The Pennsylvania State University
The Graduate School
College of Medicine

ARCUATE NUCLEUS INJECTION OF AN ANTI-INSULIN AFFIBODY PREVENTS THE SYMPATHETIC RESPONSE TO CIRCULATING INSULIN

A Thesis in
Anatomy

by
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ABSTRACT

Insulin contributes to cardiovascular dysfunction by central actions to increase sympathetic nerve activity (SNA) as presented in several diseases, notably obesity and hypertension. The arcuate nucleus (ARC) in the mediobasal hypothalamus highly expresses insulin receptors, and direct ARC injection of insulin elevates lumbar SNA. However, the area of the brain which senses circulating insulin to mediate these sympathoexcitatory effects is unknown. Therefore, the purpose of the present study was to demonstrate that peripheral insulin acts directly on ARC neurons to increase lumbar SNA and this was tested utilizing an anti-insulin affibody to neutralize insulin. First, to verify the efficacy of the anti-insulin affibody, anesthetized Sprague-Dawley rats were pretreated with bilateral ARC injection (40nL) of either an anti-insulin (1µg) or control (1µg) affibody 10 minutes before ARC injection of insulin (4µU). The anti-insulin vs. control affibody abolished the increased lumbar SNA. Second, to determine the ability of ARC neurons to detect peripheral insulin, animals received identical pretreatments with either affibody 10 minutes prior to the start of an intravenous (IV) infusion of insulin (7.5mU/kg/min) while maintaining euglycemia with 50% dextrose. ARC injection of the anti-insulin vs. control affibody prevented the increased lumbar SNA and attenuated splanchnic SNA. In marked contrast, renal SNA did not show significant changes between groups. In a third set of animals, ARC injection of the GABA<sub>A</sub> receptor antagonist gabazine (1mM, 20nL) elicited increases in lumbar, renal, and splanchnic SNA in both anti-insulin vs. control affibody pretreated animals suggesting the anti-insulin affibody specifically neutralizes insulin within ARC, rather than creating a general inhibition of neuronal activity. Anti-insulin affibody injection into the ventromedial hypothalamus (VMH) did not eliminate the lumbar sympathoexcitatory response to hyperinsulinemia-euglycemia. Collectively, these results indicate circulating insulin acts directly on ARC neurons to increase SNA regionally suggesting a possible mechanism during cardiovascular disease states.
# TABLE OF CONTENTS

List of Figures ............................................................................................................. vi

Abbreviations ............................................................................................................. vii

Acknowledgements ..................................................................................................... ix

Chapter 1: Introduction ................................................................................................ 1

1.1: Obesity and Hypertension .................................................................................. 1

1.2: Sympathetic Nervous System .......................................................................... 2

1.3: CNS Circuitry of Insulin .................................................................................. 5

1.4: Arcuate Nucleus .............................................................................................. 8

1.5: Anti-Insulin Affibody ..................................................................................... 9

1.6: Summary ......................................................................................................... 9

Chapter 2: Objectives ................................................................................................ 10

Chapter 3: Methodology ............................................................................................ 11

3.1: Animals ......................................................................................................... 11

3.2: General Surgical Procedures ......................................................................... 11

3.3: Arcuate Nucleus Microinjections .................................................................... 12

3.4: Experimental Protocols .................................................................................. 13

3.4.1: Pretreatment of Anti-Insulin Affibody in ARC followed by Direct ARC Injection of Insulin ................................................................. 13
3.4.2: Pretreatment of Anti-Insulin Affibody in ARC followed by Peripheral Hyperinsulinemia-Euglycemia………………………………………………13

3.4.3: Gabazine in ARC following Injection of Anti-Insulin Affibody……..14

3.5: Histology........................................................................................................14

3.6: Data Analysis....................................................................................................15

Chapter 4: Results....................................................................................................16

4.1: Anti-Insulin Affibody Prevents Sympathetic Response to Insulin in ARC…….16

4.2: Anti-Insulin Affibody in ARC Prevents Sympathetic Response to Peripheral Hyperinsulinemia-Euglycemia.........................................................20

4.3: Anti-Insulin Affibody Specifically Blocks the Action of Insulin……………….26

Chapter 5: Discussion.............................................................................................29

References..............................................................................................................37
LIST OF FIGURES

Figure 1: Flow diagram illustrating the ability of elevated sympathetic nervous system activity to chronically increase blood pressure.............................................4

Figure 2: Raw Recordings from Animals Receiving Insulin in ARC.................................17

Figure 3: Group Data from Animals Receiving Insulin in ARC........................................18

Figure 4: Schematic Illustration of Injection Sites of Animals Receiving Insulin in ARC…19

Figure 5: Raw Recordings from Animals Receiving Peripheral Hyperinsulinemia-Euglycemia.............................................................................................................21

Figure 6: Group Data from Animals Receiving Peripheral Hyperinsulinemia-Euglycemia.............................................................................................................22

Figure 7: Schematic Illustration of Injection Sites of Animals Receiving Peripheral Hyperinsulinemia-Euglycemia.............................................................................................................23

Figure 8: Raw Recording and Group Data from Animals Receiving Anti-Insulin Affibody in VMH.............................................................................................................24

Figure 9: Schematic Illustration of Injection Sites of Animals Receiving Anti-Insulin Affibody in VMH.............................................................................................................25

Figure 10: Group Data from Animals Receiving Gabazine in ARC.....................................27

Figure 11: Schematic Illustration of Injection Sites of Animals Receiving Gabazine in ARC.............................................................................................................28
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARC</td>
<td>Arcuate nucleus</td>
</tr>
<tr>
<td>ABP</td>
<td>Arterial blood pressure</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DMH</td>
<td>Dorsomedial hypothalamus</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
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<tr>
<td>GABAₐ</td>
<td>γ-aminobutyric acid-ₐ</td>
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<tr>
<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
</tr>
<tr>
<td>hr</td>
<td>Hour</td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
</tr>
<tr>
<td>↑</td>
<td>Increase</td>
</tr>
<tr>
<td>ICV</td>
<td>Intracerebroventricular</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
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<tr>
<td>kg</td>
<td>Kilogram</td>
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<tr>
<td>Mg/dl</td>
<td>Milligram/deciliter</td>
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<td>mg/kg</td>
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</table>
mL  Milliliter
mm  Millimeter
mmHg  Millimeters of mercury
mM  Millimolar
mU  milliunit
min  Minute(s)
mRNA  Messenger ribonucleic acid
n  Number of animals used in the study
Na+  Sodium
nL  Nanoliter
NMDA  N-Methyl-D-aspartic acid
%  Percent
PI3K  Phosphatidyl inositol 3-kinase
POMC  Proopiomelanocortin
PVN  Paraventricular nucleus
RVLM  Rostral ventrolateral medulla
SNA  Sympathetic nerve activity
SNS  Sympathetic nervous system
VMH  Ventromedial hypothalamus
3V  Third ventricle
µg  Microgram
µm  Micrometer (micron)
µU  Microunit
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Chapter 1: INTRODUCTION

