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NEURAL MECHANISMS OF SYMPATHETIC ACTIVATION DURING HYPERINSULINEMIA AND OBESITY-INDUCED HYPERTENSION

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ABSTRACT OF DISSERTATION

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By

Megan Elyse Bardgett

Lexington, Kentucky

Director: Dr. Sean D. Stocker

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Obesity afflicts more than 30% of the U.S. population and is a major risk factor for the development of hypertension, type II diabetes, and cardiovascular disease. Studies in humans and animals indicate that obesity is associated with increased sympathetic outflow to the vasculature and kidneys. One mechanism postulated to underlie the increase in sympathetic nerve activity (SNA) in obesity is hyperinsulinemia. Little is known regarding the central circuitry underlying elevated SNA and arterial blood pressure (ABP) during hyperinsulinemia and obesity or if sympathoexcitatory circuits are still responsive to insulin in obesity.

Hyperinsulinemic-euglycemic clamps elevate SNA to the hind limb vasculature in lean rodents but obesity is associated with resistance to the peripheral and anorexic effects of insulin. Therefore, the first aim was to determine whether diet-induced obesity causes development of insulin resistance in the central circuits mediating SNA. The sympathoexcitatory response to insulin was still intact in diet-induced obese rats indicating a role for insulin in the elevation in SNA and ABP in obesity.

The second aim of this project was to identify the specific receptors in the rostral ventrolateral medulla (RVLM) that mediate the elevated SNA during hyperinsulinemia. The RVLM provides basal sympathetic tone and maintains baseline ABP. Glutamate is the major excitatory neurotransmitter and glutamate receptors of the RVLM are known to mediate multiple forms of hypertension. Blockade of RVLM NMDA-specific glutamatergic receptors reverses the increased lumbar SNA associated with hyperinsulinemia. In contrast, blockade of angiotensin II type 1 or melanocortin receptors in the RVLM had no effect on the sympathoexcitatory response to insulin.

The goal of the third aim was to identify the cellular mechanisms within RVLM that mediate the elevated SNA and ABP in diet-induced obesity. Blockade of RVLM glutamate receptors reversed the elevated ABP and lumbar SNA

associated with diet-induced obesity while it had no effect on rats on a low fat diet or those resistant to weight gain on the high fat diet. Similar to the findings during hyperinsulinemia, blockade of RVLM angiotensin II type 1 or melanocortin receptors had no effect on lumbar SNA or ABP during diet-induced obesity.

KEYWORDS: Obesity, Hyperinsulinemia, Rostral Ventrolateral Medulla, Sympathetics, Blood Pressure

Megan Bardgett

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"Luck is when preparation meets opportunity" -Seneca

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Chapter I: Introduction

Obesity

1.1 Prevalence of Obesity

Obesity is a growing pandemic in industrialized as well as developing countries (Garrison et al. 1987). More than 30% of adults in the United States have a body mass index (BMI) greater than 30 while nearly 70% have a BMI greater than 25, therefore classified as overweight (Garrison et al. 1987). Obesity is associated with increased adiposity, insulin resistance, hyperinsulinemia, hyperleptinemia, and elevated cholesterol levels. Obesity is linked with decreased life expectancy of 5-20 years (Fontaine et al. 2003) and increased morbidity due to its causative association with hypertension, type II diabetes, atherosclerosis and cardiovascular diseases (Izzo et al. 2008, van Dieren et al. 2010). In addition, obesity is thought to exacerbate a number of health problems including metabolic and hormonal conditions, reproductive dysfunction, and cancer (Reeves et al. 2007). The development of obesity is determined both by genetics and lifestyle choices. Body weight is maintained by an extensive negative feedback system (Woods et al. 2004) based on stored fat, caloric intake, and energy expenditure. The majority of obesity is the result of decreased physical activity and increased caloric intake. As our society becomes more sedentary, daily energy expenditure decreases while energy intake remains unchanged or even increases due to the easy access to fast food and pre-packaged, high calorie foods. The uneven balance of excess dietary caloric intake over energy expenditure leads to glucose storage, fat mass deposition, and an increase in body weight.

1.2 Obesity-Induced Hypertension

One of the major health concerns associated with obesity is elevated arterial blood pressure (ABP) or hypertension (Izzo et al. 2008). There is a positive correlation between hypertension and adiposity and relative weight when adjusted for age in both males and females (Garrison et al. 1987). Individuals,

age 20-49, were studied over an 8 year time frame. The most common potentially modifiable characteristic predetermining individuals for hypertension was elevated body fat (Garrison et al. 1987). From this relationship, it can be garnered that as body weight increases with obesity, ABP will also rise which occurs in humans, dogs and rodents (Dobrain et al. 2000, Kassab et al. 1995). This is clinically relevant because only a ~5 mmHg increase in ABP significantly increases the risk for stroke and myocardial infarction (Izzo et al. 2008, Strazzulo et al. 2010). Convincing evidence indicates that elevated sympathetic nerve activity (SNA) leads to the pathogenesis of obesity-induced hypertension in humans, dogs, and rodents (Esler et al. 2006, Wofford and Hall 2004). Landsberg has hypothesized that obesity results in activation of the sympathetic nervous system as a compensatory mechanism. The increase in SNA helps to stabilize energy expenditure and restore body weight following overfeeding by elevating thermogenesis. But the result is an overall increase in SNA to the kidneys and vasculature raising blood pressure as a "side effect" (Landsberg 1986).

1.3 Sympathetic Nerve Activity

The sympathetic nervous system is the portion of the autonomic nervous system known as "fight or flight". Sympathetic neurons project to many organ systems where norepinephrine is released from nerve terminals. Activation of the sympathetic nervous system causes multiple physiological changes including: increased heart rate, increased blood flow to skeletal muscle, and dilated pupils. There are multiple techniques used to measure SNA in human subjects: ganglionic blockade, plasma and urinary norepinephrine levels, regional norepinephrine spillover, and microneurography. In obese humans, ganglionic blockade causes a significantly greater reduction in ABP and total peripheral resistance compared to lean subjects indicative of sympathetic activation (Shibao et al. 2007). When plasma norepinephrine is measured in obese humans, there are confounding reports (Grassi et al. 1995, Vaz et al. 1997). This could be because plasma measurements are insensitive, that norepinephrine released

from sympathetic fibers is taken back up or destroyed before entering the circulation, or that all nerve beds do not respond equally to a given stimuli (Esler et al. 1990). A more precise measure is regional norepinephrine spillover. When measured in obese vs lean subjects, obesity leads to increased renal SNA, decreased cardiac SNA, and unchanged hepatomesenteric SNA (Grassi et al. 1995, Vaz et al. 1997). These particular studies demonstrate the possibility for region specific changes in SNA associated with obesity. Measures of muscle SNA via microneurography have provided the most convincing evidence for elevated SNA associated with obesity. The first study, in 1995, demonstrated that muscle SNA is elevated in obese normotensive subjects compared to lean controls (Grassi et al. 1995). This highlighted the fact that development of obesity-induced hypertension is preceded by elevation in SNA. This might explain why weight loss decreases blood pressure even in non-hypertensive obese subjects (Neter et al. 2003). With that, a comparison of muscle SNA between obese-normotensive and obese-hypertensive subjects indicates an even greater elevation in muscle SNA (Lambert et al. 2007). Interestingly, even a modest weight gain, below what is classically considered obese, causes a significant increase in muscle SNA that is associated with a small, but significant elevation in systolic blood pressure (Gentile et al. 2007).

Similar results have been found in both dogs and rodents. Putting dogs or rodents on a high fat diet produces obesity-induced hypertension (Lohmeier et al. 2007, Stocker et al. 2007). Obese dogs have elevated plasma norepinephrine levels while obese rodents show elevated urinary catecholamines (Levin 1993, Lohmeier et al. 2007). Similar to humans, treatment with ganglionic blockers decreases blood pressure to the same level as lean animals (Truett et al. 1996, D'Angelo et al. 2006). As well, renal denervations prior to high fat feeding in dogs prevent the development of obesity-induced hypertension suggesting an important role for renal SNA (Kassab et al. 1995). In conjunction, obese Zucker rats, which have a mutation of the leptin receptor encoding gene, are hypertensive and it appears to be modestly dependent on increased SNA (Morgan et al. 1995, Carlson et al. 2000). This connection between obesity and

activation of the sympathetic nervous system provides a possible point for medical intervention but what is causing the elevation in SNA must be determined.

There are multiple factors proposed to play a role in elevating SNA and ABP in obesity. These include but are not limited to arterial baroreceptor reflex dysfunction, activation of the renin-angiotensin system, elevated circulating insulin and leptin levels, and obstructive sleep apnea (da Silva et al. 2009, Esler et al. 2006). Obesity is associated with impaired baroreflex function (Grassi et al. 2004) and although the baroreflex is traditionally thought to control ABP on a minute to minute basis, recently studies indicate a role for the baroreflex in long-term hypertension (Guyenet 2006). Chronic stimulation of the baroreflex in obese dogs causes a 20 mmHg fall in ABP associated with a parallel decrease in plasma norepinephrine with no change in either variable in lean controls (Lohmeier et al. 2007).

Angiotensin II is an important regulator of ABP and renin-angiotensin system activation appears to correlate with obesity-induced hypertension (Hall 2003, Boustany et al. 2004). In humans, angiotensin II, angiotensinogen, plasma renin activity, and angiotensin converting enzyme activity are all positively associated with BMI. And though there is a correlation between RAS activity and blood pressure, its effects on SNA are confounding. Ganglionic blockade lowers ABP to a similar level in rats that received chronic intracerebroventricular administration of angiotensin II (Bruner and Fink et al. 1986). As well, angiotensin II receptor blockade decreases muscle SNA in hypertensive humans (Grassi et al. 2003, Bechir et al. 2005). Celiac ganglionectomy, which removes neurotransmission to the splanchnic vasculature, markedly attenuates the hypertension associated with angiotensin II-salt (King et al. 2007). In contrast, chronic angiotensin II infusion in dogs does not increase renal SNA (Carroll et al. 1984) nor does it increase renal or lumbar SNA in rats on a low or high salt diet (Yoshimoto et al. 2010).

Obstructive sleep apnea is common in obesity and can result in significant activation in SNA through the night (Narkiewicz et al. 1998). It is proposed that over time this night time activation evolves into a round-the-clock increase in SNA. Obstructive sleep apnea has even been shown to raise SNA in lean individuals (Grass et al. 2005). But there is no mechanism through which a transfer of night time activation to full time activation works and treatment of obstructive sleep apnea does not decrease SNA (Mills et al. 2006).

Insulin and leptin levels both correlate positively with obesity-induced hypertension. Insulin and leptin circulate in proportion to body fat stores and gain access into the central nervous system through a transport-mediated mechanism across the blood brain barrier. Acutely, both insulin and leptin activate the sympathetic nervous system while chronic infusion in rodents increases ABP (Brands et al. 1991, Morgan DA et al. 1993, Rahmouni et al. 2009, Tallam et al. 2006). Taken together, multiple characteristics of obesity could lead to an increase in SNA and elevation of ABP and this dissertation focuses specifically on the role of insulin.

Insulin

2.1 Insulin Signaling

Two hallmark characteristics of obesity are hyperinsulinemia and insulin resistance. The peptide hormone insulin circulates in proportion to body fat stores (Woods et al. 1985) and is synthesized as a preprohormone in pancreatic β-cells. Preproinsulin is cleaved in the endoplasmic reticulum (ER) to generate proinsulin which is transported to the Golgi apparatus where it is acted on by proteolytic enzymes to generate the physiologically active form of insulin. Insulin, a 6000 Da protein, consists of 2 polypeptide chains connected by disulfide bonds (Derewenda et al. 1986). Insulin is stored as a stable, inactive hexamer, thus protecting the highly reactive monomer form. Insulin is released from pancreatic β-cells in response to elevated blood glucose levels (i.e. following a meal). Glucose enters pancreatic β-cells through the GLUT-2 transporter where it

undergoes glycolysis. The resultant shift in ATP/ADP ratio triggers ATP-sensitive K^+ channels to close. Upon depolarization, voltage gated Ca^{2+} channels open and the increased Ca^{2+} activates phospholipase C which cleaves the membrane phospholipid phosphatidyl-inositol 4, 5-bisphosphate (PIP_2) into inositol 1, 4, 5-triphosphate (IP_3). IP_3 binds to its receptor protein on the ER membrane allowing the release of Ca^{2+} from the ER. The increased intracellular Ca^{2+} concentration triggers the release of insulin stored in secretory vesicles.

Insulin is the major hormone controlling glucose homeostasis in peripheral tissues. Insulin stimulation leads to translocation of GLUT-4 transporters from an intracellular location to the cell surface (Thorell et al. 1999). It stimulates glucose uptake into skeletal muscle, glycogen synthesis in the liver and fat deposition in adipocytes (Barnard and Youngren 1992). The insulin receptor is a protein tyrosine kinase found throughout the body. When insulin binds to the extracelluar α-subunit it autophosphorylates the β-subunit giving the receptor the ability to phosphorylate other residues including insulin receptor substrates (IRS 1 and IRS 2). Tyrosine phosphorylated IRS 1 and IRS2 bind src homology 2 domain containing signaling proteins and can associate with phosphatidylinositol 3-kinase (PI_3K, mediates the metabolic actions of insulin) or mitogen-activated protein kinase (MAPK, mediates the proliferative effects of insulin) (Sale et al. 1995, Jhun et al. 1995). PI_3K and MAPK are able to activate many downstream signaling pathways including JNK, Akt/PKB, mTOR, etc (Figure 1). Insulin administration significantly increases PI_3K activation in skeletal muscle while specific inhibition of PI_3K eliminates insulin-stimulated glucose uptake (Cheatham et al. 1994, Le Marchand-Brustel et al. 1995). As well as insulin's effects on glucose uptake; insulin stimulates DNA replication and protein synthesis, increases glycogen synthesis in the liver, increases fatty acid synthesis, decreases gluconeogenesis, and modifies the activity of numerous enzymes.

2.2 Central Insulin Signaling

Insulin's actions in the brain vary greatly from that seen in peripheral tissues in that insulin is not necessary for neuronal glucose uptake, and in fact

does not influence brain glucose concentrations (Seaquist et al. 2001). Under normal conditions, insulin has three main actions in the central nervous system: 1) decrease food intake, 2) increase energy expenditure, and 3) activate the sympathetic nervous system. Most of what we know about insulin's central actions comes from the food intake field. Insulin was the first hormone implicated in control of food intake and body weight and is able to cross the blood brain barrier in a transport specific mechanism (Banks et al. 1997, Woods and Porte 1977) in direct proportion to circulating insulin levels. Insulin receptors are expressed throughout the central nervous system and are concentrated in brain regions known to control food intake such as the hypothalamus (Hill et al. 1986, Werther et al. 1987). Intracerebroventricular administration of insulin results in a profound decrease in food intake and body weight (Woods et al. 1979, Niswender et al. 2003). In contrast, injection of insulin receptor antisense into the hypothalamus significantly decreases insulin receptor expression which results in extreme hyperphagia and weight gain (Obici et al. 2002).

The signaling pathways involved centrally appear to be similar to those utilized in the periphery. Niswender (2003) demonstrated that insulin stimulates tyrosine phosphorylation of the insulin receptor, increases IRS-2 activity in the hypothalamic arcuate nucleus (ARC), and increases PI_3K activity in the mediobasal hypothalamus. As well, studies have shown that insulin's anorexic effects are dependent on activation of PI_3K as blockade abolishes the normal decrease in food intake (Carvalheira et al. 2003, Niswender et al. 2003).

The neuronal populations through which insulin acts to decrease food intake have been extensively studied. Insulin acts in the brain to activate a catabolic pathway while simultaneously inhibiting a separate anabolic pathway. These two pathways involve insulin acting on pro-opiomelanocortin (POMC) and neuropeptide-Y (NPY)/agouti related protein (AgRP) neurons, respectively (Morton et al. 2001, Schwartz et al. 2000). Insulin receptors are co-expressed with both POMC and NPY in the arcuate nucleus (Obici et al. 2001, Pardini et al. 2006) and central administration of insulin significantly increases POMC and

decreases NPY/AgRP expression. Activation of POMC neurons results in an increased release of α-melanocyte stimulating hormone (α-MSH) which binds to and activates melanocortin receptors which leads to a decrease in food intake. At the same time, inhibition of NPY/AgRP neurons decreases both NPY and AgRP release and because AgRP is a melanocortin receptor antagonist this also decreases food intake (Figure 2) (Schwartz 2000). The discovery that the hypothalamic melanocortin system is the major regulator of food intake is supported by the finding that central blockade of melanocortin receptors inhibits insulin's effects on food intake (Benoit et al. 2002) and the hyperphagia associated with type II diabetes results in an increase in NPY synthesis and release which is reversed by central insulin administration.

2.3 Insulin and Sympathetic Nerve Activity

Hyperinsulinemia activates the sympathetic nervous system (Anderson et al. 1991), is associated with obesity-induced hypertension (Modan et al. 1984), and is therefore postulated to play a role in activation of SNA in obesity-induced hypertension (Esler et al. 2006). Previous studies show a correlation between elevated plasma insulin levels and activation of the sympathetic nervous system in obese subjects (Scherrer et al. 1994, Huggett et al. 2004). Acute hyperinsulinemic-euglycemic clamps selectively elevate muscle and lumbar SNA in humans and rodents, respectively (Anderson et al. 1992, Morgan et al. 1993, Muntzel et al. 1994). This activation of SNA is known to be mediated by central circuits as intracerebroventricular administration of insulin elevates lumbar SNA to the same extent and anteroventral third ventricle lesions abolish the sympathoexcitation (Muntzel et al. 1994). Blockade of central PI_3K, but not MAPK, blunts the increase in lumbar SNA associated with hyperinsulinemia (Rahmouni et al. 2004). In addition, chronic hyperinsulinemia elevates ABP in rodents which were associated with an increase in total peripheral resistance (Brands et al. 1991).

2.4 Altered Insulin Signaling in Obesity

Plasma insulin levels are significantly elevated in obese humans and rodents (Carvalheira et al. 2003). Obesity results in an initial resistance to insulin which is compensated for by increased output of insulin from the pancreas in order to maintain euglycemia. Over time, pancreatic β cells can no longer compensate for the resistance which leads to a significant decrease in insulin-stimulated skeletal muscle glucose uptake measured by glucose tolerance tests (Storlien et al. 1986). This decrease in glucose uptake is not due to a change in the expression of GLUT-4 receptors but rather is most likely due to impairment in insulin signaling (Choi and Kim 2010). In obese humans, insulin loses its ability to stimulate skeletal muscle blood flow which could play a role in the decreased glucose uptake (Laakso et al. 1990). In fact; it has been shown that PI_3K activity is decreased in skeletal muscle of type II diabetic (Kim et al. 1999) or obese non-diabetic patients (Cusi et al. 2000). Obesity also results in central insulin resistance. Intracerebroventricular administration of insulin has a decreased anorexic effect in both diet-induced obese and Zucker obese rats (Carvalheira et al. 2003, Clegg et al. 2005, Posey et al. 2009). There is no change in hypothalamic insulin receptor expression but rather this is associated with decreased autophosphorylation of the insulin receptor, decreased phosphorylation of IRS-1 and -2 in the hypothalamus, and significantly attenuated PI_3K signal transduction (Carcalheira et al. 2003, De Souza et al. 2005, Posey et al. 2009). Very little is known in regards to insulin's ability to elevate SNA during obesity. A very recent study demonstrated that agouti obese mice, in which the agouti protein is over-expressed, which blocks melanocortin receptors increasing food intake, are selectively resistant to the central effects of insulin. Intracerebroventricular insulin is still able to increase lumbar SNA but not renal or brown adipose tissue SNA (Morgan and Rahmouni 2010).

Neural Circuitry

3.1 Rostral Ventrolateral Medulla

The rostral ventrolateral medulla (RVLM) provides basal sympathetic tone through a direct projection to preganglionic sympathetic fibers of the

intermediolateral cell column in the lumbar and thoracic spinal cord (Guyenet 2006). Electrical or chemical stimulation of the RVLM causes a significant pressor response and increase in SNA (Ross et al. 1984, Bennaroch et al. 1986) while electrolytic lesions or inhibition of the RVLM result in falls in SNA and ABP similar to that seen following spinal cord transection or ganglionic blockade (Ross et al 1984). Although other brain regions innervate sympathetic fibers and when stimulated increase ABP, the RVLM is regarded as the major vasomotor center.

In vivo electrophysiological studies have identified bulbospinal RVLM neurons that are tonically active, barosensitive, synchronized with the blood pressure pulse, and project to the spinal cord (Brown and Guyenet 1984). The tonic activity of these cells is based on either excitatory synaptic input or intrinsic pacemaker activity. To be classified as pacemaker cells, neurons must depolarize without outside input and have a highly regular discharge rate not preceded by excitatory or inhibitory post-synaptic potentials. Initial studies indicated that these cells could generate spontaneous action potentials based on their gradual depolarization (Sun et al. 1988) and ability to continually fire after application of kynurenic acid, a glutamate receptor antagonist, and hence blockade of many excitatory inputs to the RVLM (Sun et al. 1988). However, recordings of dissociated RVLM neurons revealed no such pace-maker properties (Lipski et al. 1998). Action potentials of RVLM neurons were preceded by excitatory post-synaptic potentials with no indication of gradual depolarization (Lipski et al. 1996) which signifies that tonic activity is determined by synaptic inputs under normal conditions.

Where these synaptic inputs originate and the neurotransmitters involved to maintain baseline RVLM neuronal activity are not well understood. In cats and rabbits blockade of glutamatergic input to the RVLM results in a significant decrease in ABP (Abrahams et al. 1994, Horiuchi and Dampney 2002), where as it causes no change in rats (Ito and Sved 1997), but this is believed to result due to blockade of both excitatory and inhibitory neurotransmission. There are direct monosynaptic projections to the RVLM from the caudal ventrolateral medulla

(Afarwal and Calaresu 1991), hypothalamic paraventricular nucleus (Shafton et al. 1998), nucleus of the solitary tract (Ross et al. 1985), area postrema (Shapiro and Miselis 1985), pontine reticular formation (Krassioukov and Weaver 1993) and others. Inhibition of the pontine reticular formation results in decreases in ABP and SNA similar to that seen following RVLM inhibition, but they are short lasting (Hayes and Weaver 1992). However, inhibition of any of the other regions does not lead to as significant a decrease in ABP as inhibition of the RVLM and it is most likely that a combination of regions contribute to the resting activity of RVLM neurons.

The RVLM is implicated in a number of sympathoexcitatory reflexes such as the baroreflex (Guyenet 2006), somatic pressor reflex (Stornetta et al. 1989), chemoreflex (Koshiya et al. 1993), as well as hypothalamic stimulation (Coote et al. 1998), all of which are affected by destruction or blockade of RVLM neurotransmission (Granata et al. 1985, Stornetta et al. 1989, Koshiya et al. 1993, Sun and Guyenet 1986). And although the RVLM is made up of a heterogeneous population of neurons, it is believed that it is the spontaneously active, spinally projecting neurons specifically which are responsible for maintaining baseline SNA and ABP and are the major relay station for the baroreflex involved in minute to minute as well as long term control of ABP. These neurons play a critical role in cardiovascular regulation and vasomotor tone.

3.2 Glutamate

RVLM neurons express a number of receptor types and their excitability is determined by a number of neurotransmitters. L-glutamate, the major excitatory neurotransmitter elicits profound increases in SNA and ABP when microinjected in to the RVLM. A glutamatergic connection exists between the hypothalamus and RVLM (Sun MK and Guyenet 1986). Specifically, glutamate-dependent connections have been discovered from the paraventricular nucleus (Yang et al. 2001, Stocker et al. 2006) and pontine reticular formation (Krassioukov and Weaver 1993) to the RVLM. Further evidence exists for a significant role of

RVLM glutamatergic receptors in that blockade of glutamate receptors in the RVLM eliminates multiple sympathoexcitatory reflexes including the somatic pressor reflex (Stornetta et al. 1989) and has been reported to significantly lower ABP in several experimental models of hypertension (Bergamaschi et al. 1995, Ito et al. 2000, Ito et al. 2001).

3.3 Renin-Angiotensin System

Though many sympathoexcitatory reflexes are mediated by RVLM glutamatergic receptors, not all appear to be glutamate-dependent (Kiely and Gordon 1994). The RVLM expresses an abundance of angiotensin II type 1(AT1) receptors (Song et al. 1991) that when activated increase SNA and ABP (Dampney et al. 2002). The increases in ABP and renal SNA associated with activation of the paraventricular nucleus by disinhibition appear to be mediated by RVLM AT1 receptors (Tagawa and Dampney 1999). Similar to RVLM glutamate receptors, blockade of AT1 receptors abolishes hypertension in a number of experimental models including both spontaneously and salt-sensitive hypertensive rats (Ito et al. 2002, Ito et al. 2003).

Inhibition of angiotensin converting enzyme or blockade of AT1 receptors in the brain blunts the sympathoexcitatory response to hyperinsulinemia (Muntzel et al. 1994, Nakata et al. 1998). In addition, blockade of the renin-angiotensin system prevents insulin-induced hypertension (Brands et al. 1997) and diet-induced obesity hypertension in rodents (Boustany et al. 2005). Importantly, angiotensin-converting enzyme inhibition has been reported to be an effective therapy in obese, hypertensive subjects (Reisin et al. 1997) while angiotensin II receptor blockade lowers SNA in obese-hypertensive subjects (Grassi et al. 2003, Bechir et al. 2005).

3.4 Melanocortin System

The central melanocortin system has been extensively implicated in the control of food intake (Schwartz et al. 2000) and is hypothesized to be equally as important in control of SNA. Melanocortin 3/4 receptors are expressed

throughout the brain, including the RVLM (Adan and Gispen 1997, Adan and Gispen 2000) where activation increases SNA and ABP (Kawabe et al. 2006). Interestingly, approximately 3-6% of extreme obese cases in humans are due to a mutation in the melanocortin-4 receptor. The prevalence of hypertension is significantly decreased in these subjects as is urinary norepinephrine secretion (Greenfield et al. 2009) and muscle SNA (Sayk et al. 2010). Similarly, the sympathoexcitatory response to insulin is abolished in melanocortin-4 receptor knockout mice (Rahmouni et al. 2003). While these mice are extremely obese, they do not become hypertensive despite elevated plasma insulin concentrations (Tallam et al. 2005). In addition, intracerebroventricular infusion of the melanocortin receptor antagonist SHU 9119 significantly lowers ABP in diet-induced obese rats (Dubinion et al. 2010), significantly decreases renal SNA in obese mice (Morgan et al. 2008), significantly lowers ABP in spontaneously hypertensive rats (da Silva et al. 2008), and prevents the anorexic actions of insulin (Benoit et al. 2002).

