

## Angiotensin (1-7) Receptor Antagonism Equalizes Angiotensin II–Induced Hypertension in Male and Female Spontaneously Hypertensive Rats

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**Abstract**—Females are less sensitive to the hypertensive effects of angiotensin II compared with males, although the molecular mechanisms responsible are unknown. We hypothesize that differential activation of angiotensin II, angiotensin (1-7), angiotensin II type 1, angiotensin II type 2, and *mas* levels in the renal cortex of male and female spontaneously hypertensive rats contribute to sex differences in the blood pressure response to angiotensin II infusion. Males had a greater increase in blood pressure after angiotensin II infusion than females (males:  $150 \pm 2$  to  $186 \pm 3$  mm Hg; females:  $137 \pm 3$  to  $160 \pm 4$  mm Hg;  $P < 0.05$ ). Angiotensin II infusion resulted in comparable increases in plasma and renal cortical angiotensin II levels in both sexes. Renal cortical angiotensin (1-7) levels were higher in female rats under basal conditions ( $195 \pm 10$  versus  $67 \pm 11$  ng/g of cortex;  $P < 0.05$ ) and after angiotensin II infusion ( $281 \pm 25$  versus  $205 \pm 47$  ng/g of cortex;  $P < 0.05$ ) compared with male rats. In the renal cortex of male rats, angiotensin II infusion decreased angiotensin II type 1 protein expression and increased angiotensin II type 2 expression with no change in *mas* expression. In female rats there was an increase in *mas* receptor protein expression with angiotensin II infusion, although angiotensin II type 1 and angiotensin II type 2 expressions were unchanged. Male and female rats were then treated with the angiotensin (1-7) *mas* receptor antagonist A-779 in the absence and presence of angiotensin II. A-779 equalized the blood pressure response to angiotensin II in males and females (blood pressure at the end of treatment: males,  $166 \pm 4$  mm Hg; females,  $164 \pm 5$  mm Hg). In conclusion, angiotensin (1-7) contributes to the sex difference in angiotensin II–induced increases in blood pressure in spontaneously hypertensive rats. (*Hypertension*. 2010;56:658-666.)

**Key Words:** proteinuria ■ sex ■ SHR ■ blood pressure ■ renin-angiotensin system ■ Ang (1-7) ■ *mas* receptor

Male spontaneously hypertensive rats (SHRs) have elevated blood pressure, albuminuria, and renal inflammation compared with age-matched female SHRs.<sup>1-3</sup> Reckelhoff et al<sup>3</sup> reported that treatment of male and female SHRs with the angiotensin (Ang)-converting enzyme (ACE) inhibitor enalapril reduced blood pressures to similar levels in both sexes, indicating that both the hypertension and the sex difference in blood pressure were renin-Ang system (RAS) mediated. The RAS is a key system in controlling blood pressure and kidney function, and overactivation of the RAS contributes to hypertension.<sup>3</sup> There are 2 Ang II receptors, AT<sub>1</sub> and AT<sub>2</sub>. Activation of AT<sub>1</sub> receptors, the “classic pathway,” mediate most well-known biological functions of Ang II, including vasoconstriction, oxidative stress, and inflammation.<sup>4,5</sup> Activation of the “nonclassic pathway” (Ang [1-7] and AT<sub>2</sub>, *mas* receptors) opposes AT<sub>1</sub>-mediated effects, leading to vasodilation, improved renal blood flow, and enhanced pressure natriuresis.<sup>6,7</sup> Ang (1-7) effects are thought to be primarily mediated by the G protein–coupled receptor *mas*.<sup>8,9</sup>

There are known sex differences in the expression levels of RAS components and functional responses to Ang II infusion.

Young male SHRs have higher levels of AT<sub>1</sub> mRNA and protein expression in the kidney, aorta, and mesenteric arteries,<sup>1,10</sup> whereas AT<sub>2</sub> mRNA expression is higher in females.<sup>10</sup> Although not examined previously in SHRs, Ang (1-7) levels tend to be higher in hypertensive female congenic mRen (2) Lewis rats compared with males.<sup>11</sup> There are also pronounced sex differences in the blood pressure responses of normotensive male and female experimental animals to exogenous Ang II infusion. Male Sprague-Dawley rats and C57/BL6J mice have a more robust pressor response to Ang II infusion compared with females.<sup>12-16</sup>

The molecular mechanism(s) accounting for sex differences in response to Ang II are unknown; however, a differential balance in the expression and activation of the classic and nonclassic RAS may contribute. We hypothesized that greater nonclassic RAS activation limits the hypertensive actions of Ang II in female SHRs. Thus, the first goal of this study was to assess expression levels of the primary components of the classic (AT<sub>1</sub> and Ang II) and nonclassic (AT<sub>2</sub>, *mas* receptor, Ang [1-7]) RAS in the renal cortex of male and

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female SHRs. Animals were studied under basal conditions and after Ang II infusion to determine how direct stimulation of the RAS alters that balance of the classic and nonclassic RAS. Male and female SHRs were studied as an experimental model of hypertension that mimics the human condition in that young men tend to have higher blood pressures than women and tend to become hypertensive earlier than women (based on statistics from the National Center for Health Statistics). We found that, after Ang II infusion, female SHRs had greater Ang (1-7) levels in the renal cortex and an increase in *mas* receptor expression that was not evident in males. Based on these observations, additional studies were designed to test the hypothesis that enhanced *mas* receptor activation in female SHRs contributes to a lower blood pressure in response to chronic Ang II infusion.

