Arcuate nucleus injection of an anti-insulin affibody prevents the sympathetic response to insulin

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Insulin receptors are expressed throughout the central nervous system with notably higher levels reported in the hypothalamus, including structures such as the hypothalamic paraventricular nucleus (PVH), ventromedial hypothalamus (VMH), and arcuate nucleus (ARC) (26, 48). A hypothalamic-mediated site of action for insulin is supported by studies in rats in which administration of insulin into the lateral, but not fourth, ventricle increased lumbar SNA (37). Within the hypothalamus, previous studies have demonstrated that inhibition of PVH or ARC neurons reversed the sympatoexcitatory response to insulin (8, 47); however, microinjection of insulin into the ARC, but not PVH, increased lumbar SNA (8, 47). Anatomic studies have indicated that ARC neurons densely innervate several hypothalamic structures, including the PVH (9, 11). The ARC contains an abundance of proopiomelanocortin neurons (20, 34). Blockade of melanocortin 3/4 receptors within the PVH attenuates the sympatoexcitatory and pressor response to ARC stimulation (19) and also reverses the sympatoexcitatory response to intracerebroventricular insulin and a systemic hyperinsulinemic-euglycemic clamp (47). Collectively, the results of these studies suggest that ARC neurons may detect changes in circulating insulin to initiate changes in the activity of downstream circuits through the PVH to alter sympathetic outflow (4, 28, 47).

The above studies were limited by the absence of any experimental manipulation to directly interrupt insulin-mediated actions within the ARC and definitively address whether circulating insulin acts on ARC neurons. Therefore, the purpose of the present study was to determine whether systemic insulin acts locally within the ARC to increase lumbar SNA. In these experiments, the actions of insulin were neutralized by microinjection of an anti-insulin affibody. Affibody molecules have a smaller molecular weight versus traditional antibodies. The anti-insulin affibody was recently characterized to prevent the phosphorylation of Akt in differentiated adipocytes by insulin or within VMH molecular weight versus traditional antibodies (1 ng/40 nl). To verify the efficacy of the affibody, ARC pretreatment with injection of the anti-insulin affibody completely prevented the increase in lumbar SNA produced by ARC injection of insulin. Next, ARC pretreatment with the anti-insulin affibody attenuated the lumbar sympatoexcitatory response to intracerebroventricular injection of insulin. Third, a hyperinsulinemic-euglycemic clamp increased lumbar, but not renal, SNA in animals that received ARC injection of a control affibody. However, this sympatoexcitatory response was absent in animals pretreated with the anti-insulin affibody in the ARC. Injection of the anti-insulin affibody in the adjacent ventromedial hypothalamus did not alter the sympatoexcitatory response to insulin. The ability of the anti-insulin affibody to prevent the sympathetic effects of insulin cannot be attributed to a general inactivation or nonspecific effect on ARC neurons as the affibody did not alter the sympatoexcitatory response to ARC disinhibition by gabazine. Collectively, these findings suggest that circulating insulin acts within the ARC to increase SNA.

INSULIN acts within the central nervous system to increase sympathetic nerve activity (SNA) and alter baroreflex function. This notion is supported by experimental studies that demonstrated that a hyperinsulinemic-euglycemic clamp elevated muscle or lumbar SNA in humans (3, 40) or rodents (4, 28, 47), respectively. Systemic hyperinsulinemia has also been reported to increase baroreflex gain in both humans and rodents (8, 32, 37, 50). These neural effects are attributed to a central action as intracerebroventricular injection of insulin produces similar responses (4, 31, 47). Furthermore, the sympatoexcitatory effects of insulin are prevented or attenuated by a number of central manipulations, including lesion of the anteroventral third ventricle region (30) or intracerebroventricular administration of a phosphoinositol 3-kinase (PI3K) inhibitor (39). Despite these observations, little is known regarding where circulating insulin is detected in the brain to initiate downstream changes in sympathetic outflow and baroreflex function.

