Glutamatergic Receptor Activation in the Rostral Ventrolateral Medulla Mediates the Sympathoexcitatory Response to Hyperinsulinemia

Megan E. Bardgett, John J. McCarthy, Sean D. Stocker

Abstract—Hyperinsulinemia increases sympathetic nerve activity (SNA) and has been linked to cardiovascular morbidity in obesity. The rostral ventrolateral medulla (RVLM) plays a key role in the regulation of SNA and arterial blood pressure (ABP). Many sympathoexcitatory responses are mediated by glutamatergic receptor activation within the RVLM, and both the central renin–angiotensin and melanocortin systems are implicated in the sympathoexcitatory response to hyperinsulinemia. Therefore, we hypothesized that one or more of these neurotransmitters in the RVLM mediate the sympathoexcitatory response to insulin. Hyperinsulinemic–euglycemic clamps were performed in α-chloralose anesthetized, male Sprague–Dawley rats by infusion of insulin (3.75 mU/kg per minute, IV) and 50% dextrose solution for 120 minutes. Physiological increases in plasma insulin elevated lumbar SNA, with no change in renal SNA, ABP, or blood glucose. Microinjection of the ionotropic glutamate receptor antagonist kynurenic acid into the RVLM significantly reduced lumbar SNA and ABP. Selective blockade of NMDA but not non-NMDA glutamate receptors resulted in similar reductions of lumbar SNA. In marked contrast, microinjection of the angiotensin II type 1 receptor antagonist losartan or the melanocortin 3/4 antagonist SHU9119 had no effect on lumbar SNA or ABP. Western blot analysis showed that insulin receptor expression is significantly lower in the RVLM than the hypothalamus, and direct microinjection of insulin into the RVLM did not significantly increase lumbar SNA. These findings suggest that hyperinsulinemia increases lumbar SNA by activation of a glutamatergic NMDA-dependent projection to the RVLM. (Hypertension. 2010;55:284-290.)

Key Words: insulin ■ RVLM ■ arterial blood pressure ■ sympathetic nerve activity ■ obesity

Compelling evidence in humans and rodents indicates that elevated sympathetic nerve activity (SNA) contributes to the pathogenesis of obesity-induced hypertension.1,2 Clinical studies indicate obese humans have increased norepinephrine spillover,3,4 elevated muscle SNA,5,6 and a greater drop in arterial blood pressure (ABP) in response to ganglionic blockade.7 Similar observations have been reported in rodent and dog models of obesity.8–10 One mechanism postulated to underlie the elevated SNA and ABP during obesity is hyperinsulinemia.1,2 Clinical studies have revealed a correlation between obesity, hypertension, and hyperinsulinemia.1,2 In both humans and rodents, acute hyperinsulinemic–euglycemic clamps selectively increase muscle or lumbar SNA, respectively.11–14 These actions are mediated by a central mechanism because intracerebroventricular administration of insulin causes a similar selective increase in lumbar SNA.14 In rats, chronic hyperinsulinemic–euglycemic clamps increase total peripheral resistance and ABP.15 However, the neural mechanisms and pathways that mediate the sympathoexcitatory effects of insulin are poorly understood.

The rostral ventrolateral medulla (RVLM) plays a pivotal role in the regulation of SNA and ABP.16 RVLM neurons project to sympathetic preganglionic neurons of the intermediateolateral cell column in the thoracic and lumbar spinal cord and support basal SNA.16 Electrophysiological studies in vivo have identified tonically active, bulbospinal neurons in the RVLM.16 The excitability of RVLM neurons is regulated by a number of neurotransmitters including L-glutamate. Injection of L-glutamate into the RVLM increases neuronal discharge, SNA, and ABP.16 Blockade of glutamate receptors in the RVLM eliminates many sympathoexcitatory reflexes16 and lowers ABP in multiple experimental models of hypertension.17–19 Based on this evidence, we hypothesized that glutamate receptor activation in the RVLM mediates the sympathoexcitatory response to hyperinsulinemia.

