Sex Differences in Angiotensin-Converting Enzyme Modulation of Ang (1–7) Levels in Normotensive WKY Rats

Kanchan Bhatia, Margaret A. Zimmerman, and Jennifer C. Sullivan

BACKGROUND

Levels of the vasodilatory peptide angiotensin (Ang) (1–7) have been reported to be greater in females than in males, although the molecular mechanism responsible for this is unknown. Angiotensin-converting enzyme (ACE), ACE2, and neprilysin are key enzymes regulating Ang (1–7) formation. We conducted a study to determine the effect of sex on the activities of ACE, ACE2, and neprilysin in the kidneys of normotensive rats. We hypothesized that greater ACE2 or neprilysin activity in females would result in enhanced Ang (1–7) formation as compared with that in males.

METHODS

We measured the enzymatic activities of ACE, ACE2, and neprilysin in the renal cortex and medulla of 12-week-old male and female WKY rats. We treated additional rats with vehicle or enalapril (10 mg/kg/day in drinking water) for 14 days, and measured their Ang II and Ang (1–7) levels.

RESULTS

Renal cortical activity of ACE was greater in female than in male WKY rats (P < 0.05), but the activity of ACE in the renal medulla was not significantly different in the two sexes. Renal cortical and medullary ACE2 and neprilysin activities were comparable in male and female WKY rats. Treatment with enalapril significantly decreased Ang II levels in the renal cortex and medulla of male and female WKY rats as compared with those in vehicle-treated controls (P < 0.05); enalapril did not change the plasma levels of Ang II. Cortical levels of Ang (1–7) were higher in vehicle-treated females than in vehicle-treated males (P < 0.05), and treatment with enalapril decreased Ang (1–7) levels only in females (P < 0.05).

CONCLUSIONS

Our data supports a role for ACE in the formation of renal cortical Ang (1–7) in female WKY rats that is absent in males.

Keywords: blood pressure; gender; hypertension; kidney; renin-angiotensin system; WKY.

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The renin–angiotensin system (RAS) is a critical regulator of numerous physiological processes, and there are sex differences in the expression of components of the RAS. Males have greater expression of “classical” components of the RAS including Ang II and AT1 whereas females have greater expression of “non-classical” components of the RAS including AT2 and Ang (1–7). Activation of the classical RAS pathway mediates most well-known biological functions of angiotensin (Ang) II, including vasoconstriction, oxidative stress, and inflammation. Angiotensin (1–7) (Ang (1–7)) opposes most actions of Ang II, leading to vasodilation, improved renal blood flow, and enhanced pressure natriuresis. There is growing interest in the potential role of Ang (1–7) in modulating cardiovascular function under both physiological and pathophysiological conditions. We recently reported that hypertensive female rats have significantly greater Ang (1–7) levels in their renal cortex than do hypertensive males, and others report that hypertensive female congenic mRen(2) rats have higher plasma Ang (1–7) levels than do males. However, the molecular mechanisms responsible for sex differences in Ang (1–7) levels are unknown.

Angiotensin-converting enzyme (ACE), ACE2, and neprilysin are key enzymes regulating the production and catabolism of Ang II and Ang (1–7). ACE is a zinc metallo-carboxypeptidase that functions as a carboxyl-directed dipeptidase that converts Ang I to Ang II, and the intrarenal formation of Ang II is largely mediated by ACE. Although ACE contributes to the formation of Ang (1–7) via Ang II as its substrate in the human coronary circulation, ACE2 and neprilysin are thought to be the primary enzymes catalyzing the formation of Ang (1–7). ACE2 is a monocarboxypeptidase homologue of ACE that cleaves Ang I to form Ang (1–9) and, however, the main substrate of ACE2 resulting in the formation of Ang (1–7) is Ang II. Neprilysin is a zinc metallo-endopeptidase that directly metabolizes Ang I to Ang (1–7) and contributes to the formation of Ang (1–7) in mouse podocytes.

