Role of Hypothalamic Melanocortin 3/4-Receptors in Mediating Chronic Cardiovascular, Renal, and Metabolic Actions of Leptin

Alexandre A. da Silva, Jay J. Kuo, John E. Hall

Abstract—The present study examined whether blockade of melanocortin receptors subtypes 3 and 4 (MC3/4-R) inhibits chronic cardiovascular and dietary responses to leptin infusion. A cannula was placed in the lateral ventricle of male Sprague-Dawley rats for chronic intracerebroventricular (ICV) infusion via osmotic minipump, and arterial and venous catheters were implanted for measurement of mean arterial pressure (MAP) and heart rate (HR) 24 h/d and IV infusions. After a 5-day control period, rats received (1) 0.9% saline vehicle ICV for 12 days plus leptin (1 μg/kg per minute IV, n=5) during the final 7 days; (2) MC3/4-R antagonist SHU-9119 (1 nmol/h ICV) for 12 days plus leptin (1 μg/kg per minute IV, n=6) during the final 7 days; and (3) SHU-9119 (1 nmol/h ICV, n=8) for 12 days. Leptin infusion in vehicle-treated rats caused a small increase in MAP (5±1 mm Hg) despite reduced food intake (23±1 to 10±1 g/d) and decreased body weight (−6%±1%). SHU-9119 infusion completely prevented the cardiovascular and dietary actions of leptin, leading to increased food intake (23±1 to 49±4 g/d) and body weight (+30%±2%), markedly decreased HR (−77±9 bpm), and caused a decrease in MAP (−6±1 mm Hg). Similar results were observed when SHU-9119 was infused alone in vehicle-treated rats. Leptin decreased plasma insulin to 30% of control values, an effect that was also abolished by SHU-9119 treatment, which caused a 5-fold increase in plasma insulin concentration. Thus, MC3/4-R antagonism completely blocked the chronic cardiovascular, satiety, and metabolic effects of leptin, suggesting that the hypothalamic melanocortin system plays an important role in mediating these actions of leptin. (Hypertension. 2004; 43:1312-1317.)

Key Words: hypertension ■ blood pressure ■ obesity ■ insulin

Leptin is synthesized mainly by adipocytes in proportion to the amount of body fat, and has been shown to cause weight loss by reducing appetite and increasing energy expenditure by stimulating sympathetic nervous system (SNS) activity to various tissues, including brown adipose tissue.1–3 Acute studies have also demonstrated that leptin increases SNS activity to the kidneys.4 A previous study from our laboratory showed that chronic leptin infusions, producing levels similar to those observed in morbidly obese humans, caused sustained elevations in arterial pressure in rodents.5 Moreover, the elevation in arterial pressure caused by chronic leptin infusion is prevented by combined α- and β-adrenergic blockade.6 This suggests that the adrenergic system mediates the chronic pressor actions of leptin and is consistent with the hypothesis that leptin may be an important factor linking obesity, increased SNS activity, and hypertension.

The mechanisms that mediate the long-term actions of leptin on appetite, SNS activity, and blood pressure are still not fully understood, but may involve complex interactions with hypothalamic neuropeptide systems. Among these systems, the hypothalamic proopiomelanocortin (POMC) pathway may be especially important. One important POMC bioactive post-translational cleaved peptide is α-melanocyte stimulating hormone (α-MSH), an endogenous agonist of the melanocortin receptors subtypes 3 and 4 (MC3/4-R).7 Leptin activates the POMC-containing neurons in the arcuate nucleus stimulating the production and release of α-MSH, which, in turn, activates the MC3/4-R in several nuclei in the hypothalamus to cause reduced food intake and increased energy expenditure.1,8 Moreover, acute studies have shown that blockade of MC3/4-R abolishes the effects of leptin on appetite and energy balance,9,10 as well as the effects of leptin to increase renal SNS activity.11,12 These data suggest that the MC3/4-R may mediate the acute effects of leptin on appetite and SNS activity. However, there have been no studies, to our knowledge, that have examined whether the MC3/4-R mediates the long-term actions of leptin on arterial pressure and renal function.

In the present study, we examined the cardiovascular, renal, dietary, and hormonal responses to chronic hyperlep-
tinemia in the presence or absence of central inhibition of the MC3/4-R in normal Sprague-Dawley rats.

### Methods

#### Animal Surgeries

The experimental procedures and protocols of this study conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center.

