MODULATORS OF BAROREFLEX FUNCTION IN CONDITIONS ASSOCIATED

WITH HYPERTENSION

By

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TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

CHAPTER FOUR

APPENDIX

LIST OF ABBREVIATIONS

NTS	Solitary Tract Nucleus
CVLM	Caudal Ventrolateral Medulla
RVLM	Rostral Ventrolateral Medulla
dnmX	Dorsal Motor Nucleus of the Vagus
RAS	Renin-Angiotensin System
ACE	Angiotensin Converting Enzyme
AT_1	Angiotensin Type I Receptor
AT_2	Angiotensin Type II Receptor
STAT	Signal Transducer and Activator of Transcription
MAPK	Mitogen Activated Protein Kinase
PI3K	Phosphoinositide-3 Kinase
SOCS-3	Suppressor of Cytokine Signaling 3
PTP1B	Protein Tyrosine Phosphatase 1b
IRS	Insulin Receptor Substrate
ob	Obesity Gene
db	Diabetes Gene

ABSTRACT

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MODULATORS OF BAROREFLEX FUNCTION IN CONDITIONS ASSOCIATED

WITH HYPERTENSION

Dissertation under the direction of

Debra I. Diz, Ph.D., Professor

Hypertension is associated with various physiological and pathophysiological conditions including obesity, type II diabetes and aging. Despite decades of research, the mechanisms involved in the development of hypertension are still poorly understood. Emerging evidence suggests that hypertension is mediated by an imbalance in autonomic nervous system activity. Importantly, baroreflex sensitivity for control of heart rate, an important maker of parasympathetic function, is reduced in conditions associated with hypertension and is believed to precede and contribute to the pathogenesis of hypertension. Therefore, it is critical to identify key factors that regulate brainstem areas controlling autonomic outflow in conditions associated with baroreflex dysfunction. To this end, our goal was to identify factors that modulate baroreflex sensitivity under normal conditions and during alterations of the brain renin-angiotensin system (RAS). Specifically, we determined the contribution of angiotensin peptides and leptin to baroreflex sensitivity in order to understand mechanisms contributing to baroreflex dysfunction in aged and obese individuals.

The contribution of brain angiotensin peptides to baroreflex function during aging was assessed in older ASrAOGEN rats with low brain angiotensinogen. These studies

show that maintenance of angiotensin-(1-7) and loss of angiotensin II tone within the solitary tract nucleus (NTS) may be an important mechanism contributing to preservation of baroreflex function during aging. Furthermore, we demonstrate the novel finding that exogenous leptin impairs baroreflex sensitivity for control of heart rate and alters autonomic balance at the level of the NTS in younger Sprague-Dawley rats. Leptin-mediated impairments in baroreflex sensitivity are enhanced in younger ASrAOGEN rats suggesting interactions between leptin and the brain RAS for baroreflex modulation.

Preliminary data shows a loss of sensitivity to leptin for baroreflex modulation in older Sprague-Dawley rats with elevated circulating leptin levels suggesting resistance to the cardiovascular actions of leptin during aging. Sensitivity to leptin is maintained in older ASrAOGEN rats, associated with low circulating leptin levels, suggesting that long-term reductions in the brain RAS preserves leptin sensitivity during aging. In older Sprague-Dawley rats, baroreflex sensitivity is improved by NTS administration of a leptin antagonist implicating a role for endogenous leptin in age-related baroreflex dysfunction. These findings suggest that reduction of endogenous leptin within the NTS may be a novel mechanism to improve baroreflex function and restore autonomic balance in conditions associated with elevated leptin levels. Collectively, the present findings provide insight into novel mechanisms for preservation of baroreflex function in conditions associated with hypertension, including aging and obesity.

CHAPTER ONE

INTRODUCTION

Cardiovascular disease remains the leading cause of morbidity and mortality for both men and women in the United States. Hypertension, diagnosed when systolic blood pressure is greater than 140 mm Hg and/or diastolic blood pressure is greater than 90 mm Hg,[1] is the single most important risk factor for the development of cardiovascular disease and is associated with various pathological conditions including aging, obesity and type II diabetes[2]. Despite decades of research, the mechanisms involved in the development of hypertension are still not fully understood. However, the incidence of hypertension rises substantially with increases in body mass index, a relative measure of body fat assessed from the weight and height of patients.[3] Values of body mass index greater than 40 kg/m^2, a level within the range for obesity, are associated with a 7-fold increase in the incidence of hypertension.[4] In fact, the Framingham Heart Study estimates that 65% of essential hypertension in women and 78% in men can be directly attributed to obesity.[5] In addition, the incidence of hypertension increases with age, in part due to the increased prevalence of obesity in aged populations.[6]

While there are several effective pharmacotherapies available to treat patients with hypertension, many anti-hypertensive therapies have reduced efficacy and produce undesirable side effects in aged and obese patients.[7] The increasing prevalence of elderly and obese individuals in the United States and worldwide has created the need for research into the underlying mechanisms linking these conditions with hypertension.

Importantly, the physiological and pathophysiological changes associated with aging and obesity need to be considered in order to provide optimal anti-hypertensive treatment strategies in these populations. While several mechanisms may contribute to the pathogenesis of hypertension, emerging evidence suggests that an imbalance in autonomic nervous system activity is integral to the development of hypertension associated with obesity, aging and type II diabetes, cardiovascular and cerebrovascular diseases.[2] The autonomic imbalance observed in these conditions has been linked to alterations in various factors associated with the pathogenesis of hypertension, including inflammatory cytokines, leptin, insulin, catecholamines and angiotensin peptides.[8, 9]

1. The Autonomic Nervous System

The autonomic nervous system is a part of the peripheral nervous system that controls cardiovascular, digestive and respiratory homeostasis.[2] The autonomic nervous system is divided into two opposing branches, the sympathetic and parasympathetic nervous systems, which are regulated by reflex arcs between sensory and motor neurons in the viscera and autonomic brainstem nuclei. Importantly, the sympathetic and parasympathetic nervous systems work in concert for regulation of the cardiovascular system. The sympathetic nervous system is primarily associated with energy mobilization in response to environmental stress. In terms of cardiovascular homeostasis, activation of the sympathetic nervous system increases cardiac output and vasoconstriction to elevate heart rate and blood pressure.

Opposing the sympathetic nervous system, the parasympathetic nervous system regulates vegetative and restorative functions.[2] For cardiovascular regulation, the

parasympathetic nervous system exerts tonic inhibitory control over the heart to decrease cardiac output and lower heart rate and blood pressure. Parasympathetic nervous system function is often assessed by activity of the vagus nerve, which starts in the brainstem and innervates the heart. Heart rate variability, a measure of vagal tone to the heart, and baroreflex sensitivity for control of heart in response to increases in arterial pressure are often reported as indices of vagal function.

Arterial Baroreceptor Reflex

Arterial blood pressure is regulated by feedback control systems that work in concert over varying time frames to determine the prevailing level of blood pressure and its variability.[10] The arterial baroreceptor reflex is a high gain control system primarily involved in the short-term regulation of blood pressure. The studies presented in this thesis focus on the baroreceptor reflex for control of heart rate, however, baroreflexes are also involved in the regulation of vasculature, renal function and fluid intake. Baroreceptors are stretch-sensitive mechanoreceptors located in the auricles of the heart and vena cava that detect peripheral changes in blood pressure and volume and subsequently activate feedback loops through the autonomic nervous system to maintain cardiovascular homeostasis.

The high pressure baroreceptors are located in the aortic arch and carotid sinus and are innervated by the vagus and glossopharyngeal nerves, respectively. Baroreceptor stimulation produces excitatory activity along these afferent nerves which make their first synapse in the solitary tract nucleus (NTS) in the dorsomedial medulla oblongata. The NTS is essential to the integrity of the baroreflex arc as lesions to this autonomic brainstem region eliminate baroreflex responses.[11] Cardiac vagal chemosensitive fibers

also send divergent afferent input to the NTS.[12] The NTS integrates information from afferent baro- and chemo-receptive fibers and sends input to second order autonomic brainstem nuclei involved in the modulation of sympathetic and parasympathetic nervous system activity.[10, 11]

The baroreflex for control of heart rate is a negative feedback loop in which alterations in blood pressure elicit compensatory changes in heart rate to return blood pressure back to the operating point of the system.[10] To effectively minimize blood pressure variability, the baroreflex concurrently modulates sympathetic and parasympathetic nervous system activity.[11] As shown in the diagram below, in response to increases in blood pressure the NTS sends excitatory glutamatergic input to the caudal ventrolateral medulla (CVLM), a vasodepressor brainstem region that inhibits sympathetic activity. The CVLM then sends inhibitory GABAergic input to the rostral ventrolateral medulla (RVLM), a pressor region containing pre-motor neurons that project to sympathetic preganglionic neurons in the intermediolateral cell column of the spinal cord. Inhibition of the RVLM results in reduced sympathetic tone to the heart and vasculature to lower heart rate and blood pressure. Concomitantly, the NTS activates axonal projections to parasympathetic pre-ganglionic neurons in the nucleus ambiguous and dorsal motor nucleus of the vagus (dmnX) to reduce cardiac output and lower heart rate and blood pressure. Alternatively, in response to decreases in blood pressure, baroreceptor activity is suppressed resulting in increased sympathetic and decreased parasympathetic nervous system activity to elevate blood pressure back to normal levels.

The baroreceptor reflex has two distinct components which are independently regulated: the set-point and sensitivity/gain.[10] The set-point represents the operating

4

point of the baroreflex which is often near the level of resting blood pressure. The set-point can be rapidly reset to lower or higher levels of blood pressure depending on the

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┌─────────────────────────────────────────────────────────────┐
│  Increased                                                    │
│  Blood Pressure                                               │
│                              -            -                   │
│            │           CVLM ──→ RVLM ──→  Decreased           │
│            │            ↗                 SNA                  │
│            ▼         + ╱                   ┌──────────┐        │
│                    +                       │  ↓ BP    │        │
│  Activate ──→ NTS                          │          │        │
│  Baroreceptors       +  ╲                  │  ↓ HR    │        │
│                          ↘                 └──────────┘        │
│                        +          +                           │
│              dmnX ──→ Nucleus ──→ Increased                   │
│                       Ambiguus   PSNA                         │
└─────────────────────────────────────────────────────────────┘
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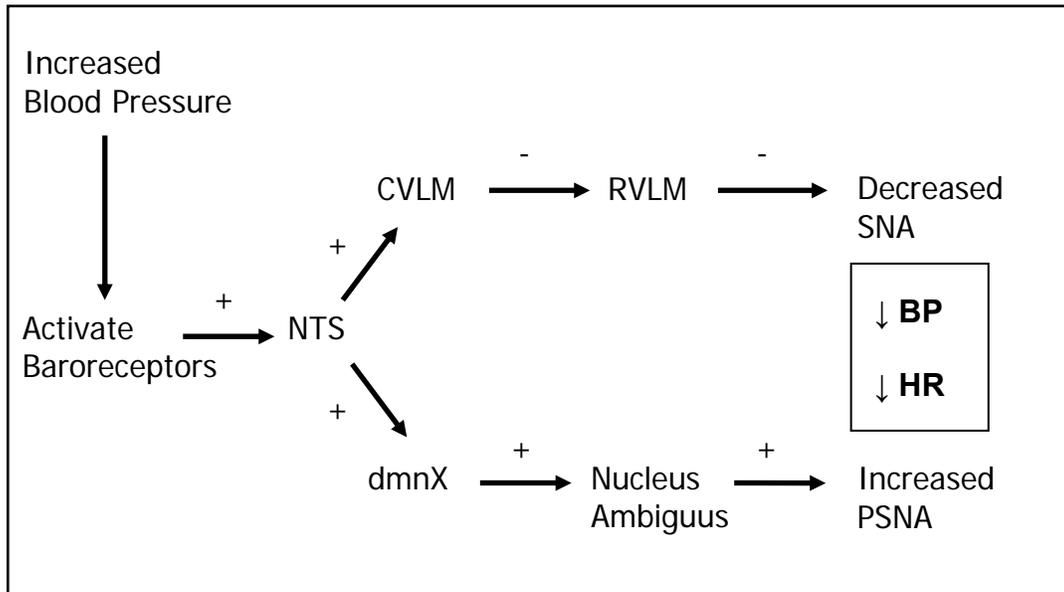
status of the cardiovascular system. The baroreflex sensitivity represents the effectiveness for correction of heart rate or sympathetic nervous system activity. The baroreflex sensitivity for control of heart rate, which influences the ability to maintain arterial pressure within a narrow range through increases in heart rate variability, is an important marker of vagal function and is often impaired in conditions associated with hypertension including obesity, aging, type II diabetes and cardiovascular and cerebrovascular diseases.[2]

Autonomic Imbalance

Under normal conditions, the sympathetic and parasympathetic arms of the autonomic nervous system are in dynamic balance and rapidly adjust based on changing environmental demands.[2] An imbalance in the autonomic nervous system in which sympathetic activity is increased and/or parasympathetic activity is decreased is

associated with various pathological conditions and may be an important mechanism underlying the development of obesity- and age-related hypertension.[13-15]

In both humans and animal models, obesity and aging are associated with an activation of the sympathetic nervous system.[8, 14] While sympathetic activation is hypothesized to be a beneficial compensatory mechanism to increase resting energy expenditure and restore energy balance in obesity,[16] increases in sympathetic outflow are also associated with the development of hypertension. Indeed, pharmacological adrenergic blockade results in greater decreases in arterial pressure in obese relative to lean hypertensive subjects confirming the importance of sympathetic activation to obesity-related hypertension.[17, 18] Independent of enhanced sympathetic activity, reductions in vagal parasympathetic function also contribute to the pathogenesis of hypertension.[2] All established risk factors for cardiovascular disease are associated with reductions in indices of parasympathetic function including heart rate variability and baroreflex sensitivity for control of heart rate.[2] Furthermore, baroreflex dysfunction is associated with negative cardiovascular outcomes including stroke, cardiac death, atherosclerosis and end organ damage and is believed to precede and contribute to the development of hypertension.[2, 19, 20] Therefore, the identification of factors that contribute to reductions in baroreflex sensitivity may be important in understanding the pathogenesis of hypertension associated with various conditions.

To date, the majority of research focuses on the contribution of the renin angiotensin system (RAS) to baroreflex function and other indices of autonomic balance. There are a few studies evaluating the role of leptin to sympathetic activation and increases in arterial pressure at brain sites involved in autonomic regulation.[13, 15]

However, the effects of leptin on indices of parasympathetic nervous system activity have yet to be considered.

2. The Classical RAS

Components and Features

As recently reviewed by Lavoie and colleagues,[21] the classical RAS is a series of enzyme-substrate interactions that generates biologically active peptides involved in cardiovascular, fluid-electrolyte and neuroendocrine homeostasis. Angiotensinogen, the hepatic-derived precursor for the RAS, is cleaved by the aspartyl protease enzyme renin into the decapeptide angiotensin I. Angiotensin I is subsequently processed by angiotensin converting enzyme (ACE), a lung-derived dipeptidyl carboxypeptidase, into the octapeptide angiotensin II. Angiotensin II is the primary effector of the RAS and exerts physiological actions by binding to angiotensin II type I (AT_1) or type II (AT_2) g-protein coupled receptors.

The ubiquitously expressed AT_1 receptor mediates the majority of biological actions attributed to angiotensin II including vasoconstriction, suppression of baroreflex function, renal sodium and water reabsorption, adrenal aldosterone release, noradrenaline release, cell proliferation and production of reactive oxygen species. These actions can be abrogated by administration of an ACE inhibitor or AT_1 receptor antagonist, to prevent the formation or actions of angiotensin II, respectively. Depending on the cell and tissue type, stimulation of the AT_1 receptor is associated with intracellular inhibition of adenylyl cyclase, activation of phospholipase C or phosphoinositide hydrolysis.[22] The majority of actions mediated by the AT_2 receptor are in opposition to the AT_1 receptor,

including vasodilation and inhibition of cellular proliferation. The AT_2 receptor is also involved in neuronal cell maturation and differentiation, growth, repair and pain threshold. [23]

Angiotensin II is degraded by aminopeptidases A or N into the smaller active fragments angiotensin III and IV, respectively. Within the central nervous system, angiotensin III binds the AT_1 receptor to mediate actions commonly attributed to angiotensin II including regulation of vasopressin release and blood pressure.[24] Angiotensin IV binds the AT_4 receptor to prevent insulin-regulated aminopeptidase-mediated metabolism of various vasoactive peptides including vasopressin, somatostatin, oxytocin, dynorphin A, lys-bradykinin and met-enkephalin.[24] The actions of angiotensin IV at AT_4 receptors within the brain are also implicated in depression, learning, memory and anxiety.[24]

Angiotensin I is also cleaved by various endopeptidases including neprilysin and prolyl endopeptidase to form the heptapeptide angiotensin-(1-7).[25] Alternatively, angiotensin II is cleaved by the ACE homologue, ACE2, to form angiotensin-(1-7). Similar to angiotensin II, angiotensin-(1-7) alters the activity of central pathways regulating blood pressure, fluid-electrolyte and neuroendocrine homeostasis. The primary biological actions of angiotensin-(1-7) include vasodilation, anti-proliferation and facilitation of baroreflex sensitivity for control of heart rate, actions opposite to angiotensin II. However, both angiotensin II and angiotensin-(1-7) can elicit similar pressor/depressor actions in specific brain nuclei[26, 27] and stimulate vasopressin release from the rat hypothalamus[28]. Since ACE degrades both angiotensin-(1-7) and bradykinin, the beneficial cardiovascular effects of ACE inhibitors are also due to the

reduced breakdown of these vasodilator peptides.[25] The actions of angiotensin-(1-7) are blocked by [D-Ala^7]-angiotensin-(1-7), but not AT_1 or AT_2 receptor antagonists, suggesting that the heptapeptide binds a distinct AT_{1-7} receptor subtype.[25]

The classical RAS exerts actions on autonomic, renal and neuroendocrine systems involved in the regulation of blood pressure. In addition, the RAS regulates various other physiological functions including learning and memory,[29] glucose homeostasis,[30] cellular proliferation,[31, 32] and oxidative stress[33]. The following sections describe the major physiological roles of the RAS, focusing on the cardiovascular actions of this system.

The Role of the RAS in Cardiovascular Regulation

It is well established that the RAS plays an integral role in cardiovascular regulation. Elevations in circulating angiotensin II are associated with vasoconstriction, sympathetic activation and baroreflex dysfunction, all of which may contribute to the development and maintenance of hypertension. The actions of angiotensin II, angiotensin III or angiotensin IV at AT_1 receptors induces potent vasoconstriction through intracellular activation of phospholipase C or calcium mobilization.[34] In opposition to the actions of angiotensin II, some studies report that angiotensin IV stimulates renal vasodilation through an AT_4 receptor-dependent production of nitric oxide.[35] In addition, low doses of angiotensin-(1-7) increase systemic vasodilation through prostaglandin and nitric oxide release.[25]

Peripheral or central administration of angiotensin II elevates arterial pressure by acting at various hypothalamic and brainstem sites within the central nervous system.[24, 36] These pressor effects are abolished by sympathectomy implicating that sympathetic activation underlies angiotensin II-mediated increases in arterial pressure. In addition to

producing sympathetic activation, systemic administration of angiotensin II reduces the vagally-mediated baroreflex sensitivity for control of heart rate in response to increases in arterial pressure.[37] The actions of angiotensin II on baroreflex function are two-fold: to shift the baroreflex set-point for control of both heart rate and sympathetic nervous system activity to higher levels of arterial pressure and to decrease baroreflex sensitivity.[10] The importance of endogenous angiotensin II to baroreflex dysfunction is illustrated by the finding that ACE inhibitors or AT_1 receptor blockers reset the baroreflex set-point to normotensive levels and improves baroreflex sensitivity in hypertensive populations.[10] In contrast, angiotensin-(1-7) facilitates baroreflex sensitivity for control of heart rate.[38] Thus, the prevailing level of baroreflex sensitivity appears to depend on the endogenous balance of these two angiotensin peptides.

Administration of angiotensin II or angiotensin-(1-7) within the NTS recapitulates the systemic effects of these peptides on baroreflex function,[39-41] implicating central sites of action for baroreflex modulation. Angiotensin peptides do not readily cross the blood-brain barrier, but may act at specialized circumventricular organs lacking a functional blood-brain barrier to modulate baroreflex function. Furthermore, recent studies suggest that AT_1 receptors within the medial NTS mediate baroreflex responses to systemic angiotensin II,[42] however, the mechanism remains unclear. Collectively, these findings implicate angiotensin peptides as important modulators of baroreflex function as well as other indices of autonomic nervous system activity.

The RAS and Regulation of Fluid-Electrolyte and Neuroendocrine Functions

As reviewed by Brewster and colleagues,[23] the kidney is integrally involved in

blood pressure regulation through alterations in sodium and water excretion. The RAS has direct renal actions to exert control over fluid and electrolyte balance. Stimulation of angiotensin II AT_1 receptors within the kidney increases sodium and water reabsorption. The anti-natriuretic and anti-diuretic effects of angiotensin II may be collectively due to increased constriction of afferent and efferent arterioles, mesangial cell contraction, decreased medullary blood flow and decreased renal interstitial pressure. Angiotensin II inhibits pressure-natriuresis,[43] reducing the ability of the kidney to compensate for increases in arterial pressure through increases in sodium and water excretion. In addition, systemic angiotensin II stimulates aldosterone release from the adrenal gland to increase sodium and water retention[36]. Collectively, the systemic actions of angiotensin II increase blood volume, through a variety of mechanisms, to elevate blood pressure.

Opposing the effects of angiotensin II, angiotensin-(1-7) acts within the kidney to increase sodium and water excretion.[44] The natriuretic and diuretic effects of angiotensin-(1-7) serve to lower blood volume and subsequently arterial pressure. However, higher levels of angiotensin-(1-7) can interact with AT_1 receptors in the proximal tubules to produce effects similar to angiotensin II.[25] Although AT_4 receptors are located in the kidneys, the physiological role of angiotensin IV to renal function is still controversial.[43]

Circulating angiotensin II is also involved in water conservation by stimulating the release of vasopressin from the posterior pituitary. Angiotensin II endogenous to the brain may also play a role in water conservation as intracerebroventricular administration of an AT_1 receptor antagonist reduces vasopressin release.[24] Peripheral administration of angiotensin II stimulates thirst and sodium appetite. The dipsogenic actions are abolished

by lesions to the subfornical circumventricular organ suggesting that angiotensin II modulates thirst central sites of action.[24] Indeed, the subfornical organ has angiotensinergic projections to the median preoptic, supraoptic and paraventricular nuclei,[45] brain regions involved in angiotensin II-mediated stimulation of thirst and vasopressin release.

3. Tissue RAS

The physiological effects of the RAS have been historically attributed to actions of the circulating hormonal system. However, all components required for the formation and degradation of angiotensin peptides are present in distinct tissue RAS systems which are independently regulated from the circulation. The experiments carried out in this dissertation focus on the brain RAS and its contribution to baroreflex function.

The Brain RAS

As early as 1961, studies demonstrated that angiotensin II acts within the central nervous system to increase blood pressure. At this time, it was hypothesized that circulating angiotensin II interacted with the central nervous system by binding AT_1 receptors in circumventricular organs. A few years later, Ganten and colleagues postulated the existence of a brain RAS independent from the circulating system.[46] Indeed, over the past 30 years all of the necessary precursors and enzymes required for the formation and metabolism of angiotensin peptides have been described in brain. However, controversy still exists regarding the cellular localization, independence from the circulating system and authenticity of brain RAS peptides.[47] The components and functions of the brain RAS have been extensively reviewed as summarized below.[24, 36]

Angiotensinogen, the precursor of both angiotensin II and angiotensin-(1-7), is ubiquitously expressed in the brain with high levels in cerebrospinal fluid, hypothalamic and brainstem regions. Angiotensinogen is produced primarily in glial cells, but is also present in neuronal cells of key cardiovascular nuclei. Renin immunoreactivity is described in discrete glial and neuronal cells of the pituitary, choroid plexus, medulla and hypothalamus providing evidence for central processing of angiotensin peptides. However, the expression of renin in the brain is low suggesting renin-independent pathways for the central production of angiotensin peptides.[47] All enzymes necessary for the processing of angiotensin I into bioactive peptides are localized to the brain, including ACE, ACE2, neprilysin and aminopeptidases. While the exact mechanisms involved in their formation remains unclear, angiotensin peptides are all reported in brain tissue. The majority of immunoreactivity is observed for angiotensin II and angiotensin-(1-7) with equal or higher levels of angiotensin-(1-7) relative to angiotensin II in the hypothalamus and medulla. Furthermore, molecular, functional and binding studies implicate the localization of angiotensin II, III, IV and 1-7 receptors in brain regions associated with autonomic outflow, fluid, neuroendocrine, gastric, respiratory, pain, stress, anxiety, motor, sensory, memory, learning and affective functions.

Angiotensin II AT_1 and angiotensin-(1-7) receptors are abundant at each synaptic relay of the sympathetic and parasympathetic nervous systems. Central infusion of angiotensin II stimulates renal sympathetic activity and increases arterial pressure in rodents. Consistent with the effects of angiotensin II to facilitate sympathetic nervous system activity, AT_1 receptors are distributed in sympathetic preganglionic neurons, sympathetic ganglia, sympathetic nerve terminals and brain sites involved in control of

sympathetic outflow including hypothalamus, dorsal medullary and ventral medullary regions. Within the hypothalamus, angiotensin II induces excitatory pressor and tachycardic responses to activate whole body and renal sympathetic outflow. At the level of the NTS, angiotensin II attenuates the ability of the baroreflexes to restrain sympathetic outflow to the heart and vasculature.[40, 41] The NTS has descending projections to brainstem vasomotor nuclei, including the RVLM and CVLM, which respond to angiotensin peptides to influence sympathetic nervous system activity, blood pressure and baroreflex function.[48] The centrally-mediated pressor actions of angiotensin II may be in part mediated by interactions with other neurotransmitter systems. For example, angiotensin II stimulates the release of dopamine, substance P and norepinephrine from central nervous system tissues,[36] all of which can contribute to elevations in arterial pressure.

Angiotensin receptors are also distributed in vagal pathways influencing control of the parasympathetic nervous system.[49] High-affinity AT_1 receptor binding sites are located on presynaptic vagal afferent terminals and on cell bodies within the NTS, with a small population of AT_2 binding sites within this brain region.[49, 50] Intracerebroventricular infusion or NTS microinjection of angiotensin II or angiotensin III impairs baroreflex sensitivity for control of heart rate in response to increases in arterial pressure,[40, 41, 51] confirming an inhibitory central effect of angiotensin II on vagal tonus. While NTS injection of low doses of angiotensin II (0.1 to 1.0 pmol) produce depressor and bradycardic responses, higher doses produce pressor and tachycardic responses[26] suggesting dose-dependent effects of angiotensin II on arterial pressure and heart rate within this brain region. Furthermore, NTS administration of the selective AT_1

receptor antagonist candesartan improves baroreflex sensitivity for control of heart rate in anesthetized Sprague-Dawley rats[52] suggesting that angiotensin II endogenous to this brain region attenuates baroreflex function. At these doses, there is no effect of candesartan on arterial pressure providing evidence that angiotensin II modulates the sensitivity without altering the set-point of the baroreflex.

In contrast to angiotensin II, intracerebroventricular infusion or NTS microinjection of angiotensin-(1-7) improves baroreflex sensitivity for control of heart rate in normotensive and hypertensive rats.[39, 51, 53] NTS injection of angiotensin-(1-7) produces depressor and bradycardic responses.[26] Furthermore, [D-Ala^7]-angiotensin-(1-7) impairs baroreflex sensitivity for control of heart rate suggesting that angiotensin-(1-7) endogenous to the brain facilitates baroreflex function.[51, 52] Since the prevailing level of baroreflex sensitivity appears to depend on the endogenous balance of these two angiotensin peptides, an increase in angiotensin II and/or a decrease in angiotensin-(1-7) actions within the NTS may result in baroreflex dysfunction.

An increasing number of studies document the importance of elevations in brain angiotensin II to the pathogenesis of hypertension.[19] Central administration of ACE inhibitors or AT$_1$ receptor antagonists lowers blood pressure in hypertensive but not normotensive animal models confirming that the brain RAS is involved in the maintenance of hypertension.[19] These pharmacotherapies also improve the set-point and sensitivity of the baroreflex in hypertensive patients and animal models.[10] Collectively, these data suggest that interruption of angiotensin II within the brain restores autonomic balance in populations exhibiting hypertension.

Importantly, reduced central angiotensin-(1-7) tone may also contribute to

impairments in baroreflex function associated with hypertension.[54] In hypertensive (mRen2)27 rats, intra-cisternal replacement of angiotensin-(1-7) lowers blood pressure and improves baroreflex sensitivity[53] suggesting that reduced central angiotensin-(1-7) tone contributes to the hypertension and baroreflex dysfunction characteristic of these animals. Recent observations by Sakima and colleagues reveal that baroreflex dysfunction during aging is also associated with reduced angiotensin-(1-7) tone within the NTS, possibly due to decreased neprilysin levels.[52] These findings suggest that maintenance of angiotensin-(1-7) actions in the NTS may be an important mechanism to preserve baroreflex function during aging. Thus, the present studies determined the contribution of angiotensin peptides within the NTS to the age-related preservation of baroreflex function observed in transgenic ASrAOGEN rats with low brain RAS activity.

Angiotensin II AT_1 receptors are also localized to cardiac vagal chemosensitive fibers that synapse within the NTS.[12] Transgenic rats with low or high brain RAS activity have enhanced or reduced depressor and bradycardic responses to cardiac vagal chemosensitive fiber activation induced by intravenous phenylbiguanide,[55] respectively, providing evidence that brain angiotensin II attenuates responses to cardiac vagal chemosensitive fiber activation. These findings are consistent with the inhibitory effects of angiotensin II on baroreflex function.[40, 41]

Finally, AT_1 receptors are localized to brain regions associated with the stimulation of salt appetite, thirst and vasopressin release, including the hypothalamic paraventricular nucleus and circumventricular subfornical organ.[24, 36] Angiotensin II also acts within the central nervous system to stimulate sympathetic outflow to the kidneys and reduce baroreflex control of renal sympathetic nervous system activity. The brain

RAS also participates in the modulation of learning, memory, cognition, stress and addiction[24] which may be in part due to the AT_2 and AT_4 receptors found at low levels in brain regions associated with motor, sensory and limbic activity.[24]

Other Tissue RAS

At the present time, the contribution of circulating versus tissue-derived angiotensin peptides to the biological actions of the RAS has not been clearly established. However, all components of the RAS have been independently identified in adipose tissue, adrenal gland, liver, kidney, heart, pancreas, vasculature and reproductive organs.[36] In humans, adipose tissue may be an independent source for angiotensin peptides as plasma renin activity and circulating levels of angiotensinogen and ACE are correlated with body mass index.[56] Indeed, the expression of all RAS components has been demonstrated in adipose tissue.[57, 58] Adipose-derived angiotensin II acts locally to increase adipocyte growth and differentiation[59] and inhibit lipolysis,[60] actions that promote negative energy balance. Illustrating the importance of the RAS to body weight, angiotensinogen deficient mice are lean, insulin sensitive and resistant to diet-induced obesity.[61] In contrast, over-expression of angiotensinogen is observed in white adipose tissue of obese humans and Zucker rats.[57, 58] Collectively, the production of angiotensin peptides by adipocytes may be an important mechanism contributing to obesity-related hypertension. In addition, adipose tissue secretes a number of other hormones, including leptin,[62] which act on receptors within central nervous system pathways involved in the regulation of cardiovascular and metabolic function[15].

4. Leptin

Body weight is relatively stable in humans implicating the existence of a physiological feedback loop to regulate energy balance. Scientists have long postulated that peripheral circulating signals convey information about the status of energy stores to brain regions involved in the regulation of appetite and energy metabolism. However, until recently the existence of these metabolic signals was widely questioned. The exciting discovery of the peptide hormone leptin in 1994 by Friedman and colleagues has provided vast information on the neuroendocrine circuitry underlying the control of appetite, energy metabolism and adiposity.[63] Leptin, a 167 amino acid product of the obesity (*ob*) gene, is produced primarily in white adipose tissue with small amounts produced in brain, ovaries, stomach, skeletal muscle, bone, bone marrow, mammary and pituitary glands.[64] Leptin is secreted into the circulation in direct proportion to adiposity in both rodents and humans.[65] Leptin undergoes renal clearance from the circulation at the same rate in lean and obese individuals[66] suggesting that obesity-related elevations in circulating leptin levels are due solely to increased leptin production.

Metabolic Actions of Leptin

Humans, mice (*ob/ob*) and rats (*fa/fa*) unable to produce leptin due to homozygous autosomal recessive mutations in the *ob* gene are obese, diabetic and exhibit neuroendocrine abnormalities which are corrected by peripheral administration of exogenous leptin.[63, 67, 68] Mutations in the leptin receptor gene also result in obesity and diabetes in humans and rodents (*db/db* mice and Zucker fatty rats).[68, 69] However, exogenous leptin does not correct abnormalities in leptin receptor-deficient populations

due to the inability of leptin to activate intracellular signaling pathways involved in metabolism.[70]

In rodents, chronic peripheral or central administration of recombinant leptin results in significant reductions in food intake and body weight.[68, 70-72] Leptin administration also increases core body temperature, metabolic rate and energy expenditure in mice.[71] Leptin levels do not change in response to meal ingestion suggesting primary involvement in long-term regulation of body weight.[65] Collectively, these findings suggest that leptin modulates energy balance through long-term regulation of appetite, adiposity and energy expenditure. In addition to serving as a satiety signal, leptin is implicated as a starvation signal in fasting and anorexic states.[73]

CNS pathways mediating leptin's metabolic actions

Peripheral leptin administration activates signal transducer and activator of transcription (STAT) and *c-fos* immunoreactivity within hypothalamic and brainstem nuclei.[74, 75] Central intracerebroventricular leptin administration inhibits food intake and reduces body weight in wild-type and *ob/ob* mice at lower doses than required for peripheral adminstration.[68] Furthermore, selective deletion of all neuronal leptin receptor isoforms results in obesity in mice.[76] These data suggest that the effects of leptin on energy balance are mediated at central sites of action. Although leptin is produced primarily in the periphery, leptin receptor immunoreactivity is observed in circumventricular organs which may provide access to the central nervous system.[77] Alternatively, a saturable uni-directional transporter and short forms of the leptin receptor can transport leptin across the blood-brain barrier.[78, 79] Leptin may also access the brain at the blood-cerebrospinal fluid barrier as immunoreactive leptin cerebrospinal fluid

levels are strongly correlated to plasma leptin levels.[80] The multi-ligand receptor megalin (low-density lipoprotein receptor-related protein 2) is localized to epithelia including the choroid plexus constituting the blood-cerebrospinal fluid barrier[81] and is involved in the polarized transport of leptin, insulin, growth hormone and angiotensin peptides.[81-83] Functionally, a decrease in choroid plexus expression of megalin is correlated with impaired leptin uptake into the brain of aged Wistar rats.[81] Although leptin gene expression is reported in multiple brain regions in rats[84] and arteriovenous blood sampling in men shows that the brain releases leptin into the circulation,[66] the physiological relevance of brain-derived leptin is currently unknown.

The majority of biological activity produced by leptin is mediated by the long form of the leptin receptor, Ob-R$_b$.[85] In rodents, Ob-R$_b$ receptors are widely distributed in the central nervous system,[84] with high levels of gene expression observed in the dorsomedial, ventromedial and arcuate hypothalamic nuclei.[84, 86] Physical or chemical ablation of any of these nuclei results in obesity suggesting that the hypothalamus is crucial to leptin's actions on energy metabolism.[87] The arcuate nucleus is a major site of neuronal transduction for afferent input from circulating leptin[88] and contains two separate populations of leptin-responsive neurons[89]. Activation of Ob-R$_b$ leptin receptors in the arcuate nucleus results in the differential regulation of anorexigenic and orexigenic genes to inhibit food intake.[89] Arcuate nucleus neurons innervate second order neuronal centers, including brainstem regions, which further integrate leptin signaling before sending efferent signals to peripheral organs.[90]

Independent of descending hypothalamic pathways, leptin receptors are localized to the nodose ganglion, on vagal afferents and within brainstem nuclei including the NTS

in normotensive rats.[84, 91] Although leptin receptor gene expression is lower in brainstem relative to hypothalamic brain regions,[92] these receptors appear functional as intravenous leptin infusion increases STAT3 phosphorylation and *c-fos* immunoreactivity within brainstem regions[75, 91] and leptin microinjection into the caudal brainstem decreases food intake and body weight in rats[93]. Furthermore, the NTS contains the same leptin-responsive neuronal populations as in the hypothalamus, however, the contribution and importance of NTS pathways to long-term regulation of energy metabolism by leptin is controversial.[93, 94] For short-term regulation of food intake, stomach-derived leptin can act synergistically with cholecystokinin to stimulate vagal afferents that synapse within the NTS.[91] In addition to effects on metabolism, leptin is involved in regulation of glucose homeostasis, neuroendocrine, sexual maturation and reproduction, thyroid, growth hormone, hematopoiesis, bone remodeling, memory and learning, development and immune functions.[95] Interestingly, recent studies implicate leptin in cardiovascular regulation as leptin levels are elevated in cardiovascular pathologies independent of obesity. The mechanisms by which leptin regulates cardiovascular function are still under investigation, but may include alterations in the balance of the autonomic nervous system.

Leptin's Cardiovascular Actions

Circulating leptin levels are an independent risk factor and diagnostic marker for hemorrhagic stroke,[96] myocardial infarction,[97] heart failure[98] and coronary heart disease[99]. In humans, circulating leptin levels correlate to both muscle sympathetic nervous system activity and renal norepinephrine spillover levels indicating a link between leptin and sympathetic activation.[100] Indeed, peripheral leptin administration

stimulates sympathetic nervous system activity to organs involved in cardiovascular regulation including the kidney, adrenal gland and hindlimb without effects on arterial pressure in normotensive rats.[101] The sympathoexcitatory effects of leptin are absent in rats lacking Ob-R$_b$ receptors providing evidence for receptor-mediated effects.[101] Transgenic skinny mice over-expressing leptin in the liver have increased arterial pressure which is normalized by intraperitoneal administration of an α adrenergic blocker[102] suggesting that leptin-induced increases in sympathetic activity may contribute to hypertension. In contrast, conscious leptin-deficient mice have reduced sympathetic activity and lower arterial pressure compared to control mice despite having increased body weight.[103]

Consistent with evidence from genetic rodent models, administration of exogenous leptin increases arterial pressure in normotensive rodents. While acute intravenous leptin administration results in no change in cardiovascular parameters in anesthetized Sprague-Dawley rats,[104] chronic intravenous infusion increases arterial pressure and heart rate in these animals.[105] Acute and chronic central intracerebroventricular infusion of leptin produces delayed increases in arterial pressure, heart rate and renal sympathetic nervous system activity in anesthetized and conscious rodents.[106, 107] These findings implicate a central mechanism of action that might include impairment of baroreflex sensitivity to allow for the delayed elevation in arterial pressure. However, the contribution of leptin to baroreflex function is currently unknown. Although depressor actions have been reported *in vivo* at low doses, at physiologically relevant doses the pressor actions of leptin predominate.[108] The pressor and tachycardic actions of leptin are reversed by pharmacological adrenergic blockade[109]

suggestive of sympathetically-mediated effects. Collectively, leptin stimulates sympathetically-mediated elevations in arterial pressure, which may contribute to cardiovascular disease in aged and obese individuals with elevated circulating leptin levels. The effect of leptin on indices of parasympathetic nervous system activity, including baroreflex sensitivity for control of heart, has yet to be assessed.

Central Pathways Mediating Leptin's Cardiovascular Actions

Microinjection of leptin into the ventromedial, dorsomedial or arcuate hypothalamic nuclei increases arterial pressure, heart rate and renal sympathetic nerve activity,[110, 111] consistent with the metabolic sites for leptin action. Leptin microinjection into the hypothalamic paraventricular nucleus has no direct effect on cardiovascular regulation possibly due to low Ob-R_b expression within this brain region.[75, 112] These hypothalamic nuclei are connected via descending pathways to brainstem nuclei involved in cardiovascular and reflex function, including the NTS and RVLM.[113, 114] Leptin receptors within the NTS mediate cardiovascular responses as microinjection of leptin (0.25 - 1.0 μg) into the caudal NTS of Sprague-Dawley rats results in dose-dependent increases in renal sympathetic nerve activity.[92] Microinjection of the highest leptin dose modestly increases arterial pressure with no significant effect on heart rate.[92] The cardiovascular effects of leptin within the NTS are delayed, occurring at two hours after the initial microinjection.[92] The prolonged time course for manifestation of leptin actions is consistent with impairments in baroreflex sensitivity for control of heart rate mediated at the level of the NTS. However, baroreflex function was not assessed in these studies. Previous studies show no effect of intravenous leptin on baroreflex for control of renal sympathetic nerve activity, a sympathetically-mediated index.[115] The effects of leptin on

baroreflex sensitivity for control of heart rate, a measure of vagal function that is often impaired in conditions associated with hypertension,[2] needs to be considered.

In addition to effects on autonomic balance, leptin promotes endothelial dysfunction, oxidative stress, inflammation, vascular smooth muscle cell proliferation and migration, atherosclerosis, angiogenesis and thrombosis.[116, 117] Any of these effects may provide key links between leptin and cardiovascular disease. The deleterious cardiovascular effects produced by leptin suggest that reduction of endogenous leptin may be protective against cardiovascular and cerebrovascular diseases. Indeed, leptin-deficient and leptin receptor-deficient mice are protected from thrombosis in arterial injury models[118, 119] and intravenous administration of a leptin antibody reduces thrombus size and protects from arterial and venous thrombosis in lean mice.[120] To determine therapeutic strategies to suppress the deleterious cardiovascular effects of leptin while maintaining its positive effects on energy balance, it will be necessary to dissect the signaling and anatomical pathways utilized by leptin for cardiovascular regulation.

Leptin Signaling

The leptin receptor is encoded by the diabetes (*db*) gene and was isolated from mouse choroid plexus by Tartaglia and colleagues in 1995.[121] Alternative splicing of the *db* gene results in mRNA variants encoding at least six isoforms (Ob-R$_{a-f}$) of the leptin receptor.[95, 122] The majority of leptin receptor isoforms are single transmembrane receptors belonging to the type I cytokine receptor superfamily.[123] All leptin receptor isoforms share identical extracellular ligand-binding domains, but short forms of the receptor (Ob-R$_{a,c,d,f}$) lack intracellular domains and do not participate in leptin signaling.[95, 122] Ob-R$_a$ and Ob-R$_c$ receptors are abundantly expressed in choroid plexus

and microvessels of the brain implicating these receptors in leptin transport.[122, 124] Indeed, Koletsky rats lacking Ob-R$_a$ receptors have decreased leptin influx from the blood to the brain.[124, 125] Ob-R$_a$ receptors are also found in the kidneys, lungs and lymph nodes where they may play a role in renal clearance and immune function.[125] While there is limited knowledge concerning other short forms of the leptin receptor, Ob-R$_e$ circulates as a soluble receptor and is believed to serve as a binding protein to antagonize leptin transport.[126]

The long form of the leptin receptor, Ob-R$_b$, is the only isoform known to initiate intracellular signal transduction pathways.[95, 127] Ob-R$_b$ receptors represent a small proportion (5-10%) of total leptin receptor mRNA expression[128] and are primarily localized to the brain with dense hybridization observed in the cerebellum and arcuate, dorsomedial and ventromedial hypothalamic nuclei.[84, 122] Leptin-responsive sites have also been identified at extra-hypothalamic sites including the dorsal raphe, periaqueductal gray, parabrachial nucleus and NTS.[84, 86] Immunoreactivity studies suggest that leptin receptors are localized to both neuronal and glial cells.[129, 130] While leptin receptor immunoreactivity is predominantly in the plasma membrane, nuclear immunoreactivity is also observed in the hypothalamus suggesting possible extra- and intra-cellular actions.[130] Ob-R$_b$ gene expression is observed in lung, kidney, adrenal and lymph node, liver, adipose and skeletal muscle, albeit at lower levels than in the central nervous system.[122] Mutations in Ob-R$_b$ receptors result in morbid obesity in humans and rodents showing the critical nature of these receptors for regulation of body weight.[131]

The Ob-R$_b$ leptin receptor lacks intrinsic kinase activity and thus associates with the constitutively expressed janus tyrosine kinase (JAK) 2 through a binding motif on the

intracellular C-terminal domain of the receptor.[64, 95] The binding of leptin to preformed Ob-R$_b$ receptor homodimers induces a conformational change in the receptor resulting in transphosphorylation and activation of JAK2.[64] The activation of JAK2 induces phosphorylation of tyrosine residues 985, 1077 and 1138 within the cytoplasmic domain of the leptin receptor.[132, 133] Upon phosphorylation, tyrosine residues 985 and 1138 recruit proteins with a src homology 2 domain to initiate intracellular signaling cascades.[64] The activation of tyrosine 1077 does not appear to contribute to leptin signaling.[64, 122]

The leptin receptor signals via three distinct intracellular signaling pathways: JAK/STAT, mitogen activated protein kinase (MAPK) and phosphoinositide-3 kinase (PI3K).[64, 95, 122] The phosphorylation of tyrosine 1138 initiates the JAK/STAT genomic signaling pathway. The binding of STAT to tyrosine 1138 results in the activation, homo-dimerization and translocation of STAT3 to the nucleus where the transcription of several genes is altered.[95] In response to leptin, STAT3 activates transcription of suppressor of cytokine signaling 3 (SOCS-3), a negative regulator of signaling.[134] Acutely, leptin signaling is also terminated by protein tyrosine phosphatase 1b (PTP1B).[135] In mice, mutations in tyrosine 1138 or neural-specific deletion of STAT3 results in obesity and diabetes implicating the JAK/STAT pathway in central regulation of appetite and body weight.[122, 136]

In addition to genomic signaling, leptin evokes rapid responses through activation of MAPK and PI3K signaling pathways. The phosphorylation of tyrosine 985 on the leptin receptor initiates the activation of downstream MAPK signaling pathways.[64, 116] Central or peripheral leptin administration increases phosphorylated extracellular signal-

related kinase (ERK) 1/2, a well studied member of the MAPK family.[137, 138] The ERK pathway is involved in the maintenance of blood pressure in the RVLM of normotensive and hypertensive rats.[139] In addition, ERK 1/2 phosphorylation is increased in kidney and aortic tissue during the early phase of leptin-induced hypertension[140] and ERK mediates renal sympathetic nerve activation in response to hypothalamic leptin administration in Sprague-Dawley rats[138]. Collectively, these findings imply that the ERK pathway is involved in leptin-mediated sympathetic and hypertensive responses.

Tyrosine residues on the intracellular domain of the $Ob-R_b$ leptin receptor also serve as docking sites for insulin receptor substrate (IRS) 1/2.[64, 122] Phosphorylation of IRS 1/2 leads to the subsequent activation of PI3K, through an association to its regulatory domain.[141] PI3K then phosphorylates downstream effectors including serine-threonine protein kinase B (Akt) to activate a number of kinases, transcription factors and regulatory molecules involved in the regulation of glucose metabolism, protein synthesis, cellular proliferation and survival.[142] Peripheral or intracerebroventricular leptin administration increases phosphorylation of IRS 1/2 and PI3K activity in the hypothalamus of normotensive rats.[137, 143] While one study found no profound effect of peripheral leptin on hypothalamic Akt phosphorylation at 3 minutes after the injection,[144] a separate study showed that central intracerebroventricular leptin increases Akt phosphorylation at 30 minutes after leptin infusion in Sprague-Dawley rats.[137] Furthermore, central pharmacological blockade of the PI3K pathway with wortmannin or LY294002 prevents the effects of leptin on food intake and renal sympathetic activity.[145, 146] Collectively, these findings suggest that the PI3K pathway is involved in both the metabolic and cardiovascular actions of leptin. Importantly, administration of PI3K

inhibitors within the NTS improves baroreflex sensitivity for control of heart rate and reduces arterial pressure in hypertensive rats implicating this pathway in baroreflex regulation.[147] However, the intracellular signaling pathways utilized by leptin for cardiovascular reflex regulation are not known. This is consistent with a general lack of knowledge concerning pathways involved in baroreflex modulation. The identification of molecular mechanisms involved in leptin signaling may help to uncover therapeutic strategies to target restoration of baroreflex function.