INSULIN receptors are expressed throughout the central nervous system with notably higher levels reported in the hypothalamus, including structures such as the hypothalamic paraventricular nucleus (PVH), ventromedial hypothalamus (VMH), and arcuate nucleus (ARC) (26, 48). A hypothalamic-mediated site of action for insulin is supported by studies in rats in which administration of insulin into the lateral, but not fourth, ventricle increased lumbar SNA (37). Within the hypothalamus, previous studies have demonstrated that inhibition of PVH or ARC neurons reversed the sympatoexcitatory response to insulin (8, 47); however, microinjection of insulin into the ARC, but not PVH, increased lumbar SNA (8, 47). Anatomic studies have indicated that ARC neurons densely innervate several hypothalamic structures, including the PVH (9, 11). The ARC contains an abundance of proopiomelanocortin neurons (20, 34). Blockade of melanocortin 3/4 receptors within the PVH attenuates the sympatoexcitatory and pressor response to ARC stimulation (19) and also reverses the sympatoexcitatory response to intracerebroventricular insulin and a systemic hyperinsulinemic-euglycemic clamp (47). Collectively, the results of these studies suggest that ARC neurons may detect changes in circulating insulin to initiate changes in the activity of downstream circuits through the PVH to alter sympathetic outflow (4, 28, 47).

The above studies were limited by the absence of any experimental manipulation to directly interrupt insulin-mediated actions within the ARC and definitively address whether circulating insulin acts on ARC neurons. Therefore, the purpose of the present study was to determine whether systemic insulin acts locally within the ARC to increase lumbar SNA. In these experiments, the actions of insulin were neutralized by microinjection of an anti-insulin affibody. Affibody molecules have a smaller molecular weight versus traditional antibodies. The anti-insulin affibody was recently characterized to prevent the phosphorylation of Akt in differentiated adipocytes by insulin or within VMH neurons in vivo during insulin-induced hypoglycemia (35).

MATERIALS AND METHODS

Animals

All experimental procedures conformed with 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 Pennsylvania State University College of Medicine. Adult male Sprague-Dawley rats (250–400 g, Charles River Laboratories) were housed in a temperature-controlled room (22–23°C) and maintained on a 12:12-h light-dark cycle for at least 1 wk before experiments. Animals had ad libitum access to laboratory chow (Harlan Teklad Global Diet no. 2018) and deionized water.
General Experimental Procedures

Rats were anesthetized with isoflurane (2–4% in 100% O₂) and prepared for recordings of arterial blood pressure (ABP), lumbar SNA, and/or renal SNA as previously described (1, 4, 47). Lumbar SNA was measured in every experiment since previous studies across multiple laboratories have reported that insulin consistently raises lumbar SNA (4, 8, 28, 47). Briefly, the lumbar sympathetic nerve was exposed through a ventral midline incision. The incision was closed with staples. After the animal had been placed into a stereotaxic frame in the prone position, the left renal sympathetic nerve was isolated through a retroperitoneal incision, placed on bipolar stainless steel electrodes, and insulated with WIKI-SIL. The incision was closed with staples. Nerve signals were amplified (10 K) and filtered (100–1,000 Hz) using a model 1700 differential alternating current amplifier (AM Systems). Signals were digitized at 5 kHz, rectified, and integrated (2-s time constant) using a Micro1401 and Spike 2 software (Cambridge Electronic Design). Animals were artificially ventilated with O₂-enriched room air. End-tidal CO₂ was maintained between 4.0% and 4.5% using a MicroCapstar End-Tidal CO₂ monitor (CWE). Body temperature was maintained at 37.0 ± 0.5°C with a rectal temperature probe (Sable Systems) and a water circulating blanket. Finally, a craniotomy was performed to remove bone overlaying the cortex to gain access to the ARC and other brain structures. After completion of the surgical procedures, isoflurane anesthesia was replaced by α-chloralose (experiments 1 and 2) or Inactin (experiments 3 and 4). α-Chloralose was initially administered as a bolus (50 mg/kg iv) followed by a continuous infusion (25 mg kg⁻¹·h⁻¹ at 0.25–0.5 ml/h). α-Chloralose was dissolved in 2% sodium borate. Inactin was administered as a bolus (120 mg/kg iv) followed by a continuous infusion (5 mg/kg·h⁻¹ at 0.5 ml/h). Inactin instead of α-chloralose was used in experiments 3 and 4 as preliminary data in our laboratory indicated that α-chloralose may interfere with the analysis of plasma insulin levels via ELISA. Both Inactin and α-chloralose solutions were dissolved in 0.45% NaCl to maintain electrolytes (Na⁺ and Cl⁻) at baseline values. The level of anesthesia was assessed by the absence of a withdrawal reflex to a toe pinch. Animals were allowed to stabilize for a minimum of 60 min before experimental protocols began.

