Hypothalamic ERK Mediates the Anorectic and Thermogenic Sympathetic Effects of Leptin

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OBJECTIVE—Leptin is an adipocyte hormone that plays a major role in energy balance. Leptin receptors in the hypothalamus are known to signal via distinct mechanisms, including signal transducer and activator of transcription-3 (STAT3) and phosphoinositol-3-kinase (PI 3-kinase). Here, we tested the hypothesis that extracellular signal-regulated kinase (ERK) is mediating leptin action in the hypothalamus.

RESEARCH DESIGN AND METHODS—Biochemical, pharmacological, and physiological approaches were combined to characterize leptin activation of ERK in the hypothalamus in rats.

RESULTS—Leptin activates ERK1/2 in a receptor-mediated manner that involves JAK2. Leptin-induced ERK1/2 activation was restricted to the hypothalamic arcuate nucleus. Pharmacological blockade of hypothalamic ERK1/2 reverses the anorectic and weight-reducing effects of leptin. The pharmacological antagonists of ERK1/2 did not attenuate leptin-induced activation of STAT3 or PI 3-kinase. Blockade of ERK1/2 abolishes leptin-induced increases in sympathetic nerve traffic to thermogenic brown adipose tissue (BAT) but does not alter the stimulatory effects of leptin on sympathetic nerve activity to kidney, hindlimb, or adrenal gland. In contrast, blockade of PI 3-kinase prevents leptin-induced sympathetic activation to kidney but not to BAT, hindlimb, or adrenal gland.

CONCLUSIONS—Our findings indicate that hypothalamic ERK plays a key role in the control of food intake, body weight, and thermogenic sympathetic outflow by leptin but does not participate in the cardiovascular and renal sympathetic actions of leptin. Diabetes 58:536–542, 2009

Leptin is a largely adipocyte-derived hormone that can act in the central nervous system to decrease appetite and increase energy expenditure, thereby leading to decreased body weight (1). Central actions of leptin play an important role in the regulation of several other physiological functions, including reproductive function (2), bone formation (3), and regional sympathetic nerve activity (SNA) subserving thermogenic metabolism and cardiovascular function (4).

Leptin exerts its effects via interaction with specific receptors located in distinct classes of neurons. While several isoforms of the leptin receptor have been identified, the Ob-Rb form that includes the long intracellular domain that has signaling capacity appears to mediate most of the biological effects of leptin (5,6). The signal transducer and activator of transcription-3 (STAT3) pathway was the first signaling mechanism associated with the leptin receptor (7). Neural-specific inactivation of STAT3 leads to hyperphagia and obesity in mice (8). In addition, disrupting the ability of the leptin receptor to activate the STAT3 pathway in mice leads to severe obesity and several other neuroendocrine abnormalities (9–11). More recently, other intracellular signaling mechanisms, including phosphoinositol-3-kinase (PI 3-kinase) (12), AMP-activated protein kinase (13), and mammalian target of rapamycin (14), have been shown to play an important role in the action of leptin on food intake.

Extracellular signal–regulated kinase (ERK), a member of the mitogen-activated protein kinase (MAPK) family, is an additional downstream pathway of the leptin receptor (15). Leptin was shown to activate ERK1/2 in a time- and dose-dependent manner in cultured cells (16–18). Activation of ERK1/2 by leptin seems to be mediated through Src homology–containing tyrosine phosphatase 2 (SHP2) emanating from the tyrosine 985 (Tyr985) of the leptin receptor (19,20). Stimulation of ERK by leptin can also be achieved by direct interaction with Jak2 (15,19,20). In turn, in cell lines, ERK appears to mediate the activation of c-fos (20) and ribosomal S6 kinase and S6 (21) by the leptin receptor.

This ERK pathway has been reported to mediate leptin effects in several tissues, including cardiomyocytes (22,23), the immune system (24,25), and kidney (26). However, the physiological significance of this pathway for the hypothalamic-mediated effects of leptin remains poorly characterized. This study depicts the effect of leptin on hypothalamic ERK and investigates the potential role of this ERK pathway in mediating the effect of leptin on food intake, body weight, and regional sympathetic outflow.

RESEARCH DESIGN AND METHODS
Male Sprague-Dawley rats and lean and obese Zucker (fa) rats were obtained from Harlan Sprague-Dawley. Rats were housed at 23°C with a 12-h light/dark cycle (light on at 6:00 A.M.) and allowed free access to standard rat chow and water. Rats receiving injections in the third cerebral ventricle were equipped with intracerebroventricular canulas at least 1 week before the experimentation as described previously (27). Ethical approval of all of the studies was granted by the University of Iowa Animal Research Committee.

