Enhanced Angiotensin II Type 2 Receptor Mechanisms Mediate Decreases in Arterial Pressure Attributable to Chronic Low-Dose Angiotensin II in Female Rats

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Abstract—The renin-angiotensin system is a far more complex enzymatic cascade than realized previously. Mounting evidence suggests sex-specific differences in the regulation of the renin-angiotensin system and arterial pressure. We examined the hemodynamic responses, angiotensin II receptor subtypes, and angiotensin-converting enzyme 2 gene expression levels after graded doses of angiotensin II in males and females. Mean arterial pressure was measured via telemetry in male and female rats in response to a 2-week infusion of vehicle, low-dose (50 ng/kg per minute SC) or high-dose (400 ng/kg per minute SC) angiotensin II. The effect of concurrent infusion of the angiotensin II type 2 receptor (AT2R) blocker (PD123319) was also examined. The arterial pressure response to high-dose angiotensin II was attenuated in females compared with males (24±8 mm Hg versus 42±5 mm Hg; P<0.002). Remarkably, low-dose angiotensin II decreased arterial pressure (11±4 mm Hg; P for the interaction between sex and treatment <0.02) at a dose that did not have an effect in males. This decrease in arterial pressure in females was abolished by AT2R blockade. Renal AT1R, angiotensin-converting enzyme 2, and left ventricular AT2R mRNA gene expressions were markedly greater in females than in males with a renal angiotensin II type 1a receptor:AT2R ratio of ~1 in females. Angiotensin II infusion did not affect renal AT1R mRNA expression but resulted in significantly less left ventricular mRNA expression. Renal angiotensin-converting enzyme 2 mRNA expression levels were greater in females than in males treated with high-dose angiotensin II (~2.5 fold; P for the interaction between sex and treatment <0.05). In females, enhancement of the vasodilatory arm of the renin-angiotensin system, in particular, AT2R and angiotensin-converting enzyme 2 mRNA expression, may contribute to the sex-specific differences in response to renin-angiotensin system activation. (Hypertension. 2008;52:666-671.)

Key Words: basic science ■ gender differences ■ gene expression/regulation ■ hypertension ■ angiotensin receptors

Cardiovascular disease is the leading cause of death in the Western world, and, contrary to popular belief, mortality rates for cardiovascular disease are greater in women than men.1,2 Significant differences in survival after a heart attack have been revealed, with 42% of women who experience heart attacks dying within 1 year, compared with 24% of men.2 The reasons for these differences in outcomes are unknown but require urgent explanation.

Hypertension is a major risk factor for cardiovascular disease, with the renin-angiotensin system (RAS) playing a predominant role in the regulation of arterial pressure in both sexes. The potent end product, angiotensin II (Ang II), acts via 2 main subtypes of receptors responsible for vasoconstriction, sodium reabsorption and cell proliferation (in the rodents there are 2 further subtypes: type 1a [AT1aR] and type 1b [AT1bR]), as well as vasodilation and antiproliferation (type 2 [AT2R]).3,4 Studies have shown that the AT2R plays a counterregulatory role in the control of arterial pressure in males, which can be seen as a vasodilatory response.5,6 Females have been shown previously to have a higher renal AT1R:Ang II type 1 receptor (AT1R) ratio than males, providing evidence for sex differences in the vasoconstrictor/vasodilator balance of the RAS.7 Of the few studies investigating the impact of Ang II in females, high doses have invariably been used to induce pressor responses, and such responses were attenuated in females as compared with males,8–10 which implies a shift in the balance of the vasoconstrictor and vasodilator elements of the RAS that may, in part, contribute to the sex differences in the incidence of cardiovascular disease.

