Sexual dimorphism in the renin-angiotensin system in aging spontaneously hypertensive rats

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Yanes, Licy L., Damian G. Romero, Joshua W. Iles, Radu Iliescu, Celso Gomez-Sanchez, and Jane F. Reckelhoff. Sexual dimorphism in the renin-angiotensin system in aging spontaneously hypertensive rats. Am J Physiol Regul Integr Comp Physiol 291: R383–R390, 2006.—In young adult spontaneously hypertensive rats (SHR), mean arterial pressure (MAP) is higher in males than in females and inhibition of the renin-angiotensin system (RAS) eliminates this sex difference. After cessation of estrous cycling in female SHR, MAP is similar to that in male SHR. The purpose of this study was to determine the role of the RAS in maintenance of hypertension in aging male and female SHR. At 16 mo of age, MAP was similar in male and female SHR (183 ± 5 vs. 193 ± 8 mmHg), and chronic losartan (40 mg·kg−1·day−1 po for 3 wk) reduced MAP by 52% (to 90 ± 8 mmHg, P < 0.05 vs. control) in males and 37% (to 123 ± 11 mmHg, P < 0.05 vs. control) in females (P < 0.05, females vs. males). The effect of losartan on angiotensin type 1 (AT1) receptor blockade was similar: MAP responses to acute doses of ANG II (62.5–250 ng/kg) were blocked to a similar extent in losartan-treated males and females. F2-isoprostane excretion was reduced with losartan more in males than in females. There were no sex differences in plasma renin activity, plasma angiotensinogen or ANG II, or renal expression of AT1 receptors, angiotensin-converting enzyme, or renin. However, renal angiotensinogen mRNA and protein expression was higher in old males than females, whereas renal ANG II was higher in old females than males. The data show that, in aging SHR, when blood pressures are similar, there remains a sexual dimorphism in the response to AT1 receptor antagonism, and the differences may involve sex differences in mechanisms responsible for oxidative stress with aging.

sex differences; angiotensin type 1 receptor; angiotensinogen; angiotensin II; oxidative stress

MEN ARE AT GREATER RISK for cardiovascular and renal disease than are women of similar ages. Men also have higher blood pressures than women (22). However, these sex differences change after menopause, when the risk for cardiovascular disease increases for women (1) and the prevalence of hypertension becomes similar in men and women (1). Reasons for the change in cardiovascular disease risk and the prevalence of hypertension after menopause are not clear.

A key system for modulating blood pressure and body fluid volume is the renin-angiotensin system (RAS). Most of the known effects of angiotensin II (ANG II) are mediated through the angiotensin type 1 (AT1) receptor, e.g., vasoconstriction, aldosterone and vasopressin release, salt and water retention, sympathetic activation, and oxidative stress. Sex differences in the RAS have been described previously for humans and animals (10, 19). For example, the spontaneously hypertensive rat (SHR) is a hypertensive model that exhibits sex differences in blood pressure similar to humans. Blood pressure is higher in male than in female SHR before 9 mo of age (16). However, after cessation of estrous cycling in females, blood pressure increases to the same level as in males (6, 15). In young adult SHR, chronic blockade of the RAS with angiotensin-converting enzyme (ACE) inhibition (ACEI) results in reductions in blood pressure to the same level, regardless of sex, thus eliminating the sex difference in blood pressure (17). These data suggest that the RAS plays an important role in the development of hypertension in SHR, regardless of sex, and that the 15- to 30-mmHg difference in blood pressure between young males and females is also mediated by the RAS. In contrast, with aging, plasma renin activity (PRA) increases in female SHR as blood pressure increases, and with aging in males, PRA decreases, such that it is similar in aged males and females (6, 15). There is evidence from other investigators that blockade of the RAS reduces blood pressure in aging male SHR (20, 21, 24), suggesting a role for the RAS in the maintenance of hypertension in these animals, but there is no information regarding the role of the RAS in the increased blood pressure in aging female SHR. We also showed previously that aging in male and female SHR is associated with oxidative stress, which impacts hypertension, because treatment with antioxidants reduces blood pressure in aging male and female SHR (6). However, there are sex differences in the depressor response to antioxidants in young and old SHR (6, 8). The role of the RAS in mediating oxidative stress in aging SHR is also not clear.

Thus the present study was performed to determine whether there was a sexual dimorphism in the role of the RAS in hypertension in aging SHR. We tested the hypothesis that the RAS would play a greater role in the hypertension of aging male than female SHR. To address this hypothesis, components of the systemic and intrarenal RAS were measured in aging male and female SHR, and the rats were given losartan, an AT1 receptor antagonist, for 3 wk to determine whether there were sex differences in the depressor response and/or oxidative stress.