1.1: Obesity and Hypertension

Obesity is rapidly increasing worldwide as a disproportionate relationship continues to develop between energy expenditure and energy gain. More frequently, the amount of daily calories are far exceeding physical activity. Many social and economic factors can contribute to the imbalance in the energy equation, including but not limited to, an increase in sedentary lifestyles with the advent of computers, motor transportation, and less physical recreational activities (Esler, Straznicky et al. 2006; Meister 2007). Adults of both sexes examined over an eight year period showed an increased risk of developing obesity-related hypertension despite normal blood pressure values at the onset of the study. Strong correlations existed between individuals with higher subscapular skin folds and increased incidences of high blood pressure, as well as a greater prevalence of hypertension for men and women between the ages of 40-49 after the eight year period when compared to those in the younger age groups (Garrison, Kannel et al. 1987).

The relationship between obesity and the occurrence of hypertension has been well established. However, the mechanisms behind this correlation remain to be determined. Obesity, hypertension, insulin resistance, hyperinsulinemia, and hyperlipidemia encompass the metabolic syndrome. These adverse health issues are the basis for much of the research regarding obesity and cardiovascular dysfunction (Esler, Straznicky et al. 2006; Kirk and Klein 2009). The presence of these multiple comorbidities significantly increases the risk for several cardiovascular diseases, such as coronary heart disease, myocardial infarction, and stroke (Meister 2007; Kirk and Klein 2009). A better understanding of these processes will
lead to more effective therapies as well as possible methods of prevention (Esler, Straznicky et al. 2006; Meister 2007).

1.2: Sympathetic Nervous System

It has become increasingly evident that obesity-related hypertension is mediated by an elevation of sympathetic nerve activity (SNA) (Esler, Straznicky et al. 2006; Guyenet 2006). The sympathetic nervous system is a division of the autonomic nervous system connecting the central nervous system (CNS) with visceral targets. Preganglionic cholinergic neurons in the CNS innervate peripheral ganglia and glands, which extend postganglionic neurons to smooth muscle and other targets of the viscera. The cardiovascular system consists of postganglionic fibers that are largely noradrenergic and primarily innervate blood vessels, the heart, the kidney, and the adrenal medulla (Guyenet 2006).

Acute changes in blood pressure are based on behavior, such as exercising, digestion, and emotional responses and do not necessarily contribute to long-term regulation of blood pressure. The clinical definition of hypertension is a chronic increase in blood pressure. Long-term regulation of blood pressure involves several regions in the CNS, including the rostral ventrolateral medulla (RVLM), the nucleus of the solitary tract, the hypothalamus, and the spinal cord (Guyenet 2006). The exact mechanism underlying how increased SNA leads to chronic hypertension is not well defined, but several factors have been implicated as shown in Figure 1. Blood pressure depends on two variables, cardiac output and total peripheral vascular resistance. Based on the blood pressure-natriuresis relationship, an elevation of SNA causes sodium reabsorption in the kidneys resulting in an increase in blood volume leading to a rise in cardiac output, causing an increase in blood pressure (Brands, Hildebrandt et al. 1991; Esler, Straznicky et al. 2006; Guyenet 2006). Another mechanism
involves increasing peripheral vascular resistance through vasoconstriction, which are both mediated by the sympathetic nervous system (Guyenet 2006).

The processes causing the activation of the sympathetic nervous system in human obesity have yet to be determined but possibilities include hyperinsulinemia, hyperleptinemia, and obstructive sleep apnea (Esler, Straznicky et al. 2006). Several studies have shown that peripheral insulin infusion while maintaining euglycemia increases SNA in both rats and humans (Anderson, Hoffman et al. 1991; Anderson, Balon et al. 1992; Morgan, Balon et al. 1993). A hyperinsulinemic-euglycemic clamp performed on spontaneously hypertensive and normotensive rats produced increases in SNA but varied across different regions with lumbar SNA significantly increasing in both groups (Morgan, Balon et al. 1993). Hyperinsulinemia has been reported to significantly increase muscle SNA in normotensive humans (Anderson, Hoffman et al. 1991) as well as borderline hypertensive subjects (Anderson, Balon et al. 1992).

Previous studies in rodents have not reported changes in mean arterial blood pressure (mean ABP) during acute peripheral or central hyperinsulinemia, which most likely is attributable to anesthetics (Morgan, Balon et al. 1993; Muntzel, Morgan et al. 1994). However, small but statistically significant increases in mean ABP have been produced during 5-7 days of hyperinsulinemic-euglycemic clamps performed in conscious rats. These findings indicate that elevated plasma insulin levels similar to those of obese individuals are capable of inducing a rise in blood pressure (Brands, Hildebrandt et al. 1991; Brands, Lee et al. 1996).
Figure 1: Flow diagram illustrating the ability of elevated sympathetic nervous system activity to chronically increase blood pressure. A strong correlation exists between over-activation of the sympathetic nervous system and elevated circulating insulin levels. This hyperinsulinemia will augment activity to the end-organs of the SNS leading to vasoconstriction of blood vessels resulting in a rise in total peripheral resistance, sodium retention in the kidneys increasing blood volume, and increased cardiac output. These factors together will ultimately contribute to vast cardiovascular dysfunction and increased risk for the metabolic syndrome. Accordingly, a high prevalence of obesity will culminate in a greater threat for developing cardiovascular disturbances as a result of these mechanisms.
1.3: CNS Circuitry of Insulin

There has been significant evidence to show that peripheral insulin increases SNA and that this is mediated centrally, but the direct CNS circuitry of insulin’s action remains unclear (Esler, Straznicky et al. 2006). Acute infusion of insulin into the third ventricle produced an increase in lumbar SNA in chloralose-anesthetized male Wistar rats, while plasma insulin levels and blood glucose levels did not change suggesting insulin acted centrally (Muntzel, Morgan et al. 1994). Insulin failed to increase adrenal or renal SNA suggesting central insulin acts regionally (Muntzel, Morgan et al. 1994) similarly to the action of peripheral insulin (Morgan, Balon et al. 1993).

