Sex-Related Differences in Hypertension and Renal Injury: Role of the Renin-Angiotensin System

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LIST OF ABBREVIATIONS

RAS	Renin-Angiotensin System
ANG	Angiotensin
ANG-(1-7)	Angiotensin (1-7)
ACE	Angiotensin Converting Enzyme
ACE2	Angiotensin Converting Enzyme 2
AT1	Angiotensin II type 1 receptor
AT2	Angiotensin II type 2 receptor
AT(1-7)	Angiotensin-(1-7)/Mas receptor
Na^+-K^+-ATPase	Sodium Potassium ATPase Pump
Na^+/H^+ exchanger	Sodium Hydrogen Exchanger

ABSTRACT

Karl Dean Pendergrass II

SEX-RELATED DIFFERENCES IN HYPERTENSION AND RENAL INJURY: ROLE OF THE RENIN-ANGIOTENSIN SYSTEM

Dissertation under the direction of

Mark C. Chappell, Ph.D., Professor

Sex differences in hypertension and tissue injury are evident in experimental models and human subjects, yet the mechanisms underlying this disparity remain equivocal. The prevalence of hypertension in the United States has increased over the past century and progresses at a faster rate in men than in premenopausal women. The renin-angiotensin system (RAS) contributes to the development and maintenance of hypertension. Angiotensin II (Ang II) and angiotensin-(1-7) are two peptides derived from the RAS that possess vasoconstrictive and vasodilatory actions, respectively. Ang II contributes to hypertension and effective antihypertensive treatments involve enzyme inhibition and receptor blockade to prevent the effects of Ang II. Estrogen may provide a protective effect in women because the hormone exerts an inhibitory action on Ang II production and activity. Therefore, the current studies sought to define the extent of female-male differences in the circulating and renal RAS of the mRen(2).Lewis, a monogenetic model of Ang II-dependent hypertension that overexpresses the mouse renin 2 (Ren-2) gene, and the control Lewis rats. Male mRen(2).Lewis rats have significantly higher blood pressure, plasma and renal Ang II than male Lewis rats. Furthermore,

mRen(2).Lewis rats also exhibit a marked sex difference both in the extent of hypertension and Ang II that is not present in the Lewis strain. Plasma renin, angiotensin converting enzyme (ACE), and Ang I are lower in female mRen(2).Lewis as compared to male mRen(2).Lewis rats. In association with lower blood pressure, female mRen(2).Lewis expressed greater Ang-(1-7) suggesting a protective vasodilatory mechanism. Moreover, evaluation of the intrarenal RAS revealed a similar sex difference in the RAS of the mRen(2).Lewis that was not present in the Lewis strain. Renal Ang II was lower, while cortical neprilysin, an enzyme that degrades Ang II and generates Ang-(1-7) from Ang I, was significantly higher in female versus male mRen(2).Lewis. Neprilysin is stimulated by estrogen and may contribute to lower blood pressure through the renal metabolism of Ang II, as well as the formation of Ang-(1-7). Therefore, we determined whether chronic administration of the neprilysin inhibitor SCH 39370 would increase blood pressure and Ang II levels in female mRen(2).Lewis rats. Following a two week treatment, blood pressure was significantly decreased by 20 mm Hg. Neither circulating nor urinary Ang II and Ang-(1-7) levels were altered by neprilysin inhibition. In conclusion, our studies revealed that the increased expression of renal neprilysin in the female mRen(2).Lewis rat does not contribute to the sex-dependent difference in blood pressure for this model of hypertension.

CHAPTER ONE

INTRODUCTION

1. Sex differences in hypertension

The prevalence of hypertension in the United States has continued to increase over the past century in association with an aging and sedentary population. Sex differences in the development and maintenance of hypertension and tissue injury have been well documented in humans (33; 51; 78). The disease has been shown to progress at a faster rate in men than women before the onset of menopause (Figure 1). Moreover, as men and women age, the protection in blood pressure afforded to women before their fourth decade appears to decline (107). The increase in blood pressure has been associated with progression into peri- and postmenopausal states. The alterations in pressure that occur with an increase in age are attributed to the decline of estrogen levels in humans (123). However, the Women's Health Initiative (WHI) data indicated hormone replacement therapy in older women does not reduce hypertension or cardiovascular incidents (127; 128). Administration of estrogen to postmenopausal women does not attenuate the onset of cardiovascular events and leads to an increase in the incidence of other diseases including cancer and stroke (127; 128). The WHI study suggests that high doses of estrogen alone are not a protective treatment regimen for postmenopausal women with cardiovascular disease. Therefore, elucidation of the factors that lead to the gradual increase in cardiovascular disease in aging women is important and may lead to better diagnostic and treatment regimens.

Clinical studies have demonstrated that a normal menstrual cycle in women is associated with lower blood pressure and cardiovascular incidents compared to postmenopausal women (26). The ovarian hormone estrogen can decrease blood pressure but the mechanisms are unknown. Estrogen is known to have interactions with important hormone systems like the renin angiotensin system (RAS). However, the specific mechanisms and conditions that contribute to estrogen's beneficial actions on RAS expression have yet to be fully elucidated. Clinical studies by Miller and colleagues have assessed the actions of cycling estrogen levels on circulating RAS activation and renal function (25; 86). Chidambaram et al. (25) found that the circulating RAS was activated or higher during the luteal phase in young women, the point of where estrogen levels are at their highest. Although the plasma RAS was activated; the mean arterial pressure was not different in women with high and low estrogen states. Furthermore, Miller and colleagues have also compared the renal response to Ang II in young women and men (86). Women exhibited lower mean arterial pressure than their male counterparts, but circulating RAS parameters were not different between sexes. Interestingly, infusion of Ang II did uncover a sex-related difference in which the filtration fraction was lower in women. The authors speculated that the lower filtration fraction in women was linked to a protective mechanism mediated through alterations of the renal microcirculation. The discrepancy in the circulating RAS components between the sexes implies that plasma concentrations should not be the only parameter evaluated in studies. Moreover, results from these studies suggest that the level of estrogen may play a role in the efficacy of RAS blockers to decrease blood pressure in women (25; 86; 87). The state of the RAS and the subsequent physiological reactivity has yet to be determined in normotensive and

hypertensive women and those results could provide insight on the protective mechanisms against the development of hypertension in younger women.

2. Components of the Renin Angiotensin System (RAS)

The renin angiotensin system (RAS) is an important hormonal system that regulates blood pressure through water and salt retention, along with other actions (15). The RAS was first characterized as a circulating endocrine system, in which angiotensinogen is converted to Ang I by renin. Angiotensinogen is a 60 kilodalton (kDa) α2-globulin serum protein that is primarily released into the circulation from the liver. Renin is an aspartyl proteolytic enzyme of approximately 40 kDa that is released from the juxtaglomerular (JG) apparatus cells of the kidneys. Ang I is then hydrolyzed to Ang II by angiotensin converting enzyme (ACE) localized in the endothelium of the vasculature, particularly in the lungs or circulating in the plasma. ACE is a zinc and chloride-dependent peptidase found throughout the body. All forms of the enzyme contain active sites at both the amino and carboxy termini except for the testicular which has an active site at the carboxy terminus. The tissue form of ACE is a type I membrane bound enzyme. Ang II binds to either the AT receptor subtype 1 or 2 found throughout the body. The predominant subtype for the known functions of Ang II is the AT1 receptor, a G protein coupled receptor (GPCR), which produces the following actions: vasoconstriction, aldosterone release, water and salt reabsorption in the kidney, stimulation of the sympathetic nervous system, cellular proliferation, and production of profibrotic and proinflammatory molecules. However, Ang II binding to the AT2 GPCR mediates effects that are contrary to the actions of Ang II activating the AT1 receptor.

AT2 receptors promote vasodilation, inhibit cellular proliferation, and mediate apoptosis (16; 120; 129). Another arm of the RAS involves the vasoactive metabolite Ang-(1-7) which lowers blood pressure and reduces renal injury in hypertensive models (5; 6). Ang-(1-7) is synthesized from Ang I and Ang II by neprilysin and ACE2, respectively (111). Neprilysin and ACE also have the dual capability of synthesizing and degrading Ang peptides. Neprilysin and ACE degrade Ang II to Ang-(1-4) and Ang-(1-7) to Ang-(1-5), respectively (Figure 2) (21; 111). Similar to ACE, ACE2 and neprilysin are metallopeptidases. In contrast to ACE, both enzymes contain one active site. The protein sequence of ACE2 shares 42% sequence homology to ACE, however, ACE2 is a monocarboxypeptidase (32). ACE2 is a type I membrane bound enzyme, while neprilysin is a type II membrane bound enzyme. Ang-(1-7) stimulates prostaglandin production through binding to the GPCR mas protein (90; 109; 110). The main documented effects of the AT(1-7) receptor are vasodilation, natriuresis and diuresis (6; 79; 119). Ang-(1-7) stimulates prostaglandins and nitric oxide which may contribute to the antihypertensive effects of the peptide.

Even though the circulating RAS can access and alter many physiological control centers in the body, local RASs have recently been characterized in some organ systems. Relevant to my studies, the presence of a complete RAS has been demonstrated in the kidney and local generation of Ang peptides can exert paracrine actions (59). RAS components have been shown in cortical proximal tubules and glomeruli (110; 113; 118). The proximal tubules in the renal cortex could be an important site for production of intrarenal Ang II due to the presence of both angiotensinogen and renin. The tubular cells synthesize and release angiotensinogen and the protein has been detected in the

urine (55; 68). Thus, urinary angiotensinogen is believed to be of tubular origin, since the protein is too large for glomerular filtration. Navar and colleagues suggest that urinary angiotensinogen is a marker for renal RAS activity (67). Renin synthesis and release has been demonstrated in proximal tubules and collecting ducts, although the major site of synthesis and release is the JG cells (23; 49; 68; 88; 101). The proximal tubules also express ACE which can synthesize Ang II and breakdown other peptides. Both the AT1 and AT2 receptors are found in the kidney. AT1 receptors are expressed throughout the kidney including the vasculature, glomeruli, and proximal tubules (47; 48; 68). The AT2 receptor is localized to the proximal tubules and glomeruli. Renal AT2 receptor density significantly decreases by adulthood in the rat (68; 97). Even though all of the RAS components are found in the kidney, their regulation and actions are not fully elucidated.

The kidney also expresses the separate arm of the RAS cascade that includes the synthesizing enzymes for Ang-(1-7), the peptide Ang-(1-7), and its receptor AT(1-7) or mas protein. The enzymes ACE2 and neprilysin, and the peptide Ang-(1-7) are found in rat proximal tubules (64; 80). ACE2 is also localized to both the glomeruli and renal arterioles (114; 134). Neprilysin is also expressed in the renal vasculature (111; 117). The renal expression of both enzymes and their presence in the urine allows access to locally-generated and filtered substrates. In contrast to the localization of enzymes that generate Ang-(1-7), the precise renal area or cell type for the AT(1-7) receptor is still under investigation. The presence of the AT(1-7) receptor has been demonstrated in the mouse kidney (100). The nonpeptide agonist Aventis 0991 displaced radiolabeled Ang-(1-7) binding which was used to detect AT(1-7) receptors in renal sections from wildtype

and mas knockout mice. The AT(1-7) mas receptor was found in the wildtype, but not the mas knockout mouse. The renal cell type was not defined in the study. However, multiple renal actions on fluid balance have been shown for the AT(1-7) receptor and these actions vary depending on the physiological state of the animal (62; 63; 105; 124). Further study into the regulation and expression of Ang-(1-7) in the kidney is needed, and Ang-(1-7) localization may convey specific actions in vascular, tubular, and interstitial areas.

3. Actions of the RAS on the kidney

The kidney is subject to regulation by both the circulating and intrarenal RAS under normal renal arterial pressures of 70 to 130 mm Hg. Studies by Guyton and colleagues showed that Ang II modifies renal function through effects on arteriole vasoactivity and glomerular filtration rate. First, Hall et al. (45) found that systemic Ang II maintains glomerular filtration rate during reduced renal artery pressure in dogs fed a normal salt diet. The glomerular filtration rate was maintained through selective vasoconstriction of the efferent arteriole through Ang II-AT1 receptor dependent mechanisms. The efferent arteriolar vasoconstriction increases or prevents a fall in glomerular filtration rate through an increase in vascular resistance and glomerular hydrostatic pressure. Moreover, Kastner and colleagues demonstrated that renal-derived Ang II is also important to the maintenance of glomerular filtration rate during decreased renal perfusion pressure (65). They used a recirculating perfusion method in an isolated canine kidney. The isolated kidney precludes systemic or non renal-derived Ang II from acting on the kidney. A decrease in renal perfusion pressure under control conditions did

not affect glomerular filtration rate; however, glomerular filtration rate fell when an ACE inhibitor was administered during the lower renal perfusion pressure, an effect likely due to the blockade of intrarenal Ang II synthesis. Recently, multiple studies have demonstrated the presence of Ang II in the tubular luminal fluid (9; 70; 76; 92; 121). Moreover, overexpression of proximal tubular angiotensinogen in mice produced a hypertensive phenotype (9; 70; 76; 92; 121). The findings by Braam et al. (9) and Navar et al. (92) showed that concentrations of Ang II in the tubular fluid of normotensive and hypertensive rats were higher than could be explained by only uptake of circulating Ang II. Ang II in the tubular lumen could activate luminal AT1 receptors to stimulate sodium reuptake. Along with sodium reuptake, fluid reabsorption accompanies the sodium transport in the nephron. Thomas et al. (121) observed a leftward shift in the Ang II dose response curve for tubular fluid reabsorption in twelve week old SHR compared to age-matched normotensive Westar-Kyoto rats and five week old SHR. The leftward shift indicated an increase in the potency of Ang II, since the maximum rates for Ang II-stimulated tubular fluid reabsorption were similar between all groups tested. Sigmund and colleagues demonstrated that an increase expression of angiotensinogen localized to renal proximal tubules caused an increase in renal injury and renal Ang II content, but did not augment plasma Ang II levels. Although renal function was not assessed in the model, this is direct evidence that the local tubular RAS can increase blood pressure in a whole animal (70; 76).

Ang II also exerts other actions in the kidney such as regulation of ion transporter activity and protein synthesis through receptor activation. AT1 receptors in proximal tubules stimulate sodium reabsorption through activation of the Na^+/H^+ exchanger and

the Na^+-K^+-ATPase, whereas the AT2 receptor inhibits Na^+-K^+-ATPase pumps (14; 44; 82; 102). However, sodium influx into the proximal tubules is activated in a biphasic manner through the AT1 receptor. Picomolar concentrations of Ang II stimulate Na^+-K^+-ATPase activity, while micromolar Ang II levels inhibit the transporter's activity (7). In contrast, the AT2 receptor agonist, CGP-42112, inhibited sodium reuptake at all tested doses in isolated rat proximal tubules and the selective AT2 receptor antagonist PD-123319 blocked the inhibition (44). The inhibitory action of the AT2 receptor is mediated through the generation of nitric oxide and cGMP (44). The decline of renal AT2 receptors in rats as they age could account for the lower natriuretic actions in older animals as compared to the sodium retentive actions of a higher renal AT1 receptor density. The aforementioned pathways evident under higher pathophysiological Ang II levels could be beneficial by attenuating excessive sodium retention.

An additional action of the renal AT1 receptor is stimulation of agents promoting fibrosis and inflammation. It was recently demonstrated that AT1 receptor blockers (ARB) decreased renal fibrosis in various animal models of hypertension (8; 81). Further exploration determined that activated AT1 receptors induced TGF-β receptors, conversion of tubular epithelial cells into fibroblasts, and fibronectin deposition (108; 130; 132; 133). Deposition of fibrotic molecules can occur throughout the nephron and lead to renal injury. Glomerular injury decreases filtration exclusion and large proteins can enter the tubular lumen. Filtered proteins can be reabsorbed by the tubules and initiate a cascade that contributes to a fibrotic response in this segment of the nephron. The accumulation of fibrotic materials damages the proximal tubules leading to an alteration in transporter activity. This change in transporter activity may further

exacerbate salt and fluid retention. In addition to the profibrotic signaling pathway of the AT1 receptor, the receptor is also known to recruit inflammatory cells to promote the release of chemokines or cytokines in the kidney. AT1 receptor signaling activates the transcription factor nuclear factor kappa B (NF-kappaB) which promotes transcription of a number of chemokines and cytokines, such as RANTES (131). Moreover, Zhuo et al. (138) demonstrated that renal tubular AT1 receptors can activate NF-kappaB. Ang II generated either in the circulation or renal tissue can act at renal AT1 receptors to increase fibrotic proteins and recruit inflammatory molecules and cells. Thus, the RAS may contribute to normal renal function or augment pathways leading to renal damage including proteinuria. Injury to the nephrons may lead to irreparable damage which severely impairs normal renal function and stimulates mechanisms that may lead to or maintain hypertension.

4. Beneficial actions of Ang-(1-7)

More recently, studies in the kidney have focused on understanding the relationship of the increase in Ang-(1-7) with certain RAS blockade treatments that may contribute to their protective mechanisms (5; 6; 20). Levels of Ang-(1-7) have been determined in the urine from humans, which could be indicative of renal-derived concentrations of the hormone (38). Essential hypertensive patients excreted a lower amount of urinary Ang-(1-7) compared to normotensive patients. The lower concentration of urinary Ang-(1-7) suggests that a deficiency in the peptide is a marker or factor in at least one form of clinical hypertension. ACE inhibition is a potential treatment to correct the low amounts of Ang-(1-7) in essential hypertensive patients since

the inhibitor has dual actions of decreasing Ang II and increasing Ang-(1-7) levels. Ang-(1-7) is a substrate for ACE and ACE inhibition increases Ang-(1-7) levels as shown by Chappell et al. (21). Furthermore, ARB treatment enhances plasma Ang-(1-7); one potential mechanism is ACE2 converting the greater concentrations of circulating Ang II to Ang-(1-7) (36). The ARB can also increase renin and angiotensinogen which would augment Ang I leading to higher Ang-(1-7) levels through neprilysin activity (36; 37). The development and implementation of new treatments to increase the renal actions of Ang-(1-7) could provide additional antihypertensive medications. While systemic blockade of Ang-(1-7) revealed that the peptide contributes to the antihypertensive effects of ACE inhibition and ARB (53; 57; 58), there is an absence of evidence that demonstrates whether a reduction in Ang II or increase in Ang-(1-7) specifically in the kidney participates in these effects.

Some of the signaling pathways and renal actions of Ang-(1-7) have been determined through the use of cell lines and *ex vivo* kidneys (6; 27; 118). Ang-(1-7) through renal AT(1-7) receptors attenuates AT1 receptor phosphorylation of mitogen-activated protein kinase (41; 118). The AT(1-7) receptor activates tyrosine phosphatases to decrease phosphorylation (41; 118). The specific signaling pathways of Ang-(1-7) in renal cells have yet to be fully elucidated; however, recent evidence indicates the peptide activates Src-homology 2-containing protein-tyrosine phosphatase-1 (SHP-1) to decrease phosphorylation of mitogen-activated protein kinases (118). Su et al. (118) found that Ang-(1-7) also reduced the Ang II-mediated phosphorylation of other proteins such as p38, ERK 1/2, and JNK in the proximal tubule cells. The inhibitory action of Ang-(1-7) was blocked by the selective AT(1-7) receptor antagonist, D-[Ala^7]Ang-(1-7) and mRNA

expression of the AT(1-7) mas receptor was evident in these cells. As previously stated, Ang II can stimulate profibrotic molecules including transforming growth factor-β1 (TGF-β). Ang-(1-7) also lowers Ang II stimulated concentrations of TGF-β from proximal tubules (118). One possible mechanism for the decrease in fibrosis could be explained by the findings of Clark and colleagues that Ang-(1-7) lowered AT1 receptor density in the tubulointerstitial region of rat kidney through a cyclooxygenase (COX) dependent pathway (27). In addition to lowering AT1 receptor density, Handa and colleagues showed that an infusion of Ang-(1-7) inhibited the Na^+-K^+-ATPase pump in the proximal tubules (46). These findings support the theory that Ang-(1-7) exerts antagonistic physiological effects to that of AT1 receptor-mediated actions which are not solely through direct blockade of the receptor.

Ang-(1-7) also exerts renoprotective actions in the intact animal (6; 27; 118). Benter et al. (6) demonstrated antihypertensive and renoprotective effects of Ang-(1-7) in the spontaneously hypertensive rat (SHR) treated with the nitric oxide synthase (NOS) inhibitor L-NAME. Infusion of Ang-(1-7) significantly lowered mean arterial pressure (MAP), urinary protein, and the severity of glomerular sclerosis in the L-NAME treated SHR. This study documented effects of Ang-(1-7) in the whole animal; however, further studies into the direct actions of the peptide in the kidney were also determined. Bell-Quilley and colleagues showed that Ang-(1-7) induced a rapid diuretic and natriuretic response in the isolated perfused kidney which was mediated through the release of PGI_2 (31; 50). These studies demonstrate that Ang-(1-7) may provide renal protection though a reduction in blood pressure which may be through an increase in prostaglandins.

5. Dysregulation of RAS in hypertension and genetic models of altered RAS

Ang II possesses stimulatory effects on intrarenal proteins and enzymes that can lead to a greater activation of the RAS. Ang II upregulates multiple renal RAS components, such as angiotensinogen, ACE, and AT1 receptors in certain cell types (68). Renal angiotensinogen is synthesized and released from the proximal tubules through an Ang II-AT1 receptor-mediated mechanism. Multiple studies have shown that infusion of exogenous Ang II into normotensive Sprague Dawley rats increased urinary excretion of angiotensinogen (67; 69). The greater levels of angiotensinogen could also raise the luminal Ang II concentration which may increase sodium and water transporter activity. An increase in AT1 receptor activity could impact renal electrolyte and fluid retention in this model through activation of transporters along the nephron which could contribute to an elevation in blood pressure. The AT1 receptor has an additional function aside from the stimulation of protein synthesis; the receptor can also bind, internalize, and protect Ang II in the kidneys. Furthermore, the internalized Ang II contributes to the intrarenal accumulation of the peptide (139). An ARB prevented internalization and accumulation of the exogenously infused Ang II in the kidney (54; 71; 139; 140). The ARB also averted the increase in urinary angiotensinogen and blood pressure (54; 71; 139; 140). Shao et al. (112) infused Valine^5-Ang II into rats and observed that endogenous Isoleucine^5-Ang II concentrations were maintained in the renal tissue. These data suggest that the endogenous Ang II production was preserved. Intrarenal Ang II generation was further supported by findings from Von Thun et al. (125) that intrarenal ACE was significantly increased in Ang II infused rats. Further exploration of renal RAS enzymes by Koka et al. (72) found that Ang II exerts differential regulatory actions on ACE and

ACE2 expression by an AT1 receptor-dependent mechanism in human tubular cells. They determined that Ang II increases ACE expression, while the peptide decreases ACE2 protein. Higher renal ACE activity is an additional mechanism to increase intrarenal Ang II content, whereas a decrease in ACE2 could diminish degradation of systemic or renal-derived Ang II. The intrarenal content of Ang II is an important parameter to assess, but the AT1 receptor density is an additional factor to evaluate for the potential renal actions. Studies that utilized radioligand binding uncovered an increase in AT1 receptor density in glomeruli and proximal tubules in the presence of high Ang II concentrations (24; 85). These studies suggest that the AT1 receptor density is an important mediator to increase blood pressure in pathophysiological states (24; 85). However, Harrison-Bernard et al. (47) demonstrated by immunoblot that a two week infusion of Ang II in rats did not alter AT1 receptor density in the whole renal cortex. The results from the previous studies suggest that radioligand binding has a higher sensitivity to detect changes in receptor density compared to the immunoblot technique (24; 47; 85). Therefore, we utilized radioligand binding instead of the immunoblot assay to determine AT1 receptor density in the mRen(2).Lewis and Lewis rats. In summary, high levels of Ang II lead to an activation of the intrarenal RAS through positive feedback loops on specific components and a vicious loop of hypertension and renal injury is maintained.

Since activation or dysregulation of the RAS is associated with hypertension and renal ischemia, transgenic hypertensive rodents were developed to evaluate the status of the RAS and the efficacy of treatments that inhibit the actions of Ang II (35; 98). An increase in the generation of Ang II or activation of the AT1 receptor in the kidney is

linked to hypertension and renal injury (91). The importance of renal-derived Ang II was demonstrated with the use of transgenic mice that express both human renin and angiotensinogen only in proximal tubules (30; 76). These transgenic mice were moderately hypertensive and administration of losartan systemically prevented the increase in blood pressure. The findings demonstrate that localized generation of Ang II in renal proximal tubules can significantly alter renal function and blood pressure in this model. Another valuable model in the study of the local RAS is the transgenic (mRen-2)27 Sprague Dawley rat (89). The (mRen-2)27 Sprague Dawley rat was created by insertion of the full length mouse submandibular gland renin 2 gene into the Sprague Dawley rat genome to generate a monogenetic model of Ang II-dependent hypertension. The homozygous strain exhibits fulminant hypertension while a lower degree of chronic hypertension develops in the heterozygous strain. Multiple labs have utilized this model of Ang II-dependent hypertension (12; 42; 56; 83; 89; 99; 115). The (mRen-2)27 Sprague Dawley animals express low levels of renal renin, but there is controversy as to whether plasma and renal levels of Ang peptides and plasma renin are higher or lower compared to Sprague Dawley rats (34). Campbell et al. (13) utilized young homozygous hypertensive (mRen-2)27 Sprague Dawley and Sprague Dawley rats to determine whether tissue levels of Ang peptides are altered in a hypertensive rat. They showed that renal Ang II levels in (mRen-2)27 Sprague Dawley rats were three-fold higher than those in the Sprague Dawley rats. The higher renal Ang II content could be derived from receptor binding and internalization of circulating Ang II as the (mRen-2)27 Sprague Dawley rats had higher plasma levels of Ang II. An increase in renal Ang II may also contribute to hypertension in (mRen-2)27 Sprague Dawley rats. Candesartan, a selective

ARB, increased plasma Ang II, but lowered both blood pressure and renal Ang II content in the (mRen-2)27 Sprague Dawley rat (73). Moreover, Zhuo and colleagues showed blockade of the AT1 receptor, but not the AT2 receptor, normalized the blood pressure of (mRen-2)27 Sprague Dawley rats to that of the Sprague Dawley rats (137). These studies indicate that in a model of Ang II-dependent hypertension, the high blood pressure could be associated with high intrarenal expression of Ang II and activation of the AT1 receptor.

It is known that prolonged shifts toward higher electrolyte reabsorption in renal function may lead to the initiation and/or maintenance of hypertension; however, it is not well understood how high renal Ang II concentrations play a significant role in blood pressure alterations. One renal mechanism that is sensitive to chronic elevations of Ang II is pressure-natriuresis, whereby an increase in blood pressure leads to an elevation in natriuresis in order to lower blood pressure to a normal range. Cowley and colleagues determined the pressure-natriuresis relationship in the isolated kidneys from hypertensive and normotensive rat strains (42). They demonstrated a rightward shift in the pressure-natriuresis curve in the (mRen-2)27 Sprague Dawley compared to Sprague Dawley rats and their findings indicate that (mRen-2)27 Sprague Dawley rats require a higher blood pressure in order to excrete a similar amount of salt and fluid as the normotensive rat. The study was designed to remove extrarenal factors from influencing the renal function and the parameters were assessed under normalized conditions where the kidneys were denervated and supplemented with hormonal factors (42). These results support the hypothesis that high amounts of intrarenal Ang II could activate AT1 receptors localized in the renal vasculature, glomeruli, and proximal tubules to induce a rightward shift of the

pressure-natriuresis relationship (137). Since ARB treatment effectively lowers blood pressure in the (mRen-2)27 Sprague Dawley rats, it would suggest that elevated levels of Ang II play a role in the maintained shift of the relationship. The Ang II-dependent dysregulation of renal function could occur through multiple pathways including an increase in sympathetic nerve activity. Higher renal sympathetic nerve activity increases renin release for Ang II production**,** and then Ang II can increase sodium retention through stimulation of the Na^+/H^+ exchanger and the Na^+-K^+-ATPase in the proximal tubule. Prolonged exposure to high levels of Ang II or hypertension can lead to damage in various areas of the nephron. A decrease in renal function from hypertension can lead to a vicious cycle of maintaining or increasing the rightward shift in the pressure-natriuresis curve.

Although the (mRen-2)27 Sprague Dawley rat is an appropriate model to study the renal RAS and dysregulation of blood pressure, the Sprague Dawley is an outbred strain of rat. The variability of different genetic traits in outbred Sprague Dawley rats could influence experimental results for comparison between the (mRen-2)27 Sprague Dawley and Sprague Dawley rats. Reports from multiple labs show differences in the phenotype of circulating Ang peptides between the (mRen-2)27 Sprague Dawley and Sprague Dawley rats, and the differences in circulating Ang II may reflect the outbred Sprague Dawley strain (1; 11; 13; 40; 77; 126; 136). A final conclusion has not been determined on whether the (mRen-2)27 Sprague Dawley has higher or lower plasma Ang II levels compared to the Sprague Dawley. These issues could stem from either the utilization of the outbred strain of rat, or the different techniques to evaluate peptides between labs. Therefore, it is imperative to generate other Ang II-dependent animal

models of hypertension with an inbred background, thereby preventing genetic variability from influencing data.

To solve the problem of the outbred background in the (mRen-2)27 Sprague Dawley rat, a new model of hypertension was developed in the Hypertension Center. This model is derived from the backcross of the transgenic (mRen-2)27 Sprague Dawley rat into the inbred normotensive Lewis rat. Similar to the (mRen-2)27 Sprague Dawley rat, the mRen(2).Lewis exhibits hypertension at an early age and RAS blockade significantly lowered blood pressure to that of the normotensive Lewis rat (60). The first published report that characterized the mRen(2).Lewis was in female rats (19). Chappell et al. (19) normalized blood pressure in young mRen(2).Lewis rats to that of the Lewis rats with a four week treatment of the selective ARB olmesartan. Moreover, in the female mRen(2).Lewis rat, blood pressure, cardiac hypertrophy, components of the circulating RAS and renal function were all increased in rats fed a high salt diet (22). Along with the exacerbation of hypertension in female mRen(2).Lewis rats fed a high salt diet, serum ACE activity was significantly elevated; however, plasma renin concentrations and Ang II levels were similar between the two groups. Renal function measured by creatinine clearance was decreased by the high salt diet in mRen(2).Lewis female rats, and protein excretion was increased suggesting greater renal damage accompanied the higher blood pressure. In association with the decreased renal function, the female rats also exhibited an increase in urine output and sodium excretion suggesting that the pressure-natriuresis relationship was still operant, although set to higher pressures. Jessup et al. (60) evaluated multiple cardiovascular and RAS parameters in male mRen(2).Lewis rats after a two week treatment with an ARB or ACE inhibitor.

Blood pressure and urinary protein excretion were significantly decreased by the two treatments compared to untreated mRen(2).Lewis. In contrast, renal ACE2 activity was significantly higher with both treatments. The higher ACE2 activity could act to lower blood pressure through an increase in the production of Ang-(1-7). When the authors assessed Ang-(1-7) levels, they found ARB treatment and ACE inhibition caused a differential effect on both the circulating and urinary Ang-(1-7) peptides. ARB treatment did not significantly increase plasma Ang-(1-7) levels above control animals and urinary Ang-(1-7) excretion declined over the two week treatment. ACE inhibition did increase plasma Ang-(1-7), but a transient decrease was observed in urinary Ang-(1-7) excretion that returned to baseline by the end of treatment. Although hypertension in both female and male mRen(2).Lewis is reduced by RAS blockade, we have not fully elucidated the specific actions that maintain an increase in blood pressure in this model.

A new component of the RAS, ACE2, converts Ang II to Ang-(1-7). The importance of the newly discovered enzyme ACE2 has been sought through the development of ACE2 knockout mice. This novel animal could indicate regulatory actions of ACE2 activity on blood pressure and Ang II concentrations. However, the use of different background mouse strains has produced conflicting reports in both blood pressure and Ang II concentration measurements in ACE2 knockout mice (28; 43). The ACE2 knockout mouse on the 129/SvEv background did not exhibit an increase in blood pressure at baseline; however, infusion of Ang II did significantly increase both blood pressure and renal Ang II over wildtype littermates (43). Although an increase in blood pressure was not evident in the $ACE2^{-/-}$ 129/SvEv strain, ACE2 knockout mice on the C57Black/6 background did exhibit an elevation in blood pressure and renal Ang II levels

compared to wildtype mice without infusion of Ang II (28; 43). C57Black/6 ACE2 knockout mice exhibited higher fibronectin deposition and glomerulosclerosis (96). ACE2 knockout mice also exhibit increased levels of the oxidative stress markers lipid peroxidation and hexanal. Moreover, irbesartan, a selective ARB, prevented the Ang II-dependent renal injury in ACE2 knockout mice. The ACE2 knockout mouse study expresses higher levels of Ang II acting through the AT1 receptor to initiate renal damage. Furthermore, the ACE2 knockout mouse model exhibits a decrease in renal Ang-(1-7) concentrations as demonstrated by Tikellis et al. (122). Therefore, the previous studies suggest that ACE2 provides renoprotective actions through the production of Ang-(1-7) and degradation of Ang II.

Another enzyme found in the kidney, neprilysin, can also metabolize multiple Ang peptides. Neprilysin converts Ang I to Ang-(1-7), but metabolizes Ang II to Ang-(1-4) (106; 111). The ability of neprilysin to degrade Ang II and synthesize Ang-(1-7) shifts the Ang peptide balance from vasoconstriction towards a vasodilatory state and may combat high blood pressure. Presently, there is not a consensus on whether inhibition of the enzyme neprilysin provides a beneficial or deleterious effect on blood pressure in all types of hypertension. Neprilysin can metabolize hormones that affect blood pressure and renal function other than Ang peptides such as natriuretic peptides (103; 117). The effects of neprilysin inhibition on blood pressure have been evaluated through alterations in ANP. Higher levels of ANP due to neprilysin inhibition could potentially increase natriuresis leading to a decrease in blood pressure through fluid volume-dependent mechanisms. Therefore, one hypothesis is that inhibition of neprilysin would lower blood pressure by increasing the concentrations of ANP in the plasma and kidneys.

Indeed, homologous recombinant deletion of neprilysin in male mice significantly lowered blood pressure compared to wild type mice; however, hypotension in the neprilysin knockout mice was not associated with chronic hypovolemia, an increase in nitric oxide release, or ANP activity (84). The neprilysin inhibitor ecadotril significantly lowered both blood pressure and plasma renin activity in male (mRen-2)27 Sprague Dawley rats (116). Although plasma ANP concentrations were not different between control and treated (mRen-2)27 Sprague Dawley rats, plasma and urinary cGMP levels were increased after ecadotril treatment compared to controls. An increase in cGMP could indicate higher levels of ANP and increased activation of the natriuretic peptide receptor. These studies suggest neprilysin is a target candidate for inhibition to produce antihypertensive and renoprotective effects in male animals. To date, the actions on blood pressure from chronic neprilysin inhibition are not fully understood and how they impact influential cardiovascular hormone systems remains to be determined.

6. Regulatory actions of sex steroids on RAS components and their actions

Sex differences in blood pressure and tissue injury are also evident in hypertensive models (78; 95; 104). These sex differences have been associated with the effects of sex steroids on multiple hormone systems that regulate blood pressure homeostasis. Moreover, evaluation of the development of tissue injury in multiple animal models of hypertension revealed that injury in females progressed at a slower rate than in males (33; 51; 78); however, the etiology of the sex differences has not been fully explained *in vivo*. The focus of the present studies is uncovering potential mechanisms for sex differences in the systemic and renal RAS. Androgens and estrogens are known

to regulate the production or activity of the RAS at different levels of the cascade (Figure 3). Two main concepts of sex steroids' regulatory actions on RAS activity have been proposed pertaining to the production of Ang II that testosterone stimulates the RAS, while estrogen can either stimulate or inhibit RAS components. Androgens may produce deleterious actions in males and estrogen elicits protective effects in females through alterations in the production and degradation of vasoactive Ang peptides. Baltatu and colleagues administered flutamide, a testosterone receptor antagonist, to male and female (mRen-2)27 Sprague Dawley rats to evaluate the actions that testosterone blockade exerts on blood pressure and RAS components (3; 4). Flutamide treatment prevented the increase in blood pressure in both female and male (mRen-2)27 Sprague Dawley rats and attenuated renal injury. The beneficial antihypertensive and renoprotective effects were associated with a decrease in both mouse and rat renin with similar levels of plasma angiotensinogen in the female (mRen-2)27 Sprague Dawley rats (3). These findings demonstrate a pathway for testosterone to exacerbate hypertension and organ damage in this model of hypertension through activation of the RAS.

Estrogen exerts stimulatory effects on certain components of the RAS. The biochemical effects of estrogens on the RAS precursor protein, angiotensinogen, have been demonstrated in *in vitro* and *in vivo* models. Multiple groups have shown that estrogen stimulates both transcription and protein synthesis of angiotensinogen in hepatic cell lines that express estrogen receptors, but testosterone did not have similar actions (18; 66; 74). Multiple studies provide evidence that ACE2 expression is also stimulated by estrogen in rodents, since renal ACE2 protein expression was increased during normal pregnancy, but decreased with preeclampsia and ovariectomy (61; 64). The normal

pregnant rat expresses higher estradiol levels compared to the model of preeclampsia further suggesting that estrogen has stimulatory actions on ACE2. Similar to angiotensinogen and ACE2, neprilysin protein expression and activity are increased in the presence of estrogen. Several labs have shown that chronic estrogen depletion significantly suppresses neprilysin protein and activity in both humans and rodents (17; 52; 135). A recent study demonstrated that estrogen replacement following ovariectomy returned neprilysin protein levels to that observed in the sham animals (52). These studies suggest enzymes that degrade Ang II and generate Ang-(1-7) are subject to a positive influence from estrogen. An additional RAS component under positive regulation by estrogen is the AT2 receptor. Armando and colleagues demonstrated a greater density of the AT2 receptor in the renal capsule and cortico-medullary region of the kidney in female Wistar-Hannover and Wistar-Kyoto rats compared to their male counterparts (2). They found that the AT2 receptor activated prostaglandin and cGMP production which may contribute to vasorelaxation or natriuresis. Armando et al. (2) also determined that ovariectomy abolished the sex difference in the renal AT2 receptors, but estrogen replacement restored the renal AT2 receptor density. These findings suggest estrogen activates the RAS cascade through an increase in angiotensinogen, ACE2, neprilysin, and the AT2 receptor; however several studies demonstrated that estrogen decreases other RAS components.

Estrogen's inhibitory actions on the RAS appear to be limited to the components that stimulate Ang II production, vasoconstriction and salt retention, such as ACE and the AT1 receptor. Studies by Gallagher and colleagues demonstrated that replacement of estrogen at supraphysiological doses in ovariectomized Sprague Dawley rats decreased

ACE activity in the serum, renal cortex and renal medulla, as well as tissue ACE mRNA (39). Furthermore, Brosnihan et al. (10) reported that estrogen replacement at a physiological dose in young ovariectomized female (mRen-2)27 Sprague Dawley and Sprague Dawley rats decreased mean arterial pressure and ACE activity in both the serum and kidneys. Plasma Ang II levels were also significantly decreased and Ang-(1-7) increased in the estrogen-replete (mRen-2)27 Sprague Dawley relative to the ovariectomized (mRen-2)27 Sprague Dawley rats. These results in estrogen-replete (mRen-2)27 Sprague Dawley were consistent with the protective actions of estrogen that produced a decrease in blood pressure and a shift in the vasoactive peptides Ang II and Ang-(1-7) compared to the ovariectomized (mRen-2)27 Sprague Dawley rats (10). Estrogen not only exerts an inhibitory action on ACE but also at the translation level for the AT1 receptor. Krishnamurthi et al. (75) demonstrated that estrogen increased the expression of cytosolic RNA proteins which bind to the 5' sequence of the AT1 receptor mRNA to prevent translation and subsequently decrease AT1 receptor protein. The authors found that tissue AT1 receptor density was decreased, while cytosolic RNA protein expression was increased, in estrogen-replete female Sprague Dawley versus ovariectomized Sprague Dawley rats. Studies by Nickenig et al. (94) demonstrated ovarian hormone-related effects on AT1 receptor regulation in rat vascular smooth muscle cells. They found estrogen reduced AT1 receptor mRNA levels and stability, whereas progesterone increased AT1 receptor mRNA expression half-life in rat vascular smooth muscle cells (93). Aortae from ovariectomized female rats exhibited higher AT1 receptor protein expression and Ang II-mediated vasoconstriction, and both receptor density and function were abolished with estrogen replacement in the rats (94). The

previous studies suggest that estrogen may act to lower the hypertensive aspects of the RAS, but the regulatory actions of other ovarian hormones such as progesterone have yet to be fully elucidated.

Studies in young female mRen(2).Lewis rats add support to the current belief that estrogen has protective effects in the presence of hypertension. The actions of estrogen on blood pressure, plasma and renal RAS activity, and renal injury have been determined in the mRen(2).Lewis rat. Studies by Chappell et al. (19; 22) in female mRen(2).Lewis rats demonstrated that ovariectomy before sexual maturation significantly elevated the blood pressure compared to age-matched sham and estrogen-replaced hypertensive rats (Figure 4). Therefore, the authors evaluated the circulating and renal RAS components in young control and ovariectomized mRen(2).Lewis rats, and found that both the plasma and renal RAS were activated. Estrogen depletion in female mRen(2).Lewis rats led to an increase in the Ang II synthesizing enzymes renin and ACE, as well as plasma and renal Ang II. These findings suggest that the balance between Ang II and Ang-(1-7) was shifted towards the vasoconstrictor arm of the RAS. Urinary isoprostanes, a marker of oxidative stress, were also elevated in association with plasma and renal Ang II. Administration of the AT1 receptor antagonist olmesartan normalized blood pressure in the ovariectomized female mRen(2).Lewis rat. All of these factors suggest the hypertension and organ injury were due to higher Ang II concentrations acting at the AT1 receptor. Next, Chappell and colleagues assessed the effect of estrogen replacement on blood pressure and RAS components (19). Physiological levels of estrogen were replaced at 5 weeks of age in ovariectomized female mRen(2).Lewis rats and blood pressure returned to levels observed in the Lewis rats at 11-12 weeks of age. The

antihypertensive effect of estrogen replacement was mediated through a decrease in Ang II production. ARB or estrogen replacement treatments were both equally effective as antihypertensive regimens in this model of hypertension. However, we do not know the renal mechanisms that maintain the hypertension in the male mRen(2).Lewis, or the antihypertensive and protective actions in the kidney afforded to female mRen(2).Lewis rats compared to their male counterparts.

7. Rationale

Despite the fact that sex differences in the development and maintenance of hypertension and tissue injury have been well documented in both humans and animal models, the etiology for these differences has not been fully explained in regards to the RAS (33; 51; 78). A better understanding of sex differences in hypertension may lead to novel antihypertensive treatment regimens in both females and males. In order to evaluate the protective sex differences afforded to females, we utilized a novel model of Ang II-dependent hypertension, the mRen(2).Lewis strain. The mRen(2).Lewis rat is similar to the SHR because RAS blockade significantly lowers hypertension in both strains; however, the mRen(2).Lewis is different due to the fact that the genesis of hypertension is known and the high blood pressure is estrogen-sensitive. The goal of the present studies is to use the mRen(2).Lewis strain to assess potential pathways that influence the antihypertensive and renoprotective actions afforded to female rats in regards to the RAS.

As previously stated, the RAS is an influential hormonal system in blood pressure regulation; however, the status of plasma and renal RAS components have not been fully

characterized in the mRen(2).Lewis strain. The substrate, enzymes, peptides, and receptors in the RAS pathway all have their own role in maintaining a homeostatic balance in electrolyte, water, and blood pressure levels in these animals. A prolonged dysregulation of the RAS that produces high concentrations of Ang II acting through the AT1 receptor may initiate and maintain hypertension. An increase in synthesis or preservation of Ang II could occur in the circulation or in local organ systems. Systemic and renal Ang II concentrations can be altered through various mechanisms that involve the generation and release of angiotensinogen, a change in the activities of enzymes such as renin, ACE, ACE2, and neprilysin, or a modification in the AT1 receptor. Moreover, renal AT1 receptors may have a higher density or binding affinity in animals with the greatest degree of hypertension. The AT1 receptor can also influence renal Ang II peptide content through receptor-mediated internalization and trafficking of the peptide into endosomes to prevent degradation. Therefore, we will evaluate plasma and renal RAS components in both sexes of the mRen(2).Lewis and Lewis rats, in order to determine the specific areas of dysregulation in the RAS.

Sex steroids regulate the RAS cascade at various sites which may provide protection through unknown mechanisms for females compared to males (Figure 3). Although not fully evaluated previously, we believe that the mRen(2).Lewis strain will exhibit a sex and strain difference in hypertension and renal injury. The female mRen(2).Lewis is proposed to have higher levels of the vasodilator Ang-(1-7) compared to male counterparts, and the hypertensive rats may express higher levels of the vasoconstrictor Ang II compared to Lewis rats, independent of sex. The concentration of vasoactive Ang peptides binding to their respective receptors could be one potential

pathway that plays a role in the level of hypertension. Thus, an evaluation of the RAS components could indicate target candidates that could shift the vasoactive peptide actions to dilation and normotension or constriction and hypertension in this model.

The kidney is an important site of long term electrolyte, water, and blood pressure homeostasis in the body and it expresses all of the RAS components. RAS peptides exert renal actions, such as water and electrolyte retention or excretion, and vasoactivity. Ang II and Ang-(1-7) display complex interactions after activating their respective renal receptors and the outcome of the interactions are dependent upon the baseline physiological state. All of the receptor-mediated mechanisms have not been characterized in the kidney, but the main renal actions associated with Ang II are antidiuresis and vasoconstriction, while those of Ang-(1-7) are diuresis and vasodilation. Therefore, the renal enzymatic activity can significantly influence the overall state of renal function by synthesis or degradation of specific Ang peptides. If an alteration in renal Ang peptides is present, renal enzymes like ACE, ACE2, and neprilysin could be involved in determining the status of renal hemodynamics to ultimately produce normal blood pressure or hypertension.

Overall Hypothesis

Differential regulation of components in the plasma and renal RAS may provide a protective sex-dependent mechanism in female mRen(2).Lewis rats through a reduction of Ang II and augmentation of Ang-(1-7) activity to lower the extent of hypertension and renal injury.

Specific Aims

Specific Aim #1: Determine whether there are differences present in plasma and renal Ang peptides, nuclear renal AT receptors, blood pressure between young male mRen(2).Lewis and Lewis rats.

Specific Aim #2: Determine if the difference in blood pressure among young adult female and male mRen(2).Lewis and Lewis rats is associated with alterations in the components of the plasma and intrarenal RAS, along with inflammatory markers and renal injury.

Specific Aim #3: Evaluate whether chronic inhibition of neprilysin will increase blood pressure and renal damage in female mRen(2).Lewis rats by shifting the Ang II to Ang-(1-7) balance towards greater Ang II activity.

Figure 1. Age-standardized incidence rate of hypertension by sex and race. A marked sex difference is present with women exhibiting a lower blood pressure into the third decade. Black patients had a higher incidence rate of hypertension independent of sex after the third decade. Women have an increase in the incidence rate of hypertension and surpass men after the fifth decade. At this age women could have progressed into menopause and lost the protective effects of estrogen. (29)

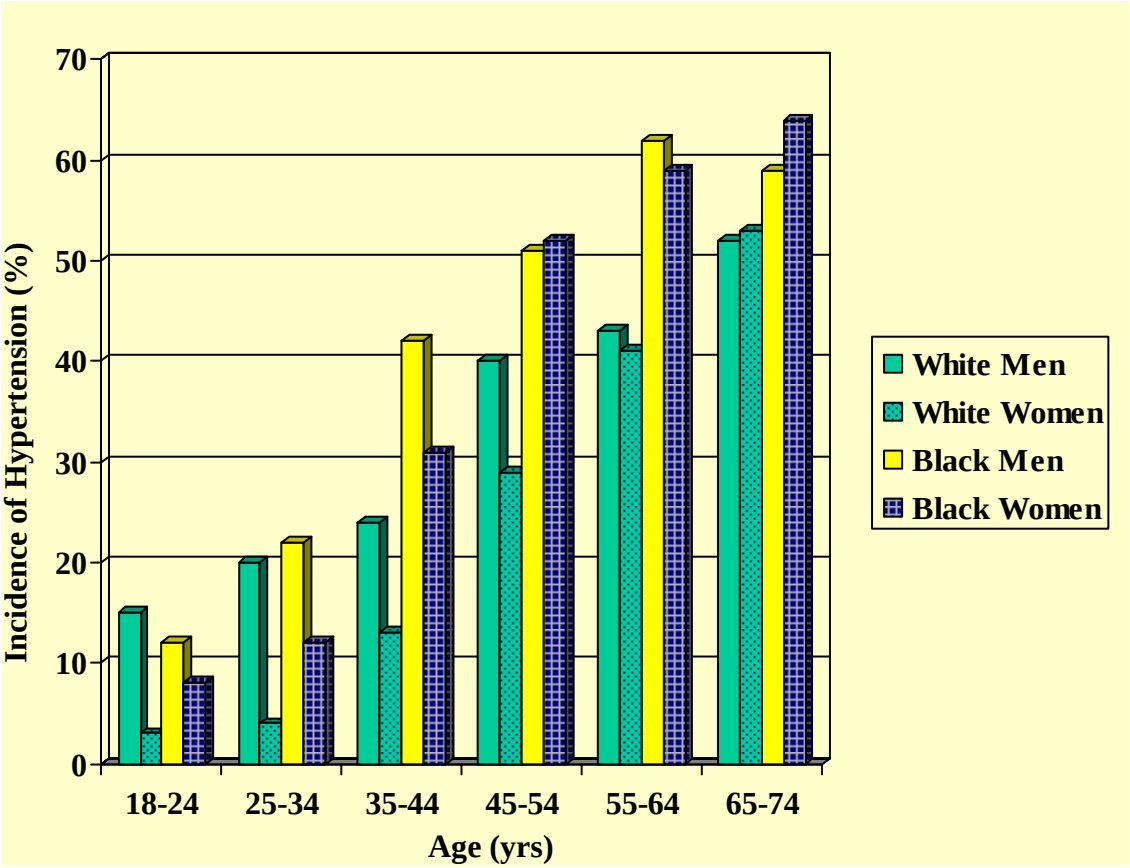
Incidence of Hypertension (%)
70
60
50
40
30
20
10
0
18-24
25-34
35-44
45-54
55-64
65-74
Age (yrs)
White Men
White Women
Black Men
Black Women

Figure 1 (29)

Figure 2. Enzymatic pathways of the Renin-Angiotensin System (RAS). Schematic representation of the RAS pathway including angiotensinogen, subsequent angiotensin (Ang) peptide products, the enzymes and their receptors. Angiotensin converting enzyme (ACE), angiotensin converting enzyme 2 (ACE2), neprilysin (NEP), angiotensin type 1 receptor (AT_1R), angiotensin type 2 receptor (AT_2R), angiotensin 1-7 receptor ($AT_{(1-7)}R$)/*Mas*.

