Sympathetic Response to Insulin Is Mediated by Melanocortin 3/4 Receptors in the Hypothalamic Paraventricular Nucleus

Kathryn R. Ward, James F. Bardgett, Lawrence Wolfgang, Sean D. Stocker

See Editorial Commentary, pp 375–376

Abstract—Hyperinsulinemia increases sympathetic nerve activity and contributes to cardiovascular dysfunction in obesity and diabetes. Neurons of the hypothalamic paraventricular nucleus (PVN) regulate sympathetic nerve activity through mono- and poly-synaptic connections to preganglionic neurons in the spinal cord. The purpose of the present study was to determine whether PVN neurons mediate the sympathetic response to insulin. Hyperinsulinemic-euglycemic clamps were performed in α-chloralose-anesthetized, male Sprague-Dawley rats (280–420 g) by an infusion of insulin (3.75 mU/kg per min) and 50% dextrose (0.75–2.0 mL/h) for 120 minutes. At 90 minutes, insulin significantly increased lumbar sympathetic nerve activity without any change in renal sympathetic nerve activity, heart rate, or blood glucose levels. Inhibition of the PVN with bilateral injection of the GABA receptor agonist muscimol completely reversed the sympathoexcitatory response. However, direct injection of insulin into the PVN did not alter lumbar sympathetic nerve activity, and thereby suggests that insulin activates neurons upstream of the PVN. Interestingly, the sympathetic response to insulin was eliminated by PVN injection of the melanocortin 3/4 receptor antagonist SHU9119, but was unaffected by the angiotensin II type 1 receptor antagonist losartan. A final set of experiments suggests activation of PVN neurons during hyperinsulinemia increases glutamatergic drive to the rostral ventrolateral medulla. Collectively, these findings indicate that insulin activates a melanocortin-dependent pathway to the PVN that increases glutamatergic drive to the rostral ventrolateral medulla and alters cardiovascular function. (Hypertension, 2011;57:435-441) • Online Data Supplement

Key Words: hyperinsulinemia ■ blood pressure ■ obesity ■ pro-opiomelanocortin ■ angiotensin II

Insulin contributes to cardiovascular dysfunction in obesity and type II diabetes.1,2 These consequences are partly mediated by the ability of insulin to act within the central nervous system to elevate sympathetic nerve activity (SNA)3–6 and alter baroreflex function.7,8 In both humans and rodents, an acute hyperinsulinemic-euglycemic clamp elevates muscle or lumbar SNA, respectively.3–6 Intracerebroventricular (ICV) administration of insulin in rodents produces similar responses.4 These sympathoexcitatory effects of insulin are prevented by lesion of the anteroventral third ventricle region,9 are attenuated by ICV administration of a phosphoinositol 3 kinase inhibitor10 or angiotensin II type I (AT₁) receptor antagonist losartan,11 and are absent in melanocortin-4 receptor deficient mice.12 Blockade of brain melanocortin receptors also prevents the central anorexic effect of insulin.13 Despite these observations, there is a paucity of knowledge regarding the central neural circuitry that mediates the sympathetic and cardiovascular effects of insulin.

Our laboratory recently reported that blockade of glutamatergic receptors in the rostral ventrolateral medulla (RVLM) completely reversed the sympathoexcitatory response to insulin.5 A primary source of glutamatergic input to the RVLM arises from neurons in the hypothalamic paraventricular nucleus (PVN).14,15 PVN neurons play a pivotal role in the regulation of SNA and arterial blood pressure (ABP) through mono- and poly-synaptic pathways via the medulla and the thoracic and lumbar segments of the spinal cord. Activation of PVN neurons elevates SNA and ABP,16–20 and previous studies have reported that altered PVN neurotransmission contributes to elevated SNA and ABP in several experimental models of hypertension.21 Interestingly, insulin receptors are widely expressed throughout the hypothalamus including in the PVN.22 Moreover, PVN neurons also express an abundance of melanocortin-423 and AT₁24 receptors. Therefore, the purpose of the present study was to determine initially whether PVN neurons mediate the sympathoexcitatory response to insulin and then subsequently identify the specific mechanism(s) within PVN that elevates SNA 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.
Animals and were approved by the Institutional Animal Care and Use Committee at the Pennsylvania State College of Medicine. Male Sprague-Dawley rats (250–420 g, Charles River Laboratories) were housed in a temperature-controlled room (22±1°C) with a 12-hour light-dark cycle. Rats were fed standard chow (Harlan Teklad Global Diet 2018) and given access to deionized water.

