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Renal Humoral, Genetic and Genomic Mechanisms Underlying Spontaneous Hypertension

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RENAL HUMORAL, GENETIC AND GENOMIC MECHANISMS UNDERLYING SPONTANEOUS HYPERTENSION

DISSERTATION

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

By
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ABSTRACT OF DISSERTATION

RENAL HUMORAL, GENETIC AND GENOMIC MECHANISMS UNDERLYING HERTIABLE HYPERTENSION

In spite of significant progress in our knowledge of mechanisms that control blood pressure, our understanding of the pathogenesis of hypertension, its genetics, and population efforts to control blood pressure, hypertension remains the leading risk factor for mortality worldwide. It's estimated that 1 out of every 3 adults has hypertension. Hypertension is a major risk factor for cardiovascular disease and stroke, and is considered a primary or contributing cause of death to more than 2.4 million US deaths each year. Although spontaneous hypertension has been the subject of substantial research, many critical questions remain unanswered.

To investigate mechanisms underlying spontaneous hypertension, a unique rodent breeding approach was used to isolate nuclear and mitochondrial genes contributing to the disease. By diluting the nuclear genome of the Spontaneously Hypertensive Rat on a normotensive Brown Norway background while maintaining the SHR mitochondrial genome, I investigated both intrinsic and extrinsic mechanisms of the kidney and its relationship to hypertension. Chapter 2 documents the dominance of the hypertensive phenotype in our rodent colony, despite the dilution of the nuclear genome of the SHR. Chapter 3 presents data indicating that the renin-angiotensin system, particularly the location and abundance of the AT_1 receptor may play an important role in the manifestation of spontaneous hypertension. Chapter 4 presents that rats in our rodent colony exhibited normal pressure-natriuresis and kidney function; however, hypertensive rats had a reduced ability to sense orally ingested sodium chloride, thus necessitating chronic elevations of arterial pressure in order to maintain sodium balance. This chronic pressure-natriuresis relationship shifts the renal function curve to the right, thus sustaining elevated blood pressure. Chapter 5 presents data that genes important for oxidative phosphorylation may play a critical role in the development of hypertension. Both nuclear and mitochondrial oxidative

phosphorylation genes were downregulated in hypertensive rats compared with normotensive rats. Data presented in every chapter highlights the importance of the kidney in the pathogenesis of hypertension. Humoral, genetic and genomic mechanisms of the kidney appear to play a dominant role in the development and maintenance of the disease.

KEYWORDS: Hypertension, Renin-Angiotensin System, Mitochondria

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June 24, 2014
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RENAL HUMORAL, GENETIC AND GENOMIC MECHANISMS UNDERLYING SPONTANEOUS HYPERTENSION

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To Mom and Dad

“Research is what I’m doing when I don’t know what I’m doing”- Wernher von Braun

“There is nothing like looking, if you want to find something. You certainly usually find something, if you look, but it is not always quite the something you were after.”- J.R.R. Tolkien, *The Hobbit*

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CHAPTER 1

KIDNEY, LONG-TERM CONTROL OF ARTERIAL PRESSURE AND HYPERTENSION

Jason Andrieu Collett

Abbreviations used: BP, blood pressure; TPR, total peripheral resistance; CO, cardiac output; RAS, Renin-Angiotensin System, ECFV, extracellular fluid volume; AVP, arginine vasopressin; MD, macula densa' cTAL, cortical thick ascending limb; JGA, juxtaglomerular apparatus; GFR, glomerular filtration rate; Ang II, angiotensin II; RPP, renal perfusion pressure; RVLM, rostral ventrolateral medulla; NTS, nucleus of the solitary tract; RSNA, renal sympathetic nerve activity; RBF, renal blood flow; SNA, sympathetic nerve activity; Ang, angiotensin; ACE, angiotensin-converting enzyme; AT_1(r), angiotensin II type 1 receptor; AT_2(r), angiotensin II type 2 receptor; CVLM, caudal ventrolateral medulla; ENaC, epithelial sodium channel; SFO, subfornical organ; OVLT, organum vasculosum of the lamina terminalis; MnPO, median preoptic nucleus; PVN, paraventricular nucleus of the hypothalamus; ADH, antidiuretic hormone; CVO, circumventricular organs; SON, supraoptic nucleus of the hypothalamus; ICV, intraerebroentricular; AP, area postrema; VSMC, vascular smooth muscle cells; CNS, central nervous system; ARB, angiotensin II receptor blocker; SHR, Spontaneously Hypertensive Rat; IV, intravenously; QTL, quantitative trait loci; CNV, copy number variant; OXPHOS, oxidative phosphorylation system; ROS, reactive oxygen species; nDNA, nuclear DNA; mtDNA, mitochondrial DNA; rRNA, ribosomal RNA; tRNA, transfer RNA; SNPs, single nucleotide polymorphisms; ETC, electron transport chain; HNF-1α, hepatocyte nuclear factor-1alpha; PGC-1α, Peroxisome proliferator-activated receptor gamma coactivator 1 alpha; NRF, nuclear respiration factor(s); Tfam, transcription factor A mitochondria; NO, nitric oxide; SOD, superoxide dismutase; NT, normotensive; HT, hypertensive;

Keywords: Spontaneous hypertension, Renin-Angiotensin System, genomics, mitochondria, sympathetic nerve activity, kidney

1.1 Introduction

Hypertension and the chronic elevation of blood pressure (BP) constitute a primary and significant factor in the development of cardiovascular disease. Despite major gains in the long-term treatment of hypertension, cardiovascular disease remains the number one cause of death and disability in developed countries. For well over 50 years, scientists have uncovered several mechanisms believed to govern the long and short-term control of arterial pressure, with much debate on one major governing factor (Cowley 1992). Spontaneous hypertension, commonly referred to as essential or primary hypertension, is a rise in blood pressure of little known cause. Spontaneous hypertension is a multifactorial disease which accounts for 95% of all cases of hypertension in humans. There is a significant positive and continuous relationship between elevated BP and cardiovascular disease (stroke, heart failure, myocardial infarction), often resulting in renal disease and even death (Carretero and Oparil 2000). Data from the National Health and Nutrition Examination Survey NHANES have indicated that 50 million or more Americans have high BP warranting some form of treatment (Burt, Whelton *et al.* 1995). Worldwide prevalence estimates for hypertension may be as much as 1 billion individuals, and approximately 7.1 million deaths per year may be attributable to hypertension (Burt, Whelton *et al.* 1995).

Understanding the mechanisms for the long-term control of BP has important clinical significance. Because movement-to-movement and long-term stability of arterial pressure is necessary for survival, mammals have evolved many redundant controllers that participate in both of these functions. It is thought that the feedback control systems that provide rapid stabilization of arterial pressure are fundamentally different than those that determine the long-term level of arterial pressure. Blood pressure, simply defined, is the pressure exerted upon the walls of blood vessels. **Figure 1.1** represents the mathematic representation (Ohm's law) of blood pressure (BP=total peripheral resistance (TPR) X cardiac output (CO). Arterial pressure is the consequence of several factors that can influence cardiovascular function including both renal intrinsic mechanisms as well as extrinsic mechanisms, such as the Renin-Angiotensin System (RAS) and the sympathetic nervous system.

1.2 The Kidney, Long-term Control of Blood Pressure and Hypertension

It has been hypothesized that the “set point” for the long-term control of BP resides in the kidney (Guyton and Coleman 1968; Guyton, Coleman *et al.* 1972; GUYTON, COLEMAN *et al.* 1974). In this model, the set-point of the chronic renal function curve (**Figure 1.2**) establishes the steady state relationship between renal perfusion pressure and urinary excretion of sodium and water, which in turn affects blood volume and CO (**Figure 1.1**). This renal-body fluid-pressure control system exhibits “infinite feedback gain”, i.e., BP will stabilize only when intake and output of sodium and water become exactly equal, which occurs at one pressure level for any given renal function curve and salt intake level (Guyton 1990). The renal-BP set-point theory predicts that the kidney controls BP to maintain its own excretory function and that long-term regulation of blood volume and CO are paramount to the regulation of BP.

The mechanism for pressure natriuresis and diuresis, developed in a theoretical analysis by Guyton and Coleman, provides the kidney with the long-term ability to detect and control arterial pressure (Guyton and Coleman 1968; Guyton, Coleman *et al.* 1972; GUYTON, COLEMAN *et al.* 1974). These seminal works demonstrated that the mechanism of pressure natriuresis and diuresis is capable of determining the long-term level of arterial pressure. Because the gain for the pressure-natriuresis relationship overrides all other regulatory systems in blood pressure control, the ultimate determinant of BP must be renal handling of sodium (Adamczak, Zeier *et al.* 2002).

The kidney exerts its powerful diuresis- natriuresis mechanism by both sensing total extracellular fluid volume (ECFV) and responding to renal perfusion pressure (Cowley 1992) (**Figure 1.3**). The quantity of NaCl in the extracellular fluid compartment and the body's efficient osmoregulatory system determine total ECFV. The regulation of ECF osmolality by both the kidney and the brain, and the rate of fluid entering and exiting the body are important determinants in blood pressure regulation. Osmoreceptors in the brain detect subtle changes in ECF sodium concentration and modify thirst and arginine vasopressin (AVP) release for restoration, conservation or contraction of body water (Andersson, Dallman *et al.* 1969; Vereerstraeten and Toussaint 1969). The mechanisms controlling sodium excretion also contribute importantly to the process of osmoregulation. The delivery of filtered sodium to the specialized cells of the renal macula densa (MD) is a major effector sensor for sodium loss in the body. MD cells in the cortical thick ascending limb (cTAL) are the sensory element of the juxtaglomerular apparatus (JGA) and play an important role in the control of renal blood flow, glomerular filtration rate (GFR) and renin release by tubuloglomerular feedback (TGF), among other things (Sipos, Vargas *et al.* 2010). It has been postulated that the MD cells sense Na^+, Cl^-, or a combination of both, however; Sipos *et al.* (2010) demonstrated that the macula densa cells of the thick ascending limb are equipped with tubular-flow sensing mechanisms that contribute to MD cell function, renin release and TGF. With increased plasma sodium filtered at the glomerulus, and more sodium (or chloride or flow) to the

MD, renin secretion from JGA is reduced. Circulating levels of Angiotensin II (Ang II) and aldosterone are therefore reduced. Because Ang II and aldosterone are intimately involved in increased sodium reabsorption, and therefore determine the rate of sodium loss, it is evident that these and other neural and endocrine pathways influence osmoregulation via changes in sodium excretion.

This influential model developed by Guyton postulates that the relationship between renal sodium excretion and BP defines the BP homeostatic set-point. According to this model, any increase in sodium retention produces an initial blood volume expansion causing BP to increase via a rise in cardiac output, as illustrated in **Figure 1.3**. Eventually, tissue over-perfusion leads to an increase in peripheral resistance (whole-body autoregulation) that returns resting cardiac output towards normal. According to this widely held theory, a resetting of the pressure–natriuresis relationship inevitably leads to hypertension regardless of the cause of the resetting, whether it be humoral, neural, degenerative or genetic (Guyton and Coleman 1968).

Renal perfusion pressure (RPP), a direct indicator of arterial pressure, can exert a powerful influence on the rate of urine excretion as evidenced by the mechanisms mentioned above (Roman and Cowley 1985; Roman, Cowley *et al.* 1988). This response can be, and is, blunted by extrinsic factors such as neural and circulation hormones. However, experiments by Roman *et al.* showed when maintaining neural and circulating hormones at constant levels, small elevations

in RPP by abdominal aortic clamping resulted in large increases of urine output *(*Roman and Cowley 1985*)* . The reflex neural and endocrine systems that effect the pressure-natriuresis relationship, and hence regulation of body volumes must be taken into account with understanding long-term BP control. For instance, the diuretic and natriuretic responses observed from carotid-occlusion or epinephrine infusion-mediated rises in BP are severely blunted compared to those observed using aortic clamps (Kirchheim, Ehmke *et al.* 1987; Sipos, Vargas *et al.* 2010). These extrinsic mechanisms include, but are not limited to Ang II, catecholamines (and renal sympathetic nerve activity), prostaglandins, renal kinins, atrial natriuetic factor, vasopressin and endothelin (Cowley 1992).

1.2.1 Sympathetic Nervous System and Blood Pressure Control

There has been much controversy over the pressure-natriuresis theory of the main controller of BP, as it states that it is not determined by the generalized level of vascular resistance of the systemic circulation (TPR). Total peripheral resistance is defined simply as the sum of the resistance of all peripheral vasculature in systemic circulation. As shown in **Figure 1.1**, a major contributor to TRP is arteriolar smooth muscle contraction via the autonomic nervous system. The neural control of circulation operates via parasympathetic neurons that innervate the heart and three main classes of sympathetic efferents; barosensitive, thermosensitive and glucosensitive that innervate blood vessels, the heart, kidneys and adrenal medulla (Guyenet 2006). The barosensitive sympathetic efferents are under the control of arterial baroreceptors. This large

group of efferents has a dominant role in both short-term and potentially long-term BP regulation (Osborn 2005; Guyenet 2006). Their level of activity at rest is postulated to be an important parameter for long-term BP control. This background activity is set by a core network of neurons that reside in the rostral ventrolateral medulla (RVLM), the spinal cord, the hypothalamus and the nucleus of the solitary tract (NTS). Both variables of BP, TPR and CO are controlled by some extent by the autonomic nervous system. CO is dependent on three regulated variables: ventricular end-diastolic volume; myocardial contractility; and heart rate. End-diastolic volume is the volume reached by the ventricular chamber before contraction and is determined by venous pressure, which is related to blood volume and venous smooth muscle tone, both of which are under sympathetic control (Osborn 2005). Myocardial contractility and heart rate are regulated by both the sympathetic and parasympathetic divisions of the autonomic nervous system. The autonomic nervous system is able to incur rapid changes via CO and regional arteriolar resistance, which can be associated with substantial increases in BP. Significant evidence has implicated the autonomic nervous system and the brain in producing long-term changes in BP (Osborn, Holdaas *et al.* 1983; Osborn, Plato *et al.* 1997; Madden and Sved 2003; Osborn 2005; Littlejohn, Siel *et al.* 2013). Additionally, peripheral efferents have shown the same effect. For instance, increased renal sympathetic nerve activity (RSNA) results in increased renin secretion rate, increased renal tubular sodium reabsorption and retention, and decreased GFR and renal blood flow (RBF) with increased renal vascular resistance (DiBona and Kopp 1997; Osborn, Plato *et al.*

1997; DiBona 2000). Thus, increased RSNA represents an important candidate as a mediator of the normal and abnormal renal function and therefore, the development of hypertension. Many reports have implicated neurally-mediated mechanisms in the development of hypertension in several animal models (Judy, Watanabe *et al.* 1976; Osborn, Holdaas *et al.* 1983; Ichihara, Inscho *et al.* 1997). Furthermore, removal of renal nerves completely prevented or delayed the onset of hypertension in numerous animal models of hypertension including humans with drug-treatment-resistant, primary hypertension (DiBona and Esler 2010). Elevated SNA is present in most forms of human hypertension (Wyss 1993) and a causal relationship is suggested by the well-documented antihypertensive efficacy of sympatholytic drugs (for example, $alpha_1$- or beta-adrenergic receptor antagonists) (James, Oparil *et al.* 2014). The sympathetic efferents that innervate the kidneys are commonly presented as the only ones that are capable of influencing the 24-h average BP (Cowley 1992). If this theory is correct, a more complete knowledge of the neural pathways that selectively regulate renal SNA could be key to understanding the contribution of the CNS to hypertension. However, elevated RSNA is probably not the sole mechanism involved in neurogenic hypertension, and the method by which an increase in SNA raises the 24-h mean BP has not been established. The most commonly invoked mechanism is resetting of the renal BP–natriuresis relationship to higher levels of BP by either a rise in sympathetic tone to the kidney or by hormones whose production is partly controlled by the autonomic nervous system (for example, Ang II) (Guyenet 2006).

1.1.2 The Kidney and its Role in Hypertension

The theoretical basis for the role of the kidney in hypertension is well established and alterations in kidney function have been identified in the established stage of every form of hypertension yet studied (Cowley and Roman 1983). Hypertension, therefore, can develop only when something impairs the ability of the kidney to excrete sodium and water and shifts the relationship between sodium excretion and arterial pressure towards higher pressures. Renal humoral mechanisms and transcriptomics related to the development and maintenance of hypertension will be presented in this doctoral thesis, highlighting the importance of the kidney in blood pressure control and pathogenesis of the disease.

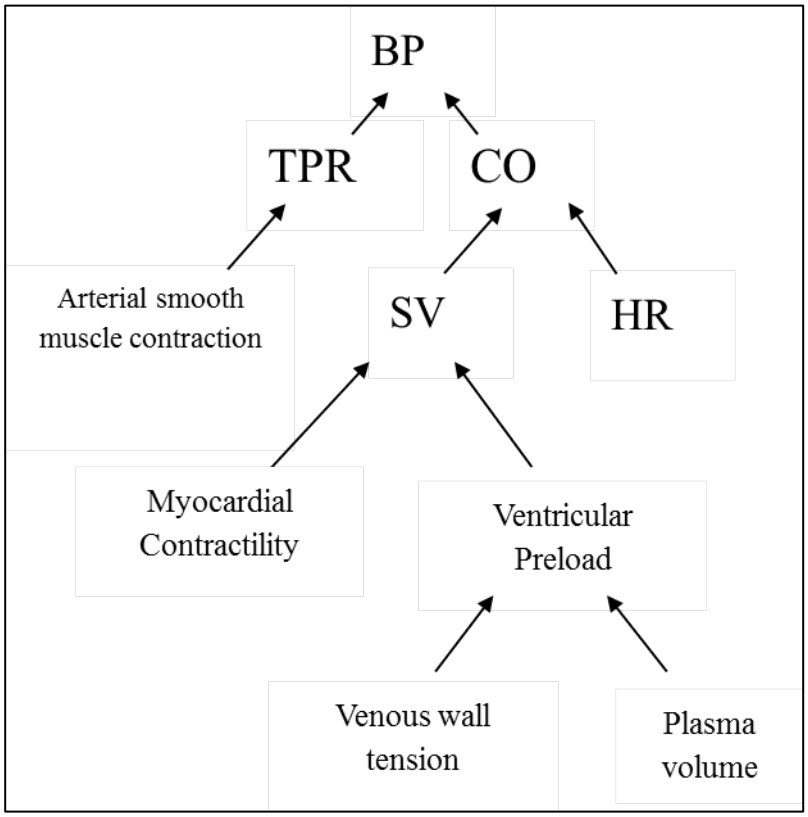

Figure 1.1: Comprehensive contributions to control of BP. Mathematically, BP control is equivalent to Ohm's law, where BP is the product of flow (CO) and resistance (TPR). Additionally, CO is the product of HR and SV. Several factors can directly influence these cardiovascular parameters, ultimately influencing to BP. BP=blood pressure, CO=cardiac output, TPR=total peripheral resistance, SV=stroke volume, HR=heart rate

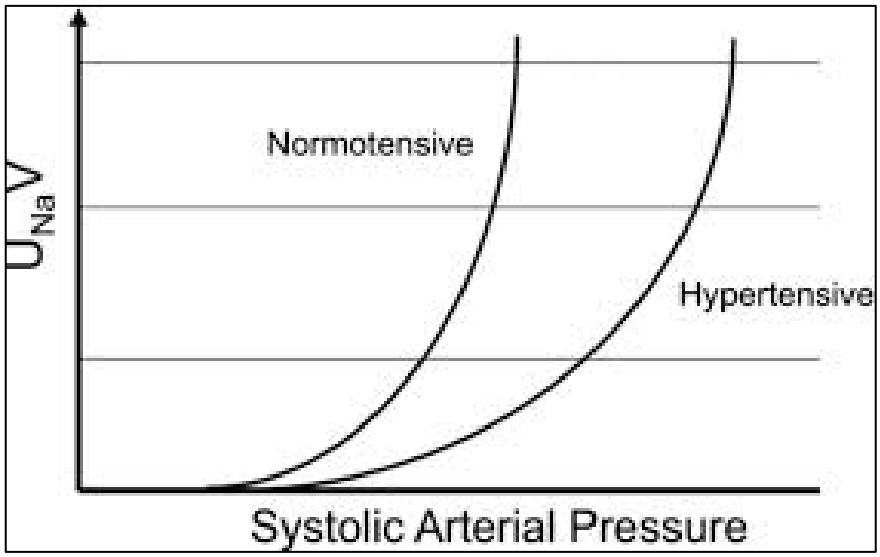

Figure 1.2: The Renal Function Cure. Systolic arterial pressure is a direct function of urine output ($U_{Na}V$), and hence kidney function. This relationship exhibits "infinite gain", i.e., BP will stabilize when intake and output of sodium are equal, which occurs at one pressure level for any given renal function curve and salt intake. It is hypothesized that the manifestation of hypertension occurs only when a rightward shift in the renal function curve occurs, requiring a higher pressure to excrete the same level of salt. A rise BP is "essential" to maintain sodium balance.

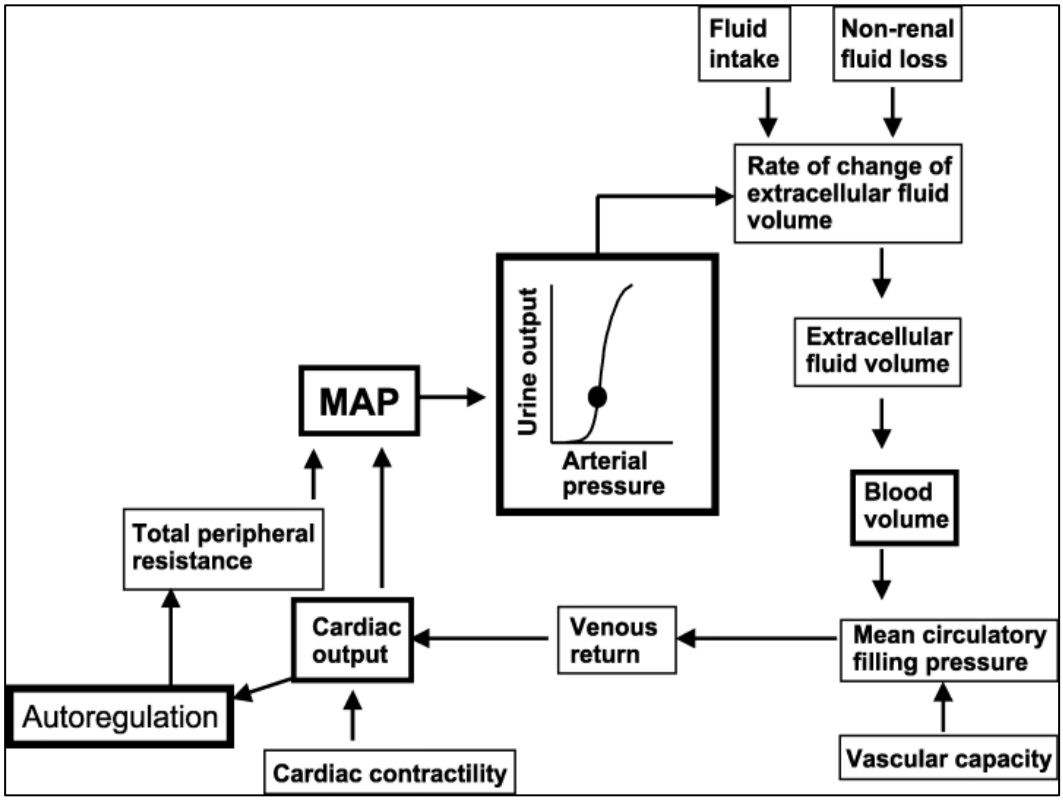

Figure 1.3: Basic renal-body fluid feedback mechanism for the long-term regulation of blood pressure and body fluid volumes. The kidney, it is hypothesized, contains the long-term homeostatic set-point.

1.3 Systemic Renin Angiotensin System (RAS): Overview of Components, Features and Actions

The RAS is an essential regulator of blood pressure, fluid balance, and cardiovascular function. The RAS produces a family of bioactive angiotensin (Ang) peptides with a variety of biological and neurobiological activities, with the most notable peptide being Ang II. The components of the RAS are present in peripheral tissues such as the vasculature, kidney, brain and heart, all of which locally produce Ang II (Peach 1977). The canonical systemic cascade begins with the release of the aspartyl protease renin from the JG cells of the kidneys leading to the conversion of circulating angiotensinogen from the liver to inactive angiotensin-I (Ang I) which, in turn, is converted to three different peptides; Ang II by the dipeptyl carboxypeptidase angiotensin-converting enzyme (ACE) located ubiquitously along vascular walls and secreted into the lungs and kidneys, Ang-(1-7) by neprilysin and Ang-(1-9) by the zinc metalloprotease ACE2 (**Figure 1.4**). Ang-(1-9) is metabolized by the metalloendopeptidase neprilysin to form Ang-(1-7), which is hydrolyzed by ACE to form Ang-(1-5). Ang II is degraded into the smaller, active peptides Ang III, Ang IV and Ang (1-7) by aminopeptidase A, aminopeptidase N and ACE2, respectively. Ang II is also converted to Ang-(1-4) by neprilysin (Lavoie and Sigmund 2003).

Ang II primarily binds two G-protein coupled receptors, Ang II type 1 (AT_1) and Ang II type 2 (AT_2) receptor, to produce both harmful and physiological effects on cardiovascular health (de Gasparo, Catt *et al.* 2000; Lavoie and Sigmund 2003).

Most of the classical actions of Ang II such as vasoconstriction, sodium retention, aldosterone release and sympathetic activation are facilitated by the AT_1 receptor. There are two AT_1 receptor isoforms in rodents, AT_{1A} and AT_{1B}, and only one in humans (de Gasparo, Catt *et al.* 2000). Activation of the AT_2 receptor generally has effects that are contrary to AT_1 receptor actions-mainly vasodilation, natriuresis and antiproliferation (Bottari, de Gasparo *et al.* 1993). In humans, the AT_1 receptor is located in blood vessels, heart, kidney, adrenal glands and liver while the AT_2 receptor is mainly expressed in fetal tissue, with relatively low amounts present in adult tissue (Matsubara 1998). Ang-(1-7), acting through the G-protein Mas receptor, has actions that are contrary to the effects of Ang II, such as vasodilation, natriuresis and antiproliferation(Santos, Simoes e Silva *et al.* 2003). Ang III (Ang 2-8) is thought to exert its actions at the AT_1 receptor and may be responsible for effects associated with Ang II, especially in the brain (Wright, Bechtholt *et al.* 1996). Ang IV binds the AT_1 and AT_2 receptors with low affinity and binds its own AT_4 receptor.

The RAS has generally been classically affiliated with blood pressure control and salt and water balance. Nevertheless, the RAS is continually emerging as a complex system involved in many physiological and pathophysiological conditions outside of the classic hemodynamic and osmoregulatory effects. The RAS contributes to oxidative damage (Sachse and Wolf 2007), insulin resistance (Henriksen 2007) and cell-growth (Pawlikowski, Melen-Mucha *et al.* 1999) and

has been implicated in the pathogenesis of the metabolic syndrome, diabetes, renal damage and hypertension.

1.3.1Cardiovascular Regulation

The body has several mechanisms to control blood pressure. These mechanisms, such as the actions of the RAS, can alter cardiac output, the blood volume in the bloodstream and vasoactivity of the arteries. The RAS has profound influence on cardiovascular and renal function due to its various actions throughout the body. Ang II is a potent vasoconstrictor, constricting arteries and veins to increase blood pressure. Ang II is also involved in fluid reabsorption.

Blood pressure regulation is a separate but not mutually exclusive process: both quick BP adjustments via baroreflexes and through long-term control involving the kidneys. The baroreflexes are mediated by the sympathetic and parasympathetic nervous systems. The baroreflex, which is a negative-feedback system, augments blood pressure in a matter of seconds to minutes. When blood pressure rises, the carotid and aortic sinuses distend resulting in activation of the baroreflex which adjusts sympathetic and parasympathetic activity accordingly. Afferent baroreceptor input suppresses sympathetic activity through a multisynaptic pathway which starts with excitatory fibers synapsing at the NTS. The NTS then sends excitatory (glutamatergic) projections to the caudal ventrolateral medulla (CVLM) and nucleus ambiguous (NA). The activated CVLM sends inhibitory (GABAergic) fibers to the RVLM, which then

decreases sympathetic nerve activity to the heart and vasculature. Activation of the NA will decrease heart rate by increasing parasympathetic nerve activity to the heart via the vagus nerve. The blood pressure is reset back to its original point. The RAS interacts with both these systems to help maintain and regulate cardiovascular function. Ang II modulates baroreflex function by decreasing the sensitivity of the gain of the baroreflex (Brooks 1995). Ang II acts at AT_1 receptors in the NTS to decrease the baroreflex sensitivity (Diz, Jessup *et al.* 2002), whereas Ang-(1-7) enhances the baroreflex sensitivity also by acting at the NTS (Ferrario, Chappell *et al.* 1997).

The kidney is involved in more long-term regulation of blood pressure and the RAS has both direct and indirect effects on the kidney. The RAS partners with the kidney to control blood pressure by maintaining salt and water balance. Ang II does this through its direct and indirect effects on water and salt reabsorption, which leads to an increase in blood pressure. Under physiological conditions, the RAS ensures that blood pressure does not drop too low and to maintain proper perfusion and blood flow to tissues and organs that need it the most under these circumstances. However, chronic activation of Ang II may lead to hypertension and damage the various organs and tissues of the body (Lavoie and Sigmund 2003). Ang II has a direct effect on the proximal tubules to increase salt reabsorption. Indirectly, Ang II causes the adrenal cortex to secrete the mineralcorticoid aldosterone which acts on the distal tubules and collecting ducts to cause conservation of sodium, water retention and potassium secretion. The

effects that Ang II has on renal blood flow and GFR vary depending on the situation. Ang II constricts the afferent and efferent arterioles and incites contraction of the mesangium, resulting in a decrease in renal blood flow, glomerular filtration rate and filtered sodium load (Brewster and Perazella 2004). Ang II decreases the surface area for glomerular filtration by constricting the mesangial cells. The effect on the efferent arteriole is greater in part due to its smaller basal diameter. Efferent arteriole constriction decreases renal blood flow and increases glomerular filtration by increasing glomerular capillary pressure. This increase in filtration fraction causes an increase in the peritubular vessel oncotic pressure and a decrease in the hydrostatic pressure, which also occurs in the renal interstitium. This pressure gradient allows sodium and water to move from the proximal tubule to the interstitium. Ang II decreases medullary blood flow and reduces renal interstitial pressure which decreases water and sodium excretion (Brewster and Perazella 2004). Ang II inhibits pressure-natriuresis and it is thought to be a TGF modulator because it augments TGF sensitivity (Vallon 2003). Furthermore, Ang II is involved in the proliferation of nephron cells (Zhang, Guo *et al.* 2004).

Ang II exerts influence on distinct ion channels along the nephron as well. Ang II enhances the sodium/hydrogen (Na^+/H^+) ion anti-porter activity on the luminal membrane of proximal tubules (Brewster and Perazella 2004) and in the early and late distal segments (Wang and Giebisch 1996). The action of Ang II to augment distal tubule sodium reabsorption complements the effect in proximal

tubules to create a greater efficiency of sodium retention. It stimulates the Na^+/HCO_3^- symporter and Na^+/K^+ pump in the proximal tubule basolateral membranes (Brewster and Perazella 2004). Ang II acts at the Na^+/K^+ pump in the medullary thick ascending limb and at the epithelial sodium channel (ENaC) of the cortical collecting tubules.

1.3.2 Neural Effects of RAS

Ang II plays a prominent role in a variety of neural mechanisms. It interacts with neurotransmitters and is itself considered a peptidergic neurotransmitter under certain conditions (McKinley, Albiston *et al.* 2003). It stimulates thirst centers in the brain, the desire for salt and is involved in vasopressin release. It facilitates norepinephrine release from sympathetic nerve endings and is known to decrease baroreflex function (DiBona 2001). Some of the central mechanisms are described in more detail below.

1.3.3 Thirst Sensation

The thirst mechanism is one way to increase fluid volume, leading to volume expansion and a subsequent increase in blood pressure. Stimulation of AT_1 receptors in the brain causes an increase in water intake, salt appetite and blood pressure. Intracerebroventricular (ICV) administration of Ang II increases the dipsogenic response and blood pressure and the subfornical organ (SFO), which lies outside the blood-brain barrier, is reported to be involved in this dipsogenic

mechanism (McKinley, Albiston *et al.* 2003). Dehydration causes a significant increase in Ang II binding sites in the SFO which could make circulating Ang II more efficient as a thirst stimulus (Saavedra 2005). Ablation studies showed that the thirst effect is facilitated by Ang II binding to receptors in the SFO and organum vasculosum of the lamina terminalis (OVLT) and Ang II injected into these forebrain areas leads to an increased blood pressure (Andersson, Eriksson *et al.* 1995) . The median preoptic nucleus (MnPO) has also been shown to be involved in thirst because Ang II micro-injected directly into the MnPO promotes drinking in rats (McKinley, Albiston *et al.* 2003). The MnPO is suggested to be an angiotensinergic synapse site that mediates thirst caused by Ang II as the SFO sends efferent projections to the MnPO. Projections are also sent to the paraventricular nucleus of the hypothalamus (PVN), which facilitates vasopressin production and secretion (McKinley, Albiston *et al.* 2003).

1.3.4 Ang II and Vasopressin Release

AVP, or anti-diuretic hormone (ADH), is a hormone secreted by the posterior pituitary gland that regulates water reabsorption in the collecting duct in kidneys as well as act as a potent vasoconstrictor. It increases water permeability of the distal convoluted and collecting tubules by inserting aquaporin channels into the apical membranes of the tubules/collecting duct epithelial cells. Circulating Ang II stimulates vasopressin release mainly by binding receptors in the SFO and possibly in the OVLT (Andersson, Eriksson *et al.* 1995). The vasopressin response to systemic Ang II is inhibited in rats following SFO ablation (Simpson,

Epstein *et al.* 1978). ICV injection of Ang II activates circumventricular organ (CVO) neurons, which directly or indirectly project to vasopressin-producing neurons in the paraventricular and supraoptic nuclei of the hypothalamus (SON) (McKinley, Albiston *et al.* 2003). Coadministration of Ang II and AT_1 receptor antagonist losartan decreases vasopressin secretion, further documenting Ang II's role in AVP release (76).

1.3.5 Ang II and the Sympathetic Nervous System

The RAS and SNA are two major extrinsic regulatory mechanisms for blood pressure and fluid homeostasis. Ang II activates the SNA at various levels and is known to be a tonic stimulus of RSNA (DiBona 2001). Both central and circulating Ang II may influence SNA. Ang II injected into the RVLM increases SNA and arterial pressure (Andersson, Eriksson *et al.* 1995). Microinjection of angiotensin II receptor blockers losartan and candesartan into the RVLM decreased the basal level of RSNA and heart rate, with candesartan also decreasing BP (DiBona and Jones 2001). Bilateral injection of Ang II receptor antagonists into the RVLM decreased BP similar to the decrease revealed subsequent to blockade of spinal sympathetic outflow (DiBona, Jones et al. 1996). ICV injection of losartan attenuated basal RSNA in proportion to the level of RAS activation (DiBona, Jones *et al.* 1996). Activation of the PVN by bicuculline leads to an increase in arterial pressure, heart rate and RSNA, which is primarily mediated by excitatory angiotensinergic projections from the PVN to

the RVLM. Furthermore, losartan injected into the ipsilateral RVLM decreases the renal sympathoexcitatory, pressor and tachycardic responses to bicuculline injected into the PVN (DiBona, Jones *et al.* 1996).

As a counter to Ang II's effects, Gironacci *et al.* (2004) showed that Ang-(1-7) decreased norepinephrine release from the hypothalamus through the Mas receptor, suggesting that Ang-(1–7) may decrease SNSA leading to an antihypertensive effect.

Ang II influences renal function through central mechanisms, mainly involving activation of the renal sympathetic nerves. RSNA and its arterial baroreflex control are mediated by changes in RAS activation. Circulating Ang II can act at areas such as the SFO and area postrema (AP) to modulate SNA (DiBona 2001). Circulating Ang II acting at the AP may increase peripheral SNA through a direct excitatory projection from the AP to RVLM (DiBona 2001). ICV injection of losartan in conscious rats did not affect basal levels of BP, but decreased basal RSNA suggesting that Ang II tonically influences basal levels of RSNA and its arterial baroreflex (DiBona, Jones *et al.* 1996). Brain Ang II activates AT_1 receptors to suppress the baroreflex control of RSNA and increases sodium reabsorption by stimulating renal nerve activity and catecholamine release within the kidney (van den Meiracker and Boomsma 2003).

The renal sympathetic nerves innervate all the major structural elements of the kidney including the afferent and efferent arterioles, JG cells, mesangium, vascular smooth muscle cells (VSMC) and tubules (proximal, distal and ascending limb of loop of Henle, collecting duct), with some areas being more innervated than others. There is also differential innervation of the three intrarenal effectors (JG cells, tubules, vasculature) such that some renal sympathetic nerve fibers only make contact with one of the three and some fibers make contact with multiple effectors (DiBona and Kopp 1997). Low frequency renal nerve stimulation stimulates renin release from the JG cells via a β-1 adrenoceptor effect, increases reabsorption of sodium and water in the tubules of the nephron, and causes renal vasoconstriction (Osborn, Holdaas *et al.* 1983). Activation of the renal nerves generally leads to a decrease in renal blood flow and GFR due to the constriction of the vasculature. Renal nerve activation is one of the more important mechanisms leading to sodium retention since it increases sodium and water retention throughout the entire nephron and can also induce sodium retention by increasing renin production and by mediating the resistance of the afferent arterioles. Increased renal nerve activity is thought to play a role in many pathologic conditions such as hypertension (Winternitz, Katholi *et al.* 1980; Hendel and Collister 2006), myocardial infarction (Souza, Mill *et al.* 2004) and renal failure. Renal denervation prevented hypertension in male SD rats with chronic renal failure (Campese, Kogosov *et al.* 1995) and in genetically hypertensive rats delays the development of hypertension (Liard 1977; Winternitz, Katholi *et al.* 1980; Diz, Nasjletti *et al.* 1982; Kline 1987). Recently,

renal denervation in humans has shown to be extremely effecting in attenuating elevated arterial pressure in treatment-resistance patients (DiBona and Esler 2010). These studies highlight the importance of renal nerves in the manifestation and maintenance of hypertension.