Given the fact that there is a greater AT2R expression in females compared with males7 and that the response to high
doses of Ang II in females is attenuated compared with males, we hypothesized that chronic administration of Ang II may affect arterial pressure in females differently from males. Therefore, we aimed to investigate the hemodynamic responses to 2 graded doses of Ang II in male and female rats and examined the effect of AT$_2$R blockade on the hemodynamic responses to chronic low-dose Ang II treatment. In addition, the relative left ventricular and renal gene expressions of AT$_1$R, AT$_2$R, AT$_3$R, and angiotensin-converting enzyme 2 (ACE2) in vehicle and Ang II-treated male and female rats were compared. We hypothesized that males would be more sensitive to the prohypertensive effects of chronic Ang II infusion. We also hypothesized that the attenuated response to Ang II in females would be mediated by a greater AT$_2$R and ACE2 gene expression, shifting the balance of the vasoconstrictor and vasodilator arms of the RAS in females compared with males.

**Methods**

**Animals**
Nine-week-old males (n=18; 300 to 350 g) and females (n=42; 200 to 250 g) Sprague-Dawley rats (Australian Research Centre) were fed a sodium-controlled diet (0.25% weight/weight sodium chloride; Glen Forrest Stockfeeders) and water ad libitum. The rats were individually housed with a 12-hour light/dark cycle at a temperature of 22°C to 25°C. Rats were acclimatized to these conditions for 1 week before entering the experimental protocol. Experiments were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved by the Monash University Standing Committee on Ethics in Animal Experimentation.

**General Procedures**
At 10 weeks of age, animals were anesthetized (isoflurane; 2% to 4% O$_2$), and a telemetry transmitter (TA11-PAC40, Data Sciences International) was implanted into the abdominal aorta. A 10-day recovery period was allowed before recording of diastolic pressure, systolic pressure, locomotor activity, and heart rate (HR; Dataquest Labpro version 3.0, Data Sciences International). After baseline recordings, drugs were delivered via an osmotic minipump (Alzet, model 2ML2) implanted subcutaneously (isoflurane; 2% to 4% O$_2$) in the subcapsular region. Arterial pressures, locomotor activity, and HR were recorded as 10-second averages every 10 minutes throughout the recording period. Mean arterial pressure (MAP) was calculated from the 10-second averages of diastolic and systolic pressures. The data were analyzed as 24-hour averages before and during drug infusion and are represented as the change in MAP from baseline for each group.

**Experimental Protocols**

**Chronic Ang II Infusion in Male and Female Rats**
In 3 groups of male and 3 groups of female rats (n=6 per group), arterial pressure, HR, and locomotor activity were measured for 3 days before and 14 days during infusion of vehicle (saline, SC), low-dose Ang II (50 ng/kg per minute SC; Sigma), or high-dose Ang II (400 ng/kg per minute SC) via an osmotic minipump. At the end of this infusion period, the rats were euthanized (sodium pentobarbital, Virbac Pty Ltd) and the left ventricle and kidneys were collected, weighed, and snap frozen.

**AT$_2$R Blockade and Chronic Ang II Infusion in Female Rats**
In 4 groups of female rats arterial pressure, HR and locomotor activity were measured for 3 days before and during the infusion of vehicle (saline)+vehicle, low-dose Ang II (50 ng/kg per minute of Ang II SC)+vehicle, vehicle+PD123319 (10 mg/kg per day SC; Sigma), or low-dose Ang II+PD123319 via osmotic minipump for a period of 10 days. The infusion of PD123319 or its vehicle (saline) was started 3 days before the 10-day Ang II infusion to establish systemic blockade of the AT$_2$R.