General Procedures
Rats were anesthetized with isoflurane (2% to 3% in 100% O₂) and prepared for recordings of ABP, lumbar, and renal SNA as described elsewhere5,25 (see the online supplement at http://hyper.ahajournals.org). Animals were artificially ventilated with oxygen-enriched room air. End-tidal CO₂ and body temperature were maintained at 4 to 4.5% and 37±1°C, respectively. Rats were placed into a stereotaxic head frame, and a craniotomy was performed to remove bone overlying the cortex to allow access to the PVN. After all surgical procedures were completed, anesthesia was replaced by α-chloralose (50 mg/kg bolus followed by 25 mg/kg per h, intravenously [IV]). The level of anesthesia was monitored by the lack of a withdrawal reflex to a foot pinch. Animals were allowed to stabilize >1 hour before experiments began.

Hyperinsulinemic-Euglycemic Clamp
Baseline values of SNA and ABP were recorded for 20 minutes. Then, insulin (3.75 mU/kg per min, IV; Humulin R) and a 50% dextrose solution (0.25–2.0 mL/h, IV) were infused for 150 minutes. This dose of insulin has been reported previously in our laboratory to produce plasma insulin levels equivalent to those of rats fed a moderately high-fat diet for 13 weeks and those of obese Zucker rats.5 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. At 90 minutes, one of several compounds was bilaterally injected into the PVN: (1) the GABA<sub>A</sub> receptor agonist muscimol (5 mmol/L), (2) the melanocortin 3/4 receptor antagonist SHU9119 (0.5 mmol/L), (3) the AT<sub>1</sub> receptor antagonist losartan (40 mmol/L), or (4) artificial cerebrospinal fluid (aCSF). Volumes (60 nL) were injected over 5 s using single-barrel glass micropipettes (OD: 436 Hypertension

Central Insulin Injection
To determine whether insulin directly acts on PVN neurons to increase lumbar SNA, various concentrations of insulin (5, 0.5, 0.05, or 0.0005 μU per nL, 60 nL) were microinjected bilaterally into the PVN. ABP and lumbar SNA were recorded for 60 minutes, and blood glucose was measured every 30 minutes. The insulin concentrations were based on previous studies using ICV injection of insulin<sup>6,10</sup> and were recalcuated because of a minimum 10-fold dilution attributable to the CSF volume of the third ventricle.

Data Analysis
All data are expressed as mean±SE. Changes in rectified and integrated (5 s time constant) SNA were calculated by subtracting background noise after hexamethonium (30 mg/kg, IV). For all variables, 5-minute segments at each time point were compared with 3 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 independent or paired t-tests with a layered Bonferroni correction. A P<0.05 was statistically significant.

Results
Inhibition of the PVN Reverses the Sympathoexcitatory Response to Insulin
A major goal of the present study was to determine whether the PVN mediates the sympathoexcitatory response to insulin during euglycemia. Figure 1 illustrates a representative example of the sympathoexcitatory response to insulin before and after PVN injection of muscimol or aCSF. Figure 2 summarizes group data. As previously reported,<sup>5,6</sup> a hyperinsulinemic-euglycemic clamp significantly increased lumbar SNA without a change in renal SNA or heart rate (data not shown). Although mean ABP was not different between groups, there was a small significant increase in mean ABP of hyperinsulinemic versus control rats at 90 minutes (Δ: 6±2 mm Hg versus 0±1 mm Hg, respectively; P<0.05). Infusion of saline did not significantly alter any variable.

Inhibition of the PVN via bilateral microinjection of the GABA<sub>A</sub> receptor agonist muscimol significantly reduced lumbar SNA and mean ABP in hyperinsulinemic rats (Figures 1 and 2). In fact, injection of muscimol reduced lumbar SNA to levels that were not different from baseline values or

Figure 1. Examples of ABP and rectified and integrated lumbar (∫ lumbar) and renal (∫ renal) SNA during PVN injection of the GABA<sub>A</sub> agonist muscimol in rats receiving (A) a hyperinsulinemic-euglycemic clamp or (B) saline infusion. Traces for raw lumbar and renal SNA represent (a) baseline, (b) 90 minutes, and (c) postinjection. Group data are presented in Figure 2.