#### Intra-Arterial and Intravenous Catheterization

Male Sprague-Dawley rats (275 to 325 g; Harlan, Indianapolis, Ind), were anesthetized with 50 mg/kg sodium pentobarbital (Nembutal), and atropine sulfate (0.1 mg/kg) was administered to prevent excess airway secretions. Arterial and venous catheters were implanted according to procedures previously described.\(^5,6\) Briefly, using aseptic techniques, a laparotomy was performed and a sterile nonocclusive polyvinyl catheter was inserted into the abdominal aorta, distal to the kidneys. Through a left femoral vein incision, a sterile catheter was placed in the vena cava. Both catheters were exteriorized through a subcutaneously implanted stainless steel button.

#### Intracerebroventricular Cannulation

Immediately after arterial and venous catheter implantation, a stainless steel cannula (26 gauge, 10 mm long) was implanted into the right lateral cerebral ventricle using the coordinates as previously described.\(^13\) The guide cannula was anchored into place with 3 stainless steel machine screws, a metal cap, and dental acrylic, and a stylet was inserted to seal the cannula until use. During stereotaxic manipulation, anesthesia was maintained with 0.5% isoflurane. Several days after recovery from surgery, accuracy of the cannula placement was tested by measuring the dipsogenic response (immediate drinking of at least 5 mL of water in 10 minutes) to an intracerebroventricular (ICV) injection of 100 ng of angiotensin II. After the experiment, the animals were euthanized and the brains removed and sectioned to confirm the placement of the cannula.

After recovery from anesthesia, the rats were housed in individual metabolic cages for determination of daily water and electrolyte balances. The arterial and venous catheters were connected to a dual-channel infusion swivel (Instech). The arterial catheter was connected to a pressure transducer (Maxxim) for continuous 24-hour measurement of mean arterial pressure (MAP) and heart rate (HR) using computerized techniques as previously described.\(^8\) The venous catheter was connected to a syringe pump for continuous infusion of saline (0.45%, 40 mL/d). The rats received food and water ad libitum throughout the study. Total sodium intake was maintained constant at \(\approx 3.1 \text{ mEq/d} \) via the continuous saline infusion combined with sodium-deficient rat chow (0.006 mmol sodium/g food, Teklad). Intravenous solutions were infused through a sterile filter (0.22 \(\mu\)m, Millipore) and the saline infusion was started immediately after placement of the rats into the metabolic cages. The rats were allowed to recover for 7 to 10 days before control measurements were initiated.

#### Experimental Protocols

MAP, HR, urine volume, urinary sodium and potassium excretion, and food and water intake were recorded daily. Blood samples (1.5 mL) were collected once during the control period and on day 11 of the experimental period for measurements of glomerular filtration rate (GFR), plasma renin activity (PRA), and plasma insulin, glucose, and leptin concentrations. The blood was replaced with 1.5 mL of saline 0.9%.

#### Chronic Leptin Infusion in 5 Control Rats

After a 5-day control period, the saline vehicle (0.9% saline, 0.5 \(\mu\)L/h) was infused ICV for 12 days via osmotic minipumps. After the first 5 days of ICV vehicle infusion, leptin (1 \(\mu\)g/kg per minute) was added to the intravenous saline infusion for the remaining 7 days. The rate of leptin infusion was based on our previous studies showing an increase in plasma leptin levels to \(\approx 45 \text{ to 90 ng/mL}\).\(^5,6\)

#### Chronic MC3/4-R Blockade

After a 5-day control period, the MC3/4-R antagonist, SHU-9119 (1 nmol/h, 0.5 \(\mu\)L/h, Polypeptide Laboratories), was infused ICV for 12 days via osmotic minipumps. These rats (n=8) only received intravenous saline infusion.

#### Chronic Leptin Infusion During MC3/4-R Blockade

After a 5-day control period, the MC3/4-R antagonist, SHU-9119 (1 nmol/h, 0.5 \(\mu\)L/hr, Polypeptide Laboratories), was infused ICV for 12 days via osmotic minipumps in 6 rats. After the first 5 days of ICV SHU-9119 infusion, leptin (1 \(\mu\)g/kg per minute) was added to the intravenous saline infusion for the remaining 7 days. The rate of SHU-9119 infusion chosen was based on our previous study showing that this dose effectively blocks the MC3/4-R.\(^13\)

#### Analytical Methods

PRA and plasma insulin and leptin concentrations were measured by radioimmunoassay. Plasma glucose concentration was measured using the glucose oxidation method (Beckman glucose analyzer 2). Urine sodium and potassium concentrations were measured using ion-sensitive electrodes (NOVA electrolyte analyzer 1+). GFR was calculated from the 24-hour clearance of \(^{[32]}\)I-iodotamol, as previously described.\(^5\)

#### Statistical Analysis

The data are expressed as mean \(\pm\) SEM and analyzed using 2-factor ANOVA with repeated measures. The Bonferroni post hoc test was used for comparisons between groups. Dunnett’s test was used for comparisons of experimental and control values within each group, when appropriate. Statistical significance was accepted at a level of \(P<0.05\).