Summary and Specific Aims

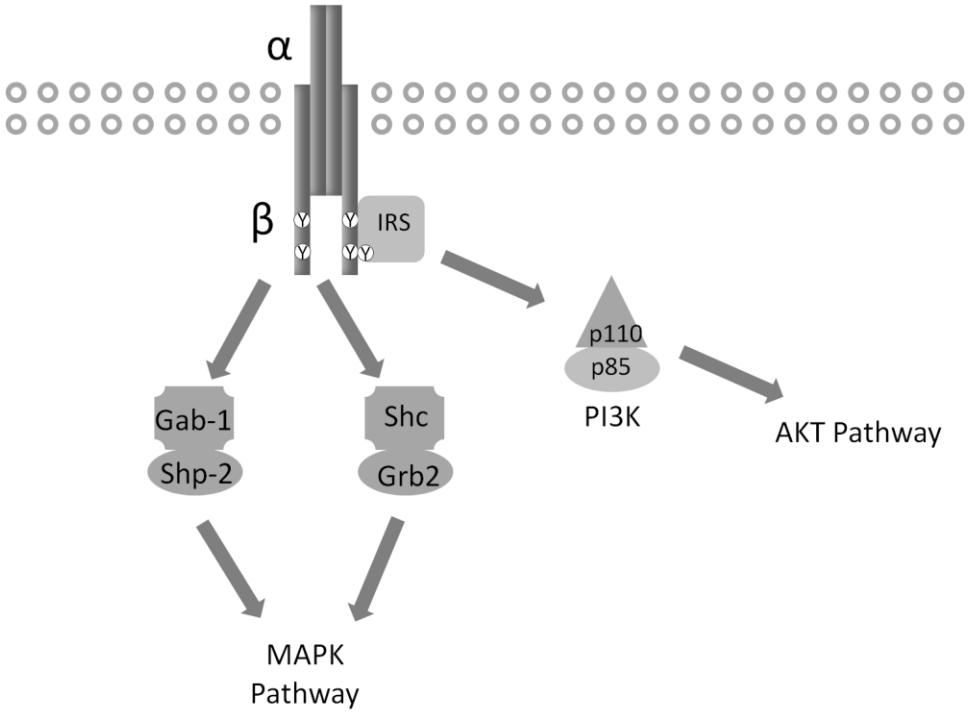
Obesity affects a large percentage of the United States population and is a major determinate in the development of essential hypertension. Multiple studies in humans and rodents indicate that obesity-induced hypertension is due to increased SNA to the hindlimb vasculature. One possible signal elevating SNA in obesity is hyperinsulinemia. Obese humans and rodents have elevated plasma insulin levels and acute hyperinsulinemic-euglycemic clamps elevate muscle and lumbar SNA, respectively. As well, chronic hyperinsulinemic-euglycemic clamps elevate arterial blood pressure in rodents. These actions are mediated by the central nervous system as acute ICV administration of insulin elevates lumbar SNA. Altogether these findings indicate that obesity causes hyperinsulinemia and the increased insulin acts centrally through an unidentified pathway to increase SNA and ABP. But it is still unknown whether insulin is able to stimulate the sympathetic nervous system in obesity or if these circuits become insulin resistant. As well, the brain regions mediating the increase in

SNA and ABP during hyperinsulinemia and obesity remain to be discovered. Therefore, this dissertation aimed to examine the central mechanisms through which insulin and obesity activate the sympathetic nervous system and elevate arterial blood pressure. Specifically these studies examined:

1. Whether diet-induced obesity results in central resistance to the sympathoexcitatory effects of insulin

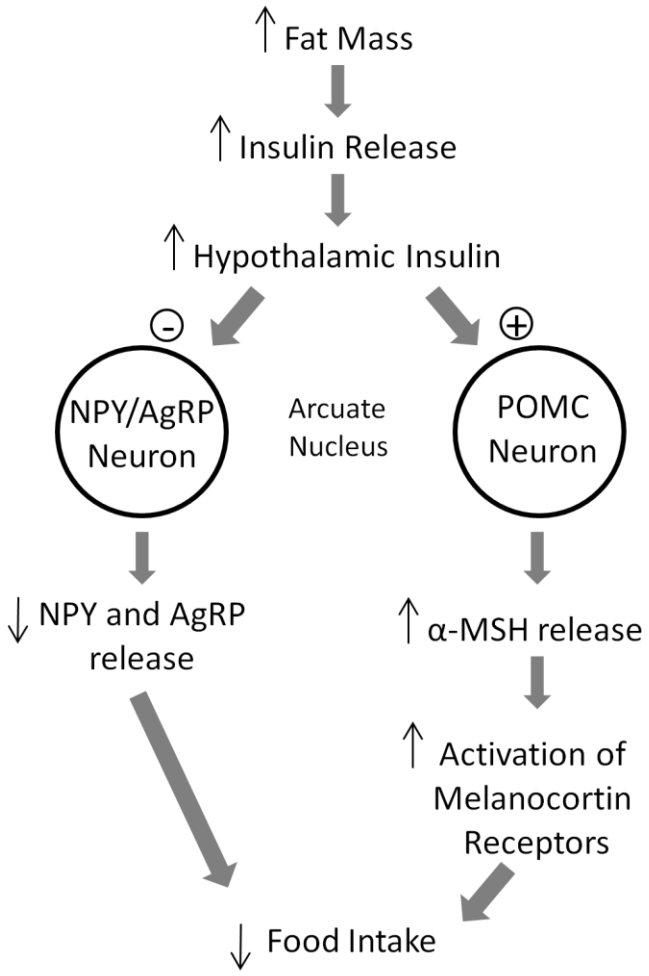
2. Whether glutamate, angiotensin II type 1, or melanocortin receptors of the RVLM mediate the sympathoexcitatory response to insulin.

3. Whether glutamate, angiotensin II type 1, or melanocortin receptors of the RVLM are involved in the development of diet-induced obesity hypertension.



<u>Figure 1.</u> Insulin signaling pathways (Adapted from Kido et al. 2001)

Insulin signaling includes a vast array of downstream pathways. Activation of the insulin receptor causes autophosphorylation of the β-subunit followed by tyrosine phosphorylation of IRS. Downstream signaling cascades include: AKT which is responsible for activation of mTOR, protein synthesis, and GLUT-4 translocation and MAPK mediates gene transcription and cell proliferation



<u>Figure 2.</u> Insulin acts within the hypothalamus to activate a relay station which results in a decrease in food intake (Adapted from Schwartz et al. 2000).

Chapter II: Diet-Induced Obesity Does Not Result in Insulin Resistance in Sympathetic Circuits

This work has previously been submitted to *Brain Research*

Specific aim 2 will examine whether diet-induced obesity causes central resistance to the sympathoexcitatory effects of insulin.

Introduction

The peptide hormone insulin is released from the pancreas and circulates in direct proportion to the amount of fat stores (Woods et al. 1985). Insulin plays a pivotal role in the regulation of glucose metabolism, food intake, and body weight (Schwartz et al. 2000). Insulin stimulates glucose uptake largely in skeletal muscle, liver, and adipocytes by eliciting translocation of GLUT-4 receptors to the membrane (Barnard and Youngren 1992, Thorell et al. 1999). In addition, circulating insulin is transported into the brain where it acts in the hypothalamus to decrease food intake and body weight (Schwartz et al. 2000, Woods and D'Alessio 2008).

Obesity and the metabolic syndrome are characterized by a marked peripheral insulin resistance defined clinically as an inability of insulin to maintain glucose homeostasis (Storlien et al. 1986, Haas and Biddinger 2009). Obesity is also associated with a reduction in the central anorexic actions of insulin (Clegg et al. 2005). In regard to the latter, high-fat feeding significantly attenuates the insulin-mediated reductions in food intake and body weight of Sprague-Dawley (Clegg et al. 2005), Osborne-Mendel (Arase et al. 1988), and out-bred and selectively-bred obesity-resistant (OR) and obesity-prone (OP) rats (Clegg et al. 2005). In fact, the ability of insulin to decrease food intake in out-bred OR and OP was inversely related to subsequent body weight gain. That is, the blunted anorexic effect of insulin was associated with, or predicted weight gain on a high energy diet (Clegg et al. 2005).

In addition to its anorexic effects, insulin works centrally to activate the sympathetic nervous system and alter cardiovascular function (Bardgett et al. 2010). Previous studies have clearly demonstrated that a hyperinsulinemic-euglycemic clamp significantly increases lumbar and/or muscle sympathetic nerve activity (SNA) in rodents and humans, respectively (Anderson et al. 1992, Morgan et al. 1993, Muntzel et al. 1994). These sympathoexcitatory actions of insulin are centrally-mediated as intracerebroventricular injection of insulin produces similar responses (Muntzel et al. 1994, Pricher et al. 2008) and interruption of central insulin signaling pathways (Rahmouni et al. 2004) or neurotransmission in the rostral ventrolateral medulla (Bardgett et al. 2010) prevents the increase in lumbar SNA. Furthermore, hyperinsulinemia is one mechanism postulated to play a role in the elevated SNA during obesity-induced hypertension (Esler et al. 2006). If hyperinsulinemia chronically elevated SNA in obesity, the central circuits that mediate the sympathoexcitatory response could not be insulin-resistant. Currently, there is not data that directly test this hypothesis.

Therefore, the purpose of the present study was to determine whether the central sympathetic circuits were insulin-resistant in diet-induced obesity. Previous studies have demonstrated that feeding rats a moderate high-fat diet results in the segregation of obesity-resistant (OR) and obesity-prone (OP) rats based on body weight gain (Stocker et al. 2007). The latter group has an elevated arterial blood pressure (ABP), activation of the renin-angiotensin system, hyperleptinemia, hyperinsulinemia, and elevated sympathetic outflow (Boustany et al. 2004, Levin et al. 1983, Dobrain et al. 2000, Levin and Keesey 1998). The segregation of OR and OP rats also permits studies to distinguish between the effects of obesity versus diet composition.

Materials and Methods

Animals

All of the experimental procedures were approved by the University of Kentucky and Penn State University Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (Charles River Laboratory) weighing 150 to 200 g were housed in a temperature-controlled room (22^{o}-23^{o} C) with a 14-hour/10-hour light-dark cycle (light on at 7:00 AM). Rats were placed on a LF diet (10% kcal from fat; Research Diets, Inc. D12489B) or a moderate high-fat diet (32% kcal from fat, Research Diets, Inc. D12266B) for 13 weeks. After 5 weeks, rats fed the moderately high-fat diet segregate into OP and OR based on body weight distribution as described previously (Stocker et al. 2007). Briefly, a body weight histogram was constructed and resulted in a distribution of rats into OP and OR groups corresponding with the upper and lower one third of rats, respectively.

Rats were anesthetized with isoflurane (2%-3%, 100% O_2) and prepared for recordings of lumbar SNA and ABP as described previously (Scislo et al. 1998, Stocker et al. 2005). Animals were artificially ventilated with oxygen-enriched room air. End-tidal CO_2 and body temperature were maintained at 4% to 5% and 37 ± 1^{o} C, respectively. After surgery, anesthesia was replaced by α-chloralose. An initial bolus (50 mg/kg, iv) was administered followed by a continuous infusion (0.017 mL/kg/min of 25 mg/mL, iv). In preliminary experiments, OP rats required a smaller dose of α-chloralose than predicted from body weight, and this dose was equivalent to the amount administered to a LF or OR rat. The level of anesthesia was assessed by the lack of a withdrawal reflex to a foot pinch. Therefore, the dose of α-chloralose for OP rats was initially based on a paired LF or OR rat studied on the same day. The α-chloralose infusion was adjusted as needed by the presence of a withdrawal reflex. Anesthesia and variables were allowed to stabilize for a minimum of 60 min before the experiment began.

Hyperinsulinemic-Euglycemic Clamps

Baseline values of ABP and lumbar SNA were recorded for 20 minutes. Then, insulin (0.017 mL/kg/min of 220 mU/mL Humulin R dissolved in α-chloralose, iv) and a 50% dextrose solution (0.25-1.0 mL/hr, IV) or an equal volume of 0.9% saline were infused for 120 minutes. This dose of insulin is equivalent to 3.75 mU/kg/min in 300-350 g Sprague-Dawley rats and has been previously reported to selectively increase lumbar SNA (Bardgett et al. 2010). Blood glucose was measured from a drop of arterial blood every 10 minutes using a standard glucometer (One Touch Ultra). The dextrose infusion rate was adjusted to maintain euglycemia. Blood (0.2 mL) was collected from the arterial line into microcentrifuge tubes (3µL, 0.5 mol/L EDTA) at baseline, 60, and 120 minutes. Samples were centrifuged, and plasma was stored at -80^{o}C. Insulin levels were determined by an ELISA using a commercially available kit (Millipore).

Data Analysis

All data are expressed as means ± SEM. Changes in integrated SNA are calculated by subtracting background noise after hexamethonium (30 mg/kg, IV). For all variables, 60-second segments at each time point were compared to three 60-second baseline period measurements. All data were analyzed by a 1- or 2-way ANOVA with repeated measures when appropriate. All post hoc tests were performed with independent or paired *t* tests with a layered Bonferroni correction. A $P<0.05$ was statistically significant.

Results

Characteristics of LF, OR, and OP Rats

Initial body weight was not significantly different between LF, OR, or OP rats (Figure 3). At week 4, OP rats weighed significantly more than LF or OR rats. The greater weight gain in OP rats was associated with a greater epididymal and retroperitoneal fat mass (Table 1). Consequently, OP versus LF or OR rats had a significantly greater adiposity index. Both ABP and lumbar SNA were significantly elevated at baseline in OP versus LF and OR rats (Table 1).

Insulin-Induced Sympathoexcitation in LF, OR, and OP Rats

The major goal of this study was to determine whether diet-induced obesity causes insulin resistance in central sympathetic circuits. To test this hypothesis, we measured lumbar SNA during a hyperinsulinemic-euglycemic clamp in LF, OR, and OP rats. Figure 4 illustrates a representative example of the response in LF, OR, and OP rats. Group data for these responses are summarized in Figure 5. A hyperinsulinemic-euglycemic clamp significantly increased lumbar SNA in LF, OR, and OP rats compared to saline infusion. In fact, the magnitude of this response was not statistically different across groups at any time. Blood glucose of LF, OR, and OP did not significantly change from baseline values despite a significantly lower glucose infusion rate of OP rats (Figure 5). Although the percent change was not significantly different between the three groups, the absolute change in lumbar SNA was significantly greater in OP compared to LF or OR rats (Figure 6). Mean ABP decreased in all three groups through the 120 min infusion whereas heart rate did not change (data not shown). Saline infusion had no effect on ABP while it slightly decreased lumbar SNA equivalently in all groups by 60 min (data not shown).

As previously reported (Bardgett ME et al. 2010), basal plasma insulin levels were significantly higher in OP versus LF or OR rats (Table 1, Figure 7A). At 60 and 120 min, plasma insulin levels of LF versus OP rats were not statistically different. Plasma insulin levels of OR rats were lower than those of OP rats. However, the change in plasma insulin levels at 60 and 120 min were not different across all three groups (Figure 7B). An equivalent change in plasma insulin resulted in a significantly greater change in lumbar SNA in OP rats.

Discussion

Previous studies have demonstrated that diet-induced obesity produces peripheral insulin resistance and a reduction in the central anorexic effect of insulin (Storlien et al. 1986, Arase et al. 1988, Clegg et al. 2005). However, insulin also acts within the brain to elevate SNA and alter cardiovascular function (Bardgett et al. 2010). It was unknown previously whether central sympathetic circuits were insulin resistant during diet-induced obesity. The present study demonstrates that a hyperinsulinemic-euglycemic clamp produced similar increases in OP versus LF or OR rats. These findings suggest that diet-induced obesity causes a selective insulin resistance in the central nervous system.

In the present study, we utilized a rodent model of diet-induced obesity characterized by a differential weight gain to a moderate high-fat diet and segregation into OP and OR rats. This model closely mimics human obesity as OP rats have elevated cholesterol, hyperinsulinemia, hyperleptinemia, activation of the renin-angiotensin and sympathetic nervous system, and an elevated ABP (Boustany et al. 2004, Boustany et al. 2005, Dobrain et al. 2000, Levin et al. 1983, Levin and Keesey 1998). Second, the segregation of OP and OR rats permits studies to differentiate between the effects of obesity/weight gain versus consumption of the moderate high-fat diet. In the present study, OP versus LF or OR rats had a significantly higher body weight, fat pad mass and adiposity index. In addition, OP versus LF or OR rats had a significantly higher plasma insulin level, lumbar SNA, and mean ABP.

Using this model, we hypothesized that central sympathetic circuits would not become insulin-resistant during diet-induced obesity. Despite marked peripheral insulin resistance as indicated by a significantly lower glucose utilization of OP versus LF or OR rats, a hyperinsulinemic-euglycemic clamp produced comparable increases in both plasma insulin levels and lumbar SNA across all three groups. These findings support the current hypothesis that diet-induced obesity does not affect insulin-sensitive sympathetic circuits.

Insulin crosses the blood-brain barrier in a transport-mediated process (Banks 1997). Previous studies have reported insulin transport into the brain either decreases (Kaiyala et al. 2000, Kern et al. 2006) or does not change (Israel et al. 1993) in response to diet-induced obesity. Although we did not measure cerebrospinal insulin levels in LF, OR, and OP rats, a decrease in insulin transport of OP rats should have resulted in a smaller increase in lumbar SNA. Since baseline lumbar SNA was significantly higher in OP rats, an equivalent percent increase in lumbar SNA would suggest that a hyperinsulinemic-euglycemic clamp produced a larger sympathoexcitatory response in OP versus LF or OR rats. In contrast, the percent increase in lumbar SNA was not statistically different across groups. In fact, an equivalent change in plasma insulin concentration resulted in a significantly greater change in absolute lumbar SNA when expressed as microvolts. These findings indicate that diet-induced obesity does not affect insulin transport into brain regions that contribute to the sympathoexcitatory response.

The present findings differ from previous studies that suggest diet-induced obesity produces insulin resistance in central neuronal networks. These studies have reported that diet-induced obesity reduces the anorexic effects of insulin administered intracerebroventricularly (Carvalheira et al. 2003, Clegg et al. 2005, Posey et al. 2009). However, the neurons that sense insulin and the downstream signaling pathways that mediate insulin's effects on food intake versus sympathetic activation have not been fully elucidated. Inhibition of phosphoinositide 3-kinase prevents the increase in lumbar SNA (Rahmouni et al. 2004) and decrease in food intake (Carvalheira et al. 2003, Niswender et al. 2003) in response to intracerebroventricular administration of insulin. Furthermore, administration of the melanocortin receptor antagonist SHU9119 prevents the anorexic effect of insulin (Benoit et al. 2002) whereas melanocortin-4 receptor knockout mice do not show a sympathoexcitatory response to intracerebroventricular injection of insulin (Rahmouni et al. 2003). Recently, our laboratory demonstrated that the sympathoexcitatory response to a hyperinsulinemic-euglycemic clamp is reversed by blockade of glutamatergic

receptors in the rostral ventrolateral medulla (Bardgett et al. 2010). Despite this evidence, it is not clear how or where the central neural pathways diverge to control food intake versus SNA. The differences between the present findings and previous studies in regard to insulin resistance in central neuronal pathways could simply be attributed to different populations of insulin-sensing neurons that control food intake versus SNA. Clearly, future studies are needed to address these questions.

Our results are in contrast to some previous findings in humans. The reasons for the confounding results could be the result of differing methods for producing hyperinsulinemia or inappropriate insulin levels. Straznicky et al found that insulin resistant human subjects show a decreased sympathoexcitatory response to an oral glucose load (Straznicky et al. 2009). In contrast to our study, they were examining the sympathetic response to an elevation in glucose as well as insulin and separated subjects based on insulin resistance as opposed to obesity. In fact, their controls, while insulin sensitive, were in fact obese as defined by a body mass index >30. Vollenweider et al. performed hyperinsulinemic-euglycemic clamps in lean and obese subjects. They found a decreased muscle SNA response to hyperinsulinemia in obese compared to lean individuals (Vollenweider et al. 1994). Though this is in contrast to our findings, they also show that lean subjects respond similarly to varying doses of insulin, the lowest dose producing insulin levels similar to that seen at baseline in obese subjects. Therefore, it is difficult to gauge whether central insulin resistance or inappropriate insulin dose results in differences between lean and obese subjects. The dose of insulin used in our study was determined by previous studies in our laboratory (Bardgett et al. 2010). This dose is slightly lower than previous studies but produces plasma insulin levels in lean rats similar to that seen in diet-induced obese and obese Zucker rats. Due to this dose producing appropriate plasma insulin levels, we did not feel it necessary to utilize a higher dose of insulin as that would produce supraphysiologic levels in OP rats.

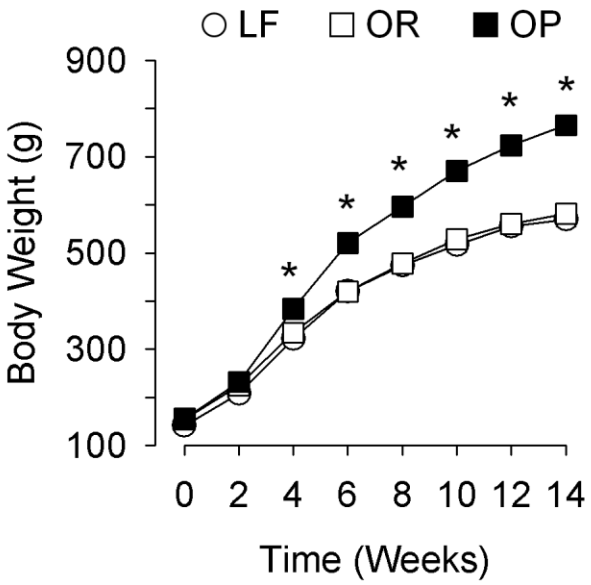
The ability of a hyperinsulinemic-euglycemic clamp to produce similar increases in SNA of LF, OR, and OP rats is reminiscent to previous studies on selective leptin resistance. Although the anorexic actions of leptin are attenuated in obesity (Clegg et al. 2005) the renal sympathoexcitatory actions of leptin are preserved (Rahmouni et al. 2005). Interestingly, only the renal but not lumbar or brown adipose SNA response to leptin was preserved in obese animals (Rahmouni et al. 2005). Altogether, this raises the possibility that insulin and leptin may act through parallel pathways to chronically elevate lumbar and renal SNA, respectively, in obesity-induced hypertension. In fact, studies in human populations using microneurography or norepinephrine spillover indicate that both muscle and renal SNA are elevated in obese individuals (Vaz et al. 1997, Grass et al. 1995). The relative contribution of insulin versus leptin (or other factors) to the elevated muscle or renal SNA in obesity-induced hypertension has not been directly assessed.

In summary, the present findings demonstrate that central sympathetic circuits are not insulin resistant in diet-induced obesity. Despite pronounced peripheral insulin resistance of OP rats, a hyperinsulinemic-euglycemic clamp produced similar increases in lumbar SNA of LF, OR, and OP rats. Together with previous reports (Morgan and Rahmouni 2010), these findings suggest that diet-induced obesity produces a selective central insulin resistance. These findings raise the possibility that hyperinsulinemia may chronically elevate lumbar SNA in obesity-induced hypertension.

Table 1. Characteristics of LF, OR, and OP Rats

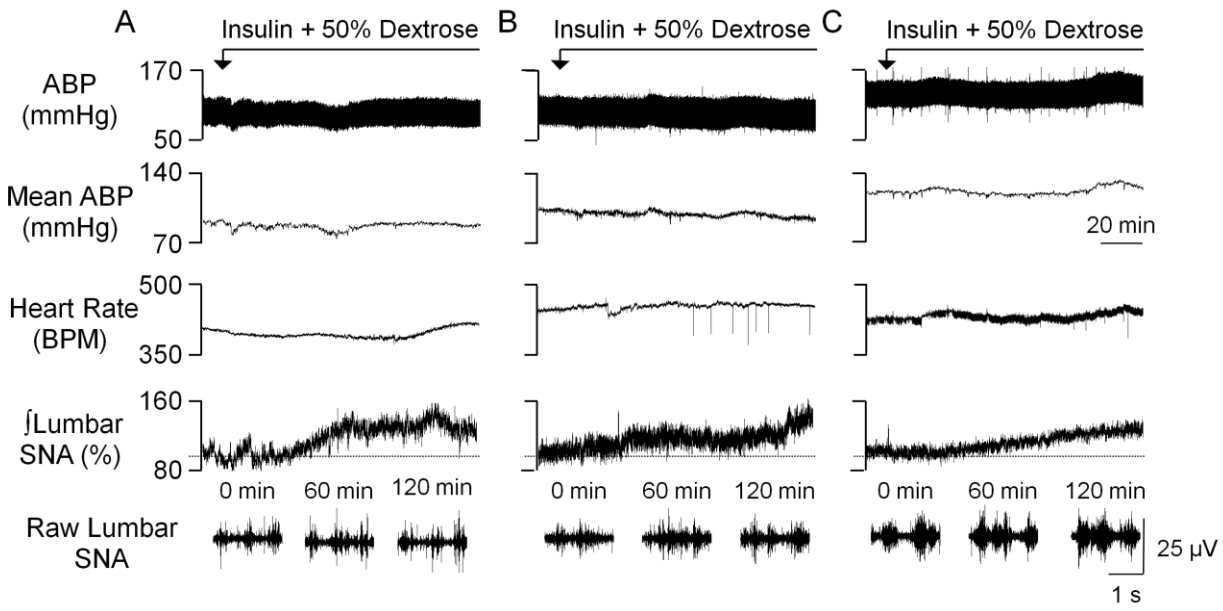
Characteristic	LF	OR	OP
Body Weight (g)	581±15	576±11	784±13*
Fat Pads (g)			
Epididymal	12.5±1.1	14.3±1.2	29.2±1.2*
Retroperitoneal	16.3±3.0	18.2±1.4	38.5±2.2*
Total	28.8±4.1	32.5±2.5	67.7±4.1*
Adiposity Index (%)	4.9±0.4	5.6±0.4	8.6±0.4*
Plasma insulin (ng/mL)	8.1±1.7	8.4±1.6	15.9±2.3*
Blood Glucose (mg/dL)	56±6	60±4	62±5
ABP (mmHg)	90.8±3.7	93.7±4.5	105.8±5.1*
Lumbar SNA (μV)	1.43±0.26	1.82±0.36	3.62±0.76*
HR (bpm)	408±10	426±9	405±13

Values are mean ± SEM. *Significant difference versus LF or OR ($P<0.05$).