In addition to glutamate, evidence from several laboratories suggests that the brain renin–angiotensin and melanocortin systems mediate the sympathoexcitatory response to insulin. In this regard, RVLM neurons express Ang II (AT₁) receptors,20 and injection of Ang II into the RVLM increases
SNA and ABP. Blockade of brain AT\(_1\) receptors blunts the pressor response to central hyperinsulinemia. Also, blockade of the renin–angiotensin system prevents insulin-induced hypertension. On the other hand, RVLM neurons express melanocortin receptors, and injection of a melanocortin agonist into the RVLM increases SNA and ABP. Interestingly, the sympathoexcitatory effect to insulin is abolished in melanocortin 4 knockout mice. Therefore, we hypothesized that one or both of these systems may contribute to the sympathoexcitatory response during hyperinsulinemia.

**Methods**

**Animals**

All of the experimental procedures conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Kentucky and Pennsylvania State College of Medicine Institutional Care and Use Committee. Male Sprague–Dawley rats (250 to 350 g; Charles River Laboratories) were housed in a temperature controlled room (22 ± 1°C) with a 14-hour:10-hour light:dark cycle. Rats were fed standard rat chow and given access to deionized water.

**General Procedures**

Rats were anesthetized with isoflurane (2% to 3%) and prepared for recordings of renal and lumbar SNA and ABP as described previously. Animals were artificially ventilated with oxygen-enriched room air. End-tidal CO\(_2\) and body temperature were maintained at 4% to 5% and 37 ± 1°C, respectively. After surgery, anesthesia was replaced by α-chloralose (50 mg/kg bolus, 25 mg/kg per hour, IV). The level of anesthesia was examined by the lack of a withdrawal reflex following a foot pinch. When a stable level of anesthesia was established, rats were paralyzed with gallamine triethiodide (20 mg/kg, IV). 

**RVLM Microinjections**

RVLM microinjections were performed as described previously in our laboratory. Initially, 1-μL (1 nmol) was injected into the RVLM at 3 different sites separated by 300 μm in the rostral–caudal plane to identify the site that produced the largest increase in ABP. Subsequent injections were performed at these coordinates. For all experiments, injections (60 nL) were performed over 5 seconds. Injection sites were marked with 0.2% rhodamine beads added to the respective drug or injected at the end of experiments.

**Hyperinsulinemic–Euglycemic Clamps**

An initial set of experiments was performed to identify a physiological dose of insulin. Animals were prepared as described above, and insulin (3.75 or 7.5 mU/kg per minute, 0.25 mL/h, IV, Humulin R) and a 50% dextrose solution (0.25 to 1.0 mL/h, IV) were infused for 120 minutes. Blood glucose was measured from a drop of arterial blood every 10 minutes using a standard glucometer (One Touch Ultra). The dextrose infusion rate was adjusted to maintain euglycemia. Control animals were infused with equal volumes of isotonic saline. Blood (0.5 mL) was collected from the arterial line into microcentrifuge tubes (10 μL, 0.5 mL/EDTA) at baseline and 60 and 120 minutes. Samples were centrifuged, and plasma was stored at −80°C.

For purposes of comparison, plasma insulin levels were analyzed from a rodent model of diet-induced obesity. Male Sprague–Dawley rats (200 to 250 g, Charles River Laboratories) were fed a low-fat (LF) diet (10% kcal from fat; Research Diets Inc, D12498B) or moderately high-fat (32% kcal from fat; Research Diets Inc, D12266B) diet for 13 weeks as described previously by our laboratory. Those on the high fat diet segregated into obesity-resistant (OR) and obesity-prone (OP) rats. Rats were anesthetized and prepared as described above. Blood samples were collected from the arterial line, and insulin levels were determined by an ELISA using a commercially available kit (Millipore).

To determine the contribution of RVLM receptors to the SNA response to the respective agonist (data not shown).

**Central Insulin Injections**

Rats were prepared as described above, and insulin (5, 0.5, 0.05, or 0.0005 μU/mL, 60 nL) was bilaterally microinjected into the RVLM. ABP and SNA were recorded for 60 minutes and blood glucose measured every 30 minutes. The insulin concentrations were based on previous studies using intracerebroventricular injection of insulin and recalculated because of a minimum 10-fold dilution attributable to the CSF volume of the lateral and 3rd ventricles. In a separate group of rats, intracerebroventricular cannulas were implanted in the lateral ventricle as described previously. Proper cannula location was verified by a positive drinking test (>3 mL in 30 minutes) to angiotensin II (20 ng/2 μL). Then, rats were prepared as described above, and insulin (100 μU/2μL) was injected into the lateral ventricle. This dose of insulin has been repeatedly demonstrated to significantly elevate lumbar SNA in rodents.

