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Leptin activates AMP-activated protein kinase in hepatic cells via a JAK2-dependent pathway

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Abstract

AMP-activated protein kinase (AMPK) plays a key role in the regulation of energy homeostasis within the individual cell. Recent reports have suggested that leptin, an adipocyte-secreted hormone, phosphorylates AMPK in skeletal muscle directly. However, little is known about the interaction between leptin signaling and AMPK activation. Here, we report that the leptin-induced phosphorylation of AMPK was detected in Huh7 cells expressing long form leptin receptor (OBRb) as well as short form leptin receptor (OBRa). In addition, we demonstrate that AMPK activation does not require the phosphorylation of either Tyr-985 or Tyr-1138 within the OBRb and may occur via a STAT3-independent signaling pathway. We also show that Huh7 cells expressing OBRb and SOCS3 (inhibitor of JAK2) resulted in a marked reduction of AMPK activation in response to leptin. These findings suggest that the activation of JAK2, but not STAT3, may play a critical role in leptin-induced AMPK activation in Huh7 cells.

Keywords: Leptin; Leptin receptor; JAK2; STAT3; AMPK

Leptin, the product of the ob gene [1], is an adipocyte-derived hormone, acts via the long form of the leptin receptor on specific nuclei of the hypothalamus [2] to regulate food intake and energy expenditure [3–5]. Besides the effects on the central nervous system, leptin may have direct effects on peripheral target tissues, such as adipocytes [6–8], skeletal muscle [6,9], and hepatocytes [6] either through OBRa or OBRb. It has been reported that leptin stimulates the phosphorylation and activation of AMPK in skeletal muscle, both directly through skeletal muscle but also indirectly through the hypothalamic-sympathetic nervous system axis [10]. AMPK is a heterotrimeric enzyme consisting of one catalytic subunit (α) and two regulatory subunits (β and γ) [11,12]. Homologues of all three subunits have been identified from yeast to humans [13]. AMPK is an intracellular energy sensor that is activated through Thr172 phosphorylation within the catalytic α-subunit in response to increased AMP and ATP depletion [14]. Once activated, AMPK phosphorylates down-stream proteins, leading to a switching off of ATP-consuming pathways, such as fatty acid biosynthesis, by the phosphorylation of acetyl-CoA carboxylase (ACC) and the switching on of ATP-regenerating pathways by stimulating fatty acid oxidation [13]. AMPK acts as a regulator of energy balance, not only within the individual cell, but also within the whole body [15]. Of clinical interest, AMPK might play possible roles in the treatment of metabolic diseases, such as type 2 diabetes and obesity [15]. Two adipocyte-derived hormones, leptin [10], and adiponectin [16], which themselves play key parts in regulating energy homeostasis, have both been demonstrated to activate AMPK in skeletal muscle. At present, little is known, however, about how leptin signaling mediates the activation of AMPK. Leptin acts via specific receptors, which belong to the family of the class I cytokine receptor family [17]. At least six different alternatively spliced isoforms have been cloned [18]. One isoform, OBRb, appears to be the dominant signaling species of the receptor [18–20]

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and is highly expressed in a limited number of sites, including key nuclei in the hypothalamus [18]. Another isoform, OBRa, is more widely expressed in the kidney, lung, and choroid plexus [17,18]; whereas, its roles remain to be defined. Although lacking the ability to activate STAT signaling [19,20], OBRa has been shown to be capable of increasing the transcription of early genes, *fos* and *jun*, in the cells stably expressing OBRa and the activation of JAK kinase in transient transfection models [21]. Therefore, we transiently transfected two representative forms of OBR, OBRa or OBRb, of the leptin receptor into Huh7 cells and to determine the signaling potential of OBR isoforms to activate AMPK. Furthermore, we investigated whether the cellular events following OBR activation play a role in AMPK activation upon leptin stimulation.