PTP1B

Reversible protein phosphorylation by tyrosine kinases is a major regulatory mechanism underlying intracellular signaling events.[148] The relative level of intracellular phosphorylation depends on the activity of kinases in concert with protein tyrosine phosphatases which catalyze the hydrolysis of phosphate monoesters.[148] Several human diseases are associated with a deregulation of phosphatase activity leading to unrestrained kinase signaling.[149] Recent evidence implicates PTP1B, a ubiquitously expressed member of the protein tyrosine phosphatase family encoded by the protein tyrosine phosphatase non-receptor 1 gene, in metabolic regulation.[149] Levels of PTP1B are elevated in conditions associated with metabolic resistance including aging, obesity and type II diabetes.[150-152]

PTP1B attenuates insulin signaling through direct dephosphorylation of the insulin receptor and IRS 1/2 substrates.[153] Single nucleotide polymorphisms in the PTP1B gene results in protection from diabetes[154] suggesting a role for this phosphatase in the disruption of glucose homeostasis. Recent studies suggest that PTP1B also attenuates leptin signaling through dephosphorylation of JAK2, a key kinase mediating

activation of the leptin receptor.[155, 156] Since the actions of insulin and leptin on energy balance require initiation of tyrosine kinase phosphorylation events, enhanced activity of PTP1B could contribute to metabolic resistance to these peptides.[149] Over-expression of PTP1B reduces phosphorylation of PI3K and ERK MAPK signaling pathway components with no acute effect on Akt activity in mouse adipocytes.[157] However, the effects of PTP1B on Akt are unclear as inhibition of PTP1B restores insulin-stimulated Akt phosphorylation in Chinese hamster ovary cells.[158] In addition, over-expression of PTP1B in a mouse hypothalamic cell line results in dose-dependent reductions in JAK2/STAT3 phosphorylation.[159] These findings suggest that PTP1B prevents activation of the major intracellular signaling pathways utilized by leptin, insulin and angiotensin II.

The interference of PTP1B with insulin and leptin signaling pathways suggests a key role for this phosphatase in the regulation of energy homeostasis.[149] Indeed, global PTP1B knockout mice have lower body weight, decreased adipose tissue mass and are resistant to diet-induced obesity relative to wild-type littermates. This phenotype is associated with increases in basal metabolic rate and total energy expenditure. PTP1B knockout mice maintain low blood glucose levels and have enhanced responses to oral glucose and intraperitoneal insulin tolerance tests in the face of lower circulating insulin and leptin levels suggesting an increased sensitivity to metabolic hormones in these animals. PTP1B knockout mice also exhibit increased insulin receptor phosphorylation in liver and muscle tissue as well as increased JAK2/STAT3 phosphorylation in fibroblasts relative to wild-type mice. There are no changes in total cellular tyrosine phosphorylation in PTP1B knockout mice,[135] however, the effect of the gene deletion on other systems is currently unknown. The beneficial metabolic effects associated with

PTP1B inhibition were initially hypothesized to be modulated by insulin-sensitive tissues including muscle, liver and adipose tissue.[160] However, targeted deletion of PTP1B in muscle, liver or fat has no effect on body weight in mice.[161] Only neuron-specific deletion of PTP1B recapitulates the phenotype observed in global knockout mice, suggesting a centrally-mediated mechanism of action.[161]

Consistent with central sites of action, PTP1B hybridization is observed in hippocampal and hypothalamic brain regions including the arcuate, ventromedial and dorsomedial hypothalamic nuclei.[162] However, the expression of PTP1B has not yet been assessed in brainstem regions involved in cardiovascular regulation. Hypothalamic levels of PTP1B are increased in aged and obese rats[150, 151] and are thought to contribute to metabolic resistance in these animals. A reduction of central PTP1B levels by intracerebroventricular delivery of a PTP1B inhibitor or central infusion of a PTP1B antisense oligonucleotide is sufficient to reduce body weight, decrease adiposity and improve leptin and insulin sensitivity in aged and obese rats.[150, 151] Therefore, targeted reduction of PTP1B within the central nervous system may be a therapeutic strategy to increase sensitivity to the metabolic actions of insulin and leptin.

As illustrated below, peptides involved in cardiovascular regulation, including angiotensin II, insulin and leptin activate kinase regulated signaling pathways to increase PTP1B expression.[149, 163] While elevated PTP1B levels attenuate the positive metabolic actions of these peptides, they may concomitantly prevent the stimulation of harmful cardiovascular signaling pathways. Indeed, in the face of reduced phosphatase activity a sensitization to the deleterious cardiovascular effects occurs. Relative to control mice, PTP1B knockout mice are hypertensive, exhibit enhanced responsiveness to the pressor

effects of leptin and have enhanced sympathetic tone associated with reduced adrenergic gene expression and reduced pressor responses to phenylephrine.[164]

Leptin
Insulin
Ang II

$Ob-R_b$
IR
AT_1

+

Intracellular Kinase
Signaling Pathways

↑ **PTP1B**

- PI3K
- MAPK
- JAK/STAT

—
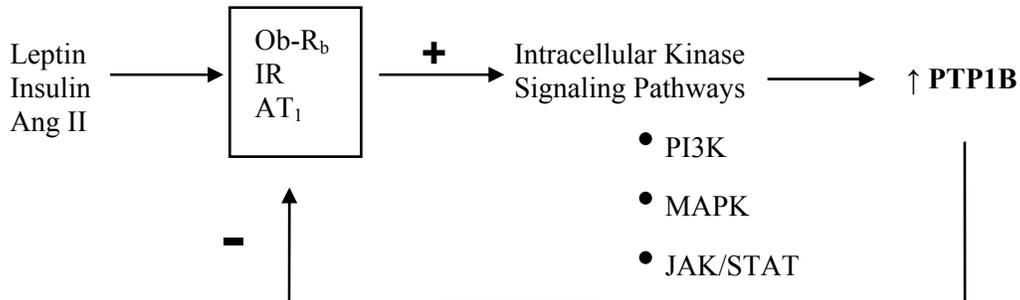
The elevations in sympathetic activity and blood pressure observed in PTP1B knockout mice may suggest a concomitant reduction in baroreflex sensitivity for control of heart rate. However, the contribution of PTP1B tone to baroreflex function has yet to be considered. If PTP1B inhibition exerts negative effects on blood pressure regulation, targeting of this phosphatase may lead to further detrimental cardiovascular outcomes.

Mechanisms of Leptin Resistance

Naturally occurring mutations in the leptin or leptin receptor genes results in morbid obesity and neuroendocrine abnormalities.[165] However, leptin mutations are only observed in a small proportion of obese individuals. Surprisingly, the majority of obese populations exhibit normal or elevated plasma leptin levels relative to lean subjects,[65] suggesting effective leptin resistance. Administration of exogenous leptin to patients with elevated leptin levels has been relatively unsuccessful as a therapeutic agent,[166] possibly due to a poor understanding of the molecular mechanisms contributing to leptin sensitivity. It has been postulated that leptin resistance may result from reduced leptin transport across the brain blood barrier[167] as well as defects in leptin receptor-mediated signaling pathways.[134, 168]

Interestingly, the ability of leptin to stimulate renal sympathetic nerve activity and arterial pressure is preserved in obese animal models suggesting possible maintenance of leptin sensitivity to cardiovascular effects, in the face of metabolic resistance.[169] Therefore, a reduction of endogenous leptin levels may prevent saturation of leptin receptors, increase leptin transport and reduce negative regulators of leptin signaling to allow for maintenance of metabolic sensitivity to leptin. Concomitantly, low endogenous leptin levels may reduce the activation of cardiovascular signaling pathways contributing to hypertension. Understanding mechanisms to preserve leptin sensitivity, in the presence of low endogenous leptin levels, may be important for maintaining sensitivity to the satiety effects while preventing the negative cardiovascular effects of this peptide. Recent studies suggest that interruption of angiotensin II may be an important mechanism to preserve the metabolic sensitivity to leptin. Whether interruption of angiotensin II actions alters the cardiovascular sensitivity to leptin has yet to be considered.

Interactions between Metabolic Systems and the RAS

The distribution of angiotensin II and angiotensin-(1-7) receptors within the central nervous system overlaps with autonomic pathways involved in the regulation of energy balance. Furthermore, angiotensin II, leptin and insulin all utilize the same intracellular signaling pathways to exert cardiovascular and metabolic actions.[95, 170] The overlap of central anatomical and signaling pathways utilized by these peptides provides a possible site for cross-talk to influence cardiovascular and metabolic function. Angiotensin II is known to interfere with insulin signaling by uncoupling the PI3K pathway to reduce glucose transport.[30] The uncoupling of PI3K is reversed by 21 day treatment with the AT_1 antagonist irbesartan in obese Zucker rats.[171] In hypertensive

patients, ACE inhibition or AT_1 receptor blockade lowers plasma insulin levels and reduces the risk of new-onset diabetes[172] providing further evidence for interactions between angiotensin II and insulin signaling pathways.

Angiotensin II also stimulates leptin release,[173] promotes leptin production[174] and increases leptin levels and Ob-R_b mRNA expression *in vitro*[175] providing evidence for stimulatory interactions between angiotensin II and leptin. Angiotensin II decreases gene and protein expression of megalin in cultured proximal tubule cells, possibly limiting leptin transport into cerebrospinal fluid.[176] However, interactions between angiotensin II and megalin are unclear as ACE inhibition also decreases megalin expression in these cells.[177] In hypertensive patients, ACE inhibitors and AT_1 receptor blockers also decrease plasma leptin levels[172] and age-related increases in circulating leptin levels are prevented in Fischer 344 rats treated long-term with an AT_1 receptor blocker.[178] Transgenic rats with lifelong low brain angiotensinogen levels maintain normal leptin levels during aging and exhibit increased leptin responsiveness to an oral glucose load.[179, 180] In contrast, hypertensive (mRen2)27 rats with high brain RAS activity exhibit reduced insulin and leptin sensitivity to an oral glucose, associated with higher serum levels of insulin and leptin during aging.[179] Thus, the beneficial effects of ACE inhibitors and AT_1 receptor blockers on leptin sensitivity for metabolism appear to be mediated by the brain RAS. Collectively, these studies suggest that chronic angiotensin II blockade maintains low endogenous leptin levels and improves leptin sensitivity for metabolic actions. Whether the brain RAS influences sensitivity to the cardiovascular actions of leptin has yet to be considered.

5. Use of Transgenic Rat Models to Address the Role of the Brain RAS to Baroreflex Modulation

The development of transgenic rodent models with systemic alterations in components of the RAS has been an important tool to study the contribution of angiotensin peptides to the pathogenesis of hypertension. The importance of the RAS to cardiovascular regulation is illustrated by the finding that systemic over-expression of angiotensinogen increases blood pressure while systemic knockout of ACE or AT_1 receptors lowers blood pressure in mice.[181] Recent studies using transgenic rodents reveal that the brain RAS plays a crucial role in the etiology of hypertension through alterations in cardiovascular, fluid-electrolyte and neuroendocrine regulation, consistent with the central distribution of angiotensin receptors in brain regions that regulate these functions.[19, 182] Over-expression of human angiotensinogen and renin in double transgenic mice reveals differential patterns for regulation of arterial pressure and baroreflex sensitivity depending on the cellular origin of the angiotensin peptides.[183, 184] Glial or neuronal over-expression increases arterial pressure, thirst and sodium appetite in these animals.[184] However, glial over-expression reduces baroreflex sensitivity while neuronal over-expression alters the baroreceptor set-point.[183] Studies in younger ASrAOGEN rats with low brain angiotensinogen extend these findings to show a glial source of angiotensinogen for angiotensin II and a non-glial source of angiotensinogen for angiotensin-(1-7) are involved in baroreflex modulation.[185] Collectively, these data show that the brain RAS is involved in the modulation of baroreflex function.

In Sprague-Dawley rats, low angiotensin-(1-7) tone within the NTS appears to contribute to age-related baroreflex dysfunction.[52] ASrAOGEN rats are spared age-

related declines in cardiovascular and metabolic function, including relative maintenance of baroreflex function during aging relative to other rat strains.[179, 180, 186] The mechanisms underlying the preservation of baroreflex function in ASrAOGEN rats during aging has yet to be evaluated, but may include alterations in the endogenous balance of angiotensin II and angiotensin-(1-7) for baroreflex modulation. Therefore, we determined the contribution of angiotensin peptides within the NTS to cardiovascular reflex regulation in older ASrAOGEN rats.

Recent studies using transgenic rodents also provide evidence that the brain RAS is involved in the regulation of energy balance. (mRen2)27 rats with high brain RAS activity are heavier and exhibit reduced insulin and leptin responsiveness to an oral glucose load relative to control Sprague-Dawley rats.[179, 180] These observations may be in part explained by the finding that angiotensin II interrupts the insulin-mediated PI3K signaling pathway.[30] In contrast, transgenic ASrAOGEN rats with low brain RAS activity are lighter and exhibit enhanced insulin and leptin responsiveness for metabolic actions.[179, 180] In hypertensive patients, ACE inhibitors and AT_1 receptor blockers lower plasma insulin and leptin levels and reduces the risk for new-onset diabetes.[172] Collectively, these findings suggest that interruption of brain angiotensin II is associated with increased sensitivity to the metabolic actions of insulin and leptin. While sensitivity to metabolic hormones is beneficial for energy and glucose homeostasis, recent studies show that leptin stimulates sympathetic nervous system activity and elevates arterial pressure at central sites of action.[15, 92, 187] Whether interruption of angiotensin II actions also results in altered sensitivity to the cardiovascular actions of leptin has yet to be determined. Therefore, we employed transgenic rats ASrAOGEN and (mRen2)27 rats

with low or high brain RAS activity, respectively, to determine interactions between the brain RAS and cardiovascular sensitivity to leptin. In addition, we used these transgenic rats to determine the influence of the brain RAS on intracellular signaling pathways utilized by leptin for modulation of baroreflex function.

Transgenic ASrAOGEN rats

In 1999, Ganten and colleagues produced a unique rodent model for under-expression of the endogenous brain RAS. Transgenic ASrAOGEN rats were created by transfection of Hannover Sprague-Dawley rats with an antisense oligonucleotide to angiotensinogen driven by a glial fibrillary acid protein promoter[188] to target expression specifically to astrocytes in both cell culture[189, 190]. Since glial cells are the main source of brain angiotensinogen, the antisense oligonucleotide results in a 90% reduction in angiotensinogen protein levels in various brain regions including medulla, hypothalamus and cerebellum.[188] ASrAOGEN rats have decreased hypothalamic tissue levels of angiotensin I with a similar trend for angiotensin II compared to Sprague-Dawley rats.[191] However, angiotensin II and angiotensin-(1-7) immunoreactivity is preserved in neuronal cells of the paraventricular nucleus in ASrAOGEN rats, consistent with extra-glial production of angiotensinogen.[192] These rats exhibit reduced drinking responses to central renin infusion,[188] consistent with a down-regulation of the brain RAS. The brain specificity of the antisense oligonucleotide is confirmed by the absence of changes in circulating angiotensinogen[188] and plasma renin activity[193] in ASrAOGEN relative to Sprague-Dawley rats. Importantly, ASrAOGEN rats have maintained a stable phenotype for over ten years and are used extensively to investigate the contribution of neuronal versus glial angiotensinogen to fluid-electrolyte, cardiovascular and metabolic

homeostasis as well as pathologies associated with altered brain RAS activity including hypertension and aging.[179, 180, 185, 186]

Conscious ASrAOGEN rats have lower blood pressure and heart rate relative to Sprague-Dawley rats[188, 194] suggestive of reduced sympathetic nervous system activity. The hypotension and bradycardia in ASrAOGEN rats is generally attributed to the reduction of glia-derived angiotensin peptides. However, these cardiovascular alterations may also be due in part to the reduced plasma vasopressin levels and resulting diabetes-insipidus like syndrome in these animals.[188] Baroreflex sensitivity for control of heart rate and heart rate variability are also higher in conscious ASrAOGEN relative to Sprague-Dawley rats suggestive of higher resting parasympathetic activity.[186, 195]

Under urethane/chloralose anesthesia a paradoxical elevation of resting arterial pressure and enhanced response to cardiac vagal chemosensitive fiber activation occurs in ASrAOGEN rats[185, 196] providing evidence for an anesthesia-induced activation of the sympathetic nervous system. ASrAOGEN rats have increased AT_1 receptor density within brainstem regions involved in descending stress pathways to the NTS and RVLM,[45] including the subfornical organ and paraventricular nucleus.[197, 198] The upregulation of AT_1 receptors is accompanied by an enhanced sensitivity to the depressor and bradycardic responses produced by NTS or RVLM microinjection of angiotensin II in anesthetized ASrAOGEN relative to Sprague-Dawley rats.[199, 200] Therefore, the sympathetic activation observed in anesthetized ASrAOGEN rats may be due to an enhanced sensitivity of these animals to anesthesia-induced elevations in plasma renin and circulating angiotensin II levels.[201] Indeed, angiotensin peptides are involved in the maintenance of arterial pressure in anesthetized younger ASrAOGEN rats as NTS

blockade of either angiotensin II or angiotensin-(1-7) receptors decreases arterial pressure in ASrAOGEN rats with no effect in Sprague-Dawley rats.[185]

Despite elevations in sympathetic activity under anesthesia, the vagally-mediated baroreflex sensitivity for control of heart rate is similar[185] or higher[196, 199] in anesthetized ASrAOGEN rats relative to Sprague-Dawley rats. In younger normotensive rats, the administration of AT_1 or angiotensin-(1-7) receptor antagonists indicates repeatedly that endogenous angiotensin II attenuates while angiotensin-(1-7) facilitates baroreflex sensitivity for control of heart rate in response to increases in arterial pressure at the level of the NTS.[39, 40, 52, 202] In younger ASrAOGEN rats, NTS blockade of AT_1 receptors has no effect while blockade of angiotensin-(1-7) receptors decreases baroreflex sensitivity suggesting a glial source of angiotensinogen for angiotensin II and a non-glial source for angiotensin-(1-7) modulation of baroreflex function.[185] These observations are consistent with studies in double transgenic mice over-expressing human renin and angiotensinogen in which glial over-expression reduces, while neuronal over-expression has no effect on baroreflex sensitivity for control of heart rate.[184] Either glial or neuronal over-expression increases arterial pressure in these animals[184] providing further evidence for independent regulation of baroreflex sensitivity and arterial pressure.

Aging is associated with increases in systolic blood pressure as well as reductions in heart rate variability and baroreflex sensitivity for control of heart rate.[2, 14] These age-related cardiovascular deficits are thought to be attributed to autonomic imbalance as well as reduced arterial distensibility.[14] Treatment with ACE inhibitors or AT_1 receptor blockers reduces age-related cardiovascular and metabolic dysfunction and improves lifespan in humans and rodents suggesting angiotensin II contributes to age-related

pathologies.[172, 178-180]   However, plasma renin activity and circulating angiotensin peptides are reduced during aging,[203] implicating that the beneficial effects of angiotensin II blockade during aging are due to actions on distinct tissue systems.

ASrAOGEN rats do not show typical cardiovascular and metabolic deficits during aging suggesting that age-related changes require an intact glial RAS.[179, 180]   Although baroreflex function is reduced in conscious older ASrAOGEN rats relative to younger rats of this strain, the level of baroreflex sensitivity in older ASrAOGEN rats is similar to that observed in younger Sprague-Dawley rats suggesting overall preservation of baroreflex function.[186]   The mechanisms responsible for the maintenance of cardiovascular function during aging in these animals have not been explored, but may involve differential regulation of angiotensin peptides within brain regions controlling autonomic outflow.   Indeed, a recent study in our laboratory reveals that age-related impairments in baroreflex sensitivity in older Sprague-Dawley rats result from reduced endogenous angiotensin-(1-7) with no alteration in angiotensin II tone within the NTS.[52] Whether the age-related preservation of baroreflex function in ASrAOGEN rats is associated with maintenance of angiotensin-(1-7) tone within the NTS has yet to be considered.

In addition to enhanced cardiovascular function during aging, ASrAOGEN rats exhibit increased insulin and leptin responsiveness to oral glucose loads relative to Sprague-Dawley rats and do not show typical age-related increases in insulin and leptin levels.[179, 180]   In the face of low levels of insulin and leptin, older ASrAOGEN rats maintain lower body weight and serum glucose levels[180] implicating preservation of sensitivity to the metabolic actions of insulin and leptin during aging. These observations

are consistent with the beneficial metabolic effects observed in rats treated chronically with ACE inhibitors or AT1 receptor blockers.[178, 204] Whether ASrAOGEN rats exhibit enhanced sensitivity to the cardiovascular actions of leptin has yet to be established.

Transgenic (mRen2)27 rats

The transgenic (mRen2)27 rat was created by Mullins and colleagues in 1990 by insertion of the mouse submandibular gland *Ren-2* renin gene into the genome of Hannover Sprague-Dawley rats.[205] Transgenic (mRen2)27 rats have shown stable expression and phenotype for approximately 20 years and have been used extensively by our laboratory.[147, 180] Rats homozygous for the Ren2 gene exhibit fulminant hypertension beginning as early as six weeks of age.[205] Hemizygous (mRen2)27 rats are used for most research studies and exhibit hypertensive systolic blood pressure levels ranging from 180 to 200 mm Hg, similar to spontaneously hypertensive rats.[180, 205] In (mRen2)27 rats, expression of the renin transgene is high in the brain and adrenal gland with reduced expression in the kidney.[205-207] Whether circulating levels of angiotensin peptides are altered in (mRen2)27 rats is controversial as studies report reduced, similar or modestly elevated circulating levels of renin, angiotensinogen, angiotensin I, angiotensin II and angiotensin-(1-7) relative to Sprague-Dawley rats.[205, 207-209] However, chronic oral treatment with ACE inhibitors or AT_1 receptor blockers reduces blood pressure in these animals implicating angiotensin II-dependent hypertension.[210]

Increasing evidence suggests that the hypertension observed in (mRen2)27 rats is of central origin. Studies show that 5 week old female (mRen2)27 rats have higher angiotensin II and angiotensin-(1-7) levels in hypothalamus and higher angiotensin I and angiotensin II with reduced angiotensin-(1-7) levels in medulla relative to Sprague-

40

Dawley rats.[206] In addition, Campbell and colleagues show an 18-fold increase in brain tissue angiotensin II levels in (mRen2)27 rats.[207] Intracerebroventricular administration of AT_1 receptor antagonists reduces blood pressure and heart rate in (mRen2)27 rats[211] suggesting that central angiotensin II is involved in the maintenance of hypertension in these animals.

Hypertensive (mRen2)27 rats display an inverted circadian rhythm of blood pressure suggestive of an imbalance in the autonomic nervous system.[212] Indeed, sympathetic transmission is increased in response to angiotensin II in (mRen2)27 relative to Sprague-Dawley rats.[213, 214] Under urethane-chloralose anesthesia, (mRen2)27 rats have equivalent resting pressure relative to Sprague-Dawley rats providing further evidence for enhanced sympathetic nervous system activity. Despite reductions in arterial pressure under anesthesia, (mRen2)27 rats still exhibit impairments in baroreflex sensitivity for control of heart rate and responses to cardiac vagal chemosensitive fiber activation indicative of baro- and chemo-reflex dysfunction.[54] Recent evidence suggests that low angiotensin-(1-7) tone within the NTS contributes to the hypertension and baroreflex dysfunction observed in (mRen2)27 rats. NTS microinjection of [D-Ala^7]-angiotensin-(1-7) has no effect of baroreflex sensitivity in these animals,[54] in contrast to the inhibitory effect of angiotensin-(1-7) receptor blockade in Sprague-Dawley and ASrAOGEN rats[185]. Furthermore, intracerebroventricular or chronic intracisternal replacement of angiotensin-(1-7) improves baroreflex sensitivity and lowers arterial pressure in (mRen2)27 rats[53, 215] providing further evidence that reduced angiotensin-(1-7) tone contributes to impaired cardiovascular regulation in these animals.

Hypertensive (mRen2)27 rats exhibit higher body weight relative to ASrAOGEN

41

rats associated with increases in food and water intake.[180, 216] Although circulating levels of insulin, leptin and glucose are similar to ASrAOGEN and Sprague-Dawley rats at 15 weeks of age, (mRen2)27 rats exhibit decreased whole body and skeletal muscle insulin sensitivity with no change in leptin sensitivity to oral glucose tolerance tests.[179, 217, 218] The cardiovascular sensitivity to metabolic hormones in (mRen2)27 rats has yet to be considered. The finding that (mRen2)27 rats exhibit metabolic resistance may suggest that these animals have alterations in pathways associated with insulin and leptin signaling. Determining factors and signaling pathways that contribute to baroreflex and metabolic dysfunction in (mRen2)27 rats may be important in further understanding the contribution of the brain RAS to insulin and leptin resistance.

	ASrAOGEN	**(mRen2)27**
Parent Strain:	Hannover Sprague-Dawley	Hannover Sprague-Dawley
Brain RAS Activity:	Low	High
Conscious BP:	Hypotensive	Hypertensive
Effect of Anesthesia on BP:	Elevates	Lowers
Baroreflex Sensitivity:	Higher	Lower
Chemoreflex Function:	Higher	Lower
Body Weight:	Light	Heavy
Leptin Sensitivity for Metabolic Actions:	Increased	Decreased
Leptin Sensitivity for Cardiovascular Actions:	**?**	**?**

6. Rationale

All established risk factors for cardiovascular disease, including aging, hypertension, diabetes and obesity, are associated with an imbalance in autonomic nervous system activity.[2] These conditions all exhibit reductions in baroreflex sensitivity for control of heart rate, an important marker of vagal parasympathetic function.[2] Importantly, baroreflex dysfunction precedes and may contribute to the development of hypertension.[19] Although the pathological implications of baroreflex dysfunction have been known for decades, only recently have studies addressed the importance of pharmacological targeting of baroreflex sensitivity. In 2007, studies demonstrated a direct link between improvement of baroreflex function and a reduced incidence of stroke in spontaneously hypertensive rats.[20] Therefore, restoration of baroreflex sensitivity may be a novel strategy to treat cardiovascular and cerebrovascular diseases. To date, the mechanisms involved in modulation of baroreflex sensitivity are poorly understood. The identification of factors that regulate brainstem areas controlling autonomic outflow is crucial in understanding the decline in baroreflex and cardiovascular function that occurs in conditions associated with hypertension, including aging and obesity.

Our ongoing studies focus on understanding factors that alter baroreflex sensitivity at the level of the NTS. The majority of evidence indicates a key role for angiotensin peptides in modulating baroreflex function within this brain region. Studies repeatedly show that angiotensin II attenuates[40, 41] and angiotensin-(1-7) facilitates[26, 39] baroreflex sensitivity for control of heart rate, suggesting that the endogenous balance of these two peptides determines the prevailing level of baroreflex function. Baroreflex dysfunction in hypertension and aging is associated with reduced angiotensin-(1-7) tone

within the NTS.[52-54] Whether maintenance of angiotensin-(1-7) actions within this brain region preserves baroreflex sensitivity during aging is currently unknown. Therefore, we employed ASrAOGEN rats with relative maintenance of baroreflex sensitivity during aging relative to control rats in the conscious state.[186] Our goal was to identify the contribution of endogenous angiotensin peptides to baroreflex function within the NTS in older ASrAOGEN rats in order to determine mechanisms underlying the age-related preservation of baroreflex sensitivity in these animals.

The increasing prevalence of obesity in the United States has prompted us to identify key peptides that are altered in obesity and may contribute to the impairments in baroreflex function characteristic of this condition[2]. Recent studies implicate a role for the leptin in mediating elevations in sympathetic activity and arterial pressure in obesity-related hypertension.[15] Leptin receptors are localized to vagal sensory and motor systems,[84, 86] however, whether leptin reduces parasympathetic activity to contribute to autonomic imbalance has yet to be established. Therefore, we determined the effect of exogenous leptin on cardiovascular reflex regulation at the level of the NTS. If leptin impairs the baroreflex sensitivity, then reduction of endogenous leptin may be a novel therapeutic strategy to improve baroreflex function in conditions with elevated levels of the peptide.

Interruption of angiotensin II actions, using ACE inhibitors or AT_1 receptor blockers, reduces plasma insulin and leptin levels and improves metabolic sensitivity to these peptides in humans and rodents.[172, 178, 179] Transgenic ASrAOGEN and (mRen2)27 rats with low or high brain RAS activity are lighter and heavier, respectively, than age-matched controls.[180] These observations implicate centrally-mediated interactions

between leptin and angiotensin II for regulation of body weight. Whether interactions between these peptides exist for cardiovascular regulation is currently unknown. Therefore, we employed transgenic ASrAOGEN rats to determine the effect of down-regulation of the endogenous brain RAS on leptin modulation of baroreflex function.

To determine therapeutic strategies to suppress the negative cardiovascular effects of leptin while maintaining its positive effects on energy metabolism, we assessed intracellular signaling pathways activated for leptin modulation of baroreflex function. In addition, we determined the contribution of PTP1B, a key phosphatase involved in negative regulation of insulin, angiotensin II and leptin signaling,[135, 163, 219] to baroreflex sensitivity. Identifying intracellular mechanisms to inhibit kinase-mediated signaling pathways involved in the cardiovascular actions of these peptides may be necessary to maintain normal baroreflex function in conditions associated with elevated insulin, leptin and angiotensin II levels. Transgenic ASrAOGEN and (mRen2)27 rats were used to determine the contribution of signaling components, including PTP1B, to the varying levels of resting baroreflex sensitivity observed in these animals. Accordingly, the specific aims are as follows:

Aim 1: Determine the contribution of angiotensin peptides within the NTS to the age-related maintenance of baroreflex function in ASrAOGEN rats.

Aim 2: Characterize the effects of exogenous and endogenous leptin at the level of the NTS on baroreflex sensitivity for control of heart rate and other indices of autonomic balance in Sprague-Dawley rats to assess the role of this peptide to parasympathetic function.

Aim 3: Determine the contribution of leptin within the NTS to baroreflex sensitivity for control of heart rate in ASrAOGEN rats, in order to identify interactions between the brain RAS and leptin sensitivity for modulation of baroreflex function.

Aim 4: Determine the intracellular signaling pathways utilized by leptin to modulate baroreflex sensitivity and the contribution of these signaling pathways to the resting level of baroreflex sensitivity in ASrAOGEN and (mRen2)27 rats with low or high brain RAS activity.

Literature Cited

(1) Ahima RS, Prabakaran D, Mantzoros C, Qu D, Lowell B, Maratos-Flier E, Flier JS. Role of leptin in the neuroendocrine response to fasting. *Nature* 1996 July 18;382(6588):250-2.

(2) Ahmad F, Goldstein BJ. Increased abundance of specific skeletal muscle protein-tyrosine phosphatases in a genetic model of insulin-resistant obesity and diabetes mellitus. *Metabolism* 1995 September;44(9):1175-84.

(3) Aileru AA, Logan E, Callahan M, Ferrario CM, Ganten D, Diz DI. Alterations in sympathetic ganglionic transmission in response to angiotensin II in (mRen2)27 transgenic rats. *Hypertension* 2004;43(2):270-5.

(4) Ailhaud G, Fukamizu A, Massiera F, Negrel R, Saint-Marc P, Teboul M. Angiotensinogen, angiotensin II and adipose tissue development. *Int J Obes Relat Metab Disord* 2000 November;24 Suppl 4:S33-S35.

(5) Aizawa-Abe M, Ogawa Y, Masuzaki H, Ebihara K, Satoh N, Iwai H, Matsuoka N, Hayashi T, Hosoda K, Inoue G, Yoshimasa Y, Nakao K. Pathophysiological role of leptin in obesity-related hypertension. *J Clin Invest* 2000 May;105(9):1243-52.

(6) Alderman MH. JNC 7: brief summary and critique. *Clin Exp Hypertens* 2004 October;26(7-8):753-61.

(7) Anderson S. Ageing and the renin-angiotensin system. *Nephrol Dial Transplant* 1997;12(6):1093-4.

(8) Andresen MC, Kunze DL. Nucleus tractus solitarius--gateway to neural circulatory control. *Annu Rev Physiol* 1994;56:93-116.

(9) Averill DB, Diz DI. Angiotensin peptides and baroreflex control of sympathetic outflow: pathways and mechanisms of the medulla oblongata. *Brain Res Bull* 2000;51(2):119-28.

(10) Bader M, Peters J, Baltatu O, Muller DN, Luft FC, Ganten D. Tissue renin-angiotensin systems: new insights from experimental animal models in hypertension research. *J Mol Med* 2001 April;79(2-3):76-102.

(11) Bahrenberg G, Behrmann I, Barthel A, Hekerman P, Heinrich PC, Joost HG, Becker W. Identification of the critical sequence elements in the cytoplasmic domain of leptin receptor isoforms required for Janus kinase/signal transducer and activator of transcription activation by receptor heterodimers. *Mol Endocrinol* 2002 April;16(4):859-72.

(12)   Baltatu O, Fontes MAP, Campagnole-Santos MJ, Caligiorni S, Ganten D, Santos RAS, Bader M. Alterations of the renin-angiotensin system at the RVLM of transgenic rats with low brain angiotensinogen.*Am.J.Physiol Regul.Integr.Comp Physiol* 2001; 280: R428-R433.

(13)   Baltatu O, Janssen BJ, Bricca G, Plehm R, Monti J, Ganten D, Bader M. Alterations in blood pressure and heart rate variability in transgenic rats with low brain angiotensinogen. *Hypertension* 2001;37(2 Part 2):408-13.

(14)   Baltatu O, Silva JA, Jr., Ganten D, Bader M. The brain renin-angiotensin system modulates angiotensin II-induced hypertension and cardiac hypertrophy. *Hypertension* 2000;35(1 Pt 2):409-12.

(15)   Banks AS, Davis SM, Bates SH, Myers MG, Jr. Activation of downstream signals by the long form of the leptin receptor. *J Biol Chem* 2000 May 12;275(19):14563-72.

(16)   Banks WA, DiPalma CR, Farrell CL. Impaired transport of leptin across the blood-brain barrier in obesity. *Peptides* 1999 November;20(11):1341-5.

(17)   Banks WA, Kastin AJ, Huang W, Jaspan JB, Maness LM. Leptin enters the brain by a saturable system independent of insulin. *Peptides* 1996;17(2):305-11.

(18)   Barr VA, Lane K, Taylor SI. Subcellular localization and internalization of the four human leptin receptor isoforms. *J Biol Chem* 1999 July 23;274(30):21416-24.

(19)   Belin de Chantemele EJ, Muta K, Mintz J, Tremblay ML, Marrero MB, Fulton DJ, Stepp DW. Protein tyrosine phosphatase 1B, a major regulator of leptin-mediated control of cardiovascular function. *Circulation* 2009 September 1;120(9):753-63.

(20)   Bence KK, Delibegovic M, Xue B, Gorgun CZ, Hotamisligil GS, Neel BG, Kahn BB. Neuronal PTP1B regulates body weight, adiposity and leptin action. *Nat Med* 2006 August;12(8):917-24.

(21)   Besnard F, Brenner M, Nakatani Y, Chao R, Purohit HJ, Freese E. Multiple interacting sites regulate astrocyte-specific transcription of the human gene for glial fibrillary acidic protein. *J Biol Chem* 1991;266(28):18877-83.

(22)   Biaggioni I. Should we target the sympathetic nervous system in the treatment of obesity-associated hypertension? *Hypertension* 2008 February;51(2):168-71.

(23)   Bjorbaek C, Elmquist JK, Frantz JD, Shoelson SE, Flier JS. Identification of SOCS-3 as a potential mediator of central leptin resistance. *Mol Cell* 1998 March;1(4):619-25.

(24) Bjorbaek C, Elmquist JK, Michl P, Ahima RS, van Bueren A, McCall AL, Flier JS. Expression of leptin receptor isoforms in rat brain microvessels. *Endocrinology* 1998 August;139(8):3485-91.

(25) Bjorbaek C, Kahn BB. Leptin signaling in the central nervous system and the periphery. *Recent Prog Horm Res* 2004;59:305-31.

(26) Bjorbaek C, Uotani S, da SB, Flier JS. Divergent signaling capacities of the long and short isoforms of the leptin receptor. *J Biol Chem* 1997 December 19;272(51):32686-95.

(27) Blendea MC, Jacobs D, Stump CS, McFarlane SI, Ogrin C, Bahtyiar G, Stas S, Kumar P, Sha Q, Ferrario CM, Sowers JR. Abrogation of oxidative stress improves insulin sensitivity in the Ren-2 rat model of tissue angiotensin II overexpression. *Am J Physiol Endocrinol Metab* 2005 February;288(2):E353-E359.

(28) Bodary PF, Westrick RJ, Wickenheiser KJ, Shen Y, Eitzman DT. Effect of leptin on arterial thrombosis following vascular injury in mice. *JAMA* 2002 April 3;287(13):1706-9.

(29) Bradley RL, Cleveland KA, Cheatham B. The adipocyte as a secretory organ: mechanisms of vesicle transport and secretory pathways. *Recent Prog Horm Res* 2001;56:329-58.

(30) Brenner M, Kisseberth WC, Su Y, Besnard F, Messing A. GFAP promoter directs astrocyte-specific expression in transgenic mice. *J Neurosci* 1994;14(3 Pt 1):1030-7.

(31) Brewster UC, Perazella MA. The renin-angiotensin-aldosterone system and the kidney: effects on kidney disease. *Am J Med* 2004 February 15;116(4):263-72.

(32) Buyse M, Ovesjo ML, Goiot H, Guilmeau S, Peranzi G, Moizo L, Walker F, Lewin MJ, Meister B, Bado A. Expression and regulation of leptin receptor proteins in afferent and efferent neurons of the vagus nerve. *Eur J Neurosci* 2001 July;14(1):64-72.

(33) Caligiorne SM, Silva AQ, Fontes MA, Silva JR, Baltatu O, Bader M, Santos RA, Campagnole-Santos MJ. Baroreflex control of heart rate and renal sympathetic nerve activity in rats with low brain angiotensinogen. *Neuropeptides* 2008 April;42(2):159-68.

(34) Campagnole-Santos MJ, Diz DI, Ferrario CM. Baroreceptor reflex modulation by angiotensin II at the nucleus tractus solitarii. *Hypertension* 1988;11(2 Pt 2):I167-I171.

(35) Campagnole-Santos MJ, Diz DI, Santos RA, Khosla MC, Brosnihan KB, Ferrario CM. Cardiovascular effects of angiotensin-(1-7) injected into the dorsal medulla of rats. *Am J Physiol* 1989;257(1 Pt 2):H324-H329.

(36) Campagnole-Santos MJ, Heringer SB, Batista EN, Khosla MC, Santos RA. Differential baroreceptor reflex modulation by centrally infused angiotensin peptides. *Am J Physiol* 1992 July;263(1 Pt 2):R89-R94.

(37) Campbell DJ, Rong P, Kladis A, Rees B, Ganten D, Skinner SL. Angiotensin and bradykinin peptides in the TGR(mRen-2)27 rat. *Hypertension* 1995 May;25(5):1014-20.

(38) Campfield LA, Smith FJ, Guisez Y, Devos R, Burn P. Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. *Science* 1995 July 28;269(5223):546-9.

(39) Campos LA, Couto AS, Iliescu R, Santos JA, Santos RA, Ganten D, Campagnole-Santos MJ, Bader M, Baltatu O. Differential regulation of central vasopressin receptors in transgenic rats with low brain angiotensinogen. *Regul Pept* 2004;119(3):177-82.

(40) Carey RM, Siragy HM. The intrarenal renin-angiotensin system and diabetic nephropathy. *Trends Endocrinol Metab* 2003 August;14(6):274-81.

(41) Carlyle M, Jones OB, Kuo JJ, Hall JE. Chronic cardiovascular and renal actions of leptin: role of adrenergic activity. *Hypertension* 2002 February;39(2 Pt 2):496-501.

(42) Carter CS, Cesari M, Ambrosius WT, Hu N, Diz D, Oden S, Sonntag WE, Pahor M. Angiotensin-converting enzyme inhibition, body composition, and physical performance in aged rats. *J Gerontol A Biol Sci Med Sci* 2004 May;59(5):416-23.

(43) Carvalheira JB, Torsoni MA, Ueno M, Amaral ME, Araujo EP, Velloso LA, Gontijo JA, Saad MJ. Cross-talk between the insulin and leptin signaling systems in rat hypothalamus. *Obes Res* 2005 January;13(1):48-57.

(44) Casto R, Phillips M. Angiotensin II attenuates baroreflexes at nucleus tractus solitarius of rats. *Am.J.Physiol*; 1986, 250: 193-198.

(45) Chaves GZ, Caligiorne SM, Santos RA, Khosla MC, Campagnole-Santos MJ. Modulation of the baroreflex control of heart rate by angiotensin-(1-7) at the nucleus tractus solitarii of normotensive and spontaneously hypertensive rats. *J Hypertens* 2000;18(12):1841-8.

(46) Cheng A, Uetani N, Simoncic PD, Chaubey VP, Lee-Loy A, McGlade CJ, Kennedy BP, Tremblay ML. Attenuation of leptin action and regulation of obesity by protein tyrosine phosphatase 1B. *Dev Cell* 2002 April;2(4):497-503.

(47) Clement K, Vaisse C, Lahlou N, Cabrol S, Pelloux V, Cassuto D, Gourmelen M, Dina C, Chambaz J, Lacorte JM, Basdevant A, Bougneres P, Lebouc Y, Froguel P, Guy-Grand B. A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. *Nature* 1998 March 26;392(6674):398-401.

(48) Cohen P, Zhao C, Cai X, Montez JM, Rohani SC, Feinstein P, Mombaerts P, Friedman JM. Selective deletion of leptin receptor in neurons leads to obesity. *J Clin Invest* 2001 October;108(8):1113-21.

(49) Correia ML, Morgan DA, Sivitz WI, Mark AL, Haynes WG. Leptin acts in the central nervous system to produce dose-dependent changes in arterial pressure. *Hypertension* 2001 March;37(3):936-42.

(50) Correia ML, Rahmouni K. Role of leptin in the cardiovascular and endocrine complications of metabolic syndrome. *Diabetes Obes Metab* 2006 November;8(6):603-10.

(51) Couto AS, Baltatu O, Santos RA, Ganten D, Bader M, Campagnole-Santos MJ. Differential effects of angiotensin II and angiotensin-(1-7) at the nucleus tractus solitarii of transgenic rats with low brain angiotensinogen. *J Hypertens* 2002;20(5):919-25.

(52) Dallaporta M, Pecchi E, Pio J, Jean A, Horner KC, Troadec JD. Expression of leptin receptor by glial cells of the nucleus tractus solitarius: possible involvement in energy homeostasis. *J Neuroendocrinol* 2009 January;21(1):57-67.

(53) Dampney RA. Functional organization of central pathways regulating the cardiovascular system. *Physiol Rev* 1994 April;74(2):323-64.

(54) Dawson R, Pelleymounter MA, Millard WJ, Liu S, Eppler B. Attenuation of leptin-mediated effects by monosodium glutamate-induced arcuate nucleus damage. *Am J Physiol* 1997 July;273(1 Pt 1):E202-E206.

(55) de Cavanagh EM, Inserra F, Toblli J, Stella I, Fraga CG, Ferder L. Enalapril attenuates oxidative stress in diabetic rats. *Hypertension* 2001 November;38(5):1130-6.

(56) DelliPizzi AM, Hilchey SD, Bell-Quilley CP. Natriuretic action of angiotensin(1-7). *Br J Pharmacol* 1994 January;111(1):1-3.

(57) Diano S, Kalra SP, Horvath TL. Leptin receptor immunoreactivity is associated with the Golgi apparatus of hypothalamic neurons and glial cells. *J Neuroendocrinol* 1998 September;10(9):647-50.

(58) Dietrich MO, Spuch C, Antequera D, Rodal I, de Yebenes JG, Molina JA, Bermejo F, Carro E. Megalin mediates the transport of leptin across the blood-CSF barrier. *Neurobiol Aging* 2007 February 26.

(59) Diz DI, Barnes KL, Ferrario CM. Contribution of the vagus nerve to angiotensin II binding sites in the canine medulla. *Brain Res Bull* 1986 October;17(4):497-505.

(60) Diz DI, Garcia-Espinosa MA, Gallagher PE, Ganten D, Ferrario CM, Averill DB. Angiotensin-(1-7) and baroreflex function in nucleus tractus solitarii of (mRen2)27 transgenic rats. *J Cardiovasc Pharmacol* 2008 June;51(6):542-8.

(61) Diz DI, Jessup JA, Westwood BM, Bosch SM, Vinsant S, Gallagher PE, Averill DB. Angiotensin peptides as neurotransmitters/neuromodulators in the dorsomedial medulla. *Clin Exp Pharmacol Physiol* 2002;29(5-6):473-82.

(62) Diz DI, Kasper SO, Sakima A, Ferrario CM. Aging and the brain renin-angiotensin system: insights from studies in transgenic rats. *Cleve Clin J Med* 2007;74 Suppl 1:S95-S98.

(63) Diz DI, Varagic J, Groban L. Aging and the brain renin-angiotensin system: relevance to age-related decline in cardiac function. *Future Cardiol* 2008 May;4(3):237-45.

(64) Dobruch J, Paczwa P, Lon S, Khosla MC, Szczepanska-Sadowska E. Hypotensive function of the brain angiotensin-(1-7) in Sprague Dawley and renin transgenic rats. *J Physiol Pharmacol* 2003 September;54(3):371-81.

(65) Dostal DE, Booz GW, Baker KM. Angiotensin II signalling pathways in cardiac fibroblasts: conventional versus novel mechanisms in mediating cardiac growth and function. *Mol Cell Biochem* 1996 April 12;157(1-2):15-21.

(66) Dunbar JC, Hu Y, Lu H. Intracerebroventricular leptin increases lumbar and renal sympathetic nerve activity and blood pressure in normal rats. *Diabetes* 1997 December;46(12):2040-3.

(67) Eikelis N, Lambert G, Wiesner G, Kaye D, Schlaich M, Morris M, Hastings J, Socratous F, Esler M. Extra-adipocyte leptin release in human obesity and its relation to sympathoadrenal function. *Am J Physiol Endocrinol Metab* 2004 May;286(5):E744-E752.

(68) El-Haschimi K, Pierroz DD, Hileman SM, Bjorbaek C, Flier JS. Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity. *J Clin Invest* 2000 June;105(12):1827-32.

(69) Elchebly M, Payette P, Michaliszyn E, Cromlish W, Collins S, Loy AL, Normandin D, Cheng A, Himms-Hagen J, Chan CC, Ramachandran C, Gresser MJ, Tremblay ML, Kennedy BP. Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. *Science* 1999 March 5;283(5407):1544-8.

(70) Elias CF, Kelly JF, Lee CE, Ahima RS, Drucker DJ, Saper CB, Elmquist JK. Chemical characterization of leptin-activated neurons in the rat brain. *J Comp Neurol* 2000 July 24;423(2):261-81.

