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Mechanisms mediating renal sympathetic activation to leptin in obesity

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The adipocyte-derived hormone, leptin, is considered as a critical signal that feeds back to inform the central nervous system about the status of peripheral energy reserves (11, 24). Knowledge of the biological actions of leptin has increased exponentially during recent years, and this hormone is now recognized as being involved in the regulation of several physiological functions that extend the role of this hormone beyond energy homeostasis control. For instance, leptin appears to be involved in bone formation (16) and immune regulation (37); it influences every aspect of reproduction, including puberty onset, fertility, and pregnancy (5, 15). Leptin also plays an important role in the modulation of the hypothalamic pituitary function and the thyroid, adrenal, and growth hormone axes (4, 20, 40).

Leptin suppresses appetite and enhances energy expenditure by activating sympathetic nerve activity (SNA) to thermogenic brown adipose tissue (BAT). Leptin also increases SNA to organs involved in cardiovascular regulation, such as the kidney (14). Consistent with its renal sympathoexcitatory effect, elevating circulating leptin levels via chronic infusion or transgenic overexpression increases arterial pressure (1, 35). Conversely, severely obese, but leptin-deficient, mice and humans display low sympathetic tone and hypotension (21, 28). These findings demonstrate that leptin plays a physiological role in maintaining sympathetic tone and blood pressure.

These findings also suggest that the elevated blood pressure associated with obesity may be caused by hyperleptinemia. A pathological role for leptin in obesity-associated hypertension is supported by the finding that leptin resistance associated with obesity may be selective and spares the sympathetic cardiovascular action of leptin. Indeed, we demonstrated that in obese mouse models, such as agouti obese and diet-induced obese (DIO) mice, renal SNA response to leptin is intact despite the impaired appetite- and weight-reducing effects of this hormone (6, 29, 33). Preservation of renal SNA response to leptin in DIO mice translates into a preservation of arterial pressure response to leptin (33). Interestingly, in these DIO mice fed a high-fat diet for 10 wk, the selectivity in leptin resistance occurs despite the modest increase in body weight, fat mass, and plasma leptin (33). However, it is not known whether the selectivity in leptin resistance occurs in the late stage of dietary obesity, which is characterized by marked defects in leptin action (7, 9, 18, 19).

The mechanisms that mediate renal sympathetic activation to leptin in obesity remain to be elucidated. We previously demonstrated that phosphoinositol-3 kinase (PI3K) plays a major role in the transduction of leptin-induced activation of renal sympathetic outflow. Indeed, leptin is known to activate hypothalamic PI3K (26), and blocking this pathway inhibits the feeding response, as well as the renal sympathoexcitatory action of leptin (30). The melanocortin receptors (MCR) also appear to be an important downstream mediator of leptin actions, including the activation of renal SNA. Intracerebroventricular administration of SHU9119, an antagonist of MC3/4R blocks the metabolic effects of leptin (34), as well as the renal sympathetic activation to leptin (13). Moreover, leptin...
failed to affect renal SNA and arterial pressure in the homozygous MC4R knockout mice, resulting in normal arterial pressure despite the high circulating leptin levels in these mice (31, 36). Whether PI3K and MCR mediate the preserved renal SNA response to leptin in obesity remain unknown.

In the present study, first, we examined whether renal sympathetic activation to leptin is preserved in DIO mice that have pronounced obesity produced by 20 wk of a high-fat diet. Next, we evaluated the role of PI3K and MCR in mediating leptin-induced renal sympathetic activation in obesity by assessing the effect of presence of PI3K inhibitor (LY294002) or MC3/4R antagonist (SHU9119) on renal SNA response to leptin in DIO and agouti obese mice.

RESEARCH DESIGN AND METHODS

Animals. Male C57BL/6j, agouti obese (KKA°) and wild-type littermate mice were obtained from the Jackson Laboratories (Bar Harbor, ME). Five- to six-week-old C57BL/6j mice were weighed then randomly assigned to standard chow or a high-fat diet (45% kcal as fat) (33). The body weight of each mouse was measured each week for the next 20 wk. The mice were housed in a room with constant temperature (23°C) and a 12:12-h light-dark cycle (lights turned off at 6:00 PM) with free access to food and tap water. Intracerebroventricular implantation of cannulae was performed as described previously (29–31). All studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by The University of Iowa Animal Research Committee.