**Variables**

For all variables, 30-second segments at each time point were compared to three 30-second baseline period measurements. All data were analyzed by a 1- or 2-way ANOVA, with repeated measures when appropriate. All post hoc tests were performed with
Results

Analysis of Plasma Insulin Levels

Initial experiments were performed to identify an insulin infusion rate, which produced physiological increases in plasma insulin levels. Both infusion rates significantly increased plasma insulin concentrations at 60 and 120 minutes (Figure 1). Plasma insulin levels were not different between OP rats and control rats infused with 7.5 mU/kg per minute. Plasma insulin levels of LF and OR rats were not different versus those of rats infused with 3.75 mU/kg per minute.

were similar to those rats infused with 7.5 mU/kg per minute and significantly higher than those rats infused with 3.75 mU/kg per minute. Plasma insulin levels of LF and OR rats were not different versus those of rats infused with 3.75 mU/kg per minute.

Blockade of Glutamatergic Receptors Reverses Sympathoexcitatory Response to Insulin

A major goal of this study was to determine whether blockade of glutamate receptors in the RVLM reversed or attenuated the sympathoexcitation during hyperinsulinemia. Figure 2 illustrates a representative example of the responses to a hyperinsulinemic–euglycemic clamp or saline infusion before and after RVLM microinjection of KYN. Group data are summarized in Figure 3. As previously reported, hyperinsulinemia selectively increased lumbar SNA,12,14 but did not affect ABP (Figure 2), blood glucose (Figure 3), renal SNA (data not shown), or heart rate (data not shown).

Microinjection of KYN significantly reduced lumbar SNA in hyperinsulinemic animals but had no effect in saline-infused animals (Figures 2 and 3). In fact, lumbar SNA after injection of KYN was not different between rats infused with insulin versus saline (P>0.3). At 120 minutes, lumbar SNA returned to preinjection values. Microinjection of aCSF had no effect on any variable in hyperinsulinemic or control rats (see online Figure S1). Figure 4 summarizes the peak changes in lumbar SNA and mean ABP after injection of aCSF or KYN in hyperinsulinemic or control rats. Although the hyperinsulinemic–euglycemic clamp did not significantly alter ABP, microinjection of KYN significantly decreased mean ABP. Microinjection of KYN or aCSF did not alter renal SNA or heart rate (data not shown).

Because lumbar SNA returned to preinjection values at 30 minutes after KYN injection, an additional set of experiments was performed to determine the time course of ionotropic receptor blockade by KYN. We compared the sympathoexcitatory response to activation of somatic afferents before and after RVLM injection of KYN. Before blockade, electrical stimulation of sciatic afferents significantly increased mean ABP, lumbar SNA, renal SNA, and heart rate (Table). As expected, microinjection of KYN into the RVLM significantly attenuated these responses at 10 and 20 minutes. However, the sympathoexcitatory responses at 30 minutes were not different from baseline responses.

Figure 1. Plasma insulin concentrations at baseline, 60, and 120 minutes during a hyperinsulinemic–euglycemic clamp (3.75 mU/kg per minute, n=9; 7.5 mU/kg per minute, n=3) or saline infusion (n=3). Plasma insulin concentrations from LF (n=5), OR (n=5) and OP (n=6) rats were analyzed for purposes of comparison. Plasma insulin levels were not different between OP rats and control rats infused with 7.5 mU/kg per minute. Plasma insulin levels of LF and OR rats were not different versus those of rats infused with 3.75 mU/kg per minute.