1.3.6 Local (Tissue) RAS

The idea of local or tissue RAS was first conceptualized upon the confirmation that there was a separate brain RAS (local Ang II production), independent of the circulating RAS. It is now well established that many tissues and organs contain their own local RAS, including the kidney, heart, vessels, adrenal gland, pancreas and brain (Lavoie and Sigmund 2003; Bader and Ganten 2008). The local systems seem to be regulated independently of the circulating RAS though interactions exist. The actions of the tissue RAS's may occur in the cell that generates the peptides (intracrine and autocrine), in neighboring cells (paracrine) or through the bloodstream to a specific organ or tissue (endocrine). In the brain, the AT_1 receptor is located in the SFO, OVLT, NTS, APS, PVN, CVLM, RVLM, dorsal medulla, the lateral parabrachial nucleus and the MnPO (Diz, Jessup *et al.* 2002; McKinley, Albiston *et al.* 2003; Veerasingham and Raizada 2003). These regions are involved in regulating cardiovascular function and/or body fluid and electrolyte balance. Though all components of the RAS are located in the brain, not a single cell contains all of the constituents of the RAS (Saavedra 2005; von Bohlen und Halbach and Albrecht 2006).

Regulation and specific mechanisms of the brain RAS in normal physiology and pathophysiology are not completely understood. Studies show that the brain RAS activates sympathetic outflow, inhibits the baroreflex, stimulates thirst, and contributes to neurogenic hypertension (Steckelings, Lebrun *et al.* 1992; Lavoie and Sigmund 2003; van den Meiracker and Boomsma 2003). In rats with chronic renal failure, the brain RAS is upregulated, resulting in sympathetic overactivity and hypertension (Nishimura, Takahashi *et al.* 2007). Transgenic mice with increased brain Ang II production developed hypertension and an increase in salt appetite and drinking volume (Morimoto, Cassell *et al.* 2001).

1.3.7 Brain RAS

It is reported that there are two RASs in the brain; an endogenous system located within the neurons inside the blood brain barrier and a system in the CVOs and cerebrovascular endothelial cells that respond to circulating Ang II of peripheral origin (Saavedra 2005). The two systems are interconnected and the brain responds to both. Angiotensinogen is produced mainly in astrocytes (McKinley, Albiston *et al.* 2003). Renin mRNA is present in the central nervous system (CNS), but concentrations are low (McKinley, Albiston *et al.* 2003). High concentrations of ACE are located in the AP, SFO, OVLT and median eminence. Aminopeptidase A and N are both located in the rodent brain. Ang I, Ang II, Ang III and Ang-(1-7) have been discovered in brain tissue, however Ang III and Ang-(1-7) are found in very low concentrations (McKinley, Albiston *et al.* 2003).

1.3.8 Kidney RAS

All of the RAS components are present within the kidney with compartmentalization in the tubules and interstitium as well as intracellular accumulation (Kobori, Nangaku *et al.* 2007). In fact, it is reported that there are two distinct intrarenal RASs; vascular (renal vessels, arterioles and glomeruli) and tubulointerstitial (proximal tubules and associated interstitium). Intrarenal Ang II, which can be formed independent of the circulation, may also be a result of circulating Ang II that is internalized into proximal tubule cells by the AT_1 receptor. In addition, Ang II may be formed from systemically delivered Ang I (Navar, Harrison-Bernard *et al.* 1999). Angiotensinogen is located mostly in the proximal tubule cells and can be secreted directly into the tubule lumen (Navar, Prieto *et al.* 2011). In general, renin from the JG cells is the primary source of both circulating and intrarenal renin. However, renin is also found in other areas of the kidney and is produced by the proximal tubule cells, as well as the distal nephron segments. Ang I and Ang II formation in the tubule lumen may occur subsequent to angiotensinogen secretion because some renin is filtered and/or secreted from juxtaglomerular or proximal tubule cells. ACE is located on the proximal tubule brush border and converts Ang I to Ang II (Kobori, Nangaku *et al.* 2007). ACE2 is present in renal endothelial and tubule cells and in glomerular podocyte and mesangial cells (Donoghue, Hsieh *et al.* 2000; Reich, Oudit *et al.* 2008). Previous work demonstrates that Ang I and Ang II are located with renin

in the juxtaglomerular apparatus cells and vascular smooth muscle cells of the afferent arteriole (Navar, Prieto *et al.* 2011). However, Ang I and Ang II are mainly located in the tubular and interstitial fluid compartments (Navar, Prieto *et al.* 2011). The AT_1 receptor is extensively dispersed throughout the kidney. It is located in the vascular smooth muscle cells of the afferent and efferent arterioles, glomeruli (mesangial cells and podocytes) and proximal tubule cells (brush border and basolateral membranes). Receptor subtypes are also found in the juxtaglomerular and macula densa cells, thick ascending limb, distal tubules, vasa recta, arcuate arteries and cortical collecting ducts (Carey and Siragy 2003; Kobori, Nangaku *et al.* 2007). The distribution of the two AT_1 receptor subtypes in rodents is different with the AT_{1A} subtype being the more prevalent of the two. The AT_{1A} is present in all nephron segments and AT_{1B} is more abundant than AT_{1A} in the glomerulus (66). The AT_2 receptor is present in the afferent arteriole, mesangium, proximal tubule, collecting ducts, parts of the renal vasculature, interstitial cells and in glomerular endothelial and epithelial cells (Bader and Ganten 2008).

The intrarenal RAS is regulated differently than the circulating RAS as shown by the fact that proximal tubule angiotensinogen, collecting duct renin and tubular AT_1 receptors are increased by intrarenal Ang II (Kobori, Nangaku *et al.* 2007). Intrarenal RAS activation may contribute to hypertension, renal injury, diabetes and metabolic syndrome. Ang II produced in the kidney directly induces

podocyte injury and apoptosis through AT_1 receptor activation independent of hemodynamic changes (Kobori, Nangaku *et al.* 2007). Ang II induces proliferation of glomerular endothelial cells, mesangial cells and fibroblasts (Rüster and Wolf 2006). Additionally, hyperglycemia, proteinuria and renal injury activate the intrarenal RAS (Rüster and Wolf 2006).

Inhibition of the RAS has proven to be renoprotective against diseases other than hypertension. The American Diabetes Association suggest that ACE inhibitors and angiotensin II receptor blockers (ARBs) be considered as first-line therapy to slow renal disease progression in hypertensive and type 2 diabetic patients (Vejakama, Thakkinstian *et al.* 2012). In hypertensive, type 2 diabetic patients, irbesartan delayed the increase in serum creatinine 24% more slowly than the placebo group and 21% slower than the group that received the calcium channel blocker amlodipine (Rossing, Schjoedt *et al.* 2005). This renoprotective effect was independent of the blood pressure lowering effect (Lewis, Hunsicker *et al.* 2001) as ARBs have been shown to benefit normotensive diabetic patients as well (Coyle, Gardner *et al.* 2004). ACE inhibitors decrease mesangial expansion, glomerulosclerosis, loss of glomeruli, tubular atrophy, interstitial fibrosis and proteinuria (Gansevoort, Sluiter *et al.* 1995; Ferder, Inserra *et al.* 2003). Proteinuria is also decreased in the presence of AT_1 receptor blockers (Mizuno, Sada *et al.* 2006). Losartan and enalapril reduced glomerular and tubulointerstitial fibrosis, tubular atrophy and increased the number mitochondria and improved mitochondrial function (Ferder, Inserra *et al.* 2003).

The RAS has been suggested to play a pathogenic role in the development of hypertension in Okamoto-Aoki Spontaneously Hypertensive Rat (SHR). These RAS implications are directly related in part to activation of sympathetic nerve activity. Numerous studies have demonstrated that administration of either angiotensin-converting ACE inhibitors or angiotensin receptor antagonists to immature SHRs prevents development of hypertension (Madeddu, Anania *et al.* 1995) . In the SHR, brain levels of Ang II and renin are elevated after the onset of adult hypertension (Phillips and Kimura 1988), hypothalamic angiotensinogen mRNA is more abundant (Yongue, Angulo *et al.* 1991), and SHRs exhibit elevated AT_1r mRNA in the brain and kidney compared to normotensive Wistar Kyoto (WKY) rats (Raizada, Sumners *et al.* 1993; Cheng, Wang *et al.* 1998). Subsequently, losartan, a potent AT_1r antagonist, normalizes blood pressure in SHRs when delivered intravenously (IV) or ICV (Medina, Cardona-Sanclemente *et al.* 1997). In addition to pharmacological blockade, Gyurko *et al.* (1993) demonstrated that inhibiting the brain RAS by antisense inhibition of angiotensinogen and AT_1 receptor genes lowers blood pressure in adult SHRs, potentially linking genetic contributions of the RAS to hypertension. Abnormalities in AT_1r function have been linked to several pathological conditions such as hypertension, cardiac and renal hypertrophy, and proliferation of vascular smooth muscle cells. Each of these pathologies alone or together may critically contribute to the onset and maintenance of hypertension. Taken

together, SHRs exhibit an exacerbated RAS, which in turn, plays a role in development of elevated arterial pressure and propagation of hypertension.

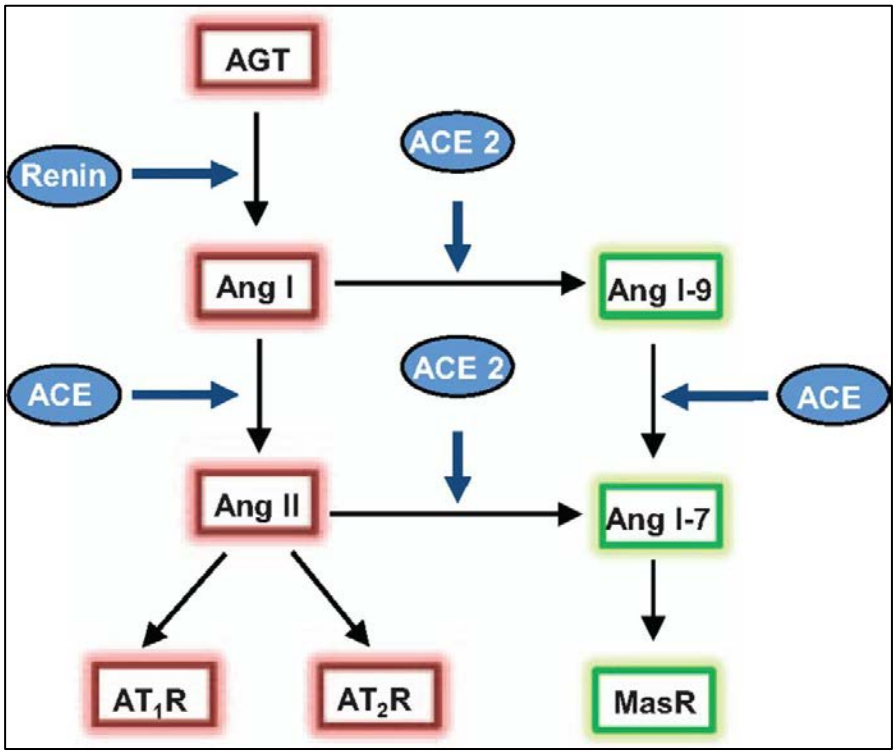

Figure 1.4: Overview of the renin-angiotensin system (RAS). AGT indicates angiotensinogen; Ang, angiotensin; ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; AT1R, angiotensin type 1 receptor; AT2R, angiotensin type 2 receptor; MasR, Mas (Conti, Cassis *et al*. 2012)

1.4 Nuclear Genetics and Genomics of Spontaneous Hypertension

Significant effort has been devoted to defining the pathogenesis of blood pressure variation. The difficulty in defining the causes of hypertension from physiological studies alone motivated the application of genetic approaches to hypertension. Identification of genes underlying blood pressure variation has the capacity to define primary physiologic mechanisms underlying this trait, thereby clarifying disease pathogenesis, identifying pathways and targets for improved therapeutic intervention, providing opportunity for preclinical diagnosis, and allowing treatment tailored to individual patients. There is substantial evidence for genetic influence on blood pressure. Twin studies document greater concordance of blood pressures of monozygotic than dizygotic twins (Feinleib, Garrison *et al.* 1977) and population studies demonstrate greater similarity of blood pressure within families than between families (Longini, Higgins *et al.* 1984). This familial aggregation of blood pressure is not simply attributable to shared environmental effects since adoption studies show greater concordance of blood pressure among biological siblings than adoptive siblings living in the same household (Biron, Mongeau *et al.* 1976) (Rice, Vogler *et al.* 1989). Single genes can impart large effects on blood pressure as demonstrated by rare Mendelian forms of high and low blood pressure (Lifton 1996). Although these Mendelian traits have quantitatively large effects in affected individuals, they are quite rare and likely account for a very small fraction of the variation in blood pressure in the human population (Lifton, Gharavi *et al.* 2001).

The polygenic nature of essential hypertension and its dependence on environmental factors complicate the identification of causative genetic factors (Garrett, Dene *et al.* 1998). Significant progress has been made to map the genes involved in blood pressure regulation. Genome scans have been carried out in rats, mice and humans and have revealed several potential chromosome regions that may contain genes involved in the pathogenesis of spontaneous hypertension. Molecular genetic analysis proves that BP regulation is polygenic and there is good evidence for several BP related quantitative trail loci (QTL) on nearly every rat chromosome (Deng, Dene *et al.* 1994; Schork, Krieger *et al.* 1995; Hopkins and Hunt 2003; Laulederkind, Hayman *et al.* 2013). Linkage studies in populations derived from the SHR indicate potential BP QTL. Recently, Atanur *et al.* (2010) sequenced the SHR/Olalpcv genome, identifying 3.6 million high quality SNPs between the SHR/Olalpcv and BN reference genome. Genomic regions containing genes that have been previously mapped as *cis*-regulated expression (QTL) contained several SNPs, short indels, and larger deletions which suggests potential functional effects on gene expression. Atanur *et al.* found 688 genes that overlap with regions showing copy number variation (CNV) that represent genes for immunological, neurological or mechanical function. The high statistical significance of these functional variants, coupled with the known metabolic, cardiovascular and neurobehaviorial phenotypes described in the SHR (Okamoto and Aoki 1963), suggests alteration of these gene classes may directly relate to the phenotypes manifested by SHR-derived strains (Rapp 2000). This sequence information supports previous

studies of experimental crosses and congenic strains that led to the identification of CNV in rat *Cd36* gene as a cause of CD36 deficiency, insulin resistance, dyslipidemia, and hypertension in the SHR strain (Aitman, Glazier *et al.* 1999; Pravenec, Landa *et al.* 2001; Pravenec, Churchill *et al.* 2008). This highlights the potential functional significance of other CNVs detected in the SHR genome.

1.4.1 Renin-Angiotensin System Genes and Hypertension

With the abundance of knowledge on the role of both circulating and tissue RAS in BP control, it is hypothesized that genetic variability in one or more of the RAS components could account for the pathogenesis of hypertension. A large number of patients with hypertension and cardiovascular disease are on ACE inhibitors or AT_1r antagonists. Recognizing a potential genetic influence on protein expression would be vital to the treatment and prevention of essential hypertension. The mouse renin gene (*Ren*) was the first gene of the RAS to be cloned (Burnham, Hawelu-Johnson *et al.* 1987). Several investigators have established transgenic models expressing the mouse Ren-2 gene (*mRen-2*). High expression of the transgene has been shown to cause severe hypertension, associated with increased renin activity and local Ang II formation, while suppressing plasma RAS (Rothermund and Paul 1998). Restriction mapping and linkage analysis have been used to study the association of renin and hypertension, but whether the renin locus is associated with hypertension is still under debate. Cloning of the human genes coding for angiotensinogen (*Agt)*

(Jeunemaitre, Soubrier *et al.* 1992), angiotensin-converting enzyme (*ACE*) (Rigat, Hubert *et al.* 1990) and the AT_1 receptor (*Agtr1a/b*) (Bonnardeaux, Davies *et al.* 1994) has led to the discovery of several polymorphisms that may play a role as risk factors for hypertension. Higher levels of angiotensinogen have been demonstrated to be associated with higher levels of Ang II (Walker, Whelton *et al.* 1979). In addition, transgenic mice with overexpression of a rat angiotensinogen gene develop hypertension, and knockout mice with a disrupted gene and absent production of angiotensinogen develop low BP (Jeunemaitre, Soubrier *et al.* 1992).

In 1990, Rigat et al (1990) produced evidence for an insertion/deletion (I/D) polymorphism (287bp insert/deletion) in intron 16 of the *ACE* gene was strongly associated with circulating ACE concentrations. In individuals that were homozygous for the D allele (D/D), plasma ACE concentrations were twice those of persons homozygous for I allele (I/I). Zhu *et al.* (Zhu, McKenzie *et al.* 2000) later reported that the functional mutation was not the I/D variant, but a variant downstream that exists in linkage disequilibrium that is responsible for changes in plasma ACE levels. Nevertheless, there is significant evidence that the *ACE* gene controls plasma ACE concentrations and therefore could have a causal relationship with hypertension.

For the *Agtr1a/b* gene, a SNP has been described in which either adenine (A) or a cytosine (C) base (A/C tranversion) in position 1166 in the 3' untranslated

region of the gene (Joe and Garrett 2005). The physiological importance of this polymorphism is uncertain because of its location in the untranslated region, however several studies have shown that it was associated with hypertension (Joe and Garrett 2005), left ventricular hypertrophy (Takami, Katsuya *et al.* 1998), coronary artery disease and myocardial infarction (Bonnardeaux, Davies *et al.* 1994), and progression of diabetic nephropathy (Tomino, Makita *et al.* 1999). The silent A1166C SNP has been associated with severe forms of hypertension, and in particular in resistant hypertensive patients taking two or more antihypertensive drugs (Bonnardeaux, Davies *et al.* 1994; Kainulainen, Perola *et al.* 1999). However, the relationship between this SNP and BP shows great diversity within human populations (Baudin 2002). The most likely scenario is that the polymorphic transversion in not functional, but may occur in linkage disequilibrium with an unidentified functional variant (Bonnardeaux, Davies *et al.* 1994).

Although significant progress has been made, the exact form of the underlying genetic mechanisms remains unanswered. Multiple approaches will be needed to achieve this assignment; however, the sequencing of the SHR nuclear genome by Atanur *et al.* (2010) will provide a great source for complete functional elucidation between hypertensive and normotensive rats of SHR origin at the molecular level. This provides substantial insight to the genetic components of human essential hypertension. It is the hope that by identifying

nuclear genes contributing to hypertension, that we can switch from treatment to prevention of hypertension and cardiovascular disease.

1.5 Mitochondrial Genomics/Dynamics and Hypertension

Eukaryotic cells contain a number of organelles with specialized functions like the mitochondria. Mitochondria are broadly known as double-membrane-bounded organelles, which perform a number of indispensable functions for the life of most eukaryotic cells. Their main function is the production of energy in the form of ATP via the citric acid cycle and the oxidative phosphorylation system (OXPHOS), but they are also involved in the biosynthesis of many metabolites like pyrimidines, amino acids or cellular iron sulphur cluster proteins (Bereiter-Hahn 1990; Attardi, Yoneda *et al.* 1995; Lill, Diekert *et al.* 1999). Mitochondria also control the ability of the cell to generate and detoxify reactive oxygen species (ROS) (Nicholls, Vesce *et al.* 2003). Beside their role as ATP generators, mitochondria have also the ability to remove Ca^{2+} ions out of the cytosol and accumulate them in their matrix (Deluca and Engstrom 1961). The release of mitochondrial pro-apoptotic factors like cytochrome *c* into the cytoplasm can induce a signaling cascade, which plays a prominent role in apoptotic cell death (Hengartner 2000).

The citric acid cycle, which takes place in the mitochondrial matrix, is a central metabolic pathway involved in the catabolic oxidation of substrates (Krebs 1970). Acetyl-CoA, which is generated by the decomposition of nutrients such as glucose, transfers two carbon acetyl groups to oxaloacetate to generate citrate. The citrate is metabolized through a series of chemical transformations and releases two carboxyl groups as CO_2. The energy-rich electrons generated by

the cycle are transferred to NAD^+/$NADP^+$ and FAD^+ to form NADH/NADPH and FADH2. Several substances like NADH, ATP and Ca^{2+} regulate the citric acid cycle (Krebs 1970).

The electrons generated via the citric acid cycle are transferred to the multisubunit enzyme complexes of the respiratory chain. The OXPHOS is embedded in the inner mitochondrial membrane. Functionally, it is composed of the five enzyme complexes: NADH dehydrogenase (Complex I), succinate dehydrogenase (Complex II), cytochrome *bc1* complex (Complex III), cytochrome c oxidase (Complex IV, COX) and ATP synthase (Complex V) as well as the two electron carriers coenzyme Q and cytochrome *c (Hatefi 1985).* The electrons are transferred to oxygen to generate water at Complex IV. The transport of electrons via the respiratory chain generates a proton gradient across the membrane which is used to synthesize ATP by Complex V (Hatefi 1985).

Molecular genetics research and hypertension has been primarily focused on the nuclear genes (nDNA), despite the fact that mitochondria are present in multiple copies in each cell, have their own genome, and mitochondrial dysfunction has recently been implicated in a wide variety of genetic disorders (Aitman, Glazier *et al.* 1999; Taylor and Turnbull 2005). Mitochondrial DNA is transmitted through the oocyte's cytoplasm at fertilization, hence, are nearly strictly maternally inherited. Alterations in mitochondrial function are observed in conjunction with

increase in age and the development of hypertension in rodents and humans (Hofmann, Jaksch *et al.* 1997; Shin, Tanaka *et al.* 2000; Schwartz, Duka *et al.* 2004; Chan, Wu *et al.* 2009; Kumarasamy, Gopalakrishnan *et al.* 2010). Mitochondria are the principal generators of cellular ATP by OXPHOS. Mammalian mtDNA encodes 37 genes, including 13 polypeptide genes, all of which encode essential components of OXPHOS. It also encodes the 12S and 16S ribosomal RNA (rRNA) genes and the 22 transfer RNA (tRNA) genes required for mitochondrial protein synthesis (**Figure 1.5**). The rat mtDNA genome varies between 16,307 and 16,315 bp in size. The size variation is due to insertions/deletions in the replication origin, D-loop, 16S rRNA, and NADH dehydrogenase subunit 2 (Schlick, Jensen-Seaman *et al.* 2006). Each mitochondrion contains one to ten copies of its genome and each cell encompasses hundreds to thousands copies of mitochondria. The faster rate of mtDNA replication, however, is not without consequences, particularly, in an environment that is high in reactive oxygen species (ROS). The rare error rates of DNA replication and editing enzymes increase in the presence of oxidative modified nucleotides and enzymes. Hence, mtDNA has ~16 times higher mutation accumulation rate than the nDNA (Richter, Park *et al.* 1988). Mutations could initiate a vicious cycle of impaired mitochondrial functions, increased ROS, higher error rates of DNA polymerases and editing enzymes and further accumulation of mutated mtDNA. Given the presence of thousands of copies of mtDNA in each cell, mutations generate an admixture of wild type and mutant mtDNA, which is referred to as heteroplasmy, as opposed to homoplasmy, when

all copies of mtDNA are identical. Heteroplasmy in mtDNA in somatic cells, as the mitochondria replicates, increases with age. Accordingly mitochondrial mutations and dysfunctions have been implicated in various age-dependent phenotypes including cellular senescence and metabolic disorders.

Base substitutions in mtDNA protein synthesis genes can result in multisystem disorders with a wide range of symptoms (Shin, Tanaka *et al.* 2000; Schwartz, Duka *et al.* 2004; Wallace 2005). Recently, Schlick *et al.* (2006) sequenced the mtDNA of 10 commonly used inbred rat strains. Analysis showed 11 single nucleotide polymorphisms (SNPs) within the tRNA genes, 6 in the 12S rRNA, and 12 in the 16S rRNA including 3 indels. Also, 14 SNPs and 2 indels were found in the D-loops. Pravenec *et al.* (Pravenec, Churchill *et al.* 2008) investigated whether naturally occurring variation in the mitochondrial genome of SHRs and BN rats could be linked to risk factors for type 2 diabetes. Sequence analysis revealed distinct differences between the 2 strains, revealing polymorphisms of functional significance in seven of 13 mRNA genes, five of 22 tRNA genes and both rRNA genes. The mRNA variants were predicted to cause amino acid substitutions in mitochondrial cytochrome oxidase subunit 1, ATP synthase subunit 6, ATP synthase subunit 8, cytochrome b, NADH dehydrogenase subunit 2, and NADH dehyrogenase 6. Some of these amino acid substitutions are located within 0-3 residues of mutation sites known to be associated with human diseases (Pravenec, Churchill *et al.* 2008). Variations in the tRNA genes were also shown to be closely located to human mutation sites

associated with mitochondrial encephalopathy (Elo, Yadavalli *et al.* 2012). Recently, reports of mitochondrial tRNA mutations were observed in a Chinese population with essential hypertension (Li, Liu *et al.* 2009; Liu, Li *et al.* 2009). Additionally, Wilson *et al.* (2004) describe a causal relationship between a T4921C transition SNP, which lies in the mitochondrial $tRNA^{Ile}$ gene and hypertension. The genetic association of mtDNA variants and tRNA mutations to type 2 diabetes and hypertension directly implicated mitochondrial defects to the etiology of cardiovascular disease and metabolic syndrome (Wallace 2005). Taken together, there is significant evidence of mtDNA mutations and altered mitochondrial genetic expression that may play a significant role in cardiovascular disease phenotypes.

The viability of individual electron transport chain (ETC) subunits and the potential for disease has been evaluated. Altered function of complex I has been linked to cardiovascular disease (Antonicka, Ogilvie *et al.* 2003; Lopez-Campistrous, Hao *et al.* 2008). Lopez-Campistrous *et al.* revealed defects in complex I in the brainstem of SHR, resulting in increased reactive oxygen species production, decreased ATP synthesis and impaired respiration in hypertension (Lopez-Campistrous, Hao *et al.* 2008). Altered function of complex III, a major site of superoxide formation and ROS production, may play an important role in renal mitochondrial ETC dysfunction and cardiovascular disease. Similar to these findings, Das *et al.* (1990) reported that the regulation of ATP synthase is abnormal in SHR cardiac cells, as demonstrated by the

inability to respond to acute increases in energy demand compared to cells from normotensive rats . Taken together, the altered function of ETC subunits derived from decreased kidney-specific mt-gene expression may have several implications to the development of hypertension.

To date, however, it is clear that phenotypes that may arise from mtDNA mutations are not uniform, and can be influenced by both environmental factors as well as the nuclear genome. Only 13 of the proteins needed to assemble the electron transport chain are coded by the mitochondrial genome. The remaining proteins are coded by the nuclear genome and transferred to the mitochondria. This interaction may have profound effects on disease phenotypes. For example, mutations in the nDNA encoded hepatocyte nuclear factor-1 alpha (HNF-1α) are associated with post-pubertal diabetes, dislipidemia and hypertension (Wallace 1999). HNF-1 is also important in regulating nDNA-encoded mitochondrial gene expression (Wang, Maechler *et al.* 2000). Patients with type II diabetes show a downregulation in the expression of nDNA encoded mitochondrial genes associated with mitochondrial biogenesis and transcription (Patti, Butte *et al.* 2003). In the SHR, the gene encoding mitochondrial coupling factor 6 was evaluated. Osanai *et al.* (2001) reported that the genetic expression and plasma concentration of coupling factor 6 was significantly higher in SHRs compared with normotensive controls. Functional analysis suggests that it acts as a potent endogenous vasoconstrictor as a circulating hormone, and thus a potential factor in the propagation of hypertension. Circulating coupling factor 6

is also elevated in human hypertensive patients compared with normotensive subjects (Osanai, Sasaki *et al.* 2003). Taken together, these findings suggest that mitochondrial dysfunction of both mitochondrial and nuclear origin may play a significant role in the pathophysiology of arterial blood pressure regulation and development of hypertension (**Figure 1.7**).

The nuclear pathway that regulates mitochondrial transcription and biogenesis is well established (Ventura-Clapier, Garnier *et al.* 2008). Peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*PGC-1α*) plays a central role in regulating mitochondrial content and function within cells, because of its ability to co-activate and augment several promoters of nuclear-encoded mitochondrial genes, as well as regulating mitochondrial transcription via the *NRF-Tfam* (Nuclear respiration factor-Transcription factor A mitochondria) pathway (**Figure 1.6**) (Wu, Puigserver *et al.* 1999). *PGC-1α* regulates NRF-dependent transcription, increases expression of both mitochondrial and nuclear encoded genes of oxidative phosphorylation and induces mitochondrial biogenesis (Scarpulla 2002). Decreased *PGC-1α* expression has been shown to decrease expression of NRF-dependent genes, leading to metabolic disturbances characteristic of type II diabetes (Patti, Butte *et al.* 2003). This linear coordinated reduction of nuclear transcription factors, that ultimately leads to decreased mitochondrial gene transcription appears to play a role in metabolic syndrome, however, it's role in the development of hypertension is largely unknown. Additionally, it has been shown that metabolic functions are controlled by *PGC-*

1α in a tissue specific manner in brown fat, muscle and liver (Puigserver 2005), however kidney regulation of *PGC-1α* and its effectors is unknown.

Mitochondrial dysfunction contributes to the pathophysiology of hypertension, renal disease, as well as other cardiovascular diseases. Significant evidence indicates that the RAS plays a role in developing mitochondriopathy. AngII stimulates mitochondrial oxidant release both directly and indirectly, leading to energy metabolism depression, and potentially modification of gene expression, ultimately contributing to cardiovascular-related pathologies (de Cavanagh, Inserra *et al.* 2007). Dikalova et. al showed that mitochondrial superoxide was directly related to the development of hypertension, and that targeting of mitochondrial superoxide scavenging significantly attenuates the development of the disease (Dikalova, Bikineyeva *et al.* 2010). Additionally, de Cavanagh *et al.* demonstrated that oxidant stress is associated with mitochondrial dysfunction in the SHR, and that blocking AngII with losartan provided mitochondrial-antioxidant actions that reversed mitochondrial dysfunction and renal impairment (de Cavanagh, Toblli *et al.* 2006). AngII can promote mitochondrial oxidative stress indirectly by stimulating both nitric oxide (NO) production and NAD(P)H oxidase-derived superoxide, which in turn enhances peroxynitrite formation (Pueyo, Arnal *et al.* 1998). Substantial evidence has shown that NAD(P)H oxidase derived reactive oxygen species are important mediators of AngII signaling (**Figure 1.7**) (de Cavanagh, Inserra *et al.* 2007). ROS has been shown to play an important role in the physiological and pathophysiological process in the

central nervous system, and has been linked to activation of sympathetic nerve activity (Gao, Wang *et al.* 2004) . SHRs exhibit increased NAD(P)H driven O_2^- generation in resistance and aortic vessels (Rodriguez-Iturbe, Zhan *et al.* 2003; Tanito, Nakamura *et al.* 2004), associated with NAD(P)H oxidase subunit overexpression and enhanced oxidase activity (Shokoji, Nishiyama *et al.* 2003; Kishi, Hirooka *et al.* 2004). Several polymorphisms in the promoter region of the $p22^{phox}$ gene have been identified in the SHR (Zalba, San Jose *et al.* 2001), and an association between $p22^{phox}$ gene polymorphism and NAD(P)H oxidase mediated O2- production in the vascular wall of humans with hypertension also has been established (Moreno, San Jose *et al.* 2003). Additionally, increased expression of $p47^{phox}$ has been reported in the renal vasculature, macula densa and distal nephron of young SHRs, suggesting renal NAD(P)H oxidase upregulation in kidney structures precedes development of hypertension, that again may be related to altered RAS activity (Kishi, Hirooka *et al.* 2004). The role played by angiotensin II in developing mitochondriopathy has been advanced recently by Benigni *et al.* (Benigni, Corna *et al.* 2009). Deletion of the *Agtr-1a* gene resulted in the reduced age-related cardio-renal complications, improved mitochondrial biogenesis, and increased longevity in mice. Treatment with antioxidants, superoxide dismutase (SOD) mimetics, and AT_1r blockers decrease vascular O_2- production and attenuate development of hypertension in the SHR and stroke-prone SHR (Park, Touyz *et al.* 2002; Rodriguez-Iturbe, Zhan *et al.* 2003; Shokoji, Nishiyama *et al.* 2003). Taken together, these findings suggest that oxidative stress plays an important role in genetic hypertension and

involves NAD(P)H oxidase activity that is regulated, in part, by the AT_1 receptor and possibly AT_1r genetic expression.

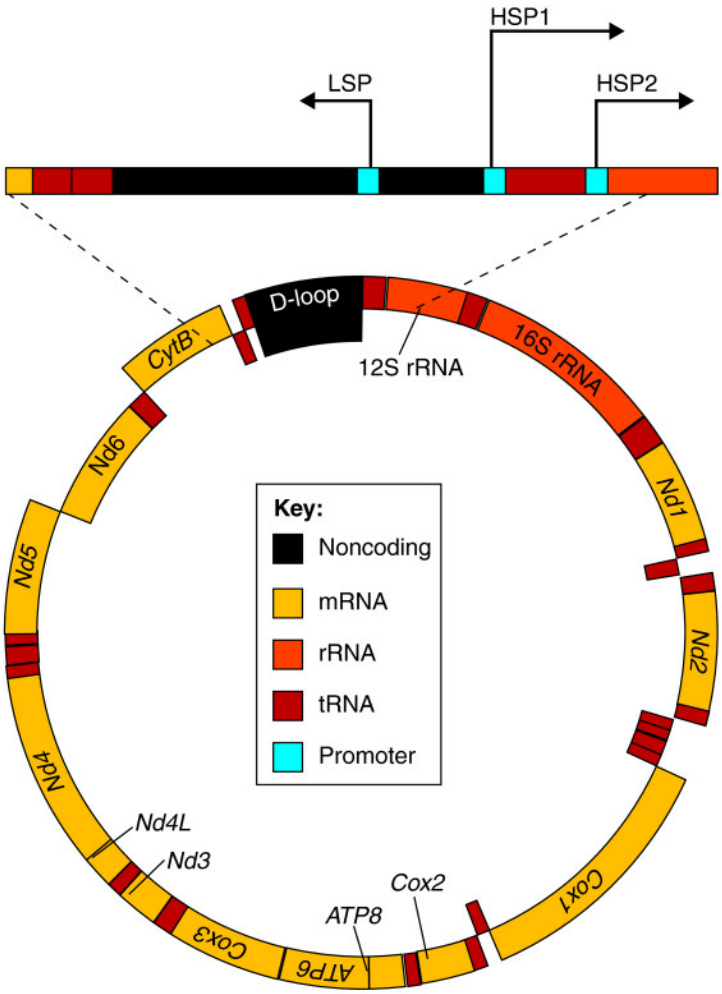

Figure 1.5: Mamalian mitochondrial genome. Mammalian mtDNA encodes 37 genes, including 13 polypeptide genes, all of which encode essential components of OXPHOS. It also encodes the 12S and 16S rRNA genes and the 22 tRNA genes required for mitochondrial protein synthesis (Leigh-Brown, Enriquez et al. 2010).

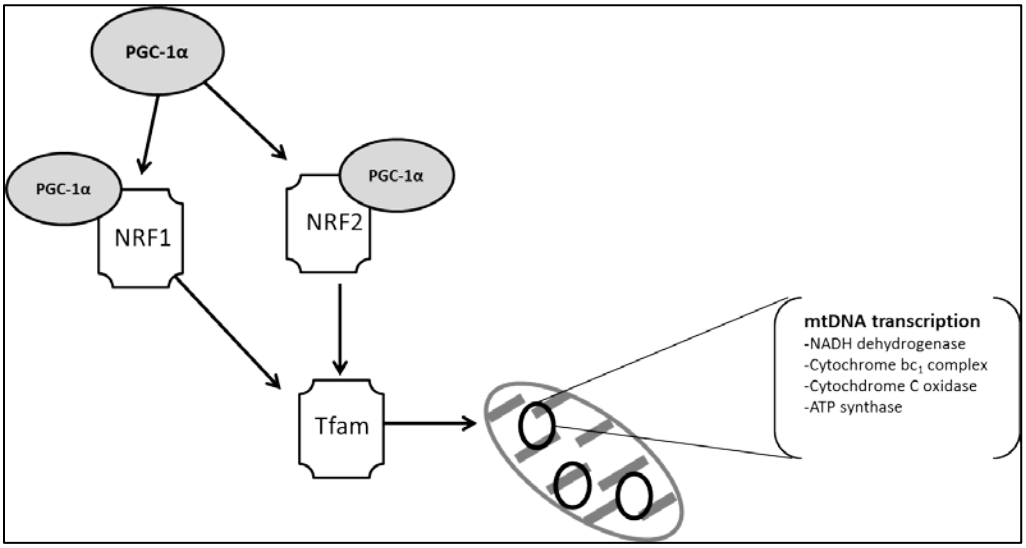

Figure 1.6: Nuclear-mitochondrial pathway driving mtDNA transcription and biogenesis. This well known pathway may be regulated in a tissue-specific manner, depending on the energetic needs of that tissue.

