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ORIGINAL RESEARCH

Acclimation to a High-Salt Diet Is Sex Dependent

Eman Y. Gohar, PhD; Carmen De Miguel, PhD; Ijeoma E. Obi, PhD; Elizabeth M. Daugherty; Kelly A. Hyndman, PhD; Bryan K. Becker, PhD; Chunhua Jin, MD, PhD; Randee Sedaka, PhD; Jermaine G. Johnston, PhD; Pengyuan Liu, PhD; Joshua S. Speed, PhD; Tanecia Mitchell, PhD; Alison J. Kriegel, PhD; Jennifer S. Pollock, PhD; David M. Pollock, PhD

BACKGROUND: Premenopausal women are less likely to develop hypertension and salt-related complications than are men, yet the impact of sex on mechanisms regulating Na^+ homeostasis during dietary salt challenges is poorly defined. Here, we determined whether female rats have a more efficient capacity to acclimate to increased dietary salt intake challenge.

METHODS AND RESULTS: Age-matched male and female Sprague Dawley rats maintained on a normal-salt (NS) diet (0.49% NaCl) were challenged with a 5-day high-salt diet (4.0% NaCl). We assessed serum, urinary, skin, and muscle electrolytes; total body water; and kidney Na^+ transporters during the NS and high-salt diet phases. During the 5-day high-salt challenge, natriuresis increased more rapidly in females, whereas serum Na^+ and body water concentration increased only in males. To determine if females are primed to handle changes in dietary salt, we asked the question whether the renal endothelin-1 natriuretic system is more active in female rats, compared with males. During the NS diet, female rats had a higher urinary endothelin-1 excretion rate than males. Moreover, Ingenuity Pathway Analysis of RNA sequencing data identified the enrichment of endothelin signaling pathway transcripts in the inner medulla of kidneys from NS-fed female rats compared with male counterparts. Notably, in human subjects who consumed an Na^+-controlled diet (3314–3668 mg/day) for 3 days, women had a higher urinary endothelin-1 excretion rate than men, consistent with our findings in NS-fed rats.

CONCLUSIONS: These results suggest that female sex confers a greater ability to maintain Na^+ homeostasis during acclimation to dietary Na^+ challenges and indicate that the intrarenal endothelin-1 natriuretic pathway is enhanced in women.

Key Words: endothelin-1 ■ natriuresis ■ nitric oxide ■ sex differences ■ sodium

Maintenance of Na^+ homeostasis has a fundamental role in blood pressure regulation, and the kidney is crucial in this process. In particular, the kidney tightly regulates natriuresis and has the capacity to acclimate to dietary salt challenges, thus ensuring that fluid-electrolyte balance is maintained.[1] Although there have been extensive studies over many years, the complex molecular interactions that control Na^+ handling in the kidney remain incompletely defined. This has severely complicated the development of therapeutic strategies to prevent salt-sensitive hypertension.

Despite evidence for male-female differences in hypertension prevalence and pathophysiology,[2] the vast majority of studies focused on Na^+ handling have been restricted to men. Therefore, a clear understanding of sex-dependent regulation of Na^+ homeostasis is critically lacking. Recent studies demonstrated that acute natriuretic responses to an intraperitoneal saline load are more rapid in female than in male rats.[3] Similarly, intravenous infusion of hypertonic saline to humans evoked a more pronounced natriuretic action in women than in men.[4] However, sex discrepancies in mechanistic pathways involved in natriuresis remain poorly characterized.

Correspondence to: Eman Y. Gohar, PhD, Division of Nephrology and Hypertension, Vanderbilt University Medical Center, Medical Research Building IV, P425B, Nashville, TN 37232. E-mail: eman.gohar@vumc.org

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CLINICAL PERSPECTIVE

What Is New?

- We report that women have a greater ability to maintain sodium balance during acclimation to dietary sodium challenges.
- This study also identifies sex-specific discrepancies in the renal endothelin system, which plays a critical role in the regulation of sodium excretion and blood pressure.

What Are the Clinical Implications?

- This study underscores the importance of studying sex-specific differences in the mechanisms regulating blood pressure and sodium excretion.
- The renal endothelin-1 system may play an important role on the advanced ability of the female kidney to excrete sodium.

Nonstandard Abbreviations and Acronyms

ET_A	endothelin receptor subtype A
ET_B	endothelin receptor subtype B
HS	high salt
IMCD	inner medullary collecting duct
IPA	Ingenuity Pathway Analysis
NCC	Na^+/Cl^- cotransporter
NHE3	Na^+/H^+ exchanger isoform 3
NOx	NO metabolites (nitrite and nitrate)
NS	normal salt