Materials and methods

Cell culture and transient transfection. All reagents for cell culture were obtained from Life Technologies (Gaithersburg, MD). Huh7 cells were grown in RPMI 1640 supplemented with 5% fetal calf serum, 50 U/ml penicillin G, and 50 μg/ml streptomycin at 37 °C under 5% CO_2. The cDNA encoding OBRa and OBRb was generated as described previously [21]. Two mutant OBRb isoforms (L985 and S1138) were generated using a site-directed mutagenesis kit from TaKaRa Bio Inc. (Shiga, Japan). The JAK2 expression vector was a generous gift of Dr. Rikiro Fukunaga (Osaka University, Osaka, Japan). Human SOCS3 expression vector was provided by Dr. Yoshimura (Kyushu University, Fukuoka, Japan) [22]. For Western blotting experiments, cells were grown in 6-well plates and transfected using 7 μl of LipofectAMINE (Life Technologies) and 2 μg DNA per well. At 48 h post-transfection, including 15–18 h of serum deprivation, the cells were incubated for 15 min with 10 nM of recombinant mouse leptin (R&D Systems, Minneapolis, MN). Cells were then washed three times with ice-cold phosphate-buffered saline (PBS), dissolved in CelLytic-M mammalian cell lysis/extraction reagent (Sigma, Saint Louis, MO), and centrifuged at 14,000 rpm at 4 °C for 20 min.

Western blotting. The cell lysates were mixed with SDS sample buffer. The mixture was boiled for 5 min before the samples were applied to SDS–PAGE. After electrophoresis, the proteins were transferred to a nitrocellulose membrane (Schleicher&Schuell, Keene, NH) and blocked by incubation for 10 min at room temperature with StartingBlock blocking buffer (PIERCE, Rockford, IL). The membranes were incubated with anti-phospho-JAK2 (Upstate Biotechnology, Lake Placid, NY), STAT3, or AMPK (Cell Signaling Technology, Beverly, MA) antibody in 5% milk in Tris-buffered saline/0.1% Tween 20 (TBST) overnight at 4 °C. After washing 3 times at room temperature, the membranes were reacted with horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Amersham Pharmacia Biotech, Piscataway, NJ) in 1% milk in TBST for 60 min at room temperature, and again swashed 6 times with TBST. The targeted proteins were detected using the SuperSignal West Pico chemiluminescent substrate or SuperSignal West Femto maximum sensitivity substrate (PIERCE, Rockford, IL) following the instructions of the manufacturer. The membranes were stripped in Restore Western Blot Stripping Buffer (PIERCE, Rockford, IL) according to the manufacturer's instructions. The membranes were then reprobed with anti-JAK2, STAT3 (Upstate Biotechnology, Lake Placid, NY) or AMPK (Cell Signaling Technology, Beverly, MA) or SOCS3 (Santa Cruz Biotechnology, Santa Cruz, CA) antibody.