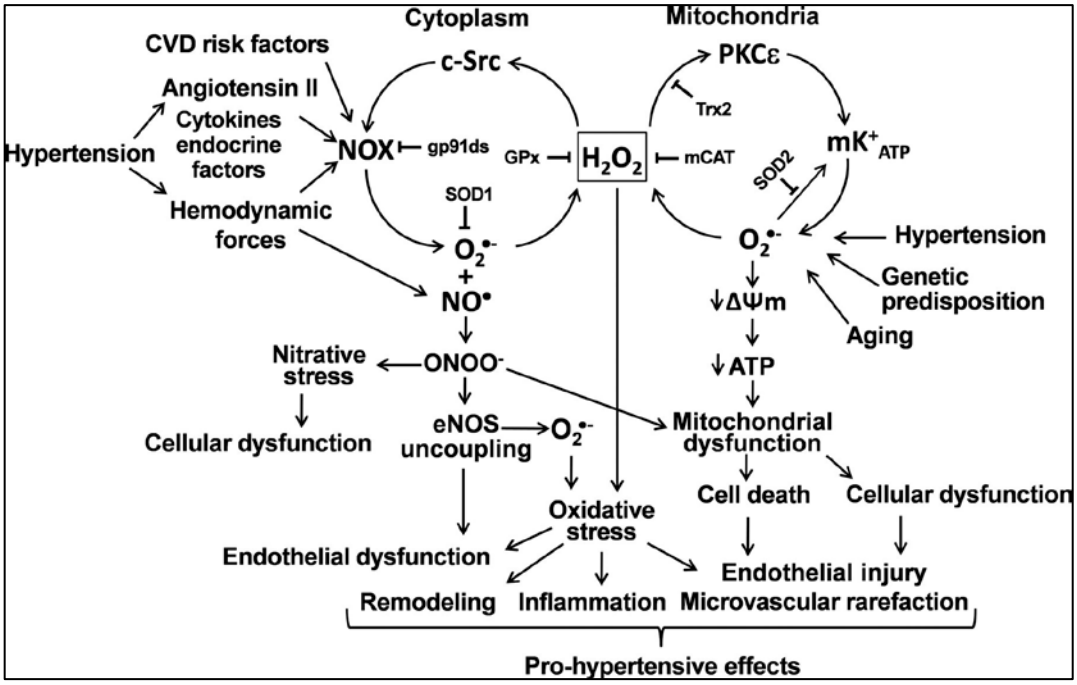

Figure 1.7: Proposed role of redox-dependent cross talk between mitochondria and NADPH oxidases (NOXs) in vascular and renal dysfunction in hypertension. CVD, cardiovascular disease; NO, nitirc oxide; ONOO−, peroxynitrite; eNOS, endothelial NO synthase; GPx, glutathione peroxidase, Trx2, thioredoxin 2; mCat, catalase targeted to mitochondria; mKATP, ATP-sensitive K+ channel; ΔΨm, mitochondrial transmembrane potential (Dikalov 2013)

Rationale, Overall Hypothesis and Specific Aims

Primary or essential hypertension is regarded as a multi-factorial disease, influenced by both genetic makeup and environmental conditions. Though several (~300) genetic loci have been identified for hypertension in the rat (Hilbert, Lindpaintner *et al.* 1991; Schork, Krieger *et al.* 1995; Lauledеrkind, Hayman *et al.* 2013), the exact form of the underlying genetic mechanism remains unanswered. In addition to being polygenic, hypertension is thought to be polygenomic, with significant evidence supporting the relationship between mtDNA dysfunction and cardiovascular disease (Wilson, Hariri *et al.* 2004; de Cavanagh, Inserra *et al.* 2007; Chan, Wu *et al.* 2009). Additionally, significant data from our lab and others have documented that angiotensin II and the renin-angiotensin system play a critical role in maintenance of arterial blood pressure and, that this hormonal system is elevated in several experimental models of hypertension, as well as human essential hypertension. Presented in this body of work is evidence of renal intrinsic and extrinsic mechanisms, including specific humoral, genetic and genomic mechanisms that appear to play a role in the manifestation and maintenance of spontaneous hypertension. This work will be laid out in the following aims:

<u>Specific Aim 1</u> established a unique rodent colony (BN/SHR-mt^{SHR}) to attempt to isolate nuclear and mitochondrial genes, and their potential interaction with the renin-angiotensin system that may be causal to hypertension in BN/SHR-mt^{SHR}

<u>Specific Aim 2</u> determined tissue angiotensin II, type 1 receptor (AT_1r) gene and protein expression in normotensive (NT) and hypertensive (HT) BN/SHR-mt^{SHR}.

- Evaluate tissue-specific Agtr1a mRNA expression and incidence of hypertension in BN/SHR-mt^{SHR}
- Evaluate kidney- and hypothalamic- specific AT1 protein expression and incidence of hypertension in BN/SHR-mt^{SHR}

<u>Specific Aim 3</u> evaluated sodium sensitivity and renal function in hypertensive and normotensive BC5 sibxsib BN/SHR-mt^{SHR}.

- Characterize renal function, pressure-natriuresis and diuresis in normotensive and hypertensive BN/SHR-mt^{SHR}

<u>Specific Aim 4</u> evaluated tissue-specific nuclear and mitochondrial genes involved in oxidative phosphorylation and their role in spontaneous hypertension in BN/SHR-mt^{SHR}.

- Evaluate tissue-specific mitochondrial gene expression and incidence of hypertension in BN/SHR-mt^{SHR}
- Evaluate tissue-specific gene expression of nuclear regulatory factors for mitochondrial transcription and regulation
- Characterize kidney-specific OXPHOS using cytochrome oxidase staining in normotensive and hypertensive BN/SHR-mt^{SHR}

CHAPTER 2

SPONTANEOUS HYPERTENSION: THE SPONTANEOUSLY HYPERTENSIVE RAT & DEVELOPMENT OF BN/SHR-mt^{SHR} CONPLASTIC RAT MODEL

Jason Andrieu Collett, Jeffrey L. Osborn

Abbreviations used: BN, Brown Norway rat; WKY, Wistar Kyoto rat; SAP, systolic arterial pressure

Keywords: Spontaneous hypertension, heritable hypertension, Spontaneously Hypertensive Rat, conplastic breeding

2.1 Introduction: Development of the Spontaneously Hypertensive Rat

The various mechanisms underlying human hypertension have been difficult to study as there is individual variation in the polygenetic/polygenomic disposition and environmental factors that are difficult to differentiate from each other. The most commonly used model of experimental hypertension is the Spontaneously Hypertensive rat (SHR). Okamoto-Aoki SHRs are descendants of an outbred Wistar male (WKY) with spontaneous hypertension from a colony in Kyoto, Japan (Okamoto and Aoki 1963). Briefly, A male rat with spontaneously high systolic blood pressures of 150 to 175 mmHg persisting for more than one month and a female rat with elevated systolic blood pressures (130 to 140 mmHg), were mated to obtain F1 rats. Of these F1 rats, males and females with hypertension, as defined as systolic BP > 150mmHg persisting for more than a month were mated to produce F2 generation rats. The procedure was repeated to obtain F3, F4, F5 and F6 rats totaling 380 animals. The incidence of the spontaneous occurrence of hypertension increased, and the development of hypertension occurred at younger ages from generation to generation. The incidence of severe hypertension (SYS>200mmHg) increased with each generation, so that among male animals it increased from only 9% in F2 to 35% in F3, 42% in F4, and 56% in F5, and in female animals from 3% in F2, 16% in F3, 33% in F4, and 37% in F5 (Okamoto and Aoki 1963). From 1968, this inbred strain of SHRs was further developed in the USA and elsewhere (Kurtz and Morris 1987). The various colonies of SHR are pre-hypertensive for the first 6–8 weeks of their lives with systolic blood pressures around 100–120 mmHg (Adams, Bobik *et al.* 1989),

and then hypertension develops over the next 12–14 weeks(Doggrell and Brown 1998). As in humans, hypertension develops more rapidly and becomes more severe in male than female SHR (Iams and Wexler 1979). *In vivo* studies have shown that, in the early stages of hypertension, SHRs have an increased cardiac output with normal total peripheral resistance. As the SHR progresses into the established hypertension state, the cardiac output returns to normal and the hypertrophied blood vessels produce an increase in the total peripheral resistance.

Researchers in hypertension have commonly used SHRs which have, within each colony, uniform polygenetic disposition that influence the cardiovascular system. It was, and continues to be the hope of geneticists and physiologists that the SHR will be pivotal in identifying genes contributing significantly to the pathophysiology of hypertension. The nuclear loci causal to chronic elevated arterial pressure have been extensively investigated in the SHR (Kurtz, Casto *et al.* 1990; Ye and West 2003; Atanur, Birol *et al.* 2010; Pravenec and Kurtz 2010; Yamamoto, Okuzaki *et al.* 2013). Translationally, the SHR follows the same progression of hypertension as human hypertension with pre-hypertensive, developing and sustained hypertensive phases with each phase lasting at least several weeks (Folkow 1993). However, the SHR differs from human hypertension in that SHRs reproducibly develop hypertension in young adulthood rather than in middle age as in humans. SHRs have a defined “pre-hypertensive

state", which makes the model invaluable to be used in studies of the cause and development of hypertension.

The WKY controls were established later, in 1971, as a normotensive control strain by the National Institutes of Health (USA) as an inbreed of the Wistar Kyoto colony via brother×sister mating (Kurtz and Morris 1987). The degree of genetic difference between the SHR and WKY strains and within different colonies of each strain is substantial and comparable to the extreme variability that exists between human populations and thus unlikely to be related solely to hypertension (Johnson, Ely *et al.* 1992; St Lezin, Simonet *et al.* 1992). Differences between SHRs and Wistar normotensive rats other than WKY may be more likely to be hypertension-related than differences between SHR and WKY because the SHRs were derived from the WKY, hypertension may develop spontaneously in the WKY, and the WKY may share some of the genes responsible for hypertension with the SHR (Louis and Howes 1990).

The SHR has been shown to exhibit lower blood pressure when administered the most common antihypertensive medications (Pinto, Paul *et al.* 1998). Thus, it is not surprising that SHRs have been used extensively and successfully for 50 years to test compounds for their effectiveness in lowering blood pressure, and to study the mechanisms of established hypertension.

To investigate mechanisms underlying spontaneous hypertension, we utilized the Okamoto-Aoki SHR in a unique breeding paradigm to isolate nuclear and mitochondrial genes responsible for the manifestation of heritable hypertension.

2.2 Development of BN/SHR-mt^{SHR} "conplastic" rat model for investigating physiological and genetic mechanisms of spontaneous hypertension

Hypertension in the Okamoto-Aoki SHR is both neurogenic and genetic in nature. The present study measured the arterial blood pressures of offspring over six generations (F1-BC5) of breeding in a "conplastic" colony derived from mating a female hypertensive, SHR with a systolic arterial pressure (SAP) averaging 188 mmHg with two normotensive Brown Norway (BN) males (SAP 100 & 104 mmHg, respectively). Hypertension was defined in all offspring as a SAP ≥ 145mmHg, normotension as SAP ≤ 124mmHg and borderline hypertension as 125mmHg < SAP < 145mmHg. F1 – BC5 female offspring with a SAP ≥ 145mmHg were back bred to the original, normotensive, founder BN males (**Figure 2.1**). Offspring at each generation were raised to adulthood and blood pressure measurements were obtained by tail cuff plethysmography at 8, 12, and 16 weeks of age, as well as into later adulthood ages. The purpose of this breeding paradigm was two-fold. First, hypertensive nuclear "loci" that are likely causal to hypertension were isolated on a normotensive, Brown Norway background. Second, as females were phenotypically selected for back breeding at each generation, all offspring of the colony had identical mitochondrial genomes of the founder SHR hypertensive female. This breeding paradigm allowed for the investigation of any potential role that the mitochondria may play in the development and maintenance of the inherited hypertension. Six generations produced more HT (n=88; 46%) than NT (n=21;11%) offspring, documenting consistent and dominant expression of the hypertensive phenotype.

These results indicate that dominant alleles of both the nuclear and mitochondrial genomes may be responsible for the development of hypertension in rats with a decreasing SHR nuclear genome that is replaced with a Brown Norway genome.

2.2.1 Methods and Materials

Rats were raised in a 12 hr light: 12 hr dark cycle in a climate at 20° to 22°C from birth. At 3 weeks of age, rats were weaned and transferred to either individual (males) or group (≤3 females of the same litter) solid-wall cages with bedding and were provided a commercial standard rodent chow and tap water *ad libitum*. Beginning at 10 weeks of age, rats were phenotyped as normotensive, borderline hypertensive or hypertensive using tail cuff plethysmography (Kent Scientific, Torrington, CT). Hypertensive female offspring were then back-crossed to the original progenitor BN males for 5 subsequent generations. All rats in the colony possessed identical mitochondrial genomes, with increasing BN nuclear genome with each subsequent backcross generation. After repeated blood pressure recordings that assured consistent determination of adult arterial pressure, rats were euthanized with an overdose of sodium pentobarbital (60 mg/kg i.p)., immediately decapitated, and organ tissues were rapidly frozen in a solution of dry ice and acetone.

2.2.2 Measurement of arterial pressure.

Since animals were to be back-bred to the founder males in the establishment of the conplastic genome, tail cuff plethysmography was used as a phenotyping methodology only to establish basic individual blood pressures. To minimize stress and improve reliability of blood pressure measurements, several steps were used in the blood pressure recording method that has been previously characterized and published (Kurtz, Griffin *et al.* 2005). Rats were exposed and acclimated to the measurement procedures and restraint equipment prior to BP recordings. A dark cover was placed over the restrained animal for the duration of the BP measurement, and BP recordings were performed at the same time each day. All equipment was thoroughly cleaned and disinfected before and after each individual rat to eliminate foreign scent. Animals were moderately warmed to dilate the ventral artery. Arterial pressures were derived from the average results of ≥5 measurements in each recording session. The average blood pressures of ≥5mmHg separate recording sessions with <5% variability were used to establish the phenotype of each animal. Both systolic and diastolic pressures were obtained and recorded. For purposes of reporting, the systolic pressures were used for the determination of the specific individual phenotype.

2.3 Results

Progenitor female SHR had a SAP of 188mmHg and progenitor BN males had SAPs of 100 (BN*) and 104 (BN^) mmHg. The BN*/SHR-mt^{SHR} produced six generations, yielding 94 total offspring, with 42.6% (n=40) expressing the hypertensive phenotype, 42.6% (n=40) expressing the BHT phenotype, and only 14.9% (n=14) expressing the normotensive phenotype (**Table 2.1**). The BN^/SHR cross/backcross also produced six generations, yielding 71 total offspring, with 52.1% (n=37) expressing the hypertensive phenotype, 39.4% (n=28) expressing the BHT phenotype, and only 8.5% (n=7) expressing the normotensive phenotype (**Table 2.2**). Together, the 6 total generations produced 190 offspring, with 110 (58%) female and 80 (42%) male offspring. There were significantly more hypertensive (n=88; 46%) than normotensive offspring (n=21;11%), while a large number of individuals expressed the intermediate phenotype (n=81; 43%). There were no differences in SAP between male and female offspring at any generation. Furthermore, comparison of systolic, diastolic and mean arterial pressures of male and female offspring across all backcross generations did not demonstrate any gender differences in arterial pressures. Hypertension was dominantly expressed and maintained across all six offspring generations of BN/SHR-mt^{SHR} rats (Figure 2.2).

With the conplastic breeding paradigm, the BN contribution to the nuclear genome at each generation is as follows: F1: 50%, BC1: 75%, BC2: 87.5%, BC3: 93.75%, BC4: 96.875%, BC5: 98.4375%. Thus, at the 6th generation, SHR only

accounts for 1.5625% of the nuclear genome, however, the hypertensive phenotype is still dominantly expressed (**Figure 2.2**). Additionally, the mitochondrial genomes of all offspring (n=190), barring any mutations, are identical to the founder SHR female.

2.4 Implications and Significance

Because of the lack of appropriate animal models, the potentially causal contributions of inherited mitochondrial genomic factors to complex traits are less well studied compared with inherited nuclear genomic factors. To evaluate the effects of these mtDNA variations in the absence of the corresponding nuclear genomic factors as confounding variables, novel conplastic BN/SHR-mt^{SHR} rats were constructed using a founder male back breeding paradigm and characterized for the existence of hypertension. Utilizing this unique rodent colony, one can begin to understand the different roles that the nuclear and mitochondrial genome may be playing in the development of chronic elevated arterial pressure, and subsequently the development of cardiovascular disease associated with arterial hypertension.

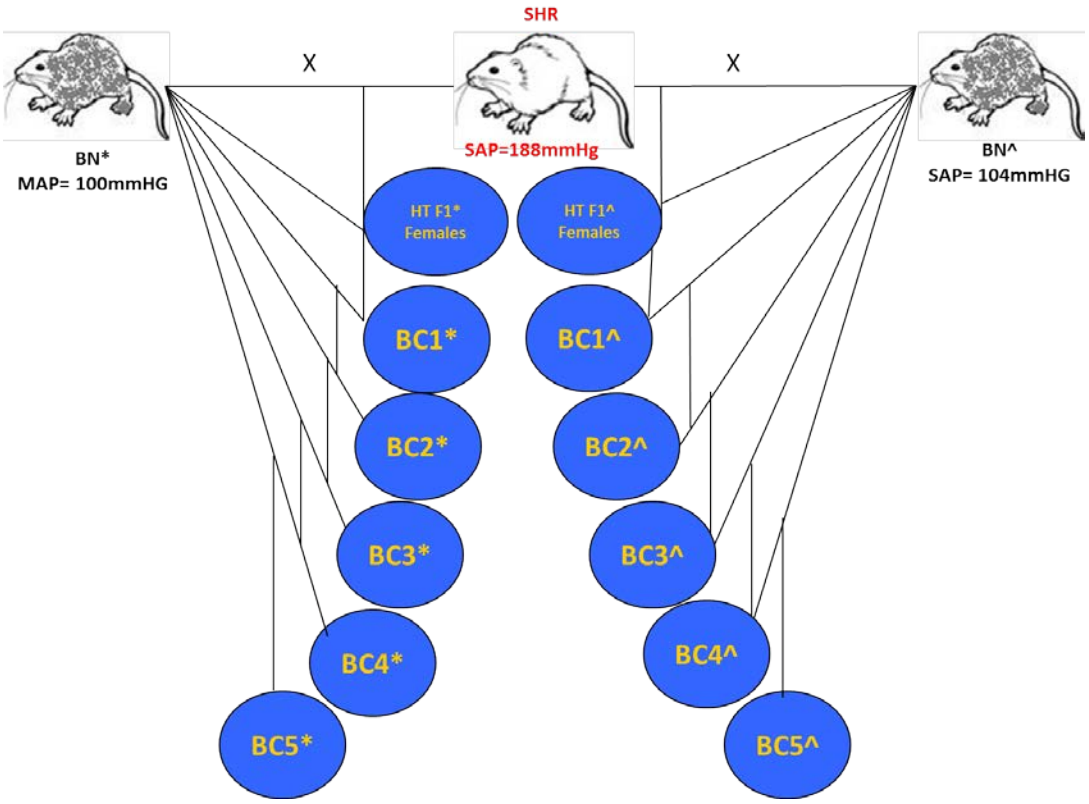

Figure 2.1: Breeding paradigm for BN/SHR-mt^{SHR} rats. Hypertensive female SHR (SAP= 188mmg) was crossed with two BN males (100mmHg* and 104mmHg^ respectively). Back-breeding female offspring using phenotypic selection of elevated arterial pressure (>145mmHg) was used to provide the second generation (BC1). Subsequent back-crossing was performed for five additional generations to create 6 total generations (F1, BC1-BC5).

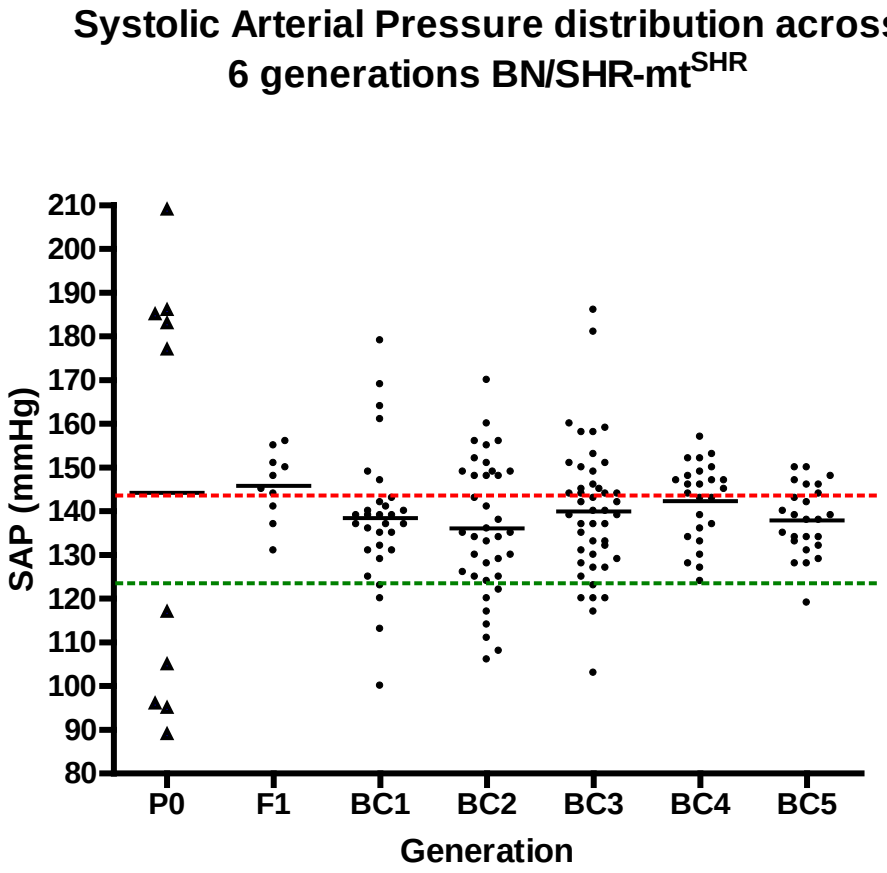

Figure 2.2: Six generations of BN/SHR-mt^{SHR} rats with corresponding average systolic pressure (SAP) values. The Po generation represents repeated measures of the founding SHR and BNs. Each individual dot in generations F1-BC5 represent average BP of individual rats. Three distinct populations persisted throughout all 6 generations (NT animals above red dotted line, NT animals below green dotted line, and BHT animals between red and green dotted lines), with hypertension being dominantly expressed and maintained. Modified from (Collett, Hart et al. 2013)

BN*(♂) SAP: 100mmHg

SHR (♀) SAP: 188 mmHg	Generation	Phenotype (SAP: mmHg)					
		Normotensive		Borderline Hypertensive		Hypertensive	
		N=7	N=7	N= 18	N=22	N= 17	N=23
		♂	♀	♂	♀	♂	♀
	F1	N=0	N=0	N=0	N=0	N=1 SAP=152	N=1 SAP=154
	BC1	N=1 SAP=114	N=1 SAP=120	N=5 SAP=135.2 ±2.4	N=5 SAP=131.6 ±2.2	N=3 SAP=146.7 ±1.8	N=6 SAP=148.5 ±4.0
	BC2	N=1 SAP=101	N=5 SAP= 116.2 ±3.1	N=3 SAP=131 ±2.6	N=2 SAP=131.5 ±1.5	N=2 SAP=146 ±1	N=4 SAP=146.8 ±2.8
	BC3	N=4 SAP=109 ±3.1	N=1 SAP=108	N=6 SAP=133.7 ±1.9	N=5 SAP=132.2 ±1.4	N=4 SAP=153 ±6.5	N=3 SAP=150.3 ±2.8
	BC4	N=0	N=0	N=3 SAP=130 ±1.2	N=0	N=5 SAP=145.6 ±3.5	N=6 SAP=145.2 ±0.95
	BC5	N=2 SAP=122	N=0	N=5 SAP=136.2 ±1.0	N=6 SAP=132.2 ±1.5	N=2 SAP=145.5 ±1.5	N=3 SAP=148.7 ±2.4

Table 2.1: Phenotypic characterization of BN*/SHR-mt^{SHR} colony. Phenotypes are separated according to gender. There was no difference in SAP between genders. SAP values are expressed as mean ± S.E.M.

BN^(♂) SAP: 104mmHg							
		Phenotype (SAP: mmHg)					
	Generation	**Normotensive**		**Borderline Hypertensive**		**Hypertensive**	
SHR (♀) SAP: 188 mmHg		**N=5**	**N=1**	**N= 12**	**N=16**	**N= 16**	**N=21**
		♂	♀	♂	♀	♂	♀
	F1	N=0	N=0	N=1 SAP=135	N=2 SAP=134 ±1.0	N=3 SAP=152 ±1.5	N=2 SAP=145.5 ±1.5
	BC1	N=0	N=0	N=2 SAP=135 ±2.1	N=1 SAP=134	N=2 SAP=145.5 ±2.5	N=2 SAP=163 ±5.0
	BC2	N=2 SAP=117 ±6	N=0	N=4 SAP=133 ±2.4	N=3 SAP=130 ±2.3	N=4 SAP=153 ±4.4	N=4 SAP=146.5 ±1.6
	BC3	N=3 SAP=116 ±3.8	N=0	N=5 SAP=132.2 ±1.7	N=5 SAP=132.6 ±1.9	N=3 SAP=146 ±4.0	N=6 SAP=149.3 ±2.6
	BC4	N=0	N=1 SAP=120	N=0	N=2 SAP=133 ±3.0	N=3 SAP=152.3 ±2.0	N=5 SAP=147.2 ±1.8
	BC5	N=0	N=0	N=1 SAP=134	N=3 SAP=133.3 ±1.9	N=1 SAP=146	N=2 SAP=146.5 ±0.5

Table 2.2: Phenotypic characterization of BN^/SHR-mt^{SHR} colony. Phenotypes are separated according to gender. There was no difference in SAPs between genderss. SAP values are expressed as mean ± S.E.M

CHAPTER 3

Renal Angiotensin II Type 1 Receptor Expression and Associated Hypertension in Rats with Minimal SHR Nuclear Genome

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Keywords: Renin-angiotensin system, AT_1 receptors, gene expression, spontaneous hypertension

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3.1 Introduction

Hypertension and the chronic elevation of blood pressure constitute a primary and significant factor in the development of cardiovascular disease. Essential hypertension is regarded as a multi-factorial disease, influenced by both genetic makeup and environmental conditions that can alter genomic expression (National Heart, Institute *et al.* 1977), (Raizada, Sumners *et al.* 1993), (Schork, Krieger *et al.* 1995). The polygenic nature of hypertension and its dependence on environmental factors complicate the clear identification of genetic factors that directly increase blood pressure (Joe and Garrett 2005). The rat is a well-established animal model for investigating human hypertension, with over 25,000 papers reported on hypertension in rats alone (Kwitek-Black and Jacob 2001). Rat models of spontaneous hypertension, in particular, the SHR, have been critical to our understanding of blood pressure control and the pathophysiology of hypertension. Significant progress has been made to map the genes involved in blood pressure regulation in these animal models. Nuclear and mitochondrial genome scans have been carried out in rats, mice and humans and have revealed several potential genomic regions that may contain genes involved in the pathogenesis of spontaneous hypertension (Rapp 2000). Molecular genetic analysis proves that BP regulation is polygenic, and there is good evidence for several BP related quantitative trait loci (QTLs) on nearly every rat chromosome (Deng, Dene *et al.* 1994), (Doris 2002), (Hilbert, Lindpaintner *et al.* 1991), (Hopkins and Hunt 2003), (Rapp, Dene *et al.* 1994), (Schork, Krieger *et al.* 1995). Linkage analyses of populations derived from the SHR shows potential BP QTLs

at numerous loci, however, the precise nature of the genetic mechanisms underlying essential hypertension remains unanswered.

Significant data from our lab and others have documented that angiotensin II and the renin-angiotensin system play a critical role in maintenance of arterial blood pressure and that this hormonal system is elevated in several experimental models of hypertension, as well as human essential hypertension (Cowley 1992), (de Gasparo, Catt *et al.* 2000), (Doris 2002), (Lenkei, Palkovits *et al.* 1997), (Lifton, Gharavi *et al.* 2001), (Reinhart, Lohmeier *et al.* 1995), (Weir and Dzau 1999). It has been suggested that the renin-angiotensin system RAS plays a pathogenic role in the development of hypertension in Aoki-Okamoto SHR. An elevated RAS impacts blood pressure directly via vasoconstriction and sodium retention, as well as indirectly, through increased reactive oxygen species, altering redox signaling and increased sympathetic outflow.

Here we demonstrate a unique rat colony developed by breeding a hypertensive female Okamoto-Aoki SHR with male, normotensive Brown Norway (BN) rats. Hypertensive female offspring were backcrossed with the original males for 5 subsequent generations. Despite the dilution of the “hypertensive” nuclear genome, hypertensive phenotype expressed by the founder female was dominantly expressed and maintained across 6 generations of BN/SHR-mt^{SHR} rats. All progeny however, have identical mitochondrial genomes. We investigated the tissue-specific mRNA expression of the RAS pathway, including angiotensinogen (*AGT*), renin (*REN*), angiotensin converting enzyme 1 (*ACE1*),

angiotensin converting enzyme 2 (*ACE2*) and angiotensin II, type 1 receptors (*Agtr1a*) in normotensive and hypertensive BN/SHR-mt^{SHR} backcross rats. It is hypothesized that tissue-specific increased RAS expression contributes to heritable hypertension in BN/SHR-mt^{SHR} rats.

3.2 Materials and Methods

3.2.1 Animals.

All experiments were carried out in accordance with the AAALAC Guide to the Care and Use of Laboratory Animals and all protocols were previously approved by the University of Kentucky Institutional Animal Care and Use Committee (UK IACUC). A congenic colony using phenotypic selection was employed. The Aoki-Okamoto SHR/Brown Norway (BN/SHR-mt^{SHR}) rat colony was developed by breeding a female SHR (Charles River Labs, Wilmington, MA) with 2 different normotensive Brown Norway males (BN* and BN^, respectively; Charles River Labs, Wilmington, MA). Rats were raised in a 12 hr light: 12 hr dark cycle in a climate at 20° to 22°C from birth. At 3 weeks of age, rats were weaned and transferred to either individual (males) or group (≤3 females of the same litter) solid-wall cages with bedding and were provided a commercial standard rodent chow and tap water *ad libitum*. Beginning at 10 weeks of age, rats were phenotyped as normotensive, borderline hypertensive or hypertensive using tail cuff plethysmography (Kent Scientific, Torrington, CT). Hypertensive female offspring were then back-crossed to the original progenitor BN males for 5 subsequent generations. All rats in the colony possessed identical mitochondrial genomes, with increasing BN nuclear genome with each subsequent backcross generation. After repeated blood pressure recordings that assured consistent determination of adult arterial pressure, rats were euthanized with an overdose of

sodium pentobarbital (60 mg/kg i.p)., immediately decapitated, and organ tissues were rapidly frozen in a solution of dry ice and acetone.

Brains were carefully removed from the skull, and hypothalami were excised using previously characterized specific landmarks: anterior – optic chiasm; lateral – optic nerve projections; dorsal – fornix.

Age and sex matched HT (N=20) and NT (N=20) animals across 6 generations of BN/SHR-mt^{SHR} were chosen for RAS mRNA evaluation. Animals in the backcross generation 3 (BC3) were chosen for protein analysis as there were an appropriate number of age and sex matched NT and HT rats within a single generation.

3.2.2 Measurement of Arterial pressure.

Systolic arterial pressure (SAP) was evaluated in parents and offspring beginning at 10-12 weeks of age. Phenotypes were assigned as either normotensive (NT: SAP ≤ 124mmHg), borderline hypertensive (BHT: 125 ≤ SAP < 145 mmHg) or hypertensive (HT: SAP ≥ 145mmHg). Since animals were to be back-bred to the founder males in the establishment of the conplastic genome, tail cuff plethysmography was used as a phenotyping methodology only to establish basic individual blood pressures. To minimize stress and improve reliability of blood pressure measurements, several steps were used in the blood pressure recording method that has been previously characterized and published (Kurtz, Griffin et al. 2005). Rats were exposed and acclimated to the measurement procedures and restraint equipment prior to BP recordings. A dark cover was

placed over the restrained animal for the duration of the BP measurement, and BP recordings were performed at the same time each day. All equipment was thoroughly cleaned and disinfected before and after each individual rat to eliminate foreign scent. Animals were moderately warmed to dilate the ventral artery. Arterial pressures was derived from the average results of ≥5 measurements in each recording session. The average blood pressures of ≥5mmHg separate recording sessions with <5% variability were used to establish the phenotype of each animal. Both systolic and diastolic pressures were obtained and recorded. For purposes of reporting, the systolic pressures were used for the determination of the specific individual phenotype.

3.2.3 RNA Extraction and RT-PCR.

Kidney, liver and lung tissue were harvested from HT and NT rats (n=20 NT; n=20 HT) as described above. Total RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, CA) and purified using RNeasy minicolumns (Qiagen Inc., Valencia, CA) according to manufacturer's protocol. Possible genomic DNA in total RNAs was digested with RNA-free DNase I (Qiagen Inc., Valencia, CA). Concentration and purity of all RNA samples was determined by the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Extracted RNA was reverse-transcribed into complementary DNA (cDNA) using qScript cDNA supermix (Quanta Biosciences, Gaithersburg, MD) in a total volume of 20µl using a MyCyler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA).

3.2.4 Quantitative Real-Time PCR.

Quantitative Real-Time PCR was performed on a StepOnePlus Real-time PCR system (Applied Biosystems). Real-time quantitative PCR amplifications were performed in triplicate in a 96-well plate. For normalization, *GAPDH* was used as the reference gene. Pre-designed primers and hydrolysis probes were purchased from Integrated DNA Technologies, Inc. (*Agtr1a*; Primer 1: 5'-CCAGCCATTTTATACCAATCTC-3', Primer 2: 5'-TCCTGTTCCACCCGATCA-3', Probe: 5'-/HEX/CAGCTCTGC/ZEN/CACATTCCCTGAGT/ 3IABkFQ/-3'.) (GAPDH; Primer 1: 5'GTAACCAGGCGTCCGATAC-3', Primer 2: 5'-GTTCTAGAGACAGCCGCATC-3', Probe: 5'-/56-FAM/ATCCGTTCA/ZEN/ CACCGACCTTCACC/3IABkFQ/-3'.) Pre-designed TaqMan primers and hydrolysis probes for AGT (Rn00593114_m1), REN (Rn00561847_m1), ACE1 (Rn00561094_m1), ACE2 (Rn01416293_m1) were purchased from Life Technologies. Relative gene expression was calculated using the comparative CT method. Primers and probes were verified and operating at similar efficiencies.

3.2.5 Membrane Protein Extraction.

Whole kidney and hypothalamic tissues were placed in an ice-cold buffer solution containing 1M Tris, 5M NaCl, 0.5M EDTA, 100% Brij 96/97 and 10% NP40 with

0.3% protease inhibitor leupeptin (50μM), aprotinin (50nM) and pepstatin (1μM). Tissue samples were immediately homogenized (PowerGen® 125 Homogenizer, Fisher Scientific, Pittsburgh, PA) at 4°C for approximately 15 seconds. After complete homogenation, samples were loaded into a centrifuge (Heraeus Biofuge 13, Baxter Scientific, Deerfield, IL) and spun at 13,000 rpm for 10 minutes at 4°C. Protein concentration was determined for each sample using colorimetric assay according to Lowry *et al.* (Lowry, Rosebrough *et al.* 1951).

3.2.6 Western blot analysis of AT_1 Receptor.

Kidney and hypothalamic protein samples were electrophoretically separated on 4% to 10% SDS-Page gels at 150V/50mA for 1 hour. Separated proteins were transferred by electroelution (200V, 1-2hrs) to polyvinylidene difluoride (PVDF) membranes (0.45μm;Millipore, Bedford, MA). Molecular weight markers (~10-190kDa; Benchmark Prestained Protein Ladder, Invitrogen, Carlsbad, CA) were used to estimate molecular mass. Blots were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline. Blots were incubated with primary antibodies AT_1r (1:200, sc-81671) and β-tubulin (loading control; 1:200, sc-23949; Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 25°C for 2 hours. Blots were washed with Tris-buffered saline/0.1% Tween-20 (TBST) and then exposed to secondary antibody conjugated to horseradish-peroxidase (1:2000, sc-2005; Santa Cruz, CA) at 25°C for 2 hours. Detection of specific proteins was accomplished using enhanced chemiluminescence (SuperSignal West Pico, Thermo Scientific, Rockford, IL) according to manufacturer's instructions, and

blots were exposed to BioMax Light Autoradiography film (Kodak #1788207). Densitometric results were reported as integrated values (area density of band) and expressed as a ratio of AT_1r to loading control (β-tubulin). Results were then compared between phenotypic groups. Lanes lacking protein were subject to AT_1r and β-tubulin primary antibody to verify antibody selectivity. Densitometry reflects mean ± S.EM. of all samples.

3.2.7 Statistical analysis.

Blood pressures, as well as tissue AT_1r protein expression between NT and HT BN/SHR-mt^{SHR} rats were analyzed using an Student's T-test. Renal *AGT*, *REN*, *ACE1*, *ACE2* and *Agtr1a* mRNA expression levels between HT and NT BN/SHR-mt^{SHR} rats were analyzed using Mann-Whitney *U* Test comparisons. The 0.05 level of probability was utilized as the criterion of significance. All statistical analyses were performed using GraphPad Prism 4 (GraphPad Software, Inc., La Jolla, CA).

3.3 Results

3.3.1 Arterial pressure phenotyping of BN/SHR-mt^{SHR} colony.

Systolic arterial pressure phenotypes were assessed weekly beginning at 10-12 weeks of age according to the following arterial blood pressure parameters: normotensive (NT: SAP ≤ 124mmHg), borderline hypertensive (BHT: 125mmHg<SAP< 145 mmHg) or hypertensive (HT: SAP > 145mmHg). Progenitor female SHR had a SAP of 188mmHg and progenitor BN males had SAPs of 105 (BN*) and 103 (BN^) mmHg. The BN*/SHR-mt^{SHR} cross/backcross produced six generations, yielding 94 total offspring, with 42.6% (n=40) expressing the hypertensive phenotype, 42.6% (n=40) expressing the BHT phenotype, and only 14.9% (n=14) expressing the normotensive phenotype. The BN$^{\wedge}$/SHR-mt^{SHR} cross/backcross also produced six generations, yielding 71 total offspring, with 52.1% (n=37) expressing the hypertensive phenotype, 39.4% (n=28) expressing the BHT phenotype, and only 8.5% (n=6) expressing the normotensive phenotype. Together, the 6 total generations produced 190 offspring, with 110 (58%) female and 80 (42%) male offspring. There were significantly more hypertensive (n=88; 46%) than normotensive offspring (n=21;11%), while a large number of individuals expressed the intermediate phenotype (n=81; 43%). There were no differences in SAP between male and female offspring at any generation. Furthermore, comparison of systolic, diastolic and mean arterial pressures of male and female offspring across all backcross generations did not demonstrate any gender differences in arterial

pressures. Hypertension was dominantly expressed and maintained across all six offspring generations of BN/SHR-mt^{SHR} rats (**Figure 3.1**).

3.3.2 Renal Agtr1a mRNA expression is higher in hypertensive BN/SHR than in normotensive BN/SHR-mt^{SHR} rats.

The mRNA levels of *AGT, REN, ACE1* and *ACE2* and *Agtr1a* in renal tissue were evaluated NT and HT BC3 (NT: n=6, HT: n=6; **Figure 3.2**) and in HT and NT rats representative of each generation of BN/SHR-mt^{SHR} rats (n=20 NT; n=20 HT; **Figure 3.3**). Animals exhibiting the most extreme phenotypes were chosen for mRNA expression analysis. *AGT*, *REN*, *ACE1* and *ACE2* mRNA levels were not different between NT and HT BN/SHR-mt^{SHR} rats. Renal *Agtr1a* was increased by ~2.5-fold ($P < 0.05$) in HT compared to NT BN/SHR-mt^{SHR} rats (**Figure 3.2**). Expression is reported as relative expression using $2^{-\Delta\Delta CT}$ method. NT values were normalized to 1.

3.3.3 Systemic RAS mRNA expression is not different in hypertensive compared to normotensive BN/SHR-mt^{SHR} rats.

The mRNA levels of liver *AGT, Agtr1a* and lung *ACE1* were evaluated in HT and NT BN/SHR-mt^{SHR} rats (n=20 NT; n=20 HT). Animals exhibiting the most extreme phenotypes were chosen for mRNA expression analysis. Liver *AGT*, *Atgr1a* and lung *ACE1* mRNA levels were not different between NT and HT

BN/SHR-mt^{SHR} rats (**Figure 3.4 A,B,C**). Expression is reported as relative expression using $2^{-\Delta\Delta CT}$ method. NT values were normalized to 1.

