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Review article

# Leptin signalling

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**Abstract**

The identification of leptin as the product of the obesity (*ob*) gene has been followed by extensive research identifying a wide spectrum of physiological effects elicited by this adipose-derived hormone. These effects are mediated via a family of cytokine-like receptor isoforms distributed in both the central nervous system and periphery. The signal transduction pathways regulated by leptin are diverse and include those characteristic of both cytokine and growth factor receptor signalling. This review describes the structure and function of leptin receptors and summarizes recent progress that has been made in characterizing the increasing number of signal transduction pathways regulated by leptin. © 2002 Elsevier Science Inc. All rights reserved.

*Keywords:* Obesity; JAK/STAT; SOCS; SHP2; PI 3-kinase; MAP kinase; PKC; Phosphodiesterase; Nitric oxide

## 1. Introduction

### 1.1. Leptin physiology

Leptin, the product of the obesity (*ob*) gene [1], is a 16-kDa circulating hormone that is best known as a regulator of food intake and energy expenditure via hypothalamic-mediated effects [2]. It is now appreciated that this hormone has many additional effects, often as a consequence of direct peripheral actions. These include angiogenesis, hematopoiesis, lipid and carbohydrate metabolism and effects on the reproductive, cardiovascular and immune systems, and these are reviewed elsewhere [3–8]. Thus, changes in plasma leptin concentrations or in leptin action have important and wide-ranging physiological implications. Lack of leptin in rodents (as evidenced by *ob/ob* mice) or functional leptin receptor (*db/db* mice and *fa/fa* rats) results in obesity and many associated metabolic complications including insulin resistance [9]. Rodent models of lipoatrophy, which lack leptin, also exhibit insulin resistance [9]. In obese humans, an increased fat mass correlates with increased plasma leptin levels [10] and is also associated with the development of insulin resistance [11].

Adipocytes are the major source of leptin synthesis and secretion, other sources include placenta, stomach and skeletal muscle [12]. Leptin expression can be induced by obesity, insulin, TNF-α and glucocorticoids and is negatively regulated by fasting, β-adrenergic agonists and thiazolidinediones [12]. Leptin acts via transmembrane receptors (obR), which show structural similarity to those of the cytokine family [13]. Recent years have seen intensified analysis of the signalling events that ensue subsequent to leptin binding to its receptor, and these are summarized here. Given the pathophysiological implications of alterations in normal leptin action, it is likely that a detailed understanding of the signalling pathways regulated by leptin will allow therapeutic exploitation and application to disease states.

### 1.2. Leptin receptors: isoforms and structure

Leptin receptors are the product of the diabetes (*db*) gene, first identified by expression cloning techniques [14]. The gene is alternatively spliced to produce at least six isoforms (obRa–obRf). Each of obRa–obRd and obRf are identical in their extracellular and transmembrane domain (Fig. 1). The extracellular domain of the leptin receptor consists of 816 amino acids and has two cytokine-like binding (Trp-Ser-X-Ser-Trp) motifs and a fibronectin Type III domain [14,15]. Analysis of mutant receptor constructs showed that only the second putative leptin binding domain mediates leptin

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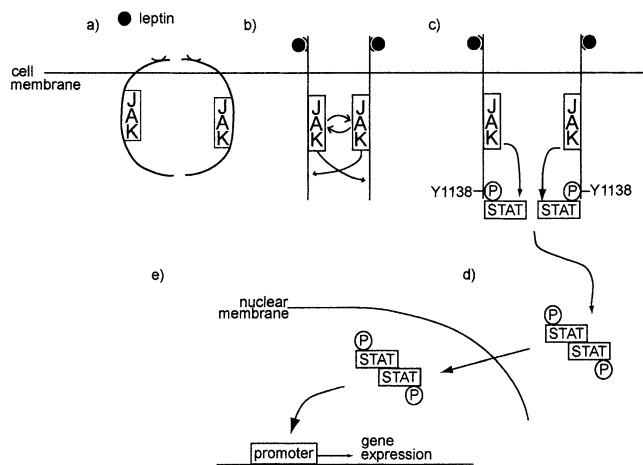


Fig. 1. Schematic illustration of the mechanism of JAK/STAT activation by long-form leptin receptor (obRb). JAK2 (Janus-family tyrosine kinase 2) associates with obR via the Box 1 motif common to all receptor isoforms (except obRe). obR may form dimers under nonstimulated conditions (a) yet the conformation of the receptor is likely to prevent close proximity of JAKs and/or function of the kinase. Upon ligand binding (b) the number of receptor dimers does not change appreciably, yet, a conformational change appears to allow juxtaposition of JAKs, which then become activated and can tyrosine phosphorylate other JAKs and tyrosine residues on obRb. Importantly, phosphorylation of Y1138 on obRb (c) allows association of STAT (signal transducers and activators of transcription), which then become substrates for receptor-associated JAKs. Phosphorylation of STAT is followed by (d) their dissociation from obRb and the formation of active dimers, which translocate to the nucleus (e) to regulate gene expression.

