUITMENT AND ERK ACTIVATION

We and others had noted the activation of the ERK kinases during signaling by the intracellular domain of LRb (Bjorbaek *et al.*, 1997; Banks *et al.*, 2000). In order to understand the mechanism for this activation, we employed our ELR isoforms containing tyrosine substitution mutations. We noted that mutation of Tyr_{985} inhibited ERK activation by approximately 70% and that the remaining

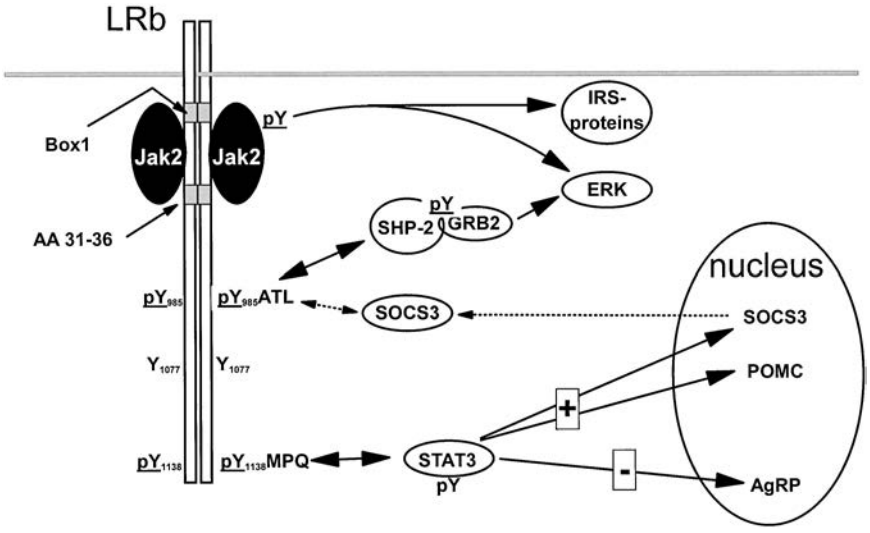

FIG. 2. Intracellular signaling by LRb. Leptin binding to the extracellular domain of the LRb dimer activates the Jak2 tyrosine kinase that associates with LRb via the Box 1 motif and intracellular amino acids 31–36 of LRb. Activated Jak2 tyrosine phosphorylates itself and Tyr_{985} and Tyr_{1138} on the intracellular tail of LRb. Phosphorylated Tyr_{1138} binds and mediates the phosphorylation-dependent activation of signal transducer and activator of transcription 3 (STAT3), which activates transcription of suppressor of cytokine signaling 3 (SOCS3) (a feedback inhibitor of LRb signaling) and pro-opiomelanocortin (POMC) (an anorexigenic neuropeptide) and inhibits the transcription of agouti-related protein (AgRP) (an orexigenic neuropeptide). Phosphorylated Tyr_{985} recruits the SH2-containing tyrosine phosphatase SHP-2, which is itself phosphorylated and binds growth factor receptor binding 2 (Grb-2) to activate the signaling pathway that culminates in extracellular signal-regulated kinase (ERK) activation. During prolonged stimulation, after induction of SOCS3 protein, phosphorylated Tyr_{985} also mediates binding of SOCS3. Signals mediated via unidentified tyrosine phosphorylation sites on LRb include the tyrosine phosphorylation of insulin receptor substrate (IRS) proteins and a minor component of ERK activation.

30% of ERK activation required Jak2 interaction but none of the tyrosine phosphorylation sites on LRb. This suggested that a minor pathway to ERK activation is mediated independently of LRb phosphorylation, presumably via tyrosine phosphorylation sites on Jak2 (Banks *et al.*, 2000).

The canonical mechanism by which tyrosine kinases activate ERK is via the SH2 domain-containing adapter protein, growth factor receptor binding (Grb)-2. Grb-2 recruitment by tyrosine kinase signaling complexes mediates the activation of the small G protein, p21ras, to stimulate the upstream activators of ERK (Lowenstein *et al.*, 1992; Myers *et al.*, 1994; Banks *et al.*, 2000). In order to understand the mechanism of ERK activation by Tyr_{985}, we examined tyrosyl phosphoproteins associated with the adapter protein, Grb-2, during ELR signal-

ing and discovered an ~ 75-kDa Grb-2-associated tyrosyl phosphoprotein (Banks *et al.*, 2000). Taking a candidate approach, we showed that this 75-kDa protein is identical to the SH2 domain-containing tyrosine phosphatase SHP-2 and that Tyr_{985} directly recruits SHP-2 via its COOH-terminal SH2 domain (Banks *et al.*, 2000; Bjorbaek *et al.*, 2001; Kloek *et al.*, 2002). Indeed, the YATL motif in which Tyr_{985} lies is predicted to bind to the COOH-terminal SH2 domain of SHP-2 (Songyang *et al.*, 1993; De Souza *et al.*, 2002). Thus, phosphorylation of LRb Tyr_{985} mediates the recruitment of SHP-2, which is then phosphorylated and, in turn, recruits Grb-2 to mediate activation of the p21ras → ERK cascade.

E. SIGNALING VIA TYR_{1138} → STAT3 MEDIATES TRANSCRIPTION OF SOCS3 AND FEEDBACK INHIBITION ON LRb SIGNALING

The role of Tyr_{1138} phosphorylation in recruiting STAT3 to the LRb/Jak2 complex was suggested by homology between LRb and other cytokine receptors of the IL-6 receptor family. Indeed, mutation of this site abrogates STAT3 signaling by LRb (Baumann *et al.*, 1996; Tartaglia, 1997; White *et al.*, 1997). We thus utilized receptors mutant for Tyr_{1138} to investigate potential roles for STAT3 in the regulation of gene expression, demonstrating that this pathway induces expression of SOCS3, which is induced by prolonged LRb activation and mediates feedback inhibition of LRb signaling (Bjorbaek *et al.*, 1998,2000; Banks *et al.*, 2000). We furthermore examined the mechanism by which SOCS3 mediates feedback inhibition on signaling by the intracellular domain of LRb. We demonstrated that SOCS3 interacts via its SH2 domain with phosphorylated Tyr_{985} of LRb and that sensitive blockade of LRb → STAT3 signaling is mediated by this interaction (Bjorbaek *et al.*, 2000). Our recent unpublished results suggest that another LRb → STAT3-dependent mechanism of feedback inhibition of LRb signaling may directly block Jak2 activation.

F. SIGNALS MEDIATED BY JAK2 AND FUTURE DIRECTIONS IN LRb SIGNALING

Jak2 tyrosine phosphorylation during LRb stimulation mediates some signals independently of tyrosine phosphorylation sites on LRb (e.g., a portion of ERK activation, IRS protein phosphorylation) (Banks *et al.*, 2000). Unfortunately, most Jak2 tyrosine phosphorylation sites have not been defined, impairing our understanding of the mechanisms by which Jak2-dependent signals are mediated. Identifying tyrosine phosphorylation sites on Jak2 and defining their roles in intracellular signaling will be critical to understanding the breadth and depth of LRb signaling.

III. Leptin Signals in the Regulation of Mammalian Physiology

A. LEPTIN ACTION IN THE HYPOTHALAMUS

While LRb is expressed at other sites, the highest levels of LRb expression in the body are found in neurons of the nuclei of the basomedial hypothalamus — including the arcuate (ARC), dorsomedial hypothalamic (DMH), and ventromedial hypothalamic (VMH) nuclei (Elmquist *et al.*, 1998). Chemical or physical ablation of these nuclei results in increased feeding and neuroendocrine abnormalities that are similar to the phenotypes of *db/db* or *ob/ob* mice, suggesting that these hypothalamic nuclei (which make up the so-called "satiety center") are critical sites of leptin action (Elmquist *et al.*, 1999; Schwartz *et al.*, 2000).

Within the nuclei of the basomedial hypothalamus, LRb is expressed at its highest levels in the ARC. Within the ARC, LRb is found in at least two distinct populations of neurons (Figure 3): 1) neurons that coexpress neuropeptide Y (NPY) and agouti-related peptide (AgRP) and 2) neurons that express pro-

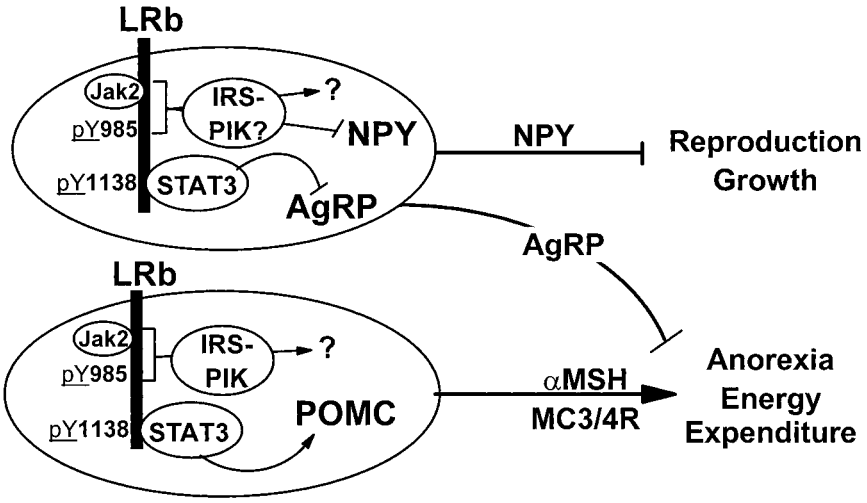

FIG. 3. Neuroendocrine regulation in the arcuate nucleus (ARC) by LRb signals. In the LRb/POMC neuron in the ARC, the $Tyr_{1138} \rightarrow$ STAT3 signal mediates the induction of POMC, which is processed to alpha melanocyte-stimulating hormone (αMSH) to activate anorexia and energy expenditure via the melanocortin receptors, MC3R and MC4R. The function of other LRb/Jak2 signals, such as IRS proteins and phosphatidylinositol 3-kinase (IRS-PI3-K), remain undefined in this neuron. In the ARC LRb/neuropeptide Y (NPY)/AgRP-expressing neuron, STAT3 signaling contributes to the repression of AgRP and the de-repression of melanocortin action, while other signals (possibly including IRS-PI3-K) function to repress NPY, disinhibiting the growth and reproductive axes. Other IRS-PI3-K-mediated signals may include the regulation of membrane potential.

opiomelanocortin (POMC) (Elmquist *et al.*, 1999; Schwartz *et al.*, 2000). POMC is processed to alpha melanocyte-stimulating hormone (αMSH) in the LRb/POMC neuron. αMSH mediates a powerful anorectic (appetite-suppressing) signal; LRb stimulates the expression of POMC and activates the LRb/POMC neuron (Schwartz *et al.*, 2000; Cowley *et al.*, 2001). AgRP is an antagonist of αMSH signaling and NPY is itself an orexigenic (appetite-stimulating) hormone that also acts to suppress the central LRb growth and reproductive axes (Erickson *et al.*, 1996b; Fan *et al*., 1997; Seeley *et al.*, 1997; Bates *et al.*, 2003). Leptin acts via LRb to inhibit the NPY/AgRP neurons and to suppress expression of these neuropeptides. Thus, LRb signaling stimulates the production of anorectic neuropeptides and suppresses levels of orexigenic peptides. Conversely, when leptin action is decreased or deficient (e.g., starvation, *ob/ob* or *db/db* mice), appetite is stimulated via the suppression of anorectic neuropeptides (e.g., POMC) and by increased expression of orexigenic peptides (e.g., NPY, AgRP) (Elmquist *et al.*, 1999; Schwartz *et al*., 2000). LRb-expressing ARC NPY/AgRP and/or POMC neurons also regulate energy expenditure and other elements of neuroendocrine function (Ahima *et al.*, 1996). Other distinct populations of LRb-expressing neurons may be found in the ARC (Jureus *et al.*, 2000). The neurochemical properties of LRb-expressing neurons in the DMR, VHM, and elsewhere (including the brainstem) are poorly defined.

B. IRS PROTEIN/PI3′-K SIGNALING IN THE CONTROL OF PHYSIOLOGY BY LEPTIN

Thus far, we have been involved in implicating two LRb signaling pathways in leptin action: STAT3 (see below) and the IRS protein-phosphatidylinositol 3-kinase (PI3′-K) pathway (Niswender *et al.*, 2001; Bates *et al.*, 2003).

First described as insulin receptor substrates, the IRS proteins (IRS 1–4) are members of a class of intracellular signaling molecules termed docking proteins that are phosphorylated by a number of activated tyrosine kinases (e.g., insulin receptor, some cytokine receptors) (Myers and White, 2002). Docking proteins, including the IRS proteins, are devoid of enzymatic activity but are phosphorylated on multiple tyrosine residues to mediate SH2-protein recruitment and downstream signaling. While IRS proteins contain tyrosine phosphorylation sites in numerous motifs that recruit several different SH2-proteins, the majority of sites lie in YMXM motifs that bind and activate PI3′-K.

The first, albeit indirect, evidence for a potential role of the IRS protein → PI3′-K pathway in leptin action came from the phenotype of the IRS-2 null (IRS-$2^{-/-}$) mouse (Withers *et al.*, 1998). In addition to other defects, IRS-$2^{-/-}$ animals display increased feeding and decreased metabolic rate in the presence of increased adiposity and circulating leptin, suggesting functional leptin resis-

tance (although not as severe as in *db/db* animals). No such phenotype has been noted in animals null for any of the other three IRS proteins (Araki *et al.*, 1994).

Blockade of PI3′-K activity abrogates leptin-mediated hyperpolarization and inhibition of (presumably) LRb/NPY/AgRP hypothalamic neurons (Spanswick *et al.*, 1997b; Harvey *et al.*, 2000). Furthermore, leptin stimulates IRS-2-associated PI3′-K activity in the hypothalamus and pharmacological blockade of PI3′-K activity in the hypothalamus blocks the anorectic effect of leptin *in vivo* (Niswender *et al.*, 2001).

Importantly, inhibition of PI3′-K does not alter the anorectic effect of melanocortin agonists that operate downstream of LRb neurons, suggesting that this effect of PI3′-K blockade is specific for LRb neurons. PI3′-K activity also is required for leptin-regulated sympathetic nervous system function (Rahmouni *et al.*, 2003).

As *in vivo* (Niswender *et al.*, 2001), we observe that LRb stimulation mediates the tyrosine phosphorylation of IRS proteins and activation of the PI3′-K pathway in cultured cells. This signal is mediated by Jak2 independently of tyrosine phosphorylation sites on LRb (M.G. Myers, Jr., *et al.*, unpublished observations).

The recruitment of IRS proteins/PI3′-K by Jak2 is less robust than by the insulin receptor, however. Indeed, animals with decreased expression of neuronal insulin receptors (NIRKO mice) display a modest obesity phenotype, similar to the phenotype of IRS-$2^{-/-}$ mice (Bruning *et al.*, 2000). Also, insulin activates the IRS-2 → PI3′-K pathway in the hypothalamus and PI3′-K activity is required for the anorectic activity of insulin in the brain (Niswender *et al.*, 2003). Thus, both leptin and insulin stimulate hypothalamic IRS-2 → PI3′-K signaling and both require PI3′-K activity for their anorectic functions. Although the relative contribution of insulin- vs. leptin-stimulated PI3′-K to functional (anorectic) hypothalamic signaling remains difficult to dissect, the importance of the IRS protein → PI3′-K pathway is clear, as will be the understanding of the potential role of this pathway in functional leptin resistance. It will be interesting to determine the neuropeptide phenotype of the neuronal population(s) in which PI3′-K activity is required for the anorectic actions of leptin and insulin as well as the role of PI3′-K in other leptin functions (e.g., neuroendocrine and immune regulation).

C. LRb → STAT3 SIGNALING IN THE CONTROL OF PHYSIOLOGY BY LEPTIN

We have directly addressed the contribution of the LRb → STAT3 pathway to physiology by studying homologously targeted “knock-in” mice in which LRb is replaced by a mutant molecule (LRb^{S1138}) that contains a substitution mutation of Tyr^{1138} (the STAT3 binding site) (Bates *et al.*, 2003). While LRb^{S1138} fails to

mediate activation of STAT3 during leptin signaling, this mutant regulates all other LRb signaling pathways normally. This approach ensures that the expression pattern and levels of LRb^{S1138} mirror that of wild-type LRb, so alterations in physiology in the resulting animals are secondary to lack of LRb → STAT3 signaling rather than due to a failure of correct LRb expression. Like *db/db* animals, mice homozygous for LRb^{S1138} *(s/s)* display hyperphagia and decreased energy expenditure, resulting in massive, early-onset obesity associated with increased serum leptin levels. Thus, the LRb → STAT3 signal is central to the regulation of body weight by leptin and dysfunction of this signal generates leptin resistance.

Important differences exist between the phenotypes of *s/s* mice and *db/db* mice, however (Bates *et al.*, 2003). While *db/db* animals are infertile and demonstrate decreased linear growth, *s/s* mice are fertile and demonstrate *increased* linear growth, compared to wild-type animals.

In order to understand the neurochemical basis for the *s/s* phenotype, we analyzed hypothalamic neuropeptide expression, revealing that, like *db/db* mice, *s/s* mice have decreased POMC and increased AgRP mRNA levels in the hypothalamus. In contrast, while *db/db* animals display dramatic induction of hypothalamic NPY mRNA, levels of NPY message are near normal in *s/s* animals. These data suggest that LRb → STAT3 signaling is a critical regulator of hypothalamic melanocortin action and that dysregulated melanocortin signaling (as opposed to alterations in NPY) accounts for the obesity of *s/s* animals. Additionally, non-STAT3 LRb signals are critical regulators of NPY expression in the LRb/NPY neuron.

These results are consistent with the proposed role for NPY in suppressing the hypothalamic growth and gonadal axes; thus, the increased NPY signaling in *ob/ob* and *db/db* mice may only modestly increase feeding but may be primarily responsible for infertility and growth retardation in these mouse models. Indeed, the phenotype of *ob/ob* mice null for NPY *(ob/ob,*$Npy^{-/-}$*)* displays important similarities with the *s/s* phenotype (Erickson *et al.*, 1996a): both show improved function of the hypothalamic/gonadal axis and increased linear growth with only modestly attenuated obesity, compared to *ob/ob* and *db/db* mice. The increased linear growth observed in these models likely results from the absolute lack of NPY in *ob/ob,*$Npy^{-/-}$ animals and the hyperactivation of non-STAT3 LRb signals (e.g., signals that inhibit the release of NPY) in hyperleptinemic *s/s* animals, as is observed in most forms of human obesity. Thus, functional leptin resistance in humans could stem from inhibition of pathways controlled by LRb → STAT3 action.

Returning to the concept of leptin resistance in common forms of obesity, one would expect that dysregulation of the NPY pathway would result in neuroendocrine abnormalities (e.g., infertility, growth retardation) that are not generally observed in obesity. In contrast, alterations in STAT3-mediated path-

ways (e.g., melanocortin action) could generate a phenotype of impaired energy balance coupled with relatively normal neuroendocrine function.

D. FUTURE DIRECTIONS IN LRb SIGNALING AND PHYSIOLOGY

Our data from *s/s* animals demonstrate that LRb-STAT3 signaling is critical for the transcriptional regulation of melanocortin action in the ARC but not for the regulation of NPY. Additionally, STAT3 is not likely to be involved in the regulation of membrane potential in ARC neurons, as leptin-regulated membrane potential is too rapid to be transcriptionally mediated (Spanswick *et al.*, 1997b; Cowley *et al.*, 2001).

Note that the phenotype of the *s/s* animals does not suggest the physiologic irrelevance of non-STAT3 pathways to the control of feeding and energy expenditure, only that STAT3 signaling is important for the regulation of energy homeostasis. We suggest that the Jak2 → IRS protein → PI3′-K pathway represents a major STAT3-independent mediator of LRb action. Data from numerous laboratories suggest that PI3′-K action regulates membrane potential in the LRb/NPY neuron (Spanswick *et al.*, 1997a; Harvey *et al.*, 2000). PI3′-K action similarly may control membrane potential in the LRb/POMC neuron. Although this possibility remains untested, one reasonable hypothesis is that PI3′-K action may control the repression of NPY by LRb. However, it is also possible that Tyr_{985}/SHP-2-mediated signals or other uncharacterized signals mediated via Jak2 tyrosine phosphorylation sites may control NPY expression and/or membrane potential. The analysis of Jak2 and Tyr_{985}-mediated signals in LRb action *in vitro* and *in vivo* will represent an important next step in understanding the mechanisms by which LRb regulates mammalian physiology.

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Leptin Signaling in the Central Nervous System and the Periphery

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ABSTRACT

The discovery of leptin in 1994 has led to astonishing advances in understanding the regulation of energy balance in rodents and humans. The demonstration of leptin receptors in hypothalamic regions known to play critical roles in regulating energy intake and body weight has produced considerable excitement in the field. Most attention has focused on the central actions of leptin. The receptor is present in several populations of neurons that express specific appetite-regulating neuropeptides for which both expression and release are regulated by leptin. Recent advances show that central leptin action is not limited to influencing energy balance. Leptin regulates a broad variety of processes and behaviors, such as blood pressure, neuroendocrine axes, bone mass, and immune function. The cloning of leptin receptors also led to parallel studies examining their signaling capacities in mammalian cell lines. The long-form receptor regulates multiple intracellular signaling cascades, including the classic janus activating kinase-signal transducer and activator of transcription (JAK-STAT) pathway, consistent with belonging to the cytokine-receptor superfamily and the phosphoinositol-3 kinase and adenosine monophosphate kinase pathways. Progress has been made in understanding the role of individual signaling pathways *in vivo* and the mechanisms by which specific neuropeptides are regulated. Regulation of the pro-opiomelanocortin (*pomc*) and the thyrotropin-releasing hormone (*trh*) genes by leptin is particularly well understood. Novel players in negative regulation of central leptin receptor signaling have been identified and open the possibility that these may be important in the development of leptin resistance and obesity. While initial focus was on the central effects of leptin, important actions have been discovered in peripheral tissues. These include roles of leptin to directly regulate immune cells, pancreatic beta cells, adipocytes, and muscle cells. Recent elucidation of a new signaling pathway in skeletal muscle affecting fatty acid metabolism has implications for regulation of insulin sensitivity and glucose metabolism. Recent progress in understanding central and peripheral leptin receptor signaling provides potential new targets for anti-obesity and anti-diabetes drug development.

I. Introduction

Leptin, the polypeptide product of the *ob* gene, acts on the brain to regulate energy balance (Elmquist *et al.*, 1998a; Friedman *et al.*, 1998; Schwartz *et al.*, 2000). The hormone, which consists of 167 amino acid residues, is produced almost exclusively in adipose tissue. Consistent with the initial prediction of leptin being a secreted protein, due to the presence of a signal sequence (Zhang *et al.*, 1994), leptin is indeed in the circulation of mice and humans at levels that

correlate with the amount of fat tissue (Frederich *et al.*, 1995; Maffei *et al.*, 1995). The discovery that administering recombinant leptin to mice resulted in marked weight loss generated considerable excitement (Campfield *et al.*, 1995; Halaas *et al.*, 1995; Pelleymounter *et al.*, 1995). The weight-reducing effect was restricted to adipose tissue without significantly affecting lean body mass. Early studies also showed that low doses of leptin, when administered directly into the brain ventricles of rodents, were effective in reducing food intake and body weight (Campfield *et al.*, 1995; Stephens *et al.*, 1995). Lack of functional leptin or leptin receptors in mice and humans results in morbid obesity (Zhang *et al.*, 1994; Montague *et al.*, 1997; Clement *et al.*, 1998). These results combined argue that leptin is an adiposity signal that acts directly on the central nervous system (CNS) via receptors expressed at this site.

Interest in leptin initially focused on its centrally mediated weight-reducing effects and on the potential for using the leptin/leptin receptor axis to develop therapeutic drugs to treat obesity. The later finding that leptin resistance is present in all but a small subset of obese humans has diminished those hopes. More-recent studies have revealed additional pleiotrophic functions of leptin, including the ability to affect neuroendocrine function, the adaptive response to fasting, reproductive function, brain size, bone development, immune function, blood cell development, blood pressure, glucose homeostasis, fatty acid metabolism, and regulation of sensory nerve input and autonomic outflow. Although many of these processes are regulated by central actions of leptin, some are mediated via direct actions in the periphery.

Through expression cloning using mouse brain and by positional cloning of the *db* locus in mice, leptin receptors (ObR) were identified in late 1995 (Tartaglia *et al.*, 1995) and early 1996 (Lee *et al.*, 1996). Consistent with leptin having structural similarity with cytokines, ObR belongs to the cytokine receptor class I superfamily and is most-closely related to gp130, the signal-transducing membrane protein of the interleukin-6 (IL-6) signaling complex, the leukemia inhibitory factor (LIF) receptor, and the granulocyte colony-stimulating factor (G-CSF) receptor (Tartaglia *et al.*, 1995). Five alternatively spliced isoforms of ObR (a, b, c, d, e) with different lengths of C-termini have been identified in mice (Lee *et al.*, 1996), with additional isoforms found in other species. This chapter will focus on the biology of the long leptin receptor (ObRb), since this isoform is fully capable of activating intracellular signaling. Its importance is demonstrated by the fact that absence of ObRb causes morbid obesity in *db/db* mice (Chen *et al.*, 1996; Lee *et al.*, 1996).

II. Central Leptin Receptor Signaling

A. EXPRESSION OF LEPTIN RECEPTORS IN THE CNS

Selective deletion of all leptin receptor isoforms in neurons leads to obesity in mice (Cohen *et al.*, 2001), underscoring the importance of leptin action in the

brain for regulating body weight. The short leptin receptor isoform, ObRa, is expressed in the brain, with the highest levels in the choroid plexus and in microvessels (Tartaglia *et al.*, 1995; Mercer *et al.*, 1996b; Bjorbaek *et al.*, 1998b). There, it may play a role in leptin uptake or efflux from the cerebrospinal fluid and in receptor-mediated transport of leptin across the blood-brain barrier into the brain (Tartaglia *et al.*, 1995; Hileman *et al.*, 2000). However, not all studies support these possibilities (Kowalski *et al.*, 2001). Less is known about biology of the other short membrane-bound isoforms also present in the brain (Guan *et al.*, 1997; Hileman *et al.*, 2002).

The long form, ObRb, is highly expressed in selected nuclear groups in the rodent and human brain. Within the hypothalamus, dense mRNA expression is detected in the arcuate (ARC), dorsomedial (DMH), ventromedial (VMH), and ventral premamillary nuclei (PMV); moderate expression is found in the periventricular hypothalamic nucleus and lateral hypothalamic area (LHA); and even-lower levels exist in the paraventricular nucleus (PVH) (Mercer *et al.*, 1996b; Fei *et al.*, 1997; Elmquist *et al.*, 1998b). Outside the hypothalamus, ObRb mRNA has been found in numerous sites, with particularly high levels in the thalamus and the Purkinje and granular cell layers of the cerebellum (Guan *et al.*, 1997; Elmquist *et al.*, 1998b; Mercer *et al.*, 1998). Surprisingly, signaling capabilities of ObRb proteins located at these extrahypothalamic regions have not been reported (see below). However, since binding of ^{125}I-leptin to brain sections containing some of these sites can be detected (Corp *et al.*, 1998; Baskin *et al.*, 1999a), leptin may not reach these regions from the circulation or ObRb may serve a novel function that has yet to be identified. In contrast, leptin administration into the bloodstream of rodents induces ObRb-dependent signaling in neurons located in the hypothalamus and in limited regions outside the hypothalamus (see below), strongly suggesting that these regions express functional ObRb proteins with access to circulating leptin and activate downstream signaling events. Specifically, using immunohistochemistry on brain sections from leptin-treated rats and mice, careful mapping studies have reported rapid and robust signal transducer and activator of transcription 3 (STAT3) activation (Hubschle *et al.*, 2001; Hosoi *et al.*, 2002; Muenzberg *et al.*, 2003) and induction of c-Fos proteins (Elmquist *et al.*, 1997; Elias *et al.*, 2000) in hypothalamic regions. Although some discrepancies are noted, the main sites generally overlap well with those reported to contain ObRb mRNA, including the ARC, DMH, VMH, LHA, PMN, and PVH. Some differences among the studies can be explained by the fact that c-Fos is induced only in the subpopulation of neurons that is activated by leptin (Elmquist *et al.*, 1997; Elias *et al.*, 1999). Sensitive signaling assays for cFos and STAT3 activation also detected leptin-responsive sites in regions outside the hypothalamus; namely, the dorsal raphae (DR), the periaqueductal gray (PAG), and the parabrachial nucleus (PBN) located in the midbrain and the nucleus of the solitary tract (NTS) in the caudal brainstem

(Figure 1). One report also demonstrates STAT3 activation in the piriform cortex (Hubschle *et al.*, 2001), consistent with the presence of dense ObRb mRNA expression at this site (Elmquist *et al.*, 1998b). However, as indicated previously, none of the immunohistochemical assays reported leptin-dependent signaling in the thalamus or cerebellum, which brings into question the presence of functional receptors at these sites. In contrast, striking progress has been made in understanding direct leptin action on neurons located in the ARC and the PVH.

B. NEURONAL SIGNALING BY THE LEPTIN RECEPTOR

Cell-based studies and *in vivo* experiments over the last 8 years have led to a relatively detailed understanding of regulation of intracellular signaling by the long form of the leptin receptor. Binding of leptin to its receptor results in rapid activation of intracellular JAK2 that is constitutively associated with conserved, membrane-proximal regions of ObRb (Ghilardi and Skoda, 1997). We demonstrated that this JAK2 activation leads to tyrosine phosphorylation of ObRb (Bjorbaek *et al.*, 1997). The phosphorylation sites later were identified as amino acid residues 985 and 1138 (Li and Friedman, 1999; Banks *et al.*, 2000), which provide binding motifs for src homology 2 (SH2)-domain containing proteins such as STAT3 and SH-2-domain-phosphotyrosine phosphatase (SHP-2) (Bjorbaek *et al.*, 2000b). STAT3 proteins bind to Y1138, become tyrosine phosphorylated by JAK2, then dissociate and form dimers in the cytoplasm, finally translocating to the nucleus to regulate gene transcription. STAT3 activation is likely a crucial component in regulation of body weight by leptin, as specific knockout (KO) of the Y1138 residue of ObRb in mice results in severe obesity (Bates *et al.*, 2003). Tyrosine 985 of ObRb binds SHP-2 and leads to activation of the extracellular signal-regulated kinase (ERK)1/2 pathway and induction of c-Fos expression in transfected cells (Banks *et al.*, 2000; Bjorbaek *et al.*, 2000b) (Figure 2).