(71) Elmquist JK, Ahima RS, Maratos-Flier E, Flier JS, Saper CB. Leptin activates neurons in ventrobasal hypothalamus and brainstem. *Endocrinology* 1997 February;138(2):839-42.

(72) Elmquist JK, Bjorbaek C, Ahima RS, Flier JS, Saper CB. Distributions of leptin receptor mRNA isoforms in the rat brain. *J Comp Neurol* 1998 June 15;395(4):535-47.

(73) Ferrari AU, Radaelli A, Centola M. Invited review: aging and the cardiovascular system. *J Appl Physiol* 2003;95(6):2591-7.

(74) Ferrario CM, Chappell MC, Tallant EA, Brosnihan KB, Diz DI. Counterregulatory actions of angiotensin-(1-7). *Hypertension* 1997 September;30(3 Pt 2):535-41.

(75) Fitzgerald SM, Evans RG, Bergstrom G, Anderson WP. Renal hemodynamic responses to intrarenal infusion of ligands for the putative angiotensin IV receptor in anesthetized rats. *J Cardiovasc Pharmacol* 1999 August;34(2):206-11.

(76) Folli F, Saad MJ, Velloso L, Hansen H, Carandente O, Feener EP, Kahn CR. Crosstalk between insulin and angiotensin II signalling systems. *Exp Clin Endocrinol Diabetes* 1999;107(2):133-9.

(77) Fontes MA, Baltatu O, Caligiorne SM, Campagnole-Santos MJ, Ganten D, Bader M, Santos RA. Angiotensin peptides acting at rostral ventrolateral medulla contribute to hypertension of TGR(mREN2)27 rats. *Physiol Genomics* 2000 April 27;2(3):137-42.

(78) Fontes MA, Martins Pinge MC, Naves V, Campagnole-Santos MJ, Lopes OU, Khosla MC, Santos RA. Cardiovascular effects produced by microinjection of angiotensins and angiotensin antagonists into the ventrolateral medulla of freely moving rats. *Brain Res* 1997 March 7;750(1-2):305-10.

(79) Fontes MA, Tagawa T, Polson JW, Cavanagh SJ, Dampney RA. Descending pathways mediating cardiovascular response from dorsomedial hypothalamic nucleus. *Am J Physiol Heart Circ Physiol* 2001 June;280(6):H2891-H2901.

(80) Ford ES, Giles WH, Mokdad AH. Increasing prevalence of the metabolic syndrome among u.s. Adults. *Diabetes Care* 2004 October;27(10):2444-9.

(81) Freeman EJ, Chisolm GM, Ferrario CM, Tallant EA. Angiotensin-(1-7) inhibits vascular smooth muscle cell growth. *Hypertension* 1996 July;28(1):104-8.

(82) Fruhbeck G. Intracellular signalling pathways activated by leptin. *Biochem J* 2006 January 1;393(Pt 1):7-20.

(83) Ganten D, Ganten U, Granger P, Hayduk K, Boucher R, Genest J. Renin-angiotensin in extrarenal tissues. *Verh Dtsch Ges Inn Med* 1972;78:1510-2.

(84) Gao Q, Wolfgang MJ, Neschen S, Morino K, Horvath TL, Shulman GI, Fu XY. Disruption of neural signal transducer and activator of transcription 3 causes obesity, diabetes, infertility, and thermal dysregulation. *Proc Natl Acad Sci U S A* 2004 March 30;101(13):4661-6.

(85) Garcia-Espinosa MA, Shaltout HA, Gallagher PE, Chappell MC, Ferrario CM, Ganten D, Reudelhuber TL, Diz DI. Angiotensin-(1-7) Replacement via Gene Transfer in the Cisterna Magna Lowers Blood Pressure and Improves Baroreflex Function in Transgenic (mRen2)27 Rats. *Hypertension* 2008, 52[4]: E36.

(86) Garrison RJ, Kannel WB, Stokes J, III, Castelli WP. Incidence and precursors of hypertension in young adults: the Framingham Offspring Study. *Prev Med* 1987 March;16(2):235-51.

(87) Giacchetti G, Faloia E, Mariniello B, Sardu C, Gatti C, Camilloni MA, Guerrieri M, Mantero F. Overexpression of the renin-angiotensin system in human visceral adipose tissue in normal and overweight subjects. *Am J Hypertens* 2002 May;15(5):381-8.

(88) Gilliam-Davis S, Payne VS, Kasper SO, Tommasi EN, Robbins ME, Diz DI. Long-term AT1 receptor blockade improves metabolic function and provides renoprotection in Fischer-344 rats. *Am J Physiol Heart Circ Physiol* 2007 September;293(3):H1327-H1333.

(89) Gonzalez-Villalobos R, Klassen RB, Allen PL, Johanson K, Baker CB, Kobori H, Navar LG, Hammond TG. Megalin binds and internalizes angiotensin-(1-7). *Am J Physiol Renal Physiol* 2006 May;290(5):F1270-F1275.

(90) Gonzalez-Villalobos R, Klassen RB, Allen PL, Navar LG, Hammond TG. Megalin binds and internalizes angiotensin II. *Am J Physiol Renal Physiol* 2005 February;288(2):F420-F427.

(91) Goossens GH, Blaak EE, Arner P, Saris WH, van Baak MA. Angiotensin II: a hormone that affects lipid metabolism in adipose tissue. *Int J Obes (Lond)* 2007 February;31(2):382-4.

(92) Goossens GH, Blaak EE, van Baak MA. Possible involvement of the adipose tissue renin-angiotensin system in the pathophysiology of obesity and obesity-related disorders. *Obes Rev* 2003 February;4(1):43-55.

(93) Grill HJ, Schwartz MW, Kaplan JM, Foxhall JS, Breininger J, Baskin DG. Evidence that the caudal brainstem is a target for the inhibitory effect of leptin on food intake. *Endocrinology* 2002 January;143(1):239-46.

(94) Grobe JL, Xu D, Sigmund CD. An intracellular Renin-Angiotensin system in neurons: fact, hypothesis, or fantasy. *Physiology (Bethesda)* 2008 August;23:187-93.

(95) Guo GB, Abboud FM. Angiotensin II attenuates baroreflex control of heart rate and sympathetic activity. *Am J Physiol* 1984 January;246(1 Pt 2):H80-H89.

(96) Hainault I, Nebout G, Turban S, Ardouin B, Ferre P, Quignard-Boulange A. Adipose tissue-specific increase in angiotensinogen expression and secretion in the obese (fa/fa) Zucker rat. *Am J Physiol Endocrinol Metab* 2002 January;282(1):E59-E66.

(97) Halaas JL, Boozer C, Blair-West J, Fidahusein N, Denton DA, Friedman JM. Physiological response to long-term peripheral and central leptin infusion in lean and obese mice. *Proc Natl Acad Sci U S A* 1997 August 5;94(16):8878-83.

(98) Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK, Friedman JM. Weight-reducing effects of the plasma protein encoded by the obese gene. *Science* 1995 July 28;269(5223):543-6.

(99) Hall JE. Pathophysiology of obesity hypertension. *Curr Hypertens Rep* 2000 April;2(2):139-47.

(100) Hall JE, Brands MW, Hildebrandt DA, Kuo J, Fitzgerald S. Role of sympathetic nervous system and neuropeptides in obesity hypertension. *Braz J Med Biol Res* 2000 June;33(6):605-18.

(101) Hall JE, Hildebrandt DA, Kuo J. Obesity hypertension: role of leptin and sympathetic nervous system. *Am J Hypertens* 2001 June;14(6 Pt 2):103S-15S.

(102) Hausberg M, Morgan DA, Chapleau MA, Sivitz WI, Mark AL, Haynes WG. Differential modulation of leptin-induced sympathoexcitation by baroreflex activation. *J Hypertens* 2002 August;20(8):1633-41.

(103) Haynes WG, Morgan DA, Walsh SA, Mark AL, Sivitz WI. Receptor-mediated regional sympathetic nerve activation by leptin. *J Clin Invest* 1997 July 15;100(2):270-8.

(104) Haynes WG, Morgan DA, Walsh SA, Sivitz WI, Mark AL. Cardiovascular consequences of obesity: role of leptin. *Clin Exp Pharmacol Physiol* 1998 January;25(1):65-9.

(105) Healy DP, Rettig R, Printz MP. Evidence of an endogenous Ang II system in the rat nucleus tractus solitarius. *Clin Exp Hypertens A* 1984;6(10-11):1743-7.

(106) Henriksen EJ, Jacob S, Kinnick TR, Teachey MK, Krekler M. Selective angiotensin II receptor receptor antagonism reduces insulin resistance in obese Zucker rats. *Hypertension* 2001 October;38(4):884-90.

(107) Heymsfield SB, Greenberg AS, Fujioka K, Dixon RM, Kushner R, Hunt T, Lubina JA, Patane J, Self B, Hunt P, McCamish M. Recombinant leptin for weight loss in obese and lean adults: a randomized, controlled, dose-escalation trial. *JAMA* 1999 October 27;282(16):1568-75.

(108) Hileman SM, Pierroz DD, Masuzaki H, Bjorbaek C, El-Haschimi K, Banks WA, Flier JS. Characterizaton of short isoforms of the leptin receptor in rat cerebral microvessels and of brain uptake of leptin in mouse models of obesity. *Endocrinology* 2002 March;143(3):775-83.

(109) Hirawa N, Uehara Y, Kawabata Y, Numabe A, Gomi T, Ikeda T, Suzuki T, Goto A, Toyo-oka T, Omata M. Long-term inhibition of renin-angiotensin system sustains memory function in aged Dahl rats. *Hypertension* 1999 September;34(3):496-502.

(110) Hosojima M, Sato H, Yamamoto K, Kaseda R, Soma T, Kobayashi A, Suzuki A, Kabasawa H, Takeyama A, Ikuyama K, Iino N, Nishiyama A, Thekkumkara TJ, Takeda T, Suzuki Y, Gejyo F, Saito A. Regulation of megalin expression in cultured proximal tubule cells by angiotensin II type 1A receptor- and insulin-mediated signaling cross talk. *Endocrinology* 2009 February;150(2):871-8.

(111) Huang BS, Ganten D, Leenen FH. Responses to central Na(+) and ouabain are attenuated in transgenic rats deficient in brain angiotensinogen. *Hypertension* 2001;37(2 Part 2):683-6.

(112) Hubert HB, Feinleib M, McNamara PM, Castelli WP. Obesity as an independent risk factor for cardiovascular disease: a 26-year follow-up of participants in the Framingham Heart Study. *Circulation* 1983 May;67(5):968-77.

(113) Hunter T. Signaling--2000 and beyond. *Cell* 2000 January 7;100(1):113-27.

(114) Huo L, Gamber KM, Grill HJ, Bjorbaek C. Divergent leptin signaling in proglucagon neurons of the nucleus of the solitary tract in mice and rats. *Endocrinology* 2008 February;149(2):492-7.

(115) Ihle JN. Cytokine receptor signalling. *Nature* 1995 October 19;377(6550):591-4.

(116) Kasper SO, Carter CS, Ferrario CM, Ganten D, Ferder LF, Sonntag WE, Gallagher PE, Diz DI. Growth, metabolism, and blood pressure disturbances during aging in transgenic rats with altered brain renin-angiotensin systems. *Physiol Genomics* 2005;23(3):311-7.

(117) Kasper SO, Ferrario CM, Ganten D, Diz DI. Central depletion of angiotensinogen is associated with elevated AT1 receptors in the SFO and PVN. *Neurotox Res* 2004;6(4):259-65.

(118) Kasper SO, Ferrario CM, Ganten D, Diz DI. Rats with low brain angiotensinogen do not exhibit insulin resistance during early aging. *Endocrine* 2006 October;30(2):167-74.

(119) Kastin AJ, Pan W, Maness LM, Koletsky RJ, Ernsberger P. Decreased transport of leptin across the blood-brain barrier in rats lacking the short form of the leptin receptor. *Peptides* 1999 December;20(12):1449-53.

(120) Kaszubska W, Falls HD, Schaefer VG, Haasch D, Frost L, Hessler P, Kroeger PE, White DW, Jirousek MR, Trevillyan JM. Protein tyrosine phosphatase 1B negatively regulates leptin signaling in a hypothalamic cell line. *Mol Cell Endocrinol* 2002 September 30;195(1-2):109-18.

(121) Kim S, Whelan J, Claycombe K, Reath DB, Moustaid-Moussa N. Angiotensin II increases leptin secretion by 3T3-L1 and human adipocytes via a prostaglandin-independent mechanism. *J Nutr* 2002 June;132(6):1135-40.

(122) Kim YB, Uotani S, Pierroz DD, Flier JS, Kahn BB. In vivo administration of leptin activates signal transduction directly in insulin-sensitive tissues: overlapping but distinct pathways from insulin. *Endocrinology* 2000 July;141(7):2328-39.

(123) Kinnick TR, Youngblood EB, O'Keefe MP, Saengsirisuwan V, Teachey MK, Henriksen EJ. Modulation of insulin resistance and hypertension by voluntary exercise training in the TG(mREN2)27 rat. *J Appl Physiol* 2002 August;93(2):805-12.

(124) Koh KK, Quon MJ, Han SH, Lee Y, Kim SJ, Koh Y, Shin EK. Distinct vascular and metabolic effects of different classes of anti-hypertensive drugs. Int J Cardiol. In press 2008.

(125) Konstantinides S, Schafer K, Koschnick S, Loskutoff DJ. Leptin-dependent platelet aggregation and arterial thrombosis suggests a mechanism for atherothrombotic disease in obesity. *J Clin Invest* 2001 November;108(10):1533-40.

(126) Konstantinides S, Schafer K, Neels JG, Dellas C, Loskutoff DJ. Inhibition of endogenous leptin protects mice from arterial and venous thrombosis. *Arterioscler Thromb Vasc Biol* 2004 November;24(11):2196-201.

(127) Koren S, Fantus IG. Inhibition of the protein tyrosine phosphatase PTP1B: potential therapy for obesity, insulin resistance and type-2 diabetes mellitus. *Best Pract Res Clin Endocrinol Metab* 2007 December;21(4):621-40.

(128) Landsberg L, Young JB. Fasting, feeding and regulation of the sympathetic nervous system. *N Engl J Med* 1978 June 8;298(23):1295-301.

(129) Lavoie JL, Sigmund CD. Minireview: overview of the renin-angiotensin system--an endocrine and paracrine system. *Endocrinology* 2003 June;144(6):2179-83.

(130) Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JI, Friedman JM. Abnormal splicing of the leptin receptor in diabetic mice. *Nature* 1996 February 15;379(6566):632-5.

(131) Lee YS. The role of leptin-melanocortin system and human weight regulation: lessons from experiments of nature. *Ann Acad Med Singapore* 2009 January;38(1):34-11.

(132) Leyva F, Anker SD, Egerer K, Stevenson JC, Kox WJ, Coats AJ. Hyperleptinaemia in chronic heart failure. Relationships with insulin. *Eur Heart J* 1998 October;19(10):1547-51.

(133) Liu AJ, Ma XJ, Shen FM, Liu JG, Chen H, Su DF. Arterial baroreflex: a novel target for preventing stroke in rat hypertension. *Stroke* 2007 June;38(6):1916-23.

(134) Logan E, Diz DI, Averill DB. Inhibition of the PI3 Kinase Signal Transduction Pathway in Nucleus Tractus Solitarii of (mRen2)27 Transgenic Rats Improves Baroreceptor Sensitivity. *FASEB J. 2008;* 22.

(135) Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S. Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat Med* 1995 November;1(11):1155-61.

(136) Mark AL, Agassandian K, Morgan DA, Liu X, Cassell MD, Rahmouni K. Leptin signaling in the nucleus tractus solitarii increases sympathetic nerve activity to the kidney. *Hypertension* 2009 February;53(2):375-80.

(137) Mark AL, Correia M, Morgan DA, Shaffer RA, Haynes WG. State-of-the-art-lecture: Obesity-induced hypertension: new concepts from the emerging biology of obesity. *Hypertension* 1999 January;33(1 Pt 2):537-41.

(138) Mark AL, Shaffer RA, Correia ML, Morgan DA, Sigmund CD, Haynes WG. Contrasting blood pressure effects of obesity in leptin-deficient ob/ob mice and agouti yellow obese mice. *J Hypertens* 1999 December;17(12 Pt 2):1949-53.

(139) Marrero MB, Fulton D, Stepp D, Stern DM. Angiotensin II-induced insulin resistance and protein tyrosine phosphatases. *Arterioscler Thromb Vasc Biol* 2004 November;24(11):2009-13.

(140) Marsh AJ, Fontes MA, Killinger S, Pawlak DB, Polson JW, Dampney RA. Cardiovascular responses evoked by leptin acting on neurons in the ventromedial and dorsomedial hypothalamus. *Hypertension* 2003 October;42(4):488-93.

(141) Massiera F, Seydoux J, Geloen A, Quignard-Boulange A, Turban S, Saint-Marc P, Fukamizu A, Negrel R, Ailhaud G, Teboul M. Angiotensinogen-deficient mice exhibit impairment of diet-induced weight gain with alteration in adipose tissue development and increased locomotor activity. *Endocrinology* 2001 December;142(12):5220-5.

(142) Matsumura K, Averill DB, Ferrario CM. Angiotensin II acts at AT1 receptors in the nucleus of the solitary tract to attenuate the baroreceptor reflex. *Am J Physiol* 1998;275(5 Pt 2):R1611-R1619.

(143) McKinley MJ, Albiston AL, Allen AM, Mathai ML, May CN, McAllen RM, Oldfield BJ, Mendelsohn FA, Chai SY. The brain renin-angiotensin system: location and physiological roles. *Int J Biochem Cell Biol* 2003 June;35(6):901-18.

(144) Mercer JG, Moar KM, Hoggard N. Localization of leptin receptor (Ob-R) messenger ribonucleic acid in the rodent hindbrain. *Endocrinology* 1998 January;139(1):29-34.

(145) Mitchell KD, Jacinto SM, Mullins JJ. Proximal tubular fluid, kidney, and plasma levels of angiotensin II in hypertensive ren-2 transgenic rats. *Am J Physiol* 1997 August;273(2 Pt 2):F246-F253.

(146) Mok A, Cao H, Zinman B, Hanley AJ, Harris SB, Kennedy BP, Hegele RA. A single nucleotide polymorphism in protein tyrosine phosphatase PTP-1B is associated with protection from diabetes or impaired glucose tolerance in Oji-Cree. *J Clin Endocrinol Metab* 2002 February;87(2):724-7.

(147) Mokdad AH, Ford ES, Bowman BA, Dietz WH, Vinicor F, Bales VS, Marks JS. Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001. *JAMA* 2003 January 1;289(1):76-9.

(148) Montague CT, Farooqi IS, Whitehead JP, Soos MA, Rau H, Wareham NJ, Sewter CP, Digby JE, Mohammed SN, Hurst JA, Cheetham CH, Earley AR, Barnett AH, Prins JB, O'Rahilly S. Congenital leptin deficiency is associated with severe early-onset obesity in humans. *Nature* 1997 June 26;387(6636):903-8.

(149) Monti J, Schinke M, Bohm M, Ganten D, Bader M, Bricca G. Glial angiotensinogen regulates brain angiotensin II receptors in transgenic rats TGR(ASrAOGEN). *Am J Physiol Regul Integr Comp Physiol* 2001;280(1):R233-R240.

(150) Moriguchi A, Brosnihan KB, Kumagai H, Ganten D, Ferrario CM. Mechanisms of hypertension in transgenic rats expressing the mouse Ren-2 gene. *Am J Physiol* 1994 April;266(4 Pt 2):R1273-R1279.

(151) Moriguchi A, Tallant EA, Matsumura K, Reilly TM, Walton H, Ganten D, Ferrario CM. Opposing actions of angiotensin-(1-7) and angiotensin II in the brain of transgenic hypertensive rats. *Hypertension* 1995 June;25(6):1260-5.

(152) Moriguchi A, Tallant EA, Matsumura K, Reilly TM, Walton H, Ganten D, Ferrario CM. Opposing actions of angiotensin-(1-7) and angiotensin II in the brain of transgenic hypertensive rats. *Hypertension* 1995 June;25(6):1260-5.

(153) Morimoto S, Cassell MD, Sigmund CD. Glia- and neuron-specific expression of the renin-angiotensin system in brain alters blood pressure, water intake, and salt preference. *J Biol Chem* 2002;277(36):33235-41.

(154) Morrison CD, White CL, Wang Z, Lee SY, Lawrence DS, Cefalu WT, Zhang ZY, Gettys TW. Increased hypothalamic protein tyrosine phosphatase 1B contributes to leptin resistance with age. *Endocrinology* 2007 January;148(1):433-40.

(155) Mullins JJ, Peters J, Ganten D. Fulminant hypertension in transgenic rats harbouring the mouse Ren-2 gene. *Nature* 1990 April 5;344(6266):541-4.

(156) Muratani H, Averill DB, Ferrario CM. Effect of angiotensin II in ventrolateral medulla of spontaneously hypertensive rats. *Am J Physiol* 1991 May;260(5 Pt 2):R977-R984.

(157) Myers MP, Andersen JN, Cheng A, Tremblay ML, Horvath CM, Parisien JP, Salmeen A, Barford D, Tonks NK. TYK2 and JAK2 are substrates of protein-tyrosine phosphatase 1B. *J Biol Chem* 2001 December 21;276(51):47771-4.

(158) Niswender KD, Gallis B, Blevins JE, Corson MA, Schwartz MW, Baskin DG. Immunocytochemical detection of phosphatidylinositol 3-kinase activation by insulin and leptin. *J Histochem Cytochem* 2003;51(3):275-83.

(159) Niswender KD, Morton GJ, Stearns WH, Rhodes CJ, Myers MG, Jr., Schwartz MW. Intracellular signalling. Key enzyme in leptin-induced anorexia. *Nature* 2001 October 25;413(6858):794-5.

(160) Paez J, Sellers WR. PI3K/PTEN/AKT pathway. A critical mediator of oncogenic signaling. *Cancer Treat Res* 2003;115:145-67.

(161) Paton JF, Kasparov S. Differential effects of angiotensin II on cardiorespiratory reflexes mediated by nucleus tractus solitarii - a microinjection study in the rat. *J Physiol* 1999;521 Pt 1:213-25.

(162) Pelleymounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T, Collins F. Effects of the obese gene product on body weight regulation in ob/ob mice. *Science* 1995 July 28;269(5223):540-3.

(163) Phillips MI. Functions of angiotensin in the central nervous system. *Annu Rev Physiol* 1987;49:413-35.

(164) Picardi PK, Calegari VC, de Oliveira PP, Contin MJ, Araujo E, Gomes Marcondes MC, Ueno M, Carvalheira JB, Velloso LA, Abdalla Saad MJ. Reduction of hypothalamic protein tyrosine phosphatase improves insulin and leptin resistance in diet-induced obese rats. *Endocrinology* 2008 August;149(8):3870-80.

(165) Plum L, Schubert M, Bruning JC. The role of insulin receptor signaling in the brain. *Trends Endocrinol Metab* 2005 March;16(2):59-65.

(166) Rahmouni K, Correia ML, Haynes WG, Mark AL. Obesity-associated hypertension: new insights into mechanisms. *Hypertension* 2005 January;45(1):9-14.

(167) Rahmouni K, Haynes WG. Leptin signaling pathways in the central nervous system: interactions between neuropeptide Y and melanocortins. *Bioessays* 2001 December;23(12):1095-9.

(168) Rahmouni K, Haynes WG. Leptin and the cardiovascular system. *Recent Prog Horm Res* 2004;59:225-44.

(169) Rahmouni K, Haynes WG, Morgan DA, Mark AL. Intracellular mechanisms involved in leptin regulation of sympathetic outflow. *Hypertension* 2003 March;41(3 Pt 2):763-7.

(170) Rahmouni K, Morgan DA. Hypothalamic arcuate nucleus mediates the sympathetic and arterial pressure responses to leptin. *Hypertension* 2007 March;49(3):647-52.

(171) Rahmouni K, Morgan DA, Morgan GM, Mark AL, Haynes WG. Role of selective leptin resistance in diet-induced obesity hypertension. *Diabetes* 2005 July;54(7):2012-8.

(172) Rahmouni K, Sigmund CD, Haynes WG, Mark AL. Hypothalamic ERK mediates the anorectic and thermogenic sympathetic effects of leptin. *Diabetes* 2009 March;58(3):536-42.

(173) Rajapurohitam V, Javadov S, Purdham DM, Kirshenbaum LA, Karmazyn M. An autocrine role for leptin in mediating the cardiomyocyte hypertrophic effects of angiotensin II and endothelin-1. *J Mol Cell Cardiol* 2006 August;41(2):265-74.

(174) Sakai K, Chapleau MW, Morimoto S, Cassell MD, Sigmund CD. Differential modulation of baroreflex control of heart rate by neuron- vs. glia-derived angiotensin II. *Physiol Genomics* 2004;20(1):66-72.

(175) Sakima A, Averill DB, Gallagher PE, Kasper SO, Tommasi EN, Ferrario CM, Diz DI. Impaired heart rate baroreflex in older rats: role of endogenous angiotensin-(1-7) at the nucleus tractus solitarii. *Hypertension* 2005;46(2):333-40.

(176) Sakima A, Averill DB, Kasper SO, Jackson L, Ganten D, Ferrario CM, Gallagher PE, Diz DI. Baroreceptor reflex regulation in anesthetized transgenic rats with low glia-derived angiotensinogen. *Am J Physiol Heart Circ Physiol* 2007;292(3):H1412-H1419.

(177) Sakima A, Averill DB, Kasper SO, Tommasi E, Gilliam-Davis S, Jackson L, Ganten D, Ferrario CM, Diz DI. Cardiovascular and autonomic control in transgenic rats with low glial-derived angiotensinogen. *Intl Soc Hypertension* 157, PM3-03-01. 2006.

(178) Salmeen A, Andersen JN, Myers MP, Tonks NK, Barford D. Molecular basis for the dephosphorylation of the activation segment of the insulin receptor by protein tyrosine phosphatase 1B. *Mol Cell* 2000 December;6(6):1401-12.

(179) Satoh N, Ogawa Y, Katsuura G, Hayase M, Tsuji T, Imagawa K, Yoshimasa Y, Nishi S, Hosoda K, Nakao K. The arcuate nucleus as a primary site of satiety effect of leptin in rats. *Neurosci Lett* 1997 March 21;224(3):149-52.

(180) Schiavone MT, Santos RA, Brosnihan KB, Khosla MC, Ferrario CM. Release of vasopressin from the rat hypothalamo-neurohypophysial system by angiotensin-(1-7) heptapeptide. *Proc Natl Acad Sci U S A* 1988 June;85(11):4095-8.

(181) Schinke M, Baltatu O, Bohm M, Peters J, Rascher W, Bricca G, Lippoldt A, Ganten D, Bader M. Blood pressure reduction and diabetes insipidus in transgenic rats deficient in brain angiotensinogen. *Proc Natl Acad Sci U S A* 1999;96(7):3975-80.

(182) Schwartz MW, Peskind E, Raskind M, Boyko EJ, Porte D, Jr. Cerebrospinal fluid leptin levels: relationship to plasma levels and to adiposity in humans. *Nat Med* 1996 May;2(5):589-93.

(183) Schwartz MW, Woods SC, Porte D, Jr., Seeley RJ, Baskin DG. Central nervous system control of food intake. *Nature* 2000 April 6;404(6778):661-71.

(184) Senanayake PD, Moriguchi A, Kumagai H, Ganten D, Ferrario CM, Brosnihan KB. Increased expression of angiotensin peptides in the brain of transgenic hypertensive rats. *Peptides* 1994;15(5):919-26.

(185) Seyedabadi M, Goodchild AK, Pilowsky PM. Differential role of kinases in brain stem of hypertensive and normotensive rats. *Hypertension* 2001 November;38(5):1087-92.

(186) Shek EW, Brands MW, Hall JE. Chronic leptin infusion increases arterial pressure. *Hypertension* 1998 January;31(1 Pt 2):409-14.

(187) Shibao C, Gamboa A, Diedrich A, Ertl AC, Chen KY, Byrne DW, Farley G, Paranjape SY, Davis SN, Biaggioni I. Autonomic contribution to blood pressure and metabolism in obesity. *Hypertension* 2007 January;49(1):27-33.

(188) Skurk T, van H, V, Blum WF, Hauner H. Angiotensin II promotes leptin production in cultured human fat cells by an ERK1/2-dependent pathway. *Obes Res* 2005 June;13(6):969-73.

(189) Smith PM, Chambers AP, Price CJ, Ho W, Hopf C, Sharkey KA, Ferguson AV. The subfornical organ: a central nervous system site for actions of circulating leptin. *Am J Physiol Regul Integr Comp Physiol* 2009 March;296(3):R512-R520.

(190) Smith PM, Mollaret V, Ferguson AV. Leptin acts in the rat hypothalamic paraventricular nucleus to induce gastric mucosal damage. *Am J Physiol* 1998 December;275(6 Pt 2):R2081-R2084.

(191) Snitker S, Pratley RE, Nicolson M, Tataranni PA, Ravussin E. Relationship between muscle sympathetic nerve activity and plasma leptin concentration. *Obes Res* 1997 July;5(4):338-40.

(192) Soderberg S, Ahren B, Jansson JH, Johnson O, Hallmans G, Asplund K, Olsson T. Leptin is associated with increased risk of myocardial infarction. *J Intern Med* 1999 October;246(4):409-18.

(193) Soderberg S, Ahren B, Stegmayr B, Johnson O, Wiklund PG, Weinehall L, Hallmans G, Olsson T. Leptin is a risk marker for first-ever hemorrhagic stroke in a population-based cohort. *Stroke* 1999 February;30(2):328-37.

(194) Stec DE, Sigmund CD. Physiological insights from genetic manipulation of the renin-angiotensin system. *News Physiol Sci* 2001 April;16:80-4.

(195) Szczepanska-Sadowska E, Paczwa P, Dobruch J. Enhanced food and water intake in renin transgenic rats. *J Physiol Pharmacol* 2003 March;54(1):81-8.

(196) Takenaka T, Suzuki H, Fujiwara K, Kanno Y, Ohno Y, Hayashi K, Nagahama T, Saruta T. Cellular mechanisms mediating rat renal microvascular constriction by angiotensin II. *J Clin Invest* 1997 October 15;100(8):2107-14.

(197) Tan PS, Killinger S, Horiuchi J, Dampney RA. Baroreceptor reflex modulation by circulating angiotensin II is mediated by AT1 receptors in the nucleus tractus solitarius. *Am J Physiol Regul Integr Comp Physiol* 2007 December;293(6):R2267-R2278.

(198) Tartaglia LA. The leptin receptor. *J Biol Chem* 1997 March 7;272(10):6093-6.

(199) Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, Richards GJ, Campfield LA, Clark FT, Deeds J, Muir C, Sanker S, Moriarty A, Moore KJ, Smutko JS, Mays GG, Wool EA, Monroe CA, Tepper RI. Identification and expression cloning of a leptin receptor, OB-R. *Cell* 1995 December 29;83(7):1263-71.

(200) Thayer JF, Lane RD. The role of vagal function in the risk for cardiovascular disease and mortality. *Biol Psychol* 2007 February;74(2):224-42.

(201) Thompson RH, Canteras NS, Swanson LW. Organization of projections from the dorsomedial nucleus of the hypothalamus: a PHA-L study in the rat. *J Comp Neurol* 1996 December 2;376(1):143-73.

(202) Tokita Y, Franco-Saenz R, Mulrow PJ, Ganten D. Effects of nephrectomy and adrenalectomy on the renin-angiotensin system of transgenic rats TGR(mRen2)27. *Endocrinology* 1994 January;134(1):253-7.

(203) Trask AJ, Ferrario CM. Angiotensin-(1-7): pharmacology and new perspectives in cardiovascular treatments. *Cardiovasc Drug Rev* 2007;25(2):162-74.

(204) Tu H, Kastin AJ, Hsuchou H, Pan W. Soluble receptor inhibits leptin transport. *J Cell Physiol* 2008 February;214(2):301-5.

(205) Veerasingham SJ, Raizada MK. Brain renin-angiotensin system dysfunction in hypertension: recent advances and perspectives. *Br J Pharmacol* 2003 May;139(2):191-202.

(206) Venable CL, Frevert EU, Kim YB, Fischer BM, Kamatkar S, Neel BG, Kahn BB. Overexpression of protein-tyrosine phosphatase-1B in adipocytes inhibits insulin-stimulated phosphoinositide 3-kinase activity without altering glucose transport or Akt/Protein kinase B activation. *J Biol Chem* 2000 June 16;275(24):18318-26.

(207) Vinsant S, Chappell MC, Ferrario CM, Ganten D, Diz DI. Low glial angiotensinogen is not associated with deficits in angiotensin peptides in neuronal pathways in transgenic ASrAogen rats (Abstract). *FASEB J.* 2005; 19.

(208) Wallace AM, McMahon AD, Packard CJ, Kelly A, Shepherd J, Gaw A, Sattar N. Plasma leptin and the risk of cardiovascular disease in the west of Scotland coronary prevention study (WOSCOPS). *Circulation* 2001 December 18;104(25):3052-6.

(209) Waring JF, Ciurlionis R, Clampit JE, Morgan S, Gum RJ, Jolly RA, Kroeger P, Frost L, Trevillyan J, Zinker BA, Jirousek M, Ulrich RG, Rondinone CM. PTP1B antisense-treated mice show regulation of genes involved in lipogenesis in liver and fat. *Mol Cell Endocrinol* 2003 May 30;203(1-2):155-68.

(210) White CL, Whittington A, Barnes MJ, Wang Z, Bray GA, Morrison CD. HF diets increase hypothalamic PTP1B and induce leptin resistance through both leptin-dependent and -independent mechanisms. *Am J Physiol Endocrinol Metab* 2009 February;296(2):E291-E299.

(211) White DW, Tartaglia LA. Leptin and OB-R: body weight regulation by a cytokine receptor. *Cytokine Growth Factor Rev* 1996 December;7(4):303-9.

(212) Wiesmann C, Barr KJ, Kung J, Zhu J, Erlanson DA, Shen W, Fahr BJ, Zhong M, Taylor L, Randal M, McDowell RS, Hansen SK. Allosteric inhibition of protein tyrosine phosphatase 1B. *Nat Struct Mol Biol* 2004 August;11(8):730-7.

(213) Wofford MR, Anderson DC, Jr., Brown CA, Jones DW, Miller ME, Hall JE. Antihypertensive effect of alpha- and beta-adrenergic blockade in obese and lean hypertensive subjects. *Am J Hypertens* 2001 July;14(7 Pt 1):694-8.

(214) Wojcicka G, Jamroz-Wisniewska A, Widomska S, Ksiazek M, Beltowski J. Role of extracellular signal-regulated kinases (ERK) in leptin-induced hypertension. *Life Sci* 2008 February 13;82(7-8):402-12.

(215) Yamada E, Endoh T, Suzuki T. Angiotensin II-induced inhibition of calcium currents via G(q/11)-protein involving protein kinase C in hamster submandibular ganglion neurons. *Neurosci Res* 2002 June;43(2):179-89.

(216) Yang R, Barouch LA. Leptin signaling and obesity: cardiovascular consequences. *Circ Res* 2007 September 14;101(6):545-59.

(217) Zabolotny JM, ce-Hanulec KK, Stricker-Krongrad A, Haj F, Wang Y, Minokoshi Y, Kim YB, Elmquist JK, Tartaglia LA, Kahn BB, Neel BG. PTP1B regulates leptin signal transduction in vivo. *Dev Cell* 2002 April;2(4):489-95.

(218) Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994 December 1;372(6505):425-32.

CHAPTER TWO

MODULATION OF REFLEX FUNCTION BY ENDOGENOUS

ANGIOTENSINS IN OLDER TRANSGENIC RATS WITH LOW GLIAL

ANGIOTENSINOGEN

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Abstract

Age-related impairments in baroreflex sensitivity in Sprague-Dawley rats are associated with low solitary tract nucleus content of angiotensin-(1-7). However, transgenic rats with low brain angiotensinogen (ASrAOGEN) due to glial over-expression of an antisense oligonucleotide to angiotensinogen are spared age-related declines in cardiovascular function characteristic of Sprague-Dawley rats. We examine whether cardiovascular and reflex actions of angiotensin-(1-7) persist in the solitary tract nucleus of older (16-22 months) ASrAOGEN rats. Baroreflex sensitivity for control of heart rate and chemosensitive vagal afferent activation in response to phenylbiguanide were measured before and after bilateral microinjection of the angiotensin II AT_1 receptor antagonist candesartan and angiotensin-(1-7) receptor antagonist [D-Ala^7]-Angiotensin-(1-7) in urethane/chloralose anesthetized rats. In older anesthetized ASrAOGEN rats, candesartan had no effect, whereas [D-Ala^7]-Angiotensin-(1-7) significantly reduced baroreflex sensitivity (1.80 ± 0.43 versus 0.50 ± 0.17 msec/ mm Hg). Phenylbiguanide responses were attenuated by injection of candesartan (-79 ± 6 versus -55 ± 12 mm Hg and -277 ± 12 versus -156 ± 27 bpm; $P < 0.05$). In addition, resting blood pressure was reduced by injection of candesartan or [D-Ala^7]-Angiotensin-(1-7). Within the solitary tract nucleus of older ASrAOGEN rats it appears that 1) glial angiotensinogen is the main source of angiotensin II attenuation of baroreflex sensitivity; 2) endogenous angiotensin-(1-7) from non-glial sources enhances baroreflex sensitivity; 3) non-glial sources of angiotensin II contribute to chemosensitive vagal afferent activation; and 4) receptors for both peptides modulate resting arterial pressure under anesthesia. These results suggest a novel mechanism for preservation of baroreflex sensitivity during aging.

Introduction

Angiotensinogen (Aogen), the precursor of both angiotensin (Ang) II and Ang-(1-7), is expressed primarily in glial cells[220] but is also present in neuronal cells in key cardiovascular nuclei.[184] Double transgenic mice over-expressing both human Aogen and renin in either glial or neuronal cells suggests that glia-derived Ang peptides are responsible for impairments in the baroreflex sensitivity (BRS) for control of heart rate (HR), whereas neuronal over-expression of Ang peptides resets the baroreceptor set-point without altering BRS.[183] Both glial and neuronal over-expression contribute to increases in resting arterial pressure (AP).[184]

Transgenic rats with low brain Aogen (ASrAOGEN) offer a targeted disruption of the endogenous renin-angiotensin system (RAS).[188] The antisense oligonucleotide targeted to glia by a glial fibrillary acidic protein (GFAP) promoter results in a 90% reduction in brain Aogen.[188] This promoter drives expression specifically to astrocytes in both cell culture[189] and in vivo experiments[190]. In ASrAOGEN rats, the antisense oligonucleotide results in decreased drinking responses to renin and lower hypothalamic tissue levels of Ang I with a trend for lower levels of Ang II compared to Sprague-Dawley (SD) rats.[191] Consistent with expression of Aogen mRNA in neurons of the paraventricular nucleus (PVN), neuronal Ang II and Ang-(1-7) are preserved in PVN of these animals compared to SD rats.[192] Functionally, conscious adult ASrAOGEN rats (3-5 months) have enhanced baroreceptor[199] and chemoreceptor[185] reflexes and lower resting mean AP (MAP) compared to age-matched SD rats.[180, 188] Solitary tract nucleus (NTS) blockade of Ang II AT_1 receptors has no effect on BRS whereas blockade of Ang-(1-7) receptors impairs BRS in younger ASrAOGEN, suggesting a glial source of Aogen for

Ang II and a non-glial source for Ang-(1-7) for modulating BRS for control of HR.[185] A

paradoxical enhanced response to cardiac vagal afferent chemosensitive fiber activation

(CVA) and elevated resting MAP occur under anesthesia compared with SD rats.[185] The

elevated MAP in anesthetized ASrAOGEN rats was reversed by blockade of either AT_1

or Ang-(1-7) receptors.[185]

In contrast to older SD rats (16 months), ASrAOGEN rats do not show typical

age-related deficits such as increases in systolic blood pressure (BP) or indices of

metabolic dysfunction.[180] Although baroreflex function is reduced in conscious older

ASrAOGEN rats compared to younger rats, the lower BRS of older ASrAOGEN is

similar to levels seen in younger SD.[186] A recent study in 16-18 month-old SD reveals

that the age-related BRS impairment may result from low production of Ang-(1-7) in the

NTS.[52] The contribution of Ang II and Ang-(1-7) at the level of the NTS to

cardiovascular and reflex function in older ASrAOGEN has not been studied. Thus, we

clarified the unique central integration of Ang II and Ang-(1-7) in the NTS of 16-22

month-old anesthetized ASrAOGEN rats. Identification of factors that regulate

brainstem areas controlling autonomic outflow is crucial in understanding the decline in

baroreflex and cardiovascular function that occurs with aging. The transgenic rat model

used in this study is a tool to evaluate the contribution of different sources of Ang

peptides in modulation of cardiovascular function.

Methods

For a detailed Methods section please see http://hyper.ahajournals.org.

Animals: Experiments were performed in 16-22 month-old male transgenic

TGR(ASrAogen)680 (ASrAOGEN) rats. The institutional animal care and use committee approved all procedures.

Surgical Procedures: As previously reported,[52, 185] rats were anesthetized with urethane-chloralose (750 mg and 35 mg per kg) via intraperitoneal injections, instrumented with femoral artery and vein catheters and placed in a stereotaxic frame with the head tilted downward (45° angle) for surgical exposure of the dorsal medulla oblongata.

Arterial Pressure and Heart Rate Measurements: Pulsatile AP and MAP were monitored, recorded and digitized using a Data Acquisition System (BIOPAC System Inc.; Acknowledge software Version 3.8.1; Santa Barbara, CA) and HR was determined from the AP wave as previously reported.[52, 185]

Reflex Testing: Baseline responses to CVA and BRS were established by bolus intravenous administration of phenylbiguanide (PBG; 10 μg/kg in 0.9% NaCl) or phenylephrine (PE; 2, 5 and 10 μg/kg in 0.9% NaCl) as previously reported.[52, 185] Peak MAP and HR responses to administration of each receptor antagonist were measured. BRS was determined by the slope of the relationship between changes in MAP and the pulse interval. CVA and BRS tests were repeated within 10 minutes of NTS microinjections so that each animal served as its own control.

Microinjections: The Ang II AT_1 receptor antagonist candesartan (CAN; CV-11974; 24 pmol/120 nL), Ang-(1-7) receptor antagonist (D-Ala^7)-Ang-(1-7) (D-Ala; 144 fmol/120 nL) or artificial cerebrospinal fluid (aCSF; pH 7.4; 120 nL) was bilaterally microinjected via pressure into the NTS [0.4 mm rostral, 0.4 mm lateral to the calamus scriptorius (caudal tip of the area postrema) and 0.4 mm below the dorsal surface] using a glass micropipette connected to a syringe.[52, 185]

Histology: The brain was removed and frozen on dry ice at the end of each experiment to assure the site of microinjections were within the medial NTS at rostro-caudal level -13.3 to -14.0 mm caudal to bregma.

Analysis of Data: Values are presented as mean ± SEM. Comparisons of changes in BRS, CVA and resting MAP and HR over time were made by repeated-measures ANOVA with post-hoc Student-Newman-Keuls multiple comparisons. Comparisons of overall absolute changes in MAP and HR in response to receptor antagonists or aCSF were analyzed by one-way ANOVA with post-hoc Dunnett multiple comparisons. Individual changes of MAP and HR in response to treatments were compared to baseline using a one-sample t-test. The criterion for statistical significance was $P < 0.05$. Tests were performed using Prism 4.0 and InStat 3 (GraphPad Software, San Diego, CA).

Results

Effect of NTS Injection of CAN, D-Ala or aCSF on Resting MAP and HR

Maximal transient changes in MAP and HR were measured after NTS injection of receptor antagonists (Figure 1). CAN alone, D-Ala in the presence of CAN, D-Ala alone and CAN in the presence of D-Ala significantly reduced MAP compared to baseline values. The effect of CAN alone was significantly greater than D-Ala alone. The combination of CAN and D-Ala were not different from treatment with either antagonist alone or each other. HR was reduced significantly from baseline values with CAN alone, D-Ala in the presence of CAN and D-Ala alone. There were no differences in HR values among the groups. Injection of aCSF (n = 3) had no effect on MAP or HR given at 10 minutes (3 ± 4 mm Hg and 3 ± 2 bpm) or 60 minutes (5 ± 4 mm Hg and 5 ± 7 bpm).

MAP and HR Immediately Prior to Reflex Tests

Values of MAP and HR immediately prior to microinjections (Table S1, please see http://hyper.ahajournals.org) remained stable throughout the study when CAN was administered first followed 60 min later by D-Ala or in aCSF experiments. In experiments where D-Ala was given first, followed 60 minutes later by CAN, the values of MAP and HR were significantly lower immediately prior to injection of CAN.

BRS after NTS Microinjection of CAN, D-Ala or aCSF

Baseline values of BRS did not differ among the groups (Figure 2). AT_1 receptor blockade by CAN did not alter BRS (Figure 2A). In contrast, blockade of Ang-(1-7) receptors following prior administration of CAN, resulted in a 72% decrease in BRS compared to baseline. When the order of administration was reversed (Figure 2B), D-Ala alone decreased BRS by 69%. BRS was significantly lower compared to baseline values following combination treatment with D-Ala and CAN 60 minutes later and was not different from BRS after D-Ala alone. NTS injection of aCSF had no significant effect on BRS (Figure S1, please see http://hyper.ahajournals.org).

Responses to CVA after NTS Microinjection of CAN, D-Ala or aCSF

CAN significantly attenuated both MAP and HR responses to CVA (Figure 3A). Responses to CVA were still attenuated when retested after D-Ala administration 60 minutes later in the presence of CAN. When the order of receptor antagonists was reversed (Figure 3B), D-Ala had no significant effect on MAP responses to CVA, but significantly attenuated HR responses. CAN, administered 60 minutes later in the presence of D-Ala, attenuated both MAP and HR responses to CVA, to a similar extent as when CAN was administered alone. Injections of aCSF had no significant effect on

responses to CVA (Figure S2, please see http://hyper.ahajournals.org).

Discussion

NTS administration of AT_1 or Ang-(1-7) receptor antagonists indicates repeatedly that endogenous Ang II attenuates and Ang-(1-7) facilitates BRS for control of HR in response to increases in MAP evoked by the α-adrenergic receptor agonist phenylephrine.[39, 40, 52, 202] In older ASrAOGEN rats, there is no effect of AT_1 receptor blockade on BRS for control of HR, while Ang-(1-7) receptor blockade impairs BRS to a greater extent than previously reported for younger ASrAOGEN or SD rats.[52, 185] The current results combined with our previous observations[185] in younger animals show that the source of endogenous Ang II modulating BRS is dependent upon expression of glial Aogen. Regulation of the BRS by glia-derived components of the RAS is consistent with studies in transgenic mice showing that glial over-expression of the RAS decreases while neuronal over-expression does not change BRS.[183] Moreover, blockade with either receptor antagonist lowered resting MAP in younger and older ASrAOGEN, indicating non-glial sources of Aogen are responsible for support of resting MAP under anesthesia in ASrAOGEN rats, which is in contrast to anesthetized SD rats.[185] While tissue peptide levels from glial and non-glial sources have not been measured to date, studies reveal lower hypothalamic Ang I with a similar trend for Ang II in ASrAOGEN rats.[191] Moreover, studies utilizing the GFAP promoter to express Aogen or renin indicate via immunostaining that the expression of these components is targeted to glia.[183, 184] Furthermore, preliminary studies in our laboratory indicate that neuronal Ang-(1-7) is preserved in neurons of the PVN of ASrAOGEN relative to SD rats.[192] Together the data

suggest a non-glial, possibly neuronal source for the preservation of the actions of endogenous Ang-(1-7) to facilitate the BRS in young and old ASrAOGEN animals.