Of the myriad Na^+-regulatory pathways, the kidney endothelin-1 signaling system plays an important role in controlling Na^+ homeostasis[5] and contributes to sex discrepancies in blood pressure control and fluid and electrolyte homeostasis.[6–10] The renal inner medulla contains the highest concentration of endothelin-1 in the body.[11] Upon release from collecting duct cells, endothelin-1 inhibits tubular Na^+ transport, promoting natriuresis through activation of endothelin receptors and production of NO.[5] Endothelin-1–induced natriuresis is mediated mainly through activation of endothelin receptor subtype B (ET_B) receptors.[5] Interestingly, evidence now indicates a contribution of medullary endothelin receptor subtype A (ET_A) receptors in facilitating endothelin-1–mediated natriuresis in female rats only.[12] However, our previous study revealed that male rats have greater expression of ET_A in inner medullary collecting duct (IMCD) cells than females do, whereas no sex difference in ET_B receptor expression was detected in these cells.[13]

The goal of the current study was 2-fold. First, we tested the hypothesis that female rats have a more efficient capacity to acclimate to increased dietary salt intake challenge. To address this question, we assessed serum, urinary, skin, and muscle electrolyte levels and the abundance of Na^+ transporters in male and female Sprague Dawley rats on a normal-salt (NS) diet and during the initial several days transitioning to a high-salt (HS) diet. Second, we determined whether the renal endothelin-1 natriuretic system is more active in female rats, compared with males. This was achieved by measurement of urinary endothelin-1 excretion, renal ET_A and ET_B receptor expression in rats during the NS or HS diet phases. We assessed sex-specific differential expression of the renal inner medullary transcriptome in NS-fed rats. To begin determining the clinical relevance of our findings in rats, we assessed sex discrepancies in urinary excretion rates of endothelin-1 and other Na^+-regulatory factors in healthy humans.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Animals

Male and female age-matched (13–15 weeks old) Sprague Dawley rats were purchased from Envigo (Indianapolis, IN). Rats were maintained on an NS diet (0.49% NaCl, TD 96208; Envigo) for 2 weeks. Then, a nutrient-matched HS diet (4% NaCl, TD 92034; Envigo) was introduced for 5 consecutive days. During the entire experimental period, animals were housed in a temperature-controlled room (22–24 °C) with a 12:12-hour light-dark cycle, with free access to water. Experiments were conducted over 4 sequential days to ensure that different stages of the estrus cycle were presented in female rats. All animal protocols were in accordance with the Guide for the Care and Use of Laboratory Animals and were approved in advance by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Clinical Studies

Healthy men and women (age, 33.6±1.8 years; body mass index, 25.8±0.8 kg/m^2) consumed controlled diets for 3 days (Na^+ intake, 3314–3668 mg/day). Specifically, Na^+ intake in all meals, snacks, and drinks was 3314 to 3668 mg/day (3668, 3314, and 3387 mg/day on the first, second, and third day, respectively). On the fourth day and after fasting overnight, the first morning void was discarded. Participants then consumed 4 cups of water. Two hours later, a second urine

void was collected to assess urinary Na^+, endothelin-1, NO metabolites (nitrite and nitrate [NOx]), aldosterone, and norepinephrine. Na^+ intake in the current human study was based on National Health and Nutrition Examination Survey 2013–2016 data, which reported that Americans consume an average of 3361 mg of sodium per day.[14] Studies involving humans were performed with approval from the University of Alabama at Birmingham Institutional Review Board for Human Use and in accordance with the Declaration of Helsinki. Study participants provided written informed consent before inclusion in the study.

Statistical Analysis

Values are presented as mean±SEM in all figures and Table 1. Values in Table 2 are presented as mean±SD. Statistical tests used for each data set are specified in the figure legend. $P<0.05$ was considered significant.

Detailed methodology for all protocols used in this study, including metabolic cage experiments, telemetry, RNA sequencing and pathway analysis, are provided in Data S1. Table S1 lists all primary antibodies used for Western blotting in the present study.

RESULTS

Urinary Electrolyte Excretion

Under steady-state conditions, no sex differences in water or Na^+ intake or in urine electrolyte excretion or flow were observed in animals on an NS diet when normalized to body weight (Figure 1A through 1D, Figure S1A through S1C). Upon challenge with an HS diet, the urinary excretion rate of Na^+ increased gradually in males over the 5-day period, whereas the urinary excretion rate of Na^+ in females reached a maximum steady state on day 1 of the HS challenge (Figure 1B). Over the 5-day HS challenge, males and females reached a positive Na^+ balance of 87.2±22.2 and 61.3±9.9 mmol/kg, respectively (P=0.3). Water intake and urine flow increased significantly in female rats (Figure 1C and 1D). In males, increases in urine flow and water intake did not reach statistical significance (Figure 1C and 1D). On day 1 of the HS, the difference between water intake and urine output was 20.3±0.9 and 15.7±2.6 mL/day in males and females, respectively (P=0.1). The urinary excretion rate of K^+ did not change in either sex during the HS challenge, whereas the urinary excretion rate of Cl^- in both sexes increased in a pattern similar to that of the urinary excretion rate of Na^+ (Figure S1B and S1C).

