Regulation of Blood Pressure, Appetite, and Glucose by Leptin After Inactivation of Insulin Receptor Substrate 2 Signaling in the Entire Brain or in Proopiomelanocortin Neurons

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Abstract—Insulin receptor substrate 2 (IRS2) is one of the 3 major leptin receptor signaling pathways, but its role in mediating the chronic effects of leptin on blood pressure, food intake, and glucose regulation is unclear. We tested whether genetic inactivation of IRS2 in the entire brain (IRS2/Nestin-cre mice) or specifically in proopiomelanocortin (POMC) neurons (IRS2/POMC-cre mice) attenuates the chronic cardiovascular, metabolic, and anti-diabetic effects of leptin. Mice were instrumented with telemetry probes for measurement of blood pressure and heart rate and with venous catheters for intravenous infusions. After a 5-day control period, mice received leptin infusion (2 μg/kg per minute) for 7 days. Compared with control IRS2flox/flox mice, IRS2/POMC-cre mice had similar body weight and food intake (33±1 versus 35±1 g and 3.6±0.5 versus 3.8±0.2 g per day) but higher mean arterial pressure (MAP) and heart rate (110±2 versus 102±2 mm Hg and 641±9 versus 616±5 bpm). IRS2/Nestin-cre mice were heavier (38±2 g), slightly hyperphagic (4.5±1.0 g per day), and had higher MAP and heart rate (108±2 mm Hg and 659±9 bpm) compared with control mice. Leptin infusion gradually increased MAP despite decreasing food intake by 31% in IRS2flox/flox and in Nestin-cre control mice. In contrast, leptin infusion did not change MAP in IRS2/Nestin-cre or IRS2/POMC-cre mice. The anorexic and anti-diabetic effects of leptin, however, were similar in all 3 groups. These results indicate that IRS2 signaling in the central nervous system, and particularly in POMC neurons, is essential for the chronic actions of leptin to raise MAP but not for its anorexic or anti-diabetic effects. (Hypertension. 2016;67:378-386. DOI: 10.1161/HYPERTENSIONAHA.115.06153.)

Online Data Supplement

Key Words: blood pressure ■ heart rate ■ insulin ■ obesity ■ proopiomelanocortin

The effects of leptin on body weight regulation and sympathetic nervous system activity are mediated by activation of the long form of the leptin receptor (LR) in the central nervous system (CNS), especially in the arcuate nucleus of the hypothalamus and the nucleus of the tractus solitarius. However, the CNS centers and signaling pathways by which leptin chronically regulates blood pressure (BP), as well as energy balance and glucose homeostasis, are only beginning to be elucidated.

The LR is a cytokine receptor that activates Janus tyrosine kinase-2, resulting in transphosphorylation of Janus tyrosine kinase-2 and phosphorylation of specific tyrosine residues located within the C-terminal domain of LR. Janus tyrosine kinase-2 tyrosine phosphorylation in the CNS also leads to phosphorylation of insulin receptor substrate 2 (IRS2) protein that binds to the regulatory subunit (P85) of phosphatidylinositol 3-kinase (PI3K) and activates the catalytic domain of this intracellular enzyme, initiating a cascade of downstream kinases that regulate ion channel activity, gene transcription, and protein translation.

The IRS2–PI3K pathway may contribute to LR signal transduction and insulin signaling in the CNS. Pharmacological blockade of PI3K partially attenuated the acute effect of leptin on food intake, reversed the inhibition of neuropeptide Y expression caused by leptin, and has been reported to attenuate the acute effects of leptin to raise renal sympathetic nervous system. In addition, previous studies showed that IRS2 signaling plays an important role in glucose regulation, as evidenced by progressive development of type 2 diabetes mellitus in mice with whole-body IRS2 deficiency, despite having similar body weight as littermate controls. However, there have been no studies, to our knowledge, that
have investigated the role of the IRS2 in the CNS in mediating the chronic effects of leptin on appetite, energy expenditure, glucose homeostasis, and BP regulation.

In this study, we developed mouse models with IRS2 inactivated in the entire CNS or specifically in POMC neurons to examine the importance of IRS2 in contributing to the chronic effects of leptin on metabolic and cardiovascular functions. Our results indicate that IRS2 signaling in POMC neurons contributes to the long-term BP actions of leptin but not the anorexic or antidiabetic effects of leptin.

**Methods**

The experimental procedures and protocols for these studies followed 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.

**Animals**

Twenty-two–week-old male IRS2/Nestin-cre (n=23), IRS2/POMC-cre (n=21), IRS2flox/flox (n=24), and Nestin-cre (n=4) control mice were used in these studies. IRS2/Nestin-cre mice were generated by expressing Nestin-cre mice that express Cre-recombinase under the control of the rat Nestin promoter and enhancer (Jackson Laboratories, stock number, 003711) with IRS2flox/flox mice (generously provided by Dr Morris White, Harvard University). IRS2/POMC-cre mice were generated by crossing POMC-cre mice that express Cre-recombinase under the control of POMC promoter (generously provided by Dr Joel Elmquist, Southwestern Texas University, Dallas, TX) with IRS2flox/flox mice. Homozygous mice for the IRS2flox/flox gene that also expressed Cre-recombinase were used as IRS2/Nestin-cre or IRS2/POMC-cre mice. Littermate homozygous IRS2flox/flox mice from our colony and Nestin-cre mice were used as controls for the chronic experiments (Figure S1A in the online-only Data Supplement).