ICV insulin studies have led to questions regarding what exact areas in the CNS are mediating the increase in SNA. Insulin receptors have been localized by in vitro autoradiography and computerized densitometry in the rat brain (Werther, Hogg et al. 1987). The highest density of insulin receptors was found in areas related to olfaction, taste, feeding and visceral responses: the olfactory bulb, limbic system and hypothalamus. The highest density of insulin receptors was noted in the choroid plexus suggesting a possible location for peripheral insulin to enter the brain. The hypothalamus showed moderate labeling in the ventromedial hypothalamus (VMH) and paraventricular nucleus (PVN), while the dorsomedial hypothalamus and the arcuate nucleus (ARC) expressed high labeling (Werther, Hogg et al. 1987). Insulin receptor mRNA measured by in situ hybridization showed similar distributions (Schulingkamp, Pagano et al. 2000). Functionality of these receptors should not be assumed, but their presence implies the possibility of insulin action at these locations, as well as a potential role in regulating metabolic function in these regions (Werther, Hogg et al. 1987).
Several areas have been implicated as having involvement in the sympathoexcitatory response to insulin, including the RVLM, the hypothalamic PVN, and the hypothalamic ARC (Bardgett, McCarthy et al. 2010; Cassaglia, Hermes et al. 2011; Ward, Bardgett et al. 2011). A potential pathway of insulin action may exist between these areas to mediate the elevation in SNA (Bardgett, McCarthy et al. 2010; Ward, Bardgett et al. 2011). Lesion of the anteroventral third ventricle attenuated the increase in lumbar SNA in response to hyperinsulinemic-euglycemic clamp, emphasizing the importance of this area, which included circumventricular organs, the preoptic-anterior hypothalamus, and median preoptic nucleus (Muntzel, Beltz et al. 1994).

RVLM is a critical area for regulation of SNA and ABP (Guyenet 2006; Bardgett, McCarthy et al. 2010). Glutamate injected into RVLM has been shown to increase SNA and mean ABP (Guyenet 2006). Blockade of glutamate receptors in RVLM by microinjection of the ionotropic glutamate receptor antagonist kynurenic acid significantly reduced the sympathoexcitation response in lumbar SNA to hyperinsulinemia. However, microinjection of insulin directly into RVLM did not result in SNA stimulation, and insulin receptor expression in RVLM was found to be significantly lower than in the hypothalamus. These results suggest RVLM plays a role in the sympathoexcitation response to insulin through glutamate receptor activation, but it is not the site of direct insulin action (Bardgett, McCarthy et al. 2010). In agreement with these results, insulin infusion into the fourth ventricle did not increase lumbar SNA when compared to an elevation seen during infusion of insulin into the lateral ventricle suggesting insulin’s action may be in the hypothalamus (Pricher, Freeman et al. 2008).

Despite the source of RVLM glutamate being relatively unknown, glutamatergic neurons in the hypothalamic PVN extensively innervate the RVLM suggesting insulin acts
Experiments performed in PVN produced similar results. Hyperinsulinemic-euglycemic clamps significantly elevated lumbar SNA without changes in mean ABP, heart rate (HR) or renal SNA, and direct inhibition of PVN by microinjection of the GABA<sub>A</sub> receptor agonist muscimol decreased lumbar SNA to levels seen in control rats (Ward, Bardgett et al. 2011). However, direct injection of insulin into PVN did not elicit a sympathetic response (Ward, Bardgett et al. 2011) despite the large amount of insulin receptor expression previously reported (Werther, Hogg et al. 1987; Unger, McNeill et al. 1989; Schulingkamp, Pagano et al. 2000; Plum, Schubert et al. 2005; Ward, Bardgett et al. 2011).

Further experimentation showed that in hyperinsulinemic-euglycemic rats, microinjection of the melanocortin 3/4 receptor antagonist SHU9119 into PVN dramatically reduced lumbar SNA down to baseline levels and decreased mean ABP. Microinjection of insulin into the third ventricle produced significant increases in lumbar SNA, and this response was also attenuated by PVN injection of SHU9119. This blockade was shown to be specific to melanocortin 3/4 receptors in PVN because SHU9119 did not reduce the increase in lumbar SNA, renal SNA, mean ABP, or HR in response to injection of NMDA or gabazine, a GABA<sub>A</sub> receptor antagonist. There are only two locations in the CNS containing proopiomelanocortin (POMC) neurons: the nucleus of the solitary tract and the arcuate nucleus (Cone 2005; Ward, Bardgett et al. 2011). POMC neurons in the ARC secrete α-melanocyte-stimulating hormone, which subsequently binds to melanocortin-4 receptors in the PVN to activate the melanocortin system (Meister 2007; Belgardt, Okamura et al. 2009). These findings suggest insulin may act in the hypothalamus upstream of PVN in the ARC to mediate sympathoexcitation (Ward, Bardgett et al. 2011).
1.4: Arcuate Nucleus

The mediobasal hypothalamus, the ARC, is of particular interest in regards to peripheral signals associated with energy regulation and feeding behavior, such as insulin and leptin, because of the relatively weak blood brain barrier in the arcuate nucleus-median eminence area (Meister 2007; Belgardt, Okamura et al. 2009). The ARC receives and integrates signals from a variety of hormones with dense projections to PVN, the lateral hypothalamic area and the brainstem (Cone, Cowley et al. 2001; Nakamura, Bhatt et al. 2009). With an abundant concentration of insulin receptors determined through quantitative binding of $^{125}$I insulin (4.3±0.4fmol/mg) in the rat brain (Werther, Hogg et al. 1987), the ARC has been implicated as a major player in the sympathoexcitatory effects of insulin (Cassaglia, Hermes et al. 2011; Ward, Bardgett et al. 2011). Blunted insulin receptor expression through infusion of antisense oligodeoxynucleotide in the third ventricle led to rapid hyperphagia and increased fat mass. The ARC showed markedly reduced insulin receptor immunoreactivity following infusion of the antisense oligodeoxynucleotide, while other areas did not appear to change, emphasizing insulin’s role in the ARC in energy regulation (Obici, Feng et al. 2002). Stimulation of the ARC by microinjection of NMDA in urethane-anesthetized rats elevated mean ABP, HR, renal SNA, and splanchnic SNA (Nakamura, Bhatt et al. 2009).