Circulating Electrolyte Concentrations

In male rats, the serum Na^+ concentration increased on day 1 of the HS challenge and remained high at day 5 (Figure 1E). In contrast, the serum Na^+ concentration in female rats did not change during the HS challenge (Figure 1F). Serum K^+ concentration increased slightly in both sexes on day 5 of HS (Figure S1D and S1E). Serum Cl^- concentration tended to increase during the HS challenge in male rats similar to serum Na^+ (Figure S1F and S1G) but did not reach statistical significance (P=0.06 and P=0.07 at day 1 and day 5, respectively; Figure S1F). Serum Cl^- concentration did not change in females during the HS challenge.

Blood Pressure

Consistent with previous reports,[2,15] mean arterial pressure was lower in female rats than in male rats during the NS diet phase (Figure S2) and mean arterial pressure did not change in either sex during the HS challenge compared with baseline values (Figure S2).

Table 1. Sex Differences in Na^+-Regulatory Factors During Acclimation to an HS Diet Challenge

Urinary excretion rate	Males (n=5–8)			Females (n=6–11)			ANOVA results
	NS	Day 1 HS	Day 5 HS	NS	Day 1 HS	Day 5 HS	
Endothelin-1, pg/day per kg	5.6±0.8	10.2±0.5*	10.6±0.7*	11.8±3.9†	14.6±2.0	14.9±2.3	$P_{interaction}$=0.9 P_{diet}=0.2 P_{sex}=0.006
NOx, µmol/day per kg	22.5±1.0	24.6±1.8	7.7±0.7*	20.1±3.5	32.7±4.7*	7.8±0.7*	$P_{interaction}$=0.08 P_{diet}<0.0001 P_{sex}=0.5
Aldosterone, µg/day per kg	14.1±2.3	5.8±0.9*	4.3±0.6*	17.3±2.9	13.3±1.5	5.3±0.7*	$P_{interaction}$=0.4 P_{diet}<0.0001 P_{sex}=0.18
Norepinephrine, µg/day per kg	5.2±0.3	4.6±0.2	4.9±0.5	4.0±1.0	6.8±1.1	5.9±1.1	$P_{interaction}$=0.1 P_{diet}=0.4 P_{sex}=0.4

Statistical comparisons were performed by repeated-measures 2-way ANOVA with Sidak's post hoc test for multiple comparisons. HS, high salt; and NS, normal salt.

*$P<0.05$ vs corresponding NS values.

†$P<0.05$ vs corresponding male values.

Table 2. Characteristics of Human Subjects

	Men (n=9–11)	Women (n=12–14)	
	Mean±SD	Mean±SD	*P* values
Age, y	34.9±7.7	32.6±10.3	0.5
Weight, kg	85.9±11.2	66.1±9.9*	0.001
Body mass index, kg/m^2	27.5±2.9	24.4±4.1*	0.04
Urine flow, mL/h per kg	0.5±0.2	0.6±0.3	0.2
UNaV, µmol/h per kg	26.6±22.1	33.6±23.6	0.7

Statistical comparisons were performed by unpaired Student *t* test. $U_{Na}V$ indicates urinary excretion of Na^+.
*P<0.05 vs corresponding values in men.

Body Weight and Total Body Water

During the NS phase, males and females weighed 406±3 and 247±3 g, respectively. Body weight did not change in either sex during the HS challenge. However, total body water relative to body weight increased in male rats on day 1 of the HS challenge (from 71.8±0.3% to 72.6±0.4%; P=0.02) and remained elevated on day 5 (72.3±0.4%; P=0.03). In contrast, total body water relative to body weight did not change significantly in females during the HS challenge (NS, 70.6±0.7%; day 1 HS, 71.2±0.8%; day 5 HS, 71.2±0.8%; P=0.3). No sex-related differences were observed in total body water relative to body weight.

Skin and Muscle Na^+

Interestingly, recent findings suggest that skin is also a site of Na^+ storage and serves as an extrarenal contributor to the maintenance of Na^+ homeostasis and blood pressure regulation as well.[16,17] In the present study, skin Na^+ concentration in male rats did not change during the HS challenge (Figure 1G). A slight but nonsignificant decline in skin Na^+ concentration occurred in female rats on day 1 of the HS challenge (P=0.07; Figure 1H). Neither sex- nor diet-related differences were observed in muscle Na^+ concentrations on day 1 of HS (Figure 2A and 2B).

Creatinine Clearance

To evaluate the effects of HS on kidney function, we determined creatinine clearance as an estimate of glomerular filtration rate. Creatinine clearance increased in both sexes on day 1 of the HS challenge, and this increase was maintained at day 5 (Figure 2C and 2D).