Figure 2. Representative examples of ABP, mean ABP, and lumbar SNA during RVLM microinjection of KYN in rats receiving a (A) hyperinsulinemic–euglycemic clamp or (B) saline infusion. Traces for raw lumbar SNA represent baseline (a), peak infusion (b), and post-KYN injection (c).
Blockade of NMDA but Not Non-NMDA Receptors Reverses the Sympathoexcitatory Response to Insulin

Because blockade of ionotropic glutamate receptors with KYN reversed the sympathoexcitatory response to insulin, an additional set of experiments was performed to identify the specific receptor subtype. RVLM microinjection of the NMDA receptor antagonist AP5 in hyperinsulinemic rats significantly reduced lumbar SNA (90 minutes: 142±6%; versus peak: 115±9%; P<0.05) and mean ABP (90 minutes: 123±6; versus peak: 116±6 mm Hg; P<0.05). Interestingly, the fall in lumbar SNA and mean ABP of hyperinsulinemic rats was similar between KYN and AP5 (Figure 4). In contrast, microinjection of the non-NMDA receptor antagonist NBQX did not affect lumbar SNA (90 minutes: 142±12; versus peak: 148±13%) or mean ABP (90 minutes: 105±7; versus peak: 110±6 mm Hg). AP5 and NBQX did not affect lumbar SNA or ABP in saline-infused animals.

RVLM AT1 and Melanocortin Receptors Do Not Mediate Insulin-Induced Sympathoexcitation

In contrast to blockade of glutamate receptors, microinjection of the AT1 receptor antagonist losartan or the melanocortin receptor antagonist SHU9119 did not affect the sympathoexcitatory response to hyperinsulinemia. Peak changes in lumbar SNA and ABP after microinjection of losartan or SHU9119 are illustrated in Figure 5. As expected, the hyperinsulinemic–euglycemic clamp significantly increased lumbar SNA at 90 minutes (P<0.01) but did not change mean ABP, renal SNA, or heart rate (data not shown). Microinjection of losartan did not decrease lumbar SNA (90 minutes: 138±12; versus peak: 147±12%) or mean ABP (90 minutes: 111±8; versus peak: 120±8 mm Hg). Similarly, microinjection of SHU9119 did not decrease lumbar SNA (90 minutes: 134±8; versus peak: 147±17%) or mean ABP (90 minutes: 96±6; versus peak: 109±5 mm Hg). Losartan and SHU9119 did not affect lumbar SNA or ABP in saline-infused animals (Figure 5).

Insulin Receptor Expression and Insulin Microinjection in the RVLM

To determine whether insulin may act directly in the RVLM to increase SNA, we analyzed insulin receptor expression and

### Table. Effect of Ionotropic Receptor Blockade on the Sympathoexcitatory Reflex to Activation of Somatic Afferents

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n</th>
<th>Baseline</th>
<th>10 Minutes</th>
<th>20 Minutes</th>
<th>30 Minutes</th>
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<tr>
<td>ΔMean ABP, mm Hg</td>
<td>9</td>
<td>31±4</td>
<td>1±4*</td>
<td>6±4*</td>
<td>23±6</td>
</tr>
<tr>
<td>ΔHeart rate, bpm</td>
<td>9</td>
<td>14±2</td>
<td>1±1*</td>
<td>5±2*</td>
<td>13±3</td>
</tr>
<tr>
<td>ΔRenal SNA, %</td>
<td>5</td>
<td>66±15</td>
<td>7±6*</td>
<td>26±9*</td>
<td>39±9</td>
</tr>
<tr>
<td>Lumbar SNA, %</td>
<td>4</td>
<td>88±22</td>
<td>40±15*</td>
<td>47±12*</td>
<td>75±25</td>
</tr>
</tbody>
</table>

Values are means±SEM and represent changes in mean ABP, heart rate, and SNA during electrical stimulation of sciatic afferents. KYN was injected bilaterally into the RVLM at time=0. Renal or lumbar SNA was recorded in each animal. Note that the sympathoexcitatory response was attenuated at 10 and 20 minutes after KYN injection but returned at 30 minutes. *P<0.05 vs baseline values of microinjection into the RVLM. Baseline mean ABP: 123±6 mm Hg; baseline heart rate: 405±11 bpm.
sympathetic responses to microinjection of insulin in the RVLM. Insulin receptor expression was significantly lower in the RVLM compared to the ventromedial hypothalamus (Figure 6). In fact, the insulin receptor band in RVLM samples was virtually absent (Figure 6) and was not altered by a hyperinsulinemic–euglycemic clamp (data not shown).