Magnetic resonance imaging. Imaging of fat by MRI was performed under anesthesia [ketamine (91 mg/kg ip) and xylazine (9.1 mg/kg ip)] using Varian Unity/Inova 4.7 T small-bore MRI system (Varian, Palo Alto, CA). The acquisition consisted of a T1-weighted fast spin-echo sequence (TR/TE = 625/12 ms) with in-plane resolution of 0.13 × 0.25 mm² and slice thickness of 1 mm acquired in the axial and coronal planes.

Effect of leptin on body weight and food intake. In a group of single-caged animals fed standard chow (lean mouse) or a high-fat diet for 20 wk (DIO mice), body weight and food intake were measured for three consecutive days. Then, each mouse was randomly treated intraperitoneally with vehicle (60 µl) or mouse leptin (30 or 60 µg, twice a day, R&D Systems, Minneapolis, MN) for the next 3 days, while measuring daily body weight and food intake. In another group of individually housed lean and DIO mice, after 3 days of baseline measurements of body weight and food intake, a single intracerebroventricular dose of vehicle (2 µl) or mouse leptin (5 µg) was injected, and the effect on body weight and food intake was recorded 24 h later. Fat pad weights (BAT, epididymal, and perirenal) were measured at the conclusion of this study.

Effect of leptin on regional SNA. Each mouse was anesthetized with an injection of ketamine (91 mg/kg ip) and xylazine (9.1 mg/kg ip) and instrumented for measurement of arterial pressure and heart rate from the carotid artery. Anesthesia was maintained with intravenous delivery of α-chloralose (initial dose; 25 mg/kg, sustaining dose: 6 mg·kg⁻¹·h⁻¹). Each mouse was intubated and allowed to spontaneously breathe oxygen-enriched air throughout the experimental procedure. The SNA to the kidney, hindlimb, or BAT was recorded from baseline and the effect on sympathetic activity was measured as described previously (31, 33). Body temperature was maintained constant at 37.5°C with a surgical heat lamp and a heating pad. Each mouse received a single intravenous injection of vehicle (60 µl) or leptin (30, 60, or 120 µg). Other groups of mice received one intracerebroventricular injection of vehicle (2 µl) or mouse leptin (0.5, 1, or 5 µg). Hemodynamic parameters and sympathetic activities were recorded at baseline and for the next 4 h after each treatment.

Effect of LY294002 and SHU9119 on renal SNA response to leptin. Anesthetized lean and DIO mice along with age-matched agouti obese and agouti lean mice were instrumented for renal SNA recording as above. Each mouse then received two intracerebroventricular injections. The first injection was either vehicle (2 µl icv) or a PI3K inhibitor (LY294002, 0.1 µg icv) or a MC3/4R antagonist (SHU9119, 30 µM icv), followed 10 min later by a second injection of vehicle (2 µl icv) or mouse leptin (5 µg icv). In one study, we tested the effect of intracerebroventricular administration of higher doses of LY294002 (0.5 µg) or SHU9119 (150 µM) alone on renal SNA. Blood pressure, heart rate, and renal SNA were continuously recorded at baseline (10 min before the first intracerebroventricular injection) and for the next 4 h following the last intracerebroventricular injection.

Leptin measurements. Leptin was measured in plasma and cerebrospinal fluid (CSF). Plasma was obtained by centrifuging blood collected from mice (5,000 rpm for 8 min). Plasma and CSF leptin were compared between untreated mice that were fed standard chow (lean) or a high-fat diet (DIO) for 20 wk. In addition, to test the effect of intravenous administration of leptin on CSF concentration of leptin, anesthetized lean and DIO mice were given vehicle or mouse leptin (30, 60, or 120 µg iv), and the CSF was collected 30 min later. To collect the CSF, each mouse was positioned in a stereotaxic apparatus with the neck flexed. An incision was then made to expose the membrane located in the area between the occipital notch and the first cervical vertebrae. Once the membrane was exposed, a 20-µl Hamilton syringe was then used to puncture the membrane, and immediately, the CSF was carefully suctioned out, and only clear CSF was used. Mice were killed after blood was collected. Concentration of murine leptin was measured using a mouse leptin ELISA kit (Catalog #90030; Crystal Chem, Downers Grove, IL).

