Contribution of Endothelin A Receptors in Endothelin 1–Dependent Natriuresis in Female Rats

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Abstract—Renal medullary endothelin B receptors contribute to blood pressure regulation by facilitating salt excretion. Premenopausal females have relatively less hypertension than males; therefore, we examined whether there is a sex difference in the natriuretic response to renal medullary infusion of endothelin peptides in the rat. All of the experiments were conducted in anesthetized wild-type (wt) or endothelin B–deficient (sl/sl) rats. Infusion of endothelin 1 (ET-1) significantly increased sodium excretion (UNaV) in female, but not male, wt rats (ΔUNaV: 0.41±0.07 versus −0.04±0.06 μmol/min, respectively). The endothelin B receptor agonist sarafotoxin 6c produced similar increases in UNaV in both male (Δ0.58±0.15 μmol/min) and female (Δ0.67±0.18 μmol/min) wt rats. Surprisingly, ET-1 markedly increased UNaV in female (Δ0.70±0.11 μmol/min) but not male sl/sl rats (Δ0.00±0.05 μmol/min). ET-1 had no effect on medullary blood flow in females, although medullary blood flow was significantly reduced to a similar extent in males of both strains. These results suggest that the lack of a natriuretic response to ET-1 in male rats is because of reductions in medullary blood flow. Treatment with ABT-627, an endothelin A receptor antagonist, or L-arginine, an NO synthase 1 inhibitor, prevented the increase in UNaV observed in female rats. Gonadectomy eliminated the sex difference in the UNaV and medullary blood flow response to ET-1. These findings demonstrate that there is no sex difference in endothelin B–dependent natriuresis, and the endothelin A receptor contributes to ET-1–dependent natriuresis in female rats, an effect that requires NO synthase 1. These findings provide a possible mechanism for why premenopausal women are more resistant to salt-dependent hypertension. (Hypertension. 2009;53[part 2]:00-00.)

Key Words: sex ■ endothelin 1 ■ sodium excretion ■ ETA ■ ETB ■ medullary blood flow

Both animal and human studies have shown that the premenopausal female is relatively protected against the development of hypertension.1,2 Experiments in normal Sprague-Dawley or spontaneously hypertensive rats have shown that the pressure-natriuresis relationship of females is shifted toward lower blood pressures for a given level of sodium intake compared with males, suggesting that females have a greater defense system against salt retention.3–5 Kawanishi et al6 reported recently that female rats were relatively resistant to hypertension produced by desoxycorticosterone acetate-salt treatment compared with male rats. Surprisingly, ET-1 markedly increased UNaV in female, but not male, wt rats (Δ0.58±0.15 μmol/min) and female (Δ0.67±0.18 μmol/min) wt rats. Surprisingly, ET-1 markedly increased UNaV in female (Δ0.70±0.11 μmol/min) but not male sl/sl rats (Δ0.00±0.05 μmol/min). ET-1 had no effect on medullary blood flow in females, although medullary blood flow was significantly reduced to a similar extent in males of both strains. These results suggest that the lack of a natriuretic response to ET-1 in male rats is because of reductions in medullary blood flow. Treatment with ABT-627, an endothelin A receptor antagonist, or L-arginine, an NO synthase 1 inhibitor, prevented the increase in UNaV observed in female rats. Gonadectomy eliminated the sex difference in the UNaV and medullary blood flow response to ET-1. These findings demonstrate that there is no sex difference in endothelin B–dependent natriuresis, and the endothelin A receptor contributes to ET-1–dependent natriuresis in female rats, an effect that requires NO synthase 1. These findings provide a possible mechanism for why premenopausal women are more resistant to salt-dependent hypertension.