Polymerase chain reaction analysis confirmed the Cre-mediated-specific recombination of the IRS2flox allele in neuronal cells of cerebral cortex, hypothalamus, and hindbrain but not in the liver of IRS2/Nestin-cre mice (Figure S1B). Fine-needle (20 gauge) puncture biopsies were obtained from the hypothalamus, arcuate nucleus, and hindbrain of the IRS2/POMC-cre mice and analyzed using a DNA/RNA microkit (Qiagen, MD) to confirm Cre-mediated recombination of the IRS2flox allele in these areas. We also analyzed samples of the brain cortex and liver to confirm that Cre-mediated recombination of the IRSflox/flox allele did not occur in these tissues. To further validate the deletion of IRS2 in the entire CNS, we performed 3,3′-diaminobenzidine staining of IRS2 in the arcuate nucleus and ventromedial hypothalamus (details are given in Figure S1C).

**Body Weight and Body Composition Analysis**

A group of male IRS2/Nestin-cre (n=5) and IRS2/POMC-cre mice (n=5) and IRS2flox/flox (n=6) and Nestin-cre controls (n=4) were individually housed and fed standard chow (Harlan Teklad, WI) for weekly determination of body weight and body composition starting at 6 weeks of age until 18 weeks of age to examine the importance of IRS2 in the entire CNS and specifically in POMC neurons for body weight regulation. Body composition was assessed weekly using magnetic resonance imaging (EchoMRI-900TM, Echo Medical System, Houston, TX) to quantify lean mass, fat mass, and free water and total water content in conscious mice.

**Telemetry Probe and Venous Catheter Implantation**

Under isoflurane anesthesia, male IRS2/Nestin-cre (n=8), IRS2/POMC-cre (n=6), IRS2flox/flox (n=6), and Nestin-cre (n=4) mice were implanted with telemetric pressure transmitter probes in the left carotid artery for determination of mean arterial pressure (MAP) and heart rate (HR) 24 hours per day using computerized methods for data collection as previously described. Daily MAP and HR were obtained from the average of 24-hour recordings using the sampling rate of 1000 Hz with duration of 10 s for every 10-minute period. A venous catheter was also implanted in the jugular vein for infusions of saline vehicle or leptin. The venous catheter was tunneled subcutaneously, exteriorized between the scapulae, and passed through a spring connected to a mouse swivel (Instech) mounted on the top of the plastic metabolic cages. The venous catheter was connected through a sterile filter to a syringe pump for continuous saline and leptin infusions. Food and water were offered ad libitum throughout the experiment, and room temperature was maintained at 23±1°C. A normal sodium intake of 460 μmol per day was kept constant by continuously infusing isotonic saline combined with a sodium-deficient rodent chow as previously described. The mice were allowed to recover for 8 to 10 days after surgery before baseline measurements were taken.

**Glucose Tolerance Test**

ν-Glucose (3 mg/kg of lean mass plus 1 mg/kg of fat mass) was administered by gavage after a 6-hour fast in 22-week-old male IRS2/Nestin-cre (n=6), IRS2/POMC-cre (n=6), and IRS2flox/flox mice (n=6). Blood samples were collected by tail snip, and blood glucose was measured at 0, 15, 30, 60, 90, and 120 minutes after the gavage using glucose strips (ReliOn).

**Experimental Design**

**Acute and Chronic Leptin Infusions**

**Acute Leptin Injection**

To determine the role of IRS2 in the entire CNS or specifically in POMC neurons in mediating the acute effects of leptin on appetite, food intake was measured 2, 4, 16, and 24 hours after intraperitoneal injection of leptin (5 mg/kg) or saline vehicle (0.3 mL) between 5:00 pm and 6:00 pm in a separate group of noninstrumented and nonfasted IRS2/Nestin-cre (n=6), IRS2/POMC-cre (n=6), and IRS2flox/flox control mice (n=6) at 22 weeks of age.

**Chronic Leptin Infusion**

After an 8- to 10-day postsurgery recovery period and 5 days of stable baseline control measurements, leptin was added to the continuous saline vehicle infusion (3.0 mL per day) at the rate of 2 μg/kg per minute in separate groups of instrumented IRS2flox/flox (n=9), Nestin-cre (n=4), IRS2/POMC-cre (n=7), and IRS2/Nestin-cre mice (n=7), which were followed by a 5-day post-treatment period during which only saline vehicle was infused intravenously. MAP, HR, urine volume, and food and water intake were recorded daily. Blood samples (100 μL) were collected via a tail snip after 6 hours of fasting (8:00 am to 2:00 pm) during the control period (day 5), on the last day of leptin infusion (day 7 of treatment), and at the end of the post-treatment period (day 5 after stopping leptin infusion) for measurements of plasma glucose, leptin, and insulin concentrations.

**Acute Air-Jet Stress Test**

To determine whether IRS2 deletion in the entire CNS or specifically in POMC neurons alters MAP response to an acute pressor stress,
mice were acclimatized to the new environment for \( \approx 1,000 \) minutes at 10-minute intervals continuously 24 hours a day using a air-jet stress testing as previously described. Details are given in the online-only Data Supplement.

**Power Spectral Analyses of Systolic Arterial Pressure and RR Interval Oscillations**

To assess the impact of chronic leptin infusion on sympathetic function in mice with IRS2 deletion in the entire CNS or specifically in POMC neurons, we used power spectral densities of systolic arterial pressure and RR interval oscillations by 512-point fast Fourier transform integrated over the specific frequency range (low frequency, 0.25–0.75 Hz; high frequency, 0.75–5.0 Hz) using Nevrokard SA-BRS software (Medistar, Ljubjana, Slovenia; details are given in the online-only Data Supplement).