**AT$_1$R, AT$_2$R, AT$_3$R, and ACE2 Gene Expression**
Total RNA was extracted from the left ventricle and kidney using RNAeasy extraction kits (Qiagen), as described previously. Gene expressions for the Ang II receptors AT$_1$R, AT$_2$R, AT$_3$R, and ACE2 were determined using the Eppendorf Mastercyler Realplex real-time machine. 18S was used as the internal housekeeping gene, and a comparative cycle of threshold fluorescence ($C_T$) was used. AT$_1$R (probe: ACCGCCTGCCTCGGCCA; primers forward: GGCCAGTCTATAACCGCTATGGAG; reverse: GCCGAAGGATCTTACATAGGGT), AT$_2$R (probe: CGGCAAGTCACCCAGGCT; primers forward: CCTCCAGCTTCTGAAATTACATTCC; reverse: GCCGAAGGATCTTACATAGGGT), and ACE2 (predesigned assay, Taqman Gene Expression Assays, Applied Biosystems) were multiplexed with 18S; however, AT$_2$R (predesigned assay, Taqman Gene Expression Assays, Applied Biosystems) was run in separate wells on the same plate, because $C_T$ interference has been determined previously when these genes are multiplexed.

**Calculations of Relative Expression**
Samples were run in duplicate, and an average $\Delta C_T$ for each sample was determined. The $C_T$ value obtained for 18S was then subtracted from the $C_T$ of the gene of interest to generate a $\Delta C_T$ for each sample. The average $\Delta C_T$ for the calibrator group (in this case, the female vehicle-treated animals) was then subtracted from each sample to generate $\Delta\Delta C_T$. This value was then substituted into the equation $2^{-\Delta\Delta C_T}$, which then generates gene expression levels relative to the calibrator. To determine fold differences in expression between sexes, the average $\Delta C_T$ values in males and females infused with vehicle were compared. One cycle greater $\Delta C_T$ value is equivalent to 2-fold lower expression levels.

**Statistical Analysis**
All of the data are presented as means±SEMs. For chronic Ang II infusion in male and female rats, differences in MAP, HR, and activity from control measurements were analyzed using a 2-way ANOVA with repeated measures (SYSTAT version 9.0) using the factors sex ($P_S$; male or female) and treatment ($P_T$; saline, low-dose Ang II or high-dose Ang II) and the interaction between sex and treatment ($P_{ST}$). For the AT$_2$R blockade and chronic Ang II infusion in female rats, differences in MAP and HR from control measurements were analyzed using a 2-way ANOVA with repeated measures (SYSTAT version 9.0), using the factors treatment ($P_T$; Ang II or vehicle) and drug ($P_D$; AT$_2$R blockade or vehicle) and the interaction between treatment and drug ($P_{DT}$). Gene expression data were analyzed using a 2-way ANOVA using $P_S$, $P_T$, and $P_D$. Statistical significance was accepted at $P<0.05$. There were no differences in resting blood pressures between the groups (Table 1). Males had a significantly lower resting HR than females (376±5 versus 395±6 bpm; $P<0.002$, unpaired $t$ test). Locomotor activity was not different in males compared with females (24-hour average of 34±4 versus 30±3 units, respectively) and was not different between the treatment groups. At the commencement of the study, age-matched males were significantly heavier than females ($P<0.001$); however, there was no difference between the treatment groups within each sex. The increase in the percentage of body weight during the study was not different between the sexes or the treatment groups. At the time of tissue collection, total wet kidney weights were significantly
less in females as compared with males (1.8 ± 0.06 versus 2.6 ± 0.07 g, respectively; \( P \leq 0.006 \)), but there was no effect of treatment.

### Chronic Ang II Infusion in Male and Female Rats

There was a significant increase in MAP evoked by high-dose Ang II in both the male- and female-treated groups, increasing by 42.5 and 24.8 ± 8 mm Hg after 13 days of infusion, respectively (\( P_T < 0.05 \); Figure 1). The increase in MAP was significantly attenuated in the females as compared with the males treated chronically with the high-dose Ang II (\( P_{ST} < 0.002 \)). In response to the chronic low-dose Ang II infusion, males showed no significant change at day 13 in MAP (2 ± 2 mm Hg), whereas MAP significantly decreased in the females (11 ± 4 mm Hg; \( P_{ST} < 0.02 \); Figure 1). There was no significant change in HR in response to the high-dose Ang II infusion in males and females. In response to treatment with low-dose Ang II, HR was slightly increased in the females but not the males as compared with the vehicle-treated groups (male HR decreased 8 ± 5 bpm posttreatment; females increased 11 ± 6 bpm; \( P_{ST} < 0.03 \)). Vehicle treatment had no significant effect on MAP, HR, or locomotor activity in either sex (Figure 1).