Experimental Protocols

Experiment 1: validation of the anti-insulin affibody in the ARC. Initial experiments were performed to test the ability of the anti-insulin affibody to prevent sympathetic responses to insulin directly injected into the ARC. Baseline values were recorded for 20 min. Rats then received a bilateral injection of the anti-insulin affibody (1 ng-40 nl⁻¹-side⁻¹, ab31906, Abcam). Control injections were performed using an anti-IG affibody molecule (1 ng-40 nl⁻¹-side⁻¹, ab31900, Abcam). As mentioned above, the dose of the anti-insulin affibody has been previously used to prevent the phosphorylation of Akt in differentiated adipocytes by insulin or within VMH neurons in vivo during insulin-induced hypoglycemia (35). Ten minutes later, insulin (4 μU/40 nl⁻¹-side⁻¹) was injected bilaterally into the ARC. Variables were recorded for 2 h after the insulin injection. Blood glucose levels were measured from a drop of arterial blood every 30 min using a standard glucometer (One Touch Ultra). ARC microprojections were performed over 5–10 s using single-barrel glass pipettes (outer diameter: 20–40 μm) connected to a pneumatic picopump and lowered into the ARC using the following coordinates in reference to the bregma: 2.4–2.9 mm caudal, 9.7–9.9 mm ventral, and 0.5–0.7 mm lateral. Bilateral injections were performed using a glass pipette for each side. Pipettes remained in the ARC for 5 min after the injections, were raised and rinsed four times with artificial cerebrospinal fluid (aCSF), and then lowered into the ARC for the second injection.

Experiment 2: gabazine in the ARC after pretreatment with the anti-insulin affibody. A second set of experiments was performed to determine whether injection of the anti-insulin affibody produced a general inhibition or had a nonspecific action on ARC neurons. Baseline values were recorded for 20 min. Rats then received a unilateral injection of the anti-insulin affibody (1 ng-40 nl⁻¹-side⁻¹, ab31906, Abcam). Control injections were performed using an anti-IG affibody molecule (1 ng-40 nl⁻¹-side⁻¹, ab31900, Abcam). Ten minutes later, the GABA_A receptor antagonist gabazine (1 mM 20 nl⁻¹-side⁻¹) was injected unilaterally into the ipsilateral ARC. Injections of the anti-insulin and anti-IGg affibodies were performed in the same animal but contralateral ARC separated by a minimum of 60 min when variables returned and stabilized at baseline values. The order was randomized.

Experiment 3: ARC anti-insulin affibody pretreatment before intracerebroventricular insulin. A third set of experiments was performed to determine whether ARC neurons mediate the sympathetic response to intracerebroventricular insulin. Inactin-anesthetized rats received a bilateral injection of the anti-insulin affibody (1 ng-40 nl⁻¹-side⁻¹, ab31906, Abcam). Control injections were performed using aCSF or an anti-IG affibody molecule (1 ng-40 nl⁻¹-side⁻¹, ab31900, Abcam). Ten minutes later, insulin (1 μU/1 μl) was administered into the lateral ventricle using a glass micropipette with the following coordinates in reference to the bregma: −1.5 mm caudal, 1.6 mm lateral, and 4.6 mm ventral. Variables were recorded for 2 h after the injection of insulin. Blood glucose was measured every 30 min. Blood samples (0.2 ml) were collected from an arterial line into microcentrifuge tubes containing EDTA (5mM) at baseline and 30, 60, and 120 min after injection of insulin. Samples were centrifuged (10,000 g, 1 min), and the plasma was stored at −80°C until insulin levels were determined via ELISA. At the end of experiments, Evan’s blue dye (0.5%, 1 μl) was injected into the lateral ventricle to verify the intracerebroventricular injection site.

Experiment 4: ARC anti-insulin affibody pretreatment during hyperinsulinemic-euglycemic clamp. A final set of experiments was performed to determine whether ARC neurons mediate the sympathetic response to a hyperinsulinemic-euglycemic clamp. Inactin-anesthetized rats received a bilateral injection of the anti-insulin affibody (1 ng-40 nl⁻¹-side⁻¹, ab31906, Abcam). Control injections were performed using aCSF or an anti-IG affibody molecule (1 ng-40 nl⁻¹-side⁻¹, ab31900, Abcam). Ten minutes later, insulin (4.0 μU·kg⁻¹·min⁻¹ iv, Humulin R) and 50% dextrose solution (0.25–2.0 ml/h) were infused for 120 min. Blood glucose was measured every 10 min through a brachial arterial line. The dextrose infusion rate was adjusted to maintain euglycemia. Blood samples (0.2 ml) were collected at baseline and 30, 60, and 120 min after the start of the insulin infusion to determine plasma insulin levels. Dextrose was dissolved in 0.45% NaCl to avoid increases in the plasma Na⁺ concentration. Plasma electrolytes were measured by an i-STAT-1 analyzer and 6+ cartridges (Abbott Laboratories). Control animals were infused with equal volumes of 0.45% saline.

