Neural Control of the Splanchnic Circulation in AngII-salt Hypertension

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Dedication

To my family, for laying the foundations.

Abstract

Sympathetic nervous system (SNS) activity is elevated in some forms of essential hypertension. What causes sympathetic tone to be elevated, and how it mediates hypertension, however, is unclear. Angiotensin II (AngII) and a high dietary salt appears to be involved since in rats, chronic peripheral infusion of AngII induces a form of hypertension that is accompanied by increased indices of peripheral sympathetic tone selectively when they are fed a high sodium diet. Studies in this model have shown that contrary to prevailing views, peripheral sympathetic tone was diminished to the kidneys, but instead, suggested that it may be elevated selectively to the splanchnic vascular bed. Based on these findings, the initial aim of this thesis was to characterize, in conscious rats, the local hemodynamics within the splanchnic vascular bed and the role of the SNS in mediating changes in splanchnic vascular hemodynamics during AngII-salt hypertension. Studies were carried out to test the hypothesis that in addition to sympathetically mediated increases in splanchnic venous tone, AngII-salt hypertension was mediated by enhanced sympathetic vasoconstriction of splanchnic arterioles occurring through its peripheral sympathetic nerve supply.

Splanchnic vascular resistance was found to be elevated in AngII-salt hypertensive rats; however, these hemodynamic changes occurred even after removal of direct sympathetic innervation to the splanchnic vascular bed by surgical denervation (celiac ganglionectomy). Furthermore, unlike previously shown, celiac ganglionectomy did not result in lowering of blood pressure during AngII-salt hypertension. Thus, contrary to the original hypothesis, changes in direct sympathetic input to the splanchnic vasculature did not mediate AngII-salt hypertension. Additional studies

in this thesis found that part of the problem with this inconsistent finding may be related to the technique commonly used to generate the model. Furthermore, studies in this thesis found, using chronic pharmacological adrenergic blockade, that the contribution of the SNS in AngII-salt hypertension may have been overestimated. Thus, the combined findings in this thesis and prior studies suggest that a fraction of AngII-salt hypertension is mediated by enhanced peripheral sympathetic tone, not through direct vasoconstrictive input to the splanchnic vasculature, but possibly via its influence on other non-renal splanchnic organs.

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Chapter 1

Introduction

1.1 Rationale

The renewed interest of targeting the sympathetic nervous system as a treatment for hypertension

It's been known through observational studies that the risk of death attributable to ischemic heart disease and stroke is related linearly to the level of blood pressure starting from a systolic blood pressure (SBP) of 115mmHg and a diastolic blood pressure (DBP) of 75mmHg (70). Because of this wide range, the definition of a high blood pressure as a "disease" is somewhat arbitrary. However, the public health burden of suboptimal blood pressure is clear; it shortens an individual's life expectancy by as much as 5 years (43), and it has been reported to be the number one attributable risk of death throughout the world (21).

The current cutoff for classifying blood pressure levels as "hypertensive" is based on observational data showing that adults at low-risk of developing cardiovascular diseases can benefit from blood pressure lowering interventions to a target SBP $<$ 140mmHg and DBP of $<$ 90 mmHg (113). Under this definition, 50 million or more Americans is afflicted by the disease, with 2009 to 2010 prevalence estimated at 30.5% among men and 28.5% among women in the United States (48). Worldwide, it is estimated that 972 million adults have hypertension, with the number predicted to rise to a total of 1.56 billion by 2025 (59). Given its current trend, hypertension is projected to be the single most important risk factor of cardiovascular diseases by the year 2020 (59).

Despite improvements in pharmacological therapy seen in the past half-century and their efficacy in lowering blood pressure in many cases of hypertension, only 40% and 56% of hypertensive men and women,

respectively, have their blood pressure effectively controlled in the United States (48). Although factors such as patient compliance may play a role to this relatively poor control rate (20), this is also likely because the current understanding on the etiology of hypertension is incomplete as multiple physiological abnormalities driven by interactions between genetic, behavioral and environmental factors can cause hypertension (16).

Although once controversial, there is now indisputable evidence that increased sympathetic nervous system activity (SNA) is one such physiological abnormality that plays a key role in the etiology of human hypertension (35). However, the clinical use of sympathetic nervous system (SNS) targeting therapies have been in decline, and has been the "forgotten pathway" in the treatment of hypertension (36). This is mostly because current pharmacotherapy not only block sympathetic control of arterial pressure, but many other functions as well, resulting in unwanted side effects and reduced patient compliance.

This view has recently changed since the exciting recent demonstration in human patients of long-term antihypertensive responses to a novel device-based method of selective renal denervation (37, 105), fueling renewed interest in therapies targeting SNS in hypertension. In order to understand the physiological impact, efficacy, and potential benefit of such therapies to a wide range of hypertensive patients, however, would require a better understanding of how elevated SNA contributes to hypertension, specifically, by elucidating the principal effector organs of elevated SNA and the specific patterns of sympathetic outflow that result in the altered hemodynamic state.

Sympathetic target organs other than the kidneys may play an important role in hypertension

It has long been thought that the principal effector organ of elevated SNA in neurogenic forms of hypertension is the kidney. Activation of sympathetic efferents to the kidney would favor salt and water retention, leading to expansion of blood volume and increase in blood pressure. Over time, autoregulatory changes in the peripheral vasculature would occur to counteract tissue overperfusion, resulting in elevated total peripheral resistance (TPR), a hallmark hemodynamic change in hypertension (72). Indeed, the rationale for targeting the renal nerves in recent clinical trials was largely based on this theory. This hypothesis initially appeared to be supported by the fact that arterial pressure is reduced for 2 years following a single renal denervation procedure (37, 105). However, in one case report in which SNA to skeletal muscle (MSNA) was measured before and after renal denervation, it was found to be reduced from 56 bursts/min before the procedure to 41 bursts/min 1 month later and 19 burst/min 1 year after the procedure (94). In addition, it has been shown in a recent case series of 35 patients with resistant hypertension that reductions in MSNA after renal denervation is more pronounced when measured at the single-unit level, compared to multi-unit MSNA (51). This observation, coupled with the fact that the magnitude of the decrease in whole body norepinephrine spillover following renal denervation cannot be explained by loss of renal efferent activity alone, suggests that the procedure decreases SNA to non-renal vascular beds as well (94).

The mechanism by which renal denervation in humans lead to a reduction in peripheral sympathetic nerve activity is currently unknown.

One possibility that has been proposed is that renal denervation results in the destruction of renal afferent nerves which drive sympathoexcitation to other cardiovascular organs (94). This raises the possibility that part or majority of the antihypertensive response to renal denervation could be secondary to withdrawal of sympathetic tone to non-renal vascular bed.

Studies in an experimental model of hypertension suggest the role of the splanchnic vascular bed as an important non-renal sympathetic target organ in hypertension

Through experiments using the AngII-salt model of hypertension in rats, a neurogenic model for human hypertension, our group has recently uncovered the potential critical role for the sympathetic regulation of splanchnic vascular bed in the development of hypertension (64). Previous reports in humans support this finding and the possible role for the splanchnic vasculature in the development of hypertension. First, surgical splanchnicectomy was an effective treatment for hypertension prior to the advent of pharmacotherapy (100); and secondly, vascular resistance has been reported to be elevated in the hepatosplanchnic circulation before any other vascular bed in humans with borderline hypertension (102). Thus, elucidating the contribution of sympathetic control of the splanchnic vasculature in the development of experimental AngII-salt hypertension may provide further insights into the role of neural control of non-renal vascular beds in hypertension, and discovery of novel targeted therapies.

1.2 AngII-induced hypertension and the AngII-salt model of experimental hypertension

(NOTE: The first two sections has been previously published in a review article I have coauthored with John W. Osborn, PhD and Gregory D. Fink, PhD (86))

AngII-induced activation of the sympathetic nervous system is dependent on salt intake

Hypertension caused by infusion of AngII in animals involves multiple control systems whose influence on arterial pressure is dependent on the dose of AngII as well as the presence of other factors such as salt intake (85, 87). Doses of AngII that increase arterial pressure slowly over a course of days to weeks produce what is commonly referred to as the "slow pressor AngII" model. It is thought that the hypertension is mediated, at least in part, by an elevated level of sympathetic nerve activity (SNA) (9, 38, 39). It has long been known that the severity of AngII-induced hypertension is directly dependent on the prevailing level of salt intake; and more recent studies suggest that the level of sympathoactivation is as well (63, 64). Thus, it is important to keep in mind that neurogenic mechanisms may not play an equally important role in "AngII-induced" hypertension in animals subjected to a normal or low salt intake as they would in those subjected to a high salt intake ("AngII-salt" hypertension).

The salt-sensitive nature of AngII-induced hypertension often has been ignored in the literature, and this could account for the contradictory conclusions about the role of the sympathetic nervous system in the model. Two different methods for assessing the role of the

sympathetic nervous system in AngII-induced hypertension have commonly been employed: 1) changes in the depressor response to ganglionic blockade, and 2) changes in tissue or plasma norepinephrine (NE) concentration and turnover. These indices serve as an indicator for AngII induced changes in peripheral SNA, which can be generated at any level of the neuraxis. Changes in responses to ganglion blockade suggest changes in SNA effects on arterial pressure. However, it is important to note that changes in plasma NE and NE turnover do not necessarily reflect changes in SNA that directly affect arterial pressure. Our group and others have reported a 5-7 day delayed increase in the acute depressor response to ganglionic blockade in AngII-induced (13) and AngII-salt (63) models a finding consistent with the hypothesis that AngII hypertension is due, in part, to delayed activation of peripheral sympathetic outflow. In contrast, tissue NE has been reported to be regionally decreased in AngII-induced rats (65) and plasma NE unchanged in AngII-induced rabbits (11). A factor that may explain these disparate findings is that the level of sympathetic outflow measured by these indices during AngII-induced hypertension is highly dependent on the level of dietary salt intake. Our studies have demonstrated that both an increase in the response to ganglionic blockade and a parallel increase in whole body NE spillover is present in rats on a high but not a normal salt diet. These increases are not observed until 5-7 days of AngII administration (63, 64). These findings suggest that administration of AngII when combined with a high salt diet leads to a delayed enhancement of peripheral sympathetic outflow that contributes to, but does not exclusively cause, the associated hypertension.

The splanchnic vascular bed is the critical neural target in AngII-salt hypertension

Another potential explanation for the disparate findings between laboratories regarding the contribution of the sympathetic nervous system to AngII-induced hypertension is a focus on the kidney as the most important sympathetic effector organ in long-term blood pressure regulation. This long-standing view stems from the kidney's role in regulation of blood volume, which has been hypothesized to be directly linked to the long-term control of arterial pressure (49). The concept is supported by reports that renal denervation prevents some forms of experimental neurogenic hypertension (29, 56) as well as by recent studies showing that renal denervation results in sustained decreases in arterial pressure in humans with drug-resistant hypertension (95). However, it is important to note that these studies have not demonstrated that renal denervation decreases arterial pressure secondary to loss of efferent neural control of kidney function and subsequent changes in blood volume. To the contrary, there is a building consensus that the response of arterial pressure to renal denervation is due to destruction of sensory fibers from the kidney resulting in decreased SNA to other vascular beds such as skeletal muscle, as was recently reported in humans (94).

In regard to the contribution of renal nerves to AngII-induced hypertension specifically, a number of studies have consistently found that renal SNA is decreased in this model, irrespective of salt intake. Indirect assessment of renal SNA in dogs suggested that it was decreased in AngII-induced hypertension (73), a finding that was later confirmed in rabbits in the first study to record SNA directly over a

period of weeks (4). We have recently reported similar results using direct long-term recording of renal SNA in AngII-salt rats (115). In addition, renal denervation does not prevent AngII-salt hypertension in the rat (64) or AngII-induced hypertension in the rabbit (13). Although these observations have been used as an argument against the role of the sympathetic nervous system in AngII-induced hypertension (82), this view assumes that the kidney is the only neural target that can result in hypertension and disregards the contribution of changes in sympathetic nerve activity to non-renal vascular beds to the pathogenesis of neurogenic hypertension.

We have addressed this issue by utilizing a number of indirect and direct methods to assess the relative importance of SNA to renal and non-renal vascular beds in AngII-salt hypertension. Based on direct long-term recording of lumbar SNA and hind limb norepinephrine spillover (61, 115), as well as lumbar sympathectomy (Fink, unpublished observation), we conclude that SNA to skeletal muscle does not contribute to AngII-salt hypertension. On the other hand, in contrast to the finding that renal denervation has no effect on this model, denervation of the splanchnic vascular bed by celiac ganglionectomy (CGX) markedly attenuates the neurogenic phase of AngII-salt hypertension (64). This finding is consistent with an earlier study in which direct recording of splanchnic SNA revealed it was increased in AngII-induced hypertensive rats compared to normotensive controls (76). Collectively these studies demonstrate that SNA is differentially regulated in AngII-salt rats and, more importantly, suggest that the splanchnic vascular bed is the primary target of the sympathetic nervous system in this model of hypertension.

Hemodynamic mechanism by which an enhanced sympathetic vasomotor tone to splanchnic vascular bed contributes to AngII-salt hypertension

The hemodynamic mechanism by which an increase in sympathetic vasomotor tone to the splanchnic vascular bed can lead to hypertension is partially uncovered and has been largely attributed to a reduction of vascular capacitance secondary to venoconstriction at the splanchnic vascular bed (62, 64). Decrease in venous capacitance would lead to a shift of blood volume from the venous to the less compliant arterial compartment of the circulation, resulting in a rise in arterial blood pressure (40). It remains currently unknown, however, whether elevated SNA to the splanchnic vascular bed also mediates constriction of splanchnic resistance arteries. It has been shown in a select cohort of human prehypertensives that splanchnic vascular resistance is elevated (102). Given anatomical evidences that the majority of postganglionic nerves in the celiac-superior mesenteric ganglia dually innervate both veins and arteries (53), it is very likely that changes in SNA to the splanchnic vascular bed will result in changes both to veins and arteries. Thus, it is hypothesized that hypertension in this model is caused by a concerted action of reduced vascular capacitance and elevated total peripheral resistance due to sympathetically mediated constriction of the splanchnic vascular bed.

1.3 Focus and organization of thesis

The goal of this thesis was to further clarify the role of sympathetic vasomotor tone to the splanchnic vascular bed in the rat model of AngII-salt hypertension. This work was motivated by 3 main prior

findings discussed above: 1) vascular capacitance, a measure of systemic venous compliance of which the majority is determined by splanchnic venous tone, was decreased during AngII-salt hypertension, 2) reduction in vascular capacitance was reversible by ganglionic blockade, and 3) sympathetic denervation of splanchnic organs by celiac ganglionectomy (CGx) attenuated AngII-salt hypertension and prevented the reduction in vascular capacitance. These 3 findings, and other related work supporting the sympathoexcitatory role of AngII, led to the working hypothesis that AngII-salt hypertension in the rat is mediated, in part, by an increased sympathetic vasomotor tone to the splanchnic vascular bed, which elevates pressure by reducing vascular capacitance and increasing total peripheral resistance. A schematic view of this hypothesis is illustrated in Figure 1.1.

The major limitation of the previous work was that the conclusions were mainly based on measures of whole body cardiovascular parameters coupled with targeted denervation to infer the role of sympathetic vasomotor tone to the splanchnic vascular bed. To overcome this limitation, I devised a surgical technique for continuous monitoring of superior mesenteric artery blood flow, in addition to arterial pressure, in conscious, freely moving animals. This allowed for the monitoring of hemodynamic changes specifically at the splanchnic vascular bed and calculation of mesenteric vascular resistance, a direct index of splanchnic arteriolar tone.

The work in this thesis was organized into 3 main chapters and a supporting chapter in the form of an appendix. In Chapter 2, I determined whether changes consistent with the hypothesis occur to splanchnic vascular resistance during AngII-salt hypertension. In

Chapter 3, I assessed whether changes in splanchnic vascular resistance were determined by sympathetic input to splanchnic vascular bed. Based on findings in Chapter 2 and 3, I reassessed the contribution of global sympathetic tone to the development of AngII-salt hypertension in Chapter 4. Finally, in Chapter 5, I provide a summary of all findings and implications to the original hypothesis.

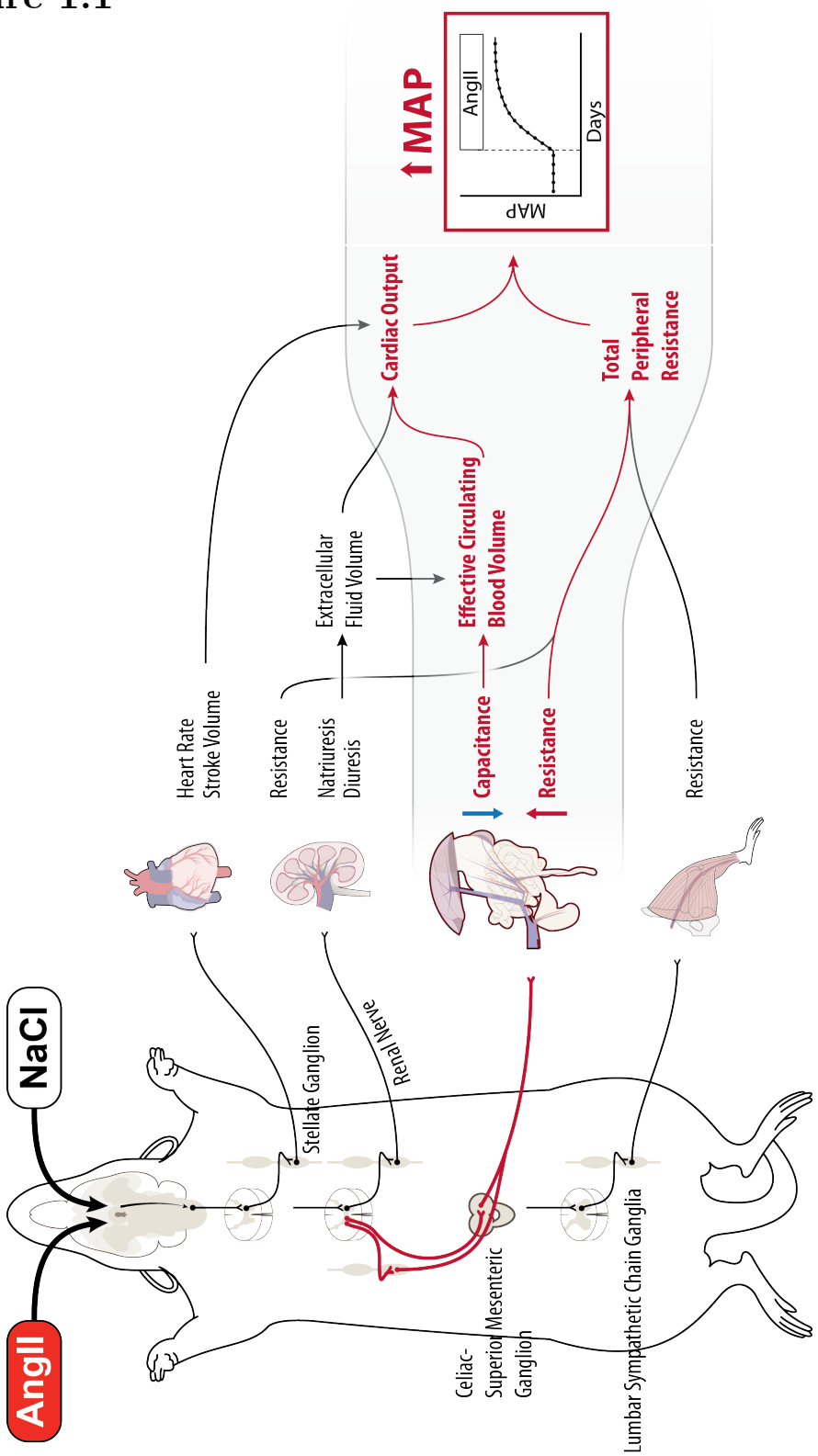
In Chapter 2, the AngII-salt model was generated by subcutaneous infusion of AngII using an implantable osmotic minipump. Observations in Chapter 2 warranted an optimization to the method of AngII delivery for improving the stability of the model. The results from this study are presented in Appendix 1. Studies in Chapters 3 and 4 were performed using the optimized method of AngII delivery based on results presented in Appendix 1.

1.4 Figures

Figure 1.1. Central Hypothesis

Diagram depicting the central and peripheral neural pathways and major sympathetic end organs thought to be involved in the neurogenic mechanism of AngII-salt hypertension. We hypothesized that AngII and salt stimulate sympathetic premotor neurons in the brain leading to activation of peripheral sympathetic pathways in a site-specific manner. Prior studies have shown that renal and lumbar sympathetic nerve activities are decreased or unchanged, respectively, during AngII-salt hypertension. Additionally, cardiac denervation by stellate ganglionectomy has no effect on AngII-salt hypertension. These results suggest that sympathetic modulation of cardiac and renal function, and skeletal muscle vascular tone plays little or no role in the neurogenic mechanism of AngII-salt hypertension (see text). Indirect evidence suggests that splanchnic sympathetic nerves may be preferentially activated during AngII-salt hypertension, causing a reduction in splanchnic vascular capacitance, which increases effective circulating blood volume. Additionally, an increase in sympathetic outflow to the splanchnic vascular bed is also thought to increase splanchnic vascular resistance. The central hypothesis of this thesis is that these combined, sympathetically mediated changes in splanchnic vascular capacitance and resistance are the primary neurogenic mechanisms responsible for the sustained increase in arterial pressure during AngII-salt hypertension.

Figure 1.1


↑MAP
AngII
Days
MAP
Cardiac Output
Total Peripheral Resistance
Effective Circulating Blood Volume
Extracellular Fluid Volume
Heart Rate
Stroke Volume
Resistance
Natriuresis
Diuresis
Capacitance
Resistance
Resistance
NaCl
AngII
Stellate Ganglion
Renal Nerve
Celiac-Superior Mesenteric Ganglion
Lumbar Sympathetic Chain Ganglia

Chapter 2

Time-dependent changes in autonomic control of splanchnic vascular resistance and heart rate in ANG II-salt hypertension

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Chapter Overview

Previous studies suggest that AngII-induced hypertension in rats fed a high salt diet (AngII-salt hypertension) has a neurogenic component dependent on an enhanced sympathetic tone to the splanchnic veins, and independent from changes in sympathetic nerve activity to the kidney or hind limb. The purpose of this study was to extend these findings and test whether altered autonomic control of splanchnic resistance arteries and the heart also contributes to the neurogenic component. Mean arterial pressure (MAP), heart rate (HR), superior mesenteric artery blood flow, and mesenteric vascular resistance (MVR) were measured during 4 control days , 14 days of AngII delivered subcutaneously (150ng/kg/min), and 4 days of recovery in conscious rats fed a high salt (HS; 2% NaCl) or low salt (LS; 0.1% NaCl) diet. Autonomic effects on MAP, HR and MVR was assessed by acute ganglionic blockade with hexamethonium (20mg/kg IV) on day 3 of control, days 1, 3, 5, 7, 10, and 13 of AngII, and day 4 of recovery. MVR increased during AngII infusion in HS and LS rats, but remained elevated only in HS rats. Additionally, the MVR response to hexamethonium was enhanced on days 10 and 13 of AngII selectively in HS rats. Compared to LS rats, heart rate in HS rats was higher during the 2nd week of AngII, and its response to hexamethonium was greater on days 7, 10 and 13 of AngII. These results suggest that AngII-salt hypertension is associated with delayed changes in autonomic control of splanchnic resistance arteries and the heart.

2.1 Introduction

Under certain conditions, hypertension resulting from systemic administration of angiotensin-II (AngII-induced hypertension) is exacerbated by activation of the sympathetic nervous system (SNS). Our group and others have shown that salt intake is one such condition (79, 87).In rats fed a relatively high salt diet (2% NaCl), the level of blood pressure achieved in AngII-induced hypertension is significantly higher than in rats fed a normal salt diet (0.4% NaCl); this is associated with an increase in whole body norepinephrine (NE) spillover (63) and enhanced mean arterial pressure (MAP) responses to ganglionic blockade (62, 64). In contrast, these measures of whole body sympathetic tone in rats fed a normal salt diet remain near control levels.

Despite increased "whole body" sympathetic tone in AngII-salt (i.e. those fed a high salt diet) hypertensive rats, we recently reported that sympathetic nerve activity (SNA) to the kidney and hind limb were reduced or unchanged, respectively (115). Suppression of renal SNA has also been directly measured during AngII-induced hypertension in rabbits (4) and indirectly in dogs (17), suggesting that this suppression is not a salt dependent effect, per se, but rather a baroreceptor mediated phenomenon. Indeed, the chronic AngII-induced decrease in renal SNA is not observed in sinoaortic denervated animals (3, 75). Additionally, AngII-induced hypertension is unaffected by sinoaortic denervation, suggesting that baroreflex mediated effects on renal SNA are not critical to the development of hypertension. Further evidence that renal SNA does not contribute to AngII-induced hypertension is that renal denervation has no effect on the final level of AngII-salt hypertension in rats (64), as well as AngII-induced hypertension in rabbits (13).

Combined, these latter results have been the major argument against the importance of the SNS in this form of hypertension (73).

In contrast to changes in sympathetic control to the kidney, relatively little attention had been given to a possible role for elevated SNA to non-renal vascular beds in the pathogenesis of AngII-induced hypertension. Recent studies by King and Fink suggest that the SNS contributes to AngII-salt hypertension via an influence to the splanchnic vascular bed. Consistent with prior studies in AngII-salt hypertensive dogs (118), mean circulatory filling pressure (MCFP) was found to be elevated in AngII-salt hypertensive rats (62). Since the increase in MCFP was not associated with increased blood volume, this finding suggests that venomotor tone is elevated in AngII-salt rats. Furthermore, the elevated MCFP was sensitive to ganglionic blockade and prevented by splanchnic sympathectomy via celiac ganglionectomy. More importantly, this latter procedure attenuated AngII-salt hypertension to levels similar to those observed in AngII-induced hypertension in rats fed a normal salt diet (64).These findings suggest that the increase in MCFP during AngII-salt hypertension is secondary to sympathetically mediated venoconstriction in the splanchnic vascular bed causing a reduction in splanchnic vascular capacitance. Based on these findings, it has been proposed that the neurogenic reduction in splanchnic vascular capacitance contributes to higher levels of AngII-induced hypertension in high salt rats by redistributing blood volume from the venous to the arterial circulation (40).

Functional consequences of enhanced splanchnic SNA, however, are not restricted to veins. A question that remains unanswered is whether sympathetic vasoconstriction to splanchnic resistance arteries also is

enhanced during AngII-salt hypertension. This seems likely since labeling studies indicate that the majority of neurons in prevertebral ganglia (a major source of splanchnic sympathetic input) dually innervate arteries and veins (53). Thus, the proposed increase in sympathetic tone to the splanchnic vascular bed in AngII-salt hypertensive rats may exert its impact on blood pressure via enhanced constriction of splanchnic resistance arteries, as well as veins.

We addressed this question in the present study by measuring arterial pressure (AP) and splanchnic blood flow continuously in conscious unrestrained rats before, during and after AngII administration in rats on a low or high salt diet. We hypothesized that AngII-induced increases in splanchnic vascular resistance, as calculated from measures of AP and splanchnic blood flow, would be greater in rats consuming a high salt diet compared to rats on a low salt diet. Moreover, we predicted that the neurogenic contribution to splanchnic vascular resistance during AngII administration, as assessed by the acute splanchnic vasodilation during ganglionic blockade, would be greater in high salt rats compared to low salt rats.

2.2 Materials and Methods

2.2.1 Animal Subjects

Male Sprague-Dawley rats (Charles River Laboratories International, Inc., Wilmington, MA) weighing ~ 270 to 370g (312±4g) were used for these experiments. Animal care and experimentation were performed in accordance with the National Institutes of Health Animals Use and Care Guideline based on a protocol submitted to, and approved by, the University of Minnesota Institutional Animal Care and Use Committee.

2.2.2 Animal Instrumentation and Care

Rats were housed in a temperature controlled environment with 12hr light-dark cycle and acclimatized to a high salt (2.0% NaCl) or low salt (0.1% NaCl) diet (Research Diets, Inc., New Brunswick, NJ) for at least 7 days. On the day of surgery, rats were atropinized (0.2mg/kg, i.p., Baxter International, Inc., Deerfield, IL) and anesthetized with isoflurane (2% mixture in 100% O2, Baxter International, Inc.). Gentamicin (0.05ml, i.m., Hospira, Inc., Lake Forest, IL) was given pre-surgically for antimicrobial prophylaxis. Surgery was performed using aseptic techniques. An arterial pressure telemeter (TA11PA-C40, Data Sciences International (DSI), Saint Paul, MN) was implanted as previously described (110). A venous catheter made from a 7cm segment of Silastic® tubing (508-001, 0.3mm I.D., 0.64mm O.D., Dow Corning, Corp., Midland, MI) attached to a 75cm Tygon S-54HL medical catheter (AAQ04103, 0.60mm I.D., 1.52mm O.D., Saint-Gobain Performance Plastics, Corp., Akron, OH) was implanted into the inferior vena cava via the left femoral vein. A 1mm Transonic flow probe (MC-1PRS-JS,

Transonic Systems, Inc., Ithaca, NY) was placed on the superior mesenteric artery (SMA) approached retroperitoneally though an incision at the left flank. The venous catheter and the flow probe cable were tunneled subcutaneously and exteriorized through an incision made at the level of the scapulae. A tether anchor made from a circular piece of surgical polyester mesh (PETKM14002, Textile Development Associates, Inc., Surgical Mesh Division, Brookfield, CN) attached to a silicone rubber catheter, (51135K78, McMaster-Carr, Co., Elmhurst, IL) was sutured to the skin at the incision over the scapulae. A 38cm stainless steel spring (Exacto Spring, Corp., Grafton, WI) was threaded halfway into the silicone portion of the tether anchor. The venous catheter and flow probe cables were externalized through this spring.

Rats were then housed individually in a custom made Plexiglass® cylindrical cage and tethered via the spring attached to an electrical swivel (SVL6C, Kent Scientific, Corp., Torrington, CT) suspended above the cage. At least 10 days were given for recovery. During this time, food and water intake were monitored along with signs of appropriate recovery from surgery. A combination of ampicillin (50mg/kg sid, i.v., Sandoz International, GmbH, Holzkirchen, Germany), tobramycin (3mg/kg sid, i.v., Teva Pharmaceuticals USA, Irvine, CA), and buprenorphine (0.05mg/kg bid, i.v., Reckitt Benckiser Pharmaceuticals, Inc., Richmond, VA) was given for antimicrobial prophylaxis and analgesia during the first 3 days of recovery.

2.2.3 Experimental Protocol

The experimental protocol was conducted in 2 groups: high salt (HS; N=10) and low salt (LS; N=10) rats. AP and superior mesenteric artery

blood flow (MBF) were continuously measured throughout the 22 day protocol. At the end of a 4 day control period, AngII was administered for 14 days at a rate of 150ng/kg/min using ALZET osmotic pumps (2ML2, DURECT, Corp., Cupertino, CA) implanted subcutaneously. On the morning after the 14th day of AngII, rats were anesthetized with isoflurane, the minipump was removed, and animals were returned to their cage for an additional 4 days of measurements (recovery period).

Assessment of autonomic control of MAP, heart rate (HR), and mesenteric vascular resistance (MVR) during AngII infusion was performed by ganglionic blockade on days 1, 3, 5, 7, 10, and 13 of AngII, and compared to values obtained on the 3rd day of control and 4th day of recovery. Ganglionic blockade was achieved by intravenous administration of hexamethonium (H0879, Sigma-Aldrich, Co., St. Louis, MO) at a dose of 20mg/kg (93). Animals were weighed in the morning on the day of the experiment and injections were performed in the afternoon between 3 and 6 PM. At least 30 min. after the injection, the i.v. catheter was flushed with saline containing 50U/mL heparin (Sagent Pharmaceuticals, Inc., Schaumburg, IL).

2.2.4 Data Acquisition and Analysis

The AP and MBF signals were collected continuously at 500Hz using DSI software (Dataquest ART v.4.0 Platinum, DSI). The AP signal was acquired using a wireless receiver (RPC-1, DSI), and the MBF signal was acquired using a dual channel flow meter (T206, Transonic Systems, Inc.) connected to an analogue to digital converter box (C11V, DSI). MAP, HR, and mean MBF was calculated on-line from consecutive 10s segments of the pulsatile waveform and stored to disk. These data were

imported into MATLAB (v.R2009b, The Mathworks, Inc., Natick, MA) for calculating MVR, averaging daily hemodynamic values, and analyzing the ganglionic blockade data. MVR was calculated off-line from the MAP and mean MBF data as MAP/MBF. 12hr averages of MAP, HR, MBF, and MVR was calculated starting from the beginning of the dark cycle on day 0 of the protocol after removal of the 4hr segments immediately following ganglionic blockade. To determine the response to ganglionic blockade, data was first smoothed with a 3rd order median filter. The peak/trough response of MAP, HR or MVR was determined on this smoothed dataset and subtracted from the average of the 10min baseline period (see Figure 1).

2.2.5 Statistical analysis

Variables in HS and LS rats were analyzed by factorial ANOVA. The effect of dietary salt intake and AngII administration, on the 12hr mean hemodynamic values for each light-dark cycle, and response to ganglionic blockade was analyzed by two-way repeated measures ANOVA using SigmaStat (v.3.5, Systat Software, Inc., San Jose, CA). A significant interaction or main effect was further analyzed by post-hoc multiple comparisons with respect to the control samples for each factor (low salt for diet, and control day 3 for day of protocol) using the Holm-Sidak method. A $p < 0.05$ was considered statistically significant.

2.3 Results

Figure 1 shows a typical trace of the pulsatile AP and MBF waveform from a single HS rat taken during control. MBF data was of high fidelity (as shown by the pulsatile flow profile at each cardiac cycle), and allowed for determination of absolute flow rate to the mesenteric vascular bed throughout the entire 22 days of the protocol. Also shown in the figure are the 10s mean AP, MBF, and HR traces calculated from the waveform data, and the MVR trace calculated from the 10s mean AP and MBF data points. These 10s mean data were used to determine the responses to ganglionic blockade and changes in 12hr hemodynamics before, during, and after AngII treatment in HS and LS rats.

2.3.1 Effect of AngII on 12hr hemodynamics in rats fed a high or low salt diet

Figure 2 shows the daily 12hr average MAP, MBF, MVR and HR for the entire 22 days of the protocol. Rats were allowed to recover from surgery (typically 10 days) until a distinct circadian rhythm was observed in every variable during baseline. There were no differences in the 12hr MAP, MBF, MVR or HR during the 4 day control period between HS and LS rats. All variables recovered to control levels following 14 days of AngII. There were no differences in the 12hr hemodynamic values during the 4 day recovery period between HS and LS rats except for the nighttime MAP on the second day of recovery.

MAP was identical in both groups during the first 24hr of AngII administration. However, whereas MAP reached a plateau in LS rats by

the first day of AngII infusion, MAP did not reach steady state until the second week of AngII in HS rats. The steady state level of MAP (averaged over days 10 thru 13 of AngII) was 162±2mmHg and 119±2mmHg for HS and LS rats, respectively, during nighttime, and 149±2mmHg and 117±2mmHg for HS and LS rats, respectively, during daytime.

MBF decreased transiently during the initial stage of AngII infusion in both HS and LS rats and this decrease was similar in magnitude. Similarly, MVR increased to the same magnitude during this time in both groups. Following the acute drop, MBF gradually returned towards control levels in both HS and LS rats. Although there were no between group differences in MBF on a day to day basis throughout the protocol, the recovery of MBF, compared to the within group control levels, took 5 days in HS rats compared to 2 days in LS rats.

Changes in MVR following the peak at day 1 of AngII deviated significantly between HS and LS rats. In HS rats, higher MVR levels persisted until removal of AngII, while in LS rats, MVR sharply dropped following the day 1 peak and gradually returned toward control levels. MVR in LS rats during AngII infusion was no longer statistically significant from control after day 6 of AngII. MVR averaged over days 10 thru 13 of AngII was 14.3±0.7mmHg*min/mL and 9.5±0.4mmHg*min/mL in HS and LS rats, respectively, during nighttime, and 14.9±0.7mmHg*min/mL and 10.5±0.5mmHg*min/mL in HS and LS rats, respectively, during daytime. Removal of the minipump on day 14 of AngII caused an acute drop in MVR toward control levels in HS rats.

Administration of AngII resulted in an initial bradycardic response that was more pronounced in HS than LS rats. There was a clear trough for this response in HS rats at the end of day 4 of AngII but then HR gradually rose toward control levels. In contrast, HR in LS rats remained at levels slightly below control thru day 11 of AngII. HR, both nighttime and daytime, on days 9, 10, 11, and 12 of AngII was higher in HS rats compared to LS rats, although these levels in HS rats were not statistically distinguishable from their own control.

2.3.2 Effect of AngII on the hemodynamic responses to acute ganglionic blockade in rats fed a high or low salt diet

Figure 3 summarizes the results from the ganglionic blockade experiments and shows the change in the contribution of autonomic tone to basal hemodynamics before, during and after AngII administration. During the control period there was no difference in the depressor response to hexamethonium between HS and LS rats. However, the depressor response to hexamethonium in HS rats increased starting at day 3 of AngII with this trend continuing until day 13 of AngII. In contrast, the depressor response to hexamethonium in LS rats was indistinguishable from control levels throughout the protocol. The depressor response to hexamethonium returned to control levels after removal of the minipumps in both HS and LS rats.

The MVR responses to hexamethonium showed a pattern similar to those observed with MAP in that responses increased during AngII administration in HS salt rats but not in LS rats. Specifically, there were no differences during the control period between HS and LS rats.

There were no between group differences in the MVR response to hexamethonium during the first week of AngII except for day 3 of AngII where the response was significantly higher in HS rats. By days 10 and 13 of AngII, the MVR response to hexamethonium was significantly higher in HS compared to LS rats. The response of MVR to hexamethonium returned to control levels by the 4th day of the recovery period and was not different between groups.

HR dropped in response to hexamethonium in both groups to a similar degree during control, and this was not different between groups. However, on day 3 of AngII, the HR response to hexamethonium switched from a bradycardia to a tachycardia in HS rats in contrast to LS rats, which did not change. As observed in the responses of MAP and MVR, the HR response to hexamethonium during the second week of AngII was significantly higher in HS rats compared to LS rats. In LS rats, the HR response to hexamethonium tended to gradually decrease in magnitude over time, however, these changes were not statistically different from control levels. The HR responses to hexamethonium in HS rats were greater than those measured in LS rats on days 7, 10, and 13 of AngII, however, these values were not statistically different from the within group control period. Finally, the HR response to hexamethonium measured on the 4th day of the recovery period was similar between HS and LS rats, and not different from their respective within group control periods.

2.4 Discussion

Rats subjected to the AngII-salt protocol, where they are fed a high salt diet during continuous infusion of AngII, have been shown to develop hypertension by multiple mechanisms including a delayed increase in "whole body" sympathetic activity (62, 63). Consistent with these previous findings, AngII-induced hypertension in our current study was significantly higher in HS compared to LS rats, and there was a delayed increase in the depressor response to hexamethonium in HS rats but not LS rats (62). Previous findings that celiac ganglionectomy attenuates the development of AngII-salt hypertension suggested that the splanchnic vascular bed is a significant contributor to the neurogenic component of hypertension in this model (64). Furthermore, direct measurement of renal and lumbar SNA, which were previously found to be slightly below or unchanged from baseline (115), suggested that the sympathoexcitatory response in the AngII-salt model is a regionally localized phenomenon. Although SNA to the splanchnic vascular bed has not been directly measured in AngII-salt rats, the evidence reported here supports a role for splanchnic sympathetic nerves in mediating peripheral hemodynamic changes that ultimately contribute to the severity of hypertension in this model.

To date, the only finding that provides a mechanistic insight into the hemodynamic effect of a localized increase in sympathetic tone to the splanchnic vascular bed during AngII-salt hypertension has been the observation that MCFP is elevated in this model, which has been shown to be progressively more sensitive to ganglionic blockade and dependent on an intact sympathetic innervation to the splanchnic vascular bed (62, 64). Our present study extends these findings by showing that

continuous infusion of AngII results in a sustained increase in MVR associated with an elevated MVR response to ganglionic blockade in HS but not LS rats (62). This new finding adds further support to the overall hypothesis that the higher level of dietary salt contributes to the severity of AngII-induced hypertension in HS rats by increasing sympathetic vasoconstrictor tone to the splanchnic vascular bed. This appears to contribute to the hypertension by increasing splanchnic vascular resistance and reducing splanchnic vascular capacitance, and through their expected overall impact on total peripheral resistance (TPR) and effective circulating blood volume. These combined effects would elevate arterial pressure by effectively translocating blood from the high compliant venous compartment to the less compliant arterial compartment as suggested in a recent review (40).

The increase in sympathetic tone during AngII-salt hypertension is delayed, as suggested by the timing at which measurable changes in whole body NE spillover occur (week 2 of AngII) (63), but more importantly, its contribution to arterial pressure is progressive, as suggested by the time course in which the depressor response to ganglionic blockade increases, starting at around day 3 of AngII. The current study suggests that the neurogenic influence on the splanchnic vasculature also occurs in a time-dependent manner. The contribution of neurogenic tone to MCFP (which is largely determined by splanchnic venous capacitance) was increased as early as one day after initiating the infusion of AngII (62). In the present study, although the magnitude of mesenteric vasodilation during ganglionic blockade was increased 3 days after the start of AngII infusion, it was not consistently elevated until the 10th day of AngII. This difference in the timing of the onset of

sustained neurogenically mediated increases in MCFP (day 1 of AngII) and MVR (day 10 of AngII) may be the result of a gradually increasing peripheral sympathetic tone in AngII-salt rats. For instance, the threshold for a functional response to increased sympathetic tone may be different in splanchnic veins and arteries. It has been reported that the frequency-response curve to nerve stimulation is shifted to the left (i.e. lower frequency) for splanchnic veins compared to splanchnic arteries (33). Thus, it is possible that splanchnic SNA increases gradually during AngII infusion, and that earlier and lower frequencies of sympathetic nerve discharge during AngII infusion in HS rats affect mostly the splanchnic veins, while later and higher frequencies of sympathetic discharge affects both veins and arteries. Thus a progressive increase in the levels of SNA and differential responses of splanchnic resistance and capacitance vessels may be the underlying mechanism for the progressive rise in MAP seen in HS rats treated with AngII. It is important to note, however, that there are several other possibilities to explain this observation, including changes in arterial reactivity to NE and release of vasodilators, which may counter the early constrictor effects of increased SNA to splanchnic resistance arteries. Further studies are necessary to establish more certainly the mechanism behind the progressive change in the sympathetic contribution to arterial pressure during AngII-salt hypertension.

In addition to changes in sympathetic vasoconstrictor tone in the splanchnic vascular bed, non-neurogenic vasoconstrictor mechanisms to the splanchnic and potentially other vascular beds appear to play a role in AngII-induced hypertension. For instance, the MVR peak on day 1 of AngII, and the early differences in MVR between HS and LS rats

starting at day 2 cannot be explained by an elevated sympathetic vasoconstrictor tone since MVR responses to ganglionic blockade were not consistently elevated until day 10 of AngII. The initial increase in MVR in both HS and LS rats may be a result of direct vasoconstrictor effects of AngII; the early differences in MVR between HS and LS rats may reflect differences in the compensatory responses triggered in response to the initial vasoconstriction and rise in MAP, or altered reactivity of mesenteric arteries to AngII. It has been shown that high salt intake can increase the reactivity of cremasteric arterioles to AngII (44). Whether similar dietary salt-dependent sensitization occurs in mesenteric arterioles is unknown, but the initial increase in MVR in response to AngII infusion was similar between HS and LS rats suggesting that any effect of sensitization to AngII mediated vasoconstriction was small. Furthermore, the sustained increase in MAP in LS rats during AngII infusion despite a return of MVR to control levels indicates that there is a persistent non-neurogenic vasoconstrictor stimulus to non-splanchnic vascular beds. The effects of AngII on the renal vascular bed are well known and likely play a prominent role in the non-neurogenic mechanisms underlying AngII-induced hypertension.

In this study, we also assessed whether autonomic tone to the heart increases during AngII-salt hypertension. Although HR was higher and the bradycardic response to ganglionic blockade more prominent in HS compared to LS rats during the second week of AngII infusion, these levels in HS rats were not statistically higher compared to their own control levels. Similarly, the HR response to ganglionic blockade in LS rats during the second week of AngII infusion were not statistically different compared to control, making it difficult to draw conclusions

from the between group differences found in this study. Nevertheless, it is possible that the difference in baseline HR between HS and LS rats during the second week of AngII is due to an inappropriate balance of sympathetic and parasympathetic cardiac autonomic activity. Although further studies are necessary to determine the role of cardiac sympathetic nerves in AngII-salt hypertension, preliminary findings in cardiac denervated rats and rats chronically treated with atenolol suggest that changes in sympathetic tone to the heart play little or no functional role in the neurogenic component of AngII-salt hypertension (52). It is clear however, that baseline HR is initially suppressed during AngII-induced hypertension, as shown by the tachycardic response to ganglionic blockade on day 3 of AngII in HS rats. This initial decrease in sympathetic tone (and/or increase in parasympathetic tone) to the heart is likely baroreflex mediated, which is consistent with previous studies showing that renal SNA is initially suppressed during AngII-induced hypertension in a baroreflex dependent manner (3, 75). Suppression of sympathetic activity to the heart and renal SNA is not seen with sympathetic effects on splanchnic resistance arteries and lumbar SNA (115), suggesting that there is a region-specific difference in the degree of baroreceptor inhibition of sympathetic tone in response to the initial phase of AngII hypertension. This hypothesis of minimal baroreflex control of SNA to the splanchnic vascular bed relative to neural control of the heart, kidney or hind limb skeletal muscle needs to be tested in further studies.

2.4.1 Strengths and Limitations of the Study

To our knowledge, this study is the first to employ direct continuous long term recording of superior mesenteric artery blood flow and AP in conscious unrestrained animals before, during and after the induction of any model of experimental hypertension. The combination of this approach, with intermittent assessment of neural control of splanchnic hemodynamics using ganglionic blockade, has generated novel results regarding the role of neural control of this vascular bed in the pathogenesis of AngII-salt hypertension. The strength of this approach is the ability to assess neural control of a specific vascular bed over a long period of time using a repeated measures experimental design. Although we did not directly measure SNA to the splanchnic vascular bed, a key variable of interest was calculated, i.e. mesenteric vascular resistance.

Ganglionic blockade has been a standard technique for determining the contribution of the SNS to AP and regional hemodynamics, and it has been shown that the peak response is relatively unaffected by compensatory release of hormones (e.g. vasopressin) in response to the rapid hypotensive effect (93). Nevertheless, results must be interpreted with caution (81). Although we feel that an increase in SNA to the splanchnic vascular bed is the most likely explanations for the obtained results, other possibilities exist. One is that the response to ganglionic blockade is a reflection of a generalized increase in sensitivity to vasoconstrictors due to "vascular amplifier" effect secondary to AngII-induced vascular hypertrophy (47). Another possibility is a withdrawal of myogenic tone in response to a drop in pressure secondary to withdrawal of sympathetic tone elsewhere. Finally, AngII may amplify nerve transmission at the sympathetic ganglia, post-ganglionic pre-

synaptic nerve terminal and vascular neuroeffector junction (90), which could result in sympathetic nerve dependent changes in the splanchnic vascular bed without an actual increase in nerve activity. Thus, further experiments, such as those measuring MVR in celiac ganglionectomized rats and directly recording splanchnic SNA, are needed to determine whether enhanced sympathetic vasomotor nerve activity to the splanchnic vascular bed was responsible for the observed changes in MVR.

However, several studies argue against these alternate possibilities. For example, it has been shown that prevention of vascular hypertrophy does not affect AngII-induced hypertension, suggesting that AngII-induced hypertension is not solely due to development of vascular amplifiers (32). Second, since sympathetic nerves to two major vascular beds, the hind limb and kidney, do not play a role in AngII-salt hypertension (64, 115), the depressor response to ganglionic blockade is most likely due to inhibition of vasomotor tone to the splanchnic vascular bed. Taken together these findings strongly support the idea that decrease in MVR following ganglionic blockade in AngII-salt rats is due to a decrease in sympathetic tone rather than autoregulatory responses secondary to withdrawal of sympathetic tone to other vascular beds.

Although the AngII-induced hypertension model has been a popular model for the study of both neurogenic and non-neurogenic mechanisms of hypertension, the protocols employed in the literature are highly variable both between species and within species. Importantly, the mechanisms that participate and predominate in the hypertension generated by infusion of AngII appears to be highly dependent on the

administered dose (97) and the level of dietary salt (79). Although our protocol generates a model of salt sensitive hypertension dependent on a neurogenic mechanism at high levels of salt intake, others, such as that of Luft and coworkers, have reported a significant neurogenic component in rats given a higher dose of AngII but fed a normal salt diet (76). It is possible that higher levels of AngII would offset the threshold for dietary salt levels required to activate the neurogenic response. Despite these differences, it appears that the splanchnic sympathetic nerves play a prominent role when a neurogenic mechanism is activated during AngII-induced hypertension as shown by our studies and studies by Luft and colleagues which reported increases in directly measured splanchnic SNA in their model of AngII-induced hypertension (76).

2.4.2 Perspectives

What is the clinical relevance of a delayed change in autonomic tone in the AngII-salt model of hypertension? In our view, it demonstrates that there is a clear role for the autonomic nervous system during established phase of salt sensitive forms of hypertension. Although several mechanisms contribute to hypertension in the AngII-salt model, the neurogenic component activated not only exacerbates the hypertension, it also likely participates in inducing end organ damage and augmenting cardiovascular risk in general. Furthermore, our studies highlight the fact that neurogenic mechanisms underlying some forms of hypertension may not be limited to effects on the kidneys alone. Our results in fact indicate that changes in sympathetic control of splanchnic vascular resistance and capacitance dominate the neurogenic component of AngII-salt hypertension (40). Whether the splanchnic vascular bed is

an important sympathetic target in other salt-sensitive forms of hypertension is yet to be determined. A better understanding of the contribution of organ specific changes in sympathetic nerve activity to neurogenic forms of hypertension could lead to development of new targeted approaches for the treatment of hypertension in the human population, such as those recently demonstrated using catheter based renal denervation (37).

Grants

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Disclosures

None

2.5 Figures

Figure 2.1. Original tracing for typical AP, MBF, MVR, and HR response to ganglionic blockade

Representative traces for the responses of AP, MBF, MVR and HR to ganglionic blockade in a single rat during control. AP and MBF signal was sampled and saved to disk continuously at 500Hz (dark grey). Mean AP, MBF and HR was calculated and saved every 10sec during acquisition (light grey for AP and MBF, dark grey for HR). MVR was calculated from the 10sec mean AP and MBF data as AP/MBF. The peak response of AP, MVR and HR after injection of hexamethonium was determined with respect to baseline (average of 10min immediately preceding injection).

Figure 2.1

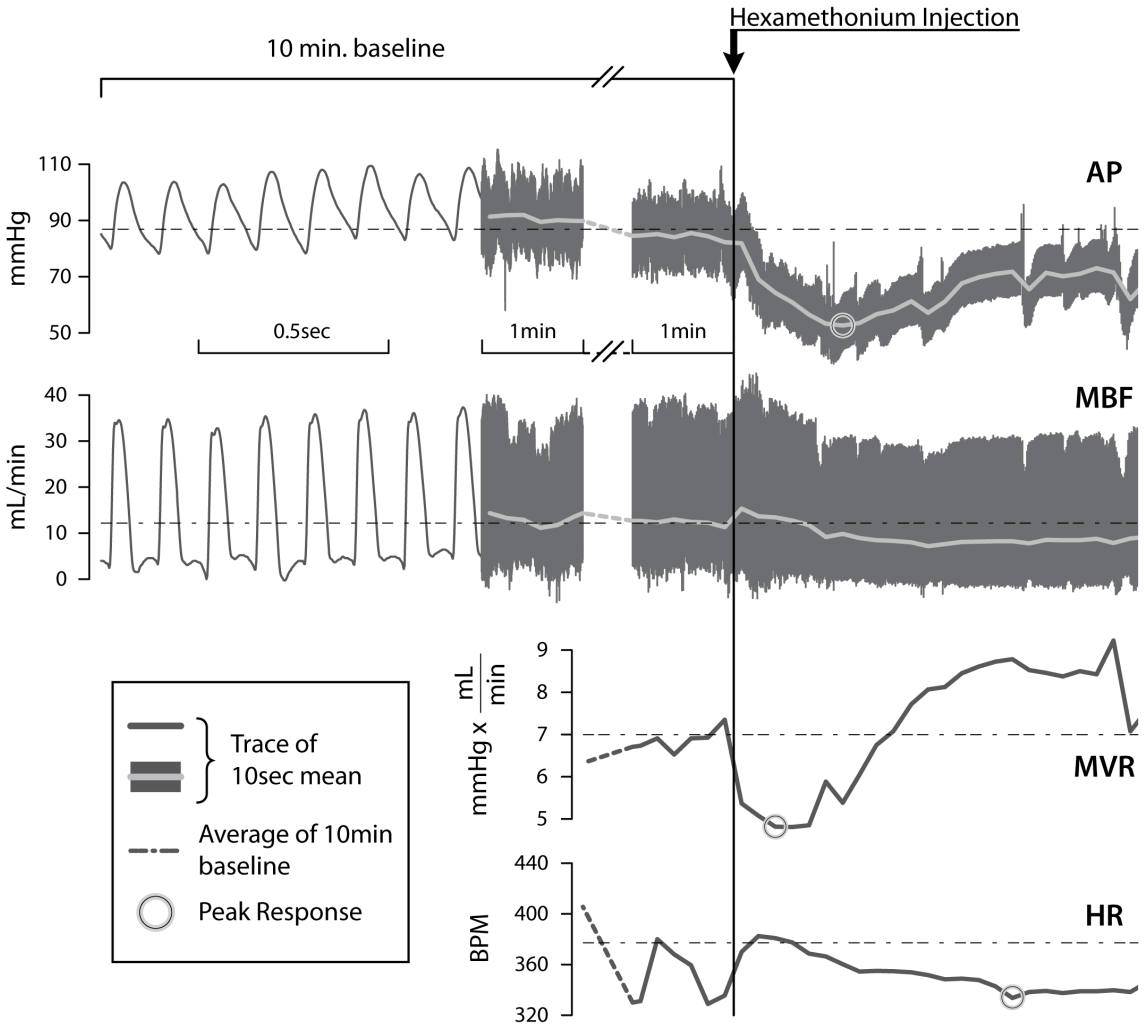

Hexamethonium Injection
10 min. baseline
AP
mmHg
110
90
70
50
0.5sec
1min
1min
MBF
mL/min
40
30
20
10
0
Trace of
10sec mean
Average of 10min
baseline
Peak Response
mL/min x mmHg
9
8
7
6
5
MVR
BPM
440
400
360
320
HR

Figure 2.2. Change in MAP, MBF, MVR, and HR during 2wk AngII infusion in high and low salt rats

12hr average HR, MAP, MBF and MVR for HS (n=10), and LS (n=10) rats during 4 days of control, 2 weeks of AngII infusion, and 4 days of recovery following cessation of AngII infusion. AngII was given via osmotic minipumps implanted subcutaneously after the control period, and removed after 14 days. Changes in MBF are comparable between HS and LS rats. (✱, ✱ denote within group statistical significance compared to daytime or nighttime values from day 4 of control at an $\alpha < 0.05$ for HS or LS rats, respectively. Statistical significance for nighttime or daytime values is denoted by]N , or]D , respectively, which are placed on the top or bottom corner at the right side of each graph. †N , †D denote between group statistical significance at an $\alpha < 0.05$ for nighttime or daytime values, respectively, compared to values from LS rats used as control.)

Figure 2.2

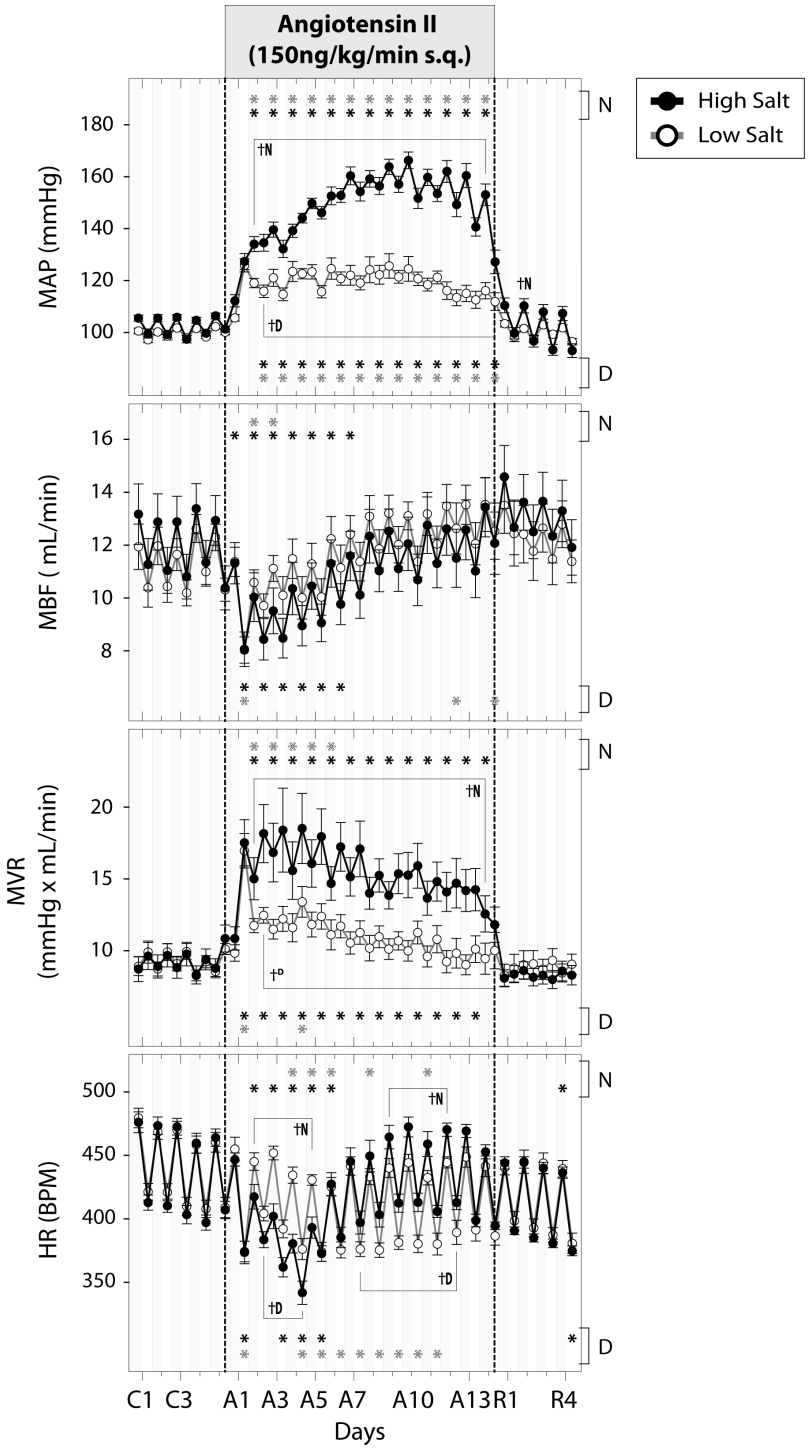
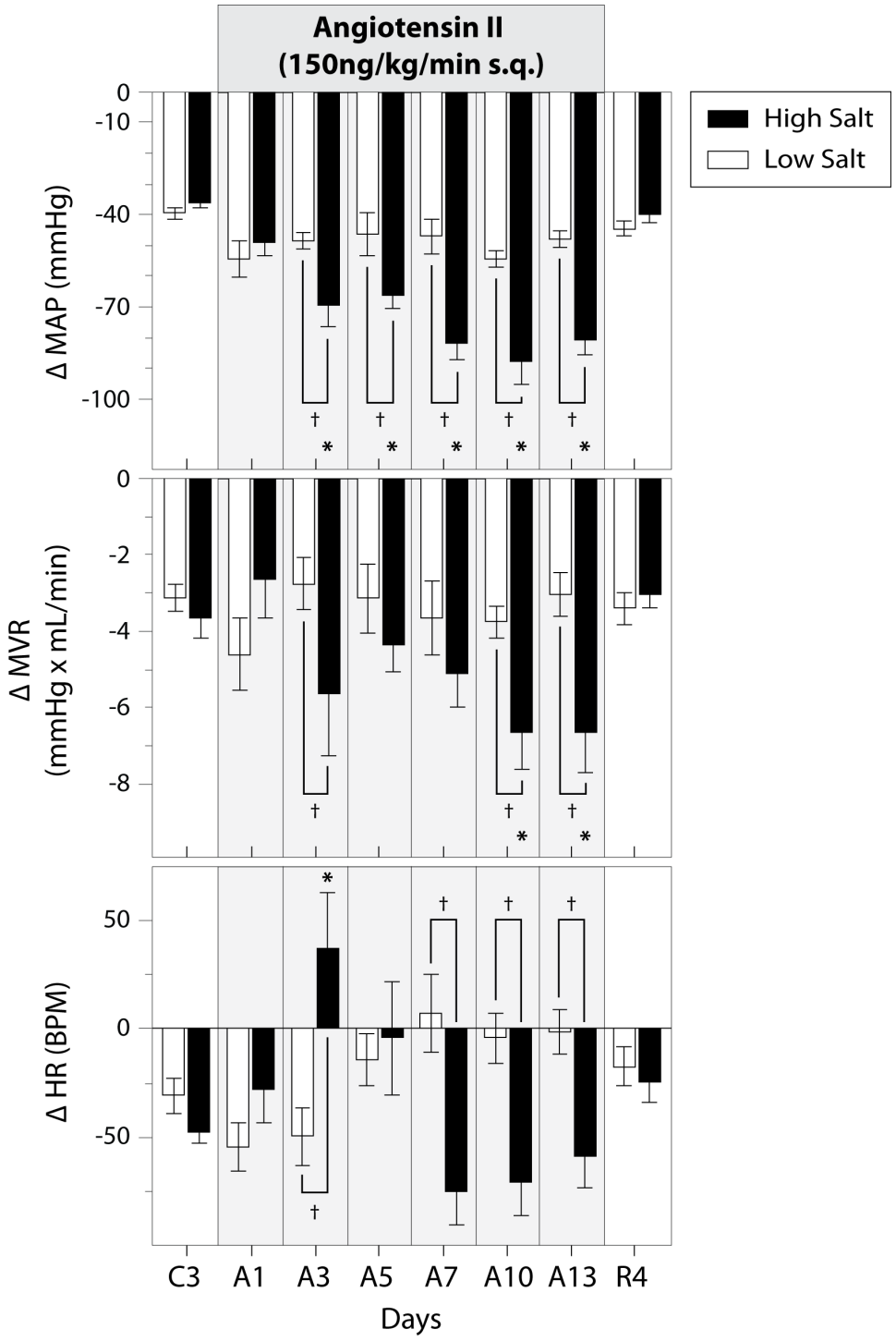

Angiotensin II
(150ng/kg/min s.q.)
High Salt
Low Salt
MAP (mmHg)
MBF (mL/min)
MVR
(mmHg x mL/min)
HR (BPM)
N
D
†N
†D
†P
C1 C3 A1 A3 A5 A7 A10 A13 R1 R4
Days

Figure 2.3. MAP, MVR, and HR response to acute ganglionic blockade during AngII induced HTN in high and low salt rats

HR, MAP, and MVR response to acute ganglionic blockade for HS (n=10) and LS (n=10) rats on control day 3, AngII days 1, 3, 5, 7, 10, and 13, and recovery day 4. ΔHR, MAP, and MVR were calculated by subtracting the peak/trough values after ganglionic blockade from the 10min. baseline period immediately preceding the injection. ✱ denote within group statistical significance compared to day 3 of control at an $\alpha < 0.05$ for HS rats † denote between group statistical significance at an $\alpha < 0.05$ compared to values from LS rats used as control.)

Figure 2.3


Angiotensin II
(150ng/kg/min s.q.)
High Salt
Low Salt
Δ MAP (mmHg)
0
-10
-40
-70
-100
Δ MVR
(mmHg x mL/min)
0
-2
-4
-6
-8
Δ HR (BPM)
50
0
-50
C3
A1
A3
A5
A7
A10
A13
R4
Days

Chapter 3

Effect of Celiac Ganglionectomy on Splanchnic Hemodynamics in AngII-Salt Hypertensive Rats

Chapter Overview

A previous study showed that celiac ganglionectomy (CGx) attenuates AngII-salt hypertension by preventing a sympathetic mediated reduction in splanchnic vascular capacitance. We have subsequently shown that splanchnic vascular resistance is elevated during AngII-salt hypertension; a response that was reversible by acute ganglionic blockade. We hypothesized that CGx attenuates AngII-salt hypertension by preventing a sympathetically mediated rise in splanchnic vascular resistance. In the present study we measured mean arterial pressure (MAP), heart rate (HR), superior mesenteric artery blood flow (MBF), and mesenteric vascular resistance (MVR) in Sham and CGx treated rats fed a 2% NaCl diet subjected to 14 days of AngII infusion. Autonomic effects on MAP, MVR, and HR was assessed by ganglionic blockade (hexamethonium) during control and days 1, 3, 5, 7, 10, and 13 of AngII. CGx rats had a lower baseline MAP and MVR, and higher HR and MBF compared to Sham. However, CGx had no effect on the rise in MAP during AngII infusion or the acute depressor response to hexamethonium. MVR rose in both Sham and CGx rats during AngII infusion. MVR stabilized at a slightly lower level in CGx rats, in part due to lower control levels, but this had no effect on the MAP response. CGx had no effect on the acute response of MVR to hexamethonium. These results suggest that changes in MVR during AngII-salt hypertension are primary mediated by non-neurogenic mechanisms, and that previously reported effect of CGx may not be due to sympathetic denervation of the splanchnic vasculature alone.

3.1 Introduction

Hypertension resulting from systemic administration of angiotensin-II (AngII) in conjunction with a high salt diet, i.e. AngII-salt hypertension, is associated with an increase in whole body norepinephrine (NE) spillover (63) and a progressive rise in the hypotensive response to acute ganglionic blockade (62, 64, 66). Combined, these results suggest that AngII-salt hypertension is mediated, in part, by an increase in peripheral sympathetic tone. However, direct recording of sympathetic nerve activity (115) in conscious rats has shown that renal and lumbar sympathetic nerve activity (SNA) is depressed or unchanged, respectively, during AngII-salt hypertension, suggesting that the peripheral target of enhanced sympathetic tone is neither the kidneys nor skeletal muscle.

Neurogenic constriction of capacitance vessels has been proposed as a potential mechanism contributing to AngII-salt hypertension (40). It has been shown that mean circulatory filling pressure (MCFP), a function of total blood volume and systemic vascular capacitance, is elevated in this model and reversible by acute ganglionic blockade. Since there are no measurable changes in total blood volume during AngII-salt hypertension, it has been hypothesized that the increase in MCFP is mediated by enhanced sympathetic tone to capacitance vessels, primarily in the splanchnic vascular bed (62). Consistent with this hypothesis, King and Fink have shown that surgical sympathectomy of the splanchnic vascular bed by celiac ganglionectomy (CGx) not only prevents the rise in MCFP in AngII-salt rats, but significantly attenuates hypertension in this model (64). These findings provided further support for the hypothesis that AngII-salt hypertension is

mediated, in part, by enhanced sympathetic tone to the splanchnic vascular bed.

We recently measured arterial pressure and superior mesenteric artery blood flow (MBF) continuously, before and during AngII-induced hypertension in rats consuming a high salt diet. Consistent with the original hypothesis, mesenteric vascular resistance (MVR) and mean arterial pressure (MAP) increased in parallel in AngII-salt rats, and both responses were acutely reversible by ganglionic blockade (66). This provided further evidence, using a more localized index of splanchnic vascular tone in conscious freely moving animals, that sympathetic tone is increased to the splanchnic resistance vessels of AngII salt rats.

Our recent finding that the *chronic* increase in MVR in AngII-salt rats can be *acutely* reversed by ganglionic blockade is consistent with the hypothesis that AngII-salt hypertension is driven by sympathetic input to the splanchnic vascular bed. If this is true, we predicted that *chronic* sympathetic denervation of the splanchnic vascular bed, by CGx, should prevent the increase in MVR in AngII-salt rats. The present study was designed to test this hypothesis.

3.2 Methods

Experiments were performed in conscious, chronically instrumented rats in accordance with NIH guidelines. All experimental procedures were conducted after approval and under supervision by the institutional animal care and use committee of the University of Minnesota.

3.2.1 Animal use and care

28 male Sprague-Dawley rats from Charles River Laboratories (Wilmington, MA) weighing 226-275g were used in this study. Upon arrival, rats were switched to a 2% NaCl diet (Research Diets Inc., New Brunswick, NJ) and housed 2 per cage in a 12-12hr light-dark cycled room (8:30/20:30 cycle). Distilled water was available ad-libidum. Animals were allowed to acclimate 7-10 days prior to surgery.

3.2.2 Surgical procedures

Average rat weight at surgery was 326±11g and 329±11g for the celiac ganglionectomy (CGx) and Sham groups, respectively. Surgery was performed under isoflurane anesthesia (2.5% isoflurane, Baxter International, Inc. in 100% O2 delivered via a nose cone at 1mL/min flow rate). After induction, rats were given atropine (0.2mg/kg, i.p., Baxter International, Inc., Deerfield, IL) for reduction of salivary and bronchial secretions, preoperative antibiotic prophylaxis (gentamicin, 0.05mL, i.m., Hospira, Inc., Lake Forest, IL), preoperative pain relief (ketoprofen, 5mg/kg, s.c., Fort Dodge Animal Health, Inc., Overland Park, KS) and placed on a heated surgical bench. Surgical instruments were heat sterilized and all implants were cold sterilized overnight in a

solution of glutaraldehyde (Cidex Plus, Johnson & Johnson Services, Inc., New Brunswick, NJ).

Rats were instrumented with a DSI pressure transmitter (TA11PA-C40, Data Sciences International (DSI), Saint Paul, MN) for measurement of arterial pressure (AP), a 1mm Transonic flow probe (MC-1PRS-JS, Transonic Systems, Inc., Ithaca, NY) for measurement of superior mesenteric artery blood flow (MBF), a subcutaneous catheter for AngII delivery, and a femoral venous catheter. Implantation of the pressure transmitter, superior mesenteric artery flow probe and femoral venous catheter was performed as described previously (66). The subcutaneous catheter (23g, 0.02" I.D. x 0.06" O.D. Tygon Micro-Bore tubing S-54-HL, Saint-Gobain Performance Plastics, Corp., Akron, OH) used in this experiment for delivery of AngII via an external infusion pump was tunneled through a small dorsal skin incision. The tip of the catheter was positioned over the animal's lower right flank. The catheter and flow probe cable were exteriorized through a skin incision over the scapulae, anchored to the underlying subcutaneous tissue using a circular surgical polyester mesh (PETKM14002, Textile Development Associates, Inc., Surgical Mesh Division, Brookfield, CN) which was implanted subcutaneously and sutured to the skin upon closure of the incision.

CGx was performed in half of the rats as described by (64). After a midline laparotomy, the intestines were exteriorized onto a wet gauze and the stomach and spleen were retracted rostrally, without exteriorizing, to expose the great vessels and the root of the superior mesenteric artery. The superior mesenteric and celiac ganglia were removed along with the connective tissue and nerve fibers comprising

the celiac plexus. Care was taken to keep the lymphatic vessel coursing along the superior mesenteric artery and abdominal aorta intact. The other half of rats were subject to sham surgery in which the celiac and superior mesenteric ganglia were exposed, but kept intact by dissecting the overlying peritoneal membrane.

Rats were given 10-14 days to recover from surgery. Ketoprofen (5mg/kg, s.c., Fort Dodge Animal Health, Inc., Overland Park, KS) was administered daily for 3 days post surgery for pain management. Fluid intake was monitored closely during recovery and supplemental fluid was given (6-8mL isotonic saline, s.c. bolus, B. Braun Medical, Inc., Irvine, CA) through the subcutaneous catheter as needed.

3.2.3 Tethering and Housing

The distal ends of the catheters and probe cable were tunneled though a stainless steel spring (Exacto Spring, Corp., Grafton, WI) used for tethering the rat to swivel. The end of the spring in contact with the rat was fitted with a 1" piece of Tygon tubing (3/16" I.D. x 5/16" O.D. Tygon S-50-HL Saint-Gobain Performance Plastics, Corp., Akron, OH) to cushion its contact with the skin. Unlike the previously described technique where the spring was affixed to the animal using a conventional subcutaneous button tether with the spring passing through the skin, the spring was allowed to sit freely over the skin. The catheters and cables were attached to the spring by placing a piece of tape over where they exited the spring. This simplified tethering technique, adapted from that used at Dr. Kenju Miki's laboratory (Nara Women's University), improved healing of the exit wound and improved

reliability of tethering compared to our previously described method (66).

Instrumented rats were housed throughout the study in a standard box cage (Allentown, Inc., Allentown, NJ) with an electrical swivel (SVL6C, Kent Scientific, Corp., Torrington, CT) mounted above the cage for the flow probe connection. The subcutaneous catheter for AngII infusion was attached to a single channel hydraulic swivel (Model 375/22PS, Instech Laboratories, Inc., Plymouth Meeting, PA) mounted above the electric swivel.

3.2.4 Experimental protocol

The experimental protocol consisted of 4 days of control followed by a 14 day AngII infusion period. AngII (A9525, Sigma-Aldrich, Co., LLC., St. Louis, MO) was administered at a dose of 150ng/kg/min at a volume flow rate of 5uL/hr through the subcutaneous catheter attached to an external Harvard infusion pump (Model 935, Harvard Apparatus, South. Natick, MA). This infusion rate was chosen to match that in Alzet osmotic minipumps (2ML2, DURECT, Corp., Cupertino, CA) used in our previous study (66). Rats were given distilled water and a pelleted high salt diet (2.0% NaCl added rodent diet, Research Diets, New Brunswick, NJ) *ad libitum* throughout the experiment. Isotonic saline was infused through the subcutaneous catheter during the control period. The femoral venous catheter was flushed every other day with 50U/mL heparinized saline (Sagent Pharmaceuticals, Inc., Schaumburg, IL). An amount of AngII infusate needed for the length of the protocol was prepared in isotonic saline on the first day of infusion and used throughout the 14days of infusion.

Sympathetic tone was assessed by measuring the responses of mean arterial pressure (MAP), heart rate (HR), and mesenteric vascular resistance (MVR) to acute ganglionic blockade with hexamethonium (20mg/kg, i.v., H0879, Sigma-Aldrich, Co., St. Louis, MO) on day 3 of the control period and days 1,3,5,7,10, and 13 of AngII infusion. The dose of hexamethonium was adjusted based on the body weight measured on the day of experiment.

3.2.5 Confirmation of successful denervation

Sympathetic denervation of the splanchnic organs following celiac ganglionectomy was determined post-mortem by measurement of norepinephrine (NE) content in the spleen. NE content was also measured in both kidneys to assess the effect of CGx on renal sympathetic innervation. Tissues were explanted on the 15^{th} day of AngII infusion. Explanted tissues were rinsed in isotonic saline and gently blotted for removal of excess blood, stored in 1.5mL microcentrifuge tubes and flash frozen in liquid nitrogen. Tissues were stored in -80°C until analytic determination of NE content using high-performance liquid chromatography with electrochemical detection (64).

3.2.6 Data collection

AP and MBF were recorded continuously throughout the experiment at a 500Hz sampling rate using acquisition software from DSI (DataQuest ART Acquisition). The acquisition hardware for AP and MBF has been described in detail previously (66). MAP, HR and mean MBF were calculated and stored every 10s using the built-in online analysis routine in the acquisition software.

3.2.7 Data analysis and Statistics

10s interval data for MAP, HR, and mesenteric blood flow (MBF) was averaged over 24hrs through the 19 days of the protocol using analysis software from DSI (Dataquest ART Analysis) and exported to JMP software (version 10, SAS Institute, Inc., Cary, NC). MVR was calculated as MAP/MBF from the 12hr averaged values. Baseline values for food intake, water intake, MAP, MBF and MVR during control were obtained from the average value of the 4 control days.

The acute response to ganglionic blockade was analyzed using LabChart Pro (ADInstruments, Dunedin, New Zealand) software after exporting DSI arterial pressure and blood flow waveform data to open source EDF file format using custom written software in C++ using reference code provided by DSI (ART Reader 4.0, available from DSI upon request). MAP, HR, MBF, and MVR were calculated from the pulsatile data on a beat-by-beat basis for determining the peak changes following ganglionic blockade. The 10min baseline value immediately prior to injection was subtracted from this value and used as index to assess changes in peripheral sympathetic tone.

3.2.8 Statistics

Baseline BW, food intake, water intake, MAP, MBF and MVR during control were compared between Sham and CGx groups by multiple unpaired t-tests. Between and within group differences in the response of the various parameters measured throughout the AngII-salt protocol were tested for statistical significance using repeated-measures analysis of variance (ANOVA) followed by a Holm-Sidak multiple

comparisons test using SigmaPlot (version 11, Systat Software, Inc., Richmond, CA)

3.3 Results

Of the 28 rats used in the study, 8 were excluded from the final analysis due to lack or loss of superior mesenteric artery blood flow (SMABF) signal after flow probe implantation. The remaining 20 rats consisted of 10 subjected to CGx and 10 Sham treated rats. Successful sympathetic denervation of splanchnic organs by CGx was confirmed by measurement of tissue NE content in the duodenum and spleen. Results are shown in Figure 1 along with tissue NE content in the left kidney to assess the degree of renal involvement in CGx. Results are consistent with previously published data and confirms that CGx resulted in effective splanchnic sympathectomy with relative renal sparing.

3.3.1 Effect of CGx on baseline parameters

Shown in Table 1 are comparisons between Sham and CGx rats for variables measured throughout the study. There were no differences between groups for body weight, food or water intake. As seen in previous studies (42, 64, 110), CGx rats had a lower MAP and slightly higher HR during the baseline period. This was associated with elevated mesenteric blood flow and reduced mesenteric vascular resistance in CGx rats.

3.3.2 Effect of CGx on the splanchnic hemodynamic profile of AngII-salt hypertension

There were no appreciable differences in body weight, food intake, and water intake between CGx and Sham treated rats during baseline and throughout the 14 day AngII infusion period (Figure 2). A

representative example of the arterial pressure and mesenteric blood flow (MBF) signals obtained over a 60-minute period in conscious freely moving Sham and CGx rats is shown in Figure 3 (bottom panel). Examination of a 3 second window from this recording period reveals the stability and high fidelity of these recordings. These signals were used to derive 24-hour averages of mean arterial pressure (MAP), MBF, mesenteric vascular resistance (MVR) and heart rate (HR) for both experimental groups.

As shown in Figure 4, AngII infusion caused a gradual and continuous rise in MAP, accompanied by a rise in MVR with no change in MBF, in both groups. HR decreased during the first week of AngII infusion in both groups and appeared to stabilize at this lower level in Sham rats, in contrast to a gradual recovery toward baseline in CGx treated rats. There were no statistically significant differences in the changes to MAP and HR during AngII infusion between CGx and Sham treated rats.

Differences in MVR between CGx and Sham treated rats were not statistically significant during days 1 through 8 of AngII. However, MVR in CGx treated rats stabilized at statistically lower values compared to Sham on days 9 and 11 through 14 of AngII. There was a corresponding, statistically significant difference in MBF between CGx and Sham treated rats on days 8 through 14 of AngII.

3.3.3 Effect of CGx on responses of MAP, MVR and HR to acute ganglionic blockade

The contribution of sympathetic activity to MAP, MVR and HR in Sham and CGx rats was assessed by measuring the response of these

variables to acute ganglionic blockade (see Figure 5). As we have previously reported (66), the depressor effect of ganglionic blockade gradually increased during the AngII infusion period in Sham rats in that the depressor response was statistically greater on days 3, 5, 7, 10 and 13 of AngII compared to control, Surprisingly, the response was similar in CGx rats and there were no statistically significant differences between groups at any time during the protocol.

The mesenteric vasodilatory response to ganglionic blockade tended to increase during AngII infusion in both Sham and CGx treated groups (Figure 5; middle panel). These responses were not statistically different from baseline however except for day 13 of AngII in CGx treated rats. There were no statistically significant between group differences at any of the tested time points.

The bradycardic response to ganglionic blockade appeared to be reduced during AngII days 5 and 7 in Sham and CGx treated rats, respectively. This change, however, was not statistically significant. There were no statistically significant between group differences in the bradycardic response to ganglionic blockade at any of the tested time points.

3.4 Discussion

The aim of this study was to extend previous findings generated by the collaboration between our two laboratories (J.W.O and G.D.F.) and further test the hypothesis that the neurogenic component of AngII-salt hypertension is primarily mediated by an increase in neurogenic tone to the splanchnic vascular bed. Our previous studies have shown that AngII-salt hypertension is accompanied by a reduction in systemic vascular capacitance, which is reversible by ganglionic blockade and prevented by sympathectomy of the splanchnic vascular bed by CGx. Most importantly, CGx attenuated AngII-salt hypertension to levels seen in AngII-hypertensive rats fed a normal NaCl diet, a model thought to have a minimal neurogenic component (63). We have recently shown by direct continuous measurement of superior mesenteric artery blood flow in conscious rats that AngII-salt hypertension is accompanied by a sustained increase in mesenteric vascular resistance (MVR), and this response is acutely reversible by ganglionic blockade (66). This finding is consistent with our initial hypothesis, and provided a more direct index of splanchnic vascular hemodynamics in AngII-salt hypertensive rats. However, this finding does not exclude the possibility that non-neurogenic mechanisms also contribute to increased MVR in AngII-salt hypertension. Therefore, the present study was designed to test the hypothesis that CGx would attenuate or abolish the increase in MVR observed during the development of AngII-salt hypertension.

There were three main findings in this study. First, CGx chronically lowered baseline MVR in normotensive rats. Second, continuous AngII administration for 2 weeks caused a similar increase in MVR in Sham and CGx rats. Finally, CGx had no effect on the development of AngII-

salt hypertension. The interpretation and significance of these findings are discussed below.

3.4.1 CGx chronically decreases mesenteric vascular resistance and arterial pressure in normotensive rats

The contribution of *organ specific* basal sympathetic tone to long-term control of arterial pressure under normal conditions has not been extensively studied with the exception of the kidney. Specifically, renal denervation has been shown to cause a sustained reduction in arterial pressure in normotensive Sprague-Dawley rats (55, 57). This response is not due to reductions in renin release or sodium balance and has been hypothesized to be secondary to a chronic reduction in renal vascular resistance (57). Although this remains a controversial topic (30), it has been shown that basal renal SNA in normal rats contributes to renal blood flow fluctuation during normal behavior (116). Malpas et.al. have shown that in rabbits, renal denervation chronically lowers renal vascular resistance (77, 78). More recently, a study in pigs has shown that catheter based radiofrequency ablation of renal nerves results in a sustained decrease in renal vascular resistance and increased renal blood flow one month post ablation, suggesting that tonic sympathetic input is an important contributor to basal levels of renal vascular tone (109).

We observed in the present study that denervation of the splanchnic vascular bed by CGx also results in sustained hypotension, similar in magnitude to renal denervation (55, 57). Although similar observation of a chronic hypotensive effect of CGx has been reported by others (64, 110), the mechanisms mediating this response are not clear. It has been suggested that the *acute* hypotensive effect of CGx in *anesthetized*

animals is primarily due to an increase in portal venous capacitance, which decreases venous return, cardiac output, and consequently arterial pressure (45). The mechanisms mediating the *chronic* hypotensive effect of CGx in conscious freely moving animals, however, appear to be driven by a different process. Total vascular capacitance, which is predominantly determined by splanchnic venous tone (33, 41), is similar in Sham and CGx rats (64). This finding suggests that either basal sympathetic activity to splanchnic veins is very low or adaptations occur to restore vascular capacitance following CGx (64). In contrast, we found in this study that MVR was chronically decreased in CGx rats suggesting that sustained vasodilatation of the splanchnic resistance vessels is partially responsible for the chronic hypotensive effect of CGx. Taken together, the studies discussed above suggest that sympathetic activity is an important long-term controller of basal renal and mesenteric vascular resistance and arterial pressure under normal conditions.

3.4.2 Effect of CGx on mesenteric vascular resistance in AngII-salt hypertensive rats

Although basal MVR was chronically decreased under basal conditions in CGx rats, the mesenteric vasoconstrictor response to AngII administration was similar to Sham control rats. Absolute values for MVR were lower in CGx compared to Sham rats on days 9 and 11-14 of AngII, in part because of a lower basal level compared to Sham rats. Despite these differences in absolute level of MVR between Sham and CGx rats, the final level of arterial pressure after 14 days of AngII was the same in both groups.

This unexpected finding suggests that the increase in MVR during AngII administration is driven by non-neurogenic mechanisms. One possibility is that MVR increases in response to elevated perfusion pressure of the splanchnic vascular bed. Such pressure sensitive mechanisms may include changes in the myogenic response of mesenteric arterioles (104), impairment of flow-mediated vasodilation (1, 103), or an increase in AngII receptor density on mesenteric arterioles due to exposure to high perfusion pressure (71) leading to an increase in direct AngII vasoconstrictor tone.

Our hypothesis that increased MVR in AngII-salt rats is sympathetically mediated was based on our recent finding that the mesenteric vasodilatory response to ganglionic blockade increases statistically over the 14 day period of AngII administration in intact rats (66). We observed the same trend in Sham rats in the present study although it was not statistically significant. More importantly, in the present study, this response was the same in CGx and Sham rats. One explanation for this finding is that the MVR response to ganglionic blockade is a secondary autoregulatory response to decreased perfusion pressure. The mechanism mediating the MVR response to ganglionic blockade in CGx rats remains to be determined. Overall, these findings suggest that AngII mediated changes in splanchnic arteriolar hemodynamics assessed by measurement of superior mesenteric artery blood flow are predominantly mediated by non-neurogenic mechanisms.

3.4.3 Effect of CGx on the development of AngII-salt HTN: Reconciliation with previous studies

We have previously reported that CGx attenuates AngII-salt hypertension (64). In the present study, however, neither the slow rising pressor response to AngII administration, or final level of arterial pressure, was affected by CGx. Furthermore, the gradual increase in the depressor response to ganglionic blockade over the 2 week period of AngII administration, an indirect indicator of "whole body" sympathetic tone, was unaffected by CGx. These findings are inconsistent with the hypothesis that the neurogenic component of AngII-salt hypertension is mediated by increased sympathetic tone to the splanchnic vascular bed. Failure of CGx to attenuate AngII-salt hypertension occurred despite successful splanchnic sympathectomy, as evidenced by the baseline hemodynamic profile of CGx rats (i.e., decreased MAP and MVR) and significantly lower tissue NE content in the duodenum and spleen. The response of tissue NE content in this study is similar to those reported by others from our group (42, 58, 64, 110).

There are at least three explanations for this unexpected finding. First, differences in the method of AngII delivery between studies may have impacted the overall characteristics of the model. In our previous studies (62, 64) AngII was delivered subcutaneously using Alzet osmotic minipumps. Although this method of delivery is standard for AngII-induced rodent models, our experience with unpredictability of pump performance led us to switch to using an external syringe pump. This decision was influenced by two factors. First, since animals needed to be tethered for externalization of the flow probe cable, exteriorizing a

subcutaneous catheter was straightforward and would not complicate the preparation. Second, we reasoned that use of an external syringe pump, which was carefully calibrated and monitored daily to insure stable long-term delivery of AngII, would be superior to the Alzet minipumps. In a recent preliminary study we discovered that, despite using the same volume flow rate and AngII infusate concentration as our previous studies, Alzet pumps result in a marked spike in plasma AngII on Day 3 of infusion compared to a traditional external syringe pump (67). The relevance of this finding is that Johnson and colleagues have recently reported that a brief period of AngII administration amplifies the hypertensive response to subsequent AngII infusion and this response is most likely to be neurally mediated (114). This would be consistent with the idea that the "spike" in plasma AngII observed with Alzet minipumps delivery may "prime" the neurogenic component of AngII-salt hypertension. Whether or not differences in the plasma AngII profile between these two methods of AngII administration is sufficient to explain the discrepancy between the present study and our previous reports remains to be established.

A second explanation is that the extent of denervation affecting the kidney was different between this study and the previous study by King and colleagues (64). As expected, tissue NE content was decreased in CGx rats in both studies in splanchnic organs. In the current study, renal NE content was not affected in CGx rats indicating innervation of the kidneys was not affected to a significant extent. This contrasts with the statistically significant decrease in NE content of the left kidney in CGx rats in the study of King et al. (64). Since the surgical approach for CGx exposes the left peritoneal cavity by exteriorizing the gut

content to the left surgical field, it is possible that in the study of King et al CGx not only removed the celiac plexus (celiac and superior mesenteric ganglia), but also extended to renal ganglia and possibly as far as the left suprarenal ganglion (101), thus affecting the innervation to the left kidney to a greater extent than the right. Although renal denervation alone did not affect AngII-salt hypertension in that study (64), it is conceivable that CGX resulted in denervation of the splanchnic vascular bed and sympathetic input to the kidneys resulting in an additive blood pressure lowering effect. We have recently reported such an additive effect of renal denervation and CGx on blood pressure in the Dahl-S rat model of hypertension (42).

A third possibility is suggested by the recent hypothesis that sympathetically mediated release of endogenous ouabain from the adrenal cortex plays a role in salt sensitive hypertension by amplifying sympathetic input to the vasculature (8). Indeed, we came to this same conclusion to explain our recent studies on neural mechanisms mediating increased arterial pressure in rats during 48hrs of water deprivation (110). Although the pressor response to water deprivation is sympathetically driven, it is not affected by targeted sympathetic ablation of the kidneys, splanchnic vascular bed or hind limb (110). However, adrenalectomy, but not adrenal demedullation, attenuated this pressor response in the same time frame as chronic adrenergic receptor blockade. We hypothesized that neural control of adrenal cortical release of endogenous ouabain may be the mechanism mediating this response (110). Similar to the AngII-salt model (87), 48 hours of water deprivation is believed to drive sympathetic activity via a central osmotic stimulus and elevated plasma AngII levels (110). This link raises

the possibility that the hypotensive response of AngII-salt rats to CGx reported by King and Fink, but not in this study, may have been the result of denervation of the adrenal cortex (i.e. endogenous ouabain release) rather than the splanchnic vasculature per se. The possibility that endogenous ouabain release mediate the hemodynamic changes observed in this model is supported by early experiments in dogs, which have shown an increase in MVR, and decrease in vascular capacitance in response to exogenously administered ouabain (46). This interesting hypothesis remains to be tested.

3.4.4 Perspectives

Our laboratories (J.W.O. and G.D.F.) have collaborated over a number of years to investigate the role of regional sympathetic activity in the rat model of AngII-salt hypertension (64, 66, 85, 86, 87, 88, 108, 115). To enable comparison of results between laboratories, we have used a standardized protocol and same sources for rats, specialized diets, and AngII. In addition, both laboratories have employed the same method of subcutaneous AngII delivery using implantable osmotic minipumps. Although this method is convenient, our experience is that it suffers from unpredictability of pump performance. In this study, AngII was delivered subcutaneously using an external syringe pump using the identical AngII infusate concentration and volume flow rate as our previous studies. We reasoned that this subtle difference in AngII delivery would not impact the model.

Contrary to our hypothesis, and a previous paper from our group, CGx had no impact on the response of MVR or arterial pressure to AngII administration in rats consuming a high salt diet. One possibility

for the difference between studies is the method of AngII administration. It has been shown that the mechanism and severity of AngII-induced hypertension is sensitive to dose and level of dietary salt intake (26, 79, 97), and prior physiological stimuli that may transiently elevate plasma AngII levels (114). Another possibility is subtle differences between laboratories for the method of CGx and target organs affected.

Although our study presents the challenge of reconciling results between two laboratories that have worked closely together using a standardize protocol, it also highlights the much greater challenge of comparing studies in the literature from different laboratories using differences species of study and vastly different protocols of AngII induced hypertension (e.g. salt diet, dose of AngII, route and method of AngII delivery). A continued collaboration between our laboratories to reconcile the effects of CGx on AngII-salt hypertension are most likely lead to new discoveries regarding both neural and non-neural mechanisms of this experimental model of hypertension.

Grants

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Disclosures

None

3.5 Figures and Table

Table 3.1. Baseline parameters

Baseline parameters in Sham and CGx rats during control period. Values for food intake, water intake, heart rate (HR), mean arterial pressure (MAP), mesenteric blood flow (MBF), and mesenteric vascular resistance (MVR) are averages of 24hr values from the 4 control days. (∗) denote significant ($p < 0.001$) between group differences.

Table 3.1

	Sham	CGx
Body Weight (g)	365 ± 7	356 ± 10
24hr Food Intake (g)	23.6 ± 0.5	24.2 ± 0.6
24hr Water Intake (mL)	37.3 ± 0.7	35 ± 1
HR (BPM)	420.7 ± 3.4	436.9 ± 2.3 *
MAP (mmHg)	103 ± 0.8	97.9 ± 0.7 *
MBF (mL/min)	11.4 ± 0.3	13.3 ± 0.5 *
MVR (mmHg•min/mL)	9.2 ± 0.3	7.8 ± 0.3 *

Figure 3.1. Tissue NE content

Tissue norepinephrine (NE) content in left kidney (LK), spleen (SP) and duodenum (DD) harvested from Sham (white bars) and CGx rats (black bars) one day after the 14 days of AngII infusion. Tissue NE was measured by HPLC. (∗) denote significant ($p<0.05$) between group differences.

Figure 3.1

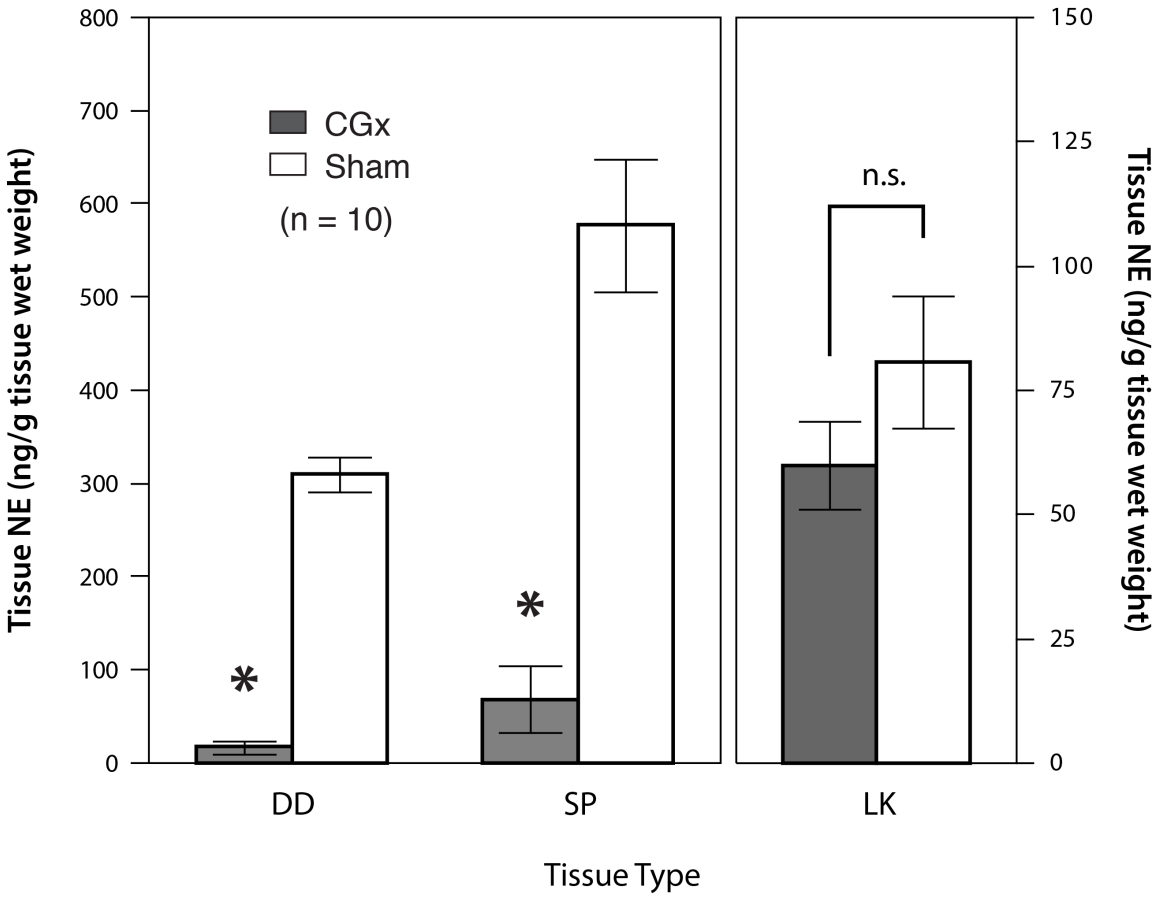

800
700
600
500
400
300
200
100
0
150
125
100
75
50
25
0
CGx
Sham
(n = 10)
n.s.
*
*
DD
SP
LK
Tissue Type
Tissue NE (ng/g tissue wet weight)
Tissue NE (ng/g tissue wet weight)

Figure 3.2. Daily food/water intake, changes in body WT during AngII-salt hypertension

Changes in body weight and daily food and water intake during control and AngII-salt hypertension in Sham (unfilled bar and circles) and CGx (filled bar and circles) rats. "C" and "A" prefixes on x-axis denote control and AngII days, respectively. (†) and (‡) denote significant within group difference compared to day 3 of control in Sham and CGx rats, respectively. Error bars are S.E.M.

Figure 3.2

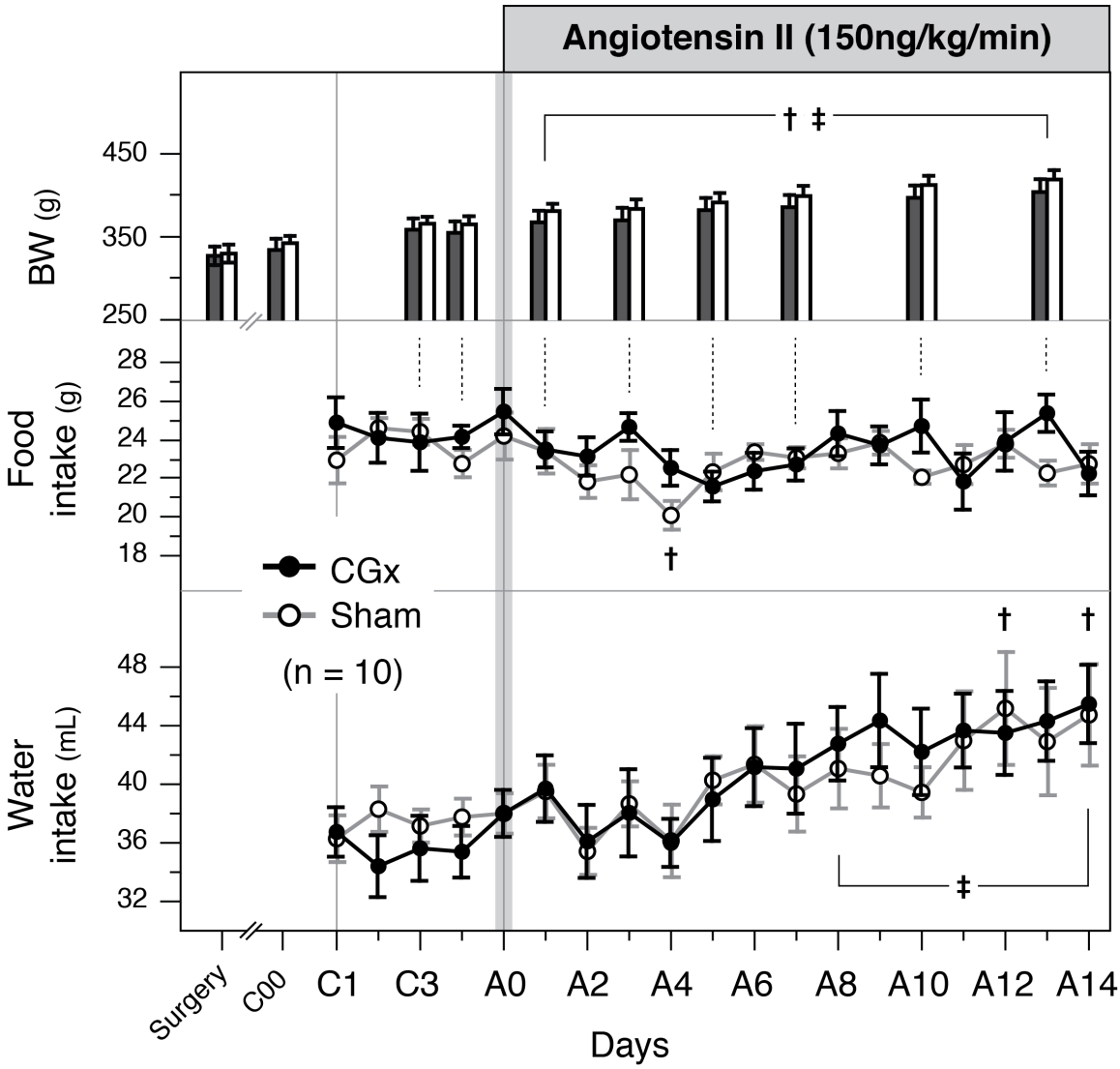

Angiotensin II (150ng/kg/min)
BW (g)
450
350
250
Food intake (g)
28
26
24
22
20
18
CGx
Sham
(n = 10)
Water intake (mL)
48
44
40
36
32
† ‡
†
† †
‡
Surgery
C00
C1
C3
A0
A2
A4
A6
A8
A10
A12
A14
Days

Figure 3.3. Representative AP, MBF Waveform

Representative arterial pressure (AP) and superior mesenteric artery blood flow (MBF) waveform data collected from Sham (left) and CGx (right) rats during the study. Bottom panel shows compressed waveform data from a 1hr period during the light cycle on control day 3. The firs 3 seconds from this 1hr segment is shown on the top panel.

Figure 3.3

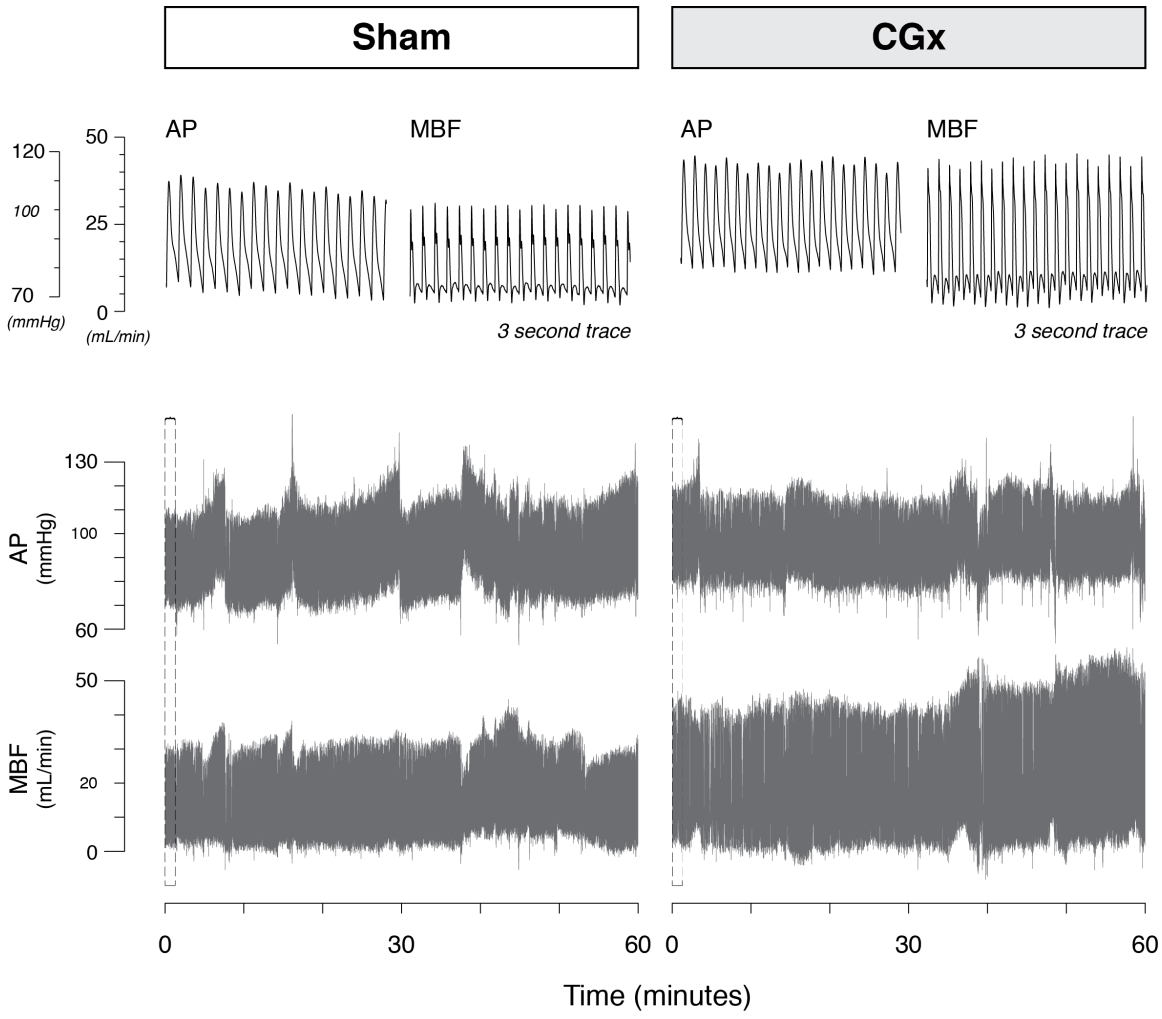

Sham
CGx
AP
MBF
AP
MBF
120
100
70
(mmHg)
50
25
0
(mL/min)
3 second trace
3 second trace
AP (mmHg)
130
100
60
MBF (mL/min)
50
20
0
0
30
60
0
30
60
Time (minutes)

Figure 3.4. 24hr hemodynamic profile of MAP, MVR, MBF, and HR during AngII-salt hypertension

24hr average mean arterial pressure (MAP), mesenteric vascular resistance (MVR), superior mesenteric artery blood flow (MBF), and heart rate (HR) during control and AngII-salt hypertension in Sham (unfilled circles) and CGx (filled circles) rats. "C" and "A" prefixes on x-axis denote control and AngII days, respectively. (†) and (‡) denote significant within group difference compared to day 3 of control in Sham and CGx rats, respectively. (∗) denote significant ($p<0.05$) between group differences. Error bars are S.E.M.

Figure 3.4

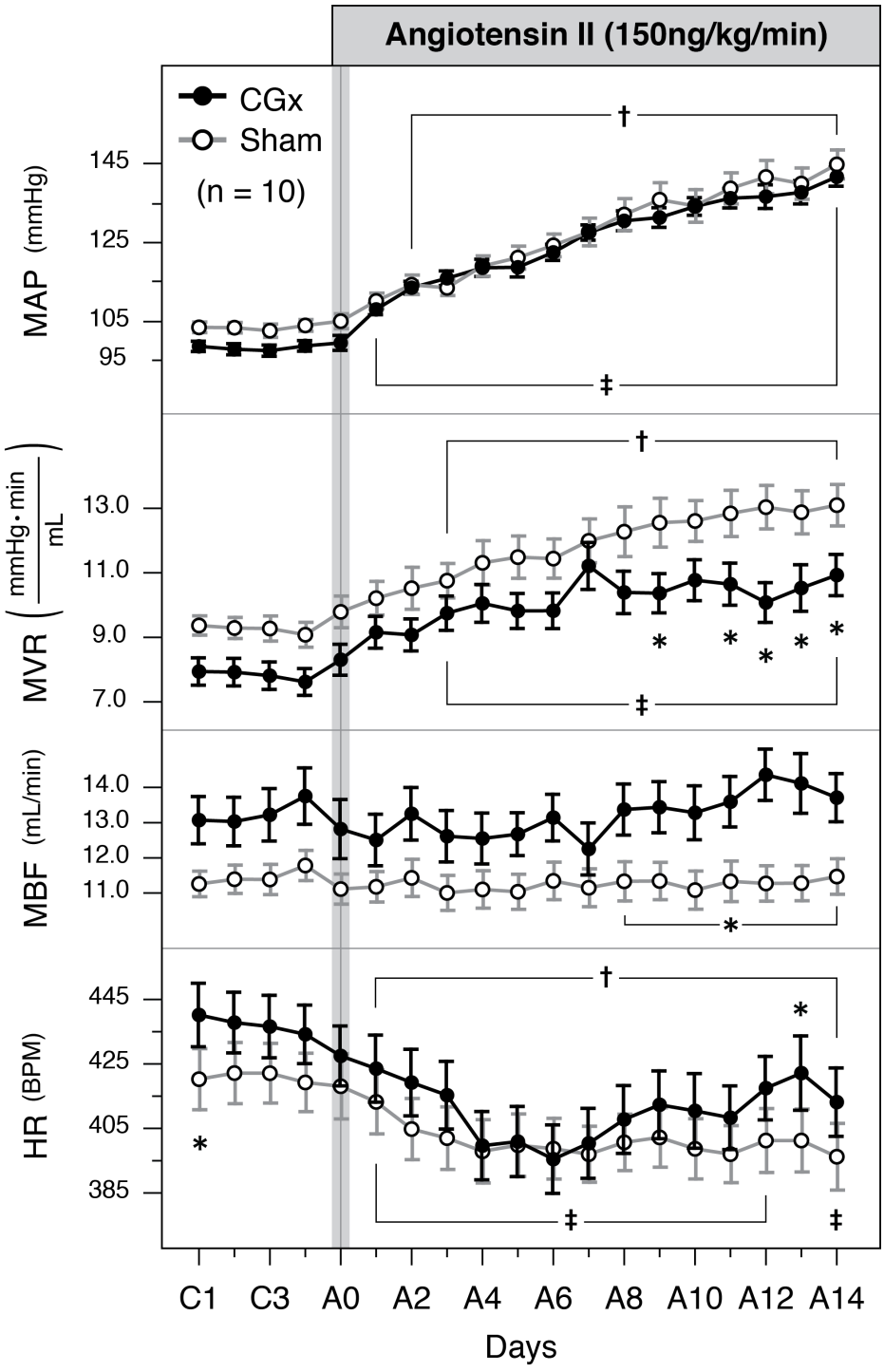
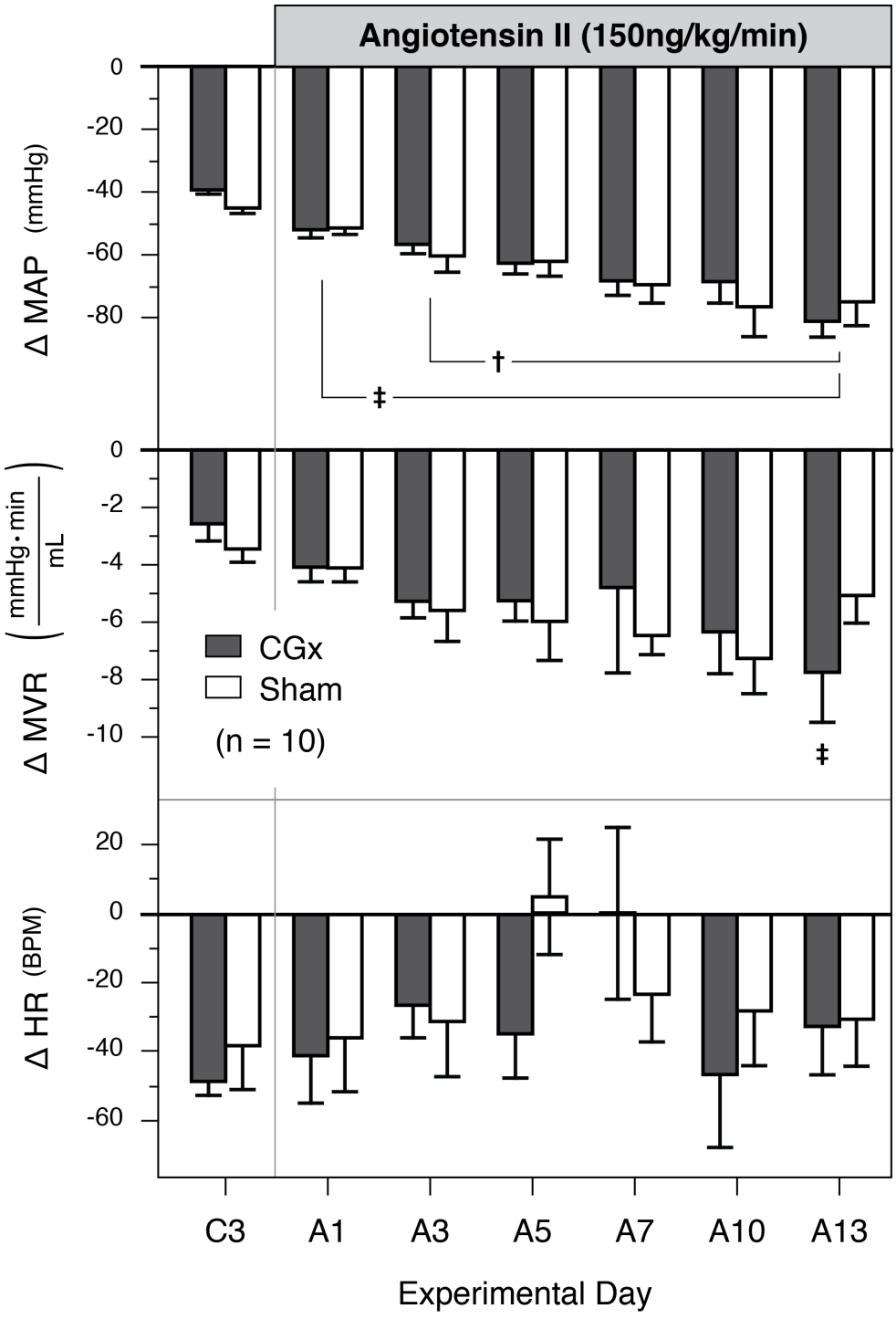

Angiotensin II (150ng/kg/min)
CGx
Sham
(n = 10)
MAP (mmHg)
145
125
105
95
MVR (mmHg·min / mL)
13.0
11.0
9.0
7.0
MBF (mL/min)
14.0
13.0
12.0
11.0
HR (BPM)
445
425
405
385
†
‡
*
C1
C3
A0
A2
A4
A6
A8
A10
A12
A14
Days

Figure 3.5. Changes in responsiveness of HR, MAP and MVR to acute ganglionic blockade during AngII-salt hypertension

Peak changes in mean arterial pressure (MAP), mesenteric vascular resistance (MVR), and heart rate (HR) after ganglionic blockade (hexamethonium, 20mg/kg, i.v.). Values represent absolute differences from a 10min baseline immediately preceding the injection. Injections were performed on control day 3 and days 1, 3, 5, 7, 10, and 13 of AngII. Unfilled and filled bars represent responses in Sham and CGx rats, respectively. "C" and "A" prefixes on x-axis denote control and AngII days, respectively. (†) and (‡) denote significant within group difference compared to day 3 of control in Sham and CGx rats, respectively. Error bars are S.E.M.

Figure 3.5


Angiotensin II (150ng/kg/min)
Δ MAP (mmHg)
0
-20
-40
-60
-80
†
‡
Δ MVR (mmHg·min/mL)
0
-2
-4
-6
-8
-10
CGx
Sham
(n = 10)
‡
Δ HR (BPM)
20
0
-20
-40
-60
C3
A1
A3
A5
A7
A10
A13
Experimental Day

Chapter 4

Effect of chronic $\alpha_{1/2}\beta_1$-adrenergic receptor blockade on the development of AngII-Salt Hypertension

Chapter Overview

The contribution of global sympathetic pressor tone in angiotensin-II salt (AngII-salt) hypertension has been inferred mostly from repeated measurements of whole body norepinephrine (NE) spillover and the depressor response to acute ganglionic blockade. Complementary evidence using chronic sympathetic blockade has been lacking for the AngII-salt model in the rat. To address this issue, we tested the effect of chronic, global sympathetic blockade on AngII-salt hypertension by continuous infusion of phentolamine and atenolol, a non-selective $\alpha_{1/2}$ and selective β_1-adrenergic receptor antagonist, respectively. We found that $\alpha_{1/2}\beta_1$-adrenergic receptor blockade attenuates AngII-induced hypertension in rats fed a high (2%) salt (NaCl) diet (AngII-salt hypertension) but not those in a low (0.1%) NaCl diet. Global sympathetic pressor tone accounted for approximately 27% of the total change in pressure in AngII-salt hypertensive rats, as estimated by the differences in pressure between chronic $\alpha_{1/2}\beta_1$-adrenergic receptor blocked and vehicle treated rats. In addition, although the salt-dependent differences in the severity of AngII-induced hypertension was blunted by chronic $\alpha_{1/2}\beta_1$-adrenergic receptor blockade, it was not completely blocked, suggesting that an increase in peripheral adrenergic tone plays some role, but is not the major determinant of the salt dependent effects on the severity of AngII-induced hypertension. We conclude that global sympathetic pressor tone contributes to AngII-salt hypertension, but to an extent that is significantly smaller than what has been previously assumed by acute ganglionic blockade.

4.1 Introduction

Hypertension that results from chronic administration of AngII (AngII-induced hypertension) is thought to be mediated, in part, by neurogenic mechanisms (86, 87). Several line of evidence supports this neurogenic hypothesis, including a progressively higher depressor response to ganglionic blockade (13, 62, 66, 79), prevention of this form of hypertension by neonatal sympathectomy (26, 98), and attenuation of hypertension by chronic adrenergic blockade (76). However, other studies employing similar techniques have found conflicting results (65, 83). These inconsistencies can be attributed in part, to the dependence of the model on the level of dietary salt intake (87). The severity of AngII-induced hypertension in dogs (24), rats (62, 63, 64, 66), and mice (22) has been shown to increase progressively with higher levels of dietary salt intake. In both rats, and rabbits, it has been shown that the depressor response to ganglionic blockade is elevated during AngII-induced hypertension selectively in animals with a high salt intake (AngII-salt hypertension) (62, 64, 66, 79). Furthermore, our group has shown that NE spillover is elevated in high salt diet rats, but not those fed a normal salt diet (63).

Although global indices of sympathetic tone were highly indicative of a rise in adrenergic activity and enhanced sympathetic vasomotor tone during AngII-salt hypertension, attempts at directly measuring increases in sympathetic nerve activity or identifying a critical peripheral sympathetic target organ has been mostly unsuccessful (85). Consistent with other studies in AngII-induced hypertension, it has been shown that renal nerves have little contribution to the rise in pressure during AngII-salt hypertension, both on the basis of denervation (13, 64), and

direct nerve recording experiments (4, 115). Direct measurement of lumbar sympathetic nerve activity has been shown to remain unchanged during AngII-salt hypertension (115), and correspondingly, hind limb NE spillover was shown to be unchanged (61). Preliminary results from our lab have shown that sympathetic input to the heart has no contribution to AngII-salt hypertension based on findings from cardiac denervation and chronic β_1-adrenergic receptor (β_1-AR) blockade experiments (52). The peripheral sympathetic target organ responsible for the neurogenic mechanism underlying AngII-salt hypertension was thought to have been finally identified when it was shown that AngII-salt hypertension is significantly attenuated by celiac ganglionectomy (CGx) (64). Most recently, however, we found conflicting results showing that CGx does not attenuate AngII-salt hypertension (see results in Chapter 3).

The expected contribution of sympathetic pressor tone on AngII-salt hypertension has largely been based on the magnitude drop in pressure following acute ganglionic blockade. Although this technique has been validate by our lab and others as an indicator of sympathetic pressor tone in the rat, repeated measures obtained under chronic experimental settings may not necessarily reflect the response when the sympathetic nervous system is inhibited in the chronic setting (23, 93). Complementary techniques for addressing this issues, such as continuous administration of adrenergic blocking agents or neonatal sympathectomy, have been used to investigate the sympathetic contribution to AngII-induced hypertension, but with mixed results as alluded to earlier (26, 76, 98). To our knowledge, there have been no

studies assessing the effect of chronic sympathetic blockade specifically during AngII-salt hypertension.

The objective of this study was to reassess the contribution of global sympathetic pressor tone during AngII-salt hypertension by chronic continuous administration of phentolamine, a competitive, non-specific α-adrenergic antagonist, and atenolol, a specific β1-AR antagonist. Phentolamine was chosen over a more selective α-adrenergic antagonist to account for α_2-adrenergic receptor (α_2-AR) mediated vasoconstrictor effects in veins (28). Atenolol was added to the infusate primarily to counteract β_1-AR mediated increases in plasma renin activity (84) expected due to increases in plasma catecholamines following non-selective α-adrenergic blockade (92). We compared the effect of $\alpha_{1/2}\beta_1$-adrenergic receptor ($\alpha_{1/2}\beta_1$-AR) blockade during AngII-induced hypertension in rats fed a high (2%) salt (NaCl) diet (AngII-salt hypertension), which is hypothesized to be highly dependent on a sympathetic pressor tone, and a low (0.1%) NaCl diet, in which the sympathetic component is thought to be less or absent.

4.2 Methods

Experiments were performed in conscious, chronically instrumented rats in accordance with NIH guidelines. All acute and chronic experimental procedures were conducted after approval and under supervision by the institutional animal care and use committee of the University of Minnesota.

4.2.1 Animal use and care

Male Sprague-Dawley rats from Charles River Laboratories (Wilmington, MA) weighing 200-225g upon transfer to our facility were used in this study. Upon arrival, rats were switched to a high (2%) or low (0.1%) salt (NaCl) diet (Research Diets, Inc., New Brunswick, NJ). Animals were housed 2 per cage in a 12-12hr light-dark cycled room (8:30/20:30 cycle). Distilled water was available ad-libidum. Animals were allowed to acclimate for at least 1 week prior to surgery.

4.2.2 Surgical procedure

Surgery was performed under isoflurane anesthesia (2.5% isoflurane in 100% O2 delivered via a nose cone at 1mL/min flow rate). After induction, rats were given atropine (0.2mg/kg, i.p., Baxter International, Inc., Deerfield, IL) for reduction of salivary and bronchial secretions, preoperative antibiotic prophylaxis (gentamicin, 0.05mL, i.m., Hospira, Inc., Lake Forest, IL), preoperative pain relief (ketoprofen, 5mg/kg, s.c., Fort Dodge Animal Health, Inc., Overland Park, KS) and placed on a heated surgical bench. Surgical instruments were heat sterilized and all implanted instruments were cold sterilized overnight in a solution of

glutaraldehyde (Cidex Plus, Johnson & Johnson Services, Inc., New Brunswick, NJ).

The left external jugular vein was cannulated via an incision slightly above the clavicles along the mid-clavicular line. A custom made jugular venous catheter was advanced 3 cm towards the right atrium. This was later used for acute injections of phenylephrine to test for the efficacy of α_1-adrenergic receptor (α_1-AR) blockade. The detailed surgical procedure and method of fabrication of the jugular venous catheter has been described previously (see Appendix 1). A custom made femoral venous catheter was advanced into the lower abdominal vena cava through the left femoral vein as described previously (66). A DSI pressure transmitter (TA11PA-C40, Data Sciences International (DSI), Saint Paul, MN) was implanted intraperitoneally through a midline laparotomy, and its catheter was inserted into the left femoral artery for measurement of arterial pressure as described previously (66). Finally, a catheter was implanted subcutaneously, through a dorsal skin incision on the right upper back of the animal, which was later used for chronic administration of AngII using an external syringe pump (model 935, Harvard Apparatus, South. Natick, MA) as previously described (see Appendix 1).

All three catheters were exteriorized through a skin incision over the scapulae, anchored to the underlying subcutaneous tissue using a circular surgical polyester mesh (PETKM14002, Textile Development Associates, Inc., Surgical Mesh Division, Brookfield, CN) which was implanted subcutaneously and sutured to the skin upon closure of the incision (see Chapter 3). The exteriorized catheters were threaded through a stainless steel spring used for tethering the rat to a dual

channel hydraulic swivel (model 375/D/22, Instech Laboratories, Inc., Plymouth Meeting, PA) mounted above their cage. The free end of the subcutaneous catheter and the femoral venous catheter were each connected to a port on the swivel.

Rats were given a minimum of 7 days to recover from surgery. Ketoprofen (5mg/kg, s.c., Fort Dodge Animal Health, Inc., Overland Park, KS) was administered daily for 3 days post surgery for pain management. The femoral and jugular venous catheters were flushed every 4 days with 50U/mL heparinated saline (Hospira, Inc., Lake Forest, IL) to maintain patency between uses.

4.2.3 Experimental protocol

The study consisted of two factors, dietary salt (NaCl) and drug treatment, each with two levels; high (2%) and low (0.1%) NaCl for "dietary NaCl", and vehicle and $\alpha_{1/2}\beta_1$-AR antagonist for "drug treatment". $\alpha_{1/2}\beta_1$-AR blockade was achieved by administration of phentolamine and atenolol (see below for dose). Experiments with all four groups were conducted in parallel. The experimental protocol consisted of 7 days of control followed by 14 days of AngII, and a 3 day recovery period. Administration of vehicle or an $\alpha_{1/2}\beta_1$-AR antagonist solution was started on day 0 of control and continued throughout the duration of the protocol. Additionally, physiological saline (B. Braun Medical, Inc., Irvine, CA) was administered continuously through the subcutaneous catheter starting on day 0 of control to maintain patency until switched to AngII on the 8^{th} day of the protocol (AngII day 0).

Vehicle or a $\alpha_{1/2}\beta_1$-AR antagonist solution was administered through the femoral venous catheter using an external syringe pump (model 975,

Harvard Apparatus) set to deliver the solutions at a rate of 2.13mL/day using a 3mL syringe (Monoject, Tyco Healthcare Group, Mansfield, MA). The 3mL syringe was coupled to a 0.2μm syringe filter (Acrodisc, Pall Corporation, Ann Arbor, MI) and connected to a dual channel hydraulic swivel using a 23g Tygon tubing (3/16" I.D. x 5/16" O.D. Tygon S-50-HL Saint-Gobain Performance Plastics, Corp., Akron, OH). The $\alpha_{1/2}\beta_1$-AR antagonist was prepared fresh, daily, from stock solutions (see section below).

We monitored the efficacy of α_1-AR blockade in $\alpha_{1/2}\beta_1$-AR antagonist treated rats by measuring the pressor response to an acute injection of phenylephrine (10ug/kg, i.v. via jugular venous catheter). The pressor response to phenylephrine was tested on day 0 of control prior to starting the $\alpha_{1/2}\beta_1$-AR antagonist or vehicle infusion, days 1 and 7 of control, and on the last day of recovery in all experimental groups.

Vehicle or AngII was infused through the subcutaneous catheter using an external syringe pump (model 935, Harvard Apparatus) calibrated to an infusion rate of 5uL/hr using a 1mL syringe (Monoject, Tyco Healthcare Group). The syringe was coupled to a port on the dual channel hydraulic swivel using 23g Tygon tubing. On the 8^{th} day of the protocol (day 0 of AngII), the syringe containing physiological saline was switched to one filled with AngII infusate. The subcutaneous catheter was primed with AngII based on the estimated catheter dead space (approximately 0.2mL) prior to restarting the infusion.

Food intake and water intake was measured daily during the protocol. Body weights were measured periodically, and dose adjustments for the $\alpha_{1/2}\beta_1$-AR antagonist solution were made weekly on days 7 of control, and days 7 and 14 of AngII based on weight. Dose

adjustment was performed only if there was an increase in weight; otherwise the dose was kept unchanged.

4.2.4 Pharmacological agents

The $\alpha_{1/2}\beta_1$-AR antagonist solution consisted of an aqueous mixture of phentolamine (methanesulfonate salt; P302560, Toronto Research Chemicals, Ontario, Canada) and atenolol (A-7655, Sigma-Aldrich, Co., LLC., St. Louis, MO) containing citric acid (C0759, Sigma-Aldrich, Co.) and sodium citrate (S4641, Sigma-Aldrich, Co.) as buffering agents. A stock solution of phentolamine was prepared in sterile water at a concentration of 50mg/mL. As atenolol is poorly soluble in water, a custom recipe was formulated to prepare a 20mg/mL stock solution. It was found that atenolol at the required concentration would solubilize in a solution of 50 parts water and 3 parts stock citrate buffer with vigorous sonication. The stock citrate buffer solution consisted of 5.5 parts citric acid and 3 parts sodium citrate in sterile water (final concentration of approximately 0.07M citric acid and 0.026M sodium citrate).

The stock solutions of phentolamine and atenolol were prepared fresh weekly. The phentolamine stock solution was stored at -80°C. The two drugs were mixed daily to prepare an infusate calculated to administer phentolamine at a rate of 3mg/kg/hr and atenolol at a rate of 2mg/kg/hr. The delivery rate of atenolol was based on a previously published study from our lab (57, 110). The delivery rate of phentolamine was based on previously reported doses (7, 106) and further titrated during a preliminary study to result in complete blockade of the pressor response to phenylephrine after a one day

infusion of phentolamine. The resulting mixture had a pH of 6.4. The osmolality of the final solution was measured using an osmometer (model 3320, Advanced Instruments, Inc., Norwood, MA) and corrected to 290mOsms/kg using a 200mg/mL hypertonic saline solution. The vehicle solution was prepared from the stock citrate buffer diluted to the same concentration as the drug mixture (approximately 16.6% v/v). The pH was adjusted to 6.4 using sodium hydroxide, and osmolality was corrected as described above.

The AngII infusate was prepared by diluting AngII (A9525, Sigma-Aldrich) in physiological saline to result in a delivery rate of 150ng/kg/min at an infusion rate of 5μL/hr. A working solution of phenylephrine was prepared in physiological saline at a concentration of 50μg/mL.

4.2.5 Data collection

Arterial pressure (AP) was collected continuously throughout the experiment at a 500Hz sampling rate using acquisition software from DSI (DataQuest ART Acquisition). The acquisition hardware has been described in detail previously (66). MAP, and HR were calculated and stored every 10s using the built-in online analysis routine in the acquisition software.

4.2.6 Data analysis and Statistics

Time dependent changes in MAP and HR was quantified using 10s interval data averaged over 24hrs using analysis software from DSI (Dataquest ART Analysis). Both original values and changes from baseline (ΔMAP and ΔHR) were used for analysis. Baseline MAP and

HR was calculated from the average of 7 days of control, and compared between groups by a t-test. ΔMAP and ΔHR for each animal were calculated by subtracting the baseline value from each data point, and used for determining the effect of chronic $\alpha_{1/2}\beta_1$ receptor blockade on AngII-induced hypertension for each level of dietary salt. The effect of $\alpha_{1/2}\beta_1$ receptor antagonist or vehicle treatment on the dietary salt dependent differences in the magnitude of AngII-induced hypertension was determined from the original MAP and HR values. Between and within group differences in MAP, ΔMAP, HR, and ΔHR during AngII infusion was tested for statistical significance by 2-way repeated-measures ANOVA followed by a Holm-Sidak multiple comparisons test when appropriate.

The pressor response to acute phenylephrine injection was analyzed using LabChart Pro (version 7, ADInstruments, Dunedin, New Zealand) and Matlab (version R2012a, Mathworks, Inc.). MAP data was extracted from the 500Hz signal and averaged over XXXs intervals using LabChart Pro. Quantification of the pressor response was carried out in Matlab. The average MAP from a 5 min. period immediately before the injection was subtracted from each trace to account for baseline MAP differences between vehicle and $\alpha_{1/2}\beta_1$ receptor antagonist treated rats. The pressor response was quantified as an area under the curve (AUC) calculated from the MAP trace starting at the beginning of PE injection to 5min after the injection. The extent of α_1 receptor blockade resulting from $\alpha_{1/2}\beta_1$ receptor antagonist infusion for each animal was determined by calculating the AUC ratio using the AUC from the PE response prior to vehicle or antagonist infusion (on control day 0) as denominator. Between group differences in AUC and within group changes in the

AUC ratio were tested for statistical significance by 2-way repeated-measures ANOVA followed by a Holm-Sidak multiple comparisons test when appropriate.

Data were tabulated and graphed using JMP software (version 10, SAS Institute, Inc., Cary, NC). Statistical analysis was performed in SigmaPlot (version 11, Systat Software, Inc., Richmond, CA). All values are shown as mean ± S.E.M unless otherwise noted.

4.3 Results

4.3.1 Effect of chronic $\alpha_{1/2}\beta_1$-AR blockade on 24hr food, water intake and body weight

Food intake was transiently lower in $\alpha_{1/2}\beta_1$-AR antagonist treated rats, especially in the low salt group, as shown in Figure 4.1. In the low salt group, rats treated with $\alpha_{1/2}\beta_1$-AR antagonist had lower food intake compared to those receiving vehicle solution during most of control through day 5 of AngII. After day 5 of AngII, food intake was, for the most part, comparable between vehicle and $\alpha_{1/2}\beta_1$-AR antagonist treated rats. In the high salt group, food intake were similar between vehicle and $\alpha_{1/2}\beta_1$-AR antagonist treated rats except for day 1 of control and days 2 and 3 of AngII.

Water intake was for the most part unaffected by $\alpha_{1/2}\beta_1$-AR blockade. Water intake in the low salt group was lower in $\alpha_{1/2}\beta_1$-AR antagonist treated rats on day 1 of control, but otherwise the same as in vehicle treated rats throughout the protocol. In the high salt group, water intake was lower in $\alpha_{1/2}\beta_1$-AR antagonist treated compared to vehicle treated rats on day 1 of control, and days 2 and 3 of AngII; corresponding to the same days that food intake was lower in the $\alpha_{1/2}\beta_1$-AR antagonist treated rats.

Body weights were comparable across all 4 groups of rats until day 7 of control (Table 4.1). From control day 7 onwards, vehicle treated rats in the low salt group showed greater weight gain compared to $\alpha_{1/2}\beta_1$-AR antagonist treated rats fed a low salt diet. The weight gain observed in vehicle treated rats fed a low salt diet was also greater compared to those seen in vehicle treated rats fed a high salt diet. The weight

difference between high and low salt rats in the vehicle treated group was statistically significant on days 7 and 14 of AngII. There were no significant weight differences between the remaining three groups of rats at any point during the protocol.

4.3.2 Effectiveness of α_1-AR blockade during chronic $\alpha_{1/2}\beta_1$-AR antagonist treatment

The acute injection of 10ug/kg phenylephrine during control day 0, prior to starting the infusion of $\alpha_{1/2}\beta_1$-AR antagonist or vehicle, resulted in a peak pressor response of approximately 50mmHg (Figure 4.1). Infusion of $\alpha_{1/2}\beta_1$-AR antagonist blocked 87% and 93% of this α_1-AR mediated pressor response after one day of treatment in high and low salt rats, respectively (Table 4.2). Prolonged treatment with $\alpha_{1/2}\beta_1$-AR antagonist resulted in a gradual loss in the efficacy of α_1-AR blockade. This was more significant in the high salt group where the efficacy of α_1-AR blockade dropped to 55% after 26 days of $\alpha_{1/2}\beta_1$-AR antagonist treatment. This drop in the efficacy of blockade in $\alpha_{1/2}\beta_1$-AR antagonist treated rats was accompanied by a 113% increase in the pressor response to PE in vehicle treated rats fed a high salt diet. In the low salt group, the efficacy of the α_1-AR blockade was 83% by the end of the protocol. There was no statistically significant change in the pressor response to the PE in the vehicle treated rats as was observed in the high salt group.

4.3.3 Effect of chronic $\alpha_{1/2}\beta_1$-AR blockade on AngII-induced hypertension in high and low salt rats

The effect of $\alpha_{1/2}\beta_1$-AR blockade on AngII-induced hypertension in high and low salt rats was compared with their respective vehicle treated control after subtraction of the baseline differences in MAP and HR (Figure 4.2). Baseline MAP and HR was calculated from the average 24hr MAP during the 7 day control period. The baseline MAP in the $\alpha_{1/2}\beta_1$-AR antagonist treated group was 87.6±1.4 and 87.7±1.4 mmHg in high and low salt rats, respectively. The baseline MAP in vehicle treated rats was 106.3±2.1 and 102.0±1.5mmHg in high and low salt rats, respectively. The difference in baseline MAP between blocked versus vehicle treated groups was 18.7 and 14.3 mmHg in high and low salt rats, respectively. Baseline HR in the $\alpha_{1/2}\beta_1$-AR antagonist treated group was 353.4±6.1 and 346.2±4.1 BPM in high and low salt rats, respectively. These HR values were lower by 76.2 and 94.0 BPM compared to high and low salt rats in the vehicle treated group, which were 429.6±5.5 and 440.2±7.6 BPM, respectively.

In high salt rats, the initial change in MAP during the first 4 days of AngII was similar between $\alpha_{1/2}\beta_1$-AR antagonist treated and vehicle treated groups. In high salt rats, the rise in MAP on the first day of AngII was 21.7±3.0mmHg and 22.0±2.4mmHg in $\alpha_{1/2}\beta_1$-AR antagonist treated and vehicle treated groups, respectively. In low salt rats, the rise in MAP on the first day of AngII was 8.7±2.0mmHg and 9.1±2.1mmHg in $\alpha_{1/2}\beta_1$-AR antagonist treated and vehicle treated groups, respectively.

The response of HR to the initial changes in MAP during AngII infusion was also minimally affected by $\alpha_{1/2}\beta_1$-AR blockade in high salt

rats. The peak drop from baseline HR was observed on the 4th day of AngII in both $\alpha_{1/2}\beta_1$-AR antagonist treated and vehicle treated groups. In low salt rats, the initial and overall change in HR was more gradual without any notable troughs.

$\alpha_{1/2}\beta_1$-AR blockade blunted the AngII-induced rise in MAP, with respect to baseline, from days 5 to 14 of AngII in high salt rats. The final change in MAP from baseline, averaged over days 11-14 of AngII when responses appeared to have plateaued, was 42.2±1.5mmHg and 58.2±1.5mmHg from baseline in $\alpha_{1/2}\beta_1$-AR blockade and vehicle treated high salt rats, respectively. This 16mmHg difference between the two groups represented 27% of the total rise in MAP seen in the vehicle group. In contrast to these effects in high salt rats, $\alpha_{1/2}\beta_1$-AR blockade in low salt rats had no effect on the level of AngII-induced rise in MAP from baseline.

High salt plus vehicle treated rats had a pronounced rise in HR following the trough on the 4th day of AngII. Absolute HR on days 7 to 14 of AngII in high salt plus vehicle treated rats was back to baseline HR despite the persistently elevated MAP. $\alpha_{1/2}\beta_1$-AR blockade significantly blunted this response. In low salt rats, $\alpha_{1/2}\beta_1$-AR blockade did not result in a consistent difference in the change in HR observed during AngII-infusion.

4.3.4 Effect of chronic $\alpha_{1/2}\beta_1$-AR blockade on the difference in MAP between high and low salt rats during control and AngII-salt hypertension

There were no differences in MAP between high and low salt rats in the vehicle or $\alpha_{1/2}\beta_1$-AR antagonist treated groups during control. AngII

infusion resulted in an immediate difference in MAP between high and low salt rats on day 1 of AngII. This between salt level differences in MAP appeared to be slowly amplified through the 14 days of AngII infusion in the vehicle treated group. The final between high and low salt group differences in MAP averaged over days 11 to 14 of AngII in the vehicle treated group was 36mmHg. A slowly increasing between salt level differences in MAP remained after $\alpha_{1/2}\beta_1$-AR blockade. The final between high and low salt group differences in MAP averaged over days 11 to 14 of AngII in the $\alpha_{1/2}\beta_1$-AR blockade group was 21mmHg.

4.4 Discussion

The increasing depressor response to acute ganglionic blockade observed during AngII-salt hypertension has been used as evidence suggesting a dominant role of the sympathetic nervous system in the pathogenesis of AngII-salt hypertension (62, 64, 66). This observation led to multiple experiments designed to identify the regional sympathetic pathways contributing to the neurogenic mechanism of the model. Many of these experiments, however, have been unsuccessful or inconsistent at identifying the sympathetic pathway(s) underlying the hypothesized neurogenic mechanism of AngII-salt hypertension. Studies from our lab and others have so far demonstrated, via chronic denervation experiments, that cardiac sympathetic nerves and renal sympathetic nerves do not play a significant role during AngII-salt hypertension (52, 64). We have also shown that lumbar sympathetic nerves are not activated during AngII-salt hypertension, and hence concluded that lumbar sympathetic nerves do not play an active role in AngII-salt hypertension (61, 115). Although celiac ganglionectomy was shown to have a significant anti-hypertensive effect during AngII-salt hypertension in one study (64), a follow up study in our laboratory showed no effect of celiac ganglionectomy (see Chapter 3). These mixed results have called into question whether neurogenic mechanisms are indeed a major component of AngII-salt hypertension as suggested by acute ganglionic blockade. To address this issue, we re-assessed the sympathetic contribution in AngII-salt hypertension by way of chronic, global sympathetic blockade by continuous administration of phentolamine and atenolol, a non-selective $\alpha_{1/2}$ and selective β_1-AR antagonist, respectively.

The main finding in this study was that chronic $\alpha_{1/2}\beta_1$-AR blockade attenuated AngII-salt hypertension. Despite the negative results so far from experiments utilizing peripheral denervation for targeted sympathetic blockade, this study confirms the presence of a sympathetic contribution to the model, and supports the results from acute ganglionic blockade. It also complements the results showing elevated norepinephrine spillover during AngII-salt hypertension (63), and together suggests that elevated $\alpha_{1/2}\beta_1$-AR signaling due to enhanced norepinephrine release from sympathetic nerve terminals contributes to AngII-salt hypertension.

Another important finding in this study was that $\alpha_{1/2}\beta_1$-AR blockade attenuated AngII-induced hypertension in rats fed a high salt diet (i.e. AngII-salt hypertension) but not those in a low salt diet. This finding was also consistent with results from our prior studies using acute ganglionic blockade (62, 66) and further supports the idea that elevated dietary salt intake and circulating plasma AngII act in a synergistic manner to elevate peripheral sympathetic outflow during AngII-salt hypertension. The mechanism by which circulating AngII and dietary salt leads to enhanced sympathetic outflow is currently not well understood. We have previously proposed that the integration of the two signals occur centrally via circumventricular organs that ultimately lead to increased output from brainstem sympathetic premotor neurons (86, 87), but further studies are necessary to elucidate the exact mechanisms involved in this interaction.

4.4.1 Mechanism of blood pressure lowering effect of chronic $\alpha_{1/2}\beta_1$-AR antagonist treatment during AngII-

salt hypertension: α versus β adrenergic receptor mediated effects

Blockade of α_1-AR mediated arteriolar vasoconstriction likely played a major role in the blood pressure lowering effect of chronic $\alpha_{1/2}\beta_1$-AR antagonist treatment during AngII-salt hypertension. Since phentolamine is a non-specific α-AR antagonist, its inhibitory effects on post-synaptic α_2-AR also likely played a role in the observed effects. Although the cardiovascular effects of α_2-AR are often associated with its effect presynaptically, post-synaptic α_2-ARs are known to exert a significant vasoconstrictive effect, especially in veins (5, 91). Although blockade of α_2-AR alone would result in peripheral sympathoactivation with a concomitant rise in arterial pressure, it has been shown that in the setting of concomitant α_1-AR blockade, α_2-AR antagonists have an additional blood pressure lowering effect (69, 74). This is thought to be secondary to venodilation, which would raise systemic vascular capacitance and lower effective circulating blood volume (28, 50, 106). It has been shown previously that vascular capacitance is reduced during AngII-salt hypertension, which appears to be sympathetically mediated based on its response to acute ganglionic blockade (62). Thus, reduction in total peripheral vascular resistance and blockade of sympathetically mediated changes in vascular capacitance likely contributed to the blood pressure lowering effect of $\alpha_{1/2}\beta_1$-AR antagonist treatment during AngII-salt hypertension.

In addition to α receptor blockade with phentolamine, atenolol, a selective β_1-AR antagonist, was also added to the adrenergic antagonist infusate in order to prevent β_1 mediated renin release from

juxtaglomerular cells (84) in response to elevated plasma catecholamine levels expected from chronic $\alpha_{1/2}$-AR blockade (92). This combined use of α/β antagonists raises the question whether the effect seen in this experiment were due primarily from $\alpha_{1/2}$ or β_1 receptor antagonism.

Consistent with cardiac β_1-AR blockade, the blood pressure lowering effect of chronic $\alpha_{1/2}\beta_1$-AR antagonist treatment was accompanied by significantly lower heart rates at baseline and during AngII infusion. In addition to its effect on baseline HR, β_1-AR blockade in high salt rats significantly attenuated the rise in HR seen in vehicle treated rats during the second week of AngII. Thus, it is possible that the blood pressure lowering effect seen in AngII-salt hypertensive rats treated with $\alpha_{1/2}\beta_1$-AR antagonist was partially due to suppression of this HR response. Although the hypotensive effect of β_1-AR blockade in normal rats is well established, the mechanism of this effect is currently not fully understood. It has been shown, however, that its effect on blocking sympathetic input to the heart alone does not account for the blood pressure lowering effect of β_1-AR blockade (117) in normal rats. The effect of β_1-AR blockade on renin release could have played some role, but it has also been shown in renal denervated rats that this mechanism does not fully account for the effect on basal blood pressure either (57). Thus, it has been hypothesized that β_1-AR blockade exerts its hypotensive effect by reducing central sympathetic outflow through its activity on central β_1-AR (117).

Although the present study cannot exclude the possibility that β_1-AR played a major role in the blood pressure lowering of AngII-salt hypertensive rats, preliminary studies in our lab have so far shown that β_1-AR blockade alone is unable to attenuate AngII-salt hypertension

(52). Furthermore, a study in cardiac denervated rats by stellate ganglionectomy has shown that interruption of sympathetic tone to the heart has no significant effects on the normal progression of AngII-salt hypertension (52).

4.4.2 Chronic $\alpha_{1/2}\beta_1$-AR blockade explains only a fraction of the total effect seen by acute ganglionic blockade in prior studies

Previous studies from our lab showed an approximate 40mmHg increase in the peak depressor response to ganglionic blockade at 2 weeks of AngII-salt hypertension[1], which suggests that approximately 60-80% of the total rise in pressure seen during AngII-salt hypertension (usually about 50-60mmHg) is sympathetically mediated (66). In contrast, $\alpha_{1/2}\beta_1$-AR blockade only reduced AngII-salt hypertension by 27%, suggesting a much smaller sympathetic contribution to the blood pressure rise seen in the model. Although this suggests that acute ganglionic blockade may have overestimated the sympathetic component of AngII-salt hypertension, the lower estimate in the present study were likely due to limitations to the method employed in this study. First, there was a gradual decrease in α_1 receptor blockade efficacy during the protocol, and second, only noradrenergic mediated sympathetic effects on blood pressure were addressed by this study.

The decreasing α_1 blockade efficiency we observed throughout the 3 week protocol is likely one reason by which chronic $\alpha_{1/2}/\beta_1$-AR blockade

[1] Estimate based on peak depressor response of ~80mmHg at 2 weeks of AngII-salt hypertension, and ~40mmHg at baseline.

underestimates the underlying sympathetic contribution during AngII-salt hypertension. Since the dosage for the $\alpha_{1/2}\beta_1$-AR antagonist was adjusted for body weight every 7 days, decreased effective drug concentration due to changes in body weight was unlikely to have played any major role in this reduction in α_1 receptor blockade efficacy. Increases in α_1-AR expression have been shown to occur in rats after chronic treatment with prazosin (119), hence changes in α_1-AR expression level in response to chronic phentolamine treatment likely contributed to the increased pressor response to phenylephrine (i.e. the decrease in α_1-AR blockade efficacy). Interestingly, the development of tolerance was more pronounced in rats treated with a high salt compared to low salt rats. In addition, a 113% increase in the pressor response to acute phenylephrine injection was seen in vehicle treated rats fed a high salt diet, suggesting that there is an underlying increase in phenylephrine sensitivity specifically in high salt rats treated with AngII. This could also explain the decrease in α_1-AR blockade efficacy in high salt rats treated with phentolamine. The mechanism and precise time course of the change in phenylephrine sensitivity, presumably secondary to an increase in α_1-AR expression, and whether these changes occur during AngII-salt hypertension remains to be addressed in a future study.

Another possible explanation why chronic $\alpha_{1/2}\beta_1$-AR blockade may underestimate the total sympathetic contribution to AngII-salt hypertension is that it does not account for the post junctional actions of other sympathetic cotransmitters, specifically, ATP and NPY (89). It has been shown, however, that ATP mediated vasoconstriction via P2X1 receptor is attenuated in isolated renal afferent arterioles from rats made

hypertensive by AngII-infusion and fed an 8% NaCl diet (54), suggesting that P2X1 mediated vasoconstriction may not play a significant role in the sympathetic component of AngII-salt hypertension. On the other hand, multiple studies have shown increases in tissue and plasma levels of NPY as well as elevated vascular responsiveness to NPY in various animal models of hypertension (112). Although results from studies looking at the effect of pharmacological blockade of Y1 receptors have so far been negative in the SHR (120) or mixed in Goldblatt hypertensive rats (80, 96), it may be possible that NPY signaling is elevated in AngII-salt hypertension. Future studies could address whether Y1 receptor mediated vasoconstriction account for the greater fall in pressure seen by acute ganglionic blockade in AngII-salt hypertensive rats, compared to blood pressure lowering effect of chronic $\alpha_{1/2}\beta_1$-AR blockade alone.

4.4.3 The salt sensitive component of AngII-salt hypertension is only partially mediated by $\alpha_{1/2}\beta_1$-AR dependent neurogenic mechanisms

Salt sensitivity of MAP during chronic administration of AngII has been thought to be primarily due to inappropriately high levels of SNA for a given level of salt intake (10, 12). Consistent with this idea, $\alpha_{1/2}\beta_1$-AR antagonist treatment reduced the incremental difference in pressure between high and low salt rats after 2 weeks of AngII infusion (i.e. the salt sensitivity of AngII-induced hypertension). However, chronic $\alpha_{1/2}\beta_1$-AR antagonist treatment did not eliminate the salt-sensitivity of AngII-induced hypertension, suggesting that a significant portion is driven by non-$\alpha_{1/2}\beta_1$-AR mediated mechanisms. As discussed previously, the remaining salt-sensitive component of pressure in AngII-infused rats

after $\alpha_{1/2}\beta_1$-AR antagonist treatment group could still have a neurogenic component that is non-adrenergic. There are, however, non-neurogenic mechanisms that likely contribute to the remaining salt-sensitivity. These salt-dependent non-neurogenic mechanisms include, but are not limited to, AngII mediated changes in renal diuretic/natriuretic function (25, 72), high-salt diet mediated changes in vascular reactivity to circulating AngII (27), and changes in the pressor effects of other circulating hormones, such as endothelin and its actions on ET-A receptors (2).

4.4.4 Summary and Perspectives

This study confirms the role of sympathetic mechanisms, specifically, a combined $\alpha_{1/2}\beta_1$-AR mediated mechanisms, to the hypertension resulting from chronic AngII infusion, and its selective effect in rats fed a high NaCl diet. In Figure 4.5, a schematized plot of AngII-induced hypertension in high and low salt rats summarizes our main findings. A question that remains unanswered is whether any specific regional sympathetic pathway contributes to the observed effect. The ineffectiveness of peripheral sympathectomy approaches at recapitulating the findings from this and other studies utilizing global sympathetic blockade, so far, suggests that targets other than cardiac, renal, splanchnic and skeletal muscle vascular beds are involved in the neurogenic mechanism of AngII-salt hypertension. One possibility, based on similar findings by Veitenheimer et.al. in the 48hr water deprivation model of acute neurogenic hypertension (110), is the adrenal gland, and the role of adrenal cortical hormones in mediating global increases in sympathetic nervous system activity. An alternative possibility,

proposed in the same study by Veitenheimer et.al., however, is that the sympathetic nervous system is activated in these settings to maintain a certain arterial pressure set point, and is able to adapt to compensate for a loss of sympathetic activity to any single vascular bed. Thus in this latter scenario, only global sympathetic blockade would be able to fully block the neurogenic component of the hypertensive response. Further studies are needed to test whether any of these possibilities hold true in the AngII-salt model of hypertension.

Although it has been suggested that the mechanism of AngII-induced hypertension, in the later phases, is almost exclusively neurogenic (6), we found that the overall contribution of sympathetic tone to the final level of pressure at 2 weeks of AngII-salt hypertension is considerably smaller than estimated by ganglionic blockade when tested using chronic $\alpha_{1/2}\beta_1$-AR blockade. The discrepancy in results obtained from ganglionic blockade and chronic $\alpha_{1/2}\beta_1$-AR blockade may have been due to the effect of tolerance to chronic pharmacologic treatment, and non-adrenergic sympathetic cotransmitters that were not tested in this study. However, our results also suggest that a significant component of hypertension during the later phase of AngII infusion may be mediated by non-neurogenic mechanisms, and that data from ganglionic blockade experiments may have overestimated the overall neurogenic contribution to the model.

4.5 Figures and Table

Figure 4.1. 24hr Food and Water Intake in $\alpha_{1/2}\beta_1$-AR antagonist treated and vehicle treated rats

24hr food and water intake in $\alpha_{1/2}\beta_1$-AR antagonist treated (light grey) and vehicle treated rats (black) fed a high (2%) or low (0.1%) salt (NaCl) diet. Measurements were carried out once a day during the light phase of the dark-light cycle. Values represent group mean±S.E.M. (†) and (‡) denote significant ($p<0.05$) within group difference compared to day 7 of control in vehicle and $\alpha_{1/2}\beta_1$-adrenergic antagonist treated rats, respectively. (∗) denote significant ($p<0.05$) between group differences n = 7 for both $\alpha_{1/2}\beta_1$-AR antagonist treated and vehicle treated groups fed a 2% NaCl diet; n = 6 for the $\alpha_{1/2}\beta_1$-AR antagonist treated group fed a 0.1% NaCl diet; n = 8 for the vehicle treated group fed a 0.1% NaCl diet.

Figure 4.1

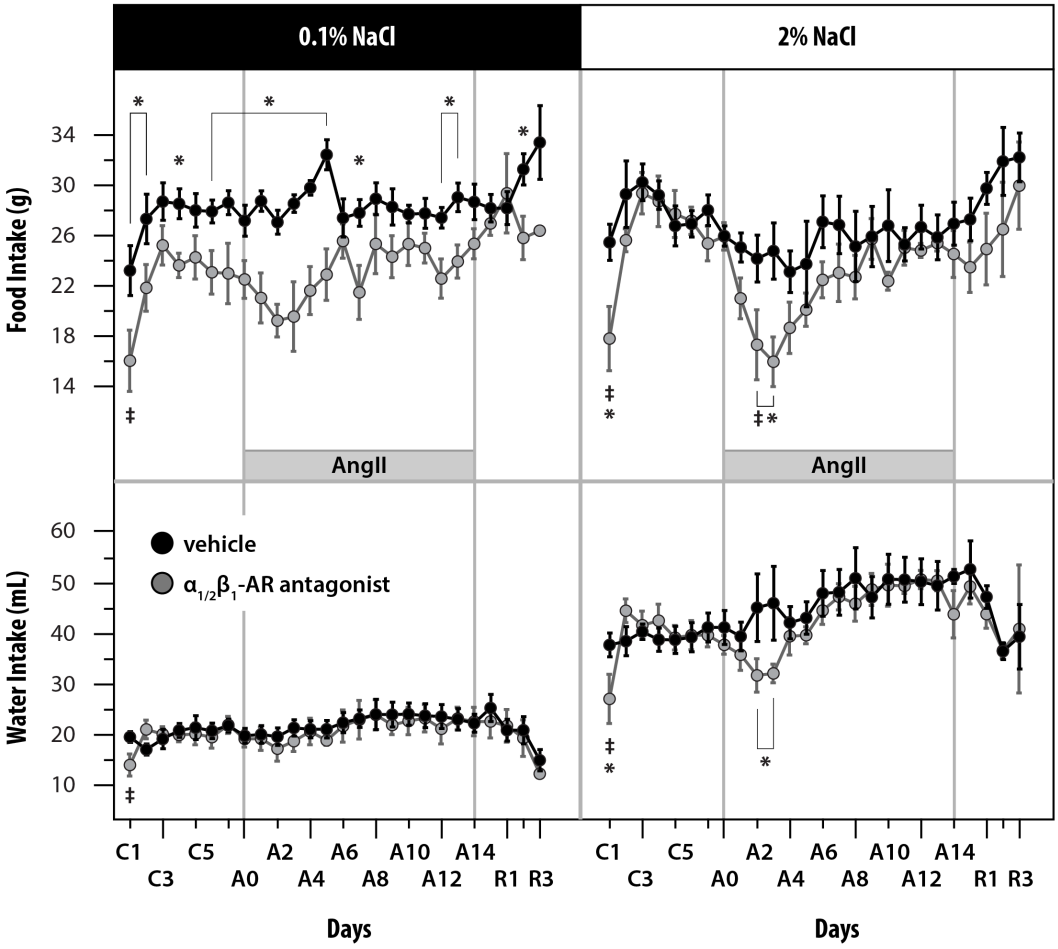

0.1% NaCl
2% NaCl
Food Intake (g)
34
30
26
22
18
14
AngII
AngII
vehicle
α1/2β1-AR antagonist
Water Intake (mL)
60
50
40
30
20
10
C1
C3
C5
A0
A2
A4
A6
A8
A10
A12
A14
R1
R3
Days
Days

Table 4.1. Change in body weight in $\alpha_{1/2}\beta_1$-AR antagonist treated and vehicle treated rats

Body weight prior to initial surgery, beginning of protocol, and weekly changes during chronic $\alpha_{1/2}\beta_1$-AR antagonist or vehicle treatment, and 2 weeks of AngII administration. Weights are reported in grams. Values represent mean±S.E.M. n = 7 for both $\alpha_{1/2}\beta_1$-AR antagonist treated and vehicle treated groups fed a 2% NaCl diet; n = 6 for the $\alpha_{1/2}\beta_1$-AR antagonist treated group fed a 0.1% NaCl diet; n = 8 for the vehicle treated group fed a 0.1% NaCl diet.

Table 4.1

				$\alpha_{1/2}\beta_1$-AR antagonist vs vehicle infusion		
					AngII infusion	
		Surgery	C00	C07	A07	A14
High	vehicle	346 ± 14	380 ± 10	434 ± 14	463 ± 16	470 ± 21
	$\alpha_{1/2}\beta_1$-AR antag	355 ± 13	386 ± 11	432 ± 13	436 ± 17	460 ± 14
Low	vehicle	372 ± 9	407 ± 8	456 ± 9	501 ± 9	530 ± 9
	$\alpha_{1/2}\beta_1$-AR antag	350 ± 12	392 ± 11	421± 14 *	428 ± 15 *	459 ± 19 *

Figure 4.2. Blood pressure response to acute phenylephrine injection in $\alpha_{1/2}\beta_1$-AR antagonist treated and vehicle treated rats

The degree of α_1-AR blockade in rats chronically treated with an $\alpha_{1/2}\beta_1$-AR antagonist was tested on days 1 and 7 of control and 3rd day after cessation of AngII infusion (Recovery; "R") by measuring the arterial pressure response to an acute injection of phenylephrine (PE; 10 μg/kg, i.v.). The tracing shown under "C00" was obtained prior to starting the vehicle or $\alpha_{1/2}\beta_1$-AR antagonist infusion. The tracings show the group averaged change in arterial pressure in response to phenylephrine in vehicle (light grey) and $\alpha_{1/2}\beta_1$-AR antagonist treated (black) rats fed a 2% (top panel) or 0.1% NaCl (bottom panel) diet. The shaded areas represent the S.E.M. The baseline arterial pressure calculated from the average of the 5min period immediately preceding the PE injection was subtracted from each tracing to account for differences in MAP between vehicle and $\alpha_{1/2}\beta_1$-AR antagonist treated rats. n = 7 for both $\alpha_{1/2}\beta_1$-AR antagonist treated and vehicle treated groups fed a 2% NaCl diet; n = 6 for the $\alpha_{1/2}\beta_1$-AR antagonist treated group fed a 0.1% NaCl diet; n = 8 for the vehicle treated group fed a 0.1% NaCl diet.

Figure 4.2

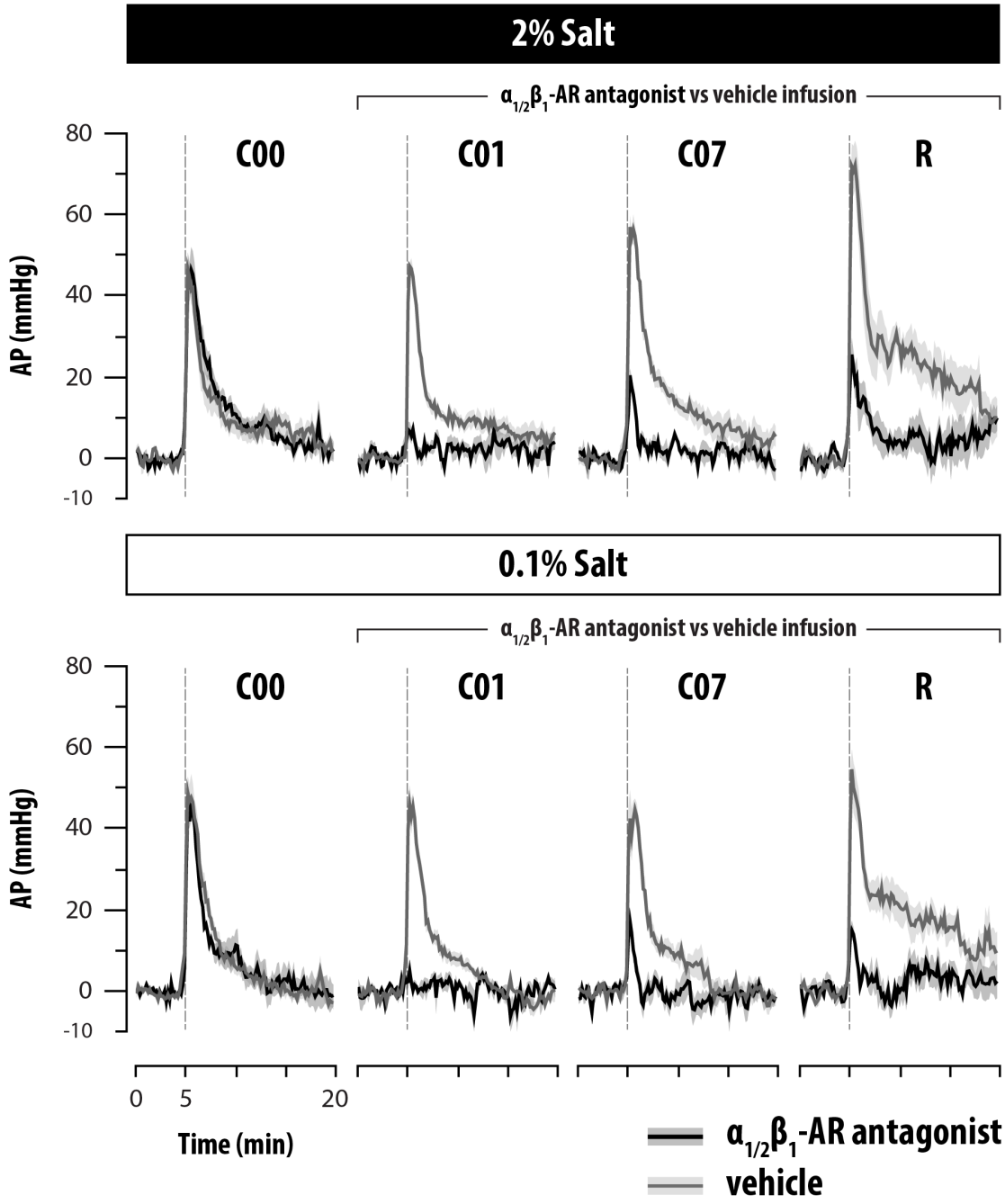

2% Salt
α1/2β1-AR antagonist vs vehicle infusion
C00
C01
C07
R
AP (mmHg)
80
60
40
20
0
-10
0.1% Salt
α1/2β1-AR antagonist vs vehicle infusion
C00
C01
C07
R
AP (mmHg)
80
60
40
20
0
-10
0
5
20
Time (min)
α1/2β1-AR antagonist
vehicle

Table 4.2. Level of α_1-AR blockade during chronic $\alpha_{1/2}\beta_1$-AR antagonist treatment

The degree of α_1-AR blockade in rats chronically treated with an $\alpha_{1/2}\beta_1$-AR antagonist was tested on days 1 and 7 of control and 3rd day after cessation of AngII infusion (Recovery; "R") by measuring the arterial pressure response to an acute injection of phenylephrine (PE; 10 μg/kg, i.v.). The degree of α_1-AR blockade was quantified by calculating the area under the curve (AUC) from the arterial pressure response tracing during the first 5 min after injection of PE. The baseline arterial pressure calculated from the average of the 5min period immediately preceding the PE injection was subtracted from each tracing prior to calculating the AUC. The values in parenthesis represent the AUC as a fraction of the PE response obtained prior to vehicle or $\alpha_{1/2}\beta_1$-AR antagonist infusion ("C00"). (†) and (‡) denote significant ($p<0.05$) within group difference compared to baseline in vehicle and $\alpha_{1/2}\beta_1$-AR antagonist treated rats, respectively. ($*$) denote significant ($p<0.05$) between group differences within each dietary salt level. Values are reported in arbitrary units (a.u.), and represent mean±S.E.M. n = 7 for both $\alpha_{1/2}\beta_1$-AR antagonist treated and vehicle treated groups fed a 2% NaCl diet; n = 6 for the $\alpha_{1/2}\beta_1$-AR antagonist treated group fed a 0.1% NaCl diet; n = 8 for the vehicle treated group fed a 0.1% NaCl diet.

Table 4.2

			$\alpha_{1/2}\beta_1$-AR antagonist vs vehicle infusion		
		C00	C01	C07	R
High	vehicle	6020 ± 857 (-)	5982 ± 331 (1.11 ± 0.15)	8598 ± 419 (1.60 ± 0.23)	11590 ± 1784 (2.13 ± 0.31)†
	$\alpha_{1/2}\beta_1$-AR antag	7277 ± 776 (-)	836 ± 382 (0.13 ± 0.07)‡*	1562 ± 316 (0.24 ± 0.05)‡*	3013 ± 918 (0.45 ± 0.15)‡*
Low	vehicle	6897 ± 582 (-)	6201 ± 507 (0.94 ± 0.11)	6310 ± 625 (0.98 ± 0.16)	8866 ± 1158 (1.31 ± 0.16)
	$\alpha_{1/2}\beta_1$-AR antag	5896 ± 405 (-)	438 ± 238 (0.07 ± 0.04)‡*	642 ± 480 (0.11 ± 0.08)‡*	942 ± 570 (0.17 ± 0.10)‡*

Figure 4.3. Effect of $\alpha_{1/2}\beta_1$-AR blockade on MAP and HR during AngII-induced hypertension in rats fed a 2% or 0.1% NaCl diet

24hr average mean arterial pressure (MAP), and heart rate (HR) during control and 2 weeks of AngII-infusion followed by 3 days of recovery in vehicle (filled circles, black line) and $\alpha_{1/2}\beta_1$-adrenergic antagonist treated (unfilled circles, grey line) rats. Data for rats fed a low (0.1%) salt (NaCl) diet are plotted on the left panel. Right panel shows data for rats fed a high (2%) NaCl diet. Values are shown after baseline subtraction. "C", "A", and "R" prefixes on x-axis denote control, AngII days, and recovery, respectively. (†) and (‡) denote significant ($p<0.05$) within group difference compared to baseline in vehicle and $\alpha_{1/2}\beta_1$-adrenergic antagonist treated rats, respectively. ($*$) denote significant ($p<0.05$) between group differences. Values are mean$\pm$S.E.M. n = 7 for both $\alpha_{1/2}\beta_1$-AR antagonist treated and vehicle treated groups fed a 2% NaCl diet; n = 6 for the $\alpha_{1/2}\beta_1$-AR antagonist treated group fed a 0.1% NaCl diet; n = 8 for the vehicle treated group fed a 0.1% NaCl diet.

Figure 4.3

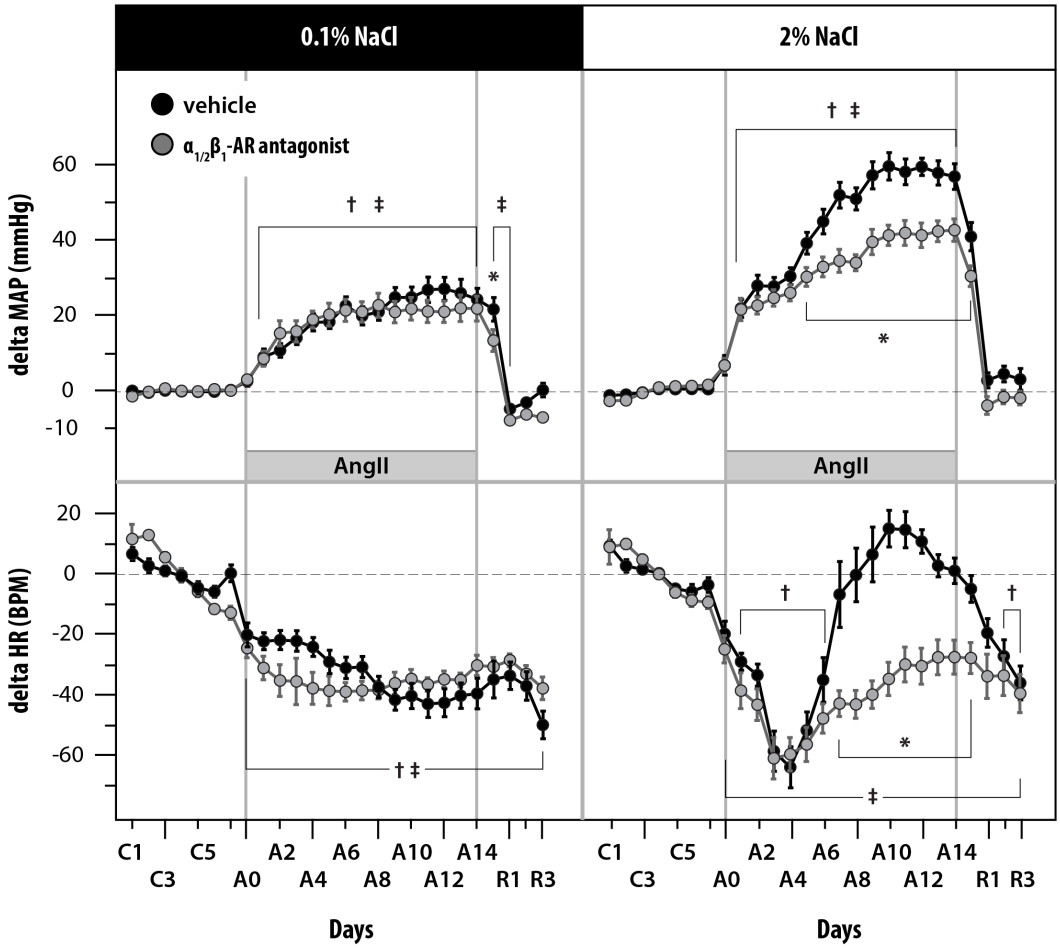

0.1% NaCl
2% NaCl
vehicle
α1/2β1-AR antagonist
delta MAP (mmHg)
delta HR (BPM)
AngII
AngII
Days
Days
C1 C3 C5 A0 A2 A4 A6 A8 A10 A12 A14 R1 R3

Figure 4.4. Effect of dietary salt on MAP and HR during AngII-induced hypertension in $\alpha_{1/2}\beta_1$-AR antagonist treated and vehicle treated rats

24hr average mean arterial pressure (MAP), and heart rate (HR) during control and 2 weeks of AngII-infusion followed by 3 days of recovery in rats fed a high (2%) salt (NaCl; black circles, black line) or low (0.1%) NaCl diet (grey circles, grey line). Data for $\alpha_{1/2}\beta_1$-AR antagonist treated rats are plotted on the left panel. Right panel shows data for vehicle treated rats. "C", "A", and "R" prefixes on x-axis denote control, AngII days, and recovery, respectively. ($*$) denote significant ($p<0.05$) between 2% NaCl and 0.1% NaCl group differences. Values are mean$\pm$S.E.M. n = 7 for both $\alpha_{1/2}\beta_1$-AR antagonist treated and vehicle treated groups fed a 2% NaCl diet; n = 6 for the $\alpha_{1/2}\beta_1$-AR antagonist treated group fed a 0.1% NaCl diet; n = 8 for the vehicle treated group fed a 0.1% NaCl diet.

Figure 4.4

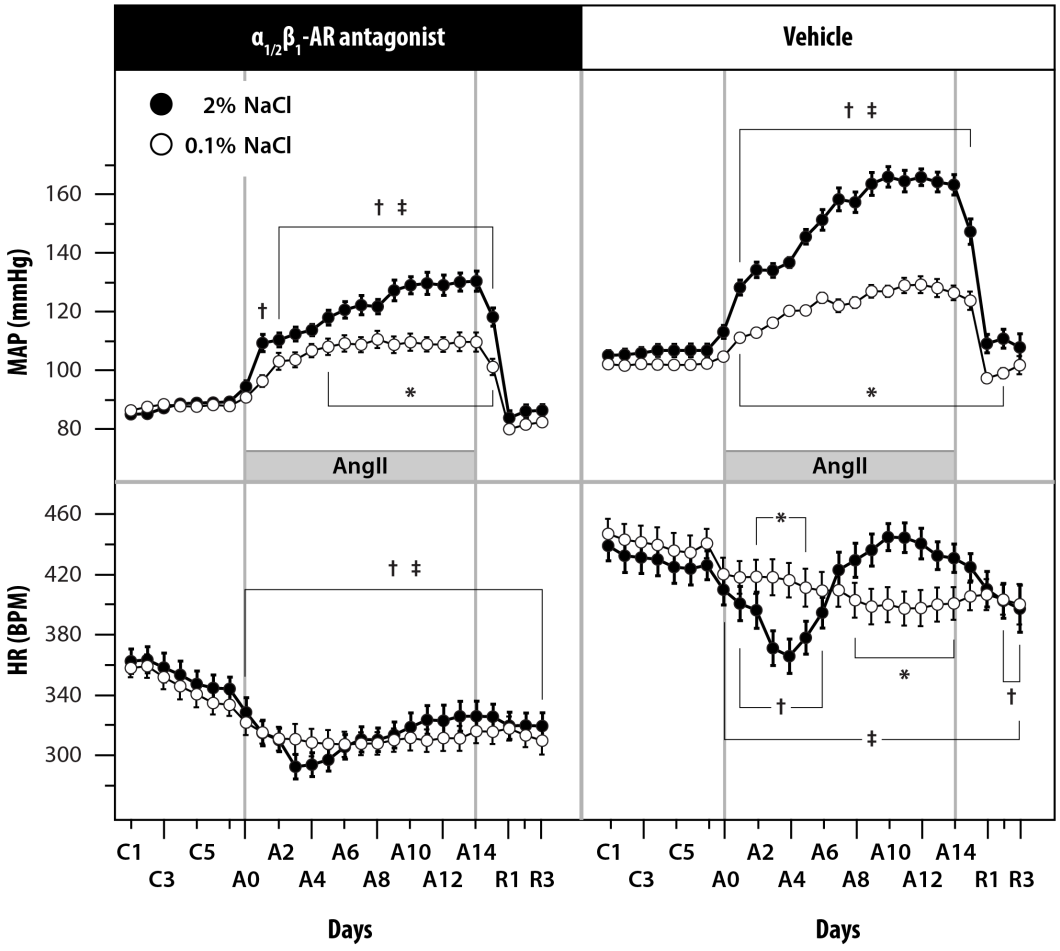
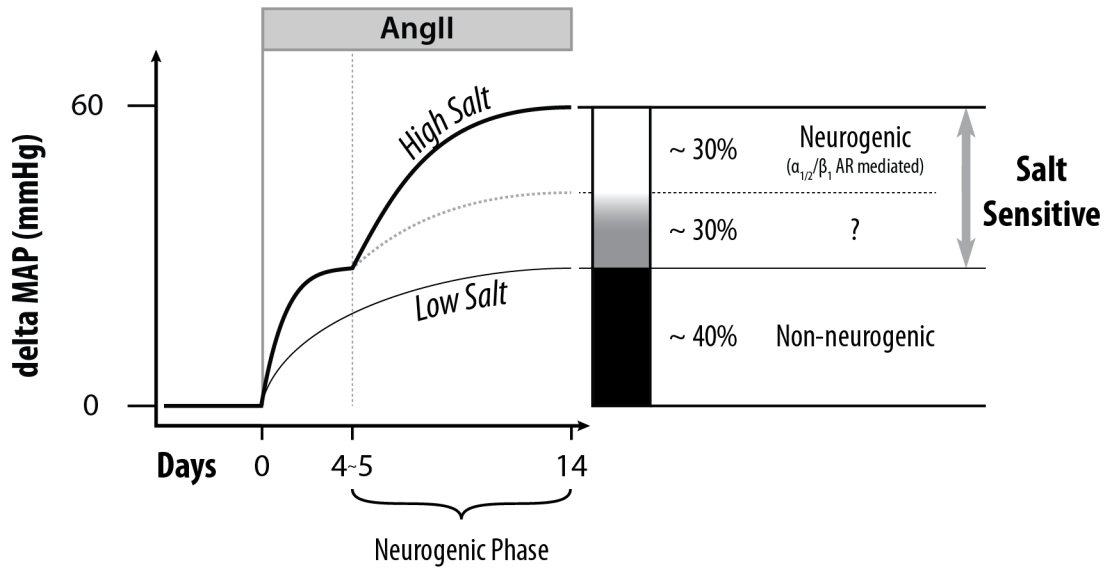

α1/2β1-AR antagonist
Vehicle
2% NaCl
0.1% NaCl
MAP (mmHg)
160
140
120
100
80
AngII
AngII
HR (BPM)
460
420
380
340
300
C1 C3 C5 A0 A2 A4 A6 A8 A10 A12 A14 R1 R3
C1 C3 C5 A0 A2 A4 A6 A8 A10 A12 A14 R1 R3
Days
Days

Figure 4.5. Estimated time course and magnitude of the neurogenic component of AngII-salt hypertension

Summary of main findings from this study depicted as schematized blood pressure response during AngII-salt hypertension. The neurogenic component of AngII-salt hypertension at 2 weeks is estimated at approximately 27% (rounded to 30%), and appears to be activated in a delayed fashion, approximately 4-5 days after AngII-infusion. At least 40% of the blood pressure response in AngII-salt hypertension is non-neurogenic. The mechanism responsible for the remaining 30% of AngII-salt hypertension is likely mediated by salt-sensitive non-neurogenic mechanisms, or possibly other sympathetic mechanisms mediated by non-adrenergic cotransmitters.

Figure 4.5


AngII
60
0
delta MAP (mmHg)
High Salt
Low Salt
Days
0
4~5
14
Neurogenic Phase
~ 30%
Neurogenic
(α1/2/β1 AR mediated)
~ 30%
?
~ 40%
Non-neurogenic
Salt
Sensitive

Chapter 5

Conclusion

The aim of this thesis was to determine whether changes in sympathetic vasomotor tone to the splanchnic vascular bed was a contributing mechanism to the neurogenic component of AngII-salt hypertension. The question to be answered was: "Is there evidence for regional hemodynamic changes that support the role of a targeted increase in sympathetic vasomotor tone to the splanchnic vascular bed?" Based on prior findings (62, 64, 76) , and then available anatomical basis for sympathetic innervation of splanchnic arteries and veins (53), we hypothesized that the neurogenic mechanism contributing to AngII-salt hypertension was mediated by an increase in sympathetic vasomotor tone to both arteries and veins, causing a rise in total peripheral resistance and a reduction in vascular capacitance (40, 86). If true, the hypothesis would predict that there would be measurable changes in mesenteric vascular resistance, a variable that could be measured in conscious rats by measuring local blood flow at the superior mesenteric artery. Therefore, studies in this thesis were conducted using a technique developed for measuring superior mesenteric artery blood flow continuously, in conscious, freely moving tethered rats. The main findings of this thesis, along with results from2 supporting studies are summarized below.

5.1 Summary of main findings

5.1.1 The sustained increase in splanchnic vascular resistance observed during AngII-salt hypertension is not mediated by an increase in sympathetic vasomotor tone (Chapters 2 & 3)

In Chapter 2, I found that AngII-salt hypertension, but not AngII-induced hypertension in low salt rats was associated with a sustained rise in mesenteric vascular resistance (MVR). Furthermore, the rise in MVR observed in AngII-salt hypertensive rats was also associated with a gradually increasing vasodilatory response to acute ganglionic blockade. These two evidences were consistent with the predicted hemodynamic changes and responsiveness to ganglionic blockade based on the original hypothesis. However, the responsiveness of MVR to ganglionic blockade alone was not conclusive evidence that the rise in MVR was due to increases in sympathetic vasomotor tone. An indirect mechanism involving withdrawal of myogenic tone in response to the depressor response to ganglionic blockade was equally likely. The study in Chapter 3 was designed to directly address the limitation in Chapter 2 by measuring changes in mesenteric vascular resistance in rats with intact or devoid of sympathetic innervation to the splanchnic vascular bed. This was achieved by surgical stripping of the celiac-superior mesenteric ganglion plexus (celiac ganglionectomy; CGx) as had been previously described (64).

Results from Chapter 3 showed that increases in MVR during AngII-salt hypertension occurred in both CGx and Sham rats, suggesting that the rise in MVR was not dependent on an intact sympathetic

innervation to the splanchnic vascular bed. Another findings in this study was that the trend for a gradually increasing vasodilatory response to ganglionic blockade was present in both CGx and the surgical sham group, suggesting that the vasodilatory response to ganglionic blockade was not an index of sympathetic vasomotor tone in the splanchnic vascular bed.

The findings from the above two studies leads to a conclusion that: "The sustained increase in splanchnic vascular resistance observed during AngII-salt hypertension is not mediated by an increase in sympathetic vasomotor tone."

5.1.2 Celiac ganglionectomy does not attenuate AngII-salt hypertension (Chapter 3)

Another major unexpected finding from the study in Chapter 3 was that CGx did not attenuate AngII-salt hypertension as previously reported (64). One likely explanation for the discrepant findings is the possibility for a difference in the extent of organ involvement of CGx between the study in this thesis and that in the previous work. As was shown by tissue norepinephrine (NE) content measurements, the renal involvement of CGx in the previous work was greater compared to CGx performed in the study presented in this thesis and works by others in our laboratory (42, 110) . The interpretation and possible mechanisms for the discrepant finding is revisited in a section below.

5.1.3 Chronic $\alpha_{1/2}\beta_1$-adrenergic receptor shows a much smaller role for sympathetic pressor tone during

AngII-salt hypertension than predicted by acute ganglionic blockade (Chapter 4)

The combined evidence from renal denervation (no effect) (64), renal sympathetic nerve recording (decreased) (115), lumbar sympathetic nerve recording (unchanged) (115), hind limb norepinephrine spillover (unchanged) (61), stellate ganglionectomy (no effect) (52), chronic β_1-adrenergic receptor blockade (no effect) (52) and the inconsistent findings with celiac ganglionectomy from Chapter 3, raised the question whether a sympathetic pressor tone had a significant contribution in AngII-salt hypertension. Although the role for a sympathetic pressor tone had been assessed by acute ganglionic blockade, thus far, no chronic peripheral sympathoinhibitory intervention had shown a role for the importance of sympathetic pressor tone to any particular vascular bed in the development and maintenance of our model of AngII-salt hypertension. In Chapter 4, I reassessed the role of global sympathetic pressor tone in the development of AngII-salt hypertension by means of a chronic infusion of a combined $\alpha_{1/2}\beta_1$-adrenergic receptor antagonist (Phentolamine and Atenolol). I found that $\alpha_{1/2}\beta_1$-adrenergic receptor mediated mechanisms contribute approximately 27% of the final level of pressure in AngII-salt hypertensive rats, far less than what could be inferred from acute ganglionic blockade studies (62, 66).

5.1.4 AngII-salt hypertension generated by Alzet osmotic minipumps can spontaneously lose its hypertensive phenotype due to pump dependent mechanisms (Appendix 1)

In addition to the findings discussed above, one important issue surfaced at the conclusion of the studies described in Chapter 2. Almost half of the rats subjected to the AngII-salt hypertension protocol failed to develop a sustained hypertension after implantation of the Alzet pump. This response was unpredictable, uncorrelated to predicted pump infusion rate (based on post-explantation infusate volume), and equally affected rats fed a high or a low salt diet. The spontaneous drops in pressure in a subset of rats was problematic because it would have made results in studies testing blood pressure lowering treatments difficult to interpret. Based on preliminary findings from our laboratory showing good repeatability of AngII-salt hypertension using a recently introduced implantable mechanical pump (iPrecio) (107), we hypothesized that the problems observed with Alzet rats were due to failure of Alzet pumps to maintain a constant level of plasma AngII, which would not be observed in mechanical infusion devices such as iPrecio or external syringe pumps.

A study was conducted to compare changes in mean arterial pressure and plasma AngII during AngII-salt hypertension generated by Alzet, iPrecio, or an external syringe pump (Harvard). This study is presented in Appendix 1, and showed that AngII-salt hypertension was generated more consistently using mechanical infusion devices (both iPrecio and Harvard pumps) compared to Alzet osmotic minipumps. Following these findings, AngII-salt hypertension in Chapters 3 and 4 were generated

using an external infusion pump connected to a subcutaneously implanted catheter. Unlike our original hypothesis, however, we did not find that differences in the arterial pressure profile observed between pumps were correlated with significant differences in plasma AngII levels. Thus, further studies may be needed to address the pump dependent differences in the arterial pressure response during AngII-salt hypertension.

5.2 Implications of combined findings

5.2.1 The neurogenic mechanism of AngII-salt hypertension is mediated by a sympathetic drive outside of the splanchnic vascular bed

The study by our group (64) that formed the basis for our initial hypothesis (86) suggested that sympathetic vasomotor tone to the splanchnic vascular bed was elevated by combining a measure of global index of venous tone with a surgical technique that, among other splanchnic organs, removed the sympathetic innervation to the splanchnic vascular bed. The results from Chapters 2 & 3, however, suggest that direct sympathetic innervation to the splanchnic vasculature is not necessary for the increases in splanchnic vasomotor tone, and that the increases can be mediated solely by non-neurogenic mechanisms. Although peripheral denervation techniques and both direct and indirect assessment of sympathetic tone to other peripheral vascular beds, combined with the negative findings from Chapter 3 suggest that peripheral sympathetic tone may not be a significant contributor to AngII-salt hypertension, global indices of sympathetic

tone, combined with results from Chapter 4 suggest that peripheral sympathetic activity is enhanced and contribute partially to the overall level of pressure in the model.

How can these apparently discrepant findings be reconciled? As previously discussed, there were slight differences in the tissue NE content profile of CGx treated rats from the study presented in this thesis and the prior study, which suggests the possibility that CGx on the prior study was more extensive, involving extrinsic innervations to the kidney from renal ganglia along rostral segments of the various branches of the renal nerve, as well as from the suprarenal ganglion. Thus, it may be possible that the hypotensive response observed in the previous work was a result from unintended damages to the aforementioned structures, creating a condition where renal and splanchnic organs were dually denervated. This dual denervation has been shown to have more than an additive hypotensive effect by a study in Dahl-S rats (42). However, in this study, both RDNx and CGx were individually shown to attenuate hypertension in Dahl-S rats, making this an unlikely explanation in the AngII-salt model.

Another organ that could have been affected by a more extensive CGx, which could provide a unifying hypothesis to the overall findings, would be the adrenal cortex (see Figure 5.1), if damages extended to the suprarenal ganglia. This possibility is suggested by the known anatomical innervation of the adrenal cortex from postganglionic neurons in the suprarenal ganglia (34), and a recent hypothesis that sympathetically mediated release of endogenous ouabain from the adrenal cortex plays a role in salt sensitive hypertension by both direct and indirect vasoconstrictor actions on the peripheral vasculature (8).

Findings from a related study in our lab, the pressor response to 48hr water deprivation (110), also supports this possibility as discussed in Chapter 2. Future studies in the AngII-salt model employing adrenalectomy and splanchnicectomy of the adrenal cortex will likely shed light to the current outstanding questions.

5.2.2 The method for generating AngII-induced hypertension by the subcutaneous route must be reconsidered for better reproducibility between and within laboratories

Although the AngII-induced model of hypertension generated by peripheral administration of AngII is a convenient model of experimental hypertension, it suffers from one glaring weakness: between and within laboratory and experimenter variability in the resulting blood pressure phenotype. The prevailing level of dietary salt and dose of AngII administration are two known factors that can contribute to this variability. It has also been speculated whether the model generated by peripheral administration using intravenous or subcutaneous routes are comparable models on the basis of the 10-20 fold differences in the dose used to generate the model (15). My experience with the AngII-salt model of hypertension using subcutaneously implanted osmotic minipumps and results from Appendix 1 suggests that the osmotic minipumps may be another source of variability to the model. These sources of variability make it difficult to compare results between studies and deter progress in uncovering the neurogenic basis of AngII-induced hypertension, a model based largely on a study by Dickinson

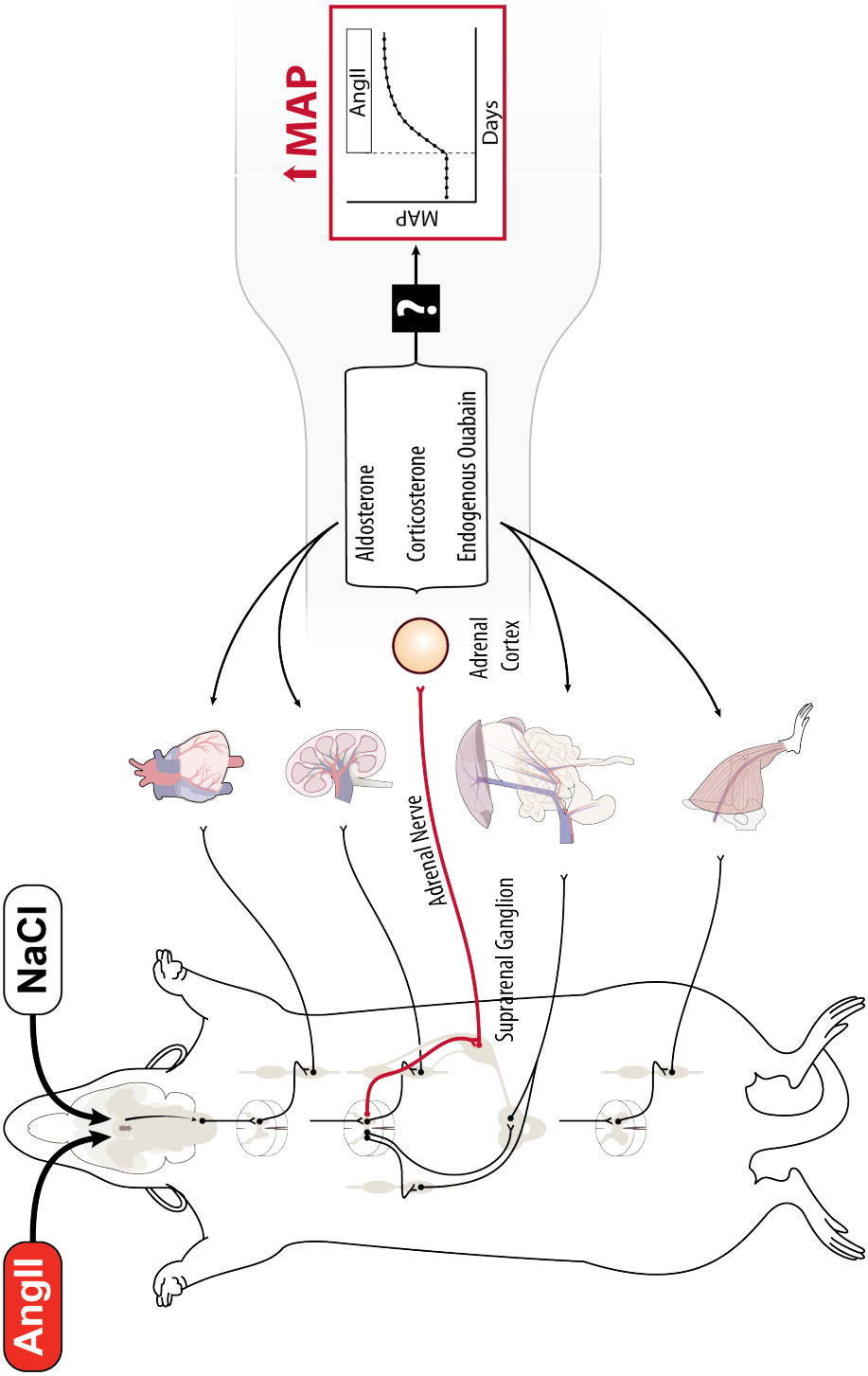
and Lawrence reported 50 years ago (31). Future investigations may benefit by adopting mechanical infusion devices for generating AngII-induced hypertension.

5.3 Figures and Table

Figure 5.1. Revised Central Hypothesis

Diagram depicting the possible role of the adrenal gland in the neurogenic mechanism of AngII-salt hypertension. One possible explanation for the discrepancy between the previously reported blood pressure lowering effect of celiac ganglionectomy from the one presented in this thesis is that there were differences in the extent of organ denervation resulting from the surgical ganglionectomy. Norepinephrine spillover data from the kidneys suggest that nerve pathways rostral to the celiac-superior mesenteric ganglia were affected in the prior study. A rostral structure other than the renal nerve that could have been affected is the suprarenal ganglion, which contains sympathetic post-ganglionic neurons that modulate adrenal cortical activity. Recent studies have highlighted the role of adrenal cortical hormones, with a focus on endogenous ouabain, in the pathogenesis of other neurogenic, salt-sensitive models of hypertension. We propose the possibility that the neurogenic mechanism of AngII-salt hypertension may involve increases in sympathetic input to the adrenal cortex leading to increased release of adrenal cortical hormones that may in turn act directly on cardiovascular end-organs, or modulate the sympathetic control of these organs which ultimately may lead to a sustained increase in blood pressure. Further studies are needed to test this hypothesis.

Figure 5.1


↑MAP
AngII
Days
MAP
?
Aldosterone
Corticosterone
Endogenous Ouabain
Adrenal Cortex
Adrenal Nerve
Suprarenal Ganglion
NaCl
AngII

Bibliography

1. **Arenas IA, Xu Y, Davidge ST.** Age-associated impairment in vasorelaxation to fluid shear stress in the female vasculature is improved by TNF-alpha antagonism. *American journal of physiology. Heart and circulatory physiology* 290: H1259-63, 2006.

2. **Ballew JR, Fink GD.** Role of ETA receptors in experimental ANG II-induced hypertension in rats. *American journal of physiology. Regulatory, integrative and comparative physiology* 281: R150--R154, 2001.

3. **Barrett CJ, Guild S, Ramchandra R, Malpas SC.** Baroreceptor denervation prevents sympathoinhibition during angiotensin II-induced hypertension. *Hypertension* 46: 168--172, 2005.

4. **Barrett CJ, Ramchandra R, Guild S, Lala A, Budgett DM, Malpas SC.** What sets the long-term level of renal sympathetic nerve activity: a role for angiotensin II and baroreflexes? *Circulation research* 92: 1330--6, 2003.

5. **Bentley GA, Widdop RE.** Postjunctional α_2-adrenoceptors mediate venoconstriction in the hindquarters circulation of anaesthetized cats. *British journal of pharmacology* 92: 121--128, 1987.

6. **Blaine EH, Cunningham JT, Hasser EM, Dale WE, Li Q, Sullivan M.** Angiotensin hypertension. *Clinical and experimental pharmacology & physiology. Supplement* 25: S16-20, 1998.

7. **Blantz RC, Tucker BJ, Gushwa LC, Peterson OW, Wilson CB.** Glomerular immune injury in the rat: The influence of angiotensin II and alpha-adrenergic inhibitors. *Kidney international* 20: 452 - 461, 1981.

8. **Blaustein MP, Leenen FH, Chen L, Golovina VA, Hamlyn JM, Pallone TL, Van Huysse JW, Zhang**

J, Wier WG. How NaCl raises blood pressure: a new paradigm for the pathogenesis of salt-dependent hypertension. *American journal of physiology. Heart and circulatory physiology* 302: H1031--H1049, 2012.

9. **Brody MJ, Haywood JR, Touw KB.** Neural mechanisms in hypertension. *Annual review of physiology* 42: 441--453, 1980.

10. **Brooks VL.** Interactions between angiotensin II and the sympathetic nervous system in the long-term control of arterial pressure. *Clinical and experimental pharmacology & physiology* 24: 83-90, 1997.

11. **Brooks VL, Hatton DC.** Chronic ANG II infusion and reflex control of norepinephrine and corticosterone in conscious rabbits. *American journal of physiology. Regulatory, integrative and comparative physiology* 272: R487-96, 1997.

12. **Brooks VL, Scrogin KE, McKeogh DF.** The interaction of angiotensin II and osmolality in the generation of sympathetic tone during changes in dietary salt intake. An hypothesis. *Annals of the New York Academy of Sciences* 940: 380-94, 2001.

13. **Burke SL, Evans RG, Moretti J, Head GA.** Levels of renal and extrarenal sympathetic drive in angiotensin II-induced hypertension. *Hypertension* 51: 878--883, 2008.

14. **Camara AK, Osborn JL.** Alpha-adrenergic systems mediate chronic central AII hypertension in rats fed high sodium chloride diet from weaning. *Journal of the autonomic nervous system* 76: 28-34, 1999.

15. **Campbell DJ.** Do intravenous and subcutaneous angiotensin II administration increase blood pressure by different mechanisms? *Clinical and experimental pharmacology & physiology* (March 30, 2013). doi:/10.1111/1440-1681.12085.

16. **Carretero OA, Oparil S.** Essential hypertension. Part I: definition and etiology. *Circulation* 101: 329-35, 2000.

17. **Carroll RG, Lohmeier TE, Brown AJ.** Chronic angiotensin II infusion decreases renal norepinephrine overflow in conscious dogs. *Hypertension* 6: 675-81, 1984.

18. **Cassis LA, Marshall DE, Fettinger MJ, Rosenbluth B, Lodder RA.** Mechanisms contributing to angiotensin II regulation of body weight. *American journal of physiology. Endocrinology and metabolism* 274: E867-76, 1998.

19. **Charles CJ, Espiner EA, Richards AM, Sybertz EJ.** Endopeptidase inhibition in angiotensin-induced hypertension. Effect of SCH 39370 in sheep. *Hypertension* 26: 89--94, 1995.

20. **Chobanian AV.** Shattuck Lecture. The hypertension paradox--more uncontrolled disease despite improved therapy. *The New England journal of medicine* 361: 878--87, 2009.

21. **Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JLJ, Jones DW, Materson BJ, Oparil S, Wright JTJ, Roccella EJ.** Seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. *Hypertension* 42: 1206--1252, 2003.

22. **Cholewa BC, Meister CJ, Mattson DL.** Importance of the renin-angiotensin system in the regulation of arterial blood pressure in conscious mice and rats. *Acta physiologica Scandinavica* 183: 309-20, 2005.

23. **Collister JP, Osborn JW.** The chronic infusion of hexamethonium and phenylephrine to effectively clamp sympathetic vasomotor tone. A novel approach. *Journal of pharmacological and toxicological methods* 42: 135 - 147, 1999.

24. Cowley AW, McCaa RE. Acute and chronic dose-response relationships for angiotensin, aldosterone, and arterial pressure at varying levels of sodium intake. *Circulation research* 39: 788-97, 1976.

25. Cowley AWJ. Long-term control of arterial blood pressure. *Physiological reviews* 72: 231--300, 1992.

26. Csiky B, Simon G. Effect of neonatal sympathectomy on development of angiotensin II-induced hypertension. *American journal of physiology. Heart and circulatory physiology* 272: H648-56, 1997.

27. Csiky B, Simon G. Synergistic vascular effects of dietary sodium supplementation and angiotensin II administration. *American journal of physiology. Heart and circulatory physiology* 273: H1275-82, 1997.

28. D'Oyley HM, Pang CC. Effects of alpha 1- and alpha 2-adrenoceptor antagonists on venous tone in conscious rats. *European journal of pharmacology* 182: 283 - 290, 1990.

29. DiBona GF. Neural control of renal function: cardiovascular implications. *Hypertension* 13: 539--548, 1989.

30. DiBona GF, Sawin LL. Effect of renal denervation on dynamic autoregulation of renal blood flow. *American journal of physiology. Renal physiology* 286: F1209-18, 2004.

31. Dickinson C, Lawrence JR. A slowly developing pressor response to small concentrations of angiotensin. Its bearing on the pathogenesis of chronic renal hypertension. *Lancet* 1: 1354-6, 1963.

32. Dilley RJ, Nataatmadja MI. Heparin inhibits mesenteric vascular hypertrophy in angiotensin II-infusion hypertension in rats. *Cardiovascular research* 38: 247--255, 1998.

33. Donald DE. Splanchnic circulation. In: *Handbook Of Physiology. Peripheral Circulation And Organ Blood Flow*, edited by

Shepherd J, Abboud F, and Geiger S. Bethesda, MD: Am. Phys. Soc., 1983, p. 219--240.

34. Engeland WC. Functional innervation of the adrenal cortex by the splanchnic nerve. *Hormone and metabolic research* 30: 311-4, 1998.

35. Esler M. The sympathetic system and hypertension. *American journal of hypertension* 13: 99S-105S, 2000.

36. Esler M. The 2009 Carl Ludwig Lecture: Pathophysiology of the human sympathetic nervous system in cardiovascular diseases: the transition from mechanisms to medical management. *Journal of applied physiology (Bethesda, Md.: 1985)* 108: 227--237, 2010.

37. Esler MD, Krum H, Sobotka PA, Schlaich MP, Schmieder RE, Böhm M. Renal sympathetic denervation in patients with treatment-resistant hypertension (The Symplicity HTN-2 Trial): a randomised controlled trial. *Lancet* 376: 1903--1909, 2010.

38. Ferrario CM. Neurogenic actions of angiotensin II. *Hypertension* 5: V73-9, 1983.

39. Fink GD. Long-term sympatho-excitatory effect of angiotensin II: a mechanism of spontaneous and renovascular hypertension. *Clinical and experimental pharmacology & physiology* 24: 91--95, 1997.

40. Fink GD. Arthur C. Corcoran Memorial Lecture. Sympathetic activity, vascular capacitance, and long-term regulation of arterial pressure. *Hypertension* 53: 307--312, 2009.

41. Fink GD, Osborn JW. The splanchnic circulation. In: *Primer On The Autonomic Nervous System*, edited by Biaggioni I and Robertson D. Amsterdam: Elsevier, 2012, p. 211--213.

42. **Foss J, Fink G, Osborn J.** Reversal of genetic salt-sensitive hypertension by targeted sympathetic ablation. *Hypertension* 61: 806--811, 2013.

43. **Franco OH, Peeters A, Bonneux L, de Laet C.** Blood pressure in adulthood and life expectancy with cardiovascular disease in men and women: life course analysis. *Hypertension* 46: 280-6, 2005.

44. **Frisbee JC, Falck JR, Lombard JH.** Contribution of cytochrome P-450 omega-hydroxylase to altered arteriolar reactivity with high-salt diet and hypertension. *American journal of physiology. Heart and circulatory physiology* 278: H1517--26, 2000.

45. **Fujita Y.** Splanchnic circulation following coeliac plexus block. *Acta anaesthesiologica Scandinavica* 32: 323--327, 1988.

46. **Goldman MR, Wolk SW, Rutlen DL, Powell WJ Jr.** Effect of ouabain on total vascular capacity in the dog. *The Journal of clinical investigation* 69: 175-84, 1982.

47. **Griffin SA, Brown WC, MacPherson F, McGrath JC, Wilson VG, Korsgaard N, Mulvany MJ, Lever AF.** Angiotensin II causes vascular hypertrophy in part by a non-pressor mechanism. *Hypertension* 17: 626-35, 1991.

48. **Guo F, He D, Zhang W, Walton RG.** Trends in prevalence, awareness, management, and control of hypertension among United States adults, 1999 to 2010. *Journal of the American College of Cardiology* 60: 599-606, 2012.

49. **Guyton AC, Coleman TG, Cowley AW, Manning RD, Norman RA, Ferguson JD.** Brief reviews: A systems analysis approach to understanding Long-Range arterial blood pressure control and hypertension. *Circulation Research* 35: 159--176, 1974.

50. Hainsworth R. Vascular capacitance: its control and importance. *Reviews of physiology, biochemistry and pharmacology* 105: 101--173, 1986.

51. Hering D, Lambert EA, Marusic P, Walton AS, Krum H, Lambert GW, Esler MD, Schlaich MP. Substantial reduction in single sympathetic nerve firing after renal denervation in patients with resistant hypertension. *Hypertension* 61: 457--464, 2013.

52. Hirsch DM, Osborn J. Cardiac sympathetic nerves do not contribute to AngII-salt hypertension. *The FASEB Journal* 25: 640.8, 2011.

53. Hsieh NK, Liu JC, Chen HI. Localization of sympathetic postganglionic neurons innervating mesenteric artery and vein in rats. *Journal of the autonomic nervous system* 80: 1--7, 2000.

54. Inscho EW, Cook AK, Clarke A, Zhang S, Guan Z. P2X1 receptor-mediated vasoconstriction of afferent arterioles in angiotensin II-infused hypertensive rats fed a high-salt diet. *Hypertension* 57: 780--787, 2011.

55. Jacob F, Ariza P, Osborn JW. Renal denervation chronically lowers arterial pressure independent of dietary sodium intake in normal rats. *American journal of physiology. Heart and circulatory physiology* 284: H2302--H2310, 2003.

56. Jacob F, Clark LA, Guzman PA, Osborn JW. Role of renal nerves in development of hypertension in DOCA-salt model in rats: a telemetric approach. *American journal of physiology. Heart and circulatory physiology* 289: H1519-29, 2005.

57. Jacob F, LaBine BG, Ariza P, Katz SA, Osborn JW. Renal denervation causes chronic hypotension in rats: role of beta1-adrenoceptor activity. *Clinical and experimental pharmacology & physiology* 32: 255--262, 2005.

58. Kandlikar SS, Fink GD. Splanchnic sympathetic nerves in the development of mild DOCA-salt hypertension. *American journal of physiology. Heart and circulatory physiology* 301: H1965--H1973, 2011.

59. Kearney PM, Whelton M, Reynolds K, Muntner P, Whelton PK, He J. Global burden of hypertension: analysis of worldwide data. *Lancet* 365: 217-23, 2005.

60. Kerenyi SZ, Hartgraves SL. Premature excess release from the Alzet osmotic pump. *Pharmacology, biochemistry, and behavior* 27: 199-201, 1987.

61. King A, Yoshimoto M, Osborn J, Fink G. Regional hind-limb (HL) hemodynamics and norepinephrine (NE) spillover in chronic angiotensin II (AngII)-salt hypertension in the rat. *Hypertension* 52: e64, 2008.

62. King AJ, Fink GD. Chronic low-dose angiotensin II infusion increases venomotor tone by neurogenic mechanisms. *Hypertension* 48: 927--933, 2006.

63. King AJ, Novotny M, Swain GM, Fink GD. Whole body norepinephrine kinetics in ANG II-salt hypertension in the rat. *American journal of physiology. Regulatory, integrative and comparative physiology* 294: R1262--R1267, 2008.

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

65. Kline RL, Chow KY, Mercer PF. Does enhanced sympathetic tone contribute to angiotensin II hypertension in rats? *European journal of pharmacology* 184: 109-18, 1990.

66. Kuroki MT, Guzman PA, Fink GD, Osborn JW. Time-dependent changes in autonomic control of splanchnic vascular

resistance and heart rate in ANG II-salt hypertension. *American journal of physiology. Heart and circulatory physiology* 302: H763--H769, 2012.

67. Kuroki MT, Osborn JW. Comparison of plasma AngII and arterial pressure responses to subcutaneous AngII administration by implantable Alzet minipumps or external syringe pump. *The FASEB Journal* 27: lb841, 2013.

68. Ledwith BJ, Cahill MK, Losse LS, Satiritz SM, Eydelloth RS, Dallob AL, Tanaka WK, Galloway SM, Nichols WW. Measurement of plasma angiotensin II: purification by cation-exchange chromatography. *Analytical biochemistry* 213: 349--355, 1993.

69. Lee JY, Brune ME, Warner RB, DeBernardis JF. Orthostatic hypotension occurs following alpha 2-adrenoceptor blockade in chronic prazosin-pretreated conscious spontaneously hypertensive rats. *Journal of autonomic pharmacology* 12: 191 - 204, 1992.

70. Lewington S, Clarke R, Qizilbash N, Peto R, Collins R. Age-specific relevance of usual blood pressure to vascular mortality: a meta-analysis of individual data for one million adults in 61 prospective studies. *Lancet* 360: 1903--1913, 2002.

71. Lindstedt I, Xu C, Zhang Y, Edvinsson L. Increased perfusion pressure enhances the expression of endothelin (ETB) and angiotensin II (AT1, AT2) receptors in rat mesenteric artery smooth muscle cells. *Blood pressure* 18: 78-85, 2009.

72. Lohmeier TE. The sympathetic nervous system and long-term blood pressure regulation. *American journal of hypertension* 14: 147S-154S, 2001.

73. Lohmeier TE, Hildebrandt DA. Renal nerves promote sodium excretion in angiotensin-induced hypertension. *Hypertension* 31: 429-34, 1998.

74. Lohmeier TE, Hildebrandt DA, Dwyer TM, Iliescu R, Irwin ED, Cates AW, Rossing MA. Prolonged activation of the baroreflex decreases arterial pressure even during chronic adrenergic blockade. *Hypertension* 53: 833--838, 2009.

75. Lohmeier TE, Lohmeier JR, Haque A, Hildebrandt DA. Baroreflexes prevent neurally induced sodium retention in angiotensin hypertension. *American journal of physiology. Regulatory, integrative and comparative physiology* 279: R1437--R1448, 2000.

76. Luft FC, Wilcox CS, Unger T, Kühn R, Demmert G, Rohmeiss P, Ganten D, Sterzel RB. Angiotensin-induced hypertension in the rat. Sympathetic nerve activity and prostaglandins. *Hypertension* 14: 396 - 403, 1989.

77. Malpas SC, Evans RG. Do different levels and patterns of sympathetic activation all provoke renal vasoconstriction? *Journal of the autonomic nervous system* 69: 72-82, 1998.

78. Malpas SC, Evans RG, Head GA, Lukoshkova EV. Contribution of renal nerves to renal blood flow variability during hemorrhage. *American journal of physiology. Regulatory, integrative and comparative physiology* 274: R1283-94, 1998.

79. McBryde FD, Guild S, Barrett CJ, Osborn JW, Malpas SC. Angiotensin II-based hypertension and the sympathetic nervous system: the role of dose and increased dietary salt in rabbits. *Experimental physiology* 92: 831--840, 2007.

80. Mezzano V, Donoso V, Capurro D, Huidobro-Toro JP. Increased neuropeptide Y pressor activity in Goldblatt hypertensive rats: in vivo studies with BIBP 3226. *Peptides* 19: 1227--32, 1998.

81. Moretti J, Burke SL, Evans RG, Lambert GW, Head GA. Enhanced responses to ganglion blockade do not reflect

sympathetic nervous system contribution to angiotensin II-induced hypertension. *Journal of hypertension* 27: 1838--1848, 2009.

82. Navar LG. Counterpoint: Activation of the intrarenal renin-angiotensin system is the dominant contributor to systemic hypertension. *Journal of applied physiology (Bethesda, Md.: 1985)* 109: 1998--2000, 2010.

83. Ohnishi A, Li P, Branch RA, Holycross B, Jackson EK. Caffeine enhances the slow-pressor response to angiotensin II in rats. Evidence for a caffeine-angiotensin II interaction with the sympathetic nervous system. *The Journal of Clinical Investigation* 80: 13, 1987.

84. Osborn JL, DiBona GF, Thames MD. Beta-1 receptor mediation of renin secretion elicited by low-frequency renal nerve stimulation. *The Journal of pharmacology and experimental therapeutics* 216: 265-9, 1981.

85. Osborn JW, Fink GD. Region-specific changes in sympathetic nerve activity in angiotensin II-salt hypertension in the rat. *Experimental physiology* 95: 61--68, 2010.

86. Osborn JW, Fink GD, Kuroki MT. Neural mechanisms of angiotensin II-salt hypertension: implications for therapies targeting neural control of the splanchnic circulation. *Current hypertension reports* 13: 221-8, 2011.

87. Osborn JW, Fink GD, Sved AF, Toney GM, Raizada MK. Circulating angiotensin II and dietary salt: converging signals for neurogenic hypertension. *Current hypertension reports* 9: 228-35, 2007.

88. Osborn JW, Hendel MD, Collister JP, Ariza-Guzman PA, Fink GD. The role of the subfornical organ in angiotensin II-salt hypertension in the rat. *Experimental physiology* 97: 80--88, 2012.

89. Pablo Huidobro-Toro J, Verónica M. Sympathetic co-transmission: the coordinated action of ATP and noradrenaline and their modulation by neuropeptide Y in human vascular neuroeffector junctions. *European journal of pharmacology* 500: 27--35, 2004.

90. Reid IA. Interactions between ANG II, sympathetic nervous system, and baroreceptor reflexes in regulation of blood pressure. *American journal of physiology. Endocrinology and metabolism* 262: E763--E778, 1992.

91. Ruffolo Jr RR, Nichols A, Stadel J, Hieble J. Pharmacologic and therapeutic applications of alpha2-adrenoceptor subtypes. *Annual review of pharmacology and toxicology* 33: 243--279, 1993.

92. Saeed M, Holtz J, Sommer O, Kuhne G, Bassenge E. Beta-adrenergic activation of the renin-angiotensin system following alpha 2-, alpha 1-, or nonselective alpha-blockade in conscious dogs: no relation to the changes in blood pressure. *Journal of cardiovascular pharmacology* 6: 683--692, 1984.

93. Santajuliana D, Hornfeldt BJ, Osborn JW. Use of ganglionic blockers to assess neurogenic pressor activity in conscious rats. *Journal of pharmacological and toxicological methods* 35: 45--54, 1996.

94. Schlaich MP, Sobotka PA, Krum H, Lambert E, Esler MD. Renal sympathetic-nerve ablation for uncontrolled hypertension. *The New England journal of medicine* 361: 932-4, 2009.

95. Schlaich MP, Sobotka PA, Krum H, Whitbourn R, Walton A, Esler MD. Renal denervation as a therapeutic approach for hypertension: novel implications for an old concept. *Hypertension* 54: 1195--1201, 2009.

96. Shin LH, Dovgan PS, Nypaver TJ, Carretero OA, Beierwaltes WH. Role of neuropeptide Y in the development

of two-kidney, one-clip renovascular hypertension in the rat. *Journal of vascular surgery* 32: 1015--1021, 2000.

97. Simon G, Abraham G, Cserep G. Pressor and subpressor angiotensin II administration. Two experimental models of hypertension. *American journal of hypertension* 8: 645--50, 1995.

98. Simon G, Csiky B. Effect of neonatal sympathectomy on the development of structural vascular changes in angiotensin II-treated rats. *Journal of hypertension* 16: 77-84, 1998.

99. Simon G, Illyes G, Csiky B. Structural vascular changes in hypertension: role of angiotensin II, dietary sodium supplementation, blood pressure, and time. *Hypertension* 32: 654--660, 1998.

100. Smithwick RH, Thompson JE. Splanchnicectomy for essential hypertension. *JAMA: the journal of the American Medical Association* 152: 1501--1504, 1953.

101. Sripairojthikoon W, Wyss JM. Cells of origin of the sympathetic renal innervation in rat. *American journal of physiology. Renal physiology* 252: F957-63, 1987.

102. Sugawara T, Noshiro T, Kusakari T, Shimizu K, Watanabe T, Akama H, Shibukawa S, Miura W, Miura Y. Preferential changes in hepatosplanchnic hemodynamics in patients with borderline hypertension. *Hypertension research: official journal of the Japanese Society of Hypertension* 20: 201--207, 1997.

103. Sun D, Huang A, Yan EH, Wu Z, Yan C, Kaminski PM, Oury TD, Wolin MS, Kaley G. Reduced release of nitric oxide to shear stress in mesenteric arteries of aged rats. *American journal of physiology. Heart and circulatory physiology* 286: H2249-56, 2004.

104. Sun D, Messina EJ, Kaley G, Koller A. Characteristics and origin of myogenic response in isolated mesenteric arterioles. *American journal of physiology. Heart and circulatory physiology* 263: H1486-91, 1992.

105. Symplicity HTN-1 I. Catheter-based renal sympathetic denervation for resistant hypertension: durability of blood pressure reduction out to 24 months. *Hypertension* 57: 911--917, 2011.

106. Tabrizchi R, Pang CC. Comparative effects of rauwolscine, prazosin, and phentolamine on blood pressure and cardiac output in anesthetized rats. *Canadian journal of physiology and pharmacology* 65: 1421 - 1427, 1987.

107. Tan T, Watts SW, Davis RP. Drug Delivery: Enabling Technology for Drug Discovery and Development. iPRECIO Micro Infusion Pump: Programmable, Refillable, and Implantable. *Frontiers in pharmacology* 2: 44, 2011.

108. Toney GM, Pedrino GR, Fink GD, Osborn JW. Does enhanced respiratory-sympathetic coupling contribute to peripheral neural mechanisms of angiotensin II-salt hypertension? *Experimental physiology* 95: 587--594, 2010.

109. Tsioufis C, Papademetriou V, Dimitriadis K, Tsiachris D, Thomopoulos C, Park E, Hata C, Papalois A, Stefanadis C. Catheter-based renal sympathetic denervation exerts acute and chronic effects on renal hemodynamics in swine. *International journal of cardiology* (November 19, 2012). doi:/10.1016/j.ijcard.2012.10.038.

110. Veitenheimer BJ, Engeland WC, Guzman PA, Fink GD, Osborn JW. Effect of global and regional sympathetic blockade on arterial pressure during water deprivation in conscious rats. *American journal of physiology. Heart and circulatory physiology* 303: H1022--H1034, 2012.

111. Vieira AA, Nahey DB, Collister JP. Role of the organum vasculosum of the lamina terminalis for the chronic cardiovascular effects produced by endogenous and exogenous ANG II in conscious rats. *American journal of physiology. Regulatory, integrative and comparative physiology* 299: R1564-71, 2010.

112. Westfall TC. Neuropeptide y and sympathetic control of vascular tone in hypertension. In: *Npy Family Of Peptides In Neurobiology, Cardiovascular And Metabolic Disorders: From Genes To Therapeutics*, edited by Zukowska Z and Feuerstein G. Birkhäuser Basel, 2006, p. 89--103.

113. Whitworth JA. 2003 World Health Organization (WHO)/International Society of Hypertension (ISH) statement on management of hypertension. *Journal of hypertension* 21: 1983--1992, 2003.

114. Xue B, Zhang Z, Johnson RF, Johnson AK. Sensitization of Slow Pressor Angiotensin II (Ang II)-Initiated Hypertension Induction of Sensitization by Prior Ang II Treatment. *Hypertension* 59: 459--466, 2012.

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

116. Yoshimoto M, Sakagami T, Nagura S, Miki K. Relationship between renal sympathetic nerve activity and renal blood flow during natural behavior in rats. *American journal of physiology. Regulatory, integrative and comparative physiology* 286: R881-7, 2004.

117. Yoshimoto M, Wehrwein EA, Novotny M, Swain GM, Kreulen DL, Osborn JW. Effect of stellate ganglionectomy on basal cardiovascular function and responses to beta1-adrenoceptor blockade in the rat. *American journal of physiology. Heart and circulatory physiology* 295: H2447-54, 2008.

118. Young DB, Murray RH, Bengis RG, Markov AK. Experimental angiotensin II hypertension. *American journal of physiology. Heart and circulatory physiology* 239: H391--H398, 1980.

119. Zhang L, Taniguchi T, Tanaka T, Shinozuka K, Kunitomo M, Nishiyama M, Kamata K, Muramatsu I. Alpha-1 adrenoceptor up-regulation induced by prazosin but not KMD-3213 or reserpine in rats. *British journal of pharmacology* 135: 1757--1764, 2002.

120. Zhao XH, Sun XY, Edvinsson L, Hedner T. Does the neuropeptide Y Y1 receptor contribute to blood pressure control in the spontaneously hypertensive rat? *Journal of hypertension* 15: 19--27, 1997.

121. Zimmerman MC, Lazartigues E, Sharma RV, Davisson RL. Hypertension caused by angiotensin II infusion involves increased superoxide production in the central nervous system. *Circulation research* 95: 210--216, 2004.

122. Zou LX, Imig JD, von Thun AM, Hymel A, Ono H, Navar LG. Receptor-mediated intrarenal angiotensin II augmentation in angiotensin II-infused rats. *Hypertension* 28: 669--677, 1996.

Appendix 1

Comparison of arterial pressure and plasma AngII responses to three methods of subcutaneous AngII administration

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Chapter Overview

Angiotensin II (AngII) induced hypertension is a commonly studied model of experimental hypertension, particularly in rodents, and is often generated by subcutaneous delivery of AngII using Alzet osmotic minipumps chronically implanted under the skin. We have observed that, in a subset of animals subjected to this protocol, mean arterial pressure (MAP) begins to decline gradually starting the second week of AngII-infusion, resulting in a blunting of the slow-pressor response and reduced final MAP. We hypothesized that this variability in the slow-pressor response to AngII was mainly due to factors unique to Alzet pumps. To test this, we compared the pressure profile and changes in plasma AngII levels during AngII-salt hypertension generated using Alzet, iPrecio implantable pumps, or a Harvard external infusion pump. At the end of 14 days of AngII, MAP was highest in iPrecio (156±3mmHg), followed by Harvard (140±3mmHg) and Alzet (122±3mmHg) groups. The rate of the slow-pressor response, measured as daily increases in pressure averaged over days 2 to 14 of AngII, was similar between iPrecio and Harvard (2.7±0.4 and 2.2±0.4 mmHg/day) groups, but was significantly blunted in the Alzet (0.4±0.4 mmHg/day) group due to a gradual decline in MAP in a subset of rats. We also found differences in the temporal profile of plasma AngII between infusion groups. We conclude that the gradual decline in MAP observed in a subset of rats during AngII-infusion using Alzet pumps is mainly due to pump dependent factors when applied in this particular context.

6.1 Introduction

Angiotensin II (AngII) induced hypertension is a commonly studied model of experimental hypertension, particularly in rodents. This popularity has been fueled by the prominent role that AngII plays in cardiovascular homeostasis and various disease states such as hypertension and heart failure. Additionally, the relative ease of generating hypertension in normal animals with a defined time of onset (allowing for a within group experimental design), without additional manipulations such as removal of a kidney and provision of salt in the drinking water that is often required in other models, have made it an attractive model. The AngII-induced model also involves multiple mechanisms spanning various research disciplines including autonomic neuroscience, nephrology, vascular biology and immunology. As a consequence, the AngII-induced model is widely used employing multiple species (e.g., mice (121), rats (64, 66), dogs (75), rabbits (79) and sheep (19)), routes of administration (e.g. intravenous (111), intraperitoneal (98), subcutaneous (64, 66), and intracerebroventricular (14)), and dosages (15).

In particular, the subcutaneous infusion model of AngII-induced hypertension in rodents (mice and rats) has been a popular model over the last two to three decades. This is likely due to the development of implantable osmotic minipumps (Alzet) that allow easy delivery of AngII without requiring a tether for connection of infusion tubing to an external infusion pump. Moreover, subcutaneous infusion in these species does not require additional surgeries to gain access to the circulation, the intracerebroventricular space or intraperitoneal cavity. The dose of AngII required to generate hypertension via the subcutaneous route is

usually ~10 fold that required for the model generated via the intravenous route (15). The reported dose for rats in the literature ranges from 50-500ng/kg/min, with a commonly used dose between 100-200ng/kg/min (15). The typical duration of AngII administration is 2 weeks (64, 66). The hypertensive response to chronic AngII administration is often characterized by a gradual rise in pressure commonly referred to as a "slow-pressor" response or the "auto-potentiating" effect of chronic AngII administration. The severity of the "slow-pressor" response and final blood pressure during chronic AngII administration is dependent both on the dose of AngII and impending level of dietary salt intake (87).

Our laboratory has been studying the neurogenic mechanisms of AngII-induced hypertension in rats fed a high salt diet (2% NaCl) using Alzet minipumps as the primary method of subcutaneous AngII administration (150ng/kg/min for 2 weeks) (64, 66, 88, 115). It has been our experience that the model generated using this method occasionally fails to demonstrate the slow-pressor effect and sustained rise in pressure that is characteristic of the model. As shown in Figure 6.1, we have found that in a subset of animals subjected to the AngII-salt protocol pressure begins to decline gradually starting the second week of AngII-infusion, resulting in a blunting of the slow-pressor response and reduced final blood pressure (labeled as "non-responders"). There have been no distinguishable physical signs between rats that showed the normal progression in pressure versus those that displayed the latter response, making this an unpredictable response. This variable response has been echoed by multiple other researchers (personal communications) conducting similar studies in mice and rats.

There are at least three explanations for the "non-responder" profile including; 1) failure of the pump to maintain a constant flow rate over the 14 day protocol, 2) degradation of AngII within the pump, or 3) failure of rats to respond to AngII (i.e. a true physiological non-responder). For the present study we focused our attention on pump performance as a contributing factor.

Until recently, the only implantable pump for rodents was the Alzet osmotic minipump (DURECT, Corp., Cupertino, CA). However, another pump has recently become available; the iPrecio implantable pump (Primetech, Corp., Tokyo, Japan). Unlike Alzet pumps, iPrecio pumps are miniaturized mechanical pumps that expel fluid from a reservoir using a motorized peristaltic rotor that is precisely controlled by an embedded microcontroller (107). A recent report has shown that slight differences exist in the temporal profile of the pressor response to AngII infusion using Alzet versus iPrecio implantable minipumps (107). Although both Alzet and iPrecio rats displayed a slow-pressor response to chronic 14 days of AngII, and the final level of pressure was comparable between the two groups, the initial rise in pressure was slightly blunted in the Alzet rats compared to iPrecio rats (107). This suggested the possibility that characteristics inherent to the Alzet pump could play a role in the unpredictable responses described above.

In the previously reported study, no other variables, such as plasma AngII concentration, were measured to provide a possible explanation for the observed differences in the profile of AngII-induced hypertension between Alzet and iPrecio groups. In addition, observed differences were based on a comparison with a device that has just recently been introduced to the scientific community, making it difficult to assess

whether these results could be generalized when compared to other traditional methods of delivery such as an external infusion pump. Furthermore, the reported study was based on a protocol using an undisclosed level of dietary salt. Our lab has shown that the neurogenic component of AngII-induced hypertension is dependent on dietary salt intake, and our primary interest was to determine whether the different methods would impact the expression of the "neurogenic phase" during the later stage of AngII infusion in rats consuming a high salt diet.

In this study, we compared the reproducibility of the AngII-salt model of hypertension generated by subcutaneous administration of AngII using three different delivery methods. We compared the differences in the blood pressure profile of AngII-salt hypertension generated using Alzet, iPrecio, and Harvard infusion pumps attached to an implanted subcutaneous infusion catheter, and tested whether any differences between groups could be explained by differences in the change of plasma AngII levels over time.

6.2 Methods

Experiments were performed in conscious, chronically instrumented rats in accordance with NIH guidelines. All acute and chronic experimental procedures were conducted after approval and by the institutional animal care and use committee of the University of Minnesota.

6.2.1 Animal use and care

Male Sprague-Dawley rats from Charles River Laboratories (Wilmington, MA) weighing 200-250g upon transfer to our facility were used in this study. Depending on the protocol (see below) rats were kept on a regular diet (Lab Diet 5012)) or switched to a special diet with variable NaCl content (Research Diets, Inc., New Brunswick, NJ). Animals were housed 2 per cage in a 12-12hr light-dark cycled room (8:30/20:30 cycle). Distilled water was available ad-libidum. Animals were allowed to acclimate for at least 1 week prior to surgery.

6.2.2 Experimental Protocol

Rats were randomly assigned to one of two experiments. In the first experiment, rats were subjected to physiological and pharmacological stimuli known to increase or decrease plasma AngII levels, in order to establish a physiological range of endogenously generated plasma AngII. In the second experiment, AngII was administered for 2 weeks using three different subcutaneous infusion methods to establish the degree to which infusion modalities affect mean arterial pressure (MAP) and plasma AngII. In both experiments, whole

blood was collected from conscious, freely moving rats via a jugular venous catheter, and plasma AngII was assayed using a commercial ELISA kit (described below).

Experiment 1: Establishment of the physiological range of endogenously generated plasma AngII

The protocol for this experiment is shown in Figure 6.2. Rats were randomly assigned to 3 groups based on dietary NaCl content: 0%, 0.1%, or 0.4%. Rats were given the respective diet and water ad-libidum throughout the study. Rats were acclimated to their diet for 7 days prior to surgical implantation of a jugular venous catheter for blood collection. Catheters were flushed daily with 50U/mL heparinized saline to maintain patency. These surgical procedures are described in detail below.

Venous blood was collected 7 days after surgery and then again 7 days later in 2 of the groups. In rats fed a 0% NaCl diet, the loop diuretic drug furosemide (F4381, Sigma Aldrich, Co., St. Louis, MO), a known stimulant for renin release and subsequent increase in plasma AngII, was administered (50mg/kg, i.p.) and blood was collected ~3hrs later. Rats fed a 0.4% NaCl diet were subjected to another stimulus for renin release, 48hr water deprivation, 5 days after the initial blood collection and blood was then collected at the end of the 48hr period.

Experiment 2: Comparing the effect of 3 infusion methods on plasma AngII and arterial pressure during AngII-salt hypertension

The protocol for this experiment is shown in 6.2. Rats were fed a 2% NaCl diet, given distilled water ad-libidum and randomly assigned to one of three groups based on the method of AngII delivery: 1) Alzet osmotic minipump (2ML2, DURECT, Corp., Cupertino, CA), 2) Harvard syringe pump (Model 935, Harvard Apparatus, South. Natick, MA) or 3) iPrecio micro infusion pump (SMP-200, Primetech, Corp., Tokyo, Japan). Rats were chronically instrumented with a DSI pressure transmitter and a jugular venous catheter. For rats in the Harvard group, a catheter was tunneled subcutaneously over the right flank area, exteriorized, and connected to a single channel hydraulic swivel (Model 375/22PS, Instech Laboratories, Inc., Plymouth Meeting, PA). This was used later for subcutaneous delivery of AngII. All other rats were tethered to an in house made swivel. Rats were given 7-10 days to recover from the surgery. The surgical procedures are described in detail below.

The study protocol consisted of 4 days of control followed by 14 days of AngII infusion. For the Alzet group, a second surgery was performed on the 5th day of the protocol for minipump implantation as previously described (66). In all groups, AngII (A9525, Sigma-Aldrich, Co.) dissolved in physiological saline was delivered at a 5uL/hr infusion rate and dosed at 150ng/kg/min based on body weight on control day 4. Blood was sampled on control day 1, and days 3, 7, and 14 of AngII for measurement of plasma AngII concentration

Arterial pressure (AP) data was collected using acquisition software from DSI (DataQuest ART Acquisition). The detailed acquisition setup has been described in detail previously (66). 10 second MAP and mean HR was calculated and stored every one minute using the built-in online analysis routine in the acquisition software.

6.2.3 Surgical procedures

Surgery was performed under isoflurane anesthesia (2.5% isoflurane in 100% O2 delivered via a nose cone at 1mL/min flow rate). After induction, rats were given atropine (0.2mg/kg, i.p., Baxter International, Inc., Deerfield, IL) for reduction of salivary and bronchial secretions, preoperative antibiotic prophylaxis (gentamicin, 0.05mL, i.m., Hospira, Inc., Lake Forest, IL), preoperative pain relief (ketoprofen, 5mg/kg, s.c., Fort Dodge Animal Health, Inc., Overland Park, KS) and placed on a heated surgical bench. Surgical instruments were heat sterilized and all implanted instruments were cold sterilized overnight in a solution of glutaraldehyde (Cidex Plus, Johnson & Johnson Services, Inc., New Brunswick, NJ).

All rats were instrumented with a jugular venous catheter made from a Tygon tubing (0.02" I.D. x 0.06" O.D. Tygon Micro-Bore tubing S-54-HL, Saint-Gobain Performance Plastics, Corp., Akron, OH) connected to a 6cm Silastic tube (0.02" I.D. x 0.037" O.D. Silastic Laboratory Tubing, Dow Corning, Corp., Midland, MI) using a 2cm polyethylene tube (PE50 Intramedic Polyethylene Tubing, Becton Dickinson and Company, Sparks, MD) coupler. A 1mm piece of Silastic tube (same as above) was slid 3cm from the tip of the Silastic portion of the catheter to serve as a cuff. A 2 cm incision was made slightly above the clavicles

along the mid-clavicular line. The external jugular vein was dissected free from the surrounding tissue. The distal end was permanently occluded with a knot and the proximal end was temporarily occluded while a small incision was made to gain access to the vascular lumen. The jugular catheter was advanced 3 cm from this incision towards the right atrium. The catheter was fixed in place by two knots above and below the cuff, tunneled subcutaneously through the chest and secured at three positions over the pectoral musculature before exteriorizing through a dorsal incision over the scapulae.

In addition to the jugular venous catheter, rats in Experiment 2 were instrumented with a DSI pressure transmitter (TA11PA-C40, Data Sciences International (DSI), Saint Paul, MN) for measurement of arterial pressure as described previously (66). In the Harvard infusion group, a catheter (23g Tygon) was implanted subcutaneously such that the tip of the catheter was positioned over the animal's right lower flank. In the iPrecio group, an iPrecio minipump filled with physiological saline was implanted subcutaneously over the animal's right flank. The tip of the pump's infusion catheter was tunneled across to the animal's left flank. The body of the minipump was secured to the underlying subcutaneous tissue via the pump's suture holes.

The catheters were exteriorized through a skin incision over the scapulae, anchored to the underlying subcutaneous tissue using a circular surgical polyester mesh (PETKM14002, Textile Development Associates, Inc., Surgical Mesh Division, Brookfield, CN) which was implanted subcutaneously and sutured to the skin upon closure of the incision. The exteriorized catheters were threaded through a stainless

steel spring used for tethering the rat to a custom made or a commercial single channel hydraulic swivel mounted above their cage.

Rats were given a minimum of 7 days to recover from surgery. Ketoprofen (5mg/kg, s.c., Fort Dodge Animal Health, Inc., Overland Park, KS) was administered daily for 3 days post surgery for pain management. Jugular venous catheters were flushed daily with lock solution (50U/mL heparinized saline; Hospira, Inc.) to ensure patency.

6.2.4 Blood collection and separation of plasma

0.35mL of whole blood was collected into a syringe containing 15uL of an inhibitor cocktail. The inhibitor cocktail consisted of a mixture of EDTA (125mM; E4884, Sigma-Aldrich, Co.), pepstatin-A (20ug/mL in methanol; 77170, Sigma-Aldrich, Co.), 1,10-Phenanthroline (8mg/mL; 131377 Sigma-Aldrich, Co.), enalaprilat (80ug/mL in 50% ethanol; E9658, Sigma-Aldrich, Co.), APMSF (800ug/mL; A6664, Sigma-Aldrich, Co.), and 2-mercapto-ethanol (2%; M6250, Sigma-Aldrich, Co.) based on a protocol described previously (68). The inhibitor cocktail was prepared fresh prior to each sample collection from previously prepared stock solutions. Blood was chilled on ice immediately after collection. Following blood collection, 1mL of physiological saline was administered intravenously and the catheter was filled with lock solution. Blood was centrifuged at 2000g for 10min. Plasma was collected and re-centrifuged at 16000g for an additional 10min and stored at -80°C until assayed for AngII. On average, 180-200μL of plasma was recovered using this technique.

6.2.5 AngII assay

Prior to the assay, plasma was thawed, acidified with 1% trifluoroacetic acid (TFA; 302031, Sigma-Aldrich, Co.) and centrifuged at 16000g for 10min. It was then purified through a C18 column (Y1000 SEP-COLUMNS, Peninsula Laboratories, LLC, San Carlos, CA), washed with 1% TFA and eluted with a 60% solution of acetonitrile (34998, Sigma-Aldrich, Co.) containing 1% TFA. Samples were dried in a speed-vac centrifuge (heated to 50°C during the initial 4hrs of a 5hr run cycle) and resuspended in 200uL EIA buffer (from EIA kit). Sample pH was adjusted to pH 6~7 by adding microliter amounts of 1M sodium phosphate buffer and NaOH. Reconstituted samples were further diluted by 15-50% with addition of EIA buffer prior to measurement of AngII levels using a commercial ELISA kit (AngII EIA Kit #589301, Cayman Chemical Company, Ann Arbor, MI). All measurements were performed in duplicates.

Absorbance at 450nm was measured using a plate reader. After loading the reporter (Ellman's reagent) to each well, absorbance was serially measured at 10min intervals for a total of 1hr to ensure linearity of reaction. Absorbance reading from the 30min time point was used for final analysis.

6.2.6 Data Analysis and Statistics

AP and HR data for individual animals were averaged over a 24hr period. Duplicate absorbance readings from plasma samples were averaged and converted to units of pg/mL based on a standard provided with the kit. Grouped data are shown as mean±S.E.M. The effect of

dietary NaCl on plasma AngII level was analyzed by a one-way analysis of variance (ANOVA) followed by multiple comparisons (Holm-Sidak) versus control (0.4% NaCl). The effect of furosemide and 48hr water deprivation on 0% and 0.4% NaCl fed rats was analyzed by a paired t-test. The effect of infusion method on plasma AngII, MAP, and HR was analyzed by a repeated measures ANOVA followed by a Holm-Sidak multiple comparisons test when appropriate. All aforementioned analysis was performed in SigmaPlot (version 11, Systat Software, Inc., Richmond, CA).

6.3 Results

6.3.1 Experiment 1: Establishment of the physiological range of endogenously generated plasma AngII

Plasma AngII responses to various stimuli showed physiologically consistent trends (Figure 6.3). Compared to plasma AngII level in rats fed a normal (0.4% NaCl) salt diet (9±2 pg/mL), plasma AngII increased ~5 fold to 50±10 pg/mL when dietary NaCl content was lowered to 0.1%. There was no further rise in plasma AngII levels (50±6 pg/mL) when dietary NaCl content was further lowered to a minimum NaCl diet (0% NaCl). Acute furosemide injection to 0% NaCl rats to stimulate renin release and plasma AngII production caused a ~3 fold increase in plasma AngII levels to 170±30 pg/mL compared to baseline, and an ~18 fold increase compared to plasma levels from 0.4% NaCl rats. 48hr water deprivation in 0.4% NaCl rats caused an ~3 fold increase in plasma AngII levels to 31±3 pg/mL. Plasma AngII level in salt loaded rats with a 2% NaCl diet was 11±1 pg/mL, which was not significantly different from levels in 0.4% NaCl rats.

6.3.2 Experiment 2: Comparing the effect of 3 infusion methods on plasma AngII and arterial pressure during AngII-salt hypertension

Arterial pressure and heart rate responses to AngII administration

The three methods for chronic subcutaneous AngII infusion resulted in a subtle difference in the initial changes in MAP, but marked

differences in the final week of AngII-infusion (Figure 6.4; top panel). MAP at day 14 of AngII was highest in the iPrecio group (156±5mmHg) followed by Harvard (140±5mmHg), and Alzet (122±4mmHg).

The largest single day rise in MAP occurred on day 1 of AngII infusion. The increase in MAP (compared to baseline) on AngII day 1 was highest in the iPrecio group (24±2mmHg) followed by Alzet (17±2mmHg), and then the Harvard (11±1mmHg) group. Pressure rose gradually following this first day. Despite differences in the AngII day 1 MAP response, the "slow pressor response" was similar between the iPrecio and Harvard group. The slow pressor response to AngII infusion was calculated by subtracting the MAP on days 1 to 14 of AngII from MAP on day 1 of AngII. This slow pressor response was markedly blunted in the Alzet group, which was due, in part, to the decrease in MAP that occurred in a subset of rats starting around day 8 of AngII. The slow pressor response from day 1 onward contributed 33±3mmHg, 29±3mmHg, and 6±3mmHg of the total change in pressure seen at day 14 of AngII in the iPrecio, Harvard, and Alzet groups respectively

The HR response to chronic AngII infusion (Figure 6.4; bottom panel) was also different among the three groups. HR decreased in all groups during the first week of AngII. The peak drop in HR occurred on days 3 and 4 of AngII for the Alzet and iPrecio groups, respectively, and on day 8 of AngII for the Harvard group. Following this nadir, HR tended to increase in all groups, and was most noticeable in the iPrecio group. HR on day 14 of AngII was 420±10BPM, 395±8BPM, and 402±7BPM in the iPrecio, Harvard, and Alzet groups respectively.

6.3.3 Plasma AngII responses to AngII administration

Figure 6.5 shows plasma AngII during control, days 3, 5, 7 and 14 of infusion and corresponding values for arterial pressure in the three groups. Plasma AngII during control was not statistically different between groups. Although the mean plasma AngII level during AngII administration for the entire infusion period (Figure 6.3; right panel), expressed as the combined average between days 3, 7, and 14 of AngII were not statistically different between the three groups (59±8, 62±7, 71±8 pg/mL for Alzet, Harvard, and iPrecio, respectively), there were differences in the temporal profile of plasma AngII during AngII infusion between the three groups (Figure 6.5; top panel). Rats in the Alzet group showed a marked peak in plasma AngII on day 3 of AngII administration followed by a marked decline on days 7 and 14. In both Harvard and iPrecio groups, this peak in plasma AngII did not occur until day 7 of AngII. Similarly to the Alzet group, plasma AngII in the Harvard group declined markedly on day 14 of AngII; however, day 14 plasma AngII levels in the iPrecio group remained near day 7 levels.

6.4 Discussion

Our collaborators and we have years of experience using Alzet minipumps to study the AngII-salt model of hypertension (62, 63, 64, 66, 88, 115). During this time we found that the arterial pressure response to AngII in random subsets of animals begins to decline gradually starting at the second week of AngII-infusion. This results in a blunting of the slow-pressor response and reduced final blood pressure in control animals, making comparison and interpretation of cardiovascular responses in the treatment groups difficult. Other researchers using this model (AngII induced hypertension with Alzet minipumps) in mice and rats have voiced similar concerns (personal communications). Indeed, we formally discussed this issue in 2011 at a workshop of the American Physiological Society on small animal instrumentation but this issue had not been addressed in the literature.

Based on a recently published article showing subtle differences in the initial arterial pressure profile of AngII-induced hypertension using Alzet pumps and a new implantable mechanical pump (iPrecio) (107), we hypothesized that the marked variability in slow-pressor response of the AngII-salt model was mainly due to factors inherent in the performance of Alzet minipumps. Therefore, we compared the arterial pressure profile of AngII-salt hypertension generated using Alzet, iPrecio, and an external infusion pump (Harvard) connected to a subcutaneously implanted catheter. We also measured plasma AngII levels during control and days 3, 7, and 14 of AngII, as we reasoned that differences in the pump's ability to maintain the expected delivery rate of AngII would be a main underlying cause for any between pump differences.

6.4.1 Response of plasma AngII to subcutaneous infusion: Comparison to endogenously generated AngII

Using a commercially available AngII ELISA assay, plasma AngII concentration in rats fed a "regular" salt diet (~0.4% NaCl) were comparable to values reported in the literature, which range between 11 – 50 pg/mL in normal rats (18, 47, 99, 122). In rats fed a 2% NaCl diet, plasma AngII levels did not decrease any further compared to 0.4% rats, despite the 5-fold increase in dietary salt intake. The absence of a statistically distinguishable difference could have been due to a limitation in the sensitivity of the assay, but it nevertheless suggests that rats fed a "regular" diet are at the tail end of a plasma AngII vs. salt intake response curve. In general, when examining all three methods of subcutaneous AngII administration at 150ng/kg/min, plasma AngII concentration in 2%NaCl rats increased ~5 fold. This response was well within the physiological range of AngII as compared to levels seen in salt deplete rats fed a 0.1% or minimum (0%) NaCl diet. Even at peak levels observed on day 3 of AngII in Alzet rats, plasma AngII values were within physiologically attainable range as values were below those seen in salt depleted rats acutely treated with furosemide.

In comparison, plasma AngII concentration reported by others in AngII-induced hypertension ranged from 26pg/mL in rats given AngII at 100ng/kg/min (99), 34pg/mL for 175ng/kg/min (18), 27-157pg/mL for 200ng/kg/min (18, 47), 79pg/mL for 350ng/kg/min (18), and 101pg/mL for 500ng/kg/min (18). In most of these cases, plasma AngII levels during AngII infusion were not statistically distinguishable from control

levels. In those that reported a statistically significant change, the fold change from control levels was 2x for 100ng/kg/min (99), 3x for 200ng/kg/min (122), and 6x for 500ng/kg/min (18). Although the rise in plasma AngII measured during AngII-salt hypertension was higher in our study for the given delivery rate of AngII, direct between study comparison is difficult to make due to differences in the level of dietary salt used in this (2% NaCl) and other studies (likely ~0.4%), and the high degree of variability in the reported values, both within and between studies. Between study variations are likely due to differences in the AngII assay.

6.4.2 Explanations for differences between methods of delivery for plasma AngII and cardiovascular responses

In the present study, the only difference between groups was the method of subcutaneous AngII delivery. The concentration and composition of the AngII infusate, and the theoretical flow rate of AngII delivery was identical in all groups. Therefore, differences in the arterial pressure (and plasma AngII response) are not likely to be explained by degradation of AngII within the pump. Indeed, other researchers have compared before implant and after explant levels of AngII and found that there were no detectable differences between the two levels (47).

Griffin and colleagues have suggested that large within study variability in plasma AngII concentration in models of AngII-induced hypertension, specifically when Alzet pumps are used, could potentially be due to intermittent pumping that result in unpredictable fluctuations in plasma AngII (47). Indeed, we found that Alzet rats had a marked

spike in plasma AngII on Day 3 of AngII administration, which rapidly trailed off over the subsequent period of AngII administration. This pattern was not observed in rats in which AngII was administered by a pump in which flow rate is mechanically fixed (iPrecio and Harvard rats). It may be that for the Alzet pumps, there is an initially higher flow rate (60) that corresponds with osmotically driven compression of the pump bladder, and this rate then decreases over time. This may be one explanation for the "non-responder" profile of the Alzet rats; a rapid fall off in pump flow rate that does not occur with a "mechanical" pump. It should be noted however that although a higher degree of variability in plasma AngII levels on day 3 of AngII was observed in Alzet rats, the variability on days 7 and 14 of AngII were comparable among the three groups. Thus, other factors may contribute to the higher variability of plasma AngII during AngII-induced hypertension.

Compared to the previously published "pilot" study (107) which showed a slight pump dependent difference in the initial profile of MAP during AngII-induced hypertension, differences between Alzet and iPrecio pumps, in this study, were larger in magnitude, and especially pronounced during the second week of AngII infusion. Based on the level of the slow-pressor response, measured in terms of day to day change in pressure averaged over days 2 to 14 of AngII, final level of MAP, and the variability in those values, the iPrecio group displayed the most "robust" AngII-salt hypertension phenotype during the 2 week protocol. The Harvard group had an intermediate final level of MAP, but a similar slow-pressor response to iPrecio. The Alzet group had the lowest final level of MAP, and a significantly blunted slow-pressor response

due, in part, to a gradual drop in MAP in a subset of rats during the second week of AngII-infusion.

There were notable between pump differences in the day 1 level of MAP, which ultimately impacted the final level of MAP at the end of the 2-week infusion protocol. Day 1 MAP in the Harvard group was lowest amongst the three groups. One likely explanation for this lower level of MAP is the difficulty in adjusting the infusion rate of the pump to match that of Alzet and iPrecio pumps. Although the Harvard pump was calibrated to an infusion rate of 5μL/hr based on accurate measurements of syringe diameter and displacement rate of the plunger, it is possible that compliance within couplings at the infusion line, and undetectable leakage in the hydraulic swivel from elevated back-pressure due to material buildup at tip of the implanted catheter or kink at the site of exteriorization, caused a lower volume to be delivered compared to the expected rate.

Although differences in day 3 plasma AngII levels between Harvard and iPrecio do not reflect a lower AngII delivery rate in the Harvard group, baroreflex and negative feedback effect of AngII on renin secretion could have resulted in lower plasma AngII levels in the iPrecio group, thus masking the initial effect of differences in the infusion rate. This uncoupling of plasma AngII-levels and MAP is also evident on day 3 values in Alzet rats compared to iPrecio. Although AngII-levels were significantly higher in the Alzet group, there were no significant differences in MAP compared to iPrecio. One possibility is a higher initial pumping rate in Alzet pumps, as our protocol has a short pre-equilibration step (~2-3hrs) and no warming step prior to pump implantation, both reported to affect initial pump performance when

used in acute settings (60). The lower day 1 change in MAP compared to iPrecio makes this scenario unlikely, however, it is possible that accelerated pumping is delayed. The lack of a difference in MAP on day 3 of AngII compared to iPrecio could be due to compensatory mechanisms that are able to buffer the pressor effect of a higher level of plasma AngII.

6.4.3 Differences in day 1 MAP response to AngII do not predict the magnitude of the subsequent slow pressor response

If differences in the infusion rate of AngII between groups were responsible for differences in the increase of MAP over time, and the infusion rate is constant, one would predict these changes should appear on the first day of AngII administration and remain for the duration of the protocol. However, this was not the case. Even though the MAP response on Day 1 of AngII administration was lower in the Harvard group compared to iPrecio, the subsequent rate of rise of MAP over time was the same. On the other hand, the MAP response on Day 1 in the Alzet group was similar to the Harvard group, but the subsequent rate of rise of MAP was significantly less. This suggests that the blunting and disappearance of the slow-pressor response after day 7 of AngII in the Alzet group is due to problems intrinsic to the use of Alzet pumps in this particular context. There are several explanations for to this result. A gradual decrease in the pumping rate or a reduction in the effective concentration of AngII in the infusate due to degradation within the pump could have resulted in a gradual reduction in the delivered dose of AngII. Alternatively, it has been proposed that fibrotic

changes or increased degradation of AngII secondary to an inflammatory reaction around the site of pump implant result in reduced AngII absorption (15). Although we did not investigate these possibilities, we reasoned that either scenario could have resulted in measurable changes in plasma AngII levels paralleling changes in arterial pressure. Consistent with this concept, there was a downward trend in plasma AngII levels in Alzet rats reflected by the significantly lower plasma AngII levels on days 7 and 14 of AngII following its day 3 peak.

6.4.4 Summary and Perspectives

AngII-induced hypertension has been a popular model due to its relative ease to generate a hypertensive phenotype. However, comparing results between laboratories are difficult due to the variety of protocols in use, ranging from differences in dose of AngII, route of AngII administration, and level of dietary salt. The findings in this study shows that Alzet pumps may add another source of variability due to characteristics inherent to the pump. The use of mechanical infusion devices, either implantable (iPrecio) or external (syringe pump), may remove this pump dependent source of variability and result in a more reproducible and consistent AngII-induced hypertension phenotype.

6.5 Figures

Figure 6.1. Spontaneous drop in MAP during AngII-salt hypertension using Alzet pumps

Data from a separate study collected from rats fed a 2% NaCl diet subjected to a 2 week infusion of AngII (150ng/kg/min, s.c.) using Alzet osmotic minipumps (model 2ML2). Half of rats showed a typical slow-pressor response ("responder", black circles; n=10). In the other half, MAP gradually dropped during the second week of AngII-infusion ("non-responder", white circles; n=9). Error bars are S.E.M.

Figure 6.1

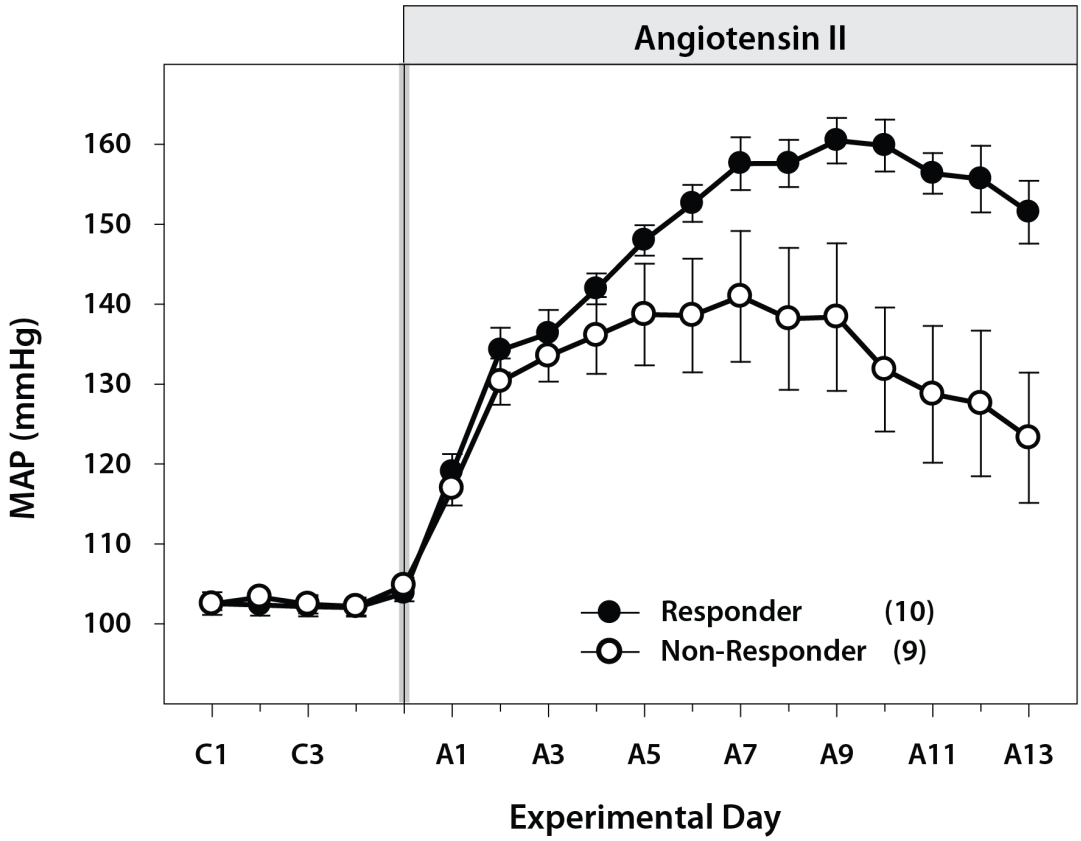

Angiotensin II
MAP (mmHg)
160
150
140
130
120
110
100
Responder (10)
Non-Responder (9)
C1
C3
A1
A3
A5
A7
A9
A11
A13
Experimental Day

Figure 6.2. Description of study protocol

(Top Panel): Protocol for experiment 1. In the first half of the protocol, blood was sampled from 3 groups of rats fed a 0.4%, 0.1%, or a minimum NaCl diet (0%) to determine the effect of dietary NaCl on plasma AngII levels. In the second half of the protocol, 0.4% rats were further subjected to a 48hr water deprivation period, and 0% rats were given an acute injection of furosemide to determine the range of plasma AngII responses attainable with further physiological and pharmacological stimuli to the renin-angiotensin-aldosterone axis. ***(Bottom Panel):*** Protocol for the main experiment in which 3 groups of rats fed a 2%NaCl diet were subjected to a 2 week subcutaneous infusion of AngII using a Harvard external infusion pump (model 935), Alzet osmotic minipumps (model 2ML2), or iPrecio pumps. Blood was collected on control day 1, and days 3, 7, and 14 of AngII for measurement of plasma AngII levels.

Figure 6.2

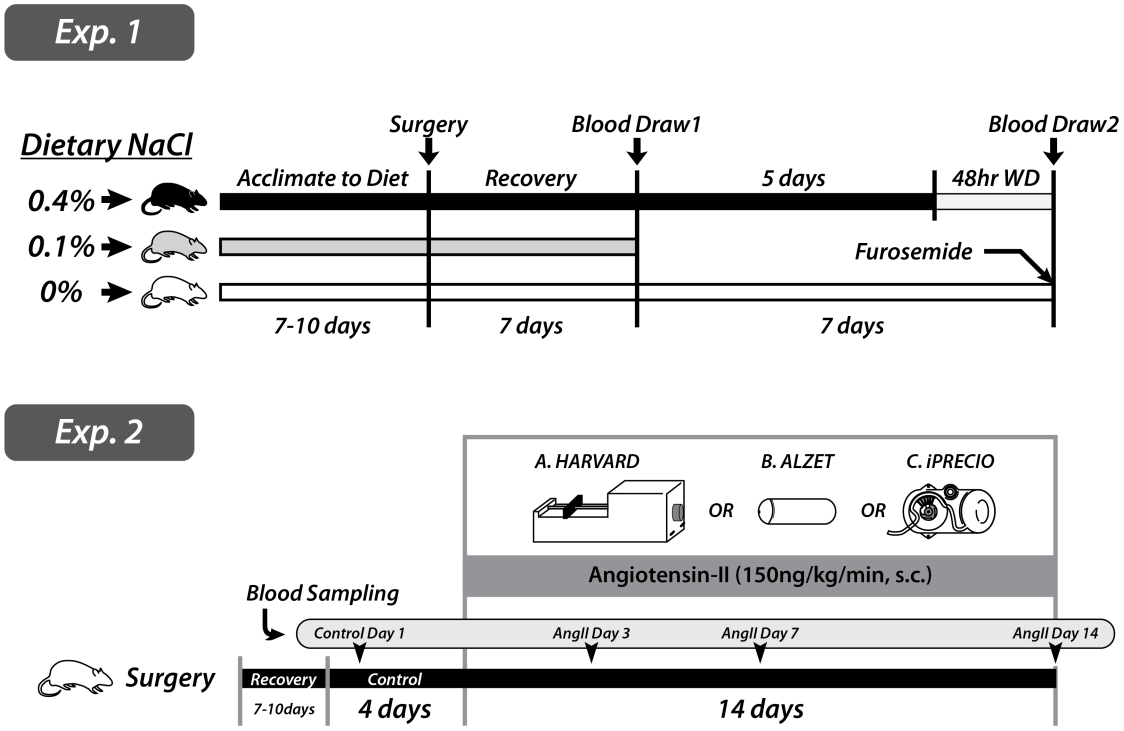

Exp. 1
Dietary NaCl
0.4%
0.1%
0%
Surgery
Blood Draw1
Blood Draw2
Acclimate to Diet
Recovery
5 days
48hr WD
Furosemide
7-10 days
7 days
7 days
Exp. 2
A. HARVARD
B. ALZET
C. iPRECIO
OR
OR
Angiotensin-II (150ng/kg/min, s.c.)
Blood Sampling
Control Day 1
AngII Day 3
AngII Day 7
AngII Day 14
Surgery
Recovery
7-10days
Control
4 days
14 days

Figure 6.3. Changes in plasma AngII levels in response to physiological salt loading, water deprivation, and pharmacological salt depletion

(Left Panel): Results from experiment 1. Data for the 2% NaCl diet group is the control period plasma AngII level from experiment 2 averaged across all 3 groups of rats (i.e. Alzet, Harvard and iPrecio). N = 26, 8, 8, 8 for 2%, 0.4%, 0.1%, and 0% salt groups, respectively. ***(Right Panel):*** Plasma AngII levels during chronic infusion of AngII (150ng/kg/min, s.c.) using Harvard (n=9), Alzet (n=12), or iPrecio (n=10) pumps. The plotted plasma AngII level represents the average from days 3, 7, and 14 of AngII infusion. There were no between group differences. (†) denotes statistical significance compared to the 0.4% NaCl group.

(∗) denotes statistically significant effect after furosemide or water deprivation within the given dietary NaCl group. (‡) denotes statistically significant increase in plasma AngII levels during AngII infusion in Harvard, Alzet, and iPrecio groups compared to their respective control levels. Statistical significance was set at $p<0.05$ or less with post-hoc comparisons using the appropriate correction for significance level. Error bars are S.E.M.

Figure 6.3

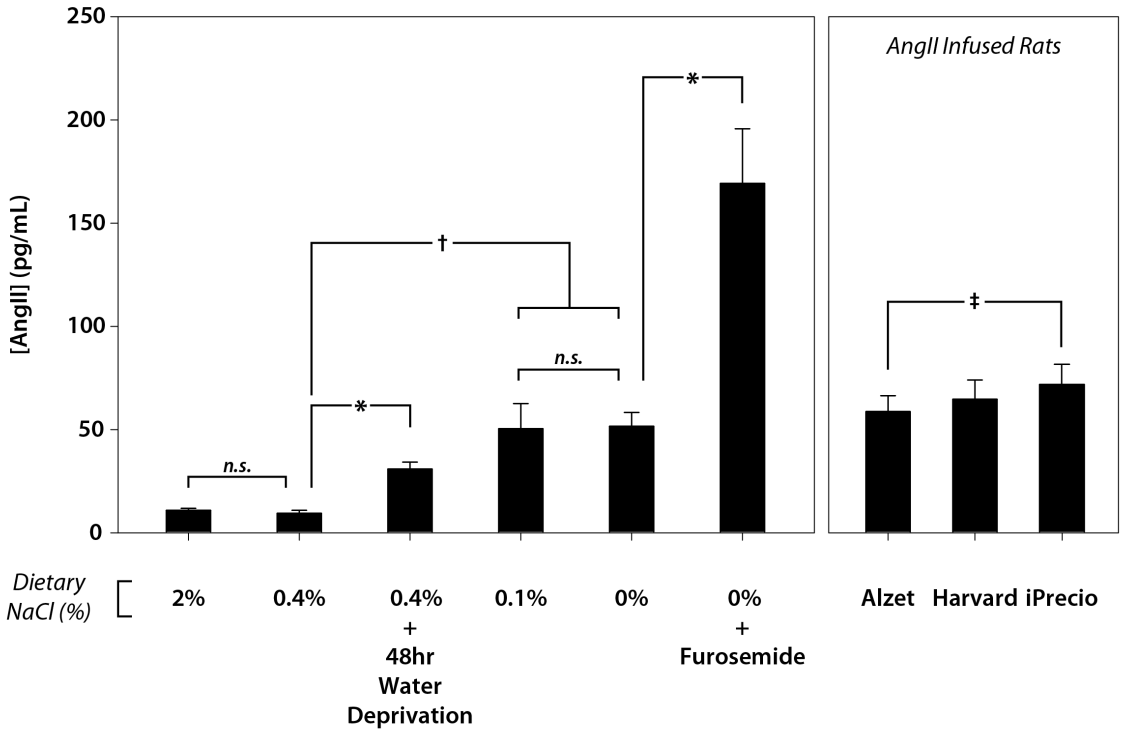

250
200
150
100
50
0
[AngII] (pg/mL)
n.s.
*
†
n.s.
*
AngII Infused Rats
‡
Dietary NaCl (%)
2%
0.4%
0.4% + 48hr Water Deprivation
0.1%
0%
0% + Furosemide
Alzet
Harvard
iPrecio

Figure 6.4. Differences in MAP profile of AngII-salt hypertension generated using Alzet, Harvard or iPrecio pumps

24hr mean arterial pressure (MAP; top panel) and heart rate (HR; bottom panel) during control and 2 weeks of AngII infusion using Harvard (white circle; n=12), Alzet (black circle; n=15), or iPrecio (black triangle; n=10) pumps in rats fed a 2% NaCl diet. MAP on days 1 through 14 of AngII was significantly elevated compared to control day 3 in all groups. (†), (‡), and (#) denote significant within group differences compared to values on control day 3 in Alzet, Harvard, and iPrecio groups, respectively. (∗), (§), and (¶) denote significant differences between iPrecio and Alzet, iPrecio and Harvard, and Harvard and Alzet, respectively. Statistical significance was set at $p<0.05$ or less in post-hoc comparisons using appropriate correction for significance level. Error bars are S.E.M.

Figure 6.4

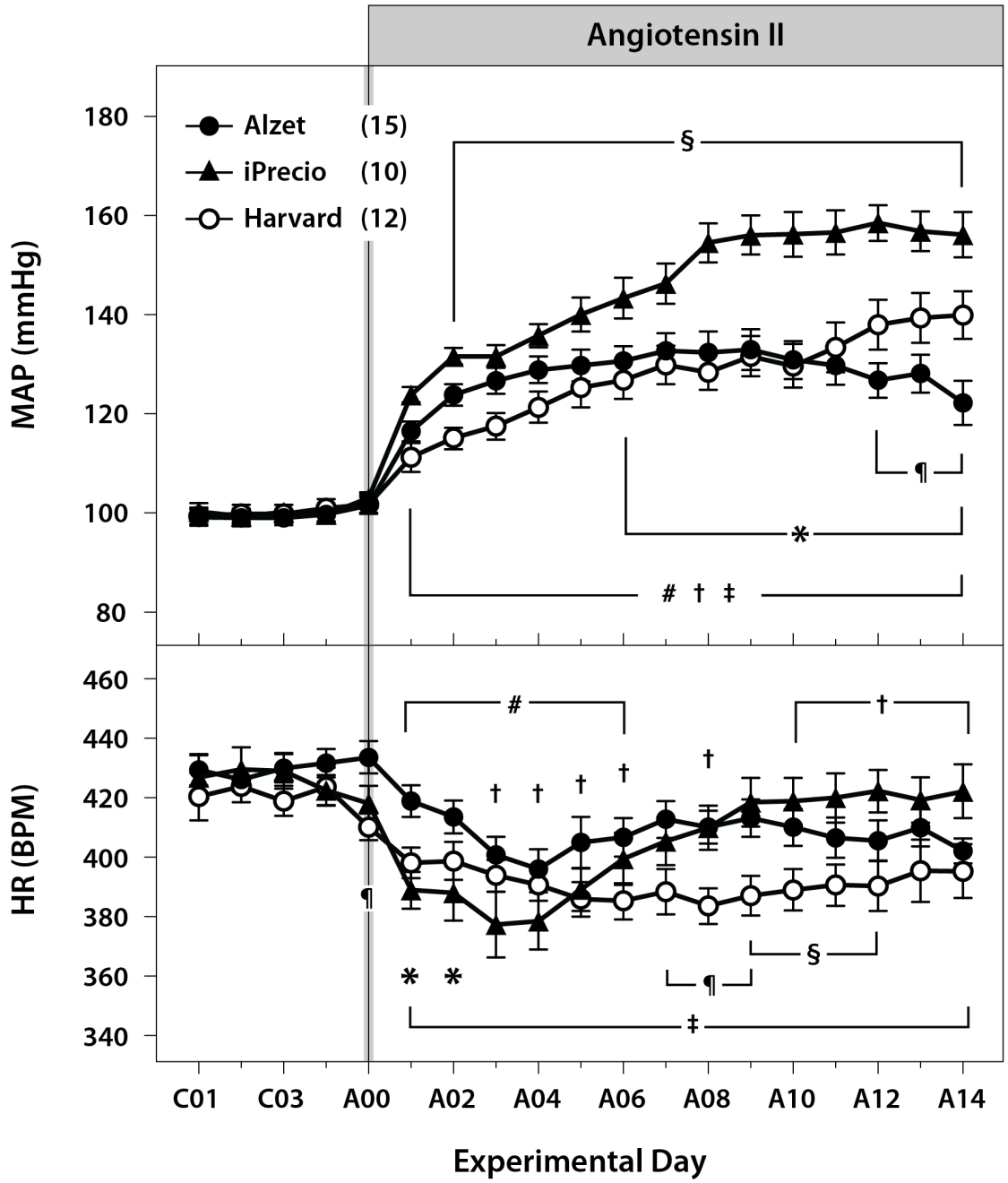
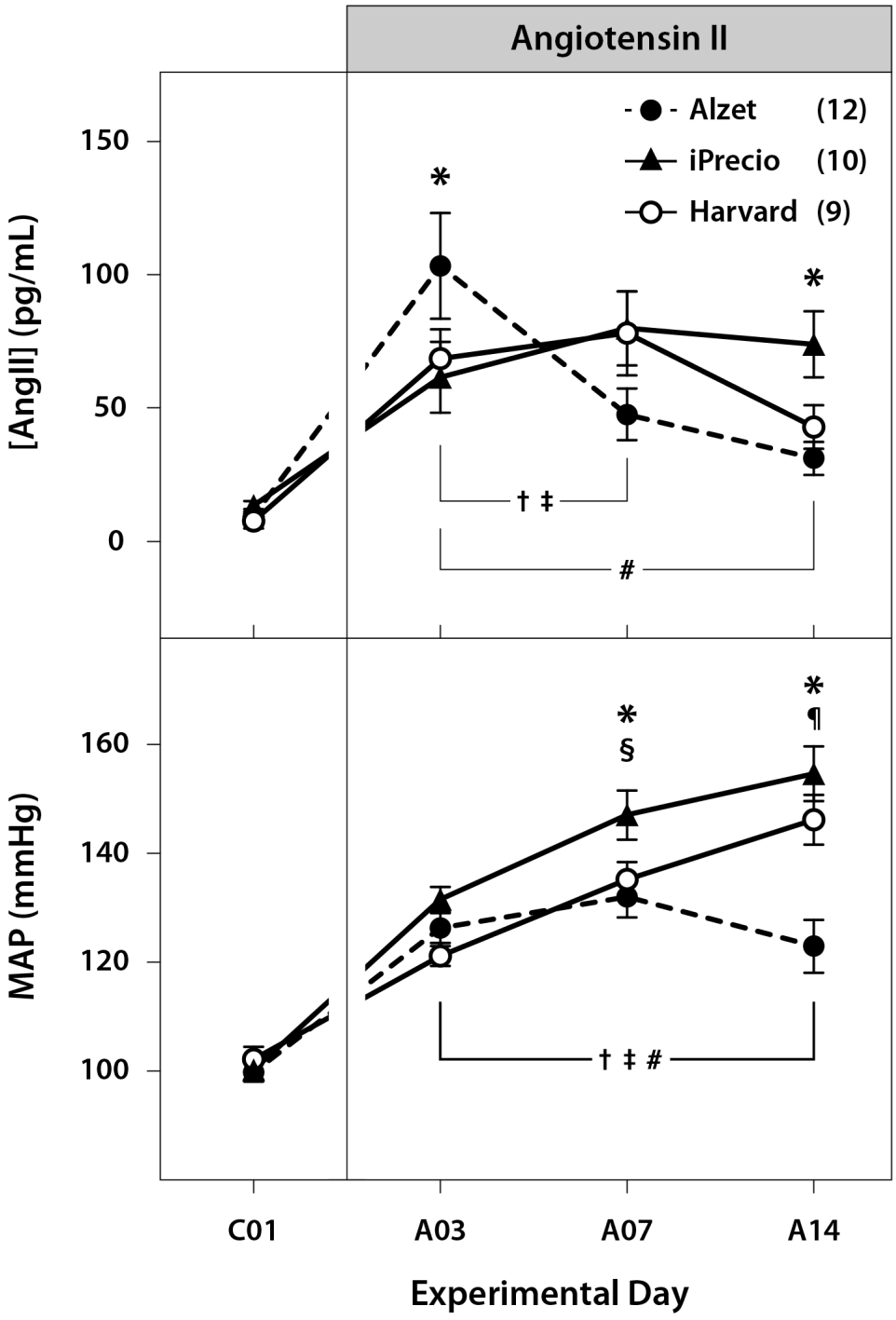

Angiotensin II
Alzet (15)
iPrecio (10)
Harvard (12)
MAP (mmHg)
HR (BPM)
Experimental Day
C01
C03
A00
A02
A04
A06
A08
A10
A12
A14

Figure 6.5. Changes in plasma AngII levels during AngII-salt hypertension generated using Alzet, Harvard or iPrecio pumps

Plasma AngII levels (top panel) measured on control day 1, and days 3, 7, and 14 of AngII in Harvard (white circle; n=9), Alzet (black circle; n=12), and iPrecio (black triangle; n=10) groups. 24hr MAP on corresponding days is also shown (bottom panel). (†), and (‡), denote significant within group change from control in Alzet, and iPrecio groups, respectively. (∗), (§), and (¶) denote significant differences between iPrecio and Alzet, iPrecio and Harvard, and Harvard and Alzet, respectively. Statistical significance was set at $p<0.05$ or less in post-hoc comparisons using appropriate correction for significance level. Error bars are S.E.M.

Figure 6.5


Angiotensin II
Alzet (12)
iPrecio (10)
Harvard (9)
[AngII] (pg/mL)
150
100
50
0
*
*
† ‡
#
MAP (mmHg)
160
140
120
100
*
§
*
¶
† ‡ #
C01
A03
A07
A14
Experimental Day