Sex Differences in the Pressor Response to Angiotensin II
When the Endogenous Renin-Angiotensin System Is Blocked

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Abstract—The present study determined whether there are sex differences in the pressor response to angiotensin II (Ang II) when the endogenous renin-angiotensin system (RAS) is blocked by enalapril (ACEI), and whether this pressor response is changed in the presence of high salt (HS). Telemetry BP was measured in rats treated with ACEI (250 mg/L drinking water) (n=6 to 7/grp), or with ACEI and Ang II (150 ng/kg/min, sc; n=5 to 6/grp), for 3 wk. For the last 2 wk of the study, rats received HS (4% NaCl). MAP was lower in females during baseline (100.8±1.1 versus 105.2±1.3; P<0.05), and with ACEI the last 3 days on normal salt diet (78.8±1.2 versus 88.5±0.9; P<0.05), but increased to higher levels than in males on day 6 of Ang II (129.0±2.2 versus 117.3±2.9; P<0.05). One week of Ang II increased albuminuria in males, but not females, and urinary 8-iso-PGF2α (F2-isoP) was not increased in either males or females. MAP was salt-sensitive in both sexes receiving ACEI, but was only salt-sensitive in males with Ang II (129.3±3.7 versus 145.1±5.7; P<0.05). Albuminuria continued to increase with HS and Ang II in males, but not in females. F2-isoP excretion increased with MAP during the last week of HS and Ang II in males but was independent of MAP in females. With ACEI, MAP in females on normal salt is more responsive to Ang II but is independent of oxidative stress or renal injury. MAP in males is salt-sensitive with Ang II, which may be mediated by oxidative stress and renal injury. (Hypertension. 2008;51:1170-1176.)

Key Words: renin-angiotensin system ■ salt sensitivity ■ sexual dimorphism ■ blood pressure

Men are at higher risk for cardiovascular disease (CVD) than are women. Normotensive men typically exhibit higher blood pressure (BP) than age-matched premenopausal women.1 Also, the incidence and severity of hypertension is greater in men than in women.2 Men also exhibit greater renal injury and progress to end stage renal failure at a more rapid rate than do women.3 Although the renin-angiotensin system (RAS) has been shown to play an important role in control of BP, whether the RAS plays a role in the sexual dimorphism of BP and renal injury is not clear.

There is evidence to suggest that components of the endogenous RAS may be different in males and females. For example, in humans, Miller and colleagues found that infusion of angiotensin II (Ang II) had similar effects on BP in men and women receiving controlled protein and sodium diet.4 However, these investigators showed in another study that women had a more rapid depressor response to irbesartan, an AT1 receptor antagonist, than did men.5 Zapater and colleagues reported that in normotensive women BP was lower than men regardless of the presence or absence of maximum angiotensin I converting enzyme inhibition (ACEI) with enalaprilat.6 In mice and rats, several investigators have addressed whether there were sex differences in the pressor response to Ang II infusion in the presence of a functional RAS. They found that Ang II caused greater increases in BP in normotensive males than in females.7–9 We and others have shown that sex steroids can modulate expression and activity of the various components of the RAS in the kidney and other tissues.10,11 Therefore, one might predict that there may be sex differences in the endogenous baseline RAS in normotensive animals and humans that could alter the BP responses to Ang II or inhibitors of the RAS.

The present studies were designed to address the following questions using normotensive Sprague-Dawley rats: (1) are there sex differences in the depressor responses to RAS blockade with the ACEI, enalapril; (2) are there sex differences in the pressor response to ACEI+Ang II; (3) does high-salt diet alter the response to ACEI or ACEI+Ang II in males and females; and (4) are there sex differences in renal injury or oxidative stress in rats given ACEI or ACEI+Ang II on normal or high salt diet?

Methods

Experimental Protocol

Male and female Sprague-Dawley rats, 11 weeks of age (Harlan; Indianapolis, Ind) were maintained on tap water and standard chow.
(Teklad; Harlan, sodium chloride content 0.4%) ad libitum and 12-hour light/dark cycle. The protocols complied with the Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee of UMMC.