Biochemical studies. Rats were fasted overnight before intracerebroventricular administration of murine leptin (R&D Systems). Rats were killed at the indicated time points by CO2 asphyxiation. The mediobasal hypothalamus was quickly removed from each rat, and the total proteins were extracted and stored at −80°C. Protein samples of homogenized tissues or immunoprecipitates [to assess the effect of leptin on PI 3-kinase, immunoprecipitates were obtained by incubating protein samples with anti-IRS-1 antibody (E-12; Santa Cruz Biotechnology) in the presence of protein A–sepharose] were resolved with 10% SDS-PAGE, and PVDF membranes were incubated with specific antibodies for STAT3 (C-20; Santa Cruz Biotechnology), phospho-STAT3...
RESULTS

To investigate the hypothesis that the ERK pathway is important for leptin action in the central nervous system, we first assessed whether leptin activates this enzyme in the hypothalamus, in vivo. The effect of intracerebroventricular administration of leptin on the activity of STAT3 and ERK1/2 in the hypothalamus was examined in Sprague-Dawley rats. As previously reported (7,12,13), leptin caused a robust activation of the STAT3 in medio-basal hypothalamic extracts (Fig. 1A). Leptin also caused a rapid activation of hypothalamic ERK1/2 with a maximum effect at 5–15 min (Fig. 1B), consistent with previous findings (29,30). This response was dose dependent (5 and 10 µg leptin increased ERK1/2 activity by 3.4- and 4.3-fold, respectively). In peripheral tissues, such as the skeletal muscle, leptin has been shown to activate different isoforms of MAPK (31). Therefore, we examined whether leptin alters the activity of another hypothalamic isoform of MAPK. Leptin did not affect the activity of p38 MAPK in the hypothalamus (Fig. 1C). Intraperitoneal (IP) administration of leptin (1 µg/g body wt) did, however, cause a 3.1-fold increase ($P < 0.01$) in p38 MAPK

Rats received first PD98059 (5 µg), U0126 (7 µg), LY294002 (5 µg), or vehicle (DMSO; 2 µl) followed 15 min later by leptin (10 µg) or saline. After intracerebroventricular administration of experimental agents, SNA measurements were made every 15 min for 6 h. The data for SNA are expressed as percentage change from baseline.

Statistical analysis. All results are expressed as means ± SE and analyzed using Student's t test, one- or two-way ANOVA. When ANOVA reached significance, a post hoc comparison was made using Bonferroni or Newman-Keuls test. A value of $P < 0.05$ was considered to be statistically significant.

FIG. 1. Leptin activation of STAT3 and ERK in the hypothalamus. Time course of activation of STAT3 (A) and ERK1/2 (B) in medio-basal hypothalamic explants after intracerebroventricular administration of murine leptin (10 µg) in rat. C: Lack of effect of intracerebroventricular leptin on the phosphorylation of hypothalamic p38 MAPK. Data represent means ± SE; n = 5 rats per group. *$P < 0.001$ vs. vehicle; †$P < 0.001$ vs. leptin alone.

(27,28). Brown adipose tissue (BAT) SNA was recorded simultaneously with SNA to kidney, hindlimb, or adrenal gland. After baseline recordings of SNA were obtained, each animal received two intracerebroventricular injections.
hypothalamic nuclei in which ERK1/2 is activated by leptin. Both systemic and central administration of leptin caused a marked increase in the immunoreactive ERK1/2 in the arcuate nucleus (Fig. 3A). In contrast, no increase in ERK activity was observed in hypothalamic nuclei other than the arcuate nucleus (in Fig. 3B, the paraventricular nucleus is shown as an example) or in extrahypothalamic nuclei, including the nucleus tractus solitarii in the brainstem (data not shown). These data suggest that leptin activation of hypothalamic ERK1/2 is selectively localized in the arcuate nucleus.