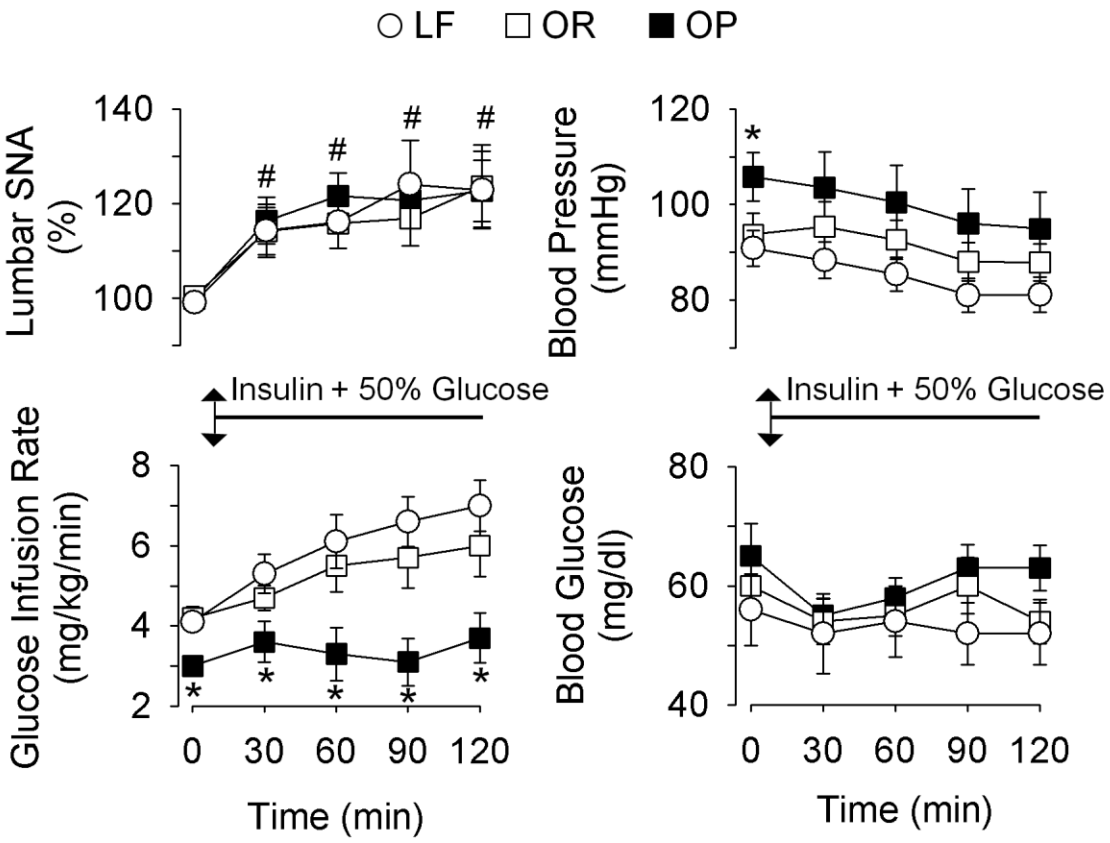
<u>Figure 3.</u> Body weight distribution of LF, OR, and OP rats

Body weight as a function of time for LF, OR, and OP rats. n=10 per group. Values are mean ± SEM. *P<0.05 OP vs LF or OR rats.



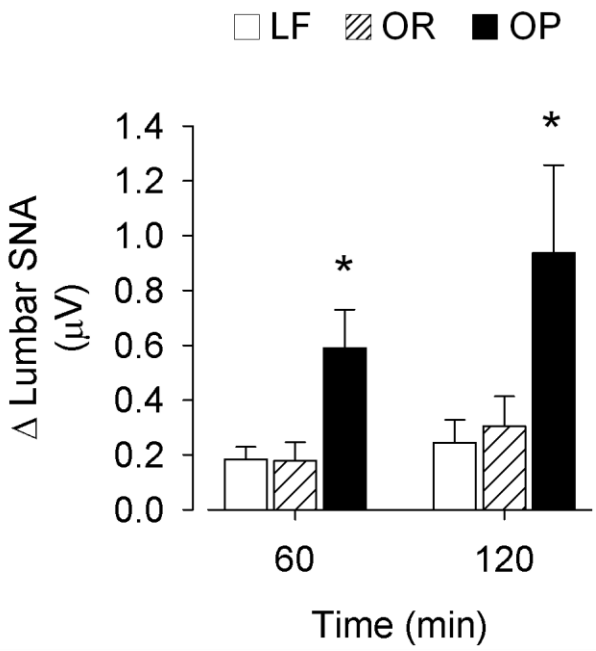
<u>Figure 4.</u> Representative traces from LF, OR, and OP rats during hyperinsulinemic-euglycemic clamps

Representative examples of ABP, mean ABP, heart rate, and lumbar SNA during a hyperinsulinemic-euglycemic clamp in (A) LF, (B) OR, and (C) OP rats.



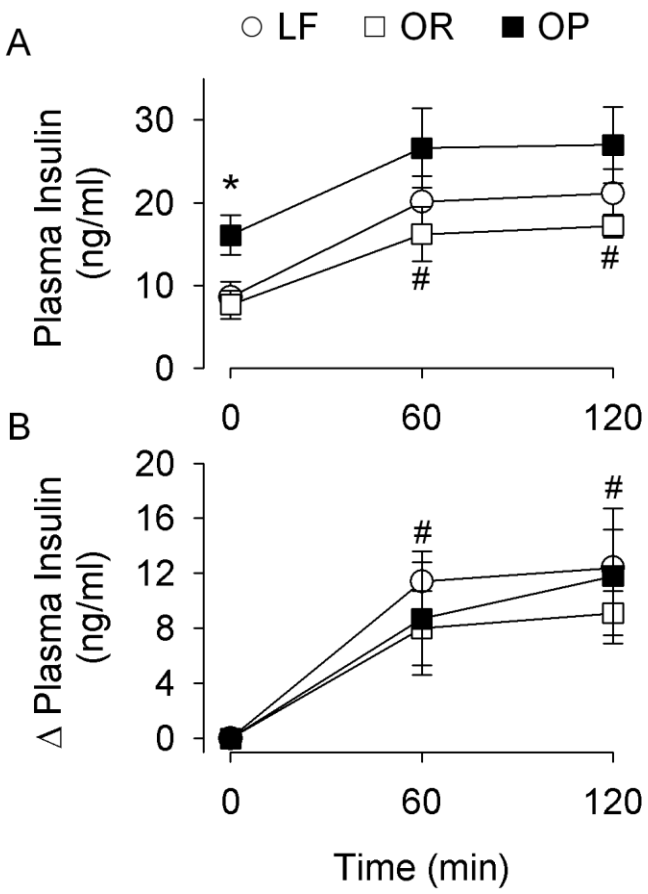
<u>Figure 5.</u> Summary data during a hyperinsulinemic-euglycemic clamps in LF, OR, and OP rats

Mean ± SEM of (A) lumbar SNA, (B) mean ABP, (C) glucose infusion rate, and (D) blood glucose during a hyperinsulinemic-euglycemic clamp in LF (ABP: n=7, SNA: n=8), OR (ABP: n=8, SNA: n=6), and OP (ABP: n=9, SNA: n=9) rats. Hyperinsulinemia significantly elevated lumbar SNA in all three groups; however, the magnitude was not statistically different across groups. * $P<0.05$ OP vs LF or OR, # $P<0.05$ vs baseline.



<u>Figure 6.</u> Change in lumbar SNA at 60 and 120 min during hyperinsulinemic-euglycemic clamps of LF, OR, and OP rats

Change in lumbar SNA of LF, OR, and OP rats during a hyperinsulinemic-euglycemic clamp. *P<0.05 OP vs LF or OR rats



<u>Figure 7.</u> Plasma insulin concentrations during hyperinsulinemic-euglycemic clamps in LF, OR, and OP rats

(A) Plasma insulin concentrations of LF, OR and OP rats at baseline, 60, and 120 min during a hyperinsulinemic-euglycemic clamp. (B) Change in plasma insulin concentration from baseline of LF, OR and OP rats at 120 min. * P<0.05 OP vs LF or OR, # P<0.05 vs baseline.

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

This chapter has previously been published: *Hypertension*. 2010 Feb; 55(2):284-290

Specific aim 2 will identify whether glutamate, angiotensin II type 1, or melanocortin receptors of the RVLM mediate the sympathoexcitatory response to hyperinsulinemia.

Introduction

Compelling evidence in humans and rodents indicates that elevated sympathetic nerve activity (SNA) contributes to the pathogenesis of obesity-induced hypertension (Esler et al. 2006, Wofford and Hall 2004). Clinical studies indicate obese humans have increased norepinephrine spillover (Rumantir et al. 1999, Vaz et al. 1999), elevated muscle SNA (Alvarez et al. 2002, Grassi et al. 1995), and a greater drop in arterial blood pressure (ABP) in response to ganglionic blockade (Shibao et al. 2007). Similar observations have been reported in rodent and dog models of obesity (Boustany et al. 2004, Levin 1993, Stocker et al. 2007). One mechanism postulated to underlie the elevated SNA and ABP during obesity is hyperinsulinemia (Esler et al. 2006, Wofford and Hall 2004). Clinical studies have revealed a correlation between obesity, hypertension, and hyperinsulinemia (Esler et al. 2006, Wofford and Hall 2004). In both humans and rodents, acute hyperinsulinemic-euglycemic clamps selectively increase muscle or lumbar SNA, respectively (Anderson et al. 1991, Morgan et al. 1993, Muntzel et al. 1994). These actions are mediated by a central mechanism because intracerebroventricular administration of insulin causes a similar selective increase in lumbar SNA (Muntzel et al. 1994). In rats, chronic hyperinsulinemic-euglycemic clamps increase total peripheral resistance

and ABP (Brands et al. 1996). However, the neural mechanisms and pathways that mediate the sympathoexcitatory effects of insulin are poorly understood.

The rostral ventrolateral medulla (RVLM) plays a pivotal role in the regulation of SNA and ABP (Guyenet 2006). RVLM neurons project to sympathetic preganglionic neurons of the intermediolateral cell column in the thoracic and lumbar spinal cord and support basal SNA (Guyenet 2006). Electrophysiological studies in vivo have identified tonically active, bulbospinal neurons in the RVLM (Guyenet 2006). The excitability of RVLM neurons is regulated by a number of neurotransmitters including L-glutamate. Injection of L-glutamate into the RVLM increases neuronal discharge, SNA, and ABP (Guyenet 2006). Blockade of glutamate receptors in the RVLM eliminates many sympathoexcitatory reflexes (Guyenet 2006) and lowers ABP in multiple experimental models of hypertension (Ito et al. 2000, Ito et al. 2001, Bergamaschi et al. 1995). Based on this evidence, we hypothesized that glutamate receptor activation in the RVLM mediates the sympathoexcitatory response to hyperinsulinemia.

In addition to glutamate, evidence from several laboratories suggests that the brain renin-angiotensin and melanocortin systems mediate the sympathoexcitatory response to insulin. In this regard, RVLM neurons express Ang II (AT_1) receptors (Song et al. 1991), and injection of Ang II into the RVLM increases SNA and ABP (Dampney et al. 2002). Blockade of brain AT_1 receptors blunts the pressor response to central hyperinsulinemia (Nakata et al. 1998). Also, blockade of the renin-angiotensin system prevents insulin-induced hypertension (Brands et al. 1997). On the other hand, RVLM neurons express melanocortin receptors, (Adan and Gispen 2000) and injection of a melanocortin agonist into the RVLM increases SNA and ABP (Kawabe et al. 2006). Interestingly, the sympathoexcitatory effect to insulin is abolished in melanocortin 4 knockout mice (Rahmouni et al. 2003). Therefore, we hypothesized that one or both of these systems may contribute to the sympathoexcitatory response during hyperinsulinemia.

Materials and Methods

Animals

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

General Procedures

Rats were anesthetized with isoflurane (2-3%) and prepared for recordings of renal and lumbar SNA and ABP as described previously (Scislo et al. 1998, Stocker et al. 2005). Animals were artificially ventilated with oxygen-enriched room air. End-tidal CO_2 and body temperature were maintained at 4-5% and 37±1^{o}C, respectively. After surgery, anesthesia was replaced by α-chloralose (50 mg/kg bolus, 25 mg/kg/hr, IV). The level of anesthesia was examined by the lack of a withdrawal reflex following a foot pinch. When a stable level of anesthesia was established, rats were paralyzed with gallamine triethiodide (20 mg/kg, 0.25 mL/hr, IV). Variables were allowed to stabilize for a minimum of 30 min before the experiment began.

RVLM Microinjections

RVLM microinjections were performed as described previously in our laboratory (Adams et al. 2007). Initially, L-glutamate (1 nmol) was injected into the RVLM at 3 different sites separated by 300 µm in the rostral-caudal plane to identify the site that produced the largest increase in ABP; subsequent injections

were performed at these coordinates. For all experiments, injections (60 nL) were performed over 5 seconds. Injection sites were marked at the end of experiments with 0.2% rhodamine beads.

Hyperinsulinemic-Euglycemic Clamps

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

For purposes of comparison, plasma insulin levels were analyzed from a rodent model of diet-induced obesity. Male Sprague-Dawley rats (200-250g, Charles River Laboratories) were fed a low fat (LF, 10% kcal from fat; Research Diets, Inc, D12489B) or moderately high fat (32% kcal from fat; Research Diets, Inc, D12266B) diet for 13 weeks as described previously by our laboratory (Stocker MD et al. 2007). Those on the high fat diet segregated into obesity resistant (OR) and obesity prone (OP). Rats were anesthetized and prepared as described above. Blood samples were collected from the arterial line, and insulin levels were determined by an ELISA using a commercially available kit (Millipore).

To determine the contribution of RVLM receptors to the SNA response during hyperinsulinemia, separate rats were prepared as described above. Baseline values of ABP, lumbar and renal SNA were recorded for 10 min. Then, insulin (3.75 mU/kg/min, 0.25 ml/hr, IV) and a 50% dextrose solution (0.25-1.0 ml/hr, IV) were infused for 120 min. At 90 min, one of several compounds was

bilaterally microinjected into the RVLM: the ionotropic glutamate receptor antagonist kynurenic acid (KYN, 5 mM), the NMDA receptor antagonist AP5 (5 mmol), the non-NMDA receptor antagonist NBQX (1 mmol), the AT_1 receptor antagonist losartan (1 nmol), the melanocortin 3/4 receptor antagonist SHU9119 (0.03 nmol), or artificial cerebrospinal fluid (aCSF, 60nL). Doses of various receptor antagonists were based on previous studies (Kawabe et al. 2006, Adams et al. 2008, Kiely and Gordon 1994) or preliminary studies in which the antagonist blocked the sympathetic and pressor responses to the respective agonist (data not shown).

To determine the time course of action for KYN, animals were prepared as described above, and the sciatic nerve was stimulated electrically (5 sec train, 500 µA, 20 Hz) before and 10, 20, and 30 min after KYN microinjection into the RVLM .

Central Insulin Injections

Rats were prepared as described above, and insulin (5, 0.5, 0.05, or 0.0005 µU/nL, 60 nL) was bilaterally microinjected into the RVLM. ABP and SNA were recorded for 60 min and blood glucose measured every 30 min. The insulin concentrations were based on previous studies using intracerebroventricular injection of insulin (Muntzel et al. 1994, Rahmouni et al. 2004) and re-calculated due to a minimum 10-fold dilution due to the CSF volume of the lateral and 3rd ventricles.

In a separate group of rats, intracerebroventricular cannulas were implanted in the lateral ventricle as described previously (Stocker et al. 2003). Proper cannula location was verified by a positive drinking test (>3 mL in 30 min) to angiotensin II (20ng/2µL) (Stocker et al. 2003). Then, rats were prepared as described above, and insulin (100 mU/2uL) was injected into the lateral ventricle. This dose of insulin has been repeatedly demonstrated to significantly elevate lumbar SNA in rodents (Muntzel et al. 1994, Rahmouni et al. 2004). Variables

were recorded for 60 min, and blood glucose was measured every 30 min. At the end of experiments, cannula placement was verified again by the spread of dye (1% Evan's Blue Dye, 2µL) to the 3rd and 4th ventricle.

Western Blot Analysis of Insulin Receptors

RVLM and hypothalamic samples were collected for western blot analysis at baseline or 60 min after a hyperinsulinemic-euglycemic clamp. Rats were deeply anesthetized with 5% isoflurane and perfused transcardially with cold oxygenated aCSF (124 mmol/L NaCl, 26 mmol/L NaHCO3, 0.6 mmol/L NaH2PO4, 3mmol/L KCl, 1.6 mmol/L MgCl2, 1.5 mmol/L CaCl2, 11 mmol/L glucose, pH 7.4). The brain was rapidly removed. A chunk of the mediobasal hypothalamus defined dorsally by the top of the 3rd ventricle, laterally by the optic tract, rostrally by the optic chiasm, and caudally by the mammillary bodies was frozen and stored at -80^{o}C. The brainstem was sectioned at 200µm in oxygenated cerebral spinal fluid (4^{o}C) using a vibratome. The RVLM was isolated under a microscope, immediately frozen on dry ice and stored at -80^{o}C. Western blots were performed using a rabbit polyclonal insulin receptor β (IR-β) antibody (1:1000, C-19; sc-711; Santa Cruz Biotechnology, Inc. Santa Cruz, CA.). The IR-β band intensity for each sample was quantified using NIH ImageJ (http://rsb.info.nih.gov/nih-image/) and normalized to the respective γ-tubulin band intensity.

Frozen RVLM or hypothalamus sections were disrupted in 150 µl of homogenization buffer [1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM NaCl, 400 mM KCl, 25 mM β-glycerophosphate, 50 mM NaF, 5 mM benzamidine, 20 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1 mM sodium orthovanadate, 5 mM N-ethylmaleimide, 1 mM PMSF) supplemented with protease inhibitor cocktail (P8340; Sigma, St Louis, MO)] using a Pyrex Potter-Elvehjem tissue grinder. To remove any insoluble particulate the tissue homogenates were centrifuged (10,000 x *g*, 10 min, 4 °C) and the supernatant

transferred to a new microcentrifuge tube. Protein concentration of each sample was determined using the Bio-Rad DC protein Assay (Hercules, CA) according to the manufacturer's directions. Five micrograms of each sample was precipitated using the methanol:chloroform procedure and then resuspended in 20 µl of 1X sample buffer. Samples were prepared for electrophoresis by heating for 5 min at 100 °C. Samples were separated by SDS-PAGE (7.5% gel) and then transferred to nitrocellulose membrane (0.2 µm) (Bio-Rad, Hercules, CA). The membrane was incubated in blocking buffer (5% nonfat dry milk in TBS plus 0.1% Tween-20 [TBS-T]) for 1 hr at room temperature and then incubated in blocking buffer overnight at 4°C with a rabbit polyclonal insulin receptor β (IR-β)antibody (1:1000, C-19; sc-711; Santa Cruz Biotechnology, Inc. Santa Cruz, CA.). After the overnight incubation, the membrane was washed (4x, 5min) in TBS-T, incubated with anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000, 1 hr) at room temperature, incubated in ECL plus for 5 min (GE Healthcare, Piscataway, NJ) and exposed to X-ray film. To determine specificity of the IR-β antibody, the membrane was stripped and reprobed with the IR-β antibody pre-absorbed with five-fold excess of a blocking peptide (sc-711P; Santa Cruz Biotechnology, Inc. Santa Cruz, CA.). The membrane was also stripped and reprobed with a mouse monoclonal γ-tubulin antibody (T6557; Sigma-Aldrich, St. Louis, MO). The IR-β band intensity for each sample was quantified using NIH ImageJ (http://rsb.info.nih.gov/nih-image/) and normalized to the respective γ-tubulin band intensity.

Data Analysis

All data are expressed as mean±SE. Changes in integrated SNA are calculated by subtracting background noise after hexamethonium (30 mg/kg IV). For all variables, 30 sec segments at each time point were compared to three 30-second baseline period measurements. All data were analyzed by a 1- or 2- way ANOVA with repeated measures when appropriate. All post hoc tests were

performed with independent or paired *t* tests with a layered Bonferonni correction. A $P<0.05$ was statistically significant.

Results

Analysis of Plasma Insulin Levels

Initial experiments were performed to identify an insulin infusion rate which produced physiological increases in plasma insulin levels. Both infusion rates significantly increased plasma insulin concentrations at 60 and 120 min (Figure 8). Plasma insulin levels were significantly greater in rats infused with 7.5 vs 3.75 mU/kg/min vs saline infusion.

To compare these infusion rates to a rodent model of obesity, we analyzed plasma insulin levels from rats maintained on a low-fat or moderate high-fat diet for 13 weeks. As previously reported (Stocker SD et al. 2007), OP rats weighed significantly more than LF or OR rats (OP: 793±13g, LF: 612±16g, OR: 596±8g). The greater body weight of OP rats was associated with a greater fat pad mass and higher adiposity index than LF or OR rats (Table 2). As expected, plasma insulin levels were significantly higher in OP versus LF or OR rats (Figure 8). In fact, plasma insulin levels of OP rats were similar to those rats infused with 7.5 mU/kg/min and significantly higher than those rats infused with 3.75 mU/kg/min. Plasma insulin levels of LF and OR rats were not different versus those of rats infused with 3.75 mU/kg/min.

Blockade of Glutamatergic Receptors Reverses Sympathoexcitatory Response to Insulin

A major goal of this study was to determine whether blockade of glutamate receptors in the RVLM reversed or attenuated the sympathoexcitation during hyperinsulinemia. Figure 9 illustrates a representative example of the responses to a hyperinsulinemic-euglycemic clamp or saline infusion before and after RVLM microinjection of KYN. Group data are summarized in Figure 10. As previously reported, hyperinsulinemia selectively increased lumbar SNA (Morgan

et al. 1993, Muntzel et al. 1994), but did not affect ABP (Figure 9), blood glucose (Figure 10), renal SNA (data not shown), or heart rate (data not shown).

Microinjection of KYN significantly reduced lumbar SNA in hyperinsulinemic animals but had no effect in saline-infused animals (Figures 9 and 10). In fact, lumbar SNA was reduced to a level not different from saline infused animals ($P>0.3$). At 120 min, lumbar SNA returned to pre-injection values. Microinjection of aCSF had no effect on any variable in hyperinsulinemic or control rats (Figure 11). Figure 12 summarizes the peak changes in lumbar SNA and mean ABP after injection of aCSF or KYN in hyperinsulemic or control rats. Although the hyperinsulemic-euglycemic clamp did not significantly alter ABP, microinjection of KYN significantly decreased mean ABP. Microinjection of KYN or aCSF did not alter renal SNA or heart rate (data not shown).

Since lumbar SNA returned to preinjection values at 30 min after KYN injection, an additional set of experiments was performed to determine the time course of ionotropic receptor blockade by KYN. We compared the sympathoexcitatory response to activation of somatic afferents before and after RVLM injection of KYN. Prior to blockade, electrical stimulation of sciatic afferents significantly increased mean ABP, renal SNA, and heart rate (Table 3). As expected, microinjection of KYN into the RVLM significantly attenuated these responses at 10 and 20 min. However, the sympathoexcitatory responses at 30 min were not different from baseline responses.

Blockade of NMDA but not non-NMDA Receptors Reverses the Sympathoexcitatory Response to Insulin

Since blockade of iontotropic glutamate receptors with KYN reversed the sympathoexcitatory response to insulin, an additional set of experiments was performed to identify the specific receptor subtype. Microinjection of the NMDA receptor antagonist AP5 significantly reduced lumbar SNA in hyperinsulinemic animals (90 min: 142±6% vs peak: 115±9 %, $P<0.05$). While AP5 did not affect

absolute values of mean ABP (90 min: 128±7 vs peak: 121 ±7 mmHg), AP5 did cause a significant drop in mean ABP (Figure 12). Interestingly, the fall in lumbar SNA and mean ABP of hyperinsulinemic rats was similar between KYN and AP5 (Figure 12). In contrast, microinjection of the non-NMDA receptor antagonist NBQX did not affect lumbar SNA (90 min: 142±12 vs peak: 148± 13 %) or mean ABP (90 min: 105± 7 vs peak: 110± 6 mmHg). AP5 and NBQX did not affect lumbar SNA or ABP in saline-infused animals.

RVLM AT_1 and Melanocortin Receptors Do Not Mediate Insulin-Induced Sympathoexcitation

In contrast to blockade of glutamate receptors, microinjection of the AT_1 receptor antagonist losartan or the melanocortin receptor antagonist SHU9119 did not affect the sympathoexcitatory response to hyperinsulinemia. Peak changes in lumbar SNA and ABP after microinjection of losartan or SHU9119 are illustrated in Figure 13. As expected, the hyperinsulinemic-euglycemic clamp significantly increased lumbar SNA at 90 min ($P<0.01$) but did not change mean ABP, renal SNA, or heart rate (data not shown). Microinjection of losartan did not decrease lumbar SNA (90 min: 138±12 vs peak: 147±12 %) or mean ABP (90 min: 111±8 vs peak: 120±8 mmHg). Similarly, microinjection of SHU9119 did not decrease lumbar SNA (90 min: 134±8 vs peak: 147±17 %) or mean ABP (90 min: 96±6 to peak: 109±5 mmHg). Losartan and SHU9119 did not affect lumbar SNA or ABP in saline-infused animals (data not shown).