To determine whether the anti-insulin affibody diffused through the pipette track dorsally to the VMH or dorsomedial hypothalamus (DMH), another set of animals was infused with insulin and dextrose as described above. However, the anti-insulin affibody was injected dorsally using the following coordinates in reference to the bregma: 2.4–2.9 mm caudal, 9.0 mm ventral, and 0.5–0.7 mm lateral.

Histology

All injection sites were marked by the addition of rhodamine beads (0.1%) to all solutions. In experiments that required a second injection, FITC beads (0.1%) were added to the second solution. Intracerebroventricular injection sites were marked by an injection of 2% Evan’s blue dye (1 μl) after recordings were complete. At the end of the experiments, animals were perfused with 4% paraformaldehyde. Brains were removed, postfixed in 4% paraformaldehyde, and sec-
tioned at 50–100 µm. Adjacent sections were counterstained with cresyl violet. Injection sites were visualized using a Nikon 90i microscope with the appropriate filters. For experiments 1, 3, and 4 with bilateral injections, data are reported for animals with both left and right injections localized to the ARC (or outside the ARC for anatomic controls). Animals with one injection in the ARC but the contralateral injection outside the ARC were omitted.

Determination of Plasma Insulin Levels

Plasma insulin levels were measured by a rat insulin ELISA (EZRMI-13K, Millipore). For purposes of comparison, insulin levels were also analyzed from plasma samples of a rodent model of diet-induced obesity previously described in our laboratory (43).

Briefly, male Sprague-Dawley rats (200–250 g, Charles River Laboratories) were fed a low-fat diet (LF group; 10% kcal from fat, D12489B, Research Diets) or a moderately high-fat diet (32% kcal from fat, D12266B, Research Diets) for 13 wk. Those on the moderately high-fat diet segregated into obesity-resistant (OR) and obesity-prone (OP) groups. After 13 wk, these rats were anesthetized with isoflurane and prepared for recordings of SNA, and isoflurane anesthesia was replaced with Inactin as described above. These animals did not receive ARC injections but were used for other experiments not described here. However, plasma insulin values of these animals are reported to verify that the plasma insulin concentrations of animals receiving an intravenous hyperinsulinemic-euglycemic clamp were physiological. The inter- and intraassay coefficients of variance were 5.2% and 7.4%, respectively.

Data Analysis

All data are expressed as means ± SE. Changes in SNA were calculated by subtracting background noise after hexamethonium (30 mg/kg iv) or physically crushing the nerve. For all variables, data were averaged over 5 min and compared with three 5-min baseline periods (30 mg/kg iv) or physically crushing the nerve. For all variables, data were averaged over 5 min and compared with three 5-min baseline periods (30 mg/kg iv) or physically crushing the nerve.

RESULTS

Pretreatment With Anti-Insulin Affibody Prevents the Sympathoexcitatory Response to Insulin Injection in the ARC

Initial experiments tested whether pretreatment with the anti-insulin affibody prevented the sympathoexcitatory response to local application of insulin in the ARC. As previously reported (8), ARC injection of insulin significantly increased lumbar SNA at 60, 90, and 120 min (Fig. 1). While values tended to increase at 30 min, this was not statistically significant (P = 0.061). However, pretreatment with the anti-insulin affibody eliminated the sympathoexcitatory response to ARC insulin (Fig. 1). In fact, lumbar SNA did not change from baseline values and was significantly lower than animals injected with the control affibody at 60, 90, and 120 min. Injection of the control or anti-insulin affibody alone did not alter any variable (Table 1). Mean ABP and heart rate (control antibody: 420 ± 10 beats/min, anti-insulin affibody: 422 ± 8 beats/min) did not change from baseline values at any time. Figure 1D shows the injection sites for each experiment. In every case, injection of the affibodies overlapped with injection of insulin as shown by the location of red and green beads. Therefore, the injection site for each animal is shown as one injection site.