Animals received 1 to 2 doses of insulin separated by a minimum of 90 minutes. For purposes of comparison, insulin (50 μU per 2 μL) was injected into the third ventricle using the following coordinates in reference to bregma: 1.0 to 1.5 mm caudal, 9.0 mm ventral, 0.5 to 0.7 mm lateral from the midline, and 4° angle from the midsagittal plane. At the end of experiments, Evan’s Blue Dye (0.5%, 1 μL) was injected into the third ventricle using the same coordinates; proper placement of the injection was confirmed by presence of the dye in the third and fourth ventricle.

In a final set of experiments, microinjections were performed in the PVN and RVLM of the same animal. The incisor bar was positioned at −11 mm. At 75 minutes after the start of the hyperinsulinemic-euglycemic clamp, muscimol (5 mmol/L) or aCSF was injected bilaterally into the PVN with a micropipette angled 15° rostral from the vertical plane using the same coordinates as described above. At 90 minutes, the ionotropic glutamate receptor antagonist kynurenic acid (KYN; 25 mmol/L) was injected into the PVN and RVLM of the same animal. The incisor bar was positioned at −11 mm. At 75 minutes after the start of the hyperinsulinemic-euglycemic clamp, muscimol (5 mmol/L) or aCSF was injected bilaterally into the PVN with a micropipette angled 15° rostral from the vertical plane using the same coordinates as described above. At 90 minutes, the ionotropic glutamate receptor antagonist kynurenic acid (KYN; 25 mmol/L) was injected into the PVN and RVLM as described previously in our laboratory.5,25

Injection sites were marked with 0.2% rhodamine or fluorescent isothiocyanate beads added to the respective drug. At the end of the experiments, animals were perfused transcardially with 4% paraformaldehyde. Brains were sectioned at 100 μm and postfixed in 4% paraformaldehyde and 0.05% sodiumcacodylate for 1 h. Brains were washed in 0.1 M phosphate buffer and stored in 0.1 M phosphate buffer with 20% sucrose. Brains were sectioned on a freezing microtome in 30 μm sections and stored in 0.1 M phosphate buffer with 20% sucrose until use.

Examples of ABP and rectified and integrated lumbar (∫ lumbar) and renal (∫ renal) SNA during PVN injection of the GABA<sub>A</sub> agonist muscimol in rats receiving (A) a hyperinsulinemic-euglycemic clamp or (B) saline infusion. Traces for raw lumbar and renal SNA represent (a) baseline, (b) 90 minutes, and (c) postinjection. Group data are presented in Figure 2.

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Inhibition of the PVN via bilateral microinjection of the GABA<sub>A</sub> receptor agonist muscimol significantly reduced lumbar SNA and mean ABP in hyperinsulinemic rats (Figures 1 and 2). In fact, injection of muscimol reduced lumbar SNA to levels that were not different from baseline values or

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those of saline-infused rats. Although muscimol significantly reduced mean ABP in both groups, the magnitude of the decrease in mean ABP was significantly greater in hyperinsulinemic versus saline rats (−21±4 mm Hg versus −10±3 mm Hg, *P*<0.05). Injection of muscimol did not significantly alter renal SNA (Figures 1 and 2) or heart rate (data not shown). It is noteworthy that injection sites located outside the PVN did not reverse the sympathoexcitatory response to insulin (lumbar SNA at 90 minutes: 141±10% versus 120 minutes: 133±9%, *n*=7; for histology, see the online supplement at http://hyper.ahajournals.org).

Bilateral injection of aCSF did not significantly alter any variable in either hyperinsulinemic or saline rats (data not shown).

**PVN Injection of Insulin Does Not Alter Lumbar SNA**

Previous studies indicate that insulin receptors are expressed within the PVN. To determine whether insulin may act directly on PVN neurons to increase SNA, we analyzed lumbar SNA responses to microinjection of insulin into the PVN. As illustrated in Figure 3, bilateral injection of various insulin concentrations into the PVN failed to produce a significant increase in lumbar SNA or mean ABP. However, injection of insulin into the adjacent third ventricle significantly elevated lumbar SNA.