## Methods

### Animals

Male and female SHRs were used in this study (Harlan Laboratories, Indianapolis, IN). All of the experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved and monitored by the Medical College of Georgia Institutional Animal Care and Use Committee. A subset of male and female SHRs ( $n=12$  and  $13$ , respectively) were implanted with telemetry transmitters (Data Sciences) at 11 weeks of age while under ketamine/xylazine ( $50$  mg/kg/ $10$  mg/kg, IP) anesthesia. Rats were allowed 1 week to recover before they were placed on telemetry receivers for measurement of baseline blood pressure. After 5 days, 6 male and 7 female SHRs received osmotic minipumps (ALZET) implanted SC while anesthetized with isoflurane ( $1.5\%$ ) to deliver Ang II (Phoenix) at a dose of  $200$  ng/kg per minute for 14 days. Separate groups of SHRs, 6 males and 6 females, received osmotic minipumps implanted SC to deliver the Ang (1-7) *mas* receptor antagonist D-alanine-[Ang-(1-7)] (A-779) at a dose of  $48$   $\mu$ g/kg per hour (Bachem). Rats were placed on telemetry receivers to measure blood pressure for 1 week and then received a second minipump to deliver Ang II ( $200$  ng/kg per minute) beginning on day 8 of the A-779 treatment. Rats receiving Ang II alone or in combination with A-779 were placed in metabolic cages weekly for 24-hour urine collection, and at the end of 2 weeks of Ang II infusion kidneys were processed for immunohistochemical analysis.

### Histological and Immunohistochemical Analysis

Briefly, kidneys were perfusion fixed and prepared as described previously for CD68 to assess macrophage infiltration (ED-1; Serotec) and CD3 for T-cell infiltration or were stained with periodic acid-Schiff or trichrome blue.<sup>1</sup> To evaluate renal lesions, stained sections were viewed with an Olympus BX40 microscope (Olympus America) on a bright-field setting fitted with a digital camera (Olympus DP70, Olympus America). To assess the histopathologic changes, we assigned a semiquantitative grade of severity (0, 1, 2, or 3, indicating that the feature was absent, mild, moderate, or severe, respectively) to each of the following morphological attributes: thickening, necrosis, thrombosis, and hyalinosis of interstitial arteries; tuft necrosis, hyalinosis, and thrombosis of glomerular capillaries; and necrosis and thrombosis of glomerular arterioles and fibrosis. Thirty randomly selected nonoverlapping fields of renal cortical interstitium and glomeruli were examined at  $\times 200$  and  $\times 400$  magnification, respectively, and the means of the values were calculated for each sample. For CD3 and CD68, to evaluate the interstitial infiltration of T cells and macrophages, the appropriate software (DPController, Olympus Optical) was used to convert the image, and a  $500\text{-}\mu\text{m} \times 500\text{-}\mu\text{m}$  grid was superimposed onto the image at  $\times 400$  magnification. Twenty grids from each slide were viewed, and positive cells were counted. For all of the studies, the examiner was unaware of the group designations during his or her evaluations.

### RT-PCR

RNA was isolated from the renal cortex of control and Ang II-infused male and female SHRs using the RNeasy Plus Mini kit (Qiagen;  $n=8$  per group). A blend of oligonucleotide and random hexanucleotide primers were used for the reverse transcription of equal amounts of total RNA ( $2$   $\mu$ g) using the iScript cDNA synthesis kit (Qiagen). RT-PCR was performed with QuantiTect SYBR Green RT-PCR kit (Qiagen) with primer pairs for AT<sub>1</sub>, AT<sub>2</sub>, and the *mas* receptor (Qiagen). GAPDH was used as an internal standard, and mRNA levels were expressed relative to male SHRs. The amplification and quantification were performed using the iCycler iQ Real-Time detection system under the following conditions: RT-PCR activation step 15 minutes at  $95^{\circ}\text{C}$ , denaturation for 15 seconds at  $94^{\circ}\text{C}$ , annealing 30 seconds at  $55^{\circ}\text{C}$ , and extension 30 seconds at  $72^{\circ}\text{C}$  for 40 cycles (Applied Biosystems). Each sample was run in duplicate, and the mean threshold cycle ( $C_t$ ) was used to calculate relative mRNA expression (fold change) using the comparative  $C_t$  method ( $2^{-\Delta\Delta C_t}$ ).

### Western Blot Analysis

Renal cortical samples ( $n=6$  per group) were homogenized as described previously, with minor modifications.<sup>1</sup> After homogenization, samples were centrifuged at  $1000g$  for 30 minutes at  $4^{\circ}\text{C}$ . The supernatant was collected and centrifuged at  $30\,000g$  for 45 minutes at  $4^{\circ}\text{C}$ . The resulting pellet fraction was resuspended in half of the original volume of homogenization buffer for use in Western blotting protocols. Protein concentrations were determined by standard Bradford assay (Bio-Rad) using BSA as the standard. Western blotting was performed as described previously.<sup>1</sup> Two-color immunoblots were performed using polyclonal primary antibodies to AT<sub>1</sub> ( $\approx 45K$ , polyclonal, Santa Cruz Biotechnology) and AT<sub>2</sub> and *mas* receptor ( $\approx 43K$  and  $50K$ , respectively, polyclonal, Alomone Labs). Specific bands were detected using the Odyssey Infrared Imager in conjunction with the appropriate IRDye secondary antibodies (LI-COR Biosciences). Actin (monoclonal, Sigma) was used to verify equal protein loading, and all of the densitometric results are reported normalized to actin.

### Peptide Analysis

Ang (1-7) levels were measured by enzyme immunoassay after methanol extraction of the renal cortex as described previously<sup>1</sup> via manufacturer's protocol III ( $n=9$  to  $15$ ; Bachem). Ang II levels were measured by enzyme immunoassay directly in the plasma immediately after collection or after methanol extraction of the renal cortex, as described previously<sup>1</sup> ( $n=6$  to  $10$  per group; Cayman Chemicals).

### Statistical Analysis

All of the data are presented as mean  $\pm$  SEM. Mean arterial pressure (MAP) and urinary protein excretion data were analyzed using ANOVA for repeated measurements. Evaluation of renal lesions in Table 1, PCR, Western blot, and peptide data were compared using a 2-way ANOVA. Differences were considered statistically significant with  $P < 0.05$ . Analyses were performed using GraphPad Prism version 4.0 software (GraphPad Software Inc).

## Results

### Ang II Infusion in Male and Female SHRs

Blood pressure was measured in male and female SHRs by telemetry. Baseline MAP was significantly higher in male SHRs compared with female SHRs (Figure 1A). Ang II infusion increased MAP in both male and female SHRs, with the increase in MAP reaching significance in males after 4 days of Ang II infusion and in females after 8 days. Male SHRs experienced a significantly greater increase in MAP compared with females at the end of 2 weeks (percentage of increase in MAP from baseline: males,  $24 \pm 1\%$ ; females,  $13 \pm 3\%$ ;  $P < 0.05$ ).