Two classes of neurons account for leptin sensitivity within the arcuate nucleus (32–34): first, a catabolic pathway represented mainly by POMC neurons that is activated by leptin; and second, an anabolic pathway represented principally by the NPY neurons that is inhibited by leptin. We therefore used double staining to determine whether activation of ERK1/2 by leptin occurs in one specific neuronal population. Interestingly, all the neurons in which the ERK1/2 immunoreactivity was increased by leptin were POMC positive (supplemental Fig. 1A, available in an online appendix at http://dx.doi.org/10.2337/db08-0822). No activation of ERK1/2 was observed in NPY neurons (supplemental Fig. 1B). These data seem to suggest that ERK mediates leptin action through an effect on POMC neurons.

To test the hypothesis that ERK is crucial for the physiological action of leptin, we assessed the effect of ERK inhibition (PD98059 and U0126) (35–37) on the feeding and body weight responses to leptin. We first

FIG. 2. Leptin activation of ERK is receptor mediated and involves JAK2. A: Intracerebroventricular leptin caused a significant increase in the phosphorylation of hypothalamic ERK1/2 in the Zucker lean (ZL) but not in the leptin receptor–deficient Zucker obese (ZO) rats. B: AG490, an inhibitor of JAK2, administered intracerebroventricularly caused a dose-dependent blockade of intracerebroventricular leptin-induced hypothalamic ERK1/2 activation. Data represent means ± SE; n = 5 rats per group. *P < 0.001 vs. vehicle; †P < 0.001 vs. leptin alone.

FIG. 3. Immunohistochemical analysis of leptin activation of ERK in the hypothalamus. Intracerebroventricular and intraperitoneal (IP) leptin activate ERK1/2 in the arcuate nucleus (ARC; A) but not in the paraventricular nucleus (PVN; B). Five rats per treatment were examined. (Please see http://dx.doi.org/10.2337/db08-0822 for a high-quality digital representation of this figure.)
verified that baseline food intake and body weight were not altered by the administration of the vehicle (DMSO) or the ERK inhibitors in the third cerebral ventricle (Fig. 4A–C). Treatment with leptin at the onset of the dark phase significantly reduced food intake at both 4 h (Fig. 4A) and 24 h (Fig. 4B). This effect was accompanied by decreased body weight at 24 h after treatment with leptin (Fig. 4C). Pretreatment with the ERK inhibitors (PD98059 or U0126) reversed the decrease in food intake induced by leptin at 4 h (Fig. 4A) and 24 h (Fig. 4B). The ability of leptin to decrease body weight was also blocked by PD98059 and U0126 (Fig. 4C). To exclude the possibility that blockade of the anorectic and weight-reducing actions of leptin by PD98059 and U0126 may be due to inhibition of other mediators of leptin action, such as STAT3 and PI 3-kinase (which are known to play an important role in leptin effects on food intake) (9,12), we tested the effect of leptin on the activity of ERK1/2, STAT3, and PI 3-kinase in the presence of PD98059 and U0126. As expected, leptin activation of hypothalamic ERK1/2 was prevented in the presence of PD98059 or U0126 (Fig. 5). In contrast, stimulation of hypothalamic PI 3-kinase and STAT3 by leptin was not affected by the presence of these inhibitors (Fig. 5). These data demonstrate that blockade of the effect of leptin on food intake and body weight by PD98059 and U0126 is due to inhibition of ERK1/2 and not to blockade of leptin-induced STAT3 or PI 3-kinase activation.

Because ERK is a key enzyme for many intracellular signaling processes, we addressed the specificity of blockade of leptin-induced anorexia and weight loss by testing the feeding- and weight-reducing actions of other stimuli, i.e., an agonist of the melanocortin receptors MTII and corticotrophin-releasing hormone. Intracerebroventricular MTII caused a significant decrease in food intake and body weight at 4 and 24 h (Fig. 4D–F; data not shown). Intracerebroventricular pretreatment with PD98059 or U0126 did not alter the effect of intracerebroventricular MTII on food intake and body weight (Fig. 4D–F). In addition, the anorectic and weight-reducing actions of intracerebroventricular administration of corticotrophin-releasing hormone were not affected by the ERK inhibitors (data not shown). Together, these findings show that the pharmacological inhibitors of ERK produce a selective blockade of the effects of leptin and do not attenuate the responses to other agonists, such as MTII and corticotrophin-releasing hormone.