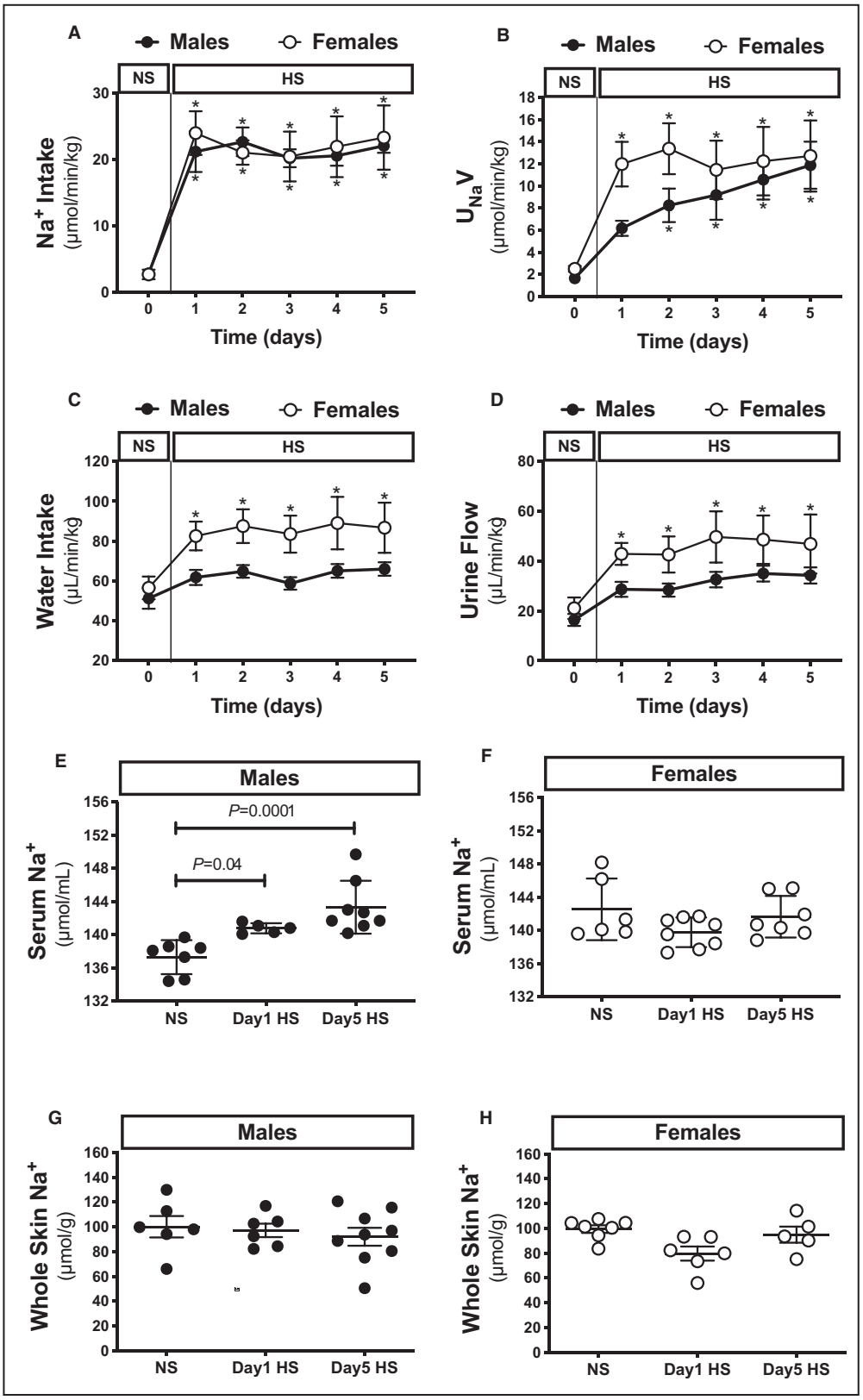
Kidney Na^+ Transporters

The actions of Na^+ transporters along the nephron determine tubular Na^+ reabsorption and, consequently, Na^+ excretion by the kidney. To assess the potential involvement of these transporters in mediating the sex differences in HS-induced natriuresis, we determined the protein abundance of Na^+/Cl^- cotransporter (NCC; total and phosphorylated), Na^+/H^+ exchanger isoform 3 (NHE3; total and phosphorylated), epithelial Na^+ channel α subunit, and Na^+, K^+-ATPase α subunit in renal cortices from rats on NS and day 1 of HS (the time at which sex differences in HS-induced natriuresis were most apparent). The abundance of total NCC and phosphorylated NCC were higher in female rats than in male rats, regardless of diet (Figure 3A and 3B). Total NHE3 was higher in females compared with males on an NS diet. However, neither sex- nor diet-related differences were observed in the ratio of phosphorylated NCC to total NCC, the abundance of phosphorylated NHE3 or Na^+, K^+-ATPase α subunit, or the ratio of phosphorylated NHE3 to total NHE3 (Figure 3A through 3E).

