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

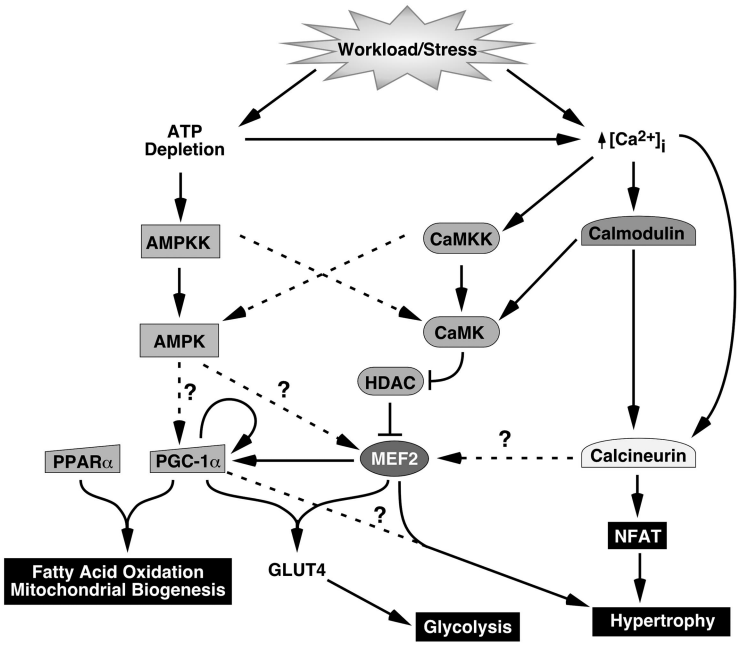

FIG. 1. Myocyte enhancer factor 2 (MEF2) participates in transducing adenosine triphosphate (ATP) and calcium signals to mediate hypertrophic gene expression. In response to increased workload or stress (e.g., inefficient coupling of excitation and contraction, inefficient contraction due to genetic 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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