3.3.4 Renal AT1 receptor protein expression is higher in hypertensive BN/SHR-mtSHR than in normotensive BN/SHR-mtSHR rats.

Western blot analysis of AT_1 receptor protein from whole kidney homogenates using the monoclonal antibody showed that AT_1 receptor protein levels were significantly higher ($p<0.05$; **Figure 3.5A**) in BC3 HT rats compared to NT rats. The ratio of renal AT_1 to β-tubulin densitometric signals were 1.000 ± 0.097 vs. 1.379 ± 0.06975 (**Figure 3.5B**). Regression analysis was also performed, indicating a positive correlation ($r^2=0.6502$, $p<0.05$) between SAP and AT_1 receptor protein expression in kidney of BC3 BN/SHR-mt^{SHR} rats (**Figure 3.5C**).

3.3.5 Hypothalamic AT_1 receptor expression is higher in hypertensive BN/SHR-mt^{SHR} than in normotensive BN/SHR-mt^{SHR} rats.

Western blot analysis of AT_1 receptor protein from hypothalamus homogenates using the same monoclonal antibody showed that AT_1 receptor protein levels were significantly higher ($p<0.05$) in BC3 HT rats compared to NT rats (**Figure 3.6A**). The ratio of hypothalamic AT_1 to β-tubulin was 2.600 ± 0.07616 (NT: N=16) vs. 3.072 ± 0.2031 (HT: N=18) respectively (**Figure 3.6B**). Regression analysis was also performed, indicating a positive correlation ($r^2=0.4781$, $p<0.05$)

between SAP and AT_1 receptor protein expression in hypothalamus of BC3 BN/SHR-mt^{SHR} rats (**Figure 3.6C**).

3.4 Discussion

The main findings of this study are: 1) elevated arterial pressure is dominant and maintained across 6 generations of BN/SHR-mt^{SHR} rats, despite the increasing nuclear genomic contribution of the NT male donor rats, 2) hypertensive BN/SHR-mt^{SHR} rats across 6 generations exhibit elevated renal *Agtr1a* mRNA expression compared to NT BN/SHR-mt^{SHR} rats, while expression of renal *REN*, liver *AGT* and *Agtr1a* and lung *ACE1* were not different 3) hypertensive BN/SHR-mt^{SHR} in the BC3 generation exhibit an elevated AT_1r protein expression in kidney and hypothalamus compared to NT BN/SHR-mt^{SHR} rats, 4) increased AT_1r expression is positively correlated with elevated SAP in BC3 BN/SHR-mt^{SHR} rats. Taken together, these results suggest that hypertension is dominant in the presence of increasing normotenive “loci” and that tissue-specific altered expression of AT_1r, but not other aspects of renal or systemic RAS, may underlie heritable hypertension.

The SHR has been the most widely studied genetic model of essential hypertension in the past four decades (Pinto, Paul *et al.* 1998). Neurogenic in nature, the underlying mechanisms of the onset and manifestation of hypertension in the SHR remains to be fully elucidated. In this experimental paradigm, an attempt to isolate genomic regions from which hypertension derives in a “conplastic” colony using phenotypic selection was employed. In these rats, the nuclear genome of the 6th generation (BC5) offspring is comprised of ~96.9% original BN males, and only 1.6% of original donor SHR. The dilution of the

hypertensive genome had seemingly little effect on the manifestation of the hypertensive phenotype from generation F1 through backcross 5 (**Figure 3.1**). It is clear, however, that the magnitude of the hypertension was never as high in the offspring, as in the progenitor SHR even from the initial F1 generation. In fact, there is approximately a 30 mmHg difference between the average SAP of the SHR and F1 generation offspring. However, from the F1 generation forward, the magnitude of elevated blood pressure did not decline toward the BN blood pressure in any of the subsequent offspring generations. Thus, although a significantly increasing influence of the BN genome is transmitted following genetic mixing with SHR of subsequent backcross generations, the impact of the SHR genome was fully maintained for 6 consecutive generations (F1-BC5) following the initial mating.

Since the discovery of “renin” in 1898 and subsequent recognition that Ang II is pressor, the RAS has been extensively studied and remains a major candidate as a causative factor in elevated arterial pressure and the pathogenesis of hypertension (Carey and Siragy 2003), (de Gasparo, Catt *et al.* 2000), (Tigerstedt and Bergman 1898). The angiotensin II, type 1 receptor has been extensively evaluated in rodents and has subsequently become a target as a causal factor in the development of essential hypertension. Reja *et al.* (2006) showed that gene expression levels of AT_1r, extracellular signal-regulated kinase 2 and phosphatidylinositol 3-kinase were significantly higher in the PVN, RVLM and adrenal medulla in SHR compared to normotensive WKY rats. Raizada *et al.*

(1993) showed that AT_1r mRNA was higher in the brains of the SHR compared to normotensive WKY rats. Furthermore, Gyurko *et al.* (1993) showed that antisense inhibition of AT_1 receptor mRNA in the brain reduces the magnitude of hypertension in adult SHR. Data from our study supports the notion that elevated AT_1 receptors may play a role in SHR-derived elevated arterial pressure. Hypertensive BN/SHR-mt^{SHR} rats across several generations exhibit elevated renal specific *Agtr1a* mRNA (**Figures 3.2 and 3.3**) expression while expression of other renal RAS components, as well as liver *AGT* and lung *ACE1* were not different (**Figure 3.4**). In the BC3 generation, where the nuclear genome of the original SHR accounts for only ~6.2%, HT rats had significantly higher AT_1r and protein expression than normotensive rats in kidney and hypothalamic tissue (**Figure 3.5 and 3.6**). Furthermore, average SAP and tissue expression of AT_1r were positively correlated (**Figure 3.5C and 3.6C**), indicating that tissue specific expression of AT_1r expression may critically impact the development and maintenance of SHR hypertension. Increased AT_1r protein could potentially have drastic effects on the cardiovascular system, including the pathogenesis of hypertension, and seems to play a role in the development of hypertension in BN/SHR-mt^{SHR} rats. Ang II's effect in the kidney would be exacerbated, increasing proximal tubular sodium reabsorption and decreasing renal blood flow. In the hypothalamus, elevated AT_1r expression could increase sympathetic nervous activity and/or vasopressin secretion, subsequently increasing vascular resistance and sodium and water retention. The hypothalamus is of particular interest, as it plays a major role in sympathetic

outflow, vasopressin production and osmoregulation (Scherrer 1959). Recently, commercially available AT_1r antibodies have come under scrutiny for lack of specificity, and therefore may lead to erroneous results (Herrera, Sparks *et al.* 2013). It is critical to note here, however, that the antibody used for this study has not previously been identified as non-specific, and that our mRNA expression data fully corroborates the protein data that AT_1r expression is elevated in kidney of BN/SHR-mt^{SHR} backcross rats.

With the abundance of knowledge on the role of both circulating and tissue RAS in BP control, it is hypothesized that genetic variability in one or more of the RAS components could account for the pathogenesis of hypertension. Common variants of the RAS genes, including those coding for angiotensinogen and angiotensin-converting enzyme were some of the first to be associated with altered blood pressure control (Norton, Brooksbank *et al.* 2010). Several linkage analysis and genome wide association studies have been performed in both rodents and humans in regards to RAS genes, producing variable results (Baudin 2002), (Bonnardeaux, Davies *et al.* 1994), (Jeunemaitre, Soubrier *et al.* 1992), (Kainulainen, Perola *et al.* 1999), (Rigat, Hubert *et al.* 1990), (Rothermund and Paul 1998), (Schmidt, Beige *et al.* 1997), (Tomino, Makita *et al.* 1999), (Zhu, McKenzie *et al.* 2000). Results from our current study highlight a renal-specific elevation of a single gene of the RAS, therefore indicating genomic polymorphisms at this allele are not causal to the manifestation of the disease. Gene expression and subsequent protein synthesis may be equally, if not more

important than heritable nucleotide differences in elucidating the causes of complex, multigenic diseases. Data from this study indicate that AT_1r mRNA and receptor protein expression may have a significantly more important role than other aspects of RAS in heritable hypertension.

Due to the breeding paradigm (i.e. backcrossing hypertensive females with founder males), the influence of the mitochondrial genome (female transmission) on the pathophysiology of hypertension should be taken into consideration. These results provide strong evidence for the dominance of loci within the SHR genome that are highly resistant to increasing normotensive influence of the BN genome. At present, there are few data that identify the specific genes located in these “SHR dominant” regions. However, it is likely that these genomic regions contain numerous blood pressure controlling gene loci (Rapp 2000), (Lowry, Rosebrough *et al.* 1951), (Kaschina and Unger 2003). As hypertensive females were phenotypically selected for backcross, all offspring (F1, BC2-BC5) should have identical mitochondrial genomes, barring any mutation(s). Mitochondrial dysfunction has recently been implicated in a wide variety of genetic disorders (Taylor and Turnbull 2005), (Wallace 1999). Alterations in mitochondrial function are observed in conjunction with the development of hypertension in rodents and humans (Chan, Wu *et al.* 2009), (Kumarasamy, Gopalakrishnan *et al.* 2010), (Pravenec, Hyakukoku *et al.* 2007). The genetic association of mtDNA variants (Kumarasamy, Gopalakrishnan *et al.* 2010), (Wilson, Hariri *et al.* 2004) and tRNA mutations (Kumarasamy, Gopalakrishnan *et al.* 2010), (Benigni, Corna *et al.*

2009), (Liu, Li *et al.* 2009), (Pravenec, Hyakukoku *et al.* 2007) to type 2 diabetes and hypertension directly implicated mitochondrial defects to the etiology of cardiovascular disease and metabolic syndrome. Mitochondrial integrity and potential dysfunction is currently being evaluated in the BN/SHR-mt^{SHR} rats.

A potentially critical avenue in investigating the underlying mechanisms of heritable hypertension in BN/SHR-mt^{SHR} is the relationship between the renin-angiotensin system and mitochondrial dysfunction. The role played by angiotensin II in developing mitochondriopathy has been advanced recently by Benigni *et al.* (2009) Deletion of the *Agtr1a* gene resulted in the reduced age-related cardio-renal complications, improved mitochondrial biogenesis, and increased longevity in mice. Additionally, treatment with antioxidants, mitochondrial superoxide dismutase mimetics, and AT_1r blockers decreased vascular O_2- production and attenuated development of hypertension in SHR (Park, Touyz *et al.* 2002), (Rodriguez-Iturbe, Zhan *et al.* 2003), (Shokoji, Nishiyama *et al.* 2003). De Cavanagh *et al.* (2006) have demonstrated that oxidative stress is associated with mitochondrial dysfunction in SHR, and that this dysfunction is attenuated with AT_1r blockade with losartan. Taken together, there is significant evidence of mtDNA mutations and/or altered mitochondrial genetic expression is influenced, at least in part, by the RAS, and that this relationship may play a significant role in the development of hypertension.

In summary, the present results indicate that hypertension is dominantly expressed in BN/SHR-mt^{SHR} rats, and that elevated arterial pressure is positively correlated with the upregulation of kidney AT_1r mRNA, as well as kidney and hypothalamic AT_1r protein expression, while other aspects of the local and systemic RAS pathways are not different. It has been reported that AT_1r mRNA is regulated in a tissue-specific manner that is distinct among the other components of the RAS, and potentially independent of changes in circulating Ang II (Sechi, Griffin *et al.* 1996). Our current study supports this finding, as well as adds insight into the heritability and expression of different aspects of RAS. The maintenance of hypertension under conditions where the proportion of the female progenitor SHR mitochondrial genome remains intact, nuclear genome is continually reduced by increasing amounts of normotensive progenitor BN genome strongly suggests a major linkage of the SHR genes to the development of hypertension. While the origins of this SHR derived hypertension remain unknown, strong physiological evidence for a major neurogenic and RAS components have been reported. Given that the maintenance of hypertension in the F1 and subsequent backcross generations, the encoding and linkage of hypertensive loci may be directly related to genomic components of the female SHR mitochondrial genome.

The presence and abundance of AT_1r in the kidney and hypothalamus appears to be related to the propagation of the HT phenotype in BN/SHR-mt^{SHR}. This work adds to the body of evidence that quantitative variations in gene expression

at loci encoding components of the renin-angiotensin-aldosterone system may be genetically linked to or associated with physiological alterations in blood pressure. Though the RAS pathway has been a major therapeutic target for decades, understanding the heritability of expression of these targets can help switch the focus from treatment to prevention. The role of AT_1 receptors in individual tissues and their differential expression provides valuable information on how personalized therapy can be used to better treat or prevent cardiovascular disease in the future.

3.5 Acknowledgments

The authors would like to acknowledge the assistance of the following student lab assistants that worked tirelessly in the development of the congenic colony and phenotype screening of each colony generation: Aaron Fain, Erin Wyatt, Samuel Carmichael II, Timothy Bradshaw and Adesuwa Ighodaro.

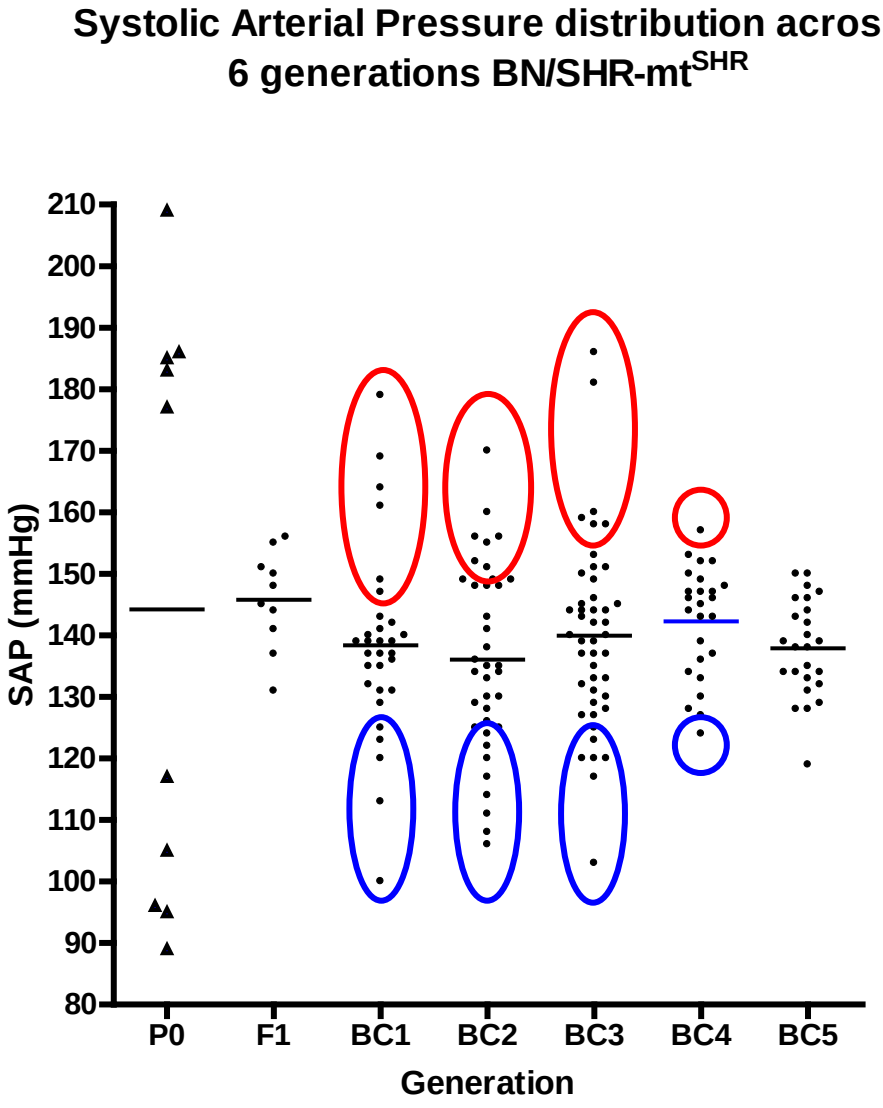

Figure 3.1: Six generations of BN/SHR-mt^{SHR} rats with corresponding average systolic pressure (SAP) values. Three distinct populations persisted throughout all 6 generations, with hypertension being dominantly expressed and maintained despite the reduction of the SHR nuclear genome. HT animals used are indicated by the red circle. NT animals used are indicated by the blue circle.

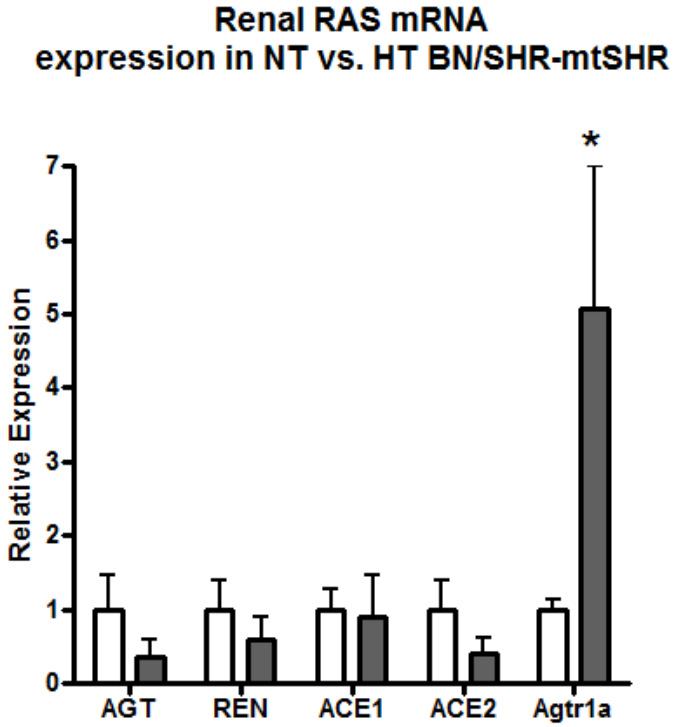

Figure 3.2: Quantitative Real-Time PCR of renal *AGT, REN, ACE1, ACE2* and *Agtr1a* in age and sex matched BC3 HT and NT rats. Data were normalized to *GAPDH* RNA from same samples (NT n=20; HT n=20). There was no difference in *AGT, REN, ACE1 and ACE2* expression HT BC3 rats compared to NT BC3 rats. *Agtr1a* mRNA levels were ~5X higher in HT rats compared to NT rats.

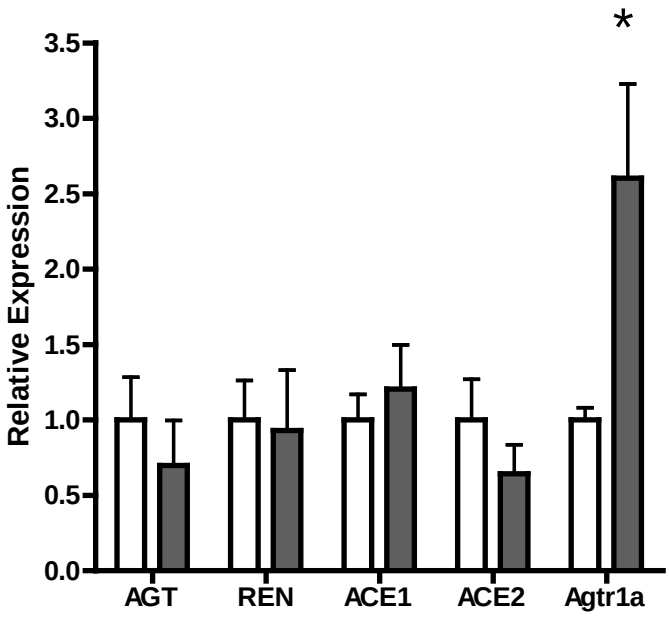

Figure 3.3: Quantitative Real-Time PCR of renal *AGT, REN, ACE1, ACE2* and *Agtr1a* in age and sex matched HT and NT rats representative of all generations in the colony. Data were normalized to *GAPDH* RNA from same samples (NT n=20; HT n=20). There was no difference in *AGT, REN, ACE1 and ACE2* expression in HT compared to NT rats. *Agtr1a* mRNA levels were ~2.5X higher in HT rats compared to NT rats.

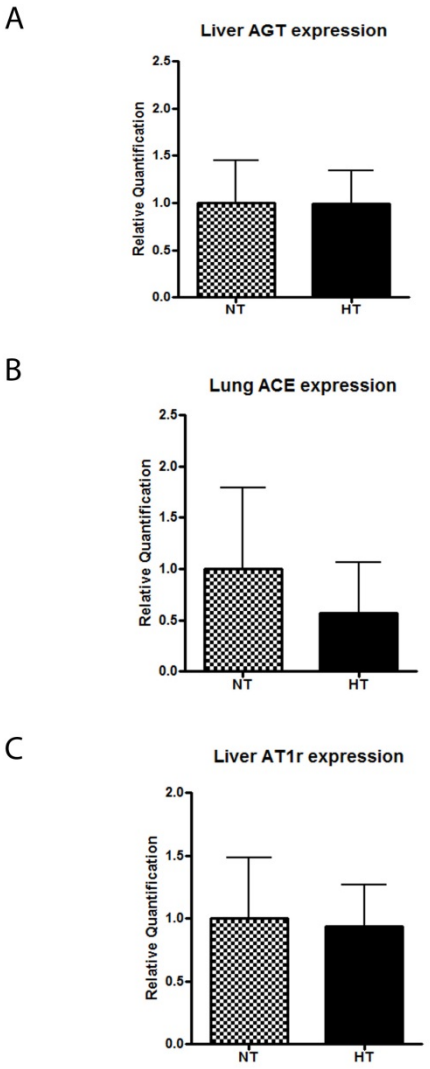

Figure 3.4: Quantitative Real-Time PCR of liver *AGT* (Figure 3.4A), lung *ACE1* (Figure 3.4B) and liver *Agtr1a* (Figure 3.4C in BC3 HT and NT rats. Data were normalized to *GAPDH* RNA from same samples (NT n=6; HT n=5). There was no difference in liver *AGT,* lung *ACE1 or* liver *Agtr1a* mRNA expression in HT BC3 rats compared to NT BC3 rats.

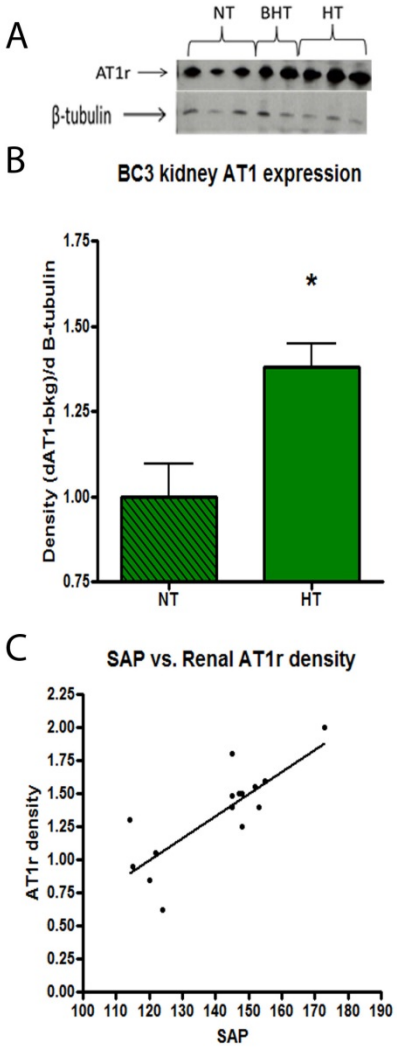

Figure 3.5: Semiquantitative immunoblotting of whole kidney tissue homogenates of BC3 HT and NT rats. A: Representative blot of AT1r and β-tubulin. B: Illustrates quantified densitometry of hypothalamic AT1r protein. HT rats exhibited significantly higher AT1r protein expression compared to NT rats ($p<0.05$). C: Regression analysis between SAP and AT1r protein expression in hypothalamic homogenates. Average SAP and AT1r protein expression were positively correlated ($r^2=0.6502$, * = $P<0.05$).

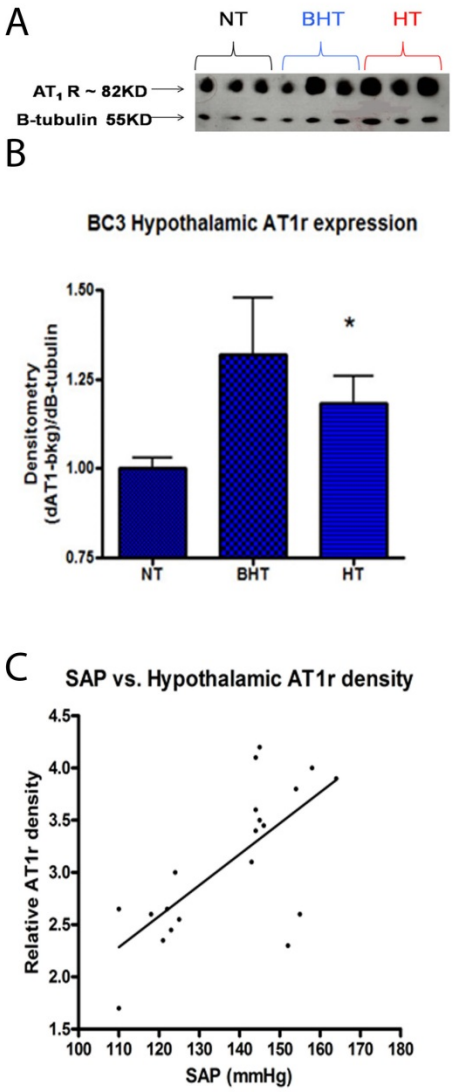

Figure 3.6: Semiquantitative immunoblotting of AT_1 receptor protein from hypothalamus homogenates of BC3 rats. A: Representative blot comparing NT, BHT and HT AT1r densities. B-tubulin was used as housekeeping protein. B: Illustrates quantified densitometry of hypothalamic AT1r protein. HT rats exhibited significantly higher AT1r protein expression compared to NT rats ($p<0.05$). C: Regression analysis between SAP and AT1r protein expression in hypothalamic homogenates. Average SAP and AT1r protein expression were positively correlated ($r^2=0.4781$, * = $P<0.05$).

CHAPTER 4

SODIUM BALANCE AND RENAL FUNCTION IN BN/SHR-mt^{SHR} RATS

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Abbreviations used: CVD, cardiovascular disease; [Na^+], sodium concentration; [Cl^-], chloride concentration

Keywords: Sodium balance, pressure-natriuresis, salt-sensitivity, kidney function

Manuscript in Preparation

4.1 Introduction

The relationship between dietary NaCl intake and the development of hypertension has been the subject of much debate for several decades (Dahl 1961; MacGregor 1985; Osborn and Camara 1997). In societies where salt intake is less than 50 to 100 mmol/d, hypertension and cardiovascular disease (CVD) are rare, and the frequency of hypertension and CVD increase at higher levels of salt intake (Weinberger 1996). Pharmacological therapy with natriuretic agents has been very successful, and is still considered a first line therapy in the treatment of hypertension (Weinberger 1996). However, epidemiological observations have established profound heterogeneity between salt intake and hypertension. There is evidence that genetic factors, particularly alterations in genes of known osmoregulatory roles, play a very significant role in the deleterious impact of hypertension on the progression of renal disease (Rostand, Kirk *et al.* 1982). For instance, the risk of developing end-stage renal disease is 10- to 20-fold greater in black Americans than in Caucasian American patients with essential hypertension, and this increased risk cannot readily be explained by differences in the severity or treatment of hypertension (Rostand, Kirk *et al.* 1982). One major difference is salt sensitivity. Greater sodium retention is thought to underlie BP determining physiology of blacks, and though the renin-angiotensin system has been implicated, the exact mechanisms are currently unknown (Tu, Eckert *et al.* 2014). The renin–angiotensin system, for example, is more likely to be suppressed in blacks than in whites, consistent with greater volume expansion from sodium accumulation and water retention (HELMER and

JUDSON 1968; Wilson, Bayer et al. 1999). In addition, BP in blacks in contrast to BP in whites typically increases in response to an increase in sodium intake, i.e. greater NaCl sensitivity (Weinberger 1996). Whether blacks have a unique renal physiology that puts them at risk for a more aggressive form of hypertension is unclear. What is clear, is that NaCl sensitivity, or possibly the inability to "sense" NaCl intake can have a profound effect on arterial pressure, the pressure-diuresis-natriuresis relationship and subsequent development of hypertension. Like humans, genetically similar rodent models responding differently to NaCl intake have been established. The SHR parallels the most common form of idiopathic hypertension and that of most Caucasian Americans, in that most SHR strains are not salt sensitive. Studies by Lundin *et al.* (1982) as well as Greenberg and Osborn (1994) have shown that renal retention of sodium in water should not be of pathogenic importance in identifying causes of hypertension in the SHR. Still, hyperosmotic saline, as well as Ang II injected ICV in the SHR and other rodents alters renal sodium handling and increases BP, which may be mediated by the activation of renal sympathetic nerve activity (Osborn and Camara 1997; Guadagnini and Gontijo 2006).

To further understand the mechanisms of sodium sensing and potential altered renal sodium handling in rats stemming from the SHR, we utilized our unique conplastic rodent model to evaluate acute renal responses to altered NaCl concentrations, when rats were able to titrate their own sodium load. It is hypothesized that hypertensive BN/SHR-mt^{SHR} will be unable to appropriately

“sense” sodium load, hence ingesting more NaCl, and potentially alter renal sodium handling compared with NT BN/SHR-mt^{SHR}.

4.2 Methods and Materials

4.2.1 Animals

Male rats generated from sibling-sibling crosses of a BC5 generation from the BN/SHR-mt^{SHR} colony described in detail previously were used for the acute sodium intake aspect of the experiment. All animals were maintained in temperature controlled rooms with 12:12 h-light/dark cycle. Rats were raised from weaning on normal NaCl chow (0.8% NaCl; Dyets, Bethlehem, PA) and given water *ad libitum*. Care of the rats before and during experimental procedures was conducted in accordance with the policies of the National Institutes of Health guidelines for the care and use of laboratory animals. All protocols had received prior approval by the Institutional Animal Care and Use Committee at the University of Kentucky.

4.2.2 Experimental Protocols

Twelve week old male rats weighing 300-400g were chosen for the experiment. Rats were housed in metabolic pens and allowed distilled water and NaCl-free chow *ad libitum* for the entirety of the 17-day experiment. Rats titrated Na^+ intake by drinking 0.9% saline for 2 acclimation days, followed by 5 experimental days. On the fifth day, BP was measured and rats were then switched to 2.0% hypertonic saline for 5 experimental days. On day 10, BP was measured and rats were switched back to 0.9% saline for 5 experimental days (**Figure 4.1A**).

4.2.3 Measurement of Arterial Pressure

Rats previously phenotyped as normotensive (NT: SAP ≤ 124mmHg; n=6) or hypertensive (HT: SAP ≥ 145mmHg; n=6) were utilized for this study. Since animals were to be back-bred to the founder males in the establishment of the conplastic genome, tail cuff plethysmography was used as a phenotyping methodology only to establish basic individual blood pressures. To minimize stress and improve reliability of blood pressure measurements, several steps were used in the blood pressure recording method that has been previously characterized and published (Kurtz, Griffin *et al.* 2005). Rats were exposed and acclimated to the measurement procedures and restraint equipment prior to BP recordings. A dark cover was placed over the restrained animal for the duration of the BP measurement, and BP recordings were performed at the same time each day. All equipment was thoroughly cleaned and disinfected before and after each individual rat to eliminate foreign scent. Animals were moderately warmed to dilate the ventral artery. Arterial pressures were derived from the average results of ≥5 measurements in each recording session. Both systolic and diastolic pressures were obtained and recorded. For purposes of reporting, the systolic pressures were used for the determination of the specific individual phenotype.

4.2.4 Determinations

Sodium and water intake were determined daily by weighing calibrated bottles every 24 hours. Sodium intake was calculated as a product of the volume ingested and sodium concentration. Urine was collected and the volume was determined in calibrated cylinders positioned directly under the pens. Urinary sodium concentration was determined by flame photometry, and urinary sodium excretion was calculated from the product of urine flow rate and sodium concentration. Sodium balance averaged over the experimental periods was determined as the difference between sodium excretion and sodium intake. Urinary protein concentration was measured by the method of Lowry *et al.* (1951), and protein excretion was calculated as the product of urine flow rate and urinary protein concentration. Bovine serum albumin was used as the standard.

4.2.5 Statistical analysis

Blood pressures, average sodium intake/excretion, water intake, sodium balance, AT_1r protein expression and urinary protein between NT and HT BN/SHR-mt^{SHR} rats were analyzed using an one-way ANOVA. Daily sodium intake/excretions were analyzed using a two-way ANOVA. The 0.05 level of probability was utilized as the criterion of significance. All statistical analyses were performed using GraphPad Prism 4 (GraphPad Software, Inc., La Jolla, CA).

4.3 Results

4.3.1 Arterial pressure did not change during acute sodium challenge.

SAP was significantly higher in the HT BN/SHR-mt^{SHR} (SAP=157.0±5.2 mmHg; n=6) compared with NT BN/SHR-mt^{SHR} (SAP=124.2±5.5 mmHg; n=6) to begin the study. SAP did not change ($p>0.05$) during any of the three phases of the experiment (**Figure 4.1B**).

4.3.2 Sodium intake was maintained when offered 2.0% saline in HT while NT BN/SHR-mt^{SHR} decreased their sodium intake.

Average daily sodium intake for NT and HT BN/SHR-mt^{SHR} rats is displayed in **Figure 4.2A**. Average 5-day sodium intake was significantly reduced in NT BN/SHR-mt^{SHR} when offered 2.0% hypertonic saline (166.3±21 to 79.554±10 µEq/day; $^{\#}p<0.05$), which remained suppressed when again offered 0.9% saline (69.14±14 µEq/day; $^{\#}p<0.05$; **Figure 4.2B**). HT BN/SHR-mt^{SHR}, however, maintained sodium intake when offered 2.0% hypertonic saline (127.5±36 to 147.9±24.9 µEq/day), that was maintained when again offered 0.9% saline (89.7±9.8 µEq/day; **Figure 4.2B**). HT BN/SHR-mt^{SHR} failed to "sense" hypertonic saline challenge, therefore failed to reduce sodium intake (**Figure 4.2B**).

4.3.3 Water intake increased in both HT and NT when offered hypertonic saline.

Average 5-day water intake was significantly increased in NT BN/SHR-mt^{SHR} when offered 2.0% hypertonic saline (4.2±0.37 to 11.1±0.628 ml/day; $^{\#}p<0.05$), and NT rats maintained an increased water intake when again offered 0.9% saline (9.14±0.864 ml/day; $^{\#}p<0.05$; **Figure 4.3**). HT BN/SHR-mt^{SHR} also increased water intake when offered 2.0% hypertonic saline (5.3±1.0 to 8.9±0.231 µEq/day; $^{\#}p<0.05$; **Figure 4.3**), however water intake values were not different in HT rats when again offered 0.9% saline (6.4±0.28 ml/day; **Figure 4.3**).

4.3.4 Sodium Excretion was higher in HT compared to NT BN/SHR-mt^{SHR}.

Average daily sodium excretion for NT and HT BN/SHR-mt^{SHR} rats is displayed in **Figure 4.4A**. In accordance with elevated NaCl intake, average 5-day sodium excretion was elevated in HT BN/SHR-mt^{SHR} when offered 2.0% hypertonic saline (271.7±25.1 to 331.8±31.7 µEq/day), and HT rats maintained an elevated sodium excretion when again offered 0.9% saline (313.22±18.1 µEq/day; **Figure 4.4B**). NT BN/SHR-mt^{SHR}, however, maintained sodium excretion when offered 2.0% hypertonic saline (220.5±57.7 to 229.6±52.2 µEq/day), and NT rats maintained same sodium excretion when again offered 0.9% saline (191.3±39.4 µEq/day; **Figure 4.4B**). HT rats did, however, excrete more NaCl during the last 0.9% phase than NT rats (*$p<0.05$; **Figure 4.4B**).

4.3.5 Sodium Balance was More Negative in HT BN/SHR-mt^{SHR}.

Cumulative daily sodium balance in illustrated in **Figure 4.5A**. HT rats exhibited greater variability in maintaining sodium balance during the three experimental phases. Average 5-day sodium balance for NT and HT BN/SHR-mt^{SHR} is displayed in **Figure 4.5B**. NT averaged (0.9%:-53.6±17.9 µEq/day; 2.0%:-120.1±8.8 µEq/day; 0.9%: -122.17±7.5 µEq/day; **Figure 4.5B**) compared to HT averages (0.9%: -151.0±29.2 µEq/day; 2.0%: -183.9±18.7 µEq/day; 0.9%: -226.9±15.5 µEq/day; **Figure 4.5B**). HT rats exhibited greater sodium natriuresis in all three experimental phases due to elevated arterial pressure (*p<0.05).

4.3.6 Urinary Protein Excretion was Not Different Between NT and HT BN/SHR-mt^{SHR}.

Urinary protein excretion was maintained and normal in NT and HT BN/SHR-mt^{SHR} for the entire experimental protocol. For the three experimental saline trials, NT rats averaged (0.9%:476.7±66 µg/day; 2.0%:557.1±71 µg/day; 0.9%: 441.7±63 µg/day; **Figure 4.6**) compared to HT averages (0.9%: 328.2±46 µg/day; 2.0%: 413.3±62 µg/day; 0.9%: 299.7±29 µg/day; **Figure 4.6**). Therefore, renal function appeared to be normal in NT and HT BN/SHR-mt^{SHR}.