Urinary Excretion of Natriuretic/ Antinatriuretic Factors

To assess sex differences in the renal endothelin-1 pathway during the HS challenge, we measured the 24-hour excretion rate of endothelin-1 and NOx) in

Figure 1. Sex differences in the natriuretic response to a HS diet challenge in rats.
Na^+ intake (**A**), $U_{Na}V$ (**B**), water intake (**C**), and urine flow (**D**) in male and female Sprague Dawley rats during the NS diet phase or the HS challenge. Serum levels of Na^+ (**E** and **F**) and whole skin Na^+ (**G** and **H**) relative to dry skin weight in male and female rats during the NS diet phase or on days 1 and 5 of the HS diet challenge (n=5–8 rats in each group). Statistical comparisons were performed by repeated measures 2-way ANOVA with Sidak's post hoc test for multiple comparisons (**A** through **D**) or two-way ANOVA with Sidak's post hoc test for multiple comparisons (**E** through **H**). *P<0.05 vs corresponding NS values. ANOVA results: Na^+ intake: $P_{interaction}$=0.9, P_{time}<0.0001, P_{sex}=0.8; $U_{Na}V$: $P_{interaction}$=0.5, P_{time}<0.0001, P_{sex}=0.2; water intake: $P_{interaction}$=0.6, P_{time}=0.002, P_{sex}=0.01; urine flow: $P_{interaction}$=0.9, P_{time}=0.001, P_{sex}=0.03; serum Na^+: $P_{interaction}$=0.002, P_{diet}=0.02, P_{sex}=0.3; skin Na^+: $P_{interaction}$=0.3, P_{time}=0.2, P_{sex}=0.3. HS indicates high salt; NS, normal salt; and $U_{Na}V$, urinary excretion of Na^+.



rats during the NS diet phase and on days 1 and 5 of HS. Among other factors involved in determining the natriuretic response to an HS diet, it is clear that reduced renal sympathetic activity (primarily mediated by norepinephrine) and aldosterone signaling inhibit natriuresis, whereas ANP (atrial natriuretic peptide) promotes natriuresis. Thus, we also measured the 24-hour excretion rate of aldosterone and norepinephrine

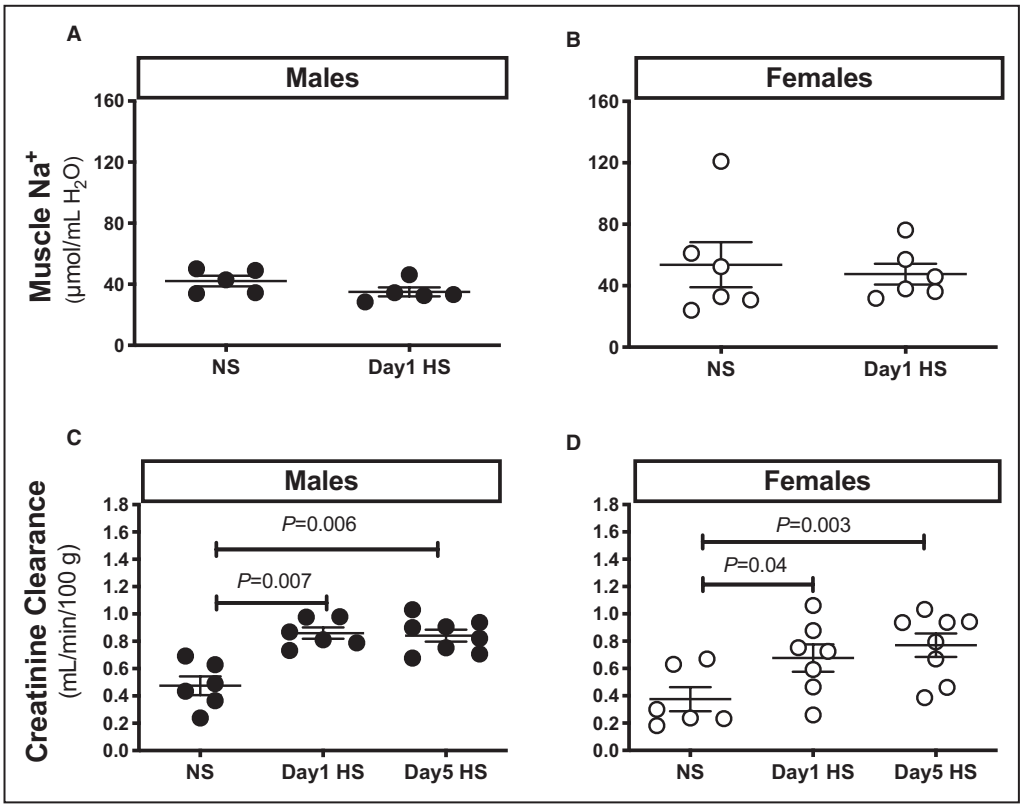


Figure 2. Muscle Na^+ and creatinine clearance during the HS diet challenge.
Ratio of muscle Na^+ to water (**A** and **B**) and creatinine clearance (**C** and **D**) in male and female Sprague Dawley rats during the NS diet phase or on day 1 of the HS challenge (n=5–8 rats in each group). Statistical comparisons were performed by 2-way ANOVA with Sidak's post hoc test for multiple comparisons. ANOVA results: muscle Na^+: $P_{interaction}$=0.9, P_{time}=0.5, P_{sex}=0.2; creatinine clearance: $P_{interaction}$=0.7, P_{time}<0.0001, P_{sex}=0.07. HS indicates high salt; and NS, normal salt.

and serum ANP during the NS and HS diet phases. Interestingly, the urinary excretion rate of endothelin-1 was higher in females than in males during the NS diet phase (Table 1). No sex differences were observed in the urinary excretion rate of NOx, aldosterone, or norepinephrine (Table 1) or in the serum ANP concentration under NS diet conditions (males, 0.12±0.04; females, 0.10±0.04 ng/mL).

