FACTORS REGULATING THE INTRARENAL RENIN-ANGIOTENSIN SYSTEM AND RENAL DAMAGE DURING AGING

By

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<td>Renin-Angiotensin System</td>
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<tr>
<td>Ang</td>
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<td>ACE</td>
<td>Angiotensin Converting Enzyme</td>
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<td>Rostral Ventrolateral Medulla</td>
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<tr>
<td>SFO</td>
<td>Subfornical Organ</td>
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<tr>
<td>OVLT</td>
<td>Organum Vasculosum Laminae Terminales</td>
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<tr>
<td>AP</td>
<td>Area Postrema</td>
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<tr>
<td>MnPO</td>
<td>Median Preoptic Nucleus</td>
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ABSTRACT

Shea Gilliam-Davis

FACTORS REGULATING THE INTRARENAL RENIN-ANGIOTENSIN SYSTEM

AND RENAL DAMAGE DURING AGING

Dissertation under the direction of

Debra I. Diz, Ph.D., Professor

The renin-angiotensin system (RAS) plays an important role in regulating blood pressure, fluid homeostasis, and it even contributes to insulin resistance. Dysfunction of this system ultimately leads to cardiovascular related diseases, such as hypertension and diabetes. Studies show that RAS blockade reduces the onset of the metabolic syndrome (MetS) and type 2 diabetes, however, the precise mechanisms underlying the beneficial effects are not entirely known. The activation of the intrarenal RAS and onset of renal dysfunction occurs over the same time span as many of the features of the MetS, while the systemic RAS declines. This suggests that suppression of the intrarenal RAS may be partly responsible for the salutary effects of RAS blockade. However, the exact timing and mechanisms underlying the increase in the intrarenal RAS are not entirely known. We used Fischer 344 (F344) and Sprague Dawley (SD) rats because both strains develop insulin resistance and kidney damage (proteinuria) during aging with the SD rats also developing an increase in blood pressure. Long-term (1 year) systemic RAS blockade with the angiotensin (Ang) II type 1 (AT₁) receptor antagonist L-158,809 in the F344 rats prevented the age-related increase in serum leptin, insulin, glucose, body weight and increased excretion of Ang peptides and protein that occur independently of blood
pressure or plasma RAS peptides. F344 rats also have increased gene expression of brain (dorsomedial medulla) angiotensinogen with a similar trend for renin, while the expression of ACE2 and neprilysin remain the same with a decrease in ACE expression compared to younger animals. There was no change in the gene expression of AT_{1a}, AT_{1b}, AT_{2} or Mas receptors in the dorsomedial medulla during aging in the F344 rats but there was a decrease in leptin receptor expression and an increase in the expression of the PI3K p85 regulatory subunit. L-158,809 increased angiotensinogen, ACE, ACE2 and neprilysin mRNA with a similar trend for renin. RAS blockade also increased AT_{1b}, AT_{2}, Mas and leptin receptor mRNA as well as the p85 subunit expression. Thus, insulin resistance, renal injury and activation of the intrarenal RAS during early aging in normotensive animals can be prevented by systemic RAS blockade in association with changes in dorsomedial medulla enzymes and receptors that would shift the balance from Ang II to Ang-(1-7) in this brain region. The prevention of age-related declines in the leptin receptor and enhancement of a leptin signaling pathway provides mechanisms for preservation of metabolic function in treated F344 rats. To better define the time course of intrarenal RAS activation relative to the elevation in proteinuria in aging SD rats and to provide information on possible mechanisms underlying intrarenal RAS activation, the intrarenal RAS, proteinuria as well as indices of metabolism were studied from 24 to 48 wks of age. In addition, bilateral renal denervations were performed at 28 weeks or 75 weeks of age in SD rats. SD rats have a significant increase in the excretion of Ang I, Ang-(1-7), and protein, but not Ang II at 38 wks of age with an increase in urinary creatinine at 27 wks of age. Bilateral renal denervation in 28 week old SD rats significantly lowered blood pressure for at least five weeks post-surgery without altering
Ang peptides or creatinine excretion. Protein excretion increased in control animals and this was prevented or delayed in denervated rats. Bilateral renal denervation in 75 week old SD rats significantly lowered blood pressure for one week post-surgery without changing protein, creatinine or Ang peptides excretion. Renal nerves appear to contribute to renal damage (proteinuria) independent of changes in Ang II excretion but only during the early stages of aging. Renal nerves do not contribute to long-term support of blood pressure in older rats with existing renal impairment. Renal denervation is not sufficient to reverse or prevent continuing declines in renal function (increases in urinary Ang II or protein) at this late time point. Based on these studies, we suggest that the intrarenal, systemic and brain RAS are independently regulated during aging, each may contribute to age-related metabolic derangements and renal damage independently of pressure, and that renal nerves may play a role in initiation of the renal damage but not regulation of the intrarenal RAS.
CHAPTER ONE

INTRODUCTION

1. Systemic Renin-Angiotensin System (RAS): Overview of Components, Features and Actions

The RAS is one of the most studied enzyme-substrate systems of the body in part due to its role in regulating 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 cascade begins with the release of the aspartyl protease renin from the juxtaglomerular (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 dipeptidyl carboxypeptidase angiotensin-converting enzyme (ACE) located ubiquitously along vascular walls, Ang-(1-7) by neprilysin and Ang-(1-9) by the zinc metalloprotease ACE2. 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. Ang II 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 salutary effects on cardiovascular health (18; 68). Most of the classical actions of Ang II such as vasoconstriction, salt retention, aldosterone release and sympathetic activation are facilitated by AT\(_1\) receptor activation. There are two AT\(_1\) receptor isoforms in rodents, AT\(_{1A}\) and AT\(_{1B}\), with AT\(_{1A}\) generating
most of the classical AT$_1$ receptor actions (8). The AT$_{1A}$ receptor is located in most organs while the AT$_{1B}$ receptor is mainly found in the adrenal and pituitary glands (68). Humans have one type of AT$_1$ receptor (68). Activation of the AT$_2$ receptor generally has effects that are contrary to AT$_1$ receptor actions, mainly vasodilation, natriuresis and antiproliferation (18; 87). 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 (92). Ang-(1-7), the second most notable peptide of the RAS, has actions that are contrary to the effects of Ang II, such as vasodilation, natriuresis and antiproliferation (18). Ang-(1-7) exerts its actions through the G-protein coupled Mas receptor (96). 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. Ang IV binds the AT$_1$ and AT$_2$ receptors with low affinity and binds its own AT4 receptor which is the insulin-regulated aminopeptidase (IRAP) (11; 20; 76). Ang IV is a potent competitive inhibitor of the insulin-IRAP because it binds the IRAP catalytic site. This may block the degradation of numerous peptides such as vasopressin, somatostatin, met-enkephalin, lys-bradykinin, dynorphin A and cholecystokinin (76).

2. Physiological Roles of the RAS

The RAS has generally been affiliated with its role in regulating blood pressure and fluid 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. As noted before, the deleterious actions are usually a result of Ang II whereas Ang-(1-7) is generally associated with more beneficial
effects. Ang IV, under certain conditions has been shown to have actions similar and contrary to Ang II. The RAS contributes to oxidative damage (95), insulin resistance (55; 56; 102) and cell-growth (9) and has been implicated in the pathogenesis of the metabolic syndrome, diabetes, renal damage and hypertension.

Cardiovascular Regulation

The body has several mechanisms to control blood pressure. These mechanisms, such as the actions of the RAS, can alter the amount of blood pumped by the heart, the blood volume in the bloodstream and the diameter of the arteries. The RAS has a large influence on governing 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. Ang IV also has vasoconstrictor activities, though not as potent as Ang II. Both peptides primarily elicit constriction by acting at the AT\textsubscript{1} receptor, though Ang IV is a weak agonist (20). Interestingly, Ang IV also causes vasorelaxation by enhancing release of endothelial intracellular calcium and augmenting eNOS activity (20). Ang-(1-7) is a vasodilator and promotes nitric oxide, vasodilator prostaglandins and can act collectively with bradykinin (18).

Blood pressure regulation may occur in a rapid time frame (baroreflexes) or through long-term control involving the kidneys. The baroreflexes are mediated by the sympathetic nervous system and as noted earlier, the juxtaglomerular cells of the kidneys are responsible for the release of renin. 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 baroreflex gain. The baroreflex, which is a negative-feedback system, modifies blood pressure vacillations 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 to the nucleus tractus solitarius (nTS) 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). The activated CVLM sends inhibitory (GABAergic) fibers to the rostral ventrolateral medulla (RVLM). This mechanism will decrease heart rate by increasing vagal activity and decreasing sympathetic nervous system activity to the heart. The blood pressure is reset back to its original point. Ang II acts at AT$_1$ receptors in the nTS to decrease the baroreflex sensitivity (36). Ang-(1-7) enhances the baroreflex sensitivity also by acting at the nTS (22; 43). Unlike the baroreflex system, 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 an adequate fluid 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, this effect is helpful to ensure 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 (sodium retention) may lead to hypertension and damage the various organs and tissues of the body. 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 Ang II has on renal blood flow and glomerular filtration rate (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 (18). 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 (13). This increase in filtration fraction (GFR/RBF), causes an increase in the peritubular vessel oncotic pressure and a decrease in the hydrostatic pressure, which also occurs in the renal interstitium (13). This pressure gradient allows sodium and water to move from the proximal tubule to the interstitium (13). Ang II decreases medullary blood flow and reduces renal interstitial pressure which decreases water and sodium excretion (13). Ang II inhibits pressure-natriuresis and it is thought to be a tubuloglomerular feedback (TGF) modulator because it augments the TGF sensitivity (18; 18; 66; 114). Ang II is also involved in the proliferation of nephron cells (9).

Ang II enhances the sodium/hydrogen ($\text{Na}^+/\text{H}^+$) ion anti-porter activity on the luminal membrane of proximal tubules (13; 18) and in the early and late distal segments (66). The action of Ang II to augment distal tubule sodium reabsorption magnifies this effect in proximal tubules, to create a greater efficiency of sodium retention. It stimulates
the sodium/bicarbonate symporter and sodium/potassium pump in the proximal tubule basolateral membranes (13; 18). Ang II acts at the sodium/potassium pump in the medullary thick ascending limb and at the sodium channel in the epithelium of the cortical collecting tubules (13).

Ang-(1-7) has actions that oppose those of Ang II, which are mainly mediated through its vasodilatory effects. Ang-(1-7) dilates blood vessels through cellular mechanisms that augment vasodilator prostaglandins, NO and by potentiating the effects of bradykinin (18; 42). In addition, Ang-(1-7) has antiproliferative actions on VSMCs (18). Ang-(1-7) elicits natriuresis, diuresis and an increase in glomerular filtration rate (26; 42). Ang-(1-7) is a potent inhibitor of \( \text{Na}^+\text{K}^-\text{ATPase} \) activity in the proximal tubules and renal cortex (51) and it causes dilation of pre-constricted afferent arterioles through a receptor-mediated process (91). In renal epithelial cells, Ang-(1-7) impedes the transcellular flux of sodium associated with phospholipase A_2 activation (42). Furthermore, intrarenal administration of Ang-(1-7) in normotensive rats decreased tubular sodium transport without altering the vascular action of exogenous Ang II (15). Ang-(1-7) may mediate renal tubule vasopressin function through its receptor interacting with the AVP V2 system, which involves adenylate cyclase activation (72).

Ang IV has been shown to have effects similar to both Ang II and Ang-(1-7) in the kidney. Ang IV is reported to act as a vasodilator or vasoconstrictor depending on the vascular bed (20). The vasoconstrictor action has been shown to be mediated via the AT_1 receptor, while the vasodilatory effects appear to be mediated through AT_4 receptor activation (20). The AT_4 receptor is located in rat proximal tubule cells, outer medulla and in the glomerular tufts, thick ascending limbs of the loop of Henle and in the
collecting duct of other species (20; 52). In isolated rat proximal tubule cells, Ang IV inhibits sodium transport (52). Hamilton et al reported that Ang IV infused into rat renal arteries causes increased renal blood flow and sodium excretion without changing urine volume, GFR, or MAP (50). An AT$_4$ receptor antagonist inhibited this effect suggesting, that Ang IV plays a role in natriuresis (50). In contrast, van Rodijnen et al showed that Ang IV decreased renal cortical blood flow through AT$_1$ receptor mediated vasoconstriction (116). The exact role of Ang IV is not entirely clear and more studies are needed to determine its primary effect in the kidney.

Neural Effects

Ang II plays a prominent role in many central mechanisms. It interacts with neurotransmitters and is itself considered a peptidergic neurotransmitter under certain conditions (29; 36). 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 (29; 36) and as mentioned earlier, is known to decrease baroreflex function. Some of the central mechanisms are described in more detail below.

Thirst Sensation

The thirst mechanism is one way to increase fluid volume, leading to an 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 drinking response and blood pressure (76) and the subfornical organ (SFO), which lies outside the blood-brain barrier, is reported to be involved in this dipsogenic mechanism. 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 (5).
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) (5) and Ang II injected into these forebrain areas leads to an increased blood pressure (24). 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 (76). The MnPO is suggested to be an angiotensinergic synapse site that mediates thirst caused by Ang II because the SFO sends efferent projections to the MnPO (76). Projections are also sent to the paraventricular nucleus (PVN) of the hypothalamus which may contain Ang II and help release vasopressin.

Ang-(1-7), unlike Ang II, does not produce thirst or salt appetite (43). ICV infusion of Ang IV did not produce increased drinking in spontaneously hypertensive (SHR), SD or Wistar-Kyoto (WKY) rats (127).

Ang II and Vasopressin Release

Vasopressin is a hormone secreted by the posterior pituitary gland that helps regulate water retention by causing the kidneys to conserve water. It increases the water permeability of the distal convoluted and collecting tubules by inserting aquaporins into the apical membranes of the tubules/collecting duct epithelial cells. Vasopressin is also a vasoconstrictor.

Circulating Ang II stimulates vasopressin release mainly by binding receptors in the SFO and possibly in the OVLT (5). The vasopressin response to systemic Ang II was inhibited in rats in which the SFO was abolished (5). Injection of Ang II into the cerebral ventricles mainly activates CVO neurons, which indirectly or directly project to vasopressin producing neurons in the paraventricular and supraoptic (SON) nuclei (24).
The SFO is one such CVO that sends these angiotensinergic projections to the PVN and SON where vasopressin is produced and secreted. Ang II produced in the brain may cause vasopressin release as suggested by the fact that transgenic rats with a deficit in brain angiotensinogen have a decreased blood level of vasopressin (99). ICV administration of Ang II promotes vasopressin release and central administration of an AT$_1$ receptor antagonist decreased vasopressin secretion (76).

Schiavone et al reported that Ang-(1-7) is equipotent to Ang II in releasing vasopressin from the hypothalamo-neurohypophysial system explants from SD rats (98). It has been suggested that Ang IV by binding the IRAP (AT$_4$ receptor) inhibits the IRAP mediated hydrolysis of vasopressin, leading to the accumulation of vasopressin (46).

Ang II and the Sympathetic Nervous System

The RAS and sympathetic nervous system (SNS) work together to maintain an adequate blood pressure and fluid homeostasis which may be beneficial in conditions such as dehydration, sodium loss or hypotension but deleterious in the scenario of excessive sodium and fluid retention. Ang II activates the SNS at various levels and is known to be a tonic stimulus of renal sympathetic nerve activity (RSNA). Both central and circulating Ang II may influence sympathetic nervous system activity (SNSA). Ang II microinjected into the RVLM increases SNSA and arterial pressure (7). Microinjection of losartan and candesartan into the RVLM decreased the basal level of RSNA and heart rate, with candesartan also decreasing MAP (32). Bilateral injection of Ang receptor blockers (ARB) into the RVLM decreased resting arterial pressure similar to the decrease revealed subsequent to blockade of spinal sympathetic outflow (58). Intracerebroventricular (ICV) injection of losartan attenuated basal RSNA in proportion
to the level of RAS activation (33). Disinhibitory activation of the PVN using 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 (30). Ang II facilitates norepinephrine release from sympathetic nerve terminals (29; 36) such as the renal nerves.

Gironacci et al showed that Ang-(1-7) decreased norepinephrine release from the hypothalamus through the Mas or AT\textsubscript{2} receptors acting via a bradykinin/nitric oxide-mediated mechanism suggesting that Ang-(1–7) may decrease SNSA leading to an antihypertensive effect (47). Relatively little is known about Ang IV functions in the CNS. Ang IV is reported to be involved in learning, memory, exploratory behavior and neuronal development (10) as well as norepinephrine release from the renal nerves (110).

Ang II and Renal Sympathetic Nerves-Control of Renal Function

Ang II both from the circulation and the central nervous system (CNS) influences renal function through central mechanisms, which mainly involves 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 (29). Circulating Ang II acting at the AP may increase peripheral SNA through a direct excitatory projection from the AP to RVLM (29). CNS Ang II is known to be a tonic stimulus of RSNA and resets the baroreflex relationship between arterial pressure and RSNA, which increases RSNA for each level of blood pressure (32). For example, ICV injection of losartan in conscious rats did not affect basal levels of MAP,
but decreased basal RSNA suggesting that Ang II tonically influences basal levels of RSNA and its arterial baroreflex (33). CNS Ang II activates AT$_1$ receptors to suppress the baroreflex control of RSNA (115) and increases sodium reabsorption by stimulating renal nerve activity and catecholamine release within the kidney.

Bilateral microinjections of the Mas receptor antagonist A-779 into the PVN produced a selective and significant decrease in RSNA, suggesting that Ang-(1-7) in the PVN neurons is involved in mediating the tonic maintenance of RSNA (104). Ang-(1-7) enhanced norepinephrine release from stroke prone SHR kidneys but not from WKY rat kidneys. Ang-(1-7) also prevented Ang II mediated alterations in renal vascular resistance more potently in stroke prone SHR than in WKY rats. The Ang II-mediated facilitation of norepinephrine release was inhibited by Ang-(1-7). These data suggest that Ang-(1-7) modulates renal vascular resistance and sympathetic neurotransmission in SHR (109). Ang IV enhanced norepinephrine release to renal nerve stimulation and induced pressure responses in the isolated rat kidney, suggesting that it regulates renal vascular resistance and norepinephrine release (110).