**Oxygen Consumption and Motor Activity**

In separate experiments, IRS2\(^{+/+}\) (n=9), Nestin-cre (n=4), IRS2/POMC-cre (n=7), and IRS2/Nestin-cre (n=7) mice at 22 weeks of age were placed individually in metabolic cages (AccuScan Instruments Inc, Columbus, OH) equipped with oxygen sensors to measure oxygen consumption (\( \text{VO}_2 \)) and motor activity. \( \text{VO}_2 \) was measured for 2 minutes at 10-minute intervals continuously 24 hours a day using a Zirconia oxygen sensor. Motor activity was determined using infrared light beams mounted in the cages in x, y, and z axes. After the mice were acclimatized to the new environment for \( \approx 4 \) to 6 days, \( \text{VO}_2 \) and motor activity were recorded for 3 consecutive days. Then, the mice were lightly anesthetized with isoflurane, and an osmotic minipump (model 1007D, Duract Corp) was placed intraperitoneally to deliver leptin (4 \( \mu \text{g/kg per minute} \)) for 7 days. Mice were followed up for an additional 5-day post-treatment period.

To further investigate the role of IRS2 signaling on glucose homeostasis and to test the hypothesis that leptin-mediated activation of IRS2 plays an important role in mediating the chronic antidiabetic actions of leptin, we performed additional chronic leptin infusion experiments in streptozotocin-diabetic IRS2/Nestin-cre and IRS2\(^{+/+}\) mice.

**Plasma Hormones and Glucose Measurements**

Plasma leptin and insulin concentrations were measured with ELISA kits (R&D Systems and Crystal Chem Inc, respectively), and plasma glucose concentrations were determined using the glucose oxidation method (Beckman glucose analyzer 2).

**Statistical Analysis**

The results are expressed as mean±SEM. Data were analyzed by paired \( t \) test or 1-way ANOVA with repeated measures followed by Dunnett post hoc test for comparisons between control and experimental values within each group when appropriate. Comparisons between different groups were made by unpaired \( t \) test or 1-way ANOVA followed by Dunnett post hoc test when appropriate. Statistical significance was accepted at a level of \( P<0.05 \).

**Results**

**IRS2 Inactivation in the Entire CNS or Specifically in POMC Neurons**

Mice were genotyped for the presence of IRS2\(^{+/+}\) transgene and cre-recombinase at 3 weeks of age using DNA obtained from a tail snip. Figure S1A and S1B show polymerase chain reaction amplification for IRS2\(^{+/+}\) and cre-recombinase from 28 tail snip samples with analyses indicating the presence or absence of cre-recombinase and IRS2\(^{+/+}\) transgenes. Nestin-Cre and POMC-Cre mice have been used to successfully delete genes of interest in the entire CNS or specifically in POMC neurons, respectively. Using polymerase chain reaction analysis, we confirmed a Cre-mediated–specific recombination of the IRS2\(^{+/+}\) allele in neuronal cells of cerebral cortex, hypothalamus, and hindbrain but not in the liver of IRS2/Nestin-cre mice (Figure S1B). Using needle puncture biopsies, we showed cre-mediated recombination of the IRS2\(^{+/+}\) allele in neuronal cells of the hypothalamus, arcuate nucleus, and hindbrain but not of the cerebral cortex and liver in IRS2/POMC-cre mice (Figure S1B). We also observed a nonspecific band \( \approx 160 \) bp from all samples examined using fine-needle puncture biopsies. We did not observe cre-mediated recombination in IRS2\(^{+/+}\) mice. We also found marked decreases in IRS2 immunostaining in the arcuate nucleus and ventromedial hypothalamic in IRS2/Nestin-cre mice (Figure S1C). The ratio of p-AKT/AKT protein content in the hypothalamus was significantly higher in mice treated chronically with leptin compared with the saline-treated group (Figure S1D) showing that IRS2 is also an important component of leptin signaling in the hypothalamus.

**Impact of IRS2 Deficiency in the Entire CNS or in POMC Neurons on Metabolic Phenotypes**

Body weight from 6 weeks until 18 weeks of age was significantly higher in IRS2/Nestin-cre mice compared with that of IRS2/POMC-cre, IRS2\(^{+/+}\) mice, and Nestin-cre mice. IRS2/POMC-cre mice were only slightly heavier than IRS2\(^{+/+}\) and Nestin-cre control mice (Figure 1A). Although the modestly higher body weight observed in IRS2/POMC-cre mice was due mainly to increased adiposity compared with control mice, the higher body weight of IRS2/Nestin-cre mice was associated with marked increases in lean and fat mass compared with the other 2 groups (Figure 1B and 1C). The higher body weight of IRS2/Nestin-cre mice was also associated with increased food intake as early as 6 weeks of age when we began monitoring daily food intake (Figure 1D). No significant differences in food intake were observed between IRS2/POMC-cre and IRS2\(^{+/+}\) mice.

Baseline \( \text{VO}_2 \) was not significantly different among the groups despite a tendency for higher \( \text{VO}_2 \) in IRS2/POMC-cre and IRS2/Nestin-cre mice compared with IRS2\(^{+/+}\) and Nestin-cre control mice (Table). Motor activity was reduced in IRS2/Nestin-cre compared with control and IRS2/POMC-cre mice (Table).

Baseline fasting plasma leptin, insulin, and glucose levels were not different between control IRS2\(^{+/+}\) and IRS2/POMC-cre mice (Table). However, IRS2/Nestin-cre mice exhibited elevated leptin and insulin levels and normal plasma glucose levels compared with the other 2 groups. Nestin-cre mice and IRS2\(^{+/+}\) control mice had reduced plasma leptin and insulin levels compared with IRS2/POMC-cre and IRS2/Nestin-cre mice (Table).