4.4 Discussion

The necessity to maintain salt and water balance is an important aspect of land dwelling animals. Alterations in this balance can significantly impact cardiovascular health and disease. Epidemiological studies have demonstrated a positive correlation between NaCl intake and elevated blood pressure (Grim, Luft *et al.* 1980; Weinberger 1996; Dahl 2005). Elevated NaCl intake may lead to the development of hypertension and cardiovascular complications by signals triggered by augmented extracellular [Na^+] , extracellular [Cl^-] , and/or osmolality of extracellular fluids (Orlov and Mongin 2007). The present study was designed to evaluate the influence of acute high NaCl on renal sodium handling and BP in rats stemming from the SHR. This study is unique, in that the experimental paradigm allowed rats to titrate their NaCl intake, providing useful information as to the rats ability to "sense" sodium load. Both the kidney and the CNS are involved in sodium sensing via at least two different mechanisms: Cl^- and Na^+ sensing in the tubular fluid and plasma osmolality in the CSF. In the kidneys, salt-sensing and the regulation of sodium excretion is provided mainly via TGF. TGF is triggered immediately after an elevation of salt concentration in the tubular fluid delivered to the JGA and results in the contraction of VSMCs of afferent arterioles, thus causing increases in the exposure of proximal tubules to high-salt fluid via the attenuation of glomerular capillary pressure and GFR. CNS [Na^+] sensors are primarily located in the circumventricular organs, separate structures that line brain ventricles and consist of the SFO, MnPO, and OVLT (Orlov and Mongin 2007). In these brain areas, the blood-brain barrier is

partially open due to the presence of fenestrated capillaries (Bourque and Oliet 1997; Hussy, Deleuze *et al.* 2000). This feature makes the circumventricular organs an optimal location for direct sensing of the ionic composition of plasma. SFO and OVLT may influence electrical activity of the magnocellular neurons in the SON and PVN of the hypothalamus, both of which secrete AVP into the circulation (Denton, McKinley *et al.* 1996; Hussy, Deleuze *et al.* 2000). Vasopressin is produced by the magnocellular neurosecretory cells of the hypothalamic SON and PVN, which both project to the median eminence in the neurohypophysis. Such regulation occurs via direct excitatory projections to the SON and PVN or indirectly via projections to the MnPO, a nucleus that also innervates both the SON and PVN (Hussy, Deleuze *et al.* 2000). Interestingly, the PVN and SON possess their own intrinsic [NaCl]-sensing mechanism, which adds additional complexity to the CNS regulation of salt intake and secretion. This mechanism involves sensing changes in CSF osmolality rather than alterations in extracellular [Na^+] (Mason 1980; Oliet and Bourque 1992).

Leenen and coworkers (Budzikowski, Huang *et al.* 1998; Huang, Amin *et al.* 2006) were the first to propose that blood pressure elevation in animal models of hypertension is triggered by augmented NaCl delivery to the CNS. Elevated NaCl diets may significantly affect the regulation of blood pressure and fluid and electrolyte balance by way of changes in the sensitivity of the brain to circulating or centrally generated humoral factors (Wilson, Bayer *et al.* 1999; Guadagnini and Gontijo 2006). For example, Andersson showed that hypertonic saline

injected into the hypothalamus of the goat induced a large dipsogenic response (Andersson, Dallman *et al.* 1969). Additionally, when hypertonic saline is administered into the CSF of the third ventricle, animals drink more water and vasopressin is released from the neurohypophysis (Andersson and Olsson 1973). Though HT BN/SHR-mt^{SHR} were able to excrete their increased sodium intake, results demonstrate a potential decreased ability to sense sodium load, likely via central mechanisms in brain. Though several mechanisms could be involved, we postulate that augmented RAS, in particular, elevated AT1r expression may be related to the reduced sodium sensing ability in HT BN/SHR-mt^{SHR} (**Figure 4.7**).

It is well documented that endogenous Ang II may contribute to the development of certain forms of experimental hypertension, including the SHR. The cardiovascular effects of centrally administered Ang II also may be related to NaCl intake. It has been reported that changes in NaCl intake may be accompanied by changes in the density and affinity of Ang II receptors in hypothalamic areas of the brain involved in cardiovascular, fluid, and electrolyte regulation (Bickerton and Buckley 1961). Systemic and brain Ang II has been shown to participate in blood pressure and salt and water balance through a variety of mechanisms including sympathetic outflow, stimulation of vasopressin release (Yang, Jin *et al.* 1992). Additionally, brain Ang II alters thirst and salt appetite, which plays a major role in fluid and electrolyte regulation (Epstein, Fitzsimons *et al.* 1970; Fluharty and Epstein 1983). Dukacz, *et al.* (2003)

previously demonstrated that long term inhibition of RAS with captopril decreased blood pressure in the SHR, resulting in a leftward shift in the pressure-natriuresis relationship. This is significant, as results from our lab demonstrate that HT BN/SHR-mt^{SHR} have significantly more hypothalamic AT_1r protein compared with NT HT BN/SHR-mt^{SHR}, indicating a potential relationship between hypothalamic AT_1r expression and reduced salt-sensing ability in HT BN/SHR-mt^{SHR} (**Figure 4.7**). An increase in both gene and protein expression of renal AT_1r has also been shown in HT BN/SHR-mt^{SHR} (Collett, Hart *et al.* 2013). Taken together, the presence and abundance of AT_1r in the hypothalamus or kidney is related to the HT phenotype in BN/SHR-mt^{SHR}, and its role may be involved in the chronic rightward shift of the renal function curve and/or a reduced ability of sodium sensing (**Figure 4.7**).

The reduced ability to sense salt in HT BN/SHR-mt^{SHR} and the compensatory elevation of blood pressure to keep the rats in sodium balance may have something to do with activation of renal sympathetic nerve activity. The elevated level of efferent sympathetic tone in the SHR may alter the renal handling of sodium and water. Renal nerve stimulation directly affects both proximal tubular sodium reabsorption and renin release (Osborn, Holdaas et al. 1983; Chen and Toney 2001). Greenberg *et al.* (2000) demonstrated the importance of the renal nerves in mediating sodium excretion in response to sodium load in SHRs. Camara and Osborn (2005) demonstrated that hypertension induced by chronic left ventricular Ang II infusion is dependent upon activation of renal sympathetic

nerve activity and is associated with sodium retention. Furthermore, Camara and Osborn showed that lifelong increases in NaCl intake may sensitize the CNS to low doses of Ang II, causing increases in renal sympathetic nerve activity mediated by brain AT_1r. Therefore, we postulate that lifetime desensitization of orally ingested NaCl causes profound pressure-natriuresis that may be mediated, in part, by activation of renal sympathetic nerves and AT_1r (**Figure 4.7**).

In summary, we present data that demonstrate that HT BN/SHR-mt^{SHR} have a reduced ability to sense elevated sodium when given the option to titrate their own NaCl load, and this may be related to tissue specific expression of AT_1r and/or elevated sympathetic nerve activity. However, it appears that HT BN/SHR-mt^{SHR} have normal kidney function and are operating within normal kidney natriuresis with increased sodium intake, which agrees with previous studies that sodium retention is not of pathogenic importance in the SHR (Lundin, Herlitz *et al.* 1982; Greenberg and Osborn 1994). It appears then, that the elevation of arterial pressure in SHR is important to maintain daily sodium balance, i.e., functional pressure-natriuresis. Hence, HT BN/SHR-mt^{SHR} is truly a model of “essential” hypertension. It is concluded then, that long-term increases in arterial pressure are necessary to maintain sodium balance, and that this is an important aspect of development and maintenance of spontaneous hypertension.

4.5 Acknowledgements

The authors would like to acknowledge the assistance of the following student lab assistants that worked tirelessly in the development of the conplastic colony and phenotype screening of each colony generation: Aaron Fain, Erin Wyatt, Samuel Carmichael II, Timothy Bradshaw and Adesuwa Ighodaro. The authors would also like to thank Kristen Blaker for her assistance in measuring urinary protein excretion.

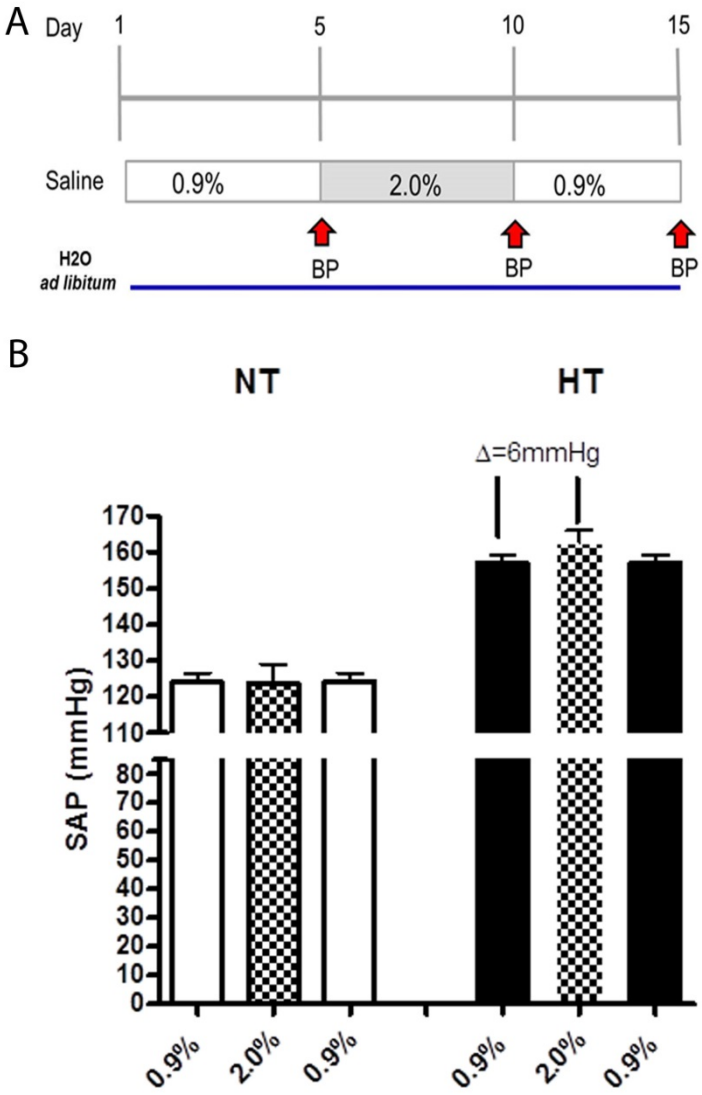

Figure 4.1: Experimental schema of the acute salt study that allowed for rats to titrate their own salt and water. A: Illustrates the experimental design, with three experimental phases. Rats were given 0.9% saline for the first 5 days, followed by hypertonic 2.0% for 5 days, and then returned to 0.9% saline. Blood pressures were taken after each of the three phases. B: Illustrates BP differences throughout the entirely of the experiment. BP was elevated in HT rats compared with NT rats, however, BP did not change with acute saline challenge ($p>0.05$).

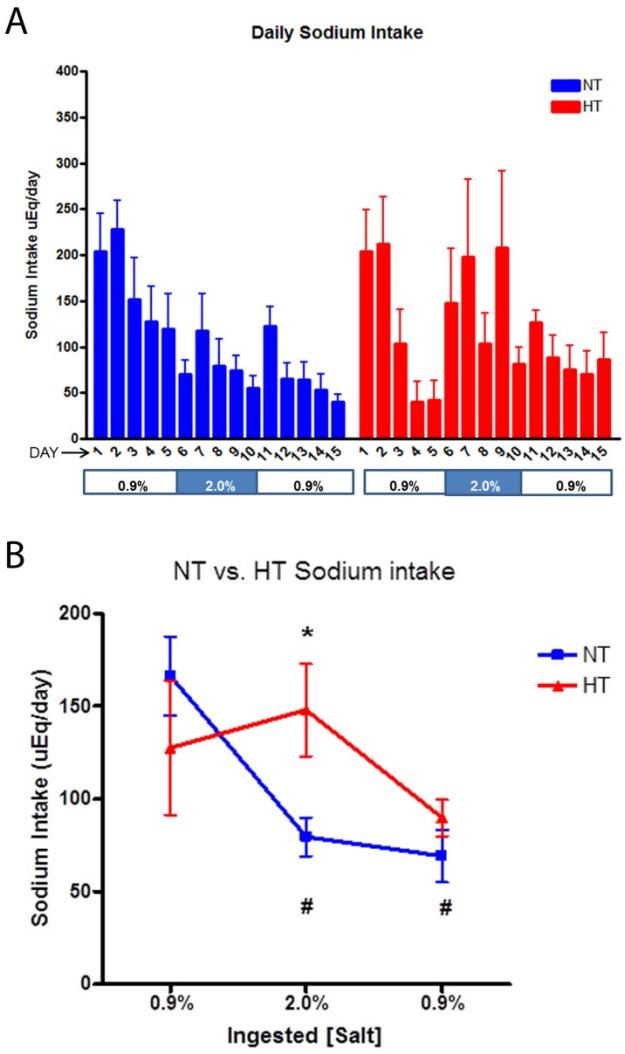

Figure 4.2: Sodium intake throughout course of the experiment. A: Illustrates the average daily sodium intake (µEq/day) for NT and HT rats. B: Illustrates the average 5-day sodium intake for NT and HT rats. HT rats ingested more sodium than NT rats when offered 2.0% saline (*p<0.05), however, HT rats ingested similar amounts of sodium in all three phases of the experiment. NT rats ingested significantly less sodium (#p<0.05) when offered 2.0% saline, and continued to eat less when again offered 0.9% saline.

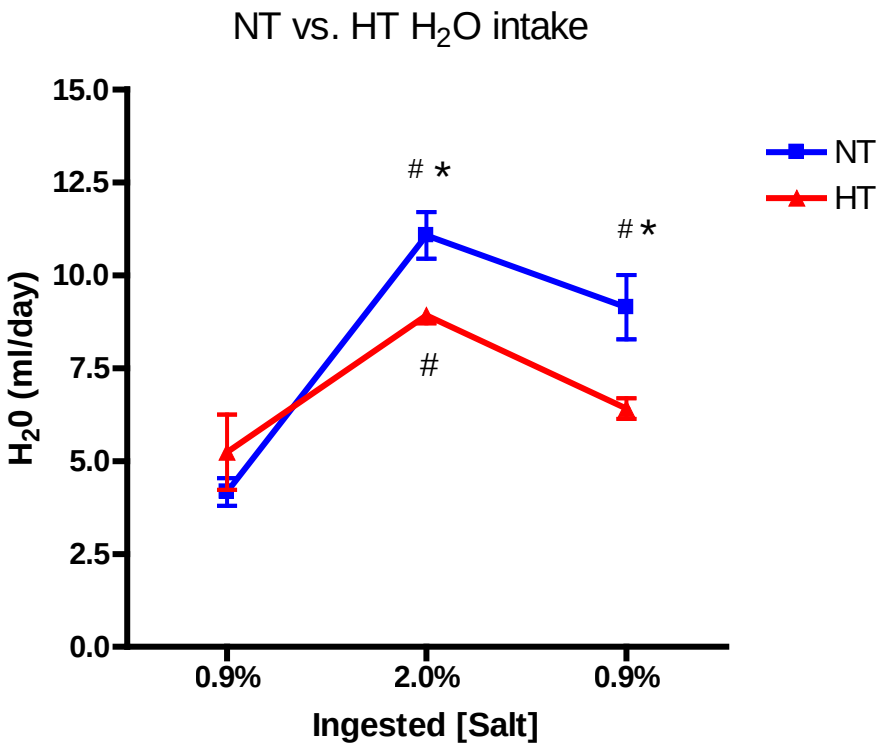

Figure 4.3: Average 5-day water intake (ml/day). NT rats ingested significantly more water (#p<0.05) when offered 2.0% saline and again offered 0.9% saline. NT rats also ingested significantly more water than HT rats (*p<0.05). HT rats ingested significantly more water than when offered 2.0% saline (#p<0.05), but ingested similar amounts when again offered 0.9% saline.

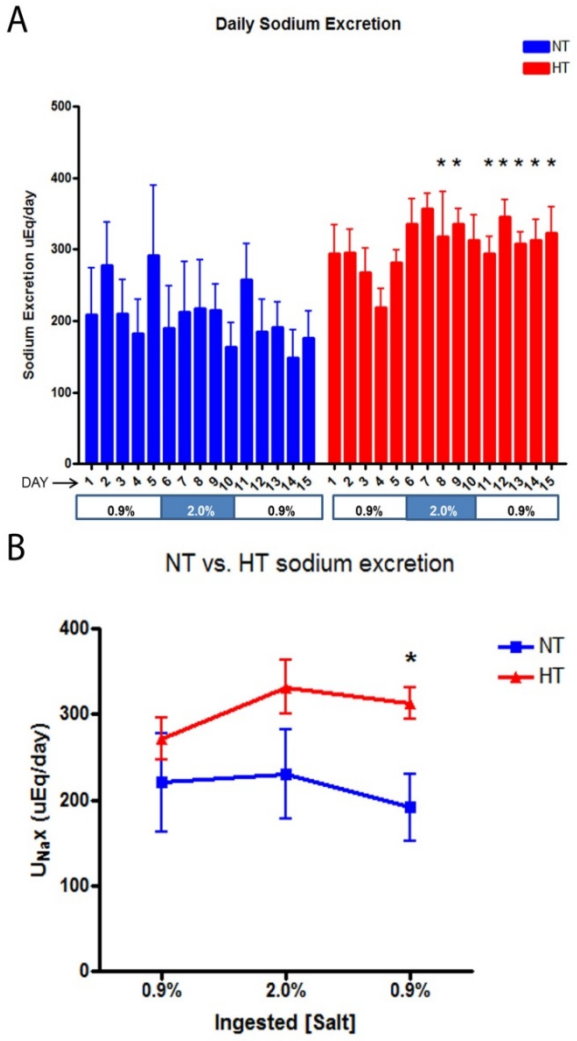

Figure 4.4: Sodium excretion throughout the course of the experiment. A: Illustrates the average daily sodium excretion (µEq/day) for NT and HT rats. B: Illustrates the average 5-day sodium excretion for NT and HT rats. HT rats excreted more sodium than NT rats when offered 2.0% saline ($p>0.05$), however, this amount did not reach statistical significance until rats were again offered 0.9% saline (*$p<0.05$). HT and NT rats maintained similar amounts of sodium in all three phases of the experiment.

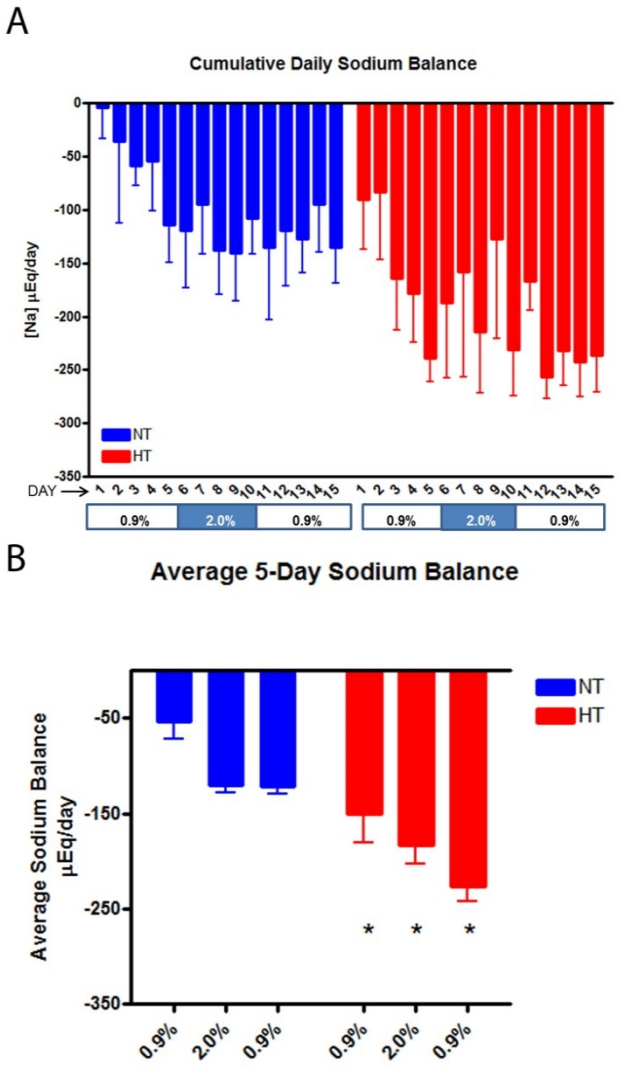

Figure 4.5: Sodium balance throughout the course of the experiment. A: Illustrates the cumulative daily sodium balance (µEq/day) for NT and HT rats. B: Illustrates the average 5-day sodium balance for NT and HT rats. Both NT and HT rats were in negative sodium balance throughout the course of the experiment, however HT rats exhibited greater variability while maintaining elevated sodium natriuresis (*$p<0.05$). This may be due to elevated arterial pressures.

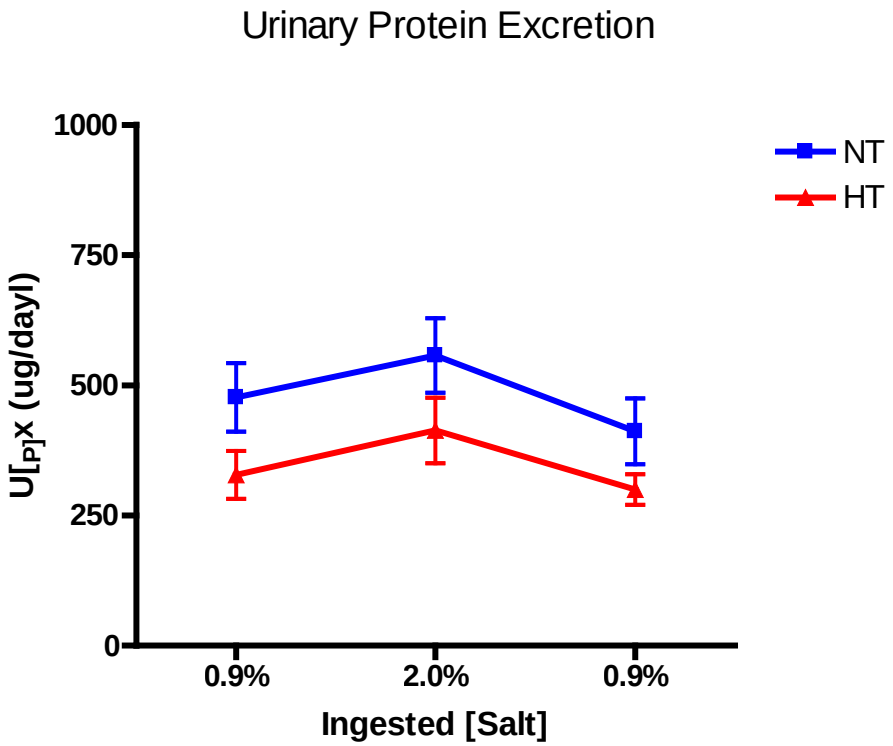

Figure 4.6: Urinary protein excretion was maintained and normal in NT and HT BN/SHR-mt^{SHR} for the entire experimental protocol. Therefore, renal function appeared to be normal and not different between NT and HT BN/SHR-mt^{SHR} ($P>0.05$).

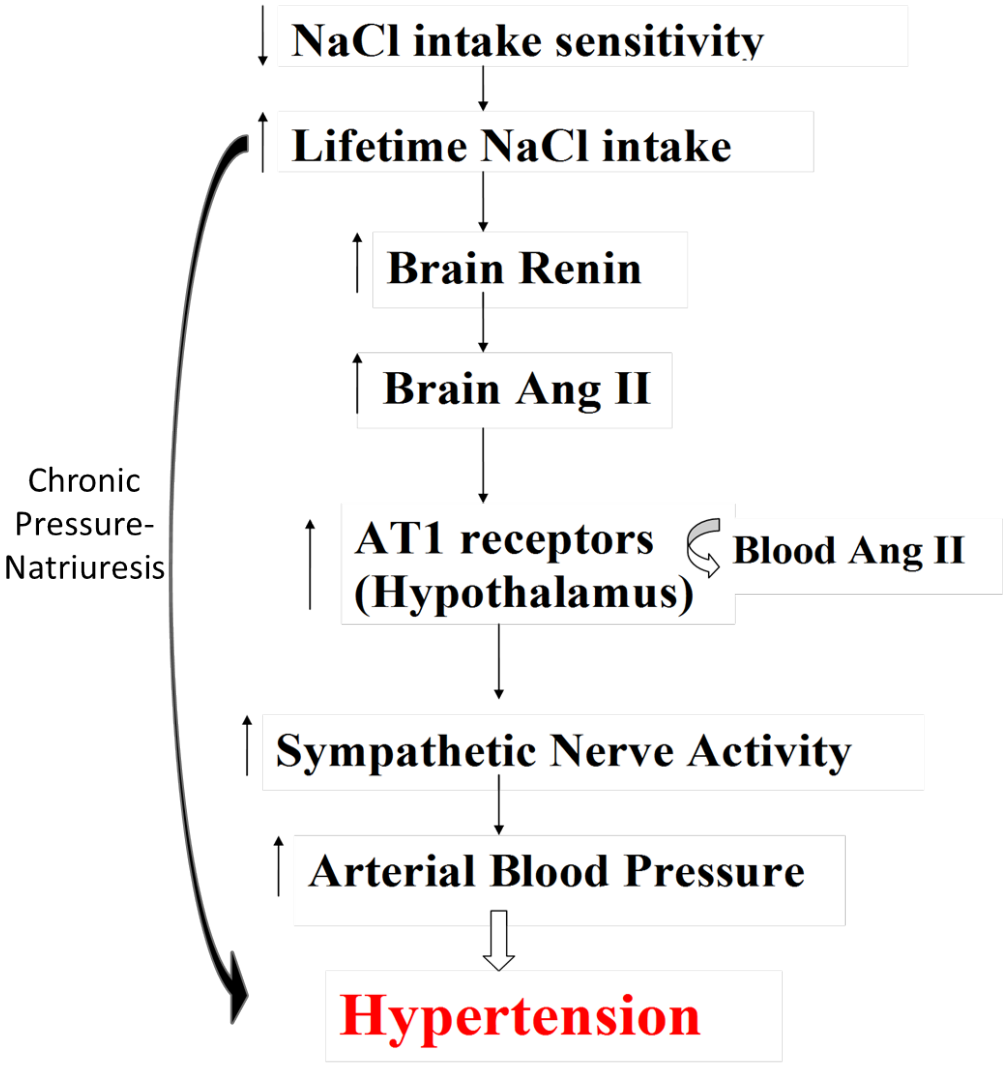

Figure 4.7: Hypothetical pathway in which long term reduced NaCl sensitivity could activate the RAS, increasing sympathetic nerve activity and increases in arterial pressure. Chronic pressure-natriuresis may be "essential" in the SHR, therefore underlie the development and maintenance of elevated arterial pressure.

CHAPTER 5

KIDNEY-SPECIFIC REDUCTION OF OXIDATIVE PHOSPHORYLATION GENES DERIVED FROM SPONTANEOUSLY HYPERTENSIVE RAT AND INCIDENCE OF HYPERTENSION

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Abbreviations used: AMPK, AMP kinase; CREB, cAMP response element-binding protein; CO, cytochrome oxidase;

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5.1 Introduction

Hypertension constitutes a primary and significant factor in the development of cardiovascular disease. Despite major gains in the long-term treatment of hypertension, cardiovascular disease remains the number one cause of death and disability in developed countries. Primary or essential hypertension is regarded as a multi-factorial disease, influenced by both genetic inheritance and environmental conditions that influence gene expression. The genetic basis of hypertension has been focused primarily on inheritance and expression of nuclear genes (Tsuchida, Liu et al. 1994), despite the fact that mitochondria are present in multiple copies in each cell and have their own genome. Additionally, and potentially of great importance, is the fact that OXPHOS depends on the coordinated expression of two separate but interactive genomes, nuclear and mitochondrial. Numerous trans-factors involved with mtDNA replication, transcription and mRNA processing are nuclear encoded, including mtRNA polymerase, mtDNA polymerase, several regulatory transcription factors and mtRNA processing proteins (Hein and Kobilka 1995). This nuclear-mitochondrial interaction is essential to cellular health and function, and therefore may play a large role in the development of disease (Nowak 2002).

Mitochondrial dysfunction has been implicated in a wide variety of genetic disorders (Wallace 1999; Taylor and Turnbull 2005) and alterations in mitochondrial function have been observed in conjunction with aging and development of hypertension in both rodents and humans (Schwartz, Duka *et al.*

2004; Wallace 2005; Chan, Wu *et al.* 2009; Kumarasamy, Gopalakrishnan *et al.* 2010). Recently, mitochondrial transfer RNA (tRNA) mutations were observed in a genetically focused population with a high incidence of essential hypertension (Osanai, Tanaka *et al.* 2001; Li, Liu *et al.* 2009). Wilson *et al.* (2004) have described a correlation between a T4921C transition SNP, which lies in the mitochondrial $tRNA^{Ile}$ gene (GenBank accession no. NC_001807) and hypertension. The genetic association of mtDNA variants and tRNA mutations (Geraldes and King 2010) to type 2 diabetes and hypertension directly implicated mitochondrial dysfunction in development of cardiovascular disease and metabolic syndrome (Wallace 2005). Taken together, there is significant evidence that altered mitochondrial genetic expression may have a significant role in the generation of cardiovascular disease phenotypes. However, the regulatory mechanisms in which mitochondria and mitochondrial genes are expressed and regulated in disease is mostly unknown.

In contrast, the role of the kidney in long-term blood pressure regulation and manifestation of hypertension is well known. The importance of kidney function to modulate blood pressure has been shown by Lifton *et al.* (2001) demonstrating genetic variants in important renal pathways underlie all of the Mendelian disorders affecting blood pressure homeostasis (Inagaki, Churchill et al. 2006). Therefore, it is postulated that altered renal expression of genes may contribute significantly to the disease. The goal of the present study was to determine the renal expression of mitochondrial protein-coding genes, the nuclear pathway that

regulates their expression and the relationship between heritable hypertension in a rat strain with localized mt-DNA from Okomoto-Aoki SHR. The “conplastic” strain was developed by crossing a hypertensive female SHR with normotensive, male BN rats (BN/SHR-mt^{SHR}) (Collett, Hart *et al.* 2013). Hypertensive female offspring then were phenotypically selected and crossed with founder males for several generations. All offspring had identical mitochondrial DNA of the progenitor SHR, barring any mutation. The results of these studies document significant reduced expression of renal mtRNA and nuclear encoded regulatory elements in hypertensive male and female offspring. The data suggest that reduced renal mtRNA expression may elicit hypertension by decreased OXPHOS.

5.2 Methods and Materials

5.2.1 Animals

All experiments were carried out in accordance with the AAALAC Guide to the Care and Use of Laboratory Animals and all protocols were previously approved by the University of Kentucky Institutional Animal Care and Use Committee. A "conplastic" colony using phenotypic selection was employed. The development and phenotypic characterization have been described in detail elsewhere (Collett, Hart *et al.* 2013). The Aoki-Okamoto SHR/Brown Norway rat colony was developed by breeding a female SHR (Charles River Labs, Wilmington, MA) with 2 different normotensive BN males (Charles River Labs, Wilmington, MA) (BN/SHR-mt^{SHR}). Beginning at 10 weeks of age, rats were phenotyped using tail cuff plethysmography (Kent Scientific, Torrington, CT). Hypertensive female offspring were then back-crossed to the original progenitor BN males for 5 subsequent generations.

After repeated blood pressure recordings that assured consistent determination of arterial pressure, rats not scheduled for rebreeding were euthanized with an overdose of sodium pentobarbital (60 mg/kg i.p)., and organ tissues were rapidly frozen and stored for later analysis.

5.2.2 Measurement of arterial pressure.

Systolic arterial pressure (SAP) was evaluated using in parents and offspring beginning at 10-12 weeks of age. Phenotypes were assigned as either normotensive (NT: SAP ≤ 124mmHg), borderline hypertensive (BHT: 125 ≤ SAP < 150 mmHg) or hypertensive (HT: SAP ≥ 150mmHg). Arterial pressure was derived from the average results of ≥5 measurements in each recording session. The average blood pressures of ≥5mmHg separate recording sessions with <5% variability were used to establish the phenotype of each animal. Systolic pressures were used for the determination of the specific individual phenotype (Collett, Hart *et al.* 2013) .

5.2.3 RNA Extraction and RT-PCR.

Renal cortex and outer medulla homogenate, liver and left ventricular cardiac tissue were selected from HT and NT BN/SHR-mt^{SHR} rats (n=20 NT; n=20 HT) as described above. Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) and purified using RNeasy minicolumns (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. Possible genomic DNA was digested with DNase I (Qiagen Inc., Valencia, CA). Concentration and purity of all RNA samples was determined by the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Extracted RNA was reverse-transcribed into complementary DNA (cDNA) using qScript cDNA Supermix

(Quanta Biosciences, Gaithersburg, MD) in a total volume of 20µl using a MyCyler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA).

5.2.4 Quantitative Real-Time PCR.

Quantitative Real-Time PCR was performed on a StepOnePlus Real-time PCR system (Applied Biosystems, Foster City, CA). Real-time quantitative PCR amplifications were performed in triplicate on a 96-well plate. Pre-designed TaqMan primers and hydrolysis probes for all genes of interest were purchased from Applied Biosystems (*mt-ND1*- Rn03296764_s1, *mt-ND2*- Rn03296765_s1, *mt-ND3*- Rn03296825_s1, *mt-ND4*- Rn03296781_s1, *mt-ND4L*- Rn03296792_s1, *mt-ND5*- Rn03296799_s1, *mt-ND6*- Rn03296815_s1, *mt-CO1*- Rn03296721_s1, *mt-CO2*- Rn03296737_s1, *mt-CO3*- Rn03296820_s1, *mt-CYB*- Rn03296746_s1, *mt-ATP6*- Rn03296710_s1, *mt-ATP8*- Rn03296716_s1, *NRF1*- Rn01455958_m1, *NRF2a*- Rn01767215_m1, *NRF2b*-Rn01514289_g1, *Pgc1α*- Rn00598552_m1, *Tfam*-Rn00580051_m1, *Cyc1*-Rn01504159_g1, *Cox6c*- Rn00820983_gH, *GAPDH*- Rn01775763_g1). Primers and probes were verified and operating at similar efficiencies. Target gene and endogenous control amplicons were labeled with either FAM or VIC. The levels of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) RNA expression were measured in all samples to normalize gene expression for sample-to-sample differences in RNA input, RNA quality and reverse transcription efficiency. Each sample was analyzed in triplicate, and the expression was calculated according to the $2^{-\Delta\Delta Ct}$ method (Bright and Mochly-Rosen 2005; Coble, Johnson et al. 2014).

5.2.5 Citrate Synthase Assay.

Citrate synthase activity was determined in homogenates prepared from kidney tissue using a citrate synthase assay kit (CS0720; Sigma-Aldrich, St. Louis, MO). Total muscle protein was determined in triplicate by the method of Bradford (Buchholz, Dundore et al. 1991) and the protein concentration of all samples was equalized. Citrate synthase activity was determined based on the formation of 2-nitro-5-thiobenzoic acid at a wavelength of 412 nm at 25°C on a microplate absorbance reader (iMark; BIO RAD, Hercules, CA). In each well, 8 µl of sample was added to a reaction medium containing 178 µl of assay buffer, 2 µl of 30 mmol/L acetyl coenzyme A, and 10 mmol/L 2-nitro-5-thiobenzoic acid. The baseline solution absorbance was recorded, reactions were initiated by the addition of 10 µl of oxaloacetic acid, and the change in absorbance measured every 15 seconds for 2 minutes.

5.2.6 Cytochrome Oxidase Histochemistry.

Cytochrome oxidase (CO) activity was determined in fresh frozen sections (20µm) in kidney, liver and heart tissue, as described previously (Whitfield-Rucker and Cassone 2000). Briefly, fresh frozen tissue was sectioned on a cryostat at 20 µm. Slides were immersed in 0.5% glutaraldehyde in 0.1% phosphate buffer for 5 minutes. Slides were then incubated for 2 hours in a diaminobenzidene (DAB)/cytochrome c solution (preceded by 5 minutes of

sparged oxygen) at 37ºC. Slides were then postfixed in 10% formalin for 15 minutes. Finally, slides were immersed in a serious of ethanol dehydration steps: 50, 70, 90, 95, 100% ethanol (30 seconds each) and xylene (2 changes, 5 minutes each). Slides were then coverslipped using Histomount (Life Technologies) and dried overnight. Colorometric change was used as a direct measurement of OXPHOS, in which darker color indicated higher metabolically active tissue. Relative density per area was calculated using ImageJ (NIH).

5.2.7 Statistical Analysis.

Blood pressures and citrate synthase activity among animals were initially analyzed by 1-way analysis of variance (ANOVA) followed by post-hoc comparisons using the Bonferroni t-test. Tissue mRNA expression levels were analyzed using Mann-Whitney *U* Test comparisons. The 0.05 level of probability was utilized as the criterion of significance. All statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software, Inc., La Jolla, CA).

5.3 Results

5.3.1 Evaluation of mt-gene expression

Quantitative real-time PCR was used to identify genes differentially expressed in renal tissue from hypertensive and normotensive BN/SHR-mt^{SHR} rats. Multiple mtDNA encoded genes of the mitochondrial respiratory chain were significantly reduced in renal, but not liver or cardiac tissue of HT BN/SHR-mt^{SHR}, including five complex I, one complex III, three complex IV and both subunits of ATP synthase. The well-established pathway in mammalian cells for mt-transcription initiation was evaluated using qPCR. *Tfam*, *NRF1*, *NRF2a*, *NRF2b* and *Pgc-1α* were all downregulated in the kidney, but not elsewhere, of HT BN/SHR-mt^{SHR} .

5.3.1.1 Complex I: NADH Dehydrogenase

Seven of the forty six genes that encode vital proteins for complex I of the electron transport chain are mt-encoded. The renal mtRNA expression of five of the seven were significantly reduced in hypertensive versus normotensive BN/SHR-mt^{SHR}. *mt-ND1* was reduced ~3.7 fold in HT BN/SHR-mt^{SHR} ($P<0.05$). mt-*ND3* was reduced ~2.6 fold in HT SHR/BN-mt^{SHR} ($P<0.01$). *mt-ND4* was reduced ~10.8 fold in HT SHR/BN-mt^{SHR} ($P<0.05$). *mt-ND4L* was reduced ~7.7 fold in HT SHR/BN-mt^{SHR} ($P<0.05$). *mt-ND5* was reduced ~2.7 fold in HT SHR/BN-mt^{SHR} ($P<0.05$). *mt-ND6* was reduced ~1.7 fold in HT SHR/BN-mt^{SHR}

(P>0.05) (**Figure 5.1A**). *mt-ND2* was not different between the two phenotypes (P>0.05).

5.3.1.2 Complex 3: Cytochrome bc_1 Complex

One of the eleven genes that encode vital proteins for complex three of the electron transport chain is mt-encoded. The renal mtRNA expression of mt-encoded cytochrome b (*mt-CYB*) was significantly reduced in hypertensive versus NT BN/SHR-mt^{SHR}. *mt-CYB* was reduced ~5 fold in HT BN/SHR-mt^{SHR} (P<0.05) (**Figure 5.1B**).

5.3.1.3 Complex 4: Cytochrome C oxidase

Three of the thirteen genes that encode vital proteins for complex IV of the electron transport chain are mt-encoded. All three genes were significantly reduced in hypertensive versus normotensive BN/SHR-mt^{SHR}. *mt-CO1* was reduced ~3.2 fold in HT BN/SHR-mt^{SHR} (P<0.05), *mt-CO2* was reduced ~3.6 fold in HT BN/SHR-mt^{SHR} (P<0.01), and *mt-CO3* was reduced 4.1 fold in HT BN/SHR-mt^{SHR} (P<0.01) (**Figure 5.1C**).