Results and discussion

The highly conserved AMPK, a family of heterotrimeric (α, β, γ) serine/threonine kinases, plays an important role in regulating energy metabolism in response to ATP depletion and signaling to downstream effector molecules that restore ATP and regulate cell growth and apoptosis [13–15]. Recent study has demonstrated that leptin selectively stimulates phosphorylation and activation of the catalytic α-subunit of AMPK in skeletal muscle [10]. Early activation of AMPK occurs by a direct effect of leptin on muscle, whereas, the mechanism by which leptin mediates the activation of AMPK has been unclear. In order to determine the signaling potential of OBR isoforms to activate AMPK, two representative forms of OBR, OBRa, or OBRb, of the leptin receptor were transiently transfected into Huh7 cells. As previously reported [23], since Huh7 cells did not have enough endogenous JAK2 protein to be detected using Western blotting, both JAK2 and OBRa or OBRb were transiently co-transfected into the cells. In order to examine the effects of forced JAK2 expression on AMPK activation, both empty vector and OBRa or OBRb were also transiently co-transfected into Huh7 cells in parallel experiments. The transfected cells were stimulated with or without 10 nM of leptin or 1 μM of oligomycin. In response to leptin, the transfection of OBRa or OBRb into Huh7 cells resulted in the phosphorylation of STAT3 and occurred in OBRb but not in OBRa. However, the phosphorylation of AMPK was detected in OBRb as well as OBRa, as determined by Western blotting using anti-phospho-AMPK-α (Thr172) antibody (Fig. 1A and B). The degree of JAK2 tyrosine phosphorylation in response to leptin appears to be correlated with the relative amount of JAK2 protein. However, leptin-induced AMPK activation in cells co-transfected with JAK2 and OBRa or OBRb was almost equal to that in cells co-transfected with empty vector and OBRa or OBRb (Fig. 1A and B). As reported earlier [23], similar results have been shown in leptin-induced STAT3 phosphorylation in the cells expressing OBRb (Fig. 1B). The expression of endogenous JAK2 might be sufficient for transducing signals downstream. As expected, oligomycin, an inhibitor of mitochondrial ATP synthesis, phosphorylated AMPK but did not affect the phosphorylation of either JAK2 or STAT3 in the cells. OBRb isoform contains several sequence elements that are required for the JAK/STAT signaling pathway [17–20,24]. Only OBRb is capable of the regulation of food intake, energy expenditure, [3–5] and endocrine function [25]. On the other hand, OBRa has no tyrosine residues or STAT binding sites in the intracellular domain. OBRa appears unable to stimulate signaling pathways that are activated by OBRb [17,18,21]. However, OBRa has been reported to capable of signaling to IRS-1 and mitogen-activated protein kinase via JAK2 in vitro systems [21]. Fig. 1A and B show that OBRa also possesses the capability of AMPK activation via JAK2 independently of receptor and STAT3 phosphorylation. However, it has been reported that the intravenous administration of leptin to db/db mice, which express OBRa but not OBRb, failed to stimulate the phosphorylation of AMPK in skeletal muscle [10]. The difference in the result is likely due to the