In both conscious and anesthetized SD rats, BRS for control of HR is highest in younger animals and declines with aging.[52, 186] The age-related decline in BRS is partly a result of low endogenous Ang-(1-7) in the NTS.[52] In conscious older ASrAOGEN, BRS is lower than in younger ASrAOGEN animals, but the level achieved is similar to that seen in younger SD rats,[186] suggesting overall maintenance of reflex function in ASrAOGEN rats relative to normotensive rats during aging. BRS as measured in older anesthetized ASrAOGEN rats in the current study is comparable to that in younger anesthetized ASrAOGEN rats.[185] Ang-(1-7) receptor blockade reduced BRS to a greater extent in older than in younger ASrAOGEN or SD rats.[52, 185] and achieved levels seen in older SD rats,[52] suggesting that maintenance of Ang-(1-7) actions within the NTS may serve to mitigate greater age-related declines in BRS in ASrAOGEN rats. Previous studies show that BRS for control of HR in response to decreases in MAP is not affected by Ang II injection or AT_1 receptor blockade in the NTS of anesthetized SD,[202] WKY or SHR rats[221] and thus reflex responses to nitroprusside were not measured in the current study.

We also evaluated responses to activation of cardiac vagal afferent chemosensitive fibers since Ang II receptors are present on these fibers as well.[222] CVA was induced by intravenous administration of phenylbiguanide, a serotonin $5-HT_3$ receptor agonist, near the heart. To minimize changes resulting from direct activation at other potential sites of action, such as NTS neurons, only immediate responses to activation were determined. Younger and older ASrAOGEN rats have an enhanced

depressor response to CVA under anesthesia. Direct activation of NTS neurons would result in a pressor response which was not observed during the time course studied.[223] In younger rats the responses to CVA were not modulated by AT_1 or Ang-(1-7) receptor blockade in the NTS.[185] In contrast, MAP responses to CVA were attenuated by AT_1 receptor blockade and HR responses were attenuated by both AT_1 and Ang-(1-7) receptor blockade in older ASrAOGEN rats. Differential effects of Ang II on BRS and responses to CVA are possible since baroreceptor and chemoreceptor modalities within vagal afferents to the NTS do not converge.[224, 225]

Furthermore, urethane-chloralose anesthesia induces 5-fold increases in plasma renin activity and increases in circulating Ang II.[226] Circulating Ang II can facilitate cardiopulmonary reflexes, effects that are blocked by AT1 receptor blockade.[227] ASrAOGEN rats have elevated AT_1 receptor binding compared to SD rats in the subfornical organ, PVN[198] and NTS[228] at the ages used in this study. Therefore, elevated AT_1 receptors in locations accessed by increased circulating Ang II could modulate CVA in the older rats. Ang-(1-7) alone does not modulate MAP responses, but may potentiate the bradycardic responses to CVA in older ASrAOGEN rats. Previous studies using antisense oligonucleotides within the NTS determined that Ang-II induced HR effects require receptors on cell bodies, whereas BP effects could be partially mediated by receptors on nerve terminals.[229] Ang II and Ang-(1-7) differentially alter neurotransmitter release in the medulla, and these effects further depend on the level of activation of the cells and fibers involved.[230] Thus, the site and mechanism of action of these peptides within the NTS may differ, allowing independent regulation of BP and HR.

The reduction in MAP and HR seen in ASrAOGEN rats in response to AT_1 or Ang-(1-7) receptor blockade in the NTS is in contrast to other rat strains.[39, 40, 202] These changes are not due to a volume stimulatory effect as control aCSF injections produced no significant change in MAP or HR. Although conscious resting MAP is lower in ASrAOGEN compared to anesthetized SD rats,[180, 188] it is elevated and to a similar extent in younger and older anesthetized ASrAOGEN rats.[185] Reductions in cerebral levels of Ang II could lead to the upregulation of AT_1 receptors seen in ASrAOGEN rats during aging.[198, 228] This may contribute in part to enhanced resting MAP under anesthesia and to greater reductions in pressure upon NTS injection of CAN in older versus younger ASrAOGEN rats. Others have shown reduced AT_1 receptor binding in the NTS of young (3-4 months) ASrAOGEN compared to age-matched SD rats[197] which may also in part explain the lack of modulation by Ang II on responses to CVA in younger rats and the lesser reduction in pressure with CAN injection compared to older ASrAOGEN rats.

Blockade of Ang-(1-7) receptors significantly reduced MAP to a similar magnitude as younger ASrAOGEN rats. We previously reported relative *mas* receptor mRNA in the dorsal medulla for younger and older SD[52] and younger ASrAOGEN rats[185], from a dataset that also included older ASrAOGEN rats. The relative value for *mas* mRNA in younger ASrAOGEN was significantly higher compared to younger SD rats as control (1.5 ± 0.1 versus 1.2 ± 0.1; $P < 0.05$)[185] with no significant difference in mRNA values between older SD (1.2 ± 0.1)[52] and older ASrAOGEN rats (1.1 ± 0.1; $P > 0.05$; unpublished data). Although *mas* receptor mRNA was higher in the dorsal medulla of younger ASrAOGEN rats, blockade of Ang-(1-7) receptors resulted in a smaller decrease in BRS in younger versus older ASrAOGEN rats. Also, there does not appear

to be an age-related difference in modulation of resting MAP by Ang-(1-7). Thus, differences in *mas* receptor mRNA do not appear to account for the functional differences observed among strains or ages in our studies.

Previous studies showed that blockade of either AT_1 or *mas* receptors lowered MAP to a similar level in younger ASrAOGEN rats.[185] To determine whether interactions between AT_1 and *mas* receptors were occurring, we administered the receptor antagonists alone and in combination in the same animals and looked for additive responses. Importantly, changes in CVA, MAP or HR do not appear to be additive, as each antagonist, alone or in combination, changed responses by approximately the same amount, suggesting a common mechanism. The contribution of Ang II and Ang-(1-7) to increases in MAP in older ASrAOGEN rats in a non-additive manner is consistent with several recent studies. Activation of pathways from PVN to NTS and rostral ventrolateral medulla (RVLM) to increase MAP involves AT_1 and Ang-(1-7) receptors at each site,[185, 231] with no evidence of additive effects.

Different mechanisms appear to be involved in modulation of resting MAP and BRS. Although AT_1 receptors are elevated in the SFO, PVN and NTS of older ASrAOGEN rats, there was no effect of AT_1 receptor blockade on BRS. In contrast, AT_1 receptor blockade significantly reduced resting MAP and HR and the responses to CVA in these animals. Ang-(1-7) continues to modulate BRS during aging in ASrAOGEN rats, and plays a role in modulating resting MAP and HR in the older animals, similar to younger ASrAOGEN rats. The source of the Aogen for both Ang II and Ang-(1-7) actions on CVA and resting MAP may be the PVN, as neuronal expression of the two peptides is preserved in ASrAOGEN rats.[192] We propose that under anesthesia, elevated

levels of circulating Ang II activate descending angiotensinergic pathways, involving both Ang II and Ang-(1-7), from the PVN to the NTS and RVLM to increase sympathetic outflow possibly contributing to the anesthesia-induced pressor responses seen in these animals.

Perspectives

Cardiovagal baroreflex function declines with age in healthy human populations.[232] Age-related impairments in BRS originate from central neuronal dysfunction and peripheral vascular changes.[14] Although plasma renin and circulating Ang peptides decrease with age[203], RAS blockade is efficacious in preventing age-related deficits and improving lifespan of normotensive rats[233] suggesting that in various tissues blockade of RAS may contribute to beneficial effects. Administration of either AT1 receptor blockers or ACE inhibitors improves BRS[52, 234] suggesting that an increase in Ang II or its actions might be responsible for impaired BRS. These treatments, however, also shift the endogenous balance of Ang peptides towards Ang-(1-7).[235] The current study indicates that neuronal preservation of Ang-(1-7) may be a mechanism to prevent the age-related decline in baroreflex function.

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Conflicts of Interest/Disclosures

None

Reference List

1. Stornetta RL, Hawelu-Johnson CL, Guyenet PG, Lynch KR. Astrocytes synthesize angiotensinogen in brain. *Science*. 1988; 242:1444-1446.

2. Morimoto S, Cassell MD, Sigmund CD. Glia- and neuron-specific expression of the renin-angiotensin system in brain alters blood pressure, water intake, and salt preference. *J Biol Chem*. 2002; 277:33235-33241.

3. Sakai K, Chapleau MW, Morimoto S, Cassell MD, Sigmund CD. Differential modulation of baroreflex control of heart rate by neuron- vs. glia-derived angiotensin II. *Physiol Genomics*. 2004; 20:66-72.

4. Schinke M, Baltatu O, Bohm M, Peters J, Rascher W, Bricca G, Lippoldt A, Ganten D, Bader M. Blood pressure reduction and diabetes insipidus in transgenic rats deficient in brain angiotensinogen. *Proc Natl Acad Sci U S A*. 1999; 96:3975-3980.

5. Besnard F, Brenner M, Nakatani Y, Chao R, Purohit HJ, Freese E. Multiple interacting sites regulate astrocyte-specific transcription of the human gene for glial fibrillary acidic protein. *J Biol Chem*. 1991; 266:18877-18883.

6. Brenner M, Kisseberth WC, Su Y, Besnard F, Messing A. GFAP promoter directs astrocyte-specific expression in transgenic mice. *J Neurosci*. 1994; 14:1030-1037.

7. Huang BS, Ganten D, Leenen FH. Responses to central Na(+) and ouabain are attenuated in transgenic rats deficient in brain angiotensinogen. *Hypertension*. 2001; 37:683-686.

8. Vinsant S, Chappell MC, Ferrario CM, Ganten D, Diz DI. Low glial angiotensinogen is not associated with deficits in angiotensin peptides in neuronal pathways in transgenic ASrAogen rats (Abstract). FASEB J 2005;19:

9. Couto AS, Baltatu O, Santos RA, Ganten D, Bader M, Campagnole-Santos MJ. Differential effects of angiotensin II and angiotensin-(1-7) at the nucleus tractus solitarii of transgenic rats with low brain angiotensinogen. *J Hypertens*. 2002; 20:919-925.

10. Sakima A, Averill DB, Kasper SO, Jackson L, Ganten D, Ferrario CM, Gallagher PE, Diz DI. Baroreceptor reflex regulation in anesthetized transgenic rats with low glia-derived angiotensinogen. *Am J Physiol Heart Circ Physiol*. 2007; 292:H1412-H1419

11. Kasper SO, Carter CS, Ferrario CM, Ganten D, Ferder LF, Sonntag WE, Gallagher PE, Diz DI. Growth, metabolism, and blood pressure disturbances during aging in transgenic rats with altered brain renin-angiotensin systems. *Physiol Genomics*. 2005; 23:311-317.

12. Diz DI, Kasper SO, Sakima A, Ferrario CM. Aging and the brain renin-angiotensin system: insights from studies in transgenic rats. *Cleve Clin J Med*. 2007; 74 Suppl 1:S95-S98

13. Sakima A, Averill DB, Gallagher PE, Kasper SO, Tommasi EN, Ferrario CM, Diz DI. Impaired heart rate baroreflex in older rats: role of endogenous angiotensin-(1-7) at the nucleus tractus solitarii. *Hypertension*. 2005; 46:333-340.

14. Matsumura K, Averill DB, Ferrario CM. Angiotensin II acts at AT1 receptors in the nucleus of the solitary tract to attenuate the baroreceptor reflex. *Am J Physiol*. 1998; 275:R1611-R1619

15. Chaves GZ, Caligiorne SM, Santos RA, Khosla MC, Campagnole-Santos MJ. Modulation of the baroreflex control of heart rate by angiotensin-(1-7) at the nucleus tractus solitarii of normotensive and spontaneously hypertensive rats. *J Hypertens*. 2000; 18:1841-1848.

16. Campagnole-Santos MJ, Diz DI, Ferrario CM. Baroreceptor reflex modulation by angiotensin II at the nucleus tractus solitarii. *Hypertension*. 1988; 11:I167-I171

17. Katsunuma N, Tsukamoto K, Ito S, Kanmatsuse K. Enhanced angiotensin-mediated responses in the nucleus tractus solitarii of spontaneously hypertensive rats. *Brain Res Bull*. 2003; 60:209-214.

18. Healy DP, Rettig R, Nguyen T, Printz MP. Quantitative autoradiography of angiotensin II receptors in the rat solitary-vagal area: effects of nodose ganglionectomy or sinoaortic denervation. *Brain Res*. 1989; 484:1-12.

19. Sevoz-Couche C, Maisonneuve B, Hamon M, Laguzzi R. Glutamate and NO mediation of the pressor response to 5-HT3 receptor stimulation in the nucleus tractus solitarii. *Neuroreport*. 2002; 13:837-841.

20. Kasparov S, Paton JF. Differential effects of angiotensin II in the nucleus tractus solitarii of the rat--plausible neuronal mechanism. *J Physiol*. 1999; 521 Pt 1:227-238.

21. Paton JF. Convergence properties of solitary tract neurones driven synaptically by cardiac vagal afferents in the mouse. *J Physiol*. 1998; 508 (Pt 1):237-252.

22. Shimokawa A, Kunitake T, Takasaki M, Kannan H. Differential effects of anesthetics on sympathetic nerve activity and arterial baroreceptor reflex in chronically instrumented rats. *J Auton Nerv Syst*. 1998; 72:46-54.

23. Veelken R, Hilgers KF, Scrogin KE, Mann JF, Schmieder RE. Endogenous angiotensin II and the reflex response to stimulation of cardiopulmonary serotonin 5HT3 receptors. *Br J Pharmacol*. 1998; 125:1761-1767.

24. Kasper SO, Ferrario CM, Ganten D, Diz DI. Central depletion of angiotensinogen is associated with elevated AT1 receptors in the SFO and PVN. *Neurotox Res*. 2004; 6:259-265.

25. Jackson L, Sakima A, Oden SD, Tommasi E, Ganten D, Ferrario CM, Diz DI. Angiotensin receptor density in dorsomedial medulla under varying local angiotensin levels during aging (Abstract). Hypertension 2004;44:

26. Diz DI, Westwood B, Averill DB. AT(1) antisense distinguishes receptors mediating angiotensin II actions in solitary tract nucleus. *Hypertension*. 2001; 37:1292-1297.

27. Diz DI, Pirro NT. Differential actions of angiotensin II and angiotensin-(1-7) on transmitter release. *Hypertension*. 1992; 19:II41-II48

28. Monti J, Schinke M, Bohm M, Ganten D, Bader M, Bricca G. Glial angiotensinogen regulates brain angiotensin II receptors in transgenic rats TGR(ASrAOGEN). *Am J Physiol Regul Integr Comp Physiol*. 2001; 280:R233-R240

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

30. Fauvel JP, Cerutti C, Mpio I, Ducher M. Aging process on spectrally determined spontaneous baroreflex sensitivity: a 5-year prospective study. *Hypertension*. 2007; 50:543-546.

31. Ferrari AU, Radaelli A, Centola M. Invited review: aging and the cardiovascular system. *J Appl Physiol*. 2003; 95:2591-2597.

32. Anderson S. Ageing and the renin-angiotensin system. *Nephrol Dial Transplant*. 1997; 12:1093-1094.

33. Basso N, Paglia N, Stella I, de Cavanagh EM, Ferder L, Rosario Lores AM, Inserra F. Protective effect of the inhibition of the renin-angiotensin system on aging. *Regul Pept*. 2005; 128:247-252.

34. Weber MA. Interrupting the renin-angiotensin system: the role of angiotensin-converting enzyme inhibitors and angiotensin II receptor antagonists in the treatment of hypertension. *Am J Hypertens*. 1999; 12:189S-194S.

35. Ferrario CM. Angiotension-(1-7) and antihypertensive mechanisms. *J Nephrol*. 1998; 11:278-283.

Figure 1. Maximal transient changes in MAP and HR in response to NTS injection of CAN or D-Ala. *Top:* All treatments, alone or in combination, significantly reduced MAP compared to baseline values. The reduction in pressure upon CAN injection was significantly larger than when D-Ala was administered alone (# $p < 0.05$). *Bottom:* Injection of CAN alone, D-Ala in the presence of CAN and D-Ala alone significantly reduced HR compared to baseline values. N = 5 for each group; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$
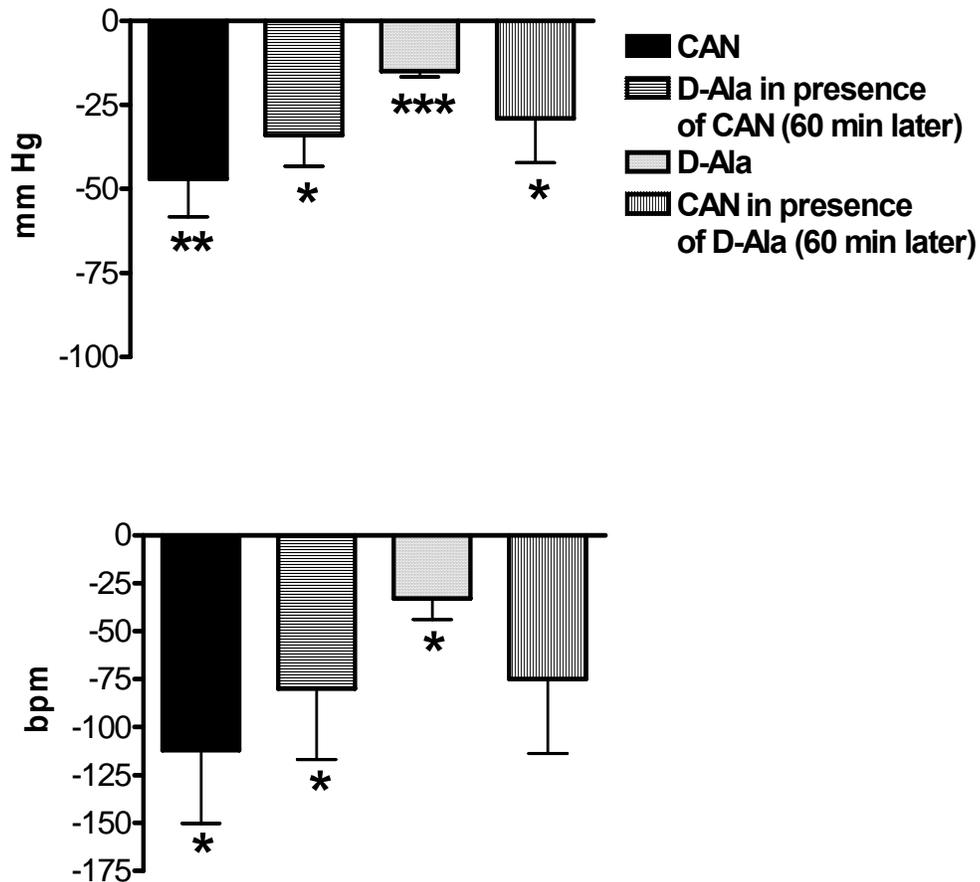
Figure 2. BRS for control of HR in response to intravenous phenylephrine before and after bilateral NTS injection of (A) CAN or (B) D-Ala. A, Administration of CAN alone (n = 5) had no effect on BRS while D-Ala in the presence of CAN (n = 5) decreased BRS by 72%. B, Reversal of receptor antagonists revealed that administration of D-Ala alone (n = 5) decreased BRS by 69%. CAN administered 60 minutes later in the presence of D-Ala (n = 5) did not have any further effect on BRS compared to D-Ala alone. * $p < 0.05$ versus baseline, & $p < 0.05$ versus after CAN, # $p < 0.01$ versus baseline
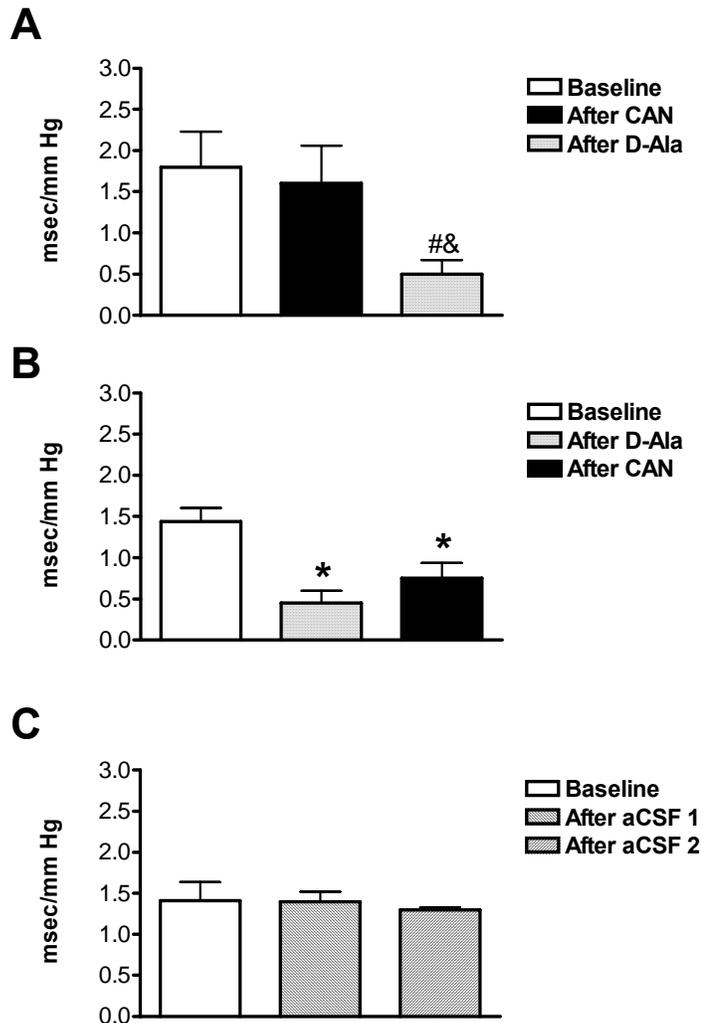
Figure 3. Responses to cardiac vagal afferent chemosensitive fiber activation induced by intravenous phenylbiguanide before and after bilateral NTS injection of (A) CAN or (B) D-Ala. A, CAN alone (n = 5) and D-Ala administered 60 minutes later in the presence of CAN (n = 5) significantly reduced MAP and HR responses to CVA. B, Upon reversal of receptor antagonists, D-Ala alone (n = 5) no longer reduced MAP but significantly reduced HR responses. CAN administered 60 minutes later in the presence of D-Ala (n = 5) still reduced both MAP and HR responses to CVA. * p < 0.05 versus baseline, & p < 0.05 versus after CAN, # p < 0.01 versus baseline, $ p < 0.001 versus baseline
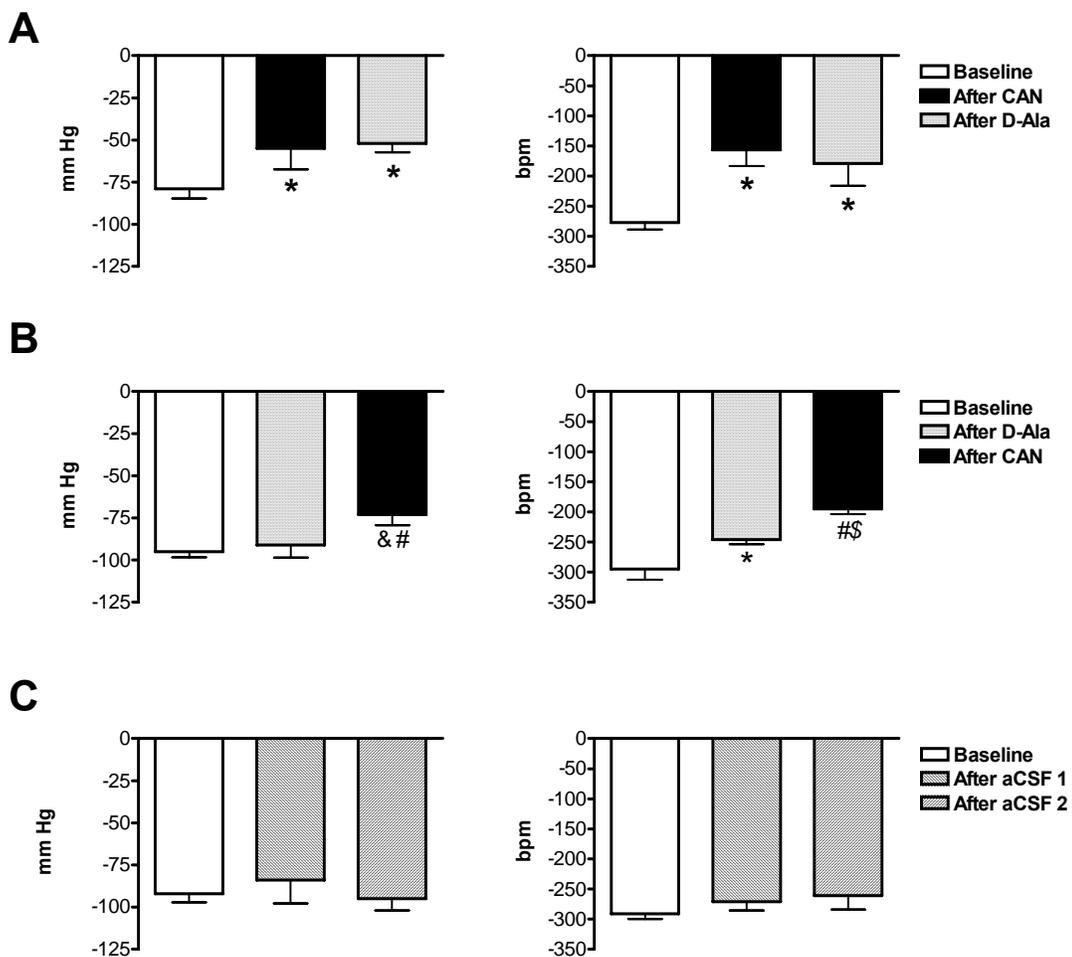
ONLINE SUPPLEMENT:

MODULATION OF REFLEX FUNCTION BY ENDOGENOUS ANGIOTENSINS IN OLDER TRANSGENIC RATS WITH LOW GLIAL ANGIOTENSINOGEN

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Methods

Animals: Experiments were performed in 16-22 month-old male transgenic TGR(ASrAogen)680 (ASrAOGEN) rats from the Hypertension and Vascular Research Center Colony, Wake Forest University School of Medicine, Winston-Salem, NC. Animals were housed in humidity- and temperature-controlled rooms in group cages (12-hour light/dark cycle) with standard rat chow and water available *ad libitum*.   The institutional animal care and use committee approved all procedures.

Surgical Procedures: As previously reported,[52, 185] rats were anesthetized with urethane-chloralose (750 mg and 35 mg per kg) via intraperitoneal injections.   Intravenous supplemental doses were given as needed.   Animals were instrumented with femoral artery and vein catheters and placed in a stereotaxic frame with the head tilted downward (45° angle) for surgical exposure of the dorsal medulla oblongata by incision of the atlanto-occipital membrane.   Rats breathed a mixture of room air and oxygen (70%:30%) and body temperature was maintained (37.0 ± 1.0°C).   A stabilization period of ≥ 30 minutes was allowed before baseline measurements were recorded.

Arterial Pressure and Heart Rate Measurements: Pulsatile AP and MAP were monitored, recorded and digitized using a Data Acquisition System (BIOPAC System Inc.; Acknowledge software Version 3.8.1; Santa Barbara, CA) and HR was determined from the AP wave as previously reported.[52, 185]

Reflex Testing: Baseline responses to CVA and BRS were established by bolus intravenous administration of phenylbiguanide (PBG; 10 μg/kg in 0.9% NaCl) or phenylephrine (PE; 2, 5 and 10 μg/kg in 0.9% NaCl) as previously reported.[52, 185]   A period of ≥ 30 minutes was allowed after baseline measurements before beginning

microinjections. Peak MAP and HR responses to administration of each receptor antagonist were determined and CVA and BRS tests were repeated within 10 minutes of NTS microinjections so that each animal was used as its own control. CVA testing (10-15 minutes) was followed immediately by baroreflex testing (15 minutes) so that reflex testing was completed within 30 minutes after microinjections. Maximum MAP responses (ΔMAP, mm Hg) and the associated reflex changes in HR (ΔHR, bpm) were recorded at each dose of PE. ΔHR was converted to changes in pulse interval (ΔPI, msec) by the formula: 60,000/HR. The slope of the line fit through the ΔMAP and corresponding ΔPI in response to graded doses of PE was used as an index of BRS for control of HR as previously reported.[52, 185]

Microinjections: Multi-barreled glass pipettes with an outer diameter of 30 to 50 μm were used as described previously.[52, 185] The Ang II AT_1 receptor antagonist candesartan (CAN; CV-11974; 24 pmol/120 nL) and Ang-(1-7) receptor antagonist (D-Ala[7])-Ang-(1-7) (D-Ala; 144 fmol/120 nL) were dissolved in artificial cerebrospinal fluid (aCSF; pH 7.4). Vehicle control injections consisted of aCSF (120 nL). Doses and volumes of receptor antagonists are similar to those found functionally effective in previous studies .[40, 52, 185, 202, 236] CAN, D-Ala or aCSF was bilaterally microinjected into the NTS [0.4 mm rostral, 0.4 mm lateral to the calamus scriptorius (caudal tip of the area postrema) and 0.4 mm below the dorsal surface] using a glass micropipette connected via PE 50 tubing to a syringe (1 mL; Becton, Dickinson and Company). Air pressure was generated by pushing on the syringe to displace the desired amount of antagonist from the pipette into the NTS. This was visualized by movement of the fluid meniscus across the calibration line of the pipette barrel. Microinjection experiments consisted of the following groups:

CAN followed 60 minutes later by D-Ala (n = 5), D-Ala followed 60 minutes later by D-Ala and then immediately by CAN (n = 5) or aCSF injections (n = 3).

Histology: The brain was removed and frozen on dry ice at the end of each experiment for histological evaluation. Serial cryostat sections (30 μm) of the frozen medulla were used to assess the site of microinjections. Experiments in which the pipette tip could be localized within the medial NTS at rostro-caudal level -13.3 to -14.0 mm caudal to bregma were included in this study.

Analysis of Data: Values are presented as mean ± SEM. Comparisons of changes in BRS, CVA and resting MAP and HR over time were made by repeated-measures ANOVA with post-hoc Student-Newman-Keuls multiple comparisons. Comparisons of overall absolute changes in MAP and HR in response to receptor antagonists or aCSF were analyzed by one-way ANOVA with post-hoc Dunnett multiple comparisons. Individual changes of MAP and HR in response to treatments were compared to baseline using a one-sample t-test. The criterion for statistical significance was $P < 0.05$. Tests were performed using Prism 4.0 and InStat 3 (GraphPad Software, San Diego, CA).

Reference List

1. Sakima A, Averill DB, Gallagher PE, Kasper SO, Tommasi EN, Ferrario CM, Diz DI. Impaired heart rate baroreflex in older rats: role of endogenous angiotensin-(1-7) at the nucleus tractus solitarii. *Hypertension.* 2005;46:333-340.

2. Sakima A, Averill DB, Kasper SO, Jackson L, Ganten D, Ferrario CM, Gallagher PE, Diz DI. Baroreceptor reflex regulation in anesthetized transgenic rats with low glia-derived angiotensinogen. *Am J Physiol Heart Circ Physiol.* 2007;292:H1412-H1419.

3. Matsumura K, Averill DB, Ferrario CM. Angiotensin II acts at AT1 receptors in the nucleus of the solitary tract to attenuate the baroreceptor reflex. *Am J Physiol.* 1998;275:R1611-R1619.

4. Fow JE, Averill DB, Barnes KL. Mechanisms of angiotensin-induced hypotension and bradycardia in the medial solitary tract nucleus. *Am J Physiol.* 1994;267:H259-H266.

5. Campagnole-Santos MJ, Diz DI, Ferrario CM. Baroreceptor reflex modulation by angiotensin II at the nucleus tractus solitarii. *Hypertension.* 1988;11:I167-I171.

Table S1. Baseline Values of MAP and HR Immediately Prior to Reflex Testing

Group	N	MAP (mm Hg)	HR (bpm)
CAN / D-Ala	5		
Baseline		115 ± 2	310 ± 25
After CAN (10 min later)		99 ± 11	259 ± 17
After D-Ala (60 min later)		95 ± 7	290 ± 29
D-Ala / CAN	5		
Baseline		119 ± 7	319 ± 16
After D-Ala (10 min later)		119 ± 7	299 ± 9
After CAN (60 min later)		100 ± 8 *	254 ± 8 *
aCSF / aCSF	3		
Baseline		113 ± 11	310 ± 41
After aCSF1 (10 min later)		114 ± 7	309 ± 14
After aCSF 2 (60 min later)		118 ± 6	314 ± 34

Values are mean ± standard error of the mean (SEM). Values were determined at baseline and immediately prior to each series of reflex testing.

N = number of animals; MAP = mean arterial pressure; HR = heart rate; CAN = candesartan; D-Ala = $(D-Ala^{7})$-Angiotensin-(1-7); aCSF = artificial cerebrospinal fluid; * $p < 0.01$ versus Baseline and After D-Ala

Figure S1. BRS for control of HR in response to intravenous phenylephrine before and after bilateral NTS microinjection of aCSF. NTS microinjection of aCSF had no effect on BRS over the time course studied (n = 3).
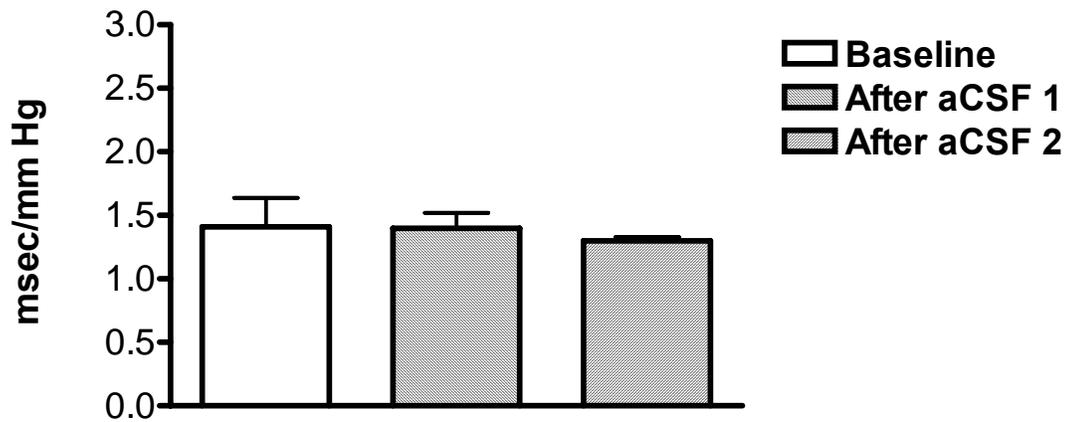
Figure S2. Responses to cardiac vagal afferent chemosensitive fiber activation induced by intravenous phenylbiguanide before and after bilateral NTS microinjection of aCSF. NTS microinjection of aCSF had no significant effect on MAP or HR responses to CVA (n = 3).
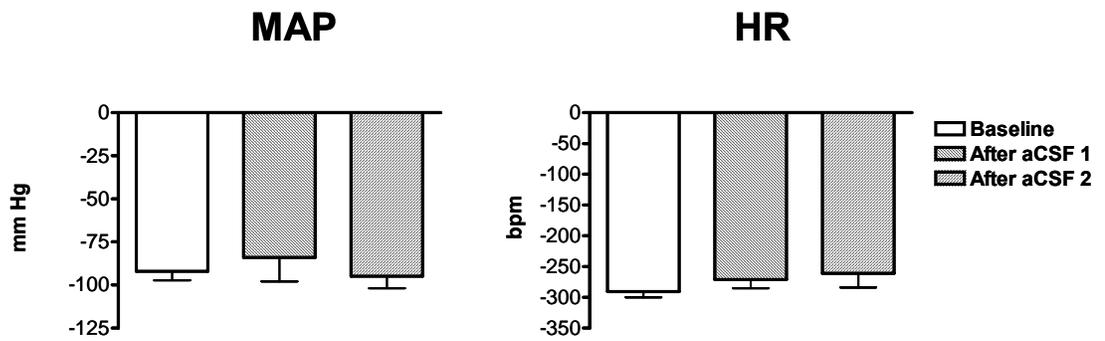
CHAPTER THREE

LEPTIN IMPAIRS CARDIOVAGAL BAROREFLEX FUNCTION AT THE LEVEL OF THE SOLITARY TRACT NUCLEUS

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Abstract

Circulating leptin is elevated in some forms of obesity-related hypertension, associated with impaired baroreflex function. Leptin receptors are present on vagal afferent fibers and neurons within the solitary tract nucleus, providing an anatomical distribution consistent with baroreflex modulation. While solitary tract nucleus microinjection of 144 fmol/60 nL leptin had no significant effect on baroreflex sensitivity for control of heart rate in urethane/chloralose anesthetized Sprague-Dawley rats, 500 fmol leptin impaired baroreflex sensitivity for bradycardia in response to increases in pressure (1.15 ± 0.04 versus 0.52 ± 0.12 msec/mm Hg; $p < 0.01$). Transgenic ASrAOGEN rats with low brain angiotensinogen have an upregulation of leptin receptor and p85 alpha mRNA in dorsal medulla relative to Sprague-Dawley rats. Consistent with these observations, the response to leptin was enhanced in ASrAOGEN rats, since both the 144 fmol (1.46 ± 0.08 versus 0.75 ± 0.10 msec/mm Hg; $p < 0.001$) and 500 fmol (1.36 ± 0.32 versus 0.44 ± 0.06 msec/mm Hg; $p < 0.05$) leptin microinjections impaired baroreflex sensitivity. At these doses, leptin microinjection had no effect on resting pressure, heart rate or the tachycardic response to decreases in pressure in Sprague-Dawley or ASrAOGEN rats. Thus, exogenous leptin at sites within the solitary tract nucleus impairs the baroreflex sensitivity for bradycardia induced by increases in arterial pressure, consistent with a permissive role in mediating increases in arterial pressure. Baroreflex inhibition was enhanced in animals with evidence of increased leptin receptor and relevant signaling pathway mRNA.

Introduction

Leptin is secreted by adipose cells in direct proportion to adiposity[1] and can cross the blood brain barrier to activate hypothalamic pathways involved in satiety and energy expenditure[2]. Leptin actions at key hypothalamic nuclei also mediate cardiovascular responses including increases in sympathetic nervous system activity (SNA) and arterial pressure (AP),[3] likely involving descending pathways to brainstem nuclei involved in direct control of AP and reflex modulation of autonomic function such as the solitary tract nucleus (NTS).[4] The active long form of the leptin receptor, Ob-Rb, has been localized to the nodose ganglion, on vagal afferent fibers and on cells within brainstem areas such as the NTS in normotensive rats[5, 6] implicating leptin in direct actions on baroreflex sensitivity (BRS) for control of heart rate (HR). While the BRS is often impaired in conditions with elevated circulating leptin levels,[7] a direct link between hyperleptinemia and brain sites mediating the effects on BRS is lacking.

Transgenic rats with low brain angiotensinogen (Aogen) resulting from glial over-expression of an antisense oligonucleotide to Aogen (ASrAOGEN) exhibit a 90% reduction in brain Aogen[8] and decreased hypothalamic tissue levels of angiotensin (Ang) I with a similar trend for Ang II.[9] ASrAOGEN rats have plasma leptin and insulin levels comparable to control Sprague-Dawley (SD) rats at 15-weeks of age,[10] but show enhanced sensitivity to both hormones as detected with a glucose challenge[11]. The sensitivity to leptin for cardiovascular actions in these rats is currently unknown.

We assessed the effect of acute, site-specific NTS microinjection of leptin on baroreflex function and indices of autonomic balance, as well as resting AP and HR in SD in comparison with ASrAOGEN rats which might be expected to show enhanced

sensitivity to leptin. The present study provides direct evidence that administration of exogenous leptin impairs BRS for control of HR in response to increases in AP within the NTS of SD rats as well as alters autonomic balance. In addition, ASrAOGEN rats with down-regulation of the endogenous brain renin-Ang system (RAS) exhibit increased sensitivity to exogenous leptin microinjection, consistent with an upregulation of leptin receptors and signaling pathways in this brain region.

Methods

The institutional animal care and use committee approved all procedures. For a detailed Methods section please see http://hyper.ahajournals.org.

Animals: Experiments were performed in 3 to 5 month-old male Hannover SD and transgenic (ASrAogen)680 rats obtained from the Hypertension and Vascular Research colony at the Wake Forest University School of Medicine.

Surgical Procedures and Hemodynamic Measures: As previously reported,[12, 13] rats were anesthetized with combination urethane-chloralose (750 mg and 35 mg per kg, respectively) via intraperitoneal injections with supplemental intravenous (IV) doses given as needed. Animals were instrumented with femoral artery and vein catheters and placed in a stereotaxic frame with the head tilted downward (45°) for surgical exposure of the dorsal medulla oblongata. Pulsatile AP and mean AP (MAP) were monitored, recorded and digitized using a Data Acquisition System (BIOPAC System Inc.; Acknowledge software Version 3.8.1; Santa Barbara, CA) and HR was determined from the AP wave. After obtaining stable measures of MAP and HR, baseline responses to BRS were established by bolus IV randomized injection of 3 doses (2, 5 and 10 µg/kg in

0.9% NaCl) of phenylephrine (PE) or sodium nitroprusside (NP), to determine the BRS for increases or decreases in AP, respectively. Assessment of BRS by bolus injections is more sensitive for detection of alterations in the bradycardic BRS relative to infusion determinations.[14] The BRS for bradycardia and tachycardia was determined for each animal as the slope of the relationship between changes in MAP and the pulse interval generated from the 3 doses of PE and NP, independently.[12, 13] Reflex testing was completed within 30 min of leptin microinjection. Maximum transient changes in MAP and HR in response to NTS microinjection of leptin were measured and BRS testing was repeated at 10 min after the leptin microinjection, with each animal serving as its own control. Indices of sympathovagal function were also analyzed using Nevrokard software (Nevrokard SA-BRS; Medistar, Ljubljana, Slovenia).[15] Consistent with the duration of recordings used in previous human and rodent studies,[15-18] spontaneous BRS was determined from a minimum of 5 min of AP recordings obtained within 10 min of leptin injection, prior to the evoked baroreflex testing. Spontaneous BRS was calculated in the time [Sequence (Seq) Up, Seq Down and Seq All] and frequency domains [Low Frequency (LF) and High Frequency (HF) alpha indices]. Time domain analysis was used to assess changes in HR variability (HRV) measured as the standard deviation of the beat-to-beat interval. Blood pressure variability (BPV) was measured in the time domain as the standard deviation of the MAP.

NTS Microinjections: Rat recombinant leptin [Sigma; 144 or 500 fmol (0.002 and 0.008 µg, respectively) in a 60 nL volume of 15 mM HCl and 7.5 mM NaOH diluted to pH 7.4 in artificial cerebrospinal fluid (CSF)] or vehicle (60 nL) was microinjected bilaterally via pressure into the NTS [0.4 mm rostral, 0.4 mm lateral to the calamus scriptorius

(caudal tip of the area postrema) and 0.4 mm below the dorsal surface] using a glass micropipette connected to a syringe as previously reported.[12, 13] The doses and volume of leptin were comparable to previous NTS microinjection studies in which the peptides effectively altered BRS.[12, 19, 20] NTS microinjection of the vehicle solution had no significant effect on MAP, HR or BRS for control of HR in SD or ASrAOGEN rats (Table S1 and Figure S1, http://hyper.ahajournals.org), similar to previous studies in our laboratory.[12, 13] The vehicle had no effect on spontaneous BRS or BPV in SD and ASrAOGEN rats. However, HRV increased after injection of vehicle (1.81 ± 0.29 versus 2.32 ± 0.17 msec after vehicle; $p < 0.05$) in SD rats, with no effect of vehicle on HRV in ASrAOGEN rats. At the end of experiments, brains were removed, frozen and sectioned (30 µM) for localization of microinjection sites (Figure S2, http://hyper.ahajournals.org). Only data from injections within the medial NTS at rostro-caudal level -13.3 to -14.0 mm caudal to bregma were used in the analysis.

Quantification of leptin receptor and p85 alpha mRNA. Leptin receptor and phosphoinositide-3 kinase (PI3K) p85 alpha mRNA were measured in dorsal medullary tissue from separate groups of naive 15-week old SD (n = 9) and ASrAOGEN (n = 7) rats. Brains were removed and placed on dry ice for excision of 2 mm^3 dorsal medullary sections. The sections were obtained from 1 mm in front of to 1 mm behind the usual placement of the pipette, corresponding to the expected injectate spread[20] and including portions of area postrema, dorsal motor nucleus and nucleus gracilis. Isolation of RNA from excised tissue was assessed for concentration and stability. Total RNA (1 µg) was reverse transcribed using AMV reverse transcriptase in a 20 µL reaction mixture containing deoxyribonucleotides, random hexamers, and Rnase inhibitor in reverse

transcriptase buffer as previously described.[12, 13] For real-time PCR, 2 µL of resultant cDNA was added to TaqMan Universal PCR Master Mix with the appropriate gene-specific primer/probe set for leptin receptor and p85 alpha (Applied Biosystems) and amplification was performed. All reactions were performed in triplicate. 18S ribosomal RNA served as the internal control. Results were quantified as Ct values, where Ct is the threshold cycle of PCR at which amplified product is first detected, and was defined as relative gene expression (ratio of target/control).

Analysis of Data: Values are presented as mean ± standard error of the mean. A 2-way ANOVA was utilized to compare data between ASrAOGEN and SD strains. Comparisons of changes in BRS and indices of sympathovagal function in response to leptin or vehicle were assessed using a one-sample paired t-test. Changes in MAP and HR over time and time-course experiments were analyzed by repeated-measures ANOVA with post-hoc Student-Newman-Keuls multiple comparisons. mRNA quantification was analyzed by an unpaired t-test between strains. The criterion for statistical significance was $p < 0.05$. Tests were performed using Prism 4.0 and InStat 3 (GraphPad Software, San Diego, CA).

Results

Effect of NTS microinjection of leptin on BRS for control of HR in SD rats

In SD rats, NTS microinjection of 144 fmol leptin impaired BRS for control of HR measured as the bradycardic response to increases in AP produced by PE by 22%, an effect that did not reach significance (Figure 1A, D). In contrast, the 500 fmol leptin dose significantly impaired BRS by 63% in SD rats (Figure 1B, D) indicative of a dose-

dependent response for leptin actions on BRS within the NTS. There were no differences in baseline BRS values or PE-induced increases in AP (Figure S3) among SD rats receiving various doses of leptin. Time-course experiments showed that the BRS was impaired at 10 and 60 min with partial recovery at 120 min after NTS microinjection of the 500 fmol leptin dose in SD rats (Figure 1C). BRS for control of HR measured as the tachycardic response to decreases in AP produced by NP was not altered by NTS microinjection of 500 fmol leptin (Figure S4, http://hyper.ahajournals.org).

