Intracellular Mechanisms Involved in Leptin Regulation of Sympathetic Outflow

Kamal Rahmouni, William G. Haynes, Donald A. Morgan, Allyn L. Mark

Abstract—Leptin acts in the hypothalamus to decrease appetite and increase sympathetic nerve activity. The leptin receptor is known to signal through the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway to modulate transcription of target genes. Alteration of the activity of phosphoinositol-3 kinase (PI3K) by leptin has also been reported, and inhibition of PI3K is known to block the leptin-induced suppression of feeding. We tested the hypothesis that leptin-induced renal sympathetic nerve activation is mediated by PI3K. We evaluated renal sympathetic nerve activity (RSNA) and feeding responses of C57BL/6J mice to intracerebroventricular (ICV) administration of leptin in the presence or absence of selective inhibitors of PI3K (LY294002 or wortmannin). As expected, ICV administration of leptin decreased food intake at 4 hours and 24 hours and increased RSNA. Pretreatment with the PI3K inhibitor LY294002 markedly attenuated both the decrease in food intake and the increase in RSNA induced by leptin. Wortmannin also inhibited the RSNA response to leptin. In contrast, PI3K inhibitors did not affect the RSNA response to MTII (melanocortin-3/4 receptor agonist). Our data demonstrate that PI3K appears to play an important role in the transduction of leptin-induced changes in renal sympathetic outflow. (Hypertension. 2003;41[part 2]:763-767.)

Key Words: hypothalamus • obesity • sympathetic nervous system • kinase

Leptin, secreted by adipocytes, promotes weight loss by reducing appetite and increasing energy expenditure through stimulation of sympathetic nerve activity.1,2 Leptin induces sympathoactivation not only to thermogenic brown adipose tissue but also to hindlimb, adrenal gland, and kidney.2,3

The leptin receptor is abundantly expressed in the hypothalamus, where this hormone is thought to act after entering the brain by a saturable specific transport mechanism.1,4,5 The leptin receptor belongs to the cytokine receptor superfamily that signals through the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway.6,7 On leptin stimulation, intracellular JAK proteins are activated, which in turn activate by phosphorylation the cytoplasmic STAT proteins. Phosphorylated STAT proteins translocate to the nucleus and stimulate transcription of target genes.8

However, the leptin receptor has divergent signaling capacities and modulates the activity of different intracellular enzymes, including phosphoinositol-3 kinase (PI3K).9-14 A role for PI3K in some peripheral effects of leptin such as on glucose uptake,9 K\textsubscript{ATP} channel activity,10 epithelial cell invasiveness,11 cholesterol ester metabolism,12 and thermogenesis13 has been reported. This enzyme also appears to mediate some central neural effects of leptin. In a recent study in rats,14 blockade of PI3K was shown to prevent the effects of leptin on food intake.

The role of PI3K in the sympathetic effects of leptin has not been reported. We therefore evaluated the role of PI3K in the control of food intake and renal sympathetic nerve activity (RSNA) in mice. We examined the feeding and RSNA responses to intracerebroventricular (ICV) administration of leptin in presence or absence of selective inhibitors of PI3K (LY294002 and wortmannin).

Methods

Animals
We studied male, 12- to 26-week-old C57BL/6J mice (Jackson Laboratories). Animals were housed in a temperature-, humidity-, and light-controlled room (light/dark cycle of 12 hours each), with free access to chow and tap water. At least 1 week before experimentation, mice were fitted with an ICV cannula in the lateral ventricle as described.15 Mice were kept in individual cages after surgery. All protocols were approved by the University of Iowa Animal Research Committee.