### AT\textsubscript{2}R Blockade and Chronic Ang II Infusion in Female Rats

There was no difference in body weight, HR, or locomotor activity among the 4 female treatment groups at the beginning or end of the experimental protocol (Table 2). Baseline blood pressures were not different between the treatment groups (Table 2), and there was no effect of AT\textsubscript{2}R blockade on baseline blood pressure.

In female rats, in response to chronic low-dose Ang II infusion, MAP decreased significantly compared with vehicle-treated females (Figure 2). On day 10 of low-dose Ang II infusion, MAP decreased by 9 ± 3 mm Hg as compared with 0 ± 1 mm Hg in the vehicle-treated group (\( P_{TD} < 0.05 \)). In the presence of the AT\textsubscript{2}R blocker PD123319, low-dose Ang II infusion did not affect MAP. MAP was 0 ± 1 mm Hg compared with baseline by day 10 of infusion in the females treated with both low-dose Ang II plus PD123319. Vehicle treatment alone did not significantly alter MAP (Figure 2).

### Left Ventricular AT\textsubscript{1a}R, AT\textsubscript{1b}R, AT\textsubscript{3}R, and ACE2 mRNA Gene Expression

mRNA expression of AT\textsubscript{1a}R in the left ventricle was not different between males and females and was not altered by low-dose Ang II treatment (Figure 3). In males, high-dose Ang II resulted in significantly greater AT\textsubscript{1a}R mRNA expres-
Renal AT1aR, AT1bR, AT2R, and ACE2 mRNA Expression

The level of AT1aR mRNA expression in the kidney was not different between males and females and was not significantly affected by Ang II treatment in either sex (Figure 4). AT1aR mRNA expression was similar in both males and females and was not altered by Ang II infusion in either sex ($P_{ST}=0.3$). AT1R mRNA expression levels were >300-fold greater in females compared with males ($P_5=0.001$), with there being ~8 cycles of difference in the $\Delta C_T$ value between females and males. Ang II treatment did not significantly affect AT1R levels in males or females ($P_T=0.4$; Figure 4). Consistent with this previous work, the current study demonstrated that females had a greater left ventricular AT1R, renal AT1R, and renal ACE2 mRNA gene expression than males. Collectively, these data demonstrate an increase in the vasodilatory arm of the RAS in females compared with males in response to Ang II infusion.

Ang II-induced hypertension has been shown previously to be attenuated in females.8,10 Xue et al8 showed a reduced pressor response to Ang II in female mice as compared with males, which has been confirmed by others in rats.9,13 Consistent with this previous work, the current study demonstrated sex differences in response to the chronic high-dose Ang II infusion with an attenuated increase in MAP in females as compared with their male counterparts. Remarkably, we were able to show a significant decrease in MAP in normotensive females in response to low-dose Ang II, and this effect was achieved without concomitant AT1R blockade.

Discussion

The main findings of these studies were that chronic low-dose infusion of Ang II decreased MAP in female rats via an AT1R-mediated effect. We have also shown that females had a greater left ventricular AT1R, renal AT1R, and renal ACE2 mRNA gene expression than males. Collectively, these data demonstrate an increase in the vasodilatory arm of the RAS in females compared with males in response to Ang II infusion.