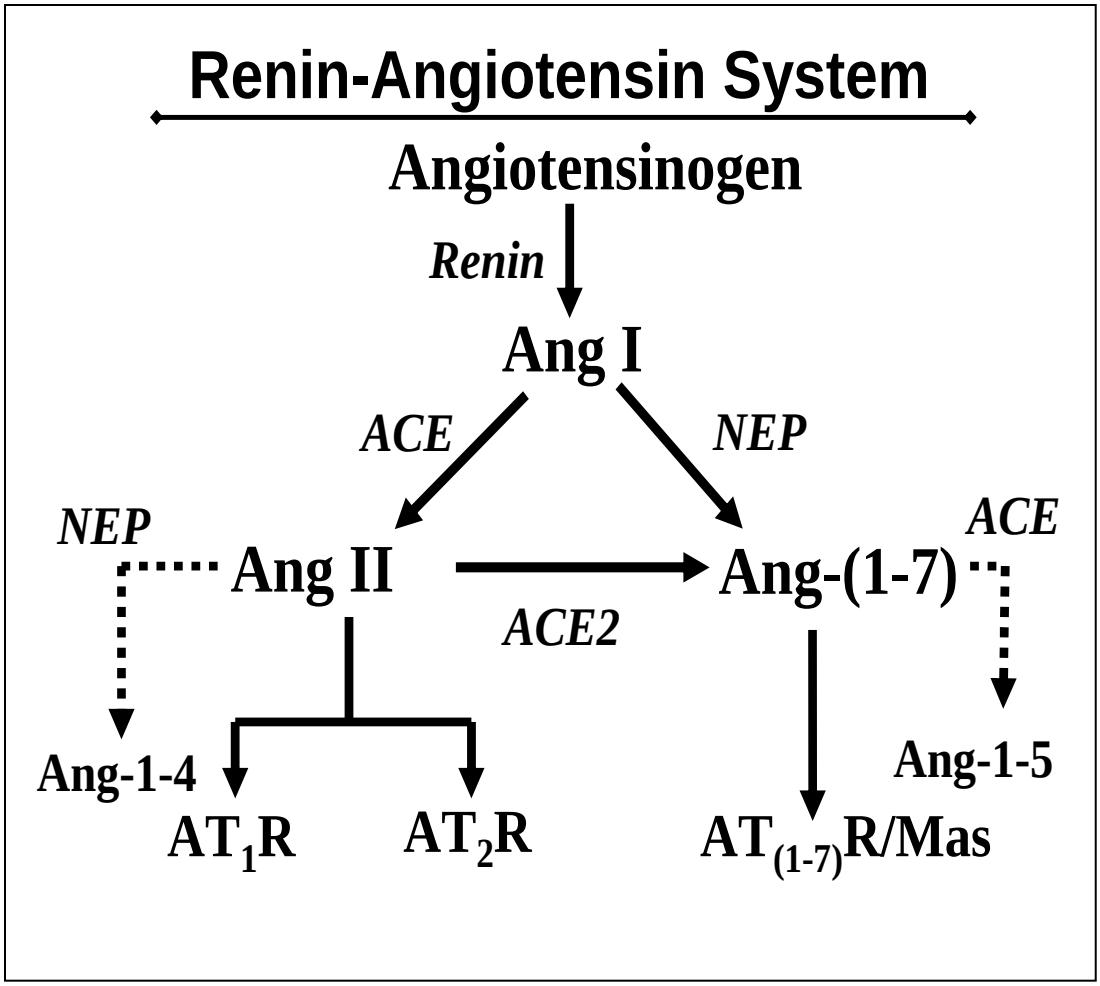
Renin-Angiotensin System
Angiotensinogen
Renin
Ang I
ACE
NEP
NEP
Ang II
Ang-(1-7)
ACE
ACE2
Ang-1-4
Ang-1-5
AT1R
AT2R
AT(1-7)R/Mas

Figure 2

Figure 3. Regulatory actions of sex steroids on the Renin Angiotensin System (RAS) cascade. Schematic representation of the RAS pathway and the levels where sex steroids can alter RAS components including angiotensinogen, the processing enzymes, subsequent angiotensin (Ang) peptide products and their receptors. Angiotensin converting enzyme (ACE), angiotensin converting enzyme 2 (ACE2), neprilysin (NEP), angiotensin type 1 receptor (AT_1R), angiotensin type 2 receptor (AT_2R), angiotensin type 1-7 receptor ($AT_{(1-7)}R$).

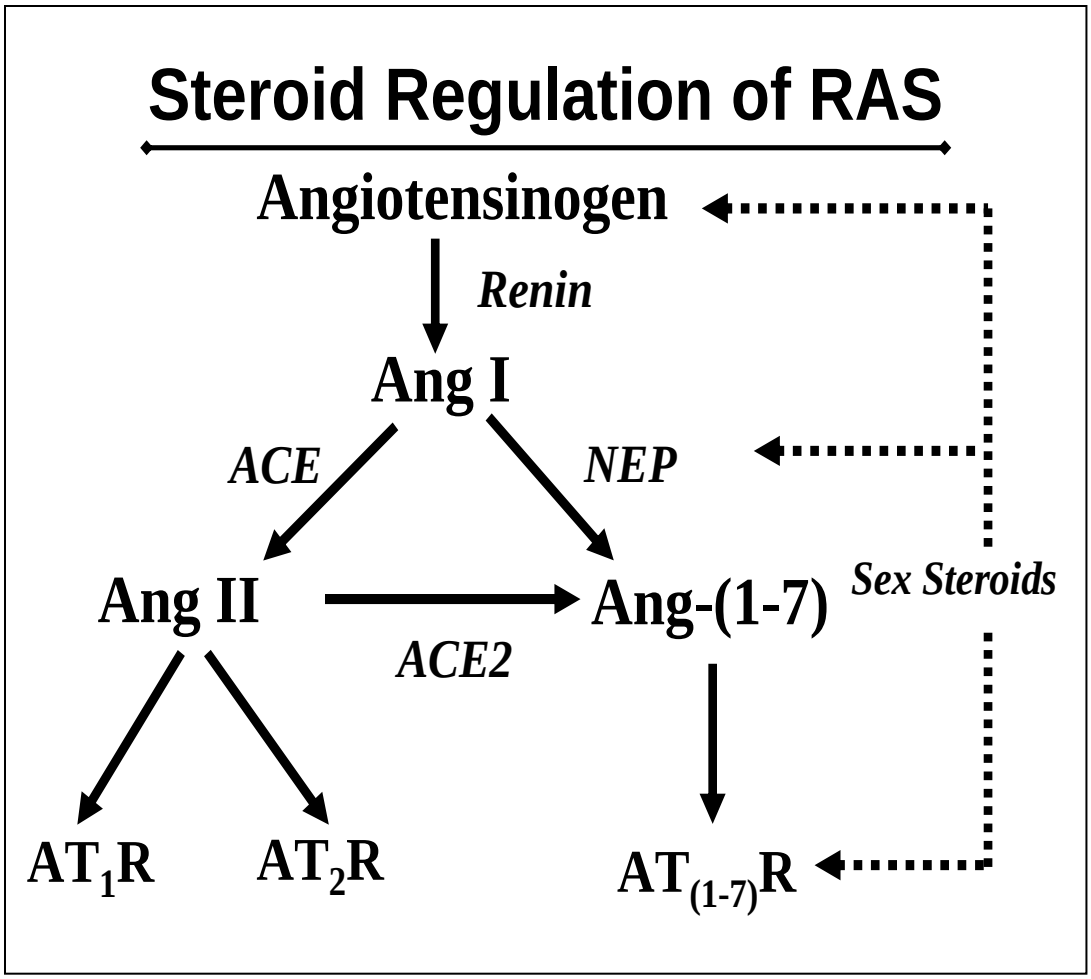
Steroid Regulation of RAS
Angiotensinogen
Renin
Ang I
ACE
NEP
Ang II
Ang-(1-7)
Sex Steroids
ACE2
AT1R
AT2R
AT(1-7)R

Figure 3

Figure 4. Effects of ovariectomy (OVX) and estrogen (E2) replacement on development of blood pressure in mRen(2).Lewis rat. Systolic blood pressure (SBP) was determined from weeks 5 through 11. Data are the mean $\pm$ SEM, *P<0.05 vs sham; #P<0.05 vs OVX+E2 (n = 6 - 7 rats per group). (19)

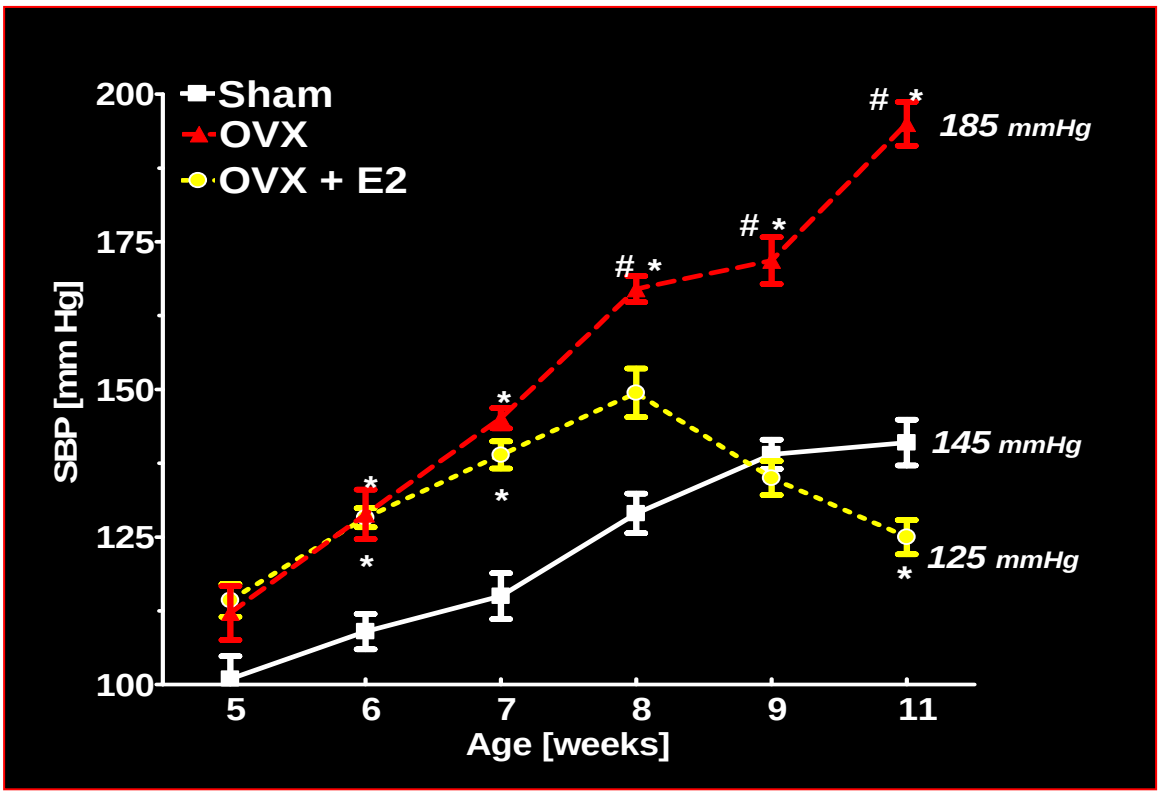
Sham
OVX
OVX + E2
200
175
150
125
100
SBP [mm Hg]
5
6
7
8
9
11
Age [weeks]
185 mmHg
145 mmHg
125 mmHg
#
*

Figure 4 (19)

Reference List

1. **Bachmann S, Peters J, Engler E, Ganten D and Mullins J**. Transgenic rats carrying the mouse renin gene -- morphological characterization of a low-renin hypertension model. *Kidney Int* 41: 24-36, 1992.

2. **Baiardi G, Macova M, Armando I, Ando H, Tyurmin D and Saavedra JM**. Estrogen upregulates renal angiotensin II AT_1 and AT_2 receptors in the rat. *Regulatory Peptides* 124: 7-17, 2005.

3. **Baltatu O, Cayla C, Iliescu R, Andreev D and Bader M**. Abolition of end-organ damage by antiandrogen treatment in female hypertensive transgenic rats. *Hypertension* 41: 830-833, 2003.

4. **Baltatu O, Cayla C, Iliescu R, Andreev D, Jordan C and Bader M**. Abolition of hypertension-induced end-organ damage by androgen receptor blockade in transgenic rats harboring the mouse Ren-2 gene. *J Am Soc Nephrol* 13: 2681-2687, 2002.

5. **Benter IF, Yousif MH, Dhaunsi GS, Kaur J, Chappell MC and Diz DI**. Angiotensin-(1-7) prevents activation of NADPH oxidase and renal vascular dysfunction in diabetic hypertensive rats. *Am J Nephrol* 28: 25-33, 2008.

6. **Benter IF, Yousif MHM, Anim JT, Cojocel C and Diz DI**. Angiotensin-(1-7) prevents development of severe hypertension and end-organ damage in spontaneously hypertensive rats treated with L-NAME. *Am J Physiol Heart Circ Physiol* 290: H684-H691, 2006.

7. **Bharatula M, Hussain T and Lokhandwala MF**. Angiotensin II AT1 receptor/signaling mechanisms in the biphasic effect of the peptide on proximal tubular Na+,K+-ATPase. *Clin Exp Hypertens* 20: 465-480, 1998.

8. **Boffa J-J, Lu Y, Placier S, Stefanski A, Dussaule JC and Chatziantoniou C**. Regression of renal vascular and glomerular fibrosis: role of angiotensin II receptor antagonism and matrix metalloproteinases. *J Am Soc Nephrol* 14: 1132-1144, 2003.

9. **Braam B, Mitchell KD, Fox J and Navar LG**. Proximal tubular secretion of angiotensin II in rats. *Am J Physiol* 264: F891-F898, 1993.

10. **Brosnihan KB, Li P, Ganten D and Ferrario CM**. Estrogen protects transgenic hypertensive rats by shifting the vasoconstrictor-vasodilator balance of RAS. *Am J Physiol* 273: R1908-R1915, 1997.

11. **Brosnihan KB, Moriguchi A, Nakamoto H, Dean RH, Ganten D and Ferrario CM**. Estrogen augments the contribution of nitric oxide to blood pressure

regulation in transgenic hypertensive rats expressing the mouse Ren-2 gene. *Am J Hypertens* 7: 576-582, 1994.

12. **Callahan MF, Li P, Ferrario CM, Ganten D and Morris M**. Salt-sensitive hypertension in (mREN-2)27 transgenic rats. *Hypertension* 27: 573-577, 1996.

13. **Campbell DJ, Rong P, Kladis A, Rees B, Ganten D and Skinner SL**. Angiotensin and bradykinin peptides in the TGR(mRen-2)27 rat. *Hypertension* 25: 1014-1020, 1995.

14. **Cano A, Preisig P and Alpern RJ**. Cyclic adenosine monophosphate acutely inhibits and chronically stimulates Na/H antiporter in OKP cells. *J Clin Invest* 92: 1632-1638, 1993.

15. **Carey RM and Siragy HM**. Newly recognized components of the Renin-Angiotensin system: potential roles in cardiovascular and renal regulation. *Endocr Rev* 24: 261-271, 2003.

16. **Carey RM, Wang ZQ and Siragy HM**. Role of the angiotensin type 2 receptor in the regulation of blood pressure and renal function. *Hypertension* 35: 155-163, 2000.

17. **Carter TL, Pedrini S, Ghiso J, Ehrlich ME and Gandy S**. Brain neprilysin activity and susceptibility to transgene-induced Alzheimer amyloidosis. *Neurosci Lett* 392: 235-239, 2006.

18. **Chang E and Perlman AJ**. Multiple hormones regulate angiotensinogen messenger ribonucleic acid levels in a rat hepatoma cell line. *Endcrinology* 121: 513-519, 1987.

19. **Chappell MC, Gallagher PE, Averill DB, Ferrario CM and Brosnihan KB**. Estrogen or the AT1 antagonist olmesartan reverses the development of profound hypertension in the congenic mRen2.Lewis rat. *Hypertension* 42: 781-786, 2003.

20. **Chappell MC, Gomez MN, Pirro NT and Ferrario CM**. Release of angiotensin-(1-7) from the rat hindlimb: influence of angiotensin-converting enzyme inhibition. *Hypertension* 35: 348-352, 2000.

21. **Chappell MC, Pirro NT, Sykes A and Ferrario CM**. Metabolism of angiotensin-(1-7) by angiotensin converting enzyme. *Hypertension* 31: 362-367, 1998.

22. **Chappell MC, Yamaleyeva LM and Westwood BM**. Estrogen and salt sensitivity in the female mRen(2).Lewis rat. *Am J Physiol Regul Integr Comp Physiol* 291: R1557-R1563, 2006.

23. **Chen M, Harris MP, Rose D, Smart A, He XR, Kretzler M, Briggs JP and Schnermann J**. Renin and renin mRNA in proximal tubules of the rat kidney. *J Clin Invest* 94: 237-243, 1994.

24. **Cheng HF, Becker BN, Burns KD and Harris RC**. Angiotensin II upregulates type-1 angiotensin II receptors in renal proximal tubule. *J Clin Invest* 95: 2012-2019, 1995.

25. **Chidambaram M, Duncan JA, Lai VS, Cattran DC, Floras JS, Scholey JW and Miller JA**. Variation in the renin angiotensin system throughout the normal menstrual cycle. *JASN* 13: 446-452, 2002.

26. **Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL, Jr., Jones DW, Materson BJ, Oparil S, Wright JT, Jr. and Roccella EJ**. The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure: The JNC 7 Report. *JAMA* 289: 2560-2571, 2003.

27. **Clark MA, Tommasi EN, Bosch SM, Tallant EA and Diz DI**. Angiotensin-(1-7) reduces renal angiotensin II receptors through a cyclooxygenase dependent pathway. *J Cardiovasc Pharmacol* 41: 276-283, 2003.

28. **Crackower MA, Sarao R, Oudit GY, Yagil C, Kozieradzki I, Scanga SE, Oliveira-dos-Santo AJ, da Costa J, Zhang L, Pei Y, Scholey J, Bray MR,**

Ferrario CM, Backx PH, Manoukian AS, Chappell MC, Yagil Y and Penninger JM. Angiotensin-converting enzyme 2 is an essential regulator of heart function. *Nature* 417: 822-828, 2002.

29. **Cutler JA, Sorlie PD, Wolz M, Thom T, Fields LE and Roccella EJ**. Trends in hypertension prevalence, awareness, treatment, and control rates in United States adults between 1988-1994 and 1999-2004. *Hypertension* 52: 818-827, 2008.

30. **Davisson RL, Ding Y, Stec DE, Catterall JF and Sigmund CD**. Novel mechanism of hypertension revealed by cell-specific targeting of human angiotensinogen in transgenic mice. *Physiol Genomics* 1: 3-9, 1999.

31. **DelliPizzi A, Hilchey SD and Bell-Quilley CP**. Natriuretic action of angiotensin (1-7). *Br J Pharmacol* 111: 1-3, 1994.

32. **Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan M, Woolf B, Robinson K, Jeyaseelan R, Breitbart RE and Acton S**. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ Res* 87: E1-E9, 2000.

33. **Dubey RK, Oparil S, Imthurn B and Jackson EK**. Sex hormones and hypertension. *Cardiovasc Res* 53: 688-708, 2002.

34. **Engler S, Paul M and Pinto YM**. The TGR(mRen2)27 transgenic rat model of hypertension. *Regul Pept* 77: 3-8, 1998.

35. **Fabiani ME, Dinh DT, Nassis L, Casley DJ and Johnston CI**. In vivo inhibition of angiotensin receptors in the rat kidney by candesartan cilexetil: A comparison with losartan. *Am J Hypertens* 13: 1005-1013, 2000.

36. **Ferrario CM, Jessup JA, Chappell MC, Averill DB, Brosnihan KB, Tallant EA, Diz DI and Gallagher PE**. Effect of angiotensin converting enzyme inhibition and angiotensin II receptor blockers on cardiac angiotensin converting enzyme 2. *Circulation* 111: 2605-2610, 2005.

37. **Ferrario CM, Jessup JA, Gallagher PE, Averill DB, Brosnihan KB, Tallant EA, Smith RD and Chappell MC**. Effects of renin angiotensin system blockade on renal angiotensin-(1-7) forming enzymes and receptors. *Kidney Int* 68: 2189-2196, 2005.

38. **Ferrario CM, Martell N, Yunis C, Flack JM, Chappell MC, Brosnihan KB, Dean RH, Fernandez A, Novikov S, Pinillas C and Luque M**. Characterization of angiotensin-(1-7) in the urine of normal and essential hypertensive subjects. *Am J Hypertens* 11: 137-146, 1998.

39. **Gallagher PE, Li P, Lenhart JR, Chappell MC and Brosnihan KB**. Estrogen regulation of angiotensin-converting enzyme mRNA. *Hypertension* 33: 323-328, 1999.

40. **Ganten D, Lindpaintner K, Ganten U, Peters J, Zimmermann F, Bader M and Mullins J**. Transgenic rats: new animal models in hypertension research. *Hypertension* 17: 843-855, 1991.

41. **Gava E, Samad-Zadeh A, Zimpelmann J, Bahramifarid N, Kitten GT, Santos RA, Touyz RM and Burns KD**. Angiotensin-(1-7) activates a tyrosine phosphatase and inhibits glucose-induced signaling in proximal tubular cells. *Nephrol Dial Transplant* 2009.

42. **Gross V, Roman RJ and Cowley AW, Jr.** Abnormal pressure-natriuresis in transgenic renin gene rats. *J Hypertens* 12: 1029-1034, 1994.

43. **Gurley SB, Allred A, Le TH, Griffiths R, Mao L, Philip N, Haystead TA, Donoghue M, Beitbart RE, Acton SL, Rockman HA and Coffman TM**. Altered blood pressure responses and normal cardiac phenotype in ACE2-null mice. *J Clin Invest* 116: 2218-2225, 2006.

44. **Hakam AC and Hussain T**. Angiotensin II AT2 receptors inhibit proximal tubular Na+-K+ATPase activity via a NO/cGMP-dependent pathway. *American*

Journal of Physiology - Renal Fluid & Electrolyte Physiology 290: F1430-F1436, 2006.

45. **Hall JE, Guyton AC, Trippodo NC, Lohmeier TE, McCaa RE and Cowley AW, Jr.** Intrarenal control of electrolyte excretion by angiotensin II. *Am J Physiol* 232: F538-F544, 1977.

46. **Handa RK, Ferrario CM and Strandhoy JW**. Renal actions of angiotensin-(1-7) in vivo and in vitro studies. *Am J Physiol* 270: F141-F147, 1996.

47. **Harrison-Bernard LM, El-Dahr SS, O'Leary DF and Navar LG**. Regulation of angiotensin II type 1 receptor mRNA and protein in angiotensin II-induced hypertension. *Hypertension* 33: 340-346, 1999.

48. **Harrison-Bernard LM, Navar LG, Ho MM, Vinson GP and El Dahr SS**. Immunohistochemical localization of ANG II AT1 receptor in adult rat kidney using a monoclonal antibody. *Am J Physiol* 273: F170-F177, 1997.

49. **Henrich WL, McAllister EA, Eskue A, Miller T and Moe OW**. Renin regulation in cultured proximal tubular cells. *Hypertension* 27: 1337-1340, 1996.

50. **Hilchey SD and Bell-Quilley CP**. Association between the natriuretic action of angiotensin-(1-7) and selective stimulation of renal prostaglandin I_2 release. *Hypertension* 25: 1238-1244, 1995.

51. **Hinojosa-Laborde C, Craig T, Zheng W, Ji H, Haywood JR and Sandberg K**. Ovariectomy augments hypertension in aging female Dahl salt-sensitive rats. *Hypertension* 44: 405-409, 2004.

52. **Huang J, Guan H, Booze RM, Eckman CB and Hersh LB**. Estrogen regulates neprilysin activity in rat brain. *Neurosci Lett* 367: 85-87, 2004.

53. **Igase M, Strawn WB, Gallagher PE, Geary RL and Ferrario CM**. Angiotensin II AT1 receptors regulate ace2 and angiotensin-(1-7) expression in aorta of spontaneously hypertensive rats. *AJP - Heart and Circulatory* 289: H1013-H1019, 2005.

54. **Imig JD, Navar GL, Zou LX, O'Reilly KC, Allen PL, Kaysen JH, Hammond TG and Navar LG**. Renal endosomes contain angiotensin peptides, converting enzyme, and AT(1A) receptors. *Am J Physiol* 277: F303-F311, 1999.

55. **Ingelfinger JR, Zuo WM, Fon EA, Ellison KE and Dzau VJ**. In situ hybridization evidence for angiotensinogen messenger RNA in the rat proximal tubule. *J Clin Invest* 85: 417-423, 1990.

56. **Isa K, Garcia-Espinosa MA, Arnold AC, Pirro NT, Tommasi EN, Ganten D, Chappell MC, Ferrario CM and Diz DI**. Chronic immunoneutralization of brain angiotensin-(1-12) lowers blood pressure in transgenic (mRen2)27 hypertensive Rats. *Am J Physiol Regul Integr Comp Physiol* 2009.

57. **Iyer SN, Chappell MC, Averill DB, Diz DI and Ferrario CM**. Vasodepressor actions of angiotensin-(1-7) unmasked during combined treatment with lisinopril and losartan. *Hypertension* 31: 699-705, 1998.

58. **Iyer SN, Ferrario CM and Chappell MC**. Angiotensin-(1-7) contributes to the antihypertensive effects of blockade of the renin-angiotensin system. *Hypertension* 31: 356-361, 1998.

59. **Jan Danser AH, Koning MMG, Admiraal PJJ, Sassen LMA, Derkx FHM, Verdouw PD and Schalekamp MADH**. Production of angiotensin I and II at tissue sites in intact pigs. *Am J Physiol* 263: H429-H437, 1992.

60. **Jessup JA, Gallagher PE, Averill DB, Brosnihan KB, Tallant EA, Chappell MC and Ferrario CM**. Effect of angiotensin II blockade on a new congenic model of hypertension derived from transgenic Ren-2 rats. *Am J Physiol Heart Circ Physiol* 291: H2166-H2172, 2006.

61. **Ji H, Menini S, Pesce C, Kim J, Wu X and Sandberg K**. Role of angiotensin-converting enzyme 2 and angiotensin(1-7) in 17beta-oestradiol regulation of renal pathology in renal wrap hypertension in rats. *Exp Physiol* 93: 648-657, 2008.

62. **Joyner J, Neves L, Ferrario C and Brosnihan K**. Administration of D-Alanine-[Ang-(1-7)] (A-779) prior to pregnancy in Sprague Dawley rats produces antidiuresis in late gestation. *J Am Soc Hypertens* 6: 430, 2008.

63. **Joyner J, Neves LA, Stovall K, Ferrario CM and Brosnihan KB**. Angiotensin-(1-7) serves as an aquaretic by increasing water intake and diuresis in association with downregulation of aquaporin-1 during pregnancy in rats. *Am J Physiol Regul Integr Comp Physiol* 294: R1073-R1080, 2008.

64. **Joyner J, Neves LAA, Granger JP, Alexander BT, Merrill DC, Chappell MC, Ferrario CM, Davis WP and Brosnihan KB**. Temporal-spatial expression of angiotensin-(1-7) and angiotensin converting enzyme 2 in the kidney of normal and hypertensive pregnant rats. *Am J Physiol Regul Integr Comp Physiol* 293: R169-R177, 2007.

65. **Kastner PR, Hall JE and Guyton AC**. Control of glomerular filtration rate: role of intrarenally formed angiotensin II. *Am J Physiol* 246: F897-F906, 1984.

66. **Klett C, Ganten D, Hellmann W, Kaling M, Ryffel GU, Weimar-Ehl T and Hackenthal E**. Regulation of hepatic angiotensinogen synthesis and secretion by steroid hormones. *Endocrinology* 130: 3660-3668, 1992.

67. **Kobori H, Harrison-Bernard LM and Navar LG**. Urinary excretion of angiotensinogen reflects intrarenal angiotensinogen production. *Kidney Int* 61: 579-585, 2002.

68. **Kobori H, Nangaku M, Navar LG and Nishiyama A**. The intrarenal renin-angiotensin system: from physiology to the pathobiology of hypertension and kidney disease. *Pharmacol Rev* 59: 251-287, 2007.

69. **Kobori H, Nishiyama A, Harrison-Bernard LM and Navar LG**. Urinary angiotensinogen as an indicator of intrarenal Angiotensin status in hypertension. *Hypertension* 41: 42-49, 2003.

70. **Kobori H, Ozawa Y, Satou R, Katsurada A, Miyata K, Ohashi N, Hase N, Suzaki Y, Sigmund CD and Navar LG**. Kidney-specific enhancement of Ang II stimulates endogenous intrarenal angiotensinogen in gene-targeted mice. *Am J Phys Renal Physiol* 293: F938-F945, 2007.

71. **Kobori H, Prieto-Carrasquero MC, Ozawa Y and Navar LG**. AT1 receptor mediated augmentation of intrarenal angiotensinogen in angiotensin II-dependent hypertension. *Hypertension* 43: 1126-1132, 2004.

72. **Koka V., Huang XR, Chung AC, Wang W, Truong LD and Lan HY**. Angiotensin II up-regulates angiotensin I-converting enzyme (ACE), but down-regulates ACE2 via the AT1-ERK/p38 MAP kinase pathway. *Am J Pathol* 172: 1174-1183, 2008.

73. **Kopkan L, Kramer HJ, Huskova Z, Vanourkova Z, Backer A, Bader M, Ganten D and Cervenka L**. Plasma and kidney angiotensin II levels and renal

functional responses to AT(1) receptor blockade in hypertensive Ren-2 transgenic rats. *J Hypertens* 22: 819-825, 2004.

74. **Krattenmacher R, Knauthe R, Parczyk K, Walker A, Hilgenfedlt U and Fritzemeier K-H**. Estrogen action on hepatic synthesis of angiotensinogen and IGF-1: direct and indirect estrogen effects. *J Steroid Biochem Mol Biol* 48: 207-214, 1994.

75. **Krishnamurthi K, Verbalis JG, Zheng W, Wu Z, Clerch LB and Sandberg K**. Estrogen regulates angiotensin AT1 receptor expression via cytosolic proteins that bind to the 5' leader sequence of the receptor mRNA. *Endocrinology* 140: 5435-5438, 1999.

76. **Lavoie JL, Lake-Bruse KD and Sigmund CD**. Increased blood pressure in transgenic mice expressing both human renin and angiotensinogen in the renal proximal tubule. *American Journal of Physiology Renal Fluid & Electrolyte Physiology* 286: F965-F971, 2004.

77. **Lee MA, Bohm M, Kim S, Bachmann S, Bachmann J, Bader M and Ganten D**. Differential gene expression of renin and angiotensinogen in the TGR(mREN-2)27 transgenic rat. *Hypertension* 25: 570-580, 1995.

78. **Lee MA, Bohm M, Paul M, Bader M, Ganten U and Ganten D**. Physiological characterization of the hypertensive transgenic rat TGR(mREN2)27. *Am J Physiol* 270: E919-E929, 1996.

79. **Lemos VS, Cortes SF, Silva DM, Campagnole-Santos MJ and Santos RA**. Angiotensin-(1-7) is involved in the endothelium-dependent modulation of phenylephrine-induced contraction in the aorta of mRen-2 transgenic rats. *Br J Pharmacol* 135: 1743-1748, 2002.

80. **Li N, Zimpelmann J, Cheng K, Wilkins JA and Burns KD**. The role of angiotensin converting enzyme 2 in the generation of angiotensin 1-7 by rat proximal tubules. *Am J Physiol Renal Physiol* 288: F353-F362, 2004.

81. **Liang B and Leenen FH**. Prevention of salt-induced hypertension and fibrosis by AT1-receptor blockers in Dahl S rats. *J Cardiovasc Pharmacol* 51: 457-466, 2008.

82. **Liu FY and Cogan MG**. Angiotensin II stimulates early proximal bicarbonate absorption in the rat by decreasing cyclic adenosine monophosphate. *J Clin Invest* 84: 83-91, 1989.

83. **Lo M, Medeiros IA, Mullins JJ, Ganten D, Barres C, Cerutti C, Vincent M and Sassard J**. High blood pressure maintenance in transgenic mRen-2 vs. Lyon genetically hypertensive rats. *Am J Physiol* 265: R180-R186, 1993.

84. **Lu B, Figini M, Emanueli C, Geppetti P, Grady EF, Gerard NP, Ansell J, Payan DG, Gerard C and Bunnett N**. The control of microvascular permeability and blood pressure by neutral endopeptidase. *Nature Medicine* 3: 904-907, 1997.

85. **Mento PF, Pica ME, Hilepo J, Chang J, Hirsch L and Wilkes BM**. Increased expression of glomerular AT1 receptors in rats with myocardial infarction. *Am J Physiol* 275: H1247-H1253, 1998.

86. **Miller JA, Anacta LA and Cattran DC**. Impact of gender on the renal response to angiotensin II. *Kidney Int* 55: 278-285, 1999.

87. **Miller JA, Cherney DZ, Duncan JA, Lai V, Burns KD, Kennedy CR, Zimpelmann J, Gao W, Cattran DC and Scholey JW**. Gender differences in the renal response to renin-angiotensin system blockade. *JASN* 17: 2554-2560, 2006.

88. **Moe OW, Ujiie K, Star RA, Miller T, Widell J and Alpein RJ**. Renin expression in renal proximal tubule. *J Clin Invest* 91: 774-779, 1993.

89. **Mullins JJ, Peters J and Ganten D**. Fulminant hypertension in transgenic rats harbouring the mouse Ren-2 gene. *Nature* 344: 541-544, 1990.

90. **Muthalif MM, Benter IF, Uddin MR, Harper JL and Malik KU**. Signal transduction mechanisms involved in angiotensin-(1-7)-stimulated arachidonic acid release and prostanoid synthesis in rabbit aortic smooth muscle cells. *J Pharm Exp Therapeutics* 284: 388-398, 1998.

91. **Navar LG, Harrison-Bernard LM, Nishiyama A and Kobori H**. Regulation of intrarenal angiotensin II in hypertension. *Hypertension* 39: 316-322, 2002.

92. **Navar LG, Lewis L, Hymel A, Braam B and Mitchell KD**. Tubular fluid concentrations and kidney contents of angiotensins I and II in anesthetized rats. *J Am Soc Nephrol* 5: 1153-1158, 1994.

93. **Nickenig G, Baumer AT, Grohe C, Kahlert S, Strehlow K, Rosenkranz S, Stablein A, Beckers F, Smits JF, Daemen MJ, Vetter H and Bohm M**. Estrogen modulates AT1 receptor gene expression in vitro and in vivo. *Circulation* 97: 2197-2201, 1998.

94. **Nickenig G, Strehlow K, Wassmann S, Baumer AT, Albory K, Sauer H and Bohm M**. Differential effects of estrogen and progesterone on AT1 receptor gene expression in vascular smooth muscle cells. *Circulation* 102: 1828-1833, 2000.

95. **Okuniewski R, Davis EA, Jarrott B and Widdop RE**. A comparison of the development of renal hypertension in male and female rats. *Clin Sci (Lond)* 95: 445-451, 1998.

96. **Oudit GY, Herzenberg AM, Kassiri Z, Wong D, Reich H, Khokha R, Crackower MA, Backx PH, Penninger JM and Scholey JW**. Loss of angiotensin-converting enzyme-2 leads to the late development of angiotensin II-dependent glomerulosclerosis. *Am J Patho* 168: 1808-1820, 2006.

97. **Ozono R, Wang Z-Q, Moore AF, Inagami T, Siragy HM and Carey RM**. Expression of the subtype 2 angiotensin (AT_2) receptor protein in rat kidney. *Hypertension* 30: 1238-1246, 1997.

98. **Page IH**. Initiation and maintenance of renal hypertension. *Am J Surg* 107: 26-34, 1964.

99. **Peters J, Hilgers KF, Maser-Gluth C and Kreutz R**. Role of the circulating renin-angiotensin system in the pathogenesis of hypertension in transgenic rats. TGR(mREN2)27. *Clin Exp Hypertens* 18: 933-948, 1996.

100. **Pinheiro SV, Simoes e Silva AC, Sampaio WO, de Paula RD, Mendes EP, Bontempo ED, Pesquero JB, Walther T, Alenina N, Bader M, Bleich M and Santos RA**. Nonpeptide AVE 0991 is an angiotensin-(1-7) receptor Mas agonist in the mouse kidney. *Hypertension* 44: 490-496, 2004.

101. **Prieto-Carrasquero MC, Harrison-Bernard LM, Kobori H, Ozawa Y, Hering-Smith KS, Hamm LL and Navar LG**. Enhancement of collecting duct

renin in angiotensin II-dependent hypertensive rats. *Hypertension* 44: 223-229, 2004.

102. **Quan A and Baum M**. Endogenous production of Angiotensin II modulates rat proximal tubule transport. *J Clin Invest* 97: 2878-2882, 1996.

103. **Rademaker MT, Charles CJ, Espiner EA, Nicholls MG, Richards AM and Kosoglou T**. Neutral endopeptidase inhibition: augmented atrial and brain natriuretic peptide, haemodynamic and natriuretic responses in ovine heart failure. *Clin Sci* 91: 283-291, 1996.

104. **Reckelhoff JF, Zhang H and Srivastava K**. Gender differences in development of hypertension in spontaneously hypertensive rats: role of the renin-angiotensin system. *Hypertension* 35: 480-483, 2000.

105. **Ren Y, Garvin JL and Carretero OA**. Vasodilator action of angiotensin-(1-7) on isolated rabbit afferent arterioles. *Hypertension* 39: 799-802, 2002.

106. **Rice GI, Thomas DA, Grant PJ, Turner AJ and Hooper NM**. Evaluation of angiotensin-converting enzyme (ACE), its homologue ACE2 and neprilysin in angiotensin peptide metabolism. *Biochem J* 383: 45-51, 2004.

107. **Rosenthal T and Oparil S**. Hypertension in women. *J Human Hypertens* 14: 691-704, 2000.

108. **Ruiz-Ortega M and Egido J**. Angiotensin II modulates cell growth-related events and synthesis of matrix proteins in renal interstitial fibroblasts. *Kidney Int* 52: 1497-1510, 1997.

109. **Santos RA, Silva ACS, Maric C, Speth R, Machado RP, Pinheiro SV, Lopes MT, Mendes EP, Bader M, Schultheiss H-P, Campagnole-Santos MJ and Walther T**. Evidence that angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor mas. *Hypertension* 40: 387, 2002.

110. **Santos RAS, Simoes e Silva AC, Maric C, Silva D.M., Machado RP, de Bul I, Heringer-Walther S, Pinheiro SV, Lopes MT, Bader M, Mendes EP, Lemos VS, Campagnole-Santos MJ, Schultheiss H-P, Speth R and Walther T**. Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci USA* 100: 8258-8263, 2003.

111. **Shaltout HA, Westwood B, Averill DB, Ferrario CM, Figueroa J, Diz DI, Rose JC and Chappell MC**. Angiotensin metabolism in renal proximal tubules, urine and serum of sheep: Evidence for ACE2-dependent processing of Angiotensin II. *Am J Physiol Renal Physiol* 292: F82-F91, 2006.

112. **Shao W, Seth DM and Navar LG**. Augmentation of endogenous intrarenal angiotensin II levels in Val5-Ang II-infused rats. *Am J Physiol Renal Physiol* 296: F1067-F1071, 2009.

113. **Soler MJ, Wysocki J, Ye M, Lloveras J, Kanwar Y and Batlle D**. ACE2 inhibition worsens glomerular injury in association with increased ACE expression in streptozotocin-induced diabetic mice. *Kid Int* 72: 614-623, 2007.

114. **Soler MJ, Ye M, Wysocki J, William J, Lloveras J and Batlle D**. Localization of ACE2 in the renal vasculature: amplification by angiotensin II type 1 receptor blockade using telmisartan. *Am J Phys Renal Physiol* 296: F398-F405, 2009.

115. **Springate JE, Feld LG and Ganten D**. Renal function in hypertensive rats transgenic for mouse renin gene. *Am J Physiol* 266: F731-F737, 1993.

116. **Stasch J-P, Dietrich CH, Ganten D and Wegner M**. Renal and antihypertensive effects of neutral endopeptidase inhibition in transgenic rats with an extra renin gene. *Am J Hypertens* 9: 795-802, 1996.

117. **Stephenson SL and Kenny AJ**. The metabolism of neuropeptides. Hydrolysis of the angiotensins, bradykinin, substance P and oxytocin by pig kidney microvillar membranes. *Biochemical Journal* 241: 237-247, 1987.

118. **Su Z, Zimpelmann J and Burns KD**. Angiotensin-(1-7) inhibits angiotensin II-stimulated phosphorylation of MAP kinases in proximal tubular cells. *Kidney Int* 69: 2212-2218, 2006.

119. **Tallant EA, Diz DI and Ferrario CM**. Antiproliferative actions of angiotensin-(1-7) in vascular smooth muscle. *Hypertension* 34: 950-957, 1999.

120. **Tea BS, Der SS, Touyz RM, Hamet P and deBlois D**. Proapoptotic and growth-inhibitory role of angiotensin II type 2 receptor in vascular smooth muscle cells of spontaneously hypertensive rats in vivo. *Hypertension* 35: 1069-1073, 2000.

121. **Thomas D, Harris PJ and Morgan TO**. Altered responsiveness of proximal tubule fluid reabsorption of peritubular angiotensin II in spontaneously hypertensive rats. *J Hypertens* 8: 407-410, 1990.

122. **Tikellis C, Bialkowski K, Pete J, Sheehy K, Su Q, Johnston C, Cooper M and Thomas M**. ACE2 deficiency modifies renoprotection afforded by ACE inhibition in experimental diabetes. *Diabetes* 57: 1018-1025, 2008.

123. **Tominaga T, Suzuki H, Ogata Y, Matsukawa S and Saruta T**. The role of sex hormones and sodium intake in postmenopausal hypertension. *J Hum Hypertens* 5: 495-500, 1991.

124. **Vallon V, Heyne N, Richter K, Khosla MC and Fechter K**. [7-D-ALA]-Angiotensin 1-7 blocks renal actions of angiotensin 1-7 in the anesthetized rat. *J Cardiovasc Pharmacol* 32: 164-167, 1998.

125. **Von T, Vari RC, El-Dahr SS and Navar LG**. Augmentation of intrarenal angiotensin II levels by chronic angiotensin II infusion. *Am J Physiol* 266: F120-F128, 1994.

126. **Wagner J, Zeh K and Paul M**. Transgenic rats in hypertension research. *J Hypertens* 10: 601-605, 1992.

127. **WHI Investigators**. Estrogen plus progestin and the risk of coronary heart disease. *N Engl J Med* 394: 523-534, 2003.

128. **WHI Investigators**. Effects of conjugated equine estrogen on stroke in the Women's Health Initiative. *Circulation* 113: 2425-2434, 2006.

129. **Wolf G**. "The road not taken": role of angiotensin II type 2 receptor in pathophysiology. *Nephrol Dial Transplant* 17: 195-198, 2002.

130. **Wolf G**. Renal injury due to renin-angiotensin-aldosterone system activation of the transforming growth factor-beta pathway. *Kidney Int* 70: 1914-1919, 2006.

131. **Wolf G, Wenzel U, Burns KD, Harris RC, Stahl RA and Thaiss F**. Angiotensin II activates nuclear transcription factor-kappaB through AT1 and AT2 receptors. *Kidney Int* 61: 1986-1995, 2002.

132. **Wolf G, Ziyadeh FN and Stahl RA**. Angiotensin II stimulates expression of transforming growth factor beta receptor type II in cultured mouse proximal tubular cells. *J Mol Med* 77: 556-564, 1999.

133. **Wolf G, Ziyadeh FN, Zahner G and Stahl RA**. Angiotensin II-stimulated expression of transforming growth factor beta in renal proximal tubular cells: attenuation after stable transfection with the c-mas oncogene. *Kidney Int* 48: 1818-1827, 1995.

134. **Ye M, Wysocki J, William J, Soler MJ, Cokic I and Batlle D**. Glomerular localization and expression of angiotensin-converting enzyme 2 and angiotensin-converting enzyme: Implications for albuminemia in diabetes. *J Am Soc Nephrol* 17: 3067-3075, 2006.

135. **Yue X, Lu M, Lancaster T, Cao P, Honda S, Staufenbiel M, Harada N, Zhong Z, Shen Y and Li R**. Brain estrogen deficiency accelerates Abeta plaque formation in an Alzheimer's disease animal model. *Proc Natl Acad Sci U S A* 102: 11811-11816, 2005.

136. **Zhao Y, Bader M, Kruetz R, Fernandez-Alfonso M, Zimmermann F, Ganten U, Metzger R, Ganten D, Mullins JJ and Peters J**. Ontogenetic regulation of mouse Ren-2d renin gene in transgenic hypertensive rats, TGR (mRen2) 27. *Am J Physiol* 265: E699-E707, 1993.

137. **Zhuo J, Ohishi M and Mendelsohn FA**. Roles of AT1 and AT2 receptors in the hypertensive Ren-2 gene transgenic rat kidney. *Hypertension* 33: 347-353, 1999.

138. **Zhuo JL, Carretero OA and Li XC**. Effects of AT1 receptor-mediated endocytosis of extracellular Ang II on activation of nuclear factor-kappa B in proximal tubule cells. *Ann N Y Acad Sci* 1091: 336-345, 2006.

139. **Zhuo JL, Imig JD, Hammond TG, Orengo S, Benes E and Navar LG**. Ang II accumulation in rat renal endosomes during Ang II-induced hypertension: Role of AT(1) receptor. *Hypertension* 39: 116-121, 2002.

140. **Zou L-X, Imig JD, Hymel A and Navar LG**. Renal uptake of circulating angiotensin II in Val^5-angiotensin II infused rats is mediated by AT_1 receptor. *Am J Hypertens* 11: 570-578, 1998.

CHAPTER TWO

DIFFERENTIAL EXPRESSION OF NUCLEAR AT1 RECEPTORS AND ANGIOTENSIN II WITHIN THE KIDNEY OF THE MALE CONGENIC mREN(2).LEWIS RAT

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ABSTRACT

We utilized a new congenic model of established hypertension, the mRen(2).Lewis rat, to assess the intracellular expression of angiotensin peptides and receptors in the kidney. The congenic strain was established from the backcross of the (mRen2)27 transgenic rat that expresses the mouse renin 2 gene onto the Lewis strain. The 20 week old male congenic rats were markedly hypertensive as compared to the Lewis controls [systolic blood pressure: 195 + 2 versus 107 + 2 mmHg, $p < 0.01$]. Although plasma Ang II levels were not different between strains, circulating levels of Ang-(1-7) were 270% higher and Ang I concentrations were 40% lower in the mRen(2).Lewis rats. In contrast, both cortical (CORT) and medullary (MED) Ang II concentrations were 60% higher in the mRen(2).Lewis rats, while tissue Ang I was 66% and 84% lower in CORT and MED. For both strains, MED Ang II, Ang I and Ang-(1-7) were significantly higher than CORT levels. Intracellular Ang II binding distinguished nuclear (NUC) and plasma membrane (PM) receptor using the Ang II radioligand ^{125}I-Sarthran. Isolated CORT nuclei exhibited a high density (B_{max} >200 fmol/mg protein) and affinity for the Sarthran ligand (K_D<0.5 nM), the majority of these sites (>95%) were the AT1 receptor subtype. CORT Ang II receptor B_{max} and K_D values in nuclei were 75% and 50% lower, respectively, for the mRen(2).Lewis versus the Lewis rats. In the MED, the PM receptor density [Lewis: 50 ± 4 versus mRen(2).Lewis: 21 ± 5 fmol/mg protein) and affinity [K_D: Lewis: 0.31 ± 0.1 versus 0.69 ± 0.1 nM] were lower in the mRen(2).Lewis. In summary, the hypertensive mRen(2).Lewis rats exhibit higher Ang II in both CORT and MED regions of the kidney. Evaluation of intracellular Ang II receptors revealed lower CORT NUC and MED PM AT1 sites in the mRen(2).Lewis.

These studies are the first demonstration of regulation of NUC Ang II receptors within the renal cortex. The down-regulation of AT1 sites in the mRen(2).Lewis may reflect a compensatory response to dampen the elevated levels of intrarenal Ang II.

Key words: angiotensin II type 1 receptors, mRen(2).Lewis, hypertension, intrarenal angiotensin, renal cortex, renal medulla, Ang II.

Introduction

The influence of the renin-angiotensin system (RAS) on the development and progression of hypertension and renal injury is without dispute. It is also well accepted that the major active components of the RAS that contribute to increased blood pressure and tissue injury are the sustained or enhanced expression of angiotensin II (Ang II) and aldosterone. Numerous studies confirm that the AT1 receptor mediates the majority of the actions of Ang II and that blockade of this receptor ameliorates the deleterious effects of the peptide. The AT1 receptor is regarded as a typical seven-transmembrane G-coupled protein residing on the cellular membrane that binds circulating or extracellular Ang II, however, several studies now suggest that intracellular Ang II has significant actions (4,19,20,22,27). These actions of Ang II are entirely consistent with evidence of intracellular and/or nuclear Ang II binding sites in various cell types including hepatocytes, vascular smooth muscles cells and neonatal neurons, as well as several tissues (7, 35, 40, 42-44, 53, 55, 62).

To our knowledge, only one study by Licea and colleagues (42) has characterized the presence of nuclear Ang II receptors in the kidney and the extent that these receptors are altered in Ang II-dependent hypertension. Although their data revealed a significant concentration of AT1 receptors in the nuclear fraction of the renal cortex, infusion of Ang II at a dose that markedly increased blood pressure did not influence the density of these nuclear sites (42). Therefore, in the present study, we determined the density and pharmacologic characteristics of nuclear and plasma membrane Ang II receptors in the renal cortex and medulla from adult hypertensive mRen2. Lewis rats, a new congenic

strain developed from the backcross of the original Ren27(2) transgenic (that overexpress renin) and the normotensive Lewis. Similar to the original transgenic strain, the mRen(2).Lewis exhibit significant hypertension, gender differences in the degree of elevated blood pressure and renal injury, as well as the normalization of blood pressure by RAS blockade (3,12,17). In addition to the characterization of renal Ang II receptors, we also assessed tissue expression of Ang II, Ang I and Ang-(1-7) in both the cortical and medullary regions of the kidneys from both strains. These studies are the first to document differential expression of both receptor and peptide levels in the renal cortex and medulla of the male mRen(2).Lewis hypertensive strain.

Materials

Experimental Animals Heterozygous male mRen(2).Lewis rats were obtained from the Hypertension and Vascular Disease Center Transgenic colony at approximately 20 weeks of age. Normotensive male Lewis rats were purchased from Charles River (Raleigh, NC) and utilized at the same age as the congenic rats. Animals were fed a powdered rat chow (Purina Mills, Richmond VA) to provide a daily intake of 17 and 28 milliequivalents of sodium and potassium, respectively per 100 gm of body weight, they had full access to water and were housed in an AALAC-approved facility in rooms maintained on a 12 hour light/dark cycle (lights on 6:00 am to 6:00 pm). Systolic blood pressure was measured in trained rats (mean of 5 determinations per data point) with a Narco Biosystems device (Houston, TX). Rats were administered heparin (1000 units, intraperitoneally) 20 minutes prior to anesthesia with halothane and a catheter (Angiocath, Sandy, Utah) was placed in the abdominal aorta (5 ml) for aortic blood

collection. Animals were immediately killed by decapitation and the tissues rapidly removed. These procedures were performed in accordance with the Wake Forest University School of Medicine AALAC guidelines for animal care. The cortex and medulla regions of the kidney were then dissected on an ice-filled plastic Petri dish, then frozen on dry ice and stored at -80ºC.