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METHODS

Rats
Female and male 8-mo-old SHR (Taconic Farms, Germantown, NY) were maintained on standard rat chow (Teklad, Harlan SD, Indianapolis, IN) and tap water in an environment with a 12:12-h light-dark cycle until 16 mo of age for all studies. All protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center and were carried out in accordance with the National Institutes of Health “Guide for the Care and Use of Laboratory Animals.”

Depressor Response to Losartan
To determine whether there was a sexual dimorphism in the depressor response to the AT1 receptor antagonist losartan, aging male and female SHR (n = 7 per group) were given losartan (40 mg·kg⁻¹·day⁻¹; a generous gift from Merck Laboratories) in the drinking water for 3 wk. A dose of 30 mg·kg⁻¹·day⁻¹ has been shown to reduce blood pressure in male SHR at this age (24). To achieve comparable and complete blockade of AT1 receptors in males at this age (24), to verify similar AT1 receptor blockade in males and females (18), we chose an even higher dose, which was tested as noted below by pressor responses to ANG II bolus injections. Controls received tap water, and water consumption was monitored daily to verify that the doses were similar between males and females.

Mean Arterial Pressure in Conscious Rats

Before the end of the losartan treatment, the rats were anesthetized by isoflurane gas, and a catheter was placed in the femoral artery for blood sampling and blood pressure monitoring. The catheter was exteriorized at the back of the neck, as we previously described (18). After 4 days of recovery, mean arterial pressure (MAP) was recorded in conscious animals confined to restraining cages. Rats had been habituated to the restraining cages before catheter placement. MAP was monitored in conscious rats with a pressure transducer connected to a PowerLab computer. After a 60-min stabilization period, MAP was recorded for two 30-min periods, and the data were averaged. Blood samples for determination of plasma renin activity (PRA) were also drawn from the catheters.

Pressor Responses to ANG II Boluses After Losartan Treatment
In another set of aging SHR treated with losartan (40 mg·kg⁻¹·day⁻¹) for 3 wk, dose-response curves to ANG II were generated with bolus injections of 62.5, 125, and 250 ng/kg body wt to verify similar AT1 receptor blockade in males and females (n = 3 per group). Briefly, PE-50 catheters were placed in the femoral vein and artery, and after 4 days of recovery, MAP was measured in resting conscious rats. After 1 h of stabilization and after steady blood pressure readings were obtained, dose-response curves to ANG II boluses (or saline) were generated. The maximal change in MAP was recorded in response to the individual doses of ANG II. Data are expressed as changes in blood pressure (mmHg) in response to the bolus injections of ANG II.

Components of Systemic and Intrarenal RAS

Systemic RAS in aging rats. Male and female 16-mo-old SHR (n = 4–6 per group) were killed by rapid decapitation with a guillotine. Blood was collected and centrifuged, and plasma was frozen at −20°C for measurement of PRA and plasma angiotensinogen concentration by the Radioimmunoassay Core of the Physiology Department at the University of Mississippi and for plasma ANG II by ELISA (see below).

Real-time RT-PCR and Western blot studies. Female and male 16-mo-old SHR (n = 7 each group) were anesthetized with isoflurane, and their kidneys were perfused with 2% heparin in saline, removed, separated into cortex and medulla, and snap frozen in liquid nitrogen.

Measurement of Intrarenal AT1, Angiotensinogen, ACE, and Renin mRNA Expression

Cortical and medullary RNA was extracted with TriReagent (MRC, Cincinnati, OH), resuspended in diethyl pyrocarbonate-H₂O, treated with DNase (DNA-free kit, Ambion, Austin, TX), and quantified by spectrophotometry. Five micrograms of RNA were reverse transcribed with 0.5 μg of T₂₇VN primer and Superscript III (Invitrogen, Carlsbad, CA) in a final volume of 20 μl. The reaction was carried out for 60 min at 50°C and terminated by incubation at 75°C for 15 min. Primers for angiotensinogen, renin, ACE, and AT1 receptors were used as described by Naito and colleagues (12). Elongation factor-1 (EF-1) primers were used as controls (12). Real-time RT-PCR contained 1 μl of RT product, primers at 0.1 μM each, 0.2 mM dNTPs, SYBR Green I (1:20,000 final concentration; Molecular Probes, Eugene, OR), and 1 μl of titanium Taq DNA polymerase (Clontech, Palo Alto, CA). Amplifications were performed in a real-time thermal cycler (iCycler, Bio-Rad Laboratories, Hercules, CA). Cycling conditions were as follows: 1 min at 95°C followed by 50 cycles of 15 s at 95°C, 15 s at 67.5°C for renin, angiotensinogen, AT1 receptor, and ACE primer pairs or 60°C for EF-1, and 60 s at 72°C. Fluorescence data were collected during the elongation step. After PCR amplification, specificity of the PCR was confirmed by determination of the melting temperature of the PCR products and electrophoretic analysis in 2% agarose gels. Standard curves were obtained with serial dilutions of pooled RT samples. Results are expressed as arbitrary units and standardized against EF-1 mRNA expression.