Effect of NTS microinjection of leptin on BRS for control of HR in ASrAOGEN rats

The bradycardic BRS was significantly higher in anesthetized ASrAOGEN relative to SD rats at baseline ($p < 0.01$), with no differences in the PE-induced increases in AP between strains (Figure S3). NTS microinjection of both 144 and 500 fmol leptin doses impaired the bradycardic BRS in ASrAOGEN rats corresponding to a 50% and 68% reduction, respectively (Figure 2A, B, D).   The 144 fmol leptin group shows data from younger (n = 3) and older (n = 5; 18 to 21 months) ASrAOGEN rats as there were no differences in BRS values at baseline or in response to leptin in younger (1.56 ± 0.15 msec/mm Hg baseline versus 0.81 ± 0.19 msec/mm Hg after leptin; $p < 0.05$) and older (1.40 ± 0.09 msec/mm Hg baseline versus 0.72 ± 0.12 msec/mm Hg after leptin injection; $p < 0.01$) rats. The 500 fmol leptin dose was only tested in younger ASrAOGEN rats. Similar to SD rats, there were no differences in baseline BRS or PE-induced increases in AP (Figure S3) among ASrAOGEN rats receiving varying leptin doses. In ASrAOGEN rats, the BRS remained suppressed at 10, 60 and 120 min after the leptin injection with no evidence of recovery over this time period (Figure 2C).   There was no significant difference in baseline values of the tachycardic BRS in response to decreases in AP

between anesthetized ASrAOGEN and SD rats. Similar to SD rats, NTS microinjection of 500 fmol leptin had no effect on the tachycardic BRS in ASrAOGEN rats (Figure S4).

Leptin influence on indices of sympathovagal function

There were no differences in baseline indices of spontaneous BRS, HRV or BPV within groups of SD or ASrAOGEN rats. Indices of spontaneous BRS were also similar at baseline between anesthetized ASrAOGEN and SD rats (Table 1). However, baseline values of HRV, a measure of cardiac vagal tone, and BPV were significantly higher in ASrAOGEN relative to SD rats (Table 2; $p < 0.001$ and $p < 0.05$, respectively). Similar to the evoked baroreflex measurements, 144 fmol leptin had no significant effect on spontaneous BRS indices, HRV or BPV in SD rats. In contrast, the 144 fmol leptin dose significantly reduced spontaneous BRS (Seq All; $p < 0.01$) as well as HRV in ASrAOGEN rats. Specifically, vagal indices of the BRS were reduced (Seq Up and HFα) with no effect on sympathetic indices of the spontaneous BRS (Seq Down and LFα) in these animals. The 500 fmol leptin reduced vagal spontaneous BRS indices (Seq Up and HFα) and HRV in both SD and ASrAOGEN rats. In addition, 500 fmol leptin reduced the LFα sympathetic index in ASrAOGEN rats only (Table 1). While 500 fmol leptin significantly increased BPV in SD, there was no effect at either dose in ASrAOGEN rats on this parameter (Table 2).

MAP and HR responses to NTS microinjection of leptin

There were no significant differences in MAP or HR within groups of SD or ASrAOGEN rats. However, as previously reported,[12] the pooled baseline MAP was significantly higher in anesthetized ASrAOGEN relative to SD rats (112 ± 5 mm Hg versus 85 ± 3 mm Hg, respectively; $p < 0.001$; n = 12 - 15 per group). The pooled

baseline HR was also significantly higher in ASrAOGEN rats (345 ± 14 bpm versus 286 ± 9 bpm; p < 0.01).   There was no significant effect of acute NTS microinjection of either 144 or 500 fmol leptin on resting MAP in SD or ASrAOGEN rats (Table S1, http://hyper.ahajournals.org).   While leptin injection had no effect on resting HR in SD rats, HR was modestly reduced following 144 fmol leptin with no effect of the 500 fmol leptin in ASrAOGEN animals.   Values of MAP and HR were not different from baseline at the time of reflex testing, at 10 minutes after the initial leptin microinjection (Table S1).

Differences in leptin receptor and PI3K mRNA in SD and ASrAOGEN rats

Relative gene expression of leptin receptor and PI3K regulatory subunit p85 alpha were measured in dorsal medullary tissue of naïve SD (n = 9) and ASrAOGEN (n = 7) rats at 15-weeks of age (Figure 3).   Leptin receptor mRNA was 3 to 4-fold higher and p85 alpha mRNA was 2-fold higher in dorsal medulla of ASrAOGEN relative to SD rats. There were no differences in control 18S ribosomal Ct values between SD (16.781 ± 0.136) and ASrAOGEN (16.587 ± 0.356) rats.

Discussion

In the present study, we determined the effects of exogenous leptin on blood pressure and baroreceptor reflex regulation at the level of the NTS.   Our results demonstrate that NTS microinjection of leptin impairs BRS for control of HR in response to increases in AP, an index of parasympathetic activity.   The leptin-mediated impairment in BRS was associated with a shift in indices of sympathovagal balance towards a decrease in parasympathetic function, with no significant acute effect on resting MAP or

106

HR. The novel finding that exogenous leptin impairs BRS for control of HR within the NTS may have implications for understanding the contribution of elevated leptin to baroreflex dysfunction. In addition, we examined whether leptin modulation of the BRS was altered in a model of enhanced leptin sensitivity to metabolic actions associated with basal differences in the central RAS by comparing leptin responses in control SD and transgenic ASrAOGEN rats. ASrAOGEN rats were more sensitive to BRS impairment in response to NTS microinjection of leptin. The enhanced sensitivity to exogenous leptin was associated with increased leptin receptor and PI3K p85 alpha mRNA in the dorsal medulla of ASrAOGEN rats, suggesting long-term reductions in brain Ang peptides or the subsequent consequences may be associated with upregulation of leptin signaling pathways.

Evidence suggests that key hypothalamic nuclei mediate increases in SNA and AP in response to exogenous leptin,[3] likely involving descending pathways to brainstem nuclei involved in cardiovascular regulation[4]. Independent of descending pathways, leptin receptors have been localized within the NTS and mediate both gastric[21] and cardiovascular responses[6]. NTS microinjection of a substantially higher leptin dose than used in the present study (1 µg) increases SNA and AP at 2 hours after the injection, consistent with baroreflex modulation.[6] However, these studies did not examine the effect of NTS microinjection of leptin on baroreceptor reflex regulation. Indeed, the localization of leptin receptors to vagal afferent fibers and within the NTS[5, 6] implicates leptin as a direct modulator of BRS for control of HR. While previous studies have shown that IV leptin does not acutely alter the sympathetically-mediated baroreflex control of renal SNA,[22] the contribution of circulating leptin to BRS for control of HR, a

vagally-mediated index that is often impaired in conditions with chronically elevated plasma leptin levels,[7] has yet to be examined.

The results of the present study provide evidence for a direct action of exogenous leptin to modulate baroreflex function as NTS microinjection of 500 fmol leptin impaired BRS for control of HR in both SD and ASrAOGEN rats. Leptin injection selectively altered BRS measured as the bradycardic response to increases in AP with no effect on BRS measured as the tachycardic response to decreases in AP in both SD and ASrAOGEN rats, similar to actions of Ang II within the NTS.[20] While there are no published studies evaluating the tachycardic BRS in anesthetized ASrAOGEN rats, the baseline tachycardic BRS values in SD rats are within the range of previously reported values using the same methods.[20] NTS microinjection of 500 fmol leptin did not alter depressor and bradycardic responses to cardiac vagal chemosensitive fiber activation (CVA) induced by IV phenylbiguanide in ASrAOGEN rats (unpublished observation). The lack of alteration in CVA responses supports specificity of leptin actions as these responses are mediated by chemoreceptor fibers that converge with baroreceptor inputs within the NTS.[23] Leptin modulation of the BRS was transient in SD rats, with partial recovery at 120 min after the initial leptin microinjection. In contrast, there was no evidence of recovery in ASrAOGEN rats at 120 min after the leptin injection. While the mechanism for the lack of recovery of BRS in ASrAOGEN rats is currently unknown, it may represent more sustained leptin actions within the NTS due to the upregulation of leptin receptor and signaling pathways.

We can not exclude the possibility that the spread of the leptin injection may have accessed the area postrema or dorsal motor nucleus of the vagus for effects on baroreflex

function. However, injection of a 100 nL of ^{125}I-Sar-Thr Ang II was mostly confined to the NTS.[20] In addition, functional assessments show that 50 nL of an AT_1 antagonist into the dorsal motor nucleus does not alter responses to NTS injection of Ang II.[24] Since the injection of leptin accessed neuronal cell bodies as well as presynaptic vagal afferents within the NTS, it is not clear which elements mediate the effects on BRS. However, Ang II is thought to exhibit its action on BRS primarily through vagal afferent fibers.[25]

In SD rats, the 144 fmol leptin dose had no significant effect on BRS, while the 500 fmol dose impaired the BRS suggesting a dose-response relationship. It appears maximal suppression of the BRS was achieved with the lower dose of leptin in ASrAOGEN rats as both the 144 fmol and 500 fmol leptin doses impaired BRS to a similar degree. These results implicate an enhanced sensitivity of ASrAOGEN rats to exogenous leptin within the NTS. Leptin impaired the BRS to approximately 0.5 msec/mm Hg in both strains, a level often observed in hypertension.[26] As a possible mechanism for the enhanced sensitivity of ASrAOGEN to exogenous leptin, we observed higher expression of leptin receptor and PI3K p85 alpha mRNA in dorsal medullary tissue of ASrAOGEN relative to SD rats at 15-weeks of age. While there are no differences in basal circulating leptin levels between strains at this age,[10] sensitivity of leptin to a glucose challenge is enhanced in ASrAOGEN rats[11].

The baseline values of MAP and HR were higher in anesthetized ASrAOGEN relative to SD rats, possibly due to an anesthesia-induced activation of the sympathetic nervous system observed in these animals[12]. NTS microinjection of leptin resulted in no significant changes in resting MAP in either SD or ASrAOGEN rats, consistent with previous NTS microinjection studies using higher doses (8 - 31 pmol) of leptin.[6, 27] Only

microinjection of 1 µg (63 pmol) leptin within the NTS results in increases in SNA and AP in SD rats.[6] However, these effects are delayed, requiring 2+ hours for manifestation. Importantly, prolonged suppression of the BRS may contribute to the delayed modest increase in AP observed with NTS injection of higher doses of leptin[6]. In the present study differences in BRS after leptin administration are not attributable to differences in resting hemodynamics, confirming that the set-point of the baroreflex is controlled independently from the sensitivity.[12, 13]

Spontaneous and spectral analysis methods for measurements of BRS,[15] revealed no difference in spontaneous BRS values between strains, in contrast to the higher BRS in ASrAOGEN rats using the pharmacologic approach. While the classical method evokes changes in AP in an open-loop system, the spontaneous method measures changes over a smaller range (beat-to-beat) in a closed-loop model. Although a highly significant correlation exists between the two methods,[15] differences have been reported in the BRS values obtained perhaps due to differences in sensitivity of the methods. Consistent with previous studies,[28] baseline HRV was significantly higher in ASrAOGEN relative to SD rats, suggestive of an increased resting vagal tone in these animals. Interestingly, baseline BPV was also higher in anesthetized ASrAOGEN relative to SD rats suggesting elevated sympathetic tone. While in the conscious state there are no reported differences in BPV in ASrAOGEN rats,[10, 29] the state of anesthesia may result in an activation of the sympathetic nervous system in these animals[12].

Similar to evoked BRS measurements, 144 fmol leptin had no effect on spontaneous BRS indices, HRV or BPV in SD rats. In ASrAOGEN rats, the 144 fmol leptin impaired Seq All, Seq Up, HFα and reduced HRV providing further evidence for

increased sensitivity to exogenous leptin in these animals. In both SD and ASrAOGEN rats, the 500 fmol leptin dose decreased Seq All as well as vagal indices of spontaneous BRS (HFα and Seq Up) and HRV further suggesting that leptin modulates BRS in response to increases but not decreases in AP. In ASrAOGEN rats, the 500 fmol leptin also reduced the LFα index with no effect in SD rats. Although this index is generally used as a marker of sympathetic activity, the spectral density of AP contained within this frequency is partially controlled by vagal tone.[30] The 500 fmol leptin dose increased BPV in SD rats further evidencing its role in altering cardiovascular autonomic balance. There was no effect of leptin on BPV in ASrAOGEN rats at either dose perhaps due to the already high basal level of this index under anesthesia. Collectively, leptin altered blood pressure regulation as assessed with either method and using a number of indices of autonomic function, similar to patterns observed in hypertension, obesity, and stroke,[31] where the circulating peptide is often elevated.

Ang II increases leptin levels and promotes leptin production *in vitro* suggesting a regulatory relationship between the peptides.[32] Ang converting enzyme inhibitors or Ang II AT$_1$ receptor blockers reduce plasma leptin levels in patients with mild/moderate hypertension.[33] Chronic AT$_1$ receptor blockade prevents age-related increases in circulating leptin levels that are associated with decreases in dorsal medullary leptin receptor mRNA in Fischer 344 rats.[34] Thus, chronic Ang II blockade maintains low endogenous leptin levels and increases leptin receptor mRNA. ASrAOGEN rats, with low endogenous brain Aogen, have decreased endogenous Ang II tone contributing to BRS suppression within the NTS[12] and therefore may also have decreased leptin levels within the NTS. While dorsal medullary leptin levels were not assessed in this study,

leptin receptors and signaling pathways in ASrAOGEN rats appear up-regulated on the basis of higher mRNA for leptin receptor and PI3K p85 alpha relative to SD rats. The enhanced BRS suppression with exogenously administered leptin in ASrAOGEN rats is functional evidence consistent with this interpretation. Enhanced sensitivity to leptin, could contribute to the overall enhanced metabolic phenotype observed in ASrAOGEN rats while maintaining low endogenous levels of leptin and thus a positive cardiovascular profile.[10, 12, 28] Whether the upregulation of leptin receptor and PI3K p85 alpha mRNA is attributed to a direct interaction with the RAS in the ASrAOGEN rats or an indirect effect is currently unknown; either low endogenous Ang II or leptin could contribute to the increased leptin receptor and PI3K mRNA expression in dorsal medulla of ASrAOGEN rats.

We examined changes in BRS, MAP and HR in response to acute, site-specific leptin administration. Effects of chronic peripheral or central leptin administration will need to be determined to further evaluate the role of leptin-mediated BRS impairments to pathophysiologies associated with elevated circulating, CSF or brain tissue leptin. Examination of the role of leptin in concert with other known modulators of cardiovascular and metabolic function such as Ang peptides, insulin and glucose, may yield differing results as recent studies show central leptin infusion may improve glucose utilization in diabetic rats to have indirect beneficial effects on BRS and sympathovagal balance.[35] Finally, examining the signaling pathways mediating the effects of leptin modulation of BRS within the NTS will help determine whether leptin utilizes different pathways for negative cardiovascular versus positive metabolic actions.

Perspectives

BRS for control of HR, a measure of vagal function, is often impaired in hypertension, obesity-related hypertension and stroke.[7, 31] Uncovering factors that modulate BRS may be important in understanding the predisposition to these conditions. Plasma leptin levels are elevated in obesity and independently in hypertension and stroke[36, 37] and exogenous leptin contributes to sympathetically-mediated elevations in AP[38]. The present data suggests that leptin impairs BRS for control of HR, an index believed to precede and contribute to the development of hypertension. Therefore, leptin-mediated impairments in BRS may be permissive towards increases in AP observed in populations with elevated leptin levels. Studies suggest that resistance develops to metabolic with maintenance of sensitivity to cardiovascular actions of leptin. Metabolic resistance to leptin is associated with reduced transport into brain and defects in intracellular signaling pathways.[39, 40] Reduction of leptin levels in patients with leptin resistance may prevent saturation of receptors to increase leptin transport as well as reduce negative regulators of leptin signaling to allow for maintenance of sensitivity to leptin's positive metabolic effects. Concomitantly, low endogenous leptin levels may reduce activation of cardiovascular signaling pathways to prevent impairments in baroreflex function, as well as increases in AP and SNA. Understanding mechanisms to preserve leptin sensitivity, in the presence of low endogenous leptin levels, may be important for maintaining satiety effects while preventing negative cardiovascular effects of the peptide.

Reference List

(1) Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S. Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat Med* 1995 November;1(11):1155-61.

(2) Campfield LA, Smith FJ, Guisez Y, Devos R, Burn P. Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. *Science* 1995 July 28;269(5223):546-9.

(3) Rahmouni K, Morgan DA. Hypothalamic arcuate nucleus mediates the sympathetic and arterial pressure responses to leptin. *Hypertension* 2007 March;49(3):647-52.

(4) Dampney RA. Functional organization of central pathways regulating the cardiovascular system. *Physiol Rev* 1994 April;74(2):323-64.

(5) Mercer JG, Moar KM, Hoggard N. Localization of leptin receptor (Ob-R) messenger ribonucleic acid in the rodent hindbrain. *Endocrinology* 1998 January;139(1):29-34.

(6) Mark AL, Agassandian K, Morgan DA, Liu X, Cassell MD, Rahmouni K. Leptin signaling in the nucleus tractus solitarii increases sympathetic nerve activity to the kidney. *Hypertension* 2009 February;53(2):375-80.

(7) Grassi G, Seravalle G, Dell'Oro R, Turri C, Bolla GB, Mancia G. Adrenergic and reflex abnormalities in obesity-related hypertension. *Hypertension* 2000 October;36(4):538-42.

(8) Schinke M, Baltatu O, Bohm M, Peters J, Rascher W, Bricca G, Lippoldt A, Ganten D, Bader M. Blood pressure reduction and diabetes insipidus in transgenic rats deficient in brain angiotensinogen. *Proc Natl Acad Sci U S A* 1999;96(7):3975-80.

(9) Huang BS, Ganten D, Leenen FH. Responses to central Na(+) and ouabain are attenuated in transgenic rats deficient in brain angiotensinogen. *Hypertension* 2001;37(2 Part 2):683-6.

(10) Kasper SO, Carter CS, Ferrario CM, Ganten D, Ferder LF, Sonntag WE, Gallagher PE, Diz DI. Growth, metabolism, and blood pressure disturbances during aging in transgenic rats with altered brain renin-angiotensin systems. *Physiol Genomics* 2005;23(3):311-7.

(11) Kasper SO, Ferrario CM, Ganten D, Diz DI. Rats with low brain angiotensinogen do not exhibit insulin resistance during early aging. *Endocrine* 2006 October;30(2):167-74.

(12) Sakima A, Averill DB, Kasper SO, Jackson L, Ganten D, Ferrario CM, Gallagher PE, Diz DI. Baroreceptor reflex regulation in anesthetized transgenic rats with low glia-derived angiotensinogen. *Am J Physiol Heart Circ Physiol* 2007;292(3):H1412-H1419.

(13) Arnold AC, Sakima A, Ganten D, Ferrario CM, Diz DI. Modulation of reflex function by endogenous angiotensins in older transgenic rats with low glial angiotensinogen. *Hypertension* 2008 May;51(5):1326-31.

(14) Korner PI. Cardiac baroreflex in hypertension: role of the heart and angiotensin II. *Clin Exp Hypertens* 1995 January;17(1-2):425-39.

(15) Shaltout HA, Abdel-Rahman AA. Mechanism of fatty acids induced suppression of cardiovascular reflexes in rats. *J Pharmacol Exp Ther* 2005 September;314(3):1328-37.

(16) Fisher JP, Ogoh S, Junor C, Khaja A, Northrup M, Fadel PJ. Spontaneous baroreflex measures are unable to detect age-related impairments in cardiac baroreflex function during dynamic exercise in humans. *Exp Physiol* 2009 April;94(4):447-58.

(17) Padley JR, Overstreet DH, Pilowsky PM, Goodchild AK. Impaired cardiac and sympathetic autonomic control in rats differing in acetylcholine receptor sensitivity. *Am J Physiol Heart Circ Physiol* 2005 November;289(5):H1985-H1992.

(18) Gerhardt U, Schafer M, Hohage H. Arterial blood pressure oscillation after active standing up in kidney transplant recipients. *J Auton Nerv Syst* 2000 April 12;80(1-2):93-100.

(19) Caligiorne SM, Santos RA, Campagnole-Santos MJ. Cardiovascular effects produced by bradykinin microinjection into the nucleus tractus solitarii of anesthetized rats. *Brain Res* 1996;720(1-2):183-90.

(20) Campagnole-Santos MJ, Diz DI, Ferrario CM. Baroreceptor reflex modulation by angiotensin II at the nucleus tractus solitarii. *Hypertension* 1988;11(2 Pt 2):I167-I171.

(21) Grill HJ, Schwartz MW, Kaplan JM, Foxhall JS, Breininger J, Baskin DG. Evidence that the caudal brainstem is a target for the inhibitory effect of leptin on food intake. *Endocrinology* 2002 January;143(1):239-46.

(22) Hausberg M, Morgan DA, Chapleau MA, Sivitz WI, Mark AL, Haynes WG. Differential modulation of leptin-induced sympathoexcitation by baroreflex activation. *J Hypertens* 2002 August;20(8):1633-41.

(23) Paton JF. Convergence properties of solitary tract neurones driven synaptically by cardiac vagal afferents in the mouse. *J Physiol* 1998;508 (Pt 1):237-52.

(24)   Fow JE, Averill DB, Barnes KL. Mechanisms of angiotensin-induced hypotension and bradycardia in the medial solitary tract nucleus. *Am J Physiol* 1994;267(1 Pt 2):H259-H266.

(25)   Diz DI, Westwood B, Averill DB. AT(1) antisense distinguishes receptors mediating angiotensin II actions in solitary tract nucleus. *Hypertension* 2001;37(5):1292-7.

(26)   Diz DI, Garcia-Espinosa MA, Gallagher PE, Ganten D, Ferrario CM, Averill DB. Angiotensin-(1-7) and baroreflex function in nucleus tractus solitarii of (mRen2)27 transgenic rats. *J Cardiovasc Pharmacol* 2008 June;51(6):542-8.

(27)   Matsumura K, Abe I, Tsuchihashi T, Fujishima M. Central effects of leptin on cardiovascular and neurohormonal responses in conscious rabbits. *Am J Physiol Regul Integr Comp Physiol* 2000 May;278(5):R1314-R1320.

(28)   Caligiorne SM, Silva AQ, Fontes MA, Silva JR, Baltatu O, Bader M, Santos RA, Campagnole-Santos MJ. Baroreflex control of heart rate and renal sympathetic nerve activity in rats with low brain angiotensinogen. *Neuropeptides* 2008 April;42(2):159-68.

(29)   Baltatu O, Janssen BJ, Bricca G, Plehm R, Monti J, Ganten D, Bader M. Alterations in blood pressure and heart rate variability in transgenic rats with low brain angiotensinogen. *Hypertension* 2001;37(2 Part 2):408-13.

(30)   Cerutti C, Barres C, Paultre C. Baroreflex modulation of blood pressure and heart rate variabilities in rats: assessment by spectral analysis. *Am J Physiol* 1994 May;266(5 Pt 2):H1993-H2000.

(31)   Thayer JF, Lane RD. The role of vagal function in the risk for cardiovascular disease and mortality. *Biol Psychol* 2007 February;74(2):224-42.

(32)   Kim S, Whelan J, Claycombe K, Reath DB, Moustaid-Moussa N. Angiotensin II increases leptin secretion by 3T3-L1 and human adipocytes via a prostaglandin-independent mechanism. *J Nutr* 2002 June;132(6):1135-40.

(33)   Koh KK, Quon MJ, Han SH, Lee Y, Kim SJ, Koh Y, Shin EK. Distinct vascular and metabolic effects of different classes of anti-hypertensive drugs. Int J Cardiol. In press 2008.

(34)   Gilliam-Davis S, Payne VS, Kasper SO, Tommasi EN, Robbins ME, Diz DI. Long-term AT1 receptor blockade improves metabolic function and provides renoprotection in Fischer-344 rats. *Am J Physiol Heart Circ Physiol* 2007 September;293(3):H1327-H1333.

(35)   do Carmo JM, Hall JE, da Silva AA. Chronic central leptin infusion restores cardiac sympathetic-vagal balance and baroreflex sensitivity in diabetic rats. *Am J Physiol Heart Circ Physiol* 2008 November;295(5):H1974-H1981.

(36) Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med* 1996 February 1;334(5):292-5.

(37) Sierra-Johnson J, Romero-Corral A, Lopez-Jimenez F, Gami AS, Sert Kuniyoshi FH, Wolk R, Somers VK. Relation of increased leptin concentrations to history of myocardial infarction and stroke in the United States population. *Am J Cardiol* 2007 July 15;100(2):234-9.

(38) Carlyle M, Jones OB, Kuo JJ, Hall JE. Chronic cardiovascular and renal actions of leptin: role of adrenergic activity. *Hypertension* 2002 February;39(2 Pt 2):496-501.

(39) Correia ML, Rahmouni K. Role of leptin in the cardiovascular and endocrine complications of metabolic syndrome. *Diabetes Obes Metab* 2006 November;8(6):603-10.

(40) Hall JE, Hildebrandt DA, Kuo J. Obesity hypertension: role of leptin and sympathetic nervous system. *Am J Hypertens* 2001 June;14(6 Pt 2):103S-15S.

(41) O'Leary DD, Kimmerly DS, Cechetto AD, Shoemaker JK. Differential effect of head-up tilt on cardiovagal and sympathetic baroreflex sensitivity in humans. *Exp Physiol* 2003 November;88(6):769-74.

(42) Iida R, Hirayanagi K, Iwasaki K, Ogawa S, Suzuki H, Yajima K. Non-invasive assessment of human baroreflex during different body positions. *J Auton Nerv Syst* 1999 February 15;75(2-3):164-70.

Table 1. Leptin Influence on Indices of Spontaneous BRS

Group	Seq Up	Seq Down	Seq All	LF α	HF α
			msec/mm Hg	msec/mm Hg	
SD 144 fmol					
Baseline	1.96 ± 0.07	2.19 ± 0.26	2.39 ± 0.29	1.53 ± 0.48	2.08 ± 0.46
After Leptin	2.05 ± 0.06	2.70 ± 0.09	2.56 ± 0.14	2.48 ± 0.23	2.25 ± 0.21
SD 500 fmol					
Baseline	1.39 ± 0.10	1.72 ± 0.33	1.52 ± 0.13	0.95 ± 0.26	1.52 ± 0.18
After Leptin	1.06 ± 0.10	0.97 ± 0.16	1.05 ± 0.12*	0.62 ± 0.13	0.89 ± 0.20*
AS 144 fmol					
Baseline	2.38 ± 0.59	1.71 ± 0.44	2.14 ± 0.45	1.26 ± 0.40	2.12 ± 0.51
After Leptin	1.28 ± 0.35†	1.45 ± 0.46	1.16 ± 0.29†	1.39 ± 0.39	1.18 ± 0.36†
AS 500 fmol					
Baseline	2.14 ± 0.46	1.58 ± 0.07	2.05 ± 0.33	1.83 ± 0.20	1.9 ± 0.32
After Leptin	0.71 ± 0.30†	0.79 ± 0.33	0.77 ± 0.32*	0.56 ± 0.21*	0.50 ± 0.24*

Values are mean ± SEM and represent indices of spontaneous BRS measured in the time (Seq Up, Seq Down, Seq All) and frequency (HFα and LFα) domains before and within10 min of NTS microinjection of leptin. BRS = baroreflex sensitivity; SD = Sprague-Dawley; AS = ASrAOGEN; Seq = Sequence; LF = low frequency; HF = high frequency; * $p < 0.05$ versus respective Baseline, † $p < 0.01$ versus respective Baseline

Table 2. Leptin Modulation of Heart Rate Variability and Blood Pressure Variability

Group	Heart Rate Variability SDRR (msec)	Blood Pressure Variability SDMAP (mm Hg)
SD 144 fmol		
Baseline	3.35 ± 0.65	2.35 ± 0.54
After Leptin	4.60 ± 1.06	2.34 ± 0.44
SD 500 fmol		
Baseline	2.46 ± 0.28	1.76 ± 0.15
After Leptin	$1.48 \pm 0.31\ddagger$	$3.61 \pm 0.61*$
AS 144 fmol		
Baseline	4.65 ± 0.87	2.56 ± 0.23
After Leptin	$2.66 \pm 0.48\dagger$	3.29 ± 0.53
AS 500 fmol		
Baseline	7.23 ± 0.78	2.85 ± 0.48
After Leptin	$1.41 \pm 0.27\dagger$	4.43 ± 0.83

Values are mean $\pm$ SEM and represent heart rate variability and blood pressure variability before and within 10 min of NTS microinjection of leptin. SDRR = standard deviation of beat-to-beat interval; SDMAP = standard deviation of mean arterial pressure; SD = Sprague-Dawley; AS = ASrAOGEN; * $p < 0.05$ versus respective Baseline, † $p < 0.01$ versus respective Baseline, ‡ $p < 0.001$ versus respective Baseline

Figure 1. Effect of NTS leptin microinjection on BRS for control of HR evoked by PE in SD rats. NTS microinjection of 144 fmol leptin impaired BRS by 22% in SD rats, an effect that did not reach significance (A; n = 4). In contrast, 500 fmol leptin injection impaired BRS by ~ 63% (B; n = 5). In SD rats, the BRS was impaired at 10 and 60 min with evidence of recovery at 120 min after NTS microinjection of 500 fmol leptin (C; n = 3). The slope of the relationship between the increases in MAP produced by phenylephrine and the corresponding reflex bradycardia [expressed as pulse interval] shows graded reductions in the linear regression slope with increasing doses of leptin [1.05 ± 0.14 msec/mm Hg baseline; 0.91 ± 0.27 msec/mm Hg after 144 fmol leptin; 0.38 ± 0.10 msec/mm Hg after 500 fmol leptin (r = 0.48 – 0.60)] in data from pooled SD rats. SD = Sprague-Dawley, MAP = mean arterial pressure, PI = pulse interval; * $p < 0.05$ versus baseline, † $p < 0.01$ versus baseline

121
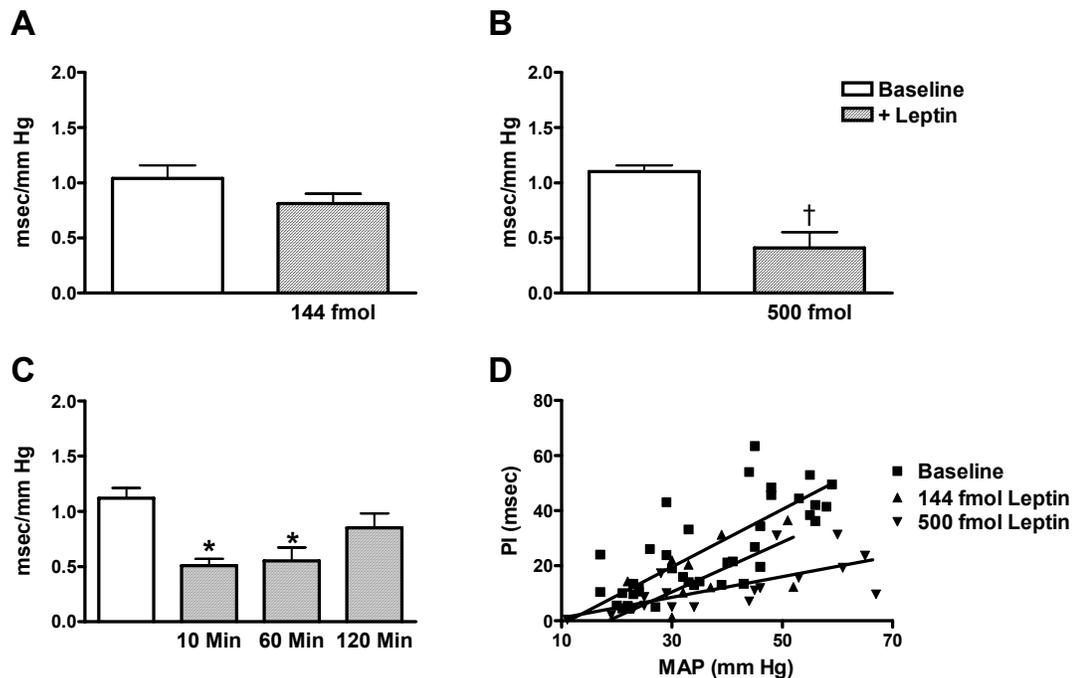
Figure 2. Effect of NTS leptin microinjection on BRS for control of HR evoked by PE in ASrAOGEN rats. In ASrAOGEN rats**,** NTS microinjection of 144 (A; n = 8) and 500 fmol (B; n = 4) leptin significantly impaired BRS by 50% and 68%, respectively. The BRS was impaired at 10, 60 and 120 min after NTS microinjection of 500 fmol leptin (C; n = 3). In ASrAOGEN rats (D), the 144 and 500 fmol leptin doses produced equivalent reductions in the slope of the regression line [1.11 ± 0.10 msec/mm Hg baseline; 0.45 ± 0.19 msec/mm Hg after 144 fmol leptin; 0.53 ± 0.08 msec/mm Hg after 500 fmol leptin (r = 0.61 – 0.89)]. AS = ASrAOGEN, MAP = mean arterial pressure; PI = pulse interval; * p < 0.05 versus baseline; ‡ = p < 0.001
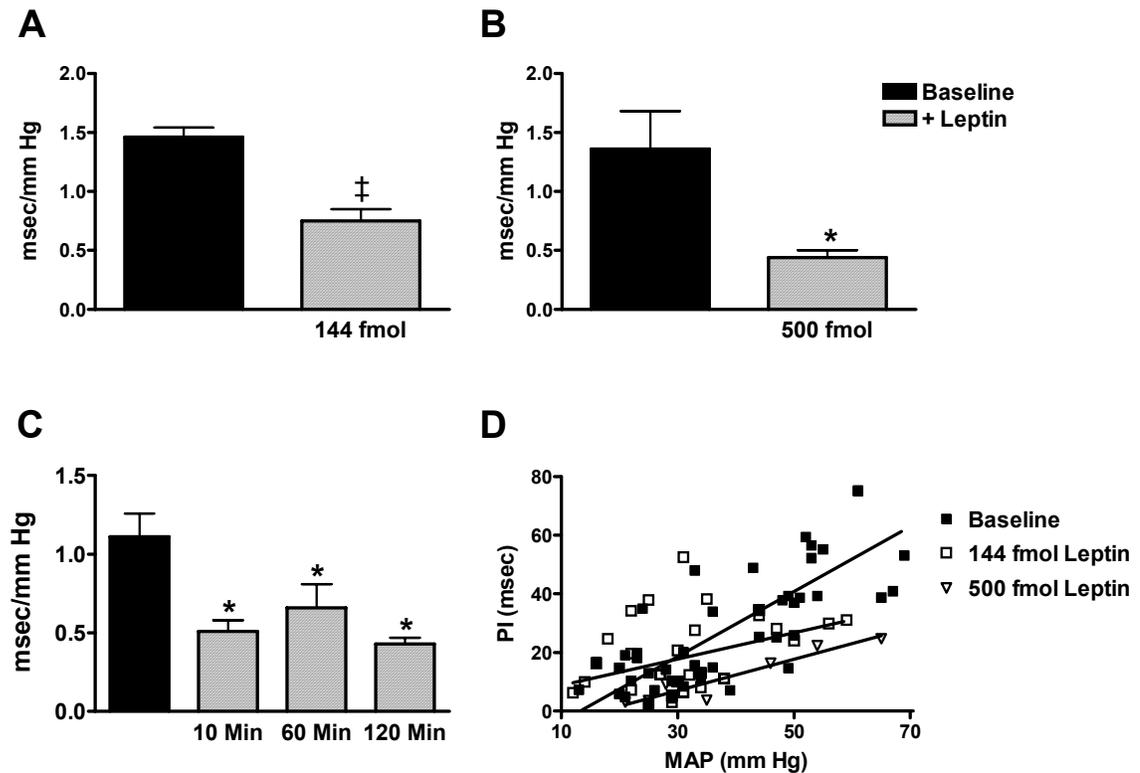
Figure 3. Leptin receptor and PI3K p85 alpha mRNA in dorsal medulla of SD and ASrAOGEN rats. Relative gene expression of leptin receptor and PI3K regulatory p85 alpha was higher in dorsal medullary tissue of 15-week old ASrAOGEN (n = 7) relative to SD (n = 9) rats. SD = Sprague-Dawley, AS = ASrAOGEN; * $p < 0.05$ versus SD; † $p < 0.01$ versus SD
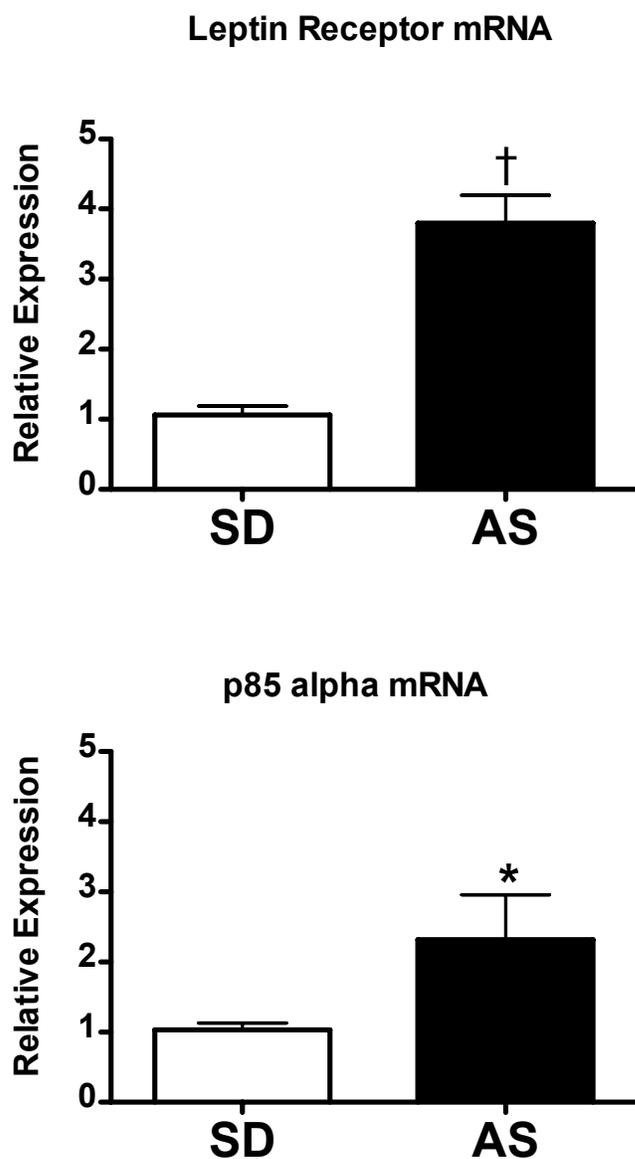
Leptin Receptor mRNA

p85 alpha mRNA

LEPTIN IMPAIRS CARDIOVAGAL BAROREFLEX FUNCTION AT THE LEVEL OF THE SOLITARY TRACT NUCLEUS

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Methods

The institutional animal care and use committee approved all procedures.

Animals: Experiments were performed in 3 to 5 month-old male Hannover Sprague-Dawley (SD) and transgenic TGR(ASrAogen)680 (ASrAOGEN) rats from the Hypertension and Vascular Research Center Colony, Wake Forest University School of Medicine, Winston-Salem, NC. The 144 fmol leptin group shows data from younger (n = 3) and older (n = 5; 18 to 21 months) ASrAOGEN rats as there were no differences in BRS values at baseline or in response to leptin in younger (1.56 ± 0.15 msec/mm Hg baseline versus 0.81 ± 0.19 msec/mm Hg after leptin; $p < 0.05$) and older (1.40 ± 0.09 msec/mm Hg baseline versus 0.72 ± 0.12 msec/mm Hg after leptin injection; $p < 0.01$) rats. The 500 fmol leptin dose was only tested in younger ASrAOGEN rats. Animals were housed in humidity- and temperature-controlled rooms in group cages (12-hour light/dark cycle) with free access to standard rat chow and water.

Surgical Procedures and Hemodynamic Measures: As previously reported,[12, 13] rats were anesthetized with combination urethane-chloralose (750 mg and 35 mg per kg, respectively) via intraperitoneal injections, with intravenous (IV) supplemental doses given as needed. Animals were instrumented with femoral arterial and venous catheters for measurement of cardiovascular parameters and administration of drugs, respectively. Rats were placed in a stereotaxic frame with the head tilted downward at a 45° angle for surgical exposure of the dorsal medulla oblongata by incision of the atlanto-occipital membrane and breathed a mixture of 70% room air and 30% oxygen with body temperature maintained at 37.0 ± 1.0°C. Approximately 30 minutes was allowed after surgical procedures before baseline measurements were recorded. Pulsatile arterial

pressure (AP) and mean AP (MAP) were monitored, recorded and digitized using a Data Acquisition System (BIOPAC System Inc.; Acknowledge software Version 3.8.1; Santa Barbara, CA) and heart rate (HR) was determined from the AP wave as previously reported.[12, 13] Baseline responses of baroreflex sensitivity (BRS) were established by bolus IV randomized injection of 3 doses (2, 5 and 10 μg/kg in 0.9% NaCl) of phenylephrine (PE) or sodium nitroprusside (NP), to determine the BRS in response to increases or decreases in AP, respectively. Bolus dose determinations were used as this method is more sensitive to parasympathetic alterations in the baroreflex relative to infusion determinations.[14] A period of $\geq$ 30 minutes was allowed after baseline measurements before beginning microinjections. Maximum MAP responses (ΔMAP, mm Hg) and the associated reflex changes in HR (ΔHR, bpm) were recorded at each dose of PE or NP. ΔHR was converted to changes in pulse interval (ΔPI, msec) by the formula: 60,000/HR. BRS for bradycardia and tachycardia was determined for each animal as the slope of the relationship between changes in MAP and the corresponding PI generated from the 3 doses of PE and NP, independently as previously reported.[12, 13] Maximum changes in MAP and HR in response to NTS microinjection of leptin were measured and BRS testing was repeated within 10 minutes of leptin microinjection so that each animal was used as its own control. Reflex testing was completed within 30 minutes of leptin microinjection. Indices of sympathovagal function at baseline and in response to leptin were also analyzed using Nevrokard software (Nevrokard SA-BRS; Medistar, Ljubljana, Slovenia).[15] Consistent with the duration of recordings used in previous human and rodent studies,[15-18] we assessed a minimum of 5 min of AP recordings obtained from immediately after leptin injection until the 10 min time point

when evoked baroreflex testing occurred. Spontaneous BRS was calculated using time and frequency domain analysis methods. For the sequence (Seq) method, we quantified sequences of at least 3 beats in which systolic AP (SAP) consecutively increases (Seq Up) or decreases (Seq Down) and beat-to-beat interval (RRI) changes in the same direction on subsequent beats (n + 1). In the present study, there was an average of 33 ± 4 up and 23 ± 2 down sequences utilized in the analysis of spontaneous BRS, well in excess of studies in humans in which the average spontaneous BRS values are based on 4 - 35 up and 4 - 11 down sequences.[18, 41, 42] A linear correlation was calculated between RRI and SAP for each sequence. The mean of all individual regression coefficients was calculated as Seq All, which was used an index of spontaneous BRS. To measure BRS in the frequency domain, power spectral densities of SAP and RRI oscillations were computed, transformed and integrated over specified frequency ranges (LF = 0.25-0.75 Hz; HF = 0.75-3.0 Hz). The square root of the ratio of RRI and SAP powers were used to calculate LFα and HFα, indices of sympathetic and parasympathetic activity of the BRS, respectively. Time domain analysis was used to assess changes in heart rate variability (HRV) as measured by the standard deviation of the beat-to-beat interval in RRI duration. Blood pressure variability was measured by time domain analysis as the standard deviation of the MAP.

NTS Microinjections: Multi-barreled glass pipettes with an outer diameter of 30 to 50 μm were used as described previously.[12, 13] Rat recombinant leptin [Sigma; 144 or 500 fmol (0.002 and 0.008 μg, respectively) in a 60 nL volume of 15 mM HCl and 7.5 mM NaOH dissolved to pH 7.4 in artificial cerebrospinal fluid (CSF)] or vehicle (60 nL) was microinjected bilaterally via pressure into the NTS [0.4 mm rostral, 0.4 mm lateral to the

calamus scriptorius (caudal tip of the area postrema) and 0.4 mm below the dorsal surface] using a glass micropipette connected via PE 50 tubing to a syringe (1 mL; Becton, Dickinson and Company). Air pressure was generated by pushing on the syringe to displace the desired amount of leptin from the pipette into the NTS. This was visualized by movement of the fluid meniscus across the calibration line of the pipette barrel as previously reported.[12, 13] The doses and volume of leptin were comparable to previous NTS microinjection studies in which the peptides effectively altered BRS.[12, 19, 20] Histology: The brain was removed and frozen on dry ice at the end of each experiment for histological evaluation. Serial cryostat sections (30 μm) of the frozen medulla were used to assess the site of microinjections (Figure S2). Only data from injections within the medial NTS at rostro-caudal level -13.3 to -14.0 mm caudal to bregma were used in the analysis. The accuracy rate for injections was > 90%.

Quantification of leptin receptor and p85 alpha mRNA: Leptin receptor and phosphoinositide-3 kinase (PI3K) p85 alpha mRNA were measured in dorsal medullary tissue from separate groups of conscious 15-week old SD (n = 9) and ASrAOGEN (n = 7) rats. Brains were removed and placed on dry ice for excision of 2 mm^3 dorsal medullary sections. The sections were obtained from 1 mm in front of to 1 mm behind the usual placement of the pipette, corresponding to the expected injectate spread[20] and including portions of area postrema, dorsal motor nucleus and nucleus gracilis. Total RNA was isolated from dorsal medullary sections of using TRIZOL reagent (GIBCO Invitrogen, Carlsbad, CA). The RNA concentration and stability was assessed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano LabChip (Agilent Technologies, Palo Alto, CA). Approximately 1 μg of total RNA was reverse transcribed using AMV

reverse transcriptase in a 20 μL reaction mixture containing deoxyribonucleotides, random hexamers, and Rnase inhibitor in reverse transcriptase buffer. Heating the reverse transcriptase reaction product at 95°C terminated the reaction. For real-time PCR, 2 μL of the resultant cDNA was added to the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) with the appropriate gene-specific primer/probe set (Applied Biosystems) and amplification was performed on an ABI 7000 Sequence Detection System. The mixtures were heated at 50°C for 2 minutes, at 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. All reactions were performed in triplicate. 18S ribosomal RNA, amplified using TaqMan Ribosomal RNA Control Kit (Applied Biosystems) served as the internal control. The results were quantified as Ct values, where Ct is defined as the threshold cycle of PCR at which amplified product is first detected, and was defined as relative gene expression (ratio of target/control).

Analysis of Data: Values are presented as mean ± standard error of the mean. A 2-way ANOVA was utilized to compare data between ASrAOGEN and SD strains. Comparisons of changes in BRS and indices of sympathovagal function in response to leptin or vehicle were compared to baseline using a one-sample paired t-test. Changes in resting MAP and HR over time and time-course experiments were analyzed by repeated-measures ANOVA with post-hoc Student-Newman-Keuls multiple comparisons. mRNA quantification was analyzed by an unpaired t-test between strains. The criterion for statistical significance was $P < 0.05$. Tests were performed using Prism 4.0 and InStat 3 (GraphPad Software, San Diego, CA).

Table S1. MAP and HR in Response to NTS Microinjection of Leptin or Vehicle

Group	N	MAP (mm Hg)	HR (bpm)
SD 144 fmol Leptin	4		
Baseline		81 ± 5	259 ± 11
Values at Peak Change		73 ± 10	248 ± 21
Value Prior to Reflex Testing		76 ± 7	244 ± 15
SD 500 fmol Leptin	5		
Baseline		91 ± 4	299 ± 14
Values at Peak Change		86 ± 5	287 ± 18
Value Prior to Reflex Testing		80 ± 9	282 ± 21
AS 144 fmol Leptin	8		
Baseline		113 ± 8	324 ± 21
Values at Peak Change		103 ± 8	303 ± 28 *
Value Prior to Reflex Testing		113 ± 8	309 ± 25
AS 500 fmol Leptin	4		
Baseline		110 ± 10	373 ± 27
Values at Peak Change		103 ± 5	367 ± 34
Value Prior to Reflex Testing		110 ± 10	368 ± 31
Vehicle in SD Rats	4		
Baseline		78 ± 1	302 ± 19
Values at Peak Change		88 ± 10	295 ± 20
Value Prior to Reflex Testing		83 ± 13	293 ± 22

Vehicle in AS Rats	3		
Baseline		110 ± 4	366 ± 13
Values at Peak Change		103 ± 6	354 ± 14
Value Prior to Reflex Testing		100 ± 7	351 ± 16

Values are mean ± SEM. Values represent baseline, peak changes in response to NTS microinjection of leptin and values at 10 minutes after the initial leptin microinjection (immediately prior to each series of reflex testing); N = number of animals; MAP = mean arterial pressure; HR = heart rate; SD = Sprague-Dawley; AS = ASrAOGEN

* = P < 0.01 versus Baseline

Figure S1. Effect of vehicle on BRS for control of HR. NTS microinjection of the vehicle solution (60 nL) had no effect on the bradycardic BRS for control of HR in response to increases in AP evoked by PE or the tachycardic BRS in response to decreases in AP evoked by NP in SD (A, n = 4) or ASrAOGEN (B, n = 3) rats.
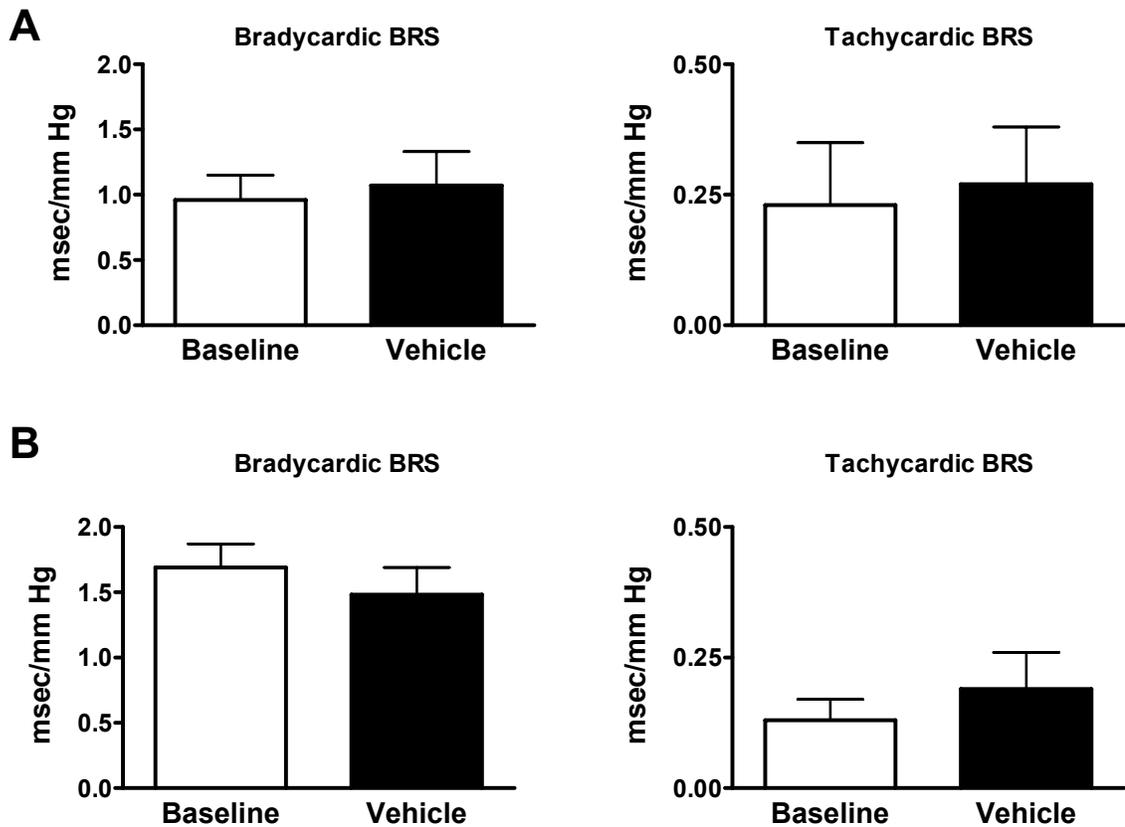
Figure S2. Histological Analysis of Microinjection Sites. Photomicrography (5X magnification) of an unstained rat medullary section (30 µM) at approximately -13.8 mm caudal to bregma showing a typical microinjection site within the NTS [0.4 mm rostral, 0.4 mm lateral to the calamus scriptorius (caudal tip of the area postrema) and 0.4 mm below the dorsal surface]. Only data from injections within the medial NTS at rostro-caudal level -13.3 to -14.0 mm caudal to bregma were used in this study. NG = nucleus gracilis, AP = area postrema, NTS = solitary tract nucleus, DMX = dorsal motor nucleus of the vagus, C = central canal.
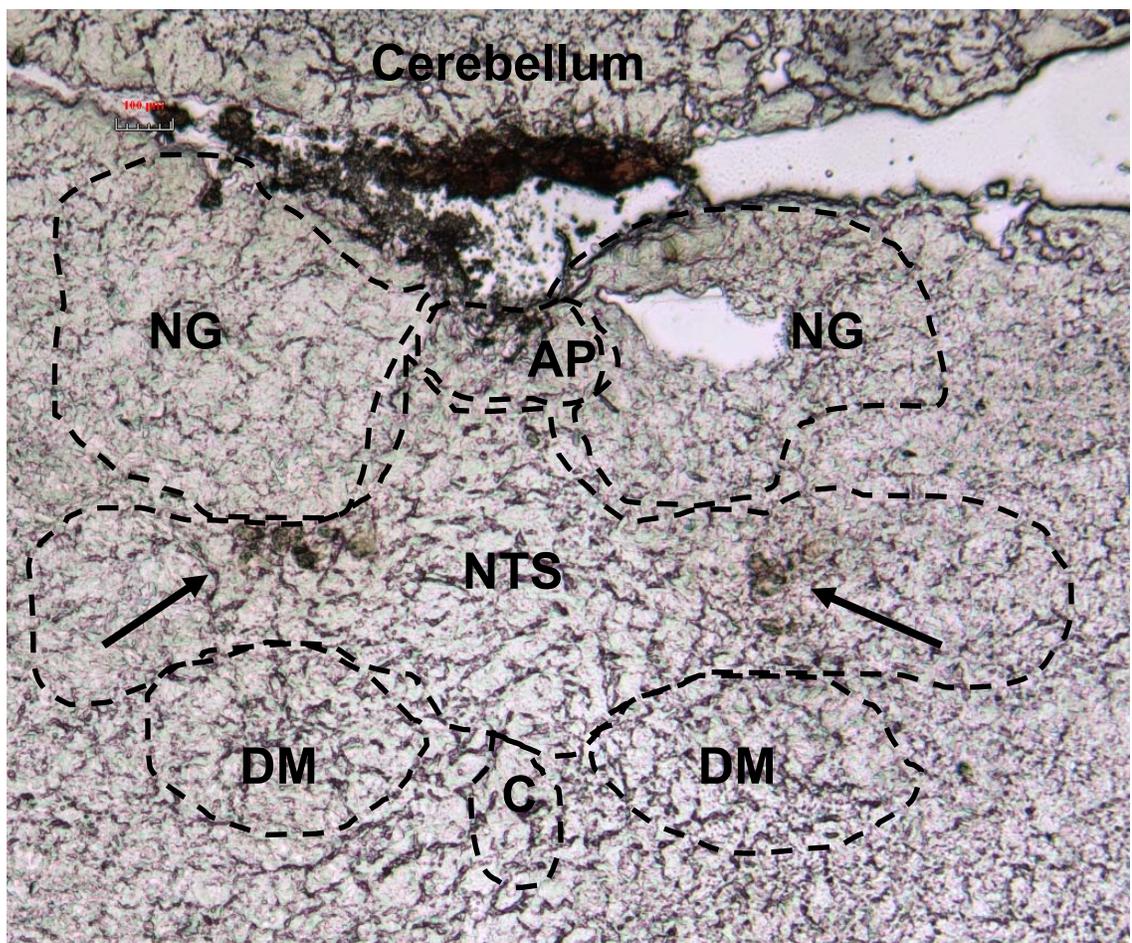
Figure S3. α-adrenergic responsiveness in SD and ASrAOGEN rats at baseline and in response to NTS microinjection of leptin. Changes in mean arterial pressure (MAP) in response to randomized, intravenous graded doses of phenylephrine were assessed in anesthetized SD and ASrAOGEN rats at baseline and in response to NTS microinjection of 144 or 500 fmol leptin. There were no significant differences in phenylephrine-induced increases in MAP within groups of SD or ASrAOGEN rats.

Change in MAP
SD Rats

■ Baseline
▲ 144 fmol Leptin
▼ 500 fmol Leptin

mm Hg

Phenylephrine (μg/kg)

Change in MAP
ASrAOGEN Rats

■ Baseline
□ 144 fmol Leptin
▽ 500 fmol Leptin

mm Hg

Phenylephrine (μg/kg)
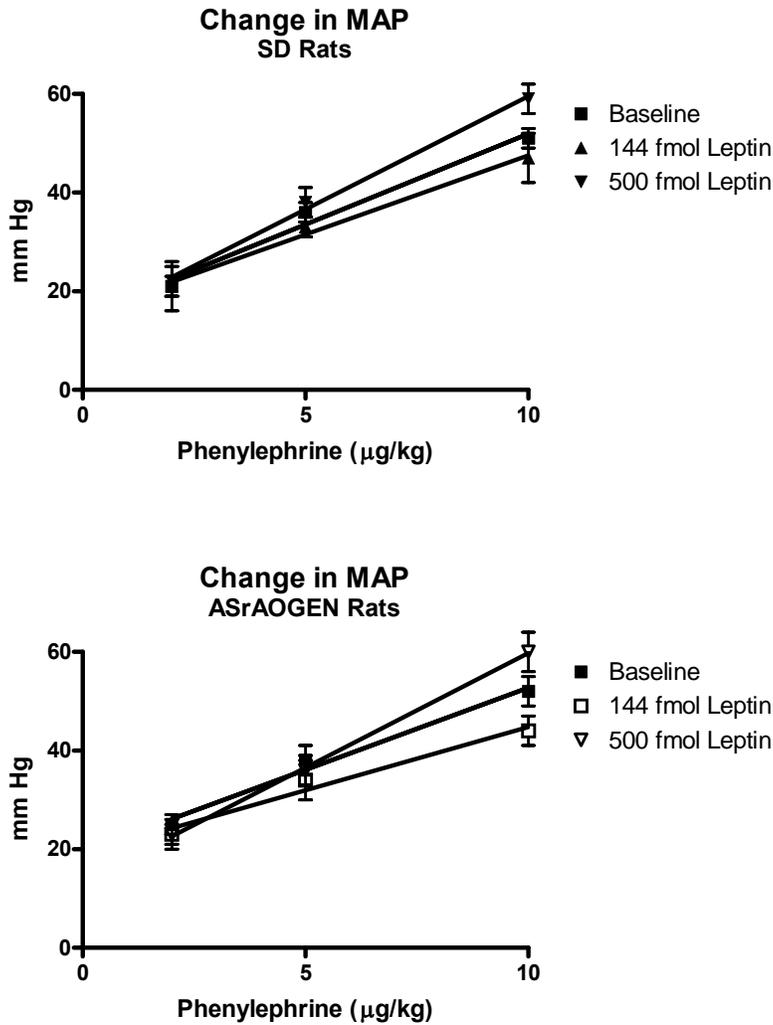
Figure S4. BRS for control of HR in response to decreases in AP. BRS for control of HR was measured as the tachycardic response to decreases in AP evoked by NP before and after NTS microinjection of 500 fmol leptin in SD (A) and ASrAOGEN (B) animals. There were no significant differences in baseline responses to NP between SD and ASrAOGEN animals (n = 8 each group). In a subset of these animals (n = 4), leptin had no significant effect on responses to NP in either strain.
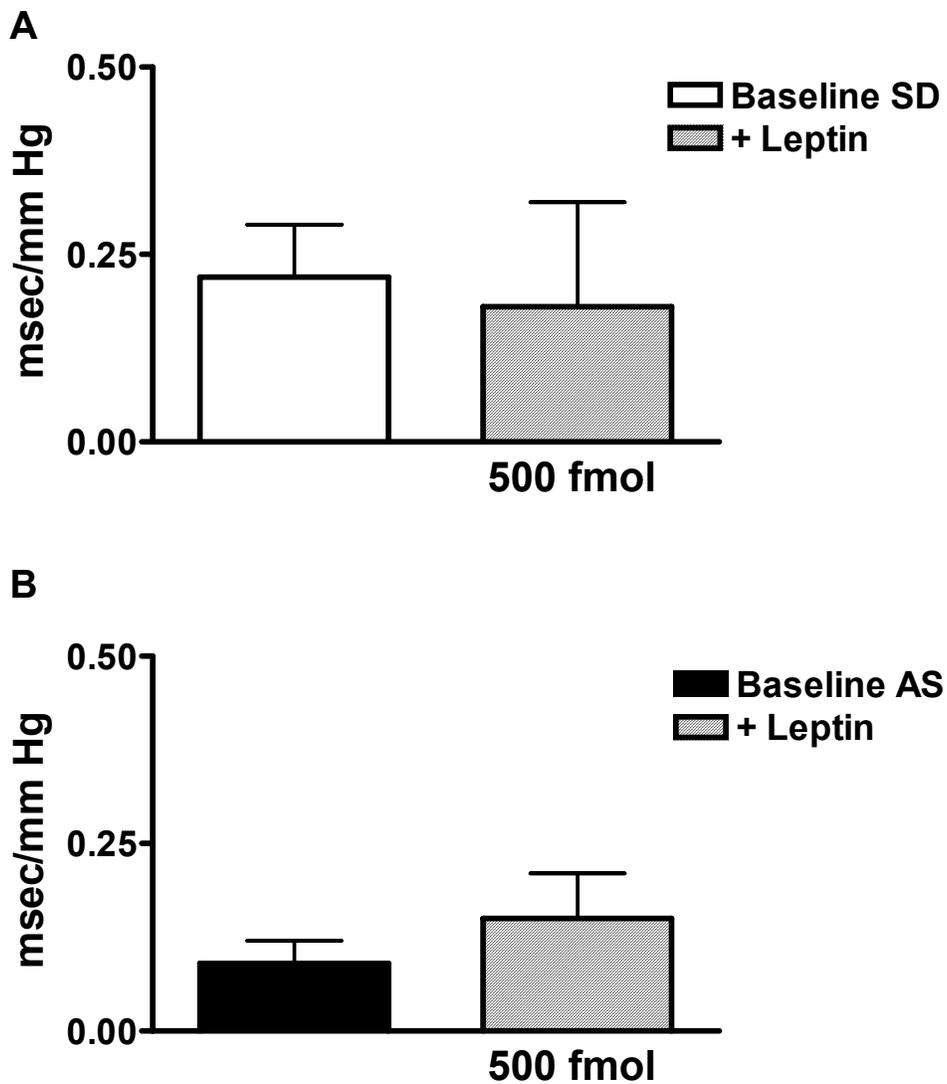
CHAPTER FOUR

PROTEIN PHOSPHATASE 1B TONE IN THE SOLITARY TRACT

NUCLEUS IS NECESSARY FOR NORMAL BAROREFLEX FUNCTION

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1032

Abstract

Protein phosphatase 1b is a molecular brake limiting activation of intracellular tyrosine kinase signaling pathways utilized by insulin, leptin and angiotensin II, which all impair baroreflex sensitivity for control of heart rate. We hypothesized that loss of protein phosphatase 1b regulation within the solitary tract nucleus would be associated with impaired baroreflex function. A sulfanamido-benzbromarone derived allosteric inhibitor of protein phosphatase 1b (100 nM/120 nL) was bilaterally microinjected within the solitary tract nucleus of urethane/chloralose anesthetized rats. Baroreflex sensitivity for control of heart rate, arterial pressure and heart rate were assessed before and at 10 and 60 min after protein phosphatase 1b inhibitor microinjection in control Sprague-Dawley and transgenic (mRen2)27 and ASrAOGEN rats with low or high resting baroreflex sensitivity, respectively. Protein phosphatase 1b inhibition impaired baroreflex sensitivity for bradycardia in Sprague-Dawley rats by 41% (0.88 ± 0.07 before vs. 0.51 ± 0.06 msec/mm Hg after; $p < 0.01$). Inhibition of protein phosphatase 1B caused greater impairment of baroreflex sensitivity for bradycardia in ASrAOGEN ($60 \pm 2\%$; $p < 0.05$) with no significant effect in (mRen2)27 rats ($15 \pm 11\%$). There were minimal effects of protein phosphatase 1b inhibition on baroreflex sensitivity for tachycardia, resting arterial pressure or heart rate across strains. Thus, protein phosphatase 1b activity within the solitary tract nucleus contributes to maintenance of resting baroreflex function in animals with normal or elevated baroreflex sensitivity. Furthermore, loss of protein phosphatase 1b tone in the solitary tract nucleus accompanies baroreflex dysfunction in a model of angiotensin II-dependent hypertension.

137

Introduction

Reversible protein phosphorylation by tyrosine kinases and dephosphorylation by tyrosine phosphatases is a major regulatory mechanism underlying intracellular signaling events. Protein phosphatase 1b (PTP1b), a ubiquitously expressed member of the protein tyrosine phosphatase family, limits the activation of intracellular kinase signaling pathways utilized by insulin, leptin and angiotensin II through direct dephosphorylation of tyrosine residues on receptors and/or signaling components used by these peptides.[135, 163, 252] Genetic deletion of PTP1b is associated with lower body weight, protection against diet-induced obesity and increases in insulin and leptin sensitivity to glucose challenge in mice.[135, 219] The beneficial effect of PTP1b deletion on energy balance implicates phosphatase inhibitors as attractive targets for the treatment of obesity and type II diabetes.

Recent studies suggest that genetic deletion of PTP1b also results in elevations in arterial pressure and enhanced sympathetic tone.[164] Angiotensin II, leptin and insulin can all independently contribute to higher sympathetic tone[8] and induce impairments in vagally-mediated baroreflex sensitivity (BRS) for control of heart rate (HR), regulated at the level of the solitary tract nucleus (NTS) in the dorsomedial medulla.[202, 253, 254] While increased PTP1b tone is associated with reduced metabolic sensitivity to insulin and leptin, endogenous PTP1b may be necessary to prevent the negative cardiovascular effects associated with elevated levels these peptides, including reductions in baroreflex function. Conversely, inhibition of PTP1b may allow for unrestrained kinase signaling to occur, resulting in baroreflex dysfunction. Therefore, we tested the hypothesis that acute inhibition of PTP1b within the NTS will impair cardiovascular reflex function in

normotensive Sprague-Dawley (SD) rats. As a secondary aim, we employed transgenic (mRen2)27 or ASrAOGEN rats with low or high BRS, respectively, to determine the contribution of PTP1b within the NTS to the prevailing levels of baroreflex function in animals with alterations in the brain renin-angiotensin and leptin systems. Collectively, these studies provide evidence that PTP1b tone within the NTS may be an important molecular mechanism participating in the maintenance of baroreflex function.

Methods

<u>Animals</u>: Experiments were performed in 3 to 5 month-old male Hannover SD and age-matched transgenic (ASrAogen)680 (ASrAOGEN) and (mRen2)27 rats obtained from the Hypertension and Vascular Research Center Transgenic Animal Facility, Wake Forest University School of Medicine, Winston-Salem, NC. Animals were housed in humidity- and temperature-controlled rooms in group cages (12-hour light/dark cycle) with free access to standard rat chow and water. All procedures were approved by the institutional animal care and use committee.

<u>Surgical Procedures and Hemodynamic Measures</u>: The following procedures have been cited in detail in recent publications.[253, 255] Rats were anesthetized with combination urethane-chloralose (750 mg and 35 mg per kg, respectively) administered by intraperitoneal injections with supplemental IV doses given as needed. Catheters were inserted into the femoral artery and vein for measurement of cardiovascular parameters and delivery of drugs, respectively. Rats were placed in a stereotaxic frame with the head tilted downward (45°) for surgical exposure of the dorsal medulla oblongata. Approximately 30 minutes were allowed after surgical procedures before baseline

measurements were recorded. Pulsatile AP and mean AP (MAP) were monitored, recorded and digitized using a Data Acquisition System (BIOPAC System Inc.; Acknowledge software Version 3.8.1; Santa Barbara, CA) and HR was determined from the AP wave. BRS responses were established by bolus IV injection of 3 randomized doses (2, 5 and 10 μg/kg in 0.9% NaCl) of phenylephrine (PE) or sodium nitroprusside (NP), to determine the BRS for bradycardia to increases or tachycardia to decreases in AP, respectively. Since angiotensin II, insulin and leptin selectively alter the BRS for bradycardic responses, we assessed BRS by bolus doses of PE and NP, which are more sensitive to parasympathetic alterations in the baroreflex relative to infusion methods.[239] Maximum MAP responses (ΔMAP, mm Hg) and the associated reflex changes in HR (ΔHR, bpm) were recorded at each dose of PE or NP and ΔHR was converted to changes in pulse interval (ΔPI, msec) by the formula: 60,000/HR. A period of 5 min was allowed for recovery of MAP and HR between each dose; thus the baseline reflex testing was completed within 30 min. BRS for bradycardia and tachycardia was determined for each animal as the slope of the relationship between changes in MAP and the corresponding PI generated from the 3 doses of PE and NP, independently. A period of ≥ 30 minutes was allowed after baseline measurements before beginning microinjections. Maximum changes in MAP and HR in response to NTS microinjection of the PTP1b inhibitor were measured and BRS testing was repeated at 10 min and again at 60 min after the microinjection so that each animal was used as its own control.

NTS Microinjections: Multi-barreled glass pipettes with an outer diameter of 30 to 50 μm were used as described.[253, 255] A selective, cell permeable allosteric inhibitor of PTP1b 3-(3,5-Dibromo-4-hydroxy-benzoyl)-2-ethyl-benzofuran-6-sulfonicacid-(4-(thiazol-2-

ylsulfamyl)-phenyl)-amide (Calbiochem; 100 nM in a 120 nL volume of artificial CSF) or vehicle (120 nL) was microinjected bilaterally via pressure into the NTS [0.4 mm rostral, 0.4 mm lateral to the calamus scriptorius (caudal tip of the area postrema) and 0.4 mm below the dorsal surface] using a glass micropipette connected to a syringe. Air pressure was generated by pushing on the syringe to displace the desired amount of drug from the pipette into the NTS. This was visualized by movement of the fluid meniscus across the calibration line of the pipette barrel as previously reported. To date, PTP1b inhibitors have not been used in site-specific brain microinjection studies. Therefore, we utilized a dose similar to that for tyrosine kinase inhibitors which were shown to modify BRS after NTS microinjection.[147]

Histology: At the end of experiments, brains were removed, frozen on dry ice and sectioned (30 μM) for the localization of microinjection sites. Only data from injections within the medial NTS at rostro-caudal level -13.3 to -14.0 mm caudal to bregma were used in the analysis.

mRNA Quantification: In separate brains from SD, ASrAOGEN and (mRen2)27 rats (n = 6 to 8 animals per strain) we measured gene expression for protein tyrosine phosphatase non-receptor 1 (PTPN1) which encodes PTP1b. The mRNA levels for PTPN1 were assessed in dorsal medullary tissue by RT-PCR using probes available from Applied Biosystems as previously reported.[253] Data from these animals was normalized to 18S ribosomal RNA and results were quantified as Ct values, where Ct is the threshold cycle of PCR at which amplified product is first detected, and was defined as relative gene expression (ratio of target/control).

Analysis of Data: Values are presented as mean ± SEM. A 2-way ANOVA was utilized

to compare data between SD, ASrAOGEN and (mRen2)27 strains. Within strains, comparisons of changes in BRS, MAP and HR over time in response to PTP1b inhibitor or vehicle were analyzed by repeated-measures ANOVA with post-hoc Student-Newman-Keuls multiple comparisons. The criterion for statistical significance was $p < 0.05$. Tests were performed using Prism 4.0 and InStat 3 (GraphPad Software, San Diego, CA).

Results

Effect of PTP1b Inhibition on Baroreflex Function in SD Rats

To determine the contribution of PTP1b within the NTS to normal baroreflex function, we assessed BRS for control of HR before and at 10 and 60 min after NTS microinjection of the PTP1b inhibitor. In SD rats, the slope of the relationship between the increases in MAP produced by PE and the corresponding reflex bradycardia shows a significant 41% reduction in pooled data from testing initiated at 10 min after the initial NTS microinjection (n = 5; Figure 1A and B; $p < 0.01$). The BRS was still impaired by 39% when retested at 60 min after the injection (Figure 1B, $p < 0.01$). There was no significant effect of PTP1b inhibition on the BRS for tachycardia (0.26 ± 0.17 before vs. 0.23 ± 0.12 msec/mm Hg after) in SD rats.

Effect of PTP1b Inhibition on Baroreflex Function in ASrAOGEN and (mRen2)27 Rats

We employed transgenic ASrAOGEN and (mRen2)27 rats to determine if differential PTP1b tone within the NTS is associated with the varying levels of BRS observed in these animals (Figure 2). In ASrAOGEN rats (Figure 2, n = 4), PTP1b inhibition caused a greater impairment of the BRS for bradycardia at 10 min after the injection ($60 \pm 2\%$, p

< 0.05) from an elevated baseline BRS (1.21 ± 0.29 msec/mm Hg, p < 0.05 vs mRen2).

The BRS was still significantly suppressed at 60 min after the injection in ASrAOGEN

rats (0.70 ± 0.23 msec/mm Hg). There was no significant effect of PTP1b inhibition on

the BRS at either time point in hypertensive (mRen2)27 rats (15 ± 11%) with low resting

BRS (n = 6; 0.35 ± 0.05 msec/mm Hg; p < 0.05 vs SD and ASrAOGEN). There were no

differences in baseline values of the BRS for tachycardia between SD, ASrAOGEN and

(mRen2)27 rats. Similar to SD rats, PTP1b inhibition produced no significant effect on

the BRS for tachycardia in ASrAOGEN (0.18 ± 0.07 before vs 0.28 ± 0.08 msec/mm Hg

after inhibition) or (mRen2)27 (0.22 ± 0.11 before vs 0.17 ± 0.04 msec/mm Hg after

inhibition) rats. There was no effect of the vehicle solution on BRS in SD, ASrAOGEN

or (mRen2)27 rats, therefore the control data was pooled (Figure 2). In a subset of SD,

ASrAOGEN and (mRen2)27 rats (n = 4) we tested the effects of PTP1b inhibition on

responses to cardiac vagal chemosensitive fiber activation (CVA) produced by IV

phenylbiguanide. There was no effect of PTP1b inhibition on the depressor (-76 ± 14

mm Hg baseline vs -68 ± 9 mm Hg after PTP1b inhibition) or bradycardic (-197 ± 34

bpm baseline vs -195 ± 25 bpm after PTP1b inhibition) responses to CVA in any of these

animals and thus the data was pooled.

Effect of PTP1b Inhibition on Resting MAP and HR

NTS microinjection of the PTP1b inhibitor resulted in a peak transient decrease in MAP

and HR in SD rats (Table 1). The MAP recovered to baseline levels at 10 min, with

recovery of HR to baseline at 60 min after the microinjection. Anesthetized ASrAOGEN

rats exhibited a significantly higher level of MAP relative to SD and (mRen2)27 rats (p <

0.001) and higher HR relative to SD rats (p < 0.05). NTS Microinjection of the PTP1b

inhibitor had no significant effect on resting MAP or HR in ASrAOGEN or (mRen2)27 rats (Table 1). Microinjection of the vehicle solution (n = 2 to 3 per strain) produced no significant change in MAP or HR in SD (81 ± 9 before vs 87 ± 1 mm Hg after and 275 ± 11 before vs 264 ± 7 bpm after), ASrAOGEN (118 ± 1 before vs 113 ± 10 mm Hg after and 349 ± 33 before vs 361 ± 33 bpm after) or (mRen2)27 (87 ± 13 before vs 88 ± 5 mm Hg after and 386 ± 5 before vs 380 ± 1 bpm after) rats.

PTPN1 mRNA in Dorsal Medulla

The observation that PTP1b inhibition had differential effects in the rat strains prompted us to assess gene expression for the PTPN1 gene which encodes PTP1b in dorsal medullary tissue from SD, ASrAOGEN and (mRen2)27 rats. We observed no significant difference in PTPN1 mRNA levels among these strains [1.04 ± 0.06 in SD vs 1.16 ± 0.06 in ASrAOGEN vs 1.10 ± 0.05 in (mRen2)27 rats].

Discussion

The goal of the present study was to determine the contribution of PTP1b to cardiovascular reflex function within the NTS. As a secondary aim, we determined whether differences in PTP1b tone within the NTS of transgenic ASrAOGEN and (mRen2)27 rats contribute to the high and low prevailing level of BRS observed in these animals, respectively. The novel findings of this study are that 1) NTS microinjection of an inhibitor of PTP1b reduces the vagally-mediated BRS for control of HR in response to increases in arterial pressure in SD rats; 2) transgenic ASrAOGEN rats showed increased PTP1b tone within the NTS, consistent with the high resting BRS observed in these animals; 3) hypertensive (mRen2)27 rats with low resting BRS show no acute PTP1b

tone within the NTS. Collectively, these studies illustrate the key finding that PTP1b tone within the NTS may be a protective molecular mechanism to maintain normal baroreflex function.

The actions of insulin, leptin and angiotensin II require initiation of multiple tyrosine kinase phosphorylation events leading to the activation of phosphoinosite-3 kinase (PI3K), mitogen-activated protein kinase (MAPK) and Janus activated kinase/signal transducer and activator of transcription (JAK/STAT) intracellular signaling pathways.[122, 149, 256] Therefore, enhanced activity of protein tyrosine phosphatases, including PTP1b, may restrain kinase signaling to contribute to the metabolic resistance associated with elevations in these peptides. Indeed, over-expression of PTP1b dose-dependently reduces phosphorylation of PI3K, extracellular signal-regulated kinase (ERK) MAPK and JAK/STAT signaling pathway components in mouse cell lines.[157, 159] Global deletion of PTP1b produces mice that are lean, resistant to diet-induced obesity and sensitive to insulin and leptin[135, 219] implicating that disruption of PTP1b regulation may be beneficial to improve energy balance.

The use of PTP1b inhibitors to treat conditions associated with metabolic resistance is confounded by recent studies implicating that genetic deletion of PTP1b in mice is also associated with negative cardiovascular effects including elevations in arterial pressure and sympathetic nervous system activity.[164] These cardiovascular effects are consistent with impairments in BRS for control of HR, an important marker of parasympathetic function that often precedes and contributes to the development of hypertension[2]. However, these studies did not directly examine the contribution of PTP1b to baroreflex function. Since PTP1b limits the activation of intracellular kinase

signaling pathways utilized by insulin, leptin and angiotensin II, all of which impair BRS,[202, 253, 254] the inhibition of the molecular brake limiting these pathways may result in unrestrained kinase signaling to promote baroreflex dysfunction. Therefore, we determined the effect of PTP1b inhibition on BRS for control of HR at the level of the NTS, an autonomic brainstem nucleus that receives afferent baroreceptor input to modulate baroreflex function[11].

The present study provides evidence that PTP1b inhibition within the NTS reduces BRS for control of HR in response to increases in pressure by 41% in SD rats. The BRS remained suppressed at 60 min after the microinjection, well within the duration of baroreflex testing. There was no effect of NTS microinjection of the PTP1b inhibitor on the BRS in response to decreases in pressure. These findings are consistent with previous studies showing a selective impairment of the BRS for bradycardia with no effect on the BRS for tachycardia after NTS microinjection of angiotensin II, insulin or leptin.[202, 253, 254] We provide direct evidence that inhibition of PTP1b within the NTS results in baroreflex dysfunction. Therefore, PTP1b may be an important molecular component participating in the maintenance of resting baroreflex function.

We also determined the contribution of PTP1b activity within the NTS to the varying BRS levels observed in transgenic ASrAOGEN and (mRen2)27 rats. Transgenic ASrAOGEN rats with low brain angiotensinogen (Aogen) due to glial over-expression of an antisense oligonucleotide to Aogen exhibit a higher conscious BRS for control of HR relative to SD rats associated with reduced brain RAS activity.[188, 194] Under anesthesia these animals have a similar or higher BRS relative to SD rats,[185, 199] consistent with the present observation of a trend for a higher BRS (27% increase) in ASrAOGEN versus SD

rats. NTS microinjection of the PTP1b inhibitor produced a greater impairment of the bradycardic BRS in ASrAOGEN compared with SD rats suggesting that these animals have enhanced PTP1b tone within the NTS. The PTP1b inhibitor reduced BRS to a similar hypertensive level (0.5 msec/mm Hg) in both SD and ASrAOGEN rats suggesting maximal suppression of the BRS was achieved with this dose. Therefore, the enhanced response in ASrAOGEN rats may be due to the higher baseline BRS in these animals. Similar to SD rats, the BRS impairment was observed at 10 and 60 min after the initial microinjection in ASrAOGEN rats. Transgenic (mRen2)27 rats with insertion of the mouse *Ren-2* renin gene into the SD genome exhibit an activation of the brain RAS, associated with hypertension and a low resting BRS relative to SD and ASrAOGEN rats in both the conscious and anesthetized state.[205, 257] In (mRen2)27 rats, NTS microinjection of the PTP1b inhibitor produced no significant effect on BRS for bradycardia at 10 and 60 min suggesting an absence of PTP1b tone within the NTS of these animals. The lack of PTP1b regulation within the NTS would be consistent with the already low baroreflex function observed in these animals, as resting BRS was ~0.4 msec/mm Hg.

There was no significant difference in the resting BRS for tachycardia to decreases in pressure among SD, ASrAOGEN and (mRen2)27 rats. The values of the tachycardic BRS were well within the range of previously reported values for anesthetized rats.[202, 253] Furthermore, NTS microinjection of the PTP1b inhibitor produced no significant effect on the evoked BRS for tachycardia in any strain. In a subset of animals, we observed no difference in the depressor and bradycardic responses to CVA induced by intravenous phenylbiguanide after the PTP1b inhibitor injection.

147

These chemoreceptor fibers converge with baroreceptor inputs within the NTS,[225] evidencing the functional specificity of the NTS microinjection of the PTP1b inhibitor to impair baroreflex function. The selectivity for impairment of the BRS for control of HR also argues that the PTP1b inhibitor was not causing a non-specific toxic effect in this brain region.

NTS microinjection of the PTP1b inhibitor produced a transient decrease in MAP and HR in SD rats with no significant effect on resting hemodynamics in ASrAOGEN and (mRen2)27 rats. Therefore, inhibition of PTP1b within the NTS produced minimal effects on pressure and HR over the time course of this study providing evidence for a pressure-independent effect of PTP1b on cardiovascular reflex regulation. PTP1b deficient mice develop hypertension associated with the long-term suppression of the phosphatase.[164] Certainly attenuation of the BRS could play a permissive role in the development of sustained increases in blood pressure in these animals. As previously reported, ASrAOGEN rats exhibited an elevation of MAP and HR under anesthesia possibly due to an enhanced sensitivity to the anesthesia-induced increase in the circulating renin-angiotensin system and subsequent activation of the sympathetic nervous system.[185] In contrast, urethane/chloralose anesthesia normalizes MAP and HR in (mRen2)27 rats through a reduction in the centrally-mediated, angiotensin II-driven sympathetic over-activity.[54] Regardless of the mechanisms, the basal levels of AP under anesthesia are clearly independent of the relative levels of baroreflex function in these animals and the effects of the PTP1b inhibition, confirming that the set-point of the baroreflex is modulated independently from the gain/sensitivity[185].

Since genetic deletion may result in unknown compensatory mechanisms or

programmed events during development, we used a cell-permeable, selective allosteric inhibitor of PTP1b to perform these studies allowing acute interruption of the pathway. The small molecule PTP1b inhibitor used in the present study reversibly binds with high affinity an allosteric site that is not conserved among phosphatases and stabilizes the inactive conformation of PTP1b.[158] The sulfanamido-benzbromarone derived inhibitor of PTP1b is selective over the closely related T cell and leukocyte antigen-related protein phosphatases.[158] The PTP1b inhibitor used in this study results in increases in phosphorylation of PI3K signaling components *in vitro* with no apparent cytotoxicity.[158] However, PTP1b regulates multiple intracellular signaling pathways in addition to those mediating the effects on metabolism including the epidermal growth factor receptor pathway.[258] Furthermore, our data clearly imply that PTP1b-regulated pathways are involved in modulation of baroreflex function.

Angiotensin II, leptin and insulin can all activate intracellular PI3K, MAP and JAK/STAT tyrosine kinase signaling pathways which are regulated by PTP1b.[122, 149, 256] While PTP1b is known to act as a molecular brake to feedback inhibit insulin and leptin signaling,[135, 252] the mechanism for PTP1b interference with angiotensin signaling is not fully understood. The intracellular signaling mechanisms responsible for impairments in BRS produced by PTP1b inhibition were not examined in the current study, but may involve unrestrained kinase signaling by angiotensin II, insulin and leptin. These peptides all impair BRS for control of HR,[202, 253, 254] suggesting the potential for similar signaling pathways. We observed no differences in PTPN1 mRNA, the gene encoding PTP1b, in dorsal medullary tissue of young SD, ASrAOGEN and (mRen2)27 rats. However, the activity of PTP1b may be decreased by post-translational modifications

including glutathionylation or oxidation.[259]   Indeed, transgenic (mRen2)27 rats exhibit

increased oxidative stress which may alter the status of PTP1b.[218]   Alternatively, resting

kinase tone may differ in these animals.   Central PI3K tone for BRS is high in

(mRen2)27 and low in SD rats[147] and MAPK tone is reported higher in some forms of

angiotensin II-dependent hypertension[139].   Therefore, differences in PTP1b tone may

reflect different regulation of kinases as opposed to direct regulation of the phosphatase

within the brain.

Further studies are necessary to determine mechanisms underlying the differential

PTP1b tone for baroreflex modulation in the NTS of ASrAOGEN and (mRen2)27 rats.

These strains exhibit similar plasma levels of angiotensin peptides, leptin and insulin at

15-weeks of age.[180]   However, there is evidence for increased central expression of AT_1

receptors, leptin receptors and p85 alpha subunit of PI3K within brainstem nuclei of

ASrAOGEN rats, associated with an increased sensitivity to insulin and leptin with

respect to glucose challenge and to exogenous angiotensin II and leptin microinjection

within the NTS.[179, 199, 253]   Therefore, increased PTP1b tone may be necessary in these

animals to counteract the upregulation of tyrosine kinase linked receptors and signaling

pathways.   In contrast, the reduced PTP1b tone in (mRen2)27 rats is associated with

increased PI3K tone and decreased sensitivity to insulin and leptin to glucose

challenges.[147, 179]   This is opposite to what might be expected for metabolic function, but

appears to be permissive for the baroreflex dysfunction observed in these animals.

Perspectives

PTP1b inhibition is an attractive target for treating obesity and type II diabetes as

genetic deletion of PTP1b lowers body weight, confers resistance to diet-induced obesity and improves insulin and leptin sensitivity in mice.[219] However, recent studies suggest that deletion of PTP1b stimulates the sympathetic nervous system and elevates arterial pressure,[164] consistent with baroreflex dysfunction. The results of the present study provide direct evidence that inhibition of PTP1b within the NTS, a key brainstem region mediating baroreflex function, results in impairments in BRS for control of HR. These data suggest that PTP1b tone within the NTS is necessary for normal baroreflex function. Collectively, while PTP1b inhibition may be an effective pharmacotherapy to improve metabolic function in insulin- and leptin-resistant populations especially at hypothalamic sites of action, reduced PTP1b tone may also lead to negative cardiovascular outcomes including baroreflex dysfunction.

Reference List

(1) Zabolotny JM, ce-Hanulec KK, Stricker-Krongrad A, Haj F, Wang Y, Minokoshi Y, Kim YB, Elmquist JK, Tartaglia LA, Kahn BB, Neel BG. PTP1B regulates leptin signal transduction in vivo. *Dev Cell* 2002 April;2(4):489-95.

(2) Dube N, Tremblay ML. Involvement of the small protein tyrosine phosphatases TC-PTP and PTP1B in signal transduction and diseases: from diabetes, obesity to cell cycle, and cancer. *Biochim Biophys Acta* 2005 December 30;1754(1-2):108-17.

(3) Marrero MB, Fulton D, Stepp D, Stern DM. Angiotensin II-induced insulin resistance and protein tyrosine phosphatases. *Arterioscler Thromb Vasc Biol* 2004 November;24(11):2009-13.

(4) Elchebly M, Payette P, Michaliszyn E, Cromlish W, Collins S, Loy AL, Normandin D, Cheng A, Himms-Hagen J, Chan CC, Ramachandran C, Gresser MJ, Tremblay ML, Kennedy BP. Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. *Science* 1999 March 5;283(5407):1544-8.

(5) Belin de Chantemele EJ, Muta K, Mintz J, Tremblay ML, Marrero MB, Fulton DJ, Stepp DW. Protein tyrosine phosphatase 1B, a major regulator of leptin-mediated control of cardiovascular function. *Circulation* 2009 September 1;120(9):753-63.

(6) Hall JE, Brands MW, Hildebrandt DA, Kuo J, Fitzgerald S. Role of sympathetic nervous system and neuropeptides in obesity hypertension. *Braz J Med Biol Res* 2000 June;33(6):605-18.

(7) Arnold AC, Shaltout HA, Gallagher PE, Diz DI. Leptin Impairs Cardiovagal Baroreflex Function at the Level of the Solitary Tract Nucleus. *Hypertension* 2009 September 21.

(8) McKernan AM, Calaresu FR. Insulin microinjection into the nucleus tractus solitarii of the rat attenuates the baroreceptor reflex. *J Auton Nerv Syst* 1996 November 6;61(2):128-38.

(9) Campagnole-Santos MJ, Diz DI, Ferrario CM. Baroreceptor reflex modulation by angiotensin II at the nucleus tractus solitarii. *Hypertension* 1988;11(2 Pt 2):I167-I171.

(10) Diz DI, Garcia-Espinosa MA, Gegick S, Tommasi EN, Ferrario CM, Ann TE, Chappell MC, Gallagher PE. Injections of angiotensin-converting enzyme 2 inhibitor MLN4760 into nucleus tractus solitarii reduce baroreceptor reflex sensitivity for heart rate control in rats. *Exp Physiol* 2008 May;93(5):694-700.

(11) Korner PI. Cardiac baroreflex in hypertension: role of the heart and angiotensin II. *Clin Exp Hypertens* 1995 January;17(1-2):425-39.

(12) Logan E, Diz DI, Averill DB. Inhibition of the PI3 Kinase Signal Transduction Pathway in Nucleus Tractus Solitarii of (mRen2)27 Transgenic Rats Improves Baroreceptor Sensitivity. *FASEB J* 2008; 22.

(13) Koren S, Fantus IG. Inhibition of the protein tyrosine phosphatase PTP1B: potential therapy for obesity, insulin resistance and type-2 diabetes mellitus. *Best Pract Res Clin Endocrinol Metab* 2007 December;21(4):621-40.

(14) Hunyady L, Catt KJ. Pleiotropic AT1 receptor signaling pathways mediating physiological and pathogenic actions of angiotensin II. *Mol Endocrinol* 2006 May;20(5):953-70.

(15) Bjorbaek C, Kahn BB. Leptin signaling in the central nervous system and the periphery. *Recent Prog Horm Res* 2004;59:305-31.

(16) Venable CL, Frevert EU, Kim YB, Fischer BM, Kamatkar S, Neel BG, Kahn BB. Overexpression of protein-tyrosine phosphatase-1B in adipocytes inhibits insulin-stimulated phosphoinositide 3-kinase activity without altering glucose transport or Akt/Protein kinase B activation. *J Biol Chem* 2000 June 16;275(24):18318-26.

(17) Kaszubska W, Falls HD, Schaefer VG, Haasch D, Frost L, Hessler P, Kroeger PE, White DW, Jirousek MR, Trevillyan JM. Protein tyrosine phosphatase 1B negatively regulates leptin signaling in a hypothalamic cell line. *Mol Cell Endocrinol* 2002 September 30;195(1-2):109-18.

(18) Thayer JF, Lane RD. The role of vagal function in the risk for cardiovascular disease and mortality. *Biol Psychol* 2007 February;74(2):224-42.

(19) Andresen MC, Kunze DL. Nucleus tractus solitarius--gateway to neural circulatory control. *Annu Rev Physiol* 1994;56:93-116.

(20)   Schinke M, Baltatu O, Bohm M, Peters J, Rascher W, Bricca G, Lippoldt A, Ganten D, Bader M. Blood pressure reduction and diabetes insipidus in transgenic rats deficient in brain angiotensinogen. *Proc Natl Acad Sci U S A* 1999;96(7):3975-80.

(21)   Caligiorne SM, Silva AQ, Fontes MA, Silva JR, Baltatu O, Bader M, Santos RA, Campagnole-Santos MJ. Baroreflex control of heart rate and renal sympathetic nerve activity in rats with low brain angiotensinogen. *Neuropeptides* 2008 April;42(2):159-68.

(22)   Sakima A, Averill DB, Kasper SO, Jackson L, Ganten D, Ferrario CM, Gallagher PE, Diz DI. Baroreceptor reflex regulation in anesthetized transgenic rats with low glia-derived angiotensinogen. *Am J Physiol Heart Circ Physiol* 2007;292(3):H1412-H1419.

(23)   Couto AS, Baltatu O, Santos RA, Ganten D, Bader M, Campagnole-Santos MJ. Differential effects of angiotensin II and angiotensin-(1-7) at the nucleus tractus solitarii of transgenic rats with low brain angiotensinogen. *J Hypertens* 2002;20(5):919-25.

(24)   Mullins JJ, Peters J, Ganten D. Fulminant hypertension in transgenic rats harbouring the mouse Ren-2 gene. *Nature* 1990 April 5;344(6266):541-4.

(25)   Ribeiro JM, Santos RA, Pesquero JB, Bader M, Krieger EM. Autonomic control in rats with overactivity of tissue renin-angiotensin or kallikrein-kinin system. *Regul Pept* 2005 July 15;129(1-3):155-9.

(26) Paton JF. Convergence properties of solitary tract neurones driven synaptically by cardiac vagal afferents in the mouse. *J Physiol* 1998;508 (Pt 1):237-52.

(27) Diz DI, Garcia-Espinosa MA, Gallagher PE, Ganten D, Ferrario CM, Averill DB. Angiotensin-(1-7) and baroreflex function in nucleus tractus solitarii of (mRen2)27 transgenic rats. *J Cardiovasc Pharmacol* 2008 June;51(6):542-8.

(28) Wiesmann C, Barr KJ, Kung J, Zhu J, Erlanson DA, Shen W, Fahr BJ, Zhong M, Taylor L, Randal M, McDowell RS, Hansen SK. Allosteric inhibition of protein tyrosine phosphatase 1B. *Nat Struct Mol Biol* 2004 August;11(8):730-7.

(29) Flint AJ, Tiganis T, Barford D, Tonks NK. Development of "substrate-trapping" mutants to identify physiological substrates of protein tyrosine phosphatases. *Proc Natl Acad Sci U S A* 1997 March 4;94(5):1680-5.

(30) Barrett WC, DeGnore JP, Konig S, Fales HM, Keng YF, Zhang ZY, Yim MB, Chock PB. Regulation of PTP1B via glutathionylation of the active site cysteine 215. *Biochemistry* 1999 May 18;38(20):6699-705.

(31) Blendea MC, Jacobs D, Stump CS, McFarlane SI, Ogrin C, Bahtyiar G, Stas S, Kumar P, Sha Q, Ferrario CM, Sowers JR. Abrogation of oxidative stress improves insulin sensitivity in the Ren-2 rat model of tissue angiotensin II overexpression. *Am J Physiol Endocrinol Metab* 2005 February;288(2):E353-E359.

(32) Seyedabadi M, Goodchild AK, Pilowsky PM. Differential role of kinases in brain stem of hypertensive and normotensive rats. *Hypertension* 2001 November;38(5):1087-92.

(33) Kasper SO, Carter CS, Ferrario CM, Ganten D, Ferder LF, Sonntag WE, Gallagher PE, Diz DI. Growth, metabolism, and blood pressure disturbances during aging in transgenic rats with altered brain renin-angiotensin systems. *Physiol Genomics* 2005;23(3):311-7.

(34) Kasper SO, Ferrario CM, Ganten D, Diz DI. Rats with low brain angiotensinogen do not exhibit insulin resistance during early aging. *Endocrine* 2006 October;30(2):167-74.

Table 1. MAP and HR in Response to NTS Microinjection of PTP1b Inhibitor

Group	N	MAP (mm Hg)	HR (bpm)
Sprague-Dawley Rats	5		
Baseline		89 ± 3	279 ± 11
Peak		78 ± 4 *	264 ± 11 †
10 Min		82 ± 5	268 ± 12 †
60 Min		93 ± 4	281 ± 7
ASrAOGEN Rats	4		
Baseline		119 ± 3 ‡	338 ± 17 §
Peak		106 ± 8	341 ± 30
10 Min		114 ± 5	342 ± 29
60 Min		111 ± 7	336 ± 18
(mRen2)27 Rats	6		
Baseline		91 ± 5	306 ± 16
Peak		86 ± 9	288 ± 13
10 Min		92 ± 4	290 ± 14
60 Min		108 ± 7	331 ± 31

Values are mean ± SEM and represent MAP and HR at baseline, peak changes in response to NTS microinjection of the PTP1b inhibitor and values at 10 minutes after PTP1b inhibitor microinjection (immediately prior to each series of reflex testing). N = number of animals; MAP = mean arterial pressure; HR = heart rate, * $p < 0.05$ vs 60 min, † $p < 0.05$ vs Baseline and 60 min, ‡ $p < 0.001$ vs SD and (mRen2)27 rats, § $p < 0.05$ vs SD rats

Figure 1. Effect of PTP1b Inhibition within the NTS on BRS for control of HR to increases in AP produced by PE in SD Rats. A, The slope of the relationship between the increases in MAP produced by PE and the corresponding reflex bradycardia [expressed as pulse interval] shows a reduction in the linear regression slope after NTS microinjection of the PTP1b inhibitor [0.87 ± 0.24 msec/mm Hg baseline vs 0.45 ± 0.16 msec/mm Hg after PTP1b inhibition ($r = 0.44 - 0.53$)] in pooled data from SD rats. B, NTS microinjection of the PTP1b inhibitor (100 nM/120 nL) impaired the BRS for bradycardia in SD rats at 10 and 60 min after the PTP1b injection. MAP = mean arterial pressure, PI = pulse interval, PTP1b = protein phosphatase 1b, # $p < 0.01$ vs baseline
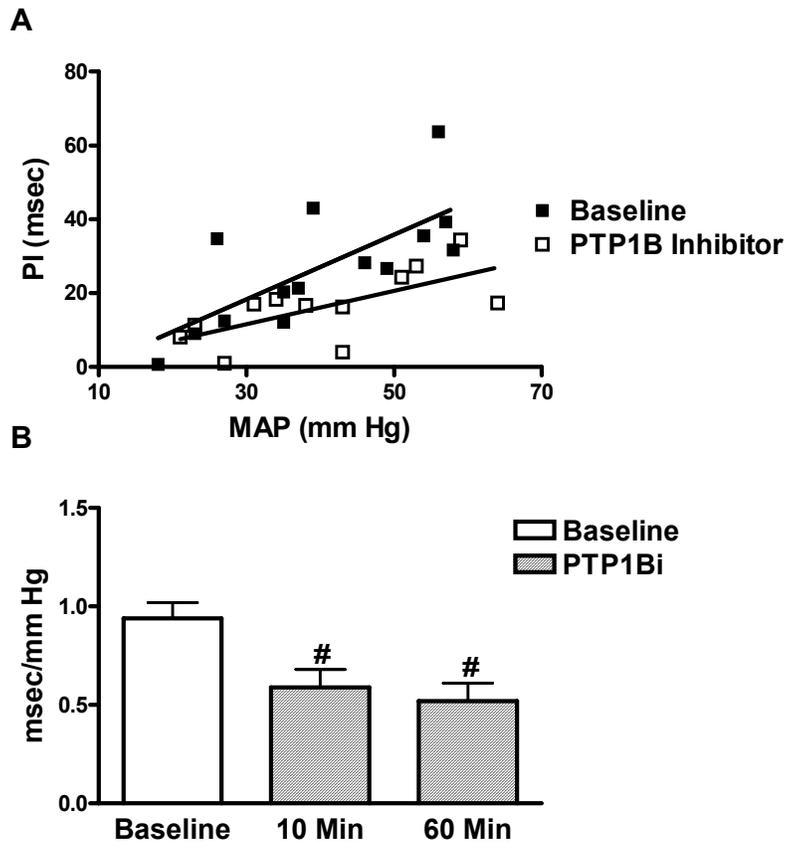
Figure 2. Evidence for Differential PTP1b tone within the NTS of SD, ASrAOGEN and (mRen2)27 Rats. As described in Figure 1, NTS microinjection of the PTP1b inhibitor impaired BRS for control of HR in response to increases in AP by 41% in SD rats when reflexes were tested starting at 10 min after injection. PTP1b inhibition impaired BRS to a greater extent of 60% in ASrAOGEN rats from an elevated resting BRS, with no significant effect on BRS in (mRen2)27 rats with low resting BRS. Pooled data from 2 SD, 2 ASrAOGEN and 3 (mRen2)27 rats shows no effect of the vehicle solution on the BRS. BRS = baroreflex sensitivity, SD = Sprague-Dawley rats, AS = ASrAOGEN rats, mRen2 = (mRen2)27 rats, * $p < 0.05$ vs (mRen2)27 rats, # $p < 0.01$ vs SD and (mRen2)27 rats
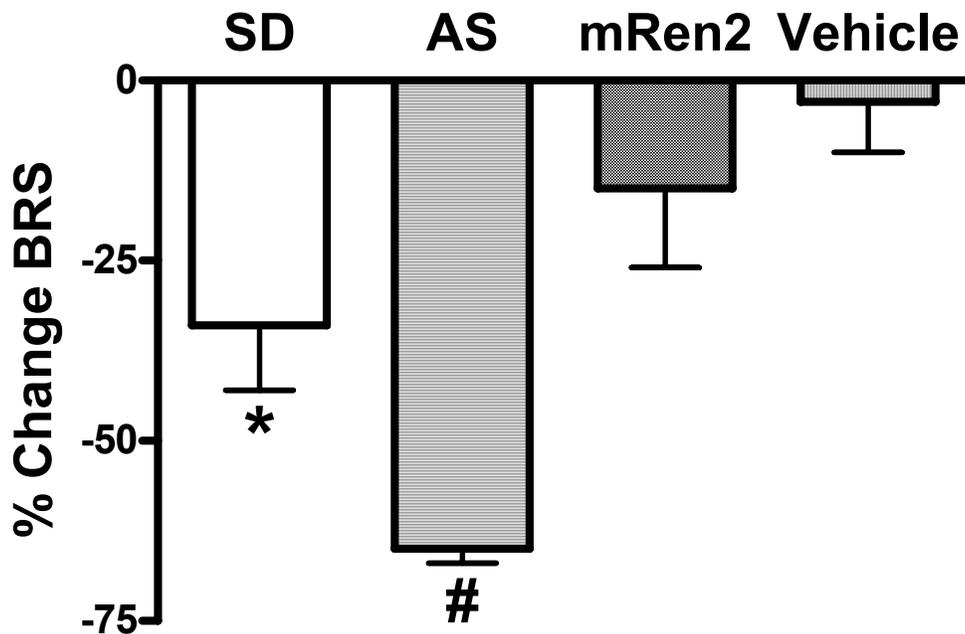
CHAPTER FIVE

SUMMARY AND CONCLUSIONS

1. Summary of Findings

The arterial baroreceptor reflex is a key homeostatic mechanism involved in the short-term regulation of blood pressure through feedback loops in the autonomic nervous system.[26] Baroreflex sensitivity for control of heart rate, an important maker of vagal function, is often reduced in conditions associated with hypertension including stroke, type II diabetes, aging and obesity.[88] Importantly, reductions in baroreflex sensitivity precede and may contribute to the development of hypertension.[90] Although the pathological importance of baroreflex sensitivity has been known for decades, the mechanisms underlying baroreflex dysfunction are poorly understood. Only recently have studies begun to address the importance of pharmacological restoration of baroreflex sensitivity as a novel method to prevent cardiovascular and cerebrovascular diseases.[54] In order to effectively target baroreflex sensitivity, it is critical to identify key factors that regulate brainstem areas controlling autonomic outflow in conditions associated with baroreflex dysfunction. To this end, the present studies identify factors that modulate baroreflex sensitivity under normal conditions and during alterations of the brain RAS. These findings implicate novel mechanisms that provide insight for preservation of baroreflex function in aged and obese individuals, populations that are increasing at an alarming rate worldwide.

In Chapter Two, we examine the contribution of brain angiotensin peptides to the age-related preservation of baroreflex function observed in older transgenic ASrAOGEN rats with low brain angiotensinogen. These studies show that maintenance of angiotensin-(1-7) and loss of angiotensin II tone within the NTS may be an important mechanism to preserve baroreflex function during aging. In addition to alterations in angiotensin peptides, both aging and obesity are characterized by elevations in circulating leptin levels.[56, 68] Whether the elevated leptin levels contribute to baroreflex dysfunction in these conditions is currently unknown. Therefore, in Chapter Three we illustrate the novel finding that exogenous leptin impairs baroreflex sensitivity for control of heart rate and alters autonomic balance at the level of the NTS in younger Sprague-Dawley rats. Furthermore, younger ASrAOGEN rats are more sensitive to leptin-mediated impairments in baroreflex sensitivity, associated with an upregulation of gene expression for relevant leptin signaling pathways in the dorsal medulla of these animals. These data implicate complex interactions between leptin and the brain RAS for modulation of baroreflex function.

In obese rodent models, there is resistance to the metabolic actions of leptin, with maintenance of sensitivity to the cardiovascular actions of the peptide.[58] Whether resistance to the cardiovascular actions of leptin accompanies other conditions exhibiting elevated leptin levels, including aging, has yet to be considered. Preliminary data are provided in the Appendix showing a loss of sensitivity to the baroreflex suppression produced by exogenous leptin in older Sprague-Dawley rats with known elevations in circulating leptin levels[45]. In contrast, microinjection of exogenous leptin continues to impair baroreflex sensitivity in older ASrAOGEN rats with maintenance of low

circulating leptin levels during aging[45]. These data suggest that long-term reductions in brain angiotensinogen levels preserve leptin sensitivity. In older Sprague-Dawley rats, baroreflex sensitivity is improved by NTS administration of a leptin antagonist suggestive of a role for endogenous leptin in baroreflex dysfunction during aging. Collectively, reduction of endogenous leptin actions within the NTS may be a novel mechanism to improve baroreflex function and restore autonomic balance in conditions associated with elevated leptin levels.

Finally, we examined the contribution of PTP1B, a protein tyrosine phosphatase involved in negative regulation of leptin, insulin and angiotensin II signaling,[29, 59, 96] to baroreflex function. We show that PTP1B activity within the NTS is necessary for normal baroreflex function, possibly due to the critical role of this phosphatase to inhibit intracellular kinase signaling pathways utilized by leptin, insulin and angiotensin II for regulation of cardiovascular function. PTP1B inhibitors are being developed as novel therapies to improve metabolic sensitivity in patient populations with insulin resistance, including type II diabetes and obesity.[51] The findings of the present study suggest that inhibition of PTP1B may also result in adverse cardiovascular outcomes, including baroreflex dysfunction.

Together these data illustrate important mechanisms for preservation of baroreflex function. The identification of factors that modulate baroreflex sensitivity are critical to the development of novel pharmacotherapies to alleviate the baroreflex dysfunction in conditions associated with hypertension, including aging and obesity, independent of effects on metabolism.

2. The Brain RAS and Baroreflex Function during Aging

Aging is associated with elevations in systolic blood pressure in part due to increases in sympathetic nervous system activity in concert with impairments in baroreflex control of heart rate, sympathetic activity and arterial pressure.[32, 88] Studies consistently show that baroreflex sensitivity for control of heart rate and heart rate variability, indices of vagal parasympathetic function, are reduced during aging.[32, 88] Importantly, the decline in vagal function associated with aging may be an important mechanism contributing to increases in cardiovascular morbidity and mortality in elderly populations.[88] Age-related impairments in baroreflex function may result from alterations in sensory, central or efferent neurons of the autonomic baroreflex arc.[32] In addition, alterations in factors involved in cardiovascular regulation, including angiotensin peptides, leptin and insulin may contribute to the age-related decline in vagal function.[27] To date, the mechanisms underlying baroreflex dysfunction associated with aging are poorly understood.

The RAS is one hormonal system in close association with brain sites involved in the regulation of both sympathetic and parasympathetic outflow.[26] Blockade of the formation or actions of angiotensin II with ACE inhibitors or AT_1 receptor blockers, respectively, improves lifespan and prevents age-related cardiovascular and metabolic pathologies in rats.[6, 27, 40, 45] However, plasma renin and circulating angiotensin peptides are reduced during aging,[1] suggesting that the beneficial effects of these pharmacotherapies on age-related pathologies are due to actions on activated tissue systems. Indeed, activation of the brain RAS is known to contribute to baroreflex dysfunction as well as the development of hypertension.[90] A high density of AT_1

receptors exists within the NTS, a brain region known to modulate baroreflex function.[26] This distribution of AT_1 receptors is consistent with the role of both exogenous and endogenous angiotensin II to impair baroreflex sensitivity for control of heart rate within the NTS.[11, 16, 62, 77] In contrast, angiotensin-(1-7) facilitates baroreflex sensitivity[13, 18, 77] and inhibition of ACE2, an enzyme with high catalytic activity for conversion of angiotensin II to angiotensin-(1-7), reduces baroreflex sensitivity[25] at the level of the NTS. Collectively, the prevailing level of baroreflex sensitivity appears to depend on an endogenous balance of these two angiotensin peptides within the NTS. Therefore, alterations in the actions of angiotensin II and angiotensin-(1-7) within this brain region may contribute to the baroreflex dysfunction during aging.

Recent studies in our laboratory focus on understanding the contribution of the brain RAS to baroreflex function during aging. In younger Sprague-Dawley rats, AT_1 receptor blockade improves and angiotensin-(1-7) receptor blockade impairs baroreflex sensitivity for control of heart rate at the level of the NTS.[77] In older Sprague-Dawley rats, there is a loss of angiotensin-(1-7) tone for modulation of baroreflex sensitivity within the NTS[77] implicating that age-related baroreflex dysfunction is associated with a differential regulation of angiotensin peptides within brain regions controlling autonomic outflow. Furthermore, the maintenance of angiotensin-(1-7) tone within the NTS may be an important mechanism to preserve baroreflex function in aged populations. Therefore, in Chapter Two we employed older ASrAOGEN rats with a glia-specific reduction in angiotensinogen to determine the role of brain angiotensin peptides to baroreflex function during aging. These animals are spared characteristic declines in cardiovascular and metabolic function during aging[27, 45] suggesting that age-related changes require an intact

glial RAS. Conscious ASrAOGEN rats maintain higher resting baroreflex sensitivity during aging relative to other rat strains.[27] Thus, we hypothesized that the preservation of resting baroreflex function in older ASrAOGEN rats would be accompanied by maintenance of endogenous angiotensin-(1-7) tone within the NTS of these animals.

Similar to observations in younger ASrAOGEN rats,[78] AT_1 receptor blockade has no effect while angiotensin-(1-7) receptor blockade impairs baroreflex sensitivity for control of heart rate at the level of the NTS in older anesthetized ASrAOGEN rats. The reduction in baroreflex sensitivity produced by blockade of angiotensin-(1-7) receptors is greater in older ASrAOGEN rats relative to younger ASrAOGEN or Sprague-Dawley rats[78] implicating the importance of angiotensin-(1-7) tone within this brain region to the support of resting baroreflex function during aging. Collectively, data from younger and older anesthetized ASrAOGEN rats suggest a glial source of angiotensinogen for angiotensin II and a non-glial source for angiotensin-(1-7) modulation of baroreflex sensitivity.[78] The findings of our studies are consistent with observations in double transgenic mice over-expressing human renin and angiotensinogen in which glial over-expression reduces baroreflex sensitivity while neuronal over-expression only alters the baroreflex set-point.[76] The non-glial source for angiotensin-(1-7) may be of neuronal origin since immunoreactivity for angiotensin peptides is preserved in the paraventricular nucleus of ASrAOGEN rats relative to Sprague-Dawley rats.[91] These findings suggest different mechanisms for angiotensin II and angiotensin-(1-7) modulation of baroreflex function, possibly involving different neuronal populations or neurotransmitters.

Importantly, these studies illustrate the novel finding that preservation of angiotensin-(1-7) tone is associated with maintenance of baroreflex sensitivity during

aging. Therefore, pharmacological therapies that increase angiotensin-(1-7) levels, including ACE inhibitors and AT_1 receptor blockers, may be important therapeutics to improve baroreflex function and restore autonomic balance in elderly populations. Other treatments which increase angiotensin-(1-7) levels, including possible direct administration of the peptide, may also improve vagal function and reduce cardiovascular morbidity and mortality in elderly patients. Future studies will focus on uncovering factors that are involved in the regulation of angiotensin peptides, processing enzymes and receptors during aging in autonomic brainstem regions to provide further critical insight into mechanisms contributing to age-related declines in baroreflex function.

3. Anesthesia-Induced Sympathetic Activation in ASrAOGEN Rats

In the conscious state, ASrAOGEN rats exhibit elevated indices of vagal function, including higher heart rate variability and better baroreflex sensitivity for control of heart rate relative to Sprague-Dawley rats.[5, 27] However, these animals do not show differences in blood pressure variability,[5] a measure of sympathetic tone to the vasculature. These data are confirmed by studies showing higher vagal tonus with no change in sympathetic tonus to the heart measured by heart rate changes in response to methyl-atropine or propranolol, respectively, in conscious ASrAOGEN relative to Sprague-Dawley rats.[10] Under anesthesia, a paradoxical elevation of arterial pressure and enhanced depressor and bradycardic responses to cardiac vagal chemosensitive fiber activation occurs in younger ASrAOGEN rats.[78] In these animals, the elevation of arterial pressure is normalized by NTS microinjection of either angiotensin II or angiotensin-(1-7) receptor antagonists with no effect of receptor blockade on responses to

cardiac vagal chemosensitive fiber activation.[78] These data suggest that angiotensin peptides from a non-glial source contribute to the maintenance of arterial pressure in younger anesthetized ASrAOGEN rats. The glial source of angiotensin II for baroreflex modulation and non-glial source for maintenance of arterial pressure provides evidence for independent regulation of baroreflex and pressor pathways.

The findings of Chapters Two and Three show an elevation of resting arterial pressure in both younger and older ASrAOGEN rats under anesthesia. In addition, we observe an elevation of resting heart rate in younger anesthetized ASrAOGEN relative to Sprague-Dawley rats. While tachycardia has not been previously reported in anesthetized ASrAOGEN rats, this finding is consistent with the known effects of anesthesia to activate the sympathetic nervous system. Despite elevations in arterial pressure and heart rate, both younger and older anesthetized ASrAOGEN rats maintain similar or higher baroreflex sensitivity for control of heart rate relative to younger Sprague-Dawley rats.[78] Consistent with previous observations,[78] angiotensin II and angiotensin-(1-7) within the NTS contribute to the maintenance of arterial pressure in older anesthetized ASrAOGEN rats. In contrast to younger ASrAOGEN rats, the depressor and bradycardic responses to cardiac vagal chemosensitive fiber activation are attenuated by angiotensin II or angiotensin-(1-7) receptor blockade in older ASrAOGEN rats.

We propose that anesthesia-induced alterations in cardiovascular function in ASrAOGEN rats are due to an activation of the sympathetic nervous system. ASrAOGEN rats have elevated AT_1 receptors in brain sites involved in the regulation of autonomic outflow, including the subfornical organ, paraventricular nucleus and NTS.[46, 67] In addition, ASrAOGEN rats have an enhanced cardiovascular sensitivity to NTS

microinjection of angiotensin II.[23] Urethane anesthesia is known to increase sympathetic activity, plasma renin activity and circulating angiotensin II levels in normotensive rats.[83] Thus, ASrAOGEN rats may have enhanced sensitivity to anesthesia-induced increases in angiotensin II due to the upregulation of AT_1 receptors within the central nervous system. Dampney and colleagues recently showed that AT_1 receptors within the NTS can mediate baroreflex responses to circulating angiotensin II.[87] Thus, elevations in circulating angiotensin II produced by anesthesia may modulate baroreflex sensitivity at central sites of action, including circumventricular organs, to stimulate descending angiotensinergic pathways, involving both angiotensins II and angiotensin-(1-7), from the paraventricular nucleus to the NTS and RVLM resulting in increases in sympathetic outflow and arterial pressure. Anesthesia also reduces baroreflex sensitivity,[83] an action permissive to increases in arterial pressure and sympathetic activity.

Studies are currently underway to elucidate the molecular mechanisms underlying the anesthesia-induced activation of the sympathetic nervous system in anesthetized ASrAOGEN rats. One possible mechanism is enhanced angiotensin II-mediated activation of intracellular signaling pathways due to the upregulation of AT_1 receptors in these animals. In addition, circulating levels of peptides known to induce sympathetic activation, including angiotensin II, insulin and leptin, may vary in response to anesthesia in ASrAOGEN rats relative to Sprague-Dawley rats.

4. Leptin Modulation of Baroreflex Sensitivity

In addition to angiotensin peptides, recent studies implicate leptin in mediating sympathetic activation and hypertension independent of obesity.[43, 72] The deleterious

cardiovascular effects of leptin are primarily attributed to the activation of Ob-R$_b$ leptin receptors within the ventromedial, dorsomedial and arcuate hypothalamic nuclei.[60, 75] However, the hypothalamic actions of leptin likely involve descending pathways to brainstem nuclei involved in direct control of arterial pressure and reflex modulation of autonomic function such as the NTS. Indeed, leptin receptors have been localized to vagal afferents and within the NTS[30, 65] implicating leptin as a direct modulator of baroreflex function. Leptin receptors within the NTS mediate cardiovascular responses as microinjection of large doses of leptin within this brain region increases renal sympathetic activity and arterial pressure in anesthetized Sprague-Dawley rats at two hours after the initial injection.[57] This delayed time-course may implicate a role for leptin to impair baroreflex sensitivity for control of heart rate prior to pressor and sympathoexcitatory effects. However, a direct link between leptin and baroreflex dysfunction is currently lacking.

The findings of Chapter Three provide evidence that NTS microinjection of exogenous leptin impairs baroreflex sensitivity for control of heart rate in anesthetized Sprague-Dawley rats. The impairments in baroreflex function are dose-dependent with the higher leptin dose (500 fmol/60 nL) reducing baroreflex sensitivity to levels observed in hypertensive rats[26]. Thus, the administration of exogenous leptin within the NTS may simulate the baroreflex dysfunction observed in conditions associated with elevated circulating leptin levels. Leptin injection selectively reduced the baroreflex sensitivity for bradycardia, similar to observations for angiotensin II and insulin within the NTS.[11, 64] Leptin-mediated impairments in baroreflex sensitivity were observed at 10 and 60 minutes, with partial recovery at 120 minutes after the initial injection. In the present

studies, NTS microinjection of leptin did not produce changes in arterial pressure or heart rate confirming pressure-independent modulation of baroreflex function. These findings provide a direct link between leptin and baroreflex dysfunction within brainstem regions mediating autonomic outflow.

In addition to the evoked pharmacological method, we used spectral analysis methods to assess changes in spontaneous baroreflex sensitivity as well as other indices of autonomic balance in response to leptin injection. In contrast to the open-loop evoked method, the closed-loop spectral analysis method provides a non-invasive measure of spontaneous BRS from post-hoc recordings of beat-to-beat fluctuations in arterial pressure and heart rate.[82] Spectral analysis methods also provide insight into other indices of autonomic regulation including heart rate variability, a measure of vagal activity to the heart, and blood pressure variability, a measure of sympathetic tone to the blood vessels.[82] These methods have been validated in humans and rodents with a highly significant correlation reported between BRS values obtained by pharmacological and spectral analysis methods.[17, 31, 82] Using spectral analysis methods, we confirmed reductions in the vagal with no effect on the sympathetic spontaneous baroreflex sensitivity in response to injection of the higher leptin dose (500 fmol) in Sprague-Dawley rats. In addition, the higher dose of leptin reduces heart rate variability and increases blood pressure variability in these animals.

Collectively, exogenous leptin alters blood pressure regulation similar to patterns observed in conditions associated with hypertension.[88] These findings may be extrapolated to suggest that reduction of endogenous leptin within the NTS may be a novel therapeutic strategy to improve baroreflex function and restore autonomic balance

in patients with elevated circulating leptin levels. Recent studies suggest that ACE inhibition or AT_1 receptor blockade lowers plasma leptin levels in hypertensive humans and improves leptin sensitivity in rats.[47, 49] Therefore, therapies that reduce angiotensin II actions may be effective to improve leptin sensitization in patients exhibiting metabolic resistance.

5. Interactions between Leptin and the Brain RAS

According to Leyva and colleagues, there is considerable variability in plasma leptin levels amongst individuals with comparable degrees of obesity suggesting that factors other than fat are involved in leptin regulation.[53] Indeed, adipose tissue secretes a number of other signaling molecules involved in energy and cardiovascular homeostasis, including insulin and angiotensin II.[41] Interestingly, numerous stimulatory interactions have been reported between leptin and angiotensin II in cellular studies. *In vitro*, angiotensin II stimulates leptin release and increases leptin and leptin receptor mRNA levels.[48, 52, 85] ACE inhibition or AT_1 receptor blockade reduces plasma leptin levels in hypertensive patients and prevents age-related increases in circulating leptin levels in Fischer 344 rats.[40, 49] Furthermore, transgenic ASrAOGEN rats with a targeted reduction in brain angiotensinogen maintain normal plasma leptin levels during aging and have increased leptin sensitivity to a glucose challenge suggesting that low brain RAS activity is associated with improved leptin sensitivity for metabolism.[45, 47] Whether low brain RAS activity also confers enhanced sensitivity to the cardiovascular actions of leptin is currently unknown.

In Chapter Three we employed transgenic ASrAOGEN rats to determine if leptin modulation of baroreflex sensitivity and associated leptin signaling pathways are altered by basal differences in brain angiotensin levels. In younger Sprague-Dawley rats, NTS microinjection of the higher leptin dose produces dose-dependent impairments in baroreflex sensitivity which recovers at 120 minutes after the leptin injection. In younger ASrAOGEN rats, NTS microinjection of either leptin dose results in maximal suppression of baroreflex sensitivity with no evidence for recovery during the time course studied. Using spectral analysis methods, we observe a decrease in vagal indices of spontaneous baroreflex sensitivity as well as heart rate variability in ASrAOGEN rats in response to either dose of leptin, similar to effects of the higher leptin dose in Sprague-Dawley rats. Collectively, these findings implicate that ASrAOGEN rats have enhanced sensitivity to exogenous leptin for baroreflex modulation.   Whether the increased sensitivity in these animals is due to a direct interaction with the brain RAS is currently unknown. However, the enhanced sensitivity to exogenous leptin is accompanied by an upregulation of leptin receptor and PI3K p85 alpha mRNA in the dorsal medulla of ASrAOGEN rats. The upregulation of leptin receptor and relevant signaling pathway gene expression may be due to low endogenous levels of angiotensin II, insulin or leptin.

ASrAOGEN rats have a loss of endogenous angiotensin II tone within the NTS contributing to baroreflex suppression.[78] We propose that the reduction in angiotensin II tone may result in decreases in leptin levels within this brain region. The upregulation of leptin signaling pathways in the dorsal medulla of ASrAOGEN rats is consistent with this interpretation.   These findings provide evidence that long-term reductions in brain angiotensinogen or the subsequent consequences are associated with enhanced sensitivity

to leptin for baroreflex modulation and increases in leptin signaling pathways. The enhanced sensitivity to leptin is associated with a positive metabolic and cardiovascular profile ASrAOGEN rats.[10, 45, 78] Therefore, therapies to enhance leptin sensitivity, perhaps allowing restoration of low endogenous leptin levels, may be important to treat conditions associated with elevated leptin levels including obesity, aging and hypertension.

The present findings may also provide novel insight into the underlying mechanisms of ACE inhibitors and AT_1 receptor blockers. Since reduction of angiotensin II actions maintains low circulating leptin levels in humans and rodents,[40, 45, 49] the beneficial cardiovascular and metabolic effects of ACE inhibitors and AT_1 receptor blockers may be in part attributed to an enhancement of leptin sensitivity. Collectively, understanding mechanisms to preserve leptin sensitivity, in the presence of low endogenous leptin levels, may be important for maintaining satiety effects of leptin while preventing the deleterious cardiovascular effects of this peptide.

6. Leptin Signaling Pathways Involved in Baroreflex Modulation

In addition to functional studies, we used molecular techniques to characterize the intracellular signaling pathways activated by leptin for modulation of baroreflex sensitivity. Dorsal medullary sections were excised from younger Sprague-Dawley or ASrAOGEN rats at 45 minutes or 2 hours after microinjection of leptin or vehicle solution for western blot analysis. Using these sections, we assessed quantitative changes in the phosphorylation of Akt and ERK, components of the PI3K and MAPK intracellular signaling pathways, respectively, that are known to be stimulated by leptin for

cardiovascular actions.[74, 94]   In preliminary studies, leptin injection produced no significant change in dorsal medullary levels of Akt or ERK phosphorylation in Sprague-Dawley or ASrAOGEN rats relative to vehicle treated animals (Appendix, Table 1).

There are several limitations that may help explain the lack of changes in phosphorylation of Akt and ERK in response to leptin injection. First, the injection may have only accessed a small population of cells. Thus, the dorsal medullary section excised might exceed the spread of the injection. Second, different time points may be necessary to see activation of intracellular signaling pathways. Previous studies show that phosphorylation of the PI3K pathway is observed at 10 minutes after NTS microinjection of insulin,[44] a metabolic peptide with similar suppressive actions on baroreflex function[64]. Finally, leptin may have activated other intracellular signaling pathways for baroreflex modulation that were not examined in the present study including JAK/STAT, c-Jun N-terminal kinase and p38 MAPK.[36]   However, these pathways have not been shown to participate in leptin-mediated pressor or sympathoexcitatory responses. Further studies are necessary to elucidate the intracellular signaling pathways involved in leptin modulation of baroreflex sensitivity.

7. Leptin Modulation of Baroreflex Sensitivity during Aging

In obesity, elevations in circulating leptin levels are associated with defects in leptin signaling pathways as well as reduced leptin transport into the brain which may collectively contribute to the metabolic leptin resistance in these populations.[22, 43] However, recent studies show maintenance of sensitivity to leptin-mediated increases in renal sympathetic activity in some obese rodent models.[58]   These findings suggest that

obesity is associated with selective resistance to the metabolic actions of leptin in the face of maintenance to the cardiovascular actions of the peptide. Whether there is maintenance of sensitivity to the cardiovascular actions of leptin in other conditions associated with hyperleptinemia, including aging, has yet to be examined.

The status of leptin sensitivity for baroreflex modulation during aging was determined in older Sprague-Dawley rats with known elevated circulating leptin levels[45]. NTS microinjection of the higher leptin dose (500 fmol) produces no significant effect on baroreflex sensitivity for control of heart rate in older Sprague-Dawley rats at 16 to 20-months of age (Appendix, Figure 1A). These findings are in contrast to the 63% reduction in baroreflex sensitivity observed in younger Sprague-Dawley rats after NTS microinjection of the same leptin dose. The effect of aging on cardiovascular sensitivity to leptin was also assessed in older ASrAOGEN rats, with maintenance of low circulating leptin levels during aging[45]. In older ASrAOGEN rats, microinjection of the higher leptin dose significantly impairs baroreflex sensitivity, to a similar extent as observed in younger rats of this strain (Appendix, Figure 1B). The preservation of leptin sensitivity for baroreflex modulation in ASrAOGEN rats is associated with a continued upregulation of leptin receptor and p85 alpha mRNA in the dorsal medulla of these animals (Appendix, Figure 2).

These data suggest that aging is associated with resistance to both the metabolic and cardiovascular actions of leptin. These findings are in contrast to some rodent models of obesity in which there is selective resistance to the metabolic actions of leptin.[58] Different mechanisms may be involved in the pathogenesis of leptin resistance in aged and obese populations. Since resting baroreflex function is often impaired during

176

aging,[19, 88] the loss of sensitivity to leptin for baroreflex modulation in older Sprague-Dawley rats may be the result of endogenous suppression of the baroreflex by elevated circulating leptin levels. Furthermore, long-term reductions in brain RAS activity result in age-related preservation of sensitivity to the cardiovascular actions of leptin, possibly due to the maintenance of low circulating leptin levels in these animals during aging.

To test the hypothesis that endogenous leptin contributes to baroreflex dysfunction in conditions associated with elevated leptin levels, we determined the effect of NTS administration of a triple mutant leptin antagonist to baroreflex sensitivity in older Sprague-Dawley rats. Previous studies show that NTS administration of an AT_1 receptor antagonist facilitates baroreflex sensitivity in Sprague-Dawley rats providing evidence that endogenous angiotensin II impairs baroreflex function.[11, 77] Similarly, NTS administration of a leptin antagonist should determine the endogenous leptin tone contributing to baroreflex suppression. The leptin antagonist used in our studies binds the leptin receptor and prevents activation of associated intracellular signaling pathways.[97] This antagonist blocks the anorexic effects of leptin, prevents leptin-mediated STAT3 phosphorylation and reverses hypertension due to central over-expression of leptin in rats.[89, 97] In preliminary studies, NTS microinjection of a leptin antagonist improves baroreflex sensitivity in older Sprague-Dawley rats at 60 minutes after the injection (Appendix, Figure 3) suggesting that leptin endogenous to the NTS contributes to baroreflex suppression during aging. There is no effect of NTS administration of the leptin antagonist on baroreflex sensitivity in younger Sprague-Dawley rats with normal circulating leptin levels (Appendix, Figure 4).

Collectively, these data suggest that aging is associated with resistance to the cardiovascular actions of leptin. The age-related loss of leptin modulation of baroreflex function is associated with endogenous suppression of the baroreflex by elevated leptin levels. These findings suggest that reduction of endogenous leptin may be a novel therapeutic approach to improve baroreflex sensitivity in conditions associated with elevated leptin levels, including aging, obesity and cardiovascular disease. Furthermore, since blockade of angiotensin II actions maintains low circulating leptin levels in humans and rodents,[40, 45, 49] ACE inhbitors and AT_1 receptor blockers may be effective therapeutics to preserve leptin sensitivity in conditions exhibiting hyperleptinemia.

8. PTP1B and Baroreflex Function

Reduction of endogenous leptin levels may also serve to maintain metabolic sensitivity to leptin by decreasing negative regulators of leptin signaling.[22, 43] Elevations in circulating leptin, insulin and angiotensin II all increase hypothalamic levels of SOCS3 and PTP1B, negative regulators of intracellular signaling.[9, 59, 92] Genetic deletion of PTP1B enhances insulin and leptin sensitivity and prevents diet-induced obesity in mice implicating PTP1B in the pathogenesis of insulin and leptin resistance.[29, 36, 96] Recently, PTP1B inhibitors have been developed as a novel treatment to improve insulin and leptin sensitization in patients with obesity and type II diabetes.[51]

Although inhibition of PTP1B is beneficial for energy balance, recent studies suggest that genetic deletion of PTP1B also produces elevations in arterial pressure and sympathetic nervous system activity,[7] cardiovascular effects often preceded by baroreflex dysfunction. Therefore, we determined the effect of PTP1B inhibition on baroreflex

sensitivity for control of heart rate at the level of the NTS. We also administered the PTP1B inhibitor to transgenic ASrAOGEN and (mRen2)27 rats with high or low resting baroreflex sensitivity,[26] respectively, to determine the endogenous contribution of this phosphatase to resting baroreflex function. Acute inhibition of PTP1B within the NTS impairs baroreflex sensitivity for control of heart rate in control Sprague-Dawley rats, implicating that PTP1B activity is required for normal baroreflex function.

In the present study, we show that ASrAOGEN rats have greater PTP1B tone within the NTS, consistent with the higher resting baroreflex sensitivity in these animals. The stimulus for increased PTP1B tone is not currently understood, however, there is evidence for upregulation of leptin receptors and PI3K signaling pathways in dorsal medulla of ASrAOGEN rats. Thus, the elevation of PTP1B tone within the NTS of ASrAOGEN rats may be a compensatory mechanism to counteract the upregulation of leptin receptors and associated enhanced sensitivity of these animals to angiotensin II and leptin. Furthermore, recent studies provide evidence for interactions between angiotensin peptides and PTP1B as angiotensin-(1-7) stimulates the expression of various phosphatases in cultured cells.[37, 39] However, the regulation of PTP1B by angiotensin peptides was not examined in these studies. The present findings extend these observations and implicate angiotensin peptides in the modulation of PTP1B activity. Since ASrAOGEN rats have sustained angiotensin-(1-7) tone for baroreflex sensitivity,[78] a role for angiotensin-(1-7) in the upregulation of PTP1B is proposed.

In contrast to ASrAOGEN rats, (mRen2)27 rats have lower PTP1B tone within the NTS, associated with low resting baroreflex sensitivity and angiotensin-(1-7) tone for baroreflex modulation[24]. The presence of low PTP1B tone is unexpected as these

animals have increased angiotensin II actions within the brain[14, 81] as well as enhanced activation of the PI3K pathway for suppression of baroreflex sensitivity.[55] Acute administration of the PTP1B inhibitor to (mRen2)27 rats may not have been sufficient to overcome the effects of long-term elevations in brain angiotensin II. Interestingly, central replacement of angiotensin-(1-7) increases PTP1B gene expression in (mRen2)27 rats[38] suggesting that the low angiotensin-(1-7) tone within the NTS of these animals may result in reduced PTP1B levels.

The differential PTP1B tone for modulation of baroreflex function in Sprague-Dawley, ASrAOGEN and (mRen2)27 rats was not accompanied by differences in dorsal medullary mRNA levels of protein tyrosine phosphatase non-receptor 1 gene which encodes PTP1B. Thus, the functional differences produced by PTP1B inhibition are not due to altered transcription of PTP1B, but may reflect different levels of activity of the phosphatase or the counterbalancing kinases. Collectively, these data suggest that that PTP1B tone within the NTS is necessary for normal baroreflex function. In addition, alterations in brain RAS activity are associated with differential PTP1B tone for baroreflex modulation.

The mechanisms underlying PTP1B-mediated impairments in baroreflex sensitivity are not currently understood. It is well established that PTP1B is involved in the direct dephosphorylation of tyrosine residues on insulin and leptin receptors and/or signaling components to terminate the intracellular kinase signaling pathways used by these peptides.[29, 70, 96] PTP1B is also activated by angiotensin II in vascular smooth muscle cells to block insulin signaling and promote insulin resistance, however, the relationship between PTP1B and angiotensin II is less clear.[59] Leptin, insulin and

angiotensin II all independently enhance sympathetic outflow and impair baroreflex sensitivity at central sites of action.[11, 64, 74] Therefore, PTP1B may serve as a molecular brake to prevent the activation of kinase signaling pathways initiated by these peptides for cardiovascular actions. Conversely, inhibition of PTP1B may result in unrestrained kinase signaling to promote deleterious cardiovascular effects, including baroreflex dysfunction.

Collectively, these studies suggest that inhibition of PTP1B within the NTS impairs the vagally-mediated baroreflex sensitivity for control of heart rate. These data are consistent with observations that genetic deletion of PTP1B elevates arterial pressure and sympathetic activity.[7] Maintenance of PTP1B tone may be a novel mechanism to prevent baroreflex dysfunction associated with hypertension. However, the utility of PTP1B to preserve baroreflex sensitivity may be confounded by the negative metabolic outcomes associated with elevations in PTP1B tone. Future studies will need to examine the mechanisms by which PTP1B regulates cardiovascular versus metabolic signaling to determine whether inhibition of PTP1B is a viable strategy to target restoration of baroreflex function without causing adverse metabolic side effects. The continued development of PTP1B inhibitors for the treatment of obesity and type II diabetes necessitates further studies to determine the long-term consequences of this pharmacotherapy on cardiovascular function. Since these patient populations exhibit elevations in insulin, leptin and angiotensin II levels, PTP1B inhibition may result in more pronounced adverse cardiovascular outcomes including exacerbation of hypertension. Indeed, while there was no effect of acute PTP1B inhibition on baroreflex

function in hypertensive (mRen2)27 rats during our studies, data at 1 hour after the injection shows a trend for elevations in arterial pressure in these animals.

In addition to possible negative cardiovascular effects, the development of successful PTP1B inhibitors has been limited by a lack of cell permeability and phosphatase specificity.[51, 93, 98] Since PTP1B resides in the endoplasmic reticulum,[35] effective inhibitors must permeate the cellular membrane. Furthermore, traditional PTP1B inhibitors bind the well conserved active catalytic site of the phosphatase domain resulting in a lack of substrate specificity.[51] For example, the closely related T cell phosphatase (TC-PTP) shares 80% homology to PTP1B in the catalytic phosphatase domain and is involved in regulation of T-cell activation.[21] Therefore, inhibition of TC-PTP results in adverse effects on the immune system.[95] The majority of PTP1B inhibitors have not achieved selectivity over TC-PTP. Furthermore, since PTP1B regulates multiple intracellular signaling pathways involving epidermal growth factor receptor,[33] cadherin,[4] integrin,[3] and cell cycle regulation,[79] inhibition of this phosphatase may produce unwanted side effects.

Recently, a small molecule inhibitor of PTP1B was designed that reversibly binds a second phosphotyrosine-binding site adjacent to the active site.[93] The sulfanamido-benzbarone inhibitor used in our studies (Compound II) binds an allosteric site that is not conserved among phosphatases and stabilizes the inactive conformation of PTP1B.[93] Compound II shows selectivity for PTP1B over closely related TC-PTP and leukocyte antigen-related protein phosphatases, is cell-permeable and binds the allosteric site with high affinity ($IC_{50} = 22$ μM).[93] Compound II increases phosphorylation of IRS-1 and Akt in chinese hamster ovary cells with no apparent cytotoxic effects providing evidence that

inhibition of PTP1B with this inhibitor results in insulin mimetic effects.[93] *In vivo*, central infusion of Compound II enhances the anorectic effects of leptin in SD rats.[86] In the present study, impairments in baroreflex sensitivity produced by PTP1B injection occurred as early as 10 minutes after injection, providing evidence for a rapid, direct effect. In addition, there was minimal effect of PTP1B inhibition on chemoreflex responses or resting arterial pressure and heart rate suggesting that the impairments in baroreflex sensitivity are not likely due to non-specific effects. To date, there have been no apparent cytotoxic or negative side effects associated with this inhibitor. Since studies using this inhibitor are limited, the duration of action for PTP1B inhibition is currently unknown.

9. General Limitations of Studies

Anesthesia

Microinjection studies have been previously performed in the conscious state in many brain areas,[63, 66] however, a fixed pipette and flexion of the neck can damage medullary tissue and eliminate baroreflex responses. Thus, the majority of NTS microinjection studies require restraint for the injection or are performed under anesthesia. In general, anesthesia can significantly affect autonomic nervous system function and suppress baroreflex sensitivity for control of heart rate.[83] Combination urethane/alpha-chloralose anesthesia is widely used to study neural control of the circulation as it preserves autonomic function to a greater extent relative to other anesthetics. In cats, administration of either urethane or alpha-chloralose anesthesia produces no changes in arterial pressure and heart rate over a 5 hour period.[61] However,

urethane-chloralose anesthesia induces 5-fold increases in plasma renin activity as well as circulating angiotensin II levels which may effect autonomic nervous system activity and cardiovascular function.[83]   As evidenced in the present studies, anesthesia alters arterial pressure and heart rate in transgenic ASrAOGEN and (mRen2)27 rats with no overall effect in Sprague-Dawley rats.[77] Under urethane-chloralose anesthesia, ASrAOGEN rats exhibit increases in arterial pressure and heart rate suggestive of an anesthesia-induced activation of the sympathetic nervous system.[78] In contrast, hypertensive (mRen2)27 rats have lower mean arterial pressure under this anesthesia.[24] Since urethane inhibits over-activity of the sympathetic nervous system, this finding provides further evidence for enhanced sympathetic activity in (mRen2)27 rats.[24]

Baroreflex sensitivity for control of heart rate is reduced in anesthetized relative to conscious rats. Regardless of this reduction in sensitivity or the change in resting arterial pressure, the relative level of baroreflex function under anesthesia mimics the patterns observed in conscious animals.[24, 27, 78] For example, conscious ASrAOGEN rats have higher and (mRen2)27 rats have lower resting baroreflex sensitivity relative to Sprague-Dawley rats, respectively.[27]   This pattern is still observed under urethane-chloralose anesthesia with ASrAOGEN rats having similar or higher BRS and (mRen2)27 rats having lower BRS relative to Sprague-Dawley rats.[24, 78] Moreover, the relative contribution of angiotensin II and angiotensin-(1-7) to baroreflex function at the level of the NTS appear maintained in conscious versus anesthetized Sprague-Dawley rats.[66, 77] These data confirm that baroreflex sensitivity is modulated independent of changes in arterial pressure. Since anesthetics produce cardiorespiratory depression,[80] anesthetized animals were supplemented with a mixture of room air and oxygen to

stabilize ventilation. The same percentage and flow rate of oxygen was used in all microinjection studies to control for respiratory variability among animals.

Microinjection Techniques

Within the NTS a heterogenous population of neurons exists, including afferent neurons, local interneurons and output neurons.[2] Determining which neurons are accessed by the spread of injectate is a consistent problem for microinjection studies. Since injections may spread to neuronal cell bodies and presynaptic vagal afferent fibers within the NTS, it is unclear which elements mediate baroreflex responses. Angiotensin II AT_1 receptors influence baroreflex sensitivity primarily through vagal afferent fibers,[28] however, the effects of angiotensin II and angiotensin-(1-7) on resting pressure are independent of tonic vagal afferent input.[12, 28] The components mediating baroreflex function in response to leptin and other neuropeptides are currently unknown.

The spread of the microinjections may also access other nuclei in close association with the NTS including the dmnX and area postrema. Several studies have addressed these issues by determining the spread of drug injections within the NTS. Andresen and colleagues report that injection of 100 nL of a typical drug spans a 300 micron radius within the NTS with a concentration gradient centered at the pipette tip.[2] However, even at small volumes it is possible that the injection will diffuse beyond a single subnucleus within the NTS. In addition, Campagnole-Santos and colleagues illustrate the spread of 100 nL of monoiodinated Sar-Thr Ang II within the NTS is limited to 462 ± 175 um rostrocaudal, 750 ± 76 um dorsoventral and 600 ± 83 um mediolateral, an area mostly confined to the NTS.[11] Although injections of this volume may reach the dmnX, functional assessments show that a 50 nL injection of an AT_1

receptor antagonist into the dmnX does not alter baroreflex responses within the NTS.[34] The injections performed in our studies utilized a volume of 60 or 120 nL and thus should be confined within the NTS. These injections are further functionally constrained by assessing effects on the evoked baro- or chemo-reflexes.

Transgenic Rat Models

Transgenic rodents with under- or over-expression of RAS components either systemically or in specific tissue systems have been useful models to investigate the role of the RAS to physiology and pathophysiology. However, it is generally unknown which systems are affected by the deletion or addition of the transgene over the long-term or developmentally. Therefore, the ability to translate our findings in transgenic rats to other animal models or humans may be limited. Previous studies in our laboratory have shown that memory and plasma levels of insulin, leptin, IGF-1 and angiotensin peptides are similar at 16-weeks of age between Sprague-Dawley, ASrAOGEN and (mRen2)27 rats.[45] Furthermore, the phenotype of ASrAOGEN rats resembles animals treated chronically with RAS inhibitors beginning at 6 months of age.[40] Together, these data suggest that transgene insertion does not result in developmental abnormalities in ASrAOGEN or (mRen2)27 rats with respect to the variables studied.

The (mRen2)27 rat is a model used to mimic essential hypertension in humans. The hypertension is of monogenetic origin, arising from the insertion of the mouse renin gene with consequent elevations in brain angiotensin II levels.[14, 69, 81] Thus, these animals are important in understanding the contribution of the brain RAS to mechanisms of hypertension. In the present study, we employed (mRen2)27 rats to investigate the contribution of PTP1B tone to baroreflex modulation and saw no effect of PTP1B

inhibition in these animals. The lack of effect of PTP1B inhibition on baroreflex function in (mRen2)27 rats may represent a general failure of acute application of antagonists to uncover the role of the peptides within the NTS due to long-term alterations in angiotensin peptides as well as hypertension in these animals. Under urethane/chloralose anesthesia, (mRen2)27 rats exhibit similar blood pressure relative to Sprague-Dawley rats, consistent with the contribution of central sympathetic activation to arterial pressure in these animals.[24] Regardless of beneficial changes in arterial pressure, the baroreflex remains suppressed in these animals under anesthesia suggesting that baroreflex modulation is independent of the resting level of pressure.

10. Concluding Statements

Pharmacological agents that reduce peripheral or central sympathetic nervous system activity are often advocated in the treatment of conditions associated with hypertension, due to the sympathetic activation observed in these patients.[8] However, sympatholytic agents are associated with an increased incidence of adverse metabolic and cardiovascular effects.[8, 71] In addition to sympathetic activation, all emerging and established risk factors for cardiovascular disease are associated with reduced vagal function.[88] Baroreflex sensitivity for control of heart rate, a measure of vagal function, is often impaired in conditions associated with hypertension, including stroke, type II diabetes, aging and obesity.[42, 88] Recently, restoration of baroreflex function was shown to be a novel target for the prevention of stroke in rodents.[54] Thus, targeting vagal function may be a novel avenue to treat cardiovascular and cerebrovascular diseases associated with hypertension. Uncovering factors that modulate baroreflex sensitivity is

critical for the development of pharmacotherapies to preserve baroreflex function. The present findings provide novel mechanisms to preserve baroreflex function in aging and obesity. It is important to note, that other factors not examined in the present study may act centrally to contribute to alterations in baroreflex function including insulin and reactive oxygen species.

Recent studies suggest that aging is associated with alterations in the brain RAS in which there is reduced angiotensin-(1-7) tone for baroreflex modulation within the NTS.[77] Whether maintenance of angiotensin-(1-7) tone within this brain region preserves baroreflex function is currently unknown. Therefore, we determined the contribution of angiotensin peptides within the NTS to the known age-related preservation of baroreflex in ASrAOGEN rats. The findings of this study implicate that maintenance of angiotensin-(1-7) tone is a novel mechanism to preserve baroreflex function during aging. These studies focus on age-related changes in baroreflex function mediated by NTS pathways that contribute to regulation of autonomic outflow. These same brain regions are involved in the regulation of energy metabolism by insulin and leptin, peptides which have been recently shown to participate in cardiovascular regulation.

Plasma leptin levels are elevated in obesity and independently in hypertension and stroke[20, 84] and exogenous leptin contributes to sympathetically-mediated elevations in arterial presure[15]. The present data extends these findings to show that leptin impairs indices of vagal function including baroreflex sensitivity for control of heart rate and heart rate variability. Leptin-mediated impairments in baroreflex sensitivity may be permissive towards the increases in arterial pressure observed in populations with elevated leptin levels. In addition, ASrAOGEN rats show enhanced sensitivity to

impairments in baroreflex sensitivity produced by exogenous leptin, associated with an upregulation of relevant leptin signaling pathway mRNA in the dorsal medulla of these animals. Since leptin and angiotensin II share similar anatomical and signaling pathways within the central nervous, there are potential sites of interaction between leptin and the brain RAS for baroreflex modulation.

Our findings further show that aging, in contrast to obesity, is associated with resistance to the cardiovascular effects of exogenous leptin for baroreflex modulation. The loss of sensitivity to exogenous leptin may be the result of increased endogenous leptin levels in older Sprague-Dawley rats contributing to baroreflex suppression. Indeed, NTS administration of a leptin antagonist improves baroreflex sensitivity in these animals. Furthermore, transgenic ASrAOGEN rats maintain sensitivity to exogenous leptin for baroreflex modulation during aging implicating that a reduction in glia-derived angiotensin peptides results in preserved leptin sensitivity, possibly in the presence of lower endogenous leptin levels. Whether we can link the preserved angiotensin-(1-7) tone in these animals with the signaling events is a subject of continued investigation. Thus, reduction of endogenous leptin or maintenance of angiotensin-(1-7), to maintain sensitivity of signaling pathways, may be novel methods to preserve baroreflex function in populations with elevated circulating leptin levels.

Despite controversy regarding the exact mechanism underlying the autonomic imbalance observed in conditions associated with hypertension, the peptides presented in these studies all participate in the regulation of autonomic outflow at central sites of action. In addition to effects on autonomic balance, leptin promotes endothelial dysfunction, oxidative stress, inflammation, vascular smooth muscle cell proliferation

and migration, atherosclerosis, angiogenesis and thrombosis all of which may provide links between leptin and cardiovascular and cerebrovascular diseases.[73] Intravenous administration of a leptin antibody reduces thrombus size and protects from arterial and venous thrombosis in lean mice.[50] Therefore, a reduction of endogenous leptin may be protective against cardiovascular and cerebrovascular diseases through a variety of sites and mechanisms.

Literature Cited

(1) Anderson S. Ageing and the renin-angiotensin system. *Nephrol Dial Transplant* 1997;12(6):1093-4.

(2) Andresen MC, Kunze DL. Nucleus tractus solitarius--gateway to neural circulatory control. *Annu Rev Physiol* 1994;56:93-116.

(3) Arregui CO, Balsamo J, Lilien J. Impaired integrin-mediated adhesion and signaling in fibroblasts expressing a dominant-negative mutant PTP1B. *J Cell Biol* 1998 November 2;143(3):861-73.

(4) Balsamo J, Arregui C, Leung T, Lilien J. The nonreceptor protein tyrosine phosphatase PTP1B binds to the cytoplasmic domain of N-cadherin and regulates the cadherin-actin linkage. *J Cell Biol* 1998 October 19;143(2):523-32.

(5) Baltatu O, Janssen BJ, Bricca G, Plehm R, Monti J, Ganten D, Bader M. Alterations in blood pressure and heart rate variability in transgenic rats with low brain angiotensinogen. *Hypertension* 2001;37(2 Part 2):408-13.

(6) Basso N, Paglia N, Stella I, de Cavanagh EM, Ferder L, Rosario Lores AM, Inserra F. Protective effect of the inhibition of the renin-angiotensin system on aging. *Regul Pept* 2005;128(3):247-52.

(7) Belin de Chantemele EJ, Muta K, Mintz J, Tremblay ML, Marrero MB, Fulton DJ, Stepp DW. Protein tyrosine phosphatase 1B, a major regulator of leptin-mediated control of cardiovascular function. *Circulation* 2009 September 1;120(9):753-63.

(8) Biaggioni I. Should we target the sympathetic nervous system in the treatment of obesity-associated hypertension? *Hypertension* 2008 February;51(2):168-71.

(9) Bjorbaek C, Elmquist JK, Frantz JD, Shoelson SE, Flier JS. Identification of SOCS-3 as a potential mediator of central leptin resistance. *Mol Cell* 1998 March;1(4):619-25.

(10) Caligiorne SM, Silva AQ, Fontes MA, Silva JR, Baltatu O, Bader M, Santos RA, Campagnole-Santos MJ. Baroreflex control of heart rate and renal sympathetic nerve activity in rats with low brain angiotensinogen. *Neuropeptides* 2008 April;42(2):159-68.

(11) Campagnole-Santos MJ, Diz DI, Ferrario CM. Baroreceptor reflex modulation by angiotensin II at the nucleus tractus solitarii. *Hypertension* 1988;11(2 Pt 2):I167-I171.

(12) Campagnole-Santos MJ, Diz DI, Ferrario CM. Actions of angiotensin peptides after partial denervation of the solitary tract nucleus. *Hypertension* 1990;15(2 Suppl):I34-I39.

(13) Campagnole-Santos MJ, Heringer SB, Batista EN, Khosla MC, Santos RA. Differential baroreceptor reflex modulation by centrally infused angiotensin peptides. *Am J Physiol* 1992 July;263(1 Pt 2):R89-R94.

(14) Campbell DJ, Rong P, Kladis A, Rees B, Ganten D, Skinner SL. Angiotensin and bradykinin peptides in the TGR(mRen-2)27 rat. *Hypertension* 1995 May;25(5):1014-20.

(15) Carlyle M, Jones OB, Kuo JJ, Hall JE. Chronic cardiovascular and renal actions of leptin: role of adrenergic activity. *Hypertension* 2002 February;39(2 Pt 2):496-501.

(16) Casto R, Phillips M. Angiotensin II attenuates baroreflexes at nucleus tractus solitarius of rats. Am.J.Physiol 250, 193-198. 1986. Ref Type: Generic

(17) Cerutti C, Barres C, Paultre C. Baroreflex modulation of blood pressure and heart rate variabilities in rats: assessment by spectral analysis. *Am J Physiol* 1994 May;266(5 Pt 2):H1993-H2000.

(18) Chaves GZ, Caligiorne SM, Santos RA, Khosla MC, Campagnole-Santos MJ. Modulation of the baroreflex control of heart rate by angiotensin-(1-7) at the nucleus tractus solitarii of normotensive and spontaneously hypertensive rats. *J Hypertens* 2000;18(12):1841-8.

(19) Cleophas TJ, van MR. Age-related decline in autonomic control of blood pressure: implications for the pharmacological management of hypertension in the elderly. *Drugs Aging* 2003;20(5):313-9.

(20) Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med* 1996 February 1;334(5):292-5.

(21) Cool DE, Tonks NK, Charbonneau H, Walsh KA, Fischer EH, Krebs EG. cDNA isolated from a human T-cell library encodes a member of the protein-tyrosine-phosphatase family. *Proc Natl Acad Sci U S A* 1989 July;86(14):5257-61.

(22) Correia ML, Rahmouni K. Role of leptin in the cardiovascular and endocrine complications of metabolic syndrome. *Diabetes Obes Metab* 2006 November;8(6):603-10.

(23) Couto AS, Baltatu O, Santos RA, Ganten D, Bader M, Campagnole-Santos MJ. Differential effects of angiotensin II and angiotensin-(1-7) at the nucleus tractus

solitarii of transgenic rats with low brain angiotensinogen. *J Hypertens* 2002;20(5):919-25.

(24) Diz DI, Garcia-Espinosa MA, Gallagher PE, Ganten D, Ferrario CM, Averill DB. Angiotensin-(1-7) and baroreflex function in nucleus tractus solitarii of (mRen2)27 transgenic rats. *J Cardiovasc Pharmacol* 2008 June;51(6):542-8.

(25) Diz DI, Garcia-Espinosa MA, Gegick S, Tommasi EN, Ferrario CM, Ann TE, Chappell MC, Gallagher PE. Injections of angiotensin-converting enzyme 2 inhibitor MLN4760 into nucleus tractus solitarii reduce baroreceptor reflex sensitivity for heart rate control in rats. *Exp Physiol* 2008 May;93(5):694-700.

(26) Diz DI, Jessup JA, Westwood BM, Bosch SM, Vinsant S, Gallagher PE, Averill DB. Angiotensin peptides as neurotransmitters/neuromodulators in the dorsomedial medulla. *Clin Exp Pharmacol Physiol* 2002;29(5-6):473-82.

(27) Diz DI, Kasper SO, Sakima A, Ferrario CM. Aging and the brain renin-angiotensin system: insights from studies in transgenic rats. *Cleve Clin J Med* 2007;74 Suppl 1:S95-S98.

(28) Diz DI, Westwood B, Averill DB. AT(1) antisense distinguishes receptors mediating angiotensin II actions in solitary tract nucleus. *Hypertension* 2001;37(5):1292-7.

(29) Elchebly M, Payette P, Michaliszyn E, Cromlish W, Collins S, Loy AL, Normandin D, Cheng A, Himms-Hagen J, Chan CC, Ramachandran C, Gresser MJ, Tremblay ML, Kennedy BP. Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. *Science* 1999 March 5;283(5407):1544-8.

(30) Elmquist JK, Bjorbaek C, Ahima RS, Flier JS, Saper CB. Distributions of leptin receptor mRNA isoforms in the rat brain. *J Comp Neurol* 1998 June 15;395(4):535-47.

(31) Fauvel JP, Cerutti C, Mpio I, Ducher M. Aging process on spectrally determined spontaneous baroreflex sensitivity: a 5-year prospective study. *Hypertension* 2007;50(3):543-6.

(32) Ferrari AU, Radaelli A, Centola M. Invited review: aging and the cardiovascular system. *J Appl Physiol* 2003;95(6):2591-7.

(33) Flint AJ, Tiganis T, Barford D, Tonks NK. Development of "substrate-trapping" mutants to identify physiological substrates of protein tyrosine phosphatases. *Proc Natl Acad Sci U S A* 1997 March 4;94(5):1680-5.

(34) Fow JE, Averill DB, Barnes KL. Mechanisms of angiotensin-induced hypotension and bradycardia in the medial solitary tract nucleus. *Am J Physiol* 1994;267(1 Pt 2):H259-H266.

(35) Frangioni JV, Beahm PH, Shifrin V, Jost CA, Neel BG. The nontransmembrane tyrosine phosphatase PTP-1B localizes to the endoplasmic reticulum via its 35 amino acid C-terminal sequence. *Cell* 1992 February 7;68(3):545-60.

(36) Fruhbeck G. Intracellular signalling pathways activated by leptin. *Biochem J* 2006 January 1;393(Pt 1):7-20.

(37) Gallagher PE, Ferrario CM, Tallant EA. MAP kinase/phosphatase pathway mediates the regulation of ACE2 by angiotensin peptides. *Am J Physiol Cell Physiol* 2008 November;295(5):C1169-C1174.

(38) Garcia-Espinosa MA, Shaltout HA, Gallagher PE, Chappell MC, Ferrario CM, Ganten D, Reudelhuber TL, Diz DI. Angiotensin-(1-7) Replacement via Gene Transfer in the Cisterna Magna Lowers Blood Pressure and Improves Baroreflex Function in Transgenic (mRen2)27 Rats. *Hypertension* 2008; 52[4], E36.

(39) Gava E, Samad-Zadeh A, Zimpelmann J, Bahramifarid N, Kitten GT, Santos RA, Touyz RM, Burns KD. Angiotensin-(1-7) activates a tyrosine phosphatase and inhibits glucose-induced signalling in proximal tubular cells. *Nephrol Dial Transplant* 2009 June;24(6):1766-73.

(40) Gilliam-Davis S, Payne VS, Kasper SO, Tommasi EN, Robbins ME, Diz DI. Long-term AT1 receptor blockade improves metabolic function and provides renoprotection in Fischer-344 rats. *Am J Physiol Heart Circ Physiol* 2007 September;293(3):H1327-H1333.

(41) Goossens GH, Blaak EE, van Baak MA. Possible involvement of the adipose tissue renin-angiotensin system in the pathophysiology of obesity and obesity-related disorders. *Obes Rev* 2003 February;4(1):43-55.

(42) Grassi G, Seravalle G, Dell'Oro R, Turri C, Bolla GB, Mancia G. Adrenergic and reflex abnormalities in obesity-related hypertension. *Hypertension* 2000 October;36(4):538-42.

(43) Hall JE, Hildebrandt DA, Kuo J. Obesity hypertension: role of leptin and sympathetic nervous system. *Am J Hypertens* 2001 June;14(6 Pt 2):103S-15S.

(44) Huang HN, Lu PJ, Lo WC, Lin CH, Hsiao M, Tseng CJ. In situ Akt phosphorylation in the nucleus tractus solitarii is involved in central control of blood pressure and heart rate. *Circulation* 2004 October 19;110(16):2476-83.

(45) Kasper SO, Carter CS, Ferrario CM, Ganten D, Ferder LF, Sonntag WE, Gallagher PE, Diz DI. Growth, metabolism, and blood pressure disturbances during aging in transgenic rats with altered brain renin-angiotensin systems. *Physiol Genomics* 2005;23(3):311-7.

(46) Kasper SO, Ferrario CM, Ganten D, Diz DI. Central depletion of angiotensinogen is associated with elevated AT1 receptors in the SFO and PVN. *Neurotox Res* 2004;6(4):259-65.

(47) Kasper SO, Ferrario CM, Ganten D, Diz DI. Rats with low brain angiotensinogen do not exhibit insulin resistance during early aging. *Endocrine* 2006 October;30(2):167-74.

(48) Kim S, Whelan J, Claycombe K, Reath DB, Moustaid-Moussa N. Angiotensin II increases leptin secretion by 3T3-L1 and human adipocytes via a prostaglandin-independent mechanism. *J Nutr* 2002 June;132(6):1135-40.

(49) Koh KK, Quon MJ, Han SH, Lee Y, Kim SJ, Koh Y, Shin EK. Distinct vascular and metabolic effects of different classes of anti-hypertensive drugs. Int J Cardiol. In press 2008.

(50) Konstantinides S, Schafer K, Neels JG, Dellas C, Loskutoff DJ. Inhibition of endogenous leptin protects mice from arterial and venous thrombosis. *Arterioscler Thromb Vasc Biol* 2004 November;24(11):2196-201.

(51) Koren S, Fantus IG. Inhibition of the protein tyrosine phosphatase PTP1B: potential therapy for obesity, insulin resistance and type-2 diabetes mellitus. *Best Pract Res Clin Endocrinol Metab* 2007 December;21(4):621-40.

(52) Leong PK, Devillez A, Sandberg MB, Yang LE, Yip DK, Klein JB, McDonough AA. Effects of ACE inhibition on proximal tubule sodium transport. *Am J Physiol Renal Physiol* 2006 April;290(4):F854-F863.

(53) Leyva F, Anker SD, Egerer K, Stevenson JC, Kox WJ, Coats AJ. Hyperleptinaemia in chronic heart failure. Relationships with insulin. *Eur Heart J* 1998 October;19(10):1547-51.

(54) Liu AJ, Ma XJ, Shen FM, Liu JG, Chen H, Su DF. Arterial baroreflex: a novel target for preventing stroke in rat hypertension. *Stroke* 2007 June;38(6):1916-23.

(55) Logan E, Diz DI, Averill DB. Inhibition of the PI3 Kinase Signal Transduction Pathway in Nucleus Tractus Solitarii of (mRen2)27 Transgenic Rats Improves Baroreceptor Sensitivity. *FASEB J* 2008; 22.

(56) Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S. Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat Med* 1995 November;1(11):1155-61.

(57) Mark AL, Agassandian K, Morgan DA, Liu X, Cassell MD, Rahmouni K. Leptin signaling in the nucleus tractus solitarii increases sympathetic nerve activity to the kidney. *Hypertension* 2009 February;53(2):375-80.

(58) Mark AL, Correia ML, Rahmouni K, Haynes WG. Selective leptin resistance: a new concept in leptin physiology with cardiovascular implications. *J Hypertens* 2002 July;20(7):1245-50.

(59) Marrero MB, Fulton D, Stepp D, Stern DM. Angiotensin II-induced insulin resistance and protein tyrosine phosphatases. *Arterioscler Thromb Vasc Biol* 2004 November;24(11):2009-13.

(60) Marsh AJ, Fontes MA, Killinger S, Pawlak DB, Polson JW, Dampney RA. Cardiovascular responses evoked by leptin acting on neurons in the ventromedial and dorsomedial hypothalamus. *Hypertension* 2003 October;42(4):488-93.

(61) Matsukawa K, Ninomiya I. Anesthetic effects on tonic and reflex renal sympathetic nerve activity in awake cats. *Am J Physiol* 1989 February;256(2 Pt 2):R371-R378.

(62) Matsumura K, Averill DB, Ferrario CM. Angiotensin II acts at AT1 receptors in the nucleus of the solitary tract to attenuate the baroreceptor reflex. *Am J Physiol* 1998;275(5 Pt 2):R1611-R1619.

(63) Mayorov DN, Head GA. Influence of rostral ventrolateral medulla on renal sympathetic baroreflex in conscious rabbits. *Am J Physiol Regul Integr Comp Physiol* 2001 February;280(2):R577-R587.

(64) McKernan AM, Calaresu FR. Insulin microinjection into the nucleus tractus solitarii of the rat attenuates the baroreceptor reflex. *J Auton Nerv Syst* 1996 November 6;61(2):128-38.

(65) Mercer JG, Moar KM, Hoggard N. Localization of leptin receptor (Ob-R) messenger ribonucleic acid in the rodent hindbrain. *Endocrinology* 1998 January;139(1):29-34.

(66) Michelini LC, Bonagamba LG. Angiotensin II as a modulator of baroreceptor reflexes in the brainstem of conscious rats. *Hypertension* 1990;15(2 Suppl):I45-I50.

(67) Monti J, Schinke M, Bohm M, Ganten D, Bader M, Bricca G. Glial angiotensinogen regulates brain angiotensin II receptors in transgenic rats TGR(ASrAOGEN). *Am J Physiol Regul Integr Comp Physiol* 2001;280(1):R233-R240.

(68) Morrison CD, White CL, Wang Z, Lee SY, Lawrence DS, Cefalu WT, Zhang ZY, Gettys TW. Increased hypothalamic protein tyrosine phosphatase 1B contributes to leptin resistance with age. *Endocrinology* 2007 January;148(1):433-40.

(69) Mullins JJ, Peters J, Ganten D. Fulminant hypertension in transgenic rats harbouring the mouse Ren-2 gene. *Nature* 1990 April 5;344(6266):541-4.

(70) Myers MP, Andersen JN, Cheng A, Tremblay ML, Horvath CM, Parisien JP, Salmeen A, Barford D, Tonks NK. TYK2 and JAK2 are substrates of protein-tyrosine phosphatase 1B. *J Biol Chem* 2001 December 21;276(51):47771-4.

(71) Pischon T, Sharma AM. Optimizing blood pressure control in the obese patient. *Curr Hypertens Rep* 2002 October;4(5):358-62.

(72) Rahmouni K, Correia ML, Haynes WG, Mark AL. Obesity-associated hypertension: new insights into mechanisms. *Hypertension* 2005 January;45(1):9-14.

(73) Rahmouni K, Haynes WG. Leptin and the cardiovascular system. *Recent Prog Horm Res* 2004;59:225-44.

(74) Rahmouni K, Haynes WG, Morgan DA, Mark AL. Intracellular mechanisms involved in leptin regulation of sympathetic outflow. *Hypertension* 2003 March;41(3 Pt 2):763-7.

(75) Rahmouni K, Morgan DA. Hypothalamic arcuate nucleus mediates the sympathetic and arterial pressure responses to leptin. *Hypertension* 2007 March;49(3):647-52.

(76) Sakai K, Chapleau MW, Morimoto S, Cassell MD, Sigmund CD. Differential modulation of baroreflex control of heart rate by neuron- vs. glia-derived angiotensin II. *Physiol Genomics* 2004;20(1):66-72.

(77) Sakima A, Averill DB, Gallagher PE, Kasper SO, Tommasi EN, Ferrario CM, Diz DI. Impaired heart rate baroreflex in older rats: role of endogenous angiotensin-(1-7) at the nucleus tractus solitarii. *Hypertension* 2005;46(2):333-40.

(78) Sakima A, Averill DB, Kasper SO, Jackson L, Ganten D, Ferrario CM, Gallagher PE, Diz DI. Baroreceptor reflex regulation in anesthetized transgenic rats with low glia-derived angiotensinogen. *Am J Physiol Heart Circ Physiol* 2007;292(3):H1412-H1419.

(79) Schievella AR, Paige LA, Johnson KA, Hill DE, Erikson RL. Protein tyrosine phosphatase 1B undergoes mitosis-specific phosphorylation on serine. *Cell Growth Differ* 1993 April;4(4):239-46.

(80) Sear JW. Practical treatment recommendations for the safe use of anaesthetics. *Drugs* 1992 January;43(1):54-68.

(81) Senanayake PD, Moriguchi A, Kumagai H, Ganten D, Ferrario CM, Brosnihan KB. Increased expression of angiotensin peptides in the brain of transgenic hypertensive rats. *Peptides* 1994;15(5):919-26.

(82) Shaltout HA, Abdel-Rahman AA. Mechanism of fatty acids induced suppression of cardiovascular reflexes in rats. *J Pharmacol Exp Ther* 2005 September;314(3):1328-37.

(83) Shimokawa A, Kunitake T, Takasaki M, Kannan H. Differential effects of anesthetics on sympathetic nerve activity and arterial baroreceptor reflex in chronically instrumented rats. *J Auton Nerv Syst* 1998 August 6;72(1):46-54.

(84) Sierra-Johnson J, Romero-Corral A, Lopez-Jimenez F, Gami AS, Sert Kuniyoshi FH, Wolk R, Somers VK. Relation of increased leptin concentrations to history of myocardial infarction and stroke in the United States population. *Am J Cardiol* 2007 July 15;100(2):234-9.

(85) Skurk T, van H, V, Blum WF, Hauner H. Angiotensin II promotes leptin production in cultured human fat cells by an ERK1/2-dependent pathway. *Obes Res* 2005 June;13(6):969-73.

(86) Sun T, Ye F, Ding H, Chen K, Jiang H, Shen X. Protein tyrosine phosphatase 1B regulates TGF beta 1-induced Smad2 activation through PI3 kinase-dependent pathway. *Cytokine* 2006 July;35(1-2):88-94.

(87) Tan PS, Killinger S, Horiuchi J, Dampney RA. Baroreceptor reflex modulation by circulating angiotensin II is mediated by AT1 receptors in the nucleus tractus solitarius. *Am J Physiol Regul Integr Comp Physiol* 2007 December;293(6):R2267-R2278.

(88) Thayer JF, Lane RD. The role of vagal function in the risk for cardiovascular disease and mortality. *Biol Psychol* 2007 February;74(2):224-42.

(89) Tumer N, Erdos B, Matheny M, Cudykier I, Scarpace PJ. Leptin antagonist reverses hypertension caused by leptin overexpression, but fails to normalize obesity-related hypertension. *J Hypertens* 2007 December;25(12):2471-8.

(90) Veerasingham SJ, Raizada MK. Brain renin-angiotensin system dysfunction in hypertension: recent advances and perspectives. *Br J Pharmacol* 2003 May;139(2):191-202.

(91) Vinsant S, Chappell MC, Ferrario CM, Ganten D, Diz DI. Low glial angiotensinogen is not associated with deficits in angiotensin peptides in neuronal pathways in transgenic ASrAogen rats (Abstract). FASEB J 19. 2005. Ref Type: Abstract

(92) White CL, Whittington A, Barnes MJ, Wang Z, Bray GA, Morrison CD. HF diets increase hypothalamic PTP1B and induce leptin resistance through both leptin-dependent and -independent mechanisms. *Am J Physiol Endocrinol Metab* 2009 February;296(2):E291-E299.

(93) Wiesmann C, Barr KJ, Kung J, Zhu J, Erlanson DA, Shen W, Fahr BJ, Zhong M, Taylor L, Randal M, McDowell RS, Hansen SK. Allosteric inhibition of protein tyrosine phosphatase 1B. *Nat Struct Mol Biol* 2004 August;11(8):730-7.

(94) Wojcicka G, Jamroz-Wisniewska A, Widomska S, Ksiazek M, Beltowski J. Role of extracellular signal-regulated kinases (ERK) in leptin-induced hypertension. *Life Sci* 2008 February 13;82(7-8):402-12.

(95) You-Ten KE, Muise ES, Itie A, Michaliszyn E, Wagner J, Jothy S, Lapp WS, Tremblay ML. Impaired bone marrow microenvironment and immune function in T cell protein tyrosine phosphatase-deficient mice. *J Exp Med* 1997 August 29;186(5):683-93.

(96) Zabolotny JM, ce-Hanulec KK, Stricker-Krongrad A, Haj F, Wang Y, Minokoshi Y, Kim YB, Elmquist JK, Tartaglia LA, Kahn BB, Neel BG. PTP1B regulates leptin signal transduction in vivo. *Dev Cell* 2002 April;2(4):489-95.

(97) Zhang J, Matheny MK, Tumer N, Mitchell MK, Scarpace PJ. Leptin antagonist reveals that the normalization of caloric intake and the thermic effect of food after high-fat feeding are leptin dependent. *Am J Physiol Regul Integr Comp Physiol* 2007 February;292(2):R868-R874.

(98) Zhang ZY. Protein tyrosine phosphatases: prospects for therapeutics. *Curr Opin Chem Biol* 2001 August;5(4):416-23.

CHAPTER SIX

APPENDIX DATA

SIGNALING PATHWAYS INVOLVED IN LEPTIN MODUATION OF

BAROREFLEX FUNCTION

AND

LEPTIN SENSITIVITY FOR BAROREFLEX MODULATION DURING AGING

Table I. Phosphorylation of Akt and ERK in Dorsal Medulla of Vehicle- or Leptin-Treated Sprague-Dawley and ASrAOGEN Rats

Group	N	Phosphorylated Akt	Phosphorylated ERK 1	Phosphorylated ERK 2
Sprague-Dawley Rats				
Vehicle Treated	4	0.73 ± 0.22	0.78 ± 0.18	0.78 ± 0.18
Leptin Treated	5	0.77 ± 0.10	0.94 ± 0.46	0.94 ± 0.46
ASrAOGEN Rats				
Vehicle Treated	4	0.75 ± 0.07	2.60 ± 1.40	0.64 ± 0.09
Leptin Treated	6	0.63 ± 0.14	2.05 ± 0.60	0.89 ± 0.24

Values are mean ± SEM. Dorsal medullary sections containing the microinjection site were excised at 45 or 120 minutes after the initial injection. Western blot analysis was performed to assess changes in the phosphorylation of PI3K and MAPK signaling pathway components. These data represent the level of phosphorylation for Akt, ERK1 and ERK2 normalized to beta-actin from dorsal medullary sections of vehicle- and leptin-treated animals. In preliminary studies, no changes in the phosphorylation of these components are observed in response to leptin treatment. N = number of animals, Akt = protein kinase B, ERK = extracellular signal-regulated kinase
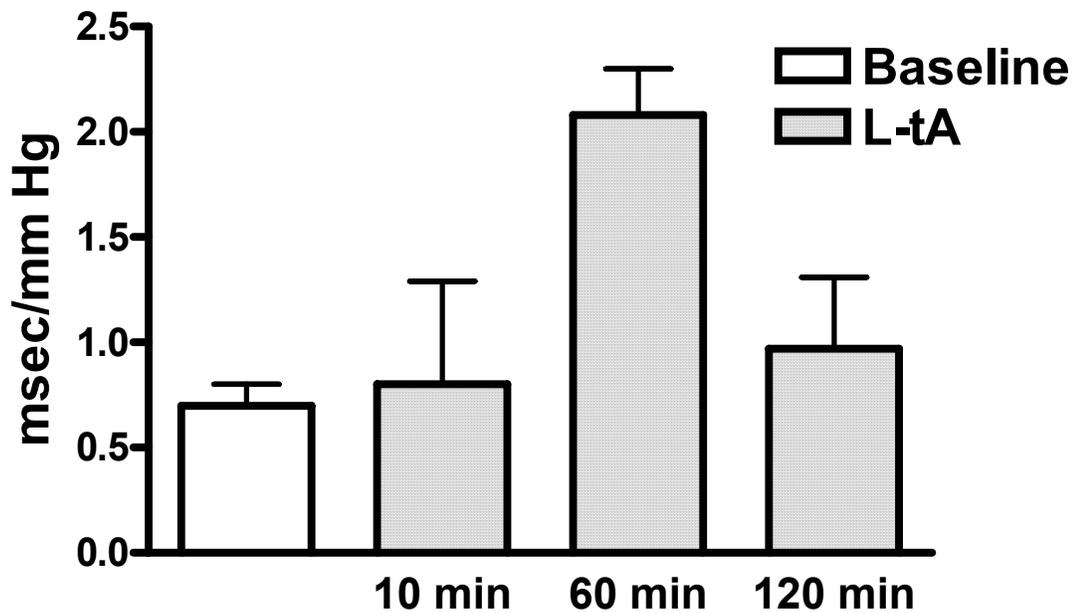
Figure 1. Leptin Sensitivity for Baroreflex Modulation in Older Sprague-Dawley and ASrAOGEN Rats. Baroreflex sensitivity for control of heart rate in response to increases in arterial pressure produced by intravenous phenylbiguanide was assessed at baseline and in response to bilateral NTS microinjection of exogenous leptin (500 fmol/60 nL) in older Sprague-Dawley and ASrAOGEN rats ranging between 64 and 80 weeks of age. A, In older Sprague-Dawley rats (n = 4), NTS microinjection of 500 fmol leptin produces no significant effect on baroreflex sensitivity. These findings are in contrast to the ~ 63% impairment in baroreflex sensitivity produced by the same dose of leptin in younger Sprague-Dawley rats. B, In older ASrAOGEN rats (n = 5), the 500 fmol dose of exogenous leptin significantly impairs baroreflex sensitivity by ~ 49%, a magnitude similar to impairments in baroreflex sensitivity produced by this dose of leptin in younger ASrAOGEN rats.
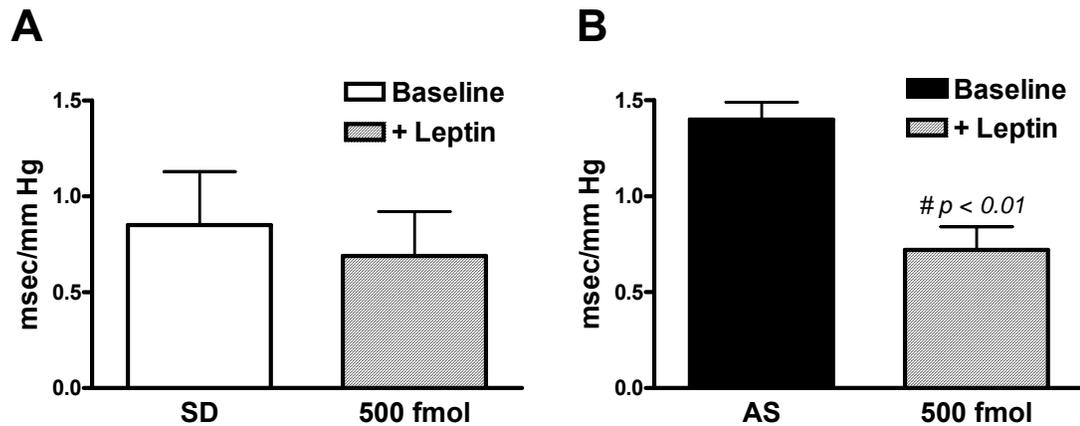
Figure 2. Leptin Receptor and p85 alpha mRNA in Dorsal Medulla of Older Sprague-Dawley and ASrAOGEN Rats. Relative gene expression of leptin receptor and phosphoinositide-3 kinase regulatory p85 alpha subunit is significantly higher in dorsal medullary tissue of 65-week old ASrAOGEN (n = 5) relative to Sprague-Dawley (n = 7) rats. SD = Sprague-Dawley, AS = ASrAOGEN, # p < 0.01 versus SD, * p < 0.05 versus SD

Leptin Receptor mRNA

p85 alpha mRNA
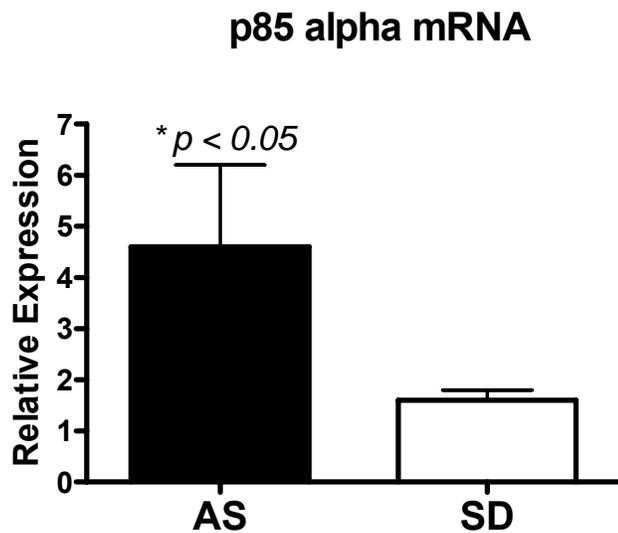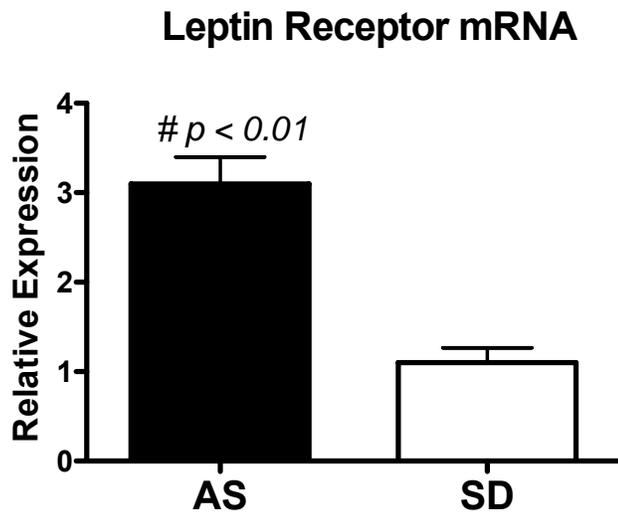
Figure 3. Leptin Antagonism within the NTS Improves Baroreflex Sensitivity for Control of Heart Rate in Older Sprague-Dawley Rats. Baroreflex sensitivity for control of heart rate in response to increases in arterial pressure produced by intravenous phenylephrine was assessed at baseline and in response to bilateral NTS microinjection of the triple-mutant leptin antagonist (L39A/D40A/F41A) in older Sprague-Dawley rats with elevated circulating leptin levels. In older Sprague-Dawley rats (n = 2, 64 to 80 weeks of age), NTS microinjection of the leptin antagonist results in a facilitation of baroreflex sensitivity at 60 minutes, with recovery to baseline values at 120 minutes after the initial injection. L-tA = leptin triple mutant antagonist

Figure 4. NTS Microinjection of a Leptin Antagonist has no Effect on Baroreflex Sensitivity in Younger Sprague-Dawley Rats. Baroreflex sensitivity for control of heart rate in response to increases in arterial pressure produced by intravenous phenylephrine was assessed at baseline and in response to bilateral NTS microinjection of the triple-mutant leptin antagonist (L39A/D40A/F41A) in younger Sprague-Dawley rats with elevated circulating leptin levels. In younger Sprague-Dawley rats (n = 3, 12 to 20 weeks of age), NTS microinjection of the leptin antagonist produces no significant changes in baroreflex sensitivity over the time course studied. LtA = leptin triple mutant antagonist
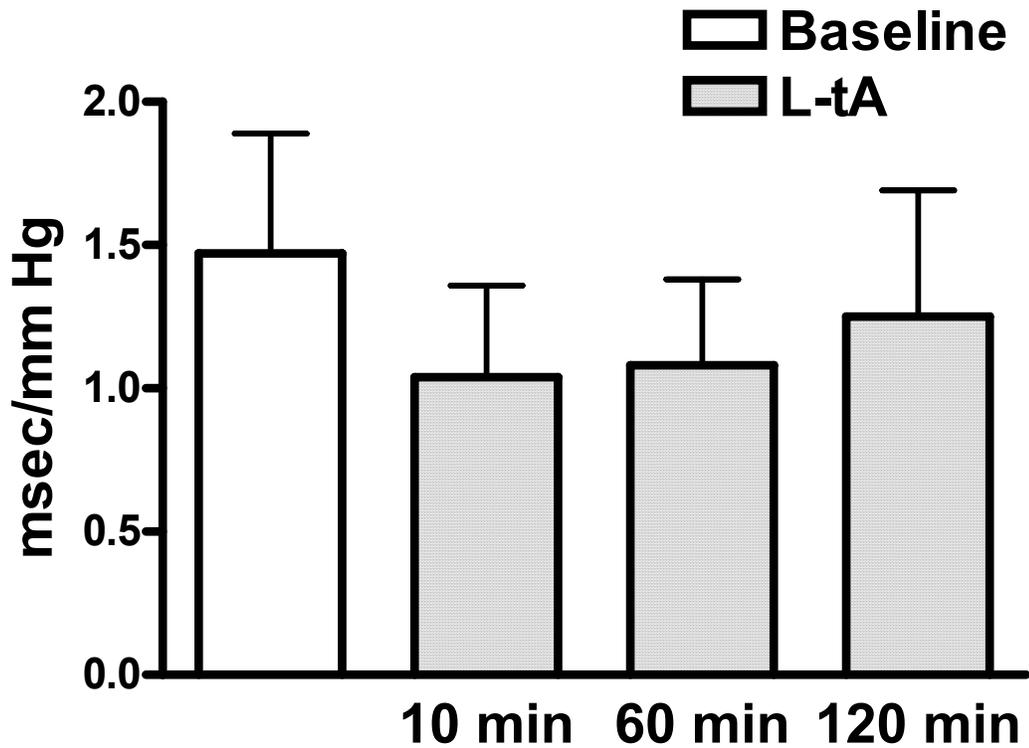
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MANUSCRIPTS:

Arnold AC, Sakima A, Ganten D, Ferrario CM, Diz DI. Modulation of Reflex Function by Endogenous Angiotensins in Older Transgenic Rats with Low Glial Angiotensinogen. *Hypertension*, 2008; 51:1326-1331.

Isa K, Garcia-Espinosa MA, **Arnold AC**, Pirro NT, Tommasi EN, Ferrario CM, Chappell MC, Diz DI. Chronic Immunoneutralization of Brain Angiotensin-(1-12) Lowers Blood Pressure in Transgenic (mRen2)27 Hypertensive Rats. *Am J Physiol Regul Integr Comp Physiol*, 2009; 297(1): R111-5.

Arnold AC, Shaltout HA, Gallagher PE, Ganten D, Ferrario CM, Diz DI. Leptin Impairs Cardiovagal Baroreflex Function at the level of the Solitary Tract Nucleus. *Hypertension*, 2009; 54: 1001-1008.

Arnold AC, Diz DI. Protein Phosphatase 1B Tone in the Solitary Tract Nucleus is Necessary for Normal Baroreflex Function. In Revision for *Hypertension*.

Arnold AC, Ferrario CM, Diz DI. Angiotensin-(1-12) has Cardiovascular Actions in the Solitary Tract Nucleus of Anesthetized Sprague-Dawley Rats. To be submitted to *The American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*.

ABSTRACTS:

Arnold AC, Sakima A, Ganten D, Ferrario CM, Diz DI. Baroreceptor Reflex Regulation in Anesthetized Older Transgenic Rats with Low Brain Angiotensinogen: Role of Endogenous Angiotensin II and Angiotensin-(1-7). *FASEB J* 2007; 21(6): A889 (752.2).

Arnold AC, Sakima A, Ganten D, Ferrario CM, Diz DI. Angiotensin II Modulates Cardiac Vagal Chemosensitive Fiber Activation in the Solitary Tract Nucleus of Older Transgenic Rats with Low Glial Angiotensinogen. Inter-American Society for Hypertension (IASH) Annual Meeting, Miami, FL, 2007.

Arnold AC, Sakima A, Ganten D, Ferrario CM, Diz DI. Ang II and Ang-(1-7) in the Solitary Tract Nucleus Participate in the Anesthesia-Induced Elevation of Mean Arterial Pressure in Older Rats with Low Glial Angiotensinogen. *Hypertension* 2007; 50: e116 (P106).

Arnold AC, Chappell MC, Ferrario CM, Diz DI. Exogenous Angiotensin-(1-12) Impairs Baroreflex Sensitivity in the Solitary Tract Nucleus in Anesthetized Sprague-Dawley Rats. *FASEB J* 2008; 22: 1171.14.

Isa K, Garcia-Espinosa MA, **Arnold AC**, Pirro NT, Tommasi EN, Ferrario CM, Chappell MC, Diz DI.   Chronic Intracerebroventricular Immunoneutralization of the Novel Angiotensin Peptide, Angiotensin-(1-12), Lowers Blood Pressure in Transgenic (mRen2)27 Hypertensive Rats. *FASEB J* 2008; 22: 738.9.

Isa K, Garcia-Espinosa MA, **Arnold AC**, Pirro NT, Tommasi EN, Ferrario CM, Chappell MC, Diz DI. Angiotensin-(1-12) Contributes to Renin-Independent Angiotensin II Activity in Brain. *Hypertension* 2008*;* 52 (4): e81 (P126).

Arnold AC, Shaltout HA, Gallagher PE, Ganten D, Ferrario CM, Diz DI. Leptin Impairs Baroreflex Sensitivity at the Solitary Tract Nucleus: Evidence for Up-Regulation of Leptin Actions in Animals with Low Brain Angiotensinogen. *Hypertension* 2008; 52 (4): e50 (077).

Arnold AC, Ferrario CM, Diz DI. Angiotensin-(1-12) has Cardiovascular Actions in the Solitary Tract Nucleus of Anesthetized Sprague-Dawley Rats. Jackson Cardiovascular-Renal Meeting, Jackson, MS, 2008.

Arnold AC, Diz DI. Baroreflex Sensitivity is Impaired in Sprague-Dawley Rats with Spontaneous Hydronephrosis Independent of Angiotensin II Actions in the Solitary Tract Nucleus. *FASEB J* 2009; 23: 957.8.

Isa K, Shaltout HA, Garcia-Espinosa MA, **Arnold AC**, Chappell MC, Ferrario CM, Diz DI. Intracerebroventricular Anti-Ang-(1-12)-IgG Improves Baroreflex Sensitivity and Heart Rate Variability in Transgenic (mRen2)27 Hypertensive Rats. *FASEB J* 2009; 23: 612.3.

Arnold AC, Shaltout HA, Gallagher PE, Diz DI. Protein phosphatase 1B activity in the solitary tract nucleus is necessary for normal baroreflex function. *Hypertension* 2009*;* 54 (4): e100 (P350).

Isa K, **Arnold AC**, Chappell MC, Diz DI. Extracellular ACE inhibition, but not AT1 receptor blockade in the solitary tract nucleus improves baroreflex sensitivity in anesthetized transgenic hypertensive (mRen2)27 rats. *Hypertension* 2009*;* 54 (4): e98 (P340).