binding and receptor activation, the first being unnecessary [16]. The affinity of leptin binding to this site is in the nanomolar range. The soluble leptin receptor isoform (obRe) does not play a direct role in leptin signalling but is likely important in determining the amount of leptin in the circulation [17]. Indeed, the proportion of leptin circulating in the free and protein-bound form may be an important physiological determinant of leptin action [18]. Other isoforms possess a transmembrane domain, which is comprised of 23 amino acids. One 'long form' of leptin receptor containing a long intracellular domain exists (obRb), whereas obRa, obRc, obRd and obRf all possess short intracellular domains. The intracellular domain of obRb is approximately 306 amino acids (longer in human than in mouse) and in the short forms is 32–40 amino acids [14].

The mutation in *db/db* mice resulting in the obese phenotype alters splicing of the long receptor isoform to yield a truncated receptor similar to obRa [19]. The phenotype of these mice suggest that the long form of leptin receptor plays an important role in regulation of food intake, energy expenditure and endocrine function. Indeed, rescue of the obesity/diabetes phenotype in *db/db* mice can be achieved by neuronal specific transgenic expression of the long-form leptin receptor [20]. Furthermore, several other obese models are associated with mutations in the leptin receptor gene [21,22]. The intracellular domain of all isoforms contains an identical 29 amino acid sequence containing a 'Box 1' Janus-family tyrosine kinase (JAK) binding domain in the juxtamembrane region while obRb also contains a 'Box 2' motif and signal transducers and activators of transcription (STAT) binding sites. Human long-form leptin receptor contains five tyrosines, and each may be associated with the activation of distinct downstream signalling pathways [13,23]. Although obRb is traditionally viewed as the 'signalling isoform', there is clear evidence that short receptor isoforms are capable of signalling (see below) and indeed show divergent signalling capacities [24–26].

Both long and short receptor isoforms are capable of forming homodimers in the absence of ligand, and the extent of this association does not change appreciably upon ligand stimulation [27–29]. Thus, dimerization does not appear to play a regulatory role in the activation of the receptor. Nevertheless, the formation of dimers between leptin receptors appears to be necessary for signalling [30]. Each obR binds leptin in a 1:1 stoichiometry, thus resulting in the formation of a tetrameric receptor/ligand complex [29]. The conformational change in receptor structure induced by the formation of this complex is deemed to be critical in activating leptin signalling [16]. Interestingly, obR heterodimers cannot be detected in the absence of ligand, even in cells where different receptor isoforms have been cotransfected to high levels [28,29]. However, heterodimers do become detectable in the presence of leptin [28]. Importantly, leptin receptors do not appear to form heterodimers with structurally similar cytokine receptors [27].

Expression of obRb is highest in hypothalamus [31], yet, it is also found in many peripheral tissues at lower levels [32–34]. The obRa is expressed fairly ubiquitously and represents the major isoform of many peripheral tissues [31,35]. Other short receptor isoforms tend to be expressed at low levels [31]. It should be noted that examination of obR isoform expression in various tissues by RT–PCR has often been inconsistent.

### *1.3. Leptin binding and internalization*

Both determination of the crystal structure of leptin and NMR studies demonstrated that leptin adopts a cytokine fold similar to the short-helix subfamily of cytokine folds [36,37]. This is in keeping with the leptin receptor exhibiting features typical of the cytokine receptor family. Members of the cytokine receptor family are generally internalized upon ligand binding via clathrin-coated vesicles into early endosomes with the receptor being processed for degradation or efficiently recycled back to the cell surface. Indeed, the dynamic process of receptor internalization and recycling or degradation is likely an important point of regulation for leptin signalling. Somewhat surprisingly, it has been estimated that, under resting conditions, only 5–25% of total cellular leptin receptor isoforms are located at the cell surface, with the majority residing in intracellular pools [38]. Recently, Lundin et al. [39] constructed a GFP-chimera

with both long and short leptin receptor isoforms and showed using fluorescent conjugated leptin that the ligand–receptor complex internalizes together. Analysis of receptor deletion mutants in CHO cells confirmed that both ObRa and obRb mediate leptin internalization and degradation by lysosomes and showed that a domain between amino acids 8 and 29 of the intracellular domain determined this process [40]. Two studies have shown that leptin internalization and down-regulation of surface receptors was greater for the obRb isoform [38,40]. In the first, COS cells overexpressing four human leptin receptor isoforms with varying cytoplasmic sequences showed that the long form (HLR-274) was most efficiently down-regulated in response to prolonged leptin exposure [38]. This was confirmed by studies of receptor down-regulation and $^{125}$I-leptin internalization in CHO cells overexpressing obRa or obRb [40]. These observations may be at least partly explained by studies using surface biotinylation in pulse chase experiments to detect receptor isoform recycling in HEK293 cells which show that the short receptor isoform recycles to the cell surface faster [39]. It is therefore possible that preferential down-regulation of obRb signalling may have important implications in human obesity (hyperleptinemia) via causing somewhat selective leptin resistance. However, there was no change in total amount of cell surface receptor in CHO cells transfected to overexpress the long-form leptin receptor after 20 h of leptin treatment [41].