Peripheral insulin has been shown to increase SNA, and inhibition of RVLM and PVN has reversed this response. However, direct injection of insulin into both RVLM and PVN did not stimulate SNA suggesting insulin acts upstream of these sites (Bardgett, McCarthy et al. 2010; Ward, Bardgett et al. 2011). Recently, the increase in lumbar SNA in response to peripheral hyperinsulinemia has been reversed by injection of muscimol into the ARC. Also, insulin at various concentrations injected directly into the ARC has been shown
to increase lumbar SNA suggesting a possible location for direct insulin action in the brain to elicit the sympathoexcitation response (Cassaglia, Hermes et al. 2011). However, further studies are needed to specifically inhibit insulin action or block insulin receptors in ARC and observe the effects of central and peripheral hyperinsulinemia on SNA.

1.5: Anti-Insulin Affibody

The anti-insulin affibody has previously been used to reduce local levels of insulin in the VMH before the start of a hyperinsulinemic-hypoglycemic clamp (Paranjape, Chan et al. 2010). This technique utilizes the affibody molecule, which mimics the action of an antibody without the limitations of large molecular sizes and instability. Affibody molecules are high affinity non-immunoglobulin-derived proteins consisting of 58 amino acids, three helices, and no internal cysteines. These 6-6.5 kDa proteins are created through engineering of the Z domain from the staphylococcal protein A. Through randomization of 13 surface residues on the first and second helices, different affibody molecules have been constructed to target a large number of proteins originating from several species, including the human insulin (Lendel, Dogan et al. 2006; Renberg, Nordin et al. 2007; Gronwall and Stahl 2009; Feldwisch, Tolmachev et al. 2010; Paranjape, Chan et al. 2010).

1.6: Summary

Several reports have contributed to explaining the mechanisms underlying the sympathoexcitation response of insulin and hypertension. However, an effective pharmacological blocker of insulin action has not been traditionally used to determine its effects on lowering SNA or blood pressure. This study is the first to neutralize insulin within ARC, thereby preventing the sympathoexcitation in response to elevated circulating insulin.
Chapter 2: OBJECTIVES

The overall hypothesis of this thesis was that an anti-insulin affibody will prevent or significantly attenuate the rise in sympathetic nerve activity following microinjection of insulin into the arcuate nucleus of the hypothalamus and beyond the proposed experiments, following IV infusion of insulin while maintaining euglycemia. It was also predicted that the anti-insulin affibody acts to specifically bind insulin preventing it from interacting with its receptor in the arcuate nucleus. This hypothesis was tested by the completion of the following specific aims.

Specific Aim 1: Determine whether an anti-insulin affibody effectively blocks the sympathoexcitatory actions of insulin in the hypothalamic arcuate nucleus of the rat.

Specific Aim 2: Establish that the anti-insulin affibody in the arcuate nucleus prevents the sympathoexcitatory actions of peripheral hyperinsulinemia during euglycemia.

Specific Aim 3: Demonstrate that the anti-insulin affibody is specific to insulin and not causing general inhibition of neuronal activity.
Chapter 3: METHODOLOGY

3.1: Animals

All experimental procedures conformed to the NIH Guide for the Health and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the Pennsylvania State University College of Medicine. Adult male Sprague-Dawley rats (250-400g, Charles River Laboratories) were housed in a controlled environment with temperature maintained at 22±1°C and a 12-hour light-dark cycle. Rats had full access to standard chow (Harlan Teklad Global Diet 2018) and deionized water prior to experimental use.

3.2: General Surgical Procedures

Rats were initially anesthetized with 4% isoflurane in 100% oxygen and maintained throughout surgical procedures with 2-2.5% isoflurane in 100% oxygen. Arterial catheters were instrumented in the femoral artery for measurement of mean ABP and HR and the brachial artery for sampling blood glucose levels. A double-lumen venous catheter was placed in the femoral vein for infusion of the post-surgical anesthetic, α-chloralose, and infusion of insulin and dextrose during the hyperinsulinemic-euglycemic clamps. Artificial ventilation with 100% oxygen was induced by placement of a tracheal tube, and end-tidal CO₂ was controlled within 4.0-4.5% using a MicroCapStar End-Tidal CO₂ Analyzer. Body temperature was maintained at 37±1°C with a rectal thermometer and a water heating pad.

Rats were prepared for lumbar SNA, renal SNA, and splanchnic SNA recordings as previously described by our laboratory (Adams, Madden et al. 2007; Bardgett, McCarthy et al. 2010; Ward, Bardgett et al. 2011).
Rats were placed in the prone position in a stereotaxic head frame (David Kopf Instruments) with the skull level between bregma and lambda. A midline incision on the dorsal surface of the head and removal of connective tissue gave access to the skull for the craniotomy. Bone was removed caudal to bregma and rostral to lambda to expose the cortical surface of the brain allowing access to the ARC (Cassaglia, Hermes et al. 2011; Ward, Bardgett et al. 2011).

After completion of all surgical procedures, anesthesia was replaced with α-chloralose. Initially, animals gradually received a bolus (50mg/kg) IV while slowly decreasing isoflurane. The rate at which both the bolus was introduced and isoflurane was withdrawn depended on the maintenance of blood pressure either above 80mmHg or at the level of baseline. For the remainder of the experiment, rats were anesthetized via continuous IV infusion of α-chloralose (25mg/kg/hr). Assessment of anesthesia was monitored by the absence of foot withdrawal following a toe pinch, and animals were allowed to stabilize for at least 60 minutes after completion of all surgical procedures before the start of experimental protocols (Ward, Bardgett et al. 2011).

3.3: Arcuate Nucleus Microinjections

For preparation of all microinjections, single-barrel glass micropipettes were positioned into the ARC bilaterally using the following coordinates in reference to bregma: 2.9 mm caudal (right ARC) and 2.4 mm caudal (left ARC), 9.8-9.9 mm ventral, and the lateral edges of the superior sagittal sinus. Stereotaxic arms were positioned at a two degree angle in the rostral/caudal direction. All coordinates were adjusted according to the size of the animal, as well as previous histological results. To minimize the variability between multiple injections, each stereotaxic manipulator held one pipette, and the same pipette for
each side was used throughout the entirety of the experiment. Injections were performed over 5-10 seconds using a picopump.