Plasma and Renal Tissue Angiotensins Blood was collected into chilled Vacutainer blood collection tubes (Becton Dickinson, Sandy, Utah) containing the following mixture of peptidase inhibitors: 25 mM ethylenediaminetetraacetic acid (EDTA), 0.44 mM o-phenanthroline (PHEN), 1 mM 4-chloromercuribenzoic acid (PCMB), 0.12 mM pepstatin A and 3 μM acetyl-His-Pro-Phe-Val-Statine-Leu-Phe, a specific rat renin inhibitor and processed for direct radioimmunoassay (RIA) of angiotensin peptides (1). Frozen renal tissue was homogenized in an acid ethanol (80% v/v 0.1 N HCl) solution containing the peptidase inhibitors described above and processed for RIA analysis as described by Allred et al. (1). A sample of homogenate was taken to determine total protein content (BioRad Protein Assay Reagent, BioRad Laboratories, Hercules, CA). Details on the RIAs have been previously described (1, 12). In brief, the Ang II RIA equally recognizes Ang-(2-8), Ang-(3-8) and Ang-(4-8), but cross-reacts less than 0.01% with Ang I and Ang-(1-7). The Ang-(1-7) fully recognizes Ang-(1-7) and Ang-(2-7), but cross-reacts less than 0.1% with Ang II or Ang I. The Ang I RIA fully recognizes angiotensin-(2-10) and angiotensin-(3-10), but cross-reacts with Ang II and Ang-(1-7) less that 0.01%. The limits of detection for each RIA were: Ang II (2.5 fmol/tube), Ang 1-7 (2.8 fmol/tube), and Ang I (1.2 fmol/tube). To verify the identity of Ang II immunoreactivity in the kidney, pooled extracts from the cortex or

medulla of the Ren2.Lewis kidney were subjected to high-performance liquid chromatography (HPLC) using the heptafluorbutyric acid – acetonitrile solvent system as described (11). Following HPLC separation, the Ang II content of each fraction was determined by Ang II RIA.

Isolation of Nuclei and Plasma Membrane The frozen tissue was placed in homogenization buffer 20 mM Tricine KOH, 25 mM Sucrose, 25 mM KCl, 5 mM $MgCl_2$, pH 7.8 (homogenization buffer) and homogenized with a Polytron Ultra Turrax T25 Basic (setting 4) for 40 seconds on ice followed by a dounce homogenizer (Barnant Mixer Series 10, setting 3) and passed through a 100 □m mesh filter (25). The homogenate was centrifuged twice at 1,000 x g at 4ºC for 10 min to obtain the nuclear pellet. The supernatant fraction was centrifuged at 25,000 x g for 20 minutes at 4ºC to obtain the plasma membrane fraction.

Density gradient separation Renal nuclei were also isolated from the cortex by iso-osmotic density gradient separation (25). For this procedure, renal cortex was homogenized in the Tricine buffer described above and centrifuged at 1,000 x g for 10 minutes at 4°C. The pellet was resuspended in 20% Optiprep media (Accurate Chemical-Long Island, NY) and layered on a density gradient medium. The gradient consisted of the 10%, 20%, 25%, 30%, and 35% Optiprep media diluted in Buffer B containing 150 mM KCl, 30 mM $MgCl_2$, 120 mM Tricine-KOH at a pH of 7.8 in a total volume of 13 ml. The gradient was centrifuged at 10,000 x g for 20 minutes at 4°C and the isolated nuclei were obtained at the 30%/35% interface.

Receptor Binding Studies The Ang II binding assay utilized the radioligand [^{125}I-Sarcosine^{1},threonine^{8}]-Ang II (^{125}I-Sarthran), as described by Chappell et al. (15). Sarthran was iodinated using chloramine T and purified by HPLC to a specific activity >2,000 Curies/mmol. The binding buffer contained 10 mM HEPES, 120 mM NaCl, 5 mM $MgCl_2$,1 mM EGTA, 0.2 % BSA 10 μM lisinopril, 200 μM phenylmethylsulfonyl fluoride (PMSF), 10 μM SCH 39370, 10 μM bestatin and 1 μM amastatin at pH 7.4. Nuclear and plasma membrane binding was performed for 45 minutes at 22ºC. Nonspecific binding was defined by the remaining counts in the presence of 10 μM unlabeled Sarthran. Competition curves were obtained over the peptide concentrations range of 100 pM – 10 μM with 0.5 nM ^{125}I-Sarthran. The binding data was analyzed with the GraphPad Prism 4 statistical and graphics program.

Immunoblot Analysis Cellular fractions were boiled in a phosphate buffer saline (PBS, pH 7.4), Laemmli with β-mercaptoethanol solution. Proteins were separated on 10% SDS polyacrylamide gels for one hour at 120 Volts in Tris-Glycine SDS, transferred onto a PVDF membrane and subsequently blocked for one hour with 5% BioRad Dry Milk and TBS with Tween prior to incubation with primary antibodies against annexin II (dilution of 1:2500, BD Transduction Laboratories, San Diego, Ca), GMP130 (1:250, BD Transduction Laboratories), nucleoporin (Nup93, 1:1000, BD PharMingen, San Diego, Ca), and the angiotensin type I receptor (AT1, 1:1000, Alpha Diagnostics International, San Antonio, TX). Immunoblots were then resolved with Pierce Super Signal West Pico Chemiluminescent substrates as described by the manufacturer and exposed to Amersham Hyperfilm ECL (Piscataway, NJ).

Statistical Analysis Data are represented as mean ± standard error of the mean (SEM). Interstrain comparisons used a paired Student's *t* test. Between strain comparisons utilized an unpaired Student's *t* test with the GraphPad Prism 4.0 plotting and statistical software (San Diego, CA). To quantify the percent of competition for specific receptor subtypes and peptide ratios, one-way ANOVA with Tukey's Multiple Comparison post test was used for the data in each renal cellular compartment. The minimum statistical significance was reached at $p \leq 0.05$. All graphs presented were constructed with GraphPad Prism 4.0

Results

Blood Pressure. We determined the systolic blood pressure by tail-cuff methods in separate groups of male Lewis and mRen(2).Lewis. Systolic blood pressure was markedly higher in the congenic rats as compared to the normotensive Lewis [194 ± 2.0 mmHg versus 107 ± 1.8 mmHg, $p<0.01$, $n = 6$].

Plasma Angiotensin Peptides. We assessed plasma peptide concentrations in the arterial blood of both strains. As shown in Figure 1A, there was no difference in plasma Ang II between the mRen(2).Lewis and Lewis rats. However Ang I was lower in the congenic rats (83 ± 14 versus 139 ± 14 pM, $p \leq 0.05$, $n = 5$), while the circulating level of Ang-(1-7) was 270% higher in the hypertensive strain (126 ± 10 versus 46 ± 2 pM, $p \leq 0.05$, $n = 5$). In the lower panel (Figure 1B), we express these plasma values as the ratio of Ang II or Ang-(1-7) to its potential immediate precursor(s). In this case, the mRen(2).Lewis Ang-(1-7)/Ang II and Ang-(1-7)/Ang I were 250% and 300% higher, respectively, in comparison to the Lewis.

Renal Angiotensin Peptides. We then determined the renal angiotensin content in the same group of animals. As shown in Figure 2A, Ang II is significantly higher in the cortex and medulla of the mRen(2).Lewis rats. Ang II content was approximately 60% higher in the mRen(2).Lewis than the control group in both cortical [$p \leq 0.05$, $n = 5 - 6$] and medullary tissue [$p \leq 0.01$, $n = 5 - 6$]. Despite the overall higher levels of Ang II in the congenic rats, the peptide concentration was greater in the renal medulla versus cortex for both the mRen(2).Lewis and Lewis strains [160 and 150%, respectively, $p \leq 0.05$]. We verified the identity of Ang II immunoreactivity in the remaining extracts pooled from either the cortical or medullary tissues of the mRen(2).Lewis kidney. The extracts were initially fractionated by high performance liquid chromatography (HPLC) on a NovaPak C-18 column with subsequent analysis by the Ang II RIA; the chromatographs revealed that Ang II was the sole immunoreactive component in both the cortical and medullary tissue extracts from the congenic rats (data not shown). We next assessed the content of Ang I, the precursor to Ang II in the renal tissues from both strains (Figure 2B). In contrast to Ang II, Ang I was significantly lower in the congenic cortex [$p \leq 0.01$, $n = 6$] and congenic medulla [$p \leq 0.01$, $n = 6$]. Ang I content was significantly higher in the medullary versus cortical tissue [280%, $p \leq 0.05$] of the Lewis, but not the mRen(2).Lewis - the Ang I levels were markedly lower in both regions. In contrast to either Ang II or Ang I, Ang-(1-7) levels were not different between strains in either cortex [$n = 6$] or medulla [$n = 6$]. As observed for Ang II and Ang I, the renal medulla exhibited significantly higher levels of Ang-(1-7) than the cortex in the Lewis and congenic strains [360% and 430%, respectively, $p \leq 0.05$]. We also expressed the tissue data as the peptide ratios for each strain (Figure 3) and find that Ang II/Ang I values for

the congenic were 650% and 740% higher in the cortical and medullary tissues, respectively, with a trend for an increase in the A7/AI ratio in both areas as well.

Receptor Binding of Purified Nuclei: In the next series of studies, we initially determined whether isolated nuclei from the renal cortex of the Lewis rats exhibited specific Ang II binding using the non-selective antagonist ^{125}I-Sarthran. For these studies, nuclei from renal cortex were isolated by differential centrifugation and density gradient separation with the Optiprep medium (25). Fractions taken from the gradient were enriched in nuclei (H&E staining, data not shown) and used for the receptor binding and immunoblot studies. In Figure 4, we show the saturation curves and Scatchard analysis of the nuclear (panel A) and plasma membrane (panel B) fractions from the renal cortex of the Lewis kidney. Both sets of binding data yielded linear plots suggesting a single population of binding sites; however, the data revealed a greater density of sites (B_{max}) for the nuclear fraction (Figure 4A). Characterization of the nuclear binding sites revealed that the AT1 antagonists losartan and candesartan competed to the same extent as Ang II (Figure 5A). The other antagonists selective for the AT_2 (PD) and Ang-(1-7) (D-ALA) receptors did not significantly displace ^{125}I-Sarthran binding from the cortical nuclei. Competition curves with Ang II, Ang III and Ang-(1-7) yielded IC_{50} values of 3, 5 and 400 nM, respectively, which are typical for binding to an AT1 receptor (Figure 5B). We next performed immunoblots on the nuclear and plasma membrane fractions using antibodies against the AT1 receptor and other cellular organelles (Figure 6A). The AT1 antibody revealed a single band at 52 kDa in the purified nuclei (N) and plasma membrane (P) fractions consistent with the binding results in these fractions. The antibody to the specific nuclear marker nucleoporin (Nup93), a nuclear pore protein,

revealed a 93 kDa band for the nuclei, but no immunoreactive band in the plasma membrane fraction. In contrast, immunoreactive bands for annexin II (33 kDa, endosomal marker) and GMP 130 (133 kDa, Golgi marker) were evident in the plasma membrane, but not the nuclear fraction.

Plasma Membrane versus Nuclear Ang II Receptors: Although use of the Optiprep density medium yielded an enriched nuclear fraction that exhibited high specific binding, this method proved laborious to determine receptor kinetics in multiple fractions from a large group of animals. Therefore, we determined whether differential centrifugation alone would yield an enriched nuclear fraction with similar binding characteristics as that obtained with the density gradient method. As shown in Figure 7, the saturation binding for renal nuclear (A) and plasma membrane fractions (B) from the congenic renal medulla yielded data consistent with a high affinity binding site. We then assessed the effects of the different antagonists on ^{125}I-Sarthran binding from both the nuclei and plasma membrane in the cortical and medullary tissues of the congenic kidney. As observed for the nuclei obtained by the density gradient method, both AT1 antagonists essentially abolished the Sarthran binding (Figure 8). Finally, the AT1 and Nup93 antibodies yielded predominant immunoreactive bands of 52 kDa and 93 kDa, respectively from the nuclear fraction obtained by the differential centrifugation (see Figure 6B). Although not shown in this figure, the annexin II and GMP130 antibodies lacked staining in the nuclear fraction. As these results were quite similar for the nuclei obtained by Optiprep density medium, potential differences between the congenic mRen(2).Lewis and Lewis rats utilized differential centrifugation to obtain the nuclear fraction. In both strains, comparison of the cortical receptor density revealed that the

nuclear fraction exhibited a significantly higher B_{max} than that of the plasma membrane fraction (Figure 9A). However, the congenic nuclear and plasma membrane B_{max} was markedly less than that of Lewis [$p \leq 0.05$, n = 4 for each group]. In Figure 9B, the K_D was significantly lower in the congenic nuclei in comparison to the Lewis [$p \leq 0.05$, n = 4]. Differences in the K_D in the plasma membrane component between the congenic and Lewis did not reach a statistical difference. In Figure 10, we show the binding data for the nuclear and plasma membrane fractions from the renal medulla. In contrast to the cortex, the B_{max} data for the nuclei were similar to the plasma membrane fraction. There were no differences in nuclear receptor density between the congenic and Lewis rats; however, the plasma membrane of the congenic exhibited a lower density than that of the Lewis rats [$p \leq 0.05$, n = 4]. Regarding the K_D values, the Lewis exhibited a lower K_D in the nuclear versus plasma membrane fraction. The congenic exhibited a lower K_D for the plasma membrane receptor than the Lewis [$p \leq 0.05$, n = 4] with no difference in the nuclear K_D values between congenic and Lewis animals.

Discussion

In the current study, we characterized a new congenic model of hypertension and demonstrate the altered renal expression of tissue angiotensins and intercellular AT1 receptors in the cortical and medullary areas of the kidney. Specifically, we show that both cortical and medullary levels of Ang II are markedly higher in adult male mRen(2).Lewis rats with established hypertension while the immediate precursor Ang I is substantially lower. We also demonstrate the predominant expression of AT1 receptors in the nuclear fraction versus the plasma membrane isolated from the renal cortex of both

the hypertensive and normotensive Lewis strains; however, the density of these nuclear sites was significantly lower in the hypertensive strain. Although AT1 receptor density values in the nuclear and plasma membrane fractions from the renal medulla of both strains were similar, the plasma membrane sites were also lower in the mRen(2).Lewis strain. Indeed, the reduced receptor expression in both cortical and medullary areas of the mRen(2).Lewis may reflect a compensatory response to dampen the high intrarenal content of Ang II and the sustained increase in blood pressure.

Circulating Angiotensins The mRen(2).Lewis was established from the backcross of the outbred (Ren2)27 strain originally developed by Mullins and colleagues (48) in Sprague Dawley rats into the inbred Lewis line across nine generations. As previously documented, the congenic mRen(2).Lewis exhibit gender-dependent differences in the extent of hypertension that is at least partially dependent on the expression of ovarian hormones (3, 12, 17). Moreover, the hypertension in both male and female mRen(2).Lewis is corrected by blockade of the RAS (12). In the adult mRen(2).Lewis with established hypertension (MAP > 190 mm Hg), the circulating levels of Ang II were similar to the Lewis strain; however, plasma Ang-(1-7) was elevated approximately three-fold and Ang I levels were significantly lower. In male heterozygous (Ren2)27, the plasma Ang II levels were either unchanged or reduced (38, 39, 47), but both male and female homozygous (Ren2)27 exhibit higher Ang II as compared to the SD strain (9, 60, 61). The extent that plasma Ang II contributes to the mRen(2).Lewis is not known, although the sustained levels of circulating Ang II are clearly inappropriate given the elevated blood pressure. The increased Ang-(1-7)/Ang I in the mRen(2).Lewis may reflect the enhanced conversion of circulating Ang I to Ang-

(1-7) as a compensatory mechanism for the elevated blood pressures. This pathway may serve to both increase Ang-(1-7) and prevent greater conversion of Ang I to Ang II. The potential enzymes that may contribute to formation of circulating Ang-(1-7) from Ang I include neprilysin and thimet oligopeptidase (2, 13, 69). Although the greater levels of Ang-(1-7) in the circulation as measured under the present conditions may provide protection in the mRen(2).Lewis, additional studies that utilize an Ang-(1-7) antagonist or inhibitor to block the peptide's production are required. Our previous studies in the (Ren2)27 maintained on a salt-restricted diet revealed that blockade with the Ang-(1-7) antagonist [D-Ala^7]-Ang-(1-7) or peptide sequestration by a monoclonal antibody lead to an increase in blood pressure suggesting a modulatory role for Ang-(1-7) in the setting of an activated RAS (14, 34).

Intrarenal Angiotensins In contrast to the circulation, we found that tissue Ang II levels were significantly increased in both the cortex and medulla of the mRen(2).Lewis kidney. In this case, the marked reduction in cortical Ang I of the hypertensive strain suggests an enhanced pathway for Ang I conversion to increase Ang II and maintain Ang-(1-7) concentrations. Indeed, the peptide ratio value for Ang II/Ang I was increased approximately 8-fold in the cortex and medulla of the mRen(2).Lewis strain. Our results are consistent with previous studies in both homozygous and heterozygous (Ren2)27 rats that demonstrate increased intrarenal levels of Ang II (9, 38, 61). Although the mRen(2).Lewis is a genetic model of enhanced renin gene expression, the present data suggest that enzymatic pathways other than renin may contribute to the increase in renal Ang II. The identity of this pathway is not known and studies are in progress to determine the status of ACE and renin expression in the kidney of the

mRen(2).Lewis, as well as other enzymes such as ACE2 and neprilysin that may participate in the degradation of Ang II (10, 16). In this regard, that medullary Ang I levels were reduced to a greater extent [5-fold] than the corresponding increase in Ang II [1.5 fold] may suggest alternative routes of Ang I metabolism that do not directly lead to Ang II or Ang-(1-7). Indeed, Burns and colleagues recently demonstrated that ACE2 participates in the formation of Ang-(1-9) from exogenous Ang I in isolated and perfused proximal tubules (41). To our knowledge, the present data are the first to document peptide expression of Ang II, Ang I and Ang-(1-7) in the cortical and medullary areas of kidney. Although Ang II levels were increased in both regions of the congenic strain, the overall tissue concentration of Ang II, as well as Ang I and Ang-(1-7) was significantly higher in the medulla. These data raise issue with the current view of the intrarenal RAS regarding the formation of tissue angiotensins (50). Renin and angiotensinogen are primarily found in cortical juxtaglomerular cells and proximal tubules, respectively which may contribute to cortical Ang II, but their role in the formation of medullary Ang II is not clear (32, 33, 37, 45). Navar and colleagues (49, 72, 73) have demonstrated that a significant portion of renal Ang II may arise from AT1 receptor-mediated internalization of the peptide, but this will not account for tissue levels of Ang I and may not for Ang-(1-7). Furthermore, the higher medullary Ang II content is at variance with the present results of an increased density of AT1 sites in the cortex versus the medulla. Regardless of the origin, the greater content of Ang II in the medulla may be particularly significant in regards to the peptide's influence on oxidative stress and medullary blood flow (21, 51, 52, 70). The Ang II-AT1 axis is a key factor in the regulation of NADPH oxidase, the production of oxidative radicals and the progression of hypertension,

inflammation and renal injury, although the status of these systems in the mRen(2).Lewis is not presently known (36, 56, 68, 70).

Intrarenal AT1 Receptors In addition to the expression of tissue angiotensin peptides, an equally important component of the intrarenal RAS is the corresponding receptor levels. Characterization of Ang II receptors is not straightforward given the extent of cellular heterogeneity within the kidney that typically necessitates the use of autoradiogrpahic methods. Moreover, Licea et al (42) recently found evidence for significant intracellular heterogeneity of Ang II receptors. These investigators demonstrated a considerable density of AT1 sites on isolated nuclei from the SD rat renal cortex by a sucrose density gradient method. Previous studies have identified nuclear Ang II receptors in the liver; however, the density of nuclear sites constituted a minor population in comparison to the plasma membrane fraction in hepatocytes (7, 23, 35, 62). Therefore, our initial studies utilized density gradient separation methods to obtain an enriched nuclei for Ang II receptor characterization. Consistent with the Licea study (42), we found a significant population of AT1 sites in the nuclear versus plasma membrane fractions of the renal cortex using Optiprep density medium. Immunoblots using an AT1 antibody revealed a single band of approximately 52 kDa in both fractions suggesting that the nuclear AT1 receptor is not an immature or un-glycosylated form of the protein. We confirmed similar binding characteristics for the renal nuclei isolated by differential centrifugation to that of nuclei prepared by the density gradient; we used the former method for comparison of Ang II receptor kinetic values between the Lewis and congenic strains. For both the nuclear and plasma membrane fractions, the binding to ^{125}I-Sarthan was essentially abolished by the AT1 antagonists losartan and candesartan.

In general, the cortex exhibited a higher density of nuclear sites versus the plasma membranes while the receptor density was similar for both fractions albeit lower in the medulla. In the renal cortex of the mRen(2).Lewis, we found a significant reduction in the nuclear AT1 sites and a trend for reduced sites in the plasma membrane. The K_D values were lower in both fractions of the congenic kidney and the nuclear fraction exhibited the highest affinity. In the congenic renal medulla, the plasma membrane density was significantly reduced and the nuclear sites tended to decline. The affinity constants were similar for all medullary fractions, except for the Lewis plasma membrane which exhibited a two-fold higher K_D. Thus, the overall trend was for reduced expression of AT1 sites in the kidney of the mRen(2).Lewis. Our data contrast those of Zhou et al (71) demonstrated increased renal AT1 sites in the glomerular, proximal tubular and inner stripe regions of the (Ren2)27 strain by *in vitro* autoradiography; however, homozygous transgenics were studied at an earlier age (12 weeks) and the intrarenal status of Ang II was not determined. Furthermore, film autoradiography lacks the resolution to distinguish nuclear versus plasma membrane receptor sites. Since Ang II exhibits distinct cell-specific regulation of renal AT1 receptors (29), we are currently assessing the cellular localization of the nuclear AT1 receptor in the mRen(2).Lewis and Lewis kidneys.

The functional significance of nuclear AT1 receptors within the kidney was not specifically addressed in the present study. Following binding, the AT1 receptor undergoes rapid internalization and a portion of the receptor complex may traffic to the nucleus prior to recycling back to the plasma membrane or undergo degradation (6, 18, 30, 43-44, 63-67, 72). Lu et al (44) blocked trafficking of the internalized Ang II-AT1

receptor complex in neuronal cultures with a decoy peptide against the nuclear localization sequence (NLS) of the receptor and the subsequent phosphorylation of the nuclear pore protein p62. In the kidney, the high density of AT1 sites in the nuclear fraction may reflect substantial internalization of the receptor complex subsequent to Ang II binding. Several reports suggest that AT1 internalization is required for activation of Ang II-dependent signaling pathways particularly in the proximal tubule; however, the participation of the nuclear receptor in these events is not known (5, 26, 28, 57, 58, 63). In the current study, the AT1 density in the nuclear fraction of the congenic strain was reduced despite higher tissue levels of Ang II and sustained plasma Ang II. Moreover, the density of the cortical nuclear Ang II sites was unchanged following a chronic infusion of Ang II that markedly increased blood pressure (42). Preliminary data in tissue ACE knockout mice which have markedly depleted intrarenal Ang II (8, 46) also showed no differences in nuclear AT1 receptor density between the wildtype and knockout mice (53). Alternatively, the nuclear AT1 receptor may constitute part of an intracellular RAS, a portion of AT1 receptors may traffic directly to the nuclei from the endoplasmic reticulum or Golgi compartments following synthesis. Indeed, the intracellular application or expression of Ang II can evoke cellular responses including increases in calcium and cellular hypertrophy (4, 19, 20, 22). Furthermore, Eggena and colleagues have shown that Ang II stimulates the levels of both renin and angiotensinogen mRNA in isolated nuclei of hepatocytes (24). One can speculate that nuclear Ang II receptors in the proximal tubule may be linked to the regulation of angiotensinogen and other RAS components that contribute to the local expression of this system. In this regard, the down regulation of nuclear AT1 sites in the kidney may reflect

a mechanism to dampen expression of the intrarenal RAS in the hypertensive mRen(2).Lewis rats.

Perspective

Despite numerous investigations of the renal RAS and the development and progression of hypertension, new genetic models are key in revealing novel aspects of this complex peptide system. The mRen(2).Lewis strain represents a unique congenic model of monogenetic Ang II-dependent hypertension. The relevance of the mRen(2).Lewis lies not in the origin of the hypertension in this strain, but the adaptive responses of the RAS components and downstream systems that mediate the sustained increased in blood pressure and tissue injury in adult or aged animals. Our studies in the mRen(2).Lewis strengthen the concept of an intracellular or "intracrine" RAS (54) and, perhaps more importantly suggest that regulation of this system is clearly evident. Blockade of the RAS increasingly constitutes the first line of treatment for hypertension and renal injury; the presence of an intracellular RAS within the kidney raises the issue of whether we are effectively or completely targeting the relevant system with these therapies.

Acknowledgement

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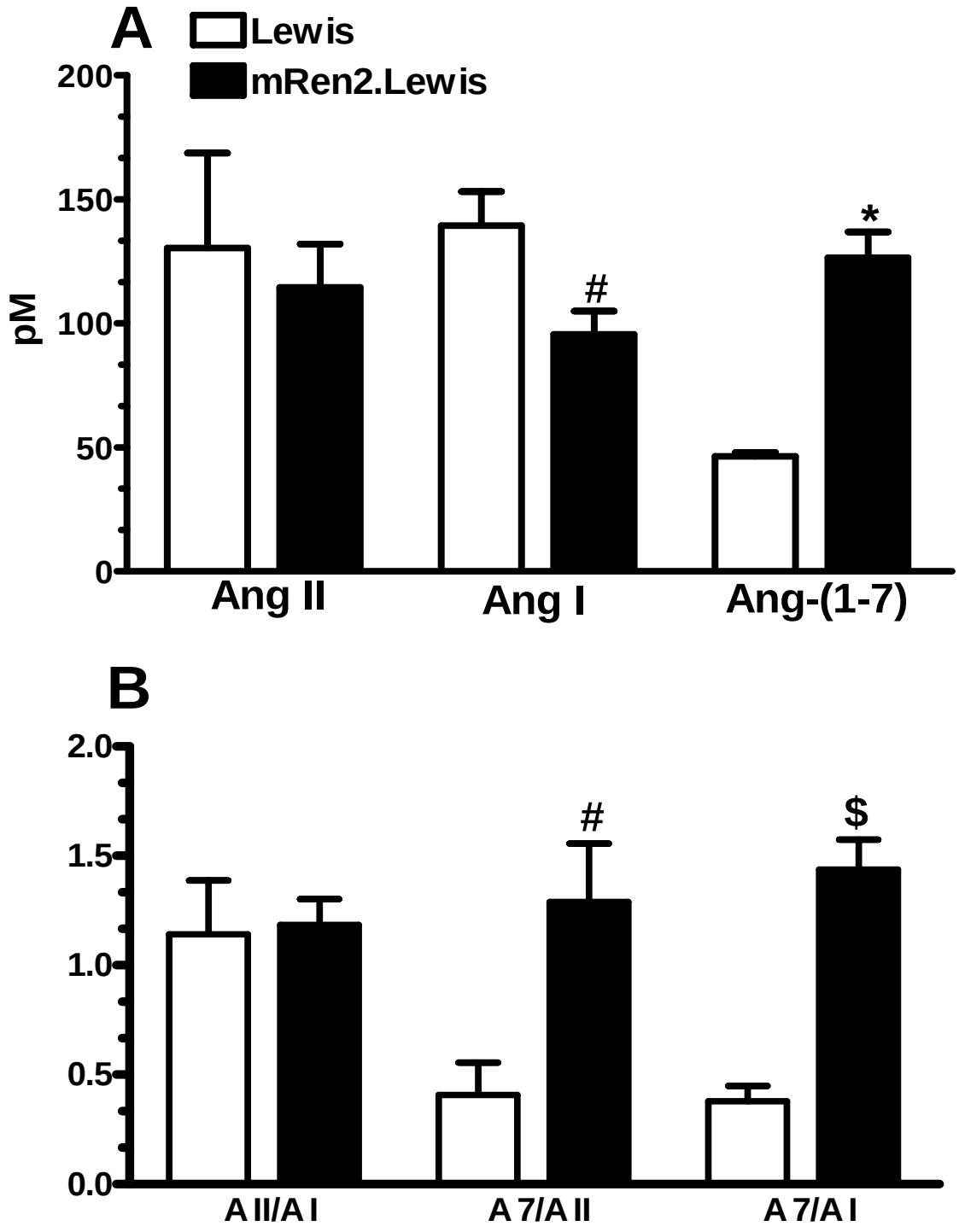
Figure Legends

Figure 1. Circulating angiotensin peptides and peptide ratios in the mRen(2).Lewis and Lewis rats. Panel A: plasma angiotensins II (Ang II), Ang I, and Ang-(1-7) were measured by separate radioimmunoassays [RIAs] and expressed as pmol/L (pM) of plasma. Panel B: plasma peptide ratios for Ang II/Ang I (AII/AI), Ang-(1-7)/Ang II (A7/AII) and Ang-(1-7)/Ang I (A7/AI) are shown. Data are the mean $\pm$ SEM, *$p<0.05$ between strains, #$p<0.01$ between strains, $^{\$}p<0.001$ between strains (n = 5 rats per group).


A
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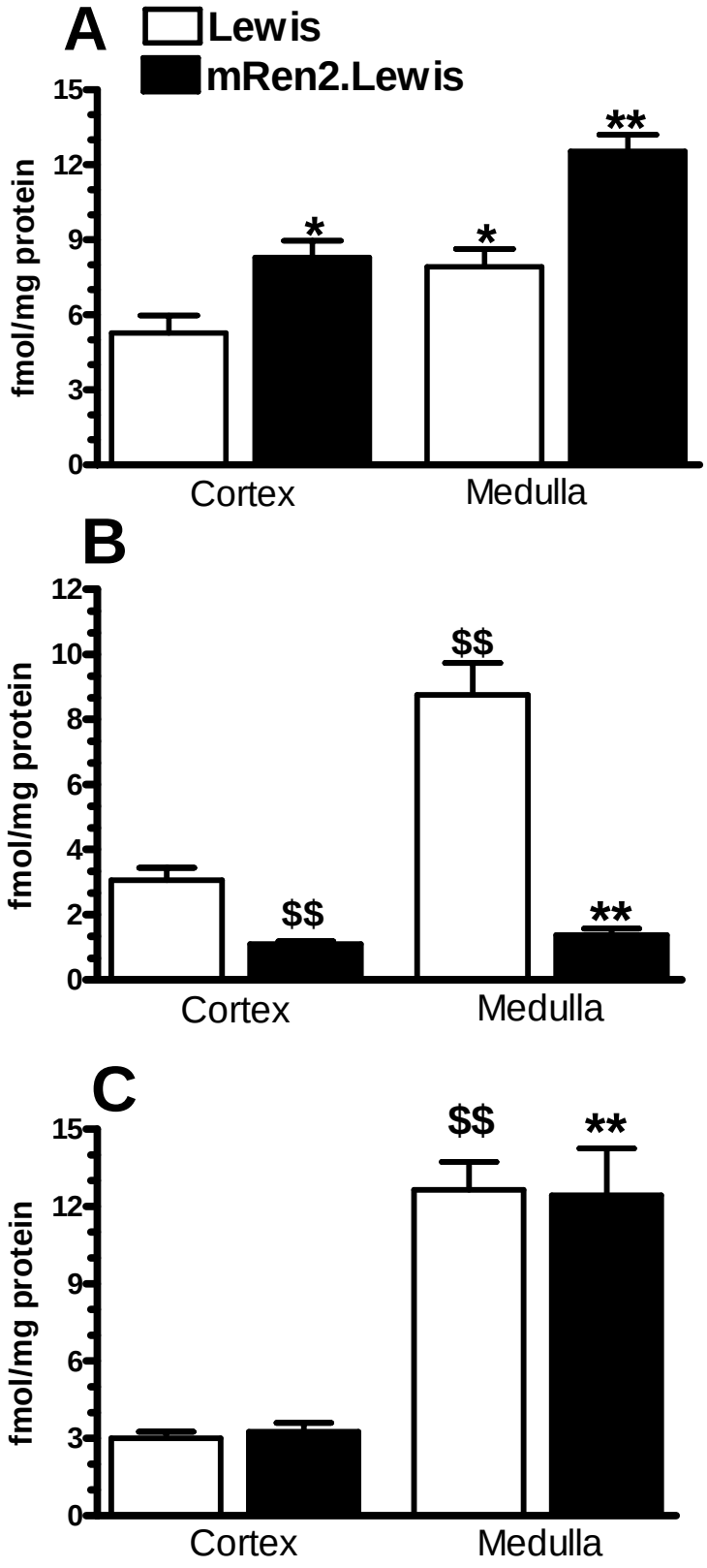
A 7/A I

Figure 1

Figure 2. Renal cortical and medullary angiotensin peptides in the mRen(2).Lewis and Lewis rats. Angiotensin II (Ang II, A), Ang I (B) and Ang-(1-7) (C) were measured by separate radioimmunoassays [RIAs] and expressed as fmol/mg protein of tissue. Data are the mean $\pm$ SEM, *$p<0.05$ versus Lewis cortex, **$p<0.01$ versus Lewis medulla, $^{\$\$}p<0.001$ versus Lewis cortex (n = 5 - 6 rats per group).


A
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mRen2.Lewis
fmol/mg protein
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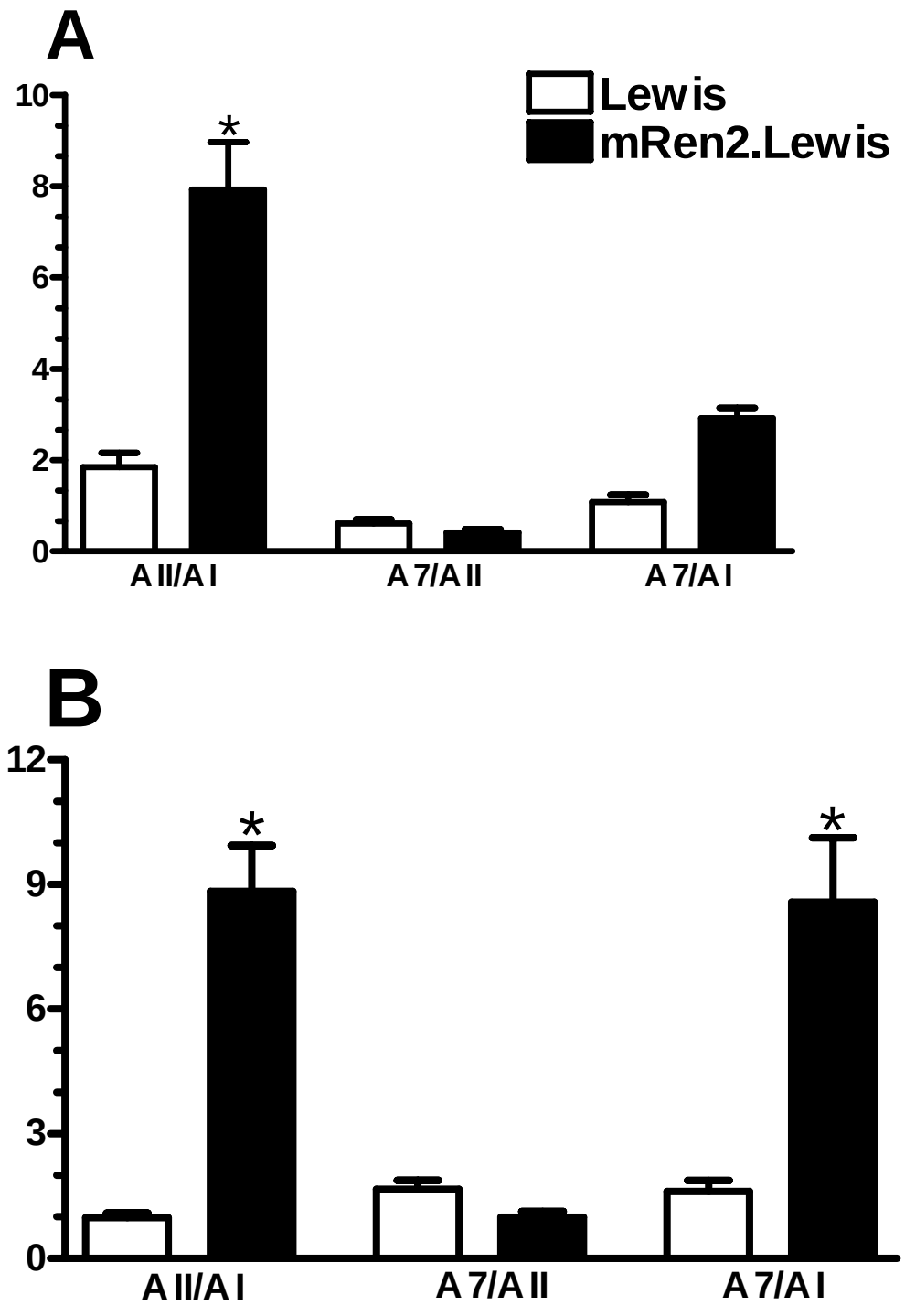
Cortex
Medulla

Figure 2

Figure 3. Comparison of the cortical (A) and medullary (B) renal angiotensin peptide ratios in the mRen(2).Lewis and Lewis rats. Peptide ratios are expressed for Ang II/Ang I (AII/AI), Ang-(1-7)/Ang II (A7/AII) and Ang-(1-7)/Ang I (A7/AI). Data are the mean $\pm$ SEM, *$p<0.05$ between strains ($n = 5 – 6$ rats per group).


A
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mRen2.Lewis
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AII/AI
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AII/AI
A7/AII
A7/AI

Figure 3

Figure 4. Representative saturation binding for Ang II receptors in the plasma membranes (A) and nuclear (B) fraction obtained from the Optiprep density gradient separation of renal cortex. Saturation binding and Scatchard analysis were performed with increasing concentrations of the specific receptor antagonist ^{125}I-[Sar^{1},thr^{8}]-Ang II [Sarthran]. Non-specific binding was obtained in the presence of 10 μM unlabeled Sarthran. The receptor affinity and density are defined as K_D and Bmax, respectively.

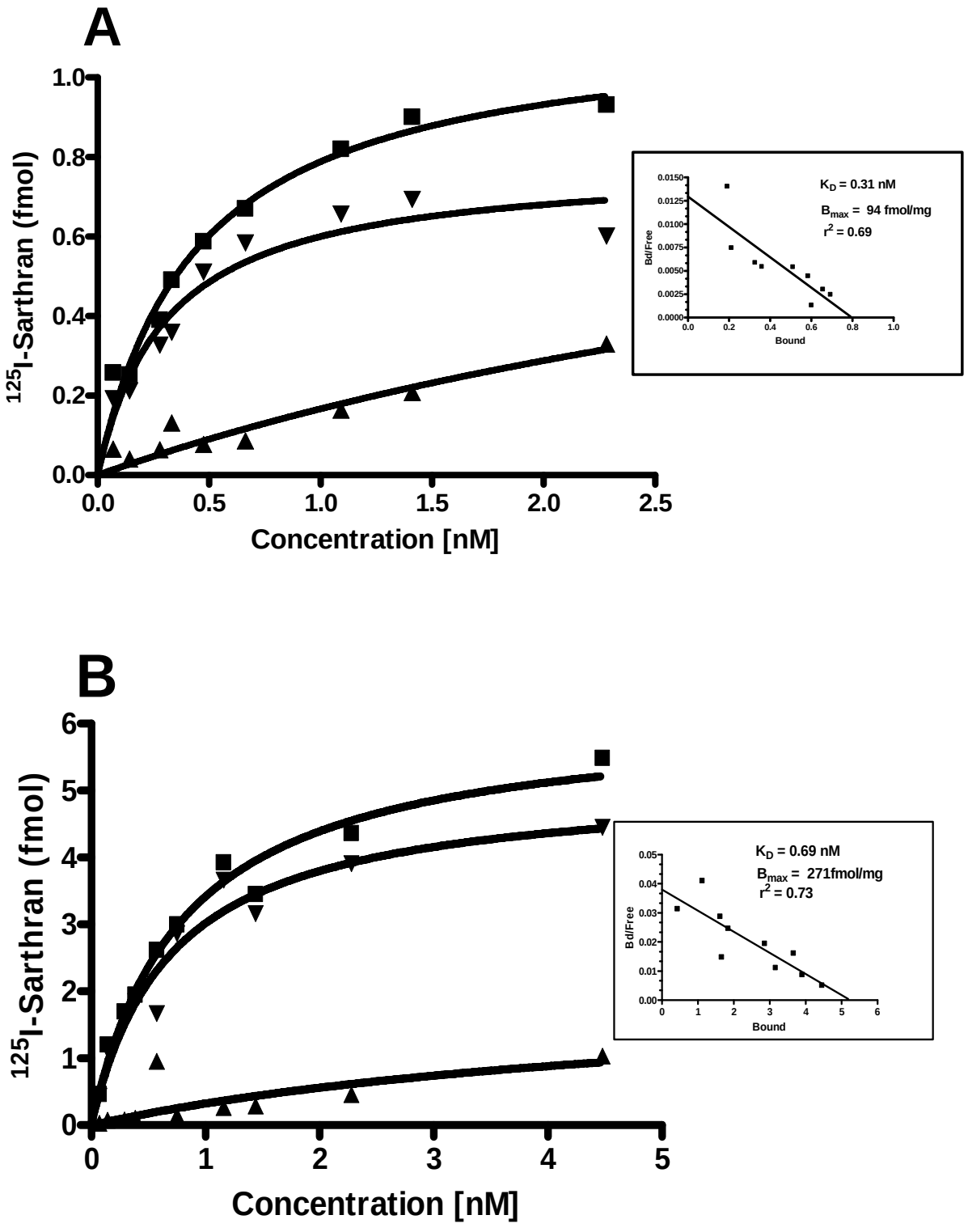

A
1.0
0.8
0.6
0.4
0.2
0.0
125I-Sarthran (fmol)
0.0
0.5
1.0
1.5
2.0
2.5
Concentration [nM]
K_D = 0.31 nM
B_max = 94 fmol/mg
r^2 = 0.69
Bd/Free
Bound
B
6
5
4
3
2
1
0
125I-Sarthran (fmol)
0
1
2
3
4
5
Concentration [nM]
K_D = 0.69 nM
B_max = 271fmol/mg
r^2 = 0.73
Bd/Free
Bound

Figure 4

Figure 5. Characterization of the receptor subtype in isolated nuclei by Optiprep density gradient. The nuclear fraction was obtained from the renal cortex of Lewis rats. Competition studies with antagonists (A) or agonists (B) utilized 0.5 nM ^{125}I-Sarthran. The antagonists included losartan (LOS), candesartan (CV), PD123319 (PD), [D-Ala^{7}]-Ang-(1-7) (D-ALA) all used at a final concentration of 10 μM. Data are the mean $\pm$ SEM, *$p<0.05$ versus specific binding in respect to Sarthran, $^{\$}p<0.05$ versus PD, $^{\#}p<0.05$ versus D-ALA (n = 3).

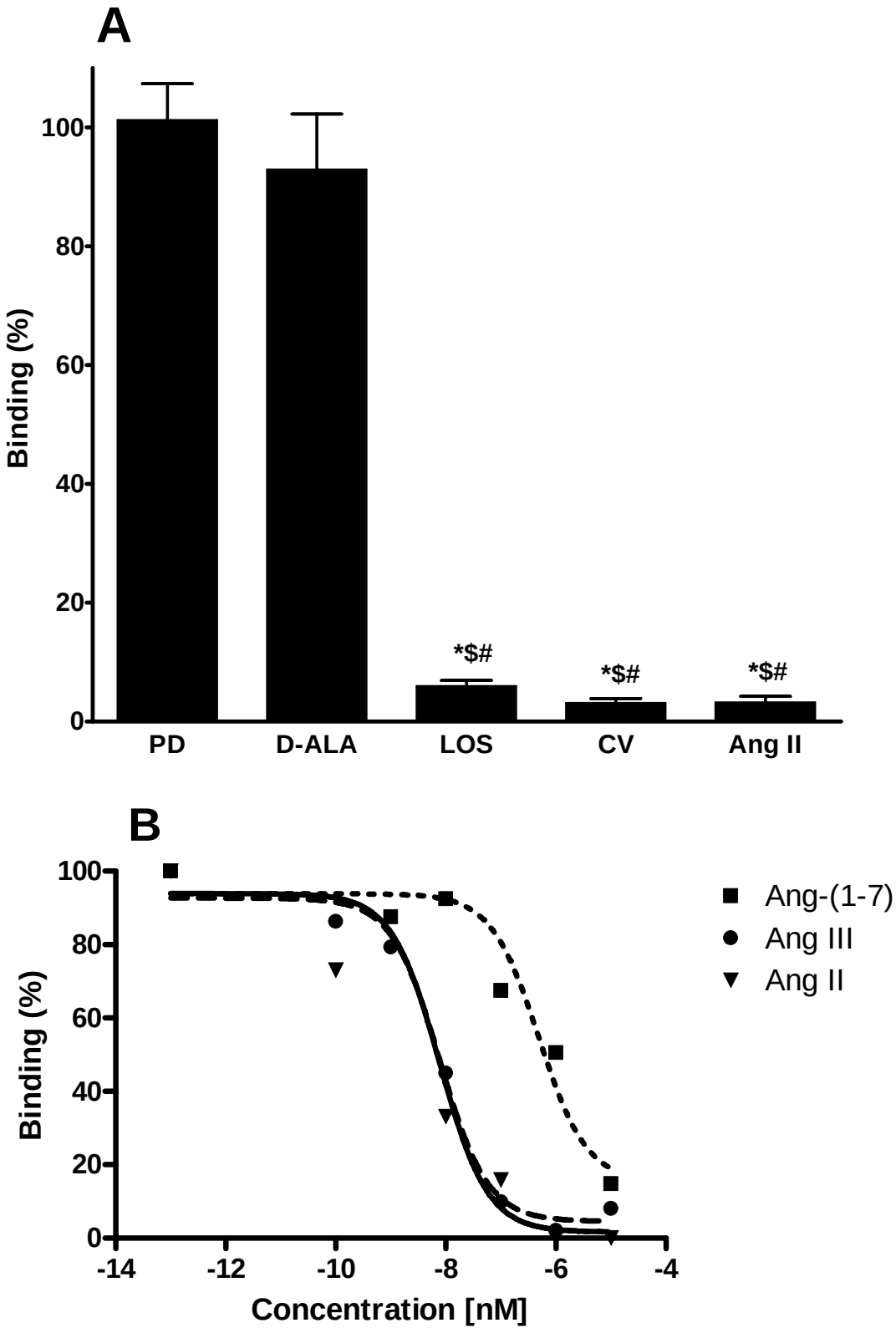
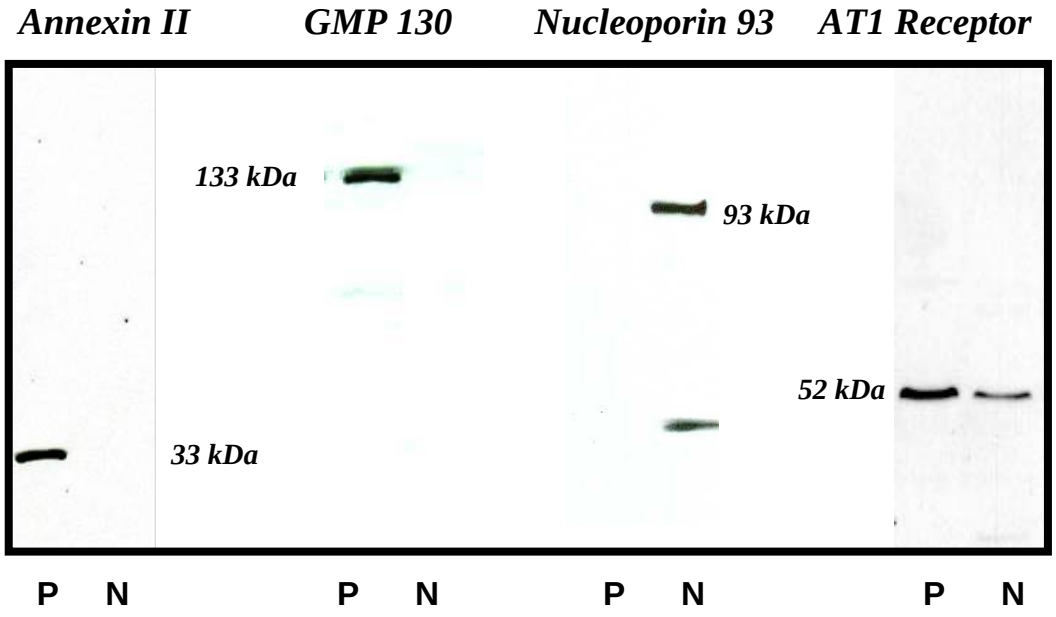

A
Binding (%)
100
80
60
40
20
0
*$#
*$#
*$#
PD
D-ALA
LOS
CV
Ang II
B
Binding (%)
100
80
60
40
20
0
-14
-12
-10
-8
-6
-4
Concentration [nM]
Ang-(1-7)
Ang III
Ang II

Figure 5

Figure 6. Immunoblot analysis of the AT1 receptor and cellular markers from the nuclear fractions obtained by either Optiprep density gradient (panel A) or differential centrifugation (panel B). Antibodies against the AT1 receptor [AT1R, 52 kDa], nucleoporin [Nuc93, 93 kDa], annexin II [33 kDa], and Golgi membrane protein [GMP130, 133 kDa] were used to analyze the nuclear (N) and plasma membrane (P) fractions. Shown are representative full-length gels from one of three density gradient preparations (A: Lewis renal cortex) and from three rats (B: mRen(2).Lewis renal medulla) by differential centrifugation.

A


Annexin II
GMP 130
Nucleoporin 93
AT1 Receptor
133 kDa
93 kDa
52 kDa
33 kDa
P N P N P N P N

B

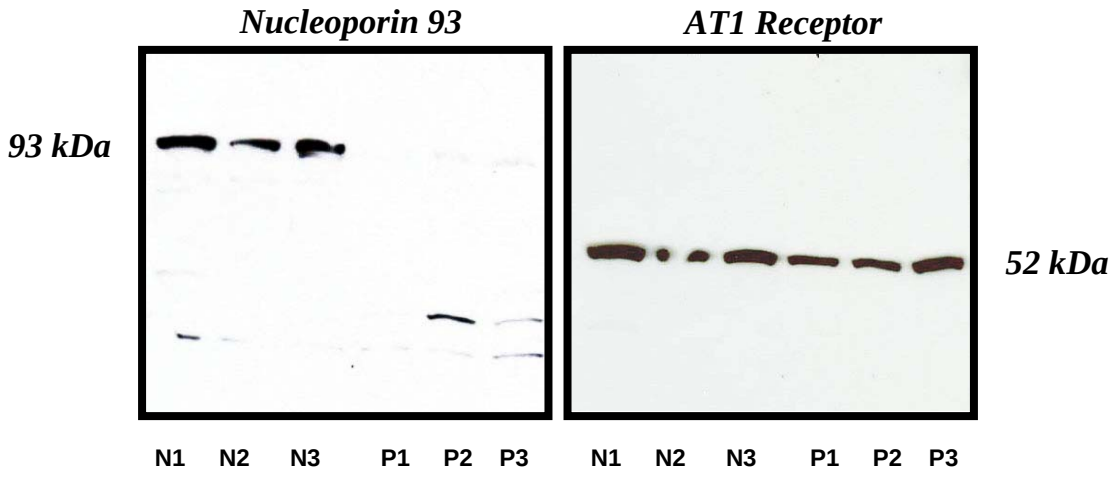

Nucleoporin 93
AT1 Receptor
93 kDa
52 kDa
N1 N2 N3 P1 P2 P3 N1 N2 N3 P1 P2 P3

Figure 6

Figure 7. Representative saturation binding for Ang II receptors in nuclear and plasma membrane fractions obtained from the density gradient separation of the renal medulla. Saturation binding and Scatchard analysis were performed with increasing concentrations of the specific receptor antagonist ^{125}I-[Sarcosine^{1},threonine^{8}]-Ang II (Sarthran). Non-specific binding was obtained in the presence of 10 μM unlabeled Sarthran. The receptor affinity and number of binding sites are defined as K_D and Bmax, respectively.

