Sex differences in ET-1 receptor expression and Ca\(^{2+}\) signaling in the IMCD

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Jin C, Speed JS, Hyndman KA, O’Connor PM, Pollock DM. Sex differences in ET-1 receptor expression and Ca\(^{2+}\) signaling in the IMCD. Am J Physiol Renal Physiol 305:F1099–F1104, 2013. First published August 14, 2013; doi:10.1152/ajprenal.00400.2013.—The inner medullary collecting duct (IMCD) is the nephron segment with the highest production of endothelin-1 (ET-1) and the greatest expression of ET-1 receptors that function to adjust Na\(^{+}\) and water balance. We have reported that male rats have reduced natriuresis in response to direct intramedullary infusion of ET-1 compared with female rats. Our aim was to determine whether alterations of ET-1 receptor expression and downstream intracellular Ca\(^{2+}\) signaling within the IMCD could account for these sex differences. IMCDs from male and female rats were isolated for radioligand binding or microdissected for intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(i\)) measurement by fluorescence imaging of fura-2 AM. IMCD from male and female rats had similar ET\(_{B}\) expression (655 ± 201 vs. 567 ± 39 fmol/mg protein, respectively), whereas male rats had significantly higher ET\(_{A}\) expression (436 ± 162 vs. 47 ± 29 fmol/mg protein, respectively; \(P < 0.05\)). The [Ca\(^{2+}\)]\(i\) response to ET-1 was significantly greater in IMCDs from male compared with female rats (288 ± 32 vs. 118 ± 32 AUC, nM \(\times\) min, respectively; \(P < 0.05\)). In IMCDs from male rats, the [Ca\(^{2+}\)]\(i\) response to ET-1 was significantly blunted by the ET\(_{A}\) antagonist BQ-123 but not by the ET\(_{B}\) antagonist BQ-788 (control: 137 ± 27; BQ-123: 53 ± 11; BQ-788: 84 ± 25 AUC, nM \(\times\) 3 min; \(P < 0.05\)), consistent with greater ET\(_{A}\) receptor function in male rats. These data demonstrate a sex difference in ET\(_{A}\) receptor expression that results in differences in ET-1 Ca\(^{2+}\) signaling in IMCD. Since activation of ET\(_{A}\) receptors is thought to oppose ET\(_{B}\) receptor activation, enhanced ET\(_{A}\) function in male rats could limit the natriuretic effects of ET\(_{B}\) receptor activation. 

inner medullary collecting duct; endothelin

IT HAS BEEN WELL ESTABLISHED that men are more susceptible to hypertension and cardiovascular disease than premenopausal women and that females are protected against high blood pressure in a number of hypertensive animal models (3, 22). For instance, male rats are more sensitive to angiotensin II (AngII)-induced hypertension than female rats, and male spontaneously hypertensive rats have higher blood pressure than their female counterparts. As the prevalence of uncontrolled hypertension becomes greater, understanding the mechanisms behind these observations becomes increasingly important to define future treatments.

Endothelin-1 (ET-1) is an important regulator of blood pressure through activation of its two receptor subtypes, ET\(_{A}\) and ET\(_{B}\) (18). Our laboratory has recently determined that sex differences in renal ET-1 signaling may mediate sex differences in blood pressure regulation (9, 11). For instance, AngII, whether stimulated by a low-salt diet or given exogenously, inhibits ET\(_{G}\) receptor function in male rats but to a much lesser degree than in female rats (9, 10). Furthermore, acute infusion of an ET\(_{B}\) agonist directly into the renal medulla enhances sodium and water excretion in both male and female rats. However, infusion of ET-1, the endogenous ligand, only produces natriuresis in female rats. Although the mechanisms for these sex differences are still speculative, one possibility is an altered functional distribution of ET receptors within the kidney.