**Table 1. Renal Injury Scores and Interstitial Infiltration of Macrophages (CD68<sup>+</sup>) and T Cells (CD3<sup>+</sup>) in Male and Female SHR Treated With Ang II Alone or in Combination With A-779**

Evaluation of Injury	Male	Male+Ang II	Male+A-779+Ang II	Female	Female+Ang II	Female+A-779+Ang II
<b>Interstitial artery</b>						
Thickening	0	1.0±0.01*	1.0±0.1	0	0.9±0.1*	0.6±0.4
Necrosis	0	2.0±0.01*	0.8±0.4‡	0	0.7±0.3*†	1.0±0.4
Thrombosis	0	1.8±0.02*	0.8±0.4	0	0.4±0.3*†	0.4±0.2
Hyalinosis	0	2.2±0.02*	1.5±0.5	0	1.4±0.2*†	1.4±0.4
<b>Glomerulus</b>						
Tuft necrosis	0	1.4±0.3*	0‡	0	0.3±0.2*†	0
Tuft hyalinosis	0	1.2±0.3*	0‡	0	0.3±0.2*†	0.2±0.2
Tuft thrombosis	0	0.7±0.2*	0‡	0	0.1±0.1*†	0
<b>Glomerular arteriole</b>						
Necrosis	0	1.3±0.3*	1.0±0.01	0	0.3±0.2*†	0.4±0.4
Thrombosis	0	1.7±0.2*	1.0±0.01	0	0.4±0.2*†	0.6±0.4
CD68 <sup>+</sup> cells per mm <sup>2</sup>	20±2	35±2*	20±2‡	12±2	20±6*†	24±3
CD3 <sup>+</sup> cells per mm <sup>2</sup>	30±4	43±2*	32±2‡	30±1	38±2*	22±6‡

Data show the renal injury scores in male and female SHR with or without Ang II infusion. Renal injury scores were assessed by an independent, blinded reviewer. Scores are as follows: 0, none; 1, mild; 2, moderate; and 3, severe. Interstitial infiltration of CD68<sup>+</sup> and CD3<sup>+</sup> cells were counted in a blinded manner (*P*<0.05; n=4 to 7).

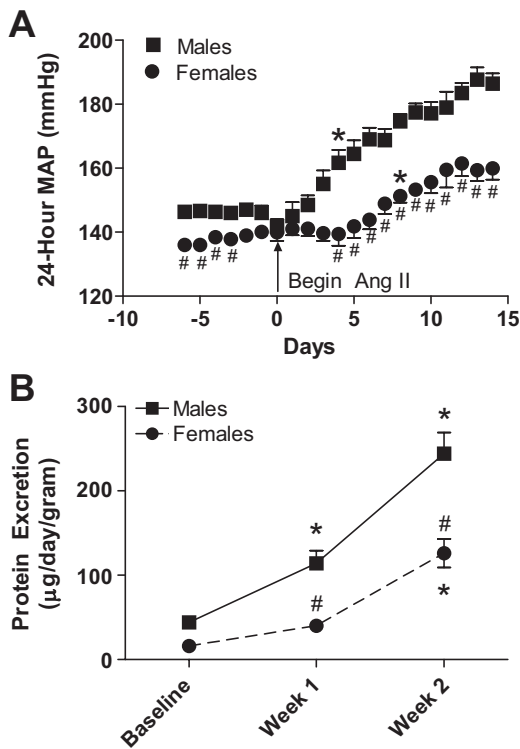
\*Data show a significant difference from same-sex control.

†Data show a significant difference between female+Ang II and male+Ang II.

‡Data show a significant differences between Ang II alone and A-779+Ang II in the same sex.

**Indices of Renal Injury**

Male SHR had greater protein excretion than females at all of the time points (Figure 1B). Proteinuria was significantly increased in male SHR after 1 week of Ang II infusion

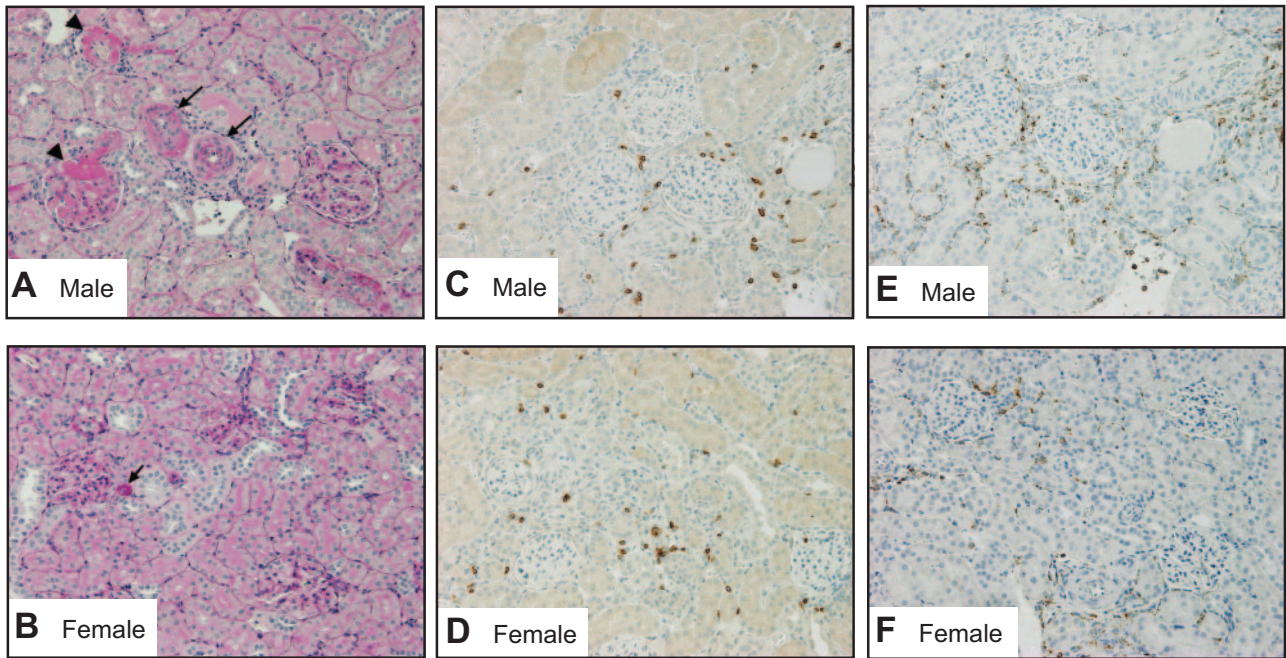


**Figure 1.** Effect of Ang II infusion on 24-hour MAP as measured by telemetry (A) and proteinuria (B) in male and female SHR. Ang II infusion was initiated on day 0. # indicates significant difference from males (*P*<0.05). \*indicates that MAP has significantly increased from baseline in the same sex (*P*<0.05). n=6 to 7.