Since leptin can activate phosphoinositol-3 kinase (PI3K) in non-neuronal tissues (Harvey *et al.*, 2000; Zhao *et al.*, 2002a), Niswender and colleagues (2001) examined possible regulation in the hypothalamus. They found a rapid activation of the enzyme, reaching maximal levels within 30 minutes. This activation appears to involve both insulin-receptor substrate (IRS)-1 (Niswender *et al.*, 2001) and IRS-2 (Zhao *et al.*, 2002b). Activation of phosphodiesterase 3B (PDE3B) by leptin, leading to reduced levels of hypothalamic cyclic adenosine monophosphate (cAMP), has been reported (Zhao *et al.*, 2002b). The exact mechanisms by which ObRb activates PI3-K and the cAMP pathway are unknown.

Determination of the biological roles of the individual signaling pathways have begun. Primarily, elegant studies of mice lacking Y1138 of ObRb, the STAT3-binding site, show that this pathway is critical for appetite and body

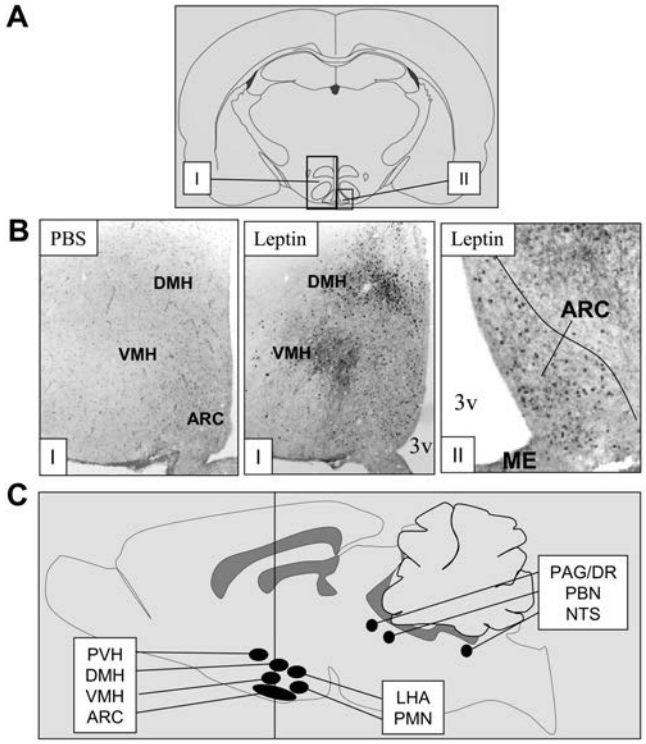

FIG. 1. Leptin-responsive regions in the rodent brain. (A) Schematic drawing of a coronal section of the rat brain. "I" and "II" depict hypothalamic nuclei that are highly leptin responsive. (B) The three microphotographs show immunohistochemistry (IHC) for phosphorylated signal transducer and activator of transcription (STAT)3 of coronal brain sections containing region "I" and "II" from (A). Rats were given a single intraperitoneal (IP) injection of recombinant leptin (1 mg/kg) or vehicle (PBS) and sacrificed 20 minutes later (Muenzberg *et al.*, 2003). The picture on the right shows a higher magnification (region "II" from (A)) where P-STAT3-positive cell nuclei in the arcuate nucleus of a leptin-treated animal can be identified. (C) Schematic drawing of a sagittal section of the rodent brain. Black areas depict approximate locations of the major leptin-responsive regions, as determined by IHC assays for STAT3 activation or c-Fos induction in brain sections from leptin-treated animals (Elmquist *et al.*, 1997; Elias *et al.*, 2000; Hubschle *et al.*, 2001; Hosoi *et al.*, 2002; Muenzberg *et al.*, 2003). Dark gray regions indicate ventricular systems. The vertical line indicates the approximate location of the coronal section shown in (A). Abbreviations: DMH, dorsomedial hypothalamic nucleus; VMH, ventromedial hypothalamic nucleus; ARC, arcuate nucleus; LHA, lateral hypothalamic area; PMN, premammilary nucleus; PVH, paraventricular hypothalamic area; PAG, periaqueductal grey; DR, dorsal raphae; PBN, parabrachial nucleus; NTS, nucleus of the solitary tract; ME, median eminence; 3v, third ventricle. [(A) modified from Schwartz MW, Woods SC, Porte D Jr, Seeley RJ, Baskin DG 2000 Central nervous system control of food intake. Nature 404:661–671; (B) modified with permission from Muenzberg H, Huo L, Nillni EA, Hollenberg AN, Bjorbaek C 2003 Role of signal transducer and activator of transcription 3 in regulation of hypothalamic proopiomelanocortin gene expression by leptin. Endocrinology 144:2121–2131. Copyright 2003 The Endocrine Society.]

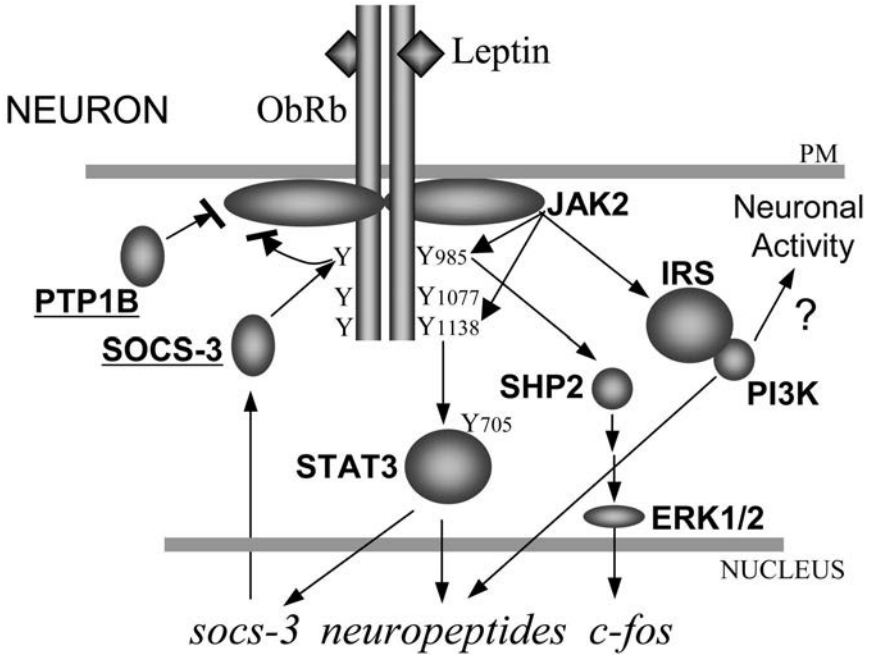

FIG. 2. Model for neuronal leptin receptor (ObRb) signaling. Activation of ObRb by leptin leads to increased activity of intracellular janus activating kinase (JAK)2 kinases associated with membrane-proximal regions of the receptor. JAK2 phosphorylates a number of cellular substrates, including Y985 and Y1138 of ObRb and Y705 of STAT3, after binding to pY1138. SH-2-domain-phosphotyrosine phosphatase (SHP-2) is important for activation of the extracellular signal-regulated kinase (ERK) pathway. STAT3, phosphoinositol-3 kinase (PI3-K), and ERK play roles in regulation of gene transcription. Suppressor of cytokine signaling (SOCS)-3 expression is regulated by the STAT3 pathway and, like protein tyrosine phosphatase (PTP)1B, is a negative regulator of ObRb signaling (indicated by underlining). PI3-K also may be involved in regulation of rapid nongenomic events affecting neuronal activity and neuropeptide release. PM, plasma membrane; IRS, insulin receptor substrate. [Modified from Bjorbaek C, Hollenberg AN 2002 Leptin and melanocortin signaling in the hypothalamus. Vitam Horm 65:288.]

weight regulation by leptin (Bates *et al.*, 2003). In addition, indirect evidence from further analyses of these mice indicates that Y986 — and thus possibly the MAPK pathway — may play a positive role in regulation of linear growth and reproductive function by leptin. Intracerebroventricular (ICV) injection of inhibitors of PI3-K, Wortmanin or LY294002 (Niswender *et al.*, 2001; Rahmouni *et al.*, 2003), or of cilostamide (Zhao *et al.*, 2002b), an inhibitor of PDE3, reverses the effect of leptin to reduce food intake, pointing to key roles of these pathways in leptin action to regulate appetite. A role for PI3-K to mediate leptin-induced stimulation of sympathetic outflow also has been reported (Rahmouni *et al.*,

2003). Finally, recent studies in our laboratory indicate that the AMP kinase pathway is involved in leptin's effect on food intake (Y. Minokoshi and B.B. Kahn, unpublished data).

Leptin also has rapid effects that lead to regulation of neuronal firing rates (Glaum *et al.*, 1996; Powis *et al.*, 1998; Cowley *et al.*, 2001; Schwartz and Moran, 2002), where some cells are found to be activated while others are inhibited (Elias *et al.*, 1998; Cowley *et al.*, 2001). The underlying mechanism for this divergent signaling capacity of ObRb is not well understood, although activation of adenosine triphosphate (ATP)-sensitive potassium (K^+) channels may play a role in inhibition (hyperpolarization) of neurons in the VMH (Spanswick *et al.*, 1997). Some evidence also indicates that activation of IRS-associated PI3-K activity is involved in this process (Spanswick *et al.*, 2000), a mechanism similar to that suggested for leptin to influence polarization of pancreatic β cells (Harvey *et al.*, 2000). However, leptin's effect to reduce food intake is not altered in mice lacking Kir6.2 K^+-ATP channels (Miki *et al.*, 2001), which are expressed in β cells and brain. Thus, the role of these K^+-ATP channels in mediating leptin's anorexigenic effects is unclear. The rapid, non-genomic actions of leptin are likely to be critical for regulating immediate release of hypothalamic neuropeptides and neurotransmitters from nerve terminals (Figure 2).

C. NEGATIVE REGULATORS OF LEPTIN SIGNALING

A novel cytokine-inducible gene was reported in 1995 (Yoshimura *et al.*, 1995) and later found to belong to a family of related genes. The protein products of these genes function as negative regulators of a wide variety of cytokine signaling systems, including those of IL-6, LIF, erythropoietin, and growth hormone (GH) (Endo *et al.*, 1997; Naka *et al.*, 1997; Starr *et al.*, 1997). The four identified proteins were named cytokine-inducible sequence (CIS) and suppressors of cytokine signaling (SOCS)-1, -2, and -3. We speculated that since leptin has a tertiary structure that strongly resembles that of cytokines and since the leptin receptor belongs to the cytokine receptor superfamily, it was likely that leptin also would regulate gene expression of members of the newly identified gene family. Indeed, by injecting *ob/ob* mice with a bolus of recombinant leptin, followed by quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of mRNA isolated from hypothalami 2 hours after administration, we found that SOCS-3 mRNA levels — but not CIS, SOCS-1, or SOCS-2 mRNA — were increased by leptin (Bjorbaek *et al.*, 1998a). This did not occur in *db/db* mice. Furthermore, *in situ* hybridization for SOCS-3 mRNA in the rat and mouse brains showed that leptin induced *socs-3* expression in nuclei of the hypothalamus that were known to express the long form of the leptin receptor (Elmquist *et al.*, 1998b). Moreover, we demonstrated that forced expression of

SOCS-3 in transfected cells ablated leptin receptor tyrosine phosphorylation and downstream signaling, altogether showing that SOCS-3 is a leptin-inducible inhibitor of leptin signaling. Subsequent studies in our lab have demonstrated that SOCS-1 potently inhibits ObRb signaling in transfected cells (C. Bjorbaek and J.S. Flier, unpublished results), despite lack of evidence that leptin can induce SOCS-1 expression (Bjorbaek *et al.*, 1998a,1999). This finding may be relevant for development of leptin resistance in obesity, if other cytokines induce SOCS-1 in leptin-responsive cells. Studies show that fasting of rats results in lower SOCS-3 mRNA levels in the ARC and DMH, as compared to fed animals, indicating that the fall of endogenous leptin during fasting regulates hypothalamic *socs-3* expression (Baskin *et al.*, 2000). Since functional STAT3 DNA binding elements are present in the *socs-3* promoter (Auernhammer *et al.*, 1999), and since lack of the STAT3 binding site on ObRb prevents induction of SOCS-3 mRNA by leptin (Banks *et al.*, 2000), we conclude that leptin stimulates *socs-3* transcription via the STAT3 pathway. Subsequent studies show that SOCS-3, via its SH-2 domain, binds to Y985 of ObRb and that this residue is required for maximal inhibition of signaling (Bjorbaek *et al.*, 2000b). However, evidence also suggests that SOCS-3 acts via binding directly to JAK2 (Bjorbaek *et al.*, 1999). SOCS-3 thus was the first identified negative regulator of leptin signaling.

Based on this evidence, we hypothesized that overreactivity of the SOCS pathway is a potential causal mechanism for leptin-resistant obesity. Consistent with this, *in situ* hybridization analyses showed that SOCS-3 mRNA levels were strongly elevated in the hypothalamus of A^y/a mice, a genetic model of leptin-resistant murine obesity (Bjorbaek *et al.*, 1998a) and is increased in aged rats (Peralta *et al.*, 2002), a state also associated with hyperleptinemia and leptin resistance (Scarpace *et al.*, 2000). *socs-1* gene expression is elevated in diet-induced obese (DIO) mice (Plut *et al.*, 2003). However, somewhat disappointing to us, PCR analysis of SOCS-3 mRNA in whole hypothalamic tissue of DIO mice has not shown that this transcript is substantially increased in this model of obesity (El-Haschimi *et al.*, 2000; Peiser *et al.*, 2000; Plut *et al.*, 2003). The finding of elevated SOCS-1 mRNA in DIO is intriguing and implies that factors other than leptin induce SOCS-1 expression in leptin-responsive neurons, leading to SOCS-1-induced leptin resistance in those cells. This mechanism could play a role in the development of obesity. Further quantitative anatomical studies of hypothalamic SOCS-1 and -3 protein levels in various obesity models are needed. Possibly, the SOCS pathways could be targets for development of anti-obesity drugs.

We and others have demonstrated that the protein tyrosine phosphatase 1B (PTP1B) is a physiological insulin receptor phosphatase and thus an important negative regulator of insulin signaling *in vivo* (Elchebly *et al.*, 1999; Klaman *et al.*, 2000). The fact that mice lacking PTP1B are resistant to developing DIO and are not hyperphagic, despite having low serum leptin levels, raised the possibility

that PTP1B might be a negative regulator of leptin signaling. To test this hypothesis, we first examined leptin receptor signaling in mammalian cell lines transiently expressing ObRb with or without PTP1B. Leptin-induced JAK2 and STAT3 tyrosine phosphorylation was reduced dramatically in cells overexpressing PTP1B (Zabolotny *et al.*, 2002). *In vitro* experiments suggested that PTP1B directly inhibits JAK2 kinase. Through *in situ* hybridization, we localized PTP1B mRNA to the hypothalamus, including the ARC nucleus. Furthermore, STAT3 phosphorylation in the hypothalamus was increased in leptin-treated mice lacking PTP1B ($PTP1B^{-/-}$), consistent with PTP1B interacting with leptin-dependent signaling pathways in leptin-responsive brain nuclei (Zabolotny *et al.*, 2002). We conclude that PTP1B is an intracellular inhibitor of central leptin receptor signaling and that the enzyme likely acts by directly dephosphorylating — and thus inhibiting – JAK2 activity. In parallel to these studies, Cheng and coworkers reported very similar findings (Cheng *et al.*, 2002).

To test whether increased leptin signaling in the hypothalamus of leptin-treated $PTP1B^{-/-}$ mice resulted in hypersensitivity to the physiological actions of leptin to reduce body weight, $PTP1B^{-/-}$, $PTP1B^{+/-}$, and wild-type (WT) mice were injected for 3 consecutive days with either leptin (three different doses) or vehicle. We found that WT mice lost weight only when given the highest dose of leptin. In contrast, $PTP1B^{-/-}$ mice responded to all doses and $PTP1B^{+/-}$ mice exhibited intermediate leptin sensitivity. Combined with the results from earlier studies (Elchebly *et al.*, 1999; Klaman *et al.*, 2000), we conclude that PTP1B deficiency in mice results in increased sensitivity to insulin and leptin and resistance to DIO. These effects of PTP1B likely occur via central and peripheral actions of the protein. Thus, PTP1B is an attractive therapeutic target for treating human obesity and insulin-resistant diabetes.

D. ROLE OF NEUROPEPTIDES IN LEPTIN ACTION

In the ARC of the hypothalamus, different leptin-responsive neuronal populations can be distinguished (Elias *et al.*, 1998; Korner *et al.*, 1999). POMC neurons, producing the anorectic peptide alpha melanocyte-stimulating hormone (α-MSH) acting via the melanocortin 3/4 receptors (MC3/4-R), are believed to be key mediators of leptin action (Schwartz *et al.*, 2000). Cocaine and amphetamine-regulated transcript (CART) is another potent inhibitor of feeding. Cells expressing this peptide overlap with POMC neurons (Elias *et al.*, 1998; Kristensen *et al.*, 1998). A separate population of cells in the ARC coexpresses two orexigenic peptides, the melanocortin receptor antagonist agouti-related protein (AgRP) and neuropeptide Y (NPY) (Ollmann *et al.*, 1997; Elias *et al.*, 1998; Bagnol *et al.*, 1999; Baskin *et al.*, 1999b; S.C. Chen *et al.*, 1999; Ebihara *et al.*, 1999). High proportions of the POMC/CART and NPY/AgRP cells express ObRb and thus are direct targets of circulating leptin (Hakansson *et al.*, 1996;

Mercer *et al.*, 1996a; Cheung *et al.*, 1997; Elmquist *et al.*, 1998a; Hahn *et al.*, 1998; Baskin *et al.*, 1999b).

The importance of these neuropeptide systems in the ARC for leptin action is supported by findings that mice lacking functional leptin or leptin receptors are morbidly obese and have significantly reduced *pomc* mRNA levels in the hypothalamus, while AgRP and NPY mRNAs are strongly increased (Thornton *et al.*, 1997; Hahn *et al.*, 1998; Mizuno *et al.*, 1998). In addition, fasting of normal mice and rats for 2–3 days leads to a fall in circulating leptin concentrations that is accompanied by a reduction of *pomc* mRNA and increases in hypothalamic NPY and AgRP that can be prevented by administration of recombinant leptin during the fasting period (Ahima *et al.*, 1996; Schwartz *et al.*, 1996,1997; Mizuno *et al.*, 1998). The molecular basis for stimulation of *pomc* gene expression likely involves STAT3 activation (Muenzberg *et al.*, 2003), while the PI3K pathway may play a specific role in repression of NPY and AgRP expression by leptin (Morrision *et al.*, 2003). Recent studies show that POMC cells respond very rapidly to leptin by increasing axonal firing rates and decreasing membrane potentials (Cowley *et al.*, 2001), leading to release of neurotransmitters and neuropeptides, including α-MSH (M. Kim *et al.*, 2000; Watanobe and Habu, 2002). In contrast, leptin hyperpolarizes NPY/AgRP neurons, presumably inhibiting release of peptides and neurotransmitters (Cowley *et al.*, 2001,2003), as supported by release measurements in hypothalamic explants (Li *et al.*, 2000). Thus, POMC/CART neurons are activated and NPY/AgRP cells are inhibited by leptin, events that are directly associated with activation of c-Fos in POMC/CART but not in NPY/AgRP neurons (Elias *et al.*, 1999). The mechanism by which ObRb has opposite signaling effects in different cells is intriguing.

It is difficult to reconcile recent findings that genetic ablation of NPY and AgRP in mice does not induce a lean phenotype (Qian *et al.*, 2002). However, these studies were carried out on a mixed genetic background and, when studied on a pure C57Bl/6 genetic background, NPY $-/-$ mice do exhibit a defect in refeeding after a fast, consistent with a deficiency of orexigenic factors. Surprisingly, however, when given food *ad libitum*, the NPY $-/-$ mice become slightly overweight and have increased adiposity, compared to control littermates. This demonstrates that genetic background must be taken into account when studying the biology of NPY (Segal-Lieberman *et al.*, 2003). Further studies are required to understand how NPY $-/-$ mice develop obesity. In contrast, powerful pharmacological and genetic evidence in rodents clearly demonstrates that the melanocortin system plays a pivotal role in regulation of body weight by leptin (Fan *et al.*, 1997; Seeley *et al.*, 1997; Brown *et al.*, 1998; Murphy *et al.*, 1998; Marks and Cone, 2001). Moreover, mutations in the *pomc* gene (Krude *et al.*, 1998; Yaswen *et al.*, 1999) and in the gene encoding the MC4-R lead to severe

obesity in rodents and humans (Huszar *et al.*, 1997; Vaisse *et al.*, 1998; Yeo *et al.*, 1998).

Compelling data show that leptin can regulate the neuroendocrine thyroid axis in rodents and humans. Thyroid hormone levels fall dramatically during short-term starvation of rodents. This mechanism is partly responsible for the conservation of energy by reducing metabolism (Blake *et al.*, 1991). The fall in thyroid hormones is associated with a reduction of hypothalamic thyrotropin-releasing hormone (TRH) mRNA expression that can be reversed by leptin treatment (Ahima *et al.*, 1996; Legradi *et al.*, 1997). In addition, the fall of thyrotropin-stimulating hormone (TSH) serum levels during 72 hours of fasting in humans can be blunted significantly by administering recombinant leptin (Chan *et al.*, 2003).

Results from rodents suggest that the effect of leptin on the thyroid axis involves both direct actions of leptin on leptin receptors expressed on the hypophysiotrophic TRH neurons located in the PVH and indirect signals from neurons that project to those TRH neurons. Consistent with direct regulation are data showing that leptin can stimulate *trh* gene expression via a STAT3 response element located in the *trh* promoter in isolated cells (Harris *et al.*, 2001) and induce STAT3 phosphorylation in TRH neurons in the PVH of leptin-treated rats (Muenzberg *et al.*, 2003; C. Bjorbaek, unpublished data). Furthermore, leptin rapidly regulates polarization of neurons in isolated brain slices of the PVH (Powis *et al.*, 1998) and can stimulate TRH peptide release from dispersed hypothalamic cultures and hypothalamic tissue *ex vivo* (M. Kim *et al.*, 2000; Nillni *et al.*, 2000). Other data support indirect regulation of TRH neurons via synaptic input from leptin-responsive POMC and NPY/AgRP neurons located in the ARC (Legradi *et al.*, 1998; Fekete *et al.*, 2000). Further studies will determine the relative physiological importance of these pathways that influence the hypophysiotrophic TRH neurons.

IV. Peripheral Leptin Receptor Signaling

A. LEPTIN RECEPTOR EXPRESSION IN THE PERIPHERY

While few studies have convincingly demonstrated protein expression of leptin receptors in any tissue, high mRNA levels of ObRa are found in lungs, kidneys, and lymph nodes, with lower levels in heart, liver, skeletal muscle, spleen, white adipose tissue (WAT), adrenals, and testes (Tartaglia *et al.*, 1995; Lee *et al.*, 1996; Fei *et al.*, 1997; Ghilardi and Skoda, 1997; Hoggard *et al.*, 1997). While some signaling capacities of ObRa have been shown in cell systems (Bjorbaek *et al.*, 1997; Murakami *et al.*, 1997), the function of ObRa in the periphery remains largely unknown.

Messenger RNA of the long-form receptor ObRb can be detected in several peripheral tissues, although to a more-limited degree and at much lower levels compared to ObRa. By applying Northern blotting, RT-PCR, or RNase-protection-assays, ObRb mRNA has been measured in lungs, kidneys, adrenals, and lymph nodes and at lower and variable levels in liver, brown adipose tissue (BAT), WAT, and skeletal muscle (Ghilardi *et al.*, 1996; Hoggard *et al.*, 1997; Kielar *et al.*, 1998; P. Chen *et al.*, 1999). In tissues where both receptor isoforms can be detected, ObRb mRNA accounts for under 5–10% of total leptin receptor expression (Ghilardi and Skoda, 1997; Lollmann *et al.*, 1997). ObRb proteins have not been demonstrated in any of the mentioned peripheral cells or tissues. One cannot, however, exclude a role of leptin signaling via ObRb in these tissues, since our experience shows that ObRb detection is difficult, even in transfected cells, mainly due to low expression levels of the ObRb protein (Bjorbaek *et al.*, 1997; DaSilva *et al.*, 1998; Uotani *et al.*, 1999). Despite being poorly expressed, we did observe leptin-dependent signaling in transfected cells, consistent with studies showing that a very limited number of erythropoietin receptors ($< 100–250$/cell) is sufficient to activate biologically relevant signal transduction (Masuda *et al.*, 1993). Finally, as will be described, rapid leptin-dependent events can be detected *in vivo*, in explants of peripheral tissues, and in cell lines derived thereof, thus supporting the presence of functional ObRb receptors at these sites.

B. LEPTIN ACTION IN PERIPHERAL TISSUES

When studying signaling by leptin directly in the periphery *in vivo*, effects that could be mediated via the CNS must be distinguished. To do this, one can investigate leptin-dependent action in cell lines derived from peripheral tissues or, even better, in primary tissues or cells, *ex vivo*. Numerous studies have utilized the first approach and reported effects on intracellular signaling and metabolism in cell lines derived from blood cells (Cioffi *et al.*, 1996; O'Rourke *et al.*, 2001), pancreatic β cells (Ahren and Havel, 1999; Morton *et al.*, 1999; Briscoe *et al.*, 2001; Okuya *et al.*, 2001), pituitary cells (Tsumanuma *et al.*, 2000), kidney (Takahashi *et al.*, 1996), and insulin-sensitive cells like hepatocytes (Szanto and Kahn, 2000; Briscoe *et al.*, 2001; Zhao *et al.*, 2002a), muscle cells (Berti *et al.*, 1997; Berti and Gammeltoft, 1999; Kellerer *et al.*, 1997; Takahashi *et al.*, 1997), and adipocytes (Bai *et al.*, 1996; Y. Kim *et al.*, 2000). Although leptin receptor-like signaling (activation of STAT3 and/or MAPK pathways) and ObRb mRNA (by RT-PCR) is reported in many of these cell lines, in only one study in a macrophage-derived cell line is the presence of functional ObRb proteins convincingly demonstrated (O'Rourke *et al.*, 2001). This suggests either very low expression levels in the other cell types or activation of unknown leptin-sensitive receptor systems. The latter seems unlikely, since leptin-dependent signaling via receptors other than ObR has not been reported.

The degree of understanding of the biological function of leptin action in these cell types varies, depending on the tissue. Strong data support a role for leptin in immune cells. *Ex vivo* studies of isolated T lymphocytes from mice and humans suggest that leptin has anti-immunosuppressive effects and promotes cellular survival (Lord *et al.*, 1998; Howard *et al.*, 1999; Martin-Romero *et al.*, 2000; Sanchez-Margalet and Martin-Romero, 2001). This provides a mechanism that may account for modulation of the change in immune function seen during starvation. Furthermore, a number of studies of isolated pancreatic islets from rodents strongly indicate that leptin can inhibit insulin secretion (Emilsson *et al.*, 1997; Fehmann *et al.*, 1997; Ishida *et al.*, 1997; Kieffer *et al.*, 1997; Kulkarni *et al.*, 1997; Roduit and Thorens, 1997; Ookuma *et al.*, 1998; Poitout *et al.*, 1998; Lupi *et al.*, 1999; Morton *et al.*, 1999; Seufert *et al.*, 1999; Islam *et al.*, 2000), although the opposite effect or no effect also has been reported (Pallett *et al.*, 1997; Shimizu *et al.*, 1997; Tanizawa *et al.*, 1997; Kawai *et al.*, 2001). Further studies are needed to resolve these discrepancies. In favor of a direct effect on pituitary cells are data showing leptin-induced release of LH and FSH from isolated pituitary explants from rats (Yu *et al.*, 1997). This is consistent with the known link between leptin and reproductive function (Ahima *et al.*, 1996,1997), although a significant part of this regulation is likely of hypothalamic origin. While leptin can stimulate sympathetic nerve activity to the kidneys via the CNS (Haynes *et al.*, 1999), direct actions of leptin to influence kidney function have not been described. One can, however, envision a potential role for short- or long-form leptin receptors to facilitate renal clearance of leptin from the circulation. We will discuss in more detail experiments from our own laboratory aimed at increasing the understanding of leptin-dependent signaling in adipose tissue, liver, and skeletal muscle, the classic insulin-sensitive tissues.

To carefully investigate leptin receptor signaling directly in insulin-sensitive tissues *in vivo*, rats were injected intravenously (IV) with a bolus of recombinant leptin (and/or insulin) and sacrificed 3 minutes later (Y. Kim *et al.*, 2000). Activation of STAT1, STAT3, MAPK, PI3-K, and Akt was examined in muscle, WAT, and liver. In adipose tissue, we found that leptin had profound effects to increase STAT1 and STAT3 phosphorylation and, to a lesser degree, activate MAPK and PI3-K. Under the same conditions, leptin did not affect signaling in *db/db* mice, which lack ObRb. Akt was not activated by leptin in any tissue and activation of signaling pathways in liver was less robust, as compared to fat tissue. Although we did not find evidence of very rapid effects in muscle on any of the measured endpoints, others have reported activation of STAT3 and Akt at later time points (Maroni *et al.*, 2003). A critical role for leptin signaling directly in liver tissue has been ruled out following demonstration that conditional KO of hepatic ObR does not lead to a discernible phenotype in terms of glucose homeostasis or other parameters (Cohen *et al.*, 2001). Insulin induced strong activation of PI3K, Akt, and MAPK in all tissues, while STAT1 and STAT3 were

activated only in adipose tissue. Since signaling was activated just 3 minutes after injection, the observed effects were unlikely to be mediated indirectly by other humoral factors. Indeed, MAPK was rapidly induced in explants of adipose tissue, demonstrating that this was a direct effect of leptin (Y. Kim *et al.*, 2000). Detection of ObRb mRNA in human and rodent WAT further supports the notion of direct actions of leptin on fat cells (Siegrist-Kaiser *et al.*, 1997; Kielar *et al.*, 1998; Kutoh *et al.*, 1998). However, since this was measured by RT-PCR, final conclusions about the presence of functionally relevant ObRbs on fat cells are premature.