Placement of Radiotelemeters
BP was measured by radiofrequency transmitters implanted in the abdominal aorta (TA11PA-C60, Transoma Medical) under isoflurane. Mean arterial pressure (MAP) was obtained during 10-second sampling periods every 5 minutes and are expressed as 24-hour averages. Rats were allowed to recover from telemeter implantation for 2 weeks before BP measurement.

Experiment 1: Sex Differences in Responses to ACEI, Ang II+ACEI Plus/Minus High-Salt Diet
Beginning on day 1, baseline BPs were recorded for 4 days in male and female rats on normal salt (0.4% NaCl) diet. Food intake was measured daily throughout the study. On day 5 of BP recording, all rats began receiving enalapril (250 mg/L drinking water, Sigma). In pilot studies we verified this dose of enalapril was equally effective in blocking conversion of Ang I to Ang II in both males and females.

On day 9 of BP recording, rats were divided into groups that would either continue on ACEI+Ang II (150 ng/kg/min s.c. via osmotic minipump (Alzet 2ML4), (ACEI+Ang II males/females, n=5 to 6/group) or ACEI+vehicle (0.9% saline), (ACEI males/females, n=6 to 7/group) for 3 weeks. On day 15, rats were placed in metabolism cages for 24 h for measurement of albumin excretion (an index of renal injury) and 8-iso-prostaglandin (PG) F2α (F2-isoprostane, an index of oxidative stress).

On day 16, all rats were started on 4% salt diet (Harlan SD) that continued until the end of the study (day 29). Rats were placed in metabolism cages again on days 22 and 29 for determination of urinary excretion of albumin and F-2isoprostanes. At the end of the protocol, rats were euthanized to obtain blood (for plasma renin activity, estradiol, and testosterone) and kidney tissue collection.

Experiment 2: Sex Differences in Pressor Response to Ang II
Based on our findings in Experiment 1 that ACEI+Ang II infusion resulted in sex differences in BP by the end of the first week of Ang II, with females having higher BP than males, we determined whether this sex difference was sustained past 1 wk when rats were maintained on normal salt diet. Male and female rats (n=6/group) were implanted with chronic aortic catheters connected to swivels for the BP measurement, as previously described. Femoral venous catheters were implanted for the infusion of Ang II (10 ng/kg/min IV) or vehicle (0.9% saline). All rats were given enalapril (250 mg/L in drinking water) beginning on the day of catheter implantation which was continued throughout the study. On day 7 after catheter implantation, Ang II (or saline) was begun and given for 14 days. MAP was measured continuously 24 h per day starting on the first day of Ang II infusion using a computerized data acquisition system.

Plasma Sex Steroids
Plasma testosterone and estradiol were measured, as previously described (Count-A-Count total testosterone kit, Diagnostic Products Corporation; Ultrasensitive estradiol kit, Diagnostic Systems Laboratories Inc).

Plasma Renin Activity
Plasma renin activity (PRA) was measured in plasma taken on day 29 of the study by radioimmunoassay (RIA), as previously described.

Urinary Albumin Excretion
Urinary albumin was measured by ELISA using a sheep polyclonal antibody raised against rat albumin. Assay sensitivity was 30 ng/mL for rat albumin.

Measurement of Urinary F2-Isoprostanes
8-iso-PGF2α (F2-isoprostanes) were measured with a kit (EA 85 Oxford Biomedical Research), as previously described by us. Data for days 15, 22, and 29 of the study are expressed as F2-isoprostanes in ng per day. Creatinine was measured in urine on day 29, using a kit (Oxford Biomedical Research, CR01). F2-isoprostanes are expressed as ng F2-isoprostanes/mg urinary creatinine.

Statistical Analyses
Data are expressed as mean±SEM. Comparisons for multigroup and multifactorial analyses were evaluated with 2-way ANOVA and Student-Newman-Keuls method for multiple comparisons. Differences in baseline BPs in the absence or presence of ACEI between males and females were determined by Student t test. Significance was defined at P<0.05.