Measurement of Kidney Angiotensinogen and AT1 Receptor Expression by Western Blot Analysis

Kidney tissue was homogenized in 8 vol of RIPA buffer and protease inhibitor cocktail (Roche). Homogenates were centrifuged at 10,000 g for 10 min at 4°C. Protein concentration was determined by the bicinchoninic acid kit (Pierce). Kidney homogenates (25 μg protein) were electrophoretically separated, and Western blots were

Table 1. Effect of losartan on body, kidney, and heart weights in 16-mo-old male and female SHR

<table>
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<tr>
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<th>n</th>
<th>Body Wt, g</th>
<th>Kidney Wt, g</th>
<th>Kidney Wt/Body Wt, ( \times 10^{-3} )</th>
<th>Heart Wt, g</th>
<th>Heart Wt/Body Wt, ( \times 10^{-3} )</th>
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<tr>
<td><strong>Females</strong></td>
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<tr>
<td>Control</td>
<td>6</td>
<td>255.6±7.5</td>
<td>1.97±0.10</td>
<td>7.71±0.90</td>
<td>1.40±0.03</td>
<td>5.50±0.09</td>
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<tr>
<td>Losartan</td>
<td>7</td>
<td>268.6±7.9</td>
<td>1.98±0.05</td>
<td>7.41±0.10</td>
<td>1.24±0.02†</td>
<td>4.62±0.09†</td>
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<tr>
<td><strong>Males</strong></td>
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<tr>
<td>Control</td>
<td>6</td>
<td>436.6±7.7*</td>
<td>3.50±0.11*</td>
<td>8.02±0.28</td>
<td>2.21±0.13*</td>
<td>5.06±0.10*</td>
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<tr>
<td>Losartan</td>
<td>7</td>
<td>405.8±17.2</td>
<td>3.31±0.06</td>
<td>8.21±0.20*</td>
<td>1.65±0.16†</td>
<td>4.06±0.23†</td>
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Values are means ± SE; n, number of spontaneously hypertensive rats (SHR). *P < 0.05 vs. females, †P < 0.05 vs. losartan.
performed as previously described (11), with the exception that polyvinylidene difluoride, instead of nitrocellulose, membranes were used. Polyclonal anti-angiotensinogen antibodies were a gift from Dr. Christoph Klett, and polyclonal anti-AT1 receptor antibodies and anti-GAPDH and -actin antibodies (loading controls) were obtained from Santa Cruz Biotechnology.

After they were washed, the polyvinylidene difluoride membranes were incubated with bovine anti-rabbit IgG or anti-mouse IgG secondary antibody conjugated with horseradish peroxidase (Amersham). Enhanced chemiluminescence (ECL System, Amersham) was used for detection. The blots were placed on X-ray film for band detection. Differences between groups were detected by densitometry scanning of experimental bands normalized to actin or GADPH bands.

**Plasma and Intrarenal ANG II Expression**

An additional set of old female and male SHR (n = 5 per group) were killed by rapid decapitation with a guillotine for ANG II measurement in kidney tissue and plasma. Briefly, blood was collected on ice with 500 mM EDTA, 125 mM phenanthroline, 1 mM PMSF, 2.0 mM pepstatin, protease inhibitor cocktail (Sigma), and 1 mM enalaprilat for plasma ANG II measurement. Kidneys were removed, decapsulated, weighed, homogenized in ice-cold methanol, and centrifuged for 30 min at 4,000 g at 4°C, and the supernatant was dried under nitrogen. Plasma and kidney ANG II were extracted by passage of the samples over solid-phase phenyl Bond-Elut columns (Varian, Palo Alto, CA), as described previously by Nishiyama and colleagues (13). The columns were activated with 90% methanol, the samples were loaded, and the columns were sequentially washed with water, hexane, and chloroform. ANG II was eluted with 90% methanol, dried under nitrogen, and resuspended in enzyme immunoassay buffer. ANG II was measured using a commercial ELISA kit (SPI-BIO, Bretonneux, France) with a lower detection limit of 0.5–1.5 pg/ml. Values are expressed as picograms of ANG II per gram of kidney tissue or picograms of ANG II per milliliter of plasma.