These signaling studies provide ample basis to speculate about the biological roles of leptin in fat cells and in possible cross-talk with insulin-signaling pathways. While leptin does not appear to directly affect glucose uptake in mouse or rat adipocytes (Zierath *et al.*, 1998), studies have shown robust and sensitive leptin-dependent induction of lipolysis in isolated fat pads or isolated adipocytes from rats (Siegrist-Kaiser *et al.*, 1997; Wang *et al.*, 1999; Rodriguez *et al.*, 2003) and mice (Fruhbeck *et al.*, 1997; Kawaji *et al.*, 2001). Consistent with this, leptin antagonizes the effects of insulin to inhibit lipolysis (Muller *et al.*, 1997). Furthermore, no increase in triglyceride breakdown could be measured in fat cells derived from Zucker *fa/fa* rats or *db/db* mice, both lacking functional leptin receptors (Fruhbeck *et al.*, 1997; Siegrist-Kaiser *et al.*, 1997; Wang *et al.*, 1999). Combined, these studies suggest opposing effects of leptin on insulin action in WAT, although one study reported that leptin had no effect on lipolysis in isolated adipocytes from humans (Elimam *et al.*, 2002), possibly indicating species differences. Based on rodent studies that suggested opposite effects of leptin and insulin on lipolysis, we speculated that since leptin and insulin activate STAT3 to a similar degree (Y. Kim *et al.*, 2000), this signaling pathway alone is unlikely to play a role in the lipolytic effect of leptin. On the other hand, one can envision that the STAT3 pathway, which leads to induction of SOCS-3 expression, may play a role in the inhibitory effect of leptin on insulin action, since SOCS-3-mediated downregulation of IRS signaling has been reported (Emanuelli *et al.*, 2001; Rui *et al.*, 2002). As GH also stimulates SOCS-3 expression, a similar mechanism has been proposed to explain the antagonistic effect of GH on insulin action in adipocytes (Ridderstrale *et al.*, 2003).

C. ROLE OF AMP-ACTIVATED PROTEIN KINASE IN LEPTIN SIGNALING IN SKELETAL MUSCLE

Although leptin stimulates glucose transport in muscle indirectly through the hypothalamus and the sympathetic nervous system (SNS) (Kamohara *et al.*, 1997), evidence does not support rapid, leptin-dependent activation of STAT3, MAPK, or PI3-K (Y. Kim *et al.*, 2000) or glucose uptake (Muoio *et al.*, 1997; Zierath *et al.*, 1998) directly in skeletal muscle. However, our recent results

provide insight into other metabolic actions of leptin in muscle. We discovered activation of a signaling pathway that has not previously been ascribed to the leptin receptor, namely, regulation of the AMP-activated protein kinase (AMPK).

AMPK is a serine/theronine kinase that is conserved from yeast to humans and is activated by an increased intracellular ratio of AMP to ATP and by upstream kinases. Considerable research has focused on the role of this enzyme in regulation of substrate metabolism. AMPK has been proposed to serve as a master fuel gauge in mammalian cells (Hardie and Carling, 1997). AMPK phosphorylates acetyl-coA carboxylase (ACC), leading to inhibition of ACC and stimulation of fatty acid oxidation in mitochondria (Winder and Hardie, 1999; Hardie and Pan, 2003). Leptin treatment of rodents reduces intracellular lipid content in muscle at doses that evidently do not affect body weight (Unger *et al.*, 1999) and leptin can increase oxidation of fatty acids in muscle tissue *ex vivo* (Muoio *et al.*, 1997). Although most leptin actions are mediated via the CNS, we speculated that at least part of the observed effects in skeletal muscle *in vivo* occur through direct activation of AMPK.

To test this hypothesis, we injected leptin IV into mice and measured AMPK activity in soleus muscle 15 minutes and up to 6 hours after administration. Leptin produced a biphasic response, with a 2-fold rise at 15 minutes, a return to baseline by 60 minutes, and a second 2-fold elevation by 6 hours (Minokoshi *et al.*, 2002). Of two known isoforms of the catalytic subunit of AMPK, $\alpha 1$ and $\alpha 2$, we detected activation of only $\alpha 2$ AMPK by leptin. To determine whether the effect of leptin was mediated via actions in the hypothalamus and SNS, several experiments were undertaken. First, injection of small doses of leptin directly into the hypothalamus resulted in a 3-fold activation of AMPK in soleus muscle at 60 minutes that remained elevated at 6 hours. This demonstrates that central action of leptin can regulate signaling events in muscle tissue and suggests that the regulation of AMPK seen after IV injection is explained partly by central actions of leptin. To directly test the relative role of central vs. direct effects, we measured AMPK activity in mice with surgical denervation of one hindlimb. Combined denervation of the femoral, sciatic, and obturator nerves blocked the ability of leptin administered either directly into the hypothalamus or IV (6 hours after administration) to stimulate $\alpha 2$ AMPK in muscle. In contrast, activation at 15 minutes was intact after IV leptin administration, altogether indicating that rapid activation involves a direct event and that slower activation of AMPK in muscle requires activation of the autonomic nervous system. To more-conclusively support the direct action, we incubated soleus muscle *ex vivo* with and without leptin and found a robust, leptin-dependent 2- to 3-fold increase in $\alpha 2$ AMPK activity. The biological effects of this pathway involve upregulation of fatty acid oxidation. Leptin phosphorylates ACC, resulting in an expected decrease in ACC enzymatic activity and increased fatty acid oxidation (Figure 3).

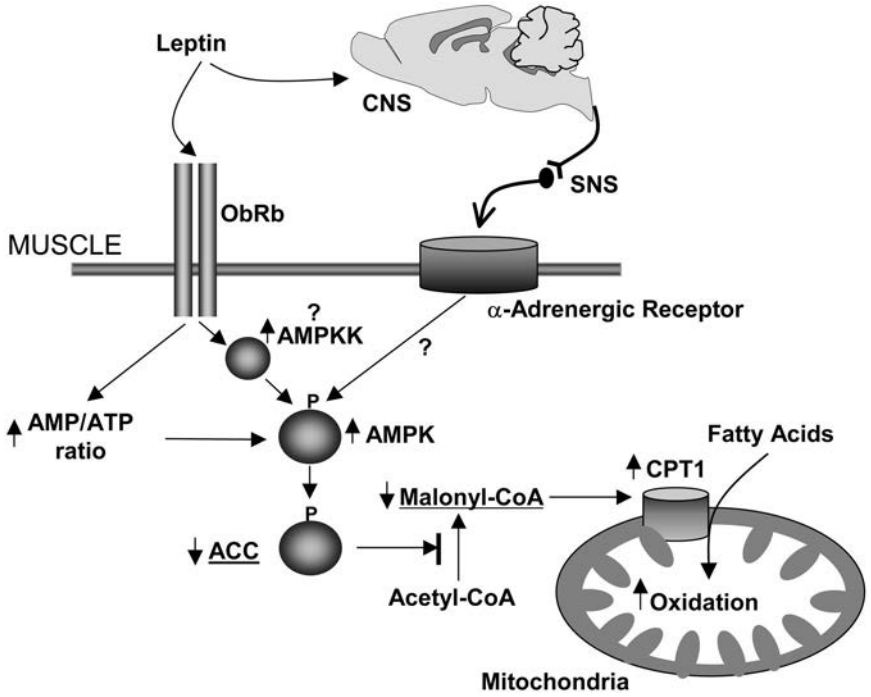

FIG. 3. Model for regulation of adenosine monophosphate-activated protein kinase (AMPK) and fatty acid oxidation by leptin in skeletal muscle. Leptin activates AMPK via two distinct mechanisms. One is rapid and occurs directly through leptin receptors expressed on muscle cells. The other occurs with slower kinetics and is mediated via actions in the central nervous system (CNS), leading to stimulation of the sympathetic nervous system (SNS) and activation of G-coupled α-adrenergic receptors expressed on muscle cells. Activation of muscle ObRb also increases AMP content, which may be the mechanism for the direct effect of leptin to activate AMPK. AMPK phosphorylates acetyl-CoA carboxylase (ACC), leading to inhibition of its activity and thus to reduced formation of malonyl-CoA. This, in turn, disinhibits the activity of carnitine palmitoyltransferase 1 (CPT-1), a required step for translocation of fatty acids into mitochondria (Minokoshi and Kahn, 2003). Underlining indicates a decrease in cellular contents (malonyl-CoA) or in enzyme activities (ACC). ATP, adenosine triphosphate. [Modified from Minokoshi Y, Kahn BB 2003 Role of AMP-activated protein kinase in leptin-induced fatty acid oxidation in muscle. Biochem Soc Trans 31:196–201.]

The mechanism for AMPK activation in muscle at 15 minutes after IV leptin may result from an increase in AMP content in muscle. However, no changes in AMP amounts were seen 6 hours after IV leptin, indicating that AMPK activation through the hypothalamic SNS axis involves upstream AMPK kinase (Hardie and Pan, 2003). Furthermore, activation of AMPK is necessary for leptin's effect on ACC activity and presumably for its effects on fatty acid oxidation (Mino-

koshi *et al.*, 2002). Other studies applying long-term leptin treatment of rats (i.e., 2 weeks) showed an increase of both α2 AMPK phosphorylation and of AMPK protein expression, compared to pair-fed control animals (Steinberg *et al.*, 2002). However, these studies could not conclude whether the effects were direct or indirect or both. We went on to show that the mechanism by which leptin indirectly activates AMPK in muscle involves α-adrenergic receptors in muscle tissue (Minokoshi *et al.*, 2002). These studies clearly suggest that leptin can directly stimulate fatty acid oxidation via AMPK and that this represents a novel pathway mediating metabolic actions of leptin in the periphery. This pathway may play a role in the fat-melting activity of leptin in peripheral tissues and also in protecting nonadipocytes from lipotoxicity by preventing upregulation of lipogenesis (Unger *et al.*, 1999).

V. Conclusion and Perspectives

Major advances have been made in understanding leptin action. A complex network of interacting signaling pathways appears to regulate food intake, fuel balance, and body weight. Compelling evidence exists that, in addition to signaling in the CNS, leptin exerts its metabolic effects by acting directly on peripheral tissues. The relative importance of this signaling in the periphery for regulating adiposity and glucose homeostasis remains to be determined. Other important challenges include determining 1) how different pathways downstream of leptin (STAT3, PI3K, MAPK, AMPK) are integrated in the regulation of body weight; 2) how leptin signaling integrates with insulin-signaling pathways in the CNS; 3) the molecular mechanisms for leptin resistance in obesity; and 4) whether insulin resistance occurs in the brain in states such as obesity and diabetes that are associated with insulin resistance in the periphery. Molecules identified over the last several years provide new potential drug targets for the prevention and treatment of obesity.

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Polymorphisms in the Glucocorticoid Receptor Gene and Their Associations with Metabolic Parameters and Body Composition

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ABSTRACT

Most actions of glucocorticoids (GCs) are mediated by the glucocorticoid receptor (GR). The interindividual response to GCs varies considerably, as demonstrated by a variable suppressive response to 0.25-mg dexamethasone (DEX). Several polymorphisms in the gene coding for the GR have been described. It is unclear to what extent the observed response variability is due to GR polymorphisms or to other factors. However, at least three polymorphisms seem to be associated with altered GC sensitivity and changes in body composition and metabolic parameters. The N363S polymorphism has been associated with increased sensitivity to GCs, increased insulin response to DEX, a tendency towards lower bone mineral density, and increased body mass index (BMI). However, other reports found no associations with BMI. Another polymorphism, previously described as a *Bcl*I restriction fragment length polymorphism, recently was identified as a C $\rightarrow$ G nucleotide change. The G allele also was associated with increased sensitivity to GCs. In middle-aged subjects, the G allele of this *Bcl*I polymorphism was associated with increased abdominal obesity, while at older age, a lower BMI was found, accompanied by a tendency towards lower lean body mass. A third polymorphism consists of two linked, single-nucleotide mutations in codons 22 and 23, of which the second mutation results in an amino acid change from arginine (R) to lysine (K). In contrast to the other polymorphisms, this ER22/23EK polymorphism was associated with a relative resistance to GCs. In line with this, ER22/23EK carriers had lower total cholesterol and low-density lipoprotein cholesterol levels as well as lower fasting insulin concentrations and a better insulin sensitivity. C-reactive protein levels were lower in ER22/23EK carriers, as was found in a different population of elderly males. In accordance with this healthy metabolic profile, we found in this population a significantly better survival in ER22/23EK carriers after a 4-year follow-up. GCs also affect the brain. Although a certain level of cortisol is essential for proper brain functioning, excessive GC levels have been shown to negatively affect brain morphology and functions. At older age, we found that the risk of dementia and white matter lesions was lower in ER22/23EK carriers. GCs are also important in the regulation of body fat distribution. At young age, we observed sex-specific differences in body composition. Male ER22/23EK carriers were taller, had more muscle mass, and were stronger than noncarriers. In young females, ER22/23EK carriers had tendencies towards smaller waist and hip circumferences and lower body weight. Another polymorphism (*TthIII*I) was not associated with altered GC sensitivity. In conclusion, these polymorphisms in the GR gene may contribute considerably to the observed variability in GC sensitivity. As a result, they are associated with several differences in body composition and metabolic factors.

I. Introduction

Glucocorticoid (GC) secretion is regulated by the hypothalamus, which receives stimuli from the central nervous system (CNS) (Chrousos and Gold, 1992). This results in a diurnal profile of cortisol secretion, with high levels in the morning and low concentrations in the afternoon and evening, with a small peak after lunch. In obese individuals, cortisol secretion is elevated and its peripheral turnover rate is higher, which results in normal, or even lower, serum cortisol concentrations (Murphy, 1968; Streeten *et al.*, 1969; Cheek *et al.*, 1981). Dysregulation of the hypothalamo-pituitary-adrenal (HPA) axis was found to be more pronounced in central obesity than in peripheral or gluteofemoral obesity (Marin *et al.*, 1992; Pasquali *et al.*, 1993; Rosmond *et al.*, 1998).

The regulation of cortisol metabolism in humans is not only centrally determined. Two key enzymes in cortisol metabolism have been identified that influence the effects of cortisol at the peripheral level: 11beta-hydroxysteroid dehydrogenase (11β-HSD) I and 11β-HSD II. The latter enzyme inactivates cortisol by conversion into cortisone, particularly in the kidney but also in other aldosterone-selective target tissues. The other enzyme, 11β-HSD I, is present predominantly in adipose tissue, liver, lung, vascular system, ovary, and CNS (Monder and White, 1993; Stewart and Krozowski, 1999). The function of 11β-HSD I is to convert cortisone into the active form, cortisol. Interestingly, in obese humans, this enzyme has been shown to have tissue-specific actions. 11β-HSD I activity is lower in the liver, while increased activity *in vitro* is observed in the subcutaneous adipose tissue of obese men. This results in higher local cortisol levels in adipose tissue, which is suggested to be an important factor in the mechanism leading to harmful metabolic consequences of obesity.

It is known that GC sensitivity, measured by a dexamethasone (DEX) suppression test, varies greatly between individuals (Huizenga *et al.*, 1998b). However, within individuals, GC sensitivity is rather stable. This suggests that, in humans, a setpoint for DEX sensitivity with respect to the feedback action exists, which might be genetically determined. An important factor in the cascade of GC action, also at the pituitary level, is binding to the GC receptor (GR).

The GR belongs to the superfamily of nuclear receptors that are present in the cytoplasm and act as transcription factors to regulate gene expression. Following cortisol binding, a conformational change occurs that leads to dissociation of the receptor from a large complex of proteins, of which heat shock protein (HSP) 90 is the most important (Pratt and Toft, 1997; Toft, 1998). This activated, ligand-bound receptor translocates to the nucleus, where it can act in several ways (Schaaf and Cidlowski, 2003). The GR can initiate transcription through binding to GC-responsive elements of the target gene. The GR also can affect gene transcription through direct protein-protein interaction and can activate, as well as repress, target gene expression (Diamond *et al.*, 1990;

Yang-Yen *et al.*, 1990; Yudt and Cidlowski, 2002). In mice in which a mutation was induced that impaired dimerization and DNA binding, these processes have been shown to be not critical for survival (Reichardt *et al.*, 1998).

Previously, some rare mutations of the *GR* gene were described that led to clinical signs and symptoms of generalized cortisol resistance (Lamberts *et al.*, 1992). Due to these receptor defects, cortisol has impaired actions through the GR. As a consequence, the central negative feedback of GCs is diminished, GC production by the adrenal is elevated, and cortisol binds with high affinity to the mineralocorticoid receptor (MR). Symptomatology in patients with cortisol resistance is the consequence of a compensatory hyperactivity of the HPA axis, which results in overproduction of mineralocorticoids, which, in turn, leads to hypertension, hypokalemic alkalosis, fatigue, and in females — due to higher adrenal production of androgens — hyperandrogenism. In normal conditions, organs that have an important mineralocorticoid function are protected from high cortisol levels by the enzyme 11β-HSD II, which rapidly inactivates cortisol into cortisone. In the situation of cortisol resistance, cortisol levels are too high for the inactivational capacity of this enzyme. The number of patients diagnosed with cortisol resistance syndrome is low (i.e., $\approx$ nine) (Brufsky *et al.*, 1990; Hurley *et al.*, 1991; Karl *et al.*, 1993,1996a; Malchoff *et al.*, 1993; Ruiz *et al.*, 2001; Mendonca *et al.*, 2002; Vottero *et al.*, 2002). Two mutations found *in vitro* could have been pre-existing acquired mutations *in vivo*, leading to Nelson syndrome and lupus nephritis (Karl *et al.*, 1996b; Jiang *et al.*, 2001). Most patients carried a mutation or defect in the ligand-binding domain; only one patient had a mutation in the DNA-binding domain (Lamberts, 2001). A possible explanation for the low number of patients is that a severe form of cortisol resistance is not compatible with life.

Hypersensitivity to endogenous cortisol has been described as well. Iida and colleagues (1990) reported a patient with symptoms of Cushing's syndrome, despite hypocortisolemia. Newfield and coworkers (2000) described a second patient with serious symptoms of Cushing's syndrome at peripubertal age but having normal cortisol levels. The lymphocytes of this second patient contained an increased number of GR per cell, with normal binding affinity. The molecular etiology of hyperreactivity to cortisol has not been clarified fully but two single-nucleotide polymorphisms of the *GR* gene seem to play an important role in determining hypersensitivity. Figure 1 shows a schematic overview of the *GR* gene, with locations of previously described mutations causing cortisol resistance and of polymorphisms shown to be associated with altered GC sensitivity. In contrast to the infrequent mutations, most polymorphisms are located in the N-terminal transactivation domain (Bray and Cotton, 2003). This review deals with these *GR* gene polymorphisms, which were not only associated with differences in GC sensitivity but also related to differences in body composition and metabolic parameters.

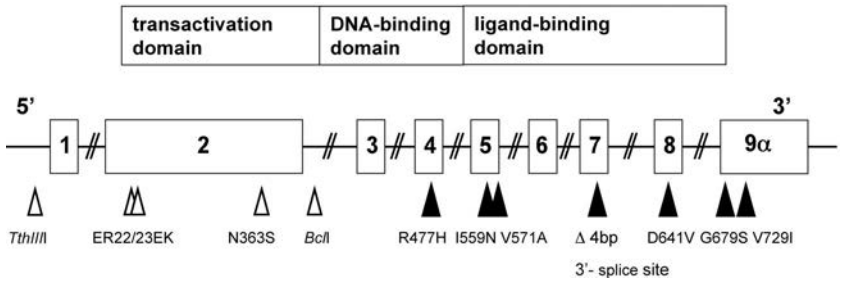

FIG. 1. Schematic overview of the *GR* gene, showing polymorphisms (white arrows), which have been shown to alter glucocorticoid (GC) sensitivity and are associated with differences in body composition, as well as mutations (black arrows) leading to the syndrome of cortisol resistance.

II. The N363S Polymorphism of the *GR* Gene

A polymorphism was identified in codon 363 of exon 2 of the *GR* gene. Table I shows an overview of the associations with body mass index (BMI) and metabolic parameters found with this polymorphism.

This AAT → AGT nucleotide change results in an asparagine → serine amino acid change. It appeared in a group of 216 normal Dutch elderly individuals known to be associated with a higher sensitivity to GCs *in vivo* (Koper *et al.*, 1997; Huizenga *et al.*, 1998a). This was shown by lower cortisol levels after administration of 0.25-mg DEX (Figure 2A) as well as a significantly greater decrease in cortisol levels (Figure 2B). Moreover, in this population, N363S carriers had an increased insulin response to exogenous DEX, which is likely to be related directly to their increased GC sensitivity. N363S carriers had a higher BMI and a tendency towards decreased bone mineral density in trabecular bone (Huizenga *et al.*, 1998a; Lin *et al.*, 1999). Lin and colleagues (1999) confirmed the association with BMI and even demonstrated an allele-dosage effect on BMI (i.e., homozygous S-allele carriers had a higher BMI than heterozygous S-allele carriers). However, some controversy arose concerning the role of this polymorphism, as reviewed by Rosmond (2002). Dobson *et al.* (2001) found an increased waist-to-hip ratio in male N363S carriers but no associations with BMI, serum lipid levels, and glucose tolerance status in a Caucasian population. In three other reports, no association was observed between the N363S polymorphism and BMI (Halsall *et al.*, 2000; Echwald *et al.*, 2001; Rosmond *et al.*, 2001). However, in a severely obese Italian population, the N363S variant was associated with increased BMI. Heterozygous carriers of both the N363S and the *Bcl*I polymorphism had higher cholesterol levels (Di Blasio *et al.*, 2003). In a recent report by Lin and coworkers (2003b), the N363S variant was associated with coronary artery disease, independent of weight. 363S allele

TABLE I
Data from Six Studies That Investigated the Association Between the N363S Polymorphism of the Glucocorticoid Receptor (GR) Gene and Body Mass Index

Reference	Population	Associations with the N363S polymorphism
Huizenga *et al.*, 1998a	216 Dutch men and women	Increased GC sensitivity, increased insulin response to DEX, increased BMI
Lin *et al.*, 1999	195 normotensive controls and 124 hypertensive subjects	Increased BMI, allele-dosage effect
Halsall *et al.*, 2000	491 subjects	No association with BMI
Dobson *et al.*, 2001	135 men and 240 women	Increased WHR in men
Rosmond *et al.*, 2001b	284 Swedish men	No association with BMI, no association with sensitivity to GCs
Echwald *et al.*, 2001	741 obese Danish men and 854 non-obese controls	No association with BMI, WHR, or weight gain
Lin *et al.*, 2003b	437 Anglo-Celtic CAD patients and 302 controls	Association with CAD, elevated cholesterol, triglycerides, total cholesterol/HDL ratio
Lin *et al.*, 2003a	951 Anglo-Celtic/Northern European subjects: 152 obese, 356 type 2 diabetes, 141 hypertensive, 302 controls	Association with obesity and overweight in several patient settings but no association with hypertension or type 2 diabetes
Di Blasio *et al.*, 2003	185 obese women, 94 obese men	Increased BMI, interaction with the *Bcl*I polymorphism: higher cholesterol levels

[Abbreviations: BMI, body mass index; CAD, coronary artery disease; DEX, dexamethasone; GC, glucocorticoid; HDL, high-density lipoprotein-cholesterol; WHR, waist-to-hip ratio.]

frequency was particularly high in patients with angina pectoris. In this population of Anglo-Celtic descent, several atherosclerosis risk factors were associated with the N363S variant: increased cholesterol and triglyceride concentrations and a higher total cholesterol/high-density lipoprotein (HDL) cholesterol ratio. The same authors showed an association between the N363S polymorphism and

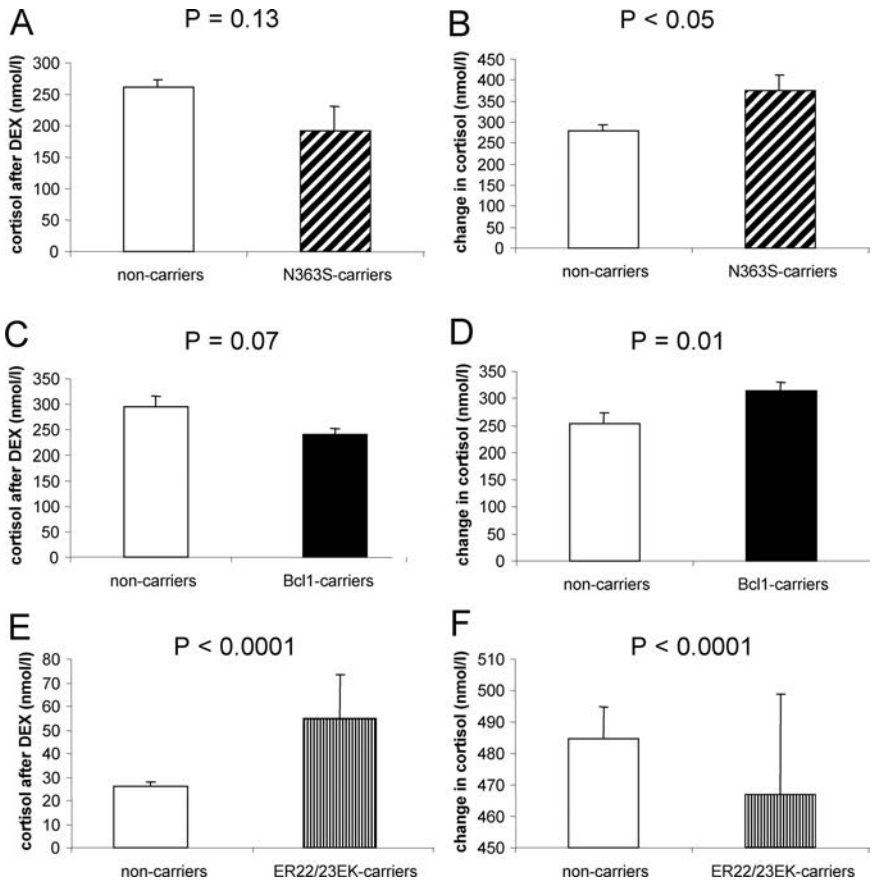

FIG. 2. Cortisol levels (nmol/l) after dexamethasone (DEX) suppression tests (graphs on the left: A, C, E) and absolute change in cortisol (nmol/l) after DEX (graphs on the right: B, D, F). Results from a 0.25-mg DEX suppression test are shown for N363S and *Bcl*I carriers. Data concerning a 1-mg test are shown for ER22/23EK carriers. (A & B) Noncarriers (white bars) were compared to N363S carriers (striped bars). Lower post-DEX cortisol and greater decrease in N363S carriers suggest a hypersensitivity to GCs. (C & D) Heterozygous and homozygous *Bcl*I G-allele carriers (black bars), also lower post-DEX cortisol and greater decrease in *Bcl*I G-allele carriers, suggesting that *Bcl*I G-allele carriers are hypersensitive to GCs. (E & F) ER22/23EK carriers (vertically striped bars), who had higher cortisol levels after 1-mg DEX and a smaller decrease in cortisol, which suggests that the ER22/23EK variant is associated with a relative resistance to GCs.

obesity as well as overweight in several groups of patients (Lin *et al.*, 2003a). However, no association was found with hypertension or type 2 diabetes. Interestingly, in a Japanese as well as in a Chinese population, the N363S variant did not occur (Ikeda *et al.*, 2001; Lei *et al.*, 2003).

Figure 3 shows the DEX concentrations necessary to achieve 50% of the maximal inhibition (IC50) in mitogen-induced, *in vitro* cell proliferation assays in noncarriers and N363S carriers (Huizenga *et al.*, 1998a). A trend was observed towards a lower IC50 in carriers of the N363S polymorphism, which supports the observation of increased sensitivity to GCs *in vivo*. We have to take in to account that in the group of noncarriers (left dots in Figure 3), carriers of the very frequent *Bcl*I polymorphism are present. This polymorphism has been associated with increased GC sensitivity. As a consequence, differences in IC50 in Figure 3 between "real noncarriers" (i.e., noncarriers of both N363S and *Bcl*I) and N363S carriers probably are underestimated. This underestimation might apply to other studies comparing noncarriers and N363S in body composition and metabolic parameters.