RVLM microinjection of insulin at any dose did not alter lumbar SNA or ABP (Figure 6). In marked contrast, injection of insulin into the lateral ventricle significantly increased lumbar SNA. Plasma glucose levels were not altered by RVLM or lateral ventricle injection of insulin (data not shown).

**Histology**

All injection sites were centered in the RVLM defined as the triangular region located 0 to 600 μm caudal to the caudal pole of the facial nucleus and bordered dorsally by nucleus ambiguous, medially by the inferior olive or pyramidal tracts, and laterally by the spinal trigeminal nucleus (online Figure S2).

**Discussion**

Previous studies have demonstrated that hyperinsulinemic–euglycemic clamps produce nonuniform increases in SNA. However, the neural mechanisms or brain regions by which insulin acts to selectively increase lumbar SNA have not been identified. The present study provides several novel findings: (1) a hyperinsulinemic clamp with physiological increases in plasma insulin levels elevated lumbar SNA; (2) blockade of glutamatergic, and more specifically NMDA, receptors reversed the sympathoexcitatory effects of hyperinsulinemia; (3) blockade of RVLM AT1 or melanocortin 3/4 receptors did not affect the sympathoexcitatory response to insulin; (4) the RVLM has a low expression of insulin receptors; and (5) microinjection of insulin into the RVLM did not elevate lumbar SNA. Collectively, these findings suggest insulin activates a NMDA-dependent glutamatergic pathway to the RVLM to increase lumbar SNA.

To identify a physiologically relevant dose of insulin, we compared plasma insulin levels between control rats infused with insulin versus diet-induced obese rats. This model of diet-induced obesity has similar characteristics to obese humans such as activation of the renin–angiotensin system, hyperleptinemia, hyperinsulinemia, elevated sympathetic outflow, and hypertension. Indeed, the plasma insulin levels of control rats infused with 3.75 mU/kg per minute were significantly lower than those of OP rats. Additional data indicate that plasma insulin levels in obese Zucker rats (13 to 15 weeks) are not different from those of control rats infused with 3.75 mU/kg per minute (see online Table S2). Although plasma insulin levels of LF rats were significantly greater than baseline levels of control rats, this difference is likely attributable to a greater adiposity index and older age of LF rats. Collectively, these data indicate that the insulin infusion rate in the present study is physiologically relevant. Whether the elevation in circulating insulin contributes to the elevated sympathetic outflow and hypertension in these rodent models of obesity is unknown.

**Figure 5.** Peak changes in lumbar SNA and mean ABP after bilateral microinjection of aCSF, KYN, losartan, or SHU 9119 into the RVLM during a saline infusion (n=4 to 9 per group) or hyperinsulinemic–euglycemic clamp (n=3 to 6 per group). *P<0.05 vs rats infused with saline within same drug treatment or rats infused with insulin plus aCSF.

**Figure 6.** A, Examples of Western blot analysis for insulin receptor β (IR-β) and γ-tubulin in the hypothalamus (H) and RVLM (R). Insulin receptor β expression as a ratio to γ-tubulin was significantly lower in the RVLM vs the hypothalamus (n=4 per group, *P<0.01). B, Change in lumbar SNA and ABP after injection of insulin into the RVLM (n=3 to 4 per group) or lateral ventricle (n=5). *P<0.01 vs aCSF or 0 insulin.
Glutamate neurotransmission in the RVLM mediates a number of sympathoexcitatory reflexes, including the responses to hypoxia and activation of somatic afferents. Blockade of RVLM ionotropic glutamate receptors also lowers ABP in a number of experimental models of hypertension associated with elevated sympathetic outflow. In the present study, RVLM injection of KYN, but not losartan or SHU 9119, completely reversed the sympathoexcitatory response to hyperinsulinemia. Although lumbar SNA returned to preinjection levels at 30 minutes after KYN injection, this response is consistent with the time course of ionotropic receptor blockade with KYN. These findings support two important conclusions: (1) insulin activates the brain renin–angiotensin and melanocortin systems outside the RVLM (ie, hypothalamus) and (2) ionotropic glutamate receptors in the RVLM mediate the sympathoexcitatory actions to hyperinsulinemia. Subsequent experiments clearly demonstrate that NMDA receptors solely mediate this response. The ability of KYN or APS to reverse the sympathoexcitatory effects of hyperinsulinemia cannot be attributed to a direct modulatory role of insulin within the RVLM as insulin receptor expression was low, and direct injection of insulin into the RVLM did not alter lumbar SNA and ABP. Therefore, insulin activates a glutamatergic NMDA-dependent pathway to the RVLM to elevate SNA.