In males, the urinary excretion rate of endothelin-1 increased on day 1 of HS, and this change was maintained on day 5 (Table 1). In contrast, the urinary excretion rate of endothelin-1 did not change in females during the HS challenge (Table 1). The urinary excretion rate of NOx increased in females only on day 1 of HS. In both sexes, however, this rate decreased on day 5 of HS, such that it was significantly lower than the rate during the NS diet phase (Table 1). The urinary excretion rate of aldosterone decreased in males on day 1 of the HS challenge, and this decrease was maintained on day 5 (Table 1). However, the urinary excretion rate of aldosterone decreased in females only on day 5 of the HS challenge (Table 1). Neither the urinary excretion rate of norepinephrine (Table 1) nor the serum ANP concentration changed on day 1 of the HS challenge in either sex (males, 0.14±0.04; females, 0.11±0.03 ng/mL).

RNA Sequencing and Ingenuity Pathway Analysis

Inner medullae, which have an important role in the fine-tuning of urinary Na^+ excretion, were collected from NS-fed rats and subjected to RNA sequencing. Raw data files, a read summary with mapping rate, and a summary file of transcripts and statistical results from RNA sequencing have been deposited in the Gene Expression Omnibus database under the accession number GSE136387. A list of transcripts that met the following criteria were uploaded to Ingenuity Pathway Analysis (IPA; Qiagen) for core analysis: (1) detected in both male and female samples, and (2) identified to be differentially expressed by Cufflinks or at least a 1.5-fold different between the sexes. Transcripts meeting these criteria are represented by color-coded points in Figure S3. Of the 16 061 annotated transcripts detected in the RNA sequencing analysis, 3.43% (551)