### Results

#### Effects of Leptin During MC3/4-R Antagonism on Food Intake and Hormones

Leptin infusion for 7 days decreased food intake from 23 \(\pm\) 1 g to an average of 10 \(\pm\) 1 g/d on the 7th day (Figure 1). The reduced food intake was accompanied by a 6% reduction in body weight (Table). During MC3/4-R blockade, leptin did not decrease food intake. In fact, during MC3/4-R blockade plus leptin infusion, food intake increased from 23 \(\pm\) 1 to an average of 45 \(\pm\) 2 g/d, and this was accompanied by \(\approx 120 \text{ g increase in body weight during the 12 days of treatment}\). These results were similar to those observed in rats receiving SHU-9119 alone in which food intake increased from 24 \(\pm\) 1 to an average of 41 \(\pm\) 1 g/d, resulting in a weight gain of \(\approx 113 \text{ g during the 12 days of treatment}\) (Figure 1 and Table).

Fasting plasma glucose concentration did not change in any of the 3 groups studied (Table). Plasma insulin levels declined from 37 \(\pm\) 12 to 12 \(\pm\) 3 \(\mu\)U/mL during leptin infusion in vehicle-treated rats and increased \(\approx 5\)-fold in MC3/4-R–blocked rats, even during leptin infusion (Table). These results confirm our previous finding that MC3/4-R antagonism causes hyperinsulinemia associated with increased food intake and weight gain, and indicates that MC3/4-R antagonism also abolished the effects of leptin to decrease plasma insulin.

Chronic leptin infusion in vehicle-infused rats increased plasma leptin concentration from 2.2 \(\pm\) 0.4 to 40.9 \(\pm\) 1.9 ng/mL (Table). MC3/4-R blockade alone increased plasma
leptin concentration from 2.2/1000 to 36.3/1000 to 3.6 ng/mL. When leptin was infused in rats pretreated with SHU-9119, plasma leptin increased even further from 2.4/1000 to 77/1000 to 2.4 ng/mL. PRA did not change significantly in any of the groups (Table).

**Effects of Leptin During MC3/4-R Antagonism on MAP and HR**

Chronic leptin infusion in vehicle-treated rats raised MAP by 5±1 mm Hg on the 7th day of infusion, whereas HR was slightly, but not significantly, elevated (Figures 1 and 2). MC3/4-R antagonism completely prevented the pressor effects of leptin. In fact, after MC3/4-R antagonism, MAP decreased by −6±1 mm Hg during leptin infusion. MC3/4-R antagonism with SHU-9119 infusion alone decreased MAP by ~3±1 mm Hg (Figures 1 and 2). SHU-9119 treatment also decreased HR by −75±2 beats per minute in rats receiving SHU-9119 plus vehicle and −78±8 beats per minute in SHU-treated rats that received leptin infusion (Figures 1 and 2).

**Discussion**

The most important finding of this study is that blockade of the MC3/4-R completely abolished the effects of chronic hyperleptinemia to reduce food intake and plasma insulin, and to increase arterial pressure. These observations support the concept that the hypothalamic melanocortin pathway may be a key mediator of leptin actions on appetite, arterial pressure, and insulin sensitivity, and are consistent with our previous finding that an intact hypothalamic MC3/4-R may be necessary for weight gain to raise arterial pressure.13

**Effects of Leptin During MC3/4-R Antagonism on Renal Function**

Leptin infusion in control rats caused no significant changes in urine volume or sodium excretion (Table). Potassium excretion, however, decreased significantly because of decreased food intake, the only source of potassium intake in this study. This resulted in a negative cumulative potassium balance of 11.6±2.3 mEq during 7 days of leptin infusion. In contrast, SHU-9119 plus vehicle-treated rats had a positive cumulative potassium balance of 5.5±1.7 mEq and SHU-9119 plus leptin-treated rats had a positive cumulative potassium balance of 6.8±2.6 mEq, paralleling the increased food intake and weight gain. MC3/4-R antagonism increased urine volume but did not alter sodium excretion.