Feeding Assays
One week after ICV cannulation, baseline measurements of 24-hour food intake were obtained during 3 consecutive days before ICV treatment. To examine the effects of PI3K inhibitor (LY294002) on the decrease of food intake induced by leptin, each mouse received 2 ICV injections (2 μL each) immediately before the onset of the dark phase. Mice received first LY294002 (0.1 or 1 μg) or its vehicle (1% methanol), followed 10 minutes later by 5 μg of leptin or vehicle (saline). Food intake was recorded at 4 and 24 hours after the injections. The mice were then anesthetized (91 mg/kg ketamine and 9.1 mg/kg xylazine IP), and blood samples were collected for plasma leptin assay by radioimmunoassay as described.2,15

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Study of RSNA

Other groups of mice were assigned to study the effect of PI3K blockade on RSNA response to leptin. One week after ICV cannulation, anesthesia was induced in these mice with 91 mg/kg ketamine and 9.1 mg/kg xylazine IP and sustained with α-chloralose (25 mg/kg per hour). A jugular vein and carotid artery were cannulated for infusion of anesthetic (α-chloralose) and for hemodynamic recording (arterial pressure and heart rate), respectively. The trachea was also cannulated, and each mouse was allowed to breathe oxygen-enriched air spontaneously. Rectal temperature was maintained near 37.5°C by means of a temperature-controlled surgical table. For direct multiferb recording of RSNA, a retroperitoneal incision was made and a nerve branch to the left kidney was carefully dissected free and placed on a bipolar 36-gauge platinum-iridium electrode. After an optimum recording of multifiber RSNA was obtained, the electrode was fixed in place using silicone gel. Nerve signals were amplified, filtered, and counted as described previously.2

After surgery was complete, animals were allowed to stabilize for 15 to 20 minutes. RSNA, mean arterial pressure, and heart rate were then collected on 3 occasions during a 10-minute control period and averaged to establish baseline. Each animal then received 2 ICV injections. Mice received first LY294002 (0.1 or 1 µg), wortmannin (0.01 µg), or their vehicles (1% methanol and DMSO, respectively) followed 10 minutes later by leptin (5 µg). MII (melanocortin-3/4 receptor agonist, 5 µg), or vehicle (saline).

Because the increase in RSNA induced by leptin was not completely blocked by a single ICV injection of the PI3K inhibitor LY294002, we performed one separate experiment in which a second ICV injection of this inhibitor or its vehicle was performed. Two groups of RSNA instrumented mice received 0.1 µg of LY294002 followed 10 minutes later by 5 µg of leptin. Two hours later, one group received a third ICV injection of 0.1 µg of LY294002, whereas the second group was given vehicle.

After ICV administration of experimental agents, mean arterial pressure, heart rate, and RSNA measurements were made every 15 minutes for 4 hours. At the end of the experiments, arterial blood was collected for plasma leptin assay. Mice were then killed by method-hexitol overdose.

Data Analysis

Results are expressed as mean±SEM. Sympathetic nerve firing rate was corrected for background noise by subtracting postmortem measurement from the measurements obtained at each time point during the experiment. The data for RSNA are expressed as percentage change from baseline. Data were analyzed by 1-way ANOVA or 2-way ANOVA on repeated measures with Bonferroni or Newman-Keuls tests for post hoc comparison. A value of \( P < 0.05 \) was considered to be statistically significant.

Results

Effect of PI3K Blockade on Feeding Response to Leptin

As expected, ICV administration of 5 µg of leptin caused a significant (\( P < 0.001 \)) decrease in food intake, measured 4 and 24 hours after the treatment (Figure 1). Pretreatment with PI3K inhibitor markedly (\( P < 0.05 \)) attenuated the decrease in food intake induced by leptin (Figure 1). Attenuation of leptin-induced anorexia by LY294002 was not dose-related, as the effects of 0.1 and 1 µg of this inhibitor were comparable at 4 (\( P = 0.81 \)) and 24 hours (\( P = 0.37 \)). LY294002 alone had no effect on food intake in the mice.

Plasma leptin, measured 24 hours after ICV injection, was not affected by any of the above treatments. Indeed, the values of plasma leptin were comparable between the vehicle control group (4.3 ± 0.5 ng/mL) and mice treated with 1 µg of LY294002 followed 10 minutes later by 5 µg of LY294002, whereas the second group was given vehicle.

Because the increase in RSNA induced by leptin was not completely suppressed by a single ICV injection of the PI3K inhibitor LY294002, we performed one separate experiment in which mice receiving 0.1 or 1 µg of leptin were given another ICV injection of leptin 2 hours after ICV injection of 0.1 µg of LY294002 or vehicle. The renal sympathoexcitatory effects of leptin as the RSNA response to leptin was substantially inhibited (\( P < 0.01 \)) by pretreatment with 0.1 µg of LY294002 (Figure 2A). As with food intake, blockade of RSNA response to leptin by LY294002 was not dose-related, as the renal sympathoexcitatory effects of leptin as the RSNA response to leptin was not statistically (\( P = 0.32 \)) different in the presence of 0.1 or 1 µg of LY294002.