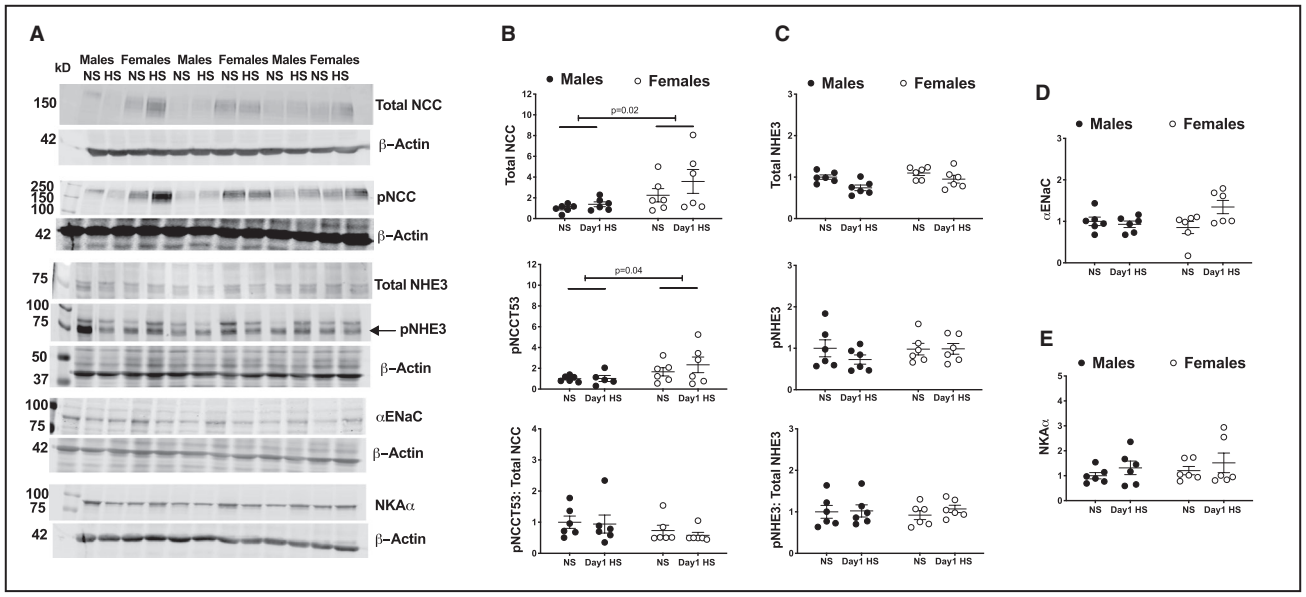


Figure 3. Na⁺ transporter protein expression during the HS diet challenge.
Representative Western blots of Na^+ transporters and loading controls are presented (**A**). Protein abundance of total NCC, pNCC, and the ratio thereof (**B**), total NHE3 and pNHE3, and the ratio thereof (**C**), αENaC (**D**) and NKAα (**E**), in renal cortices from male and female Sprague Dawley rats during the NS diet phase or on day 1 of the HS challenge (protein abundance is presented relative to corresponding levels in male NS rats (n=6 rats in each group). Statistical comparisons were performed by 2-way ANOVA with Sidak's post hoc test for multiple comparisons. ANOVA results: total NCC: $P_{interaction}$=0.5, P_{diet}=0.2, P_{sex}=0.02; pNCC: $P_{interaction}$=0.5, P_{diet}=0.2, P_{sex}=0.04; pNCC: total NCC: $P_{interaction}$=0.8, P_{diet}=0.6, P_{sex}=0.1; total NHE3: $P_{interaction}$=0.4, P_{diet}=0.01, P_{sex}=0.04; pNHE3: $P_{interaction}$=0.4, P_{diet}=0.4, P_{sex}=0.4; pNHE3: total NHE3: $P_{interaction}$=0.6, P_{diet}=0.5, P_{sex}=0.9; αENaC: $P_{interaction}$=0.04, P_{diet}=0.1, P_{sex}=0.3; NKAα: $P_{interaction}$=0.9, P_{diet}=0.2, P_{sex}=0.4. αENaC indicates epithelial Na^+ channel α subunit HS, high salt; NCC, Na^+/Cl^- cotransporter; NHE3, Na^+/H^+ exchanger isoform 3; NKAα, Na^+, K^+-ATPase α; NS: normal salt; pNCC, phosphorylated Na^+/Cl^- cotransporter; and pNHE3, phosphorylated Na^+/H^+ exchanger isoform 3.

were exclusively expressed in females, 11.10% (1782) were expressed in both sexes but significantly higher 'and' or 'or' 1.5-fold higher in females, 5.32% (855) were expressed in both sexes but significantly higher 'and' or 'or' 1.5-fold higher in males, 2.37% (381) were exclusively expressed in males, and 77.78% (12 492) appeared to be similarly expressed in both sexes (Figure 4A).

The signaling pathways with the most differentially expressed genes were ranked by –log (*P* value) in the IPA software and are reported in Table S2. The top 10 highly differentially expressed genes between sexes in the renal inner medullary transcriptome are listed in Table S3. Figure 4B presents changes in transcripts found within enriched IPA pathways that have an established role in natriuretic function, including "endothelin signaling," "NO synthase 3 signaling," and "NO signaling in the cardiovascular system." These pathways were predicted to be activated (positive *z* score) in females relative to males (Figure 4B). Other pathways that were enriched in females relative to males include G-protein coupled receptor signaling; cAMP-mediated signaling; calcium signaling; phospholipases; and interleukin-8, -9, and -10 signaling (Table S2). IPA identified an enrichment of transcripts of the endothelin-1 signaling pathway in females relative to males (*P*=0.0003), with 34 of 187 pathway transcripts being differentially expressed or with at least a 1.5-fold change in abundance between sexes (Figure 4B).

Renal Endothelin-1

Of the 34 transcripts in the endothelin-1 signaling pathway that were differentially expressed in a sex-dependent manner, 19 transcripts were experimentally determined to be involved in the endothelin-1 production/signaling cascade in IMCD cells[5] (Figure 4C). As shown, 16 of the 19 (84.2%) transcripts involved in the endothelin-1 natriuretic pathway in IMCD cells were enriched in inner medullae from females compared with those of males (Figure 4C). Of note, inner medulla tissue contains the highest concentration of endothelin-1 and ET_B in the body.[11]

Endothelin-1 evokes natriuresis primarily via activation of the ET_A and ET_B receptors.[8,12,18] Thus, we measured ET_A and ET_B receptor expression in the cortex and inner and outer medulla of the kidneys from rats on the NS diet or day 1 of the HS challenge. Neither ET_A nor ET_B mRNA expression changed significantly in the renal cortical (Figure 5A through 5D) or inner medullary (Figure 5I through 5L) tissues of either sex upon HS challenge. However, ET_B receptor expression increased within the outer medullae in males (Figure 5G),