**Blockade of PVN Melanocortin 3/4, But Not of AT₁ Receptors, Reverses the Sympathoexcitatory Response to Hyperinsulinemia**

Because inhibition of the PVN reversed the sympathoexcitatory response to insulin, but PVN injection of insulin did not raise lumbar SNA, additional experiments sought to determine the specific mechanism(s) within PVN. Bilateral injection of the melanocortin 3/4 receptor antagonist SHU9119 significantly lowered lumbar SNA and mean ABP in hyperinsulinemic rats (Figures 4 and 5). In fact, injection of SHU9119 reduced lumbar SNA to levels that were not different versus baseline values or those of control rats (109±5%, 100±1%, and 104±3%, respectively). SHU9119 did produce a small fall in mean ABP of hyperinsulinemic versus control rats (−1±5 mm Hg versus −1±1 mm Hg, respectively; *P*<0.05), but did not significantly alter renal SNA (Figures 4 and 5) or heart rate (data not shown) in hyperinsulinemic rats. Both lumbar SNA and mean ABP

![Figure 2. Mean ± SEM of mean ABP, lumbar SNA, renal SNA, and blood glucose before and after PVN injection of muscimol in rats receiving a hyperinsulinemic-euglycemic clamp or saline infusion. *P*<0.05 insulin vs saline, †*P*<0.05 vs 90 minutes values for insulin rats, #*P*<0.05 vs baseline values.](image1)

![Figure 3. Examples of ABP, j lumbar SNA, and raw lumbar SNA during injection of insulin into the (A) PVN bilaterally or (B) third ventricle. Arrow denotes injection. Traces for raw lumbar SNA represent (a) baseline and (b) 60 minutes. C. Summary data (mean ± SEM). There were no differences in baseline mean ABP (116±4 vs 116±3) or heart rate (368±9 vs 385±8) of animals receiving a PVN vs third ventricle injection, respectively. †*P*<0.01 vs aCSF.](image2)
returned to preinjection values between 120 to 140 minutes. Injection of SHU9119 did not alter any variable of control rats. It is noteworthy that injection sites located outside the PVN did not alter lumbar SNA (90 minutes: 142±11% versus 100 minutes: 144±13%, n=4; for histology, see the online supplement at http://hyper.ahajournals.org).

Figure 5. Mean±SEM of mean ABP, lumbar, and renal SNA, and blood glucose before and after PVN injection of the melanocortin 3/4 receptor antagonist SHU9119 in rats receiving a hyperinsulinemic-euglycemic clamp or saline infusion. *P<0.05 insulin vs saline, #P<0.05 vs 90 minutes values for insulin rats.

Figure 4. Examples of ABP and ∫ lumbar and renal SNA during PVN injection of SHU9119 in rats receiving (A) a hyperinsulinemic-euglycemic clamp or (B) saline infusion. Traces for raw lumbar and renal SNA represent (a) baseline, (b) 90 minutes, and (c) postinjection. Summary data are presented in Figure 5.

An additional set of experiments was performed to determine whether the sympathoexcitatory response to ICV administration of insulin was also dependent on melanocortin 3/4 receptor activation within the PVN. ICV administration of insulin (50 mU per 2 μL, n=4) significantly increased lumbar SNA (90 minutes: 142±10%, P<0.05) and heart rate (0 minutes: 404±33 bpm versus 90 minutes: 432±24 bpm, Δ: 28±10 bpm, P<0.05) without a change in mean ABP (0 minutes: 96±2 mm Hg versus 90 minutes: 99±2 mm Hg) or renal SNA (90 minutes: 99±2%). Bilateral microinjection of SHU9119 significantly reduced lumbar SNA (116±5%, P<0.05 vs 90 minutes values for insulin rats) without a significant change in mean ABP (Δ: 0±5 mm Hg), renal SNA (Δ: 1±4%) or in heart rate (Δ: −5±3 bpm). Bilateral injection of SHU9119 into the PVN did not affect any variable after ICV injection of aCSF (data not shown).

To test whether PVN injection of SHU9119 nonspecifically attenuates all sympathoexcitatory responses evoked from the PVN, N-Methyl-D-aspartic acid (5 mmol/L, 60 nL) was microinjected into the PVN before and 10 minutes after injection of SHU9119 or aCSF. Unilateral injection of N-Methyl-D-aspartic acid significantly increased lumbar and renal SNA, heart rate, and mean ABP; however, the magnitude of these responses were unaffected by PVN injection of SHU9119 (Table). In addition, PVN injection of SHU9119 did not affect the sympathoexcitatory response to PVN injection of the GABA receptor antagonist gabazine (2 mmol/L per 60 nL, data not shown). PVN injection of SHU9119 or aCSF did not alter baseline variables.