3.4: Experimental Protocols

3.4.1: Pretreatment of Anti-Insulin Affibody in ARC followed by Direct ARC Injection of Insulin.

Baseline measurements of all parameters were obtained for at least 15 minutes prior to beginning the experiment. Rats were pretreated with a microinjection of either the anti-insulin or the control (non-insulin-binding) affibody (1µg, 40nL) into the ARC following the baseline period. After 10 minutes, insulin (4µU, 40nL) was microinjected into the ARC. All parameters were recorded for at least 2 hours following the insulin injection, and blood glucose levels were measured by sampling arterial blood using a standard glucometer (One Touch Ultra) every 30 minutes.

3.4.2: Pretreatment of Anti-Insulin Affibody in ARC followed by Peripheral Hyperinsulinemia-Euglycemia.

Baseline measurements were obtained as described above. Blood samples (~200µL in 4µL EDTA) were retrieved at baseline (about -20 minutes), 30, 60, and 120 minutes. Samples were centrifuged, and plasma was stored at -20°C until analysis. Animals were pretreated with the control vs. anti-insulin affibody (1µg, 40nL) into the ARC. Ten minutes later, insulin (7.5mU/kg/min; Humulin R) and a 50% dextrose solution (0.25 to 1.0mL/hr) was continuously infused IV for the duration of two hours. Blood glucose was measured every 10 minutes and maintained at baseline by increasing the rate of dextrose infusion.
Anatomical control experiments were performed by injecting the anti-insulin affibody (1µg, 40nL) bilaterally into the ventromedial hypothalamus prior to hyperinsulinemia-euglycemia. Coordinates for the injections were similar to the ARC; however, pipettes were lowered about 8.8-8.9 mm. Blood samples and glucose measurements were performed as described above.

3.4.3: Gabazine in ARC following Injection of Anti-Insulin Affibody.

Baseline measurements were obtained as described above. The specificity of the anti-insulin affibody was determined by ARC injection of the GABA_A receptor antagonist gabazine (1mM, 20nL) about 10-15 minutes following pretreatment of the control vs. anti-insulin affibody (1µg, 40nL). In a majority of cases, animals received pretreatment of one affibody in either the left or right ARC, and then gabazine was injected in the corresponding side. All variables were recorded for about one hour or until the gabazine response decreased to baseline levels; all animals were analyzed after 50 minutes to maintain consistency between groups. In the same animal, once a new baseline was recorded, the opposite affibody and gabazine were injected into the contralateral ARC for measurement.

3.5: Histology

Following the conclusion of the experiment, rats were transcardially perfused with 50-70mL of 4% paraformaldehyde. The brain was removed, post-fixed in 4% paraformaldehyde, and stored at 4°C until sectioning. Coronal sections of 100 µm were prepared using a vibratome and placed on glass slides. All solutions contained 0.2% rhodamine or fluorescein isothiocyanate (FITC) fluorescent beads to mark the injection site. Locations of the beads were analyzed using a Nikon Eclipse 90i microscope with the appropriate filters (Bardgett, McCarthy et al. 2010; Ward, Bardgett et al. 2011). Beads
located in the ARC based on a rat brain atlas indicated a hit. Beads outside the ARC indicated a miss and were not included in the data set for evaluation.

3.6: Data Analysis

All data is expressed as mean±SEM. The level of background noise was determined at the end of each experiment by IV administration of the ganglionic blocker and neuronal nicotinic acetylcholine receptor antagonist, hexamethonium (30mg/kg). Changes in rectified and integrated SNA (one second time constant) were calculated by subtracting the background noise value from the raw nerve activity value obtained during the experiment. For all of the parameters measured, five minute segments at each time point were compared with the average of baseline measurements obtained prior to the first injection into ARC (-25 to -10 minutes). Data was analyzed by 1- or 2-way ANOVA, with repeated measures when necessary. Post-hoc tests were performed with independent or paired t-tests with a layered Bonferroni correction. A P<0.05 was considered statistically significant.
Chapter 4: RESULTS

4.1: Anti-Insulin Affibody Prevents Sympathetic Response to Insulin in ARC

The first goal of this study was to determine the extent by which the anti-insulin affibody neutralized insulin’s actions within the ARC. Figure 2 displays mean and pulsatile ABP, integrated lumbar SNA, and raw lumbar SNA at baseline and at 120 minutes from two animals receiving either the control or anti-insulin affibody pretreatment into ARC followed 10 minutes by insulin injected into ARC. Figure 3 illustrates group data. The control affibody resulted in significant sympathoexcitation from the baseline level, and in marked contrast, injection of the anti-insulin affibody prevented the increase in lumbar SNA. It is noteworthy that mean ABP and HR did not significantly change throughout the entirety of the experiments, as well as between groups, also shown in Figure 3.

Due to multiple injections into ARC throughout the protocol, brain histology was a major factor in determining the anti-insulin affibody’s effectiveness. It was imperative that both the affibody and insulin were injected into identical locations within the ARC for each animal. Upon histological examination, the presence of fluorescent beads injected with the solutions indicated that both the affibody (anti-insulin or control) was, in fact, in the same location within ARC as the following insulin injection. Figure 4 shows a diagrammatic illustration of the microinjections into ARC from all animals.
Figure 2: Raw Recordings from Animals Receiving Insulin in ARC. Mean and pulsatile ABP and integrated lumbar SNA of two animals receiving ARC pretreatment of the control (A) or anti-insulin (B) affibody 10 minutes before ARC injection of insulin. Raw lumbar SNA at (a) baseline and (b) 120 minutes.
Figure 3: Group Data from Animals Receiving Insulin in ARC. Mean±SEM ABP, lumbar SNA and HR in animals receiving ARC injection of anti-insulin vs. control affibody 10 minutes prior to ARC injection of insulin. Lumbar SNA: 99±1 vs. 123 ±5% of baseline, n=4-6. *P<0.05 anti-insulin vs. control affibody, #P<0.05 vs. baseline values.
Figure 4: Schematic Illustration of Injection Sites of Animals Receiving Insulin in ARC. Diagrammatic representation of histological brain sections from animals receiving direct ARC pretreatment of the control or anti-insulin affibody followed by direct ARC injection of insulin. Injection of the affibody did not differ in location to the insulin injection in all animals. Displayed sections are 2.56-3.14 mm caudal to bregma. (3V, third ventricle; VMH, ventromedial hypothalamus; DMH, dorsomedial hypothalamus; R, right; L, left)
4.2: Anti-Insulin Affibody in ARC Prevents Sympathetic Response to Peripheral Hyperinsulinemia-Euglycemia