Insulin Receptor Expression and Insulin Microinjection in the RVLM

To determine whether insulin may act directly in the RVLM to increase SNA, we analyzed insulin receptor expression and sympathetic responses to microinjection of insulin in the RVLM. Insulin receptor expression was significantly lower in the RVLM compared to the ventromedial hypothalamus

(Figure 14A). In fact, the IR-β band in RVLM samples was virtually absent (Figure 14A) and was not altered by a hyperinsulinemic-euglycemic clamp (data not shown).

RVLM microinjection of insulin at any dose did not alter lumbar SNA or ABP (Figure 14B). In marked contrast, injection of insulin into the lateral ventricle significantly increased lumbar SNA. Plasma glucose levels were not altered by RVLM or lateral ventricle injection of insulin (data not shown).

Histology

All injection sites were centered in the RVLM defined as the triangular region located 0 to 600 µm caudal to the caudal pole of the facial nucleus and bordered dorsally by nucleus ambiguous, medially by the inferior olive or pyramidal tracts, and laterally by the spinal trigeminal nucleus (Figure 15).

Discussion

Previous studies have demonstrated that hyperinsulinemic-euglycemic clamps produce non-uniform increases in SNA (Anderson et al. 1991, Morgan et al. 1993, Anderson et al. 1992). However, the neural mechanisms or brain regions by which insulin acts to selectively increase lumbar SNA have not been identified. The present study provides several novel findings: 1) a hyperinsulinemic clamp with physiological increases in plasma insulin levels elevated lumbar SNA, 2) blockade of glutamatergic, and more specifically NMDA, receptors reversed the sympathoexcitatory effects of hyperinsulinemia, 3) blockade of RVLM AT_1 or melanocortin 3/4 receptors did not affect the sympathoexcitatory response to insulin, 4) the RVLM has a low expression of insulin receptors, and 5) microinjection of insulin into the RVLM did not elevate lumbar SNA. Collectively, these findings suggest insulin activates a NMDA-dependent glutamatergic pathway to the RVLM to increase lumbar SNA.

To identify a physiologically relevant dose of insulin, we compared plasma insulin levels between control rats infused with insulin versus diet-induced obese rats. This model of diet-induced obesity has similar characteristics to obese humans such as activation of the renin-angiotensin system, hyperleptinemia, hyperinsulinemia, elevated sympathetic outflow, and hypertension (Boustany et al. 2004, Boustany et al. 2005, Dobrian et al. 2000, Levin and Keesey 1998, Levin 1983). Indeed, the plasma insulin levels of control rats infused with 3.75 mU/kg/min were significantly lower than those of OP rats. Although there was no difference between LF or OR rats versus control rats infused with 3.75 mU/kg/min, LF and OR rats were 13-15 weeks older and had a greater fat pad mass and higher adiposity index than the control rats. Additional data indicate that plasma insulin levels in obese Zucker rats (13-15 weeks) are not different from those of control rats infused with 3.75 mU/kg/min (Table 4). Collectively, these data indicate that the insulin infusion rate in the present study is physiologically relevant. Whether the elevation in circulating insulin contributes

to the elevated sympathetic outflow and hypertension in these rodent models of obesity is unknown.

Glutamate neurotransmission in the RVLM mediates a number of sympathoexcitatory reflexes including the responses to hypoxia and activation of somatic afferents (Guyenet 2006). Blockade of RVLM ionotropic glutamate receptors also lowers ABP in a number of experimental models of hypertension associated with elevated sympathetic outflow (Ito et al. 2000, Ito et al. 2001, Bergamaschi et al. 1995). In the present study, RVLM injection of KYN, but not losartan or SHU 9119, completely reversed the sympathoexcitatory response to hyperinsulinemia. Although lumbar SNA returned to preinjection levels at 30 min after KYN injection, this response is consistent with the time course of ionotropic receptor blockade with KYN. These findings support two important conclusions: 1) insulin activates the brain renin-angiotensin and melanocortin systems outside the RVLM (ie, hypothalamus), and 2) ionotropic glutamate receptors in the RVLM mediate the sympathoexcitatory actions to hyperinsulinemia. Subsequent experiments clearly demonstrate that NMDA receptors solely mediate this response. The ability of KYN or AP5 to reverse the sympathoexcitatory effects of hyperinsulinemia cannot be attributed to a direct modulatory role of insulin within the RVLM as insulin receptor expression is low, and direct injection of insulin into the RVLM did not alter lumbar SNA and ABP. Therefore, insulin activates a glutamatergic NMDA-dependent pathway to the RVLM to elevate SNA.

The origin of the insulin-driven glutamatergic pathway to the RVLM is not known. The sources of glutamatergic input to the RVLM have not been completely identified; however, the RVLM is densely innervated by glutamatergic neurons in the hypothalamic paraventricular nucleus (Stocker et al. 2006). Interestingly, preliminary data from our laboratory indicate that inhibition of the hypothalamic paraventricular nucleus reverses the sympathoexcitatory response to hyperinsulinemia (Stocker et al. 2007). Although previous studies have reported insulin receptor binding in the hypothalamic paraventricular nucleus

(Werther et al. 1987), it is not known whether insulin acts directly on these neurons or elsewhere to elevate SNA. A number of other hypothalamic structures also express insulin receptors including the arcuate nucleus, ventromedial hypothalamus, and circumventricular organs of the forebrain lamina terminalis (Werther et al. 1987). However, there are no available studies that have systemically examined the contribution of these various structures to the sympathoexcitatory response to insulin and whether such neurons detect circulating insulin. To date, previous studies have demonstrated that either global inhibition of hypothalamic PI_3K (Rahmouni et al. 2004) or lesion of the anteroventral third ventricular region (Muntzel et al. 1994) attenuates the increase in lumbar SNA during hyperinsulinemia. Clearly, future experiments are needed to identify the neurons that detect changes in circulating insulin and how this translates into activation of a glutamatergic pathway to the RVLM to increase lumbar SNA.

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

Perspectives

Clinical studies have revealed a correlation between obesity, hypertension, and hyperinsulinemia (Esler et al. 2006, Wofford and Hall 2004), but the role of insulin in hypertension remains controversial. In rats, acute hyperinsulinemia elevates lumbar SNA, and chronic hyperinsulinemic-euglycemic clamps increase total peripheral resistance and ABP (Morgan et al. 1993, Muntzel et al. 1994). In

contrast, studies performed in dogs have reported that peripheral infusion of insulin did not elevate ABP (Brands et al. 1991, Hildebrandt et al. 1999). The discrepancy between data from rats versus dogs may be explained by greater peripheral insulin sensitivity in dogs. Consistent with this notion, a hyperinsulinemic-euglycemic clamp in dogs increased, rather than decreased, cardiac output thereby indicating a systemic vasodilatory response and no change in ABP (Brands et al. 1991). Unfortunately, it is not known whether dogs exhibit a similar sympathoexcitatory response to insulin as previously reported in mice (Rahmouni et al. 2004, Rahmouni et al. 2004), rats (Morgan et al. 1993, Muntzel et al. 1994), and humans (Anderson et al. 1991, Anderson et al. 1992). Due to the absence of experimental tools to directly assess the contribution of insulin to these chronic diseases, the role of insulin in obesity-related hypertension or other disease states of hyperinsulinemia will likely remain controversial. Yet, the present findings provide a potential model to examine the pathways and mechanisms that may contribute or support the elevated SNA during obesity-related hypertension.

Table 2. Characteristics for LF, OR, and OP rats

	Group		
Characteristic	LF	OR	OP
Initial Body Weight, g	221±7	233±2	245±4*
Final Body Weight, g	612±16	596±8	793±13*
Fat Pads			
Epididymal, g	14±1	16±1	28±1*
Retroperitoneal, g	20±3	21±1	38±2*
Total, g	34±4	36±3	67±3*
Adiposity Index, %	5.5±0.6	6.1±0.4	8.4±0.4*

Values are mean ± SEM. *Significant difference versus LF or OR rats ($P<0.05$).

Table 3. Effect of Ionotropic Receptor Blockade on the Sympathoexcitatory Reflex to Activation of Somatic Afferents

		Stimulation Time		
Characteristic	Baseline	10 min	20 min	30 min
∆ Mean ABP (mmHg)	31±4	1±4 *	6±4 *	23±6
∆ Renal SNA (%)	66±15	7±6 *	26±9 *	39±9
∆ Heart Rate (BPM)	14±2	1±1 *	5±2 *	13±3

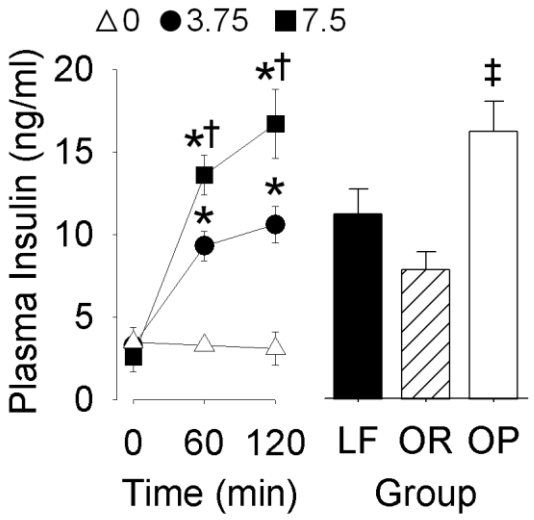
Values are mean ± SEM and represent changes in mean ABP, SNA, and heart rate during electrical stimulation of sciatic afferents. KYN was bilaterally injected into the RVLM at time = 0. Note that the sympathoexcitatory response was attenuated at 10 and 20 min after KYN injection but returned at 30 min.
*Significant difference vs baseline values microinjection into the RVLM, $P<0.05$.
Baseline mean ABP: 133±1 mmHg, baseline heart rate: 426±4 beats per minute, n=5

Table 4. Characteristics of Lean and Obese Zucker Rats

Characteristic	Lean	Obese
Age, weeks	15.3±0.5	15.8±0.4
Body Weight, g	376±9	564±21*
Insulin, ng/mL	1.1±0.2	10.5±3.5*

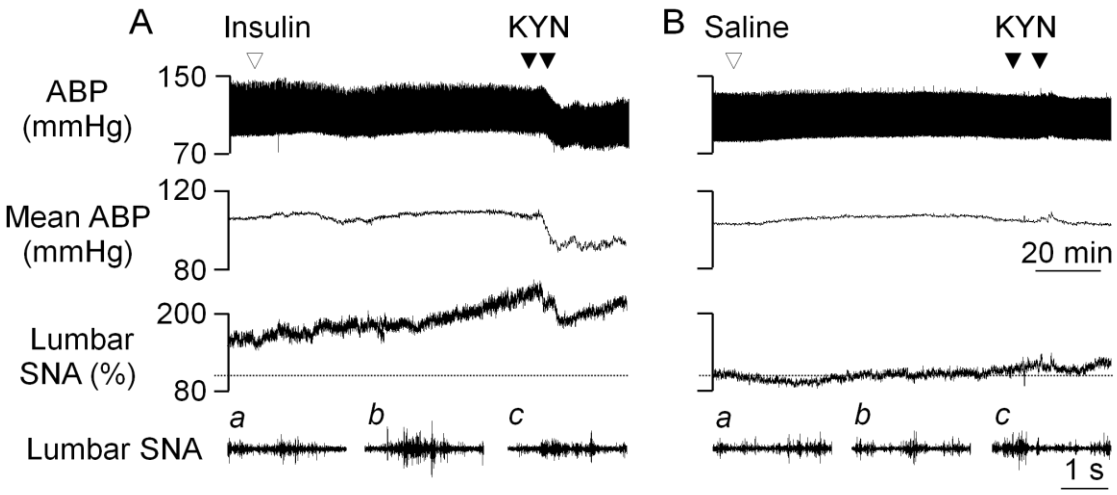
Values are mean ± SEM. *Significant difference versus lean ($P<0.05$).

Plasma insulin levels were analyzed from samples of lean and obese Zucker rats generously provided by Dr. David Stepp (Medical College of Georgia). Half the rats were fasted overnight and rats were anesthetized with isoflurane, decapitated, and trunk blood collected. Insulin levels were determined by an ELISA using a commercially available kit (Millipore).



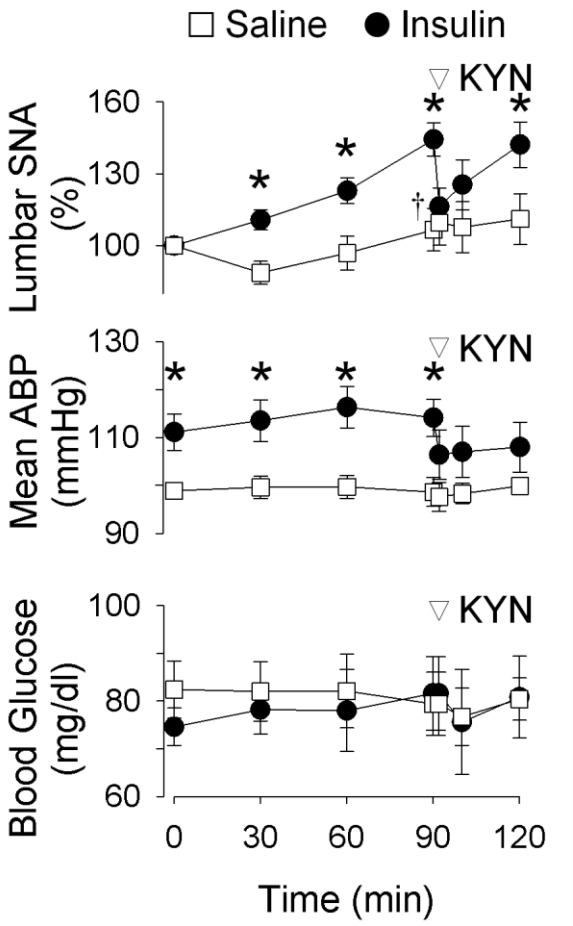
<u>Figure 8.</u> Plasma insulin concentrations during a hyperinsulinemic-euglycemic clamp compared to plasma insulin concentrations from LF, OR, and OP rats.

Plasma insulin concentrations at baseline, 60, and 120 min during a hyperinsulinemic-euglycemic clamp (3.75 mU/kg/min, n=9; 7.5 mU/kg/min, n=3) or saline infusion (n=3). Plasma insulin concentrations from LF (n=5), OR (n=5) and OP (n=6) rats were analyzed for purposes of comparison. Plasma insulin levels were not different between OP rats and control rats infused with 7.5 mU/kg/min. *Significant difference vs baseline levels ($P<0.05$), †Significant difference versus 3.75 mU/kg/min ($P<0.05$), ‡Significant difference versus LF and OR rats ($P<0.05$).



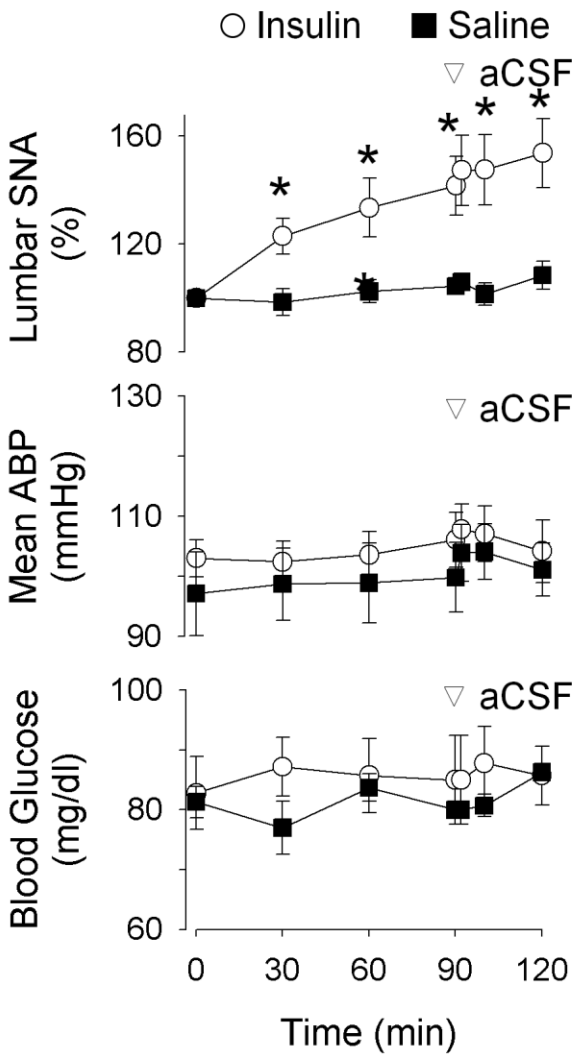
<u>Figure 9.</u> KYN significantly decreases SNA and ABP during hyperinsulinemic-euglycemic clamp

Representative examples of ABP, mean ABP, and lumbar SNA during RVLM microinjection of KYN in rats receiving a (A) hyperinsulinemic-euglycemic clamp or (B) saline infusion. Traces for raw lumbar SNA represent (*a*) baseline, (*b*) peak infusion, and (*c*) post-KYN injection.



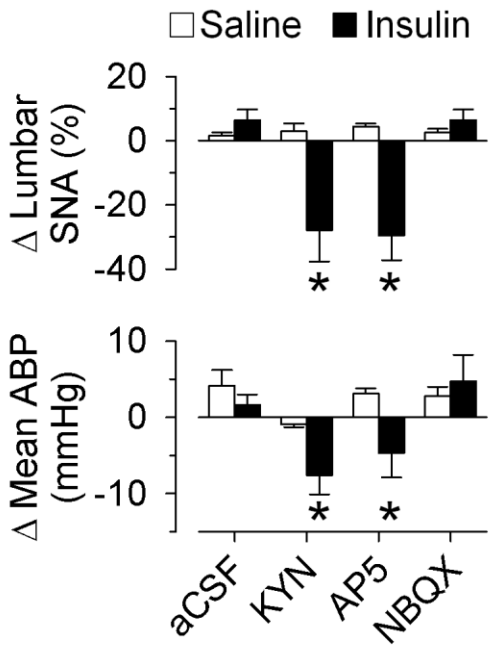
<u>Figure 10.</u> Summary data of rats receiving saline or insulin infusion during RVLM microinjection of KYN

Summary data of lumbar SNA, mean ABP and blood glucose during RVLM microinjection of KYN (▽) in rats receiving a hyperinsulinemic-euglycemic clamp (●**,** n=5) or saline infusion (□ n=5). Injection of KYN significantly reduced lumbar SNA in rats receiving a hyperinsulinemic clamp but had no effect in those receiving saline infusion. *Significant difference vs saline infused-rats (P<0.05), †Significant difference vs pre-injection or 90-min value (P<0.05).



<u>Figure 11</u>. Summary data of rats receiving saline or insulin infusion during RVLM microinjection of aCSF

Summary figures of lumbar, mean ABP, and blood glucose during 120-minute hyperinsulinemic-euglycemic clamp or saline infusion. ○ Insulin + aCSF (n=7) and ■ saline + aCSF (n=3). *Significant difference vs saline ($P<0.05$)



<u>Figure 12.</u> Peak changes in lumbar SNA and mean ABP after glutamate receptor blockade

Peak changes in lumbar SNA and mean ABP after bilateral microinjection of aCSF, KYN, AP5, or NBQX into the RVLM during a saline infusion (n=3-7 per group) or hyperinsulemic-euglycemic clamp (n=3-7 per group). *Significant difference vs rats infused with saline within same drug treatment or rats infused with insulin + aCSF ($P<0.05$)

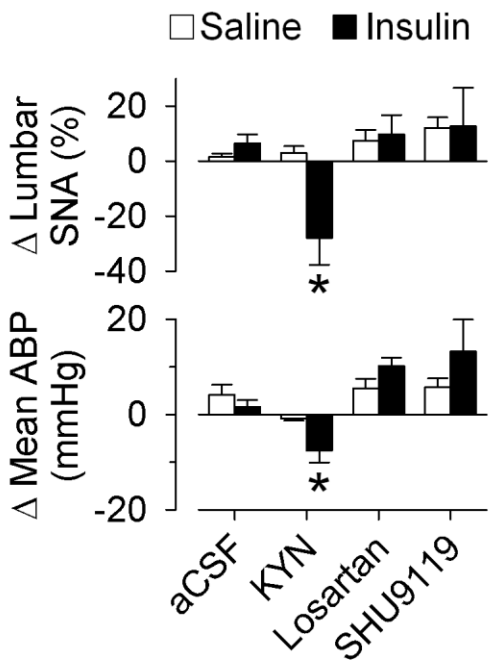


Figure 13. Peak changes in lumbar SNA and mean ABP after drug microinjection into RVLM

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

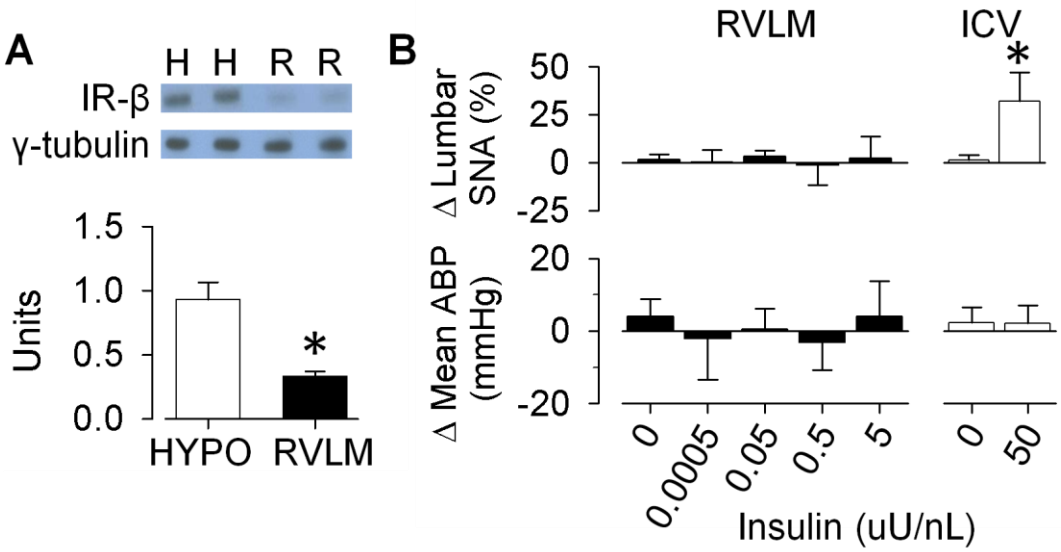


Figure 14. Insulin receptor concentration in the RVLM and lumbar SNA response to RVLM insulin microinjection

(A) Examples of western blot analysis for insulin receptor β and γ-tubulin in the hypothalamus (H) and RVLM (R). Insulin receptor β expression as a ratio to γ-tubulin was significantly lower in the RVLM versus the hypothalamus (n=4 per group, *P<0.01). (B) Change in lumbar SNA and ABP after injection of insulin into the RVLM (n=3-4 per group) or lateral ventricle (n=5). *Significant difference versus aCSF or 0 insulin (P<0.01)

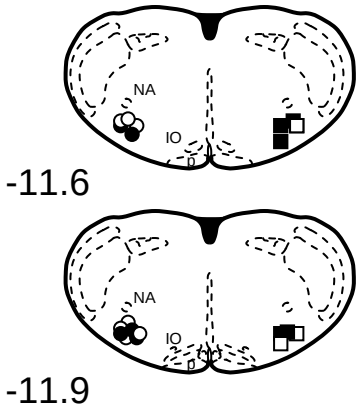


Figure 15. Microinjection sites of KYN and aCSF into the RVLM

Schematic drawings of RVLM injection sites (● Insulin + KYN, ○ insulin + aCSF, ■ saline + KYN, and □ saline + aCSF). Microinjections of AP5, NBQX, losartan and SHU9119 were similar in location (data not shown). Sections represent -11.6 mm (top) and -11.9 mm (bottom) in reference to bregma. IO indicates inferior olive; p, pyramidal tracts; NA, nucleus ambiguous; ST, spinal trigeminal nucleus

Chapter IV: Obesity-Induced Hypertension Depends on Glutamatergic Neurotransmission in the Rostral Ventrolateral Medulla

Specific aim 3 will examine whether glutamate, angiotensin II type 1, or melanocortin receptors of the RVLM are involved in the development of diet-induced obesity hypertension.

Introduction

Obesity is a major risk factor for the development of hypertension, type II diabetes, and cardiovascular disease (Izzo et al. 2008, van Dieren et al. 2010). In fact, the Framingham Heart Study suggests that adiposity is the number one potentially modifiable risk factor predetermining individuals for hypertension (Garrison et al. 1987). Strong evidence indicates that activation of the sympathetic nervous system leads to the etiology of obesity-induced hypertension (Esler et al. 2006, Wofford and Hall 2004). Obese humans have increased norepinephrine spillover to the kidneys and vasculature (Grassi et al. 1985, Vaz et al. 1997) as well as elevated muscle sympathetic nerve activity (SNA) when measured by microneurography (Grassi et al. 1995, Lambert et al. 2007). In addition, ganglionic blockade lowers arterial blood pressure (ABP) to a greater extent in obese hypertensive subjects compared to lean subjects (Shibao et al. 2007) and similar evidence was found in animal models of obesity hypertension (Truett et al. 1996, D'Angelo et al. 2006). Diet-induced obese rats have elevated urinary catecholamines while renal denervation in obese dogs prevents the development of hypertension (Levin 1993, Kassab et al. 1995). Although elevated SNA has been implicated in obesity-induced hypertension, little is known about the central circuits involved in maintaining ABP and SNA in obesity.

Our laboratory demonstrated that inhibition of the rostral ventrolateral medulla (RVLM) caused a significantly greater fall in ABP in obese hypertensive rats compared to controls highlighting a dominant role for the RVLM (Stocker et al. 2007). The RVLM plays a pivotal role in regulating SNA and ABP (Guyenet 2006). RVLM neurons are tonically active and send direct projections to preganglionic neurons of the intermediolateral cell column in the thoracic and lumbar spinal cord which control basal levels of ABP and SNA (Guyenet 2006). The excitability of RVLM neurons is mediated by a number of neurotransmitters including L-glutamate. Microinjection of glutamate into the RVLM increases both SNA and ABP (Ross et al. 1984, Bennaroch et al. 1986) while glutamate receptor blockade eliminates a number of sympathoexcitatory reflexes (Granata et al. 1985, Stornetta et al. 1989, Koshiya et al. 1993) as well as lowering blood pressure in multiple experimental models of hypertension (Bergamaschi et al. 1995, Ito et al. 2000, Ito et al. 2001). In fact, our laboratory recently demonstrated that RVLM glutamatergic receptors are responsible for the elevated lumbar SNA during hyperinsulinemia (Bardgett et al. 2010).