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 (28). The density of renal tubule innervation is greatest in the ascending limb of Henle’s loop and least in the collecting
duct (28). When the nerves are stimulated renin is released from the JG cells via a β-1 adrenoceptor effect, the renal vasculature constricts and there is increased reabsorption of sodium and water in the tubules of the nephron. 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 may be 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. Previous work showed that norepinephrine (NE), the main neurotransmitter of renal nerves, increased Na⁺/K⁺-ATPase activity in cultured renal proximal tubule epithelial cells which was inhibited by α₁B adrenoceptor antagonists (28). Increased renal nerve activity is thought to play a role in many pathologic conditions such as hypertension (54; 123), myocardial infarction (107) and renal failure. Patients with renal failure have increased muscle sympathetic nerve activity (MSNA) and bilateral renal nephrectomy corrects blood pressure and MSNA (86). Impairment of volume overload-induced natriuresis was attenuated in myocardial infarcted rats subjected to bilateral renal denervation (107). Furthermore, renal denervation prevented hypertension in male SD rats with chronic renal failure (17) and in genetically hypertensive rats delays the development of hypertension (37; 64; 70; 123). Acute renal denervation in 18-20 month old SD rats improved the pressure natriuretic response (74). In addition, data suggest that altered renal hemodynamics in the form of hyperfiltration, which may in part be due to increased renal nerve activity, is key to producing pathological changes seen in the kidney (57; 122).
3. 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 (8; 68). The local systems seem to be regulated independently of the circulating RAS but may also interact with the circulating RAS. 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). While all tissue RASs appear to have major physiological roles, the focus of this thesis involves the kidney and to a lesser extent, the brain RAS.

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 (BBB) and a system in the circumventricular organs (CVO) and cerebrovascular endothelial cells that respond to circulating Ang II of peripheral origin (94). The two systems are interconnected and the brain responds to both. Angiotensinogen is produced mainly in the astrocytes (76). Renin mRNA is present in the central nervous system (CNS), but concentrations are low (76). High concentrations of ACE are located in the area postrema, subfornical organ (SFO), organum vasculosum laminae terminalis (OVLT) and median eminence (76). Aminopeptidase A and N are both located in the rodent brain (76). 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 (76). The brain contains AT$_1$, AT$_2$ and AT$_4$.
receptors (76). The AT$_1$ receptor is located in the nucleus of the solitary tract (nTS), the hypothalamic and paraventricular nucleus, the lamina terminalis, the ventrolateral and dorsal medulla, the lateral parabrachial nucleus and the median preoptic nucleus (36; 76; 118). These regions are involved in regulating cardiovascular function and/or body fluid and electrolyte balance. The AT$_2$ receptor is present at low density in adult brain but upregulated under pathological conditions (11). Expression of the AT$_2$ receptor is found in the ventral and dorsal medulla and at the border between the nTS and the area postrema (36). The AT$_4$ receptor is broadly dispersed in the rat and human brain (76). Though all components of the RAS are located in the brain, not a single cell contains all of the constituents of the RAS (11; 94).

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 (68; 108; 115). In rats with chronic renal failure, the brain RAS is upregulated, resulting in sympathetic overactivity and hypertension (84). Transgenic mice with increased brain Ang II production developed hypertension and an increase in salt appetite and drinking volume (79; 80). In contrast, transgenic rats with a deficit in brain Aogen (ASrAogen) do not develop hypertension and have lower levels of glucose, insulin and leptin during aging compared to age-matched Sprague-Dawley (SD) rats associated with reduced sympathetic nervous system activity (62). ASrAogen rats also maintain insulin sensitivity over the course of aging compared with age-matched SD and (mRen2)27 rats (63), suggesting a role for glial-produced Ang II in the metabolic impairments that occur during aging. Though information on the brain RAS is steadily
accumulating there is still much that needs to be revealed on the changes that occur in the brain RAS during aging and disease states.

Ang-(1-7) is located throughout the amygdala, hypothalamus and medulla oblongata and sometimes has effects similar to Ang II, such as inducing vasopressin release (117). However, in general, the peptides do have opposite effects in the brain. Ang-(1-7) at the nTS of the dorsal medulla in rats caused bradycardic and depressor responses and enhanced baroreflex control of heart rate (117). Furthermore, these effects were augmented in hypertensive animals versus controls (117).

Kidney RAS

All of the RAS components are present within the kidney with compartmentalization in the tubules and interstitium as well as intracellular accumulation (66). 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 (82). Angiotensinogen is located mostly in the proximal tubule cells and can be secreted directly into the tubule lumen (83). In general, renin from the juxtaglomerular (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 (66). The distal nephron segments also form renin (66). 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 (66). ACE is located on the
proximal tubule brush border and converts Ang I to Ang II (66). Neprilysin is located in the brush border membrane of the proximal tubule and its activity is decreased in the proximal tubules of aged SD rats but in general, it is plentiful in the kidney (89; 117). Neprilysin hydrolysis of Ang-(1-7) to produce Ang-(1-4) appears to be the main pathway for Ang-(1-7) degradation in the kidney (117). ACE2 is present in renal endothelial and tubule cells and in glomerular podocyte and mesangial cells (38; 90). 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 (83). However, Ang I and Ang II are mainly located in the tubular and interstitial fluid compartments (83). 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). The receptors are also found in the juxtaglomerular and macula densa cells, thick ascending limb, distal tubules, vasa recta, arcuate arteries and cortical collecting ducts (18; 66). 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 (18; 66). The AT$_4$ receptor is found in the renal medulla, vascular smooth muscle and endothelial cells. Ang IV is involved in natriuresis and increase renal cortical blood flow (18).
The intrarenal RAS is regulated differently than the circulating RAS as shown by the fact that proximal tubule angiotensinogen, collecting duct renin and tubular AT₁ receptors are increased by intrarenal Ang II (66). Intrarenal RAS activation may contribute to hypertension, renal injury and diabetes. Ang II produced in the kidney directly induces podocyte injury and apoptosis through AT₁ receptor activation, independent of hemodynamic changes (66). Ang II induces proliferation of glomerular endothelial cells, mesangial cells and fibroblasts (93). Hyperglycemia, proteinuria and renal injury activate the intrarenal RAS (93). It is reported, that under physiological conditions, Ang II regulates the endocytosis of urinary protein in proximal tubule cells (66).

To determine intrarenal levels of RAS components, samples from the urine, renal vein, interstitial fluid and tubular fluid have been assessed (83). All of these measurements are considered indices of intrarenal RAS levels (83). In addition, processed renal tissue has also been evaluated to determine intrarenal levels of RAS constituents. Intrarenal Ang II levels are four to five times higher in the medulla than in the cortex and intratubular concentrations of Ang II are in the nanomolar range (83). More recently, components of the RAS have been measured in urine as indicators of renal function. Patients with chronic kidney disease have increased levels of urinary angiotensinogen, protein and low estimated glomerular filtration rate that correlate with renal tissue Ang II (128). Urinary excretion of angiotensinogen in Ang-II dependent hypertensive rats signifies intrarenal (tubular) production of angiotensinogen, which also has a high correlation with kidney Ang II content (65).
4. Alterations in the RAS During Aging-Insights from Studies showing the Therapeutic Benefits of RAS Blockade

Aging is associated with changes in the activity and/or responsiveness of the RAS which leads to alterations in homeostatic mechanisms and even normal RAS levels may be harmful to overall cardiovascular health. Thus, the dysregulation and/or dysfunction of the RAS plays a role in many aging-related pathological conditions and RAS blockade attenuates the effects of diseases such as hypertension, diabetes and heart failure. The therapeutic benefits of RAS blockade are known to include actions outside of the classic blood pressure lowering effects and because of this, RAS antagonist have been used to treat a wide range of conditions in both hypertensive and normotensive individuals, particularly in the senescent population.

Changes in Circulating and Tissue RAS During Aging

The circulating, cardiac and intrarenal RAS are dissimilarly regulated in aging (16; 48; 60; 61) and during short and long-term RAS blockade (16; 61). During aging, there is a decline in the circulating RAS, which suggests that the beneficial actions of RAS blockade are partly due to the suppression of Ang II in tissues. Aging is also associated with a decline in aldosterone (4). Previous studies suggest that plasma renin concentrations, kidney renin mRNA and single nephron renin content are decreased in aging rats (60; 113) associated with a decrease in plasma angiotensin (Ang) peptides in aging Sprague Dawley (SD) rats (85): plasma renin is decreased in aging humans (121). It is believed that this may be due to glomerular damage and hyperfiltration which would lead to high levels of sodium delivered to the macula densa to cause a decrease in renin (4). In contrast, there is an increase in intrarenal tissue Ang II with aging (4) as well as
an increase in the excretion of Ang peptides (85). Cardiac Ang peptides are also elevated in aging animals (48). Neprilysin (endopeptidase 24.11) activity is reported to be decreased in the proximal tubules of aged SD rats/older animals (89). In rats and humans there is an enhanced vasoconstrictor response to Ang II in the aging kidney which may be due to changes in Ang II metabolism (113). The changes that occur with the RAS during aging are mainly known due to studies that have examined the effects of RAS blockade over the course of the aging process.

RAS blockade, either with an ACE inhibitor or an ARB, alters the levels of RAS components and this alteration is partly responsible for the benefits of RAS blockade. In addition, ACE inhibitors also increase nitric oxide levels by preventing the breakdown of bradykinin. ACE inhibition has been shown to increase Ang-(1-7) and decrease Ang II in the plasma. Perindopril treatment (7 days) increased plasma renin, Ang I and Ang-(1-7) decreased plasma angiotensinogen but did not change plasma Ang II levels (16). In the kidney, Ang II decreased with no change in Ang I and Ang-(1-7) levels (16). Angiotensinogen levels were decreased and while renin mRNA increased, renal renin levels did not change (16). Long-term treatment with enalapril or losartan increased plasma Ang I and Ang-(1-7) levels while plasma Ang II levels were higher following losartan treatment only (61). This effect is consistent with the known feedback mechanism of the RAS. In contrast, urinary excretion of Ang I, Ang II and Ang-(1-7) was significantly higher after either treatment compared to controls (61). In addition, receptor density was higher in the glomeruli, tubulointerstitium and vasa recta area after enalapril treatment but lower after losartan treatment. The AT$_1$ receptor was the predominant subtype in all the mentioned areas with the AT$_2$ receptor present in the
tubulointerstitial area (61). The information provided by these studies on the differential regulation between the plasma and kidney systems suggests that the effects of ACE inhibitors and ARBs depends partly on the modulation of the tissues angiotensin systems.

*Aging, Blood Pressure Regulation and RAS Blockade*

Systolic blood pressure tends to increase during aging, often times to hypertensive levels and systemic RAS blockade lowers blood pressure in humans and animals. Enalapril treatment for three weeks in adult rats decreased blood pressure and treatment with enalapril or losartan for six months attenuated the increased in blood pressure observed in normal rats (9). Long-term treatment (weaning-22 months) with enalapril or losartan significantly decreased blood pressure in Wistar rats compared to controls (61). Studies also attempt to shed light on the possibility of dual blockade therapy being an effective treatment. Patients given a combination therapy of an ACE inhibitor-ARB were reported to have a greater reduction in blood pressure compared to monotherapy treated patients (6). Of interest is the fact that the systemic blockade lowers blood pressure even though the circulating RAS is decreased with aging. Thus, the exact mechanisms behind the beneficial effects of RAS blockade on blood pressure are not entirely known. It is hypothesized that the beneficial effects may involve blockade of the brain RAS since animals with a deficit in brain Aogen do not exhibit increased blood pressure with aging (62).

*Age-Related Changes in the Sympathetic and Parasympathetic Nervous System and Benefits of RAS Blockade*

During aging, there is a general imbalance between the sympathetic and parasympathetic activity, such that the sympathetic activity predominates. This may be
due to the reported decline in baroreflex sensitivity that occurs with age. The chronic increase in sympathetic activity during aging may also be partially due to increased activity of tissue RASs, such as that of the brain and kidney, since Ang II is known to activate the SNS and decrease baroreflex sensitivity (115). AsrAogen rats, which have low brain angiotensinogen levels, exhibit lower SNS outflow during aging compared to age-matched SD rats (62). In older humans, SNS activation may be neurally mediated (39) which could translate to the kidney via the actions of renal nerves. It is suggested that SNS activation does not uniformly affect all sympathetic outflows (39) and it is reported that there is differentiated sympathetic neural control of the kidney (27; 34).

Renal failure patients have increased muscle sympathetic nerve activity (MSNA) and bilateral renal nephrectomy corrects blood pressure and MSNA (86). Renal denervation prevented hypertension in rats with chronic renal failure (17) similar to the effects of RAS blockers in alleviating hypertension and nephropathy (13). When SNS activity is suppressed in older normotensive adults, the vascular α-adrenergic responsiveness is upregulated. Older normotensive adults have a higher SNS activity with an appropriate down-regulation of α-adrenergic receptors compared to younger adults (112). However, in older hypertensive patients, there is lack of α-adrenergic receptor down-regulation in spite of a similar level of SNS activity which could contribute to the higher blood pressure (112). Hypertensive humans and animals also have increased RSNA (31) and the kidney can be both an object of sympathetic activity and a supplier of signals that drive sympathetic tone (86). These finding suggests that the tissue RASs and renal nerves may be involved in this activation during aging.
Metabolic Dysfunction During Aging and the Benefits of RAS Blockade

Aging is often accompanied by an increase in insulin resistance, glucose, leptin and body weight. The clustering of such factors is known as the metabolic syndrome (MetS) (2; 49), which is said to be an intermediate state between normal metabolism and Type 2 diabetes (49; 88). Studies show that RAS blockade improves many of these components of aging, and reduces the onset of the MetS and type 2 diabetes (1; 25; 53; 130), however, the precise mechanisms underlying the beneficial effects are not entirely known.

In the Heart Outcomes Prevention Evaluation (HOPE) Study, treatment with ramipril decreased the development of Type 2 Diabetes in high risk patients (130). In the Losartan Intervention for Endpoint (LIFE) reduction in hypertension study, there was a 25% lower incidence of new onset Type 2 Diabetes in patients treated with losartan compared to those treated with atenolol (25). In the Valsartan Antihypertensive Long-term Use Evaluation (VALUE) trial (59), valsartan reduced new onset diabetes suggesting the antidiabetic effect is mediated through the AT₁ receptor. Valsartan treatment in KK-Ay mice, a model of type 2 diabetes, did not affect SBP, but improved insulin sensitivity, PI3K activity and glucose transporter 4 (Glut4) translocation while decreasing TNF-α expression in skeletal muscle (102). Obese Zucker rats treated with irbesartan had improved whole body insulin sensitivity and enhancement of glucose uptake into the soleus and epitrochlearis muscle. This was partially ascribed to an increase in Glut4 protein levels in the heart, soleus, and plantaris muscle (56). Insulin and leptin interact in the rat hypothalamus at the PI3 and MAP kinase pathways to decrease feeding and maintain an appropriate metabolism (19). In addition, insulin and
Ang II cross-talk at the PI3 and MAP kinase pathways. Ang II may attenuate the metabolic actions of insulin at the PI3 kinase pathway but synergistically promote its proliferative effects through the MAP kinase pathway (88). While the exact mechanisms of the beneficial effects of RAS blockade on insulin and glucose are not entirely known, it may be partly due to the decreased actions of Ang II at the kinase pathways as well as actions in the central nervous system on autonomic pathway controlling insulin and glucose metabolism.

The AsrAogen rats, which have a 90% reduction in brain (glial) angiotensinogen, have lower levels of insulin, leptin and glucose associated with reduced weight gain during aging compared to age-matched SD rats (62; 63; 85). These data suggests that the glial RAS is involved in the development of insulin resistance as well as influencing weight gain associated with early aging. Mice deficient in adipose tissue angiotensinogen gained less weight in response to a standard or high fat diet compared to controls even though food intake was similar between the groups (75).

Metabolic derangement has several negative effects on the kidney. Insulin stimulates renal cell proliferation and production of growth factors such as TGF-β (97). Insulin upregulates AT₁ receptor expression in mesangial cells (97). Furthermore, insulin resistance and hyperinsulinemia are associated with reduced endothelial production of nitric oxide and increased oxidative stress (97). Insulin effects renal function mainly at the tubular level because specific binding of insulin is greatest in the thick ascending limb and convoluted tubules (97). The antinatriuretic action of insulin is preserved in insulin resistance (44; 81) which may add to the detrimental effects of conditions such as hypertension and diabetes. Anderson et al showed that insulin and Ang II act
synergistically to enhance TGF-β1 and collagen production in cultured mesangial cells (3). Hyperglycemia causes glomerular hyperfiltration by inducing dilation of the afferent arteriole to a greater extent than that of the efferent arteriole (101) and has been shown to activate the intrarenal RAS (18; 105; 106; 119; 129). In primary mesangial cells from normal SD rats cultured in high glucose, angiotensinogen, Ang I and Ang II production increased with no change in renin and ACE levels. This suggests that hyperglycemia increases mesangial Ang II production via an increase in angiotensinogen and Ang I (106). Mouse podocytes exposed to high glucose had increased generation of angiotensinogen, Ang I, Ang II and increased AT₁ receptors. There was no difference in renin activity or levels of ACE and AT₂ receptors. Glomerular angiotensinogen levels and AT₁ receptors were also increased (129). Leptin stimulates the proliferation of glomerular endothelial cells and induces the production of transforming growth factor (TGF)-β1 (14). Leptin stimulates type 1 collagen in mesangial cells and type 4 collagen in glomerular endothelial cells which leads to extracellular matrix deposition, glomerulosclerosis, and proteinuria (14). Leptin inhibits pancreatic insulin secretion (14; 111) and insulin-induced activities are decreased when hepatic cells are exposed to leptin (14). Serum levels of the cytokine tumor necrosis factor (TNF)-α correlate with leptin levels in diabetic patients (111). Wolf et al reported that leptin stimulates glomerular endothelial cell proliferation in vitro and in vivo and that leptin administration in rats caused proteinuria and glomerular mesangial matrix expansion. This suggests that leptin is a renal growth and profibrogenic factor (126). Leptin modulates insulin sensitivity and hyperleptinemia incites insulin resistance and vice versa (73). Leptin, like insulin, via actions in the brain, activates the SNS. Activation of the SNS can lead to a reduction in
GFR and salt and water retention. This may lead to activation of the renal RAS and kidney damage. Thus, elevated levels of insulin, glucose and leptin may contribute to the declining renal function that occurs during aging.