Deriving from the results of specific aim 1, the main objective of this study was to determine whether increased circulating insulin acts on ARC neurons to mediate the observed sympathoexcitation. A hyperinsulinemic-euglycemic clamp significantly elevates lumbar SNA (Morgan, Balon et al. 1993; Bardgett, McCarthy et al. 2010; Ward, Bardgett et al. 2011). Figure 5 displays mean and pulsatile ABP, integrated lumbar, splanchnic and renal SNA, and raw lumbar and splanchnic SNA at baseline and 120 minutes from two animals receiving either the control or anti-insulin affibody pretreatment into ARC followed 10 minutes by a hyperinsulinemic-euglycemic clamp. Figure 6 illustrates group data. Hyperinsulinemia-euglycemia following the control affibody pretreatment into ARC produced significant elevations in lumbar SNA and splanchnic SNA. Interestingly, the anti-insulin affibody eliminated this response in both lumbar SNA and splanchnic SNA. In accordance with past studies, noticeable changes in mean ABP, HR, or renal SNA were not observed (Figure 6). To ensure that the anti-insulin affibody was blocking insulin within the ARC, histological examination was performed as described above. Figure 7 demonstrates the diagrammatic images of the control vs. anti-insulin affibody injections into ARC. All animals included in the data set were located within ARC.

Anatomical control experiments were performed by injecting the anti-insulin affibody into VMH 10 minutes prior to a hyperinsulinemic-euglycemic clamp. The anti-insulin affibody in VMH did not prevent an elevation in lumbar SNA in response to peripheral hyperinsulinemia (Figure 8). While splanchnic data increased above baseline levels, it does not appear to be significantly different from the ARC response, as shown in Figure 8 (B). Figure 9 illustrates histology in VMH pretreated animals.
Figure 5: Raw Recordings from Animals Receiving Peripheral Hyperinsulinemia-Euglycemia. Mean and pulsatile ABP and integrated lumbar SNA, splanchnic SNA, and renal SNA during ARC pretreatment of control (A) or anti-insulin (B) affibody 10 minutes before administration of a hyperinsulinemic-euglycemic clamp. Raw lumbar SNA and splanchnic SNA at (a) baseline and (b) 120 minutes.
Figure 6: Group Data from Animals Receiving Peripheral Hyperinsulinemia-Euglycemia. Mean±SEM of lumbar SNA, splanchnic SNA, renal SNA, ABP, HR, and blood glucose in animals receiving ARC injection of anti-insulin vs. control affibody 10 minutes prior to IV administration of insulin (7.5mU/kg/min) plus 50% dextrose. *P<0.05 anti-insulin vs. control affibody, #P<0.05 vs. baseline values.
Figure 7: Schematic Illustration of Injection Sites of Animals Receiving Peripheral Hyperinsulinemia-Euglycemia.
Diagrammatic representation of histological brain sections from animals receiving ARC pretreatment of anti-insulin or control affibody followed by hyperinsulinemic-euglycemic IV infusion. Displayed sections are 2.12-2.80 mm caudal to bregma. (3V, third ventricle; PVN, Paraventricular nucleus; VMH, ventromedial.
Figure 8: Raw Recording and Group Data from Animals Receiving Anti-Insulin Affibody in VMH. (A) Mean and pulsatile ABP and integrated lumbar SNA and splanchnic SNA during VMH pretreatment of anti-insulin affibody 10 minutes before a hyperinsulinemic-euglycemic clamp. Raw lumbar SNA and splanchnic SNA at (a) baseline and (b) 120 minutes. (B) Mean±SEM of ABP, lumbar SNA, and splanchnic SNA of animals receiving pretreatment of anti-insulin in VMH vs. ARC given 10 minutes before a hyperinsulinemic-euglycemic clamp. *P<0.05 VMH vs. ARC, #P<0.05 vs. baseline values.
Figure 9: Schematic Illustration of Injection Sites of Animals Receiving Anti-Insulin Affibody in VMH. Diagrammatic representation of histological brain sections from animals receiving anti-insulin affibody pretreatment in VMH followed by hyperinsulinemic-euglycemic clamp. Displayed sections are 2.12-2.56 mm caudal to bregma. (3V, third ventricle; PVN, Paraventricular nucleus; VMH, ventromedial hypothalamus; DMH, dorsomedial hypothalamus; R, right; L, left)
4.3: Anti-Insulin Affibody Specifically Blocks the Action of Insulin

The last aim of this study was to verify that the anti-insulin affibody was working to specifically block insulin within the ARC rather than cause a general degeneration of neuronal tissue. Group data is shown in Figure 10 from animals receiving pretreatment with the control vs. anti-insulin affibody followed by injection of the GABA<sub>A</sub> receptor antagonist, gabazine into ARC. Gabazine produced a significant sympathoexcitatory response when injected into ARC after 50 min with both pretreatments. Considerable increases resulted in all variables measured in response to gabazine in ARC: mean ABP, HR, Temperature, and end-tidal CO<sub>2</sub>, as seen in Figure 10.

Again, due to multiple injections into ARC, it was critical to note the positions of the affibodies and the gabazine as described above. Histological examination confirmed that the locations of the control and anti-insulin affibody were matching the injection of gabazine in ARC in all animals included in the data set (Figure 11).
Figure 10: Group Data from Animals Receiving Gabazine in ARC.
Mean±SEM of lumbar SNA, splanchnic SNA, renal SNA, ABP, HR, temperature, and end-tidal CO₂ of animals receiving pretreatment of anti-insulin vs. control affibody given 10 minutes prior to gabazine injection into ARC.
#P<0.05 vs. baseline values.
Figure 11: Schematic Illustration of Injection Sites of Animals Receiving Gabazine in ARC. Diagrammatic representation of histological brain sections from animals receiving control or anti-insulin affibody pretreatment followed by gabazine injection into ARC. In all animals, injection of the affibody did not differ in location when compared to the gabazine injection. Displayed sections are 2.12-2.80 mm caudal to bregma. (3V, third ventricle; PVN, Paraventricular nucleus; VMH, ventromedial hypothalamus; DMH, dorsomedial hypothalamus; R, right; L, left)
Chapter 5: DISCUSSION

From these results, new knowledge regarding insulin’s ability to elevate SNA and contribute to cardiovascular dysfunction can be concluded. The novel findings of this study are the following: (1) neutralization of insulin within ARC eliminated the sympathoexcitatory response to peripheral hyperinsulinemia, (2) direct insulin within ARC produced significant elevations in SNA, and this response was abolished with the anti-insulin affibody, (3) disinhibition of ARC neurons with gabazine resulted in amplification of SNA and mean ABP, despite blockade of insulin in ARC, (4) splanchnic SNA appears to be mediated through insulin’s actions in ARC, and (5) neutralizing insulin within the ventromedial hypothalamus did not eliminate the lumbar sympathoexcitatory response to hyperinsulinemia. Together, these findings strongly indicate that circulating insulin is sensed by ARC neurons to mediate the elevation in SNA.