The collecting duct is an important site for both endogenous ET-1 production and long-term blood pressure control. In fact, a series of publications from the Kohan laboratory has demonstrated that the absence of ET-1 and ET\(_{B}\) receptor expression in the collecting duct leads to an attenuated ability to handle a high-salt diet, resulting in salt-dependent hypertension (1, 4). Furthermore, the inner medullary collecting duct (IMCD) produces considerably more ET-1 and has a much higher expression of ET\(_{B}\) receptors compared with all other segments within the kidney (12, 13). The importance of ET-1 from the IMCD in blood pressure regulation is due in large part to ET\(_{B}\)-mediated increases in nitric oxide production, resulting in reductions in Na\(^{+}\) and H\(_{2}\)O reabsorption in the IMCD and other nephron segments (8, 15, 17, 19). In general, activation of ET\(_{A}\) receptors opposes the actions of ET\(_{B}\) receptors. Both ET\(_{A}\) and ET\(_{B}\) receptor subtypes are located on IMCD, and, given their key role in Na\(^{+}\) and water homeostasis, differences in ET-1 signaling within IMCD could potentially mediate sex differences in arterial pressure; however, little is known regarding sex differences in ET-1 signaling in IMCD.

We hypothesize that the distribution and downstream signaling of ET-1 receptors in IMCD is different in male and female rats. To determine whether the expression of ET-1 receptors could account for the functional difference between sexes, we isolated IMCD from male and female Sprague-Dawley rats and compared ET\(_{A}\) and ET\(_{B}\) receptor expression using radioligand binding assays. To assess whether any sex differences in receptor expression in IMCD were functional and result in altered cellular responses to ET-1, we determined intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(i\)) responses to ET-1 activation in live, freshly isolated IMCD from male and female Sprague-Dawley rats.

METHODS

Experimental Animals

All protocols were approved by the Institutional Animal Care and Use Committee at Georgia Regents University. Male (n = 43) and
female (n = 47) Sprague-Dawley (SD; Harlan, Indianapolis, IN) rats were obtained at 10 wk of age and maintained on a standard rat chow containing 0.4% NaCl with free access to water. Rats were housed in temperature- and humidity-controlled, 12:12-h light-cycled quarters.

**Bulk IMCD Isolation**

Renal inner medullae were dissected under sodium brevital anesthesia and chopped into 1-mm sections following the methods of Hyndman et al. (7). The minced sections from two animals were pooled and digested in sucrose buffer (250 mM sucrose and 10 mM triethanolamine, pH 7.4) plus 2 mg/ml collagenase type I, 2 mg/ml hyaluronidase type IV. The suspension was incubated in a 37°C shaking water bath for 60–90 min. After 30 min, 0.5 mg of DNAse I was added to the suspension. After digestion, the solution was filtered over a 100-μm filter (Fisher Scientific). The supernatant was centrifuged at 300 g for 2.5 min. The supernatant was removed, and the pellet was washed twice with sucrose buffer, centrifuging at 300 g for 1 min between washes to repellet the IMCD. After the final wash, the supernatant was removed, and the pellet was resuspended in Hank’s balanced saline solution (HBSS; Cellgro). It was then centrifuged for 5 min at 300 g, and the HBSS was removed. The pellet was stored at −80°C until binding assay was performed.

**ET-1 Receptor Binding in IMCD**

IMCD pellets were suspended in 200 μl of homogenization buffer (50 mM Tris-HCl, 5 mM EDTA, 250 mM sucrose, 15 μM PMSF, pH 7.4). The IMCDs were disrupted by brief sonication and spun at 1,000 g for 30 min at 4°C to pellet any large debris. To the supernatant, 1.3 mM phenylmethylsulfonfyl fluoride was added, and the supernatant was then centrifuged at 30,000 g and 4°C for 45 min to pellet membranes. The supernatant was discarded, and the pellet (cell membrane enriched) was suspended in 100 μl of homogenization buffer and disrupted. Total protein was measured using the Bradford method.

The binding assay was performed in duplicate on a 96-well Packard optiplate. Samples were diluted in binding buffer (20 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl₂, 3 mM EDTA, 0.1 mM PMSF, 5 μg/ml peptatin A, 0.025% bacitracin, 0.2% BSA) to a final protein concentration of 0.3 μg/50 μl. Sample (50 μl) and 25 μl of 40 mg/ml wheatgerm agglutinin-coated PVT SPA beads (Perkin Elmer) were added to each well. The plate was incubated on a shaker for 3 h at room temperature. Total binding was determined by adding 50 μl of 125I-ET-1 or 125I-ET-3 (Perkin Elmer, Waltham, MA) to each well at equilibrium temperature and humidity-controlled, 12:12-h light-cycled quarters.