Results
Experiment 1: Sex Differences in Response to Ang II and Salt Sensitivity
Body weights were higher in males, regardless of treatment group (day 1: ACEI alone, males versus females: 391.7±14.4 versus 261.1±10.7 g, P<0.0001; ACEI+Ang II, males versus females: 404.6±14.7 versus 249.9±5.2 g, P<0.0001 males versus females; NS compared with ACEI alone group). By the end of the study on day 29, males had gained more weight than females, but there was no difference in weight gains among groups (day 29, weight gain, ACEI alone, males: 7.2±1.6 g, P<0.001; ACEI+Ang II, males: 22.9±4.2 g, P<0.001 versus females: 48.3±2.4 versus 24.3±7.2 g, P<0.001, males versus females; NS, males/females + ACEI versus males/females ACEI+Ang II).

As shown in Figure 1, during baseline period (days 1 to 4, normal salt diet, no ACEI, no Ang II), MAP was significantly higher in males than females. ACEI, started on day 5, reduced MAP in males and females and reached a nadir by day 12. MAP at that time (days 12 through 14) was reduced by 22.9±0.8% in females with ACEI compared to baseline, but only by 16.3±1.2% in males with ACEI.

On day 9, Ang II infusion was started with ACEI in some rats (ACEI+Ang II males/females), whereas others (ACEI males/females) continued to receive ACEI alone. Ang II increased MAP in males and females during the early days of treatment, but by day 14 (6 days of Ang II), MAP was higher in ACEI+Ang II females than ACEI+Ang II males (Figure 1) with a greater change in MAP in females than males. Urinary albumin on day 15 was similar in males and females treated with ACEI alone, and Ang II infusion increased albuminuria in ACEI+Ang II males, but not females (Figure 2). F2-isoprostanes on day 15 were not different between the sexes in ACEI or ACEI+Ang II groups (Figure 3).

On day 16 (day 8 of Ang II infusion), high salt was started. MAP increased by the same percentage (9% to 10%) in males and females with ACEI alone during the first week of high salt, reaching a peak by day 20. In rats with ACEI+Ang II,
high salt increased MAP progressively in males to the level of MAP in females by day 18 and exceeded MAP in females by day 23. High salt had no effect on MAP in females treated with Ang II (Figure 1). On day 22, albumin excretion was further increased in ACEI+Ang II males, but not in ACEI+Ang II females (Figure 2). F2-isoprostanes on day 22 were not affected by 1 week of high salt in either ACEI or ACEI+Ang II males, but were increased in females of both groups (NS, females ACEI versus females ACEI+Ang II) (Figure 3).

By day 23, ACEI+Ang II males on high salt had significantly higher MAP than females (Figure 1). On day 29, after 3 wk of Ang II and 2 wk of high salt, urinary excretion of albumin was further increased in ACEI+Ang II males, but not females (Figure 2). F2-isoprostane excretion on day 29 was not changed in either ACEI or ACEI+Ang II females compared to day 22, but was increased in ACEI and ACEI+Ang II males to the levels found in females (Figure 3). When factored for urinary creatinine, F2-isoprostane levels were similar in ACEI and ACEI+Ang II females on high salt and were significantly higher than in ACEI or ACEI+Ang II males.

Other Factors That Could Affect Pressor Response to Ang II and High-Salt Diet

Sex Steroids

Plasma testosterone in males and estradiol levels in females were measured at the end of the study (day 29) and were not affected by ACEI+Ang II+high salt compared with ACEI+high salt (testosterone: 189+84 versus 144+61 ng/dL, male control versus male Ang II, respectively, p=NS; estradiol: 15.3+1.4 versus 16.9+1.5 pg/mL, female control versus female Ang II, respectively, P=NS).

Food Intake

We measured food intake daily because there was a possibility that males would eat more than females, and this could result in increased salt intake leading to a greater BP in males than females. However, ACEI+Ang II females ate more food per day when factored by body weight (females ACEI+Ang II, 70.0±1.2 g/kg, P<0.05 versus females ACEI, 62.8±2.0; versus males ACEI, 53.4±0.7, and versus males ACEI+Ang II, 54.9±0.9 g/kg BW).
PRA
PRA on day 29 was not different between males and females receiving ACEI and high-salt diet or ACEI, Ang II, and high-salt diet. Data are expressed as ng AI/mL/hr. *P<0.05, compared to rats receiving ACEI alone.