Data analysis. Results are expressed as means ± SE. Because of the animal-to-animal variability in baseline SNA, the data for SNA are expressed as a percentage change from baseline. Data were analyzed using Student’s t-test and one- or two-way ANOVA. When ANOVA reached significance, the Bonferroni test was used to compare the mean values among the different levels of mice groups and treatments. A value of P < 0.05 was considered significant.

RESULTS

The obesity induced by a high-fat diet in C57BL/6j mice was markedly pronounced after 20 wk on this diet compared with 10 wk (Fig. 1A). After 20 wk, mice on a high-fat diet weighed about 20% more than those fed the standard chow (as compared with the 10% weight gain induced after 10 wk of a high-fat feeding) (33). After 20 wk, fat mass was significantly higher in DIO mice, compared with lean counterparts (Fig. 1B and Table 1). Consistent with the increased fat mass, DIO mice had high plasma concentrations of leptin (Table 1).

Mean arterial pressure and heart rate, recorded under anesthesia, were significantly (P < 0.01) higher in the DIO mice (88 ± 1 mmHg and 310 ± 3 beats/min, respectively) compared with the lean controls (78 ± 1 mmHg and 300 ± 3 beats/min, respectively).

Effects of systemic injection of leptin. In lean mice, intraperitoneal administration of leptin (twice a day for 3 days) caused a significant and dose-dependent decrease in food intake and body weight (Table 2). In contrast, intraperitoneal leptin failed to significantly decrease food intake and body weight in DIO mice, indicating that these mice are resistant to the anorectic and weight-reducing effects of leptin.

In lean mice, intravenous administration of leptin caused a significant and dose-dependent increase in SNA to kidney, hindlimb, and BAT (Table 2). The increase in SNA to kidney,
hindlimb, and BAT following intravenous leptin were markedly attenuated in DIO mice compared with lean mice (Table 2).

Consistent with our previous study (33), the leptin-induced increase in SNA to hindlimb and BAT was less pronounced compared with renal SNA. We, therefore, tested the effect of a higher dose of leptin for these nerves. In lean mice, intravenous administration of 120 μg leptin caused a marked increase in SNA to hindlimb (128 ± 14%) and BAT (145 ± 21%). In DIO mice, the effect of 120 μg of leptin on SNA to hindlimb (41 ± 8%) and BAT (61 ± 17%) was also attenuated ($P < 0.001$ and $P = 0.015$, respectively). Together, these results indicate that DIO mice are resistant to the effect of intravenous leptin on regional SNA, including renal SNA.

**Effects of central injection of leptin.** To test whether the resistance to the catabolic and sympathetic effects of leptin in DIO mice is due to an impairment of leptin transport across the blood-brain barrier, we examined the metabolic and sympathetic responses to leptin administered directly into the cerebral ventricles.

In lean mice, intracerebroventricular leptin (5 μg) caused a significant decrease in body weight and food intake (Fig. 2). The decrease in body weight induced by intracerebroventricular leptin was associated with a 20% decrease in BAT mass ($P = 0.018$). Epididymal and perirenal fat tended to decrease in the lean mice after intracerebroventricular leptin (by 18% and 25%, respectively), but the response did not reach statistical difference ($P = 0.09$ and 0.1, respectively). In contrast to lean mice, the effects of intracerebroventricular leptin on body weight, food intake, and fat mass were attenuated or not present in the obese mice. Indeed, in DIO mice, compared with vehicle, intracerebroventricular leptin did not significantly affect body weight or food intake (Fig. 2). Furthermore, intracerebroventricular leptin did not affect BAT ($P = 0.42$), epididymal ($P = 0.27$) or perirenal ($P = 0.46$) fat depots.