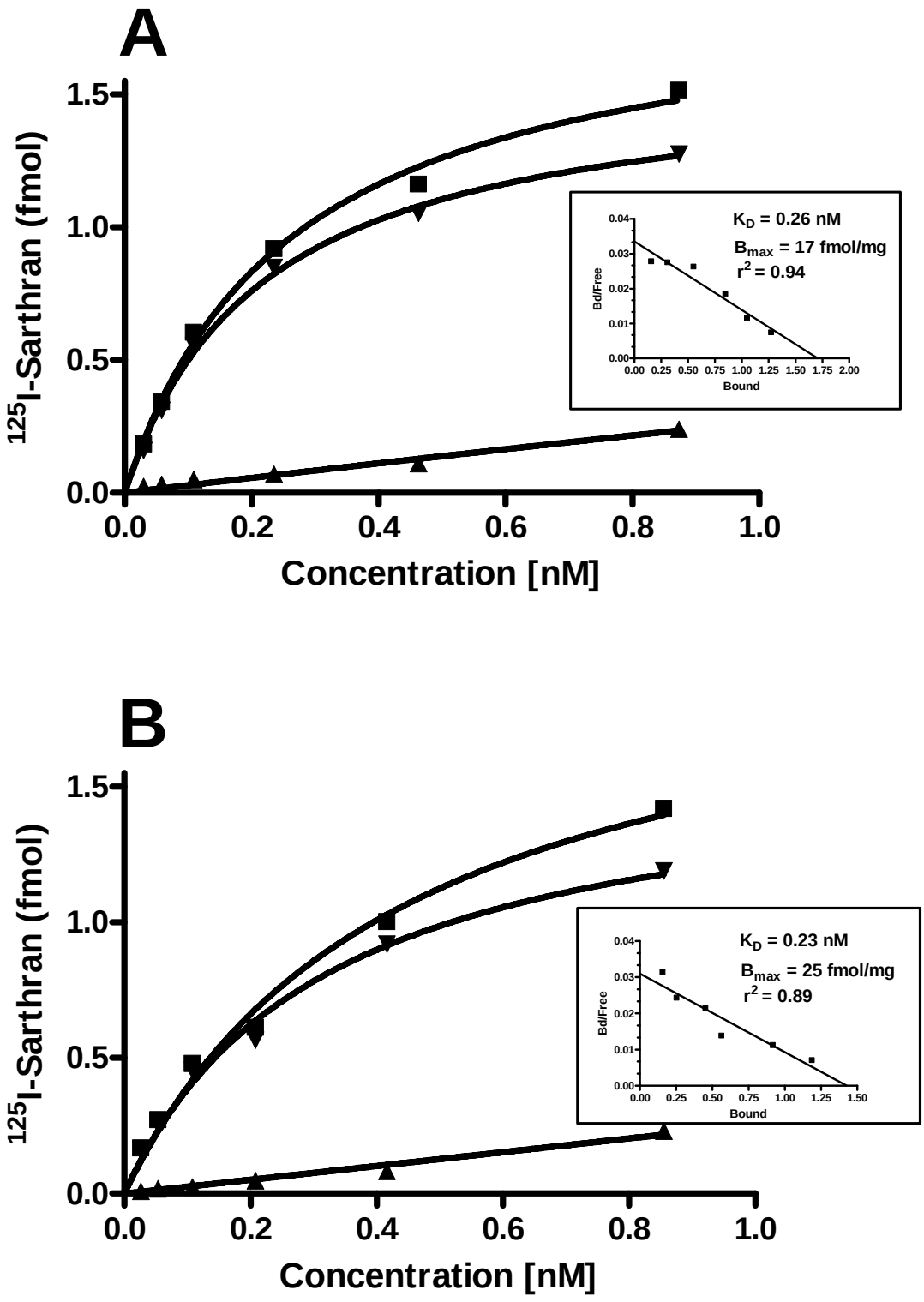

A
1.5
1.0
0.5
0.0
^{125}I-Sarthran (fmol)
0.0
0.2
0.4
0.6
0.8
1.0
Concentration [nM]
K_D = 0.26 nM
B_{max} = 17 fmol/mg
r^2 = 0.94
Bd/Free
0.04
0.03
0.02
0.01
0.00
0.00 0.25 0.50 0.75 1.00 1.25 1.50 1.75 2.00
Bound
B
1.5
1.0
0.5
0.0
^{125}I-Sarthran (fmol)
0.0
0.2
0.4
0.6
0.8
1.0
Concentration [nM]
K_D = 0.23 nM
B_{max} = 25 fmol/mg
r^2 = 0.89
Bd/Free
0.04
0.03
0.02
0.01
0.00
0.00 0.25 0.50 0.75 1.00 1.25 1.50
Bound

Figure 7

Figure 8. Characterization of renal angiotensin receptor subtype in isolated nuclei and the plasma membrane fractions obtained by differential centrifugation of the mRen(2).Lewis rat. Panels A & C: cortical and medullary nuclei. Panels B & D: cortical and medullary plasma membrane. Competition studies with receptor antagonists utilized 0.5 nM ^{125}I-Sarthran. The antagonists were losartan (LOS), candesartan (CV), PD123319 (PD), [D-Ala^{7}]-Ang-(1-7) (D-ALA) all used at a final concentration of 10 μM. Data are the mean ± SEM, *$p<0.05$ versus specific binding, $^{\$}p<0.05$ versus PD, $^{\#}p<0.05$ versus D-ALA (n = 3 for each group).

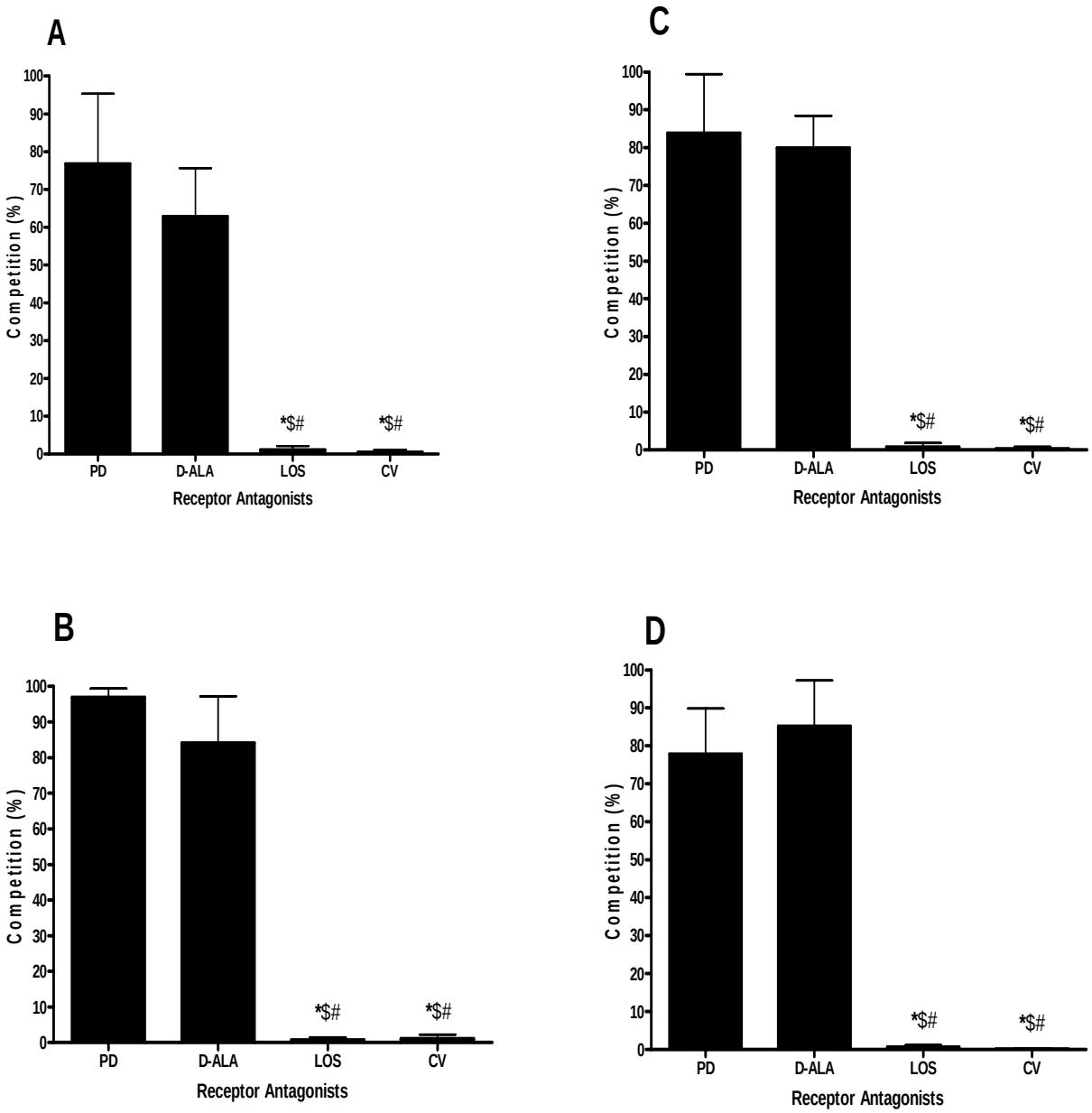


Figure 8

Figure 9. Comparison of receptor density (A) and binding affinity (B) for Ang II receptors in the nuclear and plasma membrane fractions in the renal cortex for the mRen(2).Lewis and Lewis rats. Panel A compares the receptor density or B_{max} (fmol/mg protein) in the nuclear (NUC) and plasma membrane (PM) fractions obtained by differential centrifugation. Panel B compares differences in the binding affinity or K_D (nM) in the NUC and PM fractions. Data are the mean $\pm$ SEM, *$p<0.05$ versus Lewis NUC ($n = 4$).

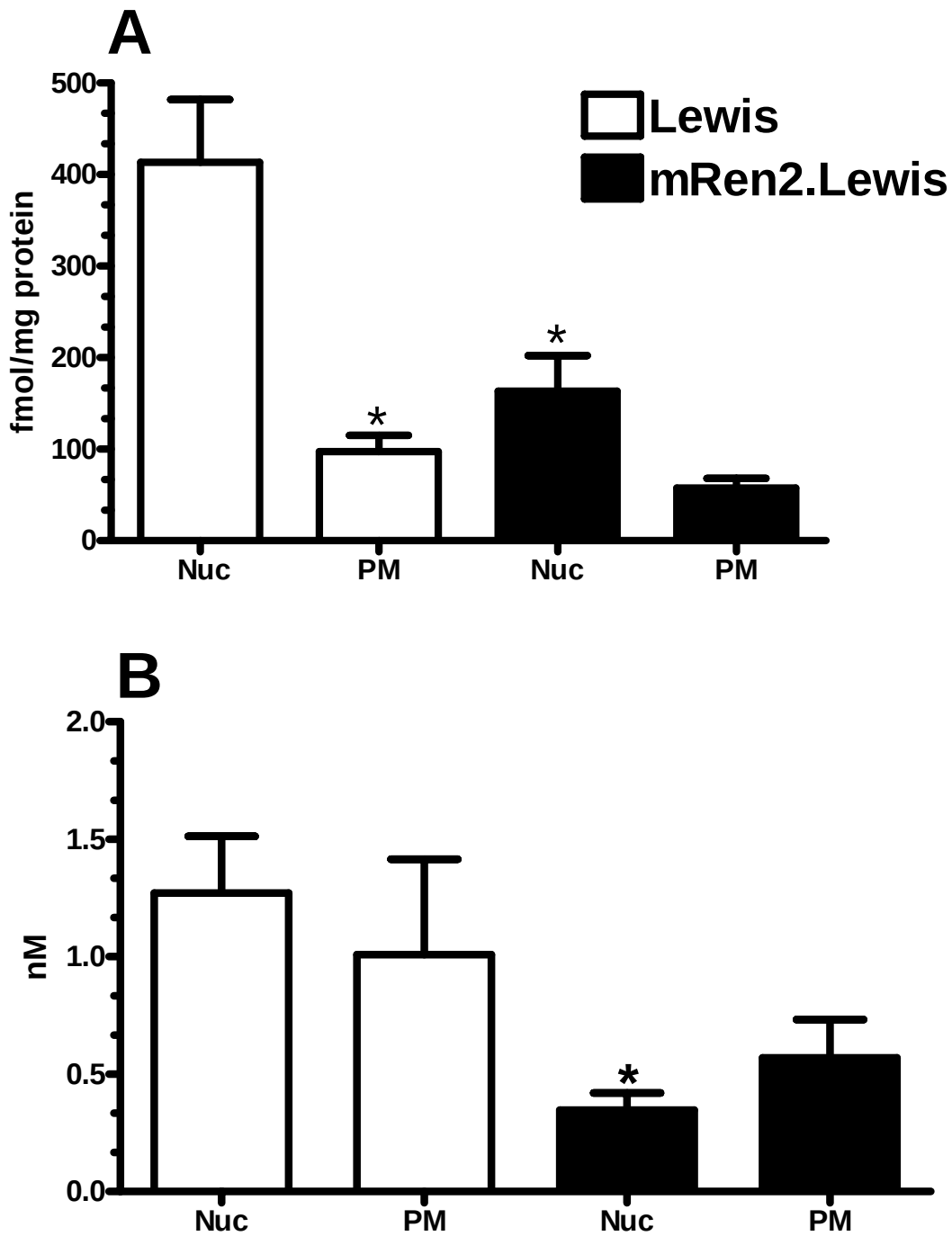

A
500
400
300
200
100
0
fmol/mg protein
Lewis
mRen2.Lewis
*
*
Nuc
PM
Nuc
PM
B
2.0
1.5
1.0
0.5
0.0
nM
*
Nuc
PM
Nuc
PM

Figure 9

Figure 10. Comparison of receptor density (A) and binding affinity (B) for Ang II receptors in the nuclear and plasma membrane fractions in the renal medulla for the mRen(2).Lewis and Lewis rats. Panel A compares the receptor density or B_{max} (fmol/mg protein) in the nuclear (NUC) and plasma membrane (PM) fractions obtained by differential centrifugation. Panel B compares differences in the binding affinity or K_D (nM) in the NUC and PM fractions. Data are the mean $\pm$ SEM, *$p<0.05$ versus Lewis PM ($n = 4$).

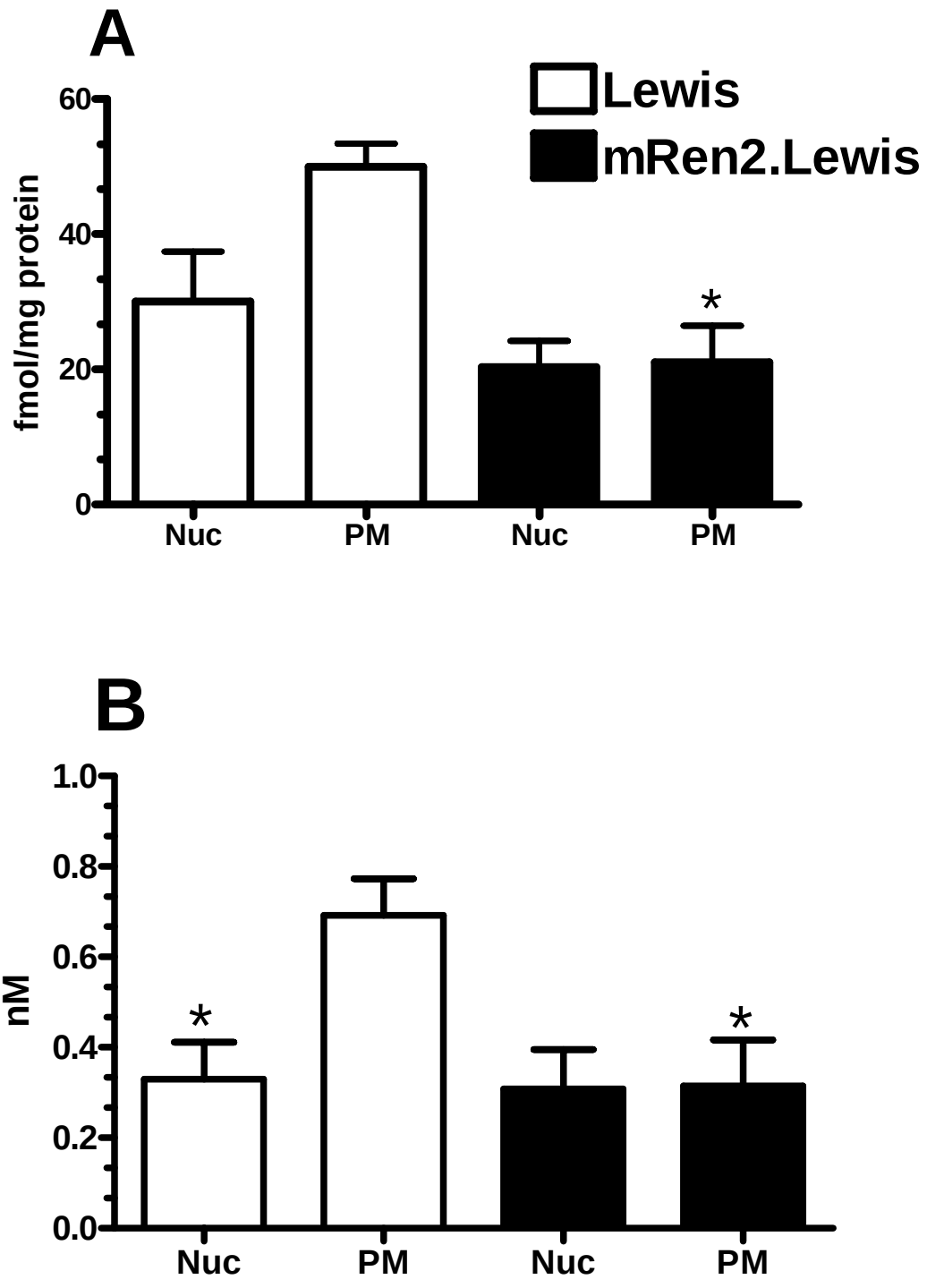

A
Lewis
mRen2.Lewis
fmol/mg protein
60
40
20
0
*
Nuc
PM
Nuc
PM
B
1.0
0.8
0.6
0.4
0.2
0.0
nM
*
*
Nuc
PM
Nuc
PM

Figure 10

Literature Cited

1. **Allred AJ, Chappell MC, Ferrario CM and Diz DI**. Differential actions of renal ischemic injury on the intrarenal angiotensin system. *Am J Physiol :Renal Physiol* 279: F636-F645, 2000.

2. **Anastasopoulos F, Leung R, Kladis A, James GM, Briscoe TA, Gorski TP and Campbell DJ**. Marked difference between angiotensin-converting enzyme and neutral endopeptidase inhibition in vivo by a dual inhibitor of both enzymes. *Journal of Pharmacology & Experimental Therapeutics* 284: 799-805, 1998.

3. **Averill DB, Sesoko S, Ganten D, and Ferrario CM.** ACE inhibition reverses hypertension of congenic mRen(2).Lewis rats. Hypertension 34; 395,. 2001. [Abstract]

4. **Baker KM, Chernin MI, Schreiber T, Sanghi S, Haiderzaidi S, Booz GW, Dostal DE and Kumar R**. Evidence of a novel intracrine mechanism in angiotensin II-induced cardiac hypertrophy. *Regul Pept* 120: 5-13, 2004.

5. **Becker BN and Harris RC**. A potential mechanism for proximal tubule angiotensin II-mediated sodium flux associated with receptor-mediated endocytosis and arachadonic acid release. *Kidney Int* 50: S66-S72, 1996.

6. **Bkaily G, Sleiman S, Stephan J, Asselin C, Choufani S, Kamal M, Jacques D, Gobeil F, Jr. and Orleans-Juste P**. Angiotensin II AT1 receptor internalization, translocation and de novo synthesis modulate cytosolic and nuclear calcium in human vascular smooth muscle cells. *Can J Physiol Pharmacol* 81: 274-287, 2003.

7. **Booz GW, Conrad KM, Hess AL, Singer HA and Baker KM**. Angiotensin-II-binding sites on hepatocyte nuclei. *Endocrinology* 130: 3641-3649, 1992.

8. **Campbell DJ, Alexiou T, Xiao HD, Fuchs S, McKinley MJ, Corvol P and Bernstein KE**. Effect of reduced angiotensin-converting enzyme gene expression and angiotensin-converting enzyme inhibition on angiotensin and bradykinin peptide levels in mice. *Hypertension* 43: 854-859, 2004.

9. **Campbell DJ, Rong P, Kladis A, Rees B, Ganten D and Skinner SL**. Angiotensin and bradykinin peptides in the TGR(mRen-2)27 rat. *Hypertension* 25: 1014-1020, 1995.

10. **Chappell MC, Allred AJ and Ferrario CM**. Pathways of angiotensin-(1-7) metabolism in the kidney. *Nephrol Dial Transplant* 16: 22-26, 2001.

11. **Chappell MC, Brosnihan KB, Diz DI and Ferrario CM**. Identification of angiotensin-(1-7) in rat brain: evidence for differential processing of angiotensin peptides. *J Biol Chem* 264: 16518-16523, 1989.

12. **Chappell MC, Gallagher PE, Averill DB, Ferrario CM and Brosnihan KB**. Estrogen or the AT1 antagonist olmesartan reverses the development of profound hypertension in the congenic mRen(2).Lewis rat. *Hypertension* 42: 781-786, 2003.

13. **Chappell MC, Gomez MN, Pirro NT and Ferrario CM**. Release of angiotensin-(1-7) from the rat hindlimb: influence of angiotensin-converting enzyme inhibition. *Hypertension* 35: 348-352, 2000.

14. **Chappell MC, Iyer SN, Diz DI and Ferrario CM**. Antihypertensive effects of angiotensin-(1-7). *Braz J Med Biol Res* 31: 1205-1212, 1998.

15. **Chappell MC, Jacobsen DW and Tallant EA**. Characterization of angiotensin II receptor subtypes in pancreatic acinar AR42J cells. *Peptides* 16: 741-747, 1995.

16. **Chappell MC, Modrall JG, Diz DI and Ferrario CM**. Novel aspects of the renal renin-angiotensin system: angiotensin-(1-7), ACE2 and blood pressure regulation. In: Kidney and Blood Pressure Regulation, edited by Suzuki H and Saruta T. Basel, Karger, 2004, p. 77-89.

17. **Chappell MC, Westwood BM, Averill DB, Ferrario CM, Brosnihan KB, and Gallagher PE.** Influence of gender on salt-sensitivity and dysregulation of the renin-angiotensin system in the mren2. Lewis rat. Hypertension 42; 430. 2003. [Abstract]

18. **Chen R, Mukhin YV, Garnovskaya MN, Thielen TE, Iijima Y, Huang C, Raymond JR, Ullian ME and Paul RV**. A functional angiotensin II receptor-GFP fusion protein: evidence for agonist-dependent nuclear translocation. *Am J Physiol Renal Physiol* 279: F440-F448, 2000.

19. **Cook JL, Re R, Alam J, Hart M and Zhang Z**. Intracellular angiotensin II fusion protein alters AT1 receptor fusion protein distribution and activates CREB. *J Mol Cell Cardiol* 36: 75-90, 2004.

20. **Cook JL, Zhang Z and Re RN**. In vitro evidence for an intracellular site of angiotensin action. *Circ Res* 89: 1138-1146, 2001.

21. **Cowley AW, Jr., Mattson DL, Lu SH and Roman RJ**. The renal medulla and hypertension. *Hypertension* 25: 663-673, 1995.

22. **De Mello WC.** Influence of intracellular renin on heart cell communication. Hypertension 25:1172-1177, 1995.

23. **Eggena P, Zhu JH, Clegg K and Barrett JD**. Nuclear angiotensin receptors induce transcription of renin and angiotensinogen mRNA. *Hypertension* 22: 496-501, 1993.

24. **Eggena P, Zhu JH, Sereevinyayut S, Giordani M, Clegg K, Andersen PC, Hyun P and Barrett JD**. Hepatic angiotensin II nuclear receptors and transcription of growth-related factors. *J Hypertens* 14: 961-968, 1996.

25. **German DC, Ng MC, Liang CL, McMahon A and Iacopino AM**. Calbindin-D28k in nerve cell nuclei. *Neuroscience* 81: 735-743, 1997.

26. **Greindling KK, Delatontaine P, Rittenhouse SE, Gimbrone MA, Alexander RW.** Correlation of receptor sequestration with sustained diacylglycerol accumulation in angiotensin II- stimulated cultured smooth muscle cells. *J Biol Chem* 262:14555-14562, 1987.

27. **Haller H, Lindschau C, Erdmann B, Quass P, Luft FC.** Effects of intracellular angiotensin II in vascular smooth muscle cells. *Circ Res* **79: 765-772, 1996.**

28. **Harris RC**. Potential mechanisms and physiologic actions of intracellular angiotensin II. *Am J Med Sci* 318: 374-379, 1999.

29. **Harrison-Bernard LM, Zhou J, Kobori H, Ohishi M and Navar LG**. Intrarenal at1 receptor and ace binding in Ang II-induced hypertensive rats. *Am J Physiol Renal Physiol* 281: F19-F25, 2002.

30. **Hein L, Meinel L, Pratt RE, Dzau VJ and Kobilka BK**. Intracellular trafficking of angiotensin II and its AT1 and AT2 receptors: evidence for selective sorting of receptor and ligand. *Mol Endocrinol* 11: 1266-1277, 1997.

31. **Imig JD, Navar GL, Zou LX, O'Reilly KC, Allen PL, Kaysen JH, Hammond TG and Navar LG**. Renal endosomes contain angiotensin peptides, converting enzyme, and AT(1A) receptors. *Am J Physiol* 277: F303-F311, 1999.

32. **Ingelfinger J, Pratt RE, Ellison KE and Dzau VJ**. Angiotensinogen gene expression in rat kidney, evidence of sodium regulation of an intrarenal renin angiotensin system. *J Clin Invest* 78: 1311-1315, 1986.

33. **Ingelfinger JR, Zuo WM, Fon EA, Ellison KE and Dzau VJ**. In situ hybridization evidence for angiotensinogen messenger RNA in the rat proximal tubule. *J Clin Invest* 85: 417-423, 1990.

34. **Iyer SN, Averill DB, Chappell MC, Yamada K, Jones AG and Ferrario CM**. Contribution of angiotensin-(1-7) to blood pressure regulation in salt-depleted hypertensive rats. *Hypertension* 36: 417-422, 2000.

35. **Jimenez E, Vinson GP, Montiel M.** Angiotensin II binding sites in nuclei from rat liver: partial characterization for the mechanism of Ang II accumulation in nuclei. *J Endocrinol* 143: 449-453, 1994.

36. **Johnson RJ, Alpers CE, Yoshimura A, Lombardi D, Pritzl P, Floege J and Schwartz SM**. Renal injury from angiotensin II-mediated hypertension. *Hypertension* 19: 464-474, 1992.

37. **Kobori H, Harrison-Bernard LM and Navar LG**. Enhancement of angiotensinogen expression in angiotensin II-dependent hypertension. *Hypertension* 37: 1329-1335, 2001.

38. **Kopkan L, Kramer HJ, Huskova Z, Vanourkova Z, Backer A, Bader M, Ganten D and Cervenka L**. Plasma and kidney angiotensin II levels and renal functional responses to AT(1) receptor blockade in hypertensive Ren-2 transgenic rats. *J Hypertens* 22: 819-825, 2004.

39. **Kreutz R, Fernandez-Alfonso MS, Paul M and Peters J**. Differential development of early hypertension in heterozygous transgenic TGR(mREN2)27 rats. *Clin Exp Hypertens* 20: 273-282, 1998.

40. **Lee DK, Lanca AJ, Cheng R, Nguyen T, Ji XD, Gobeil F, Jr., Chemtob S, George SR and O'Dowd BF**. Agonist-independent nuclear localization of the apelin, angiotensin AT1, and bradykinin B2 receptors. *J Biol Chem* 279: 7901-7908, 2004.

41. **Li N, Zimpelmann J, Cheng K, Wilkins JA and Burns KD**. The role of angiotensin converting enzyme 2 in the generation of angiotensin 1-7 by rat proximal tubules. *Am J Physiol Renal Physiol* 2004.

42. **Licea H, Walters MR and Navar G**. Renal nuclear angiotensin II receptors in normal and hypertensive rats. *Acta Physiol Hungarica* 89: 427-438, 2002.

43. **Lu D, Yang H, Shaw G and Raizada MK**. Angiotensin II-induced nuclear targeting of the angiotensin type 1 (AT1) receptor in brain neurons. *Endocrinology* 139: 365-375, 1998.

44. **Lu D, Yang H and Raizada MK**. Involvement of p62 nucleoporin in Angiotensin II-induced nuclear translocation of STAT3 in brain neurons. *J Neurosci* 18: 1329-1336, 1998.

45. **Mercure C, Ramla D, Garcia R, Thibault G, Deschepper CF and Reudelhuber TL**. Evidence for intracellular generation of angiotensin II in rat juxtaglomerular cells. *FEBS Lett* 422: 395-399, 1998.

46. **Modrall JG, Sadjadi S, Hua CH, Kramer GL, Brosnihan KB, Gallagher PE, Bernstein KE, Chappell MC**. Depletion of tissue ACE differentially influences the intrarenal and urinary expression of angiotensin peptides. Hypertension 43:849-853, 2004.

47. **Moriguchi A, Brosnihan KB, Kumagai H, Ganten D and Ferrario CM**. Mechanisms of hypertension in transgenic rats expressing the mouse Ren-2 gene. *Am J Physiol* 266: R1273-R1278, 1994.

48. **Mullins JJ, Peters J and Ganten D**. Fulminant hypertension in transgenic rats harbouring the mouse Ren-2 gene. *Nature* 344: 541-544, 1990.

49. **Navar LG, Harrison-Bernard LM, Nishiyama A and Kobori H**. Regulation of intrarenal angiotensin II in hypertension. *Hypertension* 39: 316-322, 2002.

50. **Navar LG, Imig JD, Zou L and Wang CT**. Intrarenal production of angiotensin II. [Review] [79 refs]. *Seminars in Nephrology* 17: 412-422, 1997.

51. **Onozato ML, Tojo A, Goto A, Fujita T and Wilcox CS**. Oxidative stress and nitric oxide synthase in rat diabetic nephropathy: effects of ACEI and ARB. *Kidney Int* 61: 186-194, 2002.

52. **Pallone TL, Zhang Z and Rhinehart K**. Physiology of the renal medullary microcirculation. *Am J Physiol Renal Physiol* 284: F253-F266, 2003.

53. **Pendergrass KD, Bernstein KE, Modrall JG and Chappell MC.** Chronic depletion of renal angiotensin II does not influence the intracellular distribution of the renal AT1 receptor. Hypertension 44; 558, 2004 [Abstract].

54. **Re RN**. Implications of intracrine hormone action for physiology and medicine. *Am J Physiol Heart Circ Physiol* 284: H751-H757, 2003.

55. **Robertson AL and Khailrallah PA**. Angiotensin II: rapid localization in nuclei of smooth and cardiac muscle. *Science* 72: 1138-1139, 1971.

56. **Rodriguez-Iturbe B, Vaziri ND, Herrera-Acosta J and Johnson RJ**. Oxidative stress, renal infiltration of immune cells, and salt-sensitive hypertension: all for one and one for all. *Am J Physiol Renal Physiol* 286: F606-F616, 2004.

57. **Schelling JR, Hanson AS, Marzec R and Linas SL**. Cytoskeleton-dependent endocytosis is required for apical type 1 angiotensin II receptor-mediated phospholipase C activation in cultured rat proximal tubule cells. *J Clin Invest* 90: 2472-2480, 1992.

58. **Schelling JR and Linas SL.** Angiotensin II-dependent proximal tubule sodium transport requires receptor-mediated endocytosis. *Am J Physiol* 266: C669-C675, 1994.

59. **Schunkert H, Ingelfinger JR, Jacob H, Jackson B, Bouyounes B and Dzau VJ**. Reciprocal feedback regulation of kidney angiotensinogen and renin mRNA expressions by angiotensin II. *Am J Physiol* 263: E863-E869, 1992.

60. **Senanayake PD, Moriguchi A, Kumagai H, Ganten D, Ferrario CM and Brosnihan KB**. Increased expression of angiotensin peptides in the brain of transgenic hypertensive rats. *Peptides* 15: 919-926, 1994.

61. **Senanayake PS, Smeby RR, Martins AS, Moriguchi A, Kumagai H, Ganten D and Brosnihan KB**. Adrenal, kidney, and heart angiotensins in female murine Ren-2 transfected hypertensive rats. *Peptides* 19: 1685-1694, 1998.

62. **Tang SS, Rogg H, Schumacher R and Dzau VJ**. Characterization of nuclear angiotensin-II-binding sites in rat liver and comparison with plasma membrane receptors. *Endocrinology* 131: 374-380, 1992.

63. **Thekkumkara T and Linas SL**. Role of internalization in AT(1A) receptor function in proximal tubule epithelium. *Am J Physiol Renal Physiol* 282: F623-F629, 2002.

64. **Thomas WG, Thekkumkara TJ and Baker KM**. Molecular mechanisms of angiotensin II (AT1A) receptor endocytosis. *Clinical & Experimental Pharmacology & Physiology - Supplement* 3:S74-80: S74-S80, 1996.

65. **Ullian ME and Linas SL**. Role of receptor recycling in the regulation of angiotensin II surface receptor number and angiotensin II uptake in rat vascular smooth muscle cells. *J Clin Invest.* 84:840-846, 1989.

66. **Van Kats JP, de Lannoy LM, Jan Danser A.H Van Meegan JR, Verdouw PD and Schalekamp, MA.** Angiotensin II type 1 (AT1) receptor-mediated accumulation of angiotensin II in tissues and its intracellular half-life in vivo. Hypertension 30:42-49, 1997.

67. **Van Kats, JP, Schalekamp, MA, Verdouw PD, Duncker DJ and Jan Danser A.H.** Intrarenal angiotensin II: Interstitial and cellular levels and site of production. *Kidney International* 60: 2311-2317, 2001.

68. **Wilcox CS and Gutterman D**. Focus on oxidative stress in the cardiovascular and renal systems. *Am J Physiol Heart Circ Physiol* 288: H3-H6, 2005.

69. **Yamamoto K, Chappell MC, Brosnihan KB and Ferrario CM**. In vivo metabolism of angiotensin I by neutral endopeptidase (EC 3.4.24.11) in spontaneously hypertensive rats. *Hypertension* 19: 692-696, 1992.

70. **Yuan B, Liang M, Yang Z, Rute E, Taylor N, Olivier M and Cowley AW, Jr.** Gene expression reveals vulnerability to oxidative stress and interstitial fibrosis of renal outer medulla to nonhypertensive elevations of ANG II. *Am J Physiol Regul Integr Comp Physiol* 284: R1219-R1230, 2003.

71. **Zhuo J, Ohishi M and Mendelsohn FA**. Roles of AT1 and AT2 receptors in the hypertensive Ren-2 gene transgenic rat kidney. *Hypertension* 33: 347-353, 1999.

72. **Zhuo JL, Imig JD, Hammond TG, Orengo S, Benes E and Navar LG**. Ang II accumulation in rat renal endosomes during Ang II-induced hypertension: Role of AT(1) Receptor. *Hypertension* 39: 116-121, 2002.

73. **Zou L-X, Imig JD, Hymel A and Navar LG**. Renal uptake of circulating angiotensin II in Val^5-angiotensin II infused rats is mediated by AT_1 receptor. *Am J Hypertens* 11: 570-578, 1998.

CHAPTER THREE

SEX DIFFERENCES IN CIRCULATING AND RENAL ANGIOTENSINS OF HYPERTENSIVE MREN(2).LEWIS BUT NOT NORMOTENSIVE LEWIS RATS

BY

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Short title: ***Sex differences in RAAS expression***

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ABSTRACT

Sex differences in blood pressure are evident in experimental models and human subjects, yet the mechanisms underlying this disparity remain equivocal. The current study sought to define the extent of male-female differences in the circulating and tissue renin-angiotensin aldosterone systems (RAAS) of the congenic mRen(2).Lewis and the control Lewis rats. Male congenics exhibited higher systolic blood pressure than females [200 ± 4 vs. 146 ± 7 mmHg, $p < 0.01$] or Lewis males and females [113 ± 2 vs. 112 ± 2 mmHg, $p > 0.05$]. Plasma Ang II levels were 2-fold higher in male congenics [47 ± 3 vs. 19 ± 3 pM, $p < 0.01$] and 5-fold higher than male or female Lewis [6 ± 1 vs. 6 ± 1 pM]. Ang I levels were also highest in the males; however, plasma Ang-(1-7) was higher in the female congenics. Male congenics exhibited greater circulating renin and ACE activities, as well as angiotensinogen than female littermates. Renal cortical and medullary Ang II levels were also higher in the male congenics versus all other groups; Ang I was lower in the males. Cortical ACE2 activity was higher in male congenics, yet neprilysin activity and protein were greater in the females which may contribute to reduced renal levels of Ang II. These data reveal that sex differences in both the circulating and renal RAAS are apparent primarily in the hypertensive group. The enhanced activity of the RAAS in male congenics may contribute to the higher pressure and tissue injury evident in the strain.

Key words: Ang II, ACE, Ang-(1-7), estrogen, ACE2, neprilysin, cardiac hypertrophy, proteinuria.

INTRODUCTION

Similar to other experimental models of high blood pressure, the congenic mRen(2).Lewis strain exhibits significant sex differences in the extent of hypertension and renal injury (21; 42). The mRen(2).Lewis rat was derived from the mRen2.(27) Sprague Dawley rat originally developed by Mullins et al (38) as a model of tissue renin expression and was backcrossed into the Lewis strain to yield the new congenic model. The hemizygous male mRen(2).Lewis rats exhibit systolic blood pressures 50-60 mmHg higher than their female littermates, although, blockade of the renin-angiotensin-aldosterone system (RAAS) lowers blood pressure to similar levels (17; 32). In contrast to the spontaneously hypertensive rat (SHR), estrogen depletion by ovariectomy in young mRen(2).Lewis rats significantly exacerbates the hypertension (21). Moreover, estrogen replacement or treatment with an AT_1 receptor antagonist reduce blood pressure to a similar extent and clearly supports a protective role for estrogen in this hypertensive model (17). Indeed, the cardioprotective effects of estrogen replacement in the mRen(2).Lewis, an Ang II dependent model of hypertension, are consistent with its actions to directly attenuate key components of the RAAS including ACE and the AT_1 receptor (12; 40; 45), as well as increase competing components such as ACE2 and the AT_2 receptor or influence other signaling pathways (nitric oxide, prostaglandins) that may converge on the RAAS (3; 11; 41; 60; 65). Androgens, however, may also have a significant effect on cardiovascular regulation to increase blood pressure and accelerate renal injury that is associated with alterations of the RAAS including increased expression of renin, angiotensinogen and ACE (6; 8; 9; 47). Furthermore, estrogen may

exhibit additional effects on the RAAS including stimulation of renin and angiotensinogen that in some instances may promote an increase in blood pressure (12).

Typically, the characterization of sex-based differences in experimental models of hypertension has focused on the components of the circulating RAAS such as Ang II, Ang-(1-7) and ACE (10) or receptor expression in various target tissues (28; 63). Current evidence strongly supports the existence of local or tissue RAAS in multiple organs that may have a significant if not greater impact than the circulating system. However, to our knowledge no studies have evaluated gender-dependent differences in the tissue expression of angiotensin peptides, particularly Ang II and Ang-(1-7) in the mRen(2).Lewis or its founder strain, the transgenic mRen2.(27) Sprague Dawley. Moreover, the balance of these two peptides which is likely influenced by the post-renin processing enzymes ACE, ACE2 and neprilysin within various tissues, may well contribute to the development and progression of hypertension and tissue damage (10; 19). Therefore, we tested the hypothesis as to whether there is an imbalance in the expression of Ang II and Ang-(1-7) within the circulation, heart, and renal compartments of the mRen(2).Lewis that is associated with the markedly higher blood pressure and organ injury particularly evident in the male hypertensive strain.

MATERIALS AND METHODS

Experimental animals. Hemizygous male and female mRen(2).Lewis (congenic) rats were obtained from the Hypertension and Vascular Disease Center Transgenic colony (21; 42) at 14-15 weeks of age. Normotensive male and female Lewis rats were purchased from Charles River (Raleigh, NC) and were age matched with the congenic

rats. Animals were fed a powdered rat chow (Purina Mills, Richmond,VA) to provide a daily intake of 17 and 28 meq/100 g of body wt of sodium and potassium, respectively, had full access to water, and were housed in an AALAC-approved facility in rooms maintained on a 12:12-hour light-dark cycle (lights on 6:00 A.M. to 6:00 P.M.). Animals were housed in metabolic cages (Harvard Bioscience, South Natick, MA) for a 24 hour collection period and systolic blood pressure was measured with a Narco Biosystems device (Houston, TX) (42). These procedures were approved by the Wake Forest University School of Medicine Institutional Animal Care and Use Committee.

Plasma and renal tissue hormone assays. Rats were decapitated without anesthesia. Trunk blood (3 to 5 ml) was collected into chilled Vacutainer blood collection tubes (Becton Dickinson, Sandy, UT) for plasma renin or in tubes containing peptidase inhibitors and processed for direct radioimmunoassay (RIA) of angiotensin peptides (1; 42). Trunk blood was also collected in separate tubes without inhibitors and allowed to clot to obtain the serum. Blood was spun at 1800 x g and the plasma or serum stored at -80°C. Following blood collection, tissues were rapidly collected. For hearts and kidneys, the tissue was blotted, weighed and snap frozen on dry ice. The cardiac and renal weight indices were expressed as mg organ weight/gm total body weight (mg/g). The cortical and medullary regions of the kidney were dissected on an ice-filled plastic Petri dish, and stored at -80°C. Frozen renal and cardiac tissue was homogenized in an acidic ethanol (80% vol/vol 0.1 N HCl) solution containing peptidase inhibitors described above and processed for RIA analysis as described previously (42). A sample of homogenate was taken to determine total protein content (Bio-Rad Protein Assay Reagent, Bio-Rad Laboratories, Hercules, CA). Details on the RIAs have been

previously described (42). Plasma renin concentration was determined by addition of exogenous angiotensinogen (nephrectomized rat plasma) incubated with 50 μL of plasma at either pH 6.5 (optima for rat renin) or pH 8.5 (optima for mouse renin) and the subsequent formation of Ang I as previously described (17; 21). Renal renin concentration was determined by the addition of exogenous angiotensinogen to native supernatants of homogenized renal cortex dilutions at either pH 6.5 or pH 8.5. Serum complement-reactive protein (CRP) levels were determined by an ELISA kit from Alpco Diagnostics (Salem, NH).

Immunoblot analysis. Circulating angiotensinogen was measured by immunoblot assay with an antibody directed against an epitope on the carboxy (C, residues 428-441) terminus of the protein. The antibody was produced in rabbits by coupling the C-terminus of either Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Tyr-Tyr-Ser-Cys* via an added Cys residue to keyhole limpet hemocyanin (21). For protein analysis, 0.38 μl of plasma was separated on 10% SDS polyacrylamide gels for 1 hr at 120 V in Tris-Glycine SDS, transferred onto PVDF membranes, and subsequently blocked for 1 hr with 5% Bio-Rad Dry Milk and TBS with Tween before incubation with the C (A2504, 1:2000) terminus directed antibody (21). Neprilysin expression was determined in the solubilized membranes of renal cortex (10 μg) with an antibody raised to rat neprilysin (Lot# 0702053058, Chemicon Int., Temecula, CA).

Peptidase activities. Frozen renal cortex and cardiac tissue was homogenized in enzyme reaction buffer (10 mM HEPES, 125 mM NaCl, 10 μM $ZnCl_2$, pH 7.4) with Qiagen Tissue Lyser for 1 minute at 25 Hz. The homogenate was spun at 28,000 x g for

10 minutes at 4°C, the pellet resuspended and the centrifuge step repeated. The supernatant was stored at -80°C until assayed for renin concentration. The resultant pellet was resuspended in 0.5% Triton-X 100 overnight at 4°C on ice to solubilize proteins. Following the same centrifugation step, the solubilized supernatants were utilized as the source of peptidase activity. Either the substrate ^{125}I-Ang I or ^{125}I-Ang II (2 x 10^6 cpm, 2200 Curies/mmol) was added to the supernatant with various inhibitors and incubated at 37°C for up to 2 hours. The reaction was terminated with 1.0% phosphoric acid, centrifuged at 16,000 g, and the supernatant stored at 4°C. Samples were then filtered and the enzymatic products quantified on HPLC as previously described (54). The following inhibitors comprised the inhibitor cocktail in the assay: amastatin (AM; 200 μM), bestatin (BS; 1 μM), chymostatin (CHYM; 1 μM), benzyl succinate (BSC; 1 μM), and para-chloromercuribenzoic acid (PCMB; 0.5 mM). Lisinopril was added to inhibit ACE activity, SCH39370 to block neprilysin activity and MLN-4760 to attenuate ACE2 activity (all at a final concentration of 10 μM) (54). Renal ACE activity was too low to measure by this method, particularly given the high levels of neprilysin that compete for the Ang I substrate. Thus, we used ^{3}H-Hippuryl-Histidine-Leucine (Hip-His-Leu) as the substrate for ACE as described previously (21).

Urinary markers. Urine was collected over a 24 hr period in buffer (25 mM HEPES, 125 mM NaCl, 10 μM $ZnCl_2$ pH 7.4) and stored at -20°C. Urinary protein and creatinine were measured as described (21). The oxidative marker 8-hydroxy-2'-deoxyguanosine (8OH-dG) was determined with an ELISA kit from Assay Designs (*Hayward, CA*) and normalized to body weight (35; 55).

Statistical Analyses. All measurements are expressed as the mean ± standard error of the mean (SEM) computed from an average of 5 determinations per rat for systolic blood pressure or values for the biochemical data from each rat. Comparisons between the sex and strain were evaluated using One Way ANOVA and Tukey's multi-comparison test. These analyses were performed with the GraphPad Prism IV plotting and statistical software (San Diego, CA). The associated scatter plot and the linear regression line with 95% confidence limits for cardiac hypertrophy and blood pressure were constructed with the Prism IV software (San Diego, CA).

RESULTS

Circulation: The systolic blood pressure was markedly higher in 15 week old congenic rats as compared to the age-matched normotensive Lewis for both sexes (Figure 1A). The congenic males also exhibited a greater degree of hypertension than the female congenics (200 $\pm$ 4 vs. 146 $\pm$ 7 mmHg, $p<0.001$) while there was no gender difference in systolic blood pressure for the Lewis strain. The males exhibited no weight difference between strains, however, the female mRen(2).Lewis were 11% heavier than the female Lewis rats (261 $\pm$ 5 gm vs. 233 $\pm$ 2 gm, $p<0.01$, Figure 1B). Complement-reactive protein (CRP) was determined in the serum as a circulating marker of inflammation. Male mRen(2).Lewis exhibited the highest serum CRP levels among these groups consistent with the greater extent of blood pressure and plasma Ang II (see Figure 2, below). However, we also observed a significant gender difference in serum CRP for both hypertensive and normotensive strains (Figure 1C).

We assessed plasma peptide concentrations in trunk blood collected from unanesthetized rats. As shown in Figure 2A, plasma Ang II levels were similar for the normotensive male and female Lewis rats ($p>0.05$). However, the male mRen(2).Lewis exhibited higher circulating Ang II than the female congenics (47.1 $\pm$ 2.5 pM vs. 18.6 $\pm$ 3.2 pM, $p<0.001$). Moreover, the congenic strain exhibited significantly higher levels of plasma Ang II in comparison to the Lewis strain ($p<0.01$). Consistent with the higher Ang II levels, the male mRen(2).Lewis had higher circulating levels of Ang I than both the females congenics (201 $\pm$ 21 pM vs. 56.8 $\pm$ 3.8 pM, $p<0.001$, Figure 2B) and the Lewis rats (Figure 2B). However, plasma levels of Ang-(1-7) were highest in the female congenics (Figure 2C). Aldosterone levels were similar among the groups, although there was a trend for higher plasma levels of this steroid in the male versus female mRen(2).Lewis rats (Figure 2D). Plasma angiotensinogen was measured by immunoblot using an antibody that recognizes a distinct epitope on the C terminal domain of the protein (Figure 3). The immunoblot revealed two bands for angiotensinogen at 55 kilodaltons (kDa); however, protein expression was significantly lower in plasma extracts of the female mRen(2).Lewis compared to female Lewis rats (Figure 3). Quantification of the band densities revealed a trend for lower levels of angiotensinogen for the female versus male congenics. In comparison to the male Lewis rats, the male congenics also exhibited lower expression of angiotensinogen. In addition, plasma renin concentrations were assessed at a pH level of 6.5 and 8.5 using an excess of exogenous rat angiotensinogen. The male congenics exhibited a four-fold higher level of renin at a pH of 6.5 than either the female congenics or the Lewis rat strain (Figure 4A). Renin concentration assessed at a pH of 8.5 was five-fold higher in the male versus the female

mRen(2).Lewis rats (Figure 4B). However, renin concentration for the Lewis rats were non detectable at pH 8.5 (Figure 4B). Finally, serum ACE activity was significantly higher in the male mRen(2).Lewis rats by 22% and 60% as compared to the male Lewis and female mRen(2).Lewis, respectively (Figure 4C).

Kidney: Renal hypertrophy was significantly greater in the male mRen(2).Lewis rats as compared to their female littermates or the male Lewis (Figure 5A). Proteinuria and urinary creatinine were also significantly higher in the male congenic strain as compared to all other groups ($p<0.01$, Figure 5B and 5C, respectively). A gender difference in the urinary excretion of the oxidative stress marker 8-hydroxy-2'-deoxyguanosine (8OH-dG) was evident in the congenic strain (male: 10.8 ± 1.1 vs. female: 3.7 ± 0.7 μg/kg/day, $p<0.001$, Figure 5D), while there was no difference in the Lewis. The urinary levels of 8OH-dG in the male mRen(2).Lewis were also significantly higher than the Lewis strain ($p<0.05$).

As shown in Figure 6A, Ang II content in the renal cortex was highest in the male mRen(2).Lewis with Ang II levels two-fold higher than the female congenics. Cortical levels of Ang II were similar between the female mRen(2).Lewis and both male and female Lewis rats. In contrast, Ang I was 50% lower in the male versus female mRen(2).Lewis (2.0 ± 0.2 fmol/mg protein vs. 4.7 ± 0.6 fmol/mg protein, $p<0.01$, Figure 6B). Ang-(1-7) was not significantly different between the four groups, although the female mRen(2).Lewis tended to exhibit higher peptide levels (Figure 6C). Cortical renin concentration measured at pH 6.5 was significantly lower in both genders of the mRen(2).Lewis strain compared to the Lewis rats (Figure 7A). The female rats also

exhibited higher renin levels than male littermates irrespective of strain. Renal renin concentration at pH 8.5 was significantly higher in the female versus male mRen(2).Lewis rats (Figure 7B). Cortical ACE activity was not different among all four groups, while ACE2 activity was 70% higher in male compared to female mRen(2).Lewis rats (Figures 8A and 8B, respectively). A strain difference was also evident for ACE2 activity in the male rats ($p<0.01$). As shown in Figures 8C and 8D, respectively, neprilysin activity was significantly higher (three-fold) in the female mRen(2).Lewis and Lewis as compared to their male littermates regardless of whether Ang I or Ang II was utilized as the substrate. Neprilysin protein expression in the renal cortex of the male and female mRen(2).Lewis was also assessed by immunoblots. The immunoblot revealed a predominant band at 88 kDa, identical to the molecular weight of the standard (lane 7), and neprilysin expression was noticeably more predominant in the female renal extracts (Figure 9A). A more diffuse band at 65 kDa was also evident on the full-length gel. Quantification of the gel staining revealed ten-and three-fold higher density of the 88 and 65 kDa bands, respectively for the female mRen(2).Lewis rats (Figure 9B and 9C, respectively).