compared with baseline values and further increased at week 2; however, in female SHR a significant increase in proteinuria was not detected until week 2 of Ang II infusion. Kidneys were processed for histological analysis of renal morphology and immunohistochemical quantification of macrophage (CD68) and T-cell (CD3) infiltration. There were no overt structural alterations in normal renal morphology in control SHR (Table 1). Ang II infusion resulted in marked medial thickening of interstitial arteries, necrosis, thrombosis, and hyalinosis of interstitial arteries; glomerular arterioles and glomerular tufts; and interstitial mononuclear cell infiltration in male SHR. Although mild medial thickening and hyalinosis were also noted in some interstitial arteries and glomerular arterioles in female SHR treated with Ang II, the degree of injury was much more severe in males compared with females (Table 1 and Figure 2A and 2B). The amount of renal fibrosis evident was minimal as assessed by trichrome staining (tubulointerstitial fibrosis was scored at 0 in both the male and female SHR; data not shown). Ang II infusion also resulted in a significant increase in macrophage and T-cell infiltration in the interstitial and periglomerular lesions of male and female SHR. Although macrophage infiltration was significantly greater in male SHR compared with female SHR after Ang II infusion, the increase in T-cell infiltration was comparable between the sexes (Figure 2C through 2F).

**Assessment of Classic and Nonclassic RAS Components**

We next assessed mRNA and protein expression levels of RAS components in the renal cortex of male and female SHR. Under basal conditions, there were no significant differences in AT<sub>1</sub>, AT<sub>2</sub>, or *mas* receptor mRNA expression in the renal cortex of male and female SHR (Figure 3). Ang



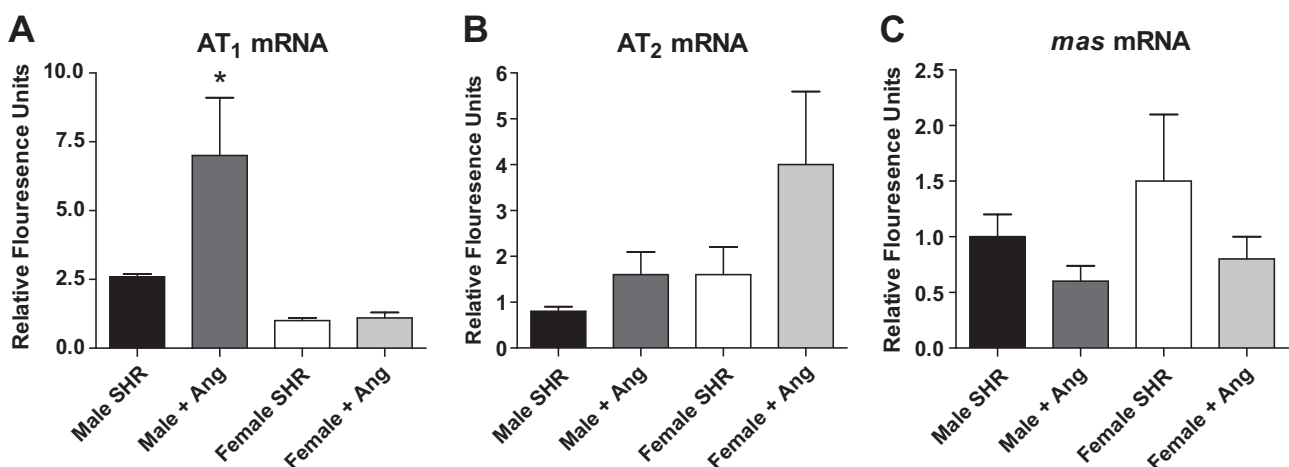
**Figure 2.** Representative pictures of the renal lesions (A and B; periodic acid-Schiff staining), intrarenal infiltration of T cells (C and D; CD3 staining), and macrophages (E and F; CD68 staining) in male (A, C, and E) and female (B, D, and F) SHR treated with Ang II. Marked medial thickening of interstitial arteries (arrows in A), fibrinoid necrosis (arrowheads in A), thrombosis and hyalinosis of interstitial arteries, glomerular arterioles, and glomerular tufts, as well as focal interstitial mononuclear cell infiltration, were noted in male SHR treated with Ang II. Although mild medial thickening and hyalinosis (arrow in B) were also noted in some interstitial arteries and glomerular arterioles in female SHR treated with Ang II, the degree of injury was much more severe in males than females. Increased interstitial and periglomerular infiltrations of T cells (C and D) and macrophages (E and F) were noted in male and female SHR treated with Ang II. Although the increase in T-cell infiltration was almost comparable between the sexes (C and D), macrophage infiltration was greater in males (E) compared with females (F). Original magnification,  $\times 200$ ;  $n=6$  to 8.

II infusion resulted in a significant increase in  $AT_1$  mRNA expression in male SHR with no change in females. In contrast,  $AT_2$  mRNA expression was not changed in male SHR after Ang II infusion ( $P=0.14$ ); however, expression tended to increase in the renal cortex of female SHR ( $P=0.06$ ). *Mas* receptor mRNA expression was comparable among all of the groups.