The first aim of the current study was to test the hypothesis that female rats have facilitated ET-B-dependent natriuretic activity to prevent salt retention compared with male rats. In contrast to the ET-B receptor, the physiological significance of the renal medullary ET A (ET-A) receptor is poorly understood, despite the fact that the ET-A receptor is expressed in medullary structures, such as vasa recta, interstitial cells, and collecting duct cells. Therefore, we also sought to determine the influence of ET-A receptors using both male and female ET-B receptor–deficient rats on the natriuretic responses to intramedullary infusion of ET-1.
Methods

Animals
Twelve- to 17-week-old wild type (wt) and homozygous (sl/sl) rats deficient of ET<sub>A</sub> receptors on a Wistar-Kyoto genetic background were obtained from our local breeding colony. Both wt and sl/sl rats carry the transgene for dopamine–β-hydroxylase that rescues the ET<sub>A</sub>-deficient rats from a lethal phenotype by expressing a functional ET<sub>B</sub> receptor in adrenergic tissues. Experimental protocols and animal care methods were approved by the Medical College of Georgia Institutional Animal Care and Use Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Surgical Preparation
Rats were anesthetized with inactin (100 mg/kg IP) and placed on a servo-controlled heating table to maintain rectal temperature constant at 37°C. The trachea was cannulated (PE-205) to facilitate respiration. The right femoral artery was catheterized for monitoring blood pressure using a MacLab data acquisition system (AD Instruments), and the right jugular vein was catheterized for infusion of phosphate-buffered 0.9% NaCl containing 6.2% BSA at a rate of 1.8 mL/h to replace fluid loss. A midline incision was made, and a stretched phycoerythrin 10 catheter was inserted into the left kidney to a depth of 5.0 mm for male and 4.5 mm for female rats. After inserting the catheter, saline was infused directly into the renal medulla (0.5 mL/h) for the duration of the experiment. Medullary blood flow (MBF) and cortical blood flow were measured by single-fiber, laser Doppler flowmetry, as described previously. Urine was collected separately from the left and right kidneys via catheters placed in each ureter. Experiments were started after an 80-minute equilibration period. At the end of each experiment, the kidneys were dissected to ensure that the catheter was in the appropriate position within the medulla.

Protocol
After two 20-minute control periods, saline or increasing dosages of ET-1 or S6c (0.15 and 0.45 µg/kg per hour; American Peptide, Inc) were infused into the renal medullary interstitium of sl/sl or wt rats for two 20-minute experimental periods at each dose. A separate group of rats received the ETA selective antagonist ABT-627 (10 mg/kg IV; Abbott Laboratories) 10 minutes before the control periods. In a third group of rats, an adjustable aortic clamp was placed around the aorta upstream of the renal arteries and tightened 10 minutes before the control period such that renal perfusion pressure was maintained at an equivalent level to that of the ABT-627-treated rats. In another group of rats, N<sup>C</sup>teropropyl-L-arginine (NPA; Calbiochem, Inc) was infused with ET-1 into the renal medulla. NPA was infused beginning 40 minutes before ET-1 infusion in female wt and sl/sl rats.

Gonadectomy
A separate group of rats underwent gonadectomy (ovariectomy for female rats and orchidectomy for male rats) at 6 weeks of age under anesthesia using an IP injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). Six to 8 weeks later, rats were prepared for intramedullary infusion and blood flow measurements as described above.

Statistical Analysis
All of the values were expressed as the means±SEMs. For statistical analysis, we used 1-way ANOVA followed by Tukey-Kramer multiple comparison tests. Student test was used to determine the effect of gonadectomy in separate groups of male or female rats. Differences were considered significant at P<0.05.

Results
Intramedullary infusion of ET-1 (0.15 and 0.45 µg/kg per hour) into the left kidney of female wt rats induced significant diuretic and natriuretic responses compared with saline infusion or compared with those in male rats (Figure 1). Infusion of ET-1 did not increase urine flow or sodium excretion in male rats compared with saline infusion. Urine flow and sodium excretion from the contralateral kidney were not significantly increased by ET-1 infusion compared with saline infusion (data not shown). Mean arterial pressure was not changed by infusion of ET-1, eliminating the possibility that increasing water and sodium excretion was a result of pressure-induced changes.

S6c, a selective ET<sub>B</sub> agonist, significantly increased urine flow rate and sodium excretion in wt rats compared with sl/sl rats (Figure 2). No difference was observed in the response to S6c between male and female rats, suggesting that the sex difference in the response to ET-1 is not dependent on the ET<sub>B</sub> receptor. As reported previously, mean arterial pressures of sl/sl rats were higher compared with wt rats.
Intramedullary infusion of S6c had no effect on mean arterial pressure.