As shown in Figure 10A, renal medullary Ang II content was significantly higher in the male mRen(2).Lewis than for all other groups ($p<0.001$). Moreover, there was no gender difference for Ang II in the Lewis strain. Medullary Ang I was significantly higher in the female Lewis compared to male Lewis (Figure 10B). Ang-(1-7) levels were lower in the mRen(2).Lewis independent of gender (Figure 10C).

Heart: As shown in Figure 11A, the male mRen(2).Lewis exhibited the highest extent of cardiac hypertrophy among all four groups with a difference in strain present only in the males (4.0 $\pm$ 0.05 mg/g vs. 2.4 $\pm$ 0.05 mg/g, $p<0.001$). Cardiac hypertrophy was strongly correlated to the systolic blood pressure among all four groups ($r^2=0.85$, $p<0.001$, Figure 11B). Neither Ang II nor Ang-(1-7) content in the ventricular tissues were different among the four groups of rats (Figure 11C and 11D, respectively). Ang I values were below the minimum detectable limit of the Ang I RIA (< 0.33 fmol/mg protein). A sex difference was not present in cardiac ACE activity among the Lewis and mRen(2).Lewis rats, although there was a trend for higher activity in the male and female congenics (Figure 11E). Sex differences for ACE2 activity were apparent only in the mRen(2).Lewis with significantly higher activity (35%) in the male congenics (Figure 11F).

DISCUSSION

The congenic mRen(2).Lewis hypertensive strain exhibits marked sex differences regarding the extent of the hypertension, cardiac hypertrophy and proteinuria that are not evident in the normotensive Lewis rats. Indeed, the gender difference in the mRen(2).Lewis is similar to that observed in other hypertensive models (12; 13; 27; 46) and may reflect the gender inequality in the progression of cardiovascular disease observed in the human population (4). Although we previously reported that the female mRen(2).Lewis is an estrogen-sensitive model whereby ovariectomy significantly exacerbates the degree of hypertension and estrogen replacement normalizes blood

pressure, the status of the RAAS that may contribute to the sex differences in this hypertensive strain is not known (17). Moreover, the assessment of the RAAS in the Lewis normotensive rats provides an appropriate control for the influence of sex alone on the regulation of these components. In this regard, our findings reveal that the male mRen(2).Lewis exhibited the highest circulating and renal tissue levels of Ang II among the four groups consistent with the greater degree of increased blood pressure, inflammation, cardiac hypertrophy, and proteinuria. The female mRen(2).Lewis rat, which exhibits moderate hypertension in comparison to their male littermates, also expressed significantly higher levels of circulating Ang II than the female or male Lewis rats. Interestingly, the female mRen(2).Lewis displayed higher levels of circulating Ang-(1-7) than either the male congenics or the Lewis control strain. The higher expression of Ang-(1-7), a peptide with vasodilatory and anti-inflammatory actions, may provide for an effective compensatory mechanism to attenuate the extent of hypertension and renal injury in the female hypertensive strain (10).

The basis for sex differences in cardiovascular disease is generally thought to involve the over expression of various components of the RAAS including ACE, the AT_1 receptor and angiotensinogen (5; 15; 19; 44). Estrogen is known to down regulate both ACE and the AT_1 receptor, as well as increase expression of the AT_2 receptor and ACE2 which may further attenuate the actions of the ACE-Ang II-AT_1 axis (3; 10; 17; 27; 33; 56; 59). In contrast, testosterone may increase ACE and the AT_1 receptor as well as angiotensinogen (9; 27). To our knowledge, the current studies are the first to document the sex-based differential expression of both circulating and tissue Ang II and Ang-(1-7) in any hypertensive strain. Importantly, we find no differences in Ang II or Ang-(1-7)

between the male and female Lewis rats suggesting that gender alone does not account for the differential expression of these peptides. Circulating Ang II was five-fold higher in the male mRen(2).Lewis than either male or female Lewis, as well as two-fold higher than the female mRen(2).Lewis. The plasma level of Ang I was also highest in the male mRen(2).Lewis - approximately four-fold greater than the three other groups. Consistent with the greater Ang I, both plasma renin concentrations at a pH of 6.5 and 8.5 were highest in the male mRen(2).Lewis and likely contribute to the greater expression of Ang I and Ang II in the male hypertensives. Although studies suggest an androgen dependent regulation of mouse and rat renin in the mRen2.(27) rats (8; 9), ovariectomy of the female mRen(2).Lewis rat results in a two-fold increase in plasma renin and Ang II, as well as a marked increase in blood pressure (17). Thus, the gender difference in circulating renin may reflect the positive influence of androgens in males, as well as the inhibitory effects of ovarian hormones in the female mRen(2).Lewis rats. The higher renin concentration at pH 8.5 is not unexpected in the male mRen(2).Lewis, however, the increase in renin concentration at pH 6.5 is surprising, particularly given the greater level of blood pressure and plasma Ang II in the male congenics. The inability to effectively downregulate rat renin may be an additional factor that contributes to the sustained elevation of blood pressure in the mRen(2).Lewis rat, although the exact mechanism(s) for the disinhibition of renin release remains to be defined. In addition to the sex differences in renin concentration, we find that the female mRen(2).Lewis expresses significantly lower levels of plasma angiotensinogen. For this analysis, we utilized an antibody that recognizes the C terminal domain of the protein. We can exclude an overall effect of gender due to no difference in plasma angiotensinogen in the Lewis rat strain.

The lower levels of the precursor could contribute to the differences in plasma Ang I and Ang II between the male and female mRen(2).Lewis strain. The lower levels of angiotensinogen in the female mRen(2).Lewis contrast with the positive influence of estrogen or estrogen agonists on angiotensinogen expression (52). It is possible that the female congenics may exhibit greater feedback control of angiotensinogen than their hypertensive male littermates that may lead to reduced circulating levels of the precursor. We also find both sex and strain differences in circulating ACE activity. The male congenics exhibited higher ACE activity than the male Lewis and the female congenics. Again, the higher levels of ACE activity suggest that the dysfunctional regulation of the endogenous RAAS likely contributes to the elevated blood pressure in the male mRen(2).Lewis rat. The lower levels of serum ACE activity, however, may also contribute to the reduced level of blood pressure in the female congenics through lower Ang II and higher levels of Ang-(1-7). ACE is the major pathway for the metabolism of Ang-(1-7) in the circulation cleaving the dipeptide His-Pro to form Ang-(1-5) (20; 64). In contrast to Ang II, Ang-(1-7) exhibits vasodilatory properties most likely by stimulating the release of prostaglandins and nitric oxide (31; 51). Indeed, we have shown that blockade of Ang-(1-7) attenuates the blood pressure lowering actions of ACE inhibitors (18; 30).

In addition to the greater circulating levels of Ang II, male and female mRen(2).Lewis exhibit higher serum levels of CRP than their respective normotensive controls. These findings are consistent with the pro-inflammatory events following chronic administration of exogenous Ang II in normotensive models (43) and the anti-inflammatory effects of RAS blockade in hypertensive rats and humans (49; 53). Sex

differences in circulating CRP were evident for both mRen(2).Lewis and Lewis strains with the males exhibiting higher levels of this inflammatory marker. The differences in CRP between the male and female Lewis, however, were apparently not associated with increased circulating Ang II or systolic blood pressure. Several clinical studies also find that males exhibit higher circulating levels of CRP than females (25; 50), although others report the opposite (36). We found that ovariectomy in older female mRen(2).Lewis (12 months of age) fed a high salt diet was associated with lower circulating CRP, as well reduced proteinuria and other indices of renal injury (65). Yang et al (66) also reported that ovariectomy reduced CRP levels in older Fisher 344 rats and that estrogen replacement restores CRP to that of the sham rats. The latter study, however, found no association between circulating CRP and the extent of complement activation suggesting that altered levels of CRP may reflect the hepatic effects of estrogen rather than an increase in inflammation per se. Although ovariectomy markedly exacerbates hypertension in young mRen(2).Lewis rats and estrogen replacement (or an AT1 antagonist) reverses this effect (17), additional studies are required to elucidate the degree of chronic inflammation and the contribution of this response to hypertension in the congenic strain.

In the kidney, the cortical levels of Ang II were significantly higher in the male mRen(2).Lewis in comparison to all other groups. The higher renal content of Ang II was associated with increased blood pressure, proteinuria, and urinary levels of the oxidative marker 8OH-dG in the male congenics. In contrast to the circulatory levels of Ang I, cortical Ang I levels were significantly reduced as compared to the female congenics and the Lewis rats. The Ang II to Ang I ratio (>4) suggests an enhanced

conversion of Ang I to Ang II in the renal cortex rather than primarily an increase in renal renin activity or angiotensinogen. However, renal renin activity (at pH 6.5) was markedly reduced in both the male and female mRen(2).Lewis in comparison to Lewis controls and likely reflects the response to the sustained increase in blood pressure and Ang II levels in the congenics. The suppressed renal renin supports earlier studies that found reduced renin mRNA and protein in the kidney of the male transgenic mRen2.(27) rats (7; 61). In contrast to plasma renin, both Lewis and mRen(2).Lewis females exhibited higher cortical renin activity (at pH 6.5 and 8.5) than their male littermates. In this regard, it is conceivable that renin from multiple organs (kidney, adrenal, etc) may be regulated differently by estrogen or androgens which may account for the gender differences in plasma and renal renin in the mRen(2).Lewis strain.

Cortical ACE activity was similar between all four groups, however, ACE2 activity was significantly higher in the male versus female mRen(2).Lewis rat and somewhat lower than that of the male Lewis as previously reported (22). Indeed, there were significant sex differences in both normotensive and hypertensive strains with higher ACE2 activity in the males. This finding is surprising given that the ACE2 gene is located on the X chromosome and females should have an additional copy of this gene that may contribute to higher activity (22). Thus, our data do not support the concept that higher ACE2 activity in the intact female may contribute to increased metabolism of Ang II to Ang-(1-7) at least in the mRen(2).Lewis strain. However, cortical neprilysin activity and protein content were markedly higher in the female hypertensives than males. Neprilysin is an endopeptidase that directly converts Ang I to Ang-(1-7), but metabolizes Ang II to Ang-(1-4) in addition to the hydrolysis of other vasoactive peptides including

endothelin, bradykinin, and natriuretic peptides (2; 54; 58; 62). Higher expression of neprilysin may contribute to the lower levels of Ang II in the renal cortex of the female mRen(2).Lewis rat, as well as to their lower pressure and proteinuria. However, neprilysin activity was also significantly higher in the female Lewis strain, and angiotensin content and blood pressure were similar between the male and female Lewis rats. Moreover, neprilysin inhibitors are generally thought to lower blood pressure, although these agents are more effective when combined with an ACE blocker (14; 34; 37). To our knowledge, experimental studies with neprilysin inhibitors have predominantly, if not exclusively utilized male hypertensive strains. In the male mRen2.(27) transgenic rat, chronic neprilysin inhibition reduced the progression of hypertension although the exact mechanism for this effect was not defined (57). Thus, studies are in progress to determine the influence of neprilysin inhibition in the female mRen(2).Lewis rat on angiotensin expression and blood pressure.

The medullary content of Ang II was also significantly higher in the male mRen(2).Lewis rats versus their female littermates and the normotensive groups. Similar to the cortex, medullary levels of Ang I and Ang-(1-7) tended to be lower, again suggesting either an enhanced conversion from Ang I by ACE or the reduced metabolism of Ang II. In this regard, Neves et al, (39) reported higher medullary levels of neprilysin activity in female Sprague Dawley and transgenic mRen2.(27) than the male strains utilizing a synthetic substrate to measure enzyme levels. Alternatively, the increased tissue content of Ang II in the medulla and possibly the cortex as well may reflect the enhanced uptake of circulating Ang II delivered to the kidney (29), particularly given the markedly lower expression of renal renin in the congenics. Indeed, the four-fold

difference in medullary Ang II content between the male and female mRen(2).Lewis may arise from the higher circulating levels of Ang II in males and the potentially lower AT_1 receptor expression in females, as well as the reduced levels of neprilysin. Estrogen is known to negatively influence AT_1 receptor density in the kidney and other tissues (28; 63). Moreover, ovariectomy markedly increased blood pressure in the female mRen(2).Lewis rat and subsequent treatment with either estrogen or the AT_1 antagonist olmesartan normalized pressure in this strain (17). The current studies lacked sufficient tissue to determine the levels of Ang receptors or whether there are differences in the processing enzymes for Ang I and Ang II in the medullary region. The renal medulla is an important target tissue for Ang II and the assessment of the mechanisms leading to gender differences in angiotensin expression is the focus of future studies, particularly in comparison to the renal cortex.

In contrast to both the circulation and kidney, the cardiac levels of Ang II and Ang-(1-7) were not different between the male and female hypertensive rats or the normotensive groups. The male mRen(2).Lewis hearts exhibited significant hypertrophy in comparison to their female littermates or male Lewis rats. In this case, cardiac hypertrophy was highly associated with blood pressure suggesting that the increased afterload contributes to the marked hypertrophy in the males. We did not determine cardiac function or other markers of cardiac damage such as the extent of fibrosis in the male and female mRen(2).Lewis rats. Consistent with the peptide data, cardiac ACE activity was not different in the male mRen(2).Lewis in comparison to the female, but tended to be higher than the male and female Lewis rats. Although ACE2 likely constitutes the major pathway for the metabolism of Ang II in the heart, the relatively

small difference in activity between the male and female mRen(2).Lewis may not significantly influence tissue peptide levels in this strain (26). Cardiac Ang I content was not detected in any of the four groups and the lack of Ang I raises the issue of whether the heart contains a complete RAAS (23; 24; 48). In this regard, receptor mediated uptake of Ang II may account for the cardiac levels of the peptide and subsequent processing by ACE2 may contribute to cardiac Ang-(1-7). Although the current studies were not designed to investigate this issue in-depth, they clearly reveal tissue-dependent differences in the expression of angiotensin peptides in the Lewis and mRen(2).Lewis rats.

In summary, the present studies utilizing the congenic mRen(2).Lewis model find significant differences in the expression of circulating and renal angiotensin peptides between the male and female strain that were not evident in the Lewis normotensive rats. Indeed, the differential expression of Ang II and Ang-(1-7) may contribute to sex-based differences in the extent of hypertension and renal injury in the mRen(2).Lewis rat. Although ongoing study of the additional components including ACE2, Ang-(1-7) and its receptor, as well as the renin receptor has redefined the regulation and functional aspects of this important hormonal system (16). Moreover, accumulating evidence that male and females clearly exhibit cardiovascular differences particularly in the setting of hypertension clearly mandates assessment of these components as a basis for sex differences and towards the continued development of effective therapeutic interventions. Finally, these data support the overall concept that sex steroids are of significant importance for both hypertensive females and males regarding the regulation of the RAAS, as well as the development and progression of cardiovascular disease.

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Figure Legends

Figure 1. Cardiovascular indices in Lewis and mRen(2).Lewis rats. Panel A: Systolic blood pressure (mm Hg) was measured by tail-cuff in conscious Lewis and hypertensive mRen(2).Lewis (mRen2) rats. Panel B: Body weight was expressed in grams (g). Panels C: Complement reactive peptide (CRP) was determined in the serum from male and female Lewis and mRen(2).Lewis rats. Values are means $\pm$ SE. #P<0.001 between gender and αP<0.01 between strains, and *P<0.001 between strains (n = 5-6 rats per group).

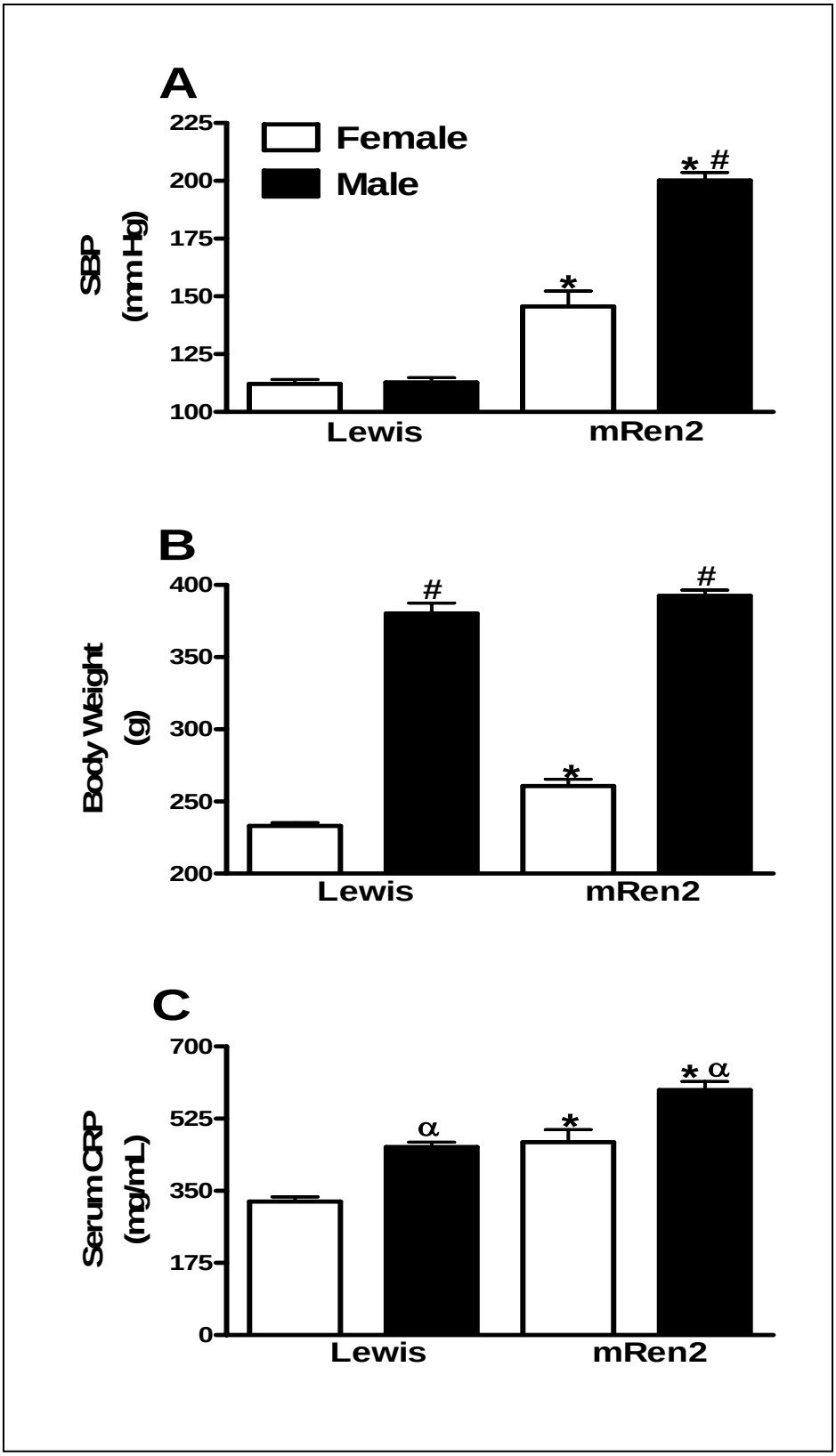
A
Female
Male
SBP (mmHg)
225
200
175
150
125
100
* #
*
Lewis
mRen2
B
Body Weight (g)
400
350
300
250
200
#
#
*
Lewis
mRen2
C
Serum CRP (mg/mL)
700
525
350
175
0
α
* α
*
Lewis
mRen2

Figure 1

Figure 2. Circulating renin-angiotensin aldosterone system hormones in Lewis and mRen(2).Lewis rats. Plasma angiotensins (Ang) and aldosterone were measured by separate radioimmunoassays (RIAs) for Ang II (Panel A), Ang I (Panel B), Ang-(1-7) (Panel C) and aldosterone (Panel D). Peptide values are expressed as picomolar concentration (pM). Plasma aldosterone is expressed as nanograms per deciliter (ng/dl). Values are means $\pm$ SE. #P<0.001 between gender, *P<0.001 between strains, εP<0.05 between strains (n = 5-6 rats per group).

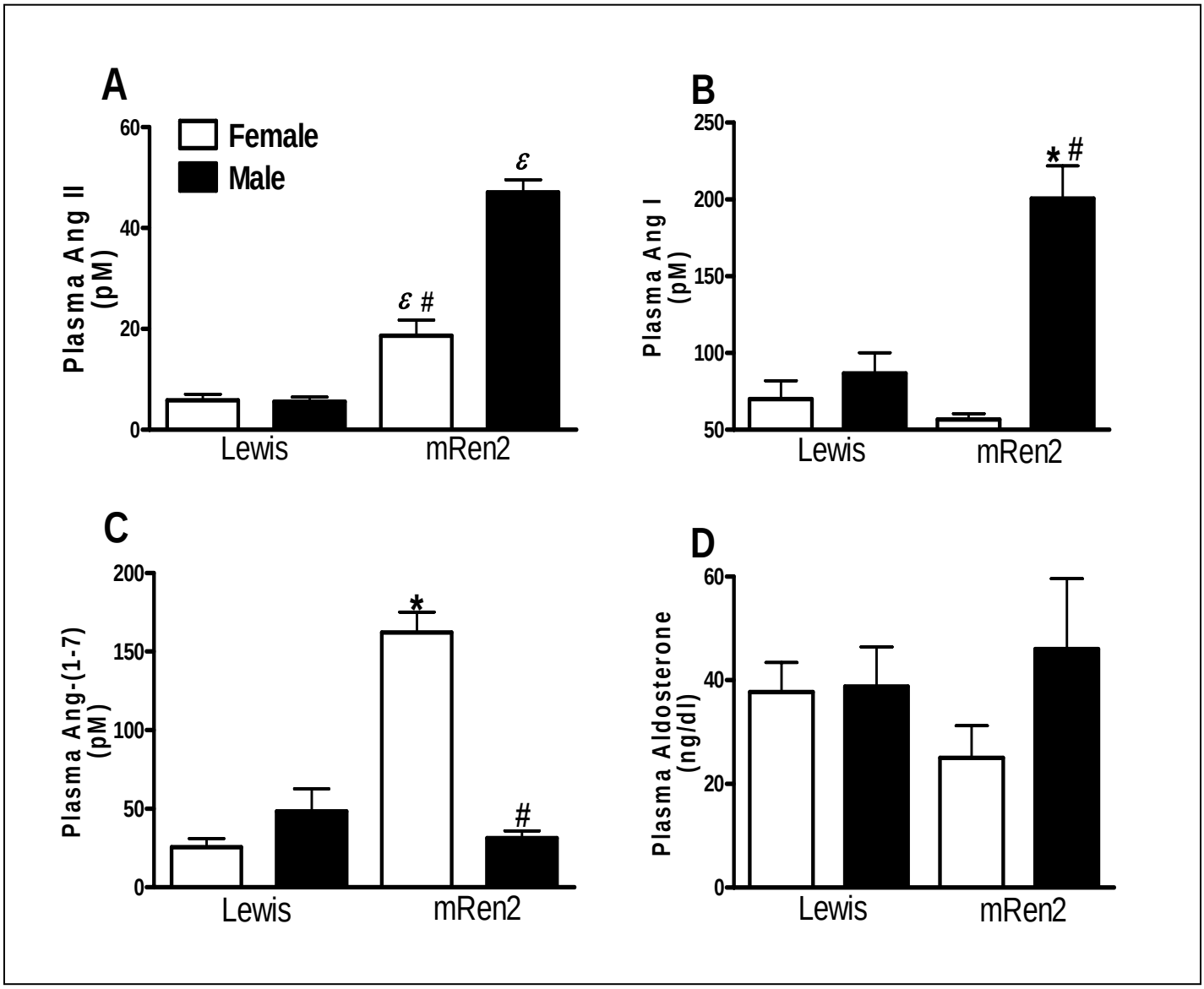
A
Female
Male
Plasma Ang II (pM)
60
40
20
0
ε
ε #
Lewis
mRen2
B
Plasma Ang I (pM)
250
200
150
100
50
* #
Lewis
mRen2
C
Plasma Ang-(1-7) (pM)
200
150
100
50
0
*
#
Lewis
mRen2
D
Plasma Aldosterone (ng/dl)
60
40
20
0
Lewis
mRen2

Figure 2

Figure 3. Representative immunoblot of plasma angiotensinogen in the Lewis and mRen(2).Lewis rats using a C-terminal antibody. Angiotensinogen was determined in plasma (0.38 μl) from the male Lewis (ML), female Lewis (FL), male mRen(2).Lewis (MC), and the female mRen(2).Lewis (FC) rats. Quantification of the bands. Protein expression was quantified by densitometry (absorbance units, AU) for the 55 kDa bands, respectively. Values are means $\pm$ SE. *$P<0.01$ between strain ($n = 4$ rats per group).

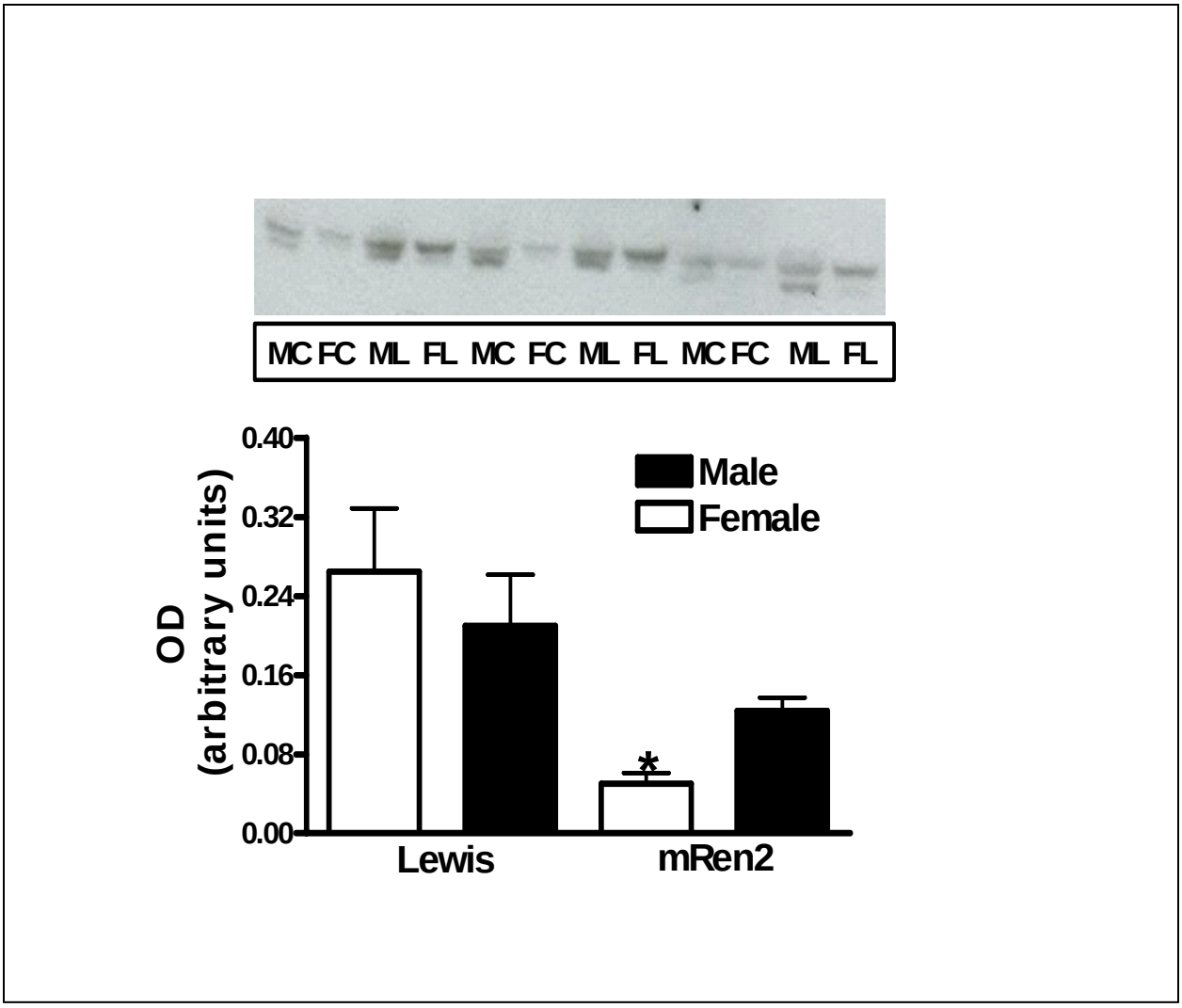
MC FC ML FL MC FC ML FL MC FC ML FL
OD
(arbitrary units)
0.40
0.32
0.24
0.16
0.08
0.00
Male
Female
*
Lewis
mRen2

Figure 3

Figure 4. Circulating renin and ACE activities in the Lewis and mRen(2).Lewis rats. Panel A: Plasma renin concentration was measured at the pH optima for rat renin (pH of 6.5). Panel B: Plasma renin concentration was measured at the pH optima of mouse remain (pH of 8.5). Panel C: Serum ACE activity was measured using the synthetic substrate ^{3}H-[Hip-His-Leu]. Plasma renin concentrations and ACE activity were expressed as ng/ml/hour and nmol/ml/minute, respectively. Values are means $\pm$ SE. #P<0.001 between gender, *P<0.001 between strains, and **P<0.001 compared to male Lewis (n = 5 rats per group).

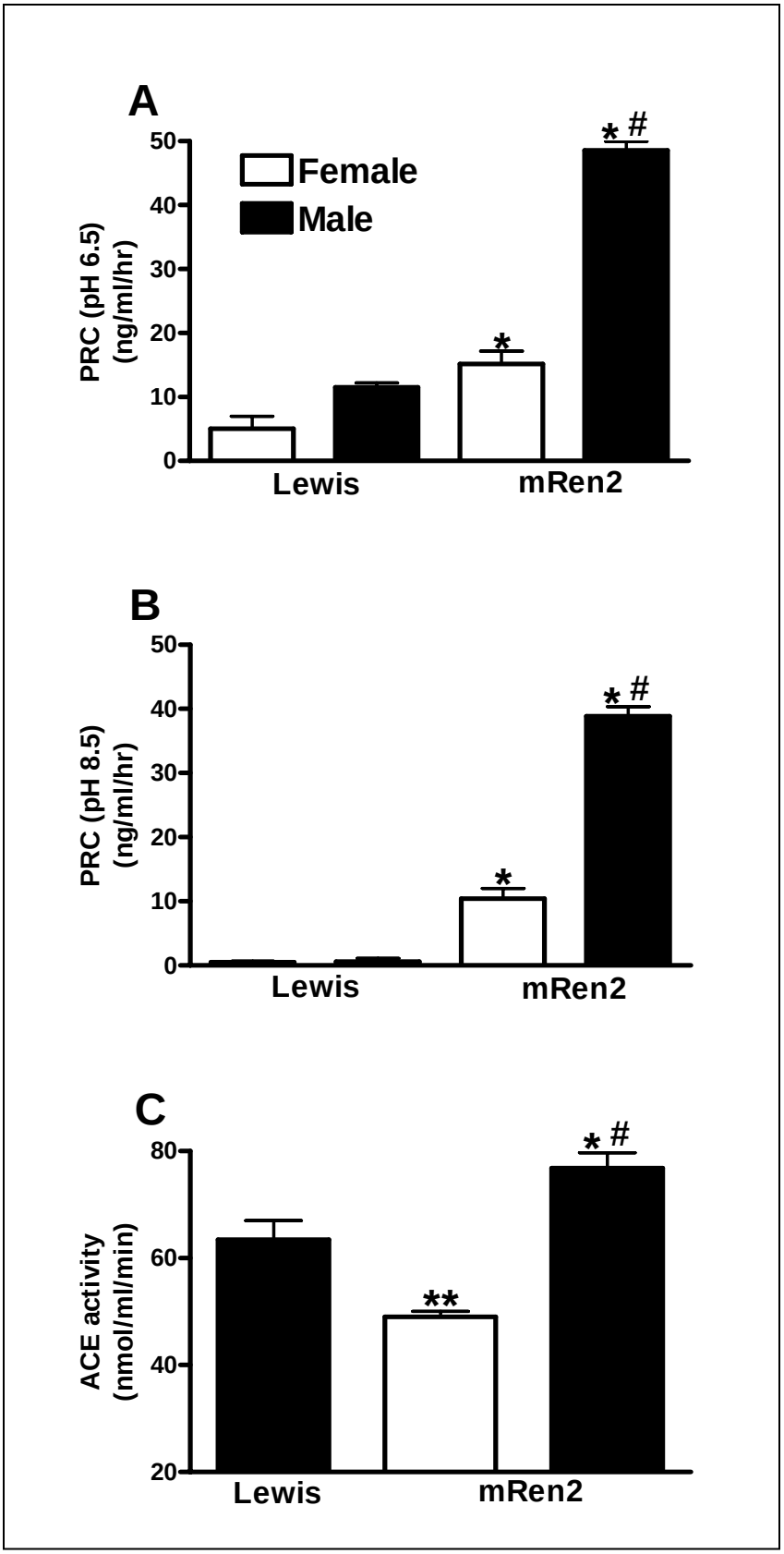
A
PRC (pH 6.5)
(ng/ml/hr)
Female
Male
Lewis
mRen2
B
PRC (pH 8.5)
(ng/ml/hr)
Lewis
mRen2
C
ACE activity
(nmol/ml/min)
Lewis
mRen2

Figure 4

Figure 5. Renal indices in the Lewis and mRen(2).Lewis rats. Panel A: Renal hypertrophy was expressed as the ratio of left kidney to body weight (mg/g). Panel B: Proteinuria was measured from a 24 hour collection of urine expressed as mg protein/kg/day. Panel C: Urinary creatinine excretion was expressed as mg/kg/day. Panel D: Urinary 8-hydroxy-2'-deoxyguanosine (8OH-dG) excretion was expressed as μg/kg/day. Values are means $\pm$ SE. #P<0.001 between gender and *P<0.001 between strains (n = 5 rats per group).

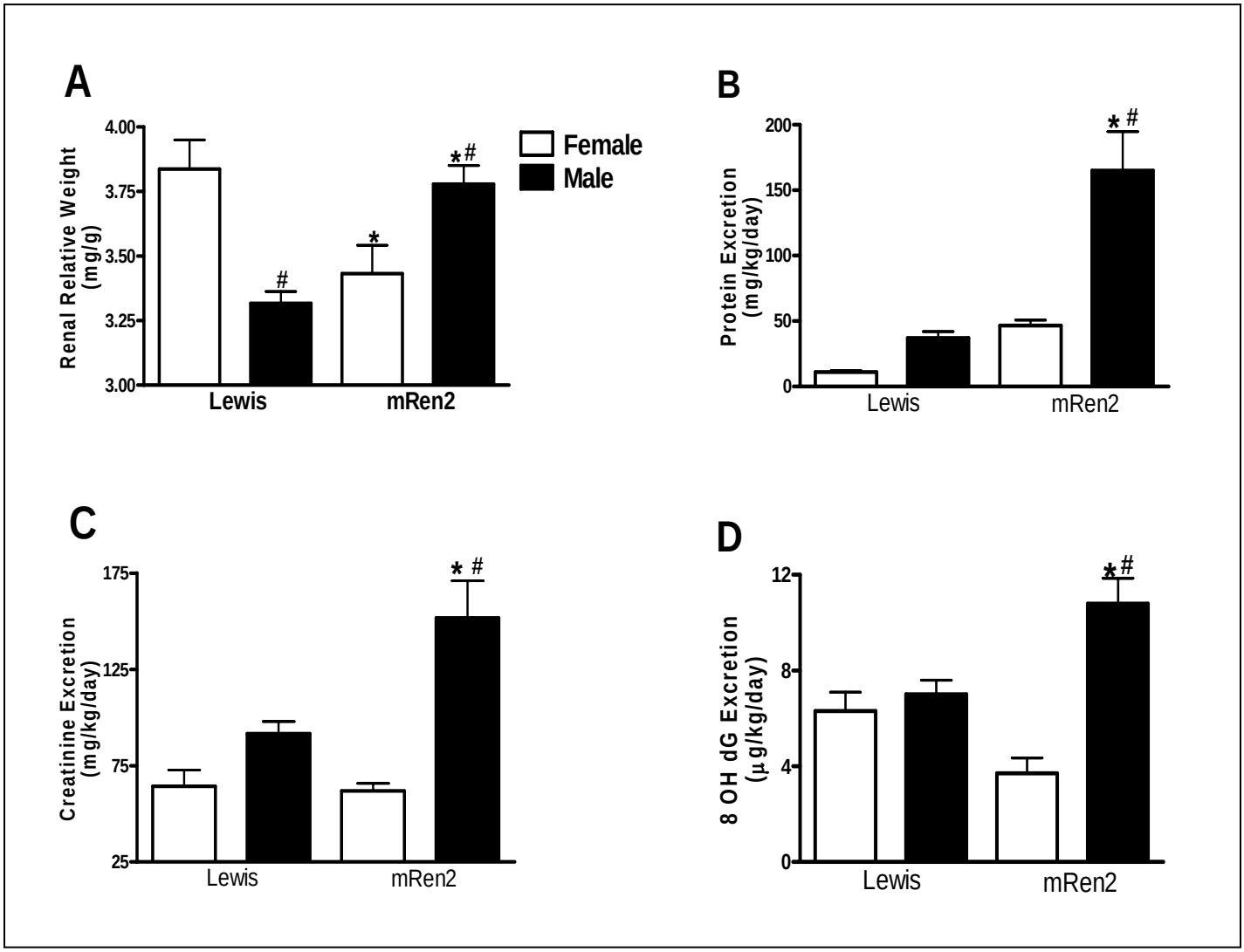
A
Renal Relative Weight (mg/g)
4.00
3.75
3.50
3.25
3.00
Lewis
mRen2
Female
Male
B
Protein Excretion (mg/kg/day)
200
150
100
50
0
Lewis
mRen2
C
Creatinine Excretion (mg/kg/day)
175
125
75
25
Lewis
mRen2
D
8 OH dG Excretion (µg/kg/day)
12
8
4
0
Lewis
mRen2

Figure 5

Figure 6. Renal cortical angiotensins in the Lewis and mRen(2).Lewis. Angiotensins (Ang) were measured by separate radioimmunoassays of cortical extracts and expressed as fmol/mg protein for Ang II (Panel A), Ang I (Panel B), Ang-(1-7) (Panel C). Values are means $\pm$ SE. #P<0.001 between gender, and εP<0.05 between strains (n = 4-6 rats per group).

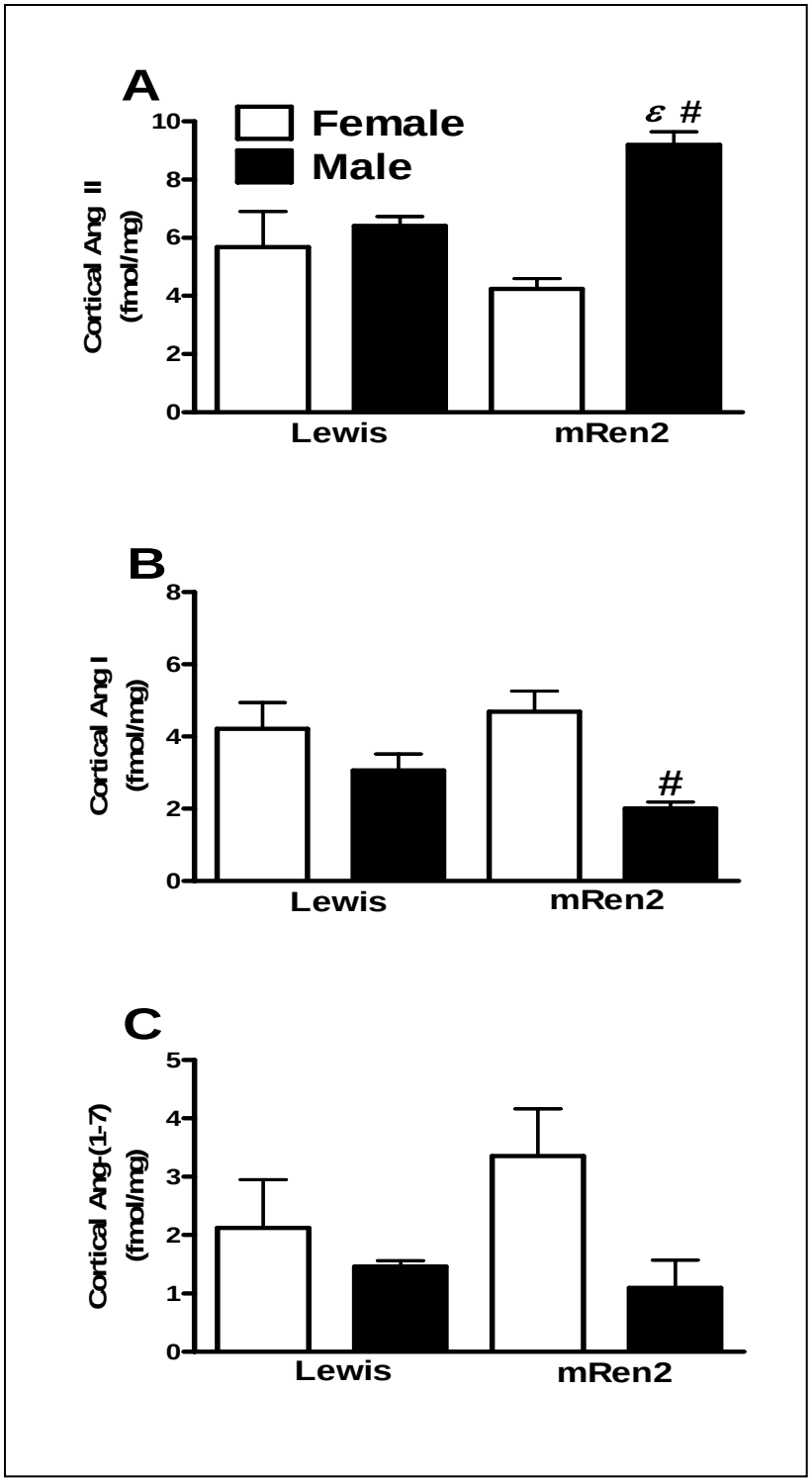
A
Female
Male
ε #
Cortical Ang II
(fmol/mg)
10
8
6
4
2
0
Lewis
mRen2
B
Cortical Ang I
(fmol/mg)
8
6
4
2
0
#
Lewis
mRen2
C
Cortical Ang-(1-7)
(fmol/mg)
5
4
3
2
1
0
Lewis
mRen2

Figure 6

Figure 7. Renal renin concentrations in the Lewis and mRen(2).Lewis rats. Panel A: Renal renin concentration was measured at the pH optima for rat renin (pH of 6.5). Panel B: Renal renin concentration was measured at the pH optima of mouse renin (pH of 8.5). Renal renin concentrations were expressed as μg/mg protein/hour. Values are means ± SE. #P<0.05 between gender and *P<0.01 between strains (n = 3-4 rats per group).

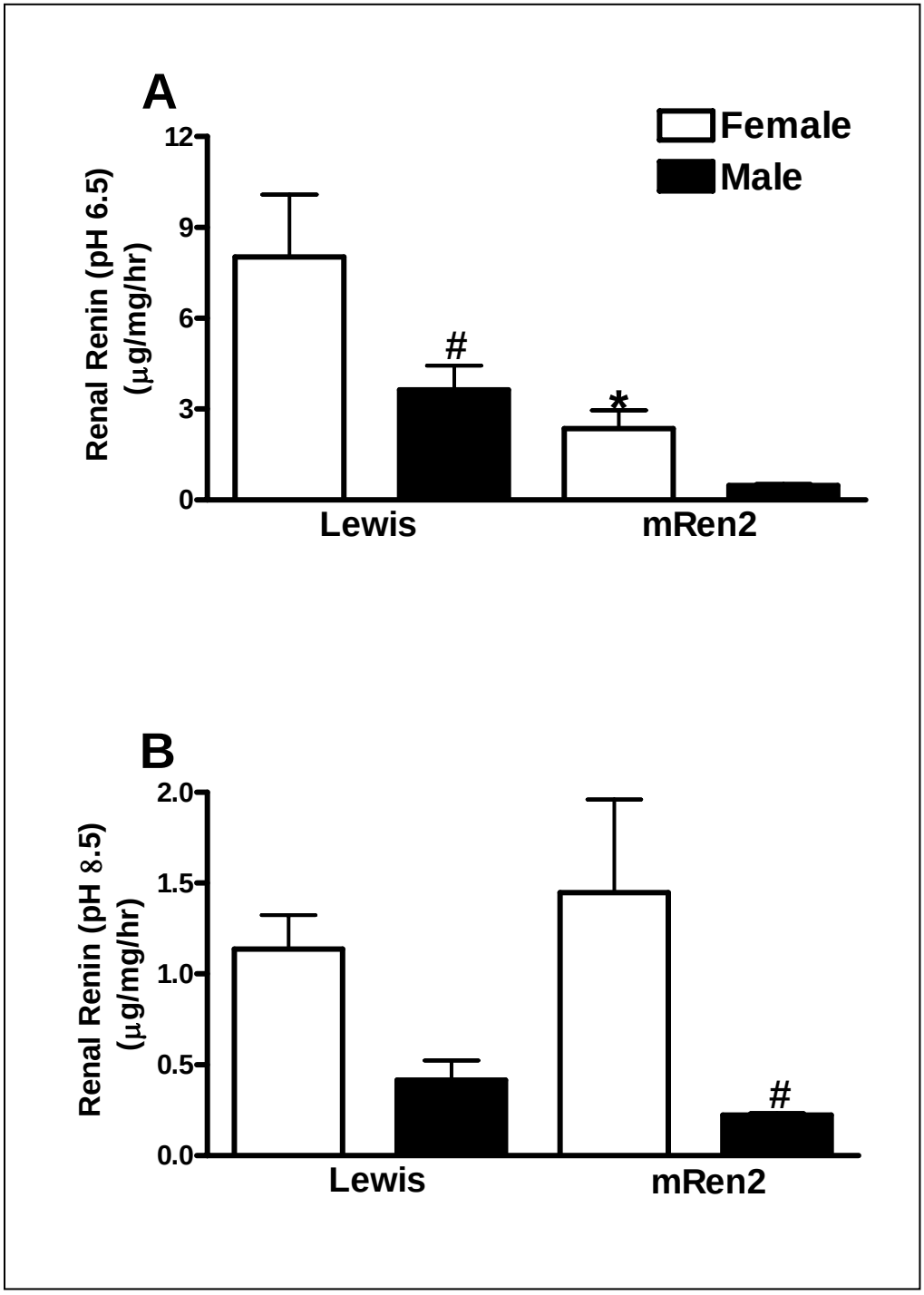
A
Female
Male
Renal Renin (pH 6.5)
(μg/mg/hr)
12
9
6
3
0
#
*
Lewis
mRen2
B
Renal Renin (pH 8.5)
(μg/mg/hr)
2.0
1.5
1.0
0.5
0.0
#
Lewis
mRen2

Figure 7

Figure 8. Renal enzymatic activities in the Lewis and mRen(2).Lewis rats. Panel A: ACE activity. Panel B: ACE2 activity. and Panels C: & D: Neprilysin activity (Ang I and Ang II as substrates) activities were determined in solubilized cortical membranes. Renal enzyme activities were expressed as nmol/mg protein/minute for ACE and fmol/mg protein/minute for ACE2 and neprilysin. Peptidase values are means $\pm$ SE. #P<0.01 between gender and *P<0.01 between strains (n = 4 rats per group).

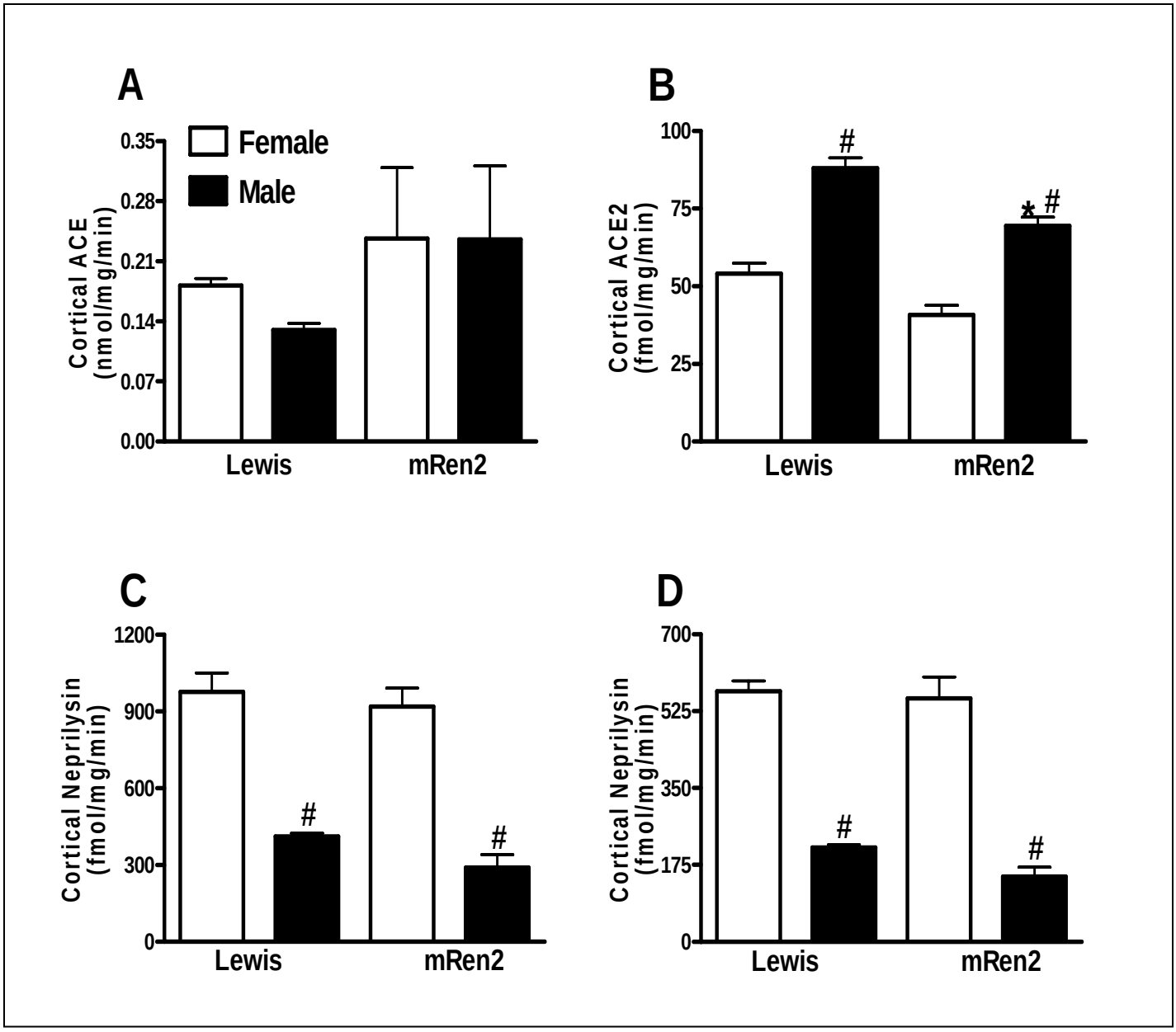
A
Female
Male
Cortical ACE
(nmol/mg/min)
0.35
0.28
0.21
0.14
0.07
0.00
Lewis
mRen2
B
Cortical ACE2
(fmol/mg/min)
100
75
50
25
0
#
*#
Lewis
mRen2
C
Cortical Neprilysin
(fmol/mg/min)
1200
900
600
300
0
#
#
Lewis
mRen2
D
Cortical Neprilysin
(fmol/mg/min)
700
525
350
175
0
#
#
Lewis
mRen2

Figure 8

Figure 9. Immunoblot analysis of cortical neprilysin in male and female mRen(2).Lewis rats. Solubilized cortical membranes (10 μg) were separated by SDS-PAGE and reacted against a neprilysin antibody. Panel A: For the full-length gel, lanes 1,3,5,7 are male cortical extracts, lanes 2,4,6,8 are female extracts and lane 9 is the neprilysin standard (88 kDa). Panels B: and C: Neprilysin protein expression was quantified by densitometry (absorbance units, AU) for the 88 kDa and 65 kDa bands, respectively. Values are means $\pm$ SE. #$P<0.01$ ($n = 4$ rats per group).