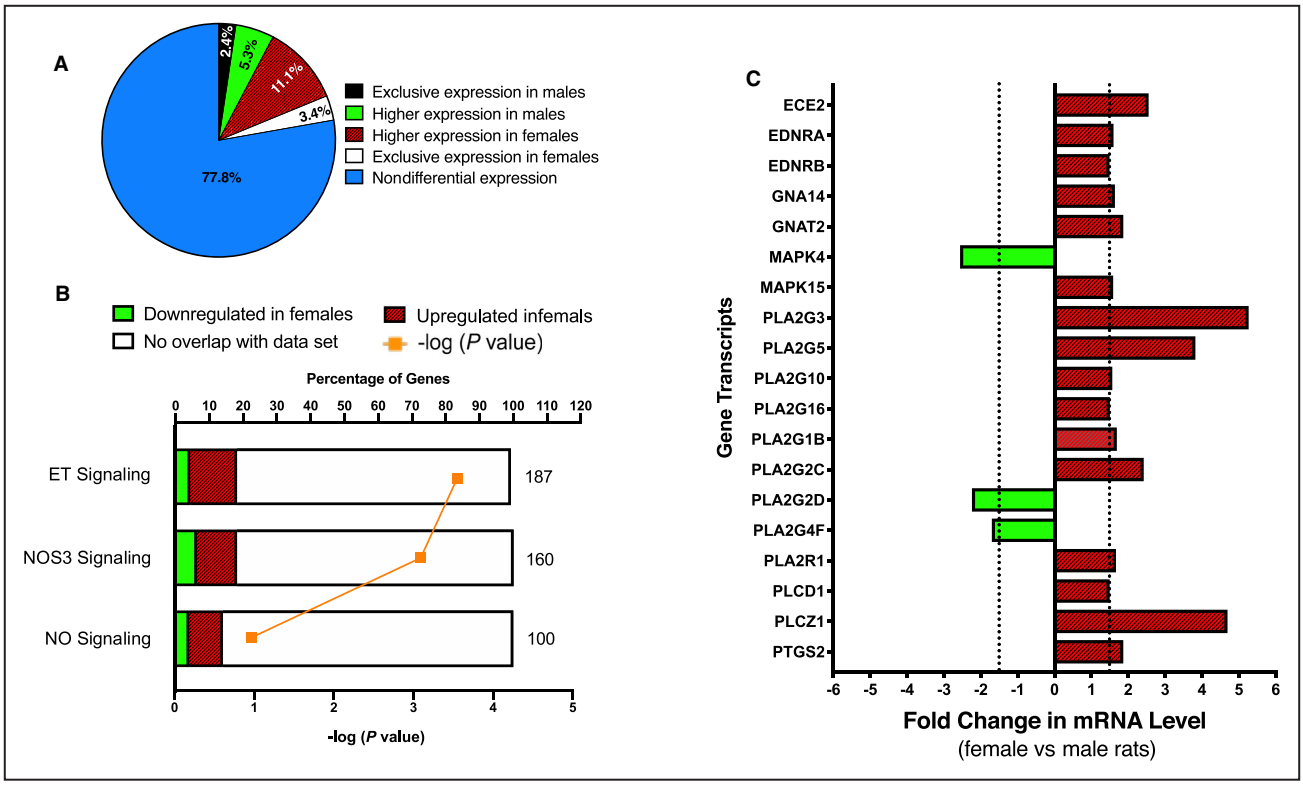


Figure 4. Sex differences in endothelin-1 signaling pathway activation in the inner medulla under NS conditions. Inner medullae were collected from NS-fed Sprague Dawley rats (n=8/sex) and pooled (n=4 rats/pool/group) by sex for RNA sequencing analysis and subsequent IPA. Graphical representation of the sex differences in the renal inner medullary transcriptome (**A**). Graphical representation of the sex differences in transcript expression found within enriched IPA pathways with established roles in natriuresis (ET signaling, NOS3 signaling, and NO signaling in the cardiovascular system) (**B**). Pathways are ranked by –log (*P* value). The total number of genes found within each pathway is shown on the right of the respective bar. Graphical represenation of the relative abundance of inner medullary gene transcripts involved in the endothelin-1 natriuretic signaling pathway in male and female rats (**C**). Gene transcripts that were differentially expressed and/or altered by at least 1.5-fold in female rats relative to males are shown. IPA indicates Ingenuity Pathway Analysis; NOS, nitric oxide synthase; and NS, normal salt.

whereas ET_A receptor expression increased within the outer medullae of females upon HS challenge (Figure 5F).

Kidney Interleukin-1β

Given that interleukin-1β stimulates renal endothelin-1 production in vivo and in vitro,[19–21] we also determined sex and dietary effects on renal interleukin-1β expression. Immunohistochemical analysis of tissues obtained from rats during the NS diet phase showed the expression of interleukin-1β tended to be elevated in the renal cortex of females relative to that of males (Figure 6A), although interleukin-1β localization in the kidney was similar in both sexes. Within the cortex of NS-fed rats, mesangial cells in the glomeruli and cortical brush border stained positive for interleukin-1β (Figure 6A). ELISA measurements revealed that renal cortical interleukin-1β expression significantly increased on day 1 of the HS challenge in males but not in females (Figure 6B). No diet-induced differences in interleukin-1β expression were observed in the inner medulla, when compared with corresponding NS values (Figure 6C). A sex difference in inner medullary interleukin-1β expression was observed (Figure 6C). However, male rats had exaggerated cortical interleukin-1β expression at the brush border and generalized staining in the tubular cell cytoplasm on day 1 of the HS challenge. In the females, tubular staining of interleukin-1β at day 1 was limited to the brush border, at levels similar to those observed on the NS diet, and punctate staining within the cortical proximal tubules.

Na^+ Regulatory Factors in Humans

To assess the relevance of our findings in humans, we measured the urinary excretion rate of endothelin-1, NOx, aldosterone, and norepinephrine in urine specimens collected from subjects maintained on an Na^+-controlled diet. Subject characteristics are provided in Table 2. Of note, women excreted significantly more endothelin-1 than men (Figure 7A).