In contrast to the response to intravenous leptin, the rise in renal SNA induced by intracerebroventricular leptin (5 μg) was of the same magnitude in lean and DIO mice (Fig. 3). However, the BAT sympathetic activation to intracerebroventricular leptin was significantly attenuated in DIO mice compared with lean controls (Fig. 4A). Lumbar SNA response to intracerebroventricular leptin was also attenuated in DIO mice (Fig. 4B).

We also compared the effect of the lower dose of intracerebroventricular leptin (1 μg) on the regional SNA between the lean and DIO mice. By the intracerebroventricular route, 1 μg seems to be the minimal dose of leptin needed to increase SNA.

**Table 1. Weight of fat depots and leptin concentrations in the plasma and CSF in lean and DIO mice**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lean Mice</th>
<th>DIO Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brown adipose tissue, g</td>
<td>0.13±0.01</td>
<td>0.43±0.03*</td>
</tr>
<tr>
<td>Epididymal fat, g</td>
<td>1.03±0.10</td>
<td>3.40±0.32*</td>
</tr>
<tr>
<td>Perirenal fat, g</td>
<td>0.42±0.06</td>
<td>1.39±0.15*</td>
</tr>
<tr>
<td>Omental fat, g</td>
<td>0.37±0.04</td>
<td>1.23±0.09*</td>
</tr>
<tr>
<td>Plasma leptin, ng/ml</td>
<td>6.0±1.3</td>
<td>49.6±1.6*</td>
</tr>
<tr>
<td>CSF leptin, ng/ml</td>
<td>0.10±0.05</td>
<td>1.15±0.1*</td>
</tr>
</tbody>
</table>

Data are means ± SE of 6 animals per group. *$P < 0.05$ vs. lean mice. CSF, cerebrospinal fluid.
because in lean mice, we found that intracerebroventricular administration of 0.5 μg leptin caused no significant change in SNA subserving kidney (−3 ± 21%, n = 4) or BAT (−2 ± 17%, n = 4). As with the higher dose, the renal SNA response to 1 μg intracerebroventricular leptin was comparable between the lean (68 ± 14%, n = 9) and DIO mice (67 ± 22%, n = 7). In contrast, the BAT and lumbar SNA were significantly (P < 0.05) attenuated in the DIO mice (11 ± 7% n = 8 and 9 ± 10% n = 7, respectively) compared with the lean controls (33 ± 11%, n = 6 and 46 ± 17%, n = 7, respectively).

Preservation of renal sympathoexcitatory effect of leptin administered intracerebroventricularly, but not intravenously, in DIO mice suggests a saturation in leptin transport across the blood-brain barrier in these animals. To test this, first, we compared CSF concentrations of leptin between the lean and DIO mice. As shown in Table 1, CSF leptin was significantly elevated in DIO mice. Next, we assessed whether intravenous administration of leptin raises CSF leptin concentration levels. In lean mice, intravenous administration of leptin increased CSF leptin in a dose-dependent manner (Fig. 5). In contrast, in the DIO mice, the increase in CSF leptin following intravenous administration of leptin was attenuated (Fig. 5).

Effects of LY294002 and SHU9119 on the renal SNA response to leptin. Consistent with our previous report (30), in lean mice, blockade of PI3K attenuated the renal sympathoexcitatory effects of leptin, as the renal SNA response to intracerebroventricular leptin was substantially inhibited by intracerebroventricular pretreatment with LY294002 (Fig. 6A). As expected, intracerebroventricular pretreatment with the MC3/4R antagonist SHU9119 also inhibited the renal SNA response to intracerebroventricular leptin in lean mice (Fig. 6A). The dose of SHU9119 we used effectively inhibited the renal sympathetic activation to melanotan II (MTII), agonist of the MC3/4R. Indeed, MTII (2 μg icv) increased renal SNA by 140 ± 41% in mice pretreated with intracerebroventricular vehicle (n = 4) and only 18 ± 9% in mice pretreated with SHU9119 (30 pM icv, n = 5, P = 0.003).