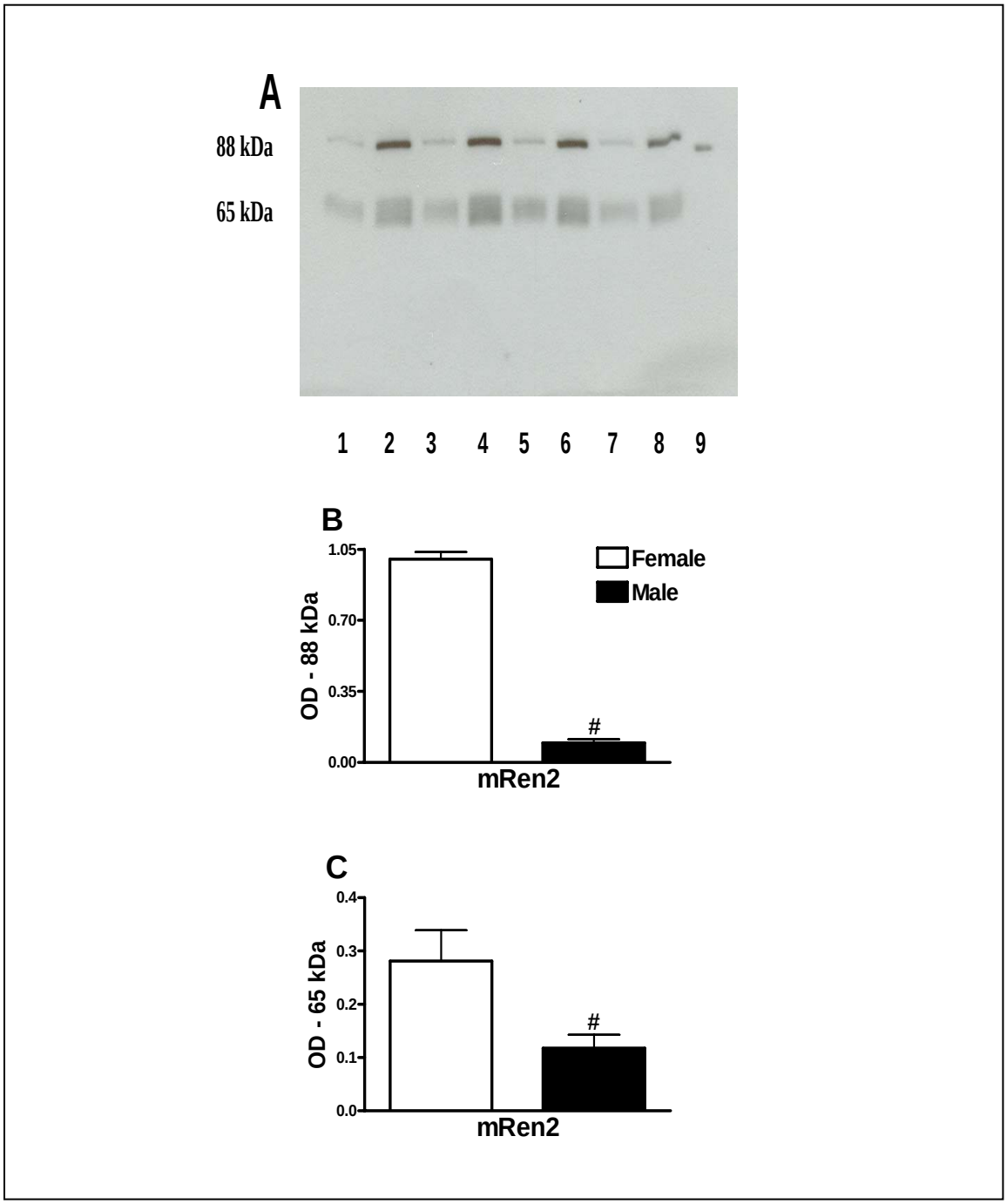
A
88 kDa
65 kDa
1 2 3 4 5 6 7 8 9
B
1.05
0.70
0.35
0.00
OD - 88 kDa
Female
Male
#
mRen2
C
0.4
0.3
0.2
0.1
0.0
OD - 65 kDa
#
mRen2

Figure 9

Figure 10. Renal medullary angiotensins in Lewis and mRen(2).Lewis rats. Angiotensins (Ang) were measured by separate radioimmunoassays and expressed as fmol/mg protein for Ang II (Panel A), Ang I (Panel B), Ang-(1-7) (Panel C). Values are means $\pm$ SE. #P<0.01 between gender and *P<0.01 between strains (n = 5 rats per group).

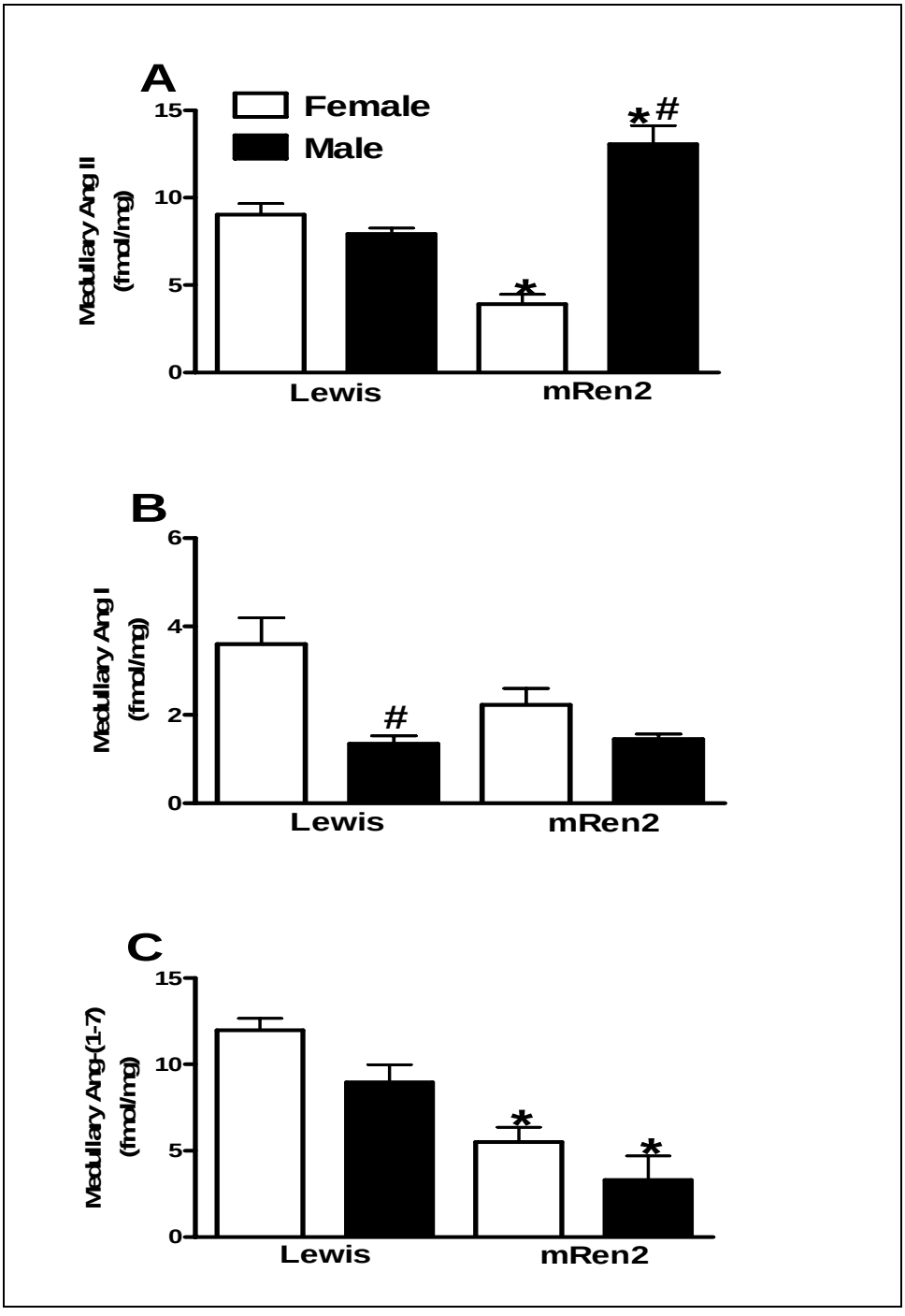
A
Female
Male
Medullary Ang II (fmol/mg)
15
10
5
0
*
*#
Lewis
mRen2
B
Medullary Ang I (fmol/mg)
6
4
2
0
#
Lewis
mRen2
C
Medullary Ang-(1-7) (fmol/mg)
15
10
5
0
*
*
Lewis
mRen2

Figure 10

Figure 11. Cardiac indices in the Lewis and mRen(2).Lewis rats. Panel A: Cardiac hypertrophy was expressed as the ratio of the left ventricle to body weight (mg/g). Panel B: Association of cardiac hypertrophy and the blood pressure data. Panel C: Cardiac Ang II. Panel D. Cardiac Ang-(1-7). Ang peptides were measured by separate radioimmunoassays and expressed as fmol/mg protein (n = 4-6 rats per group). Panels E: and F: ACE and ACE2 activities were determined in solubilized cardiac membranes. Renal enzyme activities were expressed as nmol/mg protein/minute for ACE and fmol/mg protein/minute for ACE2. Peptidase values are means $\pm$ SE. #P<0.01 between gender and *P<0.01 between strains (n = 4 rats per group).

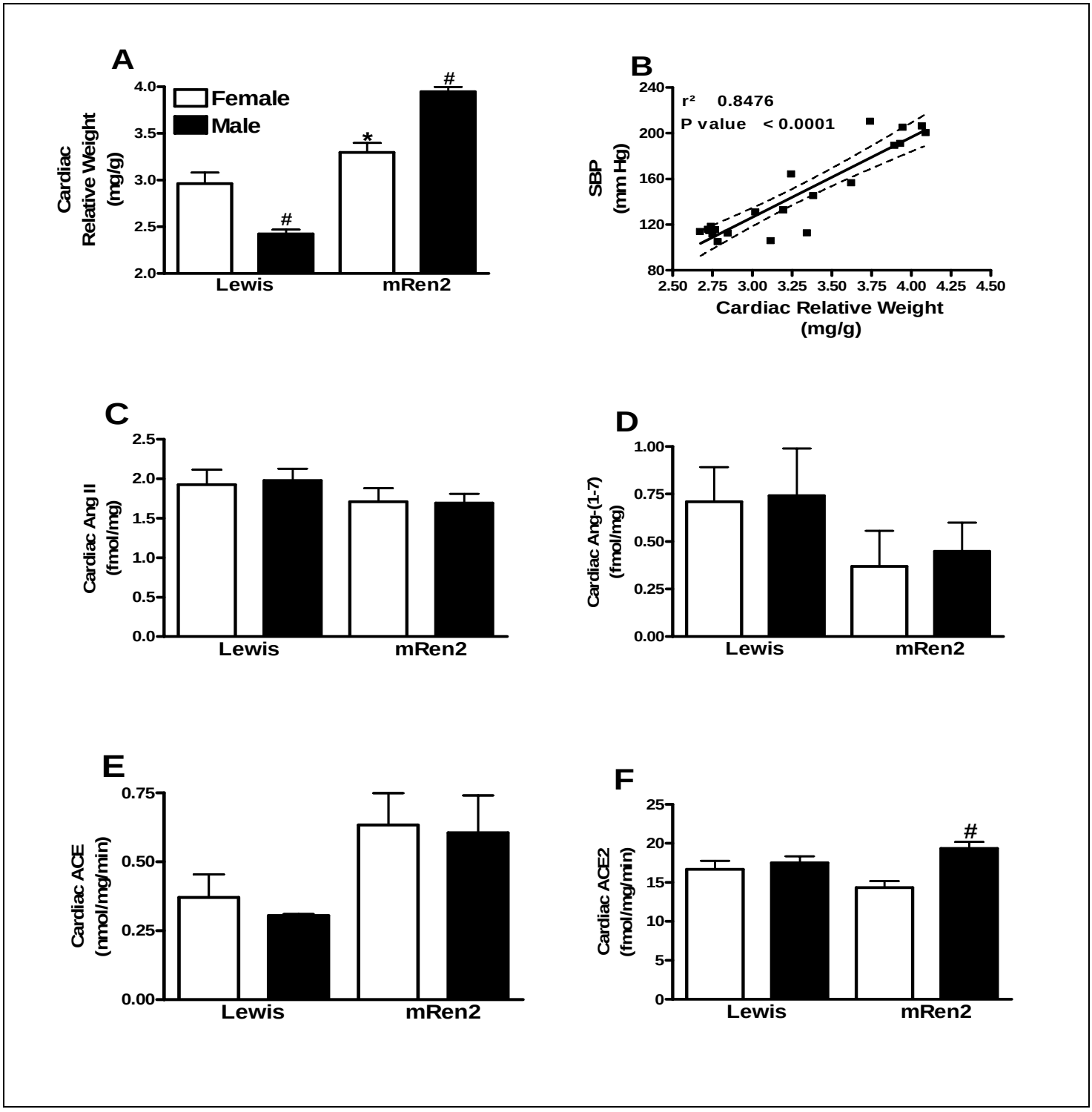
A
Female
Male
Cardiac Relative Weight (mg/g)
4.0
3.5
3.0
2.5
2.0
#
*
#
Lewis
mRen2
B
r² 0.8476
P value < 0.0001
SBP (mm Hg)
240
200
160
120
80
2.50 2.75 3.00 3.25 3.50 3.75 4.00 4.25 4.50
Cardiac Relative Weight (mg/g)
C
Cardiac Ang II (fmol/mg)
2.5
2.0
1.5
1.0
0.5
0.0
Lewis
mRen2
D
Cardiac Ang-(1-7) (fmol/mg)
1.00
0.75
0.50
0.25
0.00
Lewis
mRen2
E
Cardiac ACE (nmol/mg/min)
0.75
0.50
0.25
0.00
Lewis
mRen2
F
Cardiac ACE2 (fmol/mg/min)
25
20
15
10
5
0
#
Lewis
mRen2

Figure 11

Literature Cited

1. **Allred AJ, Chappell MC, Ferrario CM and Diz DI**. Differential actions of renal ischemic injury on the intrarenal angiotensin system. *Am J Physiol :Renal Physiol* 279: F636-F645, 2000.

2. **Allred AJ, Diz DI, Ferrario CM and Chappell MC**. Pathways for angiotensin-(1-7) metabolism in pulmonary and renal tissues. *Am J Physiol* 279: F841-F850, 2000.

3. **Armando I, Jezova M, Juorio AV, Terron JA, Falcon-Neri A, Semino-Mora C, Imboden H and Saavedra JM**. Estrogen upregulates renal angiotensin II AT_2 receptors. *Am J Physiol Renal Physiol* 283: F934-F943, 2002.

4. **August P and Oparil S**. Hypertension in women. *The Journal of Clinical Endocrinology & Metabolism* 84: 1862, 1999.

5. **Bachmann J, Feldmer M, Ganten U, Stock G and Ganten D**. Sexual dimorphism of blood pressure: Possible role of the renin-angiotensin system. *J Steroid Biochem* 40: 511-515, 1991.

6. **Bachmann J, Ganten U, Stock G and Ganten D**. Sexual dimorphism of cardiovascular function: The role of androgens. In: Sex Steroids and the Cardiovascular System, edited by Ramwell P, Rubanyi G and Schillinger E. Berlin: Springer-Verlag, 1992, p. 9-30.

7. **Bachmann S, Peters J, Engler E, Ganten D and Mullins J**. Transgenic rats carrying the mouse renin gene -- morphological characterization of a low-renin hypertension model. *Kidney Int* 41: 24-36, 1992.

8. **Baltatu O, Cayla C, Iliescu R, Andreev D and Bader M**. Abolition of end-organ damage by antiandrogen treatment in female hypertensive transgenic rats. *Hypertension* 41: 830-833, 2003.

9. **Baltatu O, Cayla C, Iliescu R, Andreev D, Jordan C and Bader M**. Abolition of hypertension-induced end-organ damage by androgen receptor blockade in transgenic rats harboring the mouse Ren-2 gene. *J Am Soc Nephrol* 13: 2681-2687, 2002.

10. **Brosnihan KB, Li P, Ganten D and Ferrario CM**. Estrogen protects transgenic hypertensive rats by shifting the vasoconstrictor-vasodilator balance of RAS. *Am J Physiol* 273: R1908-R1915, 1997.

11. **Brosnihan KB, Neves LA, Joyner J, Averill DB, Chappell MC, Sarao R, Penninger J and Ferrario CM**. Enhanced renal immunocytochemical expression of Ang-(1-7) and ACE2 during pregnancy. *Hypertension* 42: 749-753, 2003.

12. **Brosnihan KB, Senanayake PS, Li P and Ferrario CM**. Bi-directional actions of estrogen on the renin-angiotensin system. *Braz J Med Biol Res* 32: 373-381, 1999.

13. **Calhoun DA, Zhu ST, Chen YF and Oparil S**. Gender and dietary NaCl in spontaneously hypertensive and Wistar-Kyoto rats. *Hypertension* 26: 285-289, 1995.

14. **Campbell DJ**. Vasopeptidase inhibition: a double-edged sword? *Hypertension* 41: 383-389, 2003.

15. **Campbell DJ, Rong P, Kladis A, Rees B, Ganten D and Skinner SL**. Angiotensin and bradykinin peptides in the TGR(mRen-2)27 rat. *Hypertension* 25: 1014-1020, 1995.

16. **Chappell MC**. Emerging evidence for a functional angiotensin-converting enzyme 2-angiotensin-(1-7) mas receptor axis; more than regulation of blood pressure? *Hypertension* 50: 596-599, 2007.

17. **Chappell MC, Gallagher PE, Averill DB, Ferrario CM and Brosnihan KB**. Estrogen or the AT1 antagonist olmesartan reverses the development of profound hypertension in the congenic mRen(2).Lewis rat. *Hypertension* 42: 781-786, 2003.

18. **Chappell MC, Iyer SN, Diz DI and Ferrario CM**. Antihypertensive effects of angiotensin-(1-7). *Braz J Med Biol Res* 31: 1205-1212, 1998.

19. **Chappell MC, Modrall JG, Diz DI and Ferrario CM**. Novel aspects of the renal renin-angiotensin system: angiotensin-(1-7), ACE2 and blood pressure regulation.

In: Kidney and Blood Pressure Regulation, edited by Suzuki H and Saruta T. Basel, Karger, 2004, p. 77-89.

20. **Chappell MC, Pirro NT, Sykes A and Ferrario CM**. Metabolism of angiotensin-(1-7) by angiotensin converting enzyme. *Hypertension* 31: 362-367, 1998.

21. **Chappell MC, Yamaleyeva LM and Westwood BM**. Estrogen and salt sensitivity in the female mRen(2).Lewis rat. *Am J Physiol Regul Integr Comp Physiol* 291: R1557-R1563, 2006.

22. **Crackower MA, Sarao R, Oudit GY, Yagil C, Kozieradzki I, Scanga SE, Oliveira-dos-Santo AJ, da Costa J, Zhang L, Pei Y, Scholey J, Bray MR, Ferrario CM, Backx PH, Manoukian AS, Chappell MC, Yagil Y and Penninger JM**. Angiotensin-converting enzyme 2 is an essential regulator of heart function. *Nature* 417: 822-828, 2002.

23. **Danser AH and Schalekamp MA**. Is there an internal cardiac renin-angiotensin system? *Heart* 76: 28-32, 1996.

24. **De Mello WC and Danser AH**. Angiotensin II and the heart: On the intracrine renin-angiotensin system. *Hypertension* 35: 1183-1188, 2000.

25. **Dohi Y, Takase H, Sato K, and Ueda R**. Association among C-reactive protein, oxidative stress, and traditional risk factors in healthy Japanese subjects. *Int J Cardiol* 115: 63-66, 2007.

26. **Ferrario CM, Jessup JA, Chappell MC, Averill DB, Brosnihan KB, Tallant EA, Diz DI and Gallagher PE**. Effect of angiotensin converting enzyme inhibition and angiotensin II receptor blockers on cardiac angiotensin converting enzyme 2. *Circulation* 111: 2605-2610, 2005.

27. **Fischer M, Baessler A and Schunkert H**. Renin angiotensin system and gender differences in the cardiovascular system. *Cardiovasc Res* 53: 672-677, 2002.

28. **Harrison-Bernard LM, Schulman IH and Raij L**. Postovariectomy hypertension is linked to increased renal AT1 receptor and salt sensitivity. *Hypertension* 42: 1157-1163, 2003.

29. **Imig JD, Navar GL, Zou LX, O'Reilly KC, Allen PL, Kaysen JH, Hammond TG and Navar LG**. Renal endosomes contain angiotensin peptides, converting enzyme, and AT(1A) receptors. *Am J Physiol* 277: F303-F311, 1999.

30. **Iyer SN, Chappell MC, Averill DB, Diz DI and Ferrario CM**. Vasodepressor actions of angiotensin-(1-7) unmasked during combined treatment with lisinopril and losartan. *Hypertension* 31: 699-705, 1998.

31. **Iyer SN, Yamada K, Diz DI, Ferrario CM and Chappell MC**. Evidence that prostaglandins mediate the antihypertensive actions of angiotensin-(1-7) during chronic blockade of the renin-angiotensin system. *J Cardiovasc Pharmacol* 36: 109-117, 2000.

32. **Jessup JA, Gallagher PE, Averill DB, Brosnihan KB, Tallant EA, Chappell MC and Ferrario CM**. Effect of angiotensin II blockade on a new congenic model of hypertension derived from transgenic Ren-2 rats. *Am J Physiol Heart Circ Physiol* 291: H2166-H2172, 2006.

33. **Krishnamurthi K, Verbalis JG, Zheng W, Wu Z, Clerch LB and Sandberg K**. Estrogen regulates angiotensin AT1 receptor expression via cytosolic proteins that bind to the 5' leader sequence of the receptor mRNA. *Endocrinology* 140: 5435-5438, 1999.

34. **Kubota E, Dean RG, Hubner RA, Balding LC, Johnston CI and Burrell LM**. Evidence for cardioprotective, renoprotective, and vasculoprotective effects of vasopeptidase inhibitors in disease. *Current Hypertens Reports* 3(Suppl 2): S1-S5, 2001.

35. **Kushiro T, Fujita H, Hisaki R, Asai T, Ichiyama I, Kitahara Y, Koike M, Sugiura H, Saito F, Otsuka Y and Kanmatsuse K**. Oxidative stress in the Dahl salt-sensitive hypertensive rat. *Clin Exp Hypertens* 27: 9-15, 2005.

36. **Lakoski SG, Cushman M, Palmas W, Blumenthal R, D'Agostino RB, and Herrington DM**. The Relationship Between Blood Pressure and C-Reactive Protein in the Multi-Ethnic Study of Atherosclerosis (MESA). *J Am Coll Cardiol* 46: 1869-1874, 2005.

37. **Laurent S, Boutouyrie P, Azizi M, Marie C, Gros C, Schwartz JC, Lecomte JM and Bralet J**. Antihypertensive effects of fasidotril, a dual inhibitor of neprilysin and angiotensin-converting enzyme, in rats and humans. *Hypertension* 35: 1148-1153, 2000.

38. **Mullins JJ, Peters J and Ganten D**. Fulminant hypertension in transgenic rats harbouring the mouse Ren-2 gene. *Nature* 344: 541-544, 1990.

39. **Neves LAA, Chappell MC, Ferrario CM, Gallagher PE, Ganten D and Brosnihan KB**. Effect of estrogen on neprilysin expression in uterus and kidney of Sprague-Dawley normotensive and heterozygous (mRen2)27-transgenic hypertensive rats. *Peptides* 27: 2912-2918, 2007.

40. **Nickenig G, Baumer AT, Grohe C, Kahlert S, Strehlow K, Rosenkranz S, Stablein A, Beckers F, Smits JF, Daemen MJ, Vetter H and Bohm M**. Estrogen modulates AT1 receptor gene expression in vitro and in vivo. *Circulation* 97: 2197-2201, 1998.

41. **Paul M, Mehr AP and Kreutz R**. Physiology of local renin-angiotensin systems. *Physiol Rev* 86: 747-803, 2006.

42. **Pendergrass KD, Averill DB, Ferrario CM, Diz DI and Chappell MC**. Differential expression of nuclear AT1 receptors and angiotensin II within the kidney of the male congenic mRen(2).Lewis rat. *Am J Physiol Renal Physiol* 290: F1497-F1506, 2006.

43. **Peng N, Liu J, Gao D, Lin R, and Rui L**. Angiotensin II-induced C-reactive protein generation: Inflammatory role of vascular smooth muscle cells in atherosclerosis. *Atherosclerosis* 193: 292-298, 2007.

44. **Reckelhoff JF**. Gender differences in the regulation of blood pressure. *Hypertension* 37: 1199-1208, 2001.

45. **Reckelhoff JF and Fortepiani LA**. Novel mechanisms responsible for postmenopausal hypertension. *Hypertension* 43: 918-923, 2004.

46. **Reckelhoff JF, Zhang H and Srivastava K**. Gender differences in development of hypertension in spontaneously hypertensive rats: role of the renin-angiotensin system. *Hypertension* 35: 480-483, 2000.

47. **Reckelhoff JF, Zhang H, Srivastava K and Granger JP**. Gender differences in hypertension in spontaneously hypertensive rats: role of androgens and androgen receptor. *Hypertension* 34: 920-923, 1999.

48. **Reudelhuber TL, Bernstein KE, and Delafontaine P**. Is angiotensin II a direct mediator of left ventricular hypertrophy? *Hypertension* 49: 1196-1201, 2007.

49. **Ridker PM, Danielson E, Rifai N, Glynn RJ; for the Val-MARC Investigators**. Valsartan, blood pressure reduction, and C-reactive protein. Primary report of the Val-MARC Trial. *Hypertension* 48: 1-7, 2006.

50. **Saito M, Ishimitsu T, Minami J, Ono H, Masami O, and Matsuoka H**. Relations of plasma high-sensitivity C-reactive protein to traditional cardiovascular risk factors. *Atherosclerosis* 167: 73-79, 2003.

51. **Sampaio WO, dos Santos RA, Faria-Silva R, de Mata Machado LT, Schiffrin EL and Touyz RM**. Angiotensin-(1-7) through receptor mas mediates endothelial nitric oxide synthase activation via Akt-dependent pathways. *Hypertension* 49: 185-192, 2007.

52. **Seely EW, Brosnihan KB, Jeunemaitre X, Okamura K, Williams GH, Hollenberg NK and Herrington DM**. Effects of conjugated oestrogen and droloxifene on the renin-angiotensin system, blood pressure and renal blood flow in postmenopausal women. *Clin Endocrinol* 60: 315-321, 2004.

53. **Shagdarsuren E, Wellner M, Braesen JH, Park JK, Fiebeler A, Henke N, Dechend R, Gratze P, Luft FC, and Muller DN**. Complement Activation in Angiotensin II-Induced Organ Damage. *Circ Res* 97: 716-724, 2005.

54. **Shaltout HA, Westwood B, Averill DB, Ferrario CM, Figueroa J, Diz DI, Rose JC and Chappell MC**. Angiotensin metabolism in renal proximal tubules, urine and serum of sheep: Evidence for ACE2-dependent processing of Angiotensin II. *Am J Physiol Renal Physiol* 292 (1): F82-F91, 2007.

55. **Shigenaga MK, Gimeno CJ and Ames BN**. Urinary 8-hydroxy-2'-deoxyguanosine as a biological marker of in vivo oxidative DNA damage. *Proc Natl Acad Sci U S A* 86: 9697-9701, 1989.

56. **Silva-Antonialli MM, Tostes RCA, Fernandes L, Fior-Chadi DR, Akamine EH, Carvalho MHC, Fortes ZB and Nigro D**. A lower ratio of AT_1/AT_2 receptors of angiotensin II is found in female than in male spontaneously hypertensive rats. *Cardiovasc Res* 62: 587-593, 2004.

57. **Stasch J-P, Dietrich CH, Ganten D and Wegner M**. Renal and antihypertensive effects of neutral endopeptidase inhibition in transgenic rats with an extra renin gene. *Am J Hypertens* 9: 795-802, 1996.

58. **Stephenson SL and Kenny AJ**. The metabolism of neuropeptides. Hydrolysis of the angiotensins, bradykinin, substance P and oxytocin by pig kidney microvillar membranes. *Biochemical Journal* 241: 237-247, 1987.

59. **Tanaka M, Nakaya S, Watanabe M, Kumai T, Tateishi T and Kobayashi S**. Effects of ovariectomy and estrogen replacement on aorta angiotensin-converting enzyme activity in rats. *Jpn J Pharmacol* 73: 361-363, 1998.

60. **Thompson J and Khalil RA**. Gender differences in the regulation of vascular tone. *Clin Exp Pharmacol Physiol* 30: 1-15, 2003.

61. **Veniant M, Whitworth CE, Menard J, Sharp MGF, Gonzales MF, Bruneval P and Mullins JJ**. Developmental studies demonstrate age-dependent elevation of renin activity in TGR(mRen2)27 rats. *Am J Hypertens* 8: 1167-1176, 1995.

62. **Vijayaraghavan J, Scicli AG, Carretero OA, Slaughter C, Moomaw C and Hersh LB**. The hydrolysis of endothelins by neutral endopeptidase 24.11 (enkephalinase). *J Biol Chem* 265: 14150-14155, 1990.

63. **Wu Z, Zheng W and Sandberg K**. Estrogen regulates adrenal angiotensin type 1 receptors by modulating adrenal angiotensin levels. *Endocrinology* 144: 1350-1356, 2003.

64. **Yamada K, Iyer SN, Chappell MC, Ganten D and Ferrario CM**. Converting enzyme determines the plasma clearance of angiotensin-(1-7). *Hypertension* 98: 496-502, 1998.

65. **Yamaleyeva LM, Pendergrass KD, Pirro NT, Gallagher PE, Groban L, and Chappell MC**. Ovariectomy is protective against renal injury in the high-salt-fed older mRen(2).Lewis rats. *Am J Physiol Heart Circ Physiol* 293 (4): H2064-71, 2007.

66. **Yang SX, Diaz Padilla N, Zhu Q, Ma XM, Sasso D, Prestwood K, Hack CE, and Kuchel GA**. Estrogen replacement raises rat CRP without evidence of complement activation. *Endocr Res* 31 (2): 121-132, 2005.

CHAPTER FOUR

NEPRILYSIN INHIBITION LOWERS BLOOD PRESSURE IN THE FEMALE HYPERTENSIVE MREN(2).LEWIS

BY

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Short title: ***Neprilysin inhibition decreases blood pressure***

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ABSTRACT

The mRen(2).Lewis rat (mRen2) has an activated renin-angiotensin system (RAS) with increased expression of circulating and renal angiotensin II (Ang II) compared to normotensive Lewis rats. We previously demonstrated that female rats have significantly higher neprilysin (NEP) enzyme activity in the renal cortex compared to male littermates. Female mRen2 also exhibited lower blood pressure and Ang II, in association with higher concentrations of the vasodilator, Ang-(1-7) versus male mRen2. NEP can degrade Ang II to Ang-(1-4) and synthesizes Ang-(1-7) from Ang I. The aim of the present study was to determine whether NEP inhibition in female mRen2 (14 weeks of age) will increase Ang II levels and raise blood pressure, in association with a decrease in Ang-(1-7). The selective NEP inhibitor, SCH 39370 (SCH), was administered for two weeks by osmotic minipump (40 mg/kg/day). SCH significantly decreased blood pressure by 20 mm Hg in female mRen2 [130$\pm$1 vs 109$\pm$1 mm Hg, N=6, $p<0.001$]. Body weight was lower with SCH [257$\pm$5 vs 228$\pm$4 g, N=6, $p<0.01$]. Ang II and Ang-(1-7) levels in both the plasma and urine were not changed following SCH treatment. In summary, we find that SCH decreased blood pressure without an alteration in Ang II or Ang-(1-7) and these data suggest other hormonal systems are involved in the blood pressure lowering actions of the inhibitor. In conclusion, NEP inhibition is a beneficial antihypertensive treatment in a female model that exhibits an activated RAS.

Key words: Ang II, Ang-(1-7), estrogen, neprilysin, cardiac hypertrophy, proteinuria.

INTRODUCTION

The incidence of the cardiovascular disease hypertension becomes more prevalent at a later age in women compared to men (Cutler, et al., 2008). Because the incidence of hypertension increases in females after menopause or estrogen depletion, estrogen may provide a protective antihypertensive effect (Rosenthal and Oparil, 2000). It is well known that estrogen can exert regulatory effects on hormonal systems, such as the renin angiotensin system (RAS) which is an important regulator of blood pressure. Two of the vasoactive peptides in the RAS are angiotensin II (Ang II) and angiotensin-(1-7) (Ang-(1-7)); their main actions are vasoconstriction and vasodilation, respectively. The mRen(2).Lewis strain is a model of Ang II-dependent hypertension that exhibits significant sex differences in high blood pressure (Chappell, et al., 2006; Pendergrass, et al., 2006). The blood pressure of young female mRen(2).Lewis rats was 50 mm Hg lower than age-matched male mRen(2).Lewis rats (Pendergrass, et al., 2008). Assessment of renal and plasma Ang II concentrations showed lower peptide content in female versus male mRen(2).Lewis. Moreover, we also found higher levels of the vasodilatory peptide Ang-(1-7) in the cortical and medullary regions of the kidney and in the plasma of female mRen(2).Lewis compared to male littermates. Based upon our previous work, we propose that lower Ang II and higher levels of Ang-(1-7) may influence the lower degree of hypertension in female mRen2.Lewis.

The main vasoactive and blood pressure actions of the RAS are mediated through the hormones activating their receptors and the physiological status of the animal. The vasoconstrictive and salt retentive actions of Ang II are mediated through the angiotensin

type 1 (AT1) receptor, while Ang-(1-7) binds to the AT(1-7) receptor to produce prostaglandins or nitric oxide for vasodilation (Benter, et al., 2008; Clark, et al., 2003; Kobori, et al., 2007; Santos, et al., 2003). Ang-(1-7) has been shown to lower blood pressure in the spontaneously hypertensive rat (SHR) treated with the nitric oxide synthase inhibitor, L-NAME. The antihypertensive mechanism of Ang-(1-7) infusion in the L-NAME SHR was through a prostanoid-mediated pathway (Benter, et al., 2006). This study provides evidence that exogenous Ang-(1-7) can lower blood pressure.

Neprilysin, also known as enkephalinase, is a zinc metallopeptidase localized in the renal vasculature and brush border region of proximal tubules (Stephenson and Kenny, 1987). We previously reported a sex difference in renal cortical neprilysin activity and protein in which female mRen(2).Lewis rats express a 3-fold higher enzyme activity and 10-fold higher protein expression compared to male mRen(2).Lewis (Pendergrass, et al., 2008). Since neprilysin can synthesize Ang-(1-7) directly from Ang I and degrade Ang II to an inactive metabolite, it is a potential candidate for shifting Ang II/Ang-(1-7) concentrations towards vasodilation. The greater protein expression of neprilysin in female rats is most likely due to estrogen's stimulatory effect on the enzyme. Neprilysin is downregulated in ovariectomized rodents and postmenopausal women (Huang, et al., 2004; Yue, et al., 2005). Long term estrogen replacement in rodents increased neprilysin to control levels (Huang, et al., 2004; Yue, et al., 2005). In our previous study, the higher enzyme activity was positively associated with higher concentrations of Ang-(1-7) in female mRen(2).Lewis rats (Pendergrass, et al., 2008). Therefore, the aim of the present study was to determine whether chronic inhibition of

neprilysin would raise blood pressure through an increase Ang II and decrease in Ang-(1-7) levels in female mRen(2).Lewis rats.

MATERIALS AND METHODS

Experimental animals. Hemizygous female mRen(2).Lewis rats were obtained from the Hypertension and Vascular Research Center Transgenic colony (Chappell, et al., 2006; Pendergrass, et al., 2006; Pendergrass, et al., 2008) at 14 weeks of age. Animals were fed a powdered rat chow (Purina Mills, Richmond,VA) to provide a daily intake of 17 and 28 meq/100 g of body wt of sodium and potassium, respectively. The rats had full access to water, and were housed in an AALAC-approved facility in rooms maintained on a 12:12-hour light-dark cycle (lights on 6:00 A.M. to 6:00 P.M.). Female congenic rats were randomly assigned to control or SCH 39370 (SCH) treatment groups at 14 weeks of age. SCH was prepared as previously described and administered via osmotic minipump for two weeks (28 day, 2.5 μL/hr; model 2ML4, ALZET, Palo Alto, CA) intraperitoneally at a dose of 40 mg/kg/day (Iyer, et al., 2000; Yamamoto, et al., 1992). Animals were housed in metabolic cages (Harvard Bioscience, South Natick, MA) at the completion of the treatment period for a 24 hour collection period. Fluid balance was calculated by the subtraction of urine volume from water intake (mL/24 hr). Systolic blood pressure was also measured after treatment by tail cuff with a Narco Biosystems device (Houston, TX) (Pendergrass, et al., 2008). These procedures were approved by the Wake Forest University School of Medicine Institutional Animal Care and Use Committee.

Plasma and renal tissue hormone assays. Rats were decapitated without anesthesia at the completion of the two week treatment period. Trunk blood (3 to 5 ml) was collected into chilled Vacutainer blood collection tubes (Becton Dickinson, Sandy, UT) containing peptidase inhibitors, blood was spun at 1800 x g and the plasma stored at -80°C. Plasma was extracted and processed for direct radioimmunoassay (RIA) of angiotensin and endothelin peptides (Allred, et al., 2000; Chappell, et al., 2003; Pendergrass, et al., 2006; Pendergrass, et al., 2008). Details on the RIAs have been previously described (Chappell, et al., 2003; Pendergrass, et al., 2006). Following blood collection, tissues were rapidly collected. For hearts and kidneys, the tissue was blotted, weighed and snap frozen on dry ice. Cardiac and renal wet weights were expressed as mg organ weight (mg). The cardiac and renal weight indices were expressed as mg organ weight/gm total body weight (mg/g).

Urinary markers. Urine was collected in the presence of 1% HCl over a 24 hr period at the conclusion of the inhibitor treatment, stored at -20°C, and extracted for angiotensin and endothelin peptide determination by RIA.

Statistical Analyses. All measurements are expressed as the mean ± standard error of the mean (SEM) computed from systolic blood pressure or biochemical data from each rat. Comparisons between the control and treatment groups were evaluated using Student's T test. The minimum statistical significance was reached at $p \leq 0.05$. These analyses were performed with the GraphPad Prism IV plotting and statistical software (San Diego, CA).

RESULTS

Physiological Parameters: Administration of the neprilysin inhibitor, SCH 39370 (SCH), for two weeks significantly lowered systolic blood pressure in 16 week old hypertensive female mRen(2).Lewis rats. The treatment decreased blood pressure by 21±1 mm Hg (Figure 1). The body weight of the SCH-treated group was significantly less than control and the lower body weight occurred in the absence of alterations in 24 hour feeding behavior (Figures 2A-B). Moreover, no difference was observed in fluid balance between the two groups (Balance: C: 12.1 ± 2.0 mL/24 hr vs. SCH: 11.9 ± 1.1 mg; n=5, $p>0.05$). The balance was determined from the 24 hour water intake and urine output at the completion of the study (Figures 2C-D). Next, we assessed the organ weights in both groups. Hearts from the SCH-treated group were significantly smaller than control hearts (Heart: C: 0.80 ± 0.02 mg vs. SCH: 0.68 ± 0.01 mg; n=6, $p<0.001$). Kidneys were also significantly smaller with treatment (Kidney: C: 0.90 ± 0.02 mg vs. SCH: 0.78 ± 0.02 mg; n=5-6, $p<0.01$). However, the differences in cardiac and renal weights were negated when values were normalized to body weight (Figure 3A-B).

Plasma and Urinary Peptides: Angiotensins and endothelin are potent vasoactive hormones and both are metabolized by neprilysin. Therefore, we assessed Ang II, Ang-(1-7), and endothelin-1 (ET-1) in the plasma and urine (Figures 4A-F), and we found plasma and urinary levels of Ang II (Figure 4A, D) and Ang-(1-7) (Figure 4B, E) were not changed due to SCH treatment. Furthermore, no significant differences were present in the circulating or urinary concentrations of ET-1 following SCH treatment (Figure 4C,

4F). Even though, a large difference was present in the urinary ET-1 levels, the variability in the data negated significance in this comparison.

DISCUSSION

The purpose of the present study was to determine whether chronic pharmacological inhibition of neprilysin in female mRen(2).Lewis rats would increase blood pressure through alterations in the RAS. The mRen(2).Lewis rat exhibits a marked sex difference in hypertension and it is known that estrogen plays a role in the blood pressure in this model (Chappell, et al., 2003; Pendergrass, et al., 2008). The RAS plays a vital role in the development and maintenance of high blood pressure in this model, due to the fact that the hypertension can be abolished with RAS blockade (Jessup, et al., 2006). Higher levels of Ang II in the plasma were associated with a higher degree of hypertension in the male mRen(2).Lewis compared to their female littermates. However, the lower blood pressure found in female mRen(2).Lewis has been attributed to the effects of estrogen that could inhibit production of Ang II and preserve Ang-(1-7) potentially through the downregulation of ACE (Brosnihan, et al., 1997; Chappell, et al., 2006; Pendergrass, et al., 2008). Indeed, we previously reported in the mRen(2).Lewis that Ang-(1-7) levels were 3-fold higher in the plasma of females compared to males (Pendergrass, et al., 2008). Therefore, we evaluated potential Ang-(1-7) synthesizing pathways in the RAS that could produce higher levels of the vasodilator in the female rats. The RAS cascade has multiple enzymes that influence Ang peptide expression. Two enzymes that generate Ang-(1-7) from Ang II and Ang I are ACE2 and neprilysin,

respectively (Shaltout, et al., 2006). Our previous findings in the mRen(2).Lewis rat demonstrated that ACE2 was significantly higher in males, but neprilysin activity was 3-fold higher in female rats. An increase in neprilysin activity could generate higher levels of Ang-(1-7) in the females, thus providing a mechanism to lower blood pressure and renal injury. However, the current study shows that chronic SCH treatment to inhibit neprilysin did not increase blood pressure in the female rats. Indeed, we observed a 21 mm Hg decrease in blood pressure following a two week administration of SCH in female mRen(2).Lewis rats.

Neprilysin inhibition alters multiple hormone systems and one influential system is the RAS. The SCH compound is selective for neprilysin and does not have a strong affinity for ACE, unlike other neprilysin inhibitors (Stephenson and Kenny, 1987). SCH treatment in female mRen(2).Lewis rats did not significantly alter plasma or urinary Ang II levels compared to control rats; however, plasma Ang II levels were slightly higher in the SCH-treated versus control mRen(2).Lewis rats. Campbell et al. (Campbell, et al., 1998) found that in male Sprague Dawley rats increasing doses of ecadotril, a neprilysin inhibitor with ACE inhibitor qualities, caused an inhibitory bimodal effect on circulating Ang II. The 1 and 10 mg/kg doses of ecadotril were the only concentrations to significantly increase plasma Ang II in normotensive male Sprague Dawley rats, while blood pressure was significantly lowered only with the 10 mg/kg ecadotril dose. The previous study shows evidence that is consistent with our findings for a separation between blood pressure and circulating Ang II concentrations during neprilysin inhibition. Even though neprilysin inhibition lowered blood pressure in a normotensive rat; further tests were needed to determine the effects of neprilysin inhibition in other

models of cardiovascular disease such as myocardial infarction. Surprisingly, ecadotril treatment did not alter blood pressure or plasma Ang II levels in a model of myocardial infarction compared to the vehicle-treated myocardial infarcted group (Duncan, et al., 1999). Although we did not observe a significant increase in plasma Ang II levels in the female mRen(2).Lewis as seen in the normotensive male Sprague Dawley, a significant decrease in blood pressure was seen in both rat strains. Interestingly, the inhibition of neprilysin in a female or male model of cardiovascular disease does not appear to activate the RAS through a significant increase in Ang II concentrations, regardless of the effect on blood pressure.

Since, we did not observe a change in Ang II that could account for the decrease in blood pressure, we assessed an additional circulating vasoactive hormone, ET-1, that is metabolized by neprilysin. Plasma ET-1 concentrations were previously evaluated in a volume-dependent model of hypertension, the male deoxycorticosterone acetate (DOCA)-salt rat, with chronic candoxatril treatment (Newaz, et al., 2003). Candoxatril is an orally active neprilysin inhibitor. After three weeks of candoxatril treatment, plasma ET-1 levels were significantly decreased and systolic blood pressure was lowered by more than 30 mm Hg. However, in our study the plasma ET-1 levels were not different between SCH-treated and control female mRen(2).Lewis. Therefore, the antihypertensive actions of neprilysin inhibition in female mRen(2).Lewis rats did not appear to be mediated through a decrease in circulating Ang II or ET-1. Our findings demonstrate SCH is an antihypertensive medication in female mRen(2).Lewis rats and the long term hormonal mechanisms have yet to be elucidated.

We previously demonstrated that neprilysin inhibition decreased circulating Ang-(1-7) and its vasodilatory actions in male spontaneously hypertensive rats (SHR) (Iyer, et al., 2000; Yamamoto, et al., 1992). The SHR is a genetic model of hypertension. The genesis of hypertension is unknown in the SHR, although RAS blockade does normalize mean arterial pressure in the SHR to that of the Wistar Kyoto rat (Bolterman, et al., 2005). Intravenous infusion of Ang I in the SHR was rapidly metabolized to Ang-(1-7) through a neprilysin-dependent pathway (Yamamoto, et al., 1992). Vascular neprilysin can degrade circulating Ang II and synthesize Ang-(1-7) which could provide a net vasodilatory effect. Iyer et al. (Iyer, et al., 1998) found Ang-(1-7) contributes to the antihypertensive effects of RAS blockade in SHR. They found that neprilysin inhibition blocked the generation of Ang-(1-7) and mean arterial pressure was increased in male SHR; however, the effects of neprilysin inhibition are not known in a female rodent model of hypertension. In SCH-treated female mRen(2).Lewis rats, plasma levels of Ang-(1-7) were not different compared to control rats although systolic blood pressure was lowered. The maintenance of Ang-(1-7) levels suggests that we did not achieve full inhibition of neprilysin or the peptide was generated by other enzymatic pathways, such as ACE2, prolyl oligopeptidase, or thimet oligopeptidase (Gallagher and Tallant, 2004). We previously documented that renal ACE2 enzyme activity is approximately 10-fold less than renal neprilysin activity with Ang II as a substrate in female mRen(2).Lewis (Pendergrass, et al., 2008). Presently, the enzyme activities of prolyl oligopeptidase and thimet oligopeptidase are unknown in the female mRen(2).Lewis rat. ACE2 would appear to be a candidate, since circulating levels of Ang II were slightly elevated in the SCH-treated animals versus controls, but the values were not significantly different. An

additional pathway that could explain the slight increase in Ang II with SCH treatment is that the decrease in blood pressure could activate baroreceptors. The increase in baroreceptor activity could release greater concentrations of renin from the juxtaglomerular apparatus which could lead to an increase in the generation of Ang I. The greater concentration of Ang I would be converted by ACE to Ang II and not by a direct conversion to Ang-(1-7) by neprilysin due to the inhibitor. The previous mechanism would suggest that neprilysin is a main degradation pathway for Ang II. Moreover, chronic inhibition of the enzyme leads to an accumulation of Ang II which may explain the small increase of Ang II observed in the present study. Since, we find that SCH treatment significantly lowers blood pressure in the absence of a decrease in circulating Ang-(1-7); our results indicate the antihypertensive actions of SCH are not mediated through an Ang-(1-7)-dependent pathway.

Atrial natriuretic peptide (ANP) is an influential hormone that possesses vasodilatory and natriuretic properties. Neprilysin degrades natriuretic peptides, and the effects of neprilysin inhibition on natriuretic peptides have been evaluated in models of hypertension with low and high expression of renin. When a bolus dose of SCH is administered, an increase in diuresis and natriuresis is observed in hypertensive animals compared to vehicle-treated animals. The diuretic and natriuretic actions of neprilysin inhibition are transient in animals, independent of the treatment duration (Kukkonen, et al., 1992; Stasch, et al., 1996; Sybertz, et al., 1989; Sybertz, et al., 1990). Thus, the long-term antihypertensive actions of neprilysin inhibition must include pathways other than an increase in fluid excretion alone. One study did assess a non volume-dependent mechanism through a three week administration of the neprilysin inhibitor CGS 25462 in

DOCA-salt rats which significantly decreased blood pressure and remodeled mesenteric resistance arteries (Pu, et al., 2002). The antihypertensive effects of CGS 25462 were attributed to positive remodeling of the small arteries in which arteries exhibited an increase in lumen diameter from a decrease in vascular wall collagen deposition compared to the control DOCA-salt rats. The vasculature from CGS 25462-treated rats exhibited a greater lumen to media ratio and endothelium-dependent relaxation in response to acetylcholine than untreated DOCA-salt rats (Pu, et al., 2002). The authors hypothesized that ANP was associated with the beneficial structural effects in the vasculature and the increase in vasorelaxation, although the natriuretic peptide levels were not assessed in the study. Neprilysin inhibition has been assessed in various models of hypertension that exhibit different levels of RAS activation. The DOCA-salt rat is a model of low renin expression, while the (mRen-2)27 Sprague Dawley rat is a model of renin overexpression. Stasch and colleagues administered an oral dose of a neprilysin inhibitor ecadotril at 60 mg/kg/day for 13 weeks to male (mRen-2)27 Sprague Dawley rats (Stasch, et al., 1996). They observed approximately a 30 mm Hg decrease in blood pressure and approximately 30% decrease in plasma renin activity. However, plasma ANP levels and urine volumes were similar in control and SCH-treated rats as reported by Stasch et al. (Stasch, et al., 1996) and we also observed similar urine volumes with SCH treatment in female mRen(2).Lewis rats. Although, we did not assess ANP in our model, the similar urine volumes would suggest that the diuretic effects of ANP have declined by the completion of the two week treatment period. Previous studies and our work indicate that pharmacological inhibition of neprilysin is beneficial in lowering

blood pressure in various hypertensive animals, but the antihypertensive mechanism has yet to be elucidated.