There are sex differences in the activities of these enzymes. Normotensive female MF1 mice have less renal ACE2 activity than do males, whereas normotensive female Lewis rats have greater neprilysin activity and less ACE2 activity in the renal cortex than males. In contrast, ACE2 activity in female C57BL/6 mice on a high fat diet correlates with Ang (1–7) levels to a greater degree than in males. Thus, differential activation of ACE, ACE2, and neprilysin may contribute both to the formation and to sex differences in Ang (1–7) levels.
The goal of the present study was to determine the effect of sex on the activities of ACE, ACE2, and neprilysin in the kidneys of normotensive rats. We hypothesized that female normotensive rats would have greater ACE2 or neprilysin activity than males and thus enhanced Ang (1–7) formation as compared with that in males. Interestingly, we found that female WKY rats had greater ACE activity than males, and in additional studies we determined the effect of sex differences in ACE activity on renal Ang II and Ang (1–7) levels.

METHODS

Animals

The study was done with 12-week–old male and female WKY rats (Harlan Laboratories, Indianapolis, IN). All experiments in the study were done in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved and monitored by the Georgia Health Sciences University Institutional Animal Care and Use Committee. Rats were housed in temperature- and humidity-controlled, light-cycled quarters (with a 12-hour light/12-hour dark cycle) and maintained on standard rat chow (Teklad, Harlan Laboratories). A subset of rats received enalapril (10 mg/kg/day; Sigma, St Louis, MO) in their drinking water for 14 days; enalapril-treated rats were individually housed to allow for daily measurements of their water consumption. Male WKY rats were larger than females at baseline and drank more water (Table 1). Rats were weighed every 3 days, and the dose of enalapril was adjusted accordingly, to maintain comparable dosing among all rats.

Systolic blood pressure (SBP) was measured with tail-cuff plethysmography, as previously described. Rats were trained for 3 days before their SBP data were recorded, with eight readings taken for each rat and averaged. Before dosing with enalapril was begun, rats were placed in metabolic cages for 24-hour urine collection, and weekly thereafter to assess protein excretion. Protein concentrations were determined with the standard Bradford assay (Bio-Rad, Hercules, CA), and the resulting data are expressed as relative fluorescence units normalized to protein (RFU/min/mg protein).

Enzymatic activities

The activity of ACE in the kidneys of rats was determined by measuring the hydrolysis of a Hip–His–Leu substrate to a His–Leu product, as previously described. The activity of ACE was defined as the quantity of substrate whose conversion was inhibited by captopril. The activity of ACE2 was assessed with fluorogenic peptide substrate VI ((FPSVI) 7Mca–Y–V–A–D–A–PK(Dnp)–OH (50 μM)) (R&D systems, Minneapolis, MN) as previously described. The activity of ACE2 was calculated by subtracting activity in the presence of the ACE inhibitor captopril (10 μM captopril; AnaSpec, San Jose, CA) from the activity in the presence of captopril and the ACE2 inhibitor DX 600 (1 μM DX600; AnaSpec Inc). The activity of neprilysin was determined by measuring the hydrolysis of dansyl–D–Ala–Gly–p–nitro–Phe–Gly (DAGPNG; Sigma) substrate to dansyl–D–Ala–Gly as previously described, with minor modifications. Briefly, 100 μg of homogenized tissue was incubated for 30 minutes with 50 μM DAGPNG and 0.2 mM enalapril in the presence or absence of the neprilysin inhibitor thiorphan (20 nM, Sigma). All enzymatic activities were measured with a SpectraMax M2 Fluorescence Reader (Molecular Devices, Sunnyvale, CA), and the resulting data are expressed as relative fluorescence units normalized to protein (RFU/min/mg protein).