### *1.4. Signalling pathways regulated by leptin*

#### *1.4.1. JAK/STAT pathway*

The JAK/STAT signalling pathway plays a critical role in mediating the effects of many cytokines and growth factors [42]. Leptin receptors possess no intrinsic tyrosine kinase activity, and many signalling events are dependent on association with kinases such as JAK2. Ligand binding to obR and subsequent oligomerization allow juxtaposition of JAKs, which can then phosphorylate and activate each other, the leptin receptor or other substrates (Fig. 1). In response to leptin, the long form but not short form of receptor becomes tyrosine phosphorylated. Indeed, coexpression of JAK2 and obRb enhanced tyrosine phosphorylation of obRb in response to leptin [43]. Thus, JAKs are receptor-associated protein tyrosine kinases, which are utilized by leptin receptors to phosphorylate the receptor itself as well as targets such as STAT proteins. These transcription factors are recruited to activated obR/JAK complexes via SH2 and SH3 domains and become activated upon tyrosine phosphorylation. Activation involves dissociation from the receptor and the formation of homo- or heterodimers that translocate to the nucleus and interact with specific DNA elements in the promoters of target genes to regulate gene expression [44].

It has been demonstrated that the sequence surrounding Y1138 (YXXQ) in obRb represents a consensus STAT3-binding motif [45]. Studies using hybrid erythropoietin/leptin receptors confirmed that Y1138 becomes phosphorylated in response to ligand and controls subsequent activation of STAT3 [23]. Thus, while the short isoforms can bind JAK, they are not capable of STAT signalling [46,47]. A comprehensive analysis of the ability of leptin to phosphorylate and activate various STAT proteins was performed in COS cells by coexpression of the receptor long form with each of STAT1–6. In this study, ligand–receptor binding activated STAT3, STAT5 and STAT6 but not STAT1, STAT2 or STAT4 [47]. STAT3 activation has since been shown in RINm5F cells [48] and STAT3 and STAT5 DNA binding increased in CACO-2 (human small intestine epithelial), WRL68 (human liver) and BRIN-BD11 (rat insulinoma) cells [34,49,50]. In vivo studies using intraperitoneal injection of leptin to normal mice showed that STAT3 phosphorylation occurred rapidly in epididymal fat cells [51]. Intravenous leptin injection increased STAT1 and STAT3 phosphorylation in rat adipose tissue and STAT3 phosphorylation in liver and muscle [52] and pancreatic islets [48]. Peripheral but not central administration of leptin activated STAT1 and STAT3 via obRb in adipose tissue [32]. Interestingly, intravenous leptin increased STAT5 DNA binding (leading to induction of c-*fos*) in jejunum of normal and *ob/ob* but not *db/db* mice [34] and STAT1 phosphorylation occurred only in epididymal fat cells of *ob/ob* but not wild-type mice [51]. The effect of leptin on the recently identified serine phosphorylation of STAT proteins remains to be defined [53].

#### *1.4.2. Src-like homology 2 (SH2) domain containing protein tyrosine phosphatase (SHP-2)*

The often misleading impression that kinases are dominant regulators of intracellular signalling is being challenged by a growing appreciation of the importance of phosphatases such as SHP-2 [54]. SHP-2 is a tyrosine phosphatase that is activated by a conformational change induced upon interaction of one of its SH2 domains with a tyrosine phosphorylated ligand. Using phosphorylated fragments of obRb as bait, SHP-2 was found to bind to Y985 [55] (Fig. 2). Furthermore, in 293T cells, SHP-2 binding to obRb was prevented upon mutation of Y985 [55]. Stimulation of hybrid erythropoietin/obRb receptors also confirmed that phosphorylation of Y985 controls subsequent association with and activation of SHP-2 [23]. Biacore analysis of peptides corresponding to tyrosine containing intracellular domains of human leptin receptor has subsequently shown that phosphotyrosines 986 (equivalent to rodent Y985) and 974 can bind to SHP-2 [56]. This study also suggests that SHP-2 binds to these residues on the leptin receptor via different sites [56]. Binding of SHP-2 to Y985 occurs via a SH2 domain and results in the activation of the enzyme, whereas Y974 likely associates with the active site of the enzyme [56]. From these studies, a model was proposed whereby SHP-2 binds to phosphotyrosine 986, becomes activated and can thus down-regulate