Leptin stimulation of the sympathetic nervous system regulates diverse physiological processes, including energy expenditure and cardiovascular function. The possible role of hypothalamic ERK in the control of the sympathetic nerve traffic by leptin was investigated. Intracerebroventricular leptin caused regional sympathetic activation, including increases in sympathetic nerve outflow to BAT (Fig. 6A), kidney (Fig. 6C), hindlimb (Fig. 6E), and adrenal gland (Fig. 6G). Selective inhibition of ERK pre-
vented leptin-induced sympathetic activation to thermogenic BAT (Fig. 6B). This effect of ERK inhibition on BAT sympathetic activation to leptin was dose dependent (BAT sympathetic activation to leptin was 183 ± 13, 114 ± 19, and 14 ± 11% in the presence of vehicle, 3 µg U0126, and 7 µg U0126, respectively, P < 0.001). In contrast, PD98059 or U0126 did not alter renal (Fig. 6D), lumbar (Fig. 6F), and adrenal (Fig. 6G) sympathetic nerve responses to leptin. To examine whether the control of sympathetic outflow to thermogenic BAT by leptin exclusively involves ERK, we assessed the effect of inhibition of another pathway that has been shown to play a major role in the feeding and sympathetic responses to leptin, PI 3-kinase (12,38). Inhibition of PI 3-kinase with LY294002 significantly attenuated renal sympathetic activation to leptin (Fig. 6D), which is consistent with our previous report (38). In contrast, PI 3-kinase inhibition with LY294002 failed to alter BAT (Fig. 6A), lumbar (Fig. 6F), or adrenal (Fig. 6G) sympathetic nerve responses to leptin. Taken together, these results demonstrate that leptin regulates different regional sympathetic nerve activities through distinct and contrasting intracellular signaling pathways.

FIG. 6. Role of ERK in mediating the sympathetic effects of leptin is specific for the thermogenic BAT. Left panels (A, C, E, and G) show the time course of SNA responses to intracerebroventricular leptin. Right panels (B, D, F, and H) depict the effects of ERK and PI 3-kinase inhibitors on regional SNA response to leptin. B: Intracerebroventricular (ICV1) administration of ERK inhibitors PD98059 (PD; 5 µg) or U0126 (U; 7 µg), but not of PI 3-kinase inhibitor LY294002 (LY; 5 µg), 15 min before intracerebroventricular (ICV2) leptin prevents the increase in BAT SNA induced by leptin. Inhibition of ERK does not prevent the SNA increase induced by leptin to kidney (D), hindlimb (F), and adrenal gland (H). Data represent means ± SE; n = 8–20 rats per group. *P < 0.001 vs. vehicle; †P < 0.001 vs. leptin alone.
with ERK contributing to the thermogenic BAT sympathetic response but not to renal, lumbar, or adrenal sympathetic nerve responses to leptin.

**DISCUSSION**

We have characterized a new hypothalamic signaling mechanism of the leptin receptor. Our results show that leptin activates ERK1/2 in the arcuate nucleus and that this pathway contributes to leptin control of food intake, body weight, and thermogenic sympathetic outflow. Hypothalamic ERK appears to mediate selective leptin actions because its inhibition prevented some but not all actions of leptin. This suggests that modulation of the hypothalamic ERK pathway could affect the metabolic actions of leptin (appetite and thermogenic sympathetic metabolism) without altering its sympathetic cardiovascular and renal actions.

Previous studies have implicated hypothalamic ERK in the regulation of energy homeostasis. Fasting was shown to activate ERK in the hypothalamic arcuate and paraventricular nuclei in mice (39,40). Fasting-induced activation of hypothalamic ERK was reversed by re-feeding, suggesting that activation of ERK in the hypothalamus is relevant for energy balance (40). Our current findings that hypothalamic ERK contributes to the actions of leptin extend the role of this ERK pathway in the control of energy homeostasis. Our results are in line with the observation that the development of obesity in mice with neuronal-specific ablation of Shp2 is related to the inability of leptin to stimulate ERK (41). To study the role of neuronal Shp2, Zhang et al. (41) used the cre-loxP system to create a conditional Shp2 mutant allele in mice. This allowed selective deletion of Shp2 in postmitotic forebrain neurons. Surprisingly, the predominant phenotype exhibited by this mouse model was the development of early-onset obesity. In subsequent studies, Zhang et al. found that leptin-induced phosphorylation of ERK1/2 in the arcuate nucleus of hypothalamus was dramatically reduced in mice with neuronal-specific ablation of Shp2 compared with the controls (41). In contrast, the ability of leptin to induce phosphorylation of arcuate STAT3 was preserved in this mouse model. Although the signaling cascade leading to the activation of ERK by the leptin receptor remains unclear (15), the inability of leptin to stimulate ERK in the absence of Shp2 suggests that this protein mediates leptin activation of ERK. However, recent findings challenge the importance of Shp2 in mediating leptin effects (42). This is based on the observation that in mice, mutation of the Tyr985 of the leptin receptor that blocks Shp2 recruitment did not recapitulate the obesity phenotype observed in mice in which Shp2 was deleted in the forebrain neurons (42), suggesting that, in vivo, Jak2-dependent mechanism may be the predominant pathway for the stimulation of ERK by the leptin receptor.