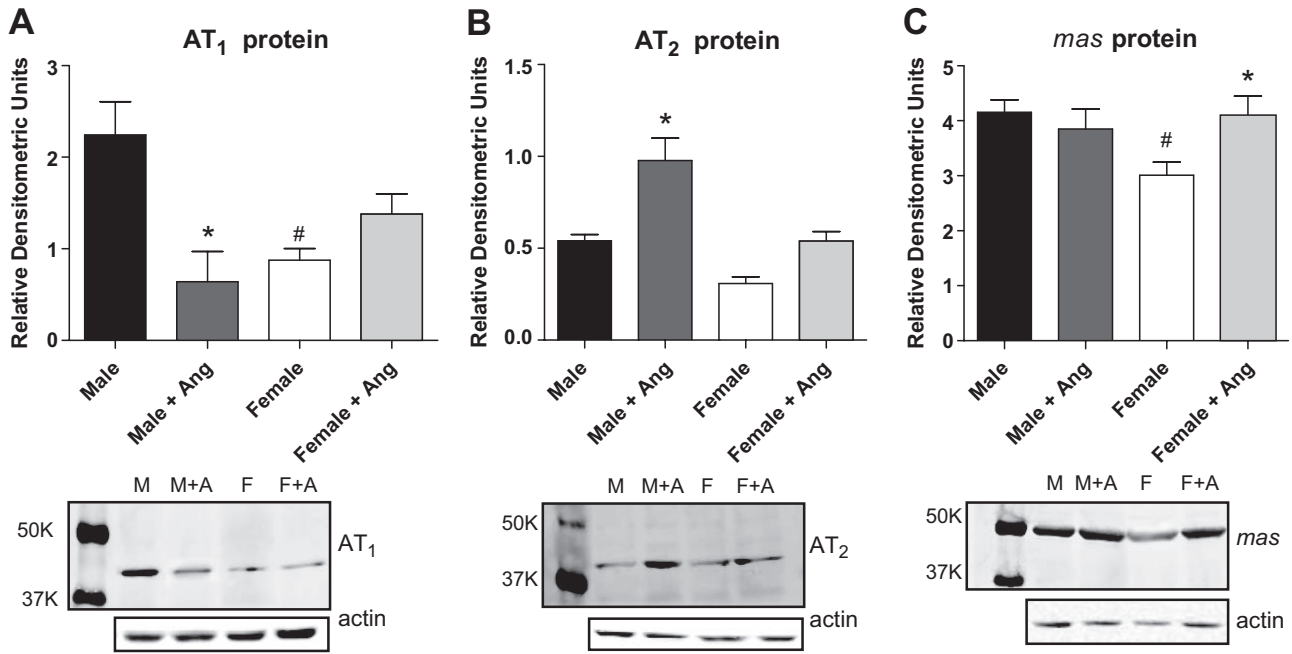
Additional experiments assessed  $AT_1$ ,  $AT_2$ , and *mas* receptor protein expression in the renal cortex of male and female SHR.  $AT_1$  and *mas* receptor protein expressions were

greater in the renal cortex of male SHR compared with female SHR under basal conditions (Figure 4).  $AT_2$  protein expression was comparable between males and females. Two weeks of Ang II infusion significantly decreased  $AT_1$  protein expression in males and significantly increased  $AT_2$  protein expression. In contrast, chronic Ang II infusion resulted in a significant increase in *mas* receptor expression in the renal cortex of female SHR.

In separate animals, plasma and renal cortical Ang II levels and renal cortical Ang (1-7) levels were measured. Basal Ang



**Figure 3.** mRNA expression levels of  $AT_1$  (A),  $AT_2$  (B), and the *mas* receptor (C) in the renal cortex of control and Ang II-infused male and female SHR. \*Indicates significant difference from same-sex control ( $P<0.05$ ).  $n=8$ .

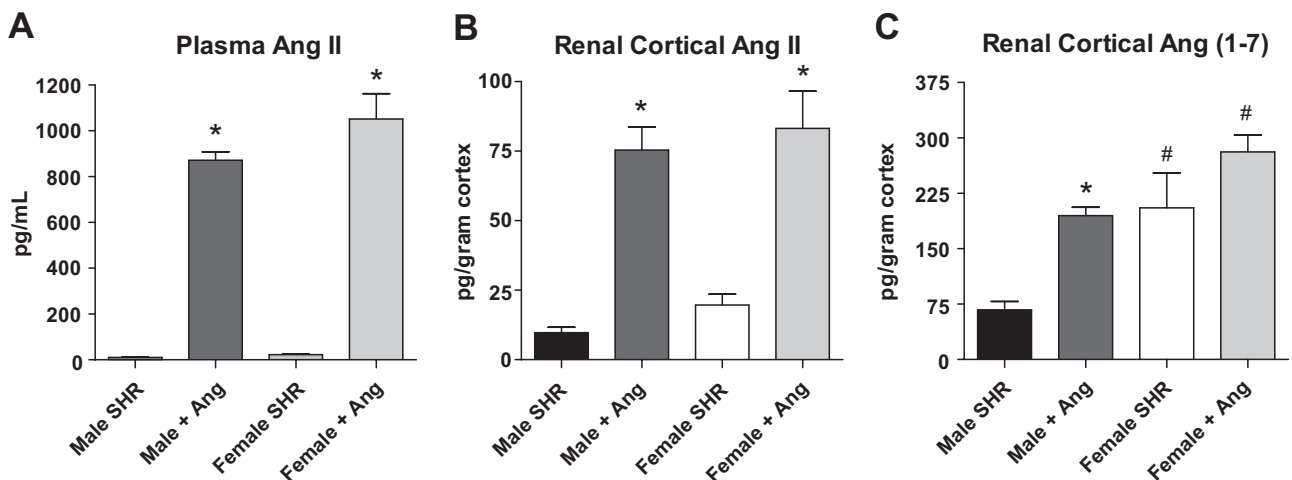


**Figure 4.** Protein expression levels of AT<sub>1</sub> (A), AT<sub>2</sub> (B), and the *mas* receptor (C) in the renal cortex of control and Ang II-infused male and female SHR. #Indicates significant difference from males ( $P < 0.05$ ). \*Indicates significant difference from same-sex control ( $P < 0.05$ ). n=6.

