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

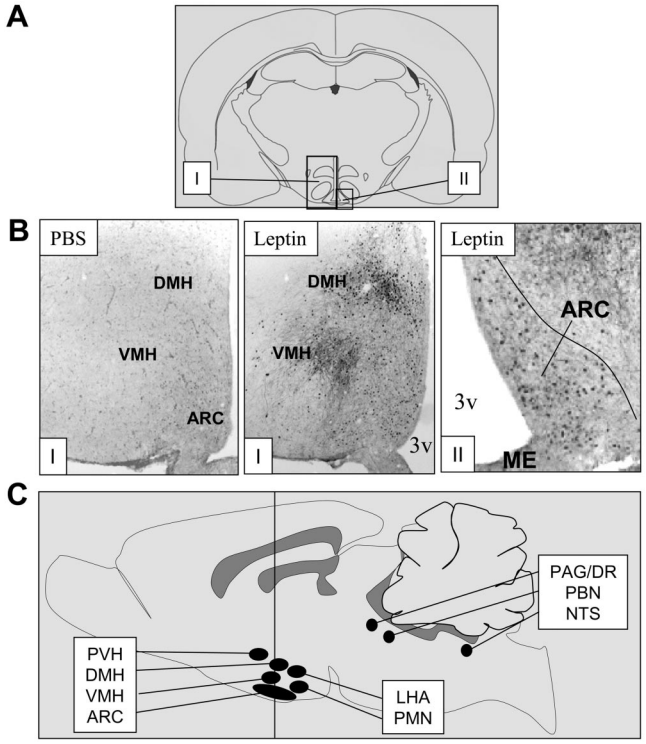

FIG. 1. Leptin-responsive regions in the rodent brain. (A) Schematic drawing of a coronal section of the rat brain. "I" and "II" depict hypothalamic nuclei that are highly leptin responsive. (B) The three microphotographs show immunohistochemistry (IHC) for phosphorylated signal transducer and activator 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.]

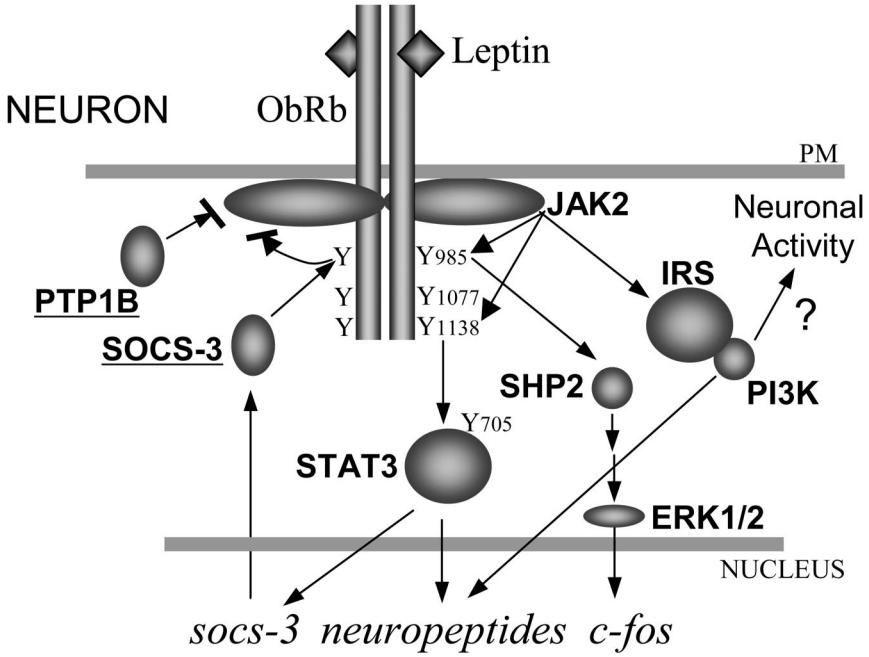

FIG. 2. Model for neuronal leptin receptor (ObRb) signaling. Activation of ObRb by leptin leads to increased activity of intracellular janus activating kinase (JAK)2 kinases associated with membrane-proximal regions of the receptor. JAK2 phosphorylates a number of cellular substrates, including Y985 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).

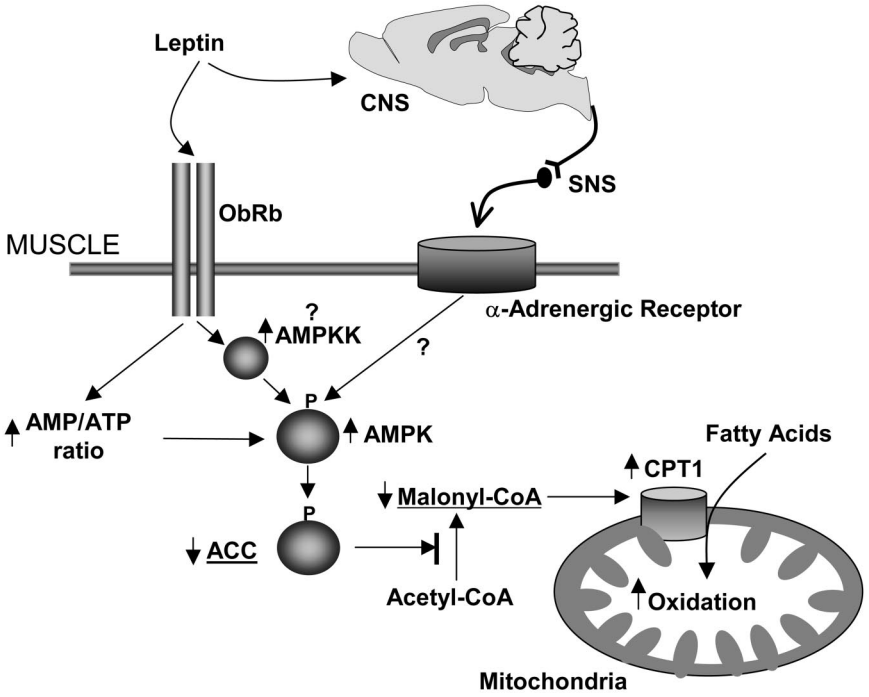

FIG. 3. Model for regulation of adenosine monophosphate-activated protein kinase (AMPK) and fatty acid oxidation by leptin in skeletal muscle. Leptin activates AMPK via two distinct mechanisms. One is rapid and occurs directly through leptin receptors expressed on muscle cells. The other occurs with 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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