The exact mechanism underlying increased sensitivity to GCs is unknown. In *in vitro* expression experiments using a mouse mammary tumor virus-driven/ luciferase expression (MMTV-LUC) system, no differences were observed

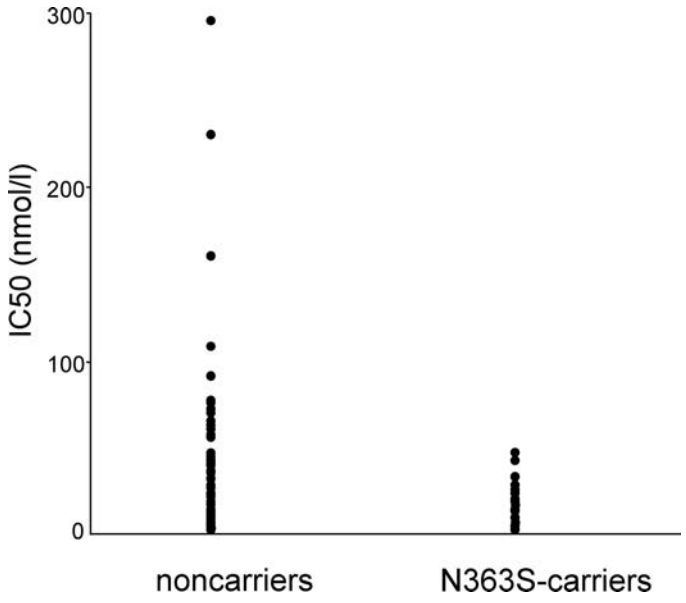

FIG. 3. Concentrations of DEX necessary to achieve 50% of the maximal inhibition (IC50) in mitogen-induced peripheral mononuclear cell proliferation assays in noncarriers and carriers of the N363S polymorphism. A trend was observed towards a lower mean IC50 in N363S carriers, compared to noncarriers. [Adapted with permission from Huizenga NA, Koper JW, De Lange P, Pols HA, Stolk RP, Burger H, Grobbee DE, Brinkmann AO, De Jong FH, Lamberts SW 1998 A polymorphism in the glucocorticoid receptor gene may be associated with and increased sensitivity to glucocorticoids *in vivo*. J Clin Endocrinol Metab 83:144–151. Copyright The Endocrine Society.]

between the N363S variant GR and wild-type GR in efficacy to activate transcription (de Lange *et al.*, 1997). However, Feng and colleagues (2000) suggested that the N363S variant modulated the phosphorylation state of the GR and might alter interactions with other transcription factors. No evidence for this hypothesis has been found. However, in two reports, GR hyperactivity was induced by *in vitro*-created mutants, resulting in increased transcriptional activity and a diminished capacity to repress activator protein-1 (AP-1) induction (Heck *et al.*, 1994; Guido *et al.*, 1996).

III. The *Bcl*I Polymorphism of the *GR* Gene

Murray *et al.* (1987) reported an intronic restriction fragment length polymorphism (RFLP) of the *GR* gene, which was described as consisting of a short fragment of 2.3 kb and a large fragment of 4.5 kb. Since then, several association studies have been performed to investigate the role of this variant in obesity, using Murray's terminology and technique. Recently, we identified the exact nucleotide alteration: a C → G mutation, 646 nucleotides downstream from exon 2, which results in fragments of 2.2 kb and 3.9 kb (van Rossum *et al.*, 2003b). The C allele is the most-frequently occuring and thus can be considered the wild-type allele (Table II). Table III displays an overview of reports of the *Bcl*I polymorphism and its associations with body composition and metabolic parameters.

The first association study of the *Bcl*I polymorphism, decribed by Weaver and coworkers (1992), showed no differences in *Bcl*I polymorphism frequency between an obese and a normal-weight population. However, within the obese group, homozygous G-allele (4.5 kb) carriers had higher insulin levels and were

TABLE II
Fragments Length of the BclI and the TthIIII Restriction Fragment Polymorphism and Their Corresponding Nucleotide Changes as Well as Allelic Frequencies

RFLP	Length restriction fragment[a]	Nucleotide change	Allele frequency[b]
*Bcl*I	2.3 kb	C	65%
	4.5 kb	G	35%
*TthIII*I	3.4 kb	C	62%
	3.8 kb	T	38%

[a]Fragment length as described in literature. After identification of the exact nucleotide change, we found that the fragments of the *Bcl*I polymorphism were 2.2 kb and 3.9 kb, respectively. [b]Allele frequency as observed in a subset of subjects from the Rotterdam study, a population-based study in the elderly. RFLP, restriction fragment length polymorphism.

TABLE III
Data from Studies That Involved the BclI Polymorphism of the GR Gene and Investigated Whether Differences Existed Between CC Carriers, CG Carriers, and GG Carriers in Body Composition, Blood Pressure, and Metabolic Parameters

Reference	Population	Associations with the G allele of the *Bcl*I polymorphism
Weaver *et al.*, 1992	56 Obese and 43 non-obese premenopausal women	Hyperinsulinemia in obese GG carriers but not in non-obese GG carriers, no association of the G allele with obesity
Watt *et al.*, 1992	864 Adults (aged 16–24 years) and their parents	Homozygosity for the G allele was more frequent in the group with personal and parental hypertension
Clement *et al.*, 1996	80 Obese families	Tendency towards linkage between the *Bcl*I marker and obesity (BMI $>$ 27), no association after replication
Panarelli *et al.*, 1998	64 Men (aged 18–40 years)	No association of the G-allele with BMI, increased *in vivo* sensitivity to budesonide in GG-carriers
Buemann *et al.*, 1997	79 Men and 73 women, middle-aged	Increased abdominal visceral fat in lean GG carriers but not in overweight GG carriers
Rosmond *et al.*, 2000	262 Swedish men	Increased abdominal obesity and higher cortisol levels in GG carriers compared to CC carriers
Ukkola *et al.*, 2001b	12 Pairs of monozygotic lean male twins (aged 21 years)	CC carriers had a greater increase in weight, abdominal visceral fat, and cholesterol levels in response to overfeeding compared to CG carriers
Ukkola *et al.*, 2001a	322 Men and 420 women (aged 42 years)	G allele associated with abdominal visceral fat and gene-gene interactions present with lipoprotein lipase gene and adrenergic receptor gene
Tremblay *et al.*, 2003	90 Male and 83 female adolescents	Female CG carriers had a greater increase in subcutaneous fat mass during a 12-year follow-up than CC carriers or GG carriers. No differences were found in males
van Rossum *et al.*, 2003c	197 Dutch elderly subjects, 1963 elderly males and females, 400 elderly males	G allele associated with hypersensitivity to both 1-mg and 0.25-mg dexamethasone (in an allele-dosage way), lower BMI, and a tendency towards lower lean mass, while fat mass was not different

CC carriers, in previous reports, were described as homozygous 2.3 kb-allele carriers, CG carriers as heterozygous 2.3/4.5 kb carriers, and GG carriers as homozygous 4.5 kb-allele carriers.

more insulin resistant, when compared to a group consisting of CC (homozygous 2.3 kb) and CG (2.3/4.5 kb) carriers. In a report by Panarelli *et al.* (1998), no association between the G allele and BMI was described. However, increased skin vasoconstriction was observed in homozygous G-allele carriers after injection with budesonide, a synthetic GC, which suggests increased i*n vivo* sensitivity to GCs. In contrast, this study showed that the *in vitro* affinity and sensitivity of leucocytes to DEX tended to be lower. Although these findings were not statistically significant, they suggest that this polymorphism might have tissue-specific effects. Three other reports, all in middle-aged individuals, showed an association between the *Bcl*I polymorphism and abdominal visceral obesity but not general obesity (Buemann *et al.*, 1997; Rosmond *et al.*, 2000b; Ukkola *et al.*, 2001a). GCs are known to induce central obesity, as observed in Cushing's disease. It is not known whether this polymorphism is associated with other features of Cushing's (e.g., easy bruising). However, the relationship between abdominal obesity and the *Bcl*I polymorphism suggests a greater effect of GCs due to alterations at the level of the GR, in particular, in visceral fat. This was confirmed in an elderly Dutch population of 197 subjects with a mean age of 67 years (van Rossum *et al.*, 2003b). The carriers of the G allele of the *Bcl*I polymorphism showed a greater suppression after 1-mg DEX as well as after 0.25-mg DEX (Figure 2D). This association was observed in an allele-dosage way and suggests a hypersensitivity to GCs with respect to the negative-feedback mechanism at the pituitary level. In a subset of 1963 subjects of the Rotterdam study, effects of the *Bcl*I variant on body composition in the elderly were further assessed (van Rossum *et al.*, 2003b). The effects appeared to be the opposite of those reported at a younger age: lower BMI in G-allele carriers. To further explore whether this lower BMI was due to a smaller amount of fat mass or less lean mass, we investigated an independent group of 370 Dutch males with a mean age of 78 years (van Rossum *et al.*, 2003b). Again, we observed a lower BMI in G-allele carriers. We did not find differences in fat mass, although lean mass tended to be lower in G-allele carriers in an allele-dosage way. Thus, the slightly lower lean mass is in line with the previously observed hypersensitivity to GCs in G-allele carriers. This suggests that, at an older age, lower BMI possibly can be ascribed to muscle atrophy, which occurs in all healthy elder individuals, but seems more pronounced in carriers of the G allele of the *Bcl*I variant than in noncarriers.

In an 100-day experiment conducted with 12 pairs of monozygotic twins at young-adult age, effects of the *Bcl*I variant were studied in relation to body composition and metabolic changes in response to overfeeding (Ukkola *et al.*, 2001b). In this study, no homozygous G-allele carriers were found. In contrast with these findings, CC carriers experienced a greater increase in body weight, visceral fat, and cholesterol levels after overfeeding than CG carriers. However, another study in adolescents showed that female heterozygous CG-allele carriers

experienced a greater increase in subcutaneous fat, as measured by skinfold, when compared to both homozygous CC carriers and GG carriers during a 12-year follow-up period (Tremblay *et al.*, 2003). No differences were found in baseline or post-follow-up subcutaneous fat mass, total fat mass, or, importantly, trunk fat mass. The authors speculated that one mutated allele could have a different effect than two mutated alleles. In the latter state, an alternative pathway might be switched on to compensate for changes resulting from two polymorphic alleles. Mechanisms supporting this theory have been reported in mouse models involving cyclooxygenase-2 and glucose transporter-4 genes (Stenbit *et al.*, 1997; Fain *et al.*, 2001). The results of Trembley *et al.* are not in line with the allele-dosage associations between the *Bcl*I polymorphism and hypersensitivity to GCs and BMI that we observed in our large elderly populations (van Rossum *et al.*, 2003b). However, at baseline, they show that female homozygous GG carriers tend to have more subcutaneous fat than CC carriers and CG carriers. Although this difference was not statistically significant, it might explain why they did not find an even greater increase in GG carriers than in CG carriers during follow-up. Thus, in this study, the GG carriers might already have been slightly fatter at preadolescent age.

The molecular mechanism of the *Bcl*I polymorphism has not been clarified. It is likely that this intronic polymorphism exerts its effects in a different way than the N363S polymorphism. No alterations in glucose and insulin metabolism have been observed in carriers of the *Bcl*I polymorphism within the normal-weight population, while N363S carriers clearly showed an increased insulin response to DEX. Only in obese carriers of the *Bcl*I polymorphism were hyperinsulinemia and relative insulin resistance observed. However, no transfection experiments are possible to elucidate the mechanism, since the *Bcl*I polymorphism is located in an intron. We cannot rule out the possibility that this intronic polymorphism is linked to another polymorphism in the promoter region of the *GR* gene, which could result in increased GR expression or a variant in the 3′-untranslated region, which could increase stability of mRNA. However, we did not observe any linkage to the polymorphisms reviewed here (data not shown). Another possibility could be linkage to another gene in the vicinity of the *GR* gene. Since in most studies, the *Bcl*I polymorphism shows clear associations with increased sensitivity to GCs, this possibility is less likely. It is also known that intronic variations can influence the splicing process. However, the point mutation in the *Bcl*I site is not located near a regulatory splice site.

In summary, contrasting data have been reported about the *Bcl*I polymorphism with respect to its association with body composition. A possible explanation is that hypersensitivity to GCs due to the *Bcl*I polymorphism has different consequences during life. It might be that early in life, fat mass — particularly abdominal fat — is predominantly affected (i.e., *Bcl*I G-allele carriers have more

fat), whereas later in life, the most-pronounced effects are observed on lean mass (i.e., *Bcl*I G-allele carriers have lower lean mass).

IV. The ER22/23EK Polymorphism of the *GR* Gene

In a previous report, we described a polymorphism consisting of two linked, single-nucleotide mutations in codons 22 and 23 (exon 2 of the *GR* gene) (Koper *et al.*, 1997). The first mutation in codon 22 did not result in an amino acid change (GAG → GAA, both coding for a glutamic acid (E)) but the mutation in codon 23 (AGG → AAG) caused a change from arginine (R) to lysine (K). In a population of 202 randomly selected individuals from the Rotterdam study, a population-based cohort study in the elderly, we found an association with higher post-DEX cortisol levels (Figure 2E) as well as less cortisol suppression after a 1-mg DEX suppression test in ER22/23EK carriers (Figure 2F). This finding suggests a relative GC resistance (van Rossum *et al.*, 2002). In the same group having a mean age of 67 years, we found that carriers of the ER22/23EK variant had lower fasting insulin levels and increased insulin sensitivity (Figure 4). Carriers of the ER22/23EK polymorphism also had lower total and low-density lipoprotein cholesterol levels (Figure 5).

These findings of lower cholesterol and insulin levels were both confirmed during a second measurement performed 2.5 years later. This suggests that ER22/23EK carriers have a lower tendency to develop impaired glucose tolerance, type 2 diabetes, or cardiovascular disease. In line with these favourable metabolic parameters, ER22/23EK polymorphism frequency was significantly

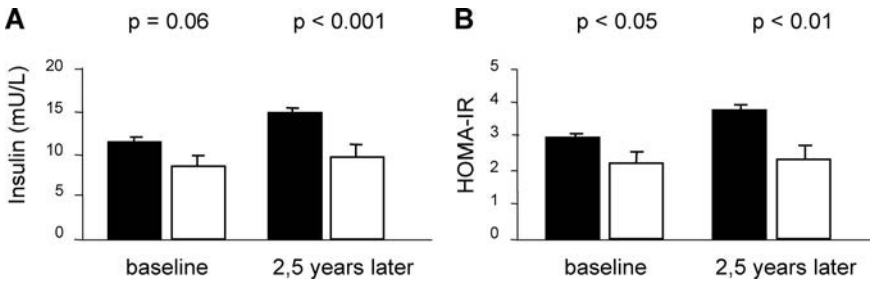

FIG. 4. (A) Fasting insulin concentrations in noncarriers and ER22/23EK carriers at first examination. Insulin concentrations tended to be lower in ER22/23EK carriers compared to noncarriers ($p = 0.06$). On the right, fasting insulin concentrations in noncarriers and ER22/23EK carriers at second examination (2.5 years later). Fasting insulin concentrations were significantly lower in ER22/23EK carriers ($p < 0.001$). (B) Homeostasis model assessment-insulin resistance (HOMA-IR) scores (index of insulin resistance) at baseline and at second examination 2.5 years later in noncarriers and ER22/23EK carriers. At both measurements, ER22/23EK carriers were significantly less insulin resistant.

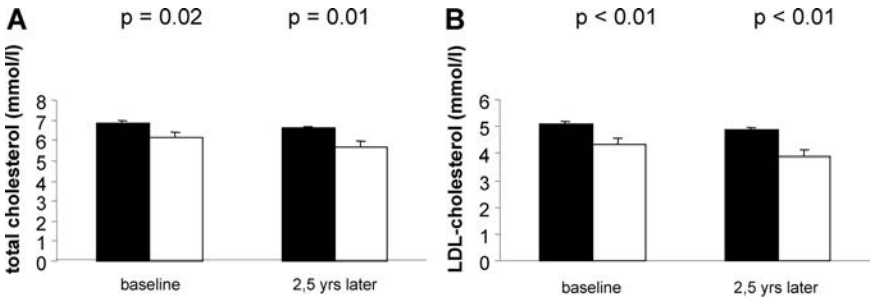

FIG. 5. Total cholesterol (A) and low-density lipoprotein (LDL) cholesterol concentrations (B) in noncarriers and ER22/23EK carriers at first examination. On the right side of each figure, cholesterol concentrations in noncarriers and ER22/23EK carriers at second examination (2.5 years later) are shown. At all measurements, ER22/23EK carriers had significantly lower total and LDL-cholesterol levels than noncarriers.

higher in the oldest half of the studied population. To further explore whether there is an effect on survival at elder age of this polymorphism, we studied a population of 402 men with a mean age of 77 years (van Rossum *et al.*, 2004a). After a follow-up of 4 years, 78 (19.2%) of the noncarriers died, while none of the 21 heterozygous ER22/23EK carriers died, a statistically significant difference. It is has been shown that high C-reactive protein (CRP) is related to increased risk of cardiovascular events (Liuzzo *et al.*, 1994; Thompson *et al.*, 1995; Haverkate *et al.*, 1997). In this population, it was shown that CRP and interleukin-6 (IL-6) were strong predictors of survival (Feelders *et al.*, 2003). We found no differences in IL-6 levels between carriers and noncarriers of the ER22/23EK variant; however, CRP levels were significantly lower in ER22/23EK carriers (van Rossum *et al.*, 2004a). This suggests that carriers of the ER22/23EK variant are relatively protected from vascular damage. Furthermore, total and LDL-cholesterol levels tended to be lower in ER22/23EK carriers, although this did not reach statistical significance. All these data together suggest that the better survival in ER22/23EK carriers might be due to a healthier metabolic profile.

We also studied the effects of this polymorphism at a younger age by investigating a cohort of 350 male and female subjects, who were followed from the age of 13 until the age of 36 years. We studied whether anthropometric parameters and body composition differed between ER22/23EK genotypes (van Rossum *et al.*, 2004b). In males aged 36 and 32 years, we found ER22/23EK carriers to be, on average, 5 cm taller. Although there were no differences in BMI or fat mass, lean body mass was significantly higher in male carriers of the ER22/23EK variant. In accordance, their muscle strength, as measured by arm pull tests and high jump from standing, also was significantly greater. These

differences tended to be already present during puberty but reached statistical significance only at young-adult age.

In females, different associations were observed with the ER22/23EK variant. Waist circumferences tended to be smaller in female ER22/23EK carriers at young-adult age but no differences in BMI were found. It is known that GCs negatively affect muscle mass and induce abdominal obesity. Thus, at young-adult age, the ER22/23EK variant is associated with beneficial changes in body composition, which can possibly be explained by a relative resistance to GCs in these tissues. In addition, effects of the ER22/23EK polymorphism seem to be gender specific.

It is known that GCs influence important brain structures and a normal level of cortisol is critical for many cerebral functions. In humans, high cortisol levels have been found to result in decreased hippocampal formation volume and memory impaiment (Starkman *et al.*, 1992; Lupien *et al.*, 1997). Disturbances in the HPA axis have been found to be related to dementia disorders (Weiner and Lourie, 1968; Gottfries *et al.*, 1994; Nasman *et al.*, 1995) In a large, population-based study in the elderly, we studied whether the ER22/23EK polymorphism was associated with hippocampal volume, dementia, and white matter lesions. We found that ER22/23EK carriers had a lower risk of dementia as well as fewer white matter lesions in the brain (van Rossum *et al.*, 2003a). In addition, the ER22/23EK polymorphism was associated with better performance on psychomotor speed tests. It has been shown that white matter lesions are associated with small vessel disease (Bots *et al.*, 1993; Breteler *et al.*, 1994). Thus, this association might be a direct result of the beneficial effects of the ER22/23EK polymorphism on metabolic risk factors for atherosclerosis. No associations were found between the ER22/23EK variant and hippocampal volume. This might be explained by the fact that, in basal conditions, most effects on the hippocampus are mediated by the MR, while the GR plays a major role only in the activated state (e.g., physical or psychological stress) (De Kloet and Reul, 1987). In other parts of the brain, the GR is more important for mediating effects of GCs, so the observed associations with dementia might be explained by a smaller direct effect of GCs on the brain due to a relative GC resistance.

The mechanism that explains the effects of this polymorphism is under study. Several possibilities exist through which this variation of the *GR* gene can lead to these effects. Since the ER22/23EK polymorphism is located in the transactivation domain, the arginine to lysine amino acid change might affect the receptor's tertiary structure, influencing the transactivational and/or transrepressional activity on target genes (de Lange *et al.*, 1997; Russcher *et al.*, 2003). Recently, it has been shown that two different methionine (M) codons in GR mRNA may be used as the initiation codon: M1 and M27, resulting in two isoforms, GR-A and GR-B, respectively. The GR-B protein has a stronger transactivating effect in transient transfection experiments but no difference in

transrepression (Yudt and Cidlowski, 2001). The nucleotide changes associated with the ER22/23EK polymorphism might affect the secondary structure of the GR mRNA, thus influencing the choice of initiation codon. Indeed, secondary structure prediction (M-*fold*) showed different structures for the wild-type and polymorphic mRNA. Another possible explanation for the decreased GC sensitivity might be that the GR transactivational activity is affected by a different GR-A/GR-B ratio (Russcher *et al.*, 2003). A third option is that the ER22/23EK variant might change mRNA stability, which is maintained when proteins responsible for this stability bind to the mRNA molecule. If the polymorphic mRNA recruits proteins in a different way, mRNA stability is affected, which might be a clue for the decreased GC sensitivity in ER22/23EK carriers.

In summary, the ER22/23EK polymorphism of the *GR* gene is associated with a relative GC resistance and a healthier metabolic condition, as evidenced by lower cholesterol levels and increased insulin sensitivity. Furthermore, this variant is associated with a beneficial body composition at young age and leads to a lower risk of dementia and better survival in the elderly.

V. The *TthIII*I Polymorphism of the *GR* Gene

In the *GR* gene promoter region, a *TthIII*I RFLP previously was reported by Detera-Wadleigh and colleagues (1991). Rosmond *et al.* (2000a) showed this polymorphism to be associated with elevated diurnal cortisol levels in a population of 284 Swedish men. No relationships were found between the *TthIII*I variant and anthropometry, glucose, and insulin metabolism or lipid spectrum. We recently identified the location of the nucleotide change: a C/T change, 3807 bp upstream of the GR mRNA start (Table II).

In the same subpopulation of the Rotterdam study in which we studied the relationship between the three other polymorphisms described in this review and feedback sensitivity to GCs, we investigated whether an association existed between the *TthIII*I polymorphism and GC sensitivity (E.F.C. van Rossum, P. Roks, F.H. de Jong, A.O. Brinkmann, H.A.P. Pols, J.W. Koper, S.W.J. Lamberts, unpublished data). In this group, we found 39.7% CC carriers, 44.5% CT carriers, and 15.8% TT carriers. No differences were found in cortisol levels between the *TthIII*I genotypes before and after 1-mg and 0.25-mg DEX suppression, nor in anthropometric parameters, glucose and insulin levels, or cholesterol concentrations. We also studied whether this *TthIII*I polymorphism interacted with the N363S, *Bcl*I, and ER22/23EK polymorphisms. No interactions with N363S or *Bcl*I were found. Interestingly, however, all carriers of the ER22/23EK polymorphism carried the *TthIII*I T variant. This T allele of the *TthIII*I polymorphism is very common and exists without the ER22/23EK variant being present. To study the effects of carrying the *TthIII*I T allele and the ER22/23EK polymorphism, we compared the following three groups: 1) noncarriers of both

polymorphisms (*TthIII*I CC and ER22/23ER); 2) carriers of one variant allele of the *TthIII*I polymorphism (*TthIII*I CT/TT and ER22/23ER); and 3) carriers of both polymorphisms (*TthIII*I CT/TT and ER22/23EK). The latter group had a significantly reduced cortisol response to 1-mg DEX as well as lower insulin and cholesterol levels, compared to the two other groups. No differences were found between the group of noncarriers of both polymorphisms and the group of carriers of only the *TthIII*I T variant. This suggests that the *TthIII*I polymorphism is not functional by itself; it might be functionally relevant only in combination with ER22/23EK. We do not know whether the *TthIII*I variant at the 5′-flanking region of the *GR* gene is essential in the associations of the ER22/23EK polymorphism or if its presence at the same allele is coincidence and does not influence the effects of the ER22/23EK variant. Possibly, the associations Rosmond and coworkers (2000a) found between alterations in cortisol levels and the *TthIII*I polymorphism could be explained by the presence of the ER22/23EK variant. However, no data have been published on the ER22/23EK polymorphism in this Swedish population.

VI. Discussion

This review focused on several *GR* gene polymorphisms that were associated with body composition and metabolic parameters. As shown in Figure 1, the three functional polymorphisms are located in exon 2 (transactivating domain) and intron 2. This is in contrast to the previously described rare mutations causing the syndrome of GC resistance, which are located predominantly in the ligand-binding domain. GCs are essential for many regulatory processes in the human body, so a mutation leading to an absolute resistance to GCs is not compatible with life. The previously described patients, carrying a mutation of the *GR* gene, have decreased negative feedback at the level of the pituitary gland, which leads to HPA axis hyperactivation. Many of the symptoms found in patients with GC resistance are the consequence of this compensatory increased HPA axis activity: hyperandrogenism (in particular, leading to symptoms in females and children before puberty) and increased mineralocorticoid effects. The latter are due to exposure of the MR to high concentrations of cortisol, which cannot be effectively inactivated by 11β HSD II.

Polymorphisms, common variations at the DNA level occurring in the normal population with a frequency of more than 1%, have much more-subtle effects. However, because of their high frequency in the population, their impact may be much greater. In several — but not all — studies, polymorphisms in the *GR* gene described here seem to correlate significantly with variation of sensitivity to endogenous GCs in normal individuals. Table IV overviews the four discussed polymorphisms and their relation with altered GC sensitivity. The N363S and *Bcl*I polymorphisms both were associated with hypersensitivity to

TABLE IV
Four Polymorphisms of the GR Gene, Studied in the Same Population in Relation to Glucocorticoid Sensitivity

Polymorphism	*Bcl*I	N363S	ER22/23EK	*TthIII*I
N	191	216	202	205
Fasting cortisol	ND	ND	ND	ND
Sensitivity to 1-mg DEX	Increased	ND	Decreased	ND
Sensitivity to 0.25-mg DEX	Increased	Increased	ND	ND

[Abbreviations: N, number; DEX, dexamethasone; ND, no differences between genotype groups of the above-mentioned polymorphism.]

GCs, while the ER22/23EK polymorphism was associated with relative resistance to GCs. No associations were found with the *TthIII*I polymorphism. However, the ER22/23EK variant was found to be linked to the *TthIII*I polymorphism. In this respect, associations with GC resistance and beneficial metabolic profile (i.e., low insulin and cholesterol levels) were observed in carriers of both the ER22/23EK and *TthIII*I polymorphisms. Considering DEX suppression test outcomes in carriers of the three functional polymorphisms, it seems that the 0.25-mg DEX suppression test is most sensitive to detect hypersensitivity to GCs, while the 1-mg DEX suppression test may be more suitable to detect a relative resistance to GCs.

Study of clinical associations of polymorphisms has several limitations. In particular, when studies are performed in rather small populations, the risk that the observed associations are based on coincidence will be increased. It is also known that the general frequency of polymorphisms varies greatly between ethnic populations. Thus, results from one population do not necessarily apply to others. For example, the N363S polymorphism has been reported in Australia with an allele frequency of 7.4% (Lin *et al.*, 1999), whereas in two Asian studies (Ikeda *et al.*, 2001; Lei *et al.*, 2003), no N363S carriers were found. Effects of polymorphisms may differ between races, due to different combinations of polymorphisms of several genes. Differences in environmental factors also play an important role. In this respect, association studies performed in nonhomogeneous populations are difficult to interpret. Within similar ethnic populations, observed associations with *GR* gene polymorphisms vary, which can be caused by differences in characteristics of the study populations, environmental or socioeconomic factors, or differences between generations. Furthermore, it is known that in large population studies, unintended errors in data collection or misclassifications occur relatively frequently, which can influence outcomes. A limitation of *GR* gene polymorphism studies is that no *in vitro* mechanisms have been clarified, while many associations have been found *in vivo*.

Observed associations with altered GC sensitivity may contribute to a better understanding of the variations in regulation of the HPA axis between normal individuals. Previous data suggest that the HPA axis setpoint in humans might be genetically determined, since the intra-individual variations in post-DEX cortisol concentrations are rather small (Huizenga *et al.*, 1998b).

These *GR* gene polymorphisms may have modifying effects on conditions such as (hereditary) atherosclerosis. It is known that some individuals survive until a great age, although they have very high cholesterol levels (Weverling-Rijnsburger *et al.*, 1997). Thus, they might be protected by a genetic variant such as the ER22/23EK. On the other hand, individuals who carry the N363S or the *Bcl*I polymorphism might be more at risk for cardiovascular disease. The N363S variant recently was found to be associated with coronary artery disease, independent of obesity, as well as with increased total cholesterol and triglyceride concentrations and an elevated total cholesterol/HDL ratio. Both the N363S and the *Bcl*I polymorphism may predispose to obesity. However, as is well known, environmental, dietary, and socioeconomic factors are also important determinants of the obesity phenotype. Associations with polymorphisms depend on many additional factors: differences in characteristics between populations, prevalence of the polymorphism, and interactions with other genetic polymorphisms. These factors, taken together, might explain the discrepancies between studies so far encountered.

In clinical practice, GCs are used widely to treat diseases (e.g., asthma, chronic inflammations, prevention of rejection of organ transplants) as well as replacement therapy. It is well known that effects of GC treatment vary considerably between patients. Some patients respond very well to therapeutical administration of GCs but also develop serious side effects, while others need a very high dose to establish any clinical effect and do not suffer from side effects. The response to GCs in the majority of patients lies between these extremes. It is likely that these polymorphisms are to some extent responsible for the variability in response to therapeutically used GCs. In the future, after appropriate additional research, it might be useful to screen for the presence of these *GR* gene variants, in order to determine an individual's dose of GCs. This dose should be adjusted to a person's need, taking into account the genetically determined GC sensitivity, in such a way that it is therapeutically effective but does not cause side effects. We do not know whether the altered sensitivity associated with these polymorphisms differs for various types of clinically used GCs and whether the manner of application (e.g., local, systemic) influences the effects of the polymorphisms.

During evolution, a selection process occurred in which some *de novo* mutations probably had beneficial effects and slowly became more frequent in the population. We found that the ER22/23EK variant in males was associated with greater lean mass and muscle strength. In this view, the ER22/23EK

polymorphism could have resulted in strong individuals with a greater chance to survive due to an advantage in food-collecting and fighting ability. The N363S and *Bcl*I carriers may have had advantages for survival through their tendency to accumulate fat, which was especially favorable in times of food deficit. In this respect, the *Bcl*I polymorphism probably arose long ago, since the allele frequency in normal population is very high. However, in modern times of food abundance, combined with increased psychological stress and lack of exercise, the N363S and *Bcl*I polymorphisms may have turned into a disadvantage. An increased sensitivity to GCs, resulting in fat accumulation, is probably a risk factor in atherosclerosis. This is supported by the findings of increased risk of coronary artery disease and obesity in N363S carriers in an Australian population (Lin *et al.*, 2003a,b).