**Urinary Isoprostanes**

Control and losartan-treated rats were placed in metabolism cages for 24 h, and isoprostanes were measured in urine purified by affinity chromatography using a commercially available kit (8-isoprostaglandin F2α, Cayman Chemical) according to the manufacturer’s directions.

**Statistics**

Values are means ± SE. For the losartan studies, comparisons among groups, males vs. females and treated vs. control, were analyzed by two-way ANOVA. For determination of differences in systemic and intrarenal RAS components (by real-time RT-PCR and Western blots) between aging males and females, Student’s t-test was used. *P < 0.05 was considered statistically significant.

**RESULTS**

**Response to AT1 Receptor Antagonism**

At 16 mo of age, female SHR were considerably smaller than male SHR, and kidney and heart weights were lower in female than in age-matched male SHR (Table 1). MAP was similar in conscious 16-mo-old male and female SHR (Fig. 1), as we showed previously (3), and losartan treatment for 3 wk reduced MAP by ∼93 mmHg (52%) in males and ∼70 mmHg (37%) in old females.

The pressor response to bolus doses of ANG II was studied in additional groups of aging SHR to determine whether losartan blockade was similarly effective in males and females. Untreated old female SHR exhibited similar pressor responses to 62.5 and 125 ng/kg ANG II, but a reduced pressor response to 250 ng/kg ANG II (*P < 0.05) was observed compared with old males (Fig. 1B). Losartan treatment attenuated the pressor response at all doses to the same extent in males and females. Losartan treatment also reduced heart weight more in males...
than in females (Table 1). There was no effect of losartan on kidney or body weight in either group. PRA was not different in control female and male SHR, and losartan increased PRA to similar levels in both groups (Fig. 1C). Urinary isoprostanes were reduced by ~30% with losartan in old male, but not female, SHR (Fig. 2).

Characterization of Systemic RAS Components

There were no sex differences in aging SHR in systemic RAS parameters, such as plasma angiotensinogen, plasma ANG II, and PRA (Fig. 3). We included additional measurements of PRA in decapitated animals to verify that there were no sex differences, because PRA values of the control rats in the losartan study were determined from plasma obtained via catheter.

Characterization of Intrarenal RAS Components

Expression of mRNA for components of the RAS in kidneys harvested from 16-mo-old SHR was measured by real-time RT-PCR. Angiotensinogen mRNA levels were significantly higher in cortex and medulla of males than females (Fig. 4A). Renin mRNA was higher in the cortex than in the medulla of males and females, and there were no sex differences in expression (Fig. 4B). Cortical and medullary mRNA expression of ACE and AT1 receptors was also similar in male and female SHR.

Renal protein expression of angiotensinogen by Western blot analysis revealed a single band at 60 kDa, and protein expression was significantly higher in old males than females (Fig. 5A). Western blots probed with anti-AT1 receptor antibodies revealed a single band at 45 kDa, and protein expression tended to be lower in females but was not significantly different between old males and females (Fig. 5B). However, renal ANG II concentration as measured by ELISA was significantly higher in female than in male SHR (Fig. 6).

DISCUSSION

The main findings of this study are as follows. 1) There is a sexual dimorphism in the depressor response to losartan in aging SHR. This is the first study in aging female SHR that has defined a role for the RAS in maintaining their hypertension. Although we found that the RAS plays a role in blood pressure in aging male and female SHR, female SHR do not exhibit as potent a depressor response as old male SHR to AT1 receptor antagonism, suggesting that the hypertension is more dependent on the RAS in aging males than in aging females. 2) Losartan is more effective in reducing urinary expression of F2-isoprostanes in old male than female SHR. This suggests that ANG II may play a more important role in mediating oxidative stress, which impacts blood pressure, in males than in females. 3) There are sex differences in the intrarenal expression of angiotensinogen and ANG II in SHR at 16 mo of age, with significantly greater expression of angiotensinogen in males than in females, whereas intrarenal concentrations of ANG II were higher in females than in males.
Previously, our laboratory reported that ACEI abolishes the sex difference in blood pressure in young adult SHR (17), suggesting that blood pressure in young SHR is mediated by the RAS independent of their sex. However, as in the present study, the depressor response to ACEI was significantly greater in young males than females, because blood pressure was higher in males than in females and blood pressure was reduced to similar levels (17). We also found that PRA was higher in young males than females. There is evidence that androgens can stimulate the synthesis of renin and angiotensinogen in the kidneys of male SHR (2, 5), and, in our study, ACEI prevented testosterone supplements from increasing blood pressure in ovariectomized female SHR (17), suggesting that androgens may increase blood pressure in young male SHR, in part by stimulating the RAS.