Fluorescence Detection

Fluorescence measurements were made using an Olympus IX81 inverted microscope with a ×40 objective lens. The signal was detected using a high-resolution digital camera (EVL0VE, Photometrics Technology). Excitation was provided by a Sutter DG-175-W xenon arc lamp (Sutter Instrument, Novato, CA) that allowed high-speed excitation wavelength switching. IMCDs isolated on coverslips were washed to remove excess dye before being placed in the imaging chamber. Fura 2 fluorescent signal was stimulated by dual-wavelength excitation at 340 and 380 nm. A 510-nm band-pass emission filter was used to collect fura 2 signals at 3-s intervals. Ratios between the fluorescence intensity stimulated by 340/380-nm excitation were calculated, and the excitation intensity was adjusted on the DG-1 to minimize fura 2 fluorescence bleaching and to balance 340/380 excitation intensities. At the end of the experiment, maximum and minimum fura 2 signals were collected for the calibration of [Ca²⁺]i concentration. The tissue bath solution was exchanged to 5 μM of the calcium ionophore 4-bromo-A23187 (Molecular Probes) with known solution of Ca²⁺ (Calcium buffer kit; Molecular Probes) to establish maximum and minimum fura 2 signals. [Ca²⁺], was calculated as equation 1, [Ca²⁺] = Kd * (R – Rmin)/(Rmax – R) * F, where Kd is the dissociation constant of fura 2, R is the actual ratio of intensities at excitation wavelengths 340 and 380 nm, Rmax and Rmin are the maximal and minimal fura 2 ratios in the presence and absence of Ca²⁺, and F is the ratio of fura 2 intensities at 380 nm in the presence and absence of Ca²⁺. Kd of fura 2 at 37°C was 224 nM as reported (6). The maximum cytosolic Ca²⁺ response (Δ[Ca²⁺],) from baseline as well as the integrated Ca²⁺ response [min of area under the curve (AUC) immediately following stimulation] were used for the comparison of the responses.

**Experimental Protocol**

Ca²⁺ imaging. Coverslips containing IMCD were placed in an imaging chamber (maintained at 37°C mounted on the stage of the inverted microscope that allowed the superfusion of the experimental buffer and buffer containing drugs. Five to eight regions, as shown in Fig. 2C, containing only collecting duct cells, were selected within each IMCD to quantify changes in fluorescent intensity of fura-2 AM dyes using Metafluor imaging software (Universal Imaging, Bedford Hills, NY). Fluorescent signals were recorded for 200 s (baseline) before bath exchange. bath media containing either IMCDs from male or female rats was then rapidly exchanged with media containing 1) buffer only or 2) ET-1 (1 nM; American Peptide) and fluorescent signal recorded for a further 300 s. Because maximal [Ca²⁺], responses occurred within the first 3 min, data are presented as AUC by 180 s. All exchanged solutions were prewarmed to 37°C using an in-line solution heater (Warner Instruments, Hamden, CT).

In experiments in which antagonists were used, IMCD were incubated with antagonists for 15 min before stimulation with ET-1. The response to ET-1 (1 nM) was determined in IMCD from both male and female rats for 300 s following incubation of IMCD with either the ET₄ receptor antagonist BQ-123 (1 μM; Calbiochem/EMD chemical, Darmstadt, Germany) or the ET₆ receptor antagonist BQ-788 (1 μM; Calbiochem/EMD chemical). In a separate group of experiments, IMCD from both male and female rats were stimulated using the ET₆ receptor-specific agonist sarafotoxin 6c (S6c;1 nM; American Peptide) in place of ET-1. To confirm [Ca²⁺], responses were mediated by receptor activation, in some experiments, IMCD were incubated with the phospholipase C (PLC) inhibitor U73122 (3 μM; Sigma) for 15 min before stimulation with ET-1 (1 nM).

**Statistical Analysis**

All data are expressed as means ± SE. Comparisons between antagonist treatments were made by one-way ANOVA, followed by Dunnett’s multiple comparison test. All other comparisons were
determined using unpaired t-test. P < 0.05 was considered statistically significant.

RESULTS

To help explain sex differences in medullary ET-1 receptor function observed in vivo (14), receptor binding was determined on freshly isolated IMCD. ETB receptor expression in IMCD as determined by ET-3 binding was similar between male (n = 4) and female (n = 5) rats (655 ± 201 vs. 567 ± 39 Bmax, respectively). In contrast, female rats had significantly reduced ETA receptor binding as measured by the difference in ET-1 and ET-3 binding (434 ± 162 vs. 47 ± 29 Bmax, male vs. female rats, P < 0.05; Fig. 1). Thus the ratio of ETB to ETA receptors in the IMCD is 60/40 for male rats but 90/10 for female rats.