A number of other neurotransmitter systems are implicated in maintaining elevated SNA and ABP in obesity. One such system is the renin-angiotensin system. The major components of the renin-angiotensin system are elevated in obese humans and rodents. Blockade of the renin-angiotensin system in obese-hypertensive humans lowers ABP (Grassi et al. 2003, Bechir et al. 2005) while treatment of diet-induced obese rats with an AT1 receptor antagonist significantly lowers ABP (Boustany et al. 2005). In addition, studies indicate a role for the melanocortin system. Melanocortin-4-receptor knockout mice are obese yet do not develop hypertension (Tallam et al. 2005) and show no sympathoexcitatory response to insulin or leptin (Benoit et al. 2002). As well, blockade of central melanocortin receptors lowers ABP in spontaneously hypertensive rats (da Silva et al. 2008). Given that the RVLM expresses both angiotensin II type 1 (AT_1) and melanocortin 3/4 receptors (Adan and Gispen 1997, Adan and Gispen 2000, Song et al 1991) and activation of either receptor increases ABP and SNA in

rodents (Dampney et al. 2002, Kawabe et al. 2006) we hypothesized that one or both systems may play a role in diet-induced obesity hypertension.

The goal of this study was to elucidate the neural circuits, and specifically to examine the role of multiple neurotransmitter systems in the RVLM, mediating ABP and lumbar SNA in obesity-hypertension. On the basis of previous findings, we hypothesized that RVLM glutamatergic receptors would be responsible for maintaining the elevated ABP and SNA associated with diet-induced obesity.

Materials and Methods

Animals

All of the experimental procedures were approved by the Penn State University Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (Charles River Laboratory) weighing 150 to 200 g were housed in a temperature-controlled room (22^{o}-23^{o} C) with a 14-hour/10-hour light-dark cycle (light on at 7:00 AM). Rats were placed on a LF diet (10% kcal from fat; Research Diets, Inc. D12489B) or a moderate high-fat diet (32% kcal from fat, Research Diets, Inc. D12266B) for 13 weeks. After 5 weeks, rats fed the moderately high-fat diet segregated into OP and OR based on body weight distribution as described previously (Stocker et al. 2007). Briefly, a body weight histogram was constructed and resulted in a distribution of rats into OP and OR groups corresponding with the upper and lower one third of rats, respectively.

Rats were anesthetized with isoflurane (2%-3%, 100% O_2) and prepared for recordings of lumbar SNA and ABP as described previously (Scislo TH et al. 1998, Stocker et al. 2005). Animals were artificially ventilated with oxygen-enriched room air. End-tidal CO_2 and body temperature were maintained at 4% to 4.5% and 37 ± 1^{o} C, respectively. After surgery, anesthesia was replaced by α-chloralose. An initial bolus (50 mg/kg, iv) was administered followed by a continuous infusion (0.017 mL/kg/min of 25 mg/mL, iv). In preliminary experiments, OP rats required a smaller dose of α-chloralose than predicted from body weight, and this dose was equivalent to the amount administered to a LF or OR rat. The level of anesthesia was assessed by the lack of a withdrawal reflex to a foot pinch. Therefore, the dose of α-chloralose for OP rats was initially based on a paired LF or OR rat studied on the same day. The α-chloralose

infusion was adjusted as needed by the presence of a withdrawal reflex. Anesthesia and variables were allowed to stabilize for a minimum of 60 min before the experiment began.

RVLM Microinjections

RVLM microinjections were performed as described previously in our laboratory (Adams et al. 2007). Initially, L-glutamate (0.1 nmol) was injected into the RVLM at 3 different sites separated by 300 µm in the rostral-caudal plane to identify the site that produced the largest increase in ABP; subsequent injections were performed at these coordinates. For all experiments, injections (60 nL) were performed over 5 seconds. Injection sites were marked at the end of experiments with 0.2% rhodamine beads.

Baseline values of ABP and lumbar were recorded for 20 minutes. Then the glutamate receptor antagonist kynurenic acid (25 mM), the AT1 receptor antagonist losartan (1 nmol), the melanocortin receptor antagonist SHU 9119 (0.5 mmol/L), or the control artificial cerebrospinal fluid (60nL) were bilaterally microinjected into the RVLM. Variables were then recorded for another 45 minutes. Doses of various receptor antagonists were based on previous studies (Bardgett et al. 2010, Kawabe et al. 2006, Adams et al. 2008, Kiely and Gordon 1994).

Data Analysis

All data are expressed as means ± SEM. Changes in integrated SNA are calculated by subtracting background noise after hexamethonium (30 mg/kg, IV). For all variables, 60-second segments at each time point were compared to three 60-second baseline period measurements. All data were analyzed by a 1- or 2-way ANOVA with repeated measures when appropriate. All post hoc tests were

performed with independent or paired *t* tests with a layered Bonferroni correction. A $P<0.05$ was statistically significant.

Results

Characteristics of LF, OR, and OP Rats

Initial body weights were not different between LF, OR, or OP rats (Figure 16). By 4 weeks, OP rats weighed significantly more than either LF or OR rats. This greater weight gain was associated with increased epididymal and retroperitoneal fat mass and consequently an elevated adiposity index (Table 5).

Blockade of Glutamate Receptors Lowers ABP and SNA in OP Rats

The major goal of this study was to determine whether blockade of glutamate receptors significantly attenuated ABP or SNA in diet-induced obese, hypertensive rats. Figure 17 illustrates a representative trace from a LF, OR, and OP rat before and after blockade of RVLM glutamatergic receptors. Group data are summarized in Figure 18. As previously reported (Stocker et al. 2007) OP rats had significantly elevated ABP compared to LF or OR rats (Figure 18A).

KYN microinjection into the RVLM significantly lowered ABP and lumbar SNA in OP rats but had no effect in either LF or OR rats (Figure 17 and 18). ABP was decreased to a level not significantly different than that seen in LF and OR rats. Microinjection of aCSF had no effect on ABP or SNA in any group. Figure 18 C, D summarize the peak changes in ABP and lumbar SNA after injection of aCSF or KYN in LF, OR, and OP rats.

RVLM AT_1 and Melanocortin 3/4 Receptors Do Not Mediate the Elevated ABP in Obesity

In contrast to blockade of glutamate receptors, microinjection of the angiotensin II type 1 receptor antagonist losartan or the melanocortin receptor antagonist SHU

9119 into the RVLM had no effect on ABP or lumbar SNA in LF, OR, or OP rats. Figures 19 and 20 illustrate representative traces from a LF, OR, and OP rat before and after blockade of AT1 and melanocortin 3/4 receptors, respectively. Peak changes in ABP and lumbar SNA after losartan or SHU 9119 injection are shown in Figure 21. Changes were not significantly different compared to aCSF injection. As expected, ABP was significantly elevated in OP rats compared to LF or OR rats in both groups. Microinjection of losartan did not affect ABP in LF (Baseline: 91±2 mmHg vs peak: 94±4 mmHg), OR (Baseline: 88±2 mmHg vs peak: 90±4 mmHg), or OP (Baseline: 103±2 mmHg vs peak: 107±3 mmHg) rats or lumbar SNA in LF (Baseline: 99±1% vs Peak: 98±1%), OR (Baseline: 100±2% vs peak: 98±3%), or OP (Baseline: 100±1% vs peak: 114±8%) rats. Similarly, microinjection of SHU 9119 did not affect ABP in LF (Baseline: 91±3 mmHg vs peak: 95±3 mmHg), OR (Baseline: 86±2 mmHg vs peak: 95±2 mmHg), or OP (Baseline: 103±3 mmHg vs peak: 107±3 mmHg) or lumbar SNA in LF (Baseline: 99±1% vs peak: 112±7%), OR (Baseline: 98±2% vs peak: 102±2%), or OP (Baseline: 98±1% vs peak: 102±3%) rats.

Glutamate Receptor Activation

Microinjection of L-glutamate (0.1 nmol) significantly elevated ABP and lumbar SNA in LF, OR, and OP rats (Figure 22). The increase in ABP and lumbar SNA were not significantly different between groups.

Histology

All injection sites were centered in the RVLM defined as the triangular region located 0 to 600 µm caudal to the caudal pole of the facial nucleus and bordered dorsally by nucleus ambiguous, medially by the inferior olive or pyramidal tracts, and laterally by the spinal trigeminal nucleus (Figure 23).

Discussion

Previous studies have demonstrated that obesity results in elevated SNA and ABP (Alvarez et al. 2002, Grassi et al. 1995, Lambert et al. 2007). Our laboratory previously demonstrated that the RVLM mediates this elevation as inhibition of RVLM neurons produced a greater fall in ABP in OP rats compared to LF or OR rats (Stocker et al. 2007). However, the neural mechanisms through which diet-induced obesity causes increased activation of RVLM neurons was previously unknown. The present study illustrates that diet-induced obesity hypertension depends on glutamatergic transmission through the RVLM.

The present study assessed the contribution of multiple RVLM receptor systems to the elevation in SNA and ABP in diet-induced obesity. We utilized a rodent model of diet-induced obesity that has previously been shown to result in segregation into OP and OR rats based on differential weight gain. This model provides multiple benefits: it closely emulates human obesity and allows differentiation between effects of obesity/weight gain versus consumption of the moderate high-fat diet. In comparison to human obesity, OP rats have elevated cholesterol, hyperinsulinemia, hyperleptinemia, activation of the renin-angiotensin and sympathetic nervous system, and elevated ABP (Boustany et al. 2004, Levin et al. 1983, Dobrain et al. 2000, Levin and Keesey 1998) due to their increased caloric intake compared to OR rats. In the present study, similar to what we have seen previously, OP rats had a significantly higher body weight, fat pad mass, adiposity index, and mean ABP.

The RVLM is the major vasomotor center with direct projections to the intermediolateral cell column of the thoracic and lumbar spinal cord. Tonic activity of RVLM neurons is known to be responsible for maintaining baseline ABP. The contribution of RVLM neurons to maintaining baseline SNA and ABP is maintained by a balance of excitatory and inhibitory inputs; and it is postulated that hypertension is a result of a shift in the balance between these excitatory and inhibitory inputs to the RVLM. In support of this notion, blockade of RVLM glutamatergic receptors lowers ABP in a number of experimental models of

hypertension. As well, our laboratory recently demonstrated that the elevation in lumbar SNA associated with hyperinsulinemia is dependent on RVLM glutamate receptors (Bardgett et al. 2010). Here, we demonstrate that glutamate neurotransmission in the RVLM is necessary for diet-induced obesity hypertension. Blockade of ionotropic glutamate receptors, using a dose of KYN we previously showed to be sufficient to eliminate the somatic pressor response (Bardgett et al. 2010), lowers lumbar SNA and ABP in OP rats compared to LF or OR rats. In addition, following glutamate receptor blockade, ABP in OP rats was not significantly different from that in LF or OR rats. From this study, it is unclear whether this response is mediated solely through NMDA vs non-NMDA RVLM glutamate receptors. Future studies will be needed in order to determine if it is specific for one or the other but given that the sympathoexcitatory response to hyperinsulinemia was mediated solely through NMDA-glutamate receptors it could be hypothesized that obesity-induced hypertension may also rely on NMDA receptors.

In contrast to the role of RVLM glutamate receptors, this study demonstrated that neither RVLM AT1 nor melanocortin 3/4 receptors mediate diet-induced obesity hypertension. Blockade of the renin-angiotensin system has been shown to reverse or block the development of obesity-induced hypertension (Brands et al. 1997, Boustany et al. 2005). In previous studies, blockade occurred systemically and it is therefore possible that renin-angiotensin system activation plays a more pivotal role in the periphery as compared to centrally. In contrast to the renin-angiotensin system, the roles of melanocortin 3/4 receptors have been implicated centrally. Given that melanocortin 3/4 receptor blockade reverses the elevated ABP in spontaneously hypertensive rats and that melanocortin-4 receptor knockout mice are obese yet not hypertensive it can be hypothesized that the melanocortin system is activated centrally but not in the RVLM. The hypothalamic paraventricular nucleus has projections to the RVLM as well as direct projections to the intermediolateral cell column. Stimulation of the hypothalamic paraventricular nucleus causes an elevation in SNA and ABP.

Therefore, it is possible that either the renin-angiotensin system or melanocortin system may be acting at the level of the hypothalamic paraventricular nucleus.

The pressor response to L-glutamate microinjection into the RVLM was not significantly different between LF, OR, or OP rats. It can therefore be inferred that diet-induced obesity does not result in an increased responsiveness of these neurons to L-glutamate. A greater response to L-glutamate has been demonstrated in rats placed on a high salt diet which was concluded to be the result of increased excitability of RVLM neurons. (Adams et al. 2007) In contrast to those studies, the pressor response in LF, OR, and OP rats was similar. It is not possible to completely rule out a change in concentration of RVLM glutamate receptors but one would expect a greater pressor response if glutamate receptor concentration was elevated in OP rats.

Blockade of glutamate receptors under normotensive conditions does not cause a decrease in SNA or ABP. This is hypothesized to occur because KYN equally blocks both an excitatory and inhibitory drive to the RVLM. In the case of obesity-induced hypertension, what is normally an equal balance is shifted to a greater excitatory drive. This change could be due to an increase in excitatory input or a decrease in inhibitory input to the RVLM. If the shift in a greater excitatory input to the RVLM was due to a decrease in the inhibition of RVLM neurons you would expect to see a greater increase in SNA and ABP during glutamate microinjection. This is based on the findings of Ito and Sved who showed that disruption of the inhibitory pathway to RVLM results in significantly greater pressor responses to glutamate stimulation (Ito and Sved 1997).

Obesity is associated with a number of altered characteristics including hyperinsulinemia, hyperleptinemia, insulin resistance, activation of the renin-angiotensin system, and dysfunction of the baroreceptor reflex. All of these factors are hypothesized to play a role in activation of the sympathetic nervous system and elevation of ABP in diet-induced obesity. From this study it is not possible to ascertain the role of these numerous characteristics. Future studies will be needed to try and determine specifically which characteristics of obesity

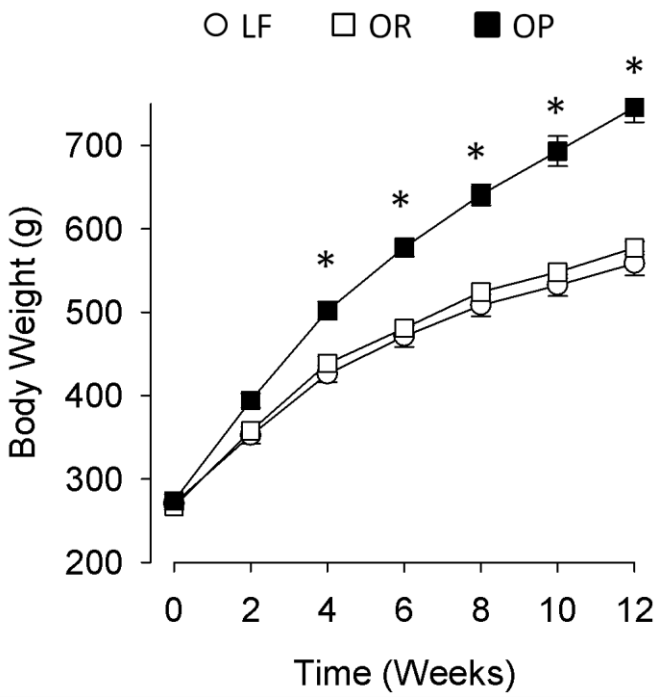
lead to activation of the sympathetic nervous system and subsequent elevation in ABP.

In summary the present findings identify a specific brain region and receptor system involved in mediating obesity-induced hypertension. The results clearly indicate that RVLM glutamatergic receptors are involved in maintaining lumbar SNA and ABP in OP rats. It is also evident that neither RVLM AT1 nor melanocortin 3/4 receptors mediate obesity-induced hypertension.

Table 5. Characteristics of LF, OR, and OP Rats

Characteristic	LF	OR	OP
Body Weight (g)	559±14	577±8	746±18*
Fat Pads (g)			
Epididymal	12.3±1.3	14.3±0.7	23.8±1.9*
Retroperitoneal	12.2±1.2	13.8±0.7	26.8±1.9*
Total	24.5±2.4	28.1±1.2	50.7±4.4*
Adiposity Index (%)	4.3±0.4	4.9±0.2	6.7±0.4*

Values are mean ± SEM. *Significant difference versus LF or OR ($P<0.05$).



<u>Figure 16.</u> Body Weight Distribution for LF, OR, and OP rats

Body weight as a function of time for LF, OR, and OP rats. Values are

Mean ±SEM. n=13 per group. *Significant difference between OP vs LF or OR rats ($P<0.05$)

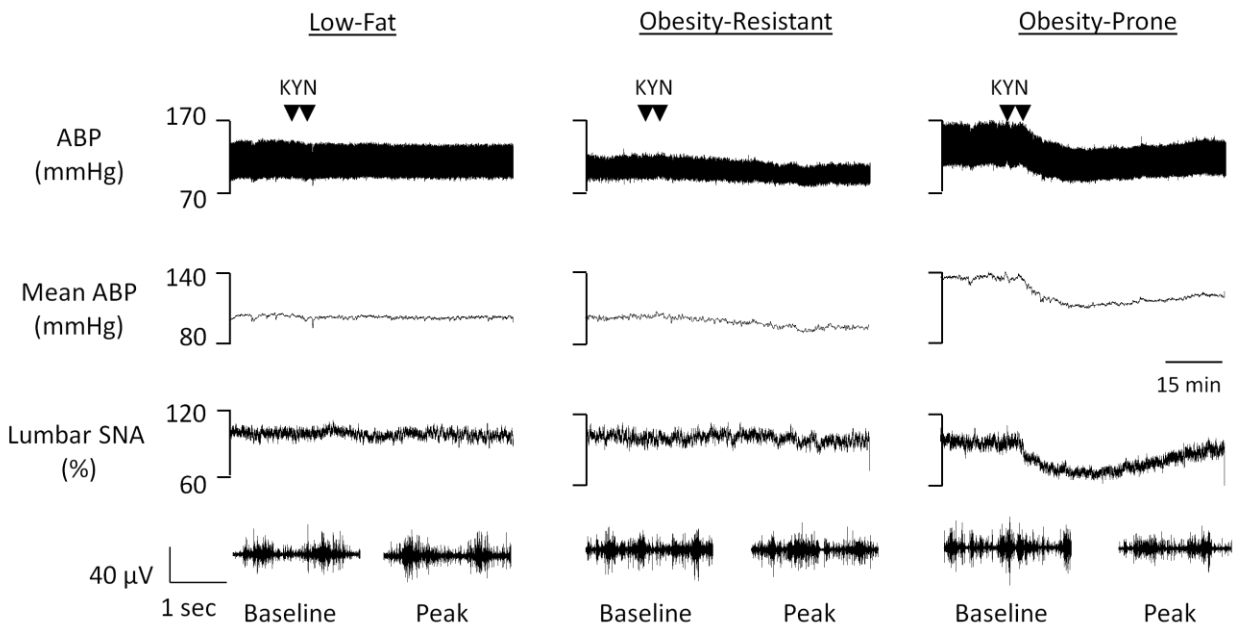
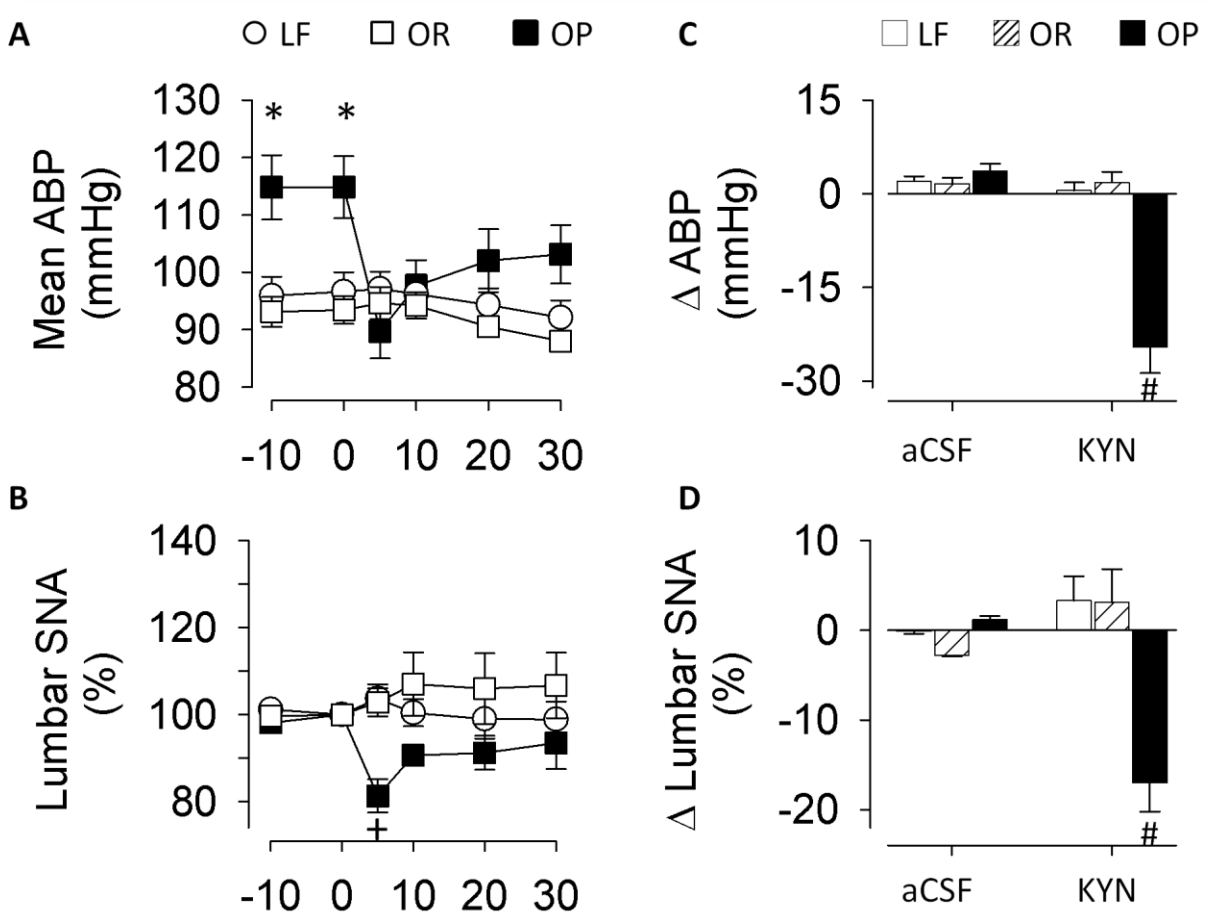


Figure 17. Representative traces of ABP and lumbar SNA from LF, OR, and OP rats before and after KYN microinjection into the RVLM

Representative examples of ABP, mean ABP, lumbar SNA, and raw lumbar SNA before and after KYN microinjection into the RVLM of LF, OR, and OP rats



<u>Figure 18.</u> Summary data before and after KYN microinjection into the RVLM of LF, OR, and OP rats

Summary data of (A) ABP and (B) lumbar SNA before and after RVLM microinjection of KYN and peak changes in (C) ABP and (D) lumbar SNA after bilateral microinjection of KYN into the RVLM of LF (ABP: n=8, SNA: n=7), OR (ABP: n=10, SNA=7), and OP (ABP: n=9, SNA: n=9) rats. * P<0.05 OP vs LF or OR rats, + P<0.05 vs baseline, # P<0.05 vs aCSF microinjection.