To further examine whether the lack of IRS2 in the entire CNS or specifically in POMC neurons impairs glucose handling, a glucose tolerance test was performed at 22 weeks of age. Although mice with IRS2 deletion in the entire CNS had the largest area under the curve during the glucose tolerance test, there were no statistical differences among the groups (Figure S2A and S2B).
Impact of IRS2 Deficiency in the Entire CNS or Specifically in POMC Neurons on Food Intake and Body Weight Responses to Acute and Chronic Leptin Infusion

Acute intraperitoneal leptin injection reduced 24-hour food intake in all groups by ≈30% compared with saline injection (Figure 2A). The acute anorexic effect of leptin was observed as early as 4 hours post injection in IRS2 flox/flox, Nestin-cre, IRS2/POMC-cre, and IRS2/Nestin-cre mice (data not shown). No changes in body weight were observed after acute leptin or saline injection (data not shown). These data indicate that activation of IRS2 in the entire CNS or specifically in POMC neurons is not required for the acute anorexic action of leptin.

Chronic leptin infusion for 7 days also markedly reduced food intake in IRS2 flox/flox, Nestin-cre, IRS2/POMC-cre, and IRS2/Nestin-cre mice (Figure 2B). The reduction in food intake was slightly, but significantly, greater in IRS2/Nestin-cre mice although the IRS2/Nestin-cre mice had a higher baseline food intake. On day 6 of leptin infusion, the reduction in food intake in IRS2/Nestin-cre mice was 48% compared with a 43% reduction in IRS2 flox/flox mice (Figure 2B), which led to a greater net deficit in food intake during leptin treatment (Figure 2C). This reduction in food intake was associated with a 10% and 8% body weight loss in IRS2 flox/flox (from 31±1 to 28±1 g), IRS2/POMC-cre (from 33±1 to 29±1 g), and IRS2/Nestin-cre mice (from 41±2 to 37±1 g; Figure 2D) and significant reduction in fat mass (Figure 2E). After leptin infusion was stopped, food intake and body weight returned to baseline values in all groups.

Impact of IRS2 Deficiency in the Entire CNS or Specifically in POMC Neurons on VO2 and Motor Activity Responses to Chronic Leptin Infusion

Chronic leptin infusion increased VO2 similarly by ≈15% to 18% in controls and IRS2/POMC-cre and IRS2/Nestin-cre mice (Figure 3A). In all groups, leptin infusion for 7 days was associated with reduced motor activity, which returned to baseline values in control and IRS2/POMC-cre mice soon after termination of leptin treatment but remained reduced in IRS2/Nestin-cre mice (Figure 3B).

Impact of IRS2 Deficiency in the Entire CNS or Specifically in POMC Neurons on Glucose and Insulin Responses to Chronic Leptin Infusion

Chronic leptin infusion caused similar increases in plasma leptin levels in all groups, although baseline levels of leptin were higher in IRS2/Nestin-cre mice compared with the other groups.

Table. Metabolic Parameters in IRS2 flox/flox, Nestin-cre, IRS2/POMC-cre, and IRS2/Nestin-cre Mice at 22 Weeks of Age

<table>
<thead>
<tr>
<th>Parameter</th>
<th>IRS2 flox/flox</th>
<th>Nestin-cre</th>
<th>IRS2/POMC-cre</th>
<th>IRS2/Nestin-cre</th>
</tr>
</thead>
<tbody>
<tr>
<td>VO2, mL/kg per min</td>
<td>44±2</td>
<td>64±4</td>
<td>52±6</td>
<td>55±2</td>
</tr>
<tr>
<td>Motor activity, m/d</td>
<td>119±14</td>
<td>110±15</td>
<td>134±7</td>
<td>64±8*</td>
</tr>
<tr>
<td>Leptin, ng/mL</td>
<td>9±1</td>
<td>3±1</td>
<td>12±1</td>
<td>22±2*</td>
</tr>
<tr>
<td>Insulin, μU/mL</td>
<td>16±1</td>
<td>6±1</td>
<td>21±2</td>
<td>28±3*</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>152±12</td>
<td>149±4</td>
<td>148±6</td>
<td>168±6</td>
</tr>
</tbody>
</table>

IRS2 indicates insulin receptor substrate 2; and POMC, proopiomelanocortin. *P<0.05 compared with IRS2 flox/flox, Nestin-cre, and IRS2/POMC-cre mice.
Hypertension
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2 groups (Figure 3C). Despite the initial mild hyperinsulinemia in IRS2/Nestin-cre mice, chronic intravenous leptin infusion significantly reduced fasting plasma glucose and insulin levels in all groups similarly (Figure 3D and 3E).

We also investigated whether IRS2 signaling in the CNS is important in mediating the antidiabetic effects of leptin in IRS2/Nestin-cre mice and in control IRS2flox/flox mice with streptozotocin-induced diabetes mellitus. Chronic leptin treatment for 7 days markedly reduced nonfasting hyperglycemia in both groups (Figure 4A). IRS2 deletion in the entire CNS also did not impair leptin’s ability to improve glucose tolerance in diabetic IRS2/Nestin-cre mice (Figure 4B), suggesting that IRS2 deficiency in the entire CNS did not significantly alter leptin’s chronic antidiabetic action.