Changes in Kidney Function During Aging and Renoprotective Effects of RAS Blockade

The number of glomeruli and GFR decline with aging mainly because of glomerulosclerosis and tubular damage that leads to kidney dysfunction (41). There is also an age-related decrease in kidney size which leads to reduced GFR and RBF (41). In addition, hypertrophy may occur in the remaining glomeruli. As more glomeruli become damaged, Ang II increases glomerular filtration rate and renal vascular resistance by increasing intraglomerular pressure via efferent arteriole constriction. Ang II also functions as renal growth factor, profibrogenic cytokine, proinflammatory promoter, and is involved in hypertrophy, apoptosis of podocytes and tubulointerstitial injury (124; 125). Ang II also mediates proteinuria and reduces protein degradation by inhibiting proteases (93). Proteinuria is considered the hallmark of renal disease and has been suggested to be both a cause and consequence of renal damage. Proteinuria may occur as a result of glomerular and/or tubular dysfunction. Levels of serum and urine creatinine are also considered markers of renal damage. Increased levels of serum creatinine indicate renal damage due to decreased creatinine clearance. However, elevated creatinine levels in urine during aging may be indicative of glomerular hyperfiltration which is an initiating factor in diabetic renal disease.

The American Diabetes Association suggest that ACE inhibitors and ARBs be considered as first-line therapy to slow renal disease progression in hypertensive and type 2 diabetic patients (23). In hypertensive, type 2 diabetic patients, irbesartan delayed the
increase in serum creatinine 24% more slowly than the placebo group and 21% more slowly than the group that received the calcium channel blocker amlodipine (69). This renoprotective effect was independent of the blood pressure lowering effect (69) as ARBs have been shown to benefit normotensive diabetic patients as well (23). ACE inhibitors decrease mesangial expansion, glomerulosclerosis, loss of glomeruli, tubular atrophy, interstitial fibrosis and proteinuria (41; 45). Proteinuria is also decreased in the presence of AT₁ receptor blockers (77; 100). Losartan and enalapril reduced glomerular and tubulointerstitial fibrosis, tubular atrophy and increased the number mitochondria and improved mitochondrial function (40). In addition to increasing mitochondria, enalapril and captopril also altered the antioxidant/oxidant balance in favor of the latter (40). The benefits of ACE inhibitors may be due to elevation of Ang-(1-7) and bradykinin levels and decrease in Ang II and aldosterone formation. The benefits of ARBs may be due to Ang II binding at AT₂ receptors to produce effects opposite of AT₁ receptor binding. The salutary effects of RAS blockade occur even though studies report suppressed plasma Ang peptides (61) plasma renin and intrarenal renin mRNA during aging (60). However, studies also suggest an increase in renal angiotensin formation (4; 61), suggesting differential regulation of the intrarenal and plasma RASs (16; 61). The exact mechanisms for the differential regulation are not known. Urine RAS components have been used in human and animal studies to assess renal RAS activity and renal function (61; 65; 67; 85; 128). As mentioned before, it is known that urine RAS components increase during aging, but the exact timing of this increase is remains to be determined. The purpose of our studies is to better define the timing and potential mechanisms of activation of the intrarenal RAS and its connection to renal injury during aging. The
renal nerves serve as a connection between the kidney and SNS and supply a tonic input to both vascular and tubular elements of the kidney. Therefore, we attempt to investigate the role of renal nerves in renal RAS activation and renal injury. Our studies utilize two strains of rats commonly used in aging studies, the Fischer 344 and Sprague Dawley rats.

5. Use of Fischer 344 and Sprague Dawley Rats to Investigate the Role and Mechanisms of the Intrarenal RAS Activation

The activation of the intrarenal RAS during aging occurs over the same time span as many of the features of the MetS, such as increased insulin resistance and glucose levels. It is not clear to what magnitude the different components of MetS are interdependent. RAS blockade in normotensive rats prevents many age-related renal pathologies, including the increase in blood pressure that occurs with age (9; 40; 41) and urinary Ang peptides remain low in animals treated long-term with RAS blockers (61). However, the extent to which the protective effects on the kidney are a result of the maintenance of low blood pressure are unclear. We used the Fischer 344 (F344) rat, which represents an interesting model since insulin resistance and kidney damage occur without an increase in blood pressure during the aging process (71; 120). Using this model will allow us to determine which factors we are interested in studying are dependent or independent of increased blood pressure during aging. In the F344 rat, aging is associated with an increase in serum leptin (78). The F344 is an inbred strain with a relatively high survival at 2 years and a median life span of 28-31 months (12). We assessed the time course of changes in indices of metabolic function, blood pressure and activation of the intrarenal RAS in older animals in comparison to young F344 rats and in the presence of long-term RAS blockade with the AT1 receptor antagonist L-
L-158,809 was chosen because it is a potent, competitive and specific AT_1 receptor antagonist (21; 103). It is reported to be 10 to 100 times more potent than losartan with long duration and antihypertensive efficacy equal to ACE inhibition after single doses (103). L-158,809 is a nonpeptide and may possibly cross the blood brain barrier. Therefore, in addition, we assessed expression of RAS components in the dorsomedial medulla of the F344 rats to offer insight on the role of the brain RAS in the changes that occur during aging and the effects of long-term RAS blockade.

The exact timing and mechanisms underlying the increase in the intrarenal RAS are not entirely known. Levels of intrarenal RAS peptides has been determined at 3 time points (16, 48 and 68 weeks) in Sprague Dawley (SD) rats, with the increase occurring sometime between 16 and 48 weeks (85). We want to further define when this increase occurs so we also used the SD rat, a model commonly used in aging studies due to an aging profile that is similar to that of the humans. The SD rats have a life span of 70 to 80 weeks (17.5 to 20 months) (35). SD rats develop insulin resistance about same time as F344 rats and have increased blood pressure and renal damage with aging (62; 63; 85). Knowing a more precise time of activation will allow us to intervene before and after this activation occurs. We aim to investigate the role of one potential activator of the intrarenal RAS, the renal nerves. Since SNSA increases with age and is implicated in many age-related pathologies, we performed bilateral renal denervations before and after intrarenal RAS activation to determine the contribution of renal nerves to renal RAS regulation and the harmful changes that may occur during aging.
6. Rationale and Aims

The circulating RAS plays a major role in preserving optimal salt and water homeostasis especially when the body is compromised in conditions such as dehydration, avid sodium loss, hypotension or any clinical scenario associated with unstable hemodynamics. Nonetheless, the influence the circulating RAS has on long term control of normal cardiovascular function and the cardiovascular pathophysiology that is associated with aging is not well known. Over the course of aging, there is an increase in blood pressure associated with a reduction in the circulating RAS and renal function in both rats and humans. In addition, aging is accompanied by impaired energy metabolism that can be alleviated with RAS blockade. RAS blockade also inhibits or decreases the age-related increase in blood pressure and improves kidney function in spite of the decline in the circulating RAS components. The decrease in circulating RAS with age may be the result of a pressure-mediated inhibition of renin release. Increased SNS activity may be one of the mechanisms behind this effect since it may lead to elevated blood pressure. The age-related increase in SNS may lead to a decline in renal function, and renal damage may drive SNS activity, creating a viscous cycle. Renal denervation has been shown to delay the development of hypertension and reduce factors associated with renal failure, similar to the actions of RAS blockers. The improvement seen with renal denervation may be partially due to the reduction in age-related increase in SNS outflow to the kidney or reduced JG cell renin release, but the exact mechanisms are not known. Overall, this information suggests the involvement of one or more tissue RASs and renal nerve activity in these detrimental changes that occur with age. The function of the intrarenal RAS in these age-related pathologies is not completely known. Our goal is
to better determine the timing and potential mechanisms of activation of the intrarenal RAS and its connection to renal injury, metabolic impairments and increased blood pressure during aging. Figure 1 explains some possible mechanisms underlying the differential regulation of the plasma and intrarenal RASs during aging, as these factors relate to the thesis.

Overall Hypothesis
Intrarenal Angiotensin II is involved in the decline in kidney function, increased blood pressure and body weight and metabolic impairment during aging. Furthermore, renal injury during aging results from increased renal nerve activity that plays a primary role in the activation of the intrarenal RAS.

Specific Aims
Aim 1: Determine if RAS blockade will prevent activation of the intrarenal RAS and metabolic dysfunction independent of blood pressure-lowering effects in a normotensive rat strain.
Aim 2: Determine the expression of RAS components in the dorsomedial medulla to offer insight into the changes that occur during aging and the effects of long-term RAS blockade.
Aim 3: Determine the age at which the intrarenal RAS of SD rats is activated in comparison with protein and creatinine excretion.
Aim 4: Determine whether renal denervation in SD rats before activation of intrarenal RAS prevents this activation and the increase in systolic blood pressure.
Aim 5: Determine if renal denervation in older animals attenuates the increase in systolic blood pressure and reduces intrarenal RAS activation.


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Figure 1. Depiction of possible mechanisms responsible for the increase in intrarenal RAS and the decrease in the plasma RAS during aging. The increase in SBP may lead to a pressure-mediated decrease in JG cell renin release and a decrease in the plasma RAS. Renal Ang II and proteinuria may augment the renal RAS components, leading to an increased intrarenal RAS. SBP, RSNA and alterations in insulin, leptin and glucose may increase intrarenal RAS activity and proteinuria. Renal Ang II may decrease JG cell renin release. Factors highlighted in blue were studied as part of the thesis. AsrAogen rats, which have decreased brain RAS, are protected from many age-related changes as shown by the red x.
CHAPTER TWO

LONG-TERM AT₁ RECEPTOR BLOCKADE IMPROVES METABOLIC FUNCTION AND PROVIDES RENOPROTECTION IN FISCHER 344 RATS

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Abstract

Fischer 344 (F344) rats exhibit proteinuria and insulin resistance in the absence of hypertension as they age. We determined the effects of long-term (1 yr) treatment with the angiotensin (Ang) II type 1 (AT\textsubscript{1}) receptor blocker L-158,809 on plasma and urinary Ang peptide levels, systolic blood pressure (SBP) and indices of glucose metabolism in 15 month old male F344 rats. Young rats 3 months of age (n = 8) were compared with two separate groups of older rats, one control group (n = 7) and one treated with L-158,809 (n = 6) orally (20 mg/L) for one year. SBP was not different between control and treated rats, but was higher in young rats. Serum leptin, insulin and glucose levels were comparable between treated and young rats, whereas controls had higher glucose and leptin with a similar trend for insulin. Plasma Ang I and Ang II were higher in treated than untreated young or older rats, as evidence of effective AT\textsubscript{1} receptor blockade. Urinary Ang II and Ang-(1-7) were higher in controls compared with young animals and treated rats failed to show age-related increases. Protein excretion was markedly lower in treated and young rats compared to control rats (Young: 8 ± 2 vs Control: 129 ± 51 vs Treated: 9 ± 3 mg/day; p < 0.05). Long-term AT\textsubscript{1} receptor blockade improves metabolic parameters and provides renoprotection. Differential regulation of systemic and intrarenal (urinary) Ang systems occurs during blockade and suppression of the intrarenal system may contribute to reduced proteinuria. Thus, insulin resistance, renal injury and activation of the intrarenal Ang system during early aging in normotensive animals can be averted by renin-angiotensin system blockade.
Introduction

Many normotensive rat strains exhibit increases in systolic blood pressure (42), insulin resistance, body weight (13; 30; 31; 41) and serum leptin (30; 39) as they age. Similar changes occur in the aging human population and a clustering of these factors is consistent with the metabolic syndrome (2; 22). The metabolic syndrome (MetS), a collection of cardiovascular risk factors, is an intermediate state between normal metabolism and type 2 diabetes (22; 43). Clinical trials have shown that renin-angiotensin system (RAS) blockade, either by angiotensin converting enzyme (ACE) inhibitors (1; 57) or AT₁ receptor blockers (17; 27), may substantially lower the risk for type 2 diabetes in hypertensive subjects. However, the exact mechanism underlying this effect is unknown. Moreover, long term ACE inhibition or AT₁ receptor blockade in rodents protects against most of these age-related changes, including the increase in blood pressure (5; 18), weight gain (11) and decline in cognitive, mitochondrial, cardiac, and renal function (19).

The circulating, cardiac and intrarenal RAS are dissimilarly regulated in the aging rat (9; 21; 28; 29) and during short and long-term RAS blockade (9; 29). Previous studies suggest that plasma renin concentrations and kidney renin mRNA are decreased in aging rats (28) associated with a decrease in plasma angiotensin (Ang) peptides in aging Sprague Dawley (SD) rats (42): plasma renin is decreased in aging humans (54). In contrast, there is an increase in intrarenal tissue Ang II with aging (4) as well as an increase in the excretion of Ang peptides (42), an indicator of activation of the intrarenal RAS. Cardiac Ang peptides are also elevated in aging animals (21). These observations suggesting activation of the cardiac and intrarenal RAS during aging in the face of a
decline in the circulating RAS raise questions about the mechanisms underlying the beneficial effects of RAS blockade during aging.

The activation of the intrarenal RAS during aging occurs over the same time frame as many of the features of the MetS, but it is not clear to what extent the different components of MetS are interdependent. The F344 rat represents an interesting model since insulin resistance and kidney damage occur without an increase in blood pressure during the aging process (53). Previous studies in normotensive rats indicate that RAS blockade will prevent many age-related renal pathologies, including the increase in blood pressure that occurs with age (5; 18; 19). Further, urinary angiotensin peptides remain low in animals treated long-term (22 months) with RAS blockers (29). However, the extent to which the protective effects on the kidney are a result of the maintenance of low blood pressure are unclear. Therefore, we assessed the time course of changes in indices of metabolic function, blood pressure and activation of the intrarenal RAS in older animals in comparison to young F344 rats and in the presence of long-term (one year) RAS blockade to provide information on the interdependence of many of the features of MetS.
Methods

Twenty-one male F344 rats were obtained from Harlan Laboratories (Indianapolis, IN) and housed under controlled conditions of light (12 hour light-dark) and temperature with free access to food and water. At 3 months of age, one group of 8 rats (young animals) were subjected to the measurements. Separate groups of animals were used as vehicle controls (n = 7) or treated with 20 mg/L of L-158,809 (n = 6) in their drinking water for a period of one year. Systolic blood pressure (SBP) was measured using the tail-cuff procedure at ~3 months of age for the group of young rats and at ~15 months of age for the control and L-158,809-treated rats. There were at least three training sessions with the tail-cuff procedure to acclimate the animals to the device. The mean of at least five blood pressure measurements were determined for each animal. Animals were then weighed and placed in metabolic cages for 24 hour urine collections on dry ice. Food and water intake were also determined. The rats were euthanized at least 3 – 4 hours after removal of food and water by quick decapitation and trunk blood was collected in the presence of inhibitors as previously described (26). Serum insulin and leptin were measured using radioimmunoassays (Linco, Inc.) and serum glucose using a Freestyle glucose monitor (TheraSense). The quantitative insulin-sensitivity check index (QUICKI) and the homeostasis model assessment (HOMA) were used as indices of insulin sensitivity as previously described (7; 15; 25; 31; 32). We used ng/ml for insulin and mg/dl for glucose as the units for calculating these values (31). Plasma and urinary Ang I, Ang II, and Ang-(1-7) were analyzed using radioimmunoassay (3; 26). Protein in urine was measured using reagent strips (Multistix 10 SG, Bayer Corporation, Elkhart IN). Urine was analyzed for creatinine (Metra creatinine assay kit, Quidel).
Corporation) and the urinary peptide data are expressed as 24-hour excretion rates normalized to creatinine excretion as detailed elsewhere (20). Serum creatinine was analyzed using the Quantichrom creatinine assay kit, Bio Assay System. Glomerular filtration rate (GFR) was determined from serum and urine creatinine values.

Statistical Analysis

All data are presented as the mean ± SEM and p values < 0.05 were considered significant. One-way ANOVA with Tukey’s Multiple Comparison post hoc tests was performed using Graphpad Prism.

Results

Mean SBP was not different between older control and treated rats, but both were significantly lower (p< 0.001) than the young group (Fig. 1). We observed similar food intake between the two older groups of rats and both were statistically higher than the young rats (p< 0.05). While both older groups gained weight over the course of the study, the older treated group maintained a significantly lower body weight (p< 0.001) than the older control rats (Fig. 2). Water intake was significantly higher (p< 0.05) in the treated rats compared to the young and control rats (Fig. 3). Urine volume was higher (p< 0.01) in the two older groups of rats compared to the young animals (Fig. 3).

As expected in this model of aging, there was a significant increase (p< 0.001) in serum leptin in the older control rats relative to the young and treated groups with a similar trend for serum insulin (Fig. 4). Serum glucose in the older control group was significantly higher (p< 0.001) than the young group, whereas values in the treated group were not different from the younger animals (Fig. 4). The older control group had a
significantly lower QUICKI value and higher HOMA value compared to the young group, and there was no difference between the young and treated rats (Fig. 5). This suggests that the older control animals have insulin resistance and that blockade of the AT$_1$ receptor may delay this from occurring.

Plasma Ang I (p< 0.05) and II (p< 0.001) were higher in the older treated animals than in the young or older control animals (Fig. 6). However, plasma Ang-(1-7) was not significantly different among the three groups (data not shown). Creatinine excretion was similar among the groups (Young: 23 ± 1 vs Control: 19 ± 2 vs Treated: 25 ± 3 mg/kg/day). Serum creatinine was not different among the groups (Young: 3.6 ± 0.5 vs Control: 4.8 ± 0.7 vs Treated: 4.6 ± 1.0 mg/dl). GFR calculated from the serum and urinary creatinine levels was similar among the groups (Young: 0.14 ± 0.02 vs Control: 0.17 ± 0.04 vs Treated: 0.16 ± 0.04 ml/min). Urinary excretion of Ang I (p< 0.001), Ang (1-7) (p< 0.01) and Ang II (p< 0.05) was greater in the older control group compared to the young animals. Urinary Ang peptide excretion in the older treated group was not different from the young animals (Fig. 7). The urinary Ang (1-7) to Ang I ratio revealed that the young group had a significantly higher ratio compared to the two older groups (Young: 4.4 ± 1.1 vs Control: 0.95 ± 0.16 vs Treated: 2.5 ± 0.64 pmol/kg/day; p < 0.05). Protein excretion was markedly elevated in the older rats (p< 0.05) compared to the young animals and this was completely prevented in the AT$_1$ receptor blocked animals (Fig. 8).
Discussion

Long-term AT\textsubscript{1} receptor blockade prevented the increases in insulin, leptin and glucose observed in older F344 rats associated with reduced weight gain. It is important to note that there were no significant differences in blood pressure between the older control and treated groups, associated with the improvements in metabolism. Proteinuria, a marker of renal damage, was completely prevented in the older treated rats compared to the older controls, and in treated rats angiotensin peptide excretion, an indicator of intra-renal RAS activity, was maintained at levels not different from those seen in the younger animals. The results are consistent with previous studies showing protective effects of long-term RAS blockade on the age-related changes in kidney and metabolic function (5; 10; 11; 19; 29; 40; 52) in rat strains where aging is accompanied by increases in systolic pressure.