The downstream hypothalamic pathways controlled by the leptin receptor–ERK axis remain to be elucidated. Importantly, leptin activation of ERK seems to occur in the POMC neurons in the arcuate nucleus of the hypothalamus, which narrows the search for the mechanisms that are controlled by the leptin receptor–ERK axis. However, additional studies are needed to analyze in more detail the role of ERK in POMC vs. NPY neurons in mediating leptin action.

A key downstream target of the POMC neurons are neurons expressing melanocortin 4 receptors (MC4Rs) that are activated by α-melanocyte stimulating hormone (product of POMC) (33,34). Pharmacological blockade of MC4Rs reverses the effect of leptin on body weight and food intake (43) and deletion of the MC4Rs leads to severe obesity in mice (44). We have previously shown that blockade of the brain MC4R inhibits the renal, but not the BAT, sympathetic nerve response to leptin (27). We also showed that leptin-induced renal sympathetic activation is absent in the homozygous MC4R knockout mice, demonstrating the importance of this receptor in the control of renal sympathetic outflow by leptin (45). In addition, blockade of PI 3-kinase also inhibits renal, but not BAT, SNA. In contrast to the role of MC4R and PI 3-kinase, blockade of ERK in this study inhibited SNA to BAT but not kidney. This supports the concept that differential intracellular mechanisms are involved in leptin-induced sympathetic activation to kidney and thermogenic BAT. Sympathetic activity to BAT is ERK dependent, whereas sympathetic activity to kidney is PI 3-kinase and MC4R dependent.

These findings are in line with the notion that leptin controls various physiological processes through a variety of signaling mechanisms. For instance, the STAT3 pathway appears to be involved in mediating the effects of leptin on food intake and energy homeostasis but not on reproductive function, growth, or glucose homeostasis (9). However, the relative role of each of the downstream pathways associated with the leptin receptor in the control of food intake by leptin remains perplexing, because disrupting any of these pathways seems to have a profound effect on leptin-induced food intake. Disruption of the leptin receptor–STAT3 pathway causes hyperphagia in mice (9,10), which demonstrates that STAT3 is important for the control of food intake by leptin. In addition, the anorectic response to leptin can be reversed by blockade of PI 3-kinase (12,38) and ERK (present study).

Some limitations of the present study need to be addressed. First, our conclusions regarding the role of ERK in mediating leptin effects were based on acute studies. Whether chronic inhibition of these signaling pathways will result in a similar effect as in the acute studies remains to be determined, but deletion of Shp2 is accompanied by increased food intake and obesity, presumably through disruption of ERK signaling. Second, the inhibitors that we used in our studies to block ERK might have other nonspecific actions. However, cell-based assays and in vitro studies have shown that both PD98059 and U0126 appear to specifically suppress ERK signaling (35–37). These inhibitors have been widely used to suppress activation of ERK and to examine the physiological roles of these enzymes. In addition, we have shown that pretreatment with ERK inhibitors did not alter leptin-induced activation of STAT3 or PI 3-kinase and did not affect the appetite- and weight-reducing actions of MTII and corticotrophin-releasing hormone. Third, our studies lack the neuroanatomical specificity regarding the brain nuclei where the ERK signaling pathway mediates the effects of leptin on food intake, body weight, and BAT sympathetic outflow, because the inhibitors were administered intracerebroventricularly. Nonetheless, using an immuno-histochemical approach, we have shown that leptin activation of ERK occurs in the arcuate nucleus.

In conclusion, our experiments provide evidence that hypothalamic ERK is a significant downstream target for the effects of leptin to regulate food intake, body weight, and thermogenic sympathetic outflow to BAT. However,
ERK does not appear to be involved in leptin activation of the sympathetic nervous system to other tissues, such as kidney, hindlimb, and adrenal gland. These findings provide new insights into the intracellular mechanisms engaged by the leptin receptor to control various physiological functions.

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