Peptide analysis

Renal cortical and medullary tissue were used for the measurement of Ang (1–7) levels by enzyme immunoassay (EIA) after methanol extraction of 0.3 g tissue specimens according to the manufacturer’s protocol III as previously described (n = 5 or 6, n refers to the number of rats from which tissue was collected; there was 1 sample per rat assayed; Bachem, San Carlos, CA). The detection limit of the Ang (1–7) kit is 1 pg/ml. Cross-reactivities for Ang peptides in the assay are as follows: Ang (1–7), 100%; Ang I, 0%; and Ang II, 0%. Levels of Ang II were measured by EIA after the methanol extraction of tissue (0.15 g tissue) as previously described (n = 7–9; Cayman Chemicals, Ann Arbor, MI). The detection limit of the Ang II kit is 1 pg/ml and the intra- and interassay variability at 20 pg/ml Ang II is 2% and 5%, respectively. Cross-reactivities for Ang peptides are as follows: Ang II, 100%; Ang I, 4%; and Ang (1–7) < 0.01%.

Table 1. Metabolic parameters in enalapril-treated male and female WKY rats

<table>
<thead>
<tr>
<th>Time</th>
<th>Body weight (g)</th>
<th>Water intake (ml)</th>
<th>Urine output (ml/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>234 ± 2</td>
<td>24 ± 1</td>
<td>13 ± 0.4</td>
</tr>
<tr>
<td>Week 1</td>
<td>238 ± 4</td>
<td>54 ± 5*</td>
<td>40 ± 6*</td>
</tr>
<tr>
<td>Week 2</td>
<td>242 ± 3*</td>
<td>53 ± 3*</td>
<td>34 ± 5*</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>152 ± 3*</td>
<td>18 ± 2*</td>
<td>10 ± 0.6*</td>
</tr>
<tr>
<td>Week 1</td>
<td>154 ± 2</td>
<td>42 ± 5*</td>
<td>30 ± 23*</td>
</tr>
<tr>
<td>Week 2</td>
<td>162 ± 2*</td>
<td>50 ± 4*</td>
<td>40 ± 4*</td>
</tr>
</tbody>
</table>

*Significant difference from same sex at baseline.

*Significant difference from male WKY rats at baseline.
Statistical analysis

All data are presented as mean ± SEM. Basal ACE, ACE2, and neprilysin activities were analyzed with Student’s t-test. Peptide data and enzyme activities in rats given vehicle and those treated with enalapril were analyzed through two-way analysis of variance (ANOVA) followed by Bonferroni post hoc analysis, with factor 1 being the sex of the animal and factor 2 being enalapril treatment. Body weight, water intake, urine output, BP, and protein excretion data were analyzed through repeated measures ANOVA. Differences were considered statistically significant at $P < 0.05$. Statistical analyses were done with GraphPad Prism version 4.0 software (GraphPad Software, La Jolla, CA).

RESULTS

Enzymatic activity of the renal RAS

Initial studies determined the effect of sex on the activities of ACE, ACE2, and neprilysin. Renal cortical ACE activity was significantly higher in female than in male WKY rats ($P = 0.0096$) (Figure 1A). Medullary ACE activity did not differ in the two sexes ($P = 0.27$) (Figure 1B). The activities of ACE2 and neprilysin did not differ significantly in male and female WKY rats in either the renal cortex or medulla (Figures 1C–F).

To assess the role of ACE in the production of Ang II and Ang (1–7), we treated additional male and female WKY rats...
with the ACE inhibitor enalapril for 2 weeks. Treatment with enalapril at 10 mg/kg/day abolished cortical ACE activity in males and significantly decreased this activity in females (effect of sex: \( P < 0.0001 \); effect of treatment: \( P = 0.0003 \); interaction: \( P = 0.5 \)) (Table 2). Enalapril did not significantly alter medullary ACE activity (effect of sex: \( P = 0.0045 \); effect of treatment: \( P = 0.29 \); interaction: \( P = 0.17 \)) (Table 2).

Enalapril did not change cortical or medullary ACE2 or neprilysin activity (Table 2). Because female WKY rats retained ACE activity after treatment with enalapril at 10 mg/kg/day, additional rats were treated with enalapril at 20 mg/kg/day, but this higher dose did not increase the inhibition of cortical ACE activity (121 ± 31 RFU/min/mg protein vs. 108 ± 26 RFU/min/mg protein; \( P = \text{NS} \)).