Leptin infusion did not significantly change GFR in vehicle-treated rats. MC3/4-R inhibition caused an increase in GFR of ~20% in vehicle and leptin-treated groups (Table).

**Effects of Leptin During MC3/4-R Antagonism on Food Intake and Hormones**

In the present study, leptin infusion for 7 days, at a rate that raised plasma leptin concentrations to levels similar to those found in obesity, decreased food intake by ~50%. This reduction in food intake was comparable to the reduction observed in our previous studies.5–6 SHU-9119 alone also increased plasma leptin markedly, paralleling the increase in food intake and weight gain. In the rats that received SHU-9119 plus leptin infusion, plasma leptin concentration increased further, reaching levels comparable to that observed in severe human obesity.14,15

Even in the presence of very high levels of leptin, MC3/4-R antagonism caused hyperphagia and abolished the appetite suppressing actions of leptin. This observation suggests that activation of the POMC pathway, and ultimately activation of MC3/4-R, is important for the long-term effects of leptin to decrease food intake.

Chronic leptin infusion markedly decreased fasting plasma insulin concentration with no alteration in plasma glucose concentration, suggesting increased insulin sensitivity as we have previously reported.5–6 MC3/4-R antagonism abolished the effect of leptin to decrease plasma insulin. Instead, MC3/4-R antagonism caused severe hyperinsulinemia, even in the presence of hyperleptinemia. This suggests that MC3/4-R antagonism may abolish the insulin-sensitizing effects of hyperleptinemia. Muzumdar et al have recently shown that the ability of leptin to acutely decrease insulin levels during hyperinsulinemic–euglycemic clamp was blocked by ICV administration of SHU-9119.16 These short-term studies also...
suggest that MC3/4-R antagonism blocks leptin’s ability to increase insulin sensitivity independent of changes in appetite, because the hyperinsulinemic-euglycemic clamp study was performed under fasting conditions. Obici et al also demonstrated that modulation of central melanocortinergic neurons has a major impact on peripheral and hepatic insulin action.17 It has also been reported that male MC4 knockout mice demonstrated that modulation of central melanocortinergic action may be mediated, at least in part, by central nervous system (CNS) effects, including stimulation of the POMC system.18 It has also been reported that male MC4 knockout mice (−/−) display diabetes mellitus and marked hyperinsulinemia.19 It is important to note that these mice are very obese and whether the metabolic disorders observed in these mice are directly caused by effects of the lack of a functional MC4-R or because of the obesity itself is unclear. The results of the present study suggest that the MC3/4-R may play an important role in mediating the long-term effects of leptin on plasma insulin levels, and presumably insulin sensitivity.

Although the mechanisms by which hyperleptinemia increases glucose utilization are not well understood, results from the present study and others16,19,20 suggest that this action may be mediated, at least in part, by central nervous system (CNS) effects, including stimulation of the POMC pathway. Previous results from our laboratory indicate that peripheral blockade of α1-adrenergic or β1+2-adrenergic receptors does not attenuate the effects of leptin to enhance insulin sensitivity.6 Therefore, the mechanism by which the effects of leptin and MC3/4-R activation on the CNS are transmitted to peripheral tissues remains to be determined and is a promising area for further investigation.

Arterial Pressure and HR Responses to Leptin During MC3/4-R Antagonism

In the present study, MC3/4-R inhibition completely abolished the effects of leptin to raise blood pressure. Moreover, MC3/4-R antagonism caused a modest decrease in blood pressure and a pronounced decrease in HR despite marked increases in food intake and body weight, which are usually associated with elevations in arterial pressure and heart rate.

These observations indicate that the long-term effects of leptin on blood pressure and HR require an intact MC3/4-R. These data also support our previous results suggesting that endogenous activity of the melanocortin system has a tonic cardiovascular action and that an intact POMC pathway may be necessary for linking obesity and hyperleptinemia to increased sympathetic activity and hypertension.

The mechanisms by which chronic inhibition of the MC3/4-R decreases HR and arterial pressure in the presence of rapid weight gain were not directly tested in the present study, but are consistent with inhibition of sympathetic activity and/or increased parasympathetic activity. However, an important finding of this study is that tonic activity of the endogenous hypothalamic melanocortin system appears to play a role in maintaining HR and arterial pressure.