Anti-Insulin Affibody Did Not Alter the Sympathoexcitatory Response to ARC Injection of Gabazine

To test whether the anti-insulin affibody produced a general nonspecific inactivation of ARC neurons, the GABA_A antagonist gabazine was co-injected with the affibody and insulin. As shown in Figure 1C, pretreatment with the anti-insulin affibody eliminated the significant sympathetic response to ARC injection of gabazine. This result provides further evidence that the sympathoexcitatory response to ARC insulin injection is GABA_A mediated.

![Fig. 1. Anti-insulin affibody prevents the sympathoexcitatory response to insulin in the arcuate nucleus (ARC). A and B: arterial blood pressure (ABP) and mean ABP (gray line) (top), integrated (∫) lumbar sympathetic nerve activity (SNA; middle), and raw lumbar SNA (bottom; 1-s segments) of rats that received an injection of the control antibody (A; n = 6) or anti-insulin affibody (B; n = 4) into the ARC 10 min before ARC injection of insulin. C: mean ± SE values of mean ABP and lumbar SNA. ∗P < 0.05, control vs. anti-insulin affibody. Arrows denote ARC injection. D: schematic illustration and photomicrographs of ARC injection sites. Coordinates are rostrocaudal levels in reference to the bregma using the atlas of Paxinos and Watson (36). The photomicrograph shows the injection site for red (i) and green (ii) beads for the same animal, although sections are 80 µm apart. Scale bars = 200 µm. DMH, dorsomedial hypothalamus; VMH, ventromedial hypothalamus; 3V, third ventricle.](http://ajpheart.physiology.org/Downloadedfromhttp://ajpheart.physiology.org/)
Table 1. Changes in mean ABP and SNA after injection of control or anti-insulin affibody into the arcuate nucleus in Inactin-anesthetized rats that received an infusion of 0.45% NaCl (0.2–2.0 ml/h iv)

<table>
<thead>
<tr>
<th></th>
<th>Time</th>
<th>Mean ABP, mmHg</th>
<th>Lumbar SNA, %</th>
<th>Renal SNA, %</th>
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<tr>
<td>Control affibody</td>
<td>–20 min</td>
<td>94 ± 3</td>
<td>100 ± 1</td>
<td>100 ± 1</td>
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<td></td>
<td>0 min</td>
<td>93 ± 2</td>
<td>101 ± 3</td>
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<td>30 min</td>
<td>95 ± 4</td>
<td>102 ± 3</td>
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<td>60 min</td>
<td>94 ± 3</td>
<td>104 ± 4</td>
<td>98 ± 5</td>
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<td></td>
<td>120 min</td>
<td>93 ± 2</td>
<td>104 ± 6</td>
<td>97 ± 5</td>
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<tr>
<td>Anti-insulin affibody</td>
<td>–20 min</td>
<td>95 ± 2</td>
<td>100 ± 1</td>
<td>100 ± 1</td>
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<td></td>
<td>0 min</td>
<td>94 ± 4</td>
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<td>60 min</td>
<td>92 ± 6</td>
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<td></td>
<td>120 min</td>
<td>92 ± 5</td>
<td>103 ± 6</td>
<td>103 ± 7</td>
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Values are means ± SE; n = 3 animals injected with control affibody and 4 animals injected with anti-insulin affibody. ABP, arterial blood pressure; SNA, sympathetic nerve activity.

Gabazine was injected into the ARC 10 min after the injection of the control or anti-insulin affibody. Unilateral injection of gabazine significantly increased mean ABP, lumbar SNA, and renal SNA (Fig. 2). Injection of the anti-insulin affibody did not significantly alter these responses. In both groups, gabazine significantly increased heart rate (0 min: 425 ± 12 beats/min vs. 20 min: 535 ± 19 beats/min for all groups, P < 0.01), end-tidal CO₂ (0 min: 2.6 ± 0.1% vs. 20 min: 3.4 ± 0.1% for all groups, P < 0.01), and core body temperature (0 min: 37.2 ± 0.1°C vs. 30 min: 37.8 ± 0.1°C for all groups, P < 0.01). In these experiments, expired CO₂ and core body temperature were permitted to change based on preliminary experiments showing that ARC disinhibition altered these variables. Figure 2B shows the injection sites for each experiment. In every case, injection of the affibodies overlapped with injection of gabazine. Therefore, the injection site for each animal is shown as one injection site.