In conclusion, the N363S, *Bcl*I, and ER22/23EK polymorphisms in the *GR* gene, but not the *TthIII*I polymorphism, are associated with altered GC sensitivity and result in a wide variety of phenotypic signs. These are not pathological *per se* but may partially explain an individual's genetically determined tendency to a certain body composition and metabolic status (Figure 6). More research is needed to elucidate the mechanisms behind these associations at a molecular level.

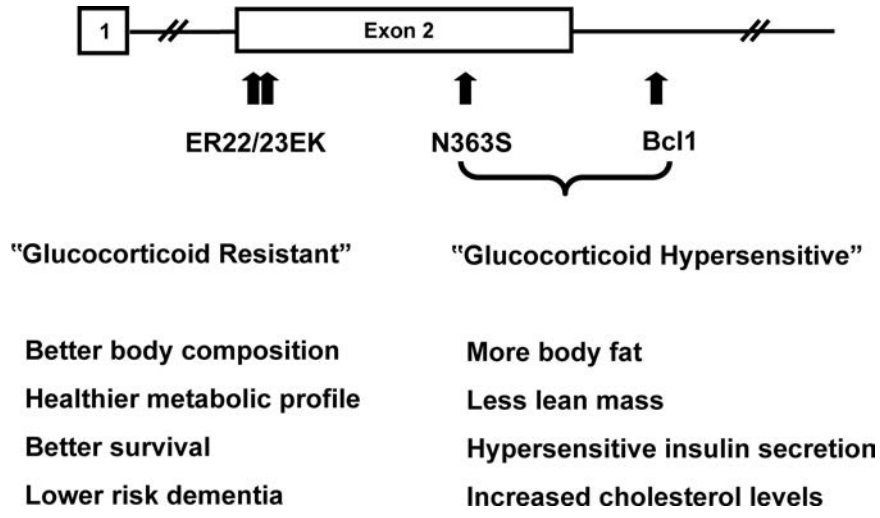

FIG. 6. A tentative scheme of the N-terminal part of the glucocorticoid receptor gene in which three functional polymorphisms are indicated as well as a summary of their clinical associations.

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Glucocorticoids and 11beta-Hydroxysteroid Dehydrogenase in Adipose Tissue

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ABSTRACT

The highly prevalent metabolic syndrome (insulin resistance, type 2 diabetes, dyslipidemia, hypertension, along with abdominal obesity) resembles Cushing's syndrome. However, in simple obesity, plasma cortisol levels are not elevated. 11beta-hydroxysteroid dehydrogenase type 1 (11β-HSD1), at least in mature adipocytes and hepatocytes, converts inactive circulating 11-keto steroids into active glucocorticoids, amplifying local glucocorticoid action. 11β-HSD1 is elevated in adipose tissue in obese humans and rodents, suggesting that adipose tissue glucocorticoid excess may explain the conundrum. Indeed, transgenic mice overexpressing 11β-HSD1 in adipose tissue faithfully replicate the metabolic syndrome. Conversely, 11β-HSD1$^{-/-}$ mice resist the metabolic consequences of stress and high-fat feeding via insulin sensitisation and other advantageous effects in the liver and adipose tissue. Adipose 11β-HSD1 deficiency contributes to a protective metabolic phenotype, supporting its role as a therapeutic target for the metabolic syndrome.

"I am resolved to grow fat and look young till forty, and then slip out of the world with the first wrinkle." — John Dryden, *The Maiden Queen*, 1668

I. Preamble

Dryden's witty 17th-century view of obesity as a convenient disguise for ageing reflects an era when the average life expectancy of a child surviving infancy was only half of today's eight decades or more in western countries. Millions of years of vertebrate evolution and most of human history are reflected in spectacular metabolic adaptations to periodic starvation. By the standards of the age, Dryden's woman was not only believed to be improving her complexion but also to be healthy and resistant to episodic famine and contagion. Today, in contrast, obesity itself has reached epidemic proportions for the world's affluent nations and, increasingly, for developing countries too. Excess fat contributes to early morbidity and mortality, bringing a latter-day irony to Dryden's line. While obesity's primary cause is a chronic imbalance between calorie intake and energy expenditure, underlying vulnerabilities within individuals modulate the likeli-

hood of its development and complications. The metabolic syndrome (Reaven's syndrome X; the insulin resistance syndrome) describes a constellation of cardiovascular risk factors — specifically, insulin resistance, type 2 diabetes, dyslipidemia, and hypertension (Reaven, 1993,2002). The relative risk of morbidities in the metabolic syndrome is increased by the co-occurrence of obesity – notably, visceral (abdominal, android, central) obesity. Despite debate about its definition, the metabolic syndrome is increasing rapidly in prevalence and represents a major burden upon health-care delivery organisations worldwide. Understanding the pathogenesis and potential treatments for visceral obesity and its cardiometabolic associations is a high priority.

Endocrinologists have long puzzled over the strong morphological and metabolic similarities between the rare Cushing's syndrome of endogenous or exogenous glucocorticoid excess and the metabolic syndrome (Walker and Seckl, 2001). Excessive cortisol secretion in Cushing's syndrome is a classic cause of secondary obesity. Effects of excess cortisol on adipose tissue are complex, with an increase in central (i.e., visceral, abdominal, facial, and nape of neck) fat deposition, while peripheral fat is reduced. This may result from opposing effects of glucocorticoids that, on the one hand, increase lipolysis and downregulate lipoprotein lipase — thereby liberating free fatty acids (FFAs) from peripheral fat — but, on the other hand, stimulate preadipocyte differentiation and enhance substrate flux in favour of gluconeogenesis and triglyceride synthesis in central fat (Samra *et al.*, 1998; Andrews and Walker, 1999). Additionally important in glucocorticoid-induced obesity is central stimulation of appetite (Udden *et al.*, 2003), mediated by complex interactions between hypothalamic responses to glucocorticoids, their effects upon target appetite-controlling neurotransmitters such as neuropeptide Y (NPY), and both central and peripheral effects upon adipose-derived endocrine mediators such as leptin and gut hormones (Solano and Jacobson, 1999). Other central effects of increased glucocorticoid action include depression (Cohen, 1980), which intriguingly is also linked specifically to idiopathic visceral obesity (Ahlberg *et al.*, 2002).

Raised plasma cortisol levels cause phenotypic changes in Cushing's syndrome. Against this background, the importance of glucocorticoids in obesity has been investigated in animal models and in humans. Many studies have focused on glucocorticoid secretion and circulating blood levels of steroids. This research has shown that cortisol levels are modestly, if at all, elevated in patients with the metabolic syndrome and even may be reduced in simple obesity (i.e., obesity without diabetes or other disease states complicating increased fat mass) (Walker and Seckl, 2001). This conundrum recently was readdressed and some progress made in disentangling the mechanisms and importance of altered tissue sensitivity to glucocorticoids. This, then, is a story of cellular corticosteroid receptors, and particularly of prereceptor metabolism by enzymes, notably by the hitherto-obscure 11beta-hydroxysteroid dehydrogenases (11β-HSDs).

II. Blood Glucocorticoid Levels in Obesity

A. GLUCOCORTICOID CONTROL: THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

Until recently, it was axiomatic that the major determinants of corticosteroid action were levels of steroid hormones in the blood, modified by binding to plasma proteins (notably, corticosteroid binding globulin (CBG)), and the densities of intracellular receptors in target organs. Plasma glucocorticoid levels are, of course, determined by the activity of the hypothalamic-pituitary-adrenal (HPA) axis, a classical short-term neuroendocrine feedback circuit. The HPA axis functions to restrict excursions of circulating glucocorticoid concentrations within fairly tight limits, with forward drive at the diurnal maximum or during stress rapidly attenuated by negative feedback of adrenal steroids upon pituitary corticotrophs, hypothalamic paraventricular nucleus (PVN) neurons that synthesise corticotropin-releasing hormone (CRH) and vasopressin (AVP), as well as a number of suprahypothalamic sites. Of the latter, the hippocampus, a part of the limbic system, has attracted much attention, as it contains a very high density of intracellular receptors for glucocorticoids and is a key locus for glucocorticoid effects upon memory and perhaps mood (de Kloet, 1991; McEwen, 1999). The hippocampus appears to act as a "brake" upon the HPA axis, since lesions interfere with glucocorticoid feedback control of HPA stress responses (Jacobson and Sapolsky, 1991). Increased HPA axis activity sufficient to cause raised blood cortisol levels would be a convenient mechanism to explain the Cushingoid aspects of the metabolic syndrome and simple visceral obesity. Data relating to glucocorticoid hypersecretion in the metabolic syndrome/visceral obesity continuum are described briefly in the following section.

B. THE HPA AXIS AND CORTISOL DYNAMICS IN OBESITY

A number of case-control studies have suggested that obesity is associated with increased urinary free cortisol (UFC) excretion (Strain *et al.*, 1980; Marin *et al.*, 1992; Pasquali *et al.*, 1993a). This association is particularly prominent in subjects with abdominal obesity, at least in women (Pasquali *et al.*, 1993b). A caveat is that UFC, though a useful marker of HPA axis activation, forms a very small fraction of total cortisol metabolite excretion. More convincingly, recent large studies confirm that total cortisol production rate is somewhat enhanced in obesity in men as well as women (Andrew *et al.*, 1998; Fraser *et al.*, 1999; Stewart *et al.*, 1999). This is further supported by evidence that HPA axis responsiveness to stimuli such as acute stress, CRH/AVP (Pasquali *et al.*, 1999), adrenergic manipulations (Pasquali *et al.*, 2000), hypoglycaemia, or a standard meal (Marin *et al.*, 1992; Hautanen and Adlercreutz, 1993) is enhanced in idiopathic obesity, particularly visceral obesity (Pasquali *et al.*, 2000). Moreover,

the cortisol response to particular types of food may differ with the locus of adiposity, as viscerally obese women show lower HPA axis responses to high-fat and protein but greater responses to high-carbohydrate meals, compared to peripherally obese and lean subjects (Vicennati *et al.*, 2002). However, in obesity, overall plasma cortisol levels are not consistently elevated. Indeed, peak plasma cortisol levels in the morning are typically *low* in the obese (Ljung *et al.*, 1996; Phillips *et al.*, 1998; Rosmond *et al.*, 1998; Walker *et al.*, 1999), though South Asians may be an exception (Ward *et al.*, 2003). While some data in humans suggest that plasma cortisol is higher during the evening nadir in obesity, flattening the diurnal variation (Ljung *et al.*, 1996; Rosmond *et al.*, 1998), evidence is lacking for marked elevations of basal plasma cortisol in obesity. The combination of increased secretion with low morning plasma levels suggests that the diurnal variation of cortisol secretion is disrupted and/or that peripheral metabolism of cortisol is enhanced.

As explanation for the increased glucocorticoid production and exaggerated cortisol responses in obesity, both increased forward drive to the HPA axis — perhaps reflecting some form of primary meal-related abnormality of cortisol release — moderate chronic stress (Rosmond *et al.*, 1998), and/or reduced sensitivity to HPA axis feedback have been suggested (Ljung *et al.*, 1996; Di Blasio *et al.*, 2003; Jessop *et al.*, 2003). Indeed, obesity has been associated with reduced sensitivity to glucocorticoid feedback (Ljung *et al.*, 1996; Jessop *et al.*, 2003), an effect thought to be mediated via altered sensitivity of glucocorticoid receptor (GR). However, increased feedback sensitivity may not be applicable to obese men (Pasquali *et al.*, 2002), although this sex is typified by a more-android adipose distribution! Several studies suggest that individuals with common GR polymorphisms demonstrate HPA axis abnormalities in addition to a peripheral phenotype of increased adiposity, insulin resistance, and hypertension (Bjorntorp *et al.*, 1999; Rosmond *et al.*, 2000; Ljung *et al.*, 2002; Di Blasio *et al.*, 2003). Whether this GR genotype is a primary cause of obesity or whether obesity reflects HPA activation remains unclear but is the topic of ongoing investigations. An additional complexity stems from recent data showing that, in the nucleus, GR homodimers are part of a macromolecular complex with specific coactivator and corepressor proteins that, in combination, determine the actions of glucocorticoids-GR upon a target gene. Variation in density or function of coactivators and corepressors may underlie aspects of interindividual variation in glucocorticoid sensitivity (Li *et al.*, 2003).

For the metabolic syndrome, case-control and cross-sectional studies have suggested that high blood pressure associates with somewhat elevated cortisol concentrations (in blood, saliva, or urine) (Watt *et al.*, 1992; Filipovsky *et al.*, 1996; Stolk *et al.*, 1996; Rosmond *et al.*, 1998; Walker *et al.*, 1998; Fraser *et al.*, 1999; Brunner *et al.*, 2002). Similarly, insulin resistance and glucose intolerance are associated with higher circulating cortisol levels (Phillips *et al.*, 1998;

Reynolds *et al.*, 2001). Higher morning plasma cortisol concentrations and increased responsiveness to corticotropin (ACTH) occur in adults having the additional cardiovascular risk factor of low birth weight (Phillips *et al.*, 1998,2000; Levitt *et al.*, 2000; Reynolds *et al.*, 2001). However, all these associations between cortisol activity and hypertension/insulin resistance are *independent* of obesity. In such studies, obesity again is associated with lower (not higher) plasma cortisol, no change in (renal) 11β-HSD2, and no difference in dermal sensitivity (vasoconstrictor responses) to glucocorticoids.

From these observational studies, it is not possible to dissect causality but they do not strongly favour a unifying hypothesis that ascribes the associations between obesity and other features of the metabolic syndrome to enhanced cortisol secretion. Rather, a primary or secondary increase in HPA axis activity may occur in subjects with hypertension and/or insulin resistance that do not directly predispose to obesity. Clearly, this syndrome cannot result from simple HPA activation or generalised alterations in tissue glucocorticoid sensitivity, yet the parallels with Cushing's remain persuasive.

Thinking this through, it is plausible that the observed combination of increased total cortisol secretion with lower plasma cortisol levels during peak secretion in obesity reflects increased peripheral cortisol clearance. More than 20 years ago, obesity was associated with increased cortisol clearance rates (Strain *et al.*, 1980; Lottenberg *et al.*, 1998). Increased glucocorticoid clearance could itself reduce plasma levels and lead to compensatory HPA axis activation. Any such activation would lead to adrenal hyperresponsivity to stress and other stimuli.

III. Corticosteroid Receptors and Obesity

Tissue sensitivity to glucocorticoids is determined by the density of receptors (and transcriptional cofactors and post-transcriptional mechanisms) in a particular cell type and, crucially, by tissue-specific metabolism by enzymes. In addition to evidence for altered sensitivity to glucocorticoids in HPA feedback sites, peripheral tissue sensitivity to glucocorticoids may be increased in the metabolic syndrome. For example, patients with essential hypertension or type 2 diabetes have enhanced tissue sensitivity to glucocorticoids, as measured by the intensity of dermal vasoconstriction following topical application of beclomethasone (Walker *et al.*, 1996,1998; Andrews *et al.*, 2002). In addressing tissue sensitivity to glucocorticoids, we will first review data on receptors and then concentrate upon enzymes.

Glucocorticoids act predominantly via nuclear receptors. There are two subtypes, GR and mineralocorticoid receptors (MR). These receptors are essential for life, since knockout (KO) of either by homologous recombination in mice is lethal at (for GR) or soon after (for MR) birth (Cole *et al.*,

1995; Berger *et al.*, 1998). GR are near ubiquitous, albeit their density varies considerably between cell types. MR are highly restricted to aldosterone target organs (i.e., distal nephron, distal colon, sweat and salivary glands) as well as some central nervous system (CNS) regions, notably, the hippocampus, and cardiovascular structures, including the heart. Metabolic effects associated with abdominal obesity are thought to originate in visceral adipose tissue and the liver, key sites determining hepatic glucose insulin and lipid homeostasis, as well as in skeletal muscle, the principal organ of glucose uptake. These tissues all highly express GR but have little or no MR. The importance of GR in obesity has been indicated by both its attenuation with adrenalectomy and GR antagonists in animal models and the effects on various mice bearing transgenic GR manipulations. However, the phenotypes are complex, since GR in the CNS also play a key role in HPA axis control such that a generalised transgenic reduction of GR causes obesity (Pepin *et al.*, 1989). This is probably because HPA axis overactivity and marked hypercorticosteronaemia override the reduced density of GR in peripheral tissues. Such outcomes are predicted by the relatively low affinity of GR, which is little occupied by basal levels of glucocorticoids but fully occupied under conditions of glucocorticoid excess (e.g., severe stress) (de Kloet, 1991).

GR density in key metabolic cells, at least in skeletal muscle and adipose tissue, shows considerable interindividual variation. Importantly, in humans, GR mRNA levels in skeletal muscle correlate with insulin sensitivity and blood pressure (Reynolds *et al.*, 2002; Whorwood *et al.*, 2002). Moreover, dermal (vascular) sensitivity to GR agonists correlates with component disorders of the metabolic syndrome (Walker *et al.*, 1996,1998; Andrews *et al.*, 2002) and is associated with a common bclI restriction fragment length polymorphism (RFLP) of the GR gene (Panarelli *et al.*, 1998), though any functional implications of this particular polymorphism are unclear. Thus, it has been hypothesised that genetic and environmental variations in GR density in specific metabolic tissues underlie the metabolic syndrome. Moreover, a mechanistic basis for such tissue-specific variation in GR expression has been provided through demonstration that the GR gene has a number of alternate untranslated exon1/promoter sequences in the 5′ region that, at least in lymphocytes and the brain, can drive tissue-specific expression under some circumstances (McCormick *et al.*, 2000). However, in obesity, GR mRNA is not consistently elevated in adipose tissue (Lindsay *et al.*, 2003; Wake *et al.*, 2003). This may reflect the fact that simple obesity and the metabolic syndrome can exist as discrete associated metabolic defects within a continuum of (visceral) obesity. Again, mechanisms pertaining to other features of the metabolic syndrome appear to differ from those that operate in obesity.

IV. Tissue Metabolism of Glucocorticoids

A. BASIC BIOCHEMISTRY

The enzymes directly metabolising cortisol include the steroid A-ring reductases (5α- and 5β-reductases), 6β-hydroxylase, 20-reductase, and 11β-HSDs. In rats and mice, which lack 17-hydroxylase in their adrenal cortex, the principal glucocorticoid is corticosterone, which is subject to analogous metabolism. This review will focus on the role of 11β-HSDs.

B. HISTORY OF 11β-HSD

50 years ago, Amelung and colleagues (1953) discovered an enzyme that catalysed the interconversion of 11-hydroxy glucocorticoids (cortisol, corticosterone) and 11-keto forms (cortisone, 11-dehydrocorticosterone): 11β-hydroxysteroid dehydrogenase (Figure 1). While 11-hydroxyglucocorticoids are active at receptors, 11-ketosteroids have very low affinity for GR and MR and appear to all intents and purposes inert. This 11β-HSD activity subsequently was described in a broad range of cells and tissues (Monder and White, 1993). In the 1980s, Monder and his coworkers in New York purified an 11β-HSD activity from rat liver (Lakshmi and Monder, 1988). Homogenates, microsomal preparations, and purified enzyme from rat liver catalysed both 11β-dehydrogenation of cortisol to inert cortisone and, typically to a lesser extent, the 11β-reduction of cortisone to active cortisol. Thus, 11β-HSD was thought to represent one of several arcane pathways for glucocorticoid clearance, so no specific function was ascribed to it.

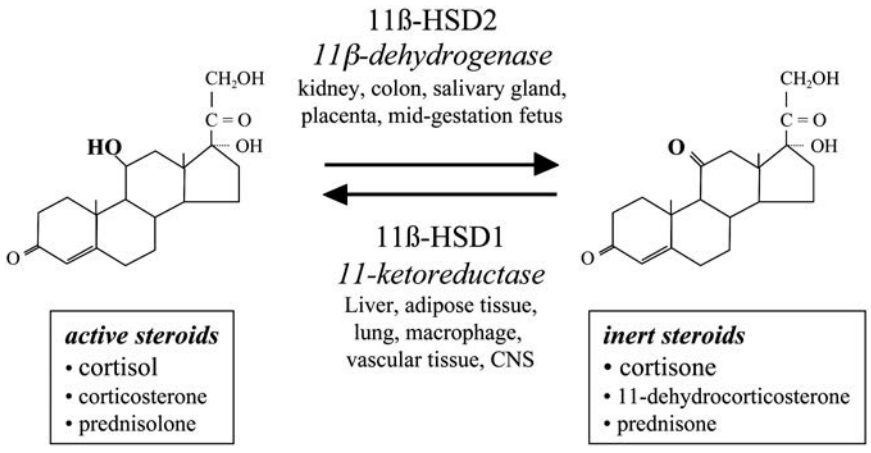

FIG. 1. 11beta-Hydroxysteroid dehydrogenase (11β-HSD). CNS, central nervous system.

Subsequently, two 11β-HSD isozymes have been characterised, isolated, and their cDNAs cloned, the products of distinct and only distantly related genes (reviewed in White *et al.*, 1997; Stewart and Krozowski, 1999; Seckl and Walker, 2001).

C. 11β-HSD TYPE 2

11β-HSD type 2 is a high-affinity (low nM Km for cortisol), nicotinamide adenine dinucleotide (NAD)-dependent dehydrogenase that rapidly converts active cortisol to inert cortisone (Albiston *et al.*, 1994; Brown *et al.*, 1996b). Although expressed in many tissues during fetal life, including the placenta (Brown *et al.*, 1996a), in adults, 11β-HSD2 is expressed principally in tissues where aldosterone induces its classical effects on sodium excretion, including distal nephron, sweat glands, salivary glands, and colonic mucosa (Smith *et al.*, 1996; Hirasawa *et al.*, 1997). There is some expression in other epithelial cells, such as in lung (Page *et al.*, 1994) and endothelium (Brem *et al.*, 1998; Hadoke *et al.*, 1998; Christy *et al.*, 2003). 11β-HSD2 functions predominantly to exclude glucocorticoids from intrinsically nonselective MR *in vivo* (Edwards *et al.*, 1988; Funder *et al.*, 1988). In an analogous action, 11β-HSD2 in placenta and fetal tissues appears to exclude glucocorticoids from GR *in utero* (Benediktsson *et al.*, 1997; Seckl *et al.*, 2000). Inhibition of this enzyme with liquorice (Stewart *et al.*, 1987) or disruption of the 11β-HSD2 gene in mice (Kotelevtsev *et al.*, 1999) or humans (Mune *et al.*, 1995; Dave-Sharma *et al.*, 1998) leads to the syndrome of apparent mineralocorticoid excess (AME). In AME, glucocorticoids illicitly occupy renal MR, causing sodium retention, hypertension, and hypokalemia. 11β-HSD2 inhibition also reduces fetal growth and alters tissue maturation (Lindsay *et al.*, 1996a,b).

D. 11β-HSD TYPE 1

1. Biochemistry and Distribution

In contrast to the well-defined functions of 11β-HSD2, although 11β-HSD1 was the first isoform to be identified and characterised, it remained until recently the Cinderella of this story. 11β-HSD1 has a much-lower affinity (i.e., low μM Km) for cortisol and corticosterone, is a nicotinamide adenine dinucleotide phosphate (NADP(H))-dependent enzyme, and is widely distributed. In cell homogenates or microsomal preparations, 11β-HSD1 readily converts cortisol to cortisone. Thus, for several years, it was thought to be responsible for inactivation of cortisol in the kidney. However, more-recent data in intact cells either transfected with 11β-HSD1 cDNA or in primary cells in culture show that in most, if not all, intact cells and organs, this enzyme is a predominant 11-ketoreductase, reactivating inert cortisone into cortisol (Low *et al.*, 1994a;

Hundertmark *et al.*, 1995; Jamieson *et al.*, 1995; Rajan *et al.*, 1996). The preponderant reductive direction *in vivo* appears to be due more to the intracellular redox context of 11β-HSD1 in the inner leaflet of the endoplasmic reticulum than to any structural feature, such as glycosylation (Agarwal *et al.*, 1995). The mechanisms determining reaction directionality have not been elucidated fully. Nonetheless, 11β-HSD1 co-precipitates with the NADPH-generating enzyme, hexose-6-phosphate dehydrogenase (Ozols, 1995), which may dictate the predominant 11-ketoreductase action of 11β-HSD1 *in vivo* (Draper *et al.*, 2003). Short-term, post-translational changes such as enzyme phosphorylation also may be important, particularly to explain the apparent instability of the 11-ketoreductase activity in homogenates despite plentiful NADPH, but remain to be clarified. Biochemical investigation of expressed 11β-HSD1 suggests that the reductase reaction has cooperative rather than Michaelis-Menten kinetics, unlike the dehydrogenase, ensuring cortisol generation across a wide range of substrate concentrations (Maser *et al.*, 2002), as observed *ex vivo* in perfused liver (Jamieson *et al.*, 2000).

In a few studies (e.g., in Leydig cells), 11β-dehydrogenase activity has been reported in apparently intact cell preparations (Gao *et al.*, 1997; Ge and Hardy, 2000). Others have found predominantly 11-ketoreduction in similar preparations (Leckie *et al.*, 1998). The discordance of reported reaction direction in intact cells, largely involving Leydig cells and preadipocytes (Bujalska *et al.*, 2002), remains unresolved but is an important issue, given the current enthusiasm to decrease/inhibit 11-ketoreductase as a therapeutic target. It has been suggested that some 11β-HSD1 may have been liberated from possible damaged cells in studies reporting 11β-dehydrogenase activity (Seckl and Walker, 2001), since this direction is more stable in homogenates and broken-cell preparations. More plausibly, adipose tissue dissociation and culture lead to early induction of adipocyte cytokine synthesis and release (Ruan *et al.*, 2003). Cytokines have potent effects upon 11β-HSD1 and other adipocyte products, which may explain some discordances in the human adipose 11β-HSD1 activities between freshly isolated tissue and cultured cells.

11β-HSD1 is expressed in kidneys of rats but negligible expression occurs in adult human kidney (Stewart *et al.*, 1994). However, 11β-HSD1 is widely expressed in other tissues in humans (Ricketts *et al.*, 1998) and rodents (Moisan *et al.*, 1990b), including liver (Agarwal *et al.*, 1989; Tannin *et al.*, 1990), adipose tissue (Bujalska *et al.*, 1997; Napolitano *et al.*, 1998), lung (Hundertmark *et al.*, 1993), skeletal muscle, cardiac and vascular smooth muscle (Walker *et al.*, 1991; Brem *et al.*, 1995; Christy *et al.*, 2003), anterior pituitary gland, brain (including hippocampus) (Moisan *et al.*, 1990a,b; Lakshmi *et al.*, 1991; Sakai *et al.*, 1992), and adrenal cortex (Shimojo *et al.*, 1996). Notably, these locations have high GR rather than MR expression, with the exception of hippocampus and heart, where MR act as high-affinity sites for physiological glucocorticoids rather than aldosterone *in vivo* (Whorwood *et al.*, 1991).

These observations in cells suggested a novel role for 11β-HSD1 involving reactivation rather than inactivation of glucocorticoid. This, indeed, occurs in intact organs, at least in the liver. Isolated perfused cat (Bush *et al.*, 1968) or rat (Jamieson *et al.*, 2000) liver models suggest that 11β-HSD1, which is the only isozyme expressed in liver, is a predominant 11β-reductase with a high capacity for reactivating 11-ketosteroid substrate over a broad range of substrate concentrations. These findings can be extrapolated to human liver *in vivo*, since historical work suggests that, on oral administration, cortisone (the first pharmacological glucocorticoid used in man) is rapidly activated to cortisol. Indeed, recent studies confirm that very little oral cortisone reaches the systemic circulation (A. Jamieson *et al.*, 1999) and that hepatic vein cortisol/cortisone ratios are very high (Walker *et al.*, 1992). 11-ketoreductase activity has been shown in other human tissues *in vivo*, including subcutaneous adipose tissue (Katz *et al.*, 1999).

2. Substrate Levels

For 11-ketoreductase to play a physiological role in regulating receptor exposure to active glucocorticoids (as opposed to a pharmacological role when cortisone is administered), there must be a substantial pool of substrate inert 11-ketosteroids in the circulation and tissues. *In vivo*, the main source of 11-ketosteroid is 11β-HSD2 dehydrogenation of cortisol and corticosterone, which predominantly occurs in the kidney (Whitworth *et al.*, 1989). In humans, cortisone circulates at levels approximating 50–100 nmol/l (Walker *et al.*, 1992). While this level is lower than diurnal peak cortisol of 400–600 nmol/l, cortisone is largely unbound, while $\approx$ 95% of cortisol is sequestered by binding to plasma proteins such as CBG (Dunn *et al.*, 1981). Moreover cortisone, unlike cortisol, shows no pronounced diurnal rhythm and so is constantly available for conversion to active glucocorticoid (Walker *et al.*, 1992). Estimates of "free" cortisol levels are rather imprecise but approximate 0.5–1 nmol/l at the diurnal nadir. In the rat, plasma concentrations of 11-dehydrocorticosterone are also $\approx$ 50 nmol/l, though in the mouse, levels are lower ($\approx$ 3–5 nmol/l) (Kotelevtsev *et al.*, 1997). Thus, for at least the quiescent part of the diurnal cycle, circulating cortisone levels equal or exceed free cortisol levels and similar ratios pertain in rodents.

E. FUNCTIONAL STUDIES OF 11β-HSD1 IN LIVER

Initial findings suggesting that 11β-HSD1 increases effective intracellular glucocorticoid action were obtained in liver. Here, glucocorticoids oppose the actions of insulin, for example, by upregulating expression of the key enzyme for gluconeogenesis, phosphoenolpyruvate carboxykinase (PEPCK). In male rats, estradiol potently downregulates 11β-HSD1 expression (Low *et al.*, 1993) and, only in the presence of glucocorticoids, downregulates PEPCK expression (P.M. Jamieson *et al.*, 1999). Such indirect studies, as well as the use of relatively

nonselective, liquorice-based inhibitors (Walker *et al.*, 1995; Andrews *et al.*, 2003), indicate that impaired activity of 11β-HSD1 in liver is associated with features of reduced glucocorticoid action and increased insulin sensitivity in hepatocytes. This has been supported by recent studies with selective 11β-HSD1 inhibitors (Alberts *et al.*, 2002,2003) (see below).