Effect of Ang II on Renal Cortical Expression of Angiotensinogen
Angiotensinogen expression was similar in kidneys of male and female ACEI rats on high salt (Figure 5). However, with ACEI/Ang II and high salt, angiotensinogen expression was increased only in males.

Experiment 2: Sex Differences in the BP Response to Ang II
Based on the above findings that there was a sex difference in BP response to ACEI/Ang II with females having a greater response than males on normal salt diet (0.4% NaCl), we performed another study in which we gave Ang II with ACEI and normal salt for 14 days to determine whether the greater increase in MAP in females was sustained beyond 1 wk. Lower doses of Ang II were used in this study because it was given intravenously as opposed to subcutaneously (by minipump), as in the previous study. As shown in Figure 6 and as we found in the previous study, ACEI reduced MAP to a lower level in ACEI females than males. In addition, by the end of week 1 of Ang II, there was a greater increase in BP in ACEI/Ang II females than males that was sustained throughout the second week of Ang II.

Discussion
Investigators showed previously that there is a sex difference in BP response to Ang II.7–9 However, in those studies the endogenous RAS was not blocked, thus leaving open the possibility that the sex differences in response to Ang II were attributable to differences in the endogenous RAS. In the present study, the major findings were as follows: (1) there is a sex difference in the BP in normotensive rats, males having higher BP; (2) females are more responsive to the depressor actions of ACEI, than males; (3) in rats receiving Ang II/Ang II, a sex difference appears by the end of 1 wk with females having higher BP; (4) in rats treated with ACEI alone, salt sensitivity of BP was seen in both males and females with similar increases in BP; however, in the presence of ACEI and Ang II, BP in females was not salt-sensitive whereas BP in males is increased with high salt; thus females are salt sensitive only when the endogenous RAS is blocked; (5) females are protected from renal injury when given Ang II and high salt, whereas the increase in renal injury in males may play a role in exacerbation of hypertension with Ang II and high salt; (6) oxidative stress, as measured by F2-isoprostanes, was independent of BP in females, but may contribute to the higher BP in males treated with high salt and Ang II; (7) angiotensinogen expression increases in cortex of kidneys of male rats treated with Ang II and high salt, but not females.

To our knowledge this is the first study showing conclusively that BP is higher in normotensive male rats than females. These data are consistent with previous data using ambulatory BP monitoring in men and women showing a sex...
difference in BP.\textsuperscript{1} The mechanisms are not clear as to why there is a sex difference in BP in normotensive humans or rats. We also found that the depressor response to ACEI was greater in normotensive females than males (shown in Figure 1). In the second study shown in Figure 6, the BP was not measured during the baseline (ie, no ACEI), thus we cannot determine whether there was a sex difference in the BP response to ACEI, although there was a sex difference in BP when rats were given ACEI with females having a lower BP as in the first study. These data are consistent with our findings that Ang II increased BP to a higher level in females than in males on normal salt. In rat models of Ang II hypertension, such as SHR, males have higher BP than females. In addition, when the endogenous Ang II is not blocked with ACEI, males are more responsive to Ang II infusion than are females.\textsuperscript{7–9} However, these sex differences are likely attributable to baseline differences in some components of the RAS upstream of Ang II receptors, a variable that was removed from our study with ACEI. Sex steroids modulate expression and activities of plasma and tissue levels of many of the components of the RAS. Therefore, changes in any of these components could affect the pressor response to Ang II. In our present study, because we gave ACEI, we eliminated any sex differences in some of the most important parameters of the RAS, such as renin activity, angiotensinogen expression, ACE activity, and also eliminated any sex differences in the efficiency of the downregulation of endogenous renin by Ang II. It is possible that there could still be sex differences in the downstream mediators of Ang II activity, such as AT\textsubscript{1} and AT\textsubscript{2} receptors and intracellular signaling cascades that could impact BP. It is possible that AT\textsubscript{1} receptor expression could have been upregulated in response to ACEI and lack of endogenous Ang II to a greater extent in females, than males, and thus when Ang II was given, females had a greater BP response. However, estradiol downregulates AT\textsubscript{1} receptor expression.\textsuperscript{16,17} Our data suggest that perhaps the effect of ACEI on AT\textsubscript{1} receptor expression could be stronger than the estradiol effect. Further investigation is necessary to determine what RAS components are important in the sex differences in the pressor responses to Ang II.