**ARC Pretreatment With Anti-Insulin Affibody Attenuates the Sympathoexcitatory Response to Intracerebroventricular Insulin**

To test whether intracerebroventricular insulin increased SNA through insulin-dependent actions on ARC neurons, the control or anti-insulin affibody was injected 10 min before an injection of insulin into the lateral ventricle. As shown in Fig. 3, intracerebroventricular injection of insulin significantly increased lumbar, but not renal, SNA over 120 min. Although intracerebroventricular insulin also increased lumbar SNA in animals pretreated with the anti-insulin affibody in the ARC, the magnitude of the effect was significantly attenuated at every time point (Fig. 3). Renal SNA, mean ABP, and heart rate did not change significantly from baseline values in any group.

**ARC Pretreatment with Anti-Insulin Affibody Prevents the Sympathoexcitatory Response to Hyperinsulinemic-Euglycemic Clamp**

The major goal of this study was to determine whether circulating insulin acts within the ARC to increase SNA. Figure 4 shows examples of raw traces. Figure 5 shows summary data. As expected (4, 47), the hyperinsulinemic-euglycemic clamp significantly increased lumbar, but not renal, SNA over 120 min in animals pretreated with the control affibody in the ARC. In marked contrast, ARC pretreatment with the anti-insulin affibody eliminated the sympathoexcitatory response to insulin. In fact, values of lumbar SNA were not different from those of animals that received an ARC injection but were infused with saline intravenously (Fig. 5 and Table 1). As noted above, a number of hypothalamic nuclei contain insulin receptors, including those located dorsal to the ARC, including the VMH and DMH. To control for diffusion of the anti-insulin affibody dorsally through the pipette track to the VMH or DMH, a hyperinsulinemic-euglycemic clamp was performed in a final set of animals pretreated with the anti-insulin affibody in the VMH. As shown in Figs. 4 and 5, insulin still produced a lumbar sympathoexcitatory response in rats pretreated with the anti-insulin affibody in the VMH. Figure 6 shows the injection sites for all three groups.

An interesting observation was the significant difference in mean ABP at 120 min between rats pretreated with the ARC anti-insulin affibody versus the ARC control affibody or VMH anti-insulin affibody. The latter two groups had a significantly higher mean ABP than the former. Heart rate did not differ across groups.

Plasma insulin values are shown in Fig. 7. The differences in the sympathoexcitatory response to insulin between animals pretreated with the anti-insulin antibody in the ARC versus those pretreated with the anti-insulin antibody in the VMH or control affibody in the ARC cannot be explained by differences in plasma insulin values. Injection of the control or anti-insulin affibody into the ARC alone did not alter plasma insulin levels (data not shown). Furthermore, the resultant plasma insulin values during the hyperinsulinemic-euglycemic clamps were not statistically different from those of OP animals fed a moderately high-fat diet for 13 wk. OP animals had significantly higher plasma insulin values than LF or OR animals. As expected (43), OP animals versus LF or OR animals had higher body weight (OP: 775 ± 16

Fig. 2. A: mean ABP, integrated lumbar SNA, and integrated renal SNA of rats injected with gabazine 10 min after injection of control or anti-insulin affibodies in the ARC. Gabazine significantly increased all variables, but the magnitudes were not different between groups. Values are means ± SE. Arrows denote ARC injection. B: schematic illustration of ARC injection sites.
Insulin ICV
2s
2s
2s
prevented the elevated lumbar SNA during a hyperinsulinemic-hyperinsulinemic-euglycemic clamp after pre-
and raw renal SNA of rats that received a
grated and raw lumbar SNA, and integrated and raw renal
Fig. 4.
broventricular insulin,
insulin affibody attenuated the sympathetic response to intracere-
tination of insulin in the ARC,
anti-insulin affibody prevented the sympathetic response to injec-
sympathetic outflow was previously unknown. The present find-
detecting circulating insulin and initiating downstream changes in
alter cardiovascular function, the primary site responsible for
DISCUSSION

Despite the ability of insulin to act within the hypothalamus to
act upon the hypothalamus to
lumbar sympathoexcitatory effect
animals injected with the control affibody,
SNA of rats injected with control or anti-
SNA in both groups. Arrows denote ARC
insulin affibodies at 10 min before an intra-
Affibody injection. *P < 0.05, control vs. anti-insulin
fibody did not alter the sympathoexcitatory response to insulin.
Taken together, these findings suggest that circulating insulin acts
metabolism.