5.3.1.4 Complex V: ATP synthase

Two of the sixteen genes that encode vital proteins for ATP synthase of the electron transport chain are mt-encoded. *mt-ATP6* was reduced ~2.3 fold in HT BN/SHR--mt^{SHR} ($P<0.05$), while *mt-ATP8* was reduced ~3.1 fold in HT BN/SHR-mt^{SHR} ($P<0.05$) (**Figure 5.1D**).

5.3.1.5 Tissue expression in Liver and Heart

Expression levels of several mt-genes were evaluated in both the liver and heart of HT and NT BN/SHR-mt^{SHR} as described above. *mt-CYB*, *mt-CO2* and *mt-ND1, mt-ATP6* were shown to be not different ($P>0.05$) between the HT and NT BN/SHR-mt^{SHR} liver or heart tissues. This is in contrast to renal tissue wherein each of these genes exhibited reduced expression in HT vs. NT animals (**Figure 5.2**).

5.3.2 Evaluation of Oxidative Phosphorylation: Cytochrome Oxidase Histochemistry

CO activity was measured densitometrically in kidney, liver and heart sections (20µm) in NT (n=6) and HT (n=6) BN/SHR-mt^{SHR}. CO activity was significantly lower ($p<0.05$) in the kidney, but not the liver or heart in HT BN/SHR-mt^{SHR} (**Figure 5.3**).

5.3.3 Evaluation of Trans-Regulatory Factors

The well-established pathway in mammalian cells for mt-transcription initiation was evaluated using qPCR. *Pgc1a, NRF1*, *NRF2a*, *NRF2b* and *Tfam* were all downregulated in the kidney, but not liver tissue, of HT compared to NT BN/SHR-mt^{SHR} (n=20). The pathway leading to reduced mitochondrial gene expression is summarized in (**Figure 5.7**).

5.3.3.1 Peroxisome Proliferator-Activated Receptor Gamma Co-Activator 1-alpha (PGC-1α)

PGC-1α regulates NRF-dependent transcription, increases expression of both mitochondrial and nuclear encoded genes of oxidative phosphorylation and induces mitochondrial biogenesis. HT BN/SHR-mt^{SHR} exhibited ~2.5 fold reduction in *PGC-1α* mRNA in kidney tissue compared with NT BN/SHR-mt^{SHR} ($P<0.05$) (**Figure 5.4A**).

5.3.3.2 Nuclear Respiratory Factors

Nuclear respiratory factors 1 and 2 are well characterized transcriptional activators of genes involved in assembly of the respiratory apparatus, as well as constituents of the mtDNA transcription and replication machinery (Kelly and Scarpulla 2004). A main factor involved in mtDNA transcription is *Tfam,* whose expression is regulated by NRF1. All three NRFs were reduced in the kidney,

but not liver of HT compared to NT BN/SHR-mt^{SHR}. Renal *NRF1* mRNA expression was reduced ~1.8 fold in HT BN/SHR-mt^{SHR} compared with NT BN/SHR-mt^{SHR} ($P<0.05$) (Figure 4B). Renal *NRF2a* mRNA expression was reduced ~2.3 fold in HT BN/SHR-mt^{SHR} compared with NT BN/SHR-mt^{SHR} ($P<0.05$) (Figure 4C). Renal *NRF2b* mRNA expression was reduced ~1.9 fold in HT BN/SHR-mt^{SHR} compared with NT BN/SHR-mt^{SHR} ($P<0.05$) (**Figure 5.4D**).

5.3.3.3 Mitochondrial Transcription Factor A (Tfam)

Tfam is a key activator of mammalian mitochondrial transcription. Kidney, but not liver tissue exhibited reduced *Tfam* mRNA expression ~2.5 fold in HT BN/SHR-mt^{SHR} compared with NT BN/SHR-mt^{SHR} ($P<0.05$) (**Figure 5.4E**).

5.3.4 Nuclear-Encoded Mitochondrial Genes: Cytochrome C-1 (Cyc1), Cytochrome C Oxidase, Subunit Vic (Cox6c)

In order to assess the downstream pathways of the nuclear encoded regulatory elements, nuclear-encoded mitochondrial gene expression were assessed. There was no difference in the renal mRNA expression of *CYC* or *Cox6c* between NT (n=10) and HT (n=10) BN/SHR-mt^{SHR} ($P>0.05$) (**Figure 5.5**).

5.3.5 Evaluating Mitochondrial Number: Citrate Synthase Assay

To quantify mitochondrial number, citrate synthase activity was measured in kidney homogenates of NT (n=10) and HT (n=10) BN/SHR-mt^{SHR} (**Figure 5.6**). There was no difference between the two phenotypes (P=0.9676), indicating that mitochondrial number was not driving the reduced transcript expression in HT BN/SHR-mt^{SHR} kidneys.

5.4 Discussion

There is significant evidence that mitochondria and the mitochondrial genome may be important in the development of hypertension (Wallace 1999; Osanai, Tanaka *et al.* 2001; Schwartz, Duka *et al.* 2004; Taylor and Turnbull 2005; de Cavanagh, Toblli *et al.* 2006; Lopez-Campistrous, Hao *et al.* 2008; Kumarasamy, Gopalakrishnan *et al.* 2010). Of particular interest may be the nature of mitochondrial inheritance, being strictly maternal, and of female SHR origin. The results of the current study provide evidence that gene expression variation in renal mitochondrial genes encoding respiratory chain complexes is related to hypertension in rats stemming from a SHR/BN conplastic breeding paradigm. This breeding method provided a continuous passing of the maternal mitochondrial genome, while mixing the inherited nuclear genome between the maternal SHR and paternal BN with each succeeding generation. We have reported the maintenance of arterial systolic hypertension for 6 consecutive generations in this conplastic strain despite the reduction in maternally derived nuclear genome (Collett, Hart *et al.* 2013). Results from the current study show mitochondrial protein-coding genes critical for OXPHOS exhibited significantly reduced expression in the kidney, but not the in the liver or heart, in HT BN/SHR-mt^{SHR} compared with NT BN/SHR-mt^{SHR}. Additionally, in HT BN/SHR-mt^{SHR}, nuclear genes involved in mitochondrial biogenesis and transcription (*PGC-1α, NRF1, NRF2a/b, Tfam*), exhibited reduced expression in the kidney, but not liver, compared with NT BN/SHR-mt^{SHR} (**Figure 5.3**). Thus, kidney-specific reduction in expression of both mitochondrial and nuclear genes critical to OXPHOS is

associated with the manifestation of hypertension in rats with intact SHR mitochondrial genome but minimal SHR nuclear genome.

Recently, Lee *et al.* (2014) postulated that increased mitochondrial activity in proximal convoluted tubule cells of young, normotensive SHR may contribute to the development of hypertension at adulthood. These studies show that various parameters of mitochondrial activity were elevated in very young SHR prior to the onset of hypertension, while mt-gene expression remained unchanged. Our study documents mitochondrial genes encoding proteins of each mitochondrial subunit of mRNA were reduced in renal tissue of HT BN/SHR-mt^{SHR} compared with NT BN/SHR-mt^{SHR}. It is possible that mitochondrial activity is elevated in renal proximal tubules of very young SHR prior to the onset of hypertension. As the development of hypertension progresses in the maturing SHR, renal mitochondrial gene expression may then decline which in turn significantly contributes to the further elevation of blood pressure. Ongoing and future studies are being conducted to address this possible relationship and mechanism specific to the SHR.

The viability of individual electron transport chain subunits and the potential for disease has been evaluated. Altered function of complex I has been linked to cardiovascular disease (Antonicka, Ogilvie *et al.* 2003; Lopez-Campistrous, Hao *et al.* 2008). Lopez-Campistrous *et al.* (2008) revealed defects in complex I in the brainstem of SHR, resulting in increased reactive oxygen species production,

decreased ATP synthesis and impaired respiration in hypertension . Results from our current study indicate reduced genetic expression of most the mt-genes coding for Complex I, suggesting that defects in complex I function may be due to reduced mitochondrial gene expression. Altered function of complex III, a major site of superoxide formation and ROS production, may play an important role in renal mitochondrial ETC dysfunction and cardiovascular disease. Similar to these findings, Das *et al.* (1990) reported that the regulation of ATP synthase is abnormal in SHR cardiac cells, as demonstrated by the inability to respond to acute increases in energy demand compared to cells from normotensive rats . Data from our study indicate that mt-gene expression is reduced in kidneys, a key organ in blood pressure control. Furthermore, kidneys of hypertensive rats had reduced CO staining, indicating reduced oxidative phosphorylation compared with NT rats (**Figure 5.3**). This could have profound effects, as the kidney has high metabolic demand, particularly in regulating salt and water balance, and hence, arterial pressure. Taken together, the altered function of ETC subunits derived from decreased kidney-specific mt-gene expression may have several implications to the development of hypertension.

PGC-1α plays a central role in regulating mitochondrial content and function within cells, because of its ability to co-activate and augment several promoters of nuclear-encoded mitochondrial genes, as well as regulating mitochondrial transcription via the *NRF-Tfam* pathway (Wu, Puigserver *et al.* 1999). *PGC-1α* regulates NRF-dependent transcription, increases expression of both

mitochondrial and nuclear encoded genes of oxidative phosphorylation and induces mitochondrial biogenesis (Scarpulla 2002). It has been shown that metabolic functions are controlled by *PGC-1α* in a tissue specific manner in brown fat, muscle and liver (Puigserver 2005), however kidney regulation of *PGC-1α* and its effectors is unknown. Results from this study show a clearly coordinated reduction of the kidney-specific expression of nuclear and mitochondrial genes vital to OXPHOS coinciding with the manifestation of hypertension (**Figure 5.7**). It is likely that this nuclear-mitochondrial gene down regulation is being driven by upstream events. Although the upstream signaling involved in the activation/reduction of *PGC-1α* is yet to be fully elucidated, several pathways have been described. Briefly, the activity of *PGC-1α* can be modulated by numerous post-translational events, including phosphorylation by AMP kinase (AMPK)(Fan, Rhee *et al.* 2004) and cAMP response element-binding protein (CREB) (Fernandez-Marcos and Auwerx 2011), among others (García-Giménez, Gimeno *et al.* 2011).

Mitochondrial biogenesis and transcription have been thought to be regulated by the same nuclear-mitochondrial pathway described above (Scarpulla, Vega *et al.* 2012). However, mitochondrial number between NT and HT rats were not different as measured by citrate synthase activity (**Figure 5.6**). Mitochondrial encoded gene ND2 of complex 1 was also not different between the NT and HT phenotypes (**Figure 5.1A**). Subunit ND2 has been shown to play a significant role in the assembly and/or stability of Complex I (Antonicka, Ogilvie *et al.* 2003).

Results from our current study reinforce the importance of ND2 in electron transport chain integrity, as it was the only subunit of complex I that showed no expression difference in kidneys between the two phenotypes. In order to further assess the downstream pathways of the nuclear-encoded regulatory elements, the expression of two separate nuclear-encoded mitochondrial genes were assessed. Interestingly, other nuclear-encoded mitochondrial genes known to be regulated by the *PGC-1α-NRF-Tfam* pathway were not different. Neither renal Cytochrome C-1 *(Cyc1)* nor cytochrome c oxidase subunit Vic *(Cox6c)* were not different between HT and NT BN/SHR-mt^{SHR} (**Figure 5.5**). This phenomenon has several implications. First, biogenesis and transcription of mitochondrial genes, though known to be regulated by the *PGC-1α* pathway, may in fact be regulated in a more complex manner than previously thought. Furthermore, mitochondrial gene expression appears to be regulated in a tissue-specific manner. If this is the case, our data indicate that tissues of high metabolic activity may have a critical role in the development of disease. Therefore, it is plausible that altered OXPHOS could impact renal function, which is the cornerstone of blood pressure regulation and development of hypertension.

One of the more interesting aspects of this study is a potential ETC dysfunction driven by transcript differences in the kidneys, but not in other tissues, of mt-genes and the nuclear *trans*-factors that regulate them. Cytochrome oxidase serves as an endogenous metabolic marker. As shown in **Figure 5.3**, CO staining was significantly reduced in the kidneys, but not liver or heart of HT

BN/SHR-mt^{SHR}, indicating that the kidneys of hypertensive rats had reduced OXPHOS. Altered renal function has been well recognized as a key factor in the development and maintenance of hypertension (Bianchi, Fox *et al.* 1974; Curtis, Luke *et al.* 1983; Dilley, Stier *et al.* 1984; Guyton 1990; Cowley and Roman 1996; Ichihara, Inscho *et al.* 1997). One such mechanism that may be responsible is altered regulation of the renin-angiotensin system. The RAS serves as one of the most powerful regulators of blood pressure. The interactions among RAS and altered mitochondrial function has been advanced recently by Benigni *et al.* (2009). Deletion of the *Agtr1a* gene resulted in the reduced age-related cardio-renal complications, improved mitochondrial biogenesis, and increased longevity in mice. Treatment with antioxidants, mitochondrial superoxide dismutase mimetics, and AT_1r blockers decreased vascular O_2- production and attenuated development of hypertension in SHR (Park, Touyz *et al.* 2002),(Rodriguez-Iturbe, Zhan *et al.* 2003),(Shokoji, Nishiyama *et al.* 2003). De Cavanagh *et al.* (2006) demonstrated that oxidative stress is associated with mitochondrial dysfunction in SHR, and that this dysfunction is attenuated with AT_1r blockade with losartan . The presence and abundance of renal AT_1r is related to the propagation of the HT phenotype in BN/SHR-mt^{SHR}. We have previously shown that HT BN/SHR-mt^{SHR} exhibit elevated AT_1r mRNA (*Agtr1a*) expression compared to NT BN/SHR-mt^{SHR}, while the renal and systemic expression of renin, angiotensinogen, and angiotensin-converting enzymes were not different (Collett, Hart *et al.* 2013). HT BN/SHR-mt^{SHR} exhibited elevated AT_1r protein compared with NT BN/SHR-mt^{SHR}, and this

increase was positively correlated with elevated systolic BP (Collett, Hart *et al.* 2013). These data highlight a renal-specific phenomenon that appears to play a role in the pathology of hypertension in these animals.

Another mechanism that may underlie reduced mt-gene expression is altered activity of the sympathetic nervous system. Elevated RSNA plays an important role in the development and maintenance of hypertension in the SHR (Judy, Watanabe et al. 1976; 1995; Karim, Defontaine et al. 1995). Increases in RSNA decrease urinary sodium and water excretion by increasing renal tubular water and sodium reabsorption throughout the nephron, decrease renal blood flow and glomerular filtration rate by constricting the renal vasculature, and increase activity of the renin-angiotensin system by stimulating renin release from juxtaglomerular granular cells (DiBona and Kopp 1997; Osborn, Plato *et al.* 1997; DiBona 2000). Activation, or overactivation of adrenergic receptors may trigger downstream effects, altering expression of *PGC-1α* through phosphorylation of AMP kinase (AMPK)(Fan, Rhee *et al.* 2004), cAMP response element-binding protein (CREB) (Fernandez-Marcos and Auwerx 2011) or others (García-Giménez, Gimeno *et al.* 2011) to ultimately decrease mt-gene expression.

Whenever mitochondrial disturbances inhibit electron transport, electrons are forwarded into an increased generation of ROS (Wallace 2005). Increased mitochondrial ROS is linked to metabolic diseases such as aging, diabetes and hypertension (Hagen, Yowe *et al.* 1997; Kristal, Jackson *et al.* 1997; Addabbo,

Montagnani *et al.* 2009). Mitochondria are a major site of oxygen consumption and oxidative stress due to generation of ROS, where complexes I and III are the main sites of mitochondrial superoxide formation (Votyakova and Reynolds 2001). Recently, mitochondrial ETC dysfunction has been shown to directly cause oxidative stress during hypertension. Chan *et al.* (2009) reported that mitochondrial ROS production in the rostral ventrolateral medulla is elevated in SHRs, and that ROS dependent inhibition of mitochondrial ETC complexes I, II and III resulted in a feed-forward production of ROS, as well as defects in anti-oxidant production. Ballinger *et al.* (2000) have demonstrated that reactive oxygen species decreased mtRNA transcripts, mitochondrial protein synthesis and decreased cellular ATP levels. Taken together, ROS decreases OXPHOS, and decreased OXPHOS increases ROS, both of which contribute to hypertension (**Figure 5.7**). Therefore, in our studies, the reduction of renal gene expression may lead to the development and maintenance of hypertension.

In summary, we present novel data documenting a decrease in a well-defined nDNA-mtDNA interactive pathway resulting in decreased mtDNA transcripts of proteins vital to OXPHOS. This coordinated reduction of nuclear-mitochondrial OXPHOS genes and its potential role in the development of hypertension until now was largely unknown (**Figure 5.7**). Though the exact mechanisms driving this reduction in gene expression is currently not known, it is clear that reduced mt-gene expression in the kidney coincides with the development of hypertension in BN/SHR-mt^{SHR}. Using our current model, where the nuclear genome is

increasingly BN while maintaining the SHR mitochondrial genome, in combination with other similar models, the relevance of the specific control of each of these genomes and how they may contribute to disease may be revealed. Furthermore, the nuclear-mitochondrial gene expression interactions may also be critically important in the manifestation of the progression of the renal disease process and ultimate development of hypertension. Future studies focused on elucidating the upstream mechanisms driving the reduced expression and subsequent potential mitochondrial dysfunction will determine if this phenomenon may directly lead to the development of heritable hypertension.

5.5 Acknowledgements

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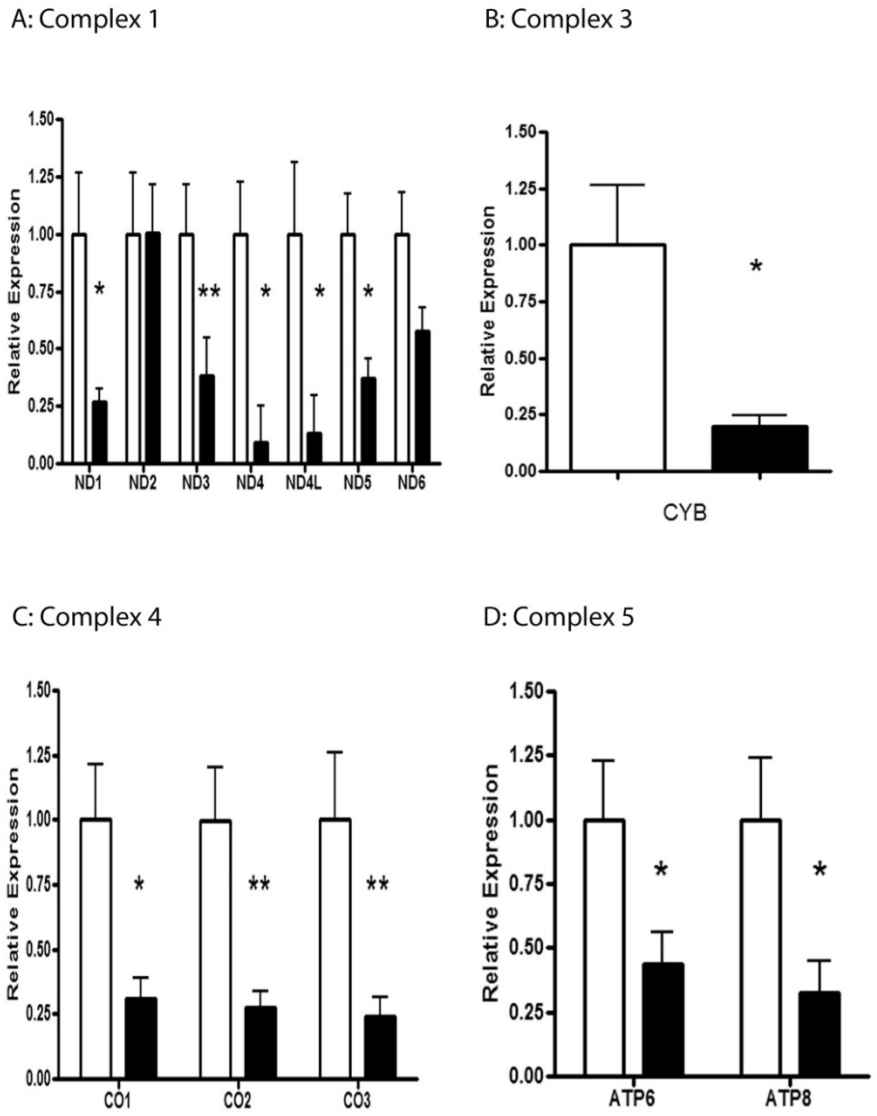

Figure 5.1: Quantitative Real-Time PCR mt-genes across 4 complexes of ETC. NT animals are in white, HT animals are in black. **A**: *mt-ND1* was reduced ~3.7 fold in HT BN/SHR-mt^{SHR} (*P<0.05). mt-*ND3* was reduced ~2.6 fold in HT SHR/BN-mt^{SHR} (**P<0.01). *mt-ND4* was reduced ~10.8 fold in HT SHR/BN-mt^{SHR} (*P<0.05). *mt-ND4L* was reduced ~7.7 fold in HT SHR/BN-mt^{SHR} (P<0.05). *mt-ND5* was reduced ~2.7 fold in HT SHR/BN-mt^{SHR} (P<0.05). *mt-ND6* was reduced ~1.7 fold in HT SHR/BN-mt^{SHR} (P>0.05). *mt-ND2* was not different between the two phenotypes (P>0.05). **B**: *mt-CYB* was reduced ~5 fold in HT BN/SHR-mt^{SHR} (*P<0.05). **C**: *mt-CO1* was reduced ~3.2 fold in HT BN/SHR-mt^{SHR} (*P<0.05), *mt-CO2* was reduced ~3.6 fold in HT BN/SHR-mt^{SHR} (P<0.05), and *mt-CO3* was reduced 4.1 fold in HT BN/SHR-mt^{SHR} (P<0.05). **D**: *mt-ATP6* was reduced ~2.3 fold in HT BN/SHR--mt^{SHR} (*P<0.05), while *mt-ATP8* was reduced ~3.1 fold in HT BN/SHR-mt^{SHR} (*P<0.05).

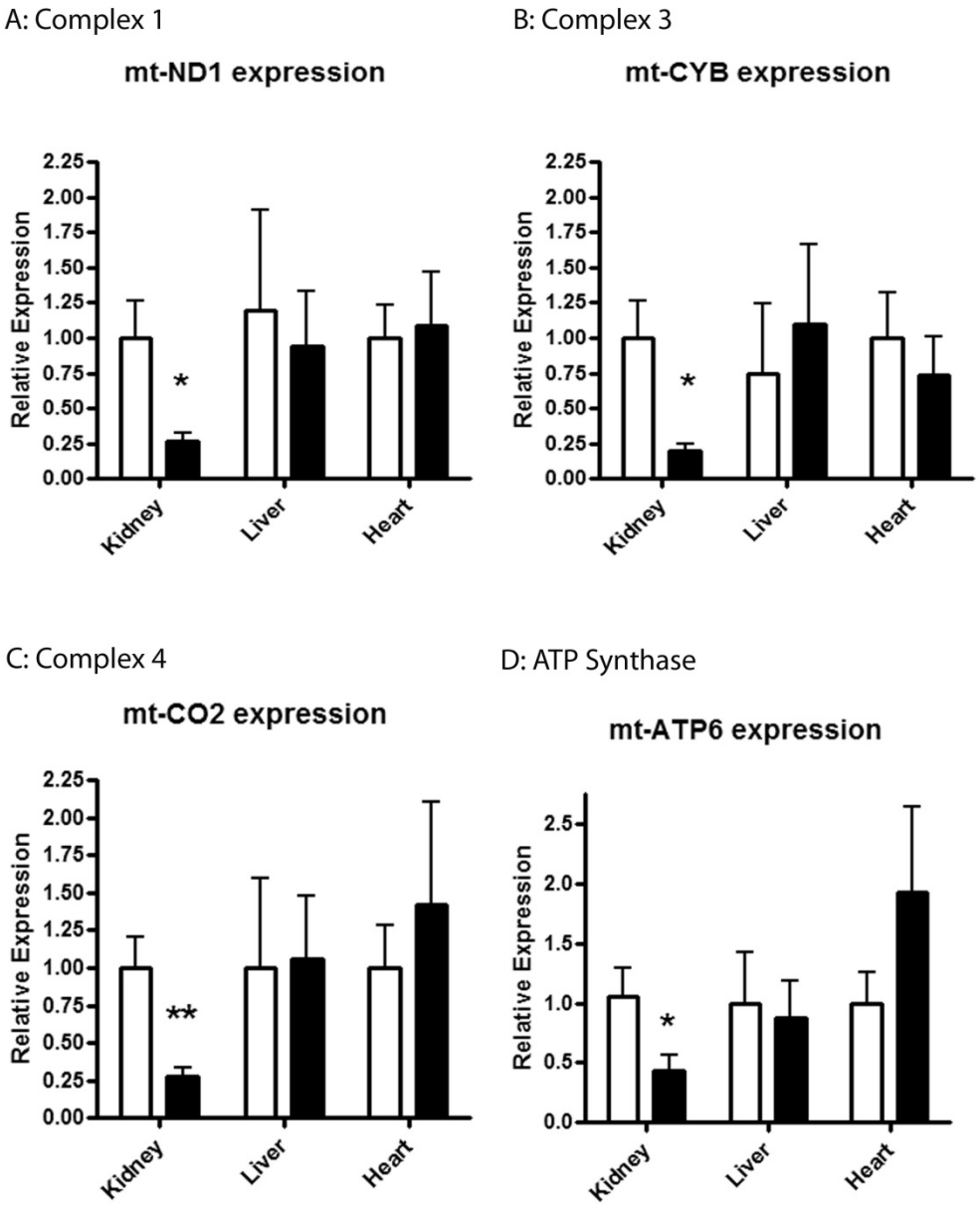

Figure 5.2: Quantitative Real-Time PCR of representative mt-genes of complex I-V were evaluated in liver and heart tissue of HT and NT BN/SHR-mt^{SHR}. NT animals are in white, HT animals are in black. **A**: *mt-CYB*, **B**: *mt-CO2*, **C**: *mt-ND1,* and **D**: *ATP6* were shown to be not different (P>0.05) between the HT and NT BN/SHR-mt^{SHR} liver and heart compared with kidney tissue.

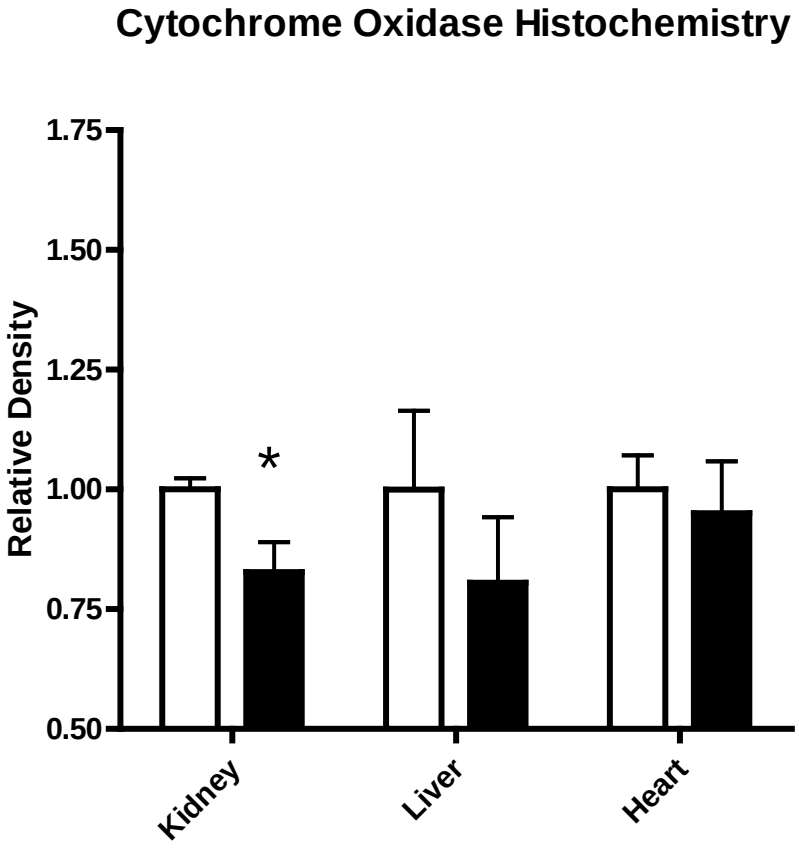

Figure 5.3: Cytochrome oxidase staining was significantly lower in the kidney of HT vs. NT BN/SHR-mt^{SHR} ($P<0.05$). However, CO staining was not different in liver ($p=0.3828$) or heart ($p=0.6664$) of HT vs. NT BN/SHR-mt^{SHR}. NT are in white, HT in black.

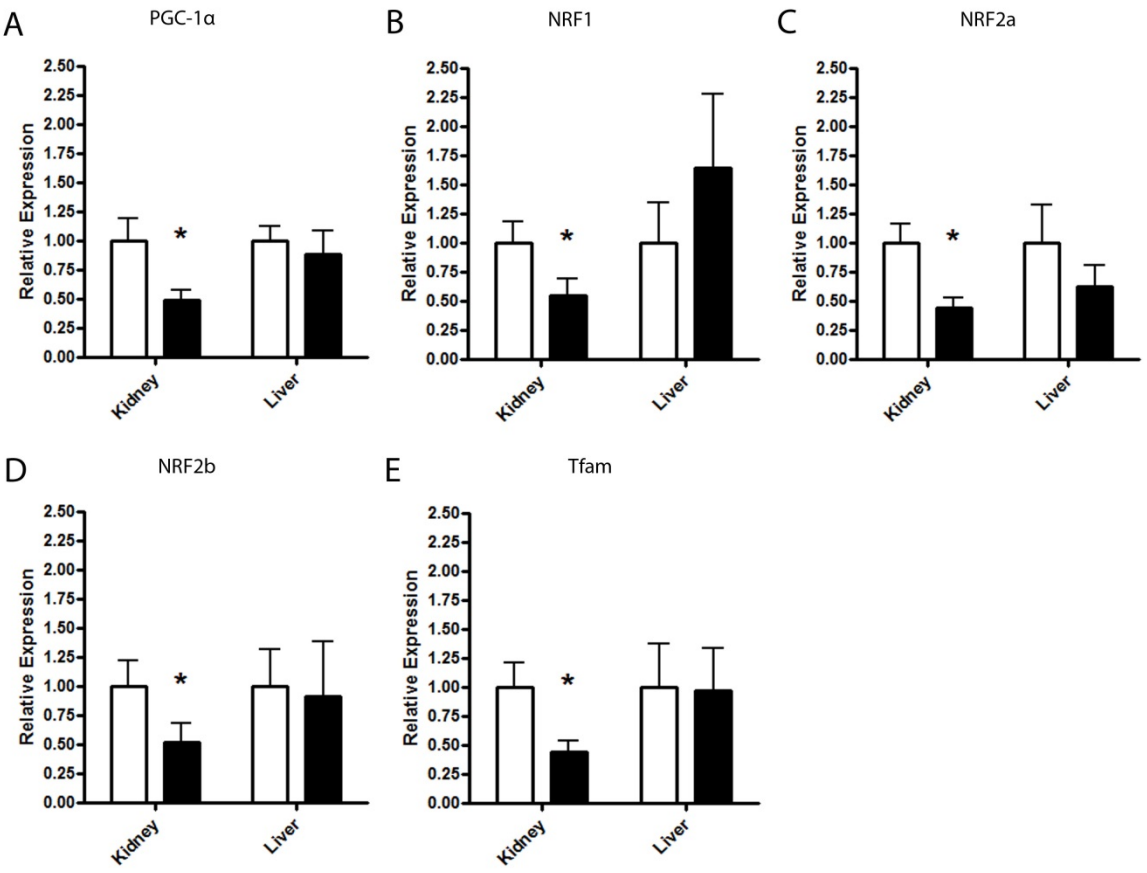

Figure 5.4: Quantitative Real-Time PCR graphs of the well-established nuclear-mitochondrial induction of mitochondrial gene transcription. NT animals are in white, HT animals are in black. **A**. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). Renal PGC-1α mRNA expression was reduced ~2.5 fold in HT BN/SHR-mtSHR compared with NT BN/SHR-mtSHR ($P<0.05$). **B**. Nuclear Respiration Factor (NRF) 1. Renal NRF1 mRNA expression was reduced ~1.8 fold in HT BN/SHR-mtSHR compared with NT BN/SHR-mtSHR ($P<0.05$). **C**. Nuclear Respiration Factor 2A. Renal NRF2A mRNA expression was reduced ~2.3 fold in HT BN/SHR-mtSHR compared with NT BN/SHR-mtSHR ($P<0.05$). **D**. Nuclear Respiration Factor 2B. Renal NR2B mRNA expression was reduced ~1.9 fold in HT BN/SHR-mtSHR compared with NT BN/SHR-mtSHR ($P<0.05$). **E**. Mitochondrial Transcription Factor A (Tfam). Renal Tfam mRNA expression was reduced ~2.5 fold in HT BN/SHR-mtSHR compared with NT BN/SHR-mtSHR ($P<0.05$) Expression levels were not different in the liver ($P>0.05$) in HT BN/SHR-mtSHR compared with NT BN/SHR-mtSHR for any of the transcription factors.

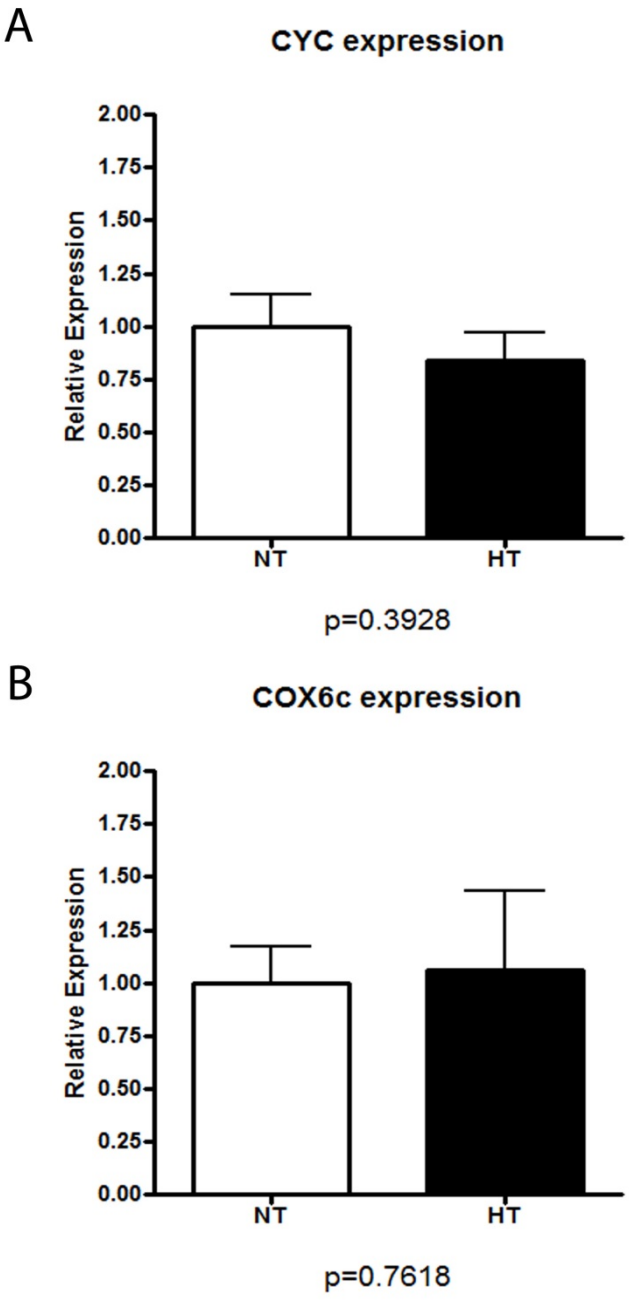

Figure 5.5: Quantitative Real-Time PCR of nuclear-encoded mitochondrial genes. NT animals are in white, HT animals are in black. Neither renal **A**: Cytochrome C-1 *(Cyc1)* (P=0.3928) nor **B**: cytochrome c oxidase, subunit Vic *(Cox6c)*(P=0.7618) were different between NT and HT BN/SHR-mt^{SHR}.

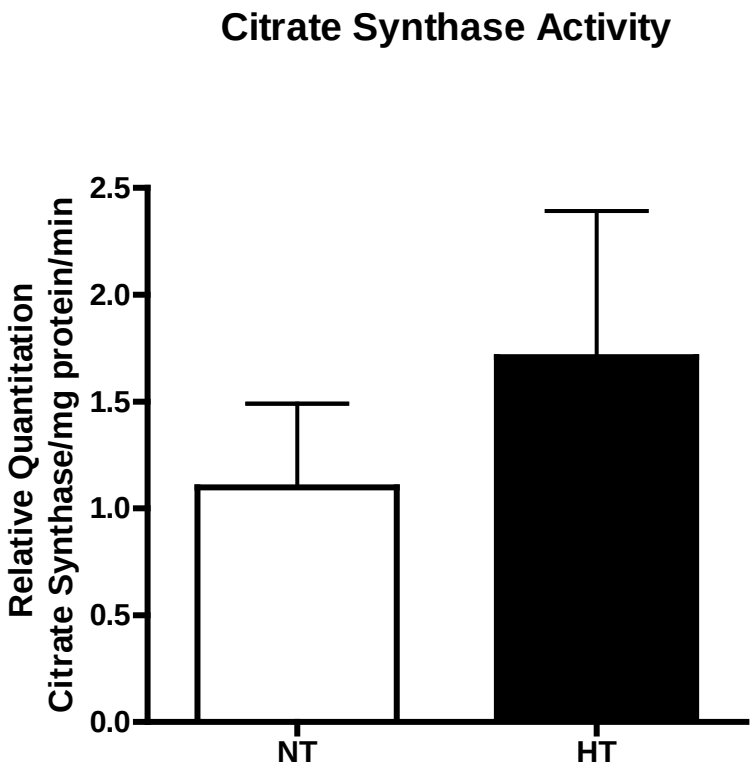

Figure 5.6: Citrate synthase activity was not different in renal tissue of NT versus HT BN/SHR-mt^{SHR}. Mitochondrial number was not different between the two phenotypes (P=0.9676).