### Blood pressure and proteinuria

Basal SBP was lower in female than in male WKY rats (\( P < 0.01 \)) (Figure 2A). Enalapril reduced SBP in males by 20%, but did not change SBP in females (\( P = 0.001 \)). As a result, male WKY rats had a significantly lower SBP than females after 2 weeks of treatment with enalapril (\( P = 0.001 \)).

Baseline proteinuria was greater in male than in female WKY rats (\( P = 0.001 \)) (Figure 2B). Enalapril treatment for 1 week did not change protein excretion in either sex. Two weeks of treatment with enalapril increased proteinuria in females but did not change it in males (\( P = 0.001 \)); however, protein excretion remained higher in enalapril-treated male than in female WKY (\( P < 0.0001 \)).

### Ang II and Ang (1–7) levels

To determine the relative contribution of ACE to the production of Ang II and Ang (1–7), we measured peptide levels in male and female WKY rats given vehicle and those treated with enalapril. Plasma levels of Ang II were greater in male than in female rats (235 ± 38 pg/ml vs. 80 ± 32 pg/ml, respectively), and treatment with enalapril did not significantly alter plasma levels of the enzyme in either (175 ± 45 pg/ml in males and 88 ± 29 pg/ml in females; two-way ANOVA: effect of sex, \( P = 0.0034 \); effect of enalapril, \( P = 0.4880 \); interaction, \( P = 0.560 \)). Renal cortical levels of Ang II were comparable in male and female WKY rats given vehicle (effect of sex: \( P = 0.71 \)) (Figure 3A). Enalapril decreased the cortical levels of Ang II in both males and females (effect of treatment: \( P = 0.025 \)), and the decrease was comparable in the two sexes (interaction: \( P = 0.99 \)). In contrast, renal medullary levels of Ang II were greater in male than in female rats (effect of sex: \( P = 0.0098 \)) (Figure 3B), and enalapril decreased Ang II levels in both sexes (effect of treatment: \( P = 0.006 \)). The decrease in Ang II levels was comparable in the two sexes (interaction: \( P = 0.17 \)), although higher levels

### Table 2. Renal enzymatic activities of ACE, ACE2, and neprilysin in male and female WKY rats given vehicle and enalapril

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>M+Enal</th>
<th>Female</th>
<th>F+Enal</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>95 ± 12</td>
<td>6 ± 4*</td>
<td>231 ± 45*</td>
<td>108 ± 26*</td>
</tr>
<tr>
<td>Medulla</td>
<td>67 ± 14</td>
<td>22 ± 6</td>
<td>101 ± 25</td>
<td>107 ± 17*</td>
</tr>
<tr>
<td>ACE2 activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>27 ± 4</td>
<td>20 ± 2</td>
<td>31 ± 6</td>
<td>39 ± 9</td>
</tr>
<tr>
<td>Medulla</td>
<td>24 ± 3</td>
<td>17 ± 2</td>
<td>30 ± 5</td>
<td>32 ± 8*</td>
</tr>
<tr>
<td>Neprilysin activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>31 ± 6</td>
<td>54 ± 9</td>
<td>48 ± 16</td>
<td>66 ± 13</td>
</tr>
<tr>
<td>Medulla</td>
<td>170 ± 34</td>
<td>183 ± 61</td>
<td>132 ± 38</td>
<td>119 ± 39</td>
</tr>
</tbody>
</table>

*Significant difference from same-sex control.
*Significant difference from vehicle-treated male WKY rats.
*Significant difference from enalapril-treated male WKY rats; \( P < 0.05 \) for all comparisons.

Abbreviations: ACE, angiotensin-converting enzyme; Enal, enalapril; RFU, relative fluorescence units.
Of Ang II persisted in male than in female WKY rats after treatment with enalapril.