To determine whether differences in ET-1 receptor expression in IMCD between male and female rats could account for functional differences, intracellular calcium responses to ET-1 were measured in freshly isolated IMCD. Baseline [Ca2+]i was 156 ± 30 and 124 ± 26 nM in male and female rats, respectively. In IMCDs from both male and female rats, addition of ET-1 (1 nM) resulted in a rapid increase in [Ca2+]i, over the following 60 s followed by a prolonged period (>200 s) in which [Ca2+]i fluctuated but remained elevated above baseline levels (Fig. 2). The total calcium response calculated as AUC was significantly greater than that of vehicle treatment in both male and female rats (treatment P < 0.0001; Fig. 3). However, [Ca2+]i responses to ET-1 were significantly greater in IMCD from male rats compared with female rats, averaging 288 ± 52 and 118 ± 32 AUC (sex P < 0.05), respectively, over 180 s following treatment with ET-1 (1 nM).

When stimulated with the potent and selective ETB receptor agonist S6c (1 nM) rather than ET-1, no significant change in [Ca2+]i was observed in either male or female rats, indicating that [Ca2+]i responses were not stimulated by activation of the ETB receptor in either sex (Fig. 4). Furthermore, prior (15 min) incubation with the phospholipase C inhibitor U-73122 (3 μM) significantly blunted the [Ca2+]i response in male (P = 0.04) but not female rats, indicating that ET-1 increases [Ca2+]i in IMCD via a G-protein-coupled receptor pathway (Fig. 6). These data together are consistent with activation of the ETA receptor.

To further elucidate which receptor on the IMCD is responsible for the increase in [Ca2+]i, in response to ET-1, IMCD taken from the same rat were treated with ET-1 in the presence of the specific antagonists BQ-123 (ETA antagonist; 1 μM) or BQ-788 (ETB antagonist; 1 μM) given 15 min before ET-1 stimulation. Baseline [Ca2+]i in IMCD was higher in male rats compared with female rats, and treatment with BQ-123 lowered baseline [Ca2+]i in IMCD from male rats but had no effect
in female rats. Treatment with BQ-788 did not affect baseline [Ca^{2+}]_{i} in either male or female rats (Table 1). Interestingly, in the presence of BQ-123, the increase in [Ca^{2+}]_{i} following addition of ET-1 was significantly blunted (P < 0.05; Fig. 5), such that, in the presence of BQ-123, the response of IMCD from male rats to ET-1 did not differ from that of IMCD from female rats. Incubation with BQ-788 did not significantly reduce the [Ca^{2+}]_{i} response to ET-1 in male rats, consistent with this response being independent of the ET_{B} receptor.

DISCUSSION

The major finding of this study is that IMCD ETA receptor expression and intracellular Ca^{2+} signaling are greater in male compared with female rats. Alterations in IMCD ET-1 signaling could explain sex differences in renal medullary ET_{B} receptor function that several laboratories, including our own, have previously observed (9, 10, 14, 21, 23). We recently reported that direct infusion of an ET_{B} agonist into the renal medulla produces diuresis and natriuresis in both male and female rats, whereas infusion of ET-1, the endogenous ligand, produces natriuresis only in female rats. Our data, together with our previous finding that male rats have a natriuretic response to an ET_{B} agonist but not to ET-1, a combined ET_{A}/ET_{B} agonist, suggest that IMCD ETA receptors oppose ET_{B} receptors and are anti-natriuretic. Although studies of collecting duct-specific knockout mice suggest that ETA receptors may contribute to the natriuretic response to ET-1, this action of the ETA receptor has only been observed in the absence of the ET_{B} receptor (5). Therefore, it is likely that removal of ET_{B} receptor function, as seen in collecting duct-specific ET_{B} knockout mice, from the ETA receptor on the IMCD may compensate to promote natriuresis, although this hypothesis has yet to be tested.