With aging, PRA decreases in male and increases in female SHR, such that PRA levels are similar in male and female SHR (6, 7), as we found in the present study. The reduction in PRA in old male SHR may be due to reduced testosterone synthesis with aging (7). The mechanism by which PRA increases with aging in females is not clear. There is little evidence that lack of estradiol (as in menopause) affects renin synthesis or release, but serum testosterone levels increase slightly with age in female SHR (6), although not to the levels found in aging male SHR. Whether the increase in androgens plays a role in the increase in PRA and/or blood pressure with aging in female SHR remains to be determined and was not the focus of the present study.

Despite similar PRA and blood pressure in old male and female SHR, losartan caused a greater reduction of blood pressure in old male (to 90 mmHg) than in old female (to 130 mmHg) SHR. These data are surprising in view of the fact that intrarenal ANG II was higher in aging females and intrarenal AT1 receptor was similar in males and females. However, we also found that losartan reduced urinary isoprostane excretion in male, but not female, SHR. It is well known that ANG II can stimulate oxidative stress (14, 18), and sex differences in the depressor responses to antioxidants have been reported in young and old SHR (6, 8). For example, tempol reduces blood pressure in young and old male SHR but has no effect in young or old female SHR (6, 8). In the present study, we found that urinary isoprostane excretion was reduced in losartan-treated male, but not female, SHR. These data suggest that ANG II maintains hypertension in aging male SHR, in part by increasing oxidative stress, whereas this is not the case in females.
The sex differences in the depressor response and the oxidative stress response to losartan suggest that mechanisms other than ANG II may play a role in the hypertension of old females. In contrast to aging male SHR (20, 21), there is a paucity of data on the mechanisms for blood pressure regulation in aging female SHR. With regard to oxidative stress, aging female SHR respond with reductions in blood pressure to chronic (8 mo) treatment with vitamins E and C (6), which suggests that oxidative stress plays a role in maintaining hypertension with aging in female SHR, although the present data do not indicate that ANG II is involved in mediating the oxidative stress in aging females. We found previously that the increase in blood pressure with aging in female SHR is associated with greater expression of intrarenal endothelin (ET) than in young females (23). In addition, a specific ET type A receptor antagonist decreased blood pressure in aging female SHR, but not to normotensive levels (23), similar to the effect of losartan.

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of losartan in the present study. In contrast, there was no effect of ET type A receptor antagonism on blood pressure in young adult females. Taken together with the data from the present study, we hypothesize that the increase in blood pressure in aging female SHR after cessation of estrous cycling is mediated by ET and the RAS, whereas the RAS is the major control system of hypertension in young adult and aging male rats. ET has also been shown to cause oxidative stress (4). Therefore, it is possible that ET, rather than ANG II, may be causing the oxidative stress that impacts blood pressure in old female SHR, a hypothesis that remains to be tested.

The mechanism responsible for the higher levels of ANG II in the kidney of females than males, despite significantly lower angiotensinogen mRNA in cortex and medulla and whole kidney angiotensinogen protein, is not clear from our present studies. Our data suggest that ACE activity may be increased in females compared with males in order for ANG II to be higher in females. However, ACE mRNA expression in cortex and medulla was also not different between the groups. It is possible that there could be translational or posttranslational modifications of the ACE protein, leading to higher ACE activity in females. This hypothesis is, in part, consistent with previous studies by Gallagher and colleagues (9), who found that estradiol replacement in ovariec-tomized female normotensive rats reduced ACE activity in kidney cortex and medulla. Therefore, in aging female SHR in which estrous cycling has stopped and estrogen is reduced, one would predict that ACE activity would be increased compared with cycling rats, leading to increased ANG II levels in the kidneys. However, Gallagher and colleagues found that ACE mRNA expression was also decreased with estradiol, whereas we found no difference between males and females. Different strains and/or different ages of the rats could account for this discrepancy. Futures studies are necessary to unravel the exact mechanisms responsible for the differences.

In conclusion, in aging male and female SHR, there is a sexual dimorphism in the depressor response to losartan, with males having a greater response than females. The depressor response in males cannot be explained by differences in ANG II, because the level of renal ANG II was decreased with estradiol, whereas we found no difference between females and males. Different strains and/or different ages of the rats could account for this discrepancy. Futures studies are necessary to unravel the exact mechanisms responsible for the differences.

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