There are reports that RAS blockade improves components of, or reduces the onset of, the MetS and type 2 diabetes (1; 17; 23; 57). Our study demonstrates the advantageous effects of RAS blockade independent of the blood pressure lowering actions in accordance with previous studies (48; 50). However, the precise mechanisms underlying the beneficial effects are not entirely known and mechanisms may involve actions at the skeletal muscle (24; 48), brain and autonomic nervous system (30; 31) or other organs such as liver and pancreas (18; 19; 51).

As expected, AT\textsubscript{1} receptor blockade led to an increase in plasma Ang I and II, consistent with previous findings (29) and the known feedback mechanism of the system. This implies that the dosing of the blocker was effective, even with the absence of a blood pressure lowering effect in these normotensive animals. Plasma Ang (1-7) levels
were not different between the two older groups of animals. Although some previous studies show an increase in the levels of this peptide with AT₁ receptor blockade or ACE inhibition (29), the failure to increase Ang-(1-7) levels in the circulation may result from the reported decline in nephrilysin activity in the circulation and kidney of older animals (44). Interestingly, the plasma levels of Ang I and Ang II were not lower in older F344 rats relative to younger animals. This was a surprising finding since the older F344 rats exhibited proteinuria with increased age and renal damage has been suggested to contribute to the loss of renin producing cells in the kidney. However, there was no reduction in GFR in the older rats in this study and the reduction in circulating levels of Ang II in other models of aging occurs coincident with the increase in systolic blood pressure (31), rather than the onset of kidney damage as assessed by proteinuria or activation of the intrarenal RAS. As the F344 rats do not have an increase in blood pressure, the maintenance of normal circulating levels of RAS peptides may reflect the absence of a pressure-mediated inhibition of renin release in these animals.

Ang II excretion was lower in the older treated animals relative to the older controls, suggesting a role for increased intrarenal Ang II production in the deleterious changes seen in renal and metabolic function with aging. The lower level of Ang II excretion in the treated animals is similar to previous studies of long-term RAS blockade in older animals (29) or tissue ACE KO mice (38). Our findings further suggest that the systemic and kidney RAS are differentially regulated, given increases in circulating peptides in this and a previous study persisting for the duration of treatment while urinary peptides remain low (29). The decrease in Ang peptide excretion in the treated group may reflect tubular RAS suppression (29), consistent with the differential regulation of the
systemic and kidney RAS (9; 38). GFR was similar among the groups. Fischer 344 rats develop age-related nephropathy while serum creatinine levels do not change until the later stages of nephropathy (8; 16; 35; 56). However, F344 rats develop proteinuria, which may manifest as protein-overload nephropathy and there may be slight changes in the glomeruli in the first couple of stages (grades) of renal disease (8; 56). Proteinuria is decreased in the presence of AT₁ receptor blockers (37; 47): our study suggests this may result from reduced activation of the intra-renal RAS as well as effects to correct hyperinsulinemia and elevated serum glucose.

Serum glucose in the older control group was significantly higher than the young group and the profile for serum insulin was similar, suggesting insulin resistance is present at this time point in the older control rats. The older treated group was indistinguishable from the young rats. The QUICKI and HOMA values were significantly different in the older control group compared to the young group, suggestive of insulin resistance which is a known feature of the F344 at this age. Both are established methods used to assess insulin resistance and correlate well with the glucose clamp technique (7; 25; 32). Rats with decreased glial angiotensinogen (ASrAogen rats) have lower levels of glucose and insulin during aging compared to age-matched Sprague-Dawley rats associated with reduced sympathetic nervous system activity, suggesting a role for glial-produced Ang II in the metabolic impairments that occur during aging (30). Insulin and leptin interact in the rat hypothalamus at the PI3 and MAP kinase pathways to decrease feeding and maintain an appropriate metabolism (12). Aged Fischer X Brown Norway rats on ad libitum and restricted diets showed a greater tendency to develop insulin resistance in association with greater adiposity compared to younger animals, suggesting
that abdominal obesity during aging may contribute to insulin resistance (13). While the exact mechanisms of the beneficial effects of RAS blockade on insulin and glucose are not known, it may be partly due to the decreased actions of Ang II at the kinase pathways as well as actions in the central nervous system on autonomic pathway controlling insulin and glucose metabolism.

In the F344 rat, aging is associated with an increase in serum leptin (39) as is the case with other rat strains (30). Our study confirms this as there was a significant increase in serum leptin in the older control rats relative to the young and L-158,809 treated animals. Leptin regulates weight loss by suppressing the appetite and food intake and enhancing energy expenditure (36; 55). Food intake was similar between the two older groups of rats and both were statistically higher than the young rats, but the treated group maintained a significantly lower body weight than the control rats. This is consistent with the reports that leptin is primarily released from adipose tissue and serves as an index of the amount of body fat mass (55). We did not measure activity in these studies and it is possible that changes in activity levels may accompany the overall improvement in metabolic function. In ASrAogen rats, leptin levels and body weight are lower and food intake higher than Sprague Dawley and (mRen2)27 rats (30) suggesting that interruption of the brain RAS may be part of the beneficial effects seen with RAS blockade. Leptin, like insulin, via actions in the brain, activates the sympathetic nervous system. Activation of the SNS can lead to a reduction in GFR and salt and water retention. This may lead to activation of the renal RAS and kidney damage. F344 rats are known to have leptin and insulin resistance (33; 34) as appears to be the case with our
animals. Thus, elevated levels of insulin and leptin may also contribute to the declining renal function that occurs during aging.

L-158809 is a competitive and specific AT$_1$ receptor antagonist (14; 49) and in this study, it decreased indices of the MetS and type 2 diabetes after treatment for one year, similar to the effects of other RAS antagonists or inhibitors, and consistent with what has been shown in previous studies (18; 19; 24; 29; 40; 48; 52). The mechanisms behind the antidiabetic effects are not well understood and there are reports that a certain subset of AT$_1$ receptor antagonists (ARA) may have such effects by acting as partial agonist of the peroxisome proliferator-activated receptor-γ (PPARγ) (6; 45; 46). Other ARAs tested, including valsartan had no effect on PPARγ. However, in the VALUE trial (27), valsartan reduced new onset diabetes suggesting the antidiabetic effect of this ARA is mediated indeed through the AT$_1$ receptor, not necessarily by interactions with the PPARγ. Valsartan treatment in KK-Ay mice, a model of type 2 diabetes, did not affect SBP, but improved insulin sensitivity, PI3K activity and Glut4 translocation while decreasing TNF-α expression in skeletal muscle (48). It is also important to note that interruption of the RAS at levels other than AT$_1$ receptor blockade has proven beneficial in reducing the new onset of diabetes (57).

We conclude that aging is associated with activation of the intra-renal RAS independent of age-related increases in SBP. Moreover, the decline in circulating RAS during aging may be a consequence of the age-related increase in SBP, since animals without the increase in SBP have no reduction in Ang II in plasma, in spite of development of MetS. Long-term blockade of the RAS reduces the onset of indices of the MetS and kidney damage that occur during aging in F344 rats independent of blood
pressure lowering effects. Moreover, the intrarenal RAS activity was decreased in the presence of the blockade suggesting that local tissue RAS blockade may contribute to the protective effects on metabolic, kidney and cardiovascular function during aging. Further investigation is needed to determine the full complement of mechanisms and other tissue RAS that may be involved in the deleterious changes that occur in the normotensive population as they age and may contribute to the beneficial effects of RAS blockade.

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Figure 1. Systolic blood pressure in young, control and L158,809 treated rats. After one year of treatment. L158,809 treated rats had a similar blood pressure to the older control rats and both older groups were lower than the young. *=p< 0.001 vs young.

Figure 2. Food intake and body weight in young, control and L158,809 treated rats. Food intake was similar between the two older groups of rats and both were statistically higher than the young rats. The older treated group maintained a significantly lower body weight than the older control rats. *=p < 0.05 vs young; +=p < 0.001 vs young; #=p < 0.001 vs control.

Figure 3. Water intake and urine volume in young, control and L158,809 treated rats. Water intake was significantly higher in the treated rats compared to the young and control rats. Urine volume was higher in the two older groups of rats. *=p < 0.05 vs L158,809; **=p < 0.01 vs L158,809; θ=p<0.01 vs young; +++ =p<0.001 vs young.

Figure 4. Serum insulin, glucose and leptin levels in young, control and L158,809 treated rats. The young and treated groups had lower levels of serum glucose and leptin, with a similar trend for insulin. **p < 0.01 vs young; ***p < 0.001 vs young and L158,809.

Figure 5. QUICKI and HOMA values in young, control and L158,809 treated rats. The older control group had a significantly lower quicki and higher homa values compared to the young group. *p < 0.05 vs young.

Figure 6. Plasma Ang peptides in young, control and L158,809 treated rats. The levels of plasma Ang I and II peptides were higher in the treated group. +p<0.05 vs control; ***p <0.001 vs young and control

Figure 7. Urinary Ang peptide excretion in young, control and L158,809 treated rats. The young and treated rats exhibited lower urinary Ang peptide levels compared to the
control group. The excretion values shown are normalized for creatinine excretion.

*p<0.05 vs young; ***p<0.001 vs young and treated; +p<0.01 vs young; #p<0.05 vs treated.

Figure 8. Protein excretion in young, control and L158,809 treated rats. Protein excretion was elevated in the control rats and this was completely prevented in the treated rats. *p< 0.05 vs control and L158,809.
Figure 1.

Systolic Blood Pressure

Young | Control | L158,809

Systolic Blood Pressure (mmHg)

*
Figure 2.

Food Intake

Body Weight
Figure 3.

Water Intake

![Water Intake Graph]

Urine Volume

![Urine Volume Graph]
Figure 4.

**Serum Insulin**

- Young: 1.0
- Control: 2.5
- L158,809: 1.5

**Serum Glucose**

- Young: 125
- Control: 150
- L158,809: 125

**Serum Leptin**

- Young: 4.0
- Control: 12.0
- L158,809: 8.0

**Legend**

- **Young** group
- **Control** group
- **L158,809** group

**Statistical Significance**

- Serum Glucose: **p < 0.01**
- Serum Leptin: ***p < 0.001**
Figure 5.
Figure 6.
Figure 7.

**Ang I Excretion**

- Young
- Control
- L158,809

**Ang II Excretion**

- Young
- Control
- L158,809

**Ang (1-7) Excretion**

- Young
- Control
- L158,809

*Significance levels:*** p < 0.001, * p < 0.05, + p < 0.1.
Figure 8.

Protein Excretion

Protein Excretion (mg/day)

Young  Control  L158,809

*
CHAPTER THREE

LONG-TERM SYSTEMIC RENIN-ANGIOTENSIN SYSTEM BLOCKADE ALTERS GENE EXPRESSION OF RENIN-ANGIOTENSIN SYSTEM COMPONENTS IN DORSOMEDIAL MEDULLA OF FISCHER 344 RATS

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Abstract

In Fischer 344 (F344) rats, renin-angiotensin system (RAS) blockade for one year with the AT$_1$ receptor blocker L-158,809 prevents age-related impairments in metabolic function (lower levels of insulin, glucose, leptin, and lower body weight relative to untreated age-matched rats), without lowering blood pressure. Treated F344 rats exhibit a metabolic profile similar to 15-18 month old transgenic rats with low glial angiotensinogen, implying that blockade of the brain RAS may contribute to the benefits of long-term systemic AT$_1$ antagonism. We assessed the gene expression of RAS components in the dorsomedial medulla of F344 rats at 3 (Y; n = 8) or 15 months of age (Old; n = 7), and in rats treated from 3 to 15 months of age with 20 mg/L of the AT$_1$ receptor antagonist L-158,809 (Old+L; n = 6), previously characterized for metabolic function and blood pressure. Angiotensinogen (Aogen) and renin mRNA were 40% (p<0.05) and 60% (p<0.01) lower in the Y compared to Old group. ACE mRNA was 55% lower in the Old compared to the Y group (p<0.01) and lower in the Old+L (p<0.05) compared to the Y group. While L-158,809 treatment did not lower Aogen or renin mRNA, gene expression of ACE2 and neprilysin was 50 – 100% higher in Old+L compared to Y or Old F344 rats suggesting that enzymes responsible for Ang-(1-7) formation are upregulated by AT$_1$ receptor blockade. AT$_{1b}$, AT$_2$ and Mas receptor mRNA levels were all significantly higher with treatment (>100%; p<0.01), but AT$_{1a}$ receptor levels were not different among the groups. Leptin receptor mRNA was 60% lower in the Old rats, and this was prevented by the L-158,809 treatment. Expression of the p85 regulatory subunit of PI3-kinase was significantly different among the groups being highest in the Old+L group and lowest in the Y group (p<0.001). The findings
provide evidence for increases in brain renin and Aogen in dorsomedial medulla during aging, and that long-term RAS blockade activates enzymes and receptors that would shift the balance from Ang II to Ang-(1-7) in this brain region. Prevention of age-related declines in the leptin receptor and its signaling pathway provide mechanisms for preservation of metabolic function in treated F344 rats.

Introduction

Systemic blockade of the RAS provides many beneficial effects outside of the classic blood pressure lowering (hemodynamic) actions. Studies show that RAS blockade improves many components of aging, and reduces the onset of the metabolic syndrome and type 2 diabetes (1; 4; 6; 21), however, the precise mechanisms underlying the beneficial effects are not entirely known. During aging, there is a decline in the circulating (systemic) RAS, which suggests that the beneficial actions of RAS blockade are partly due to the suppression of Ang II in tissues. It is well established that many tissues and organs contain their own local RAS, including the kidney, heart, vessels, adrenal gland, pancreas and brain (2; 10). The brain RAS activates sympathetic outflow, inhibits the baroreflex, stimulates thirst, and contributes to neurogenic hypertension (10; 17; 18). In rats with chronic renal failure, the brain RAS is upregulated, resulting in sympathetic overactivity and hypertension (13). Transgenic mice with increased production of brain Ang II developed hypertension and an increase in salt appetite and drinking volume (11; 12). Transgenic rats with a deficit in brain Aogen (ASrAogen) do not develop hypertension, have reduced sympathetic nervous system activity and lower levels of glucose, insulin and leptin during aging compared to age-matched Sprague-Dawley (SD) rats (8). ASrAogen rats also maintain insulin sensitivity over the course of
aging compared with age-matched SD and (mRen2)27 rats (9), suggesting a role for glial-produced Ang II in the metabolic impairments that occur during aging. In F344 rats, RAS blockade for one year with the AT$_1$ receptor blocker L-158,809 prevented age-related impairments in metabolic function (lower levels of insulin, glucose, leptin, and lower body weight relative to untreated age-matched rats)(5). The treated F344 rats exhibit a metabolic profile similar to 15-18 month old ASrAogen rats, implying that blockade of an age-induced activation of the brain RAS may contribute to the benefits of long-term systemic AT$_1$ antagonism.

Few studies have documented age-related changes in the brain RAS. Therefore, our objective was to assess the gene expression of RAS components in the dorsomedial medulla of the F344 rats that were previously characterized for metabolic function and blood pressure to offer insight on the role of the brain RAS in the changes that occur during aging and the effects of long-term RAS blockade.

**Methods**

**Animals**

All animals were obtained from Harlan Laboratories (Indianapolis, IN) and protocols were reviewed and approved by the Institutional Animal Care and Use Committee. Three groups of male F344 rats were used in these studies; a young group (n = 8) at 3 months of age, an old group (n = 7) at 15 months of age and group treated with 20 mg/L of the AT$_1$ receptor antagonist L-158,809 (n = 6) in their drinking water from 3-15 months of age (1 year).
Quantification of mRNA

Total RNA was isolated from the dorsal medulla of the three groups of rats using TRIZOL reagent followed by incubation with AMV reverse transcriptase in a mixture containing deoxynucleotides, random hexamers, and RNase inhibitor in reverse transcriptase buffer. For real-time PCR amplification of the cDNA, gene specific primers, TaqMan probes, and master mix were purchased from Applied Biosystems and the reactions performed on an ABI 7000 Sequence Detection System. 18S ribosomal RNA, amplified using TaqMan Ribosomal RNA Control Kit (Applied Biosystems) was used as the internal control. The results were quantified as Ct values, where Ct is defined as the threshold cycle of PCR at which amplified product is first detected, and defined as relative gene expression (ratio of target/control).

Statistical Analysis

All data are presented as the mean ± SEM and p values < 0.05 were considered significant. One-way ANOVA with Tukey’s Multiple Comparison post hoc tests was performed using Graphpad Prism.

Results

Angiotensinogen mRNA was significantly higher in the older animals with a similar trend for renin (p<0.01). There was a further increase in the Old+ L-158,809 group for Aogen and renin mRNA (Fig. 1). ACE mRNA was lower in the Old compared to the Young, and this was partially reversed by the treatment (Fig. 2). Neither ACE2 mRNA nor neprilysin mRNA was different between the old compared to the young animals, however, 50-100% higher values were observed in the Old+L-158,809 group compared to the Young and Old groups (p<0.001). No differences were observed in the
AT1a, AT1b, AT2 or Mas receptors mRNA between the Old and Young animals (Fig. 3 and 4). AT1b, AT2 and Mas receptor gene expression were two-fold higher in Old+ L-158,809 rats compared to Young and Old F344 rats, but AT1a receptor mRNA was not different among the groups (Fig. 3 and 4). Leptin receptor mRNA was significantly lower in the Old rats compared to the Young and L-158,809 treatment reversed this age-related change (Fig. 5). Expression of the p85 regulatory subunit of PI3K was significantly higher in the Old and Old + L-158,809 groups compared to the Young (Fig. 5).

**Discussion**

In the older F344 rats, brain medullary angiotensinogen mRNA was higher compared to the young animals, with a similar trend for renin. With respect to AT1 receptor antagonism, angiotensinogen and renin mRNA were further increased in the old treated group compared to the old group. Interestingly, increases in both Aogen and renin levels in the kidney occur with long-term AT1 receptor blockade. One mechanism for those changes is blockade of feedback inhibition of the initial components of the RAS by Ang II. Whether this is the mechanism for the changes seen in the brain medulla requires further study. ACE, ACE2 and neprilysin mRNAs were significantly higher in the old treated group compared to the old group. There was no difference in AT1a receptor gene expression between the groups, but AT1b expression was significantly increased in the old treated group. AT2 and Mas receptor mRNA levels were two-fold higher in Old+ L-158,809 rats compared to the Old rats. The findings provide evidence for increases in brain renin and angiotensinogen mRNA in dorsomedial medulla during aging, and that long-term RAS blockade activates enzymes and receptors that would shift
the balance from Ang II to Ang-(1-7) in this brain region. Expression of the leptin receptor was lower in older F344 rats and both the leptin receptor and the PI3K regulatory subunit p85 were significantly higher in the old treated rats versus the old rats. The findings provide evidence for increases in brain renin and angiotensinogen in dorsomedial medulla during aging, and that long-term RAS blockade activates enzymes and receptors that would shift the balance from Ang II to Ang-(1-7) in this brain region. The prevention of age-related declines in the leptin receptor and enhancement of a leptin and insulin signaling pathway provides mechanisms for preservation of metabolic function previously observed in the treated F344 rats (5).