Impact of IRS2 Deficiency in the Entire CNS or Specifically in POMC Neurons on BP Regulation and BP Response to Chronic Leptin Infusion and Acute Air-Jet Stress

Baseline MAP was ≈7 mm Hg higher in IRS2/POMC-cre and IRS2/Nestin-cre mice compared with IRS2flox/flox control mice but similar to Nestin-cre mice. In our previous studies, we showed that chronic leptin infusion in lean WT mice raises BP and that this effect requires LR activation in POMC neurons. Therefore, we investigated the importance of IRS2 signaling in the entire CNS and specifically in POMC neurons in contributing to leptin’s pressor actions. We found that although leptin infusion gradually increased MAP by 5 mm Hg in IRS2flox/flox and Nestin-cre control mice, despite a marked decrease in food intake and weight loss that would normally tend to lower BP, no significant increase in MAP was observed in IRS2/Nestin-cre or IRS2/POMC-cre mice (Figure 5A). Leptin did not significantly alter HR in any of the groups (Figure 5C).

These data suggest that CNS IRS2 signaling, and specifically IRS2 signaling in POMC neurons, contributes to leptin’s ability to increase BP in mice.

IRS2 deletion in the entire CNS or specifically in POMC neurons did not impair MAP responses to acute air-jet stress (Figure S3), suggesting that although these animals have higher baseline MAP compared with control mice, they have a normal response to hypertensive stimuli, such as air-jet stress.

Power Spectral Analysis of Systolic Arterial Pressure and RR Interval Oscillations During Chronic Leptin Infusion

Spectral analyses data obtained at baseline, day 7 of leptin infusion, and day 5 of the recovery period are shown in Table S1. We found that chronic leptin infusion increased the low frequency component of systolic arterial pressure in IRS2flox/flox mice but not in the mice with IRS2 deleted in the entire CNS or specifically in POMC neurons. This finding suggests that IRS2 signaling in POMC neurons is important for sympathetic activation and for BP elevation during chronic leptin infusion. No significant baseline differences were found in the low frequency or high frequency components of RR interval among groups.

Discussion

The most important novel finding of this study is that inactivation of IRS2 signaling in the entire CNS or specifically...
in POMC neurons completely abolished the chronic effects of leptin to increase BP but did not attenuate leptin’s chronic anorexic or antidiabetic effects. Previous studies have suggested that LR-mediated activation of the IRS2–PI3K pathway in the CNS may contribute to leptin’s acute effects on renal sympathetic nervous system and glucose regulation. Most of these studies, however, have investigated only the short-term effects of leptin in experiments that typically last only a few hours. To our knowledge, there have been no previous studies that have assessed the role of IRS2 signaling in mediating leptin’s chronic BP and antidiabetic effects. In this study, we investigated the impact of IRS2 deficiency in the entire CNS and specifically in POMC neurons on multiple physiological metabolic and cardiovascular parameters and their responses to chronic hyperleptinemia, lasting for 7 days.

Role of CNS IRS2 Signaling in Regulating Body Weight, Food Intake, Energy Expenditure, and Glucose Homeostasis

In a previous study, Masaki et al. showed that mice with deficient IRS2 signaling in the entire CNS had hyperphagia, elevated body weight, increased adiposity, and defective thermoregulation. In addition, arcuate nucleus mRNA POMC expression was reduced, whereas expression of Agouti-related protein and neuropeptide Y was increased. In this study, we also found that IRS2/Nestin-cre mice were hyperphagic and heavier compared with age-matched IRS2flox/flox control mice. Previous studies showed that mice with neuronal disruption of IRS2 are obese and have some characteristics of the metabolic syndrome. The slight increase in body weight observed in IRS2/POMC-cre mice was not associated with increased food intake or major alterations in oxygen consumption, but these mice had increased fat mass.

Considerable evidence indicates that POMC-cre mice when crossed with IRS2flox/flox mice produce offspring with IRS2 deficiency only in POMC neurons. However, Padilla et al. suggested that POMC is transiently expressed in several sites of the developing mouse brain and that this transient expression may be sufficient to cause recombination of floxed alleles in off-target neurons that could contribute to physiological phenotypes. However, the physiological significance of this transient expression of POMC in neurons has not, to our knowledge, been demonstrated. In this study and in
previous reports, we did not find phenotypic changes that would be consistent with POMC-cre–induced recombination occurring in multiple off-target sites that are of major importance for energy homeostasis. For example, in this study, deletion of IRS2, a major pathway for leptin and insulin signaling in the brain, did not result in major changes in body weight or appetite phenotype. However, our main goal in this study was to investigate the role of IRS2 signaling in POMC neurons, regardless of their location, in regulating metabolic and cardiovascular function. Therefore, our studies did not assess the specific brain locations of POMC neurons that contribute to the phenotypes observed in mice with POMC IRS2 deficiency.

Role of CNS IRS2 Signaling in Mediating the Chronic Metabolic Effects of Leptin

We previously showed that deletion of LR in POMC neurons did not substantially attenuate the acute or chronic effects of leptin to reduce food intake. These observations suggest that other neuronal population besides those expressing POMC mediate most of leptin’s effects to suppress appetite. The specific neuronal populations involved in mediating the chronic anorexic effects of leptin on appetite and body weight regulation, however, are still a subject of intense investigation.

In this study, we found that mice lacking IRS2 signaling in the entire CNS or only in POMC neurons exhibited normal anorexic responses to acute or chronic leptin treatment. Sadagurski et al also found that mice with IRS2 inactivation specifically in LR-containing neurons had normal responses to the acute anorexic effect of leptin. However, the effect of IRS2 deficiency in the CNS and in POMC neurons on the chronic responses to leptin has, to our knowledge, not been previously reported. Our current study suggests that IRS2 signaling in POMC neurons or in the entire CNS is not required for leptin’s acute or chronic anorexic actions.