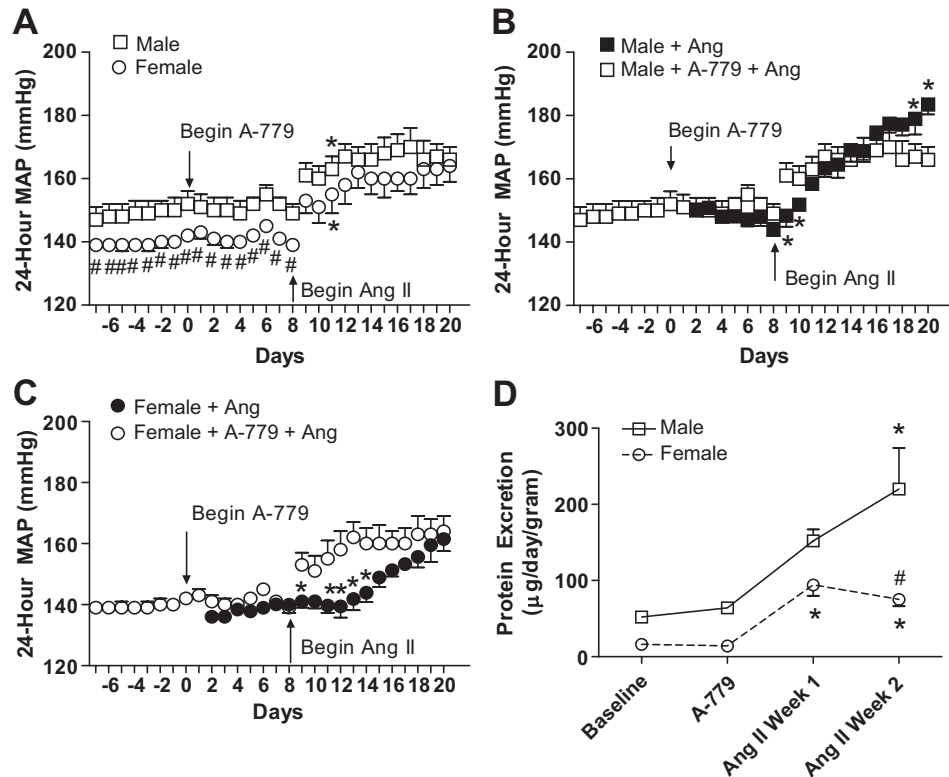
II levels in plasma were less in male SHR (11±1 pg/mL) compared with female SHR (23±2 pg/mL;  $P < 0.05$ ). Chronic Ang II infusion increased plasma Ang II to comparable levels in male and female SHR (Figure 5A). Cortical Ang II levels were comparable in male and female SHR both under basal conditions and after Ang II infusion (Figure 5B). In contrast, Ang (1-7) levels were significantly less in male SHR compared with female SHR both under basal conditions and after chronic Ang II infusion (Figure 5C). Ang II infusion increased Ang (1-7) levels in the renal cortex of both male (≈3-fold) and female SHR (1.3-fold); however, this increase was only significant in the males.

### Ang (1-7) Receptor Antagonism Alters Ang II-Induced Hypertension

Based on the findings that Ang II infusion increases *mas* receptor expression in female SHR and female SHR have higher levels of Ang (1-7) in the renal cortex, we examined the ability of Ang (1-7) antagonism to influence the blood pressure response and renal injury to chronic Ang II infusion. Infusion of A-779 did not alter basal blood pressure in either male or female SHR (Figure 6). However, there was no sex difference in the blood pressure response to Ang II in the presence of A-779 (Figure 6A). This was associated with an initial increase in sensitivity to Ang II in both males and



**Figure 5.** Plasma Ang II (A; n=6 to 8), renal cortical Ang II (B; n=6 to 10), and renal cortical Ang (1-7) levels (C; n=9 to 15) in control and Ang II-infused male and female SHR. #Indicates significant difference from males ( $P < 0.05$ ). \*Indicates significant difference from same-sex control ( $P < 0.05$ ).



**Figure 6.** Effect of A-779 on 24-hour MAP as measured by telemetry (A through C) and protein excretion (D) in male and female SHR. A-779 infusion was begun on day 0 (A). Ang II infusion was started on day 8 and continued until day 21. B and C are a direct comparison of the blood pressure response to Ang II in the absence and presence of A-779 in male and female SHR, respectively. #Indicates significant difference from males, ( $P < 0.05$ ). \*Indicates significant difference from same-sex baseline blood pressure ( $P < 0.05$ ).  $n = 6$  to 7.

females and an attenuation of the Ang II-induced increase in blood pressure in male SHR (Figure 6B and 6C).

To assess renal injury, urinary protein excretion was measured at baseline and weekly thereafter (Figure 6D and Table 2). A-779 infusion did not significantly alter protein excretion in male SHR compared with infusion with Ang II alone (Table 2). However, 1 week of Ang II infusion in female SHR resulted in a  $\approx 7.0$ -fold increase in proteinuria in the presence of A-779 as compared with a  $\approx 2.5$ -fold

increase with Ang II alone ( $P < 0.05$ ). There was not a significant change in proteinuria from week 1 of Ang II infusion to week 2 of Ang II infusion in females that had been treated with A-779 ( $P = 0.2$ ); in contrast, females treated with Ang II alone displayed a 3-fold increase in protein excretion from weeks 1 to 2 ( $P < 0.05$ ). Kidneys were also processed for histological analysis of renal morphology and immunohistochemical quantification of macrophage and T-cell infiltration. As compared with rats infused with Ang II alone, A-779 normalized glomerular arteriole morphology, glomerular tufts, and interstitial mononuclear cell infiltration in male SHR; however, there was still evidence of medial thickening of interstitial arteries, necrosis, thrombosis, and hyalinosis of interstitial arteries. In contrast, A-779 did not alter Ang II-induced morphological changes in the kidneys of female SHR, although T-cell infiltration was normalized (Table 1).

Renal cortical Ang II levels and Ang (1-7) levels were measured in rats treated with A-779 after 2 weeks of Ang II infusion. Ang II levels were comparable in male and female SHR ( $64 \pm 10$  pg/g cortex versus  $74 \pm 14$  pg/g cortex, respectively) and not significantly altered relative to SHR treated with Ang II alone. Ang (1-7) levels tended to be less in male SHR ( $212 \pm 26$  pg/g of cortex) compared with female SHR ( $269 \pm 66$  pg/g of cortex); however, again there was no significant difference in Ang (1-7) levels in rats treated with A-779 compared with rats treated with Ang II alone.