Infusion of ET-1 in female wt rats and, surprisingly, sl/sl rats markedly increased urine flow and sodium excretion (Figure 3) compared with baseline, yet no increase was observed in male rats, indicating the existence of ETB-independent effects. The diuretic and natriuretic responses in female sl/sl rats were greater than those in female wt rats. Again, infusion of ET-1 did not significantly change the mean arterial pressure.

A long-standing theory suggests that changes in renal MBF affect the sodium reabsorption in the renal medulla. Because ET-1 is a potent vasoactive peptide, we evaluated the effect of ET-1 infusion on MBF. Intramedullary infusion of ET-1 significantly decreased the MBF in male rats but not in female rats (Figure 4). The presence of a functional ETB receptor did not affect these responses, because identical changes were observed in wt and sl/sl rats. Medullary infusion of ET-1 did not induce significant effects on cortical blood flow.

Because significant ET-1–dependent natriuretic responses were observed in female sl/sl rats, we assessed the contribution of ETA receptors by administration of a selective ETα antagonist, ABT-627, before ET-1 infusion. ABT-627 prevented the ET-1–induced increase in urine flow and sodium excretion observed in female sl/sl rats (Figure 5). Because injection of ABT-627 produced a slight but significant decrease in mean arterial pressure in sl/sl rats, renal perfusion pressure was controlled by an aortic clamp to mimic the ABT-627 group in a separate group of rats. ET-1–induced responses were still observed in the clamped group, providing further support for the idea that ET-1–induced diuretic and
natriuretic responses are, at least in part, attributable to an ETA-dependent pathway.

We also examined whether NOS1 mediates the ETA-dependent increase in sodium and water excretion, similar to ETB-dependent responses.12 The diuretic and natriuretic responses to ET-1 in female rats were suppressed by coinfusion of NPA, a selective NOS1 inhibitor, indicating that ETA-dependent salt and water excretions are induced by an NOS1-dependent pathway (Figure 6).

A final series of experiments determined whether sex steroids affect the sex difference in renal medullary ET-1–dependent sodium excretion (Table). Orchidectomy potentiated the urinary sodium excretion in response to ET-1 and suppressed the ET-1–induced decrease in MBF in wt but not in sl/sl rats. Ovariectomy suppressed the natriuretic response to ET-1 in both wt and sl/sl rats and enhanced the ET-1–induced decreases in MBF only in sl/sl rats. Overall, gonadectomy diminished the sex difference in ET-1–dependent natriuresis and changes in MBF in each strain.

Discussion
The physiological importance of ET-1 in the kidney has been the subject of investigation ever since its identification in 1988. Kitamura et al18 first observed that the expression level of ET-1 in the renal medulla is markedly higher than that in the other organs. In addition, considerable in vitro and in vivo studies have shown that renal ET-1 facilitates urine production by the inhibition of tubular reabsorption.8,9,19,20 Chronic blockade of ETB receptors or genetic deletion of functional ETB receptors either generally or specifically in the renal collecting duct results in salt-dependent increases in arterial pressure.11,14,21 Our laboratory demonstrated recently that infusion of S6c into the renal medullary interstitium produces a significant diuresis and natriuresis in normal rats, indicating

![Image of Figure 4, Cortical and medullary blood flow (CBF and MBF, respectively) during renal medullary infusion of ET-1. Flow is presented as a percentage of the average flow for each animal during the initial 20-minute baseline period. Data are expressed as the means±SEs. Number of rats: male-wt, n=7; male-sl/sl, n=6; female-wt, n=6; female-sl/sl, n=8. *P<0.05 vs male rats. n.s. indicates not significant.](http://hyper.ahajournals.org/)

![Image of Figure 5, Urine flow rate, sodium excretion (UNaV), and mean arterial pressure (MAP) during medullary interstitial infusion of ET-1 in female sl/sl rats after ETA receptor blockade (ABT-627). ABT-627 was injected IV 10 minutes before the control period (10 mg/kg). In a separate group of rats, renal perfusion pressure was manually lowered (clamped) to mimic the decrease in pressure induced by ABT-627. Data are expressed as the means±SEs. Number of rats: ET-1, n=13; ABT-627, n=3; ET-1+ABT-627, n=6; ET-1 + clamped, n=5. *P<0.05 vs ET-1 group. †P<0.05 vs ET-1+ABT-627 group.](http://hyper.ahajournals.org/)
that activation of the $\text{ET}_B$ receptor in the renal medulla facilitates the excretion of salt and water.\textsuperscript{12} However, the role of the renal medullary $\text{ET}_A$ receptor has been uncertain, despite its known presence in the renal medulla.