Levels of Ang (1–7) were higher in the renal cortex of female than of male WKY rats given vehicle (effect of sex: \( P = 0.02 \)) (Figure 3C). Enalapril decreased Ang (1–7) levels only in females (effect of treatment: \( P = 0.45 \); interaction: \( P = 0.041 \)), abolishing the sex difference. Basal medullary levels of Ang (1–7) were comparable in male and female WKY rats (effect of sex: \( P = 0.29 \)), and enalapril increased these levels (effect of treatment: \( P = 0.039 \); interaction: \( P = 0.49 \)) (Figure 3D).

**DISCUSSION**

There is growing interest in the role of Ang (1–7) in modulating cardiovascular function, although the molecular mechanisms regulating differential Ang (1–7) levels in males and females are still being investigated. The primary novel finding of the present study is that female WKY rats have greater renal cortical ACE activity and greater Ang (1–7) levels than do males. After treatment with enalapril, the sex difference in renal cortical Ang (1–7) levels was abolished. In female WKY rats, this post-treatment decrease in Ang (1–7) is unlikely to have been due to sex differences in the renal uptake of Ang II, because plasma levels of Ang II were unchanged by enalapril, and renal cortical levels of Ang II did not differ in male and female rats. These data support a key role for ACE in regulating renal cortical Ang (1–7) levels in normotensive females.

ACE, ACE2, and nephrilysin are critical determinants of the relative abundance of Ang II and Ang (1–7). All three enzymes can cleave Ang I to create angiotensin peptides, including Ang II and Ang (1–7). The metabolism of Ang II is most efficiently catalyzed by ACE2, resulting in the formation of Ang (1–7). This reaction has the highest catalytic efficiency of any reaction in which an angiotensin peptide is generated by the activity of ACE, ACE2, or nephrilysin, and because of this, Ang II is the major source of Ang (1–7) in the kidney. On the basis of this, and our previous study indicating that hypertensive female rats have greater levels of Ang (1–7) than do males, we hypothesized that female WKY rats would have greater ACE2 or nephrilysin activity, driving greater generation of Ang (1–7). Instead, we found in the present study that basal ACE activity was significantly greater in the renal cortex of normotensive female than of normotensive male WKY rats. The sex difference in ACE activity was unexpected in normotensive rats because greater levels of ACE activity have typically been associated with dysregulation of the RAS and increases in BP, yet female WKY rats had lower SBPs than did males. However, most of the studies
that have linked ACE activity with increases in BP have been done with male animals.25,26,27

There is controversy in the literature about the effect of sex on enzymes of the RAS. In accord with our results, normotensive female Lewis rats tend to have greater renal Ang (1–7) levels and greater renal cortical ACE activity than do males, yet when compared in a two-way ANOVA with hypertensive mRen(2). Lewis rats, these differences did not reach statistical significance.7 In contrast to our results, female Lewis rats also had greater cortical nephrilysin activity and less ACE2 activity than did males.7 Female Sprague-Dawley rats have greater renal expression of ACE2 than do age-matched males,28 although ACE2 activity is greater in the kidneys of normotensive male than of female mice.15 Therefore, ACE2 enzyme activity is likely to depend not only on sex but also on BP status, strain, and species.

Although both ACE2 and nephrilysin are likely to contribute to Ang (1–7) formation in the kidneys of male and female WKY rats, the sex difference in these animals’ renal cortical Ang (1–7) levels was paralleled by a sex difference in ACE activity. To gain insight into the pathway by which ACE modulates Ang (1–7), we treated WKY rats with enalapril. The primary enzymatic pathways by which ACE can affect Ang (1–7) is shown as a schematic diagram in Figure 4. The primary pathway driving Ang (1–7) formation in the kidney is the ACE2-catalyzed conversion of Ang II,21,22 which depends indirectly on ACE. However, we did not detect sex differences in Ang II levels or ACE2 activity in the renal cortex.

Therefore, the sex difference in Ang (1–7) is not likely to be mediated by this pathway. Ang I can also be metabolized to Ang (1–9) by ACE2, and ACE catalyzes the formation of Ang (1–7) from Ang (1–9).29 Measurements of Ang (1–9) in the plasma of rats and humans indicate that Ang (1–9) levels are twice those of Ang II,30,31 and we therefore hypothesize that female WKY rats have greater Ang (1–9) levels than do males, leading to greater ACE-mediated formation of Ang (1–7) in females. Future studies are planned to test this hypothesis.