We and others have shown that leptin has powerful CNS-mediated antidiabetic actions capable of normalizing glyco
plasma glucose levels in type-I diabetes mellitus. Our previous studies also showed that mice with LR deficiency in POMC neurons exhibited significantly higher plasma glucose and insulin levels, suggesting that LR signaling in POMC neurons may be important in glucose homeostasis. However, the cell signaling pathways in POMC neurons that mediate these powerful antidiabetic actions of leptin are not well understood.

In this study, we found that mice with defective IRS2 signaling specifically in POMC neurons or in the entire CNS did not exhibit significant changes in fasting plasma glucose and insulin levels or tolerance to an acute glucose load. We further examined the role of IRS2 signaling in mediating the chronic effects of leptin on glucose regulation in a model of type-1 diabetes mellitus, induced by streptozotocin. We found that leptin infusion completely normalized blood glucose levels in type-1 diabetic mice even when IRS2 signaling was inactivated in the entire CNS. Taken together, our results suggest that IRS2 signaling in the CNS does not play a major role in mediating the chronic effects of leptin on glucose homeostasis in normal mice or in mice with type-1 diabetes mellitus.

Role of CNS IRS2 Signaling in BP Regulation and in Mediating Chronic Effects of Leptin

Our observation that mice with inactivation of IRS2 signaling in POMC neurons or in the entire CNS have increased BP and HR, compared with control mice, was unexpected. The elevated BP in IRS2/Nestin-cre mice was associated with increased body weight and increased adiposity, which could have activated other signaling pathways that promoted a rise in sympathetic activity and BP. However, it seems unlikely that this could explain the increased BP in IRS2/POMC-cre mice that had only a slight increase in body weight and adiposity compared with control mice. Moreover, our studies suggest that increases in leptin levels are unlikely to explain these unexpected findings because mice with IRS2 signaling inactivated in POMC neurons have similar baseline leptin levels compared with control mice and still had attenuated BP responses to chronic hyperleptinemia.

We also evaluated BP responses to air-jet stress in IRS2/POMC and IRS2/Nestin-cre mice, a stimulus that raises BP via sympathetic activation, and found that all groups exhibited normal responses. Thus, the mechanisms responsible for the effects of IRS2 deletion in the entire CNS or only in POMC neurons to increase baseline BP are unclear and will require further studies to assess the potential role of increased adiposity and activation of other signaling pathways.

We previously showed that intact LRPs on POMC neurons and intact melanocortin-4 receptors are crucial for leptin’s ability to raise BP, suggesting that CNS-POMC-MC4R axis mediates increased sympathetic nerve activity and the chronic BP effects of leptin. Leptin elicits multiple postreceptor signaling events in POMC neurons that could be involved in leptin-induced increases in sympathetic activity and BP. We previously reported that Stat3 signaling in POMC neurons contributes to leptin’s chronic BP effects. However, the role of IRS2 signaling in mediating the chronic cardiovascular effects of leptin has not, to our knowledge, been previously studied.

Rahmouni et al found that acute intracerebroventricular infusion of a pharmacological inhibitor of PI3K attenuated the effects of leptin to reduce appetite and increase renal sympathetic nervous system. Our current study indicates that IRS2 signaling in POMC neurons contributes to the chronic BP effects of leptin. In this study, we observed that chronic leptin infusion gradually increased BP in lean control mice, despite decreases in food intake and weight loss, but failed to increase BP in mice with inactivation of IRS2 signaling specifically in POMC neurons or in the entire CNS. Although our results suggest that IRS2 signaling in POMC neurons is important in mediating the chronic hypertensive effects of leptin, leptin may also influence BP via other signaling pathways.

Although our studies found that inactivation of IRS2 signaling did not markedly attenuate the chronic anorexigenic or antidiabetic effects of leptin, it is possible that inactivation of IRS2 signaling may have caused upregulation of Stat3 or Shp2 signaling pathways that may mediate some of the effects of leptin. However, acute leptin injection promoted similar expression of hypothalamic phospho-Stat3 in controls and mice lacking IRS2 signaling in LR neurons, suggesting that IRS2 signaling in LR neurons is not required for leptin to acutely activate Stat3 signaling. Also, our previous studies suggested that Stat3 and Shp2 signaling pathways in POMC neurons do not play a major role in mediating the chronic anorexigenic effects of leptin. However, Shp2 signaling in forebrain neurons does seem to be important in mediating the chronic antidiabetic effects of leptin. Although the potential effects of IRS2 inactivation on neuronal Stat3 or Shp2 signaling are still unclear, our current results clearly demonstrate that IRS2 signaling is not required for leptin’s anorexigenic or antidiabetic actions but is necessary for the full BP responses to chronic leptin infusions. These findings provide new insights into potential signaling pathways by which leptin may exert divergent control of appetite, BP, and glucose homeostasis.

Perspectives

Previous studies indicate that LRPs in POMC neurons play an important role in mediating the effects of leptin on BP regulation. Our current results indicate that intact IRS2 signaling in POMC neurons contributes to leptin’s chronic hypertensive effects but is not required for leptin’s effects on appetite and glucose regulation. This differential regulation of various metabolic and BP functions by the CNS IRS2 signaling may help explain how leptin is capable of regulating appetite independently of regulating sympathetic activity and BP in obesity. However, IRS2 deficiency in POMC neurons or in the entire CNS caused an increase in BP that was independent of leptin and may involve activation of other neuronal signaling pathways. Unraveling the signaling pathways and brain areas that contribute to sympathetic activation and hypertension in obesity is crucial to the development of better therapies to treat obesity and obesity-induced hypertension.