The origin of the insulin-driven glutamatergic pathway to the RVLM is not known. The sources of glutamatergic input to the RVLM have not been completely identified; however, the RVLM is densely innervated by glutamatergic neurons in the hypothalamic paraventricular nucleus. Interestingly, preliminary data from our laboratory indicate that inhibition of the hypothalamic paraventricular nucleus reverses the sympathoexcitatory response to hyperinsulinemia. Although previous studies have reported insulin receptor binding in the hypothalamic paraventricular nucleus, it is not known whether insulin acts directly on these neurons or elsewhere to elevate SNA. A number of other hypothalamic structures also express insulin receptors including the arcuate nucleus, ventromedial hypothalamus, and circumventricular organs of the forebrain lamina terminalis. However, there are no available studies that have systematically examined the contribution of these various structures to the sympathoexcitatory response to insulin and whether such neurons detect circulating insulin. To date, previous studies have demonstrated that either global inhibition of hypothalamic phosphatidylinositol 3-kinase or lesion of the anteroventral third ventricular region attenuates the increase in lumbar SNA during hyperinsulinemia. Clearly, future experiments are needed to identify the neurons that detect changes in circulating insulin and how this translates into activation of a glutamatergic pathway to the RVLM to increase lumbar SNA.

In summary, the present study provides the first evidence of a specific brain region that mediates the sympathoexcitatory response to hyperinsulinemia. The results clearly demonstrate the sympathoexcitatory response to insulin depends on activation of glutamatergic and, more specifically, NMDA receptors in the RVLM. Insulin likely acts at hypothalamic sites to increase glutamatergic drive to the RVLM because these neurons express a low level of insulin receptors and direct injection of insulin into the RVLM did not alter lumbar SNA or ABP.

**Perspectives**

Clinical studies have revealed a correlation between obesity, hypertension, and hyperinsulinemia, but the role of insulin in hypertension remains controversial. In rats, acute hyperinsulinemia elevates lumbar SNA, and chronic hyperinsulinemic–euglycemic clamps increase total peripheral resistance and ABP. In contrast, studies performed in dogs have reported that peripheral infusion of insulin did not elevate ABP. The discrepancy between data from rats versus dogs may be explained by greater peripheral insulin sensitivity in dogs. Consistent with this notion, a hyperinsulinemic–euglycemic clamp in dogs increased, rather than decreased, cardiac output, thereby indicating a systemic vasodilatory response and no change in ABP. Unfortunately, it is not known whether dogs exhibit a similar sympathoexcitatory response to insulin, as previously reported in mice, rats, and humans. Because of the absence of experimental tools to directly assess the contribution of insulin to these chronic diseases, the role of insulin in obesity-related hypertension or other disease states of hyperinsulinemia will likely remain controversial. Yet, the present findings provide a potential model to examine the pathways and mechanisms that may contribute or support the elevated SNA during obesity-related hypertension.

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**Disclosures**

None.