Hypertension and other disease states such as chronic renal failure are thought to be accompanied by an overactive brain RAS (13; 15). In young adult rats with aortic coarctation induced hypertension, there were marked increments in angiotensinogen and AT₁a mRNA expression in brainstem areas controlling cardiovascular function and losartan treatment for nine days blocked the increase in AT₁a mRNA expression (15). In that study, male Wistar rats treated with losartan had lower levels of brain angiotensinogen and AT₁a mRNA compared to controls. In the spontaneously hypertensive rat (SHR), candesartan treatment significantly increased brain angiotensinogen, ACE and AT₂ receptor mRNA as well as AT₂ receptor protein (22). The Wistar-Kyoto rats treated with candesartan had significantly decreased angiotensinogen and increased ACE mRNA in brain microvessels compared to vehicle controls. The increased angiotensinogen, ACE and AT₂ receptor mRNA from that study is similar to the results found in the normotensive rats in the present study in which longer-term RAS Ang II receptor blockers generally cause an increase in Ang II in
plasma due to the loss of the feedback inhibition of renin release from the kidney. With the AT₁ receptor being occupied, elevated Ang II would theoretically bind the AT₂ receptor which may be one possible mechanism that leads to beneficial effects of Ang II receptor blockade. In addition, the levels of ACE2 in the present study were two-fold higher than ACE, which suggest that the system shifts in favor of Ang-(1-7) production during AT₁ receptor blockade. Further studies to determine whether the alterations in gene expression are reflected in actual changes in receptor or enzyme protein levels or binding and activity are now warranted. However, from the above referenced studies, when measured, protein levels parallel the changes in gene expression (22).

Hyperactivity of the brain RAS may also contribute to insulin resistance since previous data revealed the rats with low brain angiotensinogen do not develop insulin resistance during aging unlike their control counterparts (9). F344 rats, though normotensive during the aging process, develop insulin resistance, and AT₁ receptor blocker treated animals have lower serum insulin, glucose and leptin levels compared to older control animals (5). While there are known interactions of Ang II and insulin in skeletal muscle (7; 16), the effects of systemic AT₁ receptor blockade on the brain RAS cannot be ruled out as contributing mechanisms. Insulin and leptin both have actions at brain sites (receptors in PVN, arcuate, dorsal vagal complex) leading to impairment in baroreflex sensitivity and increased sympathetic nervous system activity and crosstalk occurs among insulin, leptin and Ang II receptors and signaling pathways (3; 19; 20). For example, insulin, leptin and Ang II act at the PI3K pathway and the p85 regulatory subunit is an important component of the pathway. Impaired activation of the PI3K pathway also occurs in insulin resistance and Ang II activity may play a role (14; 20).
During aging there is a decline in insulin and leptin sensitivity and this does not occur in the aging AsrAogen rats with low glial derived angiotensinogen. Our results suggest that prevention of age-related decline in the leptin receptor mRNA and the increase in the p85 regulatory subunit of PI3K provide mechanisms for preservation of metabolic function in the older L-158,809 treated F344 rats.

We conclude that increases in brain renin and angiotensinogen mRNA in dorsomedial medulla occurs during aging in a normotensive rat model that develops insulin resistance and renal damage. Long-term RAS blockade promotes the gene expression of enzymes and receptors that would shift the balance from Ang II to Ang-(1-7) in this brain region. Moreover, the brain RAS may be involved in the impairments in metabolic function of the older rats since the gene expression of the leptin receptor and p85 were increased with RAS blockade.

Limitations

Interpretation of the brain RAS activity in F344 rats should be considered with caution since we measured mRNA and not protein as well. Speculation on which peptide [Ang-(1-7) or Ang II] predominates must also be carefully considered since we did not measure Ang peptide activity. It must also be taken into consideration that we measured a component of a leptin signaling pathway (p85) and thus can not make conclusive observations on what the exact effects may be. Nonetheless, we observed improvements in renal and metabolic function in these animals over the same time course as the alterations in the expression of RAS and other components that would be consistent with improved metabolic and cardiovascular function during aging. However, further studies
are required to reveal the functional consequences directly linked to each of these changes and when they occur.

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5. **Gilliam-Davis S, Payne VS, Kasper SO, Tommasi EN, Robbins ME and Diz DI.** Long-term AT1 receptor blockade improves metabolic function and provides


Figure 1. Angiotensinogen mRNA was lower in the Young group vs the Old and Old+L groups. Renin mRNA was significantly lower in the Young group compared to the Old+L group with a similar trend compared to the Old group. *P < 0.01 vs Old; @P < 0.001 vs Young and Old; #P < 0.05 vs Young

Figure 2. ACE mRNA was significantly lower in the Old vs the Young and Old+L groups and significantly lower in the Old+L compared to the Young group. Neprilysin and ACE2 mRNA were significantly higher in the Old+L group vs the Young and Old groups. $P < 0.001 vs Young and Old + L-158,809; #P < 0.05 vs Young; @P < 0.001 vs Young and Old; *P < 0.01 vs Young and Old

Figure 3. AT\textsubscript{1a} receptor mRNA did not differ among groups and the AT\textsubscript{1b} receptor mRNA was significantly higher in the Old+L group compared to the Young and Old groups. @P < 0.001 vs Young and Old

Figure 4. AT\textsubscript{2} and Mas receptor mRNA were significantly higher in the Old+L group compared to the Young and Old groups. @P < 0.01 vs Young and Old; *P < 0.01 vs Old; #P<0.001 vs Young

Figure 5. Leptin receptor mRNA was lower in Old rats and this was prevented in the Old+L rats. The p85\textalpha regulatory subunit of PI3K was significantly higher in both Old and Old+L groups. *P < 0.01 vs Old; #P<0.05 vs Old + L-158,809; @P < 0.001 vs others
Figure 1.

**Angiotensinogen mRNA**

- Young: *p* < 0.05
- Old: @
- Old + L: #

**Renin mRNA**

- Young
- Old
- Old + L
Figure 2.

ACE mRNA

ACE2 mRNA

Neprilysin mRNA
Figure 3.
Figure 4.
Figure 5.
CHAPTER FOUR

CHARACTERIZATION OF THE TIME COURSE OF INTRARENAL RAS
ACTIVATION AND RENAL DAMAGE IN SPRAGUE DAWLEY RATS

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Abstract

The intrarenal (urinary) renin-angiotensin system (RAS) increases in Sprague Dawely (SD) rats during aging while the circulating (plasma) RAS declines. The increased intrarenal RAS activity occurs prior to the elevation in blood pressure but whether activation of the intrarenal RAS is secondary to progressive renal damage or is an initiating factor is not known. Furthermore, the exact timing and mechanisms underlying the increase in the intrarenal RAS are not entirely established. Our aim was to better define the time course of activation of the intrarenal RAS relative to the elevation in proteinuria in aging SD rats. Metabolic cage studies were conducted to assess urine levels of angiotensin (Ang) peptides and proteinuria in male SD rats from ages 24 to 48 weeks of age (n≥6 for each group), with 24 weeks serving as the control time point. Serum and urine creatinine, systolic blood pressure (SBP), glomerular filtration rate (GFR) and indices of glucose metabolism were also determined. SBP was not different among the different age groups. Protein and Ang I excretion significantly increased at 38 weeks and continued with this pattern until the end time point studied. Ang-(1-7) excretion significantly increased at 38 weeks compared to 24 weeks (0.3 ± 0.03 vs 0.17 ± 0.008 pmol/mg/day; p<0.05) with a similar trend for the rest of the time points thereafter. Ang II excretion significantly decreased or remained the same over the course of the study, however, urinary Ang II was generally higher than either Ang I or Ang-(1-7) throughout the study. Serum creatinine and GFR were similar across the ages studied. The data suggest that activation of the intrarenal RAS, as reflected by Ang peptide excretion, occurs simultaneously with the increase in protein.
Introduction

The circulating and intrarenal RAS are dissimilarly regulated in the aging rat (3; 9; 11; 12). Studies suggest that plasma renin concentrations and kidney renin mRNA are decreased in aging rats (11) associated with a decrease in plasma angiotensin (Ang) peptides in aging Sprague Dawley (SD) rats (21); perhaps as a result of increased arterial pressure. Plasma renin is decreased in aging humans (26). In contrast, there is an increase in intrarenal tissue Ang II with aging (2) as well as an increase in the excretion of Ang peptides (21), whether or not circulating Ang II changes and independent of increased blood pressure (8). This suggests that the beneficial actions of RAS blockade may involve the suppression of Ang II in tissues rather than alteration of the circulating system.

Components of the renin-angiotensin system (RAS) have been measured in urine as indicators of renal function. Patients with chronic kidney disease have increased levels of urinary angiotensinogen (Aogen), proteinuria and a low estimated glomerular filtration rate (GFR) that correlate with increased renal tissue angiotensin (Ang) II (30). Urinary excretion of Aogen in Ang II-dependent hypertension in rats signifies intrarenal (tubular) production, which also has a high correlation with kidney Ang II content (16). Urinary Aogen is increased in hypertensive patients and is reduced by RAS blockade (15). In the Otsuka Long–Evans Tokushima Fatty (OLETF) rat, a model of type 2 diabetes, telmisartan decreased intrarenal Ang II and prevented proteinuria and podocyte abnormalities (20). The intrarenal RAS as assessed by urinary Ang peptide levels, is activated during aging in Wistar (12), SD (21) and Fischer 344 (F344) rats (8). The increase occurs prior to, or independent of, age-related increases in systolic blood
pressure (SBP), and prior to or independent of age-related declines in Ang II in the circulation. In SD rats, an increase in excretion of Ang I, Ang II and protein over that of 16 week old animals is seen at around 48 weeks of age (21), but whether activation of the intrarenal RAS is secondary to progressive renal damage or is an initiating factor is not known. In the present study, we aim to better define the time course of intrarenal RAS activation relative to renal damage in aging SD rats and to provide information on possible mechanisms underlying intrarenal RAS activation.

Methods

All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee. The Hannover SD rats were obtained from colonies maintained in the Hypertension and Vascular Research Center at Wake Forest University. All animals were bred and exposed to the same housing conditions (12:12-h light-dark cycle) and provided food and water ad libitum. Twenty-four hour urine collections on dry ice were conducted using metabolic cages in male SD rats every other week from 24 to 48 weeks of age. SBP was measured using the tail cuff procedure at 24, 30, 35, 40 and 46 weeks of age. There were at least three training sessions with the tail-cuff procedure to acclimate the animals to the device. The mean of at least five blood pressure measurements were determined for each animal. Not all animals were studied at every time point. Subsets of animals were euthanized at least 4 hours after removal of food and water by quick decapitation and trunk blood was collected in the presence of inhibitors as previously described (10). Serum insulin and leptin were measured using radioimmunoassays (Linco, Inc.) and serum glucose using a Freestyle glucose monitor (TheraSense). Urinary Ang I, Ang II, and Ang-(1-7) were analyzed using radioimmunoassay (1; 10). Protein in
urine was measured using reagent strips (Multistix 10 SG, Bayer Corporation, Elkhart IN). Urine was analyzed for creatinine (Metra creatinine assay kit, Quidel Corporation) and normalized to body weight. The urinary peptide data are expressed as 24-hour excretion rates normalized to creatinine excretion as detailed elsewhere (5). Serum creatinine was analyzed using the Quantichrome creatinine assay kit, Bio Assay System. Glomerular filtration rate (GFR) was determined from serum and urine creatinine values.

Statistical Analysis
All data are presented as the mean ± SEM and p values < 0.05 were considered significant. One-way ANOVA with Dunnett’s post hoc tests was performed using Graphpad InStat.

Results
SBP was not different across the age groups studied (Fig. 1). Body weight was significantly higher at 48 weeks compared to 24 weeks (p<0.05), but there were no differences in food intake, water intake or urine volume over this time period (data not shown). Ang I excretion was stable over the first 14 weeks of the study and then increased significantly at 38 weeks compared to 24 weeks (p<0.01), a pattern that continued until the last time point studied. Urinary Ang-(1-7) was significantly increased at 38 weeks only (p<0.05), with a similar trend for the time points thereafter. Ang II excretion started at a higher value than both Ang I and Ang-(1-7) but did not increase over the period of the study. In fact, significant decreases (p<0.05) were observed at 26, 27, 28, 32, 34 and 40 weeks of age relative to the 24 week time point (Fig. 2). Protein excretion showed a steady and gradual increase over time, reaching significance at 38 weeks of age; progressive increases continued until the last time point studied (Fig 3).
Creatinine excretion increased significantly (p<0.05) at 27, 28, 40, 46 and 48 weeks compared to 24 weeks (Fig. 3). The ratio of Ang II:Ang I was significantly lower at all time points studied compared to 24 weeks, indicating a steady decrease in processing over time. The ratio of Ang-(1-7):Ang I was also significantly lower at all time points studied compared to 24 weeks (p<0.01), again suggesting a steady decline over time. The ratio of Ang-(1-7):Ang II was lower than the other peptide ratios and was significantly increased at 40 and 44 weeks (p<0.05) with a similar trend at 46 weeks compared to 24 weeks (Fig. 4). Serum creatinine was similar among the age groups and there was no difference in GFR among the ages studied (Fig 5), although there is a trend for a decline as might be expected during aging in these animals. Insulin was significantly higher at 40 weeks compared to 24 weeks (Fig 6). Glucose levels were similar at all ages studied and leptin levels were not different among the age groups (Fig 6).

**Discussion**

In aging SD rats, urinary Ang I and proteinuria were significantly increased at the same time points (38-48 weeks) compared to 24 weeks, with a similar trend for Ang-(1-7). Urinary Ang II declined or remained the same over the course of the study compared to 24 weeks. In general, urinary Ang II values were higher than Ang I and Ang-(1-7) throughout the study. Creatinine excretion significantly increased at 27 weeks which was prior to the changes in urinary Ang I, Ang-(1-7) and protein levels. Ratios for Ang II:Ang I and Ang-(1-7):Ang I, often used as indices of the activity of converting enzyme (ACE) or neprilysin, respectively, were significantly decreased at all time points studied compared to 24 weeks. The ratio of Ang-(1-7):Ang II, an index of ACE2 activity was
lower than the other peptide ratios at all ages studied except at 40, 44 and 46 weeks when a significant increase occurred compared to 24 weeks (p<0.05). This suggests that RAS processing enzyme activity is reduced over the course of aging and is consistent with the reported reduction in neprilysin activity in the kidney of older rats (22). As expected, there was no difference in blood pressure among the ages studied which is consistent with previous data (13). There was no difference in serum creatinine, GFR, glucose or leptin among the age groups. Insulin was significantly increased at 40 weeks compared to 24 weeks, consistent with increases observed at 48 weeks in previous reports (8; 13).

Aging is associated with intrarenal RAS activation, as indicated by an elevation in the excretion of Ang I, Ang II and Ang-(1-7), in many normotensive rat strains, including Wistar, F344 and SD rats (8; 12; 21). From these previous studies, however, it was not possible to obtain a detailed time course of the onset of the increases in Ang peptides relative to one another or the associated increase in proteinuria. Over the time course of our study, we observed increases in Ang I and Ang-(1-7) excretion similar to these previous studies, while surprisingly, Ang II excretion was not elevated and in fact, declined or remained similar compared to the 24 week control values. It is reported that an increase in plasma or tissue Ang I primarily leads to increased Ang-(1-7) generation, which may limit the amount of substrate available for Ang II production (6), as a possible explanation for the lack of increase in Ang II during the ages studied. It is important to note that urinary Ang II has been shown to increase around the 48 week time point (21) and since this study ended at 48 weeks, the time course for the elevation in this peptide may require a longer study. However, the important point is that Ang II was not increased prior to the increase in protein. While Ang II excretion is higher than Ang I and Ang-(1-
7) throughout the study and while not activated/increased, the maintenance of even normal levels may elicit harmful effects, especially since previous studies show that RAS blockade has renoprotective effects and decreases intrarenal Ang II in spite of the decreased or unchanged plasma RAS (8; 21). Certainly, aging is associated with changes in the activity and/or responsiveness of the RAS (2; 25).

Urinary Ang I, Ang II, Ang-(1-7) and protein increased during aging in F344 rats and this was prevented by long-term ARB blockade, suggesting that the intrarenal RAS and proteinuria may be dependent factors, but this study did not clarify which occurred first (8). Urinary Ang II and Aogen were increased in Ang II-dependent hypertensive rats, but not in an Ang II-independent hypertensive model (18), confirming the notion that intrarenal Ang II has a positive feedback mechanism on urinary Aogen production (17). Increased urinary protein excretion also occurred in the Ang II-dependent hypertensive model but was dissociated from the increased urinary Aogen excretion rate (18). Thus, that study potentially ruled out proteinuria as an initiating factor in intrarenal RAS activation by suggesting that the increased Aogen is not a nonspecific consequence of proteinuria. We observed a significant increase in urinary protein at the same time as that of Ang I and Ang-(1-7). It may be that the higher urinary Ang II which is evident from the beginning of the study initiates these changes. Ang II may cause podocyte damage and apoptosis, reduce the degradation of proteins and induce cytokine expression, all of which may lead to glomerular and/or tubulointerstitial injury and proteinuria (24; 27-29). Increased proteinuria causes a decrease in oncotic pressure and intravascular volume which may stimulate Ang II production. Ang II may act preferentially at the efferent arteriole to increase intraglomerular pressure, which would
further promote proteinuria. Ang-(1-7) is generally thought to have beneficial effects since the renal actions are opposite to Ang II in terms of sodium and water excretion and promotion of vasodilation as opposed to constriction. Interestingly, however, the increase in Ang-(1-7) could be interpreted as contributing to increases in glomerular pressure, and subsequently proteinuria, since the peptide has been shown to produce dilation in the afferent arteriole (4; 23). Furthermore, an increase in Ang-(1-7) could contribute to salt and water loss, thus further activating renin and angiotensinogen to restore Ang II levels as a compensatory mechanism. Therefore, it is not possible at present to rule out elevation of any of the components of the intrarenal RAS as contributors to the proteinuria occurring during the aging process.