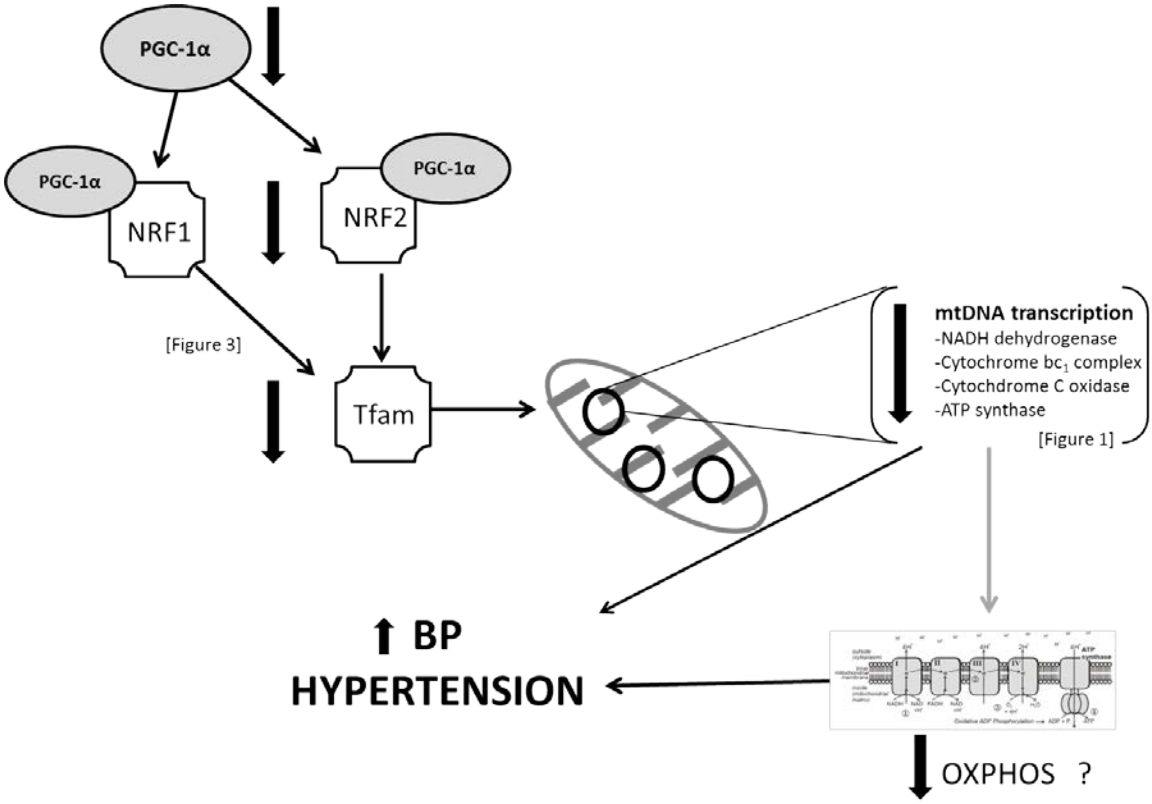

Figure 5.7: Hypothetical nuclear-mitochondrial pathway driving reduced mtDNA transcription, ultimately leading to elevated arterial pressure and hypertension. The pathway is clearly reduced in the kidney, but not liver of HT BN/SHR-mt^{SHR} compared with NT BN/SHR-mt^{SHR}.

CHAPTER 6
GENERAL CONCLUSIONS AND DISCUSSION

The goal of this dissertation was to determine the roles that specific humoral, genetic and genomic mechanisms play in the manifestation and maintenance of spontaneous hypertension. This was accomplished with two major hypotheses. One underlying hypothesis tested was that specific aspects of the renin-angiotensin system, in particular, the location and abundance of AT_1 receptors was associated with hypertension, which is supported by data presented in Chapters 3 and 4. Renal- and hypothalamic-specific expression of AT_1 receptors was associated with hypertension in rats with decreasing SHR nuclear genome. Chapter 4 data further support tissue-specific expression, suggesting that elevated hypothalamic AT_1r protein may be associated with an impaired ability to sense orally ingested sodium. The second major hypothesis tested was that the maternal inheritance of the mitochondrial genome might play a specific role in the development of hypertension. Data presented in Chapter 2 supports a dominant expression of the hypertensive phenotype stemming from a single SHR female rat, despite the continual reduction of the SHR nuclear genome. With the dominance of the hypertensive phenotype, the role of the mitochondria, mitochondrial gene expression and mitochondrial function were investigated. Data presented in Chapter 5 supports an important role that the mitochondria and the regulation of the mitochondrial genome play in the development of hypertension. Hypertensive BN/SHR-mt^{SHR} exhibited significant reductions of

mtRNA compared to NT rats of the same strain, in nearly all of the mitochondrially protein encoded genes, and this reduction coincided with a reduction of OXPHOS. Furthermore, data presented in Chapter 5 indicates an important role for tissue-specific expression and function of the mitochondria, with the reduced expression of genes critical to OXPHOS, as well as reduced OXPHOS itself, occurring in the kidney but not liver or heart in HT BN/SHR-mt^{SHR}. However, this data also implicates an important role of the nuclear factors that regulate mitochondrial gene expression and biogenesis, leading to a future hypothesis that it is not mitochondrial inheritance, but something upstream of the mitochondrion, for instance the RAS that is driving mitochondrial dysfunction, ultimately contributing to hypertension. Presented in this final chapter is a discussion of how these findings relate to each other and possible future directions to take. I postulate that Ang II binding to its type 1 receptor plays a significant role in the renal downregulation of mitochondrial gene expression and OXPHOS, thus contributing significantly to the development and maintenance of hypertension in BN/SHR-mt^{SHR} (**Figure 6.1**).

Data presented in this dissertation supports the important role of the kidney in the development and maintenance of hypertension. HT BN/SHR-mt^{SHR} exhibited elevated AT_1 gene and protein expression in the kidneys, but not the liver or heart compared with NT BN/SHR-mt^{SHR}. Transcript analyses of systemic aspects of RAS were not different between the two phenotypes in the BN/SHR-mt^{SHR} colony. This increase in kidney AT_1r expression coincided with a decrease

in mitochondrial gene expression in HT BN/SHR-mt^{SHR}. HT rats exhibited a decrease in the well-defined nDNA-mtDNA interactive pathway resulting in decreased mtDNA transcript expression of proteins vital to OXPHOS (**Figure 6.1**). This coordinated reduction of nuclear-mitochondrial OXPHOS genes and its potential relationship to the development of hypertension until now was largely unexplored. Though the exact mechanisms driving this reduction in gene expression is not known, it is clear that reduced mt-gene expression in the kidney coincides with the development of hypertension in BN/SHR-mt^{SHR}. Furthermore, data presented in Chapter 5 indicates that HT rats exhibited reduced OXPHOS, as HT rats exhibited decreased cytochrome oxidase staining compared with NT BN/SHR-mt^{SHR}, and this decrease was observed only in the kidney. What then does this mean in terms of the development of spontaneous hypertension? Does reduced mitochondrial gene transcription, and ultimately reduced OXPHOS lead to reduced renal function, which then initiates chronic elevated arterial pressure? Data presented in Chapter 4 indicates that renal function was normal between NT and HT BN/SHR-mt^{SHR}. Both NT and HT rats were in negative sodium balance, while exhibiting similar proteinuria. Since that is the case, it may be possible that reduced renal tubular mitochondrial function makes sodium handling in the kidney less efficient, therefore requiring chronic pressure-natriuresis and a resetting of the renal function curve. This hypothesis seems plausible, with a reduced ability to “sense” orally ingested NaCl, HT rats require elevated arterial pressure to maintain sodium balance, shifting the renal-function

curve to the right. Hence, the HT BN/SHR-mt^{SHR} maintain sodium balance, but at the expense of the morbidity of hypertension.

As discussed in Chapters 1, 3 and 4, Ang II plays a pivotal role in the regulation of blood pressure, volume, and electrolyte balance. The angiotensin II, type 1 receptor has been extensively evaluated in rodents and has subsequently become a target as a causal factor in the development of spontaneous hypertension. Reja *et al.* (2006) showed that gene expression levels of AT_1r, extracellular signal-regulated kinase 2 and phosphatidylinositol 3-kinase were significantly higher in the PVN, RVLM and adrenal medulla in SHR compared to normotensive WKY rats. Raizada *et al.* (1993) showed that AT_1r mRNA was higher in the hypothalamus and brainstem of hypertensive SHR compared to normotensive WKY rats. Furthermore, Gyurko *et al.* (1993) showed that antisense inhibition of AT_1 receptor mRNA in the brain reduces the magnitude of hypertension in adult SHR. Data from our studies strongly supports the notion that tissue-specific elevation of AT_1 receptors may play a role in SHR-derived hypertension. In fourth generation BN/SHR-mt^{SHR} , where the nuclear genome of the original SHR accounts for only ~6.2%, HT rats had significantly higher AT_1r protein expression than normotensive rats in both kidney and hypothalamic tissue. Average SAP and tissue expression of AT_1r were positively correlated, indicating that tissue-specific expression of AT_1r expression may critically impact the development and maintenance of spontaneous hypertension. In HT and NT BN/SHR-mt^{SHR} rats across all generations, we saw in increase in the kidney

expression of AT_1r mRNA associated with hypertension. Increased AT_1r could potentially have drastic effects on the cardiovascular system, and subsequently, the pathogenesis of hypertension. Ang II's effect in the kidney would be exacerbated, increasing proximal tubular sodium reabsorption and decreasing renal blood flow. In the hypothalamus, elevated AT_1r expression could increase sympathetic nervous activity and/or vasopressin secretion, subsequently increasing vascular resistance and sodium and water retention (Scherrer 1959; Osborn and Camara 1997; Chen and Toney 2001).

Significant evidence suggests that the RAS plays a role in altering mitochondrial function. ACE inhibitors and ARBs reduce age-related mitochondrial dysfunction and protect against cardiac mitochondrial dysfunction following acute ischemic attack (DE CAVANAGH, PIOTRKOWSKI et al. 2003; Monteiro, Duarte et al. 2005; de Cavanagh, Toblli et al. 2006). Ang II can induce oxidant stress by enhancing the generation of both NO (Pueyo, Arnal *et al.* 1998) and NAD(P)H oxidase-derived superoxide (Rueckschloss, Quinn *et al.* 2002). Ang II can also induce endothelial NO synthase uncoupling, i.e., switching from NO to superoxide production (Mollnau, Wendt *et al.* 2002). Furthermore, Ang II has been shown to stimulate mitochondrial reactive oxygen species (mtROS) production. Increased mtROS has been linked to metabolic diseases such as aging, diabetes and hypertension (Hagen, Yowe *et al.* 1997; Kristal, Jackson *et al.* 1997; Addabbo, Montagnani *et al.* 2009). Mitochondria are a major site of oxygen consumption and a major site of oxidative stress due to generation of

ROS, where complexes I and III are the main sites of mitochondrial superoxide formation (Votyakova and Reynolds 2001). There is increasing evidence that hypertension is associated with an increased mitochondria-derived production of ROS in various animal models (Doughan, Harrison *et al.* 2008; Dikalova, Bikineyeva *et al.* 2010). In mice, acute (24 h) and chronic (14 day) Ang II infusion led to a decreased cardiac expression of mitochondrial electron transport chain and Krebs cycle genes (Larkin, Frank *et al.* 2004), supporting previous observations that indicated a role for Ang II and Ang II-induced ROS in the depression of mitochondrial energy metabolism (Sanbe, Tanonaka *et al.* 1995; Casademont and Miro 2002; Sorescu and Griendling 2002). In rat endothelial cells, Ang II-induced mtROS generation activates redox-sensitive nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which is followed by a stimulation of vascular cell adhesion molecule-1 expression, a cytokine involved in atherosclerosis lesion formation (Pueyo, Gonzalez *et al.* 2000). Recent data suggest that brain activation of the RAS elicits intraneuronal signaling, which involves an increased production of mitochondrial $O_2^{\cdot -}$ (Case, Li *et al.* 2013), modulating ion channel activity and increasing neuronal firing (Yin, Yang *et al.* 2010). Furthermore, overexpression of mitochondrial superoxide dismutase in the brain effectively abolishes the central angiotensin II-induced pressor response and decreases blood pressure in rodent models of hypertension (Zimmerman, Lazartigues *et al.* 2002; Chan, Wu *et al.* 2009). It is clear then, that Ang II binding to its AT_1 receptor has profound effects on the mitochondria and metabolism, and therefore this pathway should be considered a potential target

in the treatment and/or prevention of hypertension. The RAS-mitochondria connection is strongly supported by work presented in this dissertation, demonstrating an inverse relationship between renal AT_1r expression and components of OXPHOS, despite the continual reduction of the founder SHR female nuclear genome (**Figure 6.1**).

Mitochondrial membranes, proteins, and mitochondrial DNA are particularly sensitive to oxidative damage (Yakes and Van Houten 1997; Ballinger, Patterson *et al.* 2000). ROS has been shown to posttranslationally modify mitochondrial proteins leading to their inactivation, as in the case of SOD2 and aconitase, or alter their function as occurs with cytochrome *c* (Brookes, Zhang *et al.* 2001; MacMillan-Crow, Cruthirds *et al.* 2001; Chen, Deterding *et al.* 2002). Mitochondrial ETC dysfunction has been shown to be causally related to oxidative stress during hypertension. Chan *et al.* (2009) reported that mitochondrial ROS production in the RVLM is elevated in SHRs, and that ROS-dependent inhibition of mitochondrial ETC complexes I, II and III resulted in a feed-forward production of ROS, as well as defects in anti-oxidant production. Ballinger *et al.* (2000) demonstrated that the reactive oxygen species H_2O_2 and peroxynitrite decreased mtRNA expression, mitochondrial protein synthesis and decreased cellular ATP levels. It is possible that ROS, known to be involved with the development of hypertension and mitochondrial dysfunction in SHRs, may play a role in the development of hypertension in BN/SHR-mt^{SHR} (**Figure 6.1**).

Doughan *et al.* (2008) has shown that the molecular mechanisms involved in Ang II-mediated mitochondrial dysfunction (mitochondrial H_2O_2 production, and decreased mitochondrial glutathione, ADP-stimulated respiration, and decreased membrane potential) include protein kinase C (PKC) activation. Interestingly, and of significant importance here, AT_1r and α-adrenergic receptors increase PKC activity (Tsuchida, Liu et al. 1994; Hein and Kobilka 1995; Karim, Defontaine et al. 1995). Ang II binding to its type 1 receptor causes the G-protein mediated stimulation of phospholipase C and phophoinositide 3 kinase, which in turn activates PKC. This pathway is involved with several mechanisms associated with the pathogenesis of hypertension, including vasoconstriction, catecholamine release, increased renal sodium reabsorption, as well as increased vasopressin release and salt appetite (de Gasparo, Catt et al. 2000). Buchholz et al. (1991) demonstrated that inhibition of PKC lowers blood pressure in SHRs. Pfaff and Vallon (2002) demonstrated that the renoprotective effect of ACE inhibitors in diabetic rats is mediated by the inhibition of PKC-β. Identifying the exact isozyme involved in hypertension may prove to be difficult. There are eight known homologous PKC isozymes, which are products of seven related genes (Mochly-Rosen, Das et al. 2012). Nowak (2002) demonstrated that mitochondrial dysfunction and reduced OXPHOS in renal proximal tubular cells is mediated by PKC-α and ERK1/2. Recently, Coble *et al*. (2014) demonstrated a role for central PKC-α in fluid balance by showing the role of PKC-α signaling in the SFO in fluid intake stimulated by Ang II in the brain. To date, several other isoforms have been implicated in cardiovascular pathologies, such as diabetes

(Geraldes and King 2010), heart failure (Inagaki, Churchill et al. 2006) and stroke (Bright and Mochly-Rosen 2005). Does PKC alter *PGC-1α*, thereby initiating the cascade reducing mitochondrial gene expression and OXPHOS? Does PKC directly, or through a second messenger, alter mitochondrial function in other ways? One future goal will be to elucidate what role, if any, PKC isozymes play in mitochondrial dysfunction mediated by Ang II.

I believe that data presented in this dissertation strongly supports a relationship between decreased mitochondrial gene expression, mitochondrial function and hypertension, and that this altered mitochondrial dynamics may be mediated, at least in part, by Ang II binding to its AT_1r. Another mechanism that may underlie reduced mt-gene expression is altered activity of the sympathetic nervous system. Elevated RSNA plays an important role in the development and maintenance of hypertension in the SHR (Judy, Watanabe et al. 1976; Karim, Defontaine et al. 1995). Increases in RSNA decrease urinary sodium and water excretion by increasing renal tubular water and sodium reabsorption throughout the nephron, decrease renal blood flow and glomerular filtration rate by constricting the renal vasculature, and increase activity of the renin-angiotensin system by stimulating renin release from juxtaglomerular granular cells (DiBona and Kopp 1997; Osborn, Plato *et al.* 1997; DiBona 2000). Activation, or overactivation of adrenergic receptors may trigger downstream effects, potentially altering expression of *PGC-1α* through, for instance phosphorylation AMP kinase (AMPK)(Fan, Rhee *et al.* 2004), cAMP response element-binding

protein (CREB (Fernandez-Marcos and Auwerx 2011) or others (García-Giménez, Gimeno *et al.* 2011) to ultimately decrease mt-gene expression. Adrenergic activation also increases PKC activity, which has been shown to mediate mitochondrial dysfunction in conjunction with ERK1/2 (Nowak 2002). A second future goal is to evaluate the relationship between the sympathetic nervous system and mitochondrial function, as elevated RSNA is a key component of most forms of hypertension, including primary hypertension in humans. Evaluating RAS with RSNA could provide valuable information on how each contribute to hypertension individually and in concert with one another.

PGC-1α plays a central role in regulating mitochondrial content and function within cells, because of its ability to co-activate and augment several promoters of nuclear-encoded mitochondrial genes, as well as regulating mitochondrial transcription via the *NRF-Tfam* pathway (Wu, Puigserver *et al.* 1999). *PGC-1α* regulates NRF-dependent transcription, increases expression of both mitochondrial and nuclear encoded genes of oxidative phosphorylation and induces mitochondrial biogenesis (Scarpulla 2002). It has been shown that metabolic functions are controlled by *PGC-1α* in a tissue specific manner in brown fat, muscle and liver (Puigserver 2005), however kidney regulation of *PGC-1α* and its effectors was unknown until now. Results from our studies show a clearly coordinated-reduction of the kidney-specific expression of nuclear and mitochondrial genes vital to OXPHOS coinciding with the manifestation of hypertension. As mentioned above, it is likely that this nuclear-mitochondrial

gene downregulation is being driven by upstream events. A third future goal, though related to the other two, is to elucidate the exact mechanisms that regulate *PGC-1α*, particularly in a tissue-specific manner. Understanding these mechanisms will be critical in understanding the etiology of hypertension and cardiovascular disease.

I have postulated thus far that reduced mitochondrial gene expression and OXPHOS may be driving hypertension. It is possible, however, that elevated blood pressure is driving reduced mitochondrial function. Elevated ROS can cause reduced OXPHOS, making the mitochondria less efficient. The converse is true as well, as less efficient OXPHOS can increase mtROS. Oxidative mitochondrial DNA damage may affect the synthesis of components of the respiratory chain, which in turn can further increase ROS production, initiating a feed-forward cycle (**Figure 6.1**). Chronic over activity of renal sympathetic nerves, elevated blood pressure and a rightward shift in the renal function curve may ultimately drive down the expression of mitochondrial genes. High metabolic activity in young SHRs may drive mitochondrial gene expression down in adulthood, coinciding with the chronic elevation of BP. This “chicken-or-the-egg” type question is crucial in understanding the pathology of the disease, and one that needs to be addressed. One way this could be accomplished is to trace the pathogenesis of the disease in developing SHRs, while monitoring tissue-specific mitochondrial function.

Within this dissertation, I have presented data supporting the hypotheses that the location, genetic expression and abundance of AT_1 receptors is associated with development and maintenance of spontaneous hypertension. Both renal- and hypothalamic-specific expression of AT_1 receptors was associated with hypertension in rats with decreasing SHR nuclear genome. Secondly, I presented data to support the hypothesis that the maternal inheritance of the mitochondrial genome may play a specific role in the development of hypertension, though mitochondrial function is most likely altered upstream of the well-known nuclear-mitochondrial pathway, and there is significant evidence that the RAS is involved. Most importantly, data presented here indicates both intrinsic and extrinsic mechanisms of the kidney, i.e. the RAS, as well as genetic and genomic mechanisms of the kidney may be driving that spontaneous hypertension. A major question remains: Why is there tissue-specific regulation of mitochondrial gene expression and OXPHOS, and how is this accomplished? Future directions will try to elucidate what exact mechanisms are driving decreased OXPHOS, as well as further investigate the importance of tissue-specific regulation of mitochondria, and how this may be causal to the manifestation of hypertension.

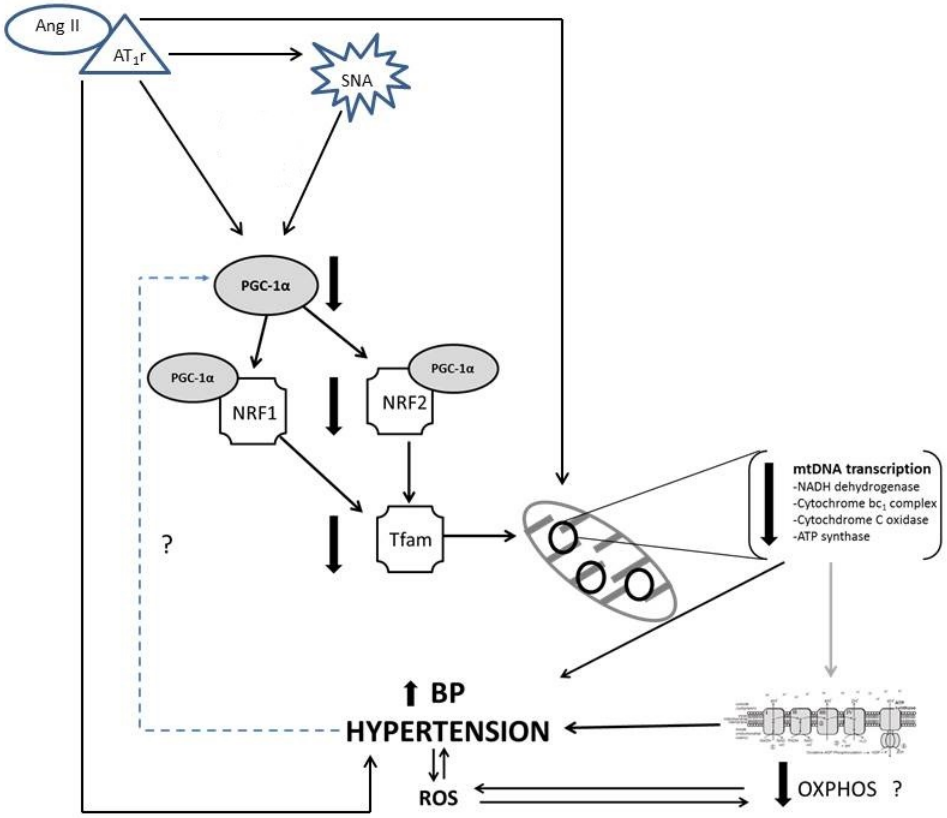

Figure 6.1: Interactions among RAS and AT_1r, reduced mitochondrial gene expression, reduced OXPHOS in the development of hypertension. There are several possible interactions presented above which ultimately lead to chronic elevation of arterial pressure.

BIBIOLOGRAPHY

Adamczak, M., M. Zeier, et al. (2002). "Kidney and hypertension." Kidney Int Suppl(80): 62-67.

Adams, M. A., A. Bobik, et al. (1989). "Differential development of vascular and cardiac hypertrophy in genetic hypertension. Relation to sympathetic function." Hypertension **14**(2): 191-202.

Addabbo, F., M. Montagnani, et al. (2009). "Mitochondria and Reactive Oxygen Species." Hypertension **53**(6): 885-892.

Aitman, T. J., A. M. Glazier, et al. (1999). "Identification of Cd36 (Fat) as an insulin-resistance gene causing defective fatty acid and glucose metabolism in hypertensive rats." Nature genetics **21**(1): 76-83.

Andersson, B., M. F. Dallman, et al. (1969). "Observations on central control of drinking and of the release of antidiuretic hormone (ADH)." Life Sci **8**(7): 425-432.

Andersson, B., S. Eriksson, et al. (1995). "Angiotensin and the brain." Acta Physiol Scand **155**(2): 117-125.

Andersson, B. and K. Olsson (1973). "On central control of body fluid homeostasis." Conditional reflex : a Pavlovian journal of research & therapy **8**(3): 147-159.

Antonicka, H., I. Ogilvie, et al. (2003). "Identification and Characterization of a Common Set of Complex I Assembly Intermediates in Mitochondria from Patients with Complex I Deficiency." Journal of Biological Chemistry **278**(44): 43081-43088.

Atanur, S. S., İ. Birol, et al. (2010). "The genome sequence of the spontaneously hypertensive rat: Analysis and functional significance." Genome Res **20**(6): 791-803.

Attardi, G., M. Yoneda, et al. (1995). "Complementation and segregation behavior of disease-causing mitochondrial DNA mutations in cellular model systems." Biochim Biophys Acta **1271**(1): 241-248.

Bader, M. and D. Ganten (2008). "Update on tissue renin-angiotensin systems." J Mol Med (Berl) **86**(6): 615-621.

Ballinger, S. W., C. Patterson, et al. (2000). "Hydrogen Peroxide– and Peroxynitrite-Induced Mitochondrial DNA Damage and Dysfunction in Vascular Endothelial and Smooth Muscle Cells." Circulation Research **86**(9): 960-966.

Baudin, B. (2002). "Angiotensin II receptor polymorphisms in hypertension. Pharmacogenomic considerations." Pharmacogenomics **3**(1): 65-73.

Benigni, A., D. Corna, et al. (2009). "Disruption of the Ang II type 1 receptor promotes longevity in mice." J Clin Invest **119**(3): 524-530.

Bereiter-Hahn, J. (1990). "Behavior of mitochondria in the living cell." Int Rev Cytol **122**: 1-63.

Bianchi, G., U. Fox, et al. (1974). "BLOOD-PRESSURE CHANGES PRODUCED BY KIDNEY CROSS-TRANSPLANTATION BETWEEN SPONTANEOUSLY HYPERTENSIVE RATS AND NORMOTENSIVE RATS." Clinical Science and Molecular Medicine **47**(5): 435-448.

Bickerton, R. K. and J. P. Buckley (1961). "Evidence for a Central Mechanism in Angiotensin Induced Hypertension." Experimental Biology and Medicine **106**(4): 834-836.

Biron, P., J. G. Mongeau, et al. (1976). "Familial aggregation of blood pressure in 558 adopted children." Can Med Assoc J **115**(8): 773-774.

Bonnardeaux, A., E. Davies, et al. (1994). "Angiotensin II type 1 receptor gene polymorphisms in human essential hypertension." Hypertension **24**(1): 63-69.

Bottari, S. P., M. de Gasparo, et al. (1993). "Angiotensin II receptor subtypes: characterization, signalling mechanisms, and possible physiological implications." Frontiers in neuroendocrinology **14**(2): 123-171.

Bourque, C. W. and S. H. Oliet (1997). "Osmoreceptors in the central nervous system." Annu Rev Physiol **59**: 601-619.

Brewster, U. C. and M. A. Perazella (2004). "The renin-angiotensin-aldosterone system and the kidney: effects on kidney disease." Am J Med **116**(4): 263-272.

Bright, R. and D. Mochly-Rosen (2005). "The role of protein kinase C in cerebral ischemic and reperfusion injury." Stroke **36**(12): 2781-2790.

Brookes, P. S., J. Zhang, et al. (2001). "Increased sensitivity of mitochondrial respiration to inhibition by nitric oxide in cardiac hypertrophy." J Mol Cell Cardiol **33**(1): 69-82.

Brooks, V. L. (1995). "Chronic Infusion of Angiotensin II Resets Baroreflex Control of Heart Rate by an Arterial Pressure–Independent Mechanism." Hypertension **26**(3): 420-424.

Buchholz, R. A., R. L. Dundore, et al. (1991). "Protein kinase inhibitors and blood pressure control in spontaneously hypertensive rats." Hypertension **17**(1): 91-100.

Budzikowski, A. S., B. S. Huang, et al. (1998). "Brain "ouabain", a neurosteroid, mediates sympathetic hyperactivity in salt-sensitive hypertension." Clin Exp Hypertens **20**(2): 119-140.

Burnham, C. E., C. L. Hawelu-Johnson, et al. (1987). "Molecular cloning of rat renin cDNA and its gene." Proceedings of the National Academy of Sciences **84**(16): 5605-5609.

Burt, V. L., P. Whelton, et al. (1995). "Prevalence of Hypertension in the US Adult Population: Results From the Third National Health and Nutrition Examination Survey, 1988-1991." Hypertension **25**(3): 305-313.

Campese, V. M., E. Kogosov, et al. (1995). "Renal afferent denervation prevents the progression of renal disease in the renal ablation model of chronic renal failure in the rat." Am J Kidney Dis **26**(5): 861-865.

Carey, R. M. and H. M. Siragy (2003). "Newly recognized components of the renin-angiotensin system: potential roles in cardiovascular and renal regulation." Endocrine reviews **24**(3): 261-271.

Carretero, O. A. and S. Oparil (2000). "Essential Hypertension: Part I: Definition and Etiology." Circulation **101**(3): 329-335.

Casademont, J. and O. Miro (2002). "Electron transport chain defects in heart failure." Heart Fail Rev **7**(2): 131-139.

Case, A. J., S. Li, et al. (2013). "Mitochondrial-localized NADPH oxidase 4 is a source of superoxide in angiotensin II-stimulated neurons." American Journal of Physiology - Heart and Circulatory Physiology **305**(1): H19-H28.

Chan, S. H., K. L. Wu, et al. (2009). "Oxidative impairment of mitochondrial electron transport chain complexes in rostral ventrolateral medulla contributes to neurogenic hypertension." Hypertension **53**(2): 217-227.

Chen, Q. H. and G. M. Toney (2001). "AT(1)-receptor blockade in the hypothalamic PVN reduces central hyperosmolality-induced renal sympathoexcitation." Am J Physiol Regul Integr Comp Physiol **281**(6): R1844-1853.

Chen, Y.-R., L. J. Deterding, et al. (2002). "Protein Oxidation of Cytochrome c by Reactive Halogen Species Enhances Its Peroxidase Activity." Journal of Biological Chemistry **277**(33): 29781-29791.

Cheng, H. F., J. L. Wang, et al. (1998). "Young SHR express increased type 1 angiotensin II receptors in renal proximal tubule." Am J Physiol **274**(1 Pt 2): F10-17.
Coble, J. P., R. F. Johnson, et al. (2014). "Activity of Protein Kinase C-alpha Within the Subfornical Organ Is Necessary for Fluid Intake in Response to Brain Angiotensin." Hypertension **64**(1): 141-148.
Collett, J. A., A. K. Hart, et al. (2013). "Renal angiotensin II type 1 receptor expression and associated hypertension in rats with minimal SHR nuclear genome." Physiol Rep **1**(5): e00104.
Conti, S., P. Cassis, et al. (2012). "Aging and the Renin-Angiotensin System." Hypertension **60**(4): 878-883.
Cowley, A. W., Jr. (1992). "Long-term control of arterial blood pressure." Physiol Rev **72**(1): 231-300.
Cowley, A. W., Jr. and R. J. Roman (1983). "Renal dysfunction in essential hypertension--implications of experimental studies." Am J Nephrol **3**(2-3): 59-72.
Cowley, A. W., Jr. and R. J. Roman (1996). "The role of the kidney in hypertension." JAMA **275**(20): 1581-1589.
Coyle, J. D., S. F. Gardner, et al. (2004). "The renal protective effects of angiotensin II receptor blockers in type 2 diabetes mellitus." Ann Pharmacother **38**(10): 1731-1738.
Curtis, J. J., R. G. Luke, et al. (1983). "REMISSION OF ESSENTIAL-HYPERTENSION AFTER RENAL-TRANSPLANTATION." New England Journal of Medicine **309**(17): 1009-1015.
Dahl, L. K. (1961). "Possible role of chronic excess salt consumption in the pathogenesis of essential hypertension." The American Journal of Cardiology **8**(4): 571-575.
Dahl, L. K. (2005). "Possible role of salt intake in the development of essential hypertension. 1960." Int J Epidemiol **34**(5): 967-972; discussion 972-964, 975-968.
Das, A. M. and D. A. Harris (1990). "Defects in regulation of mitochondrial ATP synthase in cardiomyocytes from spontaneously hypertensive rats." American Journal of Physiology - Heart and Circulatory Physiology **259**(4): H1264-H1269.
de Cavanagh, E. M., F. Inserra, et al. (2007). "From mitochondria to disease: role of the renin-angiotensin system." Am J Nephrol **27**(6): 545-553.
DE CAVANAGH, E. M. V., B. PIOTRKOWSKI, et al. (2003). "Enalapril and losartan attenuate mitochondrial dysfunction in aged rats." The FASEB Journal **17**(9): 1096-1098.
de Cavanagh, E. M. V., J. E. Toblli, et al. (2006). "Renal mitochondrial dysfunction in spontaneously hypertensive rats is attenuated by losartan but not by amlodipine." American Journal of Physiology - Regulatory, Integrative and Comparative Physiology **290**(6): R1616-R1625.
de Gasparo, M., K. J. Catt, et al. (2000). "International union of pharmacology. XXIII. The angiotensin II receptors." Pharmacological reviews **52**(3): 415-472.
Deluca, H. F. and G. W. Engstrom (1961). "Calcium uptake by rat kidney mitochondria." Proc Natl Acad Sci U S A **47**: 1744-1750.
Deng, A. Y., H. Dene, et al. (1994). "Mapping of a quantitative trait locus for blood pressure on rat chromosome 2." J Clin Invest **94**(1): 431-436.
Denton, D. A., M. J. McKinley, et al. (1996). "Hypothalamic integration of body fluid regulation." Proc Natl Acad Sci U S A **93**(14): 7397-7404.
DiBona, G. F. (2000). "Nervous Kidney: Interaction Between Renal Sympathetic Nerves and the Renin-Angiotensin System in the Control of Renal Function." Hypertension **36**(6): 1083-1088.

DiBona, G. F. (2001). "Peripheral and Central Interactions between the Renin-Angiotensin System and the Renal Sympathetic Nerves in Control of Renal Function." Ann N Y Acad Sci **940**(1): 395-406.

DiBona, G. F. and M. Esler (2010). "Translational medicine: the antihypertensive effect of renal denervation." Am J Physiol Regul Integr Comp Physiol **298**(2): R245-253.

DiBona, G. F. and S. Y. Jones (2001). "Sodium intake influences hemodynamic and neural responses to angiotensin receptor blockade in rostral ventrolateral medulla." Hypertension **37**(4): 1114-1123.

DiBona, G. F., S. Y. Jones, et al. (1996). "Effect of endogenous angiotensin II on renal nerve activity and its arterial baroreflex regulation." Am J Physiol **271**(2 Pt 2): R361-367.

DiBona, G. F. and U. C. Kopp (1997). "Neural control of renal function." Physiol Rev **77**(1): 75-197.

Dikalov, S. I. (2013). Role of mitochondrial oxidative stress in hypertension.

Dikalova, A. E., A. T. Bikineyeva, et al. (2010). "Therapeutic targeting of mitochondrial superoxide in hypertension." Circ Res **107**(1): 106-116.

Dilley, J. R., C. T. Stier, et al. (1984). "ABNORMALITIES IN GLOMERULAR FUNCTION IN RATS DEVELOPING SPONTANEOUS HYPERTENSION." American Journal of Physiology **246**(1): F12-F20.

Diz, D. I., J. A. Jessup, et al. (2002). "Angiotensin peptides as neurotransmitters/neuromodulators in the dorsomedial medulla." Clin Exp Pharmacol Physiol **29**(5-6): 473-482.

Diz, D. I., A. Nasjletti, et al. (1982). "Renal denervation at weaning retards development of hypertension in New Zealand genetically hypertensive rats." Hypertension **4**(3): 361-368.

Doggrell, S. A. and L. Brown (1998). "Rat models of hypertension, cardiac hypertrophy and failure." Cardiovascular Research **39**(1): 89-105.

Donoghue, M., F. Hsieh, et al. (2000). "A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9." Circ Res **87**(5): E1-9.

Doris, P. A. (2002). "Hypertension genetics, single nucleotide polymorphisms, and the common disease:common variant hypothesis." Hypertension **39**(2 Pt 2): 323-331.

Doughan, A. K., D. G. Harrison, et al. (2008). "Molecular Mechanisms of Angiotensin II–Mediated Mitochondrial Dysfunction: Linking Mitochondrial Oxidative Damage and Vascular Endothelial Dysfunction." Circulation Research **102**(4): 488-496.

Elo, J. M., S. S. Yadavalli, et al. (2012). "Mitochondrial phenylalanyl-tRNA synthetase mutations underlie fatal infantile Alpers encephalopathy." Hum Mol Genet **21**(20): 4521-4529.

Epstein, A. N., J. T. Fitzsimons, et al. (1970). "Drinking induced by injection of angiotensin into the rain of the rat." J Physiol **210**(2): 457-474.

Fan, M., J. Rhee, et al. (2004). "Suppression of mitochondrial respiration through recruitment of p160 myb binding protein to PGC-1α: modulation by p38 MAPK." Genes & Development **18**(3): 278-289.

Feinleib, M., R. J. Garrison, et al. (1977). "The NHLBI twin study of cardiovascular disease risk factors: methodology and summary of results." Am J Epidemiol **106**(4): 284-285.

Ferder, L. F., F. Inserra, et al. (2003). "Effects of renin-angiotensin system blockade in the aging kidney." Exp Gerontol **38**(3): 237-244.

Fernandez-Marcos, P. J. and J. Auwerx (2011). "Regulation of PGC-1α, a nodal regulator of mitochondrial biogenesis." The American Journal of Clinical Nutrition **93**(4): 884S-890S.

Ferrario, C. M., M. C. Chappell, et al. (1997). "Counterregulatory actions of angiotensin-(1-7)." Hypertension **30**(3 Pt 2): 535-541.

Fluharty, S. J. and A. N. Epstein (1983). "Sodium appetite elicited by intracerebroventricular infusion of angiotensin II in the rat: II. Synergistic interaction with systemic mineralocorticoids." Behav Neurosci **97**(5): 746-758.

Folkow, B. (1993). "Early structural changes in hypertension: pathophysiology and clinical consequences." J Cardiovasc Pharmacol **22 Suppl 1**: S1-6.

Gansevoort, R. T., W. J. Sluiter, et al. (1995). "Antiproteinuric effect of blood-pressure-lowering agents: a meta-analysis of comparative trials." Nephrol Dial Transplant **10**(11): 1963-1974.