**Table 2. Urinary Protein Excretion (Micrograms per Day per Gram of Body Weight) in Male and Female SHR Treated With Ang II Alone or in Combination With A-779**

Treatment	Male SHR	Female SHR
Ang II study		
Baseline	44 ± 4	16 ± 1
Ang II week 1	114 ± 44*	41 ± 3#
Ang II week 2	244 ± 25*	127 ± 18†*
A-779 study		
Baseline	52 ± 3	16 ± 2
A-779	64 ± 4	14 ± 1
Ang II week 1	152 ± 15	94 ± 14*†‡
Ang II week 2	220 ± 54*	75 ± 9*‡

For all comparisons,  $P < 0.05$ ;  $n = 4$  to 7.

\*Data show a significant difference from same sex at baseline.

†Data show a significant difference between female+Ang II and male+Ang II.

‡Data show significant differences between Ang II alone and A-779+Ang II in the same sex.

## Discussion

The RAS is a critical system in controlling blood pressure and renal health under physiological conditions and a contributing factor to the dysregulation of blood pressure control under numerous pathological conditions. Females have been shown both clinically and experimentally to be less sensitive to the hypertensive effects of Ang II compared with males, although the molecular mechanisms responsible are unknown. In this study, we verified that, similar to other species and strains, male SHR has a larger increase in blood pressure in response to exogenous Ang II infusion compared with females. The primary novel findings of this study are as follows: (1) Ang II infusion differentially regulates the expression of the primary RAS receptors in the renal cortex of male and female SHRs; (2) Ang (1-7) levels are greater in female SHRs under basal conditions and after Ang II infusion compared with male SHRs; and (3) Ang (1-7) receptor antagonism abolishes the sex difference in the blood pressure response to Ang II infusion. A-779 resulted in an initial increase in blood pressure sensitivity and proteinuria to Ang II, especially in females, suggesting that Ang (1-7) antagonizes the immediate Ang II-induced increases in blood pressure and renal injury. In contrast, male SHRs treated with A-779 had significantly lower blood pressure after 2 weeks of Ang II infusion, suggesting that Ang (1-7) contributes to the elevation in blood pressure with Ang II infusion in males.

Infusion of Ang II increased MAP in both sexes; however, the increase in MAP was more pronounced in males. These data agree with studies in Sprague-Dawley rats and C57/Bl6 mice in which females have an attenuated increase in MAP to Ang II infusion.<sup>13–16</sup> In contrast, in the presence of an ACE1 inhibitor, female Sprague-Dawley rats are more sensitive to Ang II-induced hypertension compared with males<sup>17</sup>; however, in C57/Bl6 mice even in the presence of an ACE inhibitor males had a greater increase in blood pressure with Ang II infusion than females.<sup>13</sup> Sex differences in response to RAS activation are also apparent in humans. Increases in Ang II in men correlate with increases in blood pressure and renal injury.<sup>18</sup> However, in women, increases in Ang II levels have been shown to decrease MAP and attenuate vasoconstrictor responses to RAS activation.<sup>19,20</sup>

Although the highest sensitivity to the vascular effects of Ang II are found in the kidney, little is known regarding how sex of the animals influences Ang II-induced renal injury.<sup>21</sup> Proteinuria, morphology, and inflammatory cell infiltration were measured to assess renal injury in this study, and Ang II-induced renal injury was more pronounced in males compared with females. Although sex differences in renal injury are likely related to sex differences in blood pressure, we have previously shown that blood pressure is not the sole factor responsible for sex differences in albuminuria.<sup>1</sup> This was verified in the present study where males had greater protein excretion after Ang II treatment in the presence of A-779 relative to female SHRs despite comparable blood pressures. In addition, Sartori-Valinotti et al<sup>17</sup> showed that treatment with an ACE1 inhibitor in conjunction with Ang II infusion increased albuminuria in male Sprague-Dawley rats, with no effect in females despite the finding that MAP was

higher in the females. Therefore, males are more sensitive to Ang II-induced renal injury compared with females.

To determine the molecular mechanisms responsible for sex differences in response to Ang II infusion, we examined how Ang II infusion altered the balance of the primary components of the classic (AT<sub>1</sub> and Ang II) and nonclassic RAS (AT<sub>2</sub>, mas, Ang [1-7]). The majority of the studies in the literature that have examined these RAS receptors have looked at mRNA expression. However, because protein levels are potentially of greater physiological relevance, we examined both mRNA and protein expression in the renal cortex of male and female SHRs. Consistent with our mRNA results, renal cortical and left ventricular AT<sub>1a</sub> receptor mRNA expressions are increased in male Sprague-Dawley rats with Ang II infusion but not in females,<sup>12,22</sup> and infusion of Ang II increased AT<sub>1</sub> mRNA and protein in the paraventricular nucleus of the hypothalamus.<sup>22</sup> In contrast, Sampson et al<sup>12</sup> published that in the whole kidney high-dose Ang II infusion had no effect on AT<sub>1</sub> or AT<sub>2</sub> mRNA expression in male and female Sprague-Dawley rats. However, this finding may be related to looking at the whole kidney level as opposed to the different regions of the kidney. In the present study, we found that Ang II decreases AT<sub>1</sub> protein expression only in males, increases AT<sub>2</sub> expression only in males, and increases *mas* receptor expression only in females, thereby underscoring the importance of determining protein expression. The fact that AT<sub>1</sub> receptor protein expression is not downregulated in the renal cortex of female SHRs may suggest an inability of the female to compensate for the increase in Ang II levels relative to the males. This may explain the comparable levels of tissue Ang II measured in male and female SHRs assuming that cortical levels of Ang II arise primarily from uptake of the high content of the circulating peptide.