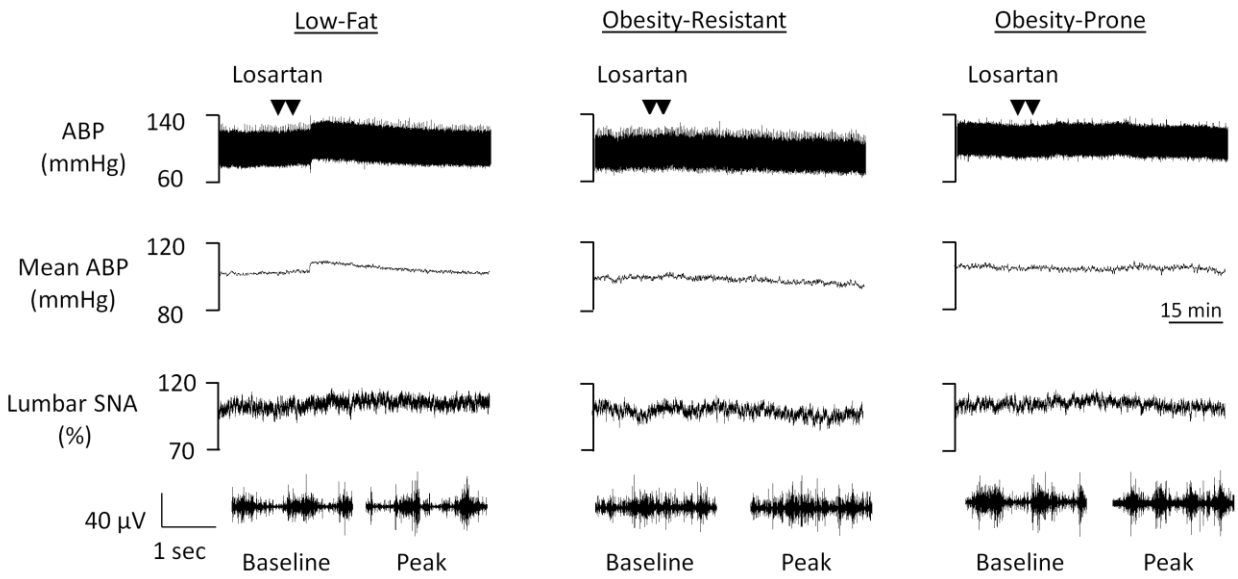
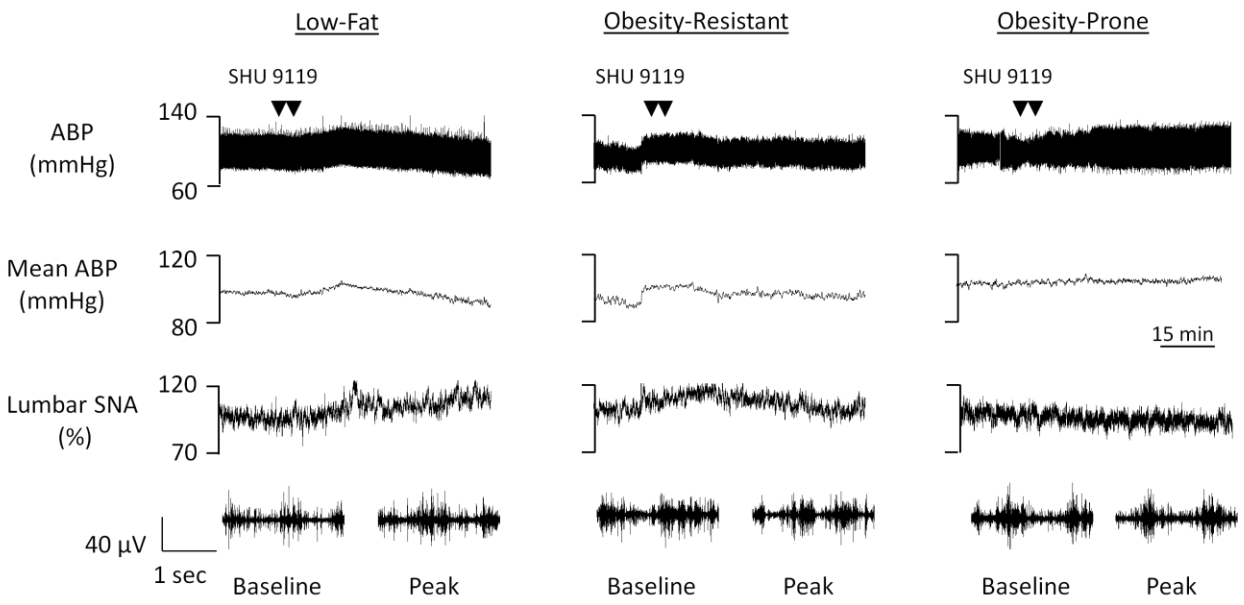


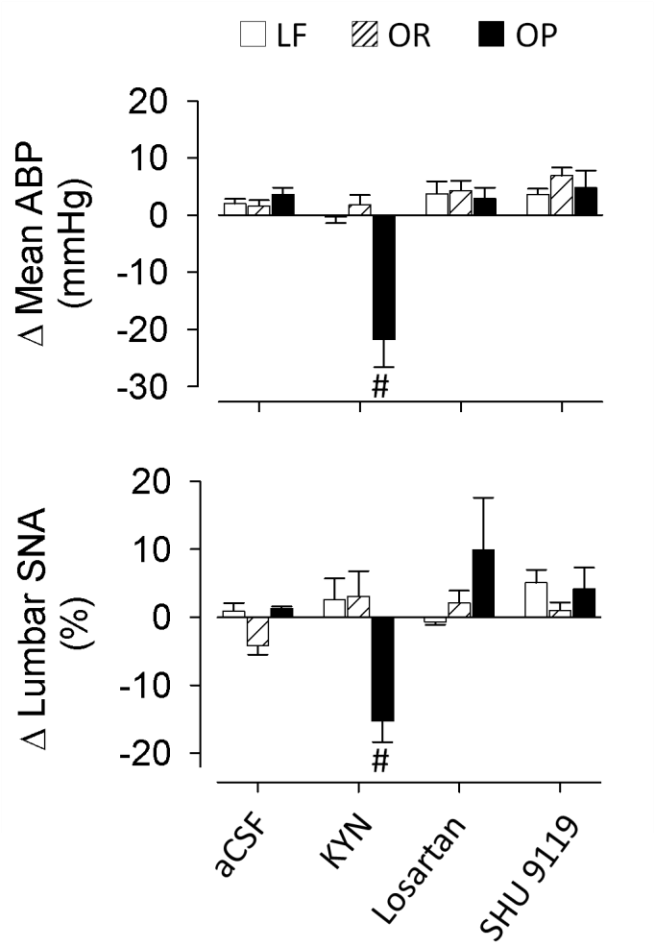
Figure 19. Representative traces from LF, OR, and OP rats before and after losartan microinjection into the RVLM

Representative examples of ABP, mean ABP, lumbar SNA, and raw lumbar SNA before and after losartan microinjection into the RVLM of LF, OR, and OP rats



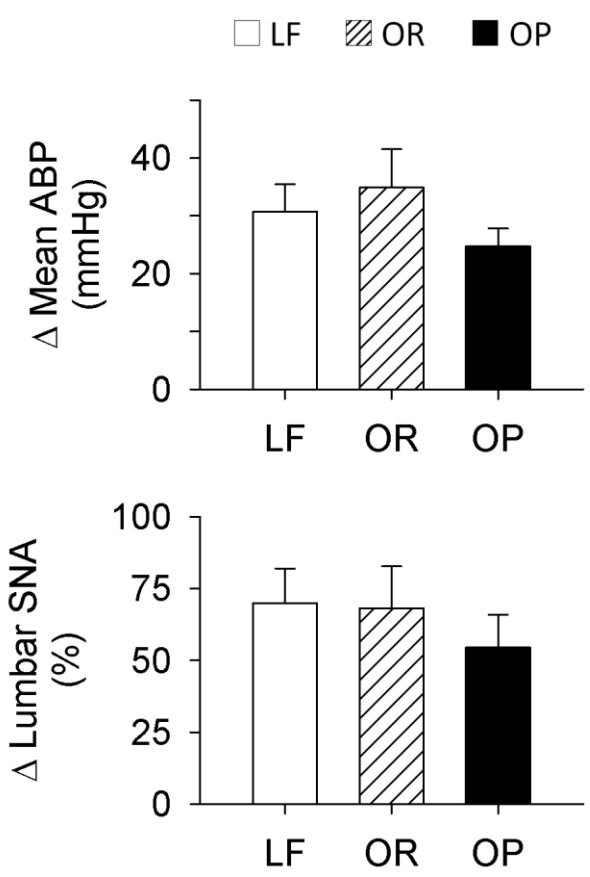
<u>Figure 20.</u> Representative traces from LF, OR, and OP rats before and after SHU 9119 microinjection into the RVLM

Representative examples of ABP, mean ABP, lumbar SNA, and raw lumbar SNA before and after SHU 9119 microinjection into the RVLM of LF, OR, and OP rats



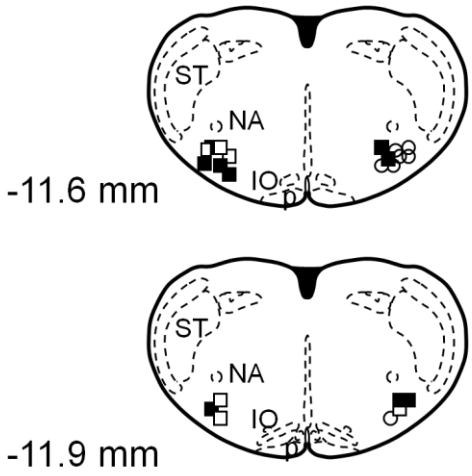
<u>Figure 21.</u> Peak changes in ABP and lumbar SNA in LF, OR, and OP rats

Peak changes in ABP and lumbar SNA after bilateral microinjection of aCSF, KYN, losartan, or SHU 9119 into the RVLM of LF (ABP: n=8-9, SNA: n=7), OR (ABP: n=7, SNA: n=3-6), and OP (ABP: n=8-10, SNA: n=7-8) rats. # $P<0.05$ vs aCSF microinjection.



<u>Figure 22.</u> Peak change in ABP and lumbar SNA following unilateral L-glutamate microinjection into the RVLM of LF, OR, and OP rats

Peak changes in ABP and lumbar SNA after unilateral microinjection of L-glutamate (0.1 nmol, 60nL) into the RVLM of LF, OR, and OP rats. There was no significant difference between changes in any of the 3 groups.



<u>Figure 23</u>. Microinjection sites of KYN into the RVLM of LF, OR, and OP rats

Schematic drawings of RVLM injection sites of KYN (○ LF, □ OR, and ■ OP rats). Microinjections of losartan, SHU 9119, and aCSF were similar in location (data not shown). Sections represent -11.6 mm (top) and -11.9 mm (bottom) in reference to bregma. IO indicates inferior olive; p, pyramidal tracts; NA, nucleus ambiguous; ST, spinal trigeminal nucleus

Chapter V: General Discussion and Conclusions

Obesity is associated with many adverse health effects including essential hypertension. This elevation in ABP is believed to stem from a central origin leading to activation of the sympathetic nervous system. Hyperinsulinemia is unequivocally linked with obesity and is known to activate the sympathetic nervous system. Therefore, my dissertation sought to identify the neural circuits that mediate insulin-induced sympathoexcitation and obesity-induced hypertension as well as to investigate the potential role that insulin might play in obesity hypertension.

These are the first studies to identify a specific brain region and receptor system involved in the elevation of SNA and ABP during hyperinsulinemia and obesity-induced hypertension. These studies present several important findings: 1) plasma insulin concentration is significantly increased in OP rats, 2) OP rats have significantly elevated lumbar SNA and ABP compared to LF or OR rats, 3) diet-induced obesity does not cause resistance to the sympathoexcitatory response to hyperinsulinemia, 4) blockade of NMDA-specific glutamatergic RVLM receptors reverses the increase in lumbar SNA associated with hyperinsulinemia, 5) blockade of RVLM AT1 or melanocortin 3/4 receptors does not affect the sympathoexcitatory response to insulin, 6) the RVLM expresses a low concentration of insulin receptors, 7) microinjection of insulin into the RVLM does not increase lumbar SNA, 8) blockade of RVLM glutamate receptors lowers lumbar SNA and ABP in diet-induced obesity hypertension, and 9) blockade of RVLM AT1 or melanocortin 3/4 receptors does not lower SNA or ABP in LF, OR, or OP rats.

Acute hyperinsulinemic-euglycemic clamps activate the sympathetic nervous system in both humans and rodents (Anderson et al. 1992, Morgan et al. 1993, Muntzel et al. 1994) though a physiologically relevant dose of insulin had never been identified. By comparison of plasma insulin levels to diet-induced obese and obese Zucker rats, we were able to identify a dose of insulin that resulted in comparable levels to that in the varying obese rodent models. It was

this dose of insulin that was subsequently used in all experiments and we did not feel it necessary to use elevated doses as that would result in supraphysiologic plasma insulin levels.

Whether insulin plays a role in activating the sympathetic nervous system and elevating ABP in diet-induced obesity has been a point of contention for many years. A major argument against a role for insulin in this process is that obese humans and rodents become hyperinsulinemic and develop insulin resistance. The efficiency with which skeletal muscle takes up glucose is severely compromised with obesity as is the reduction in food intake normally associated with centrally administered insulin. These findings lead to the belief that insulin could not be responsible for the known increase in SNA in obesity. Therefore, it was of critical importance to illustrate the capability of insulin to increase SNA during diet-induced obesity even when peripheral tissues have become insulin-resistant. The findings presented here illustrate that after development of obesity and onset of peripheral insulin resistance, central circuits are still responsive and produce an elevation in lumbar SNA in OP rats equivalent to that in both LF and OR rats.

The model of diet-induced obesity utilized in these studies produces characteristics similar to human obesity. OP rats show activation of the renin-angiotensin system, hyperleptinemia, hyperinsulinemia, elevated sympathetic outflow and elevated ABP (Boustany et al. 2004, Boustany et al. 2005, Levin et al. 1983, Dobrain et al. 2000, Levin and Keesey 1998). OP rats show elevated lumbar SNA and although renal SNA was not measured in these experiments, renal denervation prevents the development of diet-induced obesity hypertension (Kassab et al. 1995), indicating activation similar to that in obese humans (Vaz et al. 1997). Therefore, this model presents with characteristics best suited to examine that which occurs naturally in human obesity.

It is unknown whether obesity affects insulin's ability to access the central nervous system. Little or no insulin is produced within the brain (Banks 2004) and insulin crosses the blood-brain barrier in a transport specific method (Banks

WA 1997). The blood brain barrier is a mostly impenetrable barrier separating the brain from the substances circulating in the periphery. The capillary bed of the blood brain barrier is not leaky and contains many tight junctions which eliminate intercellular spaces. Blood borne substances are able to cross the blood brain barrier in one of two ways: 1) lipid soluble molecules can diffuse across the capillary membrane or 2) large or insoluble molecules utilize saturable transport systems. Molecules such as insulin, glucose, and free fatty acids cross through a transport mechanism. In the case of obesity, plasma insulin levels are elevated but whether this is associated with an increase in cerebrospinal fluid (CSF) insulin concentration is largely unknown. A multitude of studies have examined the effects of obesity on CSF insulin concentration but the results are varying. Measurement of CSF insulin in Zucker rats demonstrated that obese Zucker rats have a significant increase in baseline CSF insulin compared to lean controls (Stein et al. 1983, Stein et al. 1987), while insulin transport was not affected in a model of diet-induced obesity (Israel et al. 1993). In agreement, CSF insulin levels in obese humans are decreased following a 21 day diet (Owen et al. 1974). In contrast, a study in dogs reported that insulin transport into the CSF was decreased when dogs were placed on a high fat diet for seven weeks (Kaiyala et al. 2000). A similar study showed that the ratio of plasma/CSF insulin was negatively correlated with body mass index, though it is unclear what the relationship was between raw CSF insulin values and body mass index (Kern et al. 2006). In the present studies, if insulin transport was decreased in OP rats a blunted sympathoexcitatory response to hyperinsulinemia would have been expected. Instead; LF, OR, and OP rats showed similar sympathoexcitatory responses to hyperinsulinemia, even a significantly greater increase in lumbar SNA when expressed as microvolts, which indicates no difference in insulin transport across the blood-brain barrier though future studies could involve direct measurement of CSF insulin concentrations.

Having identified a potential role for insulin to act centrally to activate the sympathetic nervous system it was important to examine the neural circuitry involved in mediating this response. From these studies it can be ascertained

that RVLM glutamatergic receptors are mediating both obesity-induced hypertension as well as the sympathoexcitatory effect of hyperinsulinemia. There are two varieties of glutamate receptors, ionotropic and metabotropic. Ionotropic glutamate receptors are ligand-gated ion channels with rapid onset and decay. The two types of ionotropic glutamate receptors, NMDA and AMPA, are located on RVLM neurons. KYN which is a non-specific, competitive glutamate receptor antagonist blocks both NMDA and AMPA receptors. AMPA channels mediate fast synaptic transmission via Na^+ specific flow while NMDA channels are non-specific cation channels mostly permitting flow of Na^+ and Ca^{2+}. NMDA channels allow for long-lasting increases in glutamate receptors and their sensitivity and its ion channel only opens when glutamate is bound to the receptor and the cell is already depolarized removing the Mg^{2+} block (Monaghan et al. 1989). NMDA-specific channels mediate the sympathoexcitatory response to hyperinsulinemia while more studies are needed in order to determine whether NMDA or AMPA receptors specifically mediate obesity-induced hypertension.

Under normotensive conditions, blockade of RVLM ionotropic glutamatergic receptors with KYN has no effect on either SNA or ABP. Historically this was interpreted as glutamate playing no role in maintaining resting SNA and ABP although recent studies have hypothesized an alternate conclusion. Following inhibition of the caudal ventrolateral medulla, which sends a tonic inhibitory signal to the RVLM, KYN microinjection into the RVLM causes a profound decrease in ABP similar to that seen following ganglionic blockade (Ito and Sved 1997). From these findings, it is believed that glutamate is simultaneously activating both an excitatory and inhibitory pathway through the RVLM. Given that, it is believed that under normotensive conditions KYN causes an equivalent change in both excitatory and inhibitory inputs to the RVLM, though it should be noted that a very similar study found opposing results in that RVLM glutamate receptor blockade following CVLM inhibition did not lower ABP to below baseline levels and the reason for the opposing results is not known (Horiuchi et al. 2004). In the instances of hyperinsulinemia and diet-induced

obesity, KYN causes a significant decrease in SNA and ABP most likely because there is a shift from an equal balance between excitatory and inhibitory inputs to one predominated by excitation.

Based on our findings, it would be predicted that activity of pre-sympathetic RVLM excitatory neurons would be increased, which was shown to be true in spontaneously hypertensive rats (Matsuura et al. 2002), or that a larger population of said neurons are being activated. Future studies are needed to measure in vivo RVLM neuronal activity during hyperinsulinemic-euglycemic clamps and diet-induced obesity hypertension. This would also allow for differentiation between increased neuronal activity versus recruitment of once silent neurons. In addition, this would permit identification of the phenotypic characteristics of RVLM neurons activated during these conditions. There are two distinct populations of neurons comprising the RVLM: C1 and non-C1 cells. C1 cells comprise more than 50% of spinally projecting RVLM neurons, contain phenylethanolamine N-methyltransferase (PNMT), the rate limiting enzyme in production of epinephrine, and have fast and slow conduction velocities. Depletion of RVLM C-1 neurons produces either no change or a small, but significant decrease in ABP and blunts many sympathoexcitatory reflexes (Schreihofer et al. 2000, Madden and Sved 2003). While non-C1 cells do not contain PNMT and have fast conduction velocities (Schreihofer and Guyenet 1997). It is possible that non-C1 neurons are involved in generating basal sympathetic tone as depletion of C1 neurons does not cause a profound decrease in resting ABP and SNA, but there is no method to target these particular neurons.

Hyperinsulinemia increases baroreflex gain in both humans and rodents (Pricher et al. 2007, Young et al. 2010). The neural circuitry through which insulin acts to significantly alter baroreflex gain is not known. It is believed to be within the hypothalamus as lateral ventricle infusion of insulin, but not 4th ventricle infusion, increases baroreflex gain. In this study we did not examine the effect of hyperinsulinemic-euglycemic clamps on any sympathoexcitatory

reflexes. Additional studies could examine the effect of intravenous hyperinsulinemia on the arterial baroreflex, somatic pressor reflex, and chemoreflex. It would be interesting to investigate whether the pathways mediating insulin's possible effects on those sympathetic reflexes might overlap with the pathways involved in the sympathoexcitation associated with hyperinsulinemia.

Hyperinsulinemic-euglycemic clamps selectively activate lumbar SNA, though others have shown a delayed increase in renal SNA (Rahmouni et al. 2004). Renal SNA is elevated in obese humans (Esler. 2006, Vaz et al. 1997) and renal denervation prevents the development of diet-induced obesity hypertension in dogs (Kassab et al. 1997). Though the particular studies presented here did not investigate the contribution of renal SNA to diet-induced obesity or the effects that receptor blockade might have on renal SNA, future studies are needed to determine the role of renal SNA given that there is abundant evidence that renal SNA plays a critical role in the development of many forms of hypertension.

A major question that arises from the data presented here is the origin of the glutamatergic projection to the RVLM during hyperinsulinemia and diet-induced obesity hypertension. There is a glutamatergic projection from the hypothalamic paraventricular nucleus (Stocker et al. 2006) and previous evidence illustrates a role for the hypothalamic paraventricular nucleus in mediating insulin's sympathoexcitatory effect (Stocker et al. 2008). Very recent studies provide evidence that the glutamatergic projection originates from the hypothalamic paraventricular nucleus during hyperinsulinemia (Ward et al. In Press) but it is still unknown during obesity-induced hypertension. Given that nearly all neurons express ionotropic glutamate receptors, it is more useful to examine expression of vesicular glutamate transporters (V-GLUT) which are responsible for transporting glutamate into synaptic vesicles (Juge et al. 2006) prior to release. There are three major forms of V-GLUT; V-GLUT 1, V-GLUT 2, and V-GLUT 3 which are expressed in neurons though the function of V-GLUT 3

has not been elucidated. There are many brain regions which project to the RVLM including the caudal ventrolateral medulla (Afarwal and Calaresu 1991), hypothalamic paraventricular nucleus (Shafton et al. 1998), pontine reticular formation (Hayes K et al. 1994), nucleus of the solitary tract (Ross et al. 1985) area postrema (Shapiro and Miselis 1985), central amygdaloid nucleus (Takayama and Miura 1991), parabrachial nucleus (Krukoff et al. 1993), lateral hypothalamic area (Allen and Cechietto 1992), and the raphe nucleus (Ross et al. 1985), but not all are known to express V-GLUT transporters. V-GLUT 1 and 2 are expressed in the nucleus of the solitary tract (Lachamp et al. 2006), hypothalamic paraventricular nucleus (Ziegler et al. 2002) and lateral hypothalamic area (Ziegler et al. 2002) while V-GLUT 2 alone is expressed on area postrema neurons (Stornetta et al. 2002). V-GLUT 3 is expressed on raphe nucleus neurons (Yamakawa and Antle 2010). Those areas that express V-GLUT and project to the RVLM require further study in regards to the increase in glutamate receptor activation during diet-induced obesity hypertension.

Neither RVLM AT1 nor melanocortin 3/4 receptors mediate the sympathoexcitatory response to insulin or diet-induced obesity hypertension. There is abundant evidence that both systems mediate these responses but it is clear that their involvement does not occur in the RVLM. Given that inhibition of angiotensin converting enzyme with captopril blunts the sympathoexcitatory effects of insulin (Muntzel et al.1994) while intracerebroventricular blockade of AT1 receptors inhibits the pressor response to hyperinsulinemia (Nakata et al. 1998) it can be ascertained that the central RAS plays a critical role during hyperinsulinemia. As well, because blockade of the RAS is used as an effective therapy in hypertensive patients and is shown to lower SNA in obese-hypertensive subjects (Grassi et al. 2003, BechirM et al. 2005) it is likely that the RAS is activated upstream of the RVLM. In regards to the melanocortin system, melanocortin-4 receptor knockout mice are resistant to the sympathoexcitatory response of insulin but do not develop obesity-induced hypertension despite elevated plasma insulin concentrations. As well, melanocortin 4 receptors are found on ARC POMC neurons known to control food intake. Therefore, future

studies are needed to ascertain where these systems are working in the central nervous system. Similar studies to those performed here need to be completed in brain areas such as the hypothalamic paraventricular nucleus and ARC, both of which are implicated in the control of SNA and ABP and contain AT1 and melanocortin receptors (Song et al. 1991, Kishi et al. 2003).

These studies do not provide an answer to the major question of where insulin is sensed in the central nervous system to result in sympathoexcitation. Here, we demonstrate that direction injection into the RVLM does not increase lumbar SNA (Bardgett et al. 2010). The hypothalamic paraventricular nucleus has direct projections to the RVLM but microinjection of insulin directly into the hypothalamic paraventricular nucleus causes no change in lumbar SNA (unpublished). Therefore, it is likely that the increase in insulin concentration is sensed upstream of the hypothalamic paraventricular nucleus. A first step in identifying a potential brain region that senses increased insulin would be to examine the expression of c-fos immediately following ICV insulin administration. C-fos is a proto-oncogene whose transcription is up-regulated following neuronal activation. This would provide a preliminary indication of potential regions to investigate further. As well, when insulin binds to its receptor, it results in auto-phosphorylation of tyrosine residues on the β-subunit and activation of PI3K. Examining the changes in insulin receptor phosphorylation and PI3K activity would also help in identifying the brain region responsible for sensing insulin.

Based on studies investigating insulin's effects on food intake, I would hypothesize that insulin is sensed in the ARC. The ARC has a higher concentration of insulin receptors than any other brain region (Hill et al. 1986, Werther et al. 1987) and lesions of the ARC prevent the anorexic effects of insulin (Schwartz et al. 2000). In addition, PI3K activity is increased in ARC neurons following intracerebroventricular insulin in a time frame that corresponds with the increase in lumbar SNA during IV infusion of insulin (Niswender et al., 2003). Taken together, future studies need to be performed in order to determine a role for the ARC. Inhibition of the ARC, with the GABA agonist

muscimol, is needed to establish a role for the ARC in the sympathoexcitatory response, though this would not determine if the ARC is responsible for sensing insulin. Direct microinjection of insulin into the ARC would be the first step in determining its role. Rats could also be pretreated with a lentivirus for the insulin receptor in order to knock down receptor function specifically in the ARC. In combination these studies would ascertain the role of the ARC during insulin-induced sympathoexcitation. Figure 24 illustrates a hypothesized pathway through which insulin increases lumbar SNA and ABP. The ARC has a direct projection to the hypothalamic paraventricular nucleus (Cone 2005) which has a monosynaptic glutamatergic projection to the RVLM.

It is known that insulin acts in the hypothalamus to decrease food intake and hypothesized that it acts in the same or neighboring location to activate the sympathetic nervous system. Insulin activates POMC neurons, increasing α-MSH which binds melanocortin receptors resulting in a decrease in food intake while simultaneously inhibiting NPY/AgRP neurons causing a decrease in NPY/AgRP mRNA and protein release (Figure 1). Though this pathway has been implicitly studied it is still unclear how insulin binding results in such changes in protein expression and release. Direct insulin application to hypothalamic neurons *in vitro* causes hyperpolarization through opening of ATP-sensitive K^+ channels (Spanswick et al. 2000). How hyperpolarization of hypothalamic neurons which occurs within seconds translates into activation of the sympathetic nervous system and a decrease in food intake over a significantly longer period of time is unclear. In addition, although the immediate effects of insulin are hyperpolarizing, there is an increase in PI_3K and PIP_3 expression after central insulin administration, the hallmark insulin signaling pathway, whose time frame of activation correlates with the increase in lumbar SNA associated with hyperinsulinemia. To further complicate the story, leptin, which acts through a PI_3K dependent pathway similar to insulin (Hill et al. 2008) to decrease food intake, causes POMC ARC neurons to depolarize (Williams et al. 2010) and NPY/AgRP neurons to hyperpolarize (Spanswick et al. 1997). Further studies are clearly needed in order to elucidate the significance of insulin-

induced hyperpolarization and how it leads to increased POMC expression and activation of the PI_3K pathway through which insulin works. This would include recording from POMC ARC neurons *in vivo* during intracerebroventricular administration of insulin to determine if the *in vitro* effects of insulin hold true in an *in vivo* preparation.