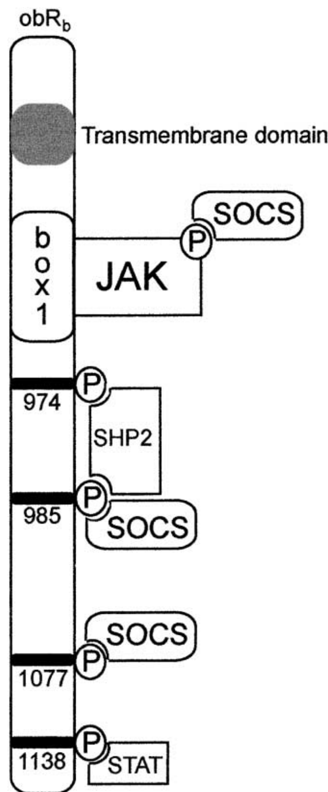


Fig. 2. Potential roles of phosphotyrosines on obRb in leptin signalling. The long-form leptin receptor (obRb) contains a JAK (Janus-family tyrosine kinase)-binding Box 1 motif, as well as four tyrosine residues. When phosphorylated, these interact with SH2 domain containing proteins such as SOCS (suppressor of cytokine signalling), SHP-2 (Src-like homology 2 (SH2) domain containing protein tyrosine phosphatase) and STAT (signal transducers and activators of transcription) as indicated (see text for details).

further tyrosine phosphorylation-dependent leptin signalling such as STAT3 activation [56,57].

### 1.4.3. Mitogen-activated protein kinases (MAPK)

The extracellular regulated kinase (ERK) members of the MAPK family are serine/threonine kinases of 44 and 42 kDa, are activated by a wide range of stimuli and are components of the well-defined Ras/MAPK signalling cascade [58] (Fig. 3). Intravenous leptin injection increased MAP kinase phosphorylation three to fourfold in rat adipose and liver tissue [52]. MAP kinase activation in response to leptin has also been observed in RINm5F (rat pancreatic β) cells [48] and $C_2C_{12}$ muscle cells [59]. Studies using hybrid receptors containing the extracellular erythropoietin and intracellular obRb domain demonstrated that tyrosine 985 becomes phosphorylated upon ligand stimulation and controls subsequent activation of ERK [23]. This process involves recruitment of SHP-2 and its phosphorylation, then association with Grb-2 [23]. In keeping with this theory, expression of a catalytically inactive mutant of SHP-2 in COS cells was shown to block ERK phosphorylation in response to leptin [60]. Furthermore, studies in COS cells coexpressing ERK1 and obRa or obRb suggested that the magnitude of ERK phosphorylation in response to leptin was significantly greater in cells transfected with obRb [43]. Recent studies overexpressing mutant leptin receptors in CHO cells showed that two independent pathways lead to ERK phosphorylation in response to leptin [60]. One required phosphorylation of tyrosine 985 of obRb, whereas the other did not require the intracellular domain of the receptor [60]. The former involved tyrosine phosphorylation of SHP-2, and both pathways required SHP-2 phosphatase activity, suggesting both a catalytic and adapter role for SHP-2 [60]. Consequences of ERK activation by leptin are many and include induction of specific gene expression, activating c-*fos* but not affecting STAT-dependent suppressors of cytokine signalling (SOCS)-3 mRNA accumulation.

Another member of the MAP kinase family, p38 MAP kinase is activated by a diverse range of stimuli including osmotic stress, heat shock, ultraviolet light and cytokines [61]. Few studies have addressed the role of p38 MAP kinase in leptin signalling. However, recently, it was shown that treatment of human mononuclear cells with leptin increased p38 MAP kinase phosphorylation [62]. Another study in L6 muscle cells suggested that leptin did not itself alter p38 MAP kinase phosphorylation but reduced insulin-stimulated p38 MAP kinase phosphorylation [63]. The NH2-terminal c-Jun kinase/stress-activated protein kinase (JNK) is activated by many cytokines, growth factors and stressors [64]. Activation of JNK by