In marked contrast to SHU9119, PVN injection of losartan did not affect the sympathoexcitatory response to insulin (data not shown). A hyperinsulinemic-euglycemic clamp significantly raised lumbar SNA at 90 minutes (133±2%, n=6), but bilateral injection of losartan did not lower lumbar SNA at 100 minutes (131±8%), 110 minutes (145±15%), or 120 minutes (151±18%). Microinjection of losartan into the PVN did not alter mean ABP in hyperinsulinemic rats (0 minutes: 115±8 mm Hg, 90 minutes: 120±9 mm Hg, and 110 minutes: 119±6 mm Hg), and losartan did not affect any variable in control rats (data not shown).

PVN Provides Glutamatergic Drive to the RVLM During Hyperinsulinemia

Our laboratory has previously reported that blockade of glutamatergic receptors in the RVLM reverses the sympathoexcitatory response to insulin. Therefore, we hypothesized that PVN neurons provide this glutamatergic drive to the RVLM. In hyperinsulinemic-euglycemic rats, PVN injection of aCSF at 75 minutes did not affect lumbar SNA or mean ABP; however, RVLM injection of KYN significantly lowered lumbar SNA and mean ABP (Figure 6). Prior inhibition of the PVN by injection of muscimol at 75 minutes significantly lowered lumbar SNA and mean ABP but also prevented any effect of subsequent injection of KYN into the RVLM at 90 minutes. In control rats (n=4), injection of muscimol into the PVN and KYN into the RVLM did not affect any variable after ICV injection of aCSF (data not shown).
Discussion

Despite the ability of insulin to elevate SNA and alter cardiovascular function, there is a paucity of knowledge regarding the central neural mechanisms that mediate these effects. The present study provides several novel findings: (1) inhibition of the PVN completely reversed the sympathoexcitatory response to insulin, (2) direct injection of insulin into the PVN did not elevate lumbar SNA, (3) blockade of PVN melanocortin 3/4, but not of AT1 receptors, eliminated the sympathoexcitatory response to insulin, and (4) inhibition of the PVN prevented the fall in lumbar SNA and mean ABP in response to RVLM injection of KYN in hyperinsulinemic rats. Collectively, these findings indicate that insulin activates a melanocortin-dependent pathway to the PVN, and activation of PVN neurons increases glutamatergic drive to the RVLM via a direct or indirect pathway to alter cardiovascular function.

The PVN plays a pivotal role in the regulation of SNA and ABP through mono- and poly-synaptic pathways to sympathetic-regulatory neurons in the medulla and in thoracic and lumbar segments of the spinal cord. The present findings clearly demonstrate that inhibition of the PVN completely reversed the sympathoexcitatory response to insulin. However, direct injection of insulin into the PVN failed to alter lumbar SNA or ABP despite the sympathoexcitatory response to insulin injections into the adjacent third ventricle. These findings indicate that structures outside or upstream of the PVN and perhaps along the third ventricle detect changes in circulating or CSF insulin. Although insulin receptors are expressed within PVN, previous studies have not localized expression to any specific cell population. Therefore, the lack of a sympathoexcitatory response to direct injection of insulin into the PVN may be explained by the expression of insulin receptors on cell groups distinct from sympathetic-regulatory neurons.

PVN neurons express a number of receptors, including those previously reported to mediate the anorexic and sympathoexcitatory and pressor effects of central insulin. Here, blockade of PVN melanocortin 3/4, but not of AT1 receptors, reversed the increase in lumbar SNA of hyperinsulinemic rats. However, injection of SHU9119 did not affect the sympathoexcitatory response to PVN injection of N-Methyl-D-aspartic acid (or gabazine). These observations suggest that the effect of SHU9119 to reverse the sympathoexcitatory

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Table. PVN Injection of SHU9119 (0.5 mmol/L, 60 nL) Did Not Alter the Sympathoexcitatory Responses Evoked by PVN Injection of NMDA (5 mmol/L, 60 nL)

<table>
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<tr>
<th>Time</th>
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<th>Mean ABP (mmHg)</th>
<th>Heart Rate (bpm)</th>
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<th>Renal SNA (%)</th>
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<td>35±7</td>
<td>122±9</td>
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<td>∆ (10 minutes post)</td>
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<td>41±8</td>
<td>131±11</td>
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<tr>
<td>SHU9119 (0.5 mmol/L, 60 nL)</td>
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<td>5</td>
<td>111±6</td>
<td>425±24</td>
<td>118±9</td>
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<td>∆ (Before treatment)</td>
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<td>∆ (10 minutes post)</td>
<td>19±2</td>
<td>55±17</td>
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Values are mean±SEM. NMDA injections were separated by >30 minutes. Injection of SHU9119 did not attenuate the sympathoexcitatory response to PVN injection of NMDA.