A potential mechanism for the antihypertensive effects of SCH treatment could arise from the decrease in body weight and/or an augmentation in metabolic activity. Compared to controls, the SCH-treated animals either did not exhibit the same increase in body weight or the treatment group lost weight, while a similar feeding behavior was observed in both groups at the completion of the study. A strong link has been reported between metabolic dysfunction leading to obesity and hypertension (Hall, et al., 1996; Singer and Setaro, 2008). Hormones that regulate metabolic homeostasis, such as insulin and glucagon, are potential targets of dysregulation that may lead to obesity. Neprilysin inactivates glucagon-like peptide 1 (GLP-1), a peptide that has a stimulatory effect on insulin sensitivity and production (Plamboeck, et al., 2005). Higher levels of GLP-1 in the circulation, heart, pancreas, or kidney may provide a pathway for these organs to improve glucose utilization. Furthermore, SCH treatment could stimulate lipolysis through an increase of GLP-1 which would subsequently raise glucagon. However, these parameters were not evaluated in the present study and warrant future evaluation in terms of obesity and hypertension.

In conclusion, we demonstrate that chronic neprilysin inhibition exerts antihypertensive actions in a female model of Ang II-dependent hypertension. Evaluation of the RAS indicated plasma and urinary Ang peptides were not changed at the conclusion of the study. Moreover, ET-1 levels were also similar between control and SCH-treated groups which may discount the importance of the circulating hormone

concentrations. The variability in urinary ET-1 concentrations suggests the kidney is a local system with a considerable capability to synthesize or degrade this peptide. A different mechanism that may explain the antihypertensive actions is through activation of the AT2, AT(1-7), or ETB receptors. Due to the observation that plasma levels of Ang II, Ang-(1-7), and ET-1 were unchanged in the presence of SCH, we propose that these peptides may act through their respective receptors to elicit vasorelaxant or natriuretic actions to initiate and maintain the lower blood pressure. Additional antihypertensive benefits of neprilysin inhibition could be mediated through an improvement in glucose utilization and metabolic regulation. The lower body weight in the SCH-treated females could provide antihypertensive effects through secondary mechanisms by affecting metabolism, due to the link between hypertension and obesity. Additional areas to evaluate are multiple hormone systems like ANP and bradykinin that are substrates of neprilysin which could mediate the beneficial findings in this model of hypertension. Future studies should assess the central nervous system, energy metabolism, and vascular resistance to develop a better understanding of the effects from neprilysin inhibition in both females and males.

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HL-56973-S1, HL-07790, GM64249), and unrestricted grants from Unifi, Inc., Greensboro, NC, and Farley-Hudson Foundation, Jacksonville, NC.

Figure Legends

Figure 1. Systolic blood pressure (SBP) was decreased in female mRen(2).Lewis rats with chronic administration of SCH 39370 (SCH, 40 mg/kg/day, intraperitoneal osmotic minipump) for 2 weeks. SBP was measured by tail cuff plysmography in control female rats (open bar) and SCH treated females (closed bar) at 16 weeks of age. Data are the mean $\pm$ SEM, #$p<0.005$ versus Control (n = 6 rats per group).

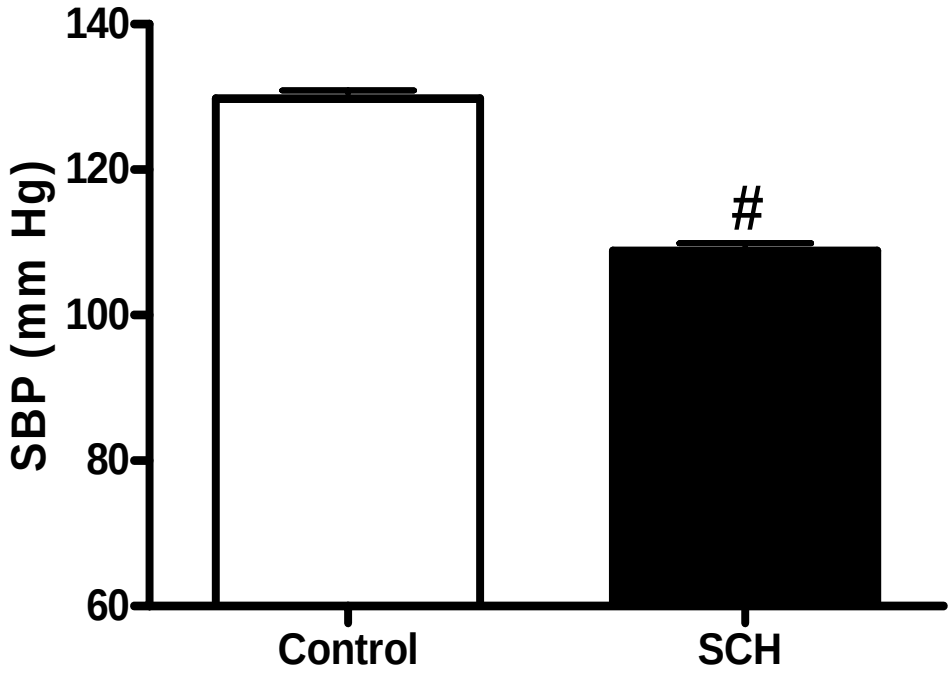

Figure 1.

Figure 2. Chronic administration of SCH 39370 decreased body weight, but not feeding behavior, fluid intake or fluid excretion in female mRen(2).Lewis rats. Effects of SCH 39370 (SCH) treatment on body weight (A), water intake (B), food intake (C), and urine excretion (D) determined on day 13. Data are the mean $\pm$ SEM, #$p<0.01$ versus Control ($n = 5 - 6$ rats per group).

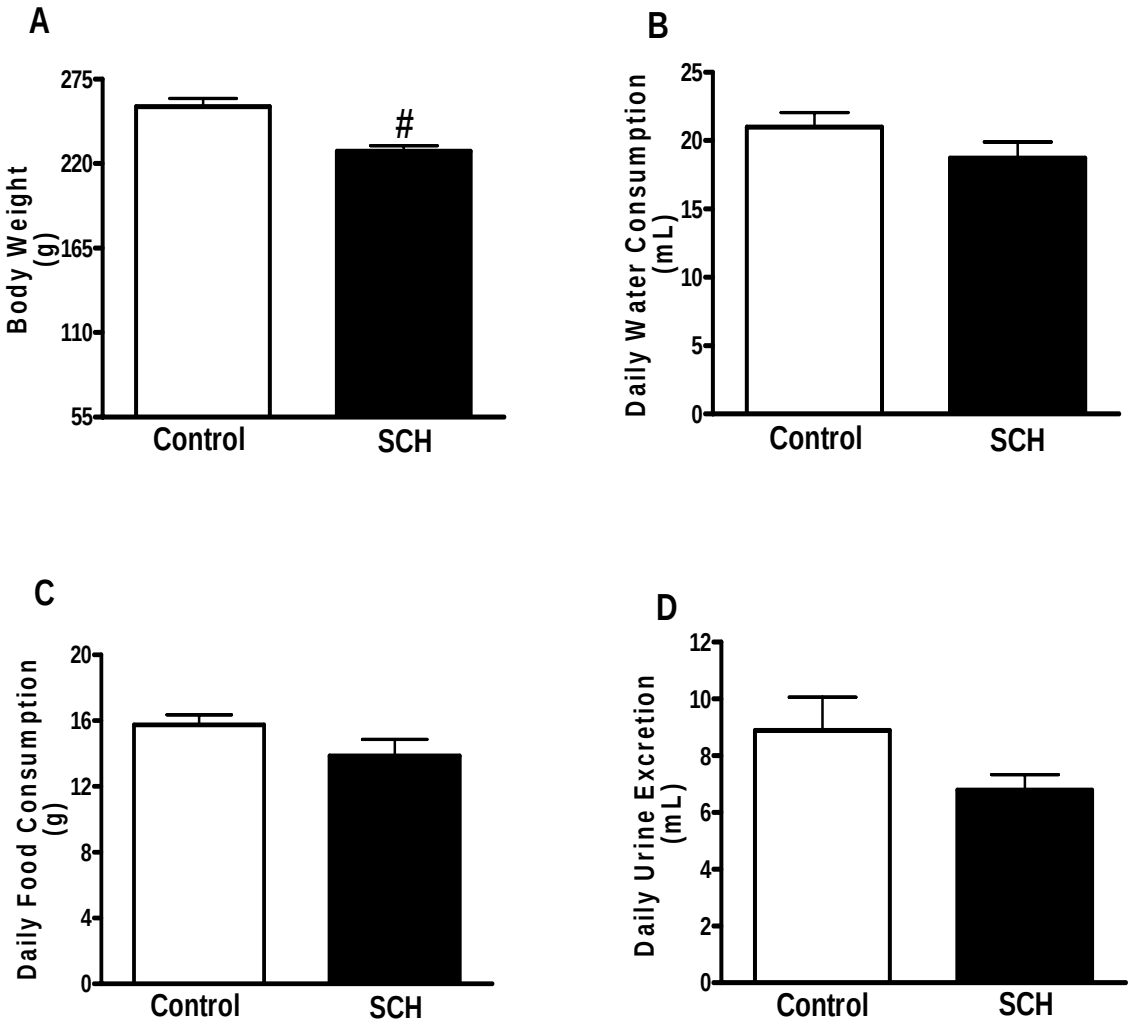
A
Body Weight (g)
275
220
165
110
55
#
Control
SCH
B
Daily Water Consumption (mL)
25
20
15
10
5
0
Control
SCH
C
Daily Food Consumption (g)
20
16
12
8
4
0
Control
SCH
D
Daily Urine Excretion (mL)
12
10
8
6
4
2
0
Control
SCH

Figure 2

Figure 3. Chronic administration of SCH 39370 (SCH) does not alter organ weight in female mRen(2).Lewis rats. Cardiac (A) and renal (B) organ weights were not different with SCH treatment. Data are the mean $\pm$ SEM, (n = 5 - 6 rats per group).

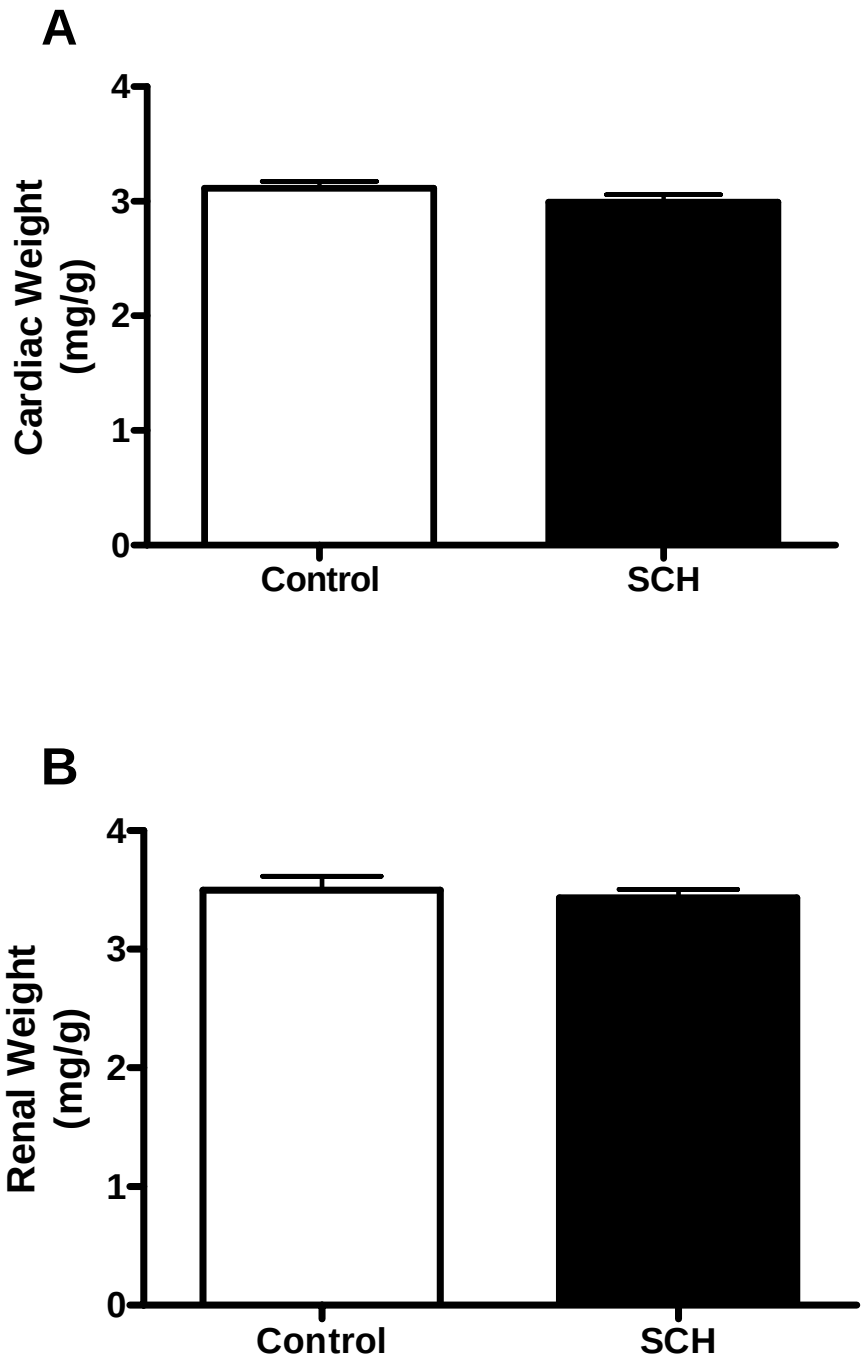
A
Cardiac Weight (mg/g)
4
3
2
1
0
Control
SCH
B
Renal Weight (mg/g)
4
3
2
1
0
Control
SCH

Figure 3

Figure 4. Evaluation of angiotensin and endothelin peptides in the plasma and urine after chronic SCH 39370 (SCH) treatment in female mRen(2).Lewis rats. Angiotensin II (Ang II, A, n=5), Angiotensin-(1-7) (Ang-(1-7), B, n=6), and Endothelin (ET-1, C, n=5) were extracted from plasma measured by separate radioimmunoassays [RIAs] and expressed as pM. Ang II (D, n=6), Ang-(1-7) (E, n=5-6), and ET-1 (F, n=5) were extracted from urine collected in acid and measured by Ang II, Ang-(1-7), and ET-1 RIAs. Data are the mean $\pm$ SEM.

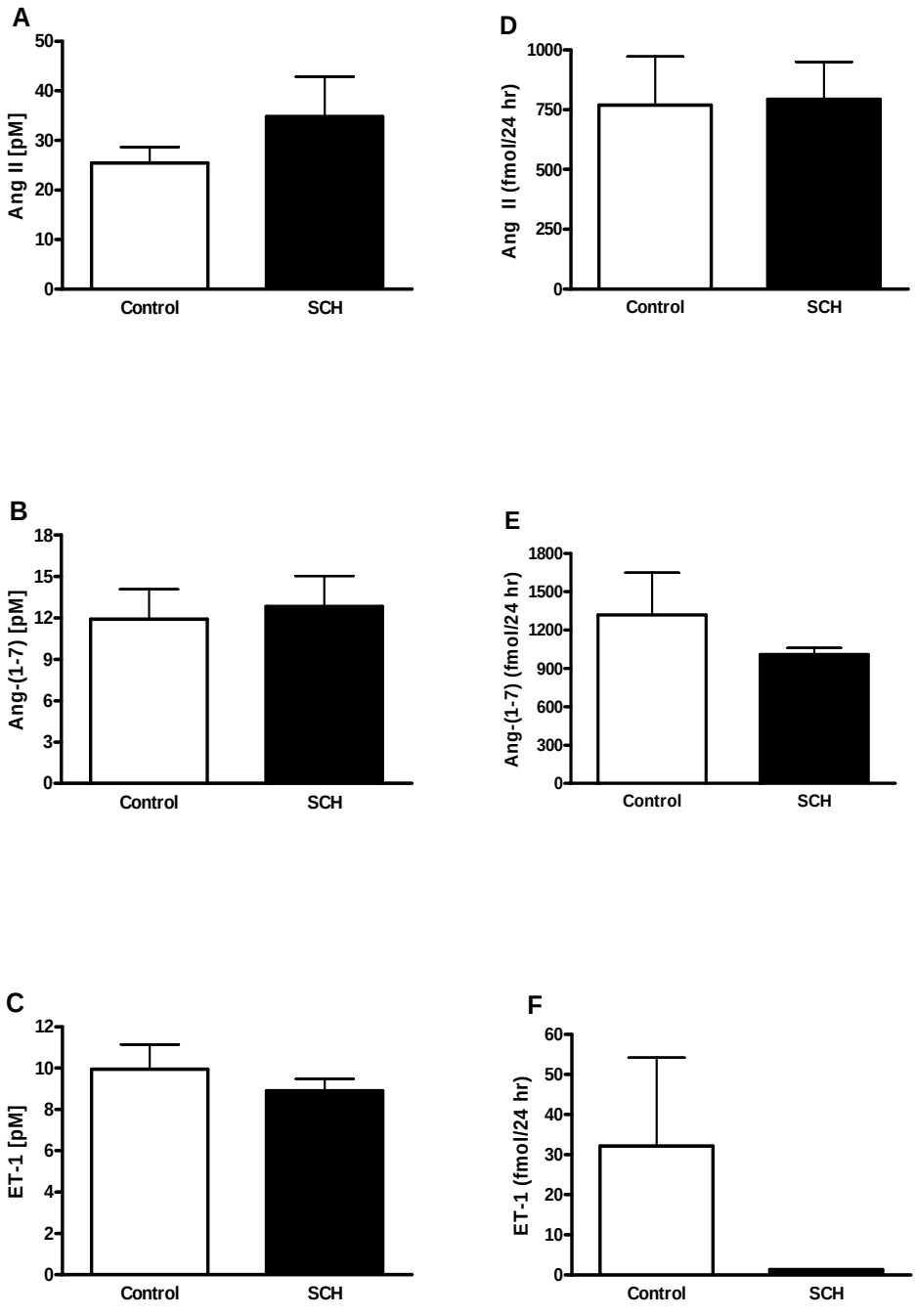
A
Ang II [pM]
50
40
30
20
10
0
Control
SCH
D
Ang II (fmol/24 hr)
1000
750
500
250
0
Control
SCH
B
Ang-(1-7) [pM]
18
15
12
9
6
3
0
Control
SCH
E
Ang-(1-7) (fmol/24 hr)
1800
1500
1200
900
600
300
0
Control
SCH
C
ET-1 [pM]
12
10
8
6
4
2
0
Control
SCH
F
ET-1 (fmol/24 hr)
60
50
40
30
20
10
0
Control
SCH

Figure 4

Reference List

Allred AJ, Chappell MC, Ferrario CM and Diz DI (2000) Differential actions of renal ischemic injury on the intrarenal angiotensin system. *Am J Physiol :Renal Physiol* **279**:F636-F645.

Benter IF, Yousif MH, Dhaunsi GS, Kaur J, Chappell MC and Diz DI (2008) Angiotensin-(1-7) prevents activation of NADPH oxidase and renal vascular dysfunction in diabetic hypertensive rats. *Am J Nephrol* **28**:25-33.

Benter IF, Yousif MHM, Anim JT, Cojocel C and Diz DI (2006) Angiotensin-(1-7) prevents development of severe hypertension and end-organ damage in spontaneously hypertensive rats treated with L-NAME. *Am J Physiol Heart Circ Physiol* **290**:H684-H691.

Bolterman RJ, Manriquez MC, Ortiz Ruiz MC, Juncos LA and Romero JC (2005) Effects of captopril on the renin angiotensin system, oxidative stress, and endothelin in normal and hypertensive rats. *Hypertension* **46**:943-947.

Brosnihan KB, Li P, Ganten D and Ferrario CM (1997) Estrogen protects transgenic hypertensive rats by shifting the vasoconstrictor-vasodilator balance of RAS. *Am J Physiol* **273**:R1908-R1915.

Campbell DJ, Anastasopoulos F, Duncan AM, James GM, Kladis A and Briscoe TA (1998) Effects of neutral endopeptidase inhibition and combined angiotensin converting enzyme and neutral endopeptidase inhibition on angiotensin and bradykinin peptides in rats. *Journal of Pharmacology & Experimental Therapeutics* **287**:567-577.

Chappell MC, Gallagher PE, Averill DB, Ferrario CM and Brosnihan KB (2003) Estrogen or the AT1 antagonist olmesartan reverses the development of profound hypertension in the congenic mRen2.Lewis rat. *Hypertension* **42**:781-786.

Chappell MC, Yamaleyeva LM and Westwood BM (2006) Estrogen and salt sensitivity in the female mRen(2).Lewis rat. *Am J Physiol Regul Integr Comp Physiol* **291**:R1557-R1563.

Clark MA, Tommasi EN, Bosch SM, Tallant EA and Diz DI (2003) Angiotensin-(1-7) reduces renal angiotensin II receptors through a cyclooxygenase dependent pathway. *J Cardiovasc Pharmacol* **41**:276-283.

Cutler JA, Sorlie PD, Wolz M, Thom T, Fields LE and Roccella EJ (2008) Trends in hypertension prevalence, awareness, treatment, and control rates in United States adults between 1988-1994 and 1999-2004. *Hypertension* **52**:818-827.

Duncan AM, James GM, Anastasopoulos F, Kladis A, Briscoe TA and Campbell DJ (1999) Interaction between neutral endopeptidase and angiotensin converting enzyme inhibition in rats with myocardial infarction: effects on cardiac hypertrophy and angiotensin and bradykinin peptide levels. *Journal of Pharmacology & Experimental Therapeutics* **289**:295-303.

Gallagher PE and Tallant EA (2004) Inhibition of human lung cancer cell growth by angiotensin-(1-7). *Carcinogenesis* **25**:2045-2052.

Hall JE, Zappe DH, Alonso-Galicia M, Granger JP, Brands MW and Kassab SE (1996) Mechanisms of obesity-induced hypertension. *News Physiol Sci* **11**:255-261.

Huang J, Guan H, Booze RM, Eckman CB and Hersh LB (2004) Estrogen regulates neprilysin activity in rat brain. *Neurosci Lett* **367**:85-87.

Iyer SN, Averill DB, Chappell MC, Yamada K, Jones AG and Ferrario CM (2000) Contribution of angiotensin-(1-7) to blood pressure regulation in salt-depleted hypertensive rats. *Hypertension* **36**:417-422.

Iyer SN, Ferrario CM and Chappell MC (1998) Angiotensin-(1-7) contributes to the antihypertensive effects of blockade of the renin-angiotensin system. *Hypertension* **31**:356-361.

Jessup JA, Gallagher PE, Averill DB, Brosnihan KB, Tallant EA, Chappell MC and Ferrario CM (2006) Effect of angiotensin II blockade on a new congenic model of hypertension derived from transgenic Ren-2 rats. *Am J Physiol Heart Circ Physiol* **291**:H2166-H2172.

Kobori H, Nangaku M, Navar LG and Nishiyama A (2007) The intrarenal renin-angiotensin system: from physiology to the pathobiology of hypertension and kidney disease. *Pharmacol Rev* **59**:251-287.

Kukkonen P, Vuolteenaho O and Ruskoaho H (1992) Basal and volume expansion-stimulated plasma atrial natriuretic peptide concentrations and hemodynamics in conscious rats: effects of SCH 39.370, an endopeptidase inhibitor, and C-ANF-(4-23), a clearance receptor ligand. *Endocrinology* **130**:755-765.

Newaz MA, Yousefipour Z, Hercule H, Truong L and Oyekan A (2003) Chronic endopeptidase inhibition in DOCA-salt hypertension: mechanism of cardiovascular protection. *Clin Exp Hypertens* **25**:335-347.

Pendergrass KD, Averill DB, Ferrario CM, Diz DI and Chappell MC (2006) Differential expression of nuclear AT1 receptors and angiotensin II within the kidney of the male congenic mRen(2).Lewis rat. *Am J Physiol Renal Physiol* **290**:F1497-F1506.

Pendergrass KD, Pirro NT, Westwood BM, Ferrario CM, Brosnihan KB and Chappell MC (2008) Sex differences in circulating and renal angiotensins of hypertensive mRen(2).Lewis but not normotensive Lewis rats. *Am J Physiol Heart Circ Physiol* **295**:H10-H20.

Plamboeck A, Holst JJ, Carr RD and Deacon CF (2005) Neutral endopeptidase 24.11 and dipeptidyl peptidase IV are both mediators of the degradation of glucagon-like peptide 1 in the anaesthetized pig. *Diabetologia* **48**:1882-1890.

Pu Q, Touyz RM and Schiffrin EL (2002) Comparison of angiotensin-converting enzyme (ACE), neutral endopeptidase (NEP) and dual ACE/NEP inhibition on blood pressure and resistance arteries of deoxycorticosterone acetate-salt hypertensive rats. *J Hypertens* **20**:899-907.

Rosenthal T and Oparil S (2000) Hypertension in women. *J Human Hypertens* **14**:691-704.

Santos RAS, Simoes e Silva AC, Maric C, Silva D.M., Machado RP, de Bul I, Heringer-Walther S, Pinheiro SV, Lopes MT, Bader M, Mendes EP, Lemos VS, Campagnole-Santos MJ, Schultheiss H-P, Speth R and Walther T (2003) Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci USA* **100**:8258-8263.

Shaltout HA, Westwood B, Averill DB, Ferrario CM, Figueroa J, Diz DI, Rose JC and Chappell MC (2006) Angiotensin metabolism in renal proximal tubules, urine and serum of sheep: Evidence for ACE2-dependent processing of angiotensin II. *Am J Physiol Renal Physiol* **292**:F82-F91.

Singer GM and Setaro JF (2008) Secondary hypertension: Obesity and the metabolic syndrome. *The Journal of Clinical Hypertension* **10**:567-574.

Stasch J-P, Dietrich CH, Ganten D and Wegner M (1996) Renal and antihypertensive effects of neutral endopeptidase inhibition in transgenic rats with an extra renin gene. *Am J Hypertens* **9**:795-802.

Stephenson SL and Kenny AJ (1987) The metabolism of neuropeptides. Hydrolysis of the angiotensins, bradykinin, substance P and oxytocin by pig kidney microvillar membranes. *Biochemical Journal* **241**:237-247.

Sybertz EJ, Chiu PJ, Vemulapalli S, Watkins R and Haslanger MF (1990) Atrial natriuretic factor-potentiating and antihypertensive activity of SCH 34826. An orally active neutral metalloendopeptidase inhibitor. *Hypertension* **15**:152-161.

Sybertz EJ, Chiu PJS, Vemulapalli S, Pitts B, Foster CJ, Watkins RW, Barnett A and Haslanger MF (1989) SCH 39370, a neutral metalloendopeptidase inhibitor, potentiates biological responses to atrial natriuretic factor and lowers blood pressure in

deoxycorticosterone acetate-sodium hypertensive rats. *J Pharmacol Exp Ther* **250**:624-631.

Yamamoto K, Chappell MC, Brosnihan KB and Ferrario CM (1992) In vivo metabolism of angiotensin I by neutral endopeptidase (EC 3.4.24.11) in spontaneously hypertensive rats. *Hypertension* **19**:692-696.

Yue X, Lu M, Lancaster T, Cao P, Honda S, Staufenbiel M, Harada N, Zhong Z, Shen Y and Li R (2005) Brain estrogen deficiency accelerates Abeta plaque formation in an Alzheimer's disease animal model. *Proc Natl Acad Sci U S A* **102**:11811-11816.

CHAPTER FIVE

CONCLUSION

1. Comparison of Male mRen(2).Lewis and Male Lewis: Circulating RAS

An increase in the circulating RAS has been associated with hypertension and inflammation (19). Higher levels of Ang II are present in models of hypertension, such as (mRen-2)27 Sprague Dawley and Ang II infused rats compared to their controls (11; 15; 50; 56; 80). Although the high levels of plasma Ang II arise from different pathways between the (mRen-2)27 Sprague Dawley rats and Ang II infusion, the circulating peptide is associated with hypertension. However, circulating Ang II levels in our first report were similar between the mRen(2).Lewis and Lewis rats. Moreover, plasma Ang II levels were higher than previous reports in the (mRen-2)27 Sprague Dawley and mRen(2).Lewis strains (11; 55; 56). Recently, Huskova et al. (29) reported that the use of anesthesia elevated circulating Ang peptides in (mRen-2)27 Sprague Dawley and Sprague Dawley rats. The use of anesthesia may likely mask any significant differences in plasma Ang II between the (mRen-2)27 Sprague Dawley and Sprague Dawley strains. Therefore, in subsequent studies, the mRen(2).Lewis and Lewis rats were decapitated without anesthesia to eliminate any potential influence on the circulating RAS. Indeed, in the absence of anesthesia, plasma Ang peptide concentrations in trunk blood were in a similar range to that previously reported for circulating Ang II (11; 56). The use of decapitation in young homozygous and heterozygous male (mRen-2)27 Sprague Dawley

demonstrated a greater than two-fold elevation in plasma Ang II levels compared to Sprague Dawley rats (11; 46). Similar to the (mRen-2)27 Sprague Dawley results, plasma Ang II concentrations from the mRen(2).Lewis rat were five-fold higher than Lewis rats. An elevation of plasma Ang II can raise blood pressure through multiple mechanisms, such as an increase in peripheral vascular resistance, or augment salt and water retention.

As previously stated, Ang II can act through multiple pathways to increase blood pressure, but in the mRen(2).Lewis rat the pathways that contribute to the higher plasma Ang II are not known. Consistent with the higher plasma Ang II, Ang I levels were significantly higher in male mRen(2).Lewis compared to male Lewis rats (56). The higher Ang I and Ang II in plasma suggest an increase in renin activity. Plasma renin circulates throughout the body in two states - active renin and an inactive state termed prorenin. Active renin contributes to the synthesis of endogenous plasma Ang I. Both forms of renin can be determined in plasma, but prorenin must be activated through an *in vitro* trypsinization step to measure the generation of Ang I from angiotensinogen. We only determined active plasma renin at different pH optima for rat and mouse renin in the mRen(2).Lewis and Lewis rat strains, as previously documented in the (mRen-2)27 Sprague Dawley (8). Rat renin activity was significantly elevated in male mRen(2).Lewis compared to male Lewis rats. Active mouse renin was present only in mRen(2).Lewis rats and its activity could also contribute to the higher circulating Ang peptides because it cleaves rat angiotensinogen (8; 63). Plasma angiotensinogen levels were not different between male mRen(2).Lewis and Lewis rats, which indicate the

mRen(2).Lewis is not substrate limited in the presence of high renin activity and elevated blood pressure.

Male mRen(2).Lewis rats had high levels of plasma rat renin in the presence of systolic blood pressures in excess of 150 mm Hg and high circulating Ang II, which should inhibit plasma renin levels. Hypertension and high plasma Ang II should attenuate release of rat renin from the kidney, likely the main source of plasma renin (36). The (mRen-2)27 Sprague Dawley rat exhibits a systolic blood pressure in excess of 170 mm Hg (40). Blood pressure at that level should decrease renin synthesis and release from juxtaglomerular (JG) cells through both the sympathetic nervous system and direct actions on the afferent arteriole and JG cells (12; 40). Indeed, an inhibitory action of hypertension on renal renin is present in young male heterozygous (mRen-2)27 Sprague Dawley rat, in which the hypertensive rats have fewer renin granules compared to Sprague Dawley rats (2). Furthermore, Ang II activates AT1 receptors on JG cells to stimulate protein kinase C (PKC) and calcium signaling pathways that decrease renin release (39). Since the (mRen-2)27 Sprague Dawley rat has a low expression of renal renin that should not be capable of supplying the high circulating concentration of rat renin, the elevated plasma levels of rat renin must be derived from a non-renal origin. As previously stated, the (mRen-2)27 Sprague Dawley rats express different species isoforms of renin similar to the mRen(2).Lewis rat. The higher plasma renin was first characterized as primarily mouse Ren-2 renin secreted from the adrenal gland, thymus, and small intestine in (mRen-2)27 Sprague Dawley rats (8; 50; 57; 64; 75). The contribution of mouse renin from these tissues would not explain the high level of rat renin observed in the mRen(2).Lewis rats, which also suggests the rat renin is of extra-

renal origin. Bader et al. (3) demonstrated that the liver has a high rat renin gene expression in the (mRen-2)27 Sprague Dawley. The generation and secretion of renin from the liver has not been evaluated in the mRen(2).Lewis rat, but the liver could be a potential non-regulated source of plasma rat renin. Although the pathway that increases the circulating renin levels is unknown, renin activity does appear to contribute to the Ang II-mediated hypertensive phenotype in the mRen(2).Lewis rat.

2. Comparison of Male mRen(2).Lewis and Male Lewis: Renal RAS

Hypertension and renal injury in the mRen(2).Lewis rat are associated with higher renal RAS peptides compared to the Lewis rat. The male mRen(2).Lewis rat exhibits both higher blood pressure and intrarenal Ang II compared to male Lewis (56). Similar to our earlier findings, male (mRen-2)27 Sprague Dawley rats also express greater cortical Ang II compared to their control strain (11; 48). In order to determine the origin of the renal Ang II, we assessed components of the renal RAS that can contribute to the greater intrarenal Ang II in male mRen(2).Lewis rats. Enzymatic production and/or degradation are potential mechanisms that could contribute to the higher intrarenal Ang II peptide levels in the mRen(2).Lewis rats. Low renal Ang I in male mRen(2).Lewis rats suggests decreased renal renin activity or Ang II synthesis in the hypertensive strain. Therefore, mouse and rat renal renin concentration and ACE activity were evaluated in the hypertensive and normotensive rat strains. The present studies are the first to assess renal renin concentrations in the mRen(2).Lewis and Lewis rats. We found that rat renal renin was three-fold lower in male mRen(2).Lewis compared Lewis rats. These results

suggest that renal renin is not elevated in the presence of the higher blood pressure and renal Ang II levels in male mRen(2).Lewis rats (2; 49; 73). A study in male (mRen-2)27 Sprague Dawley rats demonstrated that the hypertensive males have a greater deposition of smooth muscle cells and lower renin granules in the JG apparatus as shown by ultrastructural analysis of the afferent arteriole wall compared to the male Sprague Dawley (2). The alteration in the JG apparatus leads to a decrease in renal renin release (2). Presently, the JG apparatus morphology has not been determined in the mRen(2).Lewis and Lewis rats which would provide a direct comparison to data in the (mRen-2)27 Sprague Dawley. However, high renal cortical ACE activity could also decrease renal Ang I. Evidence for ACE activity contributing to renal Ang II synthesis was demonstrated through treatment with the ACE inhibitor perindopril which decreased both renal Ang II and blood pressure in (mRen-2)27 Sprague Dawley rats (72). In the present studies, we found renal ACE activity was not different between mRen(2).Lewis and Lewis rat strains. The lower levels of cortical renin, Ang I, and an absence of a difference in ACE activity all suggest that the majority of renal Ang II in hypertensive rats does not arise from intrarenal synthesis. Aside from synthesis, degradation of Ang II is an additional process that could contribute to intrarenal Ang II content. Renal neprilysin and ACE2 can degrade Ang II. Neprilysin activity was not different between the male mRen(2).Lewis and Lewis rats. Cortical ACE2 activity was significantly lower in the mRen(2).Lewis compared to the Lewis rats. The lower ACE2 activity in the mRen(2).Lewis is similar to observations in other rodent models of hypertension and their normotensive controls (18). The lower enzyme activity would suggest that a deficiency in ACE2 and not neprilysin is one mechanism that could contribute to the

higher intrarenal Ang II, but the 15% decrease in ACE2 activity does not appear to be the main factor for the approximate 40% increase in the renal peptide content.

Next, we assessed the AT1 receptor density in the renal cortex of male mRen(2).Lewis and Lewis rats since AT1 receptors can bind and internalize Ang II. One Ang II internalization mechanism is the endosomal pathway. Endosomes from the renal cortex contain Ang peptides and AT1 receptors which may lead to accumulation and protection of internalized Ang II (27; 30; 36; 79). Accumulation of renal Ang II is also associated with an increase in blood pressure (36; 37; 79). However, receptor-mediated internalization of Ang II may not be a dominant pathway in (mRen-2)27 Sprague Dawley rats because Nickenig et al. (52) found a lower AT1 receptor density in the atrium and aorta of male (mRen-2)27 Sprague Dawley versus Sprague Dawley rats. Male mRen(2).Lewis rats expressed a reduced density of AT1 receptors compared to male Lewis rats similar to the findings in the male (mRen-2)27 Sprague Dawley. The receptor density results suggest that renal Ang II concentration was not elevated due to receptor-mediated internalization in the mRen(2).Lewis. Renal AT1 receptor binding affinity was significantly higher in mRen(2).Lewis rats compared to the Lewis rat (55). Although receptor density is reduced and binding affinity is higher, the recycling pathway of the receptor and sequestration of Ang II may augment intrarenal concentrations. The origin of the higher intrarenal Ang II concentrations is an important mechanism to study because activation of renal AT1 receptors by the ligand can contribute to the development of hypertension and renal damage (51).

The measurement of markers in urine to evaluate renal damage in the presence of an activated RAS is a non-invasive and helpful method in determining the extent of organ damage in animals and people (66). Yu et al. (77) demonstrated that RAS blockade also has renoprotective qualities, as candesartan lowered blood pressure and proteinuria in SHR. Their study connects the detrimental actions of a dysregulation in the RAS with hypertension and renal injury. In our studies, chronic hypertension and increased levels of renal Ang II contributed to the significant damage sustained to the kidneys of male mRen(2).Lewis compared to male Lewis rats. The hypertensive males excreted greater concentrations of protein and the oxidative stress marker 8-hydroxy-2'-deoxyguanosine compared to their normotensive controls. The higher content of urinary injury markers was associated with high levels of renal Ang II. A possible mechanism that can contribute to renal damage is oxidative stress. Ang II is a known stimulus of NAD(P)H oxidases that creates oxidative stress and ultimately renal injury (14; 25; 67; 74). We found that renal Ang II is associated with high levels of oxidative stress in this Ang II-dependent form of hypertension. AT1 receptor blockade is known to decrease markers of oxidative stress in a rodent model that exhibits high levels of Ang II (18; 54). AT1 receptor blockade is one of the first treatment regimens administered in hypertension, but patients may still progress into renal damage and renal failure (62; 77). In conclusion, a better understanding of the origin of the higher renal Ang II in mRen(2).Lewis may lead to the development of more effective antihypertensive and renoprotective treatments.

3. Comparison of Female and Male mRen(2).Lewis: Hypertension

Few studies have evaluated the actions of sex or sex steroids in models of hypertension (23; 45; 59; 60). The present studies are the first to compare RAS components and blood pressure in females and males in the mRen(2).Lewis rat strain in regards to the potential actions sex steroids exert on blood pressure regulation and renal injury. The mRen(2).Lewis rats exhibit a marked sex difference in both the extent of hypertension and renal injury that is not present in the Lewis strain. Male mRen(2).Lewis have higher blood pressure and proteinuria, a marker of renal injury, compared to their female counterparts. Higher levels of Ang II present in the male mRen(2).Lewis rats are associated with hypertension, because ACE inhibition and AT1 receptor blockade significantly decreases systolic blood pressure (15; 35). Estrogen also provides an additional RAS-dependent antihypertensive action in female mRen(2).Lewis. Estrogen depletion in young female mRen(2).Lewis rats caused an increase in blood pressure and Ang II whereas estrogen replacement abolished these effects (15). Proteinuria may arise from sustained damage to glomeruli due to higher blood pressure. Damage to the proximal tubules which prevents protein reabsorption from the tubular fluid also increases proteinuria. Male mRen(2).Lewis rats excreted a significantly higher amount of protein in the urine compared to female mRen(2).Lewis. The mechanisms that result in the sex difference in proteinuria have not been fully elucidated in the mRen(2).Lewis rat strain, but data in the SHR would indicate that sex steroids do play a role in renal injury independent of the level of blood pressure (60). The (mRen-2)27 Sprague Dawley and SHR rats exhibit marked sex-related differences in blood pressure and renal injury and these indices are also not present in their respective normotensive controls. In summary, to control for genetic variability, the Lewis rat strain is the inbred

control for the hypertensive mRen(2).Lewis rat. This Ang II-dependent model of hypertension is a valuable tool to demonstrate the deleterious impact of Ang II on the promotion of hypertension and renal injury, whereas sex steroids may provide deleterious or protective actions.

4. Comparison of Female and Male mRen(2).Lewis: Circulating RAS

Sex-related differences in blood pressure and renal injury exist in the mRen(2).Lewis. These differences may be initiated or maintained by an alteration in the circulating RAS. Presently, there has not been a report that compared plasma Ang peptides between the sexes in the mRen(2).Lewis strain. Similar to the blood pressure data, a sex difference in plasma Ang II was evident in mRen(2).Lewis, but not the Lewis rats. Plasma Ang II levels in female mRen(2).Lewis rats were significantly lower than male mRen(2).Lewis. Evaluation of the Lewis rat strain was important to discriminate the effects of sex steroids on RAS expression in the absence of hypertension. The Lewis rats did not exhibit a sex-related difference in plasma RAS components. It is well known that sex steroids regulate RAS enzymes which may alter the peptide concentrations. Consistent with the sex-related difference in plasma Ang II, both circulating mouse and rat renin were elevated approximately 3-fold in the circulation of the male mRen(2).Lewis compared to female mRen(2).Lewis rats. The actions of sex steroids on mouse and rat renin have been evaluated in two hypertensive strains, (mRen-2)27 Sprague Dawley and mRen(2).Lewis rats (5; 6; 15). Testosterone receptor antagonism decreased mouse and rat renin in female and male (mRen-2)27 Sprague Dawley, whereas

estrogen replacement in ovariectomized female mRen(2).Lewis decreased both forms of renin in this model (5; 6; 15). The sex steroid data on renin suggest a pathway for females to have lower renin compared to males in the (mRen-2)27 Sprague Dawley and mRen(2).Lewis strains through the regulatory actions of testosterone and estrogen. To our knowledge, the actions of testosterone supplementation on renin expression in a clinical setting have not been assessed. However, women that chronically take oral contraceptives exhibit lower plasma renin activity (53). More recently, others have reported equal concentrations between plasma mouse and rat renin in young female and male (mRen-2)27 Sprague Dawley rats (5; 6). We also observed similar concentrations between plasma mouse and rat renin in mRen(2).Lewis rats which were comparable to the renin concentration values reported by Baltatu et al. (5; 6). Thus far, the testosterone receptor studies in the (mRen-2)27 Sprague Dawley and sex-related difference in mRen(2).Lewis indicate the actions of testosterone favor the production of Ang II and higher blood pressure in males.

Plasma ACE was assessed because the enzyme is subject to sex steroidal regulation and is an important component in the RAS pathway. We found circulating ACE activity was higher in male compared to female mRen(2).Lewis suggesting a stimulatory effect by testosterone. Alternatively, the lower plasma ACE levels in female mRen(2).Lewis versus their male littermates may be due to estrogen's inhibitory effect on this enzyme. Estrogen replacement in ovariectomized mRen(2).Lewis rats significantly decreased plasma ACE activity (9; 15). Furthermore, similar results of estrogen's inhibitory actions on ACE were observed in female (mRen-2)27 Sprague Dawley rats (9; 15). Estrogen may decrease Ang II production by reducing renin and ACE expression in

female mRen(2).Lewis, whereas testosterone may increase Ang II by stimulating renin activity in the males. Thus, lower circulating Ang II, ACE, and renin in female mRen(2).Lewis are possibly due to higher levels of estrogen and lower concentrations of testosterone.

Higher levels of plasma Ang-(1-7) could also provide a protective blood pressure-lowering effect in the hypertensive female rat. The vasodilatory and antihypertensive effects of circulating Ang-(1-7) are mediated through the stimulation of prostaglandins and nitric oxide (33; 65). Infusion of Ang-(1-7) into the hypertensive female Dahl salt-sensitive rats significantly lowered blood pressure and aortic vascular resistance which were associated with increased prostacyclin 6-ketoprostaglandin $F_{1\alpha}$ and nitric oxide levels in the plasma (22). However, a sustained decrease in blood pressure and an elevation in plasma prostaglandins and nitric oxide were not observed in the male Dahl rats. The precise beneficial mechanism of Ang-(1-7) is unknown in the female, but the antihypertensive actions of Ang-(1-7) are not present in male Dahl rats. The vasodilatory actions of Ang-(1-7) were demonstrated in a female model of salt-sensitive hypertension, so the data suggest that the higher concentrations of plasma Ang-(1-7) in the female mRen(2).Lewis rats could stimulate prostacyclin and nitric oxide-dependent pathways to lower blood pressure. Estrogen replacement significantly elevated plasma Ang-(1-7) which was associated with a decrease in blood pressure in the ovariectomized female (mRen-2)27 Sprague Dawley rat (9). In addition, we find that plasma Ang-(1-7) was three-fold higher in female mRen(2).Lewis compared to male mRen(2).Lewis rats. The greater circulating levels of Ang-(1-7) in female mRen(2).Lewis rats may avert the higher blood pressure exhibited by the male mRen(2).Lewis rats. These are the first studies to

document a sex-related difference in circulating Ang-(1-7) in the mRen(2).Lewis strain. The protective mechanism for a sex difference in female mRen(2).Lewis rats may, in part, be mediated through vasodilatory actions of Ang-(1-7). Further studies in which an AT(1-7) receptor antagonist such as D-[Ala^7]Ang-(1-7) is administered to female mRen(2).Lewis are required to address this mechanism.

5. Comparison of Female and Male mRen(2).Lewis: Renal RAS

The renal RAS has a strong influence on hypertension and sex steroids that affect the regulation of various RAS components in the kidney. To date, an evaluation of renal Ang peptides and enzymes between female and male Ang II-dependent hypertensive rats has not been determined. The present studies uncovered marked sex differences in renal Ang peptides and enzyme levels in this model of hypertension. Female mRen(2).Lewis rats exhibited lower cortical Ang II, while Ang I was higher in the renal cortex compared to the males. The renal levels of Ang II and Ang I in mRen(2).Lewis rats suggest a different rate of conversion in the generation of Ang II between the sexes, possibly through alterations in renal renin or ACE. Female (mRen-2)27 Sprague Dawley rats express a greater density of renin granules in JG afferent arterioles than male littermates; the renal renin was higher in females because they exhibited a lower level of blood pressure than male (mRen-2)27 Sprague Dawley (2). The renal baroreceptor reflex should exert a greater inhibitory action on renin production and release in the male versus female (mRen-2)27 Sprague Dawley. We were able to evaluate the contribution of both mouse (pH 8.5) and rat (pH 6.5) renin activities in the mRen(2).Lewis rats because the

two different isotypes of renin express different pH optima to cleave angiotensinogen (8). Renal mouse renin concentrations were significantly elevated and rat renin concentrations tended to be higher in female versus male mRen(2).Lewis rats, which could explain the higher cortical Ang I. Ang-(1-7) levels were not different in the renal cortex between sexes, although the female mRen(2).Lewis tended to have a higher concentration of this peptide. Cortical Ang I and Ang-(1-7) concentrations in female mRen(2).Lewis rats suggest a decrease in renal ACE activity in the presence of higher renal renin activity. Indeed, renal ACE activity is known to be downregulated with estrogen replacement in young ovariectomized (mRen-2)27 Sprague Dawley and mRen(2).Lewis rats (9; 15; 16). However, we found no sex difference in renal ACE activity in the mRen(2).Lewis rat strain that could substantiate the renal Ang II peptide levels. Our results do not indicate that intrarenal generation of Ang II is the predominant mechanism to explain the higher intrarenal Ang II in male versus female mRen(2).Lewis rats.

We then evaluated whether Ang II degrading enzymes could account for the peptide differences in the kidney. ACE2 is found in the kidney and converts Ang II to Ang-(1-7) (68). Interestingly, ACE2 activity was significantly higher in male mRen(2).Lewis compared to female mRen(2).Lewis rats. Higher levels of ACE2 activity in the male mRen(2).Lewis appear to be a compensatory Ang II degrading mechanism. Although Ang II through an AT1 receptor-mediated action has been shown to decrease ACE2 activity, the conversion of renal Ang II to Ang-(1-7) in hypertensive males appears to be a mechanism to attenuate the higher blood pressure (31). An additional possibility is that renal damage may induce ACE2 expression. Myocardial infarction also increases ACE2 expression in cardiac tissue of both humans and rats (10). In the present studies, it

was surprising to find the male mRen(2).Lewis rats express higher ACE2 activity compared to their female littermates since the ACE2 gene quantitative trait loci (QTL) localizes to the X chromosome in both human and rats (18). Localization of the ACE2 QTL on the X chromosome suggest female rats would have two copies of the gene and subsequently higher expression of ACE2 than males, although ACE2 activity was not higher in the mRen(2).Lewis strain.

In contrast, cortical neprilysin activity and protein expression were three- and ten-fold higher in female mRen(2).Lewis versus male littermates. The increased levels of neprilysin may provide a beneficial action by degrading renal Ang II and producing Ang-(1-7) from Ang I (68). Furthermore, chronic estrogen depletion decreases neprilysin activity and protein in rats and women (28; 78). Neprilysin activity has also been shown to be higher in female Swiss Webster mice compared to their male counterparts (13). High neprilysin activity was positively associated with high cerebral cortical estrogen levels as determined in postmortem brain tissue from women at 84 years of age diagnosed with and without Alzheimer's disease (28; 78). Neuronal aromatase mRNA and estrogen levels were negatively associated with β-amyloid plaques associated with Alzheimer's disease (28; 78). Neprilysin degrades β-amyloid plaques in the brain; therefore, a decrease in estrogen may cause an increase in plaque accumulation (28; 32; 78). Huang et al. (28) demonstrated that estrogen replacement increased neuronal neprilysin activity in rats. Moreover, potential estrogen response regions that could stimulate transcription of the enzyme have been identified (69; 76). In conclusion, higher levels of neprilysin in female mRen(2).Lewis rats could provide a pathway to prevent an accumulation of renal Ang II and decrease renal vascular resistance through an Ang-(1-

7)-dependent mechanism. The present data are the first to evaluate renal neprilysin activity in regards to the intrarenal RAS in this model of Ang II-dependent hypertension. The increased neprilysin expression in female mRen(2).Lewis rats suggests that neprilysin is protective for the blood pressure and against renal damage. Although, neprilysin inhibition has antihypertensive actions in males (70), our data indicate inhibition of neprilysin may be detrimental in females by increasing renal Ang II and ultimately blood pressure. Therefore, in the subsequent studies, we assessed the effects of chronic treatment with a neprilysin inhibitor in female mRen(2).Lewis rats.

6. Assessment of Neprilysin Inhibition in Female mRen(2).Lewis Rats

Few reports in the literature have evaluated the effect of chronic neprilysin inhibition in a model of Ang II-dependent hypertension or cardiovascular disease (21; 70). It has been demonstrated that neprilysin inhibition significantly lowered blood pressure in male (mRen-2)27 Sprague Dawley rats (70). However, the higher neprilysin activity and protein in the renal cortex of female mRen(2).Lewis rats would suggest that neprilysin inhibition could increase blood pressure and prove to be detrimental in this model of hypertension. Surprisingly, we found that a two week treatment of the neprilysin inhibitor SCH 39370 (SCH) in female mRen(2).Lewis rats significantly reduced systolic blood pressure. Since plasma Ang II and Ang-(1-7) were similar between the control SCH-treated groups, the decrease in blood pressure could be through other neprilysin substrates (natriuretic peptides or bradykinin) that reduce blood pressure (71). Stasch et al. (70) observed antihypertensive actions of chronic treatment with the

orally active neprilysin inhibitor ecadotril. The antihypertensive effects of ecadotril occurred without an alteration in natriuretic peptides in the male (mRen-2)27 Sprague Dawley rat. The authors hypothesized that the blood pressure-lowering actions could be mediated through a bradykinin-dependent pathway. Since cGMP, a product of bradykinin activity, was increased in plasma and urine of the ecadotril-treated hypertensive male rats, the data was interpreted that bradykinin activity was increased. Bradykinin activating the kinin B2 receptor could lower blood pressure through vasodilation of the peripheral and renal vasculature (4). However, Stasch and colleagues did not evaluate bradykinin or Ang peptides in the (mRen-2)27 Sprague Dawley rats. Chronic neprilysin inhibition lowered blood pressure in the male (mRen-2)27 Sprague Dawley and female mRen(2).Lewis rats, but the precise hormonal mechanisms have yet to be elucidated.