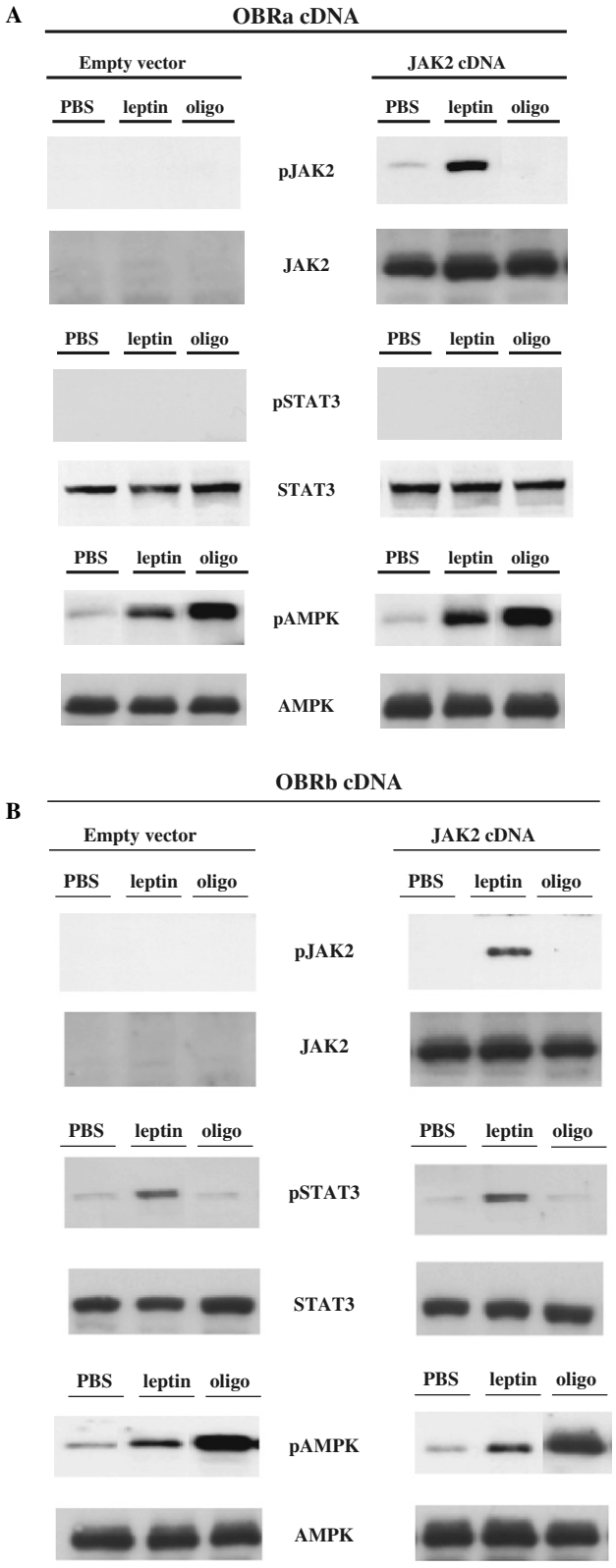

Fig. 1. Phosphorylation of AMPK in Huh7 cells expressing OBRa or OBRb. Huh7 cells were co-transfected with empty vector or JAK2 together with OBRa (A) or OBRb (B) cells were stimulated with or without 10 nM of leptin or 1 μM of oligomycin. Cell lysates were separated using 8% SDS–PAGE and analyzed by immunoblotting with an anti-phosphospecific-JAK2, -STAT3, -AMPK antibody, and then with an anti-JAK2, -STAT3, -AMPK antibody. These data are representative of at least three independent experiments.

unresponsiveness of AMPK to exogenous leptin infusion to db/db mice that have high circulating leptin concentrations. We speculate that high levels of circulating leptin may chronically activate AMPK at a submaximal level in db/db mice. Further studies in ex vivo culture experiments may be needed to investigate these possibilities. OBRb encodes three conserved tyrosines in its cytoplasmic domain at position 985, 1077, and 1138 [26]. Y985 controls ERK activation and c-fos transcription via SHP-2, but does not affect the activation of JAK2 and STAT3 during acute ligand stimulation [26]. In contrast, Y1077 does not appear to become phosphorylated or play a role in the leptin signaling pathway. Y1138 becomes phosphorylated in response to ligand and regulates the subsequent activation of JAK2 and STAT3 [26]. To investigate whether Y985 and Y1138 play a role in AMPK activation upon leptin stimulation, we treated cells expressing JAK2 and either wild type or a single point mutant OBRb isoforms (L985 or S1138) with leptin. The leptin-induced activation of AMPK was observed in cells expressing both JAK2 and OBRbL985 in addition to the phosphorylation of both JAK2 and STAT3 (Fig. 2). The stimulation of cells expressing both JAK2 and OBRbS1138 with leptin resulted in the activation of AMPK as well as JAK2, although this construct is incapable of the phosphorylation of STAT3 (Fig. 2). These results suggest that leptin-induced STAT3

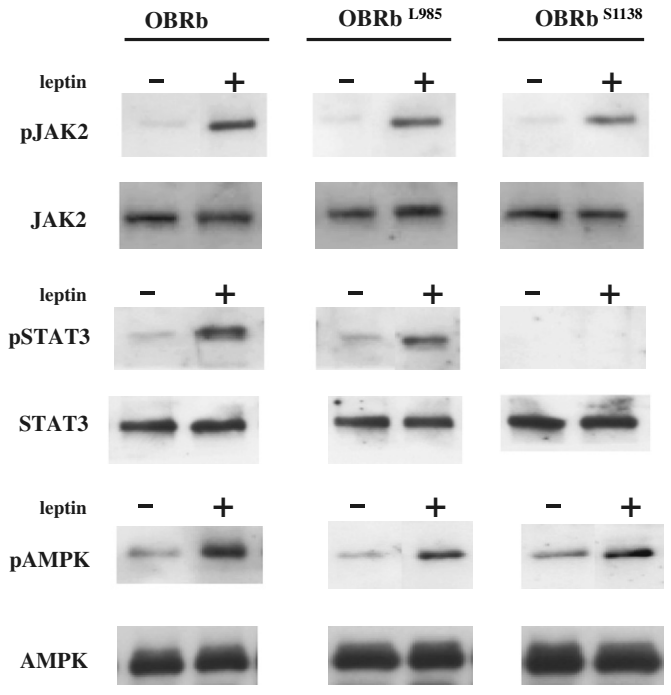

Fig. 2. Phosphorylation of AMPK in Huh7 cells expressing wild type or mutant OBRb. Huh7 cells were co-transfected with wild type or single point mutant OBRb isoforms (Y985L or Y1138S) together with JAK2. Cells were stimulated with or without 10 nM of leptin. Cell lysates were separated using 8% SDS–PAGE and analyzed by immunoblotting with an anti-phosphospecific-JAK2, -STAT3, -AMPK antibody, and then with an anti-JAK2, -STAT3, -AMPK antibody. These data are representative of at least three independent experiments.