To explore this further, 11β-HSD1 KO mice have been generated (Kotelevtsev *et al.*, 1997). These mice appear to develop normally and are viable, fertile, and apparently normotensive. This model shows that 11β-HSD1 is the sole major 11β-reductase, at least in mice, since adrenalectomised 11β-HSD1 KO mice cannot convert administered 11-dehydrocorticosterone to active corticosterone. Plasma corticosterone levels are modestly elevated at the diurnal nadir, presumably due to somewhat deficient feedback upon the HPA axis (11β-HSD1 is expressed in hippocampus, PVN, and anterior pituitary) (Harris *et al.*, 2001). However, despite slightly elevated basal plasma corticosterone levels, 11β-HSD1$^{-/-}$ mice have a phenotype compatible with impaired intracellular glucocorticoid regeneration and reduced antagonism of insulin action. They show impaired induction of the key glucocorticoid-inducible hepatic enzymes PEPCK and glucose-6-phosphatase on fasting and an attenuated hyperglycemic response to novel environment stress or chronic high-fat feeding (Kotelevtsev *et al.*, 1997). Importantly, the mice have more-than-adequate, stress-induced HPA axis responses (Harris *et al.*, 2001) and do not exhibit hypoglycaemia with prolonged fasting (Kotelevtsev *et al.*, 1997). Further aspects of the 11β-HSD1 null phenotype affect hepatic metabolic control and cardiovascular risk factors (Morton *et al.*, 2001). *Ad lib*-fed 11β-HSD1$^{-/-}$ mice have lower plasma triglyceride and elevated "cardioprotective" high-density lipoprotein (HDL) cholesterol levels (Morton *et al.*, 2001). This appears to be due to increased hepatic β oxidation of lipids, rather than to altered lipogenesis. Indeed, hepatic expression of peroxisome proliferator-activated receptor alpha (PPARα), a key glucocorticoid-sensitive transcription factor driving lipid metabolism, is elevated in fed 11β-HSD1$^{-/-}$ mice. The major murine HDL carrier, apolipoprotein AI, is increased in the null mice, whereas serum apolipoprotein CIII, which increases plasma triglycerides by inhibiting hepatic lipolysis and interfering with transfer of triglycerides to the liver, is reduced. Additionally, preliminary data suggest favourable effects upon haemostasis, as in liver, glucocorticoid-inducible Aα-fibrinogen transcript levels are reduced (Morton *et al.*, 2001).

F. 11β-HSD1 IN ADIPOSE TISSUE

1. Isozymes and Directionality

Glucocorticoids play a key role both in the regulation of adipose tissue metabolism and in the differentiation of preadipocytes into adipocytes (Gaillard

et al., 1991). 11β-HSD expression in adipose tissue was noted in the 1980s and thought to be a dehydrogenase (Monder and White, 1993). Studies in adipose cells derived from the mammary gland confirmed the presence of 11β-dehydrogenase activity *in vitro* (Quirk *et al.*, 1991). More recently, 11β-HSD in adipose tissue has been re-examined in light of the 11-ketoreductase predominance of this isozyme in intact hepatocytes and whole liver.

In terms of isozymes, 11β-HSD1, but not 11β-HSD2, mRNA is expressed in rat white (epididymal) adipose tissue (WAT) (Napolitano *et al.*, 1998) and in human adipose tissue (Bujalska *et al.*, 1997; Sun *et al.*, 1997; Paulmyer-Lacroix *et al.*, 2002). The enzyme appears to be expressed similarly in adipocytes and the stromal vascular fraction (enriched with preadipocytes and vascular cells) in the rat, though perhaps is more highly concentrated in adipose stromal cells in humans (Bujalska *et al.*, 1997). 11β-HSD also is expressed in murine fibroblast-adipocyte 3T3-F442A and 3T3-L1 cell lines, which closely reproduce preadipocyte differentiation *in vitro* when cultured in the presence of inducing agents, including dexamethasone. These respond to glucocorticoids in a similar manner to adipose tissue *in vivo* (MacDougald *et al.*, 1994; MacDougald and Lane, 1995). 3T3 cells exclusively express the 11β-HSD1 isozyme and, in intact cells, the reaction direction is solely 11-ketoreduction (Napolitano *et al.*, 1998). 11β-HSD1 mRNA and activity in 3T3 cells increases markedly with differentiation of cells from their preadipocyte to mature adipocyte state. 11β-HSD1 appears to be a late-differentiation gene in 3T3 cells. 3T3 cell *in vitro* differentiation is promoted by insulin and glucocorticoids. However, the increase in 11β-HSD1 with differentiation does not appear to be mediated directly by these hormones, since, at least in differentiated 3T3 cells, insulin and glucocorticoids reduce 11β-HSD1 expression. By contrast, intact human adipose stromal cells cultured in the presence of insulin and cortisol have been reported to show bi-directional 11β-HSD1 activity, although 11-ketoreductase activity predominates in cells derived from omental fat, with further induction by glucocorticoids in mature cells (Bujalska *et al.*, 1997).

Some recent findings suggest that 11β-HSD1 reaction direction may alter in adipose tissue with differentiation state (Bujalska *et al.*, 2002), although this was not apparent in a clonal 3T3 cell model (Napolitano *et al.*, 1998). Thus, primary cultures of human stromal and mature adipocytes taken from patients undergoing elective abdominal surgery showed predominant 11β-dehydrogenase activity in freshly isolated stromal cells from the omental compartment, whereas intact mature adipocytes had predominantly 11-ketoreductase activity. Following culture for 2 weeks, 11-ketoreductase activity predominated, despite no significant changes in 11β-HSD1 mRNA. The authors speculated that since glucocorticoids inhibit cell proliferation, dehydrogenase in immature preadipocytes facilitates proliferation. However, once

differentiation is initiated, 11-ketoreductase amplifies glucocorticoid levels, promoting adipogenesis. While these findings might be explained by cell breakage leading to liberation of enzyme into a homogenate-like state in the early phases of cell culture and/or by cytokine-inductive effects of adipose tissue dissociation and early culture *in vitro*, an intriguing biochemical mechanism for shifts in 11β-HSD1 directionality has been advanced. This advocates varying developmental expression of hexose-6-phosphate dehydrogenase, which co-precipitates with 11β-HSD1 and acts to generate NADPH inside the lumen of the endoplasmic reticulum in adipose tissue (Stegeman and Klotz, 1979). Intriguingly, while polymorphisms in the 11β-HSD1 gene (HSD11B1) correlate rather poorly (Draper *et al.*, 2002; Gelernter-Yaniv *et al.*, 2003), if at all (Caramelli *et al.*, 2001), with adipose distribution in humans, changes in the hexose-6-phosphate dehydrogenase gene may be key. Thus, in three patients with rare cortisone reductase deficiency (Phillipov *et al.*, 1996), null mutations in the HSD11B1 gene have not been found. Recent data suggest that such individuals have more-subtle mutations in both HSD11B1 and in hexose-6-phosphate dehydrogenase, resulting in both low 11β-HSD1 expression and low endoplasmic reticulum NADPH generation, with consequent loss of 11β-HSD1 reductase activity (Draper *et al.*, 2003). It remains to be established whether or not this mechanism applies to more-common cases of 11β-HSD1 dysregulation in adipose tissue with obesity and associated insulin-resistance states, such as the polycystic ovary syndrome, which phenotypically most closely resembles cortisone reductase deficiency.

2. Depot Differences

Fat depots may differ in 11β-HSD1 activity, although no clear consensus exists. Discrepancies may reflect species differences or assay conditions employed. Thus, in humans, cultures of omental stromal cells have more 11β-HSD1 than subcutaneous stromal cells (Bujalska *et al.*, 1999). In contrast, in various nonobese strains of mice, 11β-HSD1 mRNA expression and activity are higher in freshly isolated peripheral than visceral fat depots (Morton *et al.*, 2003). This is consistent with the idea that increased glucocorticoid reactivation correlates with increased differentiation potential of this depot. These interspecies differences may reflect the conditions of assay; *ex vivo* cultures in high glucocorticoid concentrations employed in humans perhaps favour increased activity in visceral fat because this depot has more GR (Masuzaki *et al.*, 2001) and/or has greater capacity for glucocorticoid-induced differentiation of immature to mature (i.e., higher 11β-HSD1-expressing) adipocytes.

3. Molecular Regulatory Mechanisms

Several studies have examined regulation of 11β-HSD1 expression in adipose tissue. Insulin appears to reduce 11β-HSD1 gene expression in fat cells. Although glucocorticoids reduce adipose 11β-HSD1 expression in some systems, in others, they stimulate its activity, with effects reflecting the assay conditions and perhaps species. Proinflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interleukin-1 (IL1) increase and anti-inflammatory cytokines reduce 11β-HSD1 in adipose cells *in vitro* (Handoko *et al.*, 2000; Friedberg *et al.*, 2003). As detailed earlier, both glucocorticoids and insulin exert effects, albeit *in vitro*. However, regulation by other nuclear receptors remains less certain. Thus, while PPARγ ligands such as thiazolidinediones downregulate 11β-HSD1 in 3T3 cells *in vitro* and in epididymal fat in mice *in vivo* (Berger *et al.*, 2001), the same was not observed in lean or obese Zucker rats (Livingstone *et al.*, 2000b). Liver X receptor (LXR) agonists also partially downregulate 11β-HSD1 *in vitro* and *in vivo* (Stulnig *et al.*, 2002). The mechanism may be indirect, which may explain the discrepancies observed in such studies. In terms of more-direct effects, CCAAT/enhancer binding protein alpha (C/EBPα) binds to several sites on the 11β-HSD1 promoter and, when transfected into human hepatoma hepG2 cells, increases 11β-HSD1 promoter activity (Williams *et al.*, 2000). In contrast, C/EBPβ acts as a dominant-negative repressor of 11β-HSD1 transcription when added to C/EBPα, although alone, it is a weak inducer. Electrophoretic mobility shift assays (EMSA) using nuclear extracts from undifferentiated and differentiated 3T3-F442A cells suggest that C/EBPα also may regulate 11β-HSD1 expression in adipocytes (Williams *et al.*, 1993). These data suggest the possibility that 11β-HSD1 regulation in the adipocyte by insulin and glucocorticoids may be mediated indirectly through changes in C/EBP-related proteins. Since under many circumstances, 11β-HSD1 regulation in liver and adipose tissue is discordant, as with other well-characterised C/EBP-regulated genes such as PEPCK, the fine details of control are likely to be tissue specific. 11β-HSD1 mRNA regulation in 3T3-F442A cells parallels glycerol-3-phosphate dehydrogenase, a key enzyme in triglyceride synthesis and a well-characterised marker of adipocyte differentiation (Moustaid *et al.*, 1990).

G. AP2–11β-HSD1 ADIPOSE OVEREXPRESSING TRANSGENIC MICE: A MODEL OF THE METABOLIC SYNDROME

The preponderance of data suggest that 11-ketoreductase activity is increased in adipose tissue in obese rodent models and humans (see below). The key question is whether increased adipose 11β-HSD1 is a cause or a consequence of obesity and any associated metabolic syndrome. To dissect this, mice over-

expressing 11β-HSD1 selectively in adipose tissue have been generated, exploiting the adipocyte fatty acid binding protein (aP2) promoter (Masuzaki *et al.*, 2001). Two lines have been selectively developed to exhibit 2- to 3-fold overexpression of 11β-HSD1 in adipose tissue, which represents the degree of enzyme increase found in obese humans and animals. These aP2–11β-HSD1 mice are viable and appear developmentally normal. The transgene is expressed in all adipose depots examined and in brown adipose tissue (BAT) but is absent from other 11β-HSD targets such as the brain, liver, skeletal muscle, and kidney. The effect of the transgene is to approximately double corticosterone levels within adipose tissue, while circulating glucocorticoid levels are unaltered, at least under basal conditions. As a consequence of local intra-adipose glucocorticoid excess, aP2–11β-HSD1 mice are modestly obese. Strikingly, the obesity is predominantly intra-abdominal, with a $>$ 3-fold increase in the mesenteric fat depot, whereas peripheral fat depots are significantly, but much less spectacularly, increased. The visceral adipose expansion in these animals (which overexpress 11β-HSD1 similarly in all adipose beds) may relate to much-greater levels of GRα in visceral adipose than peripheral depots in mice (Masuzaki *et al.*, 2001). Indeed, the transgenics show increased expression of the glucocorticoid target gene lipoprotein lipase, particularly in mesenteric adipose tissue that could drive lipid accumulation in this adipose depot. It is interesting that GRα is not downregulated in the transgenic animals. Glucocorticoid autoregulation of GR is highly tissue specific and is only seen acutely in many tissues.

In association with this localised change in glucocorticoid exposure and the consequent increased visceral fat mass, aP2–11β-HSD1 mice develop all major features of the metabolic syndrome. The animals are markedly glucose intolerant and insulin resistant, features exacerbated by high-fat feeding. The animals do not show obvious fasting hyperglycemia but this becomes markedly manifest after a glucose load, suggesting the deficit is in peripheral glucose uptake rather than a predominant increase in hepatic glucose production. They also show dyslipidemia with elevated FFA and triglyceride levels (Masuzaki *et al.*, 2001). Glucocorticoids are potent secretagogues of leptin from adipose tissue. Serum leptin levels are elevated in the transgenic animals disproportionately to their degree of obesity, suggesting leptin resistance, as observed in human obesity.

aP2–11β-HSD1 transgenic mice show adipocyte hypertrophy but not hyperplasia (Masuzaki *et al.*, 2001). This is perhaps not surprising, as aP2 is a late differentiation gene in adipocytes, so expression and effects in preadipocytes would not be expected. Within adipocytes themselves, aP2–11β-HSD1 mice show changes concordant with decreased sensitivity to insulin and/or increased corticosterone levels. Thus, transgenic adipose tissue has decreased expression of the insulin-sensitising factor adiponectin (acrp30, adipoQ) (Yamauchi *et al.*, 2002) and increased expression of TNFα, an adipose cytokine that causes insulin

resistance (Hotamisligil *et al.*, 1993). Both are commensurate with the effects of glucocorticoid excess (Fasshauer *et al.*, 2002; Viengchareun *et al.*, 2002). Serum TNFα levels also are elevated. In contrast, adipose mRNA-encoding resistin, which may lead to insulin resistance (Steppan *et al.*, 2001), is reduced in transgenic adipose tissue, perhaps as a consequence of glucocorticoid excess (Viengchareun *et al.*, 2002). Intriguingly, angiotensinogen mRNA, which normally is expressed at low levels in adipose tissue, is strikingly elevated in aP2–11β-HSD1 transgenic mouse fat. This glucocorticoid-regulated transcript may underpin the marked hypertension seen in these animals. The hypertension is associated with renin-angiotensin-aldosterone system (RAAS) activation and is strikingly sensitive to low doses of an angiotensin II receptor antagonist (Masuzaki *et al.*, 2003). Finally, the animals show intriguing hyperphagia, indicating the potential for a novel pathway, other than leptin deficiency (though leptin resistance is not excluded), of signalling between the adipocyte and CNS appetite centres. The aP2–11β-HSD1 transgenic mouse faithfully models the major features of the metabolic syndrome.

The aP2–11β-HSD1 transgenic mouse permits speculation about the subtle but important phenotypic differences between Cushing's syndrome and the metabolic syndrome. If the metabolic syndrome is, indeed, generated by local glucocorticoid excess inside adipose tissue and/or liver but with normal circulating cortisol levels, then tissue without the adipose-hepatic axis will be unaffected. We would not necessarily expect Cushingoid skin thinning, vascular fragility, delayed wound healing, myopathy, or CNS effects, which may well reflect the actions of *systemic* glucocorticoid excess upon skin, blood vessels, skeletal muscle, and brain without the compass of *local* adipose and hepatic glucocorticoid excess seen in the 11β-HSD1 transgenic models.

H. LIVER GLUCOCORTICOID EXCESS: APOE–11β-HSD1 TRANSGENIC MICE

Blood from visceral adipose tissue drains via the hepatic portal vein to the liver. Unsurprisingly, therefore, aP2–11β-HSD1 transgenic mice have elevated levels of corticosterone and FFA in the portal plasma, implying that the liver is exposed to excess glucocorticoids. To address this and to examine whether excess 11β-HSD1 in the liver *per se* can cause the metabolic syndrome, transgenic mice overexpressing 11β-HSD1 in the liver have been generated under the ApoE promoter. These mice are viable and appear normal. As adults, the mice show modest insulin resistance and hypertriglyceridemia, along with substantially increased fat deposits in the liver. However, ApoE–11β-HSD1 transgenic mice have normal weight with normal distribution and mass of fat depots and show normal glucose tolerance. Intriguingly, the animals are hypertensive, with activation of the RAAS, in this case due, apparently, to overex-

pression of angiotensinogen in the liver but not adipose tissue. Overexpression of 11β-HSD1 in liver produces an attenuated metabolic syndrome phenotype *without* visceral obesity. This might be of pathogenic relevance in cases such as patients with the insulin resistance of myotonic dystrophy (Johansson *et al.*, 2001), in which liver 11β-HSD1 activity is raised.

I. DEFICIENCY OF 11β-HSD1 IN ADIPOSE TISSUE: LESSONS FROM THE KO MOUSE

11β-HSD1 expression in adipose tissue produces a striking metabolic syndrome phenotype. But what about enzyme deficiency (Figure 2)? Inhibitors have provided some conflicting data that may relate to difficulties in access of carbenoxolone to adipose tissue *in vivo*, at least in the doses administered to rodent models (Livingstone and Walker, 2003) and perhaps humans (Andrews *et al.*, 2003). Adiposity in 11β-HSD1 KO animals has been examined on both intrinsically obesity-resistant and obesity-prone genetic backgrounds. Intra-adipose corticosterone levels are lowered substantially in the 11β-HSD1$^{-/-}$ animal

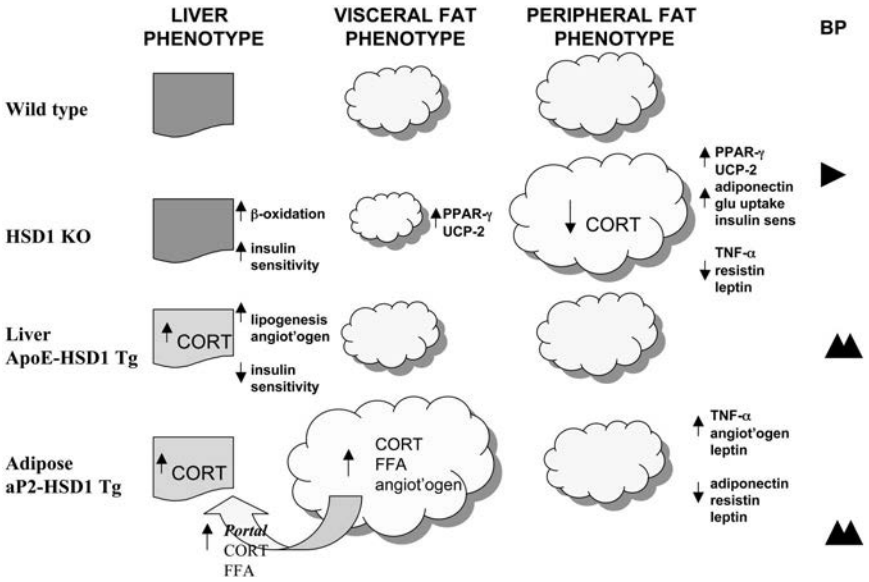

FIG. 2. Schematic of the phenotypes of mice lacking or overexpressing 11β-HSD1 in metabolic tissues. The size of the representations of fat depots reflects the mass observed. Paler liver colours represent fat accumulation. Abbreviations: BP, blood pressure; KO, knockout; Tg, transgenic; PPARγ, peroxisome proliferator-activated receptor gamma; UCP-2, uncoupling protein 2; TNFα = tumor necrosis factor alpha; HSD, hydroxysteroid dehydrogenase; FFA, free fatty acid; CORT, corticosterone.

in the face of modestly elevated plasma corticosterone concentrations (Morton *et al.*, 2003). On the obesity-prone C57Bl/6J genetic background, high-fat diet-fed 11β-HSD1 KO mice gain significantly less weight than controls, despite relative hyperphagia. This appears due to an enhanced metabolic rate, as judged by increased core body temperature (Morton *et al.*, 2002). With high-fat diet on either genetic background, 11β-HSD1 KO mice preferentially gain adipose tissue in the "metabolically safer" peripheral depots rather than in the "metabolically disadvantageous" visceral sites. While the explanation of fat redistribution in 11β-HSD1$^{-/-}$ animals is uncertain, these mice show higher expression of the thiazolidinedione target adipogenic transcription factor PPARγ receptor in all adipose tissue beds. Furthermore, 11β-HSD1$^{-/-}$ mice show a greater increase of adipose PPARγ receptors with high-fat feeding than wild-type (WT) mice. PPARγ ligands cause fat redistribution to the periphery, which may underpin the favourable morphology seen (Kelly *et al.*, 1999; Sewter *et al.*, 2002). Additionally, 11β-HSD1 null animals show greater induction of uncoupling protein-2 (UCP-2) in mesenteric adipose tissue than WT mice (Morton *et al.*, 2002), which may allow local calorie wastage rather than storage as fat (Digby *et al.*, 2000). UCP-2 is downregulated by glucocorticoids (Udden *et al.*, 2001) and upregulated by PPARγ activation (Kelly *et al.*, 1998), so its induction in the 11β-HSD1 null mouse adipose tissue is not unexpected.

In terms of adipose endocrine changes, adipose leptin mRNA and plasma leptin levels are reduced in 11β-HSD$^{-/-}$ mice, particularly in peripheral adipose. On the obesity-prone C57Bl/6J genetic background, 11β-HSD1 null animals are clearly insulin sensitised and resist hyperglycaemia that occurs with high-fat feeding in WT mice. This occurs at least partly at the adipocyte level, since isolated primary adipocytes show increased basal and insulin-stimulated glucose uptake (Morton *et al.*, 2003). Adipocyte resistin and TNFα mRNAs are reduced, whereas adiponectin is increased, again compatible with an adipose-mediated, insulin-sensitised phenotype. Thus, overall, the mouse shows improved glucose tolerance, increased insulin sensitivity, and reduced intratissue glucocorticoid levels in the face of modest hypercorticosteronaemia (Harris *et al.*, 2001). These beneficial effects of deletion in 11β-HSD1 in adipose tissue are accompanied by changes in hepatic gene expression consistent with increased β oxidation of lipids in the liver (Morton *et al.*, 2001).

Although the relative importance of liver and adipose tissues in contributing to this beneficial phenotype remains to be determined, it is clear that loss of 11β-HSD1 has few, if any, deleterious metabolic effects, at least as assessed in this mouse model. Indeed, the lifespan of this model is apparently normal and the animals resist the normal glucocorticoid-associated decline of memory and other cognitive processes with ageing (Yau *et al.*, 2001). Thus, loss of this enzyme appears to be beneficial.

1. Why Might 11β-HSD1 Exist?

However, the phenotype of the 11β-HSD1$^{-/-}$ mouse and the various transgenic and pharmacological manipulations detailed here raise the evolutionary question of the overall biological purpose of such an apparently virtueless enzyme. It cannot exist merely as a convenient target for therapy. We can only speculate. Given that our vertebrate biological antecedents had to suffer considerably more starvation than plenty, perhaps the boost of glucocorticoid from local tissue reactivation, particularly during the diurnal nadir, provided an extra lift to metabolic processes underpinning calorie generation. This might well have assisted survival during periods of starvation. Even during relative nutritional plenty, the sustenance of some key metabolic organs, while others, including the HPA system, were quiescent (i.e., during sleep), may have had an advantage. However, in modern westernised existence with an excess of calorifically rich foods available at every street corner, perhaps such activity becomes redundant, particularly as obesity and its metabolic consequences become the problem. It is an intriguing notion that an enzyme putatively required to help survive starvation becomes a metabolic "appendix" in the presence of nutritional plenty and a target for therapy of the consequences of calorific excess.

V. 11β-HSD1 and Glucocorticoid Metabolism in Obesity

The combination of increased total cortisol secretion with lower plasma cortisol levels during peak secretion suggests that peripheral cortisol clearance may be increased in obesity. Any such activation would lead to adrenal hyperresponsivity to stress and other stimuli, as observed in obesity. In the 2 decades since excess cortisol production and clearance rates were first noted in obesity (Strain *et al.*, 1980), the observation has been repeated many times (Andrew *et al.*, 1998,2002; Lottenberg *et al.*, 1998). Increased glucocorticoid clearance could reduce plasma levels and lead to compensatory HPA axis activation. The enzymes responsible for increased glucocorticoid clearance in obesity have been identified through analysis of cortisol metabolites in urine. Increased relative excretion of A-ring reduced metabolites of cortisol has been reported that is attributed to activation of the hepatic A-ring reductases (Andrew *et al.*, 1998; Fraser *et al.*, 1999). A-ring reductases (5α- and -β) are activated in both animal (Livingstone *et al.*, 2000a) and human obesity (Andrew *et al.*, 1998,2002). The underlying cause of increased enzyme activity is unknown but these enzymes may be regulated by insulin, lipids, and substrate availability. However, here, we concentrate upon another pathway, the 11β-HSDs.

A. 11β-HSD1 IN OBESITY

Measurement of 11β-HSD1 *in vivo* in humans is not straightforward (Walker, 2000). The original assessment relied upon measuring ratio of the most-abundant urinary metabolites of cortisol and cortisone (i.e., 5α- and 5β-tetrahydrocortisols/5β-tetrahydrocortisone). However, this ratio also is influenced by activities of other enzymes, including 11β-HSD2, 5α-reductase, 5β-reductase, and 3α-HSDs. For example, carbenoxolone, which inhibits both renal 11β-HSD2 and hepatic 11β-HSD1, does not alter the ratio of cortisol/cortisone metabolites (Stewart *et al.*, 1990). In obesity, the urinary cortisol/cortisone metabolite ratio has been reported to be increased (Andrew *et al.*, 1998; Rask *et al.*, 2002; Tiosano *et al.*, 2003), unchanged (Fraser *et al.*, 1999; Reynolds *et al.*, 2001), or decreased (Stewart *et al.*, 1999; Rask *et al.*, 2001). This inconsistency may depend in part upon variations — for example, with sex — in 5α- and 5β-reductase activity. Recent findings suggest that increased fat content of the liver, which constitutes a risk factor for insulin resistance over and above the effect of obesity, is associated with increased urinary excretion of 5β-reduced cortisol metabolites. Liver fat content was shown to be a more-powerful determinant of cortisol/cortisone metabolite ratios than the degree of obesity (Westerbacka *et al.*, 2003). However, this important confounder has not been accounted for in most studies in which liver fat content was not measured. Another source of inconsistency of cortisol/cortisone metabolite ratio in obesity is the possible influence of tissue-specific disruption of 11β-HSD1 activity. It is this last crucial point that turns out to be the key issue in obesity.

Studies in leptin-resistant obese Zucker rats revealed that obesity was associated with decreased 11β-HSD1 expression and activity in liver but increased 11β-HSD1 in omental adipose tissue (Livingstone *et al.*, 2000a). This led to dissection of potential tissue-specific differences in obese humans. In humans, conversion of cortisone after oral administration to cortisol in peripheral plasma, reflecting first-pass metabolism by hepatic 11β-HSD1, is impaired in obesity (Stewart *et al.*, 1999; Rask *et al.*, 2001,2002). In contrast, in subcutaneous abdominal adipose tissue, 11β-HSD1 activity is increased both *in vivo* and *in vitro* (Rask *et al.*, 2001,2002; Lindsay *et al.*, 2003; Wake *et al.*, 2003). Further studies have confirmed that the increased 11β-HSD1 activity in biopsies is accompanied by increased 11β-HSD1 mRNA (Paulmyer-Lacroix *et al.*, 2002; Lindsay *et al.*, 2003; Wake *et al.*, 2003; Westerbacka *et al.*, 2003). By analogy with the transgenic mouse having 3-fold overexpression of 11β-HSD1 in adipose tissue (Masuzaki *et al.*, 2001,2003), one would expect a Cushingoid phenotype to result from the similar magnitude of increase in adipose 11β-HSD1 documented in human obesity. Interestingly, while increased subcutaneous adipose 11β-HSD1 is associated with insulin resistance in obesity, it is not linked specifically with visceral adiposity or hypertension. In these respects, mice and

men may differ but it remains to be determined whether 11β-HSD1 mRNA or activity is increased in intact *omental* adipose tissue in obese subjects (Tomlinson *et al.*, 2002).

The mechanisms underlying increased adipose 11β-HSD1 in obesity are uncertain. 11β-HSD1 transcription is highly regulated, including by many factors that are altered in obesity (e.g., cytokines, sex steroids, growth hormone (GH), insulin, PPARα and -γ agonists). In obese Zucker rats and ob/ob mice, downregulation of hepatic 11β-HSD1 is reversible with other manipulations that induce weight loss (Livingstone *et al.*, 2000b; Liu *et al.*, 2003). Attempts to link the 11β-HSD1 genotype with obesity have not been successful (Caramelli *et al.*, 2001; Draper *et al.*, 2002), although arguably the intermediate phenotypes (i.e., anthropometric measurements or urinary cortisol/cortisone metabolite ratios) employed are too insensitive to allow clear inferences about the influence of known polymorphisms in the 11β-HSD1 gene.