Table 1. Baseline intracellular Ca^{2+} concentrations (in nM) in isolated inner medullary collecting ducts pretreated with ET_{A} (BQ-123) or ET_{B} (BQ-788) antagonists

<table>
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<tr>
<th></th>
<th>BQ-123</th>
<th>BQ-788</th>
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<tr>
<td>Male (n = 8)</td>
<td>114 ± 16*</td>
<td>75 ± 14</td>
</tr>
<tr>
<td>Female (n = 6)</td>
<td>53 ± 8</td>
<td>53 ± 10</td>
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Values are means ± SE. *Significant difference vs. females (P < 0.05).

Our finding that both ET_{A} and ET_{B} receptors are expressed in IMCD of male rats is consistent with previously published reports in both freshly isolated and cultured IMCD (20). Although it has been well established that the ET_{B} receptor within the IMCD is a major contributor to the maintenance of Na^{+} and water balance, the role of the ETA receptor in this nephron segment is largely unknown. Our data, together with our previous finding that male rats have a natriuretic response to an ET_{B} agonist but not to ET-1, a combined ET_{A}/ET_{B} agonist, suggest that IMCD ETA receptors oppose ET_{B} receptors and are anti-natriuretic. Although studies of collecting duct-specific knockout mice suggest that ETA receptors may contribute to the natriuretic response to ET-1, this action of the ETA receptor has only been observed in the absence of the ET_{B} receptor (5). Therefore, it is likely that removal of ET_{B} receptor function, as seen in collecting duct-specific ET_{B} knockout mice, from the ETA receptor on the IMCD may compensate to promote natriuresis, although this hypothesis has yet to be tested.

Fig. 3. Represents [Ca^{2+}]_{i} response as area under the curve (AUC) to either vehicle (open bars) or ET-1 (filled bars) in freshly isolated IMCD from male and female rats (n = 6–9 rats per group). *Significant difference by two-way ANOVA (P < 0.05).

Fig. 4. Represents [Ca^{2+}]_{i} response as AUC in freshly isolated IMCD from male and female rats in response to the ET_{B} agonist sarafotoxin 6c. *Significant difference by one-way ANOVA (P < 0.05).

Fig. 5. [Ca^{2+}]_{i} response to ET-1 (1 nM) in the presence or absence of an ET_{A} antagonist (BQ-123; 1 μM) or an ET_{B} antagonist (BQ-788; 1 μM) in IMCD of male (A; n = 8) or female (B; n = 6) rats. *Significant difference by one-way ANOVA (P < 0.05).
tested. Furthermore, it has yet to be directly determined whether sex differences in the renal medullary response to ET-1 exist for mice.

Previously, we reported female rats have an ET\textsubscript{A}-dependent natriuresis in response to exogenous ET-1 infusion into the renal medulla. Here, we present that females have fewer ET\textsubscript{A} receptors on the IMCD. Taken together, we postulate that ET\textsubscript{A}-dependent natriuresis in female rats is related to ET\textsubscript{A} receptors located on other cell types, such as the vasa recta, the thick ascending limb, or perhaps renomedullary interstitial cells, but the mechanism for natriuresis remains unclear (14).

To confirm that ET\textsubscript{A} receptors were responsible for enhanced Ca\textsuperscript{2+} signaling in male compared with female IMCD, we quantified [Ca\textsuperscript{2+}]\textsubscript{i} responses to pharmacological agonists/antagonists of the ET system in IMCD. Interestingly, we found that baseline [Ca\textsuperscript{2+}]\textsubscript{i} in untreated IMCD from female rats was significantly lower than in IMCD from male rats. Treatment with the potent and selective ET\textsubscript{A} receptor antagonist BQ-123 reduced baseline [Ca\textsuperscript{2+}]\textsubscript{i} in IMCD from male rats compared with levels observed in IMCD from female rats. These data are consistent with our finding that the male rats have more ET\textsubscript{A} on the IMCD than the female rats and indicate that endogenous ET-1 signaling within IMCD leads to sex differences in resting [Ca\textsuperscript{2+}]\textsubscript{i} levels within this nephron segment.