Chronic hyperinsulinemic-euglycemic clamps elevate ABP in rodents through an increase in total peripheral resistance (Brands et al. 1991) and although acute clamps increase muscle SNA in humans, ABP is not elevated. Similar results were found in dogs although SNA was not measured. In acute settings, insulin has vasodilatory effects in the periphery and it is possible that this vasodilation counteracts the increase in muscle SNA resulting in no net change in ABP. For experimental reasons, it is not possible to determine whether hyperinsulinemia would elevate ABP in a chronic setting in humans. Obesity is associated with many factors that could potentially contribute to activation of the sympathetic nervous system and elevation in ABP. Obese models in animals tend to be associated with the same physiological alterations. Therefore, it is nearly impossible to ascertain which alterations are leading to activation in SNA and elevation in ABP. From these studies highlighting insulin's continued ability to stimulate the sympathetic nervous system even after development of obesity leaves open the possibility that insulin could be one factor involved. Obesity also results in selective resistance to leptin, with an ability to still activate renal SNA but not lumbar or brown adipose tissue SNA (Rahmouni et al. 2005). From these studies, it is most likely a combination of factors that contribute to the sympathoexcitation and elevated ABP associated with obesity.

Summary

The studies within this dissertation have clearly demonstrated that physiological levels of circulating insulin activate the sympathetic nervous system under normal and diet-induced obese conditions. This is important because it highlights a potential role for insulin in mediating the sympathoexcitatory effect of

obesity as well as the elevated ABP. We were also able to begin to identify the neural pathway through which insulin and obesity work to elevate SNA and ABP. From this we discovered that both hyperinsulinemia and diet-induced obesity activate a glutamatergic pathway through the RVLM. In the case of hyperinsulinemia, it was specific to NMDA-glutamatergic receptors. In contrast, we showed that neither AT1 nor melanocortin 3/4 RVLM receptors are involved in mediating the cardiovascular response to hyperinsulinemia or diet-induced obesity.

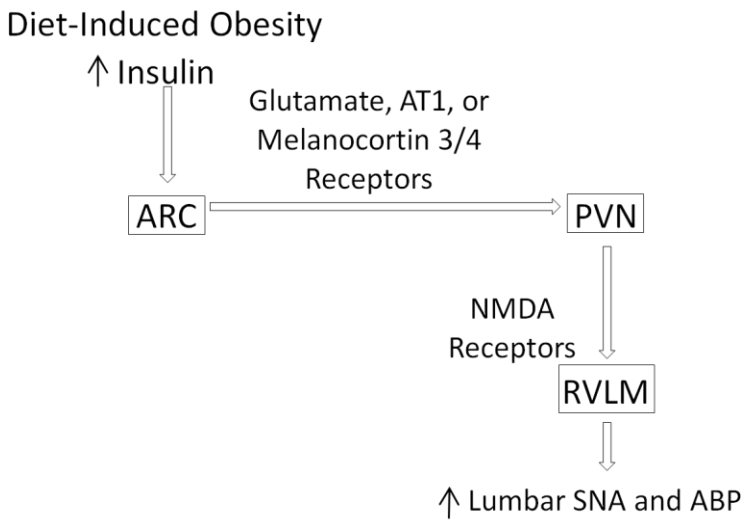


Figure 24. Hypothesized pathway through which insulin acts to elevate lumbar SNA and ABP

References

Abrahams TP, Hornby PJ, Chen K, Dasilva AM, Gillis RA. 1994. The non-NMDA subtype of excitatory amino acid receptor plays the major role in control of cardiovascular function by the subretrofacial nucleus in cats. *J Pharmacol Exp Ther*, 270(1):424-432

Adams JM, Madden CJ, Sved AF, Stocker SD. 2007. Increased dietary salt enhances sympathoexcitatory and sympathoinhibitory responses from the rostral ventrolateral medulla. *Hypertension*, 50:354-359

Adams JM, McCarthy JJ, Stocker SD. 2008. Excess dietary salt alters angiotensinergic regulation of neurons in the rostral ventrolateral medulla. *Hypertension*, 52:932-937

Adan RA, Gispen WH. 1997. Brain melanocortin receptors: from cloning to function. *Peptides*, 18(8):1279-1287

Adan RA, Gispen WH. 2000. Melanocortins and the brain: from effects via receptors to drug targets. *Eur J Pharmacol*, 405(103):13-24

Afarwal SK, Calaresu FR. 1991. Monosynaptic connection from caudal to rostral ventrolateral medulla in the baroreceptor reflex pathway. *Brain Res*, 555(1):70-74

Allen GV, Cechietto DF. 1991. Functional and anatomical organization of cardiovascular pressor and depressor sites in the lateral hypothalamic area. *J Comp Neurol*, 315(3):313-332

Alonso-Galacia MA, Brands MW, Zappe DH, Hall JE. 1996. Hypertension in obese Zucker rats. Role of Angiotensin II and Adrenergic Activity. *Hypertension*, 28:1047-1054

Alvarez GE, Beske SD, Ballard TP, Davy KP. 2002. Sympathetic neural activation in visceral obesity. *Circulation*, 106(20):2533-2536

Anderson EA, Hoffman RP, Halon TW, Sinkey CA, Mark AL. 1991. Hyperinsulinemia produces both sympathetic neural activation and vasodilation in normal humans. *J Clin Invest*, 87(6):2246-2252

Anderson EA, Balon TW, Hoffman RP, Sinkey CA, Mark AL. 1991. Insulin increases sympathetic activity but not blood pressure in borderline hypertensive humans. *Hypertension*, 19(6):621-627

Arase K, Fisler JS, Shargill NS, York DA, Bray GA. 1988. Intracerebroventricular infusion of 3-OHB and insulin in a rat model of dietary obesity. *Am J Physiol*, 255(6 Pt2):974-981

Banks WA. 2004. The source of cerebral insulin. *Eur J Pharmacol*, 490(1-3):5-12

Banks WA, Jaspan JB, Huang W, Kastin AJ. 1997. Transport of insulin across the blood brain barrier: saturability at euglycemic doses of insulin. *Peptides*, 18(9):1423-1429

Bardgett ME, McCarthy JJ, Stocker SD. 2010. Glutamatergic receptor activation in the rostral ventrolateral medulla mediates the sympathoexcitatory response to hyperinsulinemia. *Hypertension*, 55(2):284-290

Barnard RJ, Youngren JF. 1992. Regulation of glucose transport in skeletal muscle. *The FASEB Journal*, 6:3238-3244

Baura GD, Foster DM, Porte D Jr, Kohn SE, Bergman RN, Cobelli C, Schwartz MW. 1993. Saturable transport of insulin from plasma into the central nervous system of dogs in vivo. A mechanism for regulated insulin delivery to the brain. *J Clin Invest*, 92(4):1824-1830

Bechir M, Enseleit F, Chenevard R, Luscher TF, Noll G. 2005. Effect of losartan on muscle sympathetic nerve activity and baroreceptor function in systemic hypertension. *Am J Cardiol*, 95(1):129-131

Benarroch EE, Granata AR, Ruggiero DA, Park DH, Reis DJ. 1986. Neurons of C1 area mediate cardiovascular responses initiated from ventral medullary surface. *Am J Physiol*, 250(5 Pt2):R932-945

Benoit SC, Air EL, Coolen LM, Strauss R, Jackman A, Clegg DJ, Seeley RJ, Woods SC. 2002. The catabolic action of insulin in the brain is mediated by melanocortins. *J Neurosci*, 22(20):9048-9052

Bergamaschi C, Campos RR, Schor N, Lopes OU. 1995. Role of the rostral ventrolateral medulla in maintenance of blood pressure in rats with Goldblatt hypertension. *Hypertension*, 26(6 Pt2):1117-1120

Boustany CM, Bharadwaj K, Daugherty A, Brown DR, Randall DC, Cassis LA. 2004. Activation of the systemic and adipose renin-angiotensin system in rats with diet-induced obesity and hypertension. *Am J Physiol Regul Integr Comp Physiol*, 287(4):943-949

Boustany CM, Brown DR, Randall DC, Cassis LA. 2005. AT1-receptor antagonism reverses the blood pressure elevation associated with diet-induced obesity. *Am J Physiol Regul Integr Comp Physiol*, 289(1):181-186

Brands MW, Lee WF, Keen HL, Alonso-Galicia M, Zappe DH, Hall JE. 1996. Cardiac output and renal function during insulin hypertension in Sprague-Dawley rats. *Am J Physiol Regul Integr Comp Physiol*, 271:276-281

Brands MW, Harrison DL, Keen HL, Gardner A, Shek EW, Hall JE. 1997. Insulin-induced hypertension in rats depends on an intact renin-angiotensin system. *Hypertension*, 29:1014-1019

Brands MW, Hildebrandt DA, Mizelle HL, Hall JE. 1991. Sustained hyperinsulinemia increases arterial pressure in conscious rats. *Am J Physiol*, 260(4):764-768

Brands MW, Mizelle HL, Gaillard CA, Hildebrandt DA, Hall JE. 1991. The hemodynamic response to chronic hyperinsulinemia in conscious dogs. *Am J Hypertens*, 4:164-168

Brown DL, Guyenet PG. 1984. Cardiovascular neurons of brain stem with projections to spinal cord. *Am J Physiol Reg Integr Comp Physiol*, 247:1009-1016

Bruner CA, Fink GD. 1986. Neurohumoral contributions to chornic angiontensin-induced hypertension. *Am J Hypertens*, 250(1 Pt2):52-61

Carroll RG, Lohmeier TE, Brown AJ. 1984. Chronic angiotensin II infusion decreases renal norepinephrine overflow in the conscious dog. *Hypertension*, 6:675-681

Cheatham B, Vlahos CJ, Cheatham L, Wang L, Blenis J, Kahn CR. 1994. Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation. *Mol Cell Biol*, 14:4902-4911

Choudhury AI, Heffron H, Smith MA, Al-Qassab H, Xu AW, Selman C, Simmgen M, Clements M, Claret M, Maccoll G, Bedford DC, Hisadome K, Diakonov I, Moosajee V, Bell JD, Speakman JR, Batterman RL, Barsh GS, Ashford ML, Withers DJ. 2005. The role of insulin receptor substrate 2 in hypothalamic and beta cell function. *J Clin Invest*, 115(4):940-950

Carlson SH, Shelton J, White CR, Wyss JM. 2000. Elevated sympathetic activity contributes to hypertension and salt sensitivity in diabetic obese Zucker rats. *Hypertension*, 35(1 Pt 2):403-408

Clegg DJ, Benoit SC, Reed JA, Woods SC, Dunn-Meynell A, Levin BE. 2005. Reduced anorexic effects of insulin in obesity-prone rats fed a moderate-fat diet. *Am J Physiol Regul Integr Comp Physiol*, 288(4):981-986

Cone RD. 2005. Anatomy and regulation of the central melanocortin system. *Nat Neurosci*, 8(5):571-578

Coote JH, Yang Z, Pyner S, Deering J. 1998. Control of sympathetic outflows by the hypothalamic paraventricular nucleus. *Clin Exp Pharmacol Physiol*, 25(6):461-463

Cusi K, Maezono K, Osman A, Pendergrass M, Patti ME, Pratipanawatr T, DeFronzo RA, Kahn CR, Mandarino LJ. 2000. Insulin resistance differentially affects the PI 3-kinase-and MAP kinase-mediated signaling in human muscle. J Clin Invest, 105(3):311-320

Dampney RA, Fontes MA, Hirooka Y, Horiuchi J, Potts PD, Tagawa T. 2002. Role of angiotensin II receptors in the regulation of vasomotor neurons in the ventrolateral medulla. *Clin Exp Pharmacol Physiol*, 29(5-6):467-472

D'Angelo G, Mintz JD, Tidwell JE, Schreihofer AM, Pollock DM, Stepp DW. 2006. Exaggerated cardiovascular stress responses and impaired beta-adrenergic-mediated recovery in obese Zucker rats. *Hypertension*, 48(6):1109-1115

da Silva AA, Carmo J, Dubinion J, Hall JE. 2009. The role of the sympathetic nervous system in obesity-related hypertension. *Curr Hypertens Rep*, 11(3):206-211

Derewenda U, Derewenda Z, Dodson GG, Hubbard RE, Korder F. 1986. Molecular structure of insulin: the insulin monomer and its assembly. *Br Med Bull*, 45(1):4-18

De Souza CT, Araujo EP, Bordin S, Ashimine R, Zollner RL, Boschero AC, Saad MJA, Velloso LA. 2005. Consumption of a fat-rich diet activates proinflammatory response and induces insulin resistance in the hypothalamus. *Endocrinology*, 146(10):4192-4199

Dobrain AD, Davies MJ, Prewitt RL, Lauterio TJ. 2000. Development of hypertension in a rat model of diet-induced obesity. *Hypertension*, 35(4):1009-1015

Dubinion JH, da Silva AA, Hall JE. 2010. Enhanced blood pressure and appetite responses to chronic central melanocortin-3/4 receptor blockade in dietary-induced obesity. *J Hypertens*, 28(7):1466-1470

Esler M, Jennings G, Lambert G, Meredith I, Horne M, Eisenhofer G. 1990. Overflow of catecholamine neurotransmitters to the circulation: source, fate, and functions. *Physiol Rev*, 70(4):963-985

Esler M, Straznicky N, Eikelis N, Masuo K, Lambert G, Lambert E. 2006. Mechanisms of sympathetic activation in obesity-related hypertension. *Hypertension*, 48(5):787-796

Fontaine KR, Redden DT, Wang C, Westfall AO, Allison DB. 2003. Year of life lost due to obesity. *JAMA*, 289(2):187-193

Garrison RJ, Kannel WB, Stokes J 3rd, Castelli WP. 1987. Incidence and precursors of hypertension in young adults: the Framingham Offspring Study. *Prev Med*, 16(2):235-251

Gentile CL, Orr JS, Davy BM, Davy KP. 2007. Modest weight gain is associated with sympathetic neural activation in nonobese humans. *Am J Physiol Regul Integr Comp Physiol*, 292(5):1834-1838

Granata AR, Ruggiero DA, Park DH, Joh TH, Reis DJ. 1985. Brain stem area with C1 epinephrine neurons mediates baroreflex vasodepressor responses. *Am J Phsyiol Heart Circ Physiol*, 248:547-567

Grassi G, Dell'Oro R, Facchini A, Quarti Trevano F, Bolla GB, Mancia . 2004. Effect of central and peripheral obesity body fat distribution on sympathetic and baroreflex function in obese normotensives. *J Hypertens*, 22:2363-2369

Grassi G, Facchini A, Trevano FQ, Dell'Oro R, Arenare F, Tana F, Bolla G, Monzani A, Robuschi M, Mancia G. 2005. Obstructive sleep apnea-dependent and –independent adrenergic activation in obesity. *Hypertension*, 46:321-325

Grassi G, Seravalle G, Cattaneo BM, Bolla GB, Lanfranchi A, Colombo M, Giannattasio C, Brunani A, Cavagnini F, Mancia G. 1995. Sympathetic activation in obese normotensive subjects. Hypertension, 25(4 Pt 1):560-563

Grassi G, Seravalle G, Dell'Oro R, Trevano FQ, Bombelli M, Scopelliti F, Facchini A, Mancia G, CROSS Study. 2003. Comparative effects of candesartan and hydrocholorthiazide on blood pressure, insulin sensitivity, and sympathetic drive in obese hypertensive individuals: results of the CROSS study. *J Hypertens*, 21(9):1761-1769

Greenfield JR, Miller JW, Keogh JM, Henning E, Satterwhite JH, Cameron GS, Astruc B, Mayer JP, Brage S, See TC, Lomas DJ, O'Rahilly S, Farooqi IS. 2009. Modulation of blood pressure by central melanocortinergic pathways. *N Engl J Med*, 360(1):44-52

Guyenet PG. 2006. The sympathetic control of blood pressure. *Nat Rev Neurosci*, 7(5):335-346

Hall JE. 2003. The kidney, hypertension and obesity. *Hypertension*, 41(2):625-633

Hass JT, Biddinger SB. 2009. Dissecting the role of insulin resistance in the metabolic syndrome. *Curr Opin Lipidol*, 20(3):206-210

Hayes K, Calaresu FR, Weaver LC. 1994. Pontine reticular neurons provide tonic excitation to neurons in rostral ventrolateral medulla in rats. *Am J Physiol*, 266(1 Pt 2):R237-244

Hayes K, Weaver LC. 1992. Tonic sympathetic excitation and vasomotor control from pontine reticular neurons. *Am J Physiol*, 263(5 Pt 2):H1567-1575

Hildebrandt DA, Smith MJ Jr, Hall JE. 1999. Cardiovascular regulation during insulin infusion into the carotid or vertebral artery in dogs. *J Hypertens*, 17:251-260

Hill JM, Lesniak MA, Pert CB, Roth J. 1986. Autoradiographic localization of insulin receptors in rat brain: prominence in olfactory and limbic areas. *Neuroscience*, 17(4):1127-1138

Hill JW, Williams KW, Ye C, Luo J, Balthasar N, Coppari R, Cowley MA, Cantley LC, Lowell BB, Elmquist JK. 2008. Acute effects of leptin require PI3K signaling in hypothalamic proopiomelanocortin neurons in mice. *J Clin Invest*, 118(5):1796-1805

Horiuchi J, Dampney RA. 2002. Evidence for tonic disinhibition of RVLM sympathoexcitatory neurons from the caudal pressor area. *Auton Neurosci*, 99(2):102-110

Horiuchi J, Killinger S, Dampney RA. 2004. Contribution to sympathetic vasomotor tone of tonic glutamatergic inputs to neurons in the RVLM. *Am J Physiol Integr Comp Physiol*, 287(6):1335-1343

Huggett RJ, Burns J, Mackintosh AF, Mary DA. 2004. Sympathetic neural activation in nondiabetic metabolic syndrome and its further augmentation by hypertension. *Hypertension*, 44(6):847-852

Ishiki M, Klip A. 2005. Minireview: Recent Developments in the Regulation of Glucose Transporter-4 Traffic: New Signals, Locations, and Partners. *Endocrinology*, 146(2):5071-5078

Israel PA, Park CR, Schwartz MW, Green PK, Sipols AJ, Woods SC, Porte D Jr, Figlewicz DP. 1993. Effect of diet-induced obesity and experimental hyperinsulinemia on insulin uptake into CSF of the rat. *Brain Res Bull*, 30(5-6):571-575

Ito S, Sved AF. 1997. Tonic glutamate-mediated control of rostral ventrolateral medulla and sympathetic vasomotor tone. *Am J Physiol*, 273:R487-R494

Ito S, Komatsu K, Tsukamoto K, Sved AF. 2000. Excitatory amino acids in the rostral ventrolateral medulla support blood pressure in spontaneously hypertensive rats. *Hypertension*, 35(1):413-417

Ito S, Komatsu K, Tsukamoto K, Sved AF. 2001. Tonic excitatory input to the rostral ventrolateral medulla in Dahl salt-sensitive rats. *Hypertension*, 37(2):687-691

Ito S, Komatsu K, Tsukamoto K, Kanmatsuse K, Sved AF. 2002. Ventrolateral medulla AT1 receptors support blood pressure in hypertensive rats. *Hypertension*, 40(4):552-559

Ito S, Hiratsuka M, Komatsu K, Tsukamoto K, Kanmatsuse K, Sved AF. 2003. Ventrolateral medulla AT1 receptors support arterial pressure in Dahl salt-sensitive rats. *Hypertension*, 41(3):744-750

Izzo JL, Sica DA, Black HR. 2008. Hypertension Primer: The essentials of high blood pressure. Lippincott Williams and Wilkins

Jhun BH, Haruta T, Meinkoth JL, Leitner W, Draznin B, Saltiel AR, Pang L, Sasaoka T, Olefsky JM. 1995. Signal transduction pathways leading to insulin-induced early gene induction. *Biochemistry*, 34(25):7996-8004

Juge N, Yoshida Y, Yatsushiro S, Omote H, Moriyama Y. 2006. Vesicular glutamate transporter contains two independent transport machineries. *J Biol Chem*, 281(51):39499-39506

Kaiyala KJ, Prigeon RL, Kahn SE, Woods SC, Schwartz MW. 2000. Obesity induced by a high-fat diet is associated with reduced brain insulin transport in dogs. *Diabetes*, 49:1525-1533

Kassab S, Kato T, Wilkins FC, Chen R, Hall JE, Granger JP. 1995. Renal denervation attenuates the sodium retention and hypertension associated with obesity. *Hypertension*, 25(4 Pt2):893-897

Kassab S, Kato T, Wilkins FC, Mizelle L, Granger JP. 1997. Role of renal nerves in mediating the blunted natriuretic response to acute saline load in obese dogs. *Am J Hypertens*, 10(3):315-322

Kawabe T, Chitravanshi VC, Kawabe K, Sapru HN. 2006. Cardiovascular effects of adrenocorticotropin microinjections into the rostral ventrolateral medullary pressor area of the rat. *Brain Res*, 1101(1):117-126

Kern W, Benedict C, Schultes B, Plohr F, Moser A, Born J, Fehm HL, Hallschmid M. 2006. Low cerebrospinal fluid insulin levels in obese humans. *Diabetologia*, 49(11):2790-2792

Kido Y, Nakae J, Accili D. 2001. Clinical review 125: The insulin receptor and its cellular targets. J Clin Endocrinol Metab, 86(3):972-979

Kiely JM, Gordon FJ. 1993. Non-NMDA receptors in the rostral ventrolateral medulla mediate somatosympathetic pressor responses. *J Auton Nerv Syst*, 43(3):231-239

Kiely JM, Gordon FJ. 1994. Role of rostral ventrolateral medulla in centrally mediated pressor responses. *Am J Physiol Heart Circ Physiol*, 267 (4 Pt2):1549-1556

King AJ, Osborn JW, Fink GD. 2007. Splanchnic circulation is a critical neural target in angiotensin II salt hypertension in rats. *Hypertension*, 50(3):547-556

Kishi T, Aschkenasi CJ, Lee CE, Mountjoy KG, Saper CB, Elmquist JK. 2003. Expression of melanocortin 4 receptor mRNA in the central nervous system of the rat. *J Comp Neurol*, 457(3):213-235

Koshiya N, Huangfu D, Guyenet PG. 1993. Ventrolateral medulla and sympathetic chemoreflex in the rat. *Brain Res*, 609:174-184

Krassioukov AV, Weaver LC. 1993. Connections between the pontine reticular formation and rostral ventrolateral medulla. *Am J Physiol*, 265(4 Pt 2):H1386-1392

Krukoff TL, Harris KH, Jhamandas JH. 1993. Efferent projections from the parabrachial nucleus demonstrated with the anterograde tracer. *Brain Res Bull*, 30(1-2):163-172

Laakso M, Edelman SV, Brechtel G, Baron AD. 1992. Impaired insulin-mediated skeletal muscle blood flow in patients with NIDDM. *Diabetes*, 41(9):1076-1083

Lachamp P, Crest M, Kessler JP. 2006. Vesicular glutamate transporters type 1 and 2 expression in axon terminals of the rat nucleus of the solitary tract. *Neuroscience*, 137(1):73-81

Lambert E, Straznicky N, Schlaich M, Esler M, Dawood T, Hotchkin E, Lambert G. 2007. Differing pattern of sympathoexcitation in normal-weight and obesity-related hypertension. *Hypertension*, 50(5):862-868

La Marchand-Brustel Y, Gautier N, Cormont M, Can Obberghen E. 1995. Wortmannin inhibits the action of insulin but not that of okadaic acid in skeletal muscle: comparison with fat cells. *Endocrinology*, 136(8):3564-3570

Levin BE. 1993. Sympathetic activity, age, sucrose preference, and diet-induced obesity. *Obes Res*, 1:281-287

Levin BE, Triscari J, Sullivan AC. 1983. Altered sympathetic activity during development of diet-induced obesity in rat. *Am J Physiol*, 244(3):347-355

Levin BE, Keesey RE. 1998. Defense of differing body weight set points in diet-induced obese and resistant rats. *Am J Physiol*, 274(2):412-419

Li KW, Guyenet PG. 1996. Angiotensin II decreases resting K+ conductance in rat bulbospinal neurons of the C1 area. *Circ Res*, 78(2):274-282

Lipski J, Kanjhan R, Kruszewska B, Rong W. 1996. Properties of presympathetic neurons in the rostral ventrolateral medulla in the rat: an intracellular study ‘in vivo’. *J Physiol*, 490:729-744

Lipski J, Kawai Y, Qi J, Comer A, Win J. 1998. Whole cell patch-clamp study of putative vasomotor neurons isolated from the rostral ventrolateral medulla. *Am J Physiol*, 274 (4 Pt2):R1099-1110

Lohmeier TE, Dwyer TM, Irwin ED, Rossing MA, Kieval RS. 2007. Prolonged activation of the baroreflex abolishes obesity-induced hypertension. *Hypertension*, 49(6):1307-1314

Lohmeier TE, Warren S, Cunningham JT. 2003. Sustained activation of the central baroreceptor pathway in obesity hypertension. *Hypertension*, 42:96-102

Madden CJ, Sved AF. 2003. Cardiovascular regulation after destruction of the C1 cell group of the rostral ventrolateral medulla in rats. *Am J Physiol Heart Circ Physiol*, 285:2734-2748

Matsuura T, Kumagai H, Kawai A, Onimaru H, Imai M, Oshima N, Sakata K, Saruta T. 2002. Rostral ventrolateral medulla neurons of neonatal Wistar-Kyoto and spontaneously hypertensive rats. *Hypertension*, 40(4):560-565

Mills PJ, Kennedy BP, Loredo JS, Dimsdale JE, Ziegler MG. 2006. Effects of nasal continuous positive airway pressure and oxygen supplementation on norepinephrine kinetic and cardiovascular responses in obstructive sleep apnea. *J Appl Physiol*, 100:343-348

Modan M, Halkin H, Almog S, Lusky A, Eshkol A, Shefi M, Shitrit A, Fuchs Z. 1984. Hyperinsulinemia: A link between hypertension obesity and glucose intolerance. *J Clin Invest*, 75(3):809-817

Monaghan DT, Bridges RJ, Cotman CW. 1989. The excitatory amino acid receptors: Their classes, pharmacology, and distinct properties in the function of the central nervous system. *Annu Rev Pharmacol Toxicol*, 29:365-402