In the present study we found that BP in both males and females given ACEI alone increased by 9% to 10% with high-salt diet, thus BP was salt sensitive in both males and females receiving ACEI.

However, in rats that received high-salt diet and ACEI+ Ang II, BP increased in males only. Sex steroids have been shown previously to mediate salt sensitivity. For example, in women, BP becomes more salt-sensitive after menopause,\textsuperscript{18} suggesting that estradiol may protect against salt sensitivity. Males also exhibit greater salt sensitivity of hypertension than females in Dahl salt sensitive rats\textsuperscript{19} and rats given deoxycorticosterone acetate (DOCA) and salt.\textsuperscript{20} In our present study, females were not protected against salt sensitivity of BP when endogenous Ang II levels were low because of ACEI, but were protected when Ang II levels were elevated by infusion.

Hypertension causes a reduction in sex steroids in some rat models. In the present study, Ang II had no effect on plasma estradiol in females or testosterone in males. Xue and colleagues recently reported that Ang II infusion increased BP more in male than female mice,\textsuperscript{9} and that estradiol receptor alpha (ER\textalpha) played a role in the lower BP in females.\textsuperscript{21} However, in our study, in the presence of ACEI alone, females on normal salt diet exhibited higher BP than did males. One could hypothesize that there are sex differences in the endogenous RAS mediated by ER\textalpha that are blocked or inactivated when rats are given ACEI. Future studies will need to be done to determine whether this hypothesis is true.

Low doses of Ang II did not increase excretion of F2-isoprostanes in either males or females. Thus, the increase in BP in response to Ang II in females was not mediated by oxidative stress. In contrast, high salt increased urinary F2-isoprostanes in both ACEI and ACEI+ Ang II-treated females but had no effect on their BP. Thus changes in F2-isoprostanes were independent of BP in females. These data are consistent with our other studies showing that BP in female SHR is resistant to tempol, apocynin, and molsidomine, compared to males who exhibit BP responses to these drugs.\textsuperscript{22} These data are also consistent with those of other investigators who found that Ang II infusion in females failed to increase oxidative stress as measured by increases in NADPH oxidase activity\textsuperscript{7} or expression of p67\textsuperscript{phox}, a subunit of NADPH oxidase.\textsuperscript{8} We anticipate that the inclusion of high salt is the mechanism for the higher F2-isoprostane excretion in females, because both ACEI- and ACEI+ Ang II-treated females had similar increases in F2-isoprostanes. Kitiyakara and colleagues reported that high salt alone increased F2-isoprostanes in rats.\textsuperscript{23} We have no explanation as to why females may be protected from oxidative stress–induced increases in BP, despite similar or even higher levels of F2-isoprostanes than in males.\textsuperscript{22} In males the BP was higher on day 22 compared to day 15, yet F2-isoprostanes had not changed. The BP increased significantly in the following week. These data suggest that increases in renal injury, shown by increases in albuminuria, was the driving force for the increased BP between days 15 and 22, and that oxidative stress made a contribution to the BP in males in the days from 22 to 29.

In males, one week of Ang II had no effect on urinary excretion of F2-isoprostanes compared with ACEI alone. On high salt, F2-isoprostanes in males were increased at the end of the third week of Ang II and was consistent with the time course for the elevated pressor response to Ang II and high salt. These data are consistent with our previous studies showing 2 weeks of Ang II increased plasma F2-isoprostanes in males.\textsuperscript{12} The data are somewhat inconsistent with studies in rats in which short term (4 days) Ang II increased oxidative stress in males.\textsuperscript{5,24} However, these studies were performed with higher doses of Ang II (0.7 mg/kg per day), and thus the discrepancy between this study and our present results could be attributable to differences in Ang II dosage.