Results from the current study demonstrated that intramedullary infusion of ET-1 produced greater diuretic and natriuretic responses in female rats compared with male rats and that this sex difference is primarily because of an $\text{ET}_A$ receptor–dependent mechanism. To our knowledge, this study is the first evidence to show that renal medullary $\text{ET}_A$ receptors in the control of salt balance by ET-1. Considerable evidence indicates that females are more resistant in the development of hypertension compared with males\textsuperscript{2,5,22,23} and that reduced sodium retention in females is likely to account for this sex difference.\textsuperscript{24} The presence of an $\text{ET}_A$ receptor–mediated natriuretic pathway, combined with an $\text{ET}_B$ receptor–dependent natriuretic pathway, might explain why female rats are more resistant to salt-induced hypertension. We also provided evidence that the $\text{ET}_A$-dependent diuresis and natriuresis in female rats depend on NOS1, similar to previous reports of $\text{ET}_B$-mediated actions in the renal medulla,\textsuperscript{12,23} and appear to be maintained by ovarian hormones.

Infusion of ET-1 significantly decreased MBF in male rats regardless of the presence of $\text{ET}_B$ receptor, indicating the involvement of $\text{ET}_A$-dependent vasoconstriction. It is well accepted that a decrease in MBF could facilitate sodium reabsorption in the renal medulla and suppress urinary sodium excretion.\textsuperscript{17,26} Therefore, larger ET-1–induced vasoconstriction may provide an explanation for why ET-1 did not induce diuretic and natriuretic responses in male wt rats despite the presence of the $\text{ET}_B$ receptor. A contribution of hemodynamic changes in ET-1–dependent responses in females seemed to be insignificant, because we did not observe any changes in MBF in response to ET-1 in intact female rats. In addition, we observed recently that renal medullary infusion of the selective NOS1 inhibitor NPA did not change MBF, but infusion of the nonselective NOS inhibitor $N^\omega$-nitro-arginine methyl ester decreases MBF,\textsuperscript{12} suggesting that basal MBF was regulated by NOS3 and/or NOS2 but not NOS1. Taken together with the current results, we speculate that ET-1/NOS1-dependent responses observed in the current study are primarily mediated through changes in tubular reabsorption that do not involve changes in medullary hemodynamics. It is also possible that ET-1–dependent inhibition of tubular sodium reabsorption is similar between male and female rats but that the sex difference in ET-1–dependent increases in sodium excretion may be because of the counteracting effects of vasoconstriction within the renal medulla in males. Future studies will have to determine whether there is renal tubular action of $\text{ET}_A$ receptors in males independent of hemodynamic changes. The distribution of $\text{ET}_A$ and $\text{ET}_B$
receptors within the renal medulla of male and female rats should also provide further insight into the receptor-specific mechanisms.

The difference in the diuretic and natriuretic actions of ET-1 in male versus female rats could be partly because of sex differences in the receptor expression level within renal tubular and vascular structures in the renal medulla. ETB receptors are expressed in tubular cells, endothelial cells, and collecting duct cells, whereas ETA receptors are expressed in vasa recta, pericytes, interstitial cells, and collecting duct cells. However, to our knowledge, there have been no investigations into whether a sex difference exists in the distribution or expression level of each receptor. Our group has shown previously that there is no sex difference in urinary ET-1 excretion, which is generally used as an indicator of renal ET-1 production, in the same strain of wt rats used in the current study. Thus, it appears as though the sex difference in responsiveness to intramedullary ET-1 cannot be explained by endogenous peptide production. However, this may not be the best evidence for determining whether there is a sex difference in renal production of ET-1, because urinary ET-1 most likely includes production originating from both the renal cortex and medulla.