Moretti JL, Burke SL, Evnas RG, Lambert GW, Head GA. 2009. Enhanced responses to ganglionic blockade do not reflect sympathetic nervous system contribution to angiotensin-II induced hypertension. *J Hypertens*, 27(9):1838-1848

Morgan DA, Anderson EA, Mark AL. 1995. Renal sympathetic nerve activity is increased in obese Zucker rats. *Hypertension*, 25(4 Pt2):834-838

Morgan DA, Balon TW, Ginsberg BH, Mark AL. 1993. Nonuniform regional sympathetic nerve responses to hyperinsulinemia in rats. *Am J Physiol*, 264(2):423-427

Morgan DA, Rahmouni K. 2010. Differential effects of insulin on sympathetic nerve activity in agouti obese mice. *J Hypertens*, 28(9):1913-1919

Morgan DA, Thedens DR, Weiss R, Rahmouni K. 2008. Mechanisms mediating renal sympathetic activation to leptin in obesity. *Am J Physiol Regul Integr Comp Physiol*, 295(6):1730-1736

Morton GJ, Schwartz MW. 2010. The NPY/AgRP neuron and energy homeostasis. *Int J Obes Relat Metab Disord*, 25:S56-62

Morrison SF, Reis DJ. 1989. Reticulospinal vasomotor neurons in the RVL mediate the somatosympathetic reflex. *Am J Physiol*, 256(5 Pt2):1084-1097

Muntzel M, Beltz T, Mark AL, Johnson AK. 1994. Anteroventral third ventricle lesions abolish lumbar sympathetic responses to insulin. *Hypertension*, 23(6):1059-1062

Muntzel MS, Morales J, Akinsefunmi A. 2001. Converting enzyme inhibition with captopril abolishes sympathoexcitation to euglycemic hyperinsulinemia in rats. *Am J Hypertens*, 14(6 Pt1):592-598

Muntzel MS, Morgan DA, Mark AL, Johnson AK. 1994. Intracerebroventricular insulin produces nonuniform regional increases in sympathetic nerve activity. *Am J Physiol*, 267 (5 Pt2):1350-1355

Nakata T, Takeda K, Hatta T, Kiyama M, Moriguchi J, Miki S, Kawa T, Morimoto S, Makamura K, Uchida A, Itoh H, Sasaki S, Nakagawa M. 1998. Blockade of angiotensin II receptors inhibits the increase in blood pressure induced by insulin. *J Cardiovasc Pharmacol*, 31(2):248-252

Narkiewicz K, van de Borne PJH, Cooley RL, Dyken ME, Somers VK. 1998. Sympathetic activity in obese subjects with and without obstructive sleep apnea. *Circulation*, 98:772-776

Neter JE, Stam BE, Kok FJ, Grobbee DE, Geleijnse JM. 2003. Influence of weight reduction on blood pressure: a meta-analysis of randomized controlled trials. *Hypertension*, 42:878-884

Niswender KD, Morrison CD, Clegg DJ, Olson R, Baskin DG, Myers MG Jr, Seeley RJ, Schwartz MW. 2003. Insulin activation of phosphatidylinositol 3-kinase in the hypothalamic arcuate nucleus. *Diabetes*, 52(2):227-231

Niswender KD, Schwartz MW. 2003. Insulin and leptin revisited: adiposity signals with overlapping physiological and intracellular signaling capabilities. *Front Neuroendocrinol*, 24(1):1-10

Obici S, Feng Z, Karkanias G, Baskin DG, Rossetti L. 2002. Decreasing hypothalamic insulin receptors causes hyperphagia and insulin resistance in rats. *Nat Neurosci*, 5(6):566-572

Obici S, Feng Z, Tan J, Liu L, Karkanias G, Rossetti L. 2001. Central melanocortin receptors regulate insulin action. *J Clin Invest*, 108(7):1079-1085

Owen OE, Reichard GA Jr, Boden G, Shuman C. 1974. Comparative measurements of glucose, β-hydroxybutyrate, acetoacetate, and insulin in blood and cerebrospinal fluid during starvation. *Metabolism*, 23(1):7-12

Pardini AW, Nguyen HT, Figlewicz DP, Baskin DG, Williams DL, Kim F, Schwartz MW. 2006. Distribution of insulin receptor substrate-2 in brain areas involved in energy homeostasis. *Brain Res*, 1112(1):169-178

Posey KA, Clegg DJ, Printz RL, Byun J, Morton GJ, Vivekanandan-Giri A, Pennathur S, Baskin DG, Heinecke JW, Woods SC, Schwartz MW, Miswender KD. 2009. Hypothalamic proinflammatory lipid accumulation, inflammation, and insulin resistance in rats fed a high-fat diet. *Am J Physiol Endocrinol Metab*, 296(5):1003-1012

Pricher MP, Freeman KL, Brooks VL. 2008. Insulin in the brain increases gain of baroreflex control of heart rate and lumbar sympathetic nerve activity. *Hypertension*, 51(2):514-520

Rahmouni K, Sigmund CD, Haynes WG, Mark AL. 2009. Hypothalamic ERK mediates the anorectic and thermogenic sympathetic effects of leptin. *Diabetes*, 58(3):536-542

Rahmouni K, Morgan DA, Morgan DM, Liu X, Sigmund CD, Mark AL, Haynes WG. 2004. Hypothalamic PI3K and MAPK differentially mediate regional sympathetic activation to insulin. *J Clin Invest*, 114(5):652-658

Rahmouni K, Haynes WG, Morgan DA, Mark AL. 2003. Role of melanocortin-4 receptors in mediating sympathoexcitation to leptin and insulin. *J Neurosci*, 23(14):5998-6004

Rahmouni K, Morgan DA, Morgan GM, Mark AL, Haynes WG. 2005. Role of selective leptin resistance in diet-induced obesity hypertension. *Diabetes*, 54(7):2012-2018

Reeves GK, Pirie K, Breal V, Green J, Spencer E, Bull D, Million Women Study Collaboration. 2007. Cancer incidence and mortality in relation to body mass index in the Million Women Study. *BMJ*, 335(7630):1134

Reisin E, Weir MR, Falkner B, Hutchinson HG, Anzalone DA, Tuck ML. 1997. Lisinopril versus hydrochlorothiazide in obese hypertensive patients: a multicenter placebo-controlled trial. Treatment in Obese Patients with Hypertension (TROPHY) Study Group. *Hypertension*, 30(1 Pt1):40-45

Ross CA, Ruggiero DA, Park DH, Joh TH, Sved AF, Fernandez-Pardal J, Saaverda JM, Reis DJ. 1984. Tonic vasomotor control by the rostral ventrolateral medulla: effect of electrical or chemical stimulation of the area containing C1 adrenaline neurons on arterial pressure, heart rate, and plasma catecholamines and vasopressin. *J Neurosci*, 4(2):474-494

Ross CA, Ruggiero DA, Reis DJ. 1985. Projections from the nucleus tractus solitarii to the rostral ventrolateral medulla. *J Comp Neurol*, 242(4):511-534

Rumantir MS, Vaz M, Jennings GL, Collier G, Kaye DM, Seals DR, Wiesner GH, Brunner-La Rocca HP, Esler MD. 1999. Neural mechanisms in human obesity-related hypertension. *J Hypertens*, 17(8):1125-1133

Saiki A, Ohira M, Endo K, Koide N, Oyama T, Watanabe H, Miyashita Y, Shirai K. 2009. Circulating angiotensin II is associated with body fat accumulation and insulin resistance in obese subjects with type 2 diabetes mellitus. *Metabolism Clinical and Experimental*, 58:708-713

Sale EM, Atkinson PG, Sale GJ. 1995. Requirement of MAP kinase for differentiation of fibroblasts to adipocytes, for insulin activation of p90 S6 kinase and for insulin or serum stimulation of DNA synthesis. EMBO J, 15;14(4):674-684

Saltiel AR, Pessin JE. 2002. Insulin signaling pathways in time and space, *Trends Cell Biol*, 12(2):65-71

Sayk F, Heutling D, Dodt C, Iwen KA, Wellhoner JP, Scherag S, Hinney A, Hebebrand J, Lehnert H. 2010. Sympathetic function in human carriers of melanocortin-4 receptor gene mutations. *J Clin Endocrinol Metab*, 95(4):1998-2002

Scherrer U, Randin D, Tappy L, Vollenweider P, Jequier E, Nicod P. 1994. Body fat and sympathetic nerve activity in healthy subjects. *Circulation*, 89(6):2634-2640

Schreihofer AM, Guyenet PG. 1997. Identification of C1 presympathetic neurons in rat rostral ventrolateral medulla by juxtacellular labeling in vivo. *J Comp Neurol*, 387(4):524-536

Schreihofer AM, Stornetta RL, Guyenet PG. 2000. Regulation of sympathetic tone and arterial pressure by rostral ventrolateral medulla after depletion of C1 cells in rat. *J Physiol*, 529(1):221-236

Schwartz MW, Porte Jr D. 2005. Diabetes, Obesity, and the Brain. *Science*, 37:375-379

Schwartz MW, Woods SC, Porte D Jr, Seeley RJ, Baskin DG. 2000. Central nervous system control of food intake. *Nature*, 404(6778):661-671

Scislo TJ, Augustyniak RA, O'Leary DS. 1998. Differential arterial baroreflex regulation of renal, lumbar, and adrenal sympathetic nerve activity in the rat. *Am J Physiol Regul Integr Comp Physiol*, 275:995-1002

Seaquist ER, Damberg GS, Tkac I, Gruetter R. 2001. The effect of insulin on in vivo cerebral glucose concentrations and rates of glucose transport/metabolism in humans. *Diabetes*, 50(10):2203-2209

Shafton AD, Ryan A, Badoer E. 1998. Neurons in the hypothalamic paraventricular nucleus send collaterals to the spinal cord and to the rostral ventrolateral medulla in the rat. *Brain Res*, 801:239-243

Shapiro RE, Miselis RR. 1985. The central neural connections of the area postrema of the rat. *J Comp Neurol*, 234(3):344-364

Shibao C, Gamboa A, Diedrich A, Ertl AC, Chen KY, Byrne DW, Farley G, Paranjape SY, Davis SN, Biaggioni I. 2007. Autonomic contribution to blood pressure and metabolism in obesity. *Hypertension*, 49:27-33

Song K, Allen AM, Paxinos G, Mendelsohn FA. 1991. Angiotensin II receptor subtypes in rat brain. *Clin Exp Pharmacol Physiol*, 18(2):93-96

Spanswick D, Smith MA, Groppi VE, Logan SD, Ashford ML. 1997. Leptin inhibits hypothalamic neurons by activation of ATP-sensitive potassium channels. *Nature*;390:521-525

Spanswick D, Smith MA, Mirshamsi S, Routh VH, Ashford ML. 2000. Insulin activates ATP-sensitive K+ channels in hypothalamic neurons of lean, but not obese rats. *Nat Neurosci*, 3:757-758

Stein LJ, Dorsa DM, Baskin DG, Figlewicz DP, Ikeda H, Frankmann SP, Greenwood MR, Porte D Jr, Woods SC. 1983. Immunoreactive insulin levels are elevated in the cerebrospinal fluid of genetically obese Zucker rats. *Endocrinology*, 113(6):2299-2301

Stein LJ, Dorsa DM, Baskin DG, Figlewicz DP, Porte D Jr, Woods SC. 1987. Reduced effect of experimental peripheral hyperinsulinemia to elevate cerebrospinal fluid insulin concentrations of obese Zucker rats. *Endocrinology*, 121(5):1611-1615

Stocker SD, Smith CA, Kimbrough CM, Stricker EM, Sved AF. 2003. Elevated dietary salt suppresses renin secretion but not thirst evoked by arterial hypotension in rats. *Am J Physiol Regul Integr Comp Physiol*, 284:1521-1528

Stocker SD, Hunwick KJ, Toney GM. 2005. Hypothalamic paraventricular nucleus differentially supports lumbar and renal sympathetic outflow in water-deprived rat. *J Physiol*, 563:249-263

Stocker SD, Meador R, Adams JM. 2007. Neurons of the rostral ventrolateral medulla contribute to obesity-induced hypertension in rats. *Hypertension*, 49(3):640-646

Stocker SD, Simmons JR, Stornetta RL, Toney GM, Guyenet PG. 2006. Water deprivation activates a glutamatergic projection from the hypothalamic paraventricular nucleus to the rostral ventrolateral medulla. *J Comp Neurol*, 494:673-685

Stocker SD, Bardgett ME. 2007. Hypothalamic paraventricular nucleus contributes to the sympathoexcitatory effects of hyperinsulinemia. *Hypertension*, American Heart Association Council for High Blood Pressure Research Meeting Abstracts:e79

Storlien LH, James DE, Burleigh KM, Chrisholm DJ, Kraegen EW. 1986. Fat feeding causes widespread in vivo insulin resistance, decreased energy expenditure, and obesity in rats. *Am J Physiol*, 251(5 Pt1):E576-583

Stornetta RL, Morrison SF, Ruffiero DA, Reis DJ. 1989. Neurons of rostral ventrolateral medulla mediate somatic pressor reflex. *Am J Physiol Regul Integr Comp Physiol*, 256:448-462

Stornetta RL, Sevigny CP, Guyenet PG. 2002. Vesicular glutamate transporter DNPI/GLUT2 mRNA is present in C1 and several other groups of catecholaminergic neurons. *J Comp Neurol*, 444(3):191-206

Straznicky NE, Lambert GW, Masuo K, Dawood T, Eikelis N, Nestel PJ, McGrane MT, Mariani JA, Socratous F, Chopra R, Esler MD, Schlaich MP, Lambert EA. 2009. Blunted sympathetic neural response to oral glucose in obese subjects with the insulin-resistant metabolic syndrome. *Am J Clin Nutr*. 89(1):27-36

Strazzullo P, D'Elia L, Cairella G, Garbagnati F, Cappuccio FP, Scalfi L. 2010. Excess body weight and incidence of stroke: meta-analysis of prospective studies with 2 million participants. *JAMA*, 41(5):418-426

Sun MK, Guyenet PG. 1986. Hypothalamic glutamatergic input to medullary sympathoexcitatory neurons in rats. *Am J Physiol*, 251(4 Pt 2):R798-810

Sun MK, Hackett JT, Guyenet PG. 1988. Sympathoexcitatory neurons of rostral ventrolateral medulla exhibit pacemaker properties in the presence of a glutamate-receptor antagonist. *Brain Res*, 438:23-40

Sun MK, Young BS, Hackett JT, Guyenet PG. 1988. Reticulospinal pacemaker neurons of the rat rostral ventrolateral medulla with putative sympathoexcitatory function: an intracellular study in vitro. *Brain Res*, 442(2):229-239

Sved AF, Ito S, Madden CJ, Stocker SD, Yajima Y. 2001. Excitatory inputs to the RVLM in the context of the baroreceptor reflex. *Ann N Y Acad Sci*, 940:247-258

Tagawa T, Dampney RA. 1999. AT(1) receptors mediate excitatory inputs to rostral ventrolateral medulla pressor neurons from hypothalamus. *Hypertension*, 34(6):1301-1307

Takayama K, Miura M. 1991. Glutamate-immunoreactive neurons of the central amygdaloid nucleus projecting to the subretrofacial nucleus of SHR and WKY rats: a double-labeling study. *Neuroscie Lett*, 134(1):62-66

Tallam LS, da Silva AA, Hall JE. 2006. Melanocortin-4 receptor mediates chronic cardiovascular and metabolic effects of leptin. *Hypertension*, 48(1):58-64

Tallam LS, Stec DE, Willis MA, da Silva AA, Hall JE. 2005. Melanocortin-4-receptor-deficient mice are not hypertensive or salt-sensitive despite obesity, hyperinsulinemia, and hyperleptinemia. *Hypertension*, 46:326-332

Thorell A, Hirshman MF, Nygren J, Jorfeldt L, Wojtaszewski JF, Dufresne SD, Horton ES, Ljungqvist O, Goodyear LJ. 1999. Exercise and insulin cause GLUT-4 translocation in human skeletal muscle. *Am J Physiol Endocrinal Metab*, 277:733-741

Truett AA, Borne AT, Poincot MA, West DB. 1996. Autonomic control of blood pressure and heart rate in obese hypertensive dogs. *Am J Physiol*, 270(3 Pt2):541-549

Van Dieren S, Beulens JW, van der Schouw YT, Grobbee DE, Neal B. 2010. The global burden of diabetes and its complications: an emerging pandemic. *Eur J Cardiovasc Prev Rehabil*, 17:3-8

Vaz M, Jennings G, Turner A, Cox H, Lambert G, Esler M. 1997. Regional sympathetic nervous activity and oxygen consumption in obese normotensive human subjects. *Circulation*, 96(10):3423-3429

Vieira AA, Colombari E, De Luca LA Jr, Colombari DS, De Paula PM, Menani JV. 2010. Importance of angiotensinergic mechanisms for the pressor response to l-glutamate into the rostral ventrolateral medulla. *Brain Res*, 1322:72-80

Vollenweider P, Randin D, Tappy L, Jequier E, Nicod P, Scherrer U. 1994. Impaired insulin-induced sympathetic neural activation and vasodilation in skeletal muscle in obese humans. *J Clin Invest*, 93:2365-2371

Ward KR, Bardgett JF, Wolfgang L, Stocker SD. In Press. Sympathetic response to insulin is mediated by melanocortin 3/4 receptors in the hypothalamic paraventricular nucleus. *Hypertension*

Werther GA, Hogg A, Oldfield BJ, McKinley MJ, Figdor R, Allen AM, Mendelsohn FA. 1987. Localization and characterization of insulin receptors in the rat brain and pituitary gland using in vitro autoradiography and computerized densitometry. *Endocrinology*, 121(4):1562-1570

Williams KW, Margatho LO, Lee CE, Choi M, Lee S, Scott MM, Elias CF, Elmquist JK. 2010. Segregation of acute leptin and insulin effects in distinct populations of arcuate proopiomelanocortin neurons. *J Neurosci*, 30(7):2472-2479

Wofford MR, Hall JE. 2004. Pathophysiology and treatment of obesity hypertension. *Curr Pharm Des*, 10(29):3621-3637

Woods SC, D'Alessio DA. 2008. Central control of body weight and appetite. *J Clin Endocrinol Metab*, 93(11):37-50

Woods SC, D'Alessio DA, Tso P, Rushing PA, Clegg DJ, Benoit ST, Gotoh K, Liu M, Seeley RJ. 2004. Consumption of a high-fat diet alters the homeostatic regulation of energy balance. *Physiology and Behavior*, 83:573-578

Woods SC, Lotter EC, McKay LD, Porte D Jr. 1979. Chronic intracerebroventricular infusion of insulin reduces food intake and body weight of baboons. *Nature*, 282(5738):503-505

Woods SC, Porte D Jr. 1977. Relationship between plasma and cerebrospinal fluid insulin levels in dogs. *Am J Physiol*, 233:E331

Woods SC, Porte D Jr, Bobbioni E, Ionescu E, Sauter JF, Rohner-Jeanrenaud F, Jeanrenaud B. 1985. Insulin: Its relationship to the central nervous system and to the control of food intake and body weight, *Am J Clin Nutr*, 42(5):1063-1071

Xu AW, Kaelin CB, Takeda K, Akira S, Schwartz MW, Barsh GS. 2005. PI3K integrates the action of insulin and leptin on hypothalamic neurons. *J Clin Invest*, 115(4):951-958

Yamakawa GR, Antle MC. 2010. Phenotype and function of raphe projections to the suprachiasmatic nucleus. *Eur J Neurosci*, 31(11):1974-1983

Yang MJ, Wang F, Wang JH, Wu WN, Hu ZL, Cheng J, Yu DF, Long LH, Fu H, Xie N, Chen JG. 2010. PI3K integrates the effects of insulin and leptin on large-conductance Ca2+-activated K+ channels in neuropeptide Y neurons of the hypothalamic arcuate nucleus. *Am J Physiol Endocrinol Metab*, 298(2):193-201

Yang Y, Chen M, Lai Y, Gantz I, Georgeson KE, Harmon CM. 2002. Molecular determinant of human melanocortin-4 receptor responsible for antagonist SHU 9119 selective activity. *J Biol Chem*, 277(23):194-199

Yang Z, Bertram D, Coote JH. 2001. The role of glutamate and vasopressin in the excitation of RVL neurons by paraventricular neurons. *Brain Res*, 908(1):99-103

Yoshimoto M, Miki K, Fink GD, King A, Osborn JW. 2010. Chronic angiotensin II infusion causes differential responses in regional sympathetic nerve activity in rats. *Hypertension*, 55(3):644-651

Young CN, Deo SH, Chaudhary K, Thyfault JP, Fadel PJ. 2010. Insulin enhances the gain of arterial baroreflex control of muscle sympathetic nerve activity in humans. *J Physiol*, In Press

Ziegler DR, Cullinan WE, Herman JP. 2002. Distribution of vesicular glutamate transporter mRNA in rat hypothalamus. *J Comp Neurol*, 448(3):217-229

Zsombok A, Smith BN. 2008. Plasticity of central autonomic neural circuits in diabetes. *Biochim Biophys Acta*, 1792(5):423-431

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Bibliography

Peer Reviewed Manuscripts:

Adams JM, **Bardgett ME**, Stocker SD. Ventral Lamina Terminalis Mediates Enhanced Cardiovascular Responses of RVLM Neurons During Increased Dietary Salt. 2009. *Hypertension*. 54(2):308-14

Bardgett ME, McCarthy JJ, Stocker SD. Glutamatergic Receptor Activation in the Rostral Ventrolateral Medulla Mediates the Sympathoexcitatory Response to Hyperinsulinemia. *Hypertension*. 2010 Feb; 55(2):284-290

Bardgett JF, Taylor AC, **Bardgett ME**, McCarthy JJ, Stocker SD. Deletion of Both Transient Receptor Potential Vanilloid-1 (TRPV1) and TRPV4 Genes Disrupts Osmoregulatory Thirst and Central Fos Activation. 2010. *Journal of Physiology*, under revision

Bardgett ME, Ward KR, Stocker SD. Diet-Induced Obesity Does Not Result In Insulin Resistance In Sympathetic Circuits. 2010. *Brain Research*, under revision

Abstracts:

Josh Mancini, Jay Yoder, **Megan Bardgett**, Allison Maye, Matthew Collier. Germination studies on the North American dandelion testing a filamentous fungus , Cladosporium cladosporioides, isolated internally from seeds. Wittenberg University, Springfield, OH: presented at Undergraduate Research Conference Butler University, 2006

Stocker SD and **Bardgett ME**. Hypothalamic Paraventricular Nucleus Contributes to the Sympathoexcitatory Effects of Hyperinsulinemia. AHA Council for High Blood Pressure Research Meeting 2007.

Bardgett ME and Stocker SD. Rostral ventrolateral medulla (RVLM) mediates sympathetic vasomotor response to hypoglycemia. 2008 Experimental Biology Meeting Abstracts [on CD-ROM], Abstract #1169.2, 2008.

Stocker SD and **Bardgett ME**. Hypothalamic paraventricular nucleus contributes to the sympathoexcitatory effects of hyperinsulinemia. 2008 Experimental Biology Meeting Abstracts [on CD-ROM], Abstract #1167.1, 2008

Stocker SD and **Bardgett ME**. Hypothalamic paraventricular nucleus contributes to the sympathoexcitatory effects of hyperinsulinemia. 11th Annual Gill Heart Cardiovascular Research Day. Lexington, KY 2008

Bardgett JF, Taylor AC, **Bardgett ME**, McCarthy JJ, Stocker SD. Deletion of Both Transient Receptor Potential Vanilloid-1 (TRPV1) and TRPV4 Genes Disrupts Osmoregulatory Thirst and Central Fos Activation. Experimental Biology Meeting Abstracts, *FASEB J*. 2009 23:605.5

Bardgett ME and Stocker SD. Glutamatergic Receptor Activation in the Rostral Ventrolateral Medulla Contributes to the Sympathoexcitatory Response of Hyperinsulinemia. Experimental Biology Meeting Abstracts, *FASEB J*. 2009 23:958.16

Bardgett ME and Stocker SD. Dexamethasone Attenuates the Sympathoexcitatory Actions of Insulin. American Heart Association Council for High Blood Pressure Meeting Abstracts. *Hypertension* 54(4):e99, 2009

Bardgett ME and Stocker SD. Diet-Induced Obesity Causes Selective Insulin Resistance. 6th Annual Diabetes and Obesity Research Summit. Hershey, PA 2010

Bardgett ME and Stocker SD. Insulin Activates a NMDA Pathway to the RVLM to Elevate Sympathetic Nerve Activity. 2010 Experimental Biology Meeting Abstracts, *FASEB J*. 2010 24:808.4

Bardgett ME and Stocker SD. Diet-Induced Obesity Causes Selective Insulin Resistance. 2010. Experimental Biology Meeting Abstracts, *FASEB J*. 24:809.23

Bardgett ME and Stocker SD. Obesity-Induced Hypertension Depends on Glutamatergic Neurotransmission in the Rostral Ventrolateral Medulla. 2010. FASEB Summer Conference: Neural Mechanisms in Cardiovascular Regulation

Invited Talks:

Obesity-Induced Hypertension Depends on Glutamatergic Neurotransmission in the Rostral Ventrolateral Medulla. 2010. FASEB Summer Conference: Neural Mechanisms in Cardiovascular Regulation

Insulin: Its Role in Activation of the Sympathetic Nervous System. November 2010. Department of Cellular and Molecular Physiology, Penn State University

Fellowships

American Heart Association Fellowship: Neural Mechanisms of Sympathetic Activation During Hyperinsulinemia and Diet-Induced Obesity Hypertension. 2008-2010

Society Memberships

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Honors and Awards

08/02-05/06	Wittenberg Scholarship Award
08/02-05/06	Deans List Scholar
05/08	Travel Award, University of Kentucky
01/09	American Physiological Society Travel Award Scientific Writing Workshop
04/09	American Physiological Society Central Nervous System Van Harreveld

	Memorial Award, Experimental Biology
04/10	Diabetes and Obesity Research Summit, Best Poster Award, Penn State University
04/10	Caroline Tum Suden/Frances A. Hellenbrandt Professional Opportunity Award, Experimental Biology
07/10	FASEB Summer Conference Research Award