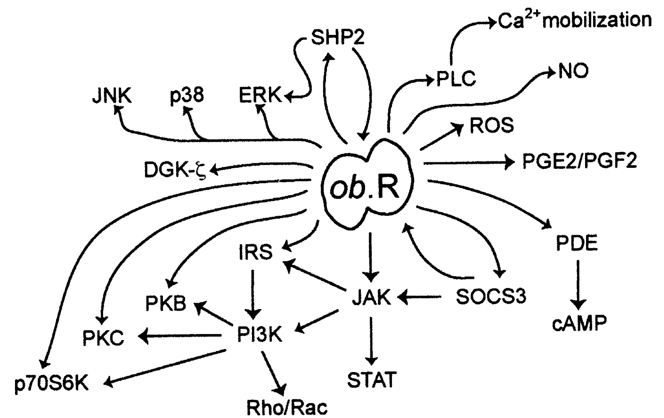


Fig. 3. Signalling pathways that have been demonstrated to be regulated by leptin. This diagram depicts the potential regulation of various signalling pathways, including JNK (NH2-terminal c-Jun kinase), p38 (p38 MAP kinase), ERK (extracellular regulated kinase), SHP-2 (Src-like homology 2 (SH2) domain containing protein tyrosine phosphatase), PLC (phospholipase C), NO (nitric oxide), DGK-ζ (diacylglycerol kinase zeta), PGE2/PGF2 (prostaglandins E2/F2), PDE (phosphodiesterase), cAMP (cyclic AMP), SOCS-3 (suppressor of cytokine signalling 3), JAK (Janus-family tyrosine kinase), STAT (signal transducers and activators of transcription), PI3K (phosphatidylinositol 3-kinase), IRS (insulin receptor substrates), PKB (protein kinase B, also known as Akt), PKC (protein kinase C), p70S6K (ribosomal p70 S6 kinase) and ROS (reactive oxygen species) by the leptin receptor (obR). Note that arrows (a) are intended to depict regulation rather than imply activation (see text for details) and (b) do not imply a direct association, as in some cases, multiple potential intermediates are as yet uncharacterized or excluded.

leptin has been demonstrated in human umbilical vein endothelial cells (HUVEC) [65].

### 1.4.4. Suppressors of cytokine signalling (SOCS)

SOCS proteins have been shown to negatively regulate cytokine-induced signalling in both a temporal and quantitative manner [66,67]. Many cytokines such as interleukin-6, growth hormone and erythropoietin induce expression of one or more SOCS isoforms, thus forming a homologous negative feedback loop, as well as creating the potential for crosstalk with signalling initiated by other ligands [41,67,68]. SOCS proteins contain a central SH2 domain, which may allow these proteins to inhibit signalling by binding to phosphorylated JAK proteins or direct interaction with tyrosine phosphorylated receptors [67]. SOCS proteins may also play a role in ubiquitination of signalling proteins or complexes and their direction for proteasome digestion [69].

Peripheral leptin administration to *ob/ob* but not *db/db* mice induced SOCS-3 mRNA expression in hypothalamus but did not increase mRNA levels of CIS, SOCS-1 or SOCS-2 [70]. Similar results are observed in CHO cells expressing the long-form leptin receptor, and this effect was shown to be mediated via stimulation of the JAK/STAT pathway [41,71]. The ability of SOCS-3 expression to inhibit leptin-stimulated phosphorylation of JAK2 and ERK suggests a role for SOCS-3 as a mediator of negative feedback control on proximal leptin signalling events [41,68]. The functional significance of increased SOCS-3 expression on leptin signalling was demonstrated by a 75% reduction in the lipopenic effect of leptin upon transgenic overexpression of SOCS-3 in islets [72]. A more detailed molecular analysis of the functional consequence of SOCS-3 induction by leptin was recently performed in HEK293 cells where SOCS3 was shown to bind directly to Y985 of obRb [68]. As expected, mutation of Y985 did not alter short-term leptin-induced STAT3 signalling, which is instead dependent on Y1138 [23]. Interestingly, mutation of Y985 did increase leptin-induced STAT3 activation at longer times ($>6$ h) [68]. This effect is explained by an inability of SOCS-3 to bind to phosphorylated Y985 and mediate feedback inhibition. The functional importance of SOCS-3 binding to Y985 of leptin receptor is in agreement with previous observations showing that SOCS-3 binds to JAK proteins with very low affinity [73]. However, at very high levels, SOCS-3 can inhibit leptin signalling in cells expressing Y985 mutant leptin receptors [68]. It has been suggested that this may be explained by binding to JAK or to Y1077 of obRb [74].

SOCS-3 levels have been reported to be increased in rats exhibiting obesity and leptin resistance, rats with acquired or diet-induced obesity and in *ob/ob* mice [70–72]. However, another study showed no change in SOCS-3 levels in rats with diet-induced obesity [75]. It is tempting to speculate that changes in SOCS-3 expression might lead to the phenomenon of leptin resistance in human obese individuals. A recent study has investigated the possible role of SOCS-3 in mediating the inhibitory effect of fatty acids on leptin signalling. However, no change in SOCS-3 levels were found following prolonged treatment of rat insulinoma (BRIN-BD11) cells with fatty acids [50]. Whether this is true for all leptin-responsive tissues is as yet unclear, and it is possible that other hormonal changes occurring in obese individuals might regulate SOCS-3 levels. Furthermore, tightly controlled regulation of SOCS-3 expression may play a role in the emerging phenomenon of selective, particularly central versus peripheral, leptin resistance. In addition, although SOCS-3 has no effect on insulin receptor tyrosine kinase activity, it binds to the insulin receptor at Y960 and prevents STAT5B binding [76]. The ability of SOCS proteins to inhibit mitogenic signalling by other growth factor receptors also suggests the potential for leptin-induced SOCS-3 to have a broader role than simply inhibition of further leptin signalling [67]. Increased expression of this potentially negative regulator of insulin signalling in response to leptin may therefore represent one possible mechanism whereby leptin induces resistance of distinct insulin signalling pathways to stimulation by the hormone and future studies to address this question should prove interesting.