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Figure 3. The relative left ventricle gene expression of RAS components in response to Ang II treatment in males and females. Left ventricle gene expression of AT1aR, AT1bR, AT2R, and ACE2 in female (solid) and male (open) rats after 14 days of vehicle (saline), low-dose Ang II (50 ng/kg per minute), and high-dose Ang II (400 ng/kg per minute) treatment. Data (means±SEM) are expressed relative to the female vehicle-treated group and were analyzed using a 2-way ANOVA with the factors $P_5$, $P_T$, and $P_{ST}$ ($n=6$ per group).

Figure 4. The relative renal gene expression of RAS components in response to Ang II treatment in males and females. Renal gene expression of AT1aR, AT1bR, AT2R, and ACE2 in female (solid) and male (open) rats after 14 days of vehicle (saline), low-dose Ang II (50 ng/kg per minute), and high-dose Ang II (400 ng/kg per minute) treatment. Data (means±SEM) are expressed relative to the female vehicle-treated group and were analyzed using a 2-way ANOVA with $P_5$, $P_T$, and $P_{ST}$ ($n>6$ per group). *$P<0.05$, †$P<0.01$ vs female saline-treated group.
This finding was confirmed in a second study in which it was demonstrated that this decrease in MAP in females was an AT1R-mediated effect, because it was abolished by AT1R blockade with PD123319.

The hypotensive response to Ang II seen in females agrees with previous evidence that the AT1R mediates vasodilation in both in vitro and in vivo studies.8,6,14–17 Evidence for a vasodepressor role of AT1R has usually been obtained from studies using male animals, where AT1R blockade is often required to uncover the vasodepressor response to an AT1R agonist.8,6,14–17 Previous studies have investigated the response to Ang II in females using only high doses.8,10,18 Therefore, this is the first study to show that not only does low-dose Ang II decrease MAP in female rats, but it did so without a background of AT1R blockade. These studies suggest that, unlike the case in males, the in vivo effect of AT1R was not masked by AT1R activity in females.

Our data also demonstrate not only significant sex differences in AT1aR, AT1bR, AT2R, and ACE2 mRNA expression in the left ventricle and kidney but also differential changes in the mRNA expression of these components of the RAS in response to Ang II infusion, which potentially push females in the direction of vasodilation and the males toward vasoconstriction. Importantly, animals in this study were maintained on a controlled sodium diet (0.25% NaCl), because undoubtedly a sodium diet affects the activation and expression levels of components of this system.19

In the current study, basal mRNA gene expression levels of AT1aR were not different between male and female rats, consistent with previous work.20 To date there is confusion as to the exact role and contribution of the AT1aR to the global AT1R response, data that are required to better interpret this finding. In contrast, basal left ventricular expression and renal AT1aR mRNA expression were markedly greater in females as compared with males. Greater renal AT1aR mRNA gene expression has been reported previously in females as compared with males, albeit in spontaneously hypertensive rats.7 The magnitude of the difference in AT1aR mRNA expression between males and females in this study was almost 300-fold, suggesting the balance between AT1aR and AT1bR would be shifted toward increased AT1aR and a vasodepressor response. For example, the relative renal ratio of AT1aR:AT1bR in females is 1. Basal ACE2 mRNA expression was also greater in the female kidney than in males, with no difference in basal left ventricular expression between the sexes. The AT2R gene is located on the X chromosome, and it is, therefore, tempting to speculate that the transcription or expression of this gene would have a greater impact in females compared with males, given that they inherently have 2 copies of the gene.21 Taken together, these data are consistent with enhancement of the vasodilator components of the RAS in females.