Another possible factor contributing to the sex difference in ETA-dependent diuretic and natriuretic responses is that the ETA receptor may regulate the balance of other hormonal factors that can influence the water and sodium reabsorption. It has been demonstrated that ET-1 antagonizes the urine concentrating effects of vasopressin (AVP), thereby facilitating water excretion. However, a recent study demonstrated that deletion of the ETA receptor in the collecting duct decreases the sensitivity of water reabsorption in response to AVP, indicating that ETA signaling would interfere with the ET-1–induced inhibition of AVP response. Moreover, men appear to have increased sensitivity to AVP–induced urine concentrating capability compared with women. Therefore, the greater AVP–induced antidiuretic effects, which could be facilitated by collecting duct ETA receptor stimulation in males, may contribute to the sex difference in ET-1– and ETA–dependent urine excretion.

The ET-1–dependent diuretic and natriuretic responses in wt rats were less than those in sl/sl rats, despite the presence of both ETA and ETB receptors. However, it is important to note that the ETB receptor also functions to clear ET-1 from the circulation, and so infusion of ET-1 in the renal medulla of wt rats might be cleared by functional ETB receptors there, resulting in reduced ETA receptor stimulation in wt rats. Alternatively, ET-1 might be accumulated in the renal medulla because of no clearance receptor in sl/sl rats. This could account for the larger ET-1–induced natriuretic response in female sl/sl rats compared with female wt rats, but this possibility has yet to be explored.

Sex hormones are known to account for a majority of functional differences between males and females in the cardiovascular and renal systems. Results from gonadectomy experiments suggest that testicular hormones enhance ET-1–induced medullary vasoconstriction by suppressing the ETB receptor–dependent pathway, thereby inhibiting sodium excretion, and that ovarian hormones weaken ET-1–induced medullary vasoconstriction by suppressing the ETA receptor–dependent pathway, thereby facilitating sodium excretion. ETA–dependent vasoconstriction may be masked by ETB–dependent vasodilation in female wt rats. Most importantly, the sex difference in the ET-1–dependent natriuretic response in intact rats was diminished by gonadectomy in each strain, indicating the importance of sex steroids on the sex difference of ET-1–induced responses in the renal medulla.

NOS1 inhibition suppressed ET-1–dependent diuresis and natriuresis in female rats. Both ETA and NOS1 are expressed in the inner medullary collecting duct cells. However, our results would appear to conflict with findings in cultured inner medullary collecting duct cells, which showed that ET-1–dependent NO release was inhibited by the ETB antagonist but not by the ETA antagonist. Aside from the obvious differences in expression that could exist in cultured cells versus intact kidneys, ETA–dependent NO production could occur within any number of cell types within the renal medulla, such as thick ascending limb or interstitial cells. Another possibility is that cell-cell interaction with surrounding cells in the renal medulla may be essential to induce ETA–dependent NOS1 stimulation.

Hypertension produced by deoxycorticosterone acetate–salt treatment is blunted in female rats compared with male rats. This difference was abolished in the absence of an ETB receptor. Therefore, we hypothesized that females might have greater ETB–dependent sodium excreting activity and that this sex difference might contribute to prevent salt retention and salt-induced blood pressure elevation. However, we could not observe the difference in the responses to S6c between male and female rats, indicating that there is no sex difference in ETB–dependent diuretic and natriuretic activities, at least via receptors located in the renal medulla. Therefore, the ETB–dependent sex difference in deoxycorticosterone acetate–salt–induced hypertension appears to result from the differences in nonmedullary tissues, such as the brain, heart, vasculature, and renal cortex.

**Perspectives**

In summary, female rats have greater renal medullary ET-1–dependent diuretic and natriuretic activity compared with male rats and involve both ETA and ETB receptor–dependent activation of NOS1. The sum of tubular and hemodynamic mechanisms results in increased ET-1–dependent natriuresis in female compared with male rats. These sex differences in response to ET-1 are, at least partly, mediated by sex hormones. This increased ET-1–dependent natriuretic capacity in females may be an important mechanism for protection from salt-dependent hypertension in premenopausal women. Furthermore, to our knowledge, this study is the first to show the existence of ETA–dependent natriuresis. This novel role of ETA receptors in the renal medulla may provide an explanation for edema observed in patients given ETA or ETB–selective antagonists.

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