B. 11β-HSD1 AND GH

In addition to dysregulation in patients with idiopathic obesity, the central adiposity of GH deficiency and hypothalamic obesity may be associated with changes in cortisol metabolism and 11β-HSD1 activation. 11β-HSD1 is inhibited by GH and insulin-like growth factor-1 (IGF-1) *in vitro* (Napolitano *et al.*, 1998; Tomlinson *et al.*, 2001). In humans with hypopituitarism and acromegaly, this effect may be important (Moore *et al.*, 1999; Trainer *et al.*, 2001; Tiosano *et al.*, 2003). Indeed, GH therapy reduces cortisol:cortisone metabolite ratios in patients with hypopituitarism (Weaver *et al.*, 1994) and in idiopathic obesity (Tomlinson *et al.*, 2003). As in studies of idiopathic obesity, the difficulty is in the interpretation of urinary cortisol:cortisone metabolite ratios, which do not reflect any tissue-specific changes. *In vivo* animal studies have shown that GH regulates liver 11β-HSD1, with the direction of change dependent upon sex-specific patterns of pulsatile GH release (Low *et al.*, 1994b). These findings may not explain changes in adiposity but may give a mechanism for improvements in insulin sensitivity in liver alone when GH is administered at the constant levels typical of female patterns that downregulate hepatic 11β-HSD1. Whether such effects pertain to humans is not fully known.

C. 11β-HSD IN THE BRAIN IN OBESITY

While not the main object of this review (for details of 11β-HSDs in the CNS, see Seckl, 1997; Yau and Seckl, 2001), specific CNS changes in 11β-HSDs in obese rodents have been reported that add to the complexity of tissue-specific dysregulation. In particular, obese Zucker rats have reduced 11β-HSD1 mRNA in the hippocampus (Mattsson *et al.*, 2003). The enzyme functions as a reductase in hippocampal neurons (Rajan *et al.*, 1996). The reduced expression of 11β-

HSD1 may, in part, underlie the reduced sensitivity to glucocorticoid feedback inhibition of the HPA axis and consequent hypercorticosteronaemia of Zucker rats. Moreover, the feedback defect appears linked more to MR than GR, both in obese rodents (Mattsson *et al.*, 2003) and humans (Jessop *et al.*, 2003). Therefore, studies with GR-selective agonists like dexamethasone may be misleading (Weyer *et al.*, 1997). Whether such effects occur in obese humans is unknown (and is probably unknowable) but 11β-HSD1 is expressed in the human hippocampus (Sandeep *et al.*, 2002) and may be targeted by 11β-HSD inhibitors (Jellinck *et al.*, 1993).

VI. 11β-HSD1 as a Drug Target for Therapy in the Metabolic Syndrome

While the various mouse models described here represent a major series of advances in understanding the role of tissue (intracrine) glucocorticoid levels in generating visceral obesity and aspects of the metabolic syndrome, several discordances in human pathophysiology remain to be adequately accounted for.

First, studies showing increased 11β-HSD1 activity in adipose tissue in humans mainly have investigated abdominal subcutaneous fat. While this is not necessarily as distinct from visceral adipose as other subcutaneous depots — and subcutaneous fat is perhaps the major source of adipose hormones and cytokines — this depot is not the same as visceral fat and probably contributes little, if anything, to the portal venous influx to the liver.

Second, mice are not humans in key aspects of their biochemistry. Thus, murine HDL cholesterol represents the majority of total cholesterol, while in humans, the preponderance is reversed, with most cholesterol transported in low-density lipoprotein (LDL) particles. Mice have corticosterone as their sole glucocorticoid, whereas in humans, cortisol predominates. Corticosterone has a higher affinity for GR and MR. Mice do not spontaneously get atheroma without a specific mutation (e.g., ApoE$^{-/-}$), whereas humans do.

Third, while human subcutaneous adipose 11β-HSD1 correlates with obesity and increased insulin resistance, it does not correlate closely with the *distribution* of body fat, notably in the visceral compartment (Westerbacka *et al.*, 2003).

Fourth, it remains unclear whether 11β-HSD1 activity is also increased in omental adipose tissue in obese humans and drives a Cushingoid phenotype of central obesity (Bujalska *et al.*, 1997). One study has reported that 11β-HSD1 in omental adipose obtained during surgery is not correlated with obesity. Indeed, when adipocytes were cultured, there was an inverse correlation between 11β-HSD1 and obesity (Tomlinson *et al.*, 2002), although the effects of anaesthesia and/or stress on the subsequent pattern of gene expression and 11β-HSD1 activity and direction are difficult to anticipate. Attempts to demonstrate a close association between increased 11β-HSD1 and intra-adipose cortisol levels or

glucocorticoid-dependent gene transcription have been inconclusive (Lindsay *et al.*, 2003; Wake *et al.*, 2003). It has been proposed that the level of 11β-HSD1 mRNA expression and total protein may not be the most-important determinant of net cortisol generation from cortisone, since the balance between reductase and dehydrogenase activities may be crucially dependent upon cofactor generation by hexose-6-phosphate dehydrogenase (Draper *et al.*, 2003). While this hypothesis has not been tested definitively, it is notable that subjects with combined 11β-HSD1 and hexose-6-phosphate dehydrogenase mutations putatively conferring impaired conversion of cortisone to cortisol are not universally lean (Phillipou *et al.*, 1996; A. Jamieson *et al.*, 1999; Draper *et al.*, 2003).

Fifth, understanding the molecular mechanisms regulating 11β-HSD1 is key. While insulin and other hormones exert indirect effects in rodents, the mechanisms in humans are poorly understood. However, recent data suggest that patients with glucose intolerance have paradoxically normal cortisol secretion, which is perhaps inappropriately high, given enhanced central and peripheral tissue sensitivity to glucocorticoids (Andrews *et al.*, 2002). In these subjects, "normal" 11β-HSD1 activity in adipose tissue with reduced hepatic activity suggests that tissue-specific changes in 11β-HSD1 in hyperglycemia differ from those in primary obesity (Andrews *et al.*, 2002). Thus, increased adipose 11β-HSD1 appears to relate specifically to obesity, rather than to hyperglycaemia or insulin resistance (Westerbacka *et al.*, 2003) *per se*.

Despite these caveats, 11β-HSD1 inhibition is a tempting target for treatment of the metabolic syndrome and its complications. In this line, at least one selective inhibitor has been formulated and doubtless others will be developed.

A. INHIBITOR STUDIES: OLD AND NEW AGENTS

Carbenoxolone, the hemisuccinate of glycyrrhetinic acid, which is the main active derivative of liquorice, has been a prototype, though nonselective, 11β-HSD1 inhibitor (Stewart *et al.*, 1990). Carbenoxolone enhances insulin sensitivity, as measured by a euglycaemic hyperinsulinaemic clamp (Walker *et al.*, 1995), an effect attributed to enhanced hepatic insulin sensitivity. Stable isotope glucose tracers have been used to demonstrate that, in lean patients with type 2 diabetes, carbenoxolone inhibits hepatic glucose production (Andrews *et al.*, 2003). However, in contrast with enhanced adipose insulin sensitivity and glucose disposal in 11β-HSD1 KO mice, carbenoxolone has no effect on glucose disposal, perhaps because carbenoxolone poorly inhibits adipose 11β-HSD1 (Livingstone and Walker, 2003). Moreover, liver 11β-HSD1 is downregulated in obesity, so that the incremental effect of inhibition may be smaller in the obese (Stewart *et al.*, 1999; Rask *et al.*, 2001,2002). Whether inhibition in liver alone, and resulting enhancement of hepatic insulin sensitivity, is sufficient to confer clinically useful metabolic benefits is uncertain.

Recently, a new, potent, nonsteroidal class of selective inhibitors of 11β-HSD1 has been described, the arylsulfonamidothiazoles (Barf *et al.*, 2002). These agents inhibit 11β-HSD1 and enhance insulin action in liver, decreasing PEPCK and glucose-6-phosphatase expression and lowering blood glucose concentrations in KKA^Y diabetic and ob/ob obese mice (Alberts *et al.*, 2002,2003). However, euglycaemic hyperinsulinaemic clamps in mice suggested that the selective 11β-HSD1 inhibitor did not increase peripheral glucose uptake (Alberts *et al.*, 2003). What remains to be established for 11β-HSD1 inhibitors is whether their effects extend beyond the liver and include benefits in adipose tissue, where the most-beneficial target is likely to be.

VII. Summary

Close phenotypic similarities exist between rare Cushing's syndrome, caused by excess circulating glucocorticoids, and the much more-common visceral obesity (with or without the metabolic syndrome), although plasma cortisol levels in the latter typically are normal. This chapter reviews recent data suggesting that this paradox may be explained by increased local tissue activity of glucocorticoids in simple obesity syndromes. In particular, we concentrate on the emerging role of 11β-HSD1, an enzyme that catalyses the regeneration of active glucocorticoids in adipose tissue and liver as both a cause and a therapeutic target in this increasingly prevalent group of disorders.

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The Central Melanocortin System and the Integration of Short- and Long-term Regulators of Energy Homeostasis

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ABSTRACT

The importance of the central melanocortin system in the regulation of energy balance is highlighted by studies in transgenic animals and humans with defects in this system. Mice that are engineered to be deficient for the melanocortin-4 receptor (MC4R) or pro-opiomelanocortin (POMC) and those that overexpress agouti or agouti-related protein (AgRP) all have a characteristic obese phenotype typified by hyperphagia, increased linear growth, and metabolic defects. Similar attributes are seen in humans with haploinsufficiency of the MC4R. The central melanocortin system modulates energy homeostasis through the actions of the agonist, α-melanocyte-stimulating hormone (α-MSH), a POMC cleavage product, and the endogenous antagonist AgRP on the MC3R and MC4R. POMC is expressed at only two locations in the brain: the arcuate nucleus of the hypothalamus (ARC) and the nucleus of the tractus solitarius (NTS) of the brainstem. This chapter will discuss these two populations of POMC neurons and their contribution to energy homeostasis. We will examine the involvement of the central melanocortin system in the incorporation of information from the adipostatic hormone leptin and acute hunger and satiety factors such as peptide YY ($PYY_{3–36}$) and ghrelin via a neuronal network involving POMC/cocaine and amphetamine-related transcript (CART) and neuropeptide Y (NPY)/AgRP neurons. We will discuss evidence for the existence of a similar network of neurons in the NTS and propose a model by which this information from the ARC and NTS centers may be integrated directly or via adipostatic centers such as the paraventricular nucleus of the hypothalamus (PVH).

I. Introduction

Pro-opiomelanocortin (POMC) modulates energy homeostasis principally through one of its cleavage products, α-melanocyte-stimulating hormone (α-MSH), which exerts a tonic inhibitory control on food intake and energy storage though its actions in the central nervous system (CNS) at two of the five known melanocortin receptors, melanocortin-3 receptor (MC3R) and melanocortin-4 receptor (MC4R) (for a review, see Cone, 1999). While the contribution of the agonist α-MSH is important, it is the endogenous antagonist at these receptors, agouti-related protein (AgRP) (Ollmann *et al.*, 1997), whose mRNA shows a greater degree of regulation by extremes of negative or positive energy balance such as fasting and diet-induced obesity in rodents (Mizuno and Mobbs, 1999;

Ziotopoulou *et al.*, 2000). The most-compelling evidence, however, for a pivotal role for the central melanocortin system in the regulation of energy homeostasis comes from studies in transgenic mice (for a review, see Butler and Cone, 2001). POMC and MC4R knockout mice and mice that overexpress the agouti gene (A^y/a) or AgRP all have a characteristic obese phenotype typified by hyperphagia, increased linear growth, and metabolic defects (Yen *et al.*, 1994; Huszar *et al.*, 1997; Ollmann *et al.*, 1997; Yaswen *et al.*, 1999). Similar attributes are seen in humans with mutations in genes of the central melanocortin system. Defects in the MC4R gene have been linked to obesity, particularly severe early-onset obesity in children (Farooqi *et al.*, 2000). Significantly, alterations in this gene have been linked to up to 5% of cases in children and adults (Farooqi *et al.*, 2003).

Although there are five melanocortin receptors, it is the MC3R and MC4R subtypes that have been implicated in the regulation of energy balance (for a review, see Adan *et al.*, 1997). While these receptors both have a fairly widespread distribution in the rodent brain (Roselli-Rehfuss *et al.*, 1993; Mountjoy *et al.*, 1994; Kishi *et al.*, 2003), POMC has a limited distribution, with only two neuronal populations described: one in the arcuate nucleus of the hypothalamus (ARC) and the other in the nucleus of the tractus solitarius (NTS) of the brainstem (Joseph *et al.*, 1983; Palkovits *et al.*, 1987; Bronstein *et al.*, 1992). Of these two populations, the ARC neurons have drawn the most attention from researchers. The ARC and other hypothalamic nuclei have classically been associated with the actions of leptin and the regulation of body weight in the long term, while the NTS and other brainstem nuclei predominantly are linked to the regulation of meal initiation and termination (Grill and Kaplan, 2002). We will review evidence for the involvement of both populations of POMC neurons in the regulation of energy homeostasis, both in the long term and short term, and discuss the potential for the integration of information from these two sites by adipostatic centers.

II. POMC Neurons and the ARC Neuronal Network

A. THE ARC NEURONAL NETWORK AND LONG-TERM REGULATORS OF ENERGY HOMEOSTASIS

The POMC neurons of the ARC are known to be responsive to leptin via leptin receptors (Ob-R) expressed on their surface (Cheung *et al.*, 1997). In addition to the POMC neurons, another important element of the melanocortin system in the hypothalamus is the neurons that express the melanocortin receptor antagonist AgRP, which also express the orexigenic peptide, neuropeptide Y (NPY) (Hahn *et al.*, 1998) and are leptin sensitive (Wilson *et al.*, 1999). These NPY/AgRP-containing neurons are able to form synapses with POMC neurons

of the ARC and exert regulatory effects, producing a neuronal network that is responsive to the modulatory actions of leptin (Figure 1) (Cowley *et al.*, 2001). In this model, leptin causes hyperpolarization of NPY/AgRP neurons, leading to a reduction in the release of gamma aminobutyric acid (GABA) that, in turn, causes disinhibition of the POMC neurons with which they synapse. In addition to its indirect actions on the POMC neurons via NPY/AgRP cells, leptin appears to act on the POMC system directly by causing a depolarization of the ARC neurons, increasing their firing rate. This model demonstrates how leptin may serve as an overall modulator of energy homeostasis by altering the firing rate of orexigenic and anorexigenic neurons. The fact that serum leptin levels do not vary after meals (Korbonits *et al.*, 1997) but generally are proportional to adipose mass (Maffei *et al.*, 1995) suggests that leptin is not acting as an anorectic factor

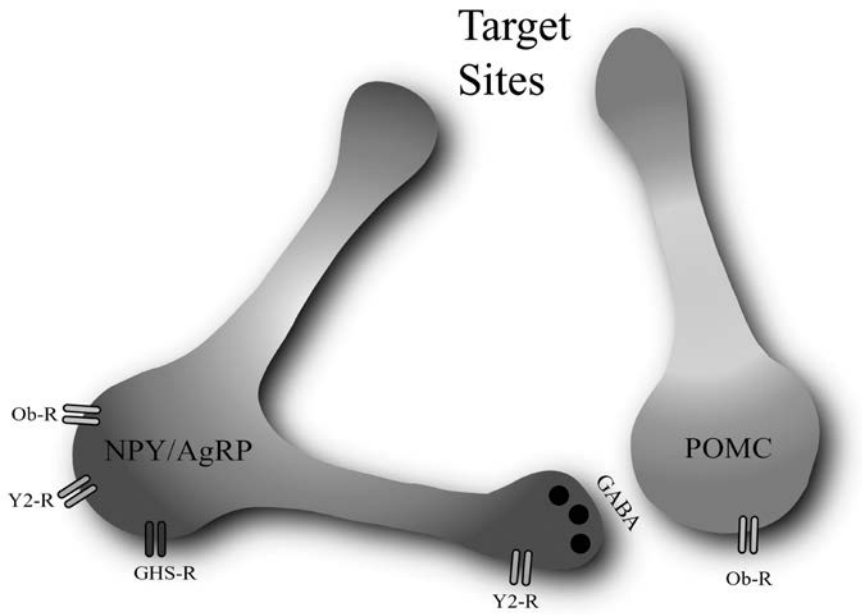

FIG. 1. The arcuate nucleus (ARC) neuronal network. Neuropeptide Y/agouti-related protein (NPY/AgRP) neurons form synapses with the pro-opiomelanocortin/cocaine and amphetamine-regulated transcript (POMC/CART) neurons in the ARC, forming a regulatory network that is responsive to leptin via leptin receptors (Ob-R) present on their surface. Leptin acts on POMC neurons directly and indirectly via a reduction in the release of gamma aminobutyric acid (GABA) from NPY/AgRP neurons. The circuit is able to respond, via growth hormone secretagogue receptor (GHS-R) and Y2-R, to signals from ghrelin and $PYY_{3–36}$.

but rather as an indicator of the long-term energy status of the animal. Thus, modulation of the firing rate of neurons of the ARC and other hypothalamic sites may be a means by which the body weight of an animal is maintained and adjusted over extended periods of time in response to variations in leptin levels.

B. THE MELANOCORTIN SYSTEM AND SHORT-TERM REGULATORS OF ENERGY HOMEOSTASIS

In addition to the mediation of long-term changes in energy balance via signals from leptin, we have shown that POMC neurons of the ARC may be able to respond to signals from the gut hormone peptide $YY_{3–36}$ ($PYY_{3–36}$) (Batterham *et al.*, 2002). $PYY_{3–36}$, an N-terminal truncated form of PYY, is released from the lower intestine following a meal in proportion to the number of calories ingested (Pedersen-Bjergaard *et al.*, 1996). ARC POMC neurons are activated by administration of $PYY_{3–36}$ via Y2 receptors (Y2-R) on NPY/AgRP neurons that, in turn, causes modulation of the hypothalamic ARC network previously described. This evidence suggests that the ARC network actually may integrate information from both long-term signals of nutritional status and satiety signals that are released postprandially from the gut. While the finding that direct injection of $PYY_{3–36}$ into the ARC causes a reduction in food intake, it remains to be established whether the ARC is the functional site of action of $PYY_{3–36}$ *in vivo* or whether, in common with other postprandially released gastric peptides, it acts via the brainstem and sites in the gut itself. In addition to $PYY_{3–36}$, there is evidence to suggest that the network may be activated by other gut hormones. The satiety signal cholecystokinin (CCK) has been shown to electrically (Burdakov and Ashcroft, 2002) modulate the activity of ARC neurons, although these have not been identified as containing POMC or NPY/AgRP. This indicates that there is potential for an interaction between the central melanocortin system and other postprandially released gut peptides.

Another more-recently identified gut-derived peptide is ghrelin. Ghrelin is the endogenous peptide for the growth hormone secretagogue receptor (GHS-R), the mRNA for which is expressed in a number of sites in the hypothalamus (Guan *et al.*, 1997). Ghrelin originally was described as being produced by the oxyntic cells of the stomach (Kojima *et al.*, 1999) but since has been shown to be expressed at low levels in the small intestine (Date *et al.*, 2000), kidney (Mori *et al.*, 2000), testis (Tanaka *et al.*, 2001), placenta (Gualillo *et al.*, 2001), brain (Lu *et al.*, 2002; Cowley *et al.*, 2003), lymphocytes (Hattori *et al.*, 2001), pituitary (Korbonits *et al.*, 2001), and pancreas (Volante *et al.*, 2002). Perhaps unsurprisingly, due to the close association between growth and energy homeostasis and what was already known about the effects of synthetic growth hormone secretagogue (Bercu *et al.*, 1992), ghrelin peptide and mRNA levels were shown to be

regulated by changes in energy balance such as fasting (Tschop *et al.*, 2000; Cummings *et al.*, 2001), hypoglycemia (Toshinai *et al.*, 2001), and diet-induced obesity (Tschop *et al.*, 2000) in rodents. However, the effects of ghrelin are independent of GH secretion (Tschop *et al.*, 2000; Wren *et al.*, 2000; Nakazato *et al.*, 2001).

NPY/AgRP neurons of the ARC have been implicated in mediating ghrelin's effects on energy homeostasis (Kamegai *et al.*, 2001; Nakazato *et al.*, 2001; Shintani *et al.*, 2001; Lawrence *et al.*, 2002b; Wang *et al.*, 2002). Ghrelin has a unique distribution in the brain, encompassing the internuclear space between the ARC, ventromedial (VMH), dorsomedial (DMH), and paraventricular hypothalamic nuclei (PVH) (Cowley *et al.*, 2003). The discovery of this network led to questions about whether the effects of ghrelin on the ARC NPY/AgRP neurons were due to centrally or peripherally derived ghrelin, or both. Indeed, axons from ghrelin-containing neurons form synaptic contact with NPY/AgRP and POMC neurons of the ARC (Cowley *et al.*, 2003). Electrical recording from these ARC neurons indicates that ghrelin is able to cause depolarization of ARC NPY/AgRP neurons and hyperpolarization of POMC neurons. When considered in conjunction with studies showing that c-fos is activated in NPY/AgRP but not in POMC neurons following peripheral ghrelin administration (Wang *et al.*, 2002), the data would suggest that the effect of ghrelin on POMC neurons is probably inhibitory, mediated by the action of GABA released by NPY/AgRP neurons (Cowley *et al.*, 2003).

In the same study, it was demonstrated that in addition to its effects in the ARC, ghrelin is able to influence the activity of PVH corticotropin-releasing hormone (CRH) neurons, possibly via an increase in release of GABA from NPY/AgRP neurons, in a similar manner to its effects on POMC neurons. The interaction between the ARC neuronal network and the neurons of the PVH will be discussed further in this review. In addition to interacting with NPY and the central melanocortin system, evidence suggests that ghrelin interacts with the orexigenic peptide orexin/hypocretin in the brain. Central administration of ghrelin in rats causes activation of orexin-containing neurons of the lateral hypothalamic area (LHA) (Lawrence *et al.*, 2002b). Ghrelin-immunoreactive terminals make contact with orexin neurons in the LHA (Toshinai *et al.*, 2003). The blockade of orexin-A and -B receptors by injection of antisera attenuates the effects of centrally administered ghrelin on food intake, providing *in vivo* evidence for an interaction between the two peptides. The wide distribution of ghrelin-immunoreactive neurons (Cowley *et al.*, 2003), GHS-R mRNA (Guan *et al.*, 1997), and the data outlined earlier suggest that, in common with leptin, ghrelin may serve as an overall modulator of a number of anorexigenic and orexigenic pathways directly and via the ARC neuronal network.

III. POMC Neurons of the NTS

A. THE INVOLVEMENT OF NTS POMC NEURONS IN THE REGULATION OF FOOD INTAKE

In recent years, while most of the attention in the field of energy homeostasis has been concentrated on the hypothalamus, the importance of the brainstem largely has been neglected. As such, comparatively little is known about the POMC neurons of the brainstem. An extensive network of fibers immunoreactive for POMC-derived peptides exists in the brainstem. This network includes immunoreactivity in the NTS, lateral reticular nucleus (A5-C1 groups), ventrolateral medulla (A1 cell group), and nucleus ambiguus. The only POMC cell bodies present in the brainstem are found in the commissural region of the NTS. Interestingly, POMC neurons have been shown to send a number of projections within the brainstem, particularly to the ventral lateral medulla and onto the spinal cord. However, studies involving lesioning of hypothalamic connections indicate that only 30–50% of the POMC-derived immunoreactivity in the brainstem originates from cell bodies in the commissural NTS (Palkovits *et al.*, 1987; Joseph and Michael, 1988). The remainder of the immunoreactivity seen is derived from projections from the hypothalamic POMC neurons. Hypothalamic POMC fibers innervate the brainstem via two distinct pathways: one that travels via the periaqueductal gray and the dorsomedial tegmentum to innervate the rostral NTS and lateral reticular nucleus (A5-C1 groups) and a second, more-dominant pathway through the ventrolateral tegmentum, believed to be the route of the majority of descending pathways, that innervates the rostral NTS, ventrolateral medulla (A1 cell group), nucleus ambiguus, and the descending spinal bundle. The MC4R is expressed at a number of these sites, indicating that hypothalamic POMC may exert some of its effects on energy homeostasis via receptors in the brainstem (Kishi *et al.*, 2003).

The work of Grill and colleagues has demonstrated that the melanocortin system of the brainstem plays a role in regulation of energy homeostasis. Administration of MTII, a synthetic melanocortin receptor agonist, or SHU9119, an MC3R and MC4R antagonist, into the fourth ventricle or directly into the dorsal vagal complex (DMX) causes a reduction in food intake in the case of MTII and an increase in food intake in the case of SHU9119 (Williams *et al.*, 2000). Following fourth ventricular administration, changes seen are comparable with those following administration of MTII or SHU9119 into the lateral ventricle (Grill *et al.*, 1998).

A potentially important consideration when studying the melanocortin system of the brainstem is the low level of expression of the endogenous antagonist AgRP. In contrast to the hypothalamus, where there is a relatively high level of expression of both the AgRP- and POMC-immunoreactive fibers and terminals

that project to identical areas of the brain (Bagnol *et al.*, 1999), the brainstem has few, if any, AgRP-immunoreactive cell bodies and receives limited terminals from the ARC. Given the lack of AgRP in the brainstem, it is unknown what regulates melanocortinergic tone in this area. It is unlikely that much regulation comes from AgRP projections from the hypothalamus but it is feasible that the system in this area is regulated by other mechanisms such as differences in POMC processing or post-translational modification (for a review of POMC processing, see Pritchard *et al.*, 2002).

B. EVIDENCE FOR THE EXISTENCE OF A REGULATORY NEURONAL NETWORK IN THE BRAINSTEM: COMPARISON WITH THE ARC NEURONAL NETWORK

Taking into account all the evidence to suggest the existence of a POMC-NPY/AgRP neuronal network in the ARC, it is interesting to speculate whether a similar network may be involved in modulating energy homeostasis via the brainstem. While the ARC network is able to respond to what are considered long-term as well as short-term modulators of energy homeostasis, is there any reason that a similar network should not exist in the NTS?

A number of similarities between the ARC and the NTS make the existence of such a network possible. First, they both lie in close anatomical proximity to a circumventricular organ, the median eminence in the case of the ARC and the area postrema in the case of the NTS. Although AgRP cell bodies are absent, the NTS contains cell bodies that show immunoreactivity for NPY and POMC-derived peptides. In common with the ARC, the NTS contains leptin receptors (Hakansson *et al.*, 1998; Mercer *et al.*, 1998). These neurons have been shown to be able to mediate the inhibitory effects of leptin on food intake and body weight gain following fourth ventricle administration (Grill *et al.*, 2002). In addition to causing activation of hypothalamic sites, peripheral administration of leptin activates neurons of the NTS, as measured by the expression of signal transducer and activator of transcription (STAT)-3 (Hosoi *et al.*, 2002; Munzberg *et al.*, 2003) or c-fos (Elmquist *et al.*, 1997). The NTS receives projections from numerous centers in the brain but is also the site at which vagal afferents terminate, making it an important site in mediating the vago-vagal reflex.

IV. The PVH as a Site of Integration of Hypothalamic and Brainstem Signals

The PVH is an important hypothalamic nucleus in the integration of autonomic and neuroendocrine information (for a review, see Palkovits, 1999). The PVH receives projections from a number of sites in the brain, including the ARC and NTS (Sawchenko and Swanson, 1983). Both melanocortin and NPY/

AgRP terminals are present in this area (Bagnol *et al.*, 1999). Indeed, NTS NPY neurons have been shown to project to the PVH (Sawchenko *et al.*, 1985). The PVH may serve as a site of integration of information from melanocortin, NPY/AgRP, and possibly other orexigenic and anorexigenic neurons via GABAergic interneurons. Evidence for this model comes from *in vivo* and electrophysiological studies. Direct injection of the melanocortin agonist MTII into the PVH results in a reduction in food intake. MTII at this site is able to functionally antagonize the orexigenic effects of NPY, indicating the potential for interactions between the two systems in the PVH *in vivo*. Electrophysiological studies have shown that neurons expressing NPY/AgRP and POMC have opposing actions on neurons of the medial PVH, potentiating and inhibiting GABAergic currents, respectively (Cowley *et al.*, 1999). Modulation of the central melanocortin system following intracerebroventricular administration of MTII, α-MSH, or AgRP activates a number of hypothalamic and extrahypothalamic sites in rats, including in the PVH (Thiele *et al.*, 1998; McMinn *et al.*, 2000; Hagan *et al.*, 2001). Indeed, the PVH seems to be a site that is activated following administration of a number of orexigenic and anorexigenic peptides, reinforcing the hypothesis that it is a key site for the integration of information regarding energy homeostasis (Hamamura *et al.*, 1991; Lambert *et al.*, 1995; Van Dijk *et al.*, 1996; Elmquist *et al.*, 1997; Edwards *et al.*, 1999; Lawrence *et al.*, 2002a).

The ARC melanocortin and NPY neurons innervate neurosecretory neurons of both the parvocellular and magnocellular subdivisions of the PVH (Piekut, 1985,1987; Liposits *et al.*, 1988; Sawchenko and Pfeiffer, 1988; Li *et al.*, 2000). The innervation of the thyrotrophin-releasing hormone (TRH) neurons has been particularly well characterized. Neurons containing immunoreactivity for both AgRP and NPY or α-MSH innervate TRH neurons in the PVH directly through projections from the ARC and indirectly via projections from the medial preoptic nucleus (Legradi and Lechen, 1999; Fekete *et al.*, 2000; Kawano and Masuko, 2000). These and numerous other anatomical studies highlight the importance of the PVH as a site for the integration of information from a number of systems and demonstrate how the regulation of energy balance may modulate other neuroendocrine processes such as the growth, reproductive, and stress axes (Schioth and Watanobe, 2002; Smith and Grove, 2002).

As discussed earlier, in addition to the PVH acting as a site of integration, the neurons of the ARC and NTS may communicate via direct projections between the two sites. ARC POMC neurons have been shown to project to a number of sites in the brainstem, including the NTS, periaqueductal gray, dorsal raphe nucleus, nucleus raphe magnus, nucleus raphe pallidus, locus coeruleus, parabrachial nucleus, nucleus reticularis gigantocellularis, and DMX (Chronwall, 1985; Sim and Joseph, 1991). Many of these regions contain MC4R mRNA (Mountjoy *et al.*, 1994; Kishi *et al.*, 2003), raising the possibility that these

receptors in the brainstem may be served by projections from the ARC neurons in addition to or in place of projections from the POMC neurons of NTS.