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Disclosures
None.

References


Novelty and Significance

What Is New?

• Insulin receptor substrate 2 (IRS2) signaling in proopiomelanocortin neurons contributes to the chronic effects of leptin on blood pressure but not leptin’s anorectic effects.

• IRS2 deficiency in proopiomelanocortin neurons or in the entire central nervous system raises blood pressure through mechanisms that are still unclear but seem to be independent of leptin.

• IRS2 signaling in the central nervous system or proopiomelanocortin neurons is not required for chronic glucose and insulin regulation by leptin, demonstrating that IRS2 signaling in the central nervous system permits differential regulation of metabolic and cardiovascular actions of leptin.

What Is Relevant?

• Unraveling the areas of the brain and signaling pathways by which leptin regulates body weight, glucose, and blood pressure will provide important and novel information that could lead to new therapeutic approaches for the treatment of metabolic diseases, including obesity and high blood pressure.

Summary

IRS2 signaling in proopiomelanocortin neurons is important in mediating the long-term blood pressure actions of leptin but is not required for leptin’s ability to reduce appetite, insulin, and glucose levels.
Regulation of Blood Pressure, Appetite, and Glucose by Leptin After Inactivation of Insulin Receptor Substrate 2 Signaling in the Entire Brain or in Proopiomelanocortin Neurons
Jussara M. do Carmo, Alexandre A. da Silva, Zhen Wang, Nathan J. Freeman, Ammar J. Alsheik, Ahmad Adi and John E. Hall

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REGULATION OF BLOOD PRESSURE, APPETITE AND GLUCOSE BY LEPTIN AFTER INACTIVATION OF INSULIN RECEPTOR SUBSTRATE 2 (IRS2) SIGNALING IN THE ENTIRE BRAIN OR IN POMC NEURONS

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Running head: IRS2 signaling in POMC neurons and BP regulation

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SUPPLEMENTAL METHODS

Power spectral analysis of systolic arterial pressure (SAP) and RR interval (RRI) oscillations

Power spectral densities of SAP and RRI oscillations were computed by 512-point fast Fourier transform and integrated over the specific frequency range (low frequency: LF, 0.25-0.75 Hz; high frequency: HF, 0.75-5.0 Hz oscillations by using Nevrokard SA-BRS software (Medistar, Ljubjana, Slovenia). A Hanning window was applied, and the square roots of the ratio of SAP and RRI powers were computed to calculate LF and HF components, which LF is a marker of sympathetic tone to the heart and BP, whereas HF component reflects parasympathetic the to the heart and the influence of respiratory rhythm on Bp

Acute air-jet stress test

To determine whether IRS2 deletion in the entire CNS or specifically in POMC neurons alters MAP response to an acute pressor stress, IRS2\textsuperscript{flox/flox}, IRS2/POMC-cre and IRS2/Nestin-cre mice were placed in a special cage used for air-jet stress testing. Briefly, after a 2-hour period for acclimatization to the cage, MAP was continuously measured, using telemetry, for 30 minutes before the air-jet stress test was applied. The air-jet stress test consisted of 2-second pulses of air jet delivery every 5 seconds during 5 consecutive minutes aimed at the forehead of the mice at an approximately distance of 5 cm using a 14 gauge needle opening at the front of the tube connected to compressed air. After the 5-minute air-jet stress, MAP was measured for an additional 30-minute recovery period. BP response during the air-jet stress and recovery period following the air-jet stress were calculated as the changes compared to baseline period (average of the last 10 minutes of baseline measurements before air-jet stress was initiated). We also calculated the areas under the MAP curve (AUC) during the air-jet stress and recovery periods using the following parameters: average change in MAP for each minute during the 5-minute air-jet stress test and for each 5 minutes during the 30-minute recovery period.

Analytical methods

Polymerase chain reaction

Genotyping was performed as previously described. Briefly, after weaning at 4 weeks of age, mice were genotyped using tail snips to perform PCR across the IRS2\textsuperscript{flox/flox} exon 6 and for cre-recombinase using the following primers: 5’-ACTTTGAAGGAAGCCACAGTCG -3’, 5’- AGTCCACTTTTCCCTGACAAGC -3’; and for Cre positive 5’- CTGCCACGACCA AGTGACAGC -3’, and Cre negative 5’- CTTCCTACACCTGCAGTGCT -3’. To detect the IRS2\textsuperscript{flox} allele, a forward primer 5’GGGAACCTGACAAGTGAATG -3’ and reverse primer 5’AGTCCACTTTTCCCTGACAAGC -3 were used. Only animals that tested positive for the IRS2\textsuperscript{flox/flox} and cre (homozygous) were used as IRS2/POMC-cre and IRS2/Nestin-cre mice (Figure S1).

Immunohistochemistry

To confirm deletion of IRS2 in IRS2/Nestin-cre mice, we used immunohistochemistry to label IRS2 in sections of the arcuate nucleus (ARC) and
ventromedial hypothalamus (VMH). Briefly, frozen brain coronal sections of IRS2$^{\text{flox/flox}}$ and IRS2/Nestin-cre mice (30 µm thick) were prepared, free-floating sections were rinsed in PBS and then incubated in blocking solution (PBS, 0.3% Triton X-100) and then incubated with 5% normal horse serum in PBS for 1h at room temperature. Sections were incubated with rabbit anti-IRS2 (Cell Signaling, MA) at a dilution 1:700 for 24 hrs at 4°C. After rinses with PBS, sections were incubated with secondary antibody 1:400 anti-rabbit (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Sections were rinsed, stained with 3’3’-Diaminobenzidine (DAB) peroxidase substrate (Vector Laboratories), mounted on slides and examined in a microscope.

**Western Blot for pAKT and AKT**

IRS2$^{\text{flox/flox}}$ mice at 20 wks of age were infused with leptin (n=4) or saline (n=4) for 5 days and the hypothalamus were homogenized in lysis buffer (KPO$_4$, pH 7.4) and cleared by centrifugation (1,000g, 5 min at 4°C). After protein concentration of supernatant was determined by the Bradford method (Bio-Rad, Hercules, CA), 50 µg of protein was separated in a 4-15% precast linear gradient polyacrylamide gel (Bio-Rad). After being transferred to a nitrocellulose membrane, blots were rinsed in PBS and blocked in Odyssey blocking buffer (LI-COR, Lincoln, NE) for 1 h at room temperature, and incubated with rabbit monoclonal anti-AKT or phospho-AKT (p-AKT) antibody (1:2,000; Cell Signaling, Danvers, MA) overnight at 4°C. The membrane was probed for mouse anti β-actin (1:3,000, Abcam) as a loading control. The membrane was then incubated with IR700-conjugated donkey anti-mouse IgG and IR800-conjugated donkey anti-rabbit (1: 2,000, Rockland Immunologicals, Gilbertsville, PA). Antibody labeling was visualized using the Odyssey infrared scanner (LI-COR) for simultaneous detection of two probes. Fluorescence intensity analyses after subtracting the background were performed using Odyssey software (LI-COR). Measurements of p-AKT and AKT were normalized to β-actin.

**References**


SUPPLEMENTAL RESULTS

Table S1. Spectral analysis data of RR interval (RRI) and systolic arterial pressure (SAP) during control, on day 7\textsuperscript{th} of leptin infusion, and recovery periods in IRS2\textsuperscript{flox/flox}, IRS2/POMC and IRS2/Nestin-cre mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>RRI</th>
<th></th>
<th>SAP</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LF (Hz)</td>
<td>HF (Hz)</td>
<td>LF (nu)</td>
<td>HF (nu)</td>
</tr>
<tr>
<td>IRS2\textsuperscript{flox/flox} (n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.38±0.01</td>
<td>0.94±0.02</td>
<td>28.6±2.0</td>
<td>19.9±2.0</td>
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<tr>
<td>Leptin</td>
<td>0.32±0.03</td>
<td>1.40±0.21</td>
<td>32.4±2.3</td>
<td>20.5±3.2</td>
</tr>
<tr>
<td>Recovery</td>
<td>0.39±0.06</td>
<td>0.85±0.15</td>
<td>25.6±3.2</td>
<td>20.5±2.7</td>
</tr>
<tr>
<td>IRS2/POMC-cre (n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.48±0.05</td>
<td>1.31±0.22</td>
<td>27.7±2.0</td>
<td>30.3±4.2</td>
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<tr>
<td>Leptin</td>
<td>0.39±0.32</td>
<td>1.32±0.11</td>
<td>25.6±1.8</td>
<td>29.5±3.1</td>
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<tr>
<td>Recovery</td>
<td>0.37±0.02</td>
<td>1.42±0.25</td>
<td>24.7±2.3</td>
<td>28.9±5.1</td>
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<td>IRS2/Nestin-cre (n=5)</td>
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<tr>
<td>Control</td>
<td>0.55±0.02</td>
<td>1.93±0.23</td>
<td>28.8±2.0</td>
<td>30.3±1.9</td>
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<td>Leptin</td>
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<td>1.58±0.22</td>
<td>25.9±2.7</td>
<td>23.6±2.5</td>
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<tr>
<td>Recovery</td>
<td>0.48±0.03</td>
<td>1.70±0.27</td>
<td>23.5±1.8</td>
<td>28.2±3.8</td>
</tr>
</tbody>
</table>
Values are mean±SEM. LF, low frequency; HF, high frequency; nu, normalized units (represent the relative value of each power component in proportion to the total power minus VLF (very low frequency) component. Values were obtained on day 5 of control, day 7 of chronic IV leptin infusion (2 μg/kg/min), and recovery periods. * p<0.05 compared to control period.
Figure S1. (A) Polymerase chain reaction of tail-snip samples from IRS2<sup>flox/flox</sup> (+), heterozygous (+/-), and Nestin-cre or POMC-cre positive (+) and Nestin-cre or POMC-cre negative (-) mice. Positive (+) and negative (-) DNA samples and wild-type (WT) were used as controls. (B) PCR analysis confirmed a Cre-mediated specific recombination of the IRS<sup>flox/flox</sup> allele in neuronal cells of cerebral cortex (C), hypothalamus (H), hindbrain (HB), and liver (L) in IRS2/Nestin-cre, IRS2/POMC-cre and IRS2<sup>flox/flox</sup> mice. (C) 20x view of representative immunohistochemical stain of IRS2 (brown staining) in the arcuate nucleus of IRS2/Nestin-cre and IRS2<sup>flox/flox</sup> mice. (D) Ratio of pAKT/AKT in the hypothalamus of mice treated with leptin and in saline treated control mice. *p<0.05 compared to saline-treated group.
Figure S2. (A) Glucose tolerance test (GTT), and (B) area under the curve of the GTT in IRS2<sup>flox/flox</sup>, IRS2/POMC-cre and IRS2/Nestin-cre mice. The number of animals varied from 7-9 mice per group.
Figure S3. Area under curve (AUC) for blood pressure during acute air-jet stress and recovery period in IRS2\textsuperscript{flox/flox} (n=5), IRS2/POMC-cre (n=6) and IRS2/Nestin-cre (n=5) mice.