Because the RSNA response to leptin was not completely reversed in the presence of LY294002 and because RSNA started to increase after 2 hours after ICV leptin, we performed a separate experiment in which mice receiving 0.1 µg of LY294002 and 5 µg of leptin were given another ICV injection of leptin 0.1 µg of LY294002 or vehicle 2 hours after ICV injection of leptin (Figure 2B). A second injection of LY294002 completely suppressed the RSNA response to leptin (24 ± 14%, \( P = 0.012 \) versus the group receiving vehicle as third injection).

Arterial pressure and heart rate did not change significantly in any of the groups as compared with the control group (receiving vehicle+vehicle) during the experimental period. For example, the robust increase in RSNA observed in mice treated with leptin alone was not associated by changes in

Figure 1. Effects of PI3K blockade on the decrease in food intake induced by leptin. Presence of PI3K inhibitor LY294002 (at a dose of 0.1 or 1 µg) attenuates the 4-hour (A) and 24-hour (B) decrease in food intake induced by leptin. Data represent mean±SEM, n = 9 mice per group. \( ^{\dagger} P < 0.05 \) vs control group (vehicle+vehicle).
hemodynamics as mean arterial pressure and heart rate did not differ significantly in baseline conditions (81±2 mm Hg and 285±7 bpm, respectively) and 4 hours after ICV treatment (83±5 mm Hg and 279±65 bpm). Plasma concentrations of leptin, measured 4 hours after the ICV injection, were also not affected by any ICV treatments (data not shown).

In a different group of mice, we used wortmannin instead of LY294002 to confirm the inhibition of leptin-induced sympathoexcitation by PI3K blockade (Figure 2C). Like LY294002, wortmannin also substantially inhibited the RSNA response to leptin (Figure 2C, P<0.001). Wortmannin alone had no effect on RSNA.

**Effect of PI3K Blockade on RSNA Response to MTII**
To examine whether the RSNA response to another stimulus is attenuated by inhibition of PI3K, we tested the effects of MTII (melanocortin-3/4 receptor agonist) on RSNA in the presence or absence of PI3K inhibitors. ICV administration of 5 μg of MTII caused a significant increase in RSNA (173±50%, P=0.006). As shown in Figure 3, the renal sympathoactivation induced by MTII was not reduced by 0.1 or 1 μg of LY294002. Pretreatment with 0.01 μg of wortmannin also had no effect on the RSNA response to MTII (161±56%, P=0.88, n=6).

No significant change in mean arterial pressure and heart rate was observed after ICV administration of MTII in presence or absence of LY294002 or wortmannin (data not shown).

**Discussion**
We investigated the intracellular pathways involved in the transduction of leptin signaling in the central nervous system. Our major new findings relate to effects of PI3K inhibitors on renal sympathetic response to leptin. Our results show that PI3K has a pivotal role in mediating the effects of leptin on renal sympathetic nerve outflow and confirm a previous report that PI3K is also involved in effects of leptin on food intake.

The importance of PI3K as a key link between leptin receptor activation and responses is provided by our present data and a previous report. Niswender et al reported that ICV pretreatment with the PI3K inhibitors completely abol-
ished the decrease in food intake induced by leptin in rat. In the present study, the decrease in food intake induced by leptin was significantly reduced by the LY294002 compound. The sympathoexcitatory effects of leptin to the kidney were also significantly reduced by a single ICV pretreatment with the LY294002 compound, and a second injection of LY294002 or a single ICV injection of wortmannin completely suppressed the RSNA response to leptin. This is consistent with the characteristics of these 2 inhibitors because, in contrast to wortmannin, the LY294002 compound is a reversible inhibitor.9,19

It has been shown that by engaging JAK2, the leptin receptor is able to stimulate IRS-2, which in turn activates PI3K through an association to its regulatory subunit.9,19 This provides a potential signaling cascade between leptin receptor and PI3K. Activation of PI3K by leptin has been demonstrated, in several cell lines and tissues, to mediate many effects of leptin including regulation of glucose uptake,9 K ATP channels,10 epithelial cell invasiveness,11 cholesterol ester metabolism,12 and thermogenesis.13 Niswender et al14 showed that in the brain, leptin induces a rapid increase in hypothalamic PI3K activity, with a peak at 30 minutes. Our demonstration that PI3K inhibitors (LY294002 and wortmannin) significantly attenuated the leptin-induced decrease in food intake and renal sympatoexcitation confirms that the activity of PI3K is crucial for leptin receptor signaling in the central nervous system.