### 1.4.5. Phosphatidylinositol (PI) 3-kinase and insulin receptor substrate (IRS) proteins

PI 3-kinase activity is regulated by a wide spectrum of ligands, in particular growth factors such as insulin [77]. PI 3-kinase is activated upon binding of its regulatory subunit to tyrosine-phosphorylated proteins, which induce a conformational change allowing the activation of its catalytic subunit. The function of PI 3-kinase is to add a phosphate to the 3′ position of the inositol ring of phosphatidylinositols, thus allowing switching of protein kinase-dependent cascades to lipid-dependent signalling cascades [77]. PI 3-kinase products typically stimulate protein kinases such as Akt (protein kinase B (PKB)) and atypical protein kinase C (PKC) isoforms [77]. An important role of PI 3-kinase in signalling is therefore likely to be targeting kinase activation to particular intracellular compartments.

Regulation of PI 3-kinase activity by leptin was first demonstrated in HepG2 cells [78]. Stimulation of PI 3-kinase by leptin has since been implicated in the regulation of phosphodiesterase (PDE)3B in pancreatic β cells [79], Na,K-pump in fibroblasts [80], invasiveness of colon epithelial cells [81], hormone-sensitive lipase activity in J774.2 macrophages [82], glucose uptake in $C_2C_{12}$ cells [83] and actin reorganization and activation of $K_{ATP}$ channels in CRI-G1 insulinoma cells [84,85]. Nevertheless, caution in interpreting many studies using PI 3-kinase inhibitors is advisable given potential nonspecific effects of such compounds. For example, PI 3-kinase inhibitors attenuate the ability of leptin to activate $K_{ATP}$ channels in CRI-G1 insulinoma cells, yet, leptin did not increase total cellular PI(3,4,5)$P_3$ levels [84]. It is, of course, possible

that leptin may stimulate specific isoforms of PI 3-kinase and lead to generation of specific PI 3-kinase products. In addition, the ability of leptin to stimulate PI 3-kinase is likely tissue specific since leptin had no effect on PI 3-kinase activity in Fao cells [86] or L6 muscle cells [63].

The mechanism underlying the stimulation of PI 3-kinase by leptin often involves phosphorylation of IRS proteins. In some [24,43,78,81], but not all [86], cells leptin can induce phosphorylation of IRS proteins, often via the activation of JAK. For example, tyrosine phosphorylation of IRS-1 was increased by leptin in HEK293 cells coexpressing the long or short form of leptin receptor, JAK2 and IRS1 [43]. This may represent the mechanism of PI 3-kinase activation by leptin, and it was shown that leptin increased PI 3-kinase activity in $C_2C_{12}$ cells via a JAK2- and IRS-2-dependent mechanism [24]. Another study in $C_2C_{12}$ cells showed increased PI 3-kinase activity in response to leptin associated with p85$\alpha$, but not IRS-1, immunoprecipitates [83]. Leptin-stimulated PI 3-kinase activity has also been measured in both phosphotyrosine and JAK2 immunoprecipitates [80,81].

Interestingly, most insulin-dependent actions involve the activation of PI 3-kinase [87], making this an important potential point of crosstalk between the insulin and leptin signalling pathways. However, leptin did not affect the ability of insulin to stimulate PI 3-kinase activity associated with phosphotyrosine immunoprecipitates and IRS-1 or IRS-2 phosphorylation in muscle cells [63]. In addition, the magnitude of PI 3-kinase stimulation in response to leptin tends to be less than that seen with insulin. For example, intravenous injection of leptin to rats increased IRS1- and IRS2-associated PI 3-kinase activity by 2- and 1.7-fold over basal, respectively, whereas stimulation by insulin was 10- and 6-fold, respectively [52].

#### *1.4.6. Protein kinase B (PKB, also called Akt)*

PKB is a serine/threonine kinase that plays an important role in many cellular processes including cell survival and carbohydrate metabolism [88]. The activation of PKB involves binding of its PH domain to D3-phosphorylated phosphoinositides and subsequent phosphorylation of PKB on T308 and S473 [88]. An ability of leptin to stimulate PKB was suggested by results showing that treatment of Fao cells with leptin for 10 or 15 min increased Akt phosphorylation [86]. However, in keeping with the results discussed above for PI 3-kinase, the magnitude of this effect was very small and much less than that produced by insulin. Intravenous administration of leptin to rats for 3 min did not change Akt activity in adipocytes, despite stimulating small increases in the association of PI 3-kinase with IRS1 and IRS2 [52]. Similarly, treatment of L6 muscle cells with leptin for up to 30 min did not cause an increase in Akt phosphorylation [63]. These results suggest that leptin has little or no effect on Akt. Studies in primary rat hepatocytes, which express little or no long form of leptin receptor, may explain this apparent lack of effect [89]. In these cells, leptin (30 min) increased IRS1 and IRS2 phosphorylation and associated PI 3-kinase activity without a corresponding increase in Akt activity or phosphorylation [89]. However, an increase in Akt activity and phosphorylation was induced by leptin in these cells if pretreated for 15 min with glucagon [89]. This study suggests that the ability of leptin to stimulate Akt is dependent on high intracellular cAMP levels, which the authors speculate, may inhibit a phosphatase that allows leptin to increase Akt phosphorylation. Therefore, studies to date suggest that experimental conditions and cell type will play an important role in determining the effect of leptin on Akt activation. Interestingly, there is no additive or inhibitory effect of leptin on insulin-stimulated Akt phosphorylation in studies to date, suggesting that crosstalk between these two hormones does not occur at this level [63,86].