V. Summary

The evidence presented herein reinforces the importance of the POMC-NPY/AgRP system in the regulation of energy homeostasis and a number of other neuroendocrine processes. Localization of the POMC-NPY/AgRP neuronal networks in the ARC and possibly the NTS and the diversity of their neuronal projections from these sites make them well placed to respond to and coordinate both long-term adipostatic and short-term hunger/satiety signals between the periphery and the brain.

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Monogenic Human Obesity Syndromes

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ABSTRACT

Over the past decade, we have witnessed a major increase in the scale of scientific activity devoted to the study of energy balance and obesity. This explosion of interest has, to a large extent, been driven by the identification of genes responsible for murine obesity syndromes and the novel physiological pathways revealed by those genetic discoveries. We and others recently have identified several single-gene defects causing severe human obesity. Many of these defects have occurred in molecules identical or similar to those identified as a cause of obesity in rodents. This chapter will consider the human monogenic obesity syndromes that have been characterized to date and discuss how far such observations support the physiological role of these molecules in the regulation of human body weight and neuroendocrine function.

I. Introduction

The concept that mammalian body fat mass is likely to be regulated has its underpinning in experimental science going back over 50 years. The adipostatic theory of Kennedy (1953), which emerged in the 1950s, was based on his observations of responses of rodents to perturbations of food intake, together with the hypothalamic lesioning studies of Hetherington (Hetherington and Ranson, 1940) and Anand (Anand and Brobeck, 1951) and the parabiosis experiments of Hervey (1959). The subsequent emergence of several murine genetic models of obesity (Bray and York, 1971) and their study in parabiosis experiments by Coleman (1973) led to the consolidation of the concept that a circulating factor might be involved in mediation of energy homeostasis. However, it was not until the 1990s, when the precise molecular basis for the *agouti*, *ob/ob*, *db/db*, and *fat/fat* mouse emerged, that the molecular components of an energy balance regulatory network began to be pieced together (Leibel *et al.*, 1997). The use of gene-targeting technology has gone on to demonstrate the critical roles of certain other key molecules in that network, such as the melanocortin 4 receptor (MC4R) (Huszar *et al.*, 1997) and melanin-concentrating hormone (MCH) (Shimada *et al.*, 1998; Chen *et al.*, 2002).

A critical question raised by these discoveries is the extent to which these regulatory pathways are operating to control human body weight. Over the past few years, a number of novel monogenic disorders causing human obesity have emerged (Barsh *et al.*, 2000). In many cases, the mutations are found in components of the regulatory pathways identified in rodents. The importance of these human studies is several-fold. First, they established for the first time that humans can become obese due to a simple inherited defect. Second, it has been notable that in all cases, the principle effect of the genetic mutation has been to disrupt mechanisms regulating food intake. Third, some defects, though rare, are amenable to rational therapy. Fourth, although the physiological consequences of mutations in the same gene in humans and mice are frequently very similar, there are certain key interspecies differences in phenotype.

This review will describe certain recent advances in our understanding of single-gene defects causing human obesity. It will not consider genetic diseases such as Bardet-Biedl, Cohen's, Alstrom's, and Prader-Willi, where obesity is only one feature in a complex developmental disorder. Indeed, considerable advances have been made in the identification of genetic defects underlying many of these syndromes. However, in all cases, the link between the molecular defect and the clinical phenotype remains unclear (reviewed in O'Rahilly and Farooqi, "The Genetics of Obesity in Humans," at www.endotext.org.).

II. Congenital Leptin Deficiency

In 1997, we reported two severely obese cousins from a highly consanguineous family of Pakistani origin (Montague *et al.*, 1997). Both children had undetectable levels of serum leptin and were found to be homozygous for a frameshift mutation in the *ob* gene (ΔG133), which resulted in a truncated protein that was not secreted (Montague *et al.*, 1997; Rau *et al.*, 1999). We since have identified three more affected individuals from two other families (Farooqi *et al.*, 2002; I.S. Farooqi and S. O'Rahilly, unpublished observations) who are homozygous for the same mutation in the leptin gene. All the families are of Pakistani origin but are not known to be related over five generations. A large Turkish family who carries a homozygous missense mutation also has been described (Strobel *et al.*, 1998). All subjects in these families are characterised by severe, early-onset obesity and intense hyperphagia (Farooqi *et al.*, 1999,2002; Ozata *et al.*, 1999). Hyperinsulinaemia and an advanced bone age are also common features (Farooqi *et al.*, 1999,2002). Some of the Turkish subjects are adults with hypogonadotropic hypogonadism (Ozata *et al.*, 1999). Although normal pubertal development did not occur, there was some evidence of a delayed but spontaneous pubertal development in one person (Ozata *et al.*, 1999).

We demonstrated that children with leptin deficiency had profound abnormalities of T-cell number and function (Farooqi *et al.*, 2002), consistent with

high rates of childhood infection and a high reported rate of childhood mortality from infection in obese Turkish subjects (Ozata *et al.*, 1999). Most of these phenotypes closely parallel those seen in murine leptin deficiency (Table I). However, some phenotypes do not have as clear-cut parallels between human and mouse. Thus, while *ob/ob* mice are stunted (Dubuc and Carlisle, 1988), it appears that growth retardation is not a feature of human leptin deficiency (Farooqi *et al.*, 1999,2002), although abnormalities of dynamic growth hormone (GH) secretion have been reported in one human subject (Ozata *et al.*, 1999). *ob/ob* mice have marked activation of the hypothalamic-pituitary-adrenal axis, with very elevated corticosterone levels (Dubuc, 1977). In humans, abnormalities of cortisol secretion are, if present, much more subtle (Farooqi *et al.*, 2002). The contribution of reduced energy expenditure to the obesity of the *ob/ob* mouse is reasonably well established (Trayhurn *et al.*, 1977). In leptin-deficient humans, we found no detectable changes in resting or free-living energy expenditure (Farooqi *et al.*, 2002), although it was not possible to examine how such systems adapted to stressors such as cold. Ozata and colleagues reported abnormalities of sympathetic nerve function in leptin-deficient humans, consistent with defects in the efferent sympathetic limb of thermogenesis (Ozata *et al.*, 1999).

III. Response to Leptin Therapy

Recently, we reported the dramatic and beneficial effects of daily subcutaneous injections of leptin for reducing body weight and fat mass in three congenitally leptin-deficient children (Farooqi *et al.*, 2002). We have commenced therapy in two other children and seen comparably beneficial results (I.S. Farooqi and S. O'Rahilly, personal observations). All children showed a response to initial leptin doses that were designed to produce plasma leptin levels at only 10% of those predicted by height and weight (i.e., $\approx$ 0.01 mg/kg of lean body mass) (Farooqi *et al.*, 2002). The most-dramatic example of leptin's effects was in a 3-year-old boy, severely disabled by gross obesity (weight, 42 kg), who now weighs 32 kg (75th centile for weight) after 48 months of leptin therapy (Figure 1).

The major effect of leptin was on appetite, with normalisation of hyperphagia. Leptin therapy reduced energy intake during an 18-megajoule (MJ) *ad libitum* test meal by up to 84% (5 MJ ingested pretreatment vs 0.8 MJ post-treatment in the child with the greatest response) (Farooqi *et al.*, 2002). We were unable to demonstrate a major effect of leptin on basal metabolic rate or free-living energy expenditure (Farooqi *et al.*, 2002). However, as weight loss by other means is associated with a decrease in basal metabolic rate (BMR) (Rosenbaum *et al.*, 2002), the fact that energy expenditure did not fall in our leptin-deficient subjects is notable.

TABLE I
Phenotypes Associated with Leptin Deficiency in Rodents (ob/ob) and Humans

Phenotype	*ob/ob*	Human leptin deficiency
Total body weight	3X Normal	Mean BMI sds = 6.2
Body composition		
Fat mass	> 50%	Mean 57% of body weight
Lean mass	Decreased	Normal for age
Bone mineral content	Decreased	Normal for age
Food intake	Increased meal size	Increased meal size and frequency
Energy expenditure		
Body temperature	Decreased in response to cold	Normal in basal state
Basal metabolic rate	Decreased oxygen consumption	Appropriate for body composition
Physical activity	Reduced	Reduced
SNS activation	Basal decreased and refractory to cold exposure	Reduced in response to cold
Metabolic responses		
Diabetes	Fasting hyperglycaemia	Normoglycaemia
Hyperinsulinaemia	Severe; resistance to exogenous insulin	Appropriate for degree of obesity
T cell-mediated immunity	Decreased CD4 cells, reduced T-cell proliferation	Decreased CD4 cells, reduced T-cell proliferation
Neuroendocrine function		
Reproductive	Hypogonadotropic hypogonadism	Hypogonadotropic hypogonadism
Thyroid	Hypothalamic and ?peripheral effects	Mild hypothalamic hypothyroidism
Growth	Stunted	Normal linear growth and IGF-1 levels
Adrenal	Corticosterone excess	Normal cortisol and ACTH levels

[Abbreviations: BMI, body mass index; SNS, sympathetic nervous system; IGF-1, insulin-like growth factor-1; ACTH, corticotropin.]

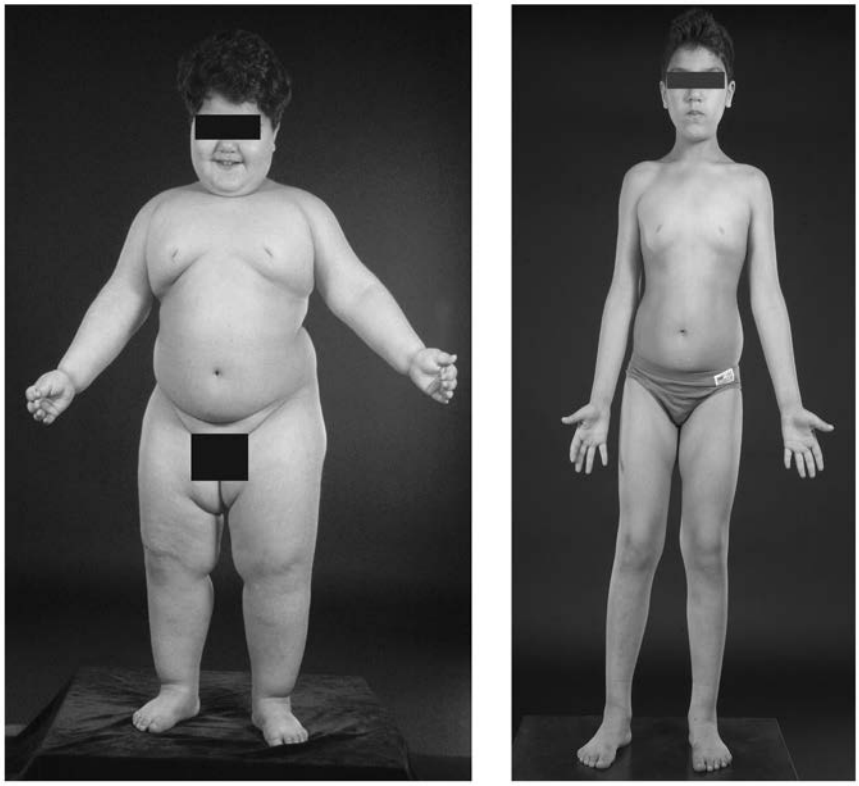

FIG. 1. Effects of recombinant human leptin treatment in a child with leptin deficiency.

Leptin administration permitted progression of appropriately timed pubertal development in the single child of appropriate age and did not cause the early onset of puberty in the younger children (Farooqi *et al.*, 2002). Free thyroxine and thyroid-stimulating hormone (TSH) levels, although in the normal range before treatment, consistently increased at the earliest post-treatment time point and subsequently stabilized at this elevated level (Farooqi *et al.*, 2002). These findings are consistent with evidence from animal models that leptin influences thyrotropin-releasing hormone (TRH) release from the hypothalamus (Legradi *et al.*, 1997; Nillni *et al.*, 2000; Harris *et al.*, 2001) and from studies illustrating the effect of leptin deficiency on TSH pulsatility in humans (Mantzoros *et al.*, 2001).

Throughout the trial of leptin administration, weight loss continued in all subjects, albeit with refractory periods that were overcome by increases in leptin dose (Farooqi *et al.*, 2002). The families in the United Kingdom harbour a

mutation that leads to a prematurely truncated form of leptin; thus, wild-type (WT) leptin is a novel antigen to them. Thus, all subjects developed antileptin antibodies after ≈ 6 weeks of leptin therapy, which interfered with interpretation of serum leptin levels and, in some cases, were capable of neutralising leptin in a bioassay (Farooqi *et al.*, 2002). These antibodies are the likely cause of refractory periods that occur during therapy. The fluctuating nature of the antibodies probably reflects the complicating factor that leptin deficiency is itself an immunodeficient state (Lord *et al.*, 1998; Matarese, 2000). Leptin administration leads to a change from the secretion of predominantly Th2 to Th1 cytokines, which may directly influence antibody production. Thus far, we have been able to regain control of weight loss by increasing the dose of leptin.

IV. Does a Heterozygous Phenotype Exist?

The major question with respect to the potential therapeutic use of leptin in more-common forms of obesity relates to the shape of the leptin dose/response curve. We have shown clearly that at the lower end of plasma leptin levels, raising leptin levels from undetectable to detectable has profound effects on appetite and weight (Farooqi *et al.*, 2002). Heymsfield and coworkers (1999) administered supraphysiological doses (i.e., 0.1–0.3 mg/kg body weight) of leptin to obese subjects for 28 weeks. On average, subjects lost significant weight, but the extent of weight loss and the intersubject variability has led many to conclude that the leptin resistance of common obesity cannot be usefully overcome by leptin supplementation, at least when administered peripherally. However, on scientific rather than pragmatic grounds, it is of interest that there was a significant effect on weight, suggesting that plasma leptin can continue to have a dose/response effect on energy homeostasis across a wide plasma concentration range.

To test this hypothesis, we studied the heterozygous relatives of our leptin-deficient subjects. Serum leptin levels in the heterozygous subjects were found to be significantly lower than expected for % body fat and they had a higher prevalence of obesity than seen in a control population of similar age, sex, and ethnicity (Farooqi *et al.*, 2001). Additionally, % body fat was higher than predicted from height and weight of the heterozygous subjects, compared to control subjects of the same ethnicity (Farooqi *et al.*, 2001). These findings closely parallel those in heterozygous *ob*/+ and *db*/+ mice (Coleman, 1979; Chung *et al.*, 1998). These data provide further support for the possibility that leptin can produce a graded response in terms of body composition across a broad range of plasma concentrations.

All heterozygous subjects had normal thyroid function and appropriate gonadotropins, normal development of secondary sexual characteristics, and normal menstrual cycles and fertility, suggesting that low leptin levels are

sufficient to preserve these functions (Farooqi *et al.*, 2001). This is consistent with the data of Ioffe and colleagues, who demonstrated that several of the neuroendocrine features associated with leptin deficiency were abolished in low-level leptin transgenic mice, which were fertile with normal corticosterone levels (Ioffe *et al.*, 1998). However, these low-level leptin transgenic mice still exhibited abnormal thermoregulation in response to cold exposure and had mildly elevated plasma insulin concentrations, suggesting that different thresholds exist for the various biological responses elicited by changes in serum leptin concentration and that these could be reversed by leptin administration (Ioffe *et al.*, 1998).

Our findings in the heterozygous individuals have implications for treating common forms of obesity. While serum leptin concentrations correlate positively with fat mass, considerable interindividual variation exists at any particular fat mass. Leptin is inappropriately low in some obese individuals. The relative hypoleptinemia in these subjects may be contributing actively to their obesity and may be responsive to leptin therapy (Ravussin *et al.*, 1997). Heymsfield and colleagues (1999) found no relationship between baseline plasma leptin levels and therapeutic response. However, study subjects were not preselected for relative hypoleptinemia. A therapeutic trial in a subgroup of subjects selected for disproportionately low circulating leptin levels would be of great interest.

V. Leptin Receptor Deficiency

A mutation in the leptin receptor has been reported in one consanguineous family with three affected subjects (Clement *et al.*, 1998). Affected individuals were found to be homozygous for a mutation that truncates the receptor before the transmembrane domain. The mutant receptor ectodomain is shed from cells and circulates bound to leptin. The phenotype has similarities to leptin deficiency. Leptin receptor-deficient subjects were of normal birthweight but exhibited rapid weight gain in the first few months of life, with severe hyperphagia and aggressive behaviour when food was denied (Clement *et al.*, 1998). Basal temperature and resting metabolic rate were normal, cortisol levels were in the normal range, and all individuals were normoglycaemic, with mildly elevated plasma insulin similar to leptin-deficient subjects. Leptin receptor-deficient subjects had some unique neuroendocrine features not seen with leptin deficiency. Evidence of mild growth retardation in early childhood, with impaired basal and stimulated GH secretion and decreased insulin-like growth factor (IGF)-1 and IGF-binding protein (BP)3 levels, alongside features of hypothalamic hypothyroidism in these subjects, suggest that loss of the leptin receptor results in a more-severe neuroendocrine phenotype than loss of leptin itself (Clement *et al.*, 1998). The most-likely explanation for this is that the leptin

receptor has some constitutive ligand-independent activity that is lost when the leptin receptor is absent but retained in leptin deficiency.

VI. Pro-opiomelanocortin

Two unrelated obese German children have been reported with homozygous or compound heterozygous mutations in pro-opiomelanocortin (POMC) (Krude *et al.*, 1998). Both children were hyperphagic and developed early-onset obesity, presumably due to impaired melanocortin signaling in the hypothalamus (Krude *et al.*, 1998). Presentation was in neonatal life, with adrenal crisis due to isolated corticotrophin (ACTH) deficiency. (POMC is a precursor of ACTH in the pituitary.) The children had pale skin and red hair from the lack of MSH function at MC1Rs in the skin (Krude *et al.*, 1998). Three additional subjects with homozygous or compound heterozygous complete loss-of-function mutations of the POMC gene have been described (Krude and Gruters, 2000). Recently, several groups have identified a heterozygous missense mutation (Arg236Gly) in POMC that disrupts the dibasic amino acid processing site between β-MSH and β-endorphin (Echwald *et al.*, 1999; Del Giudice, 2001a; Challis *et al.*, 2002). This results in an aberrant β-MSH/β-endorphin fusion peptide, which binds to MC4R with an affinity identical to that of α- and β-MSH but has a markedly reduced ability to activate the receptor (Challis *et al.*, 2002). Therefore, this cleavage site mutation in POMC may confer susceptibility to obesity through a novel molecular mechanism. Mutations affecting this processing site have been reported in obese children from several different populations and therefore may be a relatively common contributor to early-onset obesity.

VII. Prohormone Convertase 1 Deficiency

Further evidence for the role of the melanocortin system in human body weight regulation comes from the description of a 47-year-old woman with severe childhood obesity, abnormal glucose homeostasis, very low plasma insulin, but elevated levels of proinsulin, hypogonadotropic hypogonadism, and hypocortisolaemia associated with increased levels of POMC (Jackson *et al.*, 1997). She was found to be a compound heterozygote for mutations in prohormone convertase 1 (PC1), which cleaves prohormones at pairs of basic amino acids, leaving C-terminal basic residues that are excised by carboxypeptidase E (CPE) (Jackson *et al.*, 1997). We recently identified a child with severe early-onset obesity who was compound heterozygote for complete loss-of-function mutations in PC1 (Jackson *et al.*, 2003). Although inability to cleave POMC is a likely mechanism for obesity in these patients, PC1 cleaves a number of other neuropeptides in the hypothalamus, including glucagon-like-peptide 1, which may influence feeding behaviour. The phenotype of these subjects is very similar

to that of the CPE-deficient *fat/fat* mouse (Naggert *et al.*, 1995), implying that this part of the pathway may be important in controlling body weight in humans. To date, however, no humans with CPE defects have been described.

VIII. Human MC4R Deficiency

Of the five known melanocortin receptors, MC4R has been most-closely linked to control of energy balance in rodents (Yeo *et al.*, 2000). Mice homozygous for a deleted MC4R become severely obese; heterozygotes have body weights intermediate between WT and homozygote null animals (Huszar *et al.*, 1997). In 1998, two groups reported heterozygous MC4R mutations in humans that were associated with dominantly inherited obesity (Vaisse *et al.*, 1998; Yeo *et al.*, 1998). Since then, heterozygous mutations in MC4R have been reported in obese humans from various ethnic groups (Hinney *et al.*, 1999; Farooqi *et al.*, 2000; Vaisse *et al.*, 2000).

We have studied over 500 severely obese probands and found that $\approx$ 5–6% have pathogenic MC4R mutations that are nonconservative in nature, not found in control subjects from the background population, and that co-segregate with obesity in families (Farooqi *et al.*, 2003). MC4R deficiency represents the most-commonly known monogenic cause of human obesity. Some studies have observed a lower prevalence, which may be explained by the differing prevalence in certain ethnic groups. However, it is more likely to reflect the later onset and reduced severity of obesity of the subjects in these studies (Jacobson *et al.*, 2002). While we found a 100% penetrance of early-onset obesity in heterozygous probands, others have described obligate carriers who were not obese (Vaisse *et al.*, 2000). Given the large number of potential influences on body weight, it perhaps is not surprising that both genetic and environmental modifiers will have important effects in some pedigrees. Indeed, we have now studied six families in which the probands were homozygotes. In all of these, the homozygotes were more obese than the heterozygotes (Farooqi *et al.*, 2003). Interestingly, in these families, some heterozygous carriers were not obese. This may reflect ethnic-specific effects, as all these families were of Indo origin. Taking into account all these observations, co-dominance, with modulation of expressivity and penetrance of the phenotype, is the most-appropriate descriptor for the mode of inheritance. This finding is supported by the pattern of inheritance of obesity seen in heterozygous and homozygous MC4R knockout (KO) mice (Huszar *et al.*, 1997).

We now have studied over 70 MC4R mutant carriers in our Clinical Research Facility. In addition to increased fat mass, MC4R mutant subjects have increased lean mass that is not seen in leptin deficiency (Farooqi *et al.*, 2003). Linear growth of these subjects is striking, with affected children having a height standard deviation score (SDS) of $+2$, compared to population standards (mean

height SDS of other obese children in our cohort = + 0.5) (Farooqi *et al.*, 2003). MC4R-deficient subjects also have higher levels of fasting insulin than age, sex, and BMI SDS-matched children (Farooqi *et al.*, 2003). The accelerated linear growth and disproportionate early hyperinsulinaemia are consistent with observations in the MC4R KO mouse (Fan *et al.*, 2000).

Affected subjects are objectively hyperphagic but not as severely as seen with leptin deficiency (Farooqi *et al.*, 2003). Of particular note is the finding that the severity of receptor dysfunction seen in *in vitro* assays can predict the amount of food ingested at a test meal by the subject harbouring that particular mutation (Figure 2). One notable feature of this syndrome is that the severity of many of the phenotypic features appears to partially ameliorate with time. Thus, obese adult mutation carriers report less-intense feelings of hunger and are less hyperinsulinaemic than children with the same mutation (I.S. Farooqi and S. O'Rahilly, personal observations). We have studied in detail the signaling properties of many of these mutant receptors. This information should help advance the understanding of structure/function relationships within the receptor (Yeo *et al.*, 2003). Importantly, we have been unable to discover evidence for dominant negativity associated with these mutants, which suggests that MC4R mutations are more likely to result in a phenotype through haploinsufficiency (Yeo *et al.*, 2003).

MC4R mutations appear to be the most-common monogenic cause of obesity thus far described in humans. Maintenance of this reasonably high disease frequency is likely to be due partly to the fact that obesity is expressed in heterozygotes and no evidence exists of any apparent effect of the mutations on reproductive function.

IX. Other Possible Monogenic Syndromes

Identification of a leptin-regulated melanocortin pathway has provided a molecular and neuroanatomical link between peripheral signals and central nervous system (CNS) circuits but leaves open the question of how these melanocortin signals produce downstream effects on appetite, energy expenditure, and neuroendocrine function. Mutations in a number of other genes have been found in association with severe obesity in a small number of individuals; however, the significance of these findings often remains unclear.

Two groups have found missense mutations in the cocaine- and amphetamine-regulated transcript (CART), a neuropeptide implicated in the control of feeding behaviour in rodents. We identified a Ser66Thr mutation in heterozygous form in two unrelated U.K. probands. However, this did not co-segregate with obesity in family studies (Challis *et al.*, 2000). In an Italian study, the Leu34Phe CART mutation was identified in the heterozygous state in a 10-year-old obese

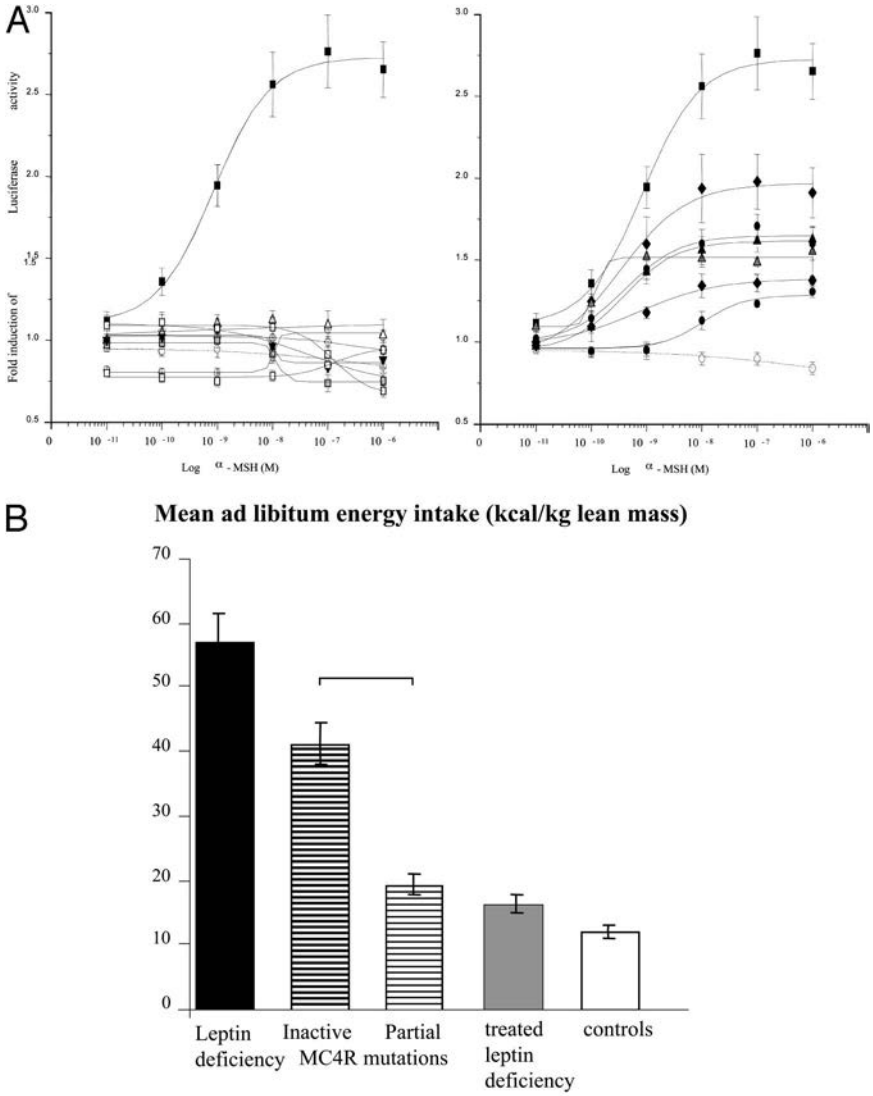

FIG. 2. Genotype-phenotype correlations in human melanocortin 4 receptor (MC4R) deficiency. (A) Cyclic adenosine monophosphate (cAMP) response to alpha-melanocyte-stimulating hormone (α-MSH) in transiently transfected human embryonic kidney (HEK)293 cells for mutant MC4Rs, indicating complete (left panel) or partial (right panel) loss of function *in vitro*. (B) *Ad libitum* food intake at an 18-megajoule (MJ) test meal for leptin-deficient subjects and for MC4R-deficient subjects with complete/partial loss-of-function mutations.

boy and a number of obese family members but no functional data were provided (del Giudice *et al.*, 2001b).

Holder and colleagues (2000) studied a girl with early-onset obesity and a balanced translocation between 1p22.1 and 6q16.2. The child displayed an aggressive, voracious appetite. The obesity was thought to be due to increased energy intake, as measured energy expenditure was normal. The translocation did not appear to affect any transcription unit on 1p but it disrupted the SIM1 gene on 6q (Holder *et al.*, 2000). The *Drosophila* single-minded (sim) gene is a regulator of fruit fly neurogenesis. In the mouse, Sim1 is expressed in the developing kidney and CNS and is essential for formation of the supraoptic and paraventricular (PVN) nuclei, which express the MC4R (Michaud *et al.*, 2001). It thus could be hypothesized that haploinsufficiency of SIM1, possibly acting upstream or downstream of MC4R in the PVN, was responsible for severe obesity in this patient.

It is of note that in all the genetic syndromes thus far described, the major physiological perturbance appears to be in appetite and energy intake. One possible exception is the description of three German subjects with mutations in the N terminus of the nuclear hormone receptor peroxisome proliferator-activated receptor gamma (PPARγ), an important determinant of adipogenesis (Ristow *et al.*, 1998). Receptors containing this mutation are more-powerful inducers of adipogenesis when transfected into cultured cells. Unfortunately, however, no information is available regarding the co-segregation of this mutation with obesity in pedigrees. Since this mutation appears to be unique to the original population, it has not been possible to test this issue in independent families.

X. Summary

Several monogenic forms of human obesity have been identified by searching for mutations homologous to those causing obesity in mice. Although such monogenic obesity syndromes are rare, the successful use of murine models to study human obesity indicates that substantial homology exists across mammalian species in the functional organisation of the weight regulatory system. More importantly, identification of molecules that control food intake has generated new targets for drug development in the treatment of obesity and related disorders. These considerations indicate that an expanded ability to diagnose the pathophysiological basis of human obesity will have direct applications to its treatment. A more-detailed understanding of the molecular pathogenesis of human obesity ultimately may guide treatment of affected individuals.

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