Although AT<sub>2</sub> mRNA expression has been reported to be greater in kidneys from females compared with males,<sup>10</sup> of more potential relevance are reports that normotensive females have greater AT<sub>2</sub>-dependent regulation of the vasculature and blood pressure. Low-dose (50 ng/kg per minute) Ang II results in an AT<sub>2</sub> receptor-dependent decrease in MAP in female, but not male, Sprague-Dawley rats.<sup>12</sup> Greater AT<sub>2</sub> activity has also been shown in female mice where treatment with an AT<sub>1</sub> receptor blocker attenuates vascular injury to a greater extent in arteries from female mice because of increased AT<sub>2</sub> receptor expression in females.<sup>23</sup> We report in this study that male SHRs after chronic Ang II infusion have an increase in AT<sub>2</sub> receptor expression that is not evident in female SHRs. However, we did not assess AT<sub>2</sub> receptor activity. A difference in receptor expression alone is insufficient to conclude that a parallel sex difference exists in the physiological contribution of the receptor to blood pressure regulation. Alternatively, this increase in AT<sub>2</sub> expression in males may be a compensatory response in conjunction with a decrease in AT<sub>1</sub> receptor expression to limit the rise in blood pressure with Ang II. There is recent evidence in the literature supporting a functional role for the AT<sub>2</sub> receptor in male SHRs to offer neuroprotection against ischemic stroke<sup>24</sup> and lower blood pressure when the AT<sub>1</sub> receptor is blocked.<sup>25</sup> Therefore, it is possible that, under certain conditions, such as

in the presence of high levels of circulating and tissue Ang II, the AT<sub>2</sub> receptor contributes to blood pressure regulation in male SHRs; however, additional work is needed to determine the role of the AT<sub>2</sub> receptor in Ang II-mediated hypertension in SHRs. Because the *mas* receptor was the only RAS receptor regulated by Ang II in females, the remainder of this study focused on the effect of Ang II infusion on Ang (1-7) to test the hypothesis that greater Ang (1-7) and *mas* receptor activation attenuate Ang II-induced hypertension in female SHRs accounting for the sex difference in response to Ang II infusion.

We published previously that plasma levels of Ang II are greater in female SHRs compared with males, and renal cortical Ang II levels under basal conditions are comparable in males and females.<sup>1</sup> We verified our previous finding; however, there were no sex differences in Ang II levels after Ang II infusion. We next measured Ang (1-7) levels in the renal cortex and we found female SHRs to have significantly higher levels of Ang (1-7) in the renal cortex after Ang II infusion. This is consistent with reports in the literature in congenic mRen (2) Lewis rats in which plasma Ang (1-7) levels are higher in females than in males.<sup>11</sup> Previous findings also reported that female Dahl rats are more sensitive to the hypotensive effects of Ang (1-7).<sup>26</sup> It is interesting to note that, whereas Ang II infusion increased Ang (1-7) levels in the renal cortex of both male and female SHRs, in female SHRs there was a 1.3-fold increase in Ang (1-7) levels after chronic Ang II infusion, whereas in males there was a 2.7-fold increase. These data raise the possibility that Ang (1-7) may play an important role as a compensatory inhibitor of increases in blood pressure in males, however, only in female SHRs was there also an increase in *mas* receptor expression with Ang II infusion.

To examine the functional implications of the increase in *mas* receptor expression with Ang II infusion and higher Ang (1-7) levels in female SHRs, rats were treated with the Ang (1-7) receptor antagonist A-779. A-779 had no effect on baseline blood pressure in either male or female SHRs, which is consistent with previous reports in male control and diabetic SHRs, Wistar-Kyoto rats, and 2-kidney, 1-clip Goldblatt hypertensive rats.<sup>27–29</sup> However, with the initiation of Ang II infusion, female SHRs experienced a much more robust increase in MAP as compared with infusion of Ang II alone. Similarly, female SHRs had a significantly larger increase in proteinuria after the first week of Ang II infusion when pretreated with A-779 as opposed to Ang II infusion alone. These data suggest that Ang (1-7) normally acts to buffer the immediate increase in MAP and renal injury with Ang II in female SHRs. However, at the end of the 2-week Ang II infusion there was no significant difference in blood pressure in females treated with A-779 compared with those infused with Ang II alone. In male SHRs, although there was an increase in sensitivity to Ang II initially, over time the males treated with Ang (1-7) maintained a lower blood pressure compared with males infused with Ang II alone. Although A-779 had no effect on Ang II-induced proteinuria in male SHRs, there was an improvement in structural damage to the kidney. These data suggest that Ang (1-7) has opposite effects in males and females and that, in males, Ang

(1-7) may contribute to Ang II-mediated hypertension. Ang (1-7) effects are thought to be primarily mediated by the G protein-coupled receptor *mas*.<sup>8,9</sup> However, there are also reports that Ang (1-7) binds and activates the AT<sub>2</sub> receptor in male SHRs,<sup>30</sup> and there was an increase in AT<sub>2</sub> receptor expression in the renal cortex of male SHRs after Ang II infusion. If the lower blood pressure in male SHRs treated with A-779 is because of loss of Ang (1-7), activation of the *mas* receptor or increased AT<sub>2</sub> receptor activation is not known. It is also possible that there is a sex difference in the time course of the increase in Ang (1-7) in males and females such that it took males longer to increase Ang (1-7) levels. Alternatively, additional vasoactive Ang peptides may be present and contribute to Ang II-induced hypertension. Additional studies are planned to further investigate the mechanism by which A-779 differentially influences the blood pressure responses to Ang II in male and female SHRs. Our study demonstrates that Ang (1-7) contributes to sex differences in the physiological responses to Ang II infusion and adds to our knowledge of how sex of the animal influences the balance of the classic and nonclassic RAS.

### Perspectives

ACE inhibitors and ARBs are among the most commonly prescribed drugs to help control blood pressure in hypertensive patients, regardless of sex of the patient. There is accumulating evidence in the literature, at both the clinical and basic science levels, to support the idea that the RAS of males is not the same as the RAS of females. Our studies support this notion and further show that not only may males and females respond differently to Ang II but it is also likely that they respond differently to Ang (1-7). Better understanding of the components of the RAS that are being inappropriately activated or suppressed in hypertension and after activation of the RAS may lead to the development of more targeted therapies for more efficient blood pressure regulation.

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### Disclosures

None.

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## Angiotensin (1-7) Receptor Antagonism Equalizes Angiotensin II–Induced Hypertension in Male and Female Spontaneously Hypertensive Rats

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