#### *1.4.7. Protein kinase C (PKC)*

The serine/threonine kinase family of PKC isoforms have been implicated in a wide range of cellular effects [90]. Leptin appears to have both stimulatory and inhibitory effects on PKC. Insulin release from pancreatic islets of *ob/ob* mice in response to stimulation of PKC was decreased by leptin [91]. In another study, the ability of leptin to decrease glucose-induced insulin secretion was correlated with an ability to decrease the activity of $Ca^{2+}$-dependent PKC [92]. These observations may at least partly explain the decreased insulin secretion often observed in vivo after leptin administration [93]. However, this latter study [92] contrasts with others [79,94], since it suggested that leptin does not decrease insulin secretion induced by agents that increase intracellular cAMP levels. Nevertheless, there is evidence to suggest that leptin action in pancreatic islets may inhibit the PKC-regulated component of the phospholipase C (PLC)–PKC signalling system that normally elicits insulin secretion. Conversely, activation of PKC by leptin is implied by the ability of PKC inhibitors (GF109203X and Go6976) to block leptin-stimulated invasiveness of colon epithelial cells [81].

#### *1.4.8. Cyclic AMP PDE*

Cyclic AMP activates protein kinase A and plays a pivotal role in the crosstalk between many signalling systems [95,96]. Metabolism of cAMP, and cGMP, is controlled by a family of PDE enzymes [97]. Several years ago, leptin was shown to decrease cAMP levels in pancreatic $\beta$-cells via the activation of PDE3B [79]. One important physiological consequence of this response to leptin was an inhibition of GLP-1-stimulated insulin secretion [79]. In contrast, the ability of leptin to decrease glucose-induced insulin secretion was not blocked by the PDE3 inhibitor milrinone [98]. A similar ability of leptin to increase cAMP PDE3B activity was observed in primary rat hepatocytes, which express little or no long form of receptor [89]. Normal leptin receptor, but not the mutated form present in *fa/fa* rats, has been shown to lead to changes in PDE2

expression and activity in brown fat [99]. Therefore, regulation of PDE isoforms by leptin is apparent in various tissues and further work will help understand the mechanism and consequences of this action.

### *1.4.9. Nitric oxide (NO)*

NO interacts with soluble guanylate cyclase to evoke a surprisingly large array of physiological responses [100]. The widespread implications of NO have become even more pronounced with the realization that NO can also interact with molecular oxygen and superoxide radicals to produce reactive nitrogen species, which can then modify proteins, lipids and nucleic acids [101]. Many studies of NO production depend on the use of inhibitors of NO production such as the competitive inhibitor of NO synthase (NOS), L-$N^{G}$-nitro-methyl-arginine (L-NAME), and are therefore implied rather than direct proof. The last few years have seen many studies focused on the effects of leptin on NO production or expression of NOS' and the physiological implications of these effects, with contrasting observations being reported.

Two independent studies provide the most direct confirmation that leptin increases NO production. First, intravenous administration of leptin to Wistar rats increased serum NO concentrations by up to 90%, an effect not observed in *fa/fa* rats [102]. It was subsequently shown that preincubation of endothelial cells with leptin enhanced NO production as measured using 4,5-diaminofluorescein-2 diacetate staining and measurement of nitrate and nitrite concentrations [103]. A traditional pharmacological approach also produced evidence of an ability of leptin to cause NO release from endothelial cells [104]. In this study, the ability of leptin to relax rat arterial rings preconstricted with phenylephrine was blocked by the removal of the endothelium, L-NAME or L-arginine [104]. Interestingly, these studies also demonstrated that the concentration of $Cl^{-}$ plays an important role in determining the effect of leptin on NO release [104]. An ability of leptin infusion into rats to induce NO production was implied by the fact that L-NAME markedly amplifies the tachycardia caused by hyperleptinemia and slightly enhances the chronic renal hemodynamic and hypertensive effects of leptin [105]. Further studies using L-NAME suggested that leptin can modulate cardiac contraction by increasing NO production in ventricular myocytes [106]. Therefore, a significant mass of evidence suggests that leptin contributes positively to the NO system. This may have important physiological implications since it is known that leptin can increase sympathetic nerve activity to peripheral tissues and is therefore likely to increase blood pressure [107]. However, the attractive suggestion that leptin may also release NO to oppose sympathetically induced vasoconstriction has recently been refuted in studies where leptin perfusion had no effect on changes in renal or hind limb blood flow induced by sympathetic trunk stimulation [108]. The inability of L-NAME to modify the effect of leptin on whole body blood pressure also suggests that NO release by leptin may not play a physiologically important role [109]. In contrast, the ability of leptin to increase blood pressure [107] may be at least partly attributed to an increase in endothelium-derived hyperpolarizing factor (EDHF), as was shown in response to leptin in preconstricted rat aortic or mesenteric rings [109]. Finally, it should be noted that L-NAME inhibited leptin-stimulated glucose uptake in skeletal muscles, but had no effect in adipose tissue [110], that the ability of leptin to stimulate corticotrophin-releasing hormone (CRH) secretion from rat hypothalamic cells was not blocked by L-NAME [111] and that leptin did not stimulate NO production in rat hypothalamic cells [111]. It is therefore important to keep in perspective that NO is a potential, but certainly not a ubiquitous mediator of leptin action.