Fig. 3. A: mean ABP, integrated and raw lumbar SNA, and integrated and raw renal SNA of rats injected with control or anti-
insulin affibodies at 10 min before an intracerebroventricular injection of insulin. In animals injected with the control affibody, intracerebroventricular injection of insulin significantly increased lumbar, but not renal, SNA. This lumbar sympathoexcitatory effect was attenuated in animals pretreated with ARC anti-insulin affibody. B: mean ± SE values of mean ABP, lumbar SNA, and renal SNA in both groups. Arrows denote ARC injection. *P < 0.05, control vs. anti-insulin affibody.

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Interestingly, direct injection of insulin into the ARC increases lumbar SNA (8), and the sympathoexcitatory effects of insulin are prevented in melanocortin 4 receptor-deficient mice (38) or by PVH injection of the melanocortin 3/4 antagonist SHU-9119 (47). Finally, inhibition of ARC neurons with injection of the GABA\textsubscript{A} agonist muscimol reversed the sympathoexcitatory response to systemic insulin (8). Taken together, these observations suggest that insulin raises SNA through a pathway originating from the ARC to the PVH and rostral ventrolateral medulla; however, these studies were limited by the absence of any direct experimental manipulation of insulin actions within the ARC.

The present study used an anti-insulin affibody to locally neutralize the actions of insulin within a specific brain region. The efficacy of this affibody has been previously reported to block the phosphorylation of Akt in differentiated adipocytes by insulin or within VMH neurons in vivo during insulin-induced hypoglycemia (35). While we did not perform similar measures within the ARC, our initial experiments validated the use of the affibody as ARC injection of the anti-insulin affibody prevented the sympathoexcitatory response to local ARC application of insulin and partially attenuated the response to intracerebroventricular insulin. These effects cannot be attributed to a nonspecific action of the anti-insulin affibody as local ARC injection did not affect the sympathoexcitatory response to ARC-injected gabazine. Although there were differences in the baseline mean ABP of animals injected with ARC insulin, this difference is not likely to explain the absence of a sympathetic response to the anti-insulin affibody-treated group. The discrepancy between a complete prevention of the sympathetic response to local ARC insulin versus a partial attenuation to intracerebroventricular insulin can be attributed to one of two explanations: 1) intracerebroventricular insulin can also act outside of the ARC to increase SNA or 2) the dose of intracerebroventricular insulin exceeded the neutralizing ability of the anti-insulin affibody. Indeed, the amount of insulin administered intracerebroventricularly was high. On the other hand, it remains plausible that insulin administered intracerebroventricularly may act on other structures to increase SNA as insulin receptors are expressed widely throughout the hypothalamus (26, 48). To the extent that it has been tested, insulin injection into these other hypothalamic areas, such as the PVH, does not increase SNA (4, 8, 47).

The main goal of the present study was to use the anti-insulin affibody to directly test whether circulating insulin acts within the ARC to increase SNA. As previously reported by a number of laboratories (4, 28, 47), a hyperinsulinemic-euglycemic clamp after pretreatment with the control affibody in the ARC, anti-insulin affibody in the ARC, or anti-insulin affibody in the VMH. Injection sites are shown in Fig. 6. Insuffusion of insulin increased lumbar SNA in rats that received a control antibody injection in the ARC or anti-insulin injection in the VMH. However, the lumbar sympathoexcitatory response was absent in rats pretreated with the anti-insulin affibody in the ARC. Arrows denote ARC injection. *P < 0.05, ARC control affibody vs. ARC anti-insulin affibody; †P < 0.05, VMH anti-insulin vs. ARC anti-insulin.

Fig. 6. A: photomicrograph of a bilateral ARC injection site with left (i) and right (ii) sides. In this animal, the injection sites were 120 μm apart in the rostral-caudal plane. Scale bars = 100 μm. B and C: schematic illustration of ARC or VMH injection sites for animals injected with intracerebroventricular insulin (B) or that received a hyperinsulinemic-euglycemic clamp (C).
lumbar SNA in response to ARC insulin or intracerebroventricular insulin.

The absence of any change in renal SNA during insulin administration is in marked contrast to the findings of Rahmouni and colleagues (29, 38, 39). These studies reported that large doses of insulin administered intracerebroventricularly increased renal SNA of rats or mice at 3–6 h after insulin injection. In these studies, renal and lumbar SNA increased 100–200% without any change in mean ABP (29, 38, 39). Second, the time course of the renal sympathoexcitatory response to intracerebroventricular insulin appears variable with a statistical increase at 120 or 240 min.