Concomitant with the increase in BP with Ang II in males was the increase in albuminuria. In rats with ACEI alone, albumin excretion was similar between males and females throughout the experimental period. In the first week of Ang II infusion, when rats were maintained on normal salt,
albumin excretion was similar in males and females, yet BP was higher in females, thus the increase in BP in females was independent of renal injury. By the end of the second week of Ang II, when rats were also receiving high salt, BP remained similar between males and females, but males excreted more albumin. During week 3, males exhibited higher BP and further increases in albumin, whereas BP in females plateaued and albumin excretion did not change. Thus renal injury may play a role in the higher BP in males on high salt and Ang II, because the increase in albuminuria preceded the increase in BP in males. It is well known that an increase in renal injury, ie, loss of filtering glomeruli attributable to damage, increases BP because of the reduction in GFR which causes an increase in sodium reabsorption in the proximal tubule. BP at day 29 is indeed higher in males than on day 22. Because we have not measured renal function, we cannot comment on sex differences in intraglomerular pressure in response to Ang II. Miller and colleagues did report that Ang II caused an increase in filtration fraction in women, but not men.5 Filtration fraction differences are often used as a surrogate of glomerular capillary pressure. One could speculate that Ang II may impact efferent arteriolar resistance more in females than afferent arteriolar resistance because of the estrogen-mediated increase in NO that would preferentially affect afferent resistance, leading to an increase in glomerular capillary pressure.

Men and male animals, whether normotensive or hypertensive, are more susceptible to renal injury than females.3 Additionally, the renal response to Ang II in sodium-replete women is different than men, with women exhibiting a blunted increase in intraglomerular pressure than men; and therefore, less renal damage.4

Kobori and colleagues showed that angiotensinogen expression is increased in kidney with Ang II infusion.25 This is not a possibility that could account for the sex differences in the response to Ang II in our study because we blocked endogenous Ang II production with ACEI. However, if there are sex differences in angiotensinogen expression in response to Ang II, it could explain why when endogenous production of Ang II is not blocked as in other studies of sex differences in Ang II infusion,7–9 BP in males is more responsive to Ang II than females. In fact, in our present studies we found that males given Ang II did indeed have a greater upregulation of intrarenal angiotensinogen expression than did females. When we measured PRA, renin was elevated in rats receiving ACEI alone, because of lack of negative feedback from endogenous Ang II. With Ang II + ACEI + high salt, PRA was very low because of negative feedback on renin of both exogenous Ang II and high salt. Neither of these changes in renin could have affected our studies. However, any sex difference in PRA when the endogenous Ang II is not blocked could play a role in the higher BP response to exogenous Ang II. Because renin in rats does not work at Vmax, an increase in substrate (angiotensinogen) will increase renin enzyme activity leading to an increase in endogenous Ang II. So in males, the fact that angiotensinogen is increased in the presence of Ang II infusion, suggests that when the endogenous RAS is not blocked, as in the studies of Drs Hay, Touyz, or Martin,7–9 there is a higher level of Ang II developed in males than females (endogenous + infused Ang II) which could be responsible for the greater BP response they found in males. Ang II infusion should reduce renin release, but it is not clear whether there are sex differences in the negative feedback response of endogenous Ang II on renin. Sex differences in negative feedback of Ang II on renin release could account in part for the increased responsiveness of males to Ang II when the endogenous production of Ang II is not blocked. In addition, because we do not have a baseline PRA level, we do not know whether renin was different in our study in males and females before Ang II was given. It is also not clear whether PRA adequately represents intrarenal tissue renin, and thus there could be sex differences in intrarenal renin that could impact the BP is the endogenous Ang II production is not blocked.

Perspectives
In this study we evaluated the basic response to Ang II infusion in males and females when Ang II is clamped at low (with ACEI) or high (Ang II infusion) levels. These data suggest that BP in women may be more responsive under conditions in which Ang II is increased, such as low-salt diet, low volume states, congestive heart failure, cirrhosis, renovascular hypertension, or hemorrhage. The data also suggest that men may be more salt sensitive when levels of Ang II are elevated.

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Disclosures
None.

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