Obesity and its associated risks have steadily become a leading cause of severe health defects. The energy equation normally maintained at equilibrium has been shifted with an increase in energy gain and a decrease in energy loss. Unfortunately, an imbalance between energy expenditure and intake has the potential to result in enormous adverse health effects, specifically, an increased body weight. Obesity has the profound ability to induce a wide range of metabolic and cardiac abnormalities: diabetes mellitus, hypertension, coronary heart disease, and left ventricular hypertrophy (Meister 2007; Bombelli, Facchetti et al. 2011). The underlying cause of these obesity-related health risks has gone well beyond increased high-fat diets, inactive daily routines, and genetics (Lambert, Straznicky et al. 2010).

Hypertension, although particularly frequent and dangerous to end-organ function, remains a mystery in its relationship to obesity (Lambert, Straznicky et al. 2010). Despite
strong evidence linking hyperinsulinemia and hypertension in obesity, a clear mechanism by which insulin mediates chronic increases in blood pressure has not been established. Numerous studies have proposed the activation of the sympathetic nervous system as a method of inducing cardiovascular dysfunction (Landsberg 1989; Guyenet 2006). In the present study, the dose of insulin administered in the hyperinsulinemic-euglycemic clamps is comparable to plasma insulin levels measured in obesity-prone animals fed a moderately high-fat diet over a 13 week period (Bardgett, McCarthy et al. 2010). In the normal state, insulin plasma levels in systemic circulation generally reflect that of brain circulation suggesting most insulin within the brain is transported from the periphery, rather than locally synthesized in the central nervous system. Thus, under conditions of peripheral hyperinsulinemia, the ratio between central and systemic plasma insulin levels will increase (Unger, Livingston et al. 1991). Subsequently, these results led to questions regarding transport of insulin into the central nervous system. Central neural pathways taken by insulin to shape this sympathoexcitation have been the focus of an abundance of research in the last few decades. Several areas have been indicated as having involvement in the sympathoexcitatory response stimulated by hyperinsulinemia, such as RVLM and PVN (Bardgett, McCarthy et al. 2010; Ward, Bardgett et al. 2011). However, the distinct area within the brain that senses circulating insulin to mediate these effects still remained unknown.

Nuclei within the hypothalamus have been suggested for years to play a major role in this mechanism, predominantly due to the well-established notion of its involvement in appetite regulation and high expression of insulin receptors (Werther, Hogg et al. 1987). Of prominent interest, the ARC in the mediobasal hypothalamus vastly expresses insulin receptors in comparison to other moderately labeled nuclei, particularly in rostral medial
proopiomelanocortin (POMC) neurons as demonstrated in whole-cell patch-clamp electrophysiological recordings (Werther, Hogg et al. 1987; Williams, Margatho et al. 2010). Positioned immediately lateral to the third ventricle and median eminence, the ARC is in a prime location to sense both blood and CSF hormone levels (Cone 2005).

The most novel finding of the present study is that peripheral insulin acts directly on ARC neurons to mediate a sympathoexcitatory response. By neutralizing insulin within the ARC with the anti-insulin affibody, elevated circulating insulin during euglycemia did not produce an increase in lumbar SNA, as well as attenuated the amplified splanchnic SNA observed in control animals. Notably, insulin directly injected into ARC did not produce a rise in lumbar SNA following blockade with the anti-insulin affibody, which indicates insulin works within ARC to modify SNA. Previous work revealed similar results when inhibiting neurons in PVN; however, SNA was not elevated in response to insulin directly injected into PVN in spite of high expression of insulin receptors in this area. This suggested insulin was acting upstream of PVN (Ward, Bardgett et al. 2011). Accordingly, central proopiomelanocortin expression is primarily in the ARC, and dense fibers project downstream to PVN (Cone 2005). Consequently, the present study supports the possibility that insulin may work in a pathway mediated by neurons in the ARC that project to PVN to eventually alter cardiovascular function. However, the mechanism and specific pathway downstream of PVN remains unknown, but perhaps involves increasing glutamergic drive to RVLM (Bardgett, McCarthy et al. 2010; Ward, Bardgett et al. 2011).

Due to the nearby location and high expression of insulin receptors in VMH, it was critical to determine whether the anti-insulin affibody was also working in this nucleus to mediate the elevation in SNA. Neutralization of insulin within VMH with the anti-insulin affibody did not prevent an increase in lumbar SNA, while splanchnic SNA appeared to be
attenuated to similar levels as animals pretreated in ARC. These anatomical control experiments confirm that ARC neurons are sensing circulating insulin to mediate lumbar sympathoexcitation. Further experimentation will be needed to verify the effects on splanchnic SNA.