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Figure 6. Examples of ABP and integrated lumbar SNA during PVN injection of (A) aCSF or (B) muscimol at 75 minutes followed by RVLM injection of KYN at 90 minutes in hyperinsulinemic rats. Note that the hyperinsulinemic-euglycemic clamp began 60 minutes earlier. Traces for raw lumbar SNA represent (a) 60 minutes, (b) post-PVN injection, and (c) post-RVLM injection. C. Mean±SEM of mean ABP, lumbar SNA, and blood glucose before and after PVN and RVLM injections. Mus indicates muscimol; *P<0.05 vs baseline, †P<0.05 vs 60 minutes (PVN muscimol group), #P<0.05 vs 75 minutes (PVN aCSF group).
response to insulin cannot be attributed to a general inactivation of PVN neurons, but it reflects the antagonism of melanocortin 4 receptors. Furthermore, injections of SHU9119 (or muscimol) located outside the PVN (~200 to 400 μm) did not reduce SNA or mean ABP, thereby suggesting that the actions of these drugs can be attributed to receptors on PVN neurons. Interestingly, lumbar SNA and mean ABP did return to preinjection levels at 120 to 140 minutes. This observation is consistent with previous studies and unpublished data in our laboratory, which show that injection of SHU9119 effectively blocks melanocortin receptors for ~30 to 40 minutes.

Proopiomelanocortin neurons are confined to the arcuate nucleus and nucleus of the solitary tract. Although insulin receptor binding/expression is more abundant within the arcuate nucleus, the contribution of these structures to the sympathetic-cardiovascular actions of insulin has not been determined. Manipulation of hypothalamic insulin receptors does attenuate the impact of insulin on energy homeostasis, thereby suggesting that insulin may act on neurons within the arcuate nucleus. This notion is supported by several recent electrophysiological studies in vitro, although acute insulin application was reported to hyperpolarize proopiomelanocortin cells through a phosphoinositol 3 kinase-dependent mechanism. It is noteworthy that inhibition of hypothalamic phosphoinositol 3 kinase has been reported to prevent insulin-induced increases in SNA. Moreover, preliminary data in our laboratory suggests that inhibition of the arcuate nucleus reverses the sympathoexcitatory response to insulin, and direct injection of insulin into the arcuate nucleus raises SNA. However, future studies that directly antagonize insulin receptors or knockdown insulin receptor expression will be needed to determine whether arcuate neurons detect changes in circulating insulin levels to subsequently alter cardiovascular function.

We have previously reported that ~85% of RVLM-projecting PVN neurons express the vesicular glutamate transporter-2 mRNA. Since blockade of RVLM ionotropic glutamate receptors reversed the sympathetic response to insulin, we hypothesized that this increased glutamatergic drive originated from the PVN. Indeed, the present findings are consistent with this hypothesis, as inhibition of the PVN prevented the fall in lumbar SNA after RVLM injection of KYN. While these data highlight the importance of the PVN and RVLM as a circuit to mediate the sympathoexcitatory effects of insulin, these findings do not conclusively prove that a direct pathway from the PVN to the RVLM is involved.

Several studies clearly indicate that insulin acutely alters SNA and baroreflex function in animals and humans. However, the role of insulin in cardiovascular dysfunction in obesity-induced hypertension or type II diabetes remains controversial. In rodents, a hyperinsulinemic-euglycemic clamp acutely raises SNA and chronically raises ABP. However, a chronic infusion of insulin in dogs produced opposite hemodynamic changes in cardiac output and peripheral resistance and does not elevate ABP. In humans, findings from the Normative Aging Study suggest a correlation between obesity, insulin levels and blood pressure, and plasma insulin concentrations have been correlated with muscle SNA. However, other studies have not observed a relationship between plasma insulin concentrations versus muscle SNA and renal norepinephrine spillover in obese populations or after modest weight gain in nonobese individuals. Therefore, the precise role of insulin in cardiovascular dysfunction during obesity-induced hypertension or type II diabetes remains unclear. However, it is unlikely that 1 factor, by itself, mediates the pathogenesis of complex diseases, such as the metabolic syndrome.