Leptin may at least partly influence NO concentrations by playing an important role in the regulation of NOS levels. Central administration of leptin to rats prevented the fasting-induced reduction of nNOS mRNA in the brain [112], suggesting that leptin normally maintains a 'tonic' level of nNOS expression. This may be because nNOS plays an important physiological role in leptin action, as suggested by the observation that the ability of leptin to reduce food intake was severely blunted in nNOS knockout mice [113]. In addition, the ability of leptin to accelerate gastric ulcer healing in rats was reduced by L-NAME since leptin acted at least partially by inducing expression of cNOS and iNOS [114]. However, contrasting effects of leptin on NOS expression have again been reported. Intracerebroventricular or intraperitoneal injection of leptin for 5 days decreased nNOS activity in mice [113]. A decrease in NOS was also observed upon subcutaneous leptin injection BY 37% in the hypothalamus and 69% in the brown adipose tissue (no effect in the white adipose tissue), and this correlated with a decrease in weight of *ob/ob* mice [115]. This is in agreement with the fact that *ob/ob* mice display elevated NOS levels in hypothalamus [116], yet, it is in contrast to the results discussed above [112,113].

### *1.4.10. Rho family proteins and the actin cytoskeleton*

Rho family GTPases are involved in many cellular processes such as apoptosis and regulation of the actin–myosin cytoskeleton [117]. The latter effect has wide-ranging implications in cell signalling, cell–cell or cell–matrix adhesion and cell migration [118]. Remodelling of the actin cytoskeleton may play a role in promoting invasiveness of colon epithelial cells in response to leptin since the effect of leptin was potentiated by constitutively active RhoA and decreased by dominant-negative RhoA, Rac1 or the p110$\alpha$ catalytic subunit of PI 3-kinase [81]. In addition, activation of $K_{ATP}$ channels by leptin in CRI-G1 insulinoma cells was blocked by the actin filament stabilizer phalloidin or PI 3-kinase inhibitors, suggesting that leptin-induced disassembly of actin filaments mediated the effect on $K_{ATP}$ channels [84,85].

*1.4.11. Others*

There are several other signalling proteins regulated by leptin that should be mentioned here. Two studies have demonstrated an ability of leptin to increase production of reactive oxygen species (ROS) in human umbilical vein endothelial cells (HUVEC) or bovine aortic endothelial cells (BAEC) [65,119]. These may be relevant in the pathogenesis of cardiovascular complications associated with obesity, such as atherosclerosis, and demand further investigation. Leptin stimulates prostaglandin E2 and prostaglandin F2$\alpha$ production in rat hypothalamic cells [111]. Yeast two hybrid and protein precipitation experiments identified binding of diacylglycerol kinase-$\zeta$ interacts via ankyrin repeats with the cytoplasmic domain of obRb (not obRa) [120]. The p90 ribosomal protein S6 and p70S6K are activated by leptin in human mononuclear cells [62]. Finally, leptin activates phospholipase C and voltage-dependent $Ca^{2+}$ channels, most likely L-type and N-type $Ca^{2+}$ channels, an effect that might underlie leptin-induced catecholamine secretion [121].

## 2. Conclusions

Our appreciation of the physiological significance of leptin has grown from a hormone that regulated food intake to one that has a surprisingly large number of diverse effects. Associated with this is a need for leptin to regulate many signalling pathways, often in a tissue-specific manner, as summarized above. The pathophysiological role played by leptin in many disease states, in particular, Type 2 diabetes, often occurs as a result of its ability to influence the effect of other endogenous hormones. With this in mind, the ability of leptin-regulated signals to crosstalk with other signalling systems promises to be a revealing area of research. For example, the ability of SOCS-3 to associate with insulin receptor might represent a mechanism whereby leptin induces insulin resistance and Type 2 diabetes.

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