In response to exogenous ET-1 (1 nM), an initial Ca\textsuperscript{2+} spike followed by a prolonged increase in [Ca\textsuperscript{2+}]\textsubscript{i} was observed. As observed following ET-1 administration, initial Ca\textsuperscript{2+} spikes were quite variable in magnitude and were sometimes absent all together; therefore, we determined [Ca\textsuperscript{2+}]\textsubscript{i} as AUC, integrating the [Ca\textsuperscript{2+}]\textsubscript{i} signal over the duration of the response to better quantify Ca\textsuperscript{2+} responses in IMCD. In IMCD from male rats, ET\textsubscript{A} receptor antagonism blunted Ca\textsuperscript{2+} responses to ET-1. Confirming that [Ca\textsuperscript{2+}]\textsubscript{i} responses were mediated by receptor signaling, the phospholipase C inhibitor U-73122 also greatly blunted the response to ET-1 (Fig. 6). Our data indicating that ET\textsubscript{A} receptor activation drives increased intracellular Ca\textsuperscript{2+} in IMCD of rats is in contrast to data from porcine IMCD indicating Ca\textsuperscript{2+} responses are mediated by ET\textsubscript{B} receptors. Our data do not completely exclude the possibility that ET\textsubscript{B} receptors participate in Ca\textsuperscript{2+} signaling in rat IMCD, because neither ET\textsubscript{A} nor ET\textsubscript{B} receptor antagonism completely abolished responses to ET-1 in freshly isolated rat IMCD. Furthermore, in some individual preparations, we did observe reduced [Ca\textsuperscript{2+}]\textsubscript{i} responses following ET\textsubscript{B} receptor inhibition with BQ-778. This, however, did not reach statistical significance (Fig. 5A).

Although we cannot rule out Ca\textsuperscript{2+} signaling through the ET\textsubscript{B} receptor, our data is in agreement with reports that there is increased ET\textsubscript{A}-mediated calcium signaling in the thin limb of Henle’s loop of hypertensive rats (2), and we are confident that sex differences in [Ca\textsuperscript{2+}]\textsubscript{i} are mediated by ET\textsubscript{A} receptor signaling, because differences in the response of male and female rats were completely abolished in the presence of an ET\textsubscript{A} antagonist BQ-123 (AUC [Ca\textsuperscript{2+}]\textsubscript{i} = 53 ± 11 vs. 34 ± 19 N·S in male and female rats, respectively). Furthermore, S6c (1 nM), a potent ET\textsubscript{B} selective agonist, did not elicit measureable [Ca\textsuperscript{2+}]\textsubscript{i} responses in either male or female rats, indicating that Ca\textsuperscript{2+} signaling by ET\textsubscript{B} receptors is minimal.

Our data clearly demonstrate a sex difference in ET-1 receptor expression and signaling in rats; however, it is unknown whether ET-1 peptide levels are different within the IMCD of male and female rats. Although tissue concentrations have proven difficult to accurately measure, previous data from our laboratory demonstrates that urinary ET-1 levels, an indicator of renal production, are not different between male and female ET\textsubscript{B}-deficient rats or wild-type rats (23). Thus it is unlikely that IMCD ET-1 concentrations differ among the sexes.

Our data demonstrate a clear sex difference in this important cell type for ET\textsubscript{A} expression and signaling that affects both renal function and ET signaling. Since ET\textsubscript{B} receptor expression was not different in IMCD of male and female rats, nor was the [Ca\textsuperscript{2+}]\textsubscript{i} response to ET-1 affected by ET\textsubscript{B} blockade, these data prompt us to examine the role and the functional consequences of ET\textsubscript{A} receptor activation at both the cellular and whole animal level. Although several mechanisms are implicated in fluid and electrolyte balance malfunction, differences in ET\textsubscript{A} signaling could potentially explain sex differences in associated pathologies such as hypertension and may provide a clinical target for sex-specific treatment of these diseases. Moreover, there is a need to determine whether sex differences observed in this study play a role in experimental models of hypertension. In perspective, these results provide further mechanistic information that could potentially explain the reduced incidence of salt-sensitive hypertension in women vs. men.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

Author contributions: C.J., J.S.S., K.A.H., P.M.O., and D.M.P. conception and design of research; C.J., J.S.S., K.A.H., and P.M.O. performed experiments; C.J., J.S.S., K.A.H., P.M.O., and D.M.P. analyzed data; C.J., J.S.S., K.A.H., P.M.O., and D.M.P. interpreted results of experiments; C.J., J.S.S., K.A.H., P.M.O., and D.M.P. prepared figures; C.J., J.S.S., K.A.H., P.M.O., and D.M.P. drafted manuscript; C.J., J.S.S., K.A.H., P.M.O., and D.M.P. edited and revised manuscript; C.J., J.S.S., K.A.H., P.M.O., and D.M.P. approved final version of manuscript.
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