Interestingly, in DIO mice, intracerebroventricular pretreatment with LY294002 or SHU9119 significantly inhibited renal sympathetic activation induced by intracerebroventricular leptin (Fig. 6B). As indicated above, we have previously showed that agouti obese mice have preserved renal sympathetic activation to leptin (6, 29). To test the role of PI3K and MC3/4R in the renal sympathoexcitatory effects of leptin, we examined the effect of the presence of LY294002 and SHU9119 on the renal SNA response to leptin in this mouse model of obesity. As shown in Fig. 6C, intracerebroventricular pretreatment with LY294002 blocked renal sympathetic activation to ICV leptin in the agouti obese mice. Noticeably, pretreatment with ICV SHU9119 significantly reduced the renal SNA response to intracerebroventricular leptin, but to a lesser extent compared with the lean and DIO mice.

Intracerebroventricular administration of LY294002 or SHU9119 alone, at the doses used with leptin (0.1 μg and 30 pM), had no effect on baseline renal SNA. For instance, injection of LY294002 (0.1 μg icv) caused no significant

Fig. 2. Change in food intake (A) and body weight (B) after intracerebroventricular administration of vehicle or leptin (5 μg) in lean and DIO mice. Data are expressed as means ± SE of 11 to 12 animals per group. There was a significant difference (P < 0.05) between lean and DIO mice in the effects of leptin on body weight and food intake. *P < 0.05 vs. vehicle; †P < 0.05 vs. DIO mice.

Fig. 3. Effect of intracerebroventricular administration of vehicle and leptin (5 μg) on renal sympathetic nerve activity (SNA) in lean and DIO mice. A: time course of renal SNA response to leptin vs. vehicle in lean mice. B: time course of renal SNA response to leptin vs. vehicle in DIO mice. C: comparison of renal SNA response (average of the 4th h) to vehicle and leptin between lean and DIO mice. Data are expressed as means ± SE of 14 to 21 animals per group. There was no significant difference (P = 0.71) between lean and DIO mice in the effects of leptin on renal SNA. *P < 0.01 vs. vehicle.
change in renal SNA in lean (6 ± 5%, n = 8) and DIO (0 ± 7%, n = 8) mice. However, higher doses of LY294002 (0.5 μg) and SHU9119 (150 pM) caused a significant (P < 0.05) decrease in renal SNA in the DIO mice (−46 ± 6%, n = 4 and −14 ± 11%, n = 7, respectively) compared with lean controls (4 ± 14%, n = 4 and 7 ± 12%, n = 7, respectively).

In accordance with our previous reports (13, 29, 30), compared with vehicle, leptin (administered intravenously or intracerebroventricularly), LY294002 and SHU9119 did not alter baseline mean arterial pressure or heart rate recorded during the SNA studies in the lean, DIO, or agouti obese mice (data not shown).

**DISCUSSION**

In the present study, there are two primary findings. First, we show that in late-stage dietary obesity, induced by 20 wk of a high-fat diet in mice, selective leptin resistance occurs with central neural administration of leptin. Preservation of renal sympathetic response to intracerebroventricular leptin in the presence of high CSF leptin levels could explain an adverse effect of leptin on blood pressure in obesity, despite resistance to the metabolic actions of leptin. Second, we reveal the mechanisms underlying the effect of leptin on renal SNA in obesity by demonstrating the pivotal role of PI3K and melanocortin receptors in mediating the renal sympathetic activation to leptin in DIO and agouti obese mice. In addition, the decrease in baseline renal SNA in the DIO mice following PI3K and MC3/4R blockade underscores the importance of these pathways for renal sympathetic tone in obesity.

