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 (Elmqquist 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; Hallas 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-terminals 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.
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, pre mammillary 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, Niltni EA, Hollenberg AN, Bjerbaek 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.]
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.,
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 Aβ/α 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 (Scarpas 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 (Morrison 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
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 (Muioio 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/threonine 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, α1 and α2,
we detected activation of only α2 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 α2 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 α2
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).
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 (Minokoshi and Kahn, 2003).
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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