phosphorylation is not required for the subsequent activation of AMPK. Next, we examined the roles of leptin-induced JAK2 activation in the subsequent activation of AMPK. SOCS are a family of proteins containing a central SH2 domain initially described as an antagonist of cytokine signaling [27–29]. The systemic leptin administration to ob/ob mice induced SOCS-3 mRNA in the hypothalamus expressing high levels of the leptin receptor long form [30]. In mammalian cell lines, the forced expression of SOCS-3 blocked the leptin-induced phosphorylation of JAK2 and the phosphorylation of substrates of JAK2, including OBR and STAT3 by binding to JAK2 in a leptin-dependent fashion [31]. To examine the possible contribution of JAK2 in regulating leptin-induced AMPK activation, Huh7 cells were transiently co-transfected with JAK2 and OBRb together with an empty vector or SOCS3 expression vector. As previously observed, we detected the leptin-induced phosphorylation of AMPK in cells co-transfected with JAK2 and OBRb together with empty vector (Fig. 3). In contrast, the co-transfection of SOCS3 along with JAK2 and OBRb into cells resulted in the suppression of JAK2, as well as AMPK activation upon leptin stimulation (Fig. 3). These data suggest that JAK2, but not STAT3, may play a critical role in leptin-induced AMPK activation in Huh7 cells, and AMPK activation via JAK2 may be impaired in a state of leptin resistance mediated by SOCS3.

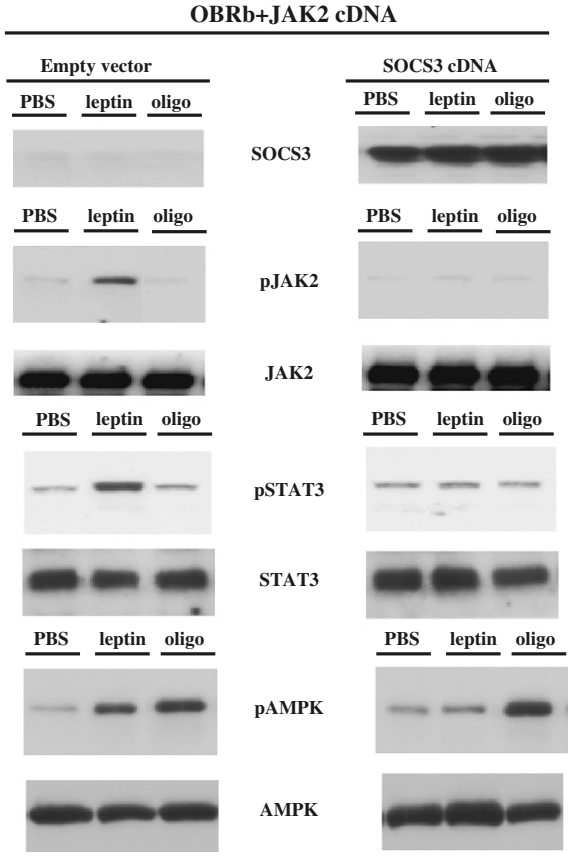

Fig. 3. Effects of forced expression of SOCS3 on AMPK phosphorylation in Huh7 cells. Huh7 cells were co-transfected with empty vector or SOCS3 together with JAK2 and OBRb. Cells were stimulated with or without 10 nM of leptin or 1 μM of oligomycin. Cell lysates were separated using 8% SDS–PAGE and analyzed by immunoblotting with SOCS3 or an anti-phosphospecific-JAK2, -STAT3, -AMPK antibody, and then with an anti-JAK2, -STAT3, -AMPK antibody. These data are representative of at least three independent experiments.

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