Ultimately, there is little knowledge regarding the mechanism of the anti-insulin affibody (Paranjape, Chan et al. 2010). The three aims of this study together provide clear insight in its ability to effectively neutralize insulin. By eliminating an increase in lumbar SNA in response to insulin directly injected into the ARC, as well as during peripheral hyperinsulinemia, in marked contrast to animals treated with the control (non-insulin binding) affibody, it can be concluded that it successfully works to block insulin. The third experiment made certain the anti-insulin affibody was functioning only to bind insulin, thereby preventing insulin from interacting with its receptor within the ARC. The inhibitory neurotransmitter GABA is synthesized by the action of the enzyme glutamic acid decarboxylase (GAD) within the hypothalamus. Distinctively, mRNA hybridization of two isoforms of the enzyme, GAD65 and GAD67, has been shown to be highly localized within the ARC (Schwartz, Sipols et al. 1993; Hentges, Otero-Corchon et al. 2009). Therefore, injection of the GABA_A receptor antagonist, gabazine, into ARC through disinhibition results in excitation of neuronal synapses. Pretreatment with the anti-insulin affibody in ARC followed by gabazine produced a considerable activation of all three sympathetic nerves, in addition to elevations in mean ABP and HR, demonstrating ARC neurons were still viable. Thus, the anti-insulin affibody eliminating the sympathoexcitatory response is truly reflective of its specificity to block insulin, rather than producing general neuronal inhibition within ARC.
The membrane-bound insulin receptor consists of four subunits: two extracellular alpha and two intracellular beta subunits. Insulin binds to the alpha subunits allowing the receptor to undergo a conformational change. As a member of the tyrosine kinase family, this alteration activates the beta subunit tyrosine kinase activity to result in receptor autophosphorylation. This leads to phosphorylation of tyrosine residues on insulin receptor substrates (IRS) 1-4, which provides binding sites for the initiation of several intracellular cascades ultimately leading to protein synthesis, gene transcription, and gene expression (Unger, Livingston et al. 1991; Plum, Schubert et al. 2005). The described events are particularly well-known for insulin receptors in the periphery, such as those located in adipose tissue and liver cells. Reports have indicated that insulin receptors in the CNS are functionally similar to those in peripheral tissue, and CNS receptors contain subunits with only slightly smaller molecular weights (Unger, Livingston et al. 1991).

Insulin receptor mediated events in the hypothalamus are under investigation in regards to feeding behavior and energy homeostasis. Administration of insulin systemically and into the third ventricle activated the phosphatidylinositol 3-kinase (PI3K) signaling cascade. The p85 subunit of PI3K binds to phosphorylated IRS proteins. Specifically, IRS-2 is a chief regulator of insulin action in the periphery and showed immense immunoreactivity within ARC. Under normal conditions, insulin circulates in the periphery in direct proportion to adipose stores and produces an anorexic effect leading to feeding regulation. Inhibition of PI3K in the hypothalamus prevented insulin’s ability to decrease both food intake and body weight. Consequently, this suggests insulin activation of the PI3K cascade is essential for its proper anorexic function and provides a possible intracellular pathway that may be disrupted in obesity (Niswender, Morrison et al. 2003).
It is well documented that obese humans and rodents experience peripheral insulin resistance due to the inability of insulin to decrease glucose (Brands, Hall et al. 1995; Esler, Straznicky et al. 2006), but controversy exists whether central sympathetic pathways are also resistant to insulin. Agouti obese mice displayed apparent peripheral insulin resistance, but lumbar SNA elevated significantly in response to ICV insulin (Morgan and Rahmouni 2010). In marked contrast, obese humans undergoing acute hyperinsulinemic-euglycemic clamps experienced blunted activation of muscle SNA when compared to lean controls. Although this may suggest central insulin resistance, a major limitation of this study could be the comparison of basal muscle SNA between the lean and obese groups (17±3 vs. 37±5 bursts/min). The lean group showed significant increases in muscle SNA after two hours of hyperinsulinemia-euglycemia (32±5 burst/min) (Vollenweider, Randin et al. 1994). Due to a higher obese basal rate of SNA comparable to the maximum increase seen in lean humans, obese subjects could be experiencing impaired elevations following hyperinsulinemia because they have reached a plateau. Therefore, further elevations of plasma insulin would no longer contribute to increases in SNA. This was illustrated in dose-response curves generated in the same study on the lean subjects, which determined that despite three increasing doses of insulin, muscle SNA elevations were comparable between groups (Vollenweider, Randin et al. 1994). Essentially, there remains to be a general lack of conclusive understanding surrounding insulin resistance in sympathetic circuits.

Acute blood pressure changes occur throughout the diurnal cycle in response to fluctuating behaviors; however, long-term management of blood pressure is firmly regulated by several mechanisms. As a consequence of one or all of these mechanisms going awry, a chronic elevation of blood pressure can result. Arterial baroreceptors located at the aortic arch and carotid sinus are high-pressure receptors with some level of tonic activity, which
has shown to be crucial in the regulation of long-term blood pressure (Guyenet 2006). During normal function, the baroreceptors are activated by increased blood pressure due to stretching of the blood vessel, information is integrated within RVLM and the nucleus of the solitary tract, and compensatory mechanisms are initiated through lowering SNA, as well as increasing parasympathetic nerve activity. The end-result is a decrease in blood pressure, and this system allows for regulation and maintenance of blood pressure in a vast number of situations (Guyenet 2006). Hence, impairment of the baroreflex has been strongly linked to obese and hypertensive patients and previous evidence has indicated that baroreceptor sensitivity is reduced with elevated SNA (Grassi, Seravalle et al. 1998; Skrapari, Tentolouris et al. 2007). Thus, hyperinsulinemia producing high levels of SNA and activating the proposed pathways in the hypothalamus can lead to a direct impairment of baroreflex (Hong and Hsieh 2007; Pricher, Freeman et al. 2008; Young, Deo et al. 2010; Cassaglia, Hermes et al. 2011).

Deriving from results of this work, several experiments may be implemented in the future, the most novel of which is the use of conscious animals and chronic sympathetic nerve recordings. Anesthesia most likely creates discrepancies in mean ABP and HR, but this can be avoided with the use of awake animals. Logically, the next step in this series would be to target insulin or insulin receptor expression specifically in ARC and perform hyperinsulinemic-euglycemic clamps in conscious rats implanted with chronic nerve recordings, particularly lumbar and splanchnic SNA. Given the lack of knowledge regarding central insulin resistance, another useful direction would be to perform clamps in a model of diet-induced obesity in conscious animals. An additional set of experiments to perform next include in vitro studies targeting exact signaling mechanisms within ARC that insulin initiates to mediate these sympathoexcitatory effects. The results of the present study do not
indicate whether insulin works through POMC or neuropeptide Y neurons, the two neuronal populations in ARC. Additionally, it is unclear whether downstream events in the insulin cascade are involved in the sympathetic response.

The overall conclusion of this study is that circulating insulin acts directly on neurons of the hypothalamic ARC to mediate the elevation of SNA. It is important to understand that a number of mechanisms are contributing to obesity and hypertension. While this study and previous data support the idea that hyperinsulinemia raises SNA to then exacerbate obesity, the exact distinction of cause and effect have yet to be determined. The presentations of these processes have the potential to work in opposite directions simultaneously. Also, hyperinsulinemia is presumably not functioning alone, and several cardiovascular, autonomic, and renal abnormalities working additively are the most probable culprit for increasing the presence of obesity-related health risks.
REFERENCES