Proteinuria is considered the hallmark of renal disease and has been suggested to be both a cause and consequence of renal damage. Proteinuria may occur as a result of glomerular and/or tubular dysfunction. Intrarenal RAS components may also be considered markers of renal damage or dysfunction in light of information provided from previous studies. In a rat model of type 2 diabetes, telmisartan decreased intrarenal Ang II and prevented proteinuria and podocyte abnormalities (20). Urinary Aogen to urinary creatinine levels were significantly higher in hypertensive patients compared to normotensive patients and the levels were decreased to normotensive levels by RAS blockade (15). In general, it appears that proteinuria and the intrarenal RAS peptides may act as co-partners, each contributing to the other and not necessarily one initiating the other.

We observed a significant increase in creatinine excretion at 27, 28, 40, 46 and 48 weeks when compared to 24 weeks. Therefore, we assessed serum creatinine and GFR
at various ages to see if hyperfiltration may contribute to the increase in urine creatinine. Serum creatinine was not different among the ages studied. Likewise, GFR was similar among the groups. Therefore, the renal injury resulting in proteinuria is not associated with a significant decline in GFR over the time course of our study. Since there is increasing support for loss of the active process of tubular protein reabsorption as a contributing factor to proteinuria, rather than a greater filtered load, perhaps these results argue for a role of tubular dysfunction during aging.

Insulin resistance is known to occur in normotensive, non-diabetic rat strains during aging (8; 14). Part of our investigation also involved assessing indices of body metabolism since previous data suggests that intrarenal RAS activation may contribute to metabolic dysfunction (8; 21). As expected, there were no differences in food intake, water intake or urine volume. Body weight was also similar throughout the study consistent with previous data in aging SD rats (13), except at 48 weeks, which was significantly higher than 24 weeks. We observed similar serum insulin concentration values at all ages studied except at 40 weeks when a significant increase occurred compared to 24 weeks. The insulin value in the 40 weeks old animals is similar to that seen in 68 weeks old SD rats (13) and the reason behind the transient elevation at 40 weeks is unclear. The serum insulin at the other ages is similar to previously reported values in rats over the course of aging (8; 13; 19). Serum leptin concentration was not different in the ages studied and was higher than those previously reported in SD rats (13) but similar to values reported in F344 rats (8). Serum glucose concentration was similar among the different ages studied and consistent with previously reported values in rats over the course of aging (7; 8). The timing of the transient increase in insulin and relative
stability of the other measures over the time frame of study would not support a major role of these factors as contributing to either the intra-renal RAS activation or the increase in protein excretion.

We conclude that urinary Ang II is generally higher than Ang I and Ang-(1-7) and that changes in Ang I and Ang-(1-7) excretion but not Ang II during early aging in SD rats are associated in a time-dependent manner with the increase in urinary protein. The changes in the intrarenal RAS and protein excretion are independent of or occur prior to any changes in SBP, serum creatinine, insulin, leptin or glucose, or any decline in GFR. Further studies are needed to decipher the complete scope of mechanisms behind the specific time related changes in the intrarenal RAS (urinary Ang peptides) during aging and to establish the link if any to the gradually increasing proteinuria that develops over the same time course.

Acknowledgments

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Reference List


Figure 1. Systolic blood pressure in SD rats at the various ages. There was no difference in blood pressure among the age groups.

Figure 2. Urinary Ang peptides in SD rats at the various ages studied. Ang I was significantly increased beginning at 38 weeks. Ang-(1-7) was significantly increased at 38 weeks only. Ang II was significantly decreased at 26, 27, 28, 32, 34 and 40 weeks of age. Urinary peptide data are expressed as 24-hour excretion rates normalized to creatinine excretion.*p<0.05 vs 24 weeks; ^p<0.01 vs 24 weeks

Figure 3. Urinary protein and creatinine in SD rats at the various ages. Proteinuria was significantly increased beginning at 38 weeks. Urine creatinine significantly increased at 27, 28, 42, 46 and 48 weeks. Urinary creatinine data are normalized to body weight.*p<0.05 vs 24 weeks; ^p<0.01 vs 24 weeks

Figure 4. Urinary Ang peptide ratios in SD rats at the various ages. Urinary peptide data are expressed as 24-hour excretion rates normalized to creatinine excretion.*p<0.05 vs 24 weeks; ^p<0.01 vs 24 weeks; #p<0.01 vs all

Figure 5. Serum creatinine and GFR in SD rats at the various ages. There was no difference in serum creatinine among the ages. GFR was similar among the age groups studied.

Figure 6. Serum insulin, glucose and leptin in SD rats at the various ages. Insulin was significantly higher at 40 weeks compared to 24 weeks. Glucose levels were similar at all ages studied and leptin levels were not different among the age groups. *p<0.05 vs 24 weeks
Figure 1.

Systolic Blood Pressure

Age (weeks)

mmHg

25 30 35 40 46

120 126
Figure 2.

**Urinary Angiotensin Peptides**

**Ang I**

**Ang II**

**Ang (1-7)**
Figure 3.

Protein Excretion

Urine Creatinine
Figure 4.

**Urinary Angiotensin Peptide Ratios**

- **Ang II:Ang I**
- **Ang-(1-7):Ang I**
- **Ang-(1-7):Ang II**

Graphs showing peptide ratios over age (weeks) with statistical significances marked (e.g., *).
Figure 5.

**Serum Creatinine**

![Bar chart showing serum creatinine levels across different age weeks.](image)

**GFR**

![Bar chart showing GFR levels across different age weeks.](image)
Figure 6.
CHAPTER FIVE

BILATERAL RENAL DENERVATION LOWERS BLOOD PRESSURE AND DELAYS THE DEVELOPMENT OF PROTEINURIA BUT DOES NOT ALTER LEVELS OF INTRARENAL RENIN-ANGIOTENSIN SYSTEM PEPTIDES IN AGING SPRAGUE-DAWLEY RATS

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Abstract

Increased renal sympathetic nerve activity is associated with hypertension and the general decline in kidney function with advancing age. Renal denervation is known to lower blood pressure in hypertensive rats during development of the hypertension, but evidence of the chronic effects of renal denervation on blood pressure and kidney damage during aging is not well documented. In addition, the exact mechanisms behind the beneficial effects of renal denervation are not completely established. We investigated the effects of bilateral renal denervation on blood pressure, intrarenal renin-angiotensin system (RAS) activation, proteinuria and creatinine excretion during aging. Male SD rats were subjected to either bilateral renal denervation or sham operation at 28 weeks of age (adult) and studied over a 6 month period or at 75 weeks of age (older adult) and studied for a month. SBP was lower in the 28 week denervated vs sham rats for at least 5 weeks after surgery (one wk post surgery: 106 ± 5 vs 126 ± 6 mm Hg; n= 8-11; p<0.005) and lower in the 75 week denervated rats for 1 week post-surgery compared to their baseline values (93 ± 3 vs 133 ± 5 mm Hg; n=7; p<0.05). Proteinuria was not different between the older sham and denervated groups. Proteinuria increased in the sham but not the denervated adult rats starting at 6 weeks after surgery. Angiotensin (Ang) peptide (Ang I, Ang-(1-7) and Ang II) and creatinine excretion were not different between the adult denervated and sham groups or between the older adult sham and denervated groups. Renal denervation decreased blood pressure and delayed the onset and reduced the severity of proteinuria without altering Ang peptides or creatinine excretion in the adult rat. Renal denervation transiently decreased blood pressure without effecting proteinuria or Ang peptides and creatinine excretion in the older adult rats. The data suggests that
during aging renal nerve activity contributes to renal damage (proteinuria) associated with a reduction in blood pressure, but independent of changes in the intrarenal RAS.

**Introduction**

Increased sympathetic nervous system (SNS) activity is implicated in the pathogenesis of hypertension and renal failure. Renal denervation prevented hypertension in rats with chronic renal failure (4) and in genetically hypertensive rats delayed the development of hypertension (8; 18; 21; 31). Hyperinsulinemia-induced hypertension in SD rats is prevented by bilateral renal denervation and fully established hypertension is reversed returning blood pressure to normotensive levels within 2 weeks (14). Glomerular enlargement, hyperfiltration and hyperperfusion were prevented in streptozotocin-induced diabetic rats in response to bilateral renal denervation (22). These data suggest that renal nerve activity may play a prominent role in the alterations of kidney function that occurs in response to disease states and aging. In addition, data suggest that altered renal hemodynamics in the form of hyperfiltration, which may be a result of increased renal sympathetic nerve activity (RSNA), is key to producing pathological changes seen in the kidney (13; 30). It is reported that RAS blockade decreases the effects of increased RSNA on renal function and that renal denervation reduces the effects of intrarenal administration of Ang II (7). In the Otsuka Long-Evans Tokushima Fatty (OLETF) rat, a model of type 2 diabetes, bilateral renal denervation impeded the progression of glomerulosclerosis and the increase in blood pressure (29) and the angiotensin receptor blocker telmisartan decreased intrarenal Ang II and prevented podocyte abnormalities and proteinuria (25). Proteinuria leads to the reduction in oncotic pressure and decreased intravascular volume which may activate the RAS and
renal nerves to produce avid salt and water retention. In addition, renal nerves may
differentially influence glomerular hemodynamics and promote proteinuria in diabetes
and hypertension (6). Ang II can cause podocyte damage and loss leading to proteinuria
(28). Previous studies suggest that intrarenal RAS activation and blood pressure
contribute to proteinuria (10; 26) and it is possible the renal nerves may contribute to
intrarenal RAS activation. These are just a few possible mechanisms by which the renal
nerves, Ang II and proteinuria may interact to cause renal dysfunction. However, the
chronic effects of renal nerves on the intrarenal RAS and proteinuria during aging are not
well documented. Which if any of these mechanisms is the initiating factor and to what
extent each contributes to the development and/or maintenance of renal dysfunction
during aging is not fully known and may be dependent on the condition or disease state.
Therefore, we assessed the effects of bilateral renal denervation on blood pressure,
intrarenal RAS levels and proteinuria in adult and older adult SD rats to provide
information on the role of renal nerves on these factors during aging.

Methods
All animal protocols were reviewed and approved by the Institutional Animal Care and
Use Committee. The male Hannover SD rats were obtained from colonies maintained in
the Hypertension and Vascular Research Center at Wake Forest University. All animals
were bred and exposed to the same housing conditions (12:12-h light-dark cycle) and
provided food and water ad libitum. Twenty-four hour urine collections on dry ice were
conducted using metabolic cages to determine excretion values for protein, creatinine and
Ang peptides. Urinary Ang I, Ang II, and Ang-(1-7) were analyzed using
radioimmunoassay (2; 15). Protein in urine was measured using reagent strips (Multistix
Urine was analyzed for creatinine (Metra creatinine assay kit, Quidel Corporation) and the urinary peptide data are expressed as 24-hour excretion rates normalized to creatinine excretion as detailed elsewhere (9). Food and water intake and body weight and urine volume were also determined. SBP was measured using the tail cuff procedure and there were at least three training sessions to acclimate the animals to the device. The mean of at least five blood pressure measurements were determined for each animal. The metabolic cage and SBP determinations were conducted prior to and after surgery. At the end of the study, serum insulin and leptin were measured using radioimmunoassays (Linco, Inc.) and serum glucose using a Freestyle glucose monitor (TheraSense). Bilateral renal denervation was performed in 28 week old rats that were studied for 6 months after surgery and in 75 week old rats that were studied for 1 month after surgery.

Bilateral Renal Denervation Procedure

Both kidneys were exposed by a retroperitoneal flank incision and each renal artery was surgically stripped. An absorbable suture thread soaked in a 10% phenol in ethanol solution was looped around the artery and allowed to remain for 10 minutes. Sham rats were subjected to exposure only. Denervation was assessed by measuring norepinephrine (NE) levels in each kidney (ALPCO Diagnostics). Renal tissue norepinephrine was measured because it represents 90-95% of total rat renal catecholamine content (8). Complete denervation is generally considered to be a tissue NE content that is ~ 10% of the mean value in the sham operated groups.
Statistical Analysis

All data are presented as the mean ± SEM and p values < 0.05 were considered significant. Two-way ANOVA of GraphPad Prism was used to assess overall interactions across time and denervation. One-way ANOVA with Dunnett’s post hoc test was performed using GraphPad Prism. Unpaired t-test with Welch’s corrected post-hoc test was performed using GraphPad InStat.

Results

28 week old (adult) animals

There was an effect of time and denervation on SBP as determined by two way analysis of variance. SBP was significantly lower (p<0.05) five weeks post-surgery in the denervated animals compared to the shams and then at eight, ten and eighteen weeks post-surgery (Fig. 1). There was no difference in Ang I, Ang II or Ang-(1-7) excretion between the groups (Fig. 2) but there was an effect of time on all three urinary Ang peptides. Two way analysis of variance revealed that protein excretion increased over time in the sham rats but not the denervated animals. Creatinine excretion was not significantly different between the groups over the time period of study. Urine volume was effected by time and denervation (Fig. 3), with the volume being lower in the denervated rats throughout the study. Food and water intake were similar between sham and denervated rats (data not shown) and increased over time in both groups. There was an effect of time and denervation on body weight (Fig. 4), with the denervated animals maintaining a slightly lower body weight beginning at week 34. At the end of the study, the kidney was removed to determine renal tissue NE content; renal NE levels were significantly lower in the denervated rats compared to the sham rats (p<0.02). Plasma
renin concentration was not different between groups (Fig. 5) in samples obtained at the end of the study, and serum insulin, leptin and glucose were similar between the two groups at this time point (Table 1).

75 week old (older adult) animals

Two way analysis of variance revealed an effect of time on SBP which was significantly lower for one week post-surgery in the older denervated rats compared to their baseline values (Fig 6). There was an effect of time on urinary Ang II and Ang-(1-7) with no difference between groups. Ang I excretion was similar between the sham and denervated groups (Fig. 7), and was not altered over time. There was an effect of time on proteinuria, urine volume and urine creatinine with no differences in these factors between the treatment groups (Fig. 8). Food and water intake were similar between sham and denervated rats (data not shown). Body weight was similar between the groups (Fig. 9). At the end of the study, samples were obtained to determine renal tissue NE content and plasma renin concentration, which were not different between the groups (Fig 10). Serum insulin, leptin and glucose levels assessed at the end of the study were similar between the groups (Table 1).

Discussion

Bilateral denervation decreased blood pressure in adult and older adult rats without altering the excretion of the angiotensins peptides or creatinine. Already established proteinuria, a marker of renal damage in the older rats, was not different between the older adult denervated and sham rats, but progression towards increased proteinuria starting at 6 weeks post-surgery in the adult sham-operated animals was prevented or at least delayed in the adult denervated group. Renal tissue NE levels were
significantly lower in the adult denervated rats compared to their sham counterparts, but not different between the older adult sham and denervated groups. Plasma renin concentration was not significantly different between the sham and denervated, although a trend for lower values was evident in the denervated groups of both the adult and older adult rats. There were no effects of the denervation at either age on insulin, leptin, glucose, food or water intake.

There are reports, mainly from studies using hypertensive rats, that renal denervation delays or prevents increases in blood pressure (8; 21; 31). Renal denervation in 5 week old SHR delayed hypertension by 2-3 weeks but did prevent the development of hypertension (21). Similarly, renal denervation delayed the onset of stable adult hypertension but did not prevent its development in genetically hypertensive rat subjected to denervation at weaning (8). The renal denervated adult rats had a significantly lower blood pressure compared to shams for 5 weeks post-surgery and then at 8, 10 and 18 weeks post-surgery. We observed a sustained effect of renal denervation on blood pressure in the adult group, possibly because we used a normotensive rat strain or because in older rats, denervation may persist longer. SD rats have increased blood pressure as they age (17) and it is possible that in the adult rats, we would have observed an increase in blood pressure at a time point beyond the ages studied. In the older adult group, there was a significant decrease in blood pressure in the denervated rats compared to their baseline values 2 weeks post-surgery. This transient effect may be due to likely impairments in cardiac function which may occur at this age in rats and the fact that the older adult animals did not have hypertension at any time point studied suggests possible heart failure (11; 12). Since the drop in blood pressure was not accompanied by a
reduction in urinary protein, there did not appear to be reversal of the already established renal injury. In contrast, the lower pressure in the adult rats can not be ruled out as a factor in the delayed progression towards proteinuria during aging in the adult denervated rats.

Activation of the intrarenal RAS is thought to play a role in altering renal function by effecting renal hemodynamics and activating factors and mechanisms that lead to renal damage (20). The mechanisms behind the activation are not completely understood and may involve increased SNSA since animals with a decreased SNS outflow do not have intrarenal RAS activation with aging (17; 26). In addition, renal nerves innervate the juxtaglomerular (JG) cells and stimulate renin release (7) and it is reported that the JG cells are the main source of both systemic and intrarenal renin levels (20). This suggests that renal innervation may regulate the intrarenal RAS. In addition, Ang II facilitates the SNS at various levels and is known to be a tonic stimulus of renal sympathetic nerve activity (RSNA) both in brain and at sympathetic ganglia (3). The present study demonstrates that in SD rats, renal innervation does not contribute to intrarenal RAS regulation since urinary Ang peptides were not altered by renal denervation in the adult or older adult rats when compared to their sham counterparts either immediately following denervation or with respect to the 50-100% increase in Ang peptide excretion by the end of our study.

Proteinuria, which is considered the hallmark of renal damage, is a result of and contributes to altered renal hemodynamic, RAS activation and promotion of factors that lead to the decline in renal function (1; 16; 27; 28). Increased proteinuria, which may be caused by scenarios such as elevated intraglomerular pressure or podocyte loss, leads to a
decrease in oncotic pressure and intravascular volume. This decrease in pressure and volume mimics a state of fluid loss and thus may lead to activation of the RAS and renal sympathetic nerves. Likewise, increased renal nerve activity may contribute to glomerular hyperfiltration (6) leading to proteinuria. An indicator of glomerular hyperfiltration is increased levels of urinary creatinine. In the adult and older adult animals, renal denervation did not alter creatinine excretion and levels were similar to sham rats throughout the study. Proteinuria appeared at about 32 weeks of age in the sham-operated adult rats and this was prevented by denervation. Proteinuria was already evident in the older adult animals at the start of our study and there was no difference between the older adult denervated and sham rats. Thus, since proteinuria starts at an elevated level in the older adult group as would be expected, the renal denervation was not capable of reversing the renal injury leading to loss of protein. It is interesting to note that the proteinuria in the sham-operated adult rats occurs independently of changes in urinary Ang II or other Ang peptides. Importantly, the renal denervation in the adult rats had an influence on proteinuria in the absence of changes in the excretion of Ang peptides.