To address whether intravenous insulin influences renal SNA over longer time periods, we did extend the hyperinsulinemic-euglycemic clamps to 4 h in a subset of animals that received an ARC injection of the control or anti-insulin affibody. In control affibody-injected animals, lumbar SNA continued to increase (2 h: 145 ± 10% vs. 4 h: 161 ± 14%, n = 4) but renal SNA did not change (2 h: 96 ± 8% vs. 4 h: 102 ± 11, n = 4). However, the ARC anti-insulin affibody injection still prevented the increase in lumbar SNA (2 h: 107 ± 3% vs. 4 h: 110 ± 5, n = 3) with no change in renal SNA (2 h: 103 ± 4% vs. 4 h: 109 ± 6%, n = 3).

To our knowledge, there are limited data on the impact of intravenous hyperinsulinemic-euglycemic clamps on renal SNA (4, 28, 47), and all of these studies examined the renal SNA response over 2 h. In humans, a hyperinsulinemic-euglycemic clamp has been reported to increase muscle SNA but failed to significantly alter renal norepinephrine spillover (15). Collectively, the above observations indicate that intravenous insulin administered acutely at physiological concentrations has limited impact on renal SNA.

An interesting observation in the present study was that the injection of gabazine increased lumbar SNA, renal SNA, and ABP, but also increased end-tidal CO₂ and body temperature. Although the SNA and ABP responses to ARC gabazine have been previously reported (19), the latter effect may be attributed to increased metabolism via elevated brown adipose tissue SNA. Indeed, unpublished data in our laboratory indicate that gabazine microinjection into the ARC increases brown adipose tissue SNA. Since a source of GABA in the ARC originates from local neuropeptide Y neurons projecting onto proopiomelanocortin neurons (19, 20), gabazine may block this inhibitory input to produce the sympathetic and cardiovascular responses. In addition, recent reports (23, 44) have suggested that GABergic ARC neurons are important to mediate the thermogenic actions of leptin. Given the differences in the hemodynamic and thermogenic responses between insulin and gabazine in the ARC, it is likely that blockade of GABAergic receptors targets a much larger population of neurons than those affected by insulin.

Within the ARC nucleus, in vitro patch-clamp studies (16, 49) have reported that insulin hyperpolarized and leptin depolarized distinct populations of proopiomelanocortin neurons. In both instances, the electrophysiological responses were prevented by inhibition of PI3K (16, 49). The role of PI3K as a key mediator of insulin’s actions is supported by the results of an in vivo study (39) in which intracerebroventricular administration of the PI3K inhibitors LY-294002 or wortmannin attenuated the lumbar sympathoexcitatory response to intracerebroventricular insulin in rats. While these studies underscore the potential importance of PI3K in leptin and insulin’s actions, it also questions how PI3K can have two diametric actions
within ARC proopiomelanocortin neurons. Furthermore, there is no evidence regarding the electrophysiological responses of ARC neurons to insulin or leptin during synaptic blockade or to leptin and insulin under much longer timeframes (minutes to hours) that may relate to its effects on feeding and sympathetic regulation.

Numerous laboratories have reported that insulin acutely increases SNA and enhances baroreflex gain (3, 4, 8, 28, 32, 37, 40, 47, 50). Yet, the role of insulin in cardiovascular dysfunction in diabetes or obesity remains controversial. Obesity is characterized by elevated SNA to the kidney and muscle (12, 45), with the latter consistent with a sympathoexcitatory role for insulin. However, baroreflexes are attenuated (14, 42, 51). Whereas some studies (24, 27, 41, 46) have reported a direct correlation between obesity, plasma insulin levels, and blood pressure or muscle SNA, others (2, 13, 45) have failed to do so. One complicating factor is that insulin works centrally to exert its effects on SNA and baroreflex function. Therefore, the interpretation of these studies is potentially confounded by changes in insulin transport across the blood-brain barrier in obese animals/subjects (5, 18, 22, 33). These latter studies have clearly indicated that insulin is transported into the brain via a saturable transporter, but pathological disease states (such as obesity) decrease insulin delivery into the central nervous system. Furthermore, there is little information regarding the insulin responsiveness or sensitivity of ARC neurons regulating sympathetic outflow in obesity. Interestingly, a recent study (25) in high-fat diet-fed rabbits reported that acute intracerebroventricular administration of an insulin receptor antagonist lowered ABP but not renal SNA. Baroreflex function was not assessed. Clearly, future studies are needed to address the contribution of brain insulin and ARC neurons to altered SNA and baroreflex function in diabetes and/or obesity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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REFERENCES