Our finding that mice fed a high-fat diet for 20 wk are resistant to the effect of systemic, as well as central injection of leptin on food intake, body weight, and SNA to BAT and hindlimb, is consistent with our previous finding in modestly obese mice on a high-fat diet for 10 wk (33). However, with regard to renal SNA response to leptin, our findings in the present study differ from our previous report (33). The modest obesity caused by feeding mice a high-fat diet for 10 wk was associated with preservation of renal SNA response to systemic, as well as central administration of leptin. Here, we found that the pronounced obesity caused by 20 wk of a...
high-fat diet was associated with reduced ability of systemic injection of leptin to increase renal SNA. However, renal SNA response to central injection of leptin was intact. The differential effect of systemic injection of leptin on renal SNA between mice fed a high-fat diet for 10 and 20 wk indicates that there is saturation in transport of exogenous leptin across the blood-brain barrier, as the obesity induced by a high-fat diet progresses. A decreased ability of leptin to cross the blood-brain barrier has been suggested as an important mechanism of leptin resistance (10). This is supported by our finding of attenuated increase in CSF concentration of leptin in the DIO mice 30 min after intravenous administration of leptin. Although not tested here, a more pronounced increase in CSF leptin in the DIO mice may occur at later time points than 30 min, which could explain the slight, but significant, increase in SNA in DIO mice after intravenous administration of leptin. We also found that in the DIO mice, the high circulating levels of leptin are associated with an increase in CSF leptin, suggesting that endogenous leptin crosses the blood-brain barrier in these animals. Renal SNA is important for the regulation of arterial pressure and particularly in the pathogenesis of obesity-induced hypertension (12, 17). Therefore, the preserved ability of leptin to stimulate renal SNA associated with high CSF leptin could explain the role of this hormone in obesity-associated hypertension.

It was previously thought that STAT3 was the primary mediator of leptin actions (3, 38). In recent years, however, additional hypothalamic leptin signaling pathways have been identified, including PI3K. By engaging JAK2, the leptin receptor is able to stimulate insulin receptor substrates which, in turn, activates PI3K through an association to its regulatory subunit (24, 26). This pathway appears to be involved in the modulation by leptin of neuronal firing rate (23) and gene transcription (39). PI3K is a crucial signaling pathway for the control of appetite by leptin. Indeed, the effect of leptin on food intake is reversed by blockade of this enzyme (26, 30). We previously obtained evidence that PI3K mediates the renal SNA response to leptin in lean mice (30). Here, we extend our findings to demonstrate that PI3K mediates renal sympathetic activation to leptin in obesity as well. Indeed, pretreatment with PI3K inhibitor, LY294002, blocked the renal sympathetic activation to leptin in DIO and agouti obese mice. The melanocortin system is an essential mediator of leptin action in the central nervous system (8, 24). Proopiomelanocortin neurons releases α-melanocyte-stimulating hormone, which acts mainly on MC4R located in second-order hypothalamic targets (2, 8). Renal sympathetic activation to leptin seems mediated by the melanocortin system because pharmacological blockade of the MC3/4R (13) or genetic disruption of MC4R (31) inhibits the renal SNA response to leptin. Interestingly, our results indicate that the MC3/4R mediate the preserved renal SNA response to leptin in obesity. Indeed, pretreatment with SHU9119 inhibited the leptin-induced increase in renal SNA in both DIO and agouti obese mice. Blockade of renal SNA response to leptin with SHU9119 in agouti obese mice is particularly intriguing because in this mouse model, obesity is caused by ubiquitous overexpression of agouti protein that blocks hypothalamic melanocortin receptors (27). Our data seem to suggest that in the agouti obese mice the melanocortin system involved in the control of renal SNA is functional, at least partially.

Perspectives and Significance

Understanding the mechanisms of selective leptin resistance will provide valuable insight into the pathophysiological role of leptin in the cardiovascular diseases associated with obesity. Selective leptin resistance may result from the differential levels of leptin resistance found in distinct brain nuclei or postreceptor intracellular signaling mechanisms associated with the leptin receptor. Indeed, activation of renal sympathetic activity by leptin can be evoked by the action of this hormone in the arcuate nucleus (32) and the ventromedial and dorsomedial hypothalamus (22). In the obese mice, the inability of leptin to activate intracellular signaling pathways such as the signal transducer and activator of transcription 3 protein appears to be restricted to the arcuate nucleus of the hypothalamus (25). In contrast, other hypothalamic and extrahypothalamic nuclei remain leptin sensitive (25). Therefore, in obesity, the cardiovascular sympathetic effects of leptin may be mediated by those nuclei that remain sensitive to leptin.

REFERENCES