Although the chronic effects of leptin on arterial pressure, HR, appetite, and insulin were all completely blocked by inhibiting the MC3/4-R in our studies, previous short-term studies have suggested that some of the effects of leptin, such as stimulation of sympathetic activity to brown adipose tissue, were not attenuated by MC3/4-R inhibition.11 Leptin may also influence activity of other neuropeptide systems, such as NPY. However, the importance of these systems in mediating the long-term cardiovascular and sympathetic effects of leptin has, to our knowledge, not been tested.

An observation that may, at first glance, seem inconsistent with the results of the present study is the finding that acute leptin administration can increase renal sympathetic activity in the agouti yellow mouse, a model of obesity caused by overexpression of agouti protein that inhibits melanocortin receptors.21 However, there may be additional changes in the agouti mouse that could influence blood pressure besides inhibition of hypothalamic melanocortin receptors. For example, the agouti mouse has increased cocaine- and amphetamine-regulated transcript (CART) expression in the hypothalamus22 and excess agouti protein has been reported to directly increase intracellular Ca²⁺ concentration in many tissues, including vascular smooth muscle.23 Our observations in the present and previous studies13 suggest that pharmacological inhibition of the MC3/4-R alone completely

<table>
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<tr>
<th>Experimental Groups</th>
<th>Body weight, g</th>
<th>Glucose, mg/100 mL</th>
<th>Insulin, μU/mL</th>
<th>Leptin, ng/mL per hour</th>
<th>PRA, mL/min</th>
<th>GFR, mL/min</th>
<th>Urine Volume, mL/d</th>
<th>UNaV, mmol/d</th>
<th>UKV, mmol/d</th>
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<td>Control</td>
<td>379±5</td>
<td>149±3</td>
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<tr>
<td>Leptin day 7</td>
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<td>147±12</td>
<td>12±3*†</td>
<td>40.9±1.9*</td>
<td>3.5±1.3</td>
<td>3.0±0.2</td>
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<td>Control</td>
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<tr>
<td>Control</td>
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UnV indicates urinary sodium excretion; UKV, urinary potassium excretion.

* P<0.05 compared with control; †P<0.05 compared with SHU plus leptin day 7.
abolishes the effects of leptin on HR and arterial pressure. Consistent with our results is the observation of Haynes et al that inhibition of MC3/4-R completely prevented the acute effects of leptin to stimulate renal sympathetic nerve activity. Thus, the effects of leptin on renal sympathetic activity, arterial pressure, and HR all appear to be mediated primarily through pathways that lead to activation of MC3/4-R.

Effects of Leptin During MC3/4-R Antagonism on Renal Function

In the present study and in previous studies from our laboratory, we found no significant changes in sodium excretion or urine volume during chronic leptin infusion at rates that raised plasma leptin concentrations to physiological and pathophysiological levels. The absence of significant changes in sodium excretion despite a 4- to 5-mm Hg increase in arterial pressure during chronic leptin infusion suggests that leptin may have caused a slight shift in the renal-pressure natriuresis relationship to higher arterial pressures. On the other hand, treatment with the MC3/4-R antagonist caused a shift in the renal-pressure natriuresis toward lower blood pressure, as evidenced by normal sodium excretion and increased diuresis despite lower arterial pressure, even in leptin-infused rats. This suggests that the MC3/4-R mediates, at least in part, the effects of leptin on renal function, probably by preventing increases in renal sympathetic activity normally elicited by chronic hyperleptinemia. Previous acute studies indicate that MC3/4-R antagonism completely abolishes the effect of leptin to increase renal SNS activity. However, further studies are needed to test the importance of the MC3/4-R in mediating the long-term effects of leptin on renal SNS activity.

The changes in GFR and potassium excretion in MC3/4-R–blocked rats are likely to be related to increased food intake and weight gain observed in these groups. Leptin infusion by itself, however, decreased potassium excretion and caused a negative potassium balance, mainly because of its effect on food intake, the only source of potassium in this study. These effects were also prevented by blockade of the MC3/4-R.

Perspectives

Our results indicate that chronic inhibition of the MC3/4-R completely prevented the cardiovascular, dietary, metabolic, and renal actions of chronic leptin infusion. Moreover, this study provides further evidence that the melanocortin system may be an important link for obesity, hyperleptinemia, and increased blood pressure. Whether this is related to changes in sympathetic activity and whether drugs that block the melanocortin system pathway may protect against cardiovascular injury associated with obesity require further investigation. Leptin also appears to markedly enhance insulin sensitivity and this action is also prevented by central MC3/4-R blockade. The mechanisms involved in the association between leptin, MC3/4-R, and insulin sensitivity are still unclear and remain an important area for further investigation.

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References


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