In addition, our results suggest that, in females, this vasodepressor arm can be further upregulated. Although renal AT1aR mRNA expression was not altered by Ang II infusion in males or females, we demonstrated for the first time that renal ACE2 mRNA expression was upregulated by Ang II treatment in females. The rise in ACE2 mRNA expression occurred in both sexes in response to Ang II but was enhanced to a greater level in females. Treatment with Ang II had no enhancing effect on left ventricular or renal AT1aR, AT1bR, or AT2R mRNA expression in males or females, consistent with previous work in males.22 The complex role of the kidney in arterial pressure regulation is well recognized. AT1R and AT2R have been localized in the vasculature, with differential expression of the AT1R in different vessels, low expression of AT2R in large vessels, and high expression in microvessels.23 The potentially important role of the vasculature in the response to Ang II was not investigated in this study and deserves attention in future research. In the current study, mRNA gene expression was determined in whole-tissue homogenates, a method that may mask important mRNA gene expression differences in individual segments of the kidney, and, therefore, future investigation of mRNA gene expression in specific renal segments is required. Also, the present study measured mRNA gene expression, which may not always reflect protein expression or activity. Therefore, further investigation of the protein and activity levels of these genes should be conducted to confirm the current hypothesis. Our results suggest that, in response to Ang II infusion, the balance of the RAS in females has further shifted toward vasodilation because of increased renal ACE2 mRNA expression.

One possible reason for the shift in balance from vasoconstriction to vasodilation in females is because of the role of the sex hormones. It has been shown previously that estrogen upregulates AT1R expression24 and downregulates AT1R expression and binding.25,26 It is, therefore, tempting to speculate that estrogen is involved in the upregulation of ACE2 expression. Indeed, ACE2 gene expression is significantly increased in pregnancy in rats, which is consistent with upregulation of ACE2 gene expression by estrogen.27 Recently it has been shown that the attenuation of Ang II–induced hypertension in females is mediated by estrogen receptor–α,28 further suggesting the importance of the interaction between estrogen and RAS in this response in females.

Given the fact that the ligand affinity of Ang II for the receptor subtypes is the same3,13,20 and AT1R are highly expressed, why should low-dose Ang II infusion decrease MAP? As suggested previously, the altered AT1R to AT1R balance in the kidney of females may contribute to this finding. Ang II is readily converted to Ang 1-7 by the enzyme ACE2,29 which we have shown was upregulated in females. Ang 1-7 is known to cause NO release and, hence, vasodilation.30 In this context, we have shown recently that short-term infusion of Ang 1-7 lowered MAP in conscious, male rats, albeit during AT1R blockade.31 More importantly, this vasodilator effect of Ang 1-7 was mediated via AT1R.31 Certainly work by others has shown that stimulation of the AT1R leads to a decrease in systemic arterial pressure, a response that is mediated by the production of bradykinin and NO.5,32 Therefore, in females, it is possible that Ang II, either directly or indirectly, via conversion to Ang 1-7 by ACE2, caused AT1R-mediated vasodilation. Certainly, we have demonstrated that ACE2 mRNA gene expression is markedly enhanced in the kidney of females (∼2.5-fold). Our study suggests that, on balance, the RAS in females is tipped toward vasodilation. Another possible explanation stems from the known actions of ACE2 to convert Ang II to Ang 1-7 and, hence, decrease the circulating levels of Ang II. Therefore, a
greater ACE2 expression in females would lead to a greater conversion to Ang 1-7 and a decrease in circulating Ang II, which likely contributes to the attenuated pressor response and enhanced depressor response to Ang II infusion. Future studies are required to further test these hypotheses.

In summary, the present study has shown that low-dose Ang II significantly decreases MAP in female rats at a dose that has a negligible effect in males and that this response was mediated by AT-R. We have also shown an increase in the mRNA gene expression of vasodilatory components of the RAS, namely, AT2R and ACE2, in females, and we suggest that this altered balance of the vasodilator and vasoconstrictor components of the RAS may contribute to the sex-specific differences observed in response to RAS activation. This study provides an impetus for further investigation of the sex differences in the RAS.

**Perspectives**

We have shown that the contribution of the vasodilator pathway of the RAS is enhanced in females. We suggest that the enhancement of this pathway may underlie the cardiovascular protection seen in females. Additional elucidation of this pathway and the implications for therapeutic stimulation of this pathway provide targets for future cardiovascular treatments.

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**Disclosures**

None.

**References**

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