Determining renal NE content, which is an indirect marker of SNSA, is a standard measure for assessing whether renal denervation has occurred. Interestingly, adult and older adult sham rats had similar tissue NE content. While there was not a difference between the older adult sham and denervated rats, renal NE content was significantly lower in the adult denervated rats compared to the adult sham rats. However, the levels were not reduced by ~90% which is considered to be evidence of complete denervation. In spite of only ~50% reduction in tissue NE levels, differences in SBP and proteinuria
in the functionally denervated adult rats suggests that renal denervation may have been complete in the initial stages of the study and reinnervation may have occurred. It is reported that renal NE levels in denervated rats return to levels similar to sham rats (5) probably due to functional reinnervation (19). Reinnervation may occur as early as 14 days after denervation in younger rats (19) and may take longer to occur in older animals. Though tissue NE is an accepted standard of determining renal denervation, it is reported to be an inaccurate index of return of nerve function since nerve function may return to normal when tissue NE is low (19). There is also the possibility of partial denervation in which case the data suggest that even some interruption of renal nerve activity may be effective in reducing blood pressure and possibly delaying renal damage. The plasma renin concentration was similar between the sham and denervated groups in the adult rats with a trend towards lower levels in the denervated rats. This was the pattern observed in the older adult rats as well. Plasma renin concentration was measured because of the role of renal nerves in stimulating renin release from the JG cells. It is important to note that renin secretion is governed by other factors such as the renal baroreceptors and the macula densa (23; 24; 32).

These data reveal that, in adult rats bilateral renal denervation, producing a sustained ~ 50% reduction in renal NE content, lowered blood pressure for at least five weeks post-surgery and delayed the onset and reduced the severity of proteinuria. In older adult rats with renal impairment as assessed by significant proteinuria at the time of denervation and likely impairments in cardiac function, renal denervation did not contribute to long-term support of blood pressure or alter protein excretion. The
intrarenal RAS, as indicated by Ang peptide excretion, was not altered by the renal
denervation at either age.

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technical assistance.


Figure 1. Systolic blood pressure in SD rats subjected to bilateral renal denervation or sham operation at 28 weeks of age. Blood pressure was significantly lower in the denervated vs shams rats for 6 weeks post-surgery and at 8, 10 and 18 weeks post-surgery. p<0.05 vs shams

Figure 2. Urinary Ang I, Ang II and Ang-(1-7) in SD rats subjected to bilateral renal denervation or sham operation at 28 weeks of age. There was no difference in urine Ang peptide levels between the denervated and sham rats.

Figure 3. Protein and creatinine excretion and urine volume in SD rats subjected to bilateral renal denervation or sham operation at 28 weeks of age. Creatinine excretion was similar between the groups. Proteinuria and urine volume were effected by time and denervation. @p< 0.0003 for an effect of time and denervation; *p< 0.0004 for an effect of time; #p< 0.03 for an effect of denervation.

Figure 4. Renal norepinephrine and plasma renin concentration in SD rats subjected to bilateral renal denervation or sham operation at 28 weeks of age. Renal norepinephrine levels were significantly lower in the denervated rats compared to the shams. Plasma renin concentration was similar between the sham and denervated rats. *p<0.02 vs shams

Figure 5. Body weight in SD rats subjected to bilateral renal denervation or sham operation at 28 weeks of age. p< 0.0002 for an effect of time and denervation

Figure 6. Systolic blood pressure in SD rats subjected to bilateral renal denervation or sham operation at 75 weeks of age. Blood pressure was significantly lower for 2 weeks post-surgery in the denervated rats vs their baseline values. *p<0.05 vs baseline
Figure 7. Urinary Ang I, Ang II and Ang-(1-7) in SD rats subjected to bilateral renal denervation or sham operation at 75 weeks of age. There was no difference in levels of urinary Ang peptides between the sham and denervated rats.

Figure 8. Protein and creatinine excretion and urine volume in SD rats subjected to bilateral renal denervation or sham operation at 75 weeks of age. There was no difference between levels of proteinuria between the denervated and sham rats. Creatinine excretion and urine volume were similar between the groups.

Figure 9. Renal norepinephrine and plasma renin concentration in SD rats subjected to bilateral renal denervation or sham operation at 75 weeks of age. Renal norepinephrine levels were similar between the sham and denervated rats. There was no difference in plasma renin concentration between sham and denervated rats.

Figure 10. Body weight in SD rats subjected to bilateral renal denervation or sham operation at 75 weeks of age. There were no differences at any time point in body weight between sham and denervated rats.
Figure 1.

Systolic Blood Pressure

mmHg

Age (weeks)

Dnx
Sham

* * * * *
Figure 2.

Urinary Angiotensin Peptides

Ang I

Ang II

Ang-(1-7)
Figure 3.
Figure 4.
Figure 5.

![Graph of Tissue Norepinephrine][1]

![Graph of Plasma Renin Concentration][2]
Figure 6.

Systolic Blood Pressure

![Graph showing systolic blood pressure over age (weeks). The graph compares Dnx and Sham groups. The Dnx group shows a significant increase in blood pressure compared to the Sham group.](image-url)
Figure 7.

**Urinary Angiotensin Peptides**

**Ang I**

[Graph showing changes in Ang I levels with age for Dnx and Sham groups.]

**Ang II**

[Graph showing changes in Ang II levels with age for Dnx and Sham groups.]

**Ang-(1-7)**

[Graph showing changes in Ang-(1-7) levels with age for Dnx and Sham groups.]
Figure 8.

Protein Excretion

Urine Creatinine

Urine Volume
Figure 9.

**Body Weight**

- **Dnx**
- **Sham**

![Graph showing body weight over age (weeks)]

- Age (weeks) range from 74 to 80.
- Body weight in grams, ranging from 400 to 700 grams.
Figure 10.

Tissue Norepinephrine

Plasma Renin Concentration
Table 1. Serum insulin, glucose and leptin determined at the end of the study in SD rats subjected to bilateral renal denervation or sham operation at 28 or 75 weeks of age.

<table>
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<th></th>
<th>Insulin</th>
<th>Glucose</th>
<th>Leptin</th>
</tr>
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<tr>
<td>28 weeks</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>2.07 ± 0.4</td>
<td>120 ± 6</td>
<td>7.55 ± 1.4</td>
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<tr>
<td>Dnx</td>
<td>2.09 ± 0.7</td>
<td>115 ± 2</td>
<td>6.57 ± 0.7</td>
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<td>75 weeks</td>
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<td>1.39 ± 1</td>
<td>115 ± 18</td>
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<tr>
<td>Dnx</td>
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CHAPTER SIX

SUMMARY AND CONCLUSION

1. Summary of Results

The use of Fischer 344 (F344) and Sprague Dawley (SD) rats offers insight into the interrelationships of the intrarenal RAS with blood pressure control, renal nerve activity, body metabolism and kidney function as well as the interactions of these factors during aging. Both the F344 and SD rats develop insulin resistance and renal damage during aging but unlike what is reported in SD rats, F344 rats do not exhibit increased SBP as they age. During aging, the F344 rats have higher serum leptin, insulin, glucose, body weight and increased excretion of Ang peptides and protein without an increase in blood pressure or plasma RAS peptides when compared to young animals. RAS blockade with the AT$_1$ receptor antagonist L-158,809 significantly improved overall body metabolism, decreased intrarenal RAS activation and proteinuria and increased the plasma RAS without altering blood pressure. During aging, the F344 rats also have increased gene expression of dorsomedial medulla angiotensinogen with a similar trend for renin while the expression of ACE2 and neprilysin remain the same with a decrease in ACE expression compared to younger animals. There was no change in the gene expression of AT$_{1a}$, AT$_{1b}$, AT$_2$ or Mas receptors in the dorsomedial medulla during aging in the F344 rats but there was a decrease in leptin receptor expression and an increase in the expression of the PI3K p85 regulatory subunit. RAS blockade with L-158,809 increased the gene expression of angiotensinogen, ACE, ACE2 and neprilysin with a similar trend for renin. RAS blockade also increased AT$_{1b}$, AT$_2$, Mas and leptin receptor
mRNA as well as the p85 subunit expression. The data provide evidence for AT\textsubscript{1} mediated feedback inhibition of expression of Aogen and renin in medulla, similar to renin in the kidney. Long-term RAS blockade activates enzymes and receptors that would shift the balance from Ang II to Ang-(1-7) in this brain region. The prevention of age-related declines in the leptin receptor and enhancement of a leptin signaling pathway provides mechanisms for preservation of metabolic function in treated F344 rats. The brain RAS may be involved in the impairments in metabolic function of the older rats since the decreased gene expression of the leptin receptor and p85 were reversed with RAS blockade. This concept is consistent with previous data showing that ~ 18 month old transgenic rats with a deficit in brain (glial) angiotensinogen have lower body weight and insulin, leptin and glucose levels compared to age-matched SD rats (30; 31).

SD rats also have a significant increased excretion of Ang I, Ang-(1-7), protein and creatinine during early aging. Ang II excretion is known to increase during aging in SD rats (47) but, our studies did not show a significant increase during early aging probably because our study ended around the time of the reported increase. These events occur in advance of increases in blood pressure supporting the independent regulation of these factors in SD rats as in the F344 rats. Bilateral renal denervation in 28 week old SD rats significantly lowered SBP for at least five weeks post-surgery without altering Ang II or creatinine excretion. Protein excretion was effected by time and denervation such that there was no increase at the later time points studied in the denervated rats when compared to the sham rats. Thus, bilateral renal denervation may delay the onset and reduce the severity of proteinuria associated with increasing age at time points prior to age-related increases in SBP in SD rats but does not alter intrarenal RAS levels. Bilateral
renal denervation in 75 week old SD rats significantly lowered SBP for one week post-surgery without changing protein, creatinine or Ang II excretion. This implies that renal nerves do not contribute to long-term support of blood pressure in older rats with existing renal impairment as assessed by significant proteinuria at the time of denervation and likely impairments in cardiac function. Renal denervation is not sufficient to reverse or prevent continuing declines in renal function (increases in Ang II or protein) occurring in older normotensive rats.

Collectively, the data from the F344 and SD rat studies reveal that the intrarenal RAS is activated during aging with or without a subsequent increase in blood pressure. Results from the F344 rats suggest that the brain RAS is activated during aging and that it may act with the intrarenal RAS to contribute to declining health. RAS blockade improved many parameters of aging in F344 rats, in spite of unchanging circulating RAS levels during aging. This implies that blockade of the renal and brain RAS, as evident in these studies, may contribute to the beneficial effects. Increased proteinuria appears to occur at the same time as intrarenal RAS activation in SD rats subsequent to increased creatinine excretion. Renal nerves appear to contribute to renal damage (proteinuria) independent of changes in Ang II excretion but only during the early stages of aging.

2. The Intrarenal RAS and Blood Pressure Regulation

Previous evidence suggests a role for the intrarenal RAS in regulating blood pressure (47; 51) and these studies often involve subjects with increased blood pressure or hypertension over the course of aging. In SD rats, increased excretion of Ang II, an index of intrarenal RAS activation, precedes the elevation in blood pressure (47). Transgenic mice overexpressing renal angiotensinogen exhibited an increase in blood
pressure which was prevented by RAS blockade (51). Urinary angiotensinogen levels were significantly higher in hypertensive patients not receiving RAS blockers compared with normotensive patients and RAS blockade in hypertensive patients prevented the increase (35). These data imply that increased blood pressure may occur secondarily to or is dependent upon intrarenal RAS activation and blocking the intrarenal RAS activation may promote decreased blood pressure by reducing tubular salt and water reabsorption. The F344 rats in our study had an age-related increase in the intrarenal RAS without an increase in blood pressure. In addition, RAS blockade decreased the age-related activation of the intrarenal RAS without lowering blood pressure. This suggests that increased blood pressure may occur independently of intrarenal RAS activation. Certainly other tissue RAS, such as the brain may be involved in regulating blood pressure during aging. Transgenic rats (ASrAogen) with a 90% reduction in brain (glial) angiotensinogen do not exhibit increased blood pressure during aging suggesting the this reduction in pressure is secondary to the decrease in brain RAS activity (30). We found an age-related increase in the expression of dorsomedial medulla angiotensinogen in the F344 rats implying that the brain RAS may be activated without leading to increased blood pressure during aging. However, we do not know which tissue RAS is activated first in the F344 rats or if they both are activated around the same time. Certainly alterations in the brain medullary RAS may influence reflex regulation of pressure independent of an actual increase in resting blood pressure.

Overall, these data show independence of the intrarenal and brain RASs on blood pressure regulation during aging and that RAS blockade has several beneficial effects independently of changes in blood pressure as previously noted. There may be control of
blood pressure by the specific tissue systems according to the model used or based on the
disease state, treatment regimen, condition or age of the subjects. Neither independence
nor dependence of blood pressure regulation on tissue RAS should be ruled out at this
time as there is conflicting evidence suggesting either may be the case. The information
here only focuses on two tissue RAS. It is well established that other tissues and organs
contain their own local RAS, including the heart, vessels, adrenal gland and pancreas (4;
38). Further studies are needed to clarify the exact mechanisms and in which situations
activation of the tissue systems may lead to increased blood pressure and if other tissue
RAS play a role in blood pressure regulation.

3. Interactions between the Intrarenal RAS and Brain RAS During Aging: Insights
from Data showing the Protective effects of Systemic RAS Blockade in F344 rats

During aging, the circulating RAS declines or remains the same, which suggests
that the beneficial actions of RAS blockade are partly due to the suppression of Ang II in
tissues. The local systems, which appear to increase during aging, seem to be regulated
independently of the circulating RAS but may also interact with the circulating RAS and
each other. 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). The exact mechanisms that cause
the increase in the intrarenal RAS are not entirely known and previous data suggests that
the brain RAS may play a role (47). The AsrAogen rats, which have a 90% reduction in
brain angiotensinogen, do not have intrarenal RAS activation during aging, implying
that an age-induced activation of the brain RAS may mediate the intrarenal RAS
activation (47). The overall profile of the AsrAogen resembles what we observed in the
F344 rats and L-158,809 is a non-peptide and may potentially cross the blood brain barrier to act at AT₁ receptors. Therefore, we investigated the brain RAS components in an area that is involved in regulating baroreflex sensitivity and cardiovascular function to offer insight on the role of the brain RAS in the changes that occur during aging and the effects of long-term RAS blockade in the F344 rats. We observed an age-related increase in the gene expression of angiotensinogen in the dorsomedial medulla of F344 rats with a similar trend for renin. The gene expression of ACE2 and neprilysin remained the same with a decrease in ACE expression compared to younger animals. There was no change in the expression of AT₁a, AT₁b, AT₂ or Mas receptors during aging. RAS blockade with L-158,809 increased the gene expression of angiotensinogen, ACE, ACE2 and neprilysin with a similar trend for renin. RAS blockade also increased AT₁b, AT₂ and Mas receptor mRNA. The data provide evidence for AT₁ mediated feedback inhibition of gene expression of angiotensinogen and renin in medulla, similar to the circulating RAS. Long-term RAS blockade activates enzymes and receptors that would shift the balance from Ang II to Ang-(1-7) in the dorsomedial medulla. In addition, the data suggest that the blocker possibly works at more than one tissue and that there appears to be a brain RAS-kidney RAS interaction. However, the F344 rat study does not offer insight into which tissue RAS is activated first or if they are activated around the same time.

4. Timeline of Alterations in Intrarenal RAS levels and Protein Excretion During Aging in SD rats

Aging is associated with intrarenal RAS activation in normotensive rat strains, including Wistar and SD rats (29; 30). In SD rats, an increase in excretion of Ang I, Ang II and protein over that of 16 week old animals is seen near 48 weeks of age (47) and
from our F344 rat study, we know there is an age-related increase in intrarenal RAS (Ang I, Ang II and Ang-(1-7) excretion) and proteinuria somewhere between 3 and 15 months (12 and 60 weeks) of age. Nevertheless, whether activation of the intrarenal RAS is secondary to progressive renal damage or is an initiating factor is not known. Since the timing of intrarenal RAS activation was partially characterized in SD rats, we wanted to further define the timing and consider a few potential mechanisms of activation, mainly whether renal damage is an initiating factor.

The SD rats had an age-related increase in Ang I and Ang-(1-7) excretion similar to previous studies (29; 30), while surprisingly, Ang II excretion was not elevated as previously reported, but, in fact, declined or remained similar compared to the control Ang II values. It is important to note that Ang II excretion has been shown to increase around the 48 week time point (47) and since this study ended at 48 weeks, it’s possible that studying ages slightly beyond 48 weeks would have revealed the increase. An increase in plasma or tissue Ang I primarily leads to increased Ang-(1-7) generation limiting the amount of substrate available for Ang II production (19). This may also be a possible explanation for why we did not see an increase in Ang II excretion during the ages studied. The increased Ang-(1-7) may be a protective mechanism to counteract the higher levels of Ang II. Urine Ang II starts out much higher than Ang I and Ang-(1-7) levels (0.42 ± 0.06 vs 0.02 ± 0.002 vs 0.17 ± 0.008 pmol/mg/day; respectively) and throughout the study tends to be higher or somewhat similar to Ang I and Ang-(1-7) values. Though Ang II may not appear to be activated/increased, the levels observed are generally maintained and may still be enough to elicit harmful effects, especially since previous studies show that RAS blockade has renoprotective effects and decreases
intrarenal Ang II in spite of the decreased or unchanged plasma RAS (47). Aging is associated with changes in the activity and/or responsiveness of the RAS which leads to alterations in homeostatic mechanisms and even normal RAS levels may be harmful to overall cardiovascular health. It is also possible that the kidney may also be more sensitive to the effects of Ang II, perhaps through upregulation of AT$_1$ receptors. We assessed the ratios of urinary Ang peptides to gain some perspective on enzymatic activity occurring in the kidney. Ang II:Ang I was significantly lower at all ages studied compared to 24 weeks. However, the levels were generally higher than the other peptide ratios over the course of the study except at a few time points. Ang-(1-7):Ang I was significantly lower at all age studied compared to 24 weeks. There was a general decline in the Ang II:Ang I and Ang-(1-7):Ang I ratios over the course of the study. This suggests that RAS enzyme activity is reduced over the course of aging and is consistent with the reported decline in neprilysin activity in the kidney of older rats (49). Ang-(1-7):Ang II remained similar and relatively low throughout most of the study except at 40, 44 and 46 weeks in which there were significant increases. The reason for the transient increase is not known and may be an attempt to degrade Ang II and produce more Ang-(1-7) in light of the overall higher levels of urinary Ang II production.