Because PI3K is a central enzyme for many cellular effects, it raises questions about the specificity of blockade of leptin-induced anorexia and sympatoexcitation by inhibition of this enzyme. The feeding response to stimulation of melanocortin receptors by α-melanocyte-stimulating hormone (α-MSH) was not affected by the presence of PI3K inhibitors.14 Similarly, we have shown that the same doses of LY294002 and wortmannin that block the effects of leptin do not alter the RSNA response to stimulation of melanocortin receptors with MTII. Thus, in the central nervous system, the role of PI3K in the feeding and sympathetic effects of leptin is not a nonspecific effect.

The JAK/STAT pathway was thought to be the main pathway that mediates the leptin action in the hypothalamus.

The previous report14 and our present data demonstrate that the regulation of food intake and renal sympathetic outflow by leptin are PI3K-dependent, but so far the role of PI3K in the anorectic and sympathetic nerve effects of leptin has been studied only in a relatively short-term (4 to 24 hours). Thus, a role for the JAK/STAT pathway in the long-term regulation of food intake and sympathetic nerve activity by leptin cannot be excluded. Furthermore, leptin action in the central nervous system is known to induce regional sympathoexcitation.2,3 It is very likely that leptin controls sympathetic nervous system activity in a tissue-specific manner as this is one of the characteristics of the autonomic nervous system. Further experiments on the role of PI3K and JAK/STAT pathways in leptin-induced sympathoexcitation to different other beds including brown adipose tissue, hindlimb, and adrenal gland should shed some light in this regard.

Some potential limitations for the present study need to be addressed. First, the potential role of PI3K in the sympathoexcitatory effects of leptin to other organs than the kidney was not examined because of difficulty in recording sympathetic nerve activity to other tissues in the mice. Second, the exact sites in the brain where PI3K inhibitors act to block the responses to leptin were not investigated. The arcuate nucleus of the hypothalamus is considered the major site of leptin action in the central nervous system. This is supported by the feeding response induced by local injection of leptin in this area1,20 and the inability of leptin to affect food intake1 or sympathetic nerve activity21 after the arcuate nucleus has been destroyed. Microinjection of PI3K inhibitors in the arcuate nucleus in presence or absence of leptin should reveal a potential role of this area in the blockade of leptin response by PI3K inhibition. Third, the downstream pathways that link activation of PI3K and the leptin responses were not identified. PI3K is known to regulate several downstream effectors such as protein kinases B and C, Jun kinase, serine phosphatase, and K ATP channels.10,22 The role of these downstream pathways in leptin-induced increases in RSNA remains to be determined.

Perspectives
A detailed understanding of the integrated neuronal circuits and intracellular pathways involved in leptin action will help in understanding the pathophysiology of obesity and related diseases, including obesity-associated hypertension. For example, we have previously reported that in some models of obesity, leptin resistance is selective with preservation of renal sympathetic effects despite the loss of metabolic action of leptin.15,23 This implies that differential effects of leptin on metabolic function and renal sympatoexcitation result from divergent central pathways underlying leptin-induced decreases in appetite and increases renal sympathetic nerve outflow in much the same way that the selectivity in insulin resistance is due to the inability of insulin to stimulate the PI3K pathway while activation of mitogen activated protein kinase pathway by insulin is preserved24 that might explain some of the detrimental effects of chronic hyperinsulinemia on cardiovascular function. The molecular basis of selective leptin resistance remains unknown. Our present data suggest that the PI3K pathway is unlikely to explain this selectivity in
leptin resistance because this enzyme appears to mediate both the feeding and renal sympathetic nerve response to leptin.

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