Because of the limited number of studies that have directly measured both the activities of RAS enzymes and Ang peptides in the same experimental animals, it is difficult to know how ACE, ACE2, and nephrilysin affect the relative balance of Ang II and Ang (1–7). More studies are needed to better define the role of each enzyme. The current study did not assess the localization of ACE or co-localization of other RAS enzymes with substrate. Although ACE is predominantly expressed in the proximal tubule brush border and endothelial cells of both renal blood vessels and glomerular capillaries,19 we are not aware of reports of sex differences in the renal localization of ACE. The local enzyme and substrate environment in the renal cortex of female WKY rats may uniquely favor the formation of Ang (1–7).

ACE is essential to the control of BP and kidney function. In accord with reports from other laboratories,32 we found that female WKY rats had a lower basal BP and less proteinuria than did males, and that treatment with enalapril reduced SBP in male WKY rats.33 In the present study, enalapril did not alter SBP in female WKY rats as measured with tail-cuff plethysmography. However, the use of tail cuff plethysmography was a limitation of the study, in that telemetry may have detected an effect of enalapril on mean arterial pressure in female rats. Irrespective of this, our results imply a greater role for ACE in the control of basal SBP in normotensive males than in females. This is consistent with the genetic linkage of the ACE locus to hypertension and diastolic BP in men but not in women.34 In addition, a comparison of BP in heterozygous male and female ACE knockout mice found that only male mice had BPs that were below normal.23

There is controversy in the literature about the effect of sex on the efficacy of ACE inhibitors. Although basal plasma ACE activity has been reported to be lower in young, healthy women than in comparable men, both sexes have also been shown to be equally sensitive to ACE inhibition.35,36 However, little is known about the relative effect of enalapril on tissue ACE activity in either sex. In the present study, enalapril abolished renal cortical ACE activity and decreased renal medullary ACE activity in male WKY rats, but only blunted renal cortical ACE activity in females and had no effect on their medullary ACE activity. Yet despite the more effective decrease by enalapril of renal ACE activity in male than in female WKY rats, the percent decrease in renal Ang II levels was comparable in the two sexes. These data suggest that there was no functional difference in the sensitivity of male and female WKY rats to ACE inhibition. Additionally, enalapril decreased Ang (1–7) levels in female WKY rats, further supporting its efficacious inhibition of ACE. The absence of significant residual ACE activity in the kidneys of female WKY rats after treatment with enalapril suggests the presence of a unique metalloproteinase enzyme capable of producing the same enzymatic cleavage as produced by ACE in female WKY rats. A potential candidate for this unique metalloproteinase enzyme is meprin A, a metalloendopeptidase found in renal proximal tubules that colocalizes with ACE. However, male mice have been reported to have a greater content of active meprin protein in their kidneys than females.37-39 Although the identity of the metalloproteinase enzyme that acts analogously to ACE is currently unknown, it is unlikely to be significantly related to renal Ang II metabolism in female WKY rats because Ang peptide levels are effectively decreased by enalapril.

In summary, the present study demonstrates that there are sex differences in ACE activity and Ang (1–7) levels in

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**Figure 4.** Schematic pathway depicting potential role of ACE in the formation of Ang (1–7).
the renal cortex of normotensive rats, with females having greater ACE activity and Ang (1–7) levels than do males. Inhibition of ACE significantly decreased Ang (1–7) formation only in female WKY rats, abolishing the observed sex difference. These data are consistent with the expanding body of literature documenting the sex differences in the expression, activity, and physiological roles of components of the RAS. On the basis of the central role of the RAS in regulating normal physiological function, our work underscores the importance of better defining the RAS in both sexes under both physiological and pathological conditions.

ACKNOWLEDGMENT

K Bhatia and M Zimmerman contributed equally as first authors.

DISCLOSURE

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REFERENCES