It is not completely clear whether intrarenal RAS activation or increased proteinuria occurs first. In addition, it has been reported that intrarenal Ang II has a positive feedback on tubular angiotensinogen and AT$_1$ receptors and collecting duct renin (36). Urinary Ang II and Aogen were increased in Ang II-dependent hypertensive rats, but not in an Ang II-independent hypertensive model (37), confirming the notion from previous studies that intrarenal Ang II has a positive feedback mechanism on urinary
Aogen production (36). Increased urinary protein excretion also occurred in the Ang II-dependent hypertensive model but was dissociated from the increased urinary Aogen excretion rate (37). Thus, that study potentially ruled out proteinuria as an initiating factor in intrarenal RAS activation by suggesting that the increased Aogen is not a nonspecific consequence of proteinuria. We observed a significant increase in protein excretion at the same time as that of Ang I and Ang-(1-7). Ang II may cause podocyte damage and apoptosis, reduce the degradation of proteins and induce cytokine expression, all of which may lead to glomerular and/or tubulointerstitial injury and proteinuria (52; 58-60). Increased proteinuria may cause a decrease in oncotic pressure and intravascular volume which may stimulate Ang II production. Ang II may act preferentially at the efferent arteriole to increase intraglomerular pressure, which would further promote proteinuria. Proteinuria, which is considered the hallmark of renal disease, has been suggested to be both a cause and consequence of renal damage. Proteinuria may occur as a result of glomerular and/or tubular dysfunction. Levels of intrarenal RAS components may also be considered markers of renal damage or dysfunction in light of information provided from previous studies. Telmisartan decreased intrarenal Ang II and prevented proteinuria and podocyte abnormalities in a rat model of type 2 diabetes (46). Urinary Aogen to urinary creatinine levels were significantly higher in hypertensive patients compared to normotensive patients and the levels were decreased to normotensive levels by RAS blockade (35). Thus, it appears that proteinuria and intrarenal RAS components may act as co-partners, each facilitating the other.
We observed a significant increase in creatinine excretion at 27, 28, 40, 46 and 48 weeks when compared to 24 weeks and though the other values were not significantly increased, there was a trend towards that. Since this data suggest appears to indicate a transient hyperfiltration as a contributing initiating factor to the proteinuria and intrarenal RAS activation, we determined the serum creatinine and GFR at various ages. Serum creatinine was similar among the ages studied and GFR was not different among the groups. The data appears to eliminate hyperfiltration as a cause of the increased urinary protein, Ang I and Ang-(1-7).

Early aging in SD rats is associated with urinary Ang II that is generally higher than Ang I and Ang-(1-7) which may contribute to the subsequent increase in urinary protein, Ang I and Ang-(1-7). Further studies are needed to elucidate the complete set of mechanisms behind the changes in the intrarenal RAS during aging and to establish role how other tissue RASs may regulate the intrarenal RAS.

5. The Influence of Renal Nerves on Blood Pressure, Intrarenal RAS levels and Proteinuria During Aging in SD rats

Renal sympathetic nerves help control renal function by regulating the functions of the tubules, vasculature, and the renin-containing JG cells. Intrarenal Ang II facilitates renal venous outflow of NE during renal nerve stimulation and this effect is blocked by an AT₁ receptor antagonist (14). It is reported that RAS blockade decreases the effects of increased RSNA on renal function and that renal denervation reduces the effects of intrarenal administration of Ang II (15). The exact mechanisms behind the regulation and activation of intrarenal RAS are not entirely known and may involve increased renal nerve activity. Therefore, we investigated the effects of bilateral renal
denervation on blood pressure, intrarenal RAS levels and proteinuria in adult (28 weeks) and older adult (75 weeks) SD rats to provide information on the role of renal nerves on these factors during aging.

Renal denervation prevented or delayed hypertension in hypertensive rats (6; 16; 25; 33; 39; 57) and in diabetic rats impedes the increase in blood pressure (55). The renal denervated adult rats had a significantly lower blood pressure compared to shams for 5 weeks post-surgery and then at 8, 10 and 18 weeks post-surgery. SD rats have increased blood pressure with aging (30) and it is possible that in the adult group, we would have observed an increase in blood pressure beyond the ages studied. In addition, the data suggest that early intervention may lead to a sustained effect of renal denervation on decreasing blood pressure in the adult group, possibly because we used a normotensive rat strain. In the older adult group, there was a significant decrease in blood pressure in the denervated rats compared to sham rats 2 weeks post-surgery. This transient effect may be due to possible impairments in cardiac function which may occur at this age in rats and the fact that the older adult animals did not have hypertension at any time point studied may suggest possible heart failure.

Activation of the intrarenal RAS is thought to play a role in altering renal function by effecting renal hemodynamics and activating factors and mechanisms that lead to renal damage (36). Animals with a decreased SNS outflow do not have intrarenal RAS activation with aging (30; 47) suggesting that increased SNSA may activate the intrarenal RAS. In addition, renal nerves innervate the juxtaglomerular (JG) cells and stimulate renin release (15) and it is reported that the JG cells are the main source of both systemic and intrarenal renin levels (36). This suggests that renal nerve activity may activate the
intrarenal RAS. In addition, Ang II activates the SNS at various levels and is known to be a tonic stimulus of renal sympathetic nerve activity (RSNA) (3). Our findings demonstrate that in SD rats, renal nerve activity does not contribute to intrarenal RAS activation since the levels of urinary Ang I, Ang II and Ang-(1-7) were not altered by renal denervation in the adult or older adult rats when compared to their sham counterparts. There appears to be dissociation between renal nerve activity and intrarenal RAS activation during aging in SD rats.

Renal denervation prevents hypertension in rats with chronic renal failure (6) and proteinuria is considered the hallmark of renal damage. Proteinuria is a result of and contributes to altered renal hemodynamic, RAS activation and promotion of factors that lead to the decline in renal function (2; 26; 50; 52). Increased proteinuria, which may be caused by scenarios such as elevated intraglomerular pressure or podocyte loss, leads to a decrease in oncotic pressure and intravascular volume. This decrease in pressure and volume mimics a state of fluid loss and thus may lead to activation of the RAS and renal nerves. Likewise, increased renal nerve activity may contribute to glomerular hyperfiltration (13) leading to proteinuria. An indicator of glomerular hyperfiltration is increased levels of urinary creatinine. In diabetic rats, renal denervation impeded the progression of glomerulosclerosis (55) and prevented glomerular enlargement, hyperfiltration and hyperperfusion (40). In the adult and older adult animals in our study, renal denervation did not alter creatinine excretion and levels were similar to sham rats. However, our denervation was performed right at the timepoint where hyperfiltration may occur. Thus, complications of the anesthesia and surgery may have masked an early effect. Nonetheless, there were lower levels of protein excretion in the denervated rats,
with no increase in proteinuria over the time points studied in the denervated rats. In contrast, there was no difference in levels of proteinuria between the older adult denervated and sham rats, where proteinuria was already established. These data suggest that renal nerves do not contribute to sustained alterations in protein or creatinine excretion in SD rats, a normotensive rat model.

Renal NE content, which is an indirect marker of SNSA, is a standard measure for assessing whether renal denervation has occurred. The tissue NE content in the present study in both age groups was not as markedly reduced in denervated rats relative to sham rats in our studies. Among the factors measured in this study, there was clear evidence that functional denervation occurred. Moreover, the factors measured have the same pattern, regardless of the magnitude of the reduction in NE, with all animals included. This suggests that renal denervation was complete in the initial stages of the study and reinnervation may have occurred to account for the incomplete reduction of tissue NE at the end of the study. It is reported that renal NE levels in denervated rats return to levels similar to sham rats (11) probably due to functional reinnervation (34). Reinnervation may occur as early as 14 days after denervation in younger rats (34) and may take longer to occur in older animals. Though tissue NE is an accepted standard of determining renal denervation, it is reported to be an inaccurate index of return of nerve function since nerve function may return to normal when tissue NE is low (34). There is also the possibility of partial denervation in which case the data suggest that even some interruption of renal nerve activity may be effective in reducing blood pressure and possibly delaying renal damage. The plasma renin concentration was similar between the sham and denervated groups in the adult rats with a trend towards lower levels in the
denervated rats. This was the pattern observed in the older adult rats as well. Plasma renin concentration was measured because of the role of renal nerves in stimulating renin release from the JG cells. It is important to note that renin secretion is governed by other factors such as the renal baroreceptors and the macula densa (43; 45; 62).

Overall, these data reveal that, in adult rats bilateral renal denervation lowered blood pressure for at least five weeks post-surgery and delayed the onset and reduced the severity of proteinuria. In older adult rats with renal impairment as assessed by significant proteinuria at the time of denervation and likely impairments in cardiac function, renal nerves do not contribute to long-term support of blood pressure. The intrarenal RAS, as indicated by Ang peptide excretion, was not altered by the renal denervation. Renal denervation did not alter protein excretion in the older adult rats and is not sufficient to reverse or prevent continuing declines in renal function occurring in older adult normotensive rats.

6. Insulin, Glucose and Body Metabolism: Influence of the Intrarenal RAS During Aging

Insulin resistance is known to occur in normotensive, non-diabetic rat strains during aging (31). Part of our investigation also involved assessing indices of body metabolism since previous data suggests that intrarenal RAS activation may contribute to metabolic dysfunction (47). Again, the timing of increases in insulin or glucose relative to the renal damage is not presently known.

Both older groups of F344 rats gained weight over the course of the study, however, the L-158,809 group maintained a significantly lower body weight compared to the older control group even though the food intake was similar between them. The
young group had a significantly lower food intake and body weight compared to the two older groups. These data show an age-related and treatment effect on these factors and is consistent with previous reports. Body weight was similar in SD rats in our timeline study, consistent with previous data in aging SD rats (30), except at 48 weeks, which was significantly higher than 24 weeks. As expected, there were no differences in food intake, water intake and urine volume in SD rats between the ages of 24 to 48 weeks. ASrAogen rats have lower body weight over the course of aging compared to age-matched SD rats (30) and mice with systemic angiotensiongen deficiency have lower body weights when given a standard or high fat diet compared to wild type mice (41). Rats treated with enalapril have a lower body fat mass compared with lean mass (8) and lower body weight during aging (21). The lower body weight in the treated F344 rats despite a similar food intake compared to the older controls and an intact circulating RAS implies a connection between intrarenal Ang II and possibly brain RAS and body energy metabolism.

The F344 rat data are consistent with reports that RAS blockade improves components of, or reduces the onset of, the MetS and type 2 diabetes (1; 12; 22; 61). Our study demonstrates the advantageous effects of RAS blockade independent of the blood pressure lowering actions in accordance with previous studies (53; 54). However, the precise mechanisms underlying the beneficial effects are not entirely known and mechanisms may involve actions at the skeletal muscle (23; 53), brain and autonomic nervous system (30; 31) or other organs such as liver and pancreas (17; 18; 56). In the HOPE study, treatment with ramipril decreased the development of Type 2 Diabetes in high risk patients (61). In the LIFE and VALUE studies, there was a lower incidence of new onset Type 2 Diabetes with improved insulin sensitivity in patients due to RAS
blockade (12; 27). Valsartan treatment in KK-Ay mice, a model of type 2 diabetes, did not affect SBP, but improved insulin sensitivity, PI3K activity and glucose transporter 4 (Glut4) translocation while decreasing TNF-α expression in skeletal muscle (53). Obese Zucker rats treated with irbesartan had improved whole body insulin sensitivity and enhancement of glucose uptake into the soleus and epitrochlearis muscle. This was partially ascribed to an increase in Glut4 protein levels in the heart, soleus, and plantaris muscle (23). Insulin and leptin interact in the rat hypothalamus at the PI3 and MAP kinase pathways to decrease feeding and maintain an appropriate metabolism (9). In addition, there is cross-talk between insulin and Ang II at the PI3 and MAP kinase pathways. Ang II may reduce the metabolic actions of insulin at the PI3 kinase pathway but synergistically promote its proliferative effects through the MAP kinase pathway (48). While the exact mechanisms of the beneficial effects of RAS blockade on insulin and glucose are not entirely known, it may be partly due to the decreased actions of Ang II at the kinase pathways as well as actions in the central nervous system on autonomic pathway controlling insulin and glucose metabolism. In addition, the beneficial effects may be due to suppressing the intrarenal or brain Ang II as our data and previous data (30; 31) implies. However, the issue of timing was unresolved in our studies of the F344 rats.

Aging is associated with an increased serum leptin in F344 rats (42) and this is consistent with our data. Serum leptin was significantly higher in the older control rats compared to the treated and young groups. In the SD rats, leptin levels were not different in the ages studied and were higher than those previously reported in SD rats (30) but similar to values in our young and older control F344 rats. Serum glucose in the older
control F344 group was significantly higher than the young group and the profile for serum insulin was similar, suggesting insulin resistance is present at this time point in the older control rats. The older AT$_1$ receptor blocker treated group was indistinguishable from the young rats. In the experiments providing a detailed timeline, we observed similar insulin values in our SD rats at the ages studied except at 40 weeks when a significant increase occurred compared to 24 weeks. The insulin levels in the 40 weeks old animals is similar to those seen in 68 weeks old SD rats (30) and the reason behind this transient increase seen at 40 weeks is unclear. However, this occurs slightly after the spike in urinary protein, Ang I and Ang-(1-7). The insulin levels at the other ages are similar to previously reported values in rats over the course of aging (30; 44) and to our young and older control F344 rats. Glucose in our SD rats was similar among the different ages studied and consistent with previously reported values in rats over the course of aging (20). QUICKI and HOMA assessments, in our F344 rats, which are indexes of insulin sensitivity (5; 10; 24; 31; 32), further suggests that the older controls have insulin resistance and that AT$_1$ receptor blockade may delay or prevent this from occurring. However, from our data, we are not able to discern which tissue RAS, the intrarenal or brain, is the initial culprit or if they work simultaneously to cause these changes. In addition, treated F344 rats exhibit a metabolic profile similar to 15-18 month old transgenic rats with low glial angiotensinogen (30; 31), further implying that blockade of the brain RAS may contribute to the benefits of long-term systemic AT$_1$ antagonism. In the dorsomedial medulla of the F344 rats, leptin receptor mRNA was significantly lower in the old rats compared to the young and L-158,809 treatment reversed this age-related change. Gene expression of the p85 regulatory subunit of PI3K
was significantly higher in the Old and Old + L-158,809 groups compared to the Young. The data imply that prevention of age-related declines in the leptin receptor and signaling pathway in the dorsomedial medulla provide mechanisms for preservation of metabolic function in the treated F344 rats. The brain RAS may be involved in the impairments in metabolic function of the older rats since the gene expression of the leptin receptor and p85 were increased with RAS blockade.

7. Limitations

Though the utilization of two different rat strains was performed to investigate certain potential mechanisms behind the age-related changes in metabolic and renal dysfunction, it also causes some reservation in data interpretation. Studying various possible mechanisms does shed some light on what factors may interact with the intrarenal RAS but also leaves some questions unanswered. For instance, we do not know the exact timing of increased proteinuria or intrarenal RAS activation in F344 rats but have a better sense of that in SD rats. It is not clear if the intrarenal or brain RAS is activated first in the F344 rats or if they are activated around the same time. Speculation of the brain RAS activity in F344 rats should be considered with caution since we only measured levels of mRNA and not protein. The role of the brain RAS in SD rats during aging was not assessed and therefore we can not determine when it is activated and if it interacts with the intrarenal RAS and the other factors studied. We do not know what effects chronic renal denervation has on F344 rats, which would be interesting since they do not have increased blood pressure with aging. Likewise, we do not know what effects long-term RAS blockade started early in the SD rats would have on proteinuria and intrarenal RAS levels. It should be noted that even though two different rat strains were
used, both are normotensive with many similarities in their aging profile, such as the occurrence of insulin resistance and renal damage. Thus, some extrapolation on the data between the strains should be allowed.

8. Concluding Remarks

In this study, the timing and potential mechanisms of intrarenal RAS activation were assessed using two rat strains commonly employed in aging studies. F344 rats do not have increased blood pressure during aging but SD rats do. However, both have insulin resistance and kidney damage with aging, suggesting that these changes are not necessarily dependent on the hemodynamic dysfunction that occurs during aging. The F344 rats maintain plasma RAS levels similar to their young counterparts unlike SD rats, which are known to have decreased plasma RAS levels during aging. Long-term treatment with L-158,809 in F344 rats demonstrated that there is a connection among the intrarenal RAS, brain RAS, proteinuria and metabolic dysfunction. The timeline study in SD rats revealed that there is a connection between the increased urinary Ang I, Ang-(1-7) and proteinuria and that urinary Ang II did not increase, though the values were higher than that of Ang I and Ang-(1-7).

Renal denervation in SD rats revealed that renal nerves play a major role in maintaining systolic blood pressure during aging but not in regulating the intrarenal RAS. Therefore, the data do not support the hypothesis that renal nerve activity plays a primary role in intrarenal RAS activation but offers some support to the idea that renal nerve activity contributes to the initiation of renal damage leading to proteinuria. It appears that interrupting renal nerve activity must occur during early aging in order to have an effect on lessening proteinuria.
Altogether, the data provide some support to the overall hypothesis that the intrarenal RAS is associated with the decline in kidney function, increased blood pressure and body weight and metabolic impairment during aging. However, our finding clearly show that each of these factors can be regulated independently. In addition, the data indirectly suggest that the brain RAS is involved and this is further supported by the fact that ASrAogen rats have a profile similar to the treated F344 rats (lower insulin, glucose, leptin, body weight and levels of intrarenal RAS components) during aging (30; 31; 47). These data imply that suppressing tissue RASs, in particular the intrarenal and brain RASs, are important markers of the salutary effects seen with long-term systemic RAS blockade, whether or not a causative role is confirmed. Figure 1 shows what may occur in association with the differential regulation of the plasma and renal RASs based on the aging profiles of the AsrAogen, F344 and SD rats.


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Figure 1. Proposed mechanisms for the increased proteinuria and differential regulation of the plasma and intrarenal RAS during aging. The proposed mechanisms are based on previous data from AsrAogen rats and the data from the F344 and SD rats. The brain RAS (activation of the brain RAS) contributes to increased blood pressure, autonomic dysfunction and increases in insulin and leptin (insulin and leptin resistance) during aging. The increased SBP may lead to a pressure mediated inhibition of renin release. The common increase in SNS that occurs during aging may result in increased sympathetic drive to the kidney via renal nerves which may lead to proteinuria. Insulin and leptin may influence RSNA as well as contribute to renal RAS activation. All of these actions may be blocked by angiotensin receptor blockers and ACE inhibitors whose
actions reduce the effects of Ang II. Similarly, the AsrAogen rats, which have a reduced brain RAS, are protected from these changes during aging.
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