The antihypertensive effects of SCH in female mRen(2).Lewis rats could be mediated through various pathways. Gardiner et al. (24) addressed the influence of AT1 receptors in the presence of neprilysin inhibition. Gardiner and colleagues sought to improve upon the antihypertensive and vasodilatory actions of neprilysin inhibition by concomitantly administering an AT1 receptor blocker. Neprilysin inhibition and AT1 receptor blockade had additive effects to significantly enhance renal and hindquarter regional blood flow in male SHR. These data provide evidence for a balance of vasoactivity in blood pressure effects between the Ang II-AT1 receptor and other vasoactive hormones. It is also possible that neprilysin can affect metabolic regulation because the female mRen(2).Lewis rats may also exhibit symptoms of metabolic dysregulation or syndrome. The SCH-treated mRen(2).Lewis rats had a lower body

weight compared to controls. Neprilysin has many substrates including glucagon-like peptide 1 which stimulates adipolysis and hepatic glucose storage (58). An increase in glucagon-like peptide 1 levels may decrease body weight in the SCH-treated female mRen(2).Lewis rats. Future studies should evaluate insulin, glucose, and other markers of metabolic syndrome dysregulation, particularly during neprilysin inhibition. It is evident from our study that systemic treatment with the neprilysin inhibitor produces an antihypertensive effect in female mRen(2).Lewis rats. While this does not appear to be through alterations in the circulating RAS, future studies should evaluate whether inhibition of renal neprilysin is protective or detrimental for blood pressure homeostasis in a female animal model of hypertension.

7. Conclusion and Future Directions

In summary, female and male mRen(2).Lewis exhibit a phenotypic trait of hypertension compared to the Lewis rats, but the mechanisms and degree of hypertension and renal injury are different between the sexes. The present studies sought to characterize the sex-related differences in the RAS and potential components and pathways that can contribute to the different level of hypertension in mRen(2).Lewis rats. We propose that the female mRen(2).Lewis rats exhibit lower Ang II activity and minimal renal damage, while the male mRen(2).Lewis display higher Ang II activity and significant renal damage that may account for their higher blood pressure. Evidence to support this hypothesis in the females is based on data in the (mRen-2)27 Sprague Dawley and mRen(2).Lewis rats. Female (mRen-2)27 Sprague Dawley have actions that

attenuate renal damage through a decrease in renal blood flow in response to an injection of Ang II into the renal artery as compared to Sprague Dawley rats (34). The female (mRen-2)27 Sprague Dawley rat may display an enhanced Ang II-mediated vasoconstrictive response in the afferent arteriole to dampen the force from the higher blood pressure that could severely damage the glomeruli. Furthermore, female (mRen-2)27 Sprague Dawley rats have an exaggerated hypertensive response to a bolus injection of Ang II in the periphery compared to Sprague Dawley. Also, female mRen(2).Lewis rats exhibit similar intrarenal Ang II content, but higher plasma Ang II levels versus female Lewis rats (56). These data suggest the hypertensive actions of Ang II on vascular resistance in renal and peripheral beds in female mRen(2).Lewis rats could arise from alterations in multiple regulatory cardiovascular areas or pathways, such as the central nervous system, the kidneys, regulatory actions of sex steroids, circulating and local peptide concentrations, and Ang receptor density and function. These alterations may ultimately decrease the amount of damage inflicted on the female mRen(2).Lewis rats' kidneys. On the other hand, the greater level of hypertension in male mRen(2).Lewis could be maintained by a higher activation of Ang II activity compared to other groups, as well as significant renal damage. A combination of higher Ang II activity in multiple cardiovascular control areas or organs and the constant damage inflicted upon renal homeostatic mechanisms may permanently shift the blood pressure and organ function to a higher pathophysiological state. Additionally, males exhibit a greater level of renal injury than females in the absence of hypertension. The degree of renal damage between the sexes may also arise from the concentration of testosterone and/or estrogen. Further evaluation of the activity of sex steroids and comparison of both

females and males in this model of Ang II-dependent hypertension should potentially uncover additional novel mechanisms that regulate blood pressure and renal injury.

The sex-related blood pressure difference in the hypertensive mRen(2).Lewis strain is influenced by the expression of renal neprilysin, Ang peptides, and renal AT receptor subtype. The expression of cortical neprilysin in the renal vasculature and proximal tubules could decrease the concentration of Ang II leading to lower activity of renal AT1 receptors. Lower Ang II-AT1 receptor-mediated actions would increase renal blood flow and sodium excretion to subsequently decrease blood pressure (47). Ang II could also bind to intrarenal AT2 receptors to elicit vasodilatory or natriuretic actions. The AT2 receptor is a target for sex steroid regulation, since estrogen upregulates renal AT2 receptor mRNA in the vasculature and glomeruli (1; 44). These regulatory actions on the AT2 receptor could improve renal blood flow and filtration. In the neprilysin inhibition study, we observed similar plasma Ang II concentrations between groups in the presence of a decrease in blood pressure. The results from the administration of SCH could indicate that renal Ang II expression is differentially regulated compared to the plasma, although urinary Ang II levels were also similar between the treatment and control groups. Our findings would argue against a change in the renal peptide content, since urinary Ang peptide concentrations are indices of the intrarenal peptide levels. However, assessment of Ang II content may not always reflect the extent of enzyme inhibition since inhibitors can have varying degrees of accessibility to organ compartments. The unique attribute of the inhibitor to easily diffuse throughout organs may alter Ang II in various tissues. SCH, on the other hand, should have access to renal neprilysin localized to the brushborder region of proximal tubules after the inhibitor is

filtered by the glomeruli. SCH inhibition should prevent renal neprilysin from generating Ang-(1-7), a peptide that promotes natriuresis through a prostaglandin-mediated pathway in the kidney (17; 20). The favorable actions of Ang-(1-7) are known in one model of hypertension due to infusion of the peptide in the SHR (7). Ang-(1-7) decreased blood pressure and renal injury in SHR. The female mRen(2).Lewis expressed higher amounts of both neprilysin and Ang-(1-7) which may contribute to their lower blood pressure as compared to males. Following SCH administration in female mRen(2).Lewis rats, plasma and urinary Ang-(1-7) levels were not different, but renal Ang-(1-7) was not determined. Moreover, our hypothesis that chronic neprilysin inhibition would decrease Ang-(1-7), as well as increase Ang II and blood pressure, was not substantiated. Indeed, blood pressure fell by an unknown mechanism following administration of the neprilysin inhibitor.

Finally, the total renal AT receptor density and affinity may also contribute to the sex-related differences in blood pressure and intrarenal Ang II in the mRen(2).Lewis rat strain. Although the density and affinity of nuclear and plasma membrane AT1 receptors are known in male mRen(2).Lewis and Lewis rats, assessment of the density and affinity of renal AT receptors in female mRen(2).Lewis and Lewis rats has yet to be determined. The lower renal AT1 receptor density was associated with high intrarenal Ang II concentrations in male mRen(2).Lewis compared to Lewis rats (55). However, AT1 receptors in male mRen(2).Lewis rats exhibited a significantly higher binding affinity compared to male Lewis rats. The higher binding affinity coupled with a faster receptor recycling process could contribute to the internalization of greater amounts of intrarenal Ang II (41; 42). A future study to determine whether the higher intrarenal Ang II content

in male mRen(2).Lewis arises from AT1 receptor internalization is the assessment of renal Ang II content after chronic losartan treatment. If renal Ang II is reduced with losartan that would suggest AT1 receptor-mediated uptake is the main pathway. However, Jessup et al. (35) showed that a two week treatment of losartan in young male mRen(2).Lewis rats stimulated synthesis of renal renin and angiotensinogen mRNA. An increase in the production of these two components may maintain renal Ang II concentrations through an intrarenal synthesis pathway. In contrast, Ang II internalization pathways may not be different in females, since a strain difference in renal Ang II was not present between the female rats. These results suggest a similar density of renal AT1 receptors in the female rats, if receptor internalization is the main pathway that contributes to intrarenal Ang II content. Moreover, the female mRen(2).Lewis rats may have a higher density of other AT receptor subtype(s) compared to the male mRen(2).Lewis since estrogen downregulates AT1 receptors, but upregulates AT2 receptors (1; 38). Lastly, a different pathway that may contribute to the renal Ang II accumulation is the internalization of Ang II by the protein megalin (26). Megalin is localized in proximal tubules and binds Ang II. It is currently unknown whether megalin internalizes Ang II for a preservation or degradation pathway. The internalization of Ang II by the AT1 receptor and megalin are potential uptake pathways that may contribute to the intrarenal Ang II content.

In light of the antihypertensive actions of systemic SCH treatment in female mRen(2).Lewis rats and the absence of decreased plasma Ang-(1-7), additional studies are required to elucidate the actions of a renal neprilysin-Ang-(1-7) mechanism. One antihypertensive pathway involving neprilysin results from potential higher synthesis of

Ang-(1-7) versus the degradation of Ang II, since Ang I is a better substrate for the enzyme (56). Plasma and renal Ang-(1-7) levels were two- to three-fold higher in female mRen(2).Lewis rats compared to their male counterpart. Ang-(1-7) binds to the AT(1-7)/*mas* receptor localized in the vasculature to elicit nitric oxide or prostaglandin-mediated relaxation or in the proximal tubules to prevent sodium reabsorption (17; 36; 61). Ang-(1-7) concentrations were not different between the SCH and control groups, the beneficial effect of Ang-(1-7) in female mRen(2).Lewis rats can also be assessed by infusion of the AT(1-7) receptor antagonist D-[Ala^7]Ang-(1-7). The selective receptor antagonist will prevent the higher concentrations of Ang-(1-7) in the plasma or kidney from binding to its receptor and eliciting a vasodilatory or renoprotective effect in female mRen(2).Lewis rats.

An assortment of techniques is available to determine how blood pressure and renal Ang peptides are altered during neprilysin inhibition in a female model of hypertension. The kidney is an organ of interest for neprilysin inhibition because it is an important regulator of blood pressure homeostasis and exhibits higher neprilysin expression and activity in females compared to males (13). Systemic neprilysin inhibition may not provide complete blockade of the renal enzyme due to the extensive and potentially higher expression of neprilysin throughout the body. Therefore, infusion of a selective neprilysin inhibitor with a small catheter directly into the kidney could provide optimal inhibition. Direct renal infusion should prevent a large concentration of the inhibitor from traveling throughout the body and affecting extra-renal neprilysin in other cardiovascular regulatory areas such as the central nervous system. An additional model to evaluate the actions of neprilysin inhibition is the neprilysin knockout mouse

(43). The neprilysin knockout mouse is a model of hypotension that has an inbred background control strain for comparison. The hypotension is not bradykinin-dependent since administration of the B2 kinin receptor antagonist HOE 140 did not abolish the lower blood pressure compared to the control mice. Presently, the hypotensive mechanism has yet to be elucidated in the neprilysin knockout mouse. To date, studies have only evaluated male mice, but female knockout mice infused with a pressor dose of Ang II would aid in determining the effects of complete neprilysin inhibition in the presence of Ang II-dependent hypertension. These additional studies will help determine if renal neprilysin and the degradation of Ang II contribute to the lower degree of blood pressure and renal injury in females.

Reference List

1. **Armando I, Jezova M, Juorio AV, Terron JA, Falcon-Neri A, Semino-Mora C, Imboden H and Saavedra JM**. Estrogen upregulates renal angiotensin II AT_2 receptors. *Am J Physiol Renal Physiol* 283: F934-F943, 2002.

2. **Bachmann S, Peters J, Engler E, Ganten D and Mullins J**. Transgenic rats carrying the mouse renin gene -- morphological characterization of a low-renin hypertension model. *Kidney Int* 41: 24-36, 1992.

3. **Bader M, Zhao Y, Sander M, Lee MA, Bachmann J, Bohm M, Djavidani B, Peters J, Mullins JJ and Ganten D**. Role of tissue renin in the pathophysiology of hypertension in TGR (mREN2) 27 rats. *Hypertension* 19: 681-686, 1992.

4. **Bagate K, Grima M, Imbs J-L, Jong WD, Helwig JJ and Barthelmebs M**. Signal transduction pathways involved in kinin B(2) receptor-mediated vasodilation in the rat isolated perfused kidney. *Br J Pharmacol* 132: 1735-1742, 2001.

5. **Baltatu O, Cayla C, Iliescu R, Andreev D and Bader M**. Abolition of end-organ damage by antiandrogen treatment in female hypertensive transgenic rats. *Hypertension* 41: 830-833, 2003.

6. **Baltatu O, Cayla C, Iliescu R, Andreev D, Jordan C and Bader M**. Abolition of hypertension-induced end-organ damage by androgen receptor blockade in transgenic rats harboring the mouse Ren-2 gene. *J Am Soc Nephrol* 13: 2681-2687, 2002.

7. **Benter IF, Yousif MHM, Anim JT, Cojocel C and Diz DI**. Angiotensin-(1-7) prevents development of severe hypertension and end-organ damage in spontaneously hypertensive rats treated with L-NAME. *Am J Physiol Heart Circ Physiol* 290: H684-H691, 2006.

8. **Bohlender J, Menard J, Edling O, Ganten D and Luft FC**. Mouse and rat plasma renin concentration and gene expression in (mRen2)27 transgenic rats. *Am J Physiol* 274: H1450-H1456, 1998.

9. **Brosnihan KB, Li P, Ganten D and Ferrario CM**. Estrogen protects transgenic hypertensive rats by shifting the vasoconstrictor-vasodilator balance of RAS. *Am J Physiol* 273: R1908-R1915, 1997.

10. **Burrell LM, Risvanis J, Kubota E, Dean RG, Macdonald PS, Lu S, Tikellis C, Grant SL, Lew RA, Smith AI, Cooper ME and Johnston CI**. Myocardial infarction increases ace2 expression in rat and humans. *Eur Heart J* 26: 369-375, 2005.

11. **Campbell DJ, Rong P, Kladis A, Rees B, Ganten D and Skinner SL**. Angiotensin and bradykinin peptides in the TGR(mRen-2)27 rat. *Hypertension* 25: 1014-1020, 1995.

12. **Carey RM, McGrath HE, Pentz ES, Gomez RA and Barrett PQ**. Biomechanical coupling in renin-releasing cells. *J Clin Invest* 100: 1566-1574, 1997.

13. **Carter TL, Pedrini S, Ghiso J, Ehrlich ME and Gandy S**. Brain neprilysin activity and susceptibility to transgene-induced Alzheimer amyloidosis. *Neurosci Lett* 392: 235-239, 2006.

14. **Chabrashvili T, Kitiyakara C, Blau J, Karber A, Aslam S, Welch WJ and Wilcox CS**. Effects of Ang II type 1 and 2 receptors on oxidative stress, renal NADPH oxidase, and SOD expression. *Am J Physiol Regul Integr Comp Physiol* 285: R117-R124, 2003.

15. **Chappell MC, Gallagher PE, Averill DB, Ferrario CM and Brosnihan KB**. Estrogen or the AT1 antagonist olmesartan reverses the development of profound hypertension in the congenic mRen2.Lewis rat. *Hypertension* 42: 781-786, 2003.

16. **Chappell MC, Yamaleyeva LM and Westwood BM**. Estrogen and salt sensitivity in the female mRen(2).Lewis rat. *Am J Physiol Regul Integr Comp Physiol* 291: R1557-R1563, 2006.

17. **Clark MA, Tommasi EN, Bosch SM, Tallant EA and Diz DI**. Angiotensin-(1-7) reduces renal angiotensin II receptors through a cyclooxygenase dependent pathway. *J Cardiovasc Pharmacol* 41: 276-283, 2003.

18. **Crackower MA, Sarao R, Oudit GY, Yagil C, Kozieradzki I, Scanga SE, Oliveira-dos-Santo AJ, da Costa J, Zhang L, Pei Y, Scholey J, Bray MR, Ferrario CM, Backx PH, Manoukian AS, Chappell MC, Yagil Y and Penninger JM**. Angiotensin-converting enzyme 2 is an essential regulator of heart function. *Nature* 417: 822-828, 2002.

19. **de Vinuesa SG, Goicoechea M, Kanter J, Puerta M, Cachofeiro V, Lahera V, Gomez-Campdera F and Luno J**. Insulin resistance, inflammatory biomarkers, and adipokines in patients with chronic kidney disease: Effects of angiotensin II blockade. *J Am Soc Nephrol* 17: S206-S212, 2006.

20. **DelliPizzi A, Hilchey SD and Bell-Quilley CP**. Natriuretic action of angiotensin (1-7). *Br J Pharmacol* 111: 1-3, 1994.

21. **Duncan AM, James GM, Anastasopoulos F, Kladis A, Briscoe TA and Campbell DJ**. Interaction between neutral endopeptidase and angiotensin converting enzyme inhibition in rats with myocardial infarction: effects on cardiac hypertrophy and angiotensin and bradykinin peptide levels. *Journal of Pharmacology & Experimental Therapeutics* 289: 295-303, 1999.

22. **Eatman D, Wang M, Socci RR, Thierry-Palmer M, Emmett N and Bayorh MA**. Gender differences in the attenuation of salt-induced hypertension by angiotensin (1-7). *Peptides* 22: 927-933, 2001.

23. **Fortepiani LA, Zhang H, Racusen L, Roberts II LJ and Reckelhoff JF**. Characterization of an animal model of postmenopausal hypertension in spontaneously hypertensive rats. *Hypertension* 41: 640-645, 2003.

24. **Gardiner SM, March JE, Kemp PA, Ballard SA and Bennett T**. Regional hemodynamic effects of neutral endopeptidase inhibition and angiotensin (AT(1)) receptor antagonism alone or in combination in conscious spontaneously hypertensive rats. *J Pharm Exp Therapeutics* 319: 340-348, 2006.

25. **Gill PS and Wilcox CS**. NADPH oxidases in the kidney. *Antioxid Redox Signal* 8: 1597-1607, 2006.

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

27. **Harrison-Bernard LM, Zhou J, Kobori H, Ohishi M and Navar LG**. Intrarenal AT_1 receptor and ACE binding in ANG II-induced hypertensive rats. *Am J Physiol Renal Physiol* 281: F19-F25, 2002.

28. **Huang J, Guan H, Booze RM, Eckman CB and Hersh LB**. Estrogen regulates neprilysin activity in rat brain. *Neurosci Lett* 367: 85-87, 2004.

29. **Huskova Z, Kramer HJ, Thumova M, Vanourkova Z, Burgelova M, Teplan V, Maly J and Cervenka L**. Effects of anesthesia on plasma and kidney Ang II levels in normotensive and Ang II-dependent hypertensive rats. *Kidney Blood Press Res* 29: 74-83, 2006.

30. **Imig JD, Navar GL, Zou LX, O'Reilly KC, Allen PL, Kaysen JH, Hammond TG and Navar LG**. Renal endosomes contain angiotensin peptides, converting enzyme, and AT(1A) receptors. *Am J Physiol* 277: F303-F311, 1999.

31. **Ishiyama Y, Gallagher PE, Averill DB, Tallant EA, Brosnihan KB and Ferrario CM**. Upregulation of angiotensin-converting enzyme 2 after myocardial infarction by blockade of angiotensin II receptors. *Hypertension* 43: 970-976, 2004.

32. **Iwata N, Tsubuki S, Takaki Y, Shirotani K, Lu B, Gerard NP, Gerard C, Hama E, Lee H and Saido TC**. Metabolic Regulation of Brain Aβ by Neprilysin. *Science* 292: 1550-1552, 2001.

33. **Iyer SN, Yamada K, Diz DI, Ferrario CM and Chappell MC**. Evidence that prostaglandins mediate the antihypertensive actions of angiotensin-(1-7) during

chronic blockade of the renin-angiotensin system. *J Cardiovasc Pharmacol* 36: 109-117, 2000.

34. **Jacinto SM, Mullins JJ and Mitchell KD**. Enhanced renal vascular responsiveness to angiotensin II in hypertensive ren-2 transgenic rats. *American Journal of Physiology - Renal Fluid & Electrolyte Physiology* 276: F315-F322, 1999.

35. **Jessup JA, Gallagher PE, Averill DB, Brosnihan KB, Tallant EA, Chappell MC and Ferrario CM**. Effect of angiotensin II blockade on a new congenic model of hypertension derived from transgenic Ren-2 rats. *Am J Physiol Heart Circ Physiol* 291: H2166-H2172, 2006.

36. **Kobori H, Nangaku M, Navar LG and Nishiyama A**. The intrarenal renin-angiotensin system: from physiology to the pathobiology of hypertension and kidney disease. *Pharmacol Rev* 59: 251-287, 2007.

37. **Kobori H, Prieto-Carrasquero MC, Ozawa Y and Navar LG**. AT1 receptor mediated augmentation of intrarenal angiotensinogen in angiotensin II-dependent hypertension. *Hypertension* 43: 1126-1132, 2004.

38. **Krishnamurthi K, Verbalis JG, Zheng W, Wu Z, Clerch LB and Sandberg K**. Estrogen regulates angiotensin AT1 receptor expression via cytosolic proteins

that bind to the 5' leader sequence of the receptor mRNA. *Endocrinology* 140: 5435-5438, 1999.

39. **Krutz A**. Intracellular control of renin release - An Overview. *Klin Wochenschr* 64: 838-846, 1986.

40. **Lee MA, Bohm M, Paul M, Bader M, Ganten U and Ganten D**. Physiological characterization of the hypertensive transgenic rat TGR(mREN2)27. *Am J Physiol* 270: E919-E929, 1996.

41. **Li XC and Zhuo JL**. Selective knockdown of AT1 receptors by RNA interference inhibits Val5-ANG II endocytosis and NHE-3 expression in immortalized rabbit proximal tubule cells. *Am J Physiol Cell Physiol* 293: C367-C378, 2007.

42. **Li XC and Zhuo JL**. In vivo regulation of AT1a receptor-mediated intracellular uptake of [125I]Val5-ANG II in the kidneys and adrenals of AT1a receptor-deficient mice. *Am J Physiol Renal Physiol* 294: F293-F302, 2008.

43. **Lu B, Figini M, Emanueli C, Geppetti P, Grady EF, Gerard NP, Ansell J, Payan DG, Gerard C and Bunnett N**. The control of microvascular permeability and blood pressure by neutral endopeptidase. *Nature Medicine* 3: 904-907, 1997.

44. **Macova M, Armando I, Zhou J, Baiardi G, Tyurmin D, Larrayoz-Roldan IM and Saavedra JM**. Estrogen reduces aldosterone, upregulates adrenal angiotensin II AT2 receptors and normalizes adrenomedullary Fra-2 in ovariectomized rats. *Neuroendocrinology* 88: 276-286, 2009.

45. **Mazancova K, Miksik I, Kunes J, Zicha J and Pacha J**. Sexual dimorphism of 11beta-hydroxysteroid dehydrogenase in hypertensive and normotensive rats. *Hypertens Res* 26: 333-338, 2003.

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

47. **Mitchell KD and Navar LG**. Interactive effects of angiotensin II on renal hemodynamics and tubular reabsorptive function. *Kidney Int Suppl* 36: S43-S48, 1990.

48. **Moriguchi A, Brosnihan KB, Kumagai H, Ganten D and Ferrario CM**. Mechanisms of hypertension in transgenic rats expressing the mouse Ren-2 gene. *Am J Physiol* 266: R1273-R1278, 1994.

49. **Müller MW, Todorov V, Kramer BK and Kurtz A**. Angiotensin II inhibits renin gene transcription via the protein kinase C pathway. *Pflugers Arch* 444: 499-505, 2002.

50. **Mullins JJ, Peters J and Ganten D**. Fulminant hypertension in transgenic rats harbouring the mouse Ren-2 gene. *Nature* 344: 541-544, 1990.

51. **Navar LG**. The role of the kidneys in hypertension. *J Clin Hypertens (Greenwich)* 7: 542-549, 2005.

52. **Nickenig G, Laufs U, Schnabel P, Knorr A, Paul M and Bohm MP**. Down-regulation of aortic and cardiac AT1 receptor gene expression in transgenic (mRen-2) 27 rats. *Br J Pharmacol* 121: 134-140, 1997.

53. **Oelkers WKH**. Effects of estrogens and progestogens on the renin-aldosterone system and blood pressure. *Steroids* 61: 166-171, 1996.

54. **Oudit GY, Herzenberg AM, Kassiri Z, Wong D, Reich H, Khokha R, Crackower MA, Backx PH, Penninger JM and Scholey JW**. Loss of angiotensin-converting enzyme-2 leads to the late development of angiotensin II-dependent glomerulosclerosis. *Am J Patho* 168: 1808-1820, 2006.

55. **Pendergrass KD, Averill DB, Ferrario CM, Diz DI and Chappell MC**. Differential expression of nuclear AT1 receptors and angiotensin II within the kidney of the male congenic mRen2.Lewis rat. *Am J Physiol Renal Physiol* 290: F1497-F1506, 2006.

56. **Pendergrass KD, Pirro NT, Westwood BM, Ferrario CM, Brosnihan KB and Chappell MC**. Sex differences in circulating and renal angiotensins of hypertensive mRen(2).Lewis but not normotensive Lewis rats. *Am J Physiol Heart Circ Physiol* 295: H10-H20, 2008.

57. **Peters J, Munter K, Bader M, Hackenthal E, Mullins JJ and Ganten D**. Increased adrenal renin in transgenic hypertensive rats, TGR (mREN2) 27, and its regulation by cAMP, angiotensin II, and calcium. *J Clin Invest* 91: 742-747, 1993.

58. **Plamboeck A, Holst JJ, Carr RD and Deacon CF**. Neutral endopeptidase 24.11 and dipeptidyl peptidase IV are both mediators of the degradation of glucagon-like peptide 1 in the anaesthetised pig. *Diabetologia* 48: 1882-1890, 2005.

59. **Podesser BK, Jain M, Ngoy S, Apstein CS and Eberli FR**. Unveiling gender differences in demand ischemia: a study in a rat model of genetic hypertension. *Eur J Cardiothorac Surg* 31: 298-304, 2007.

60. **Reckelhoff JF, Zhang H and Srivastava K**. Gender differences in development of hypertension in spontaneously hypertensive rats: role of the renin-angiotensin system. *Hypertension* 35: 480-483, 2000.

61. **Ren Y, Garvin JL and Carretero OA**. Vasodilator action of angiotensin-(1-7) on isolated rabbit afferent arterioles. *Hypertension* 39: 799-802, 2002.

62. **Riegersperger M and Sunder-Plassmann G**. How to prevent progression to end stage renal disease. *J Ren Care* 33: 105-107, 2007.

63. **Rong P, Campbell DJ and Skinner SL**. Hypertension in the (mRen-2)27 rat is not explained by enhanced kinetics of transgenic Ren-2 renin. *Hypertension* 42: 523-527, 2003.

64. **Rong P, Wilkinson-Berka JL and Skinner SL**. Control of renin secretion from adrenal gland in transgenic Ren-2 and normal rats. *Mol Cell Endocrinol* 173: 203-212, 2001.

65. **Sampaio WO, dos Santos RA, Faria-Silva R, de Mata Machado LT, Schiffrin EL and Touyz RM**. Angiotensin-(1-7) through receptor mas mediates endothelial nitric oxide synthase activation via Akt-dependent pathways. *Hypertension* 49: 185-192, 2007.

66. **Segura J, Campo C and Ruilope LM**. Influence of chronic kidney disease development and renin-angiotensin system inhibition on cardiovascular prognosis. *Curr Med Chem Cardiovasc Hematol Agents* 3: 55-60, 2005.

67. **Seshiah PN, Weber DS, Rocic P, Valppu L, Taniyama Y and Griendling KK**. Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators. *Circ Res* 91: 406-413, 2002.

68. **Shaltout HA, Westwood B, Averill DB, Ferrario CM, Figueroa J, Diz DI, Rose JC and Chappell MC**. Angiotensin metabolism in renal proximal tubules, urine and serum of sheep: Evidence for ACE2-dependent processing of Angiotensin II. *Am J Physiol Renal Physiol* 292: F82-F91, 2006.

69. **Shen R, Sumitomo M, Dai J, Hardy DO, Navarro D, Usmani B, Papandreou CN, Hersh LB, Shipp MA, Freedman LP and Nanus DM**. Identification and characterization of two androgen response regions in the human neutral endopeptidase gene. *Mol Cell Endocrinol* 170: 131-142, 2000.

70. **Stasch J-P, Dietrich CH, Ganten D and Wegner M**. Renal and antihypertensive effects of neutral endopeptidase inhibition in transgenic rats with an extra renin gene. *Am J Hypertens* 9: 795-802, 1996.

71. **Stephenson SL and Kenny AJ**. The metabolism of neuropeptides. Hydrolysis of the angiotensins, bradykinin, substance P and oxytocin by pig kidney microvillar membranes. *Biochemical Journal* 241: 237-247, 1987.

72. **Tokita Y, Franco-Saenz R and Mulrow PJ**. Reversal of the suppressed kidney renin level in the hypertensive transgenic rat TGR(mRen-2)27 by angiotensin converting enzyme inhibition. *Am J Hypertens* 8: 1031-1039, 1995.

73. **Veniant M, Whitworth CE, Menard J, Sharp MGF, Gonzales MF, Bruneval P and Mullins JJ**. Developmental studies demonstrate age-dependent elevation of renin activity in TGR(mRen2)27 rats. *Am J Hypertens* 8: 1167-1176, 1995.

74. **Welch WJ, Blau J, Xie H, Chabrashvili T and Wilcox CS**. Angiotensin-induced defects in renal oxygenation: role of oxidative stress. *Am J Physiol Heart Circ Physiol* 288: H22-H28, 2005.

75. **Wilkinson-Berka JL, Kelly DJ, Rong P, Campbell DJ and Skinner SL**. Characterisation of a thymic renin-angiotensin system in the transgenic m(Ren-2)27 rat. *Mol Cell Endocrinol* 194: 201-209, 2002.

76. **Xiao ZM, Sun L, Liu YM, Zhang JJ and Huang J**. Estrogen regulation of the neprilysin gene through a hormone-responsive element. *Journal Molecular Neuroscience* Epub ahead of print: 2009.

77. **Yu C, Gong R, Rifai A, Tolbert EM and Dworkin LD**. Long-term, high dosage candesartan suppresses inflammation and injury in chronic kidney disease: nonhemodynamic renal protection. *J Am Soc Nephrol* 18: 750-759, 2007.

78. **Yue X, Lu M, Lancaster T, Cao P, Honda S, Staufenbiel M, Harada N, Zhong Z, Shen Y and Li R**. Brain estrogen deficiency accelerates Abeta plaque formation in an Alzheimer's disease animal model. *Proc Natl Acad Sci U S A* 102: 11811-11816, 2005.

79. **Zhuo JL, Imig JD, Hammond TG, Orengo S, Benes E and Navar LG**. Ang II accumulation in rat renal endosomes during Ang II-induced hypertension: Role of AT(1) receptor. *Hypertension* 39: 116-121, 2002.

80. **Zou L-X, Imig JD, Hymel A and Navar LG**. Renal uptake of circulating angiotensin II in Val^5-angiotensin II infused rats is mediated by AT_1 receptor. *Am J Hypertens* 11: 570-578, 1998.

Curriculum Vitae

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EDUCATION

Graduate:

2004 - Present — **Candidate for Ph.D. in Physiology & Pharmacology, Wake Forest University School of Medicine, Winston-Salem, North Carolina**
Advisor: Dr. Mark C. Chappell
Description: Investigating the effect of gender on the circulating and renal renin angiotensin system (RAS) and consequences of pharmacological inhibition of various RAS components in the hypertensive congenic mRen2.Lewis rat.

Post Baccalaureate:

2003 - 2004 — **Post Baccalaureate Research Education Program (PREP), Wake Forest University, Winston-Salem, North Carolina.**
Advisor: Dr. Mark C. Chappell
Description: Characterizing the circulating and local intrarenal angiotensin peptides in a novel hypertensive congenic male rat model.

Undergraduate:

1998 - 2002 — **Bachelor of Science: Health & Exercise Science, Minor: Chemistry, Wake Forest University, Winston-Salem, North Carolina.**
Major Advisor: Dr. Paul M. Ribisl

RESEARCH EXPERIENCE

2004 – Present — **Hypertension and Vascular Disease Center, Physiology & Pharmacology Department, Wake Forest University School of Medicine, Winston-Salem, NC.**
Dissertation Research Advisor: Dr. Mark C. Chappell
Description: Investigating the effect of pharmacological inhibition of angiotensin receptors and enzymes on various RAS

components in hypertensive female and male congenic mRen2.Lewis rats. Performed surgical implantation of osmotic pumps and indwelling catheters, enzyme activity assays, ELISAs, renal dissections, peptide extraction, protein assay, and tail cuff sphygmomanometry.

2003 – 2004 **Post Baccalaureate Research Education Program (PREP), Wake Forest University, Winston-Salem, North Carolina.**
Research Advisor: Dr. Mark C. Chappell
Description: Studied the expression of angiotensin peptides in the plasma and kidneys of male hypertensive congenic mRen2.Lewis rats. Determined plasma membrane and nuclear angiotensin receptor density and subtype between control Lewis and congenic mRen2.Lewis rats. Performed renal dissections, peptide extraction, density gradient cellular fractionation, protein assay, tail cuff sphygmomanometry, and radioligand receptor binding studies.

Summer 2003 **Excellence in Cardiovascular Sciences (EICS) Internship, Wake Forest University, Winston-Salem, North Carolina.**
Research Advisor: Dr. Mark C. Chappell
Description: Determined plasma membrane and nuclear angiotensin receptor density and subtype between control Lewis and congenic mRen2.Lewis rats. Performed renal dissections, density gradient cellular fractionation, protein assay, and radioligand receptor binding studies.

Fall 2001 **Department of Chemistry, Wake Forest University, Winston-Salem, North Carolina.**
Research: Chemistry Faculty
Description: Analyzed unknown samples on ^{1}H and ^{13}C nuclear magnetic resonance spectrometers.

2000 – 2001 **Department of Health and Exercise Science, Wake Forest University, Winston-Salem, North Carolina.**
Independent Study Advisor: Dr. Patricia A. Nixon
Description: Evaluated the efficacy of adolescents' exercise ability before and after asthma treatment. Techniques employed – ECG machine and MedGraphic Metabolic Cart.

COMPUTER PROFICIENCY

Sound working knowledge in scientific, statistical and reference management software such as Microsoft Excel, Flowjo – Flow cytometry, Bruker SHELXTL – crystallized compounds, X-WIN-NMR – nuclear magnetic resonance, Graph Pad Prism, Graph Pad Instat, and Reference Manager.

AWARDS AND HONORS

2009 Caroline tum Suden/Frances A. Hellebrandt Professional Opportunity Award. FASEB April 2009.

2009	APS/NIDDK Minority Travel Award. FASEB April 2009.
2008	Alumni Student Travel Award to attend 62nd Annual Fall Conference and Scientific Sessions of the Council for High Blood Pressure Research Meeting at Atlanta, Ga. Wake Forest University School of Medicine.
2008	FASEB MARC Program Poster/Oral Presentation Travel Award. FASEB April 2008.
2008	APS Professional Writing Skills Workshop and Travel Award. January 2008.
2007	Alumni Student Travel Award to attend 61st Annual Fall Conference and Scientific Sessions of the Council for High Blood Pressure Research Meeting at Tucson, Az. Wake Forest University School of Medicine.
2007	Research Recognition Award for Outstanding Abstract. APS Conference: Sex Steroids and Gender in Cardiovascular-Renal Physiology and Pathophysiology. August 2007.
2007	APS/NIDDK Minority Travel Award. APS Conference: Sex Steroids and Gender in Cardiovascular-Renal Physiology and Pathophysiology. August 2007.
2007	APS/NIDDK Minority Travel Award. FASEB April 2007.
2007	APS Professional Presentation Skills Workshop and Travel Award. January 2007.
2006	COSEHC Travel Fellowship Award. Abstract presentation at Consortium for Southeastern Hypertension Control. September 2006.
2006	APS/NIDDK Minority Travel Award. FASEB April 2006.
2005	Alumni Student Travel Award to attend the 13th Annual Cardiovascular Diversity Research Supplement Awardee conference at Dallas, Texas. Wake Forest University School of Medicine.
2005	APS/NIDDK Minority Travel Award. FASEB April 2005.
2004	COSEHC Travel Fellowship Award. Abstract presentation at Consortium for Southeastern Hypertension Control. August 2004.
2004	APS/NIDDK Minority Travel Award. FASEB April 2004.

2003	Guy Parks Educational Scholarship Award from Phi Beta Sigma Fraternity Incorporated. Winston-Salem State University.
2002	Thomas K. Hearns Presidential Scholarship. Wake Forest University.
1998 - 2002	Black American Scholarship. Wake Forest University.
1998	Who's Who Among American Colleges and Universities. Wake Forest University.
1998	National Dean's List. Wake Forest University.

TEACHING EXPERIENCE

Spring 2007 & 2008	Lectured on Adrenal Cortical and Androgen Hormones to Master's Physical Therapy students in Physiology & Pharmacology at Winston-Salem State University, Winston-Salem, NC.
2002 – 2004	Tutored Biology, General Chemistry, Organic Chemistry, Biochemistry, Microbiology, Botany and Anatomy & Physiology to undergraduates at Winston-Salem State University, Winston-Salem, NC.

Committees

2006- Present	Curriculum Development. Physiology & Pharmacology Department, Wake Forest University School of Medicine.
2005- Present	Graduate Student Recruitment. Physiology & Pharmacology Department, Wake Forest University School of Medicine.

MEMBERSHIP IN SCIENTIFIC SOCIETIES

- American Heart Association (AHA).
- American Physiological Society (APS).
- Graduate Student Association – Class Representative.
- Black Graduate Student Association – Historian/Photographer.

PEER-REVIEWED PUBLICATIONS

1. **Pendergrass KD**, Gwathmey TM, Michalek RD, Grayson JM, Chappell MC. Nuclear Angiotensin II (AT1) Receptors Mediate the Formation of Reactive Oxygen Species. *Epub May 3, 2009*. Biochemical Biophysical Research Communications 2009; 384(2): 149-154.

2. Gwathmey TM, Shaltout HA, **Pendergrass KD**, Pirro NT, Figueroa JP, Rose JC, Diz DI and Chappell MC. Nuclear Angiotensin II – Type 2 (AT2) Receptors Are Functionally Linked to Nitric Oxide Production. *Epub Feb 25, 2009*. American Journal of Physiology, Renal Physiology 2009; 296: F1484-1493.

3. **Pendergrass KD**, Pirro NT, Westwood BM, Ferrario CM, Brosnihan KB, Chappell MC. Sex differences in circulating and renal angiotensins of hypertensive mRen(2).Lewis but not normotensive Lewis rats. *Epub May 2, 2008*. American Journal of Physiology, Heart and Circulatory Physiology 2008; 295: H10-20.

4. Yamaleyeva LM, **Pendergrass KD**, Pirro NT, Gallagher PE, Groban L, Chappell MC. Ovariectomy is protective against renal injury in the high salt fed older mRen2.Lewis rat. *Epub July 13, 2007*. American Journal of Physiology, Heart and Circulatory Physiology 2007; 293: H2064-2071.

5. **Pendergrass KD**, Averill DB, Ferrario CM, Diz DI, Chappell MC. Differential expression of nuclear AT1 receptors and Angiotensin II within the kidney of the male congenic mRen2.Lewis rat. *Epub Jan 10, 2006.* American Journal of Physiology, Renal Physiology 2006; 290: F1497-1506.

MANUSCRIPTS SUBMITTED/IN PREPARATION

1. Gwathmey TM, **Pendergrass KD**, Reid SD, Rose JC, Diz DI and Chappell MC. A functional Angiotensin-(1-7)-ACE2 pathway within the cell nucleus attenuates reactive oxygen species formation to Angiotensin II. (Submitted)

SCIENTIFIC ABSTRACTS

1. Gwathmey TM, **Pendergrass KD**, Rose JC, Diz DI and Chappell MC. Nuclear Angiotensin-(1-7) Receptors Mediate the Production of Nitric Oxide. Accepted to the 63rd Annual Fall Conference and Scientific Sessions of the Council for High Blood Pressure Research, 2009.

2. Gwathmey TM, **Pendergrass KD**, Shaltout HA, Reid SD, Rose JC, Diz DI and Chappell MC. Steroid-induced Fetal Programming Differentially Alters Nuclear Angiotensin II Receptor Expression and Function. Accepted to the 63rd Annual Fall Conference and Scientific Sessions of the Council for High Blood Pressure Research, 2009.

3. **Pendergrass KD**, Gwathmey TM, Grayson JM and Chappell MC., (2009) Protein Kinase C and PI3 Kinase Mediate Ang II-Dependent Generation of Reactive Oxygen Species in Renal Nuclei. FASEB J. 23, 803.3.

4. Gwathmey TM, **Pendergrass KD**, Pirro NT, Shaltout HA, Reid SD, Rose JC and Chappell MC., (2009) Nuclear AT2 Receptors Mediate Angiotensin II-Dependent Generation of Nitric Oxide. FASEB J. 23, 606.9.

5. Diz H, **Pendergrass KD,** Westwood B, Chappell MC., (2009) Chronic Angiotensin II infusion in Lewis rats does not reveal sex differences in blood pressure or renal injury apparent in the mRen2.Lewis strain. FASEB J. 23, 1013.3.

6. **Pendergrass KD**, Gwathmey TM, Michalek RD, Grayson JM and Chappell MC. Nuclear Angiotensin II AT1 Receptors Mediate the Formation of Reactive Oxygen Species. Hypertension 52:4 [e48] (O67), 2008.

7. Gwathmey TM, **Pendergrass KD**, Reid SD, Rose JC and Chappell MC. Evidence that the ACE2-Angiotensin-(1-7) Pathway Counterbalances Angiotensin II-Induced Formation of Reactive Oxygen Species in Renal Nuclei. Hypertension 52:4 [e124] (LB030), 2008.

8. **Pendergrass KD** and Chappell MC. Female mRen2.Lewis Exhibit Higher Intrarenal Renin But Lower Renal and Circulating Angiotensin II Than Male Littermates. FASEB J 22; 940.4, 2008.

9. **Pendergrass KD**, Westwood BM and Chappell MC. Differential Expression of Neprilysin and Angiotensin Converting Enzyme 2 (ACE2) May Contribute to the Gender Disparity in the Hypertensive mRen2.Lewis Strain. Hypertension 50:4 [e78] (O16), 2007.

10. Chappell MC, Westwood BM, **Pendergrass KD**, Jessup J and Ferrario CM. Distinct Processing Pathways for the Novel Peptide Angiotensin-(1-12) in the Serum and Kidney of the Hypertensive mRen2.Lewis Rat. Hypertension 50:4 [e139] (P228), 2007.

11. **Pendergrass KD**, Westwood BM, Brosnihan KB and Chappell MC. Differential Expression of Neprilysin and Angiotensin Converting Enzyme 2 (ACE2) May Contribute to Decreased Organ Damage in the Female Hypertensive mRen2.Lewis Rat. *APS Sex Steroids and Gender in Cardiovascular-Renal Physiology and Pathophysiology, 2007.* The Physiologist 50:6 [P19] (10.3), 2007.

12. **Pendergrass KD**, Brosnihan KB and Chappell MC. Evidence that Intrarenal Angiotensin II May Influence the Gender Disparity in Blood Pressure of the Hypertensive mRen2.Lewis Rat. FASEB J 21(6); A1415 (971.2), 2007.

13. **Pendergrass KD**, Grayson J and Chappell MC. Novel Use of Flow Cytometry Demonstrates Evidence of Multiple Subpopulations of Nuclei that Express Angiotensin II Type 1 Receptors in the Renal Cortex of Lewis Rats. FASEB J 20(4); A754 (470.4), 2006.

14. **Pendergrass KD**, Diz DI, Averill DB, Ferrario CM, Pirro NT and Chappell MC. Differential Expression of Ang II and the AT1 Receptor in Renal Cortex and Medulla of the Hypertensive mRen2.Lewis Rat. FASEB J 19(4); A201 (135.5), 2005.

15. **Pendergrass KD**, Bernstein KE, Modrall JG and Chappell MC. Chronic Depletion of Angiotensin II Does Not Influence the Intracellular Distribution of the Renal AT_1 Receptor. 58th Annual Fall Conference and Scientific Sessions of the Council for High Blood Pressure Research. Hypertension 44:588 [P220], 2004.

16. **Pendergrass KD**, Westwood BM, Averill DB, Ferrario CM, Diz DI and Chappell MC. Increased Angiotensin II and Sustained Ang II Receptor Expression in Nuclear and Plasma Membrane Compartments in the Kidney of the mRen2.Lewis Rat. Consortium for Southeastern Hypertension Control (COSEHC) August 2004. The American Journal of The Medical Sciences 329:6 [P274], 2005.

17. **Pendergrass KD**, Pirro N, Westwood BM, Averill DB, Ferrario CM, Diz DI and Chappell MC. Sustained levels of the nuclear AT1 receptor in the hypertensive mRen2.Lewis rat. FASEB J 18(4); A299 (208.7), 2004.

GRANT SUPPORT

Title: "Estrogen, ACE2 and Salt Sensitivity"

Goals: Characterize the renal renin angiotensin system components in hypertensive female mRen2.Lewis rats, which can contribute to gender differences observed in this model.

Dates: July 2005- March 2009

Funding source: NHLBI, Washington, D.C.

ORAL PRESENTATIONS

2009 "Nuclear Angiotensin II AT1 Receptors Mediate the Formation of Reactive Oxygen Species." Hypertension and Vascular Research Division Seminar Series at Henry Ford Hospital. **Karl Pendergrass**.

2009 "Sex-Related Differences in Hypertension and Renal Injury: Role of the Renin-Angiotensin System." Vascular Biology Center Seminar Series at Medical College of Georgia. **Karl Pendergrass**.

2009 "Nuclear Angiotensin II AT1 Receptors Mediate the Formation of Reactive Oxygen Species." Vascular Biology Seminar Series at Emory University. **Karl Pendergrass**.

2008 "Nuclear Angiotensin II AT1 Receptors Mediate the Formation of Reactive Oxygen Species." 62nd Annual Fall Conference and Scientific Sessions of the Council for High Blood Pressure Research. **Karl Pendergrass**, TanYa Gwathmey, Ryan Michalek, Jason Grayson, and Mark Chappell.

2007 "Differential Expression of Neprilysin and Angiotensin Converting Enzyme 2 (ACE2) May Contribute to the Gender Disparity in the Hypertensive mRen2.Lewis Strain." 61st Annual Fall Conference and Scientific Sessions of the Council for High Blood Pressure Research. **Karl Pendergrass**, Brian Westwood, and Mark Chappell.

2007 "Differential Expression of Neprilysin and Angiotensin Converting Enzyme 2 (ACE2) May Contribute to the Gender Disparity in the Hypertensive mRen2.Lewis Strain." 61st Annual Fall Conference and Scientific Sessions of the Council for High Blood Pressure Research. "ACE2: More Than an Angiotensin-Degrading Enzyme?" Workshop. **Karl Pendergrass** and Mark Chappell.

2007 "Differential Expression of Neprilysin and Angiotensin Converting Enzyme 2 (ACE2) May Contribute to Decreased Organ Damage in the Female Hypertensive mRen2.Lewis Rat." APS Sex Steroids and Gender in Cardiovascular Renal Physiology and Pathophysiology. **Karl Pendergrass**, Brian Westwood, Bridget Brosnihan and Mark Chappell.

2006 "An Enhanced Intrarenal Renin Angiotensin System Contributes to the Gender Disparity in Blood Pressure of the Hypertensive mRen2.Lewis Rat." 14th Annual NIH/NHLBI Cardiovascular Diversity Research Supplement Awardee Session. **Karl Pendergrass**, Bridget Brosnihan and Mark Chappell.

POSTER PRESENTATIONS

2008 Surgical Sciences Research Day Poster Presentation, Wake Forest University School of Medicine. "Nuclear Angiotensin II AT1 Receptors Mediate the Formation of Reactive Oxygen Species." Karl Pendergrass, TanYa Gwathmey, Ryan Michalek, Jason Grayson and Mark Chappell.

2007 Surgical Sciences Research Day Poster Presentation, Wake Forest University School of Medicine. "Differential Expression of Neprilysin and Angiotensin Converting Enzyme 2 (ACE2) May Contribute to Decreased Organ Damage in the Female Hypertensive mRen2.Lewis Rat." Karl Pendergrass, Brian Westwood, Bridget Brosnihan and Mark Chappell.

2006 Surgical Sciences Research Day Poster Presentation, Wake Forest University School of Medicine. "Circulating versus Renal Tissue Angiotensin Expression in the Hypertensive mRen2.Lewis Strain." Karl Pendergrass, Bridget Brosnihan, Carlos Ferrario and Mark Chappell.

2006 Consortium For Southeastern Hypertension Control (COSEHC) Poster Presentation. "Circulating versus Renal Tissue Angiotensin Expression in the Hypertensive mRen2.Lewis Strain." Karl Pendergrass, Bridget Brosnihan, Carlos Ferrario and Mark Chappell.

2005 13th Annual Cardiovascular Diversity Research Supplement Awardee Session. "Differential Expression of Nuclear AT1 Receptors and Angiotensin II Levels within the Kidney of Congenic mRen2.Lewis Rat." Karl Pendergrass, David Averill, Carlos Ferrario, Jason Grayson, Debra Diz and Mark Chappell.

2005 Surgical Sciences Research Day Poster Presentation, Wake Forest University School of Medicine. "Differential Expression of Nuclear AT1 Receptors and Angiotensin II Levels within the Kidney of Congenic mRen2.Lewis Rat." Karl Pendergrass, Jason Grayson and Mark Chappell.

2004 Surgical Sciences Research Day Poster Presentation, Wake Forest University School of Medicine. "Evidence for An Intracellular Angiotensin-AT1 Receptor System in ACE(-/-) Mice." Karl Pendergrass and Mark Chappell.

2004 Post-Baccalaureate Research Education Program (PREP) Scholars Wake Forest University Graduate School of Arts and Sciences "Characterization of Nuclear Angiotensin II Receptors in Kidneys of Normotensive and Hypertensive Rat Strains. Advisor: Mark Chappell.

2003 Surgical Sciences Research Day Poster Presentation, Wake Forest University Graduate School of Arts and Sciences. "Predominance of Nuclear Angiotensin II Receptors in the Kidney." Karl Pendergrass, Nancy Pirro, Brian Westwood, Debra I. Diz and Mark Chappell.

2003 Undergraduate Summer Research Poster Presentations (EICS), Wake Forest University Graduate School of Arts and Sciences. "Evidence of Nuclear Angiotensin II AT1 Receptors in the Rat Renal Cortex." Karl Pendergrass, Nancy Pirro, Brian Westwood, Debra I. Diz and Mark Chappell.

REFERENCES

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7) **Paul M. Ribisl, Ph.D.**
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