**Perspective**

Accumulating evidence indicates the important of the central melanocortin system as a pivotal mediator of obesity-induced hypertension. First, the sympathoexcitatory responses to acute leptin and insulin administration are abolished by ICV administration of SHU9119 or in melanocortin-4 deficient mice. Interestingly, melanocortin-4 deficient mice are normotensive despite obesity, hyperinsulinemia, and hyperleptinemia. Chronic ICV administration of SHU9119 lowers ABP in a rodent model of diet-induced obesity. Finally, the prevalence of hypertension is significantly lower in obese humans with melanocortin-4 receptor deficiency. These observations together with the present findings suggest that melanocortin receptors on PVN sympathetic-regulatory neurons may represent a novel therapeutic target for obesity-related hypertension. However, future studies are needed to determine specifically whether such mechanisms in the PVN mediate elevated SNA and ABP in obesity and to identify the specific populations of PVN neurons involved.

**Sources of Funding**

This work was supported by a National Institutes of Health National Heart, Lung, and Blood Institute Grant HL090826 (S.D.S.).

**Disclosures**

None.

**References**


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Hypertension. 2011;57:435-441; originally published online January 24, 2011;
doi: 10.1161/HYPERTENSIONAHA.110.160671

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Methods

General Procedures. On the day of the experiment, rats were anesthetized with isoflurane (2-3% in 100% O₂) and prepared for recordings of lumbar and/or renal SNA. Briefly, a lumbar sympathetic nerve was isolated through a midline laparotomy, placed on a bipolar stainless steel electrode, and insulated with KWIK-SIL (World Precision Instruments). A renal sympathetic nerve was isolated through a retroperitoneal incision. Nerve signals were amplified (10 K) and filtered (100-1000 Hz). Then, signals were rectified, integrated (5s time constant), and digitized (5000 Hz) using Spike 2 software (Cambridge Electronic Design). Arterial and venous catheters were implanted in the femoral vessels for ABP recording and administration of drugs. An additional brachial arterial catheter was used for blood glucose sampling. Animals were artificially-ventilated with oxygen-enriched room air. End-tidal CO₂ and body temperature were maintained at 4-4.5% and 37±1 °C, respectively. Rats were placed into a stereotaxic head frame with the skull leveled between bregma and lambda. A craniotomy was performed to remove bone overlying the cortex to allow access to the PVN. After all surgical procedures were completed, anesthesia was replaced by α-chloralose (50 mg/kg bolus followed by 25 mg/kg/hr, IV). The level of anesthesia was examined by the lack of a withdrawal reflex to a foot pinch. Animals were allowed to stabilize >1 hr before experiments began.

Effect of Melanocortin Receptor Blockade on Sympathetic-Cardiovascular Responses Evoked from the PVN. Animals were prepared as described above except isoflurane anesthesia was replaced by urethane (1.2 g/kg, iv). Urethane anesthesia was used instead of α-chloralose as more consistent NMDA-evoked sympathoexcitatory responses are evoked from the PVN under urethane versus α-chloralose. At least 1 h later, NMDA (5 mM / 60 nL) was unilateral injected into the PVN. Approximately 20 min later, SHU9119 (0.5 mM / 60 nL) or aCSF (60 nL) was injected into the same PVN site. At 10 min after SHU9119 or aCSF, NMDA was unilaterally injected into PVN.
Results

Figure S1. Schematic illustration of muscimol injection sites for animals infused with insulin + dextrose or vehicle.

Figure S2. Schematic illustration of insulin injection sites (□) in the PVN.
Figure S3. Schematic illustration of SHU9119 injection sites for animals infused with insulin + dextrose or vehicle.

Figure S4. Schematic illustration of SHU9119 injection sites for animals that received an ICV injection of insulin or aCSF.
Figure S5. Schematic illustration of injection sites of SHU9119 or aCSF during injection of NMDA.
Figure S6. Schematic illustration of (A) PVN and (B) RVLM injection sites for animals infused with insulin + dextrose or vehicle.