**References**


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Sympathoexcitatory Response to Hyperinsulinemia

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Material and Methods for Western Blot Analysis of Insulin Receptor β

Frozen RVLM or hypothalamus sections were disrupted in 150 μl of homogenization buffer [1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM NaCl, 400 mM KCl, 25 mM β-glycerophosphate, 50 mM NaF, 5 mM benzamidine, 20 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1 mM sodium orthovanadate, 5 mM N-ethylmaleimide, 1 mM PMSF] supplemented with protease inhibitor cocktail (P8340; Sigma, St Louis, MO) using a Pyrex Potter-Elvehjem tissue grinder. To remove any insoluble particulate the tissue homogenates were centrifuged (10,000 x g, 10 min, 4 °C) and the supernatant transferred to a new microcentrifuge tube. Protein concentration of each sample was determined using the Bio-Rad DC protein Assay (Hercules, CA) according to the manufacturer’s directions. Five micrograms of each sample was precipitated using the methanol:chloroform procedure and then resuspended in 20 μl of 1X sample buffer. Samples were prepared for electrophoresis by heating for 5 min at 100 °C. Samples were separated by SDS-PAGE (7.5% gel) and then transferred to nitrocellulose membrane (0.2 μm) (Bio-Rad, Hercules, CA). The membrane was incubated in blocking buffer (5% nonfat dry milk in TBS plus 0.1% Tween-20 [TBS-T]) for 1 hr at room temperature and then incubated in blocking buffer overnight at 4°C with a rabbit polyclonal insulin receptor β (IR-β) antibody (1:1000, C-19; sc-711; Santa Cruz Biotechnology, Inc. Santa Cruz, CA.). After the overnight incubation, the membrane was washed (4x, 5min) in TBS-T, incubated with anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000, 1 hr) at room temperature, incubated in ECL plus for 5 min (GE Healthcare, Piscataway, NJ) and exposed to X-ray film. To determine specificity of the IR-β antibody, the membrane was stripped and reprobed with the IR-β antibody pre-absorbed with five-fold excess of a blocking peptide (sc-711P; Santa Cruz Biotechnology, Inc. Santa Cruz, CA.). The membrane was also stripped and reprobed with a mouse monoclonal γ-tubulin antibody (T6557; Sigma-Aldrich, St. Louis, MO). The IR-β band intensity for each sample was quantified using NIH ImageJ (http://rsb.info.nih.gov/nih-image/) and normalized to the respective γ-tubulin band intensity.
Table S1. Characteristics for LF, OR, and OP rats

<table>
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<th>Characteristic</th>
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<th>OR</th>
<th>OP</th>
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<td>Initial Body Weight, g</td>
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<td>233±2</td>
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<td>Final Body Weight, g</td>
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<td>Fat Pads</td>
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<td>Retroperitoneal, g</td>
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<td>Total, g</td>
<td>34±4</td>
<td>36±3</td>
<td>67±3*</td>
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<tr>
<td>Adiposity Index, %</td>
<td>5.5±0.6</td>
<td>6.1±0.4</td>
<td>8.4±0.4*</td>
</tr>
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</table>

Values are mean ± SEM. *Significant difference versus LF or OR rats (P<0.05).
Table S2. Characteristics of Lean and Obese Zucker Rats

<table>
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<th>Obese (n=8)</th>
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<td>Age (weeks)</td>
<td>15.3±0.5</td>
<td>15.8±0.4</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>376±9</td>
<td>564±21*</td>
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<tr>
<td>Insulin (ng/mL)</td>
<td>1.1±0.2</td>
<td>10.5±3.5*</td>
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</tbody>
</table>

Values are mean ± SEM. *Significant difference versus lean (P<0.05).

Plasma insulin levels were analyzed from samples of lean and obese Zucker rats generously provided by Dr. David Stepp (Medical College of Georgia). Half the rats were fasted overnight. Then, rats were anesthetized with isoflurane, decapitated, and trunk blood collected. Insulin levels were determined by an ELISA using a commercially available kit (Millipore).
Figure S1. Summary figures of lumbar, mean ABP, and blood glucose during 120-minute hyperinsulinemic-euglycemic clamp or saline infusion. ○ Insulin + aCSF (n=7) and ■ saline + aCSF (n=3). *Significant difference vs saline (P<0.05)
Figure S2. Schematic drawings of RVLM injection sites (● Insulin + KYN, ○ insulin + aCSF, ■ saline + KYN, and □ saline + aCSF). Microinjections of AP5, NBQX, losartan and SHU9119 were similar in location (data not shown). Sections represent -11.6 mm (top) and -11.9 mm (bottom) in reference to bregma. IO indicates inferior olive; p, pyramidal tracts; NA, nucleus ambiguous; ST, spinal trigeminal nucleus.