Gao, L., W. Wang, et al. (2004). "Superoxide mediates sympathoexcitation in heart failure: roles of angiotensin II and NAD(P)H oxidase." Circ Res **95**(9): 937-944.

García-Giménez, J. L., A. Gimeno, et al. (2011). "Differential Expression of PGC-1α and Metabolic Sensors Suggest Age-Dependent Induction of Mitochondrial Biogenesis in Friedreich Ataxia Fibroblasts." PLoS One **6**(6): e20666.

Garrett, M. R., H. Dene, et al. (1998). "Genome Scan and Congenic Strains for Blood Pressure QTL Using Dahl Salt-Sensitive Rats." Genome Res **8**(7): 711-723.

Geraldes, P. and G. L. King (2010). "Activation of protein kinase C isoforms and its impact on diabetic complications." Circ Res **106**(8): 1319-1331.

Gironacci, M. M., M. S. Valera, et al. (2004). "Angiotensin-(1-7) inhibitory mechanism of norepinephrine release in hypertensive rats." Hypertension **44**(5): 783-787.

Greenberg, S. and J. L. Osborn (1994). "Relationship between sodium balance and renal innervation during hypertension development in the spontaneously hypertensive rat." J Hypertens **12**(12): 1359-1364.

Grim, C. E., F. C. Luft, et al. (1980). "Racial differences in blood pressure in Evans County, Georgia: relationship to sodium and potassium intake and plasma renin activity." J Chronic Dis **33**(2): 87-94.

Guadagnini, D. and J. A. Gontijo (2006). "Altered renal sodium handling in spontaneously hypertensive rats (SHR) after hypertonic saline intracerebroventricular injection: role of renal nerves." Life Sci **79**(17): 1666-1673.

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

Guyton, A. C. (1990). "Renal function curves and control of body fluids and arterial pressure." Acta Physiol Scand Suppl **591**: 107-113.

Guyton, A. C. and T. G. Coleman (1968). "Regulation on interstitial fluid volume and pressure." Ann N Y Acad Sci **150**(3): 537-547.

Guyton, A. C., T. G. Coleman, et al. (1972). "Arterial pressure regulation. Overriding dominance of the kidneys in long-term regulation and in hypertension." Am J Med **52**(5): 584-594.

GUYTON, A. C., T. G. COLEMAN, et al. (1974). "Brief Reviews: A Systems Analysis Approach to Understanding Long-Range Arterial Blood Pressure Control and Hypertension." Circulation Research **35**(2): 159-176.

Gyurko, R., D. Wielbo, et al. (1993). "Antisense inhibition of AT1 receptor mRNA and angiotensinogen mRNA in the brain of spontaneously hypertensive rats reduces hypertension of neurogenic origin." Regul Pept **49**(2): 167-174.

Hagen, T. M., D. L. Yowe, et al. (1997). "Mitochondrial decay in hepatocytes from old rats: Membrane potential declines, heterogeneity and oxidants increase." Proceedings of the National Academy of Sciences **94**(7): 3064-3069.

Hatefi, Y. (1985). "The mitochondrial electron transport and oxidative phosphorylation system." Annu Rev Biochem **54**: 1015-1069.
Hein, L. and B. K. Kobilka (1995). "Adrenergic receptor signal transduction and regulation." Neuropharmacology **34**(4): 357-366.
HELMER, O. M. and W. E. JUDSON (1968). "Metabolic Studies on Hypertensive Patients with Suppressed Plasma Renin Activity Not Due to Hyperaldosteronism." Circulation **38**(5): 965-976.
Hendel, M. D. and J. P. Collister (2006). "Renal denervation attenuates long-term hypertensive effects of Angiotensin ii in the rat." Clin Exp Pharmacol Physiol **33**(12): 1225-1230.
Hengartner, M. O. (2000). "The biochemistry of apoptosis." Nature **407**(6805): 770-776.
Henriksen, E. J. (2007). "Improvement of insulin sensitivity by antagonism of the renin-angiotensin system." Am J Physiol Regul Integr Comp Physiol **293**(3): R974-980.
Herrera, M., M. A. Sparks, et al. (2013). "Lack of specificity of commercial antibodies leads to misidentification of angiotensin type 1 receptor protein." Hypertension **61**(1): 253-258.
Hilbert, P., K. Lindpaintner, et al. (1991). "Chromosomal mapping of two genetic loci associated with blood-pressure regulation in hereditary hypertensive rats." Nature **353**(6344): 521-529.
Hofmann, S., M. Jaksch, et al. (1997). "Population genetics and disease susceptibility: characterization of central European haplogroups by mtDNA gene mutations, correlation with D loop variants and association with disease." Hum Mol Genet **6**(11): 1835-1846.
Hopkins, P. N. and S. C. Hunt (2003). "Genetics of hypertension." Genet Med **5**(6): 413-429.
Huang, B. S., M. S. Amin, et al. (2006). "The central role of the brain in salt-sensitive hypertension." Curr Opin Cardiol **21**(4): 295-304.
Hussy, N., C. Deleuze, et al. (2000). "Osmotic regulation of neuronal activity: a new role for taurine and glial cells in a hypothalamic neuroendocrine structure." Prog Neurobiol **62**(2): 113-134.
Iams, S. G. and B. C. Wexler (1979). "Inhibition of the development of spontaneous hypertension in SH rats by gonadectomy or estradiol." The Journal of laboratory and clinical medicine **94**(4): 608-616.
Ichihara, A., E. W. Inscho, et al. (1997). "Role of renal nerves in afferent arteriolar reactivity in angiotensin-induced hypertension." Hypertension **29**(1 Pt 2): 442-449.
Inagaki, K., E. Churchill, et al. (2006). "Epsilon protein kinase C as a potential therapeutic target for the ischemic heart." Cardiovasc Res **70**(2): 222-230.
James, P. A., S. Oparil, et al. (2014). "2014 evidence-based guideline for the management of high blood pressure in adults: Report from the panel members appointed to the eighth joint national committee (jnc 8)." JAMA **311**(5): 507-520.
Jeunemaitre, X., F. Soubrier, et al. (1992). "Molecular basis of human hypertension: role of angiotensinogen." Cell **71**(1): 169-180.
Joe, B. and M. Garrett (2005). Substitution Mapping. Cardiovascular Genomics. M. Rai, J. R. Paton, S. Kasparov and M. Katovich, Humana Press**:** 41-58.
Johnson, M. L., D. L. Ely, et al. (1992). "Genetic divergence between the Wistar-Kyoto rat and the spontaneously hypertensive rat." Hypertension **19**(5): 425-427.
Judy, W. V., A. M. Watanabe, et al. (1976). "Sympathetic nerve activity: role in regulation of blood pressure in the spontaenously hypertensive rat." Circ Res **38**(6 Suppl 2): 21-29.

Kainulainen, K., M. Perola, et al. (1999). "Evidence for involvement of the type 1 angiotensin II receptor locus in essential hypertension." Hypertension **33**(3): 844-849.
Karim, Z., N. Defontaine, et al. (1995). "Protein kinase C isoforms in rat kidney proximal tubule: acute effect of angiotensin II." American Journal of Physiology - Cell Physiology **269**(1): C134-C140.
Kaschina, E. and T. Unger (2003). "Angiotensin AT1/AT2 receptors: regulation, signalling and function." Blood pressure **12**(2): 70-88.
Kelly, D. P. and R. C. Scarpulla (2004). "Transcriptional regulatory circuits controlling mitochondrial biogenesis and function." Genes & development **18**(4): 357-368.
Kirchheim, H., H. Ehmke, et al. (1987). "Sympathetic Modulation of the Pressure-Dependent Renin Release in Conscious Dogs." Clinical and Experimental Hypertension **a9**(s1): 167-180.
Kishi, T., Y. Hirooka, et al. (2004). "Increased reactive oxygen species in rostral ventrolateral medulla contribute to neural mechanisms of hypertension in stroke-prone spontaneously hypertensive rats." Circulation **109**(19): 2357-2362.
Kline, R. L. (1987). "Renal nerves and experimental hypertension: evidence and controversy." Can J Physiol Pharmacol **65**(8): 1540-1547.
Kobori, H., M. Nangaku, et al. (2007). "The intrarenal renin-angiotensin system: from physiology to the pathobiology of hypertension and kidney disease." Pharmacol Rev **59**(3): 251-287.
Krebs, H. A. (1970). "The history of the tricarboxylic acid cycle." Perspect Biol Med **14**(1): 154-170.
Kristal, B. S., C. T. Jackson, et al. (1997). "Defects at Center P Underlie Diabetes-Associated Mitochondrial Dysfunction." Free Radical Biology and Medicine **22**(5): 823-833.
Kumarasamy, S., K. Gopalakrishnan, et al. (2010). "Mitochondrial polymorphisms in rat genetic models of hypertension." Mammalian genome : official journal of the International Mammalian Genome Society **21**(5-6): 299-306.
Kurtz, T. W., R. Casto, et al. (1990). "Biometric genetic analysis of blood pressure in the spontaneously hypertensive rat." Hypertension **16**(6): 718-724.
Kurtz, T. W., K. A. Griffin, et al. (2005). "Recommendations for Blood Pressure Measurement in Animals: Summary of an AHA Scientific Statement From the Council on High Blood Pressure Research, Professional and Public Education Subcommittee." Arteriosclerosis, Thrombosis, and Vascular Biology **25**(3): 478-479.
Kurtz, T. W. and R. C. Morris (1987). "Biological variability in Wistar-Kyoto rats. Implications for research with the spontaneously hypertensive rat." Hypertension **10**(1): 127-131.
Kwitek-Black, A. and H. Jacob (2001). "The use of designer rats in the genetic dissection of hypertension." Current Hypertension Reports **3**(1): 12-18.
Larkin, J. E., B. C. Frank, et al. (2004). "Cardiac transcriptional response to acute and chronic angiotensin II treatments." Physiological Genomics **18**(2): 152-166.
Laulederkind, S. J., G. T. Hayman, et al. (2013). "The Rat Genome Database 2013--data, tools and users." Brief Bioinform **14**(4): 520-526.
Lavoie, J. L. and C. D. Sigmund (2003). "Minireview: overview of the renin-angiotensin system--an endocrine and paracrine system." Endocrinology **144**(6): 2179-2183.
Lee, H., Y. Abe, et al. (2014). "Increased mitochondrial activity in renal proximal tubule cells from young spontaneously hypertensive rats." Kidney Int **85**(3): 561-569.
Leigh-Brown, S., J. A. Enriquez, et al. (2010). "Nuclear transcription factors in mammalian mitochondria." Genome Biol **11**(7): 215.

Lenkei, Z., M. Palkovits, et al. (1997). "Expression of angiotensin type-1 (AT1) and type-2 (AT2) receptor mRNAs in the adult rat brain: a functional neuroanatomical review." Frontiers in neuroendocrinology **18**(4): 383-439.

Lewis, E. J., L. G. Hunsicker, et al. (2001). "Renoprotective effect of the angiotensin-receptor antagonist irbesartan in patients with nephropathy due to type 2 diabetes." N Engl J Med **345**(12): 851-860.

Li, R., Y. Liu, et al. (2009). "Failures in mitochondrial tRNAMet and tRNAGln metabolism caused by the novel 4401A>G mutation are involved in essential hypertension in a Han Chinese Family." Hypertension **54**(2): 329-337.

Liard, J. F. (1977). "Renal denervation delays blood pressure increase in the spontaneously hypertensive rat." Experientia **33**(3): 339-340.

Lifton, R. P. (1996). "Molecular genetics of human blood pressure variation." Science **272**(5262): 676-680.

Lifton, R. P., A. G. Gharavi, et al. (2001). "Molecular mechanisms of human hypertension." Cell **104**(4): 545-556.

Lill, R., K. Diekert, et al. (1999). "The essential role of mitochondria in the biogenesis of cellular iron-sulfur proteins." Biol Chem **380**(10): 1157-1166.

Littlejohn, N. K., R. B. Siel, Jr., et al. (2013). "Hypertension in mice with transgenic activation of the brain renin-angiotensin system is vasopressin dependent." Am J Physiol Regul Integr Comp Physiol **304**(10): R818-828.

Liu, Y., R. Li, et al. (2009). "Mitochondrial transfer RNAMet 4435A>G mutation is associated with maternally inherited hypertension in a Chinese pedigree." Hypertension **53**(6): 1083-1090.

Longini, I. M., Jr., M. W. Higgins, et al. (1984). "Environmental and genetic sources of familial aggregation of blood pressure in Tecumseh, Michigan." Am J Epidemiol **120**(1): 131-144.

Lopez-Campistrous, A., L. Hao, et al. (2008). "Mitochondrial dysfunction in the hypertensive rat brain: respiratory complexes exhibit assembly defects in hypertension." Hypertension **51**(2): 412-419.

Louis, W. J. and L. G. Howes (1990). "Genealogy of the spontaneously hypertensive rat and Wistar-Kyoto rat strains: implications for studies of inherited hypertension." J Cardiovasc Pharmacol **16 Suppl 7**: S1-5.

Lowry, O. H., N. J. Rosebrough, et al. (1951). "PROTEIN MEASUREMENT WITH THE FOLIN PHENOL REAGENT." Journal of Biological Chemistry **193**(1): 265-275.

Lundin, S., H. Herlitz, et al. (1982). "Sodium balance during development of hypertension in the spontaneously hypertensive rat (SHR)." Acta Physiol Scand **115**(3): 317-323.

MacGregor, G. A. (1985). "Sodium is more important than calcium in essential hypertension." Hypertension **7**(4): 628-640.

MacMillan-Crow, L. A., D. L. Cruthirds, et al. (2001). "Mitochondrial tyrosine nitration precedes chronic allograft nephropathy." Free Radic Biol Med **31**(12): 1603-1608.

Madden, C. J. and A. F. Sved (2003). "Cardiovascular regulation after destruction of the C1 cell group of the rostral ventrolateral medulla in rats." Am J Physiol Heart Circ Physiol **285**(6): H2734-2748.

Madeddu, P., V. Anania, et al. (1995). "Prevention by blockade of angiotensin subtype1-receptors of the development of genetic hypertension but not its heritability." Br J Pharmacol **115**(4): 557-562.

Mason, W. T. (1980). "Supraoptic neurones of rat hypothalamus are osmosensitive." Nature **287**(5778): 154-157.

Matsubara, H. (1998). "Pathophysiological Role of Angiotensin II Type 2 Receptor in Cardiovascular and Renal Diseases." Circulation Research **83**(12): 1182-1191.
McKinley, M. J., A. L. Albiston, et al. (2003). "The brain renin-angiotensin system: location and physiological roles." Int J Biochem Cell Biol **35**(6): 901-918.
Medina, R., L. E. Cardona-Sanclemente, et al. (1997). "Effect of captopril and losartan on blood pressure and accumulation of LDL and fibrinogen by aortic wall and other tissues in normotensive and hypertensive rats." J Cardiovasc Pharmacol **29**(1): 125-129.
Mizuno, M., T. Sada, et al. (2006). "The effect of angiotensin II receptor blockade on an end-stage renal failure model of type 2 diabetes." J Cardiovasc Pharmacol **48**(4): 135-142.
Mochly-Rosen, D., K. Das, et al. (2012). "Protein kinase C, an elusive therapeutic target?" Nat Rev Drug Discov **11**(12): 937-957.
Mollnau, H., M. Wendt, et al. (2002). "Effects of angiotensin II infusion on the expression and function of NAD(P)H oxidase and components of nitric Oxide/cGMP signaling." Circulation Research **90**(4): E58-E65.
Monteiro, P., A. I. Duarte, et al. (2005). "Valsartan improves mitochondrial function in hearts submitted to acute ischemia." Eur J Pharmacol **518**(2-3): 158-164.
Moreno, M. U., G. San Jose, et al. (2003). "Preliminary characterisation of the promoter of the human p22(phox) gene: identification of a new polymorphism associated with hypertension." FEBS Lett **542**(1-3): 27-31.
Morimoto, S., M. D. Cassell, et al. (2001). "Elevated blood pressure in transgenic mice with brain-specific expression of human angiotensinogen driven by the glial fibrillary acidic protein promoter." Circ Res **89**(4): 365-372.
National Heart, L., B. Institute, et al. (1977). Spontaneous hypertension, its pathogenesis and complications: proceedings of the 2d International Symposium on the Spontaneously Hypertensive Rat, Dept. of Health, Education, and Welfare, Public Health Service, National Institutes of Health.
Navar, L. G., L. M. Harrison-Bernard, et al. (1999). "Intrarenal angiotensin II generation and renal effects of AT1 receptor blockade." J Am Soc Nephrol **10 Suppl 12**: S266-272.
Navar, L. G., M. C. Prieto, et al. (2011). "Intrarenal angiotensin II and its contribution to the genesis of chronic hypertension." Curr Opin Pharmacol **11**(2): 180-186.
Nicholls, D. G., S. Vesce, et al. (2003). "Interactions between mitochondrial bioenergetics and cytoplasmic calcium in cultured cerebellar granule cells." Cell Calcium **34**(4-5): 407-424.
Nishimura, M., H. Takahashi, et al. (2007). "Upregulation of the brain renin-angiotensin system in rats with chronic renal failure." Acta Physiol (Oxf) **189**(4): 369-377.
Norton, G. R., R. Brooksbank, et al. (2010). "Gene variants of the renin-angiotensin system and hypertension: from a trough of disillusionment to a welcome phase of enlightenment?" Clinical science (London, England : 1979) **118**(8): 487-506.
Nowak, G. y. (2002). "Protein Kinase C-α and ERK1/2 Mediate Mitochondrial Dysfunction, Decreases in Active Na+ Transport, and Cisplatin-induced Apoptosis in Renal Cells." Journal of Biological Chemistry **277**(45): 43377-43388.
Okamoto, K. and K. Aoki (1963). "Development of a strain of spontaneously hypertensive rats." Japanese circulation journal **27**: 282-293.
Oliet, S. H. and C. W. Bourque (1992). "Properties of supraoptic magnocellular neurones isolated from the adult rat." J Physiol **455**: 291-306.
Orlov, S. N. and A. A. Mongin (2007). "Salt-sensing mechanisms in blood pressure regulation and hypertension." American Journal of Physiology - Heart and Circulatory Physiology **293**(4): H2039-H2053.

Osanai, T., S. Sasaki, et al. (2003). "Circulating coupling factor 6 in human hypertension: role of reactive oxygen species." J Hypertens **21**(12): 2323-2328.
Osanai, T., M. Tanaka, et al. (2001). "Mitochondrial coupling factor 6 as a potent endogenous vasoconstrictor." J Clin Invest **108**(7): 1023-1030.
Osborn, J. L. and A. K. S. Camara (1997). "Renal Neurogenic Mediation of Intracerebroventricular Angiotensin II Hypertension in Rats Raised on High Sodium Chloride Diet." Hypertension **30**(3): 331-336.
Osborn, J. L., H. Holdaas, et al. (1983). "Renal adrenoceptor mediation of antinatriuretic and renin secretion responses to low frequency renal nerve stimulation in the dog." Circ Res **53**(3): 298-305.
Osborn, J. L., C. F. Plato, et al. (1997). "LONG-TERM INCREASES IN RENAL SYMPATHETIC NERVE ACTIVITY AND HYPERTENSION." Clinical and Experimental Pharmacology and Physiology **24**(1): 72-76.
Osborn, J. W. (2005). "Hypothesis: set-points and long-term control of arterial pressure. A theoretical argument for a long-term arterial pressure control system in the brain rather than the kidney." Clin Exp Pharmacol Physiol **32**(5-6): 384-393.
Park, J. B., R. M. Touyz, et al. (2002). "Chronic treatment with a superoxide dismutase mimetic prevents vascular remodeling and progression of hypertension in salt-loaded stroke-prone spontaneously hypertensive rats." Am J Hypertens **15**(1 Pt 1): 78-84.
Patti, M. E., A. J. Butte, et al. (2003). "Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1." Proceedings of the National Academy of Sciences **100**(14): 8466-8471.
Pawlikowski, M., G. Melen-Mucha, et al. (1999). "The involvement of the renin-angiotensin system in the regulation of cell proliferation in the rat endometrium." Cell Mol Life Sci **55**(3): 506-510.
Peach, M. J. (1977). "Renin-angiotensin system: biochemistry and mechanisms of action." Physiol Rev **57**(2): 313-370.
Pfaff, I. L. and V. Vallon (2002). "Protein Kinase C Beta Isoenzymes in Diabetic Kidneys and Their Relation to Nephroprotective Actions of the ACE Inhibitor Lisinopril." Kidney and Blood Pressure Research **25**(5): 329-340.
Phillips, M. I. and B. Kimura (1988). "Brain angiotensin in the developing spontaneously hypertensive rat." J Hypertens **6**(8): 607-612.
Pinto, Y. M., M. Paul, et al. (1998). "Lessons from rat models of hypertension: from Goldblatt to genetic engineering." Cardiovasc Res **39**(1): 77-88.
Pravenec, M., P. C. Churchill, et al. (2008). "Identification of renal Cd36 as a determinant of blood pressure and risk for hypertension." Nature genetics **40**(8): 952-954.
Pravenec, M., M. Hyakukoku, et al. (2007). "Direct linkage of mitochondrial genome variation to risk factors for type 2 diabetes in conplastic strains." Genome Res **17**(9): 1319-1326.
Pravenec, M. and T. W. Kurtz (2010). "Recent advances in genetics of the spontaneously hypertensive rat." Curr Hypertens Rep **12**(1): 5-9.
Pravenec, M., V. Landa, et al. (2001). "Transgenic rescue of defective Cd36 ameliorates insulin resistance in spontaneously hypertensive rats." Nature genetics **27**(2): 156-158.
Pueyo, M. E., J. F. Arnal, et al. (1998). "Angiotensin II stimulates the production of NO and peroxynitrite in endothelial cells." Am J Physiol **274**(1 Pt 1): C214-220.
Pueyo, M. E., W. Gonzalez, et al. (2000). "Angiotensin II Stimulates Endothelial Vascular Cell Adhesion Molecule-1 via Nuclear Factor-κB Activation Induced by

Intracellular Oxidative Stress." Arteriosclerosis, Thrombosis, and Vascular Biology **20**(3): 645-651.
Puigserver, P. (2005). "Tissue-specific regulation of metabolic pathways through the transcriptional coactivator PGC1-alpha." Int J Obes (Lond) **29 Suppl 1**: S5-9.
Raizada, M. K., C. Sumners, et al. (1993). "Angiotensin II type 1 receptor mRNA levels in the brains of normotensive and spontaneously hypertensive rats." J Neurochem **60**(5): 1949-1952.
Rapp, J. P. (2000). "Genetic Analysis of Inherited Hypertension in the Rat." Physiological Reviews **80**(1): 135-172.
Rapp, J. P., H. Dene, et al. (1994). "Seven renin alleles in rats and their effects on blood pressure." J Hypertens **12**(4): 349-355.
Reich, H. N., G. Y. Oudit, et al. (2008). "Decreased glomerular and tubular expression of ACE2 in patients with type 2 diabetes and kidney disease." Kidney Int **74**(12): 1610-1616.
Reinhart, G. A., T. E. Lohmeier, et al. (1995). "Hypertension induced by chronic renal adrenergic stimulation is angiotensin dependent." Hypertension **25**(5): 940-949.
Reja, V., A. K. Goodchild, et al. (2006). "Upregulation of angiotensin AT1 receptor and intracellular kinase gene expression in hypertensive rats." Clin Exp Pharmacol Physiol **33**(8): 690-695.
Rice, T., G. P. Vogler, et al. (1989). "Cardiovascular risk factors in a French Canadian population: resolution of genetic and familial environmental effects on blood pressure using twins, adoptees, and extensive information on environmental correlates." Genet Epidemiol **6**(5): 571-588.
Richter, C., J. W. Park, et al. (1988). "Normal oxidative damage to mitochondrial and nuclear DNA is extensive." Proc Natl Acad Sci U S A **85**(17): 6465-6467.
Rigat, B., C. Hubert, et al. (1990). "An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels." J Clin Invest **86**(4): 1343-1346.
Rodriguez-Iturbe, B., C. D. Zhan, et al. (2003). "Antioxidant-rich diet relieves hypertension and reduces renal immune infiltration in spontaneously hypertensive rats." Hypertension **41**(2): 341-346.
Roman, R. J. and A. W. Cowley, Jr. (1985). "Characterization of a new model for the study of pressure-natriuresis in the rat." Am J Physiol **248**(2 Pt 2): F190-198.
Roman, R. J., A. W. Cowley, Jr., et al. (1988). "Pressure-diuresis in volume-expanded rats. Cortical and medullary hemodynamics." Hypertension **12**(2): 168-176.
Rossing, K., K. J. Schjoedt, et al. (2005). "Enhanced renoprotective effects of ultrahigh doses of irbesartan in patients with type 2 diabetes and microalbuminuria." Kidney Int **68**(3): 1190-1198.
Rostand, S. G., K. A. Kirk, et al. (1982). "Racial differences in the incidence of treatment for end-stage renal disease." N Engl J Med **306**(21): 1276-1279.
Rothermund, L. and M. Paul (1998). "Hypertension and the renin-angiotensin system--evidence from genetic and transgenic studies." Basic research in cardiology **93 Suppl 2**: 1-6.
Rueckschloss, U., M. T. Quinn, et al. (2002). "Dose-Dependent Regulation of NAD(P)H Oxidase Expression by Angiotensin II in Human Endothelial Cells: Protective Effect of Angiotensin II Type 1 Receptor Blockade in Patients With Coronary Artery Disease." Arteriosclerosis, Thrombosis, and Vascular Biology **22**(11): 1845-1851.
Rüster, C. and G. Wolf (2006). "Renin-Angiotensin-Aldosterone System and Progression of Renal Disease." Journal of the American Society of Nephrology **17**(11): 2985-2991.

Saavedra, J. M. (2005). "Brain angiotensin II: new developments, unanswered questions and therapeutic opportunities." Cell Mol Neurobiol **25**(3-4): 485-512.
Sachse, A. and G. Wolf (2007). "Angiotensin II–Induced Reactive Oxygen Species and the Kidney." Journal of the American Society of Nephrology **18**(9): 2439-2446.
Sanbe, A., K. Tanonaka, et al. (1995). "Effects of long-term therapy with ACE inhibitors, captopril, enalapril and trandolapril, on myocardial energy metabolism in rats with heart failure following myocardial infarction." J Mol Cell Cardiol **27**(10): 2209-2222.
Santos, R. A., A. C. Simoes e Silva, et al. (2003). "Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas." Proc Natl Acad Sci U S A **100**(14): 8258-8263.
Scarpulla, R. C. (2002). "Transcriptional activators and coactivators in the nuclear control of mitochondrial function in mammalian cells." Gene **286**(1): 81-89.
Scarpulla, R. C., R. B. Vega, et al. (2012). "Transcriptional integration of mitochondrial biogenesis." Trends Endocrinol Metab **23**(9): 459-466.
Scherrer, H. (1959). "Hypothalamic stimulation and blood pressure homeostasis." Acta Neurovegetativa **20**(2): 205-218.
Schlick, N. E., M. I. Jensen-Seaman, et al. (2006). "Sequence analysis of the complete mitochondrial DNA in 10 commonly used inbred rat strains." Am J Physiol Cell Physiol **291**(6): C1183-1192.
Schmidt, S., J. Beige, et al. (1997). "A polymorphism in the gene for the angiotensin II type 1 receptor is not associated with hypertension." J Hypertens **15**(12 Pt 1): 1385-1388.
Schork, N. J., J. E. Krieger, et al. (1995). "A biometrical genome search in rats reveals the multigenic basis of blood pressure variation." Genome Res **5**(2): 164-172.
Schwartz, F., A. Duka, et al. (2004). "Mitochondrial genome mutations in hypertensive individuals." Am J Hypertens **17**(7): 629-635.
Sechi, L. A., C. A. Griffin, et al. (1996). "Tissue-Specific Regulation of Type 1 Angiotensin II Receptor mRNA Levels in the Rat." Hypertension **28**(3): 403-408.
Shin, W. S., M. Tanaka, et al. (2000). "A novel homoplasmic mutation in mtDNA with a single evolutionary origin as a risk factor for cardiomyopathy." American journal of human genetics **67**(6): 1617-1620.
Shokoji, T., A. Nishiyama, et al. (2003). "Renal Sympathetic Nerve Responses to Tempol in Spontaneously Hypertensive Rats." Hypertension **41**(2): 266-273.
Simpson, J. B., A. N. Epstein, et al. (1978). "Localization of receptors for the dipsogenic action of angiotensin II in the subfornical organ of rats." Journal of Comparative and Physiological Psychology **92**(4): 581-608.
Sipos, A., S. Vargas, et al. (2010). "Direct demonstration of tubular fluid flow sensing by macula densa cells." Am J Physiol Renal Physiol **299**(5): F1087-1093.
Sorescu, D. and K. K. Griendling (2002). "Reactive oxygen species, mitochondria, and NAD(P)H oxidases in the development and progression of heart failure." Congest Heart Fail **8**(3): 132-140.
Souza, D. R., J. G. Mill, et al. (2004). "Chronic experimental myocardial infarction produces antinatriuresis by a renal nerve-dependent mechanism." Braz J Med Biol Res **37**(2): 285-293.
St Lezin, E., L. Simonet, et al. (1992). "Hypertensive strains and normotensive 'control' strains. How closely are they related?" Hypertension **19**(5): 419-424.
Steckelings, U., C. Lebrun, et al. (1992). "Role of brain angiotensin in cardiovascular regulation." J Cardiovasc Pharmacol **19 Suppl 6**: S72-79.

Takami, S., T. Katsuya, et al. (1998). "Angiotensin II type 1 receptor gene polymorphism is associated with increase of left ventricular mass but not with hypertension." Am J Hypertens **11**(3 Pt 1): 316-321.
Tanito, M., H. Nakamura, et al. (2004). "Enhanced oxidative stress and impaired thioredoxin expression in spontaneously hypertensive rats." Antioxid Redox Signal **6**(1): 89-97.
Taylor, R. W. and D. M. Turnbull (2005). "Mitochondrial DNA mutations in human disease." Nat Rev Genet **6**(5): 389-402.
Tigerstedt, R. and P. Q. Bergman (1898). "Niere und Kreislauf1." Skandinavisches Archiv Für Physiologie **8**(1): 223-271.
Tomino, Y., Y. Makita, et al. (1999). "Relationship between polymorphism in the angiotensinogen, angiotensin-converting enzyme or angiotensin II receptor and renal progression in Japanese NIDDM patients." Nephron **82**(2): 139-144.
Tsuchida, A., Y. Liu, et al. (1994). "alpha 1-adrenergic agonists precondition rabbit ischemic myocardium independent of adenosine by direct activation of protein kinase C." Circulation Research **75**(3): 576-585.
Tu, W., G. J. Eckert, et al. (2014). "Racial Differences in Sensitivity of Blood Pressure to Aldosterone." Hypertension.
Vallon, V. (2003). "Tubuloglomerular Feedback and the Control of Glomerular Filtration Rate." Physiology **18**(4): 169-174.
van den Meiracker, A. H. and F. Boomsma (2003). "The angiotensin II-sympathetic nervous system connection." J Hypertens **21**(8): 1453-1454.
Veerasingham, S. J. and M. K. Raizada (2003). "Brain renin-angiotensin system dysfunction in hypertension: recent advances and perspectives." Br J Pharmacol **139**(2): 191-202.
Vejakama, P., A. Thakkinstian, et al. (2012). "Reno-protective effects of renin-angiotensin system blockade in type 2 diabetic patients: a systematic review and network meta-analysis." Diabetologia **55**(3): 566-578.
Ventura-Clapier, R., A. Garnier, et al. (2008). "Transcriptional control of mitochondrial biogenesis: the central role of PGC-1α." Cardiovasc Res **79**(2): 208-217.
Vereerstraeten, P. and C. Toussaint (1969). "Effects of plasmapheresis on renal hemodynamics and sodium excretion in dogs." Pflügers Archiv **306**(1): 92-102.
von Bohlen und Halbach, O. and D. Albrecht (2006). "The CNS renin-angiotensin system." Cell Tissue Res **326**(2): 599-616.
Votyakova, T. V. and I. J. Reynolds (2001). "ΔΨm-Dependent and -independent production of reactive oxygen species by rat brain mitochondria." Journal of Neurochemistry **79**(2): 266-277.
Walker, W. G., P. K. Whelton, et al. (1979). "Relation between blood pressure and renin, renin substrate, angiotensin II, aldosterone and urinary sodium and potassium in 574 ambulatory subjects." Hypertension **1**(3): 287-291.
Wallace, D. C. (1999). "Mitochondrial diseases in man and mouse." Science **283**(5407): 1482-1488.
Wallace, D. C. (2005). "A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine." Annu Rev Genet **39**: 359-407.
Wang, H., P. Maechler, et al. (2000). "Hepatocyte Nuclear Factor 4α Regulates the Expression of Pancreatic β-Cell Genes Implicated in Glucose Metabolism and Nutrient-induced Insulin Secretion." Journal of Biological Chemistry **275**(46): 35953-35959.
Wang, T. and G. Giebisch (1996). "Effects of angiotensin II on electrolyte transport in the early and late distal tubule in rat kidney." Am J Physiol **271**(1 Pt 2): F143-149.

Weinberger, M. H. (1996). "Salt Sensitivity of Blood Pressure in Humans." Hypertension **27**(3): 481-490.

Weir, M. R. and V. J. Dzau (1999). "The renin-angiotensin-aldosterone system: a specific target for hypertension management." American journal of hypertension **12**(12 Pt 3): 205S-213S.

Whitfield-Rucker, M. and V. M. Cassone (2000). "Photoperiodic regulation of the male house sparrow song control system: gonadal dependent and independent mechanisms." Gen Comp Endocrinol **118**(1): 173-183.

Wilson, D. K., L. Bayer, et al. (1999). "The prevalence of salt sensitivity in an African-American adolescent population." Ethnicity & disease **9**(3): 350-358.

Wilson, F. H., A. Hariri, et al. (2004). "A Cluster of Metabolic Defects Caused by Mutation in a Mitochondrial tRNA." Science **306**(5699): 1190-1194.

Winternitz, S. R., R. E. Katholi, et al. (1980). "Role of the renal sympathetic nerves in the development and maintenance of hypertension in the spontaneously hypertensive rat." J Clin Invest **66**(5): 971-978.

Wright, J. W., A. J. Bechtholt, et al. (1996). "Angiotensin III and IV activation of the brain AT1 receptor subtype in cardiovascular function." Peptides **17**(8): 1365-1371.

Wu, Z., P. Puigserver, et al. (1999). "Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1." Cell **98**(1): 115-124.

Wyss, J. M. (1993). "The role of the sympathetic nervous system in hypertension." Curr Opin Nephrol Hypertens **2**(2): 265-273.

Yakes, F. M. and B. Van Houten (1997). "Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress." Proceedings of the National Academy of Sciences **94**(2): 514-519.

Yamamoto, H., D. Okuzaki, et al. (2013). "Genetic analysis of genes causing hypertension and stroke in spontaneously hypertensive rats." Int J Mol Med **31**(5): 1057-1065.

Yang, R. H., H. Jin, et al. (1992). "Blocking hypothalamic AT1 receptors lowers blood pressure in salt-sensitive rats." Hypertension **20**(6): 755-762.

Ye, P. and M. J. West (2003). "Cosegregation analysis of natriuretic peptide genes and blood pressure in the spontaneously hypertensive rat." Clin Exp Pharmacol Physiol **30**(12): 930-936.

Yin, J.-X., R.-F. Yang, et al. (2010). "Mitochondria-produced superoxide mediates angiotensin II-induced inhibition of neuronal potassium current." American Journal of Physiology - Cell Physiology **298**(4): C857-C865.

Yongue, B. G., J. A. Angulo, et al. (1991). "Brain and liver angiotensinogen messenger RNA in genetic hypertensive and normotensive rats." Hypertension **17**(4): 485-491.

Zalba, G., G. San Jose, et al. (2001). "Polymorphisms and promoter overactivity of the p22(phox) gene in vascular smooth muscle cells from spontaneously hypertensive rats." Circ Res **88**(2): 217-222.

Zhang, S. L., J. Guo, et al. (2004). "Angiotensin II stimulates Pax-2 in rat kidney proximal tubular cells: impact on proliferation and apoptosis." Kidney international **66**(6): 2181-2192.

Zhu, X., C. A. McKenzie, et al. (2000). "Localization of a small genomic region associated with elevated ACE." American journal of human genetics **67**(5): 1144-1153.

Zimmerman, M. C., E. Lazartigues, et al. (2002). "Superoxide Mediates the Actions of Angiotensin II in the Central Nervous System." Circulation Research **91**(11): 1038-1045.

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Publications

Peer Refereed

1. Bradshaw, T.J., S.P. Carmichael II, **J.A. Collett**, A.F. Ferrier, & J.L. Osborn. Using Vernier Equipment to Convert Didactically Taught Human Respiration Lab to Inquiry Based Human Respiration Lab. Tested Studies for Laboratory Teaching: *Proceedings of the Annual Conference of the Association for Biology Laboratory Education*, 2007.

2. **Collett**, J.A., A. Hart, E. Patterson, J. Kretzer, J.L. Osborn.. Renal Angiotensin II Type 1 Receptor Expression and Associated Hypertension in Rats with Minimal SHR Nuclear Genome. *Physiological Reports* 1 (5), 2013.

Abstracts

1. **Jason A. Collett**, Samuel P. Carmichael II, Aaron Fain, Erin A. Wyatt and Jeffrey L. Osborn
   Expression of hypertension over six generations in Spontaneous Hypertension/ Brown Norway (SHR/BN) congenic rats. *FASEB Journal 21; 6, 1017.34. 2007*.

2. **Jason A. Collett**, Kirsten Blaker, Hersh Patel and Jeffrey L. Osborn. Sodium Intake Sensitivity in Hypertensive and Normotensive Congenic, Spontaneously Hypertensive Rats. *FASEB Journal 21; 5, A507. 2007*.

3. **Jason A. Collett**, Brandon M. Franklin and Jeffrey L. Osborn
   Evidence for andrenergic receptor activation in control of hemolymph flow in Procambarus clarkii. *FASEB Journal 25, 20011*.

4. **Jason A. Collett**, Adesuwa Ighodaro and Jeffrey L. Osborn
   Tissue Angiotensin II Type 1 Receptor Expression in Development of Hypertension in SHR/Brown Norway Backcross Rats. *FASEB Journal 26, 2012*.

5. **Jason A. Collett**, Jiffin K. Paulose, Vincent M. Cassone and Jeffrey L.Osborn
   Maternally Derived Mitochondria in SHR Exhibits Significant Reduction in Oxidative Phosphorylation Gene Expression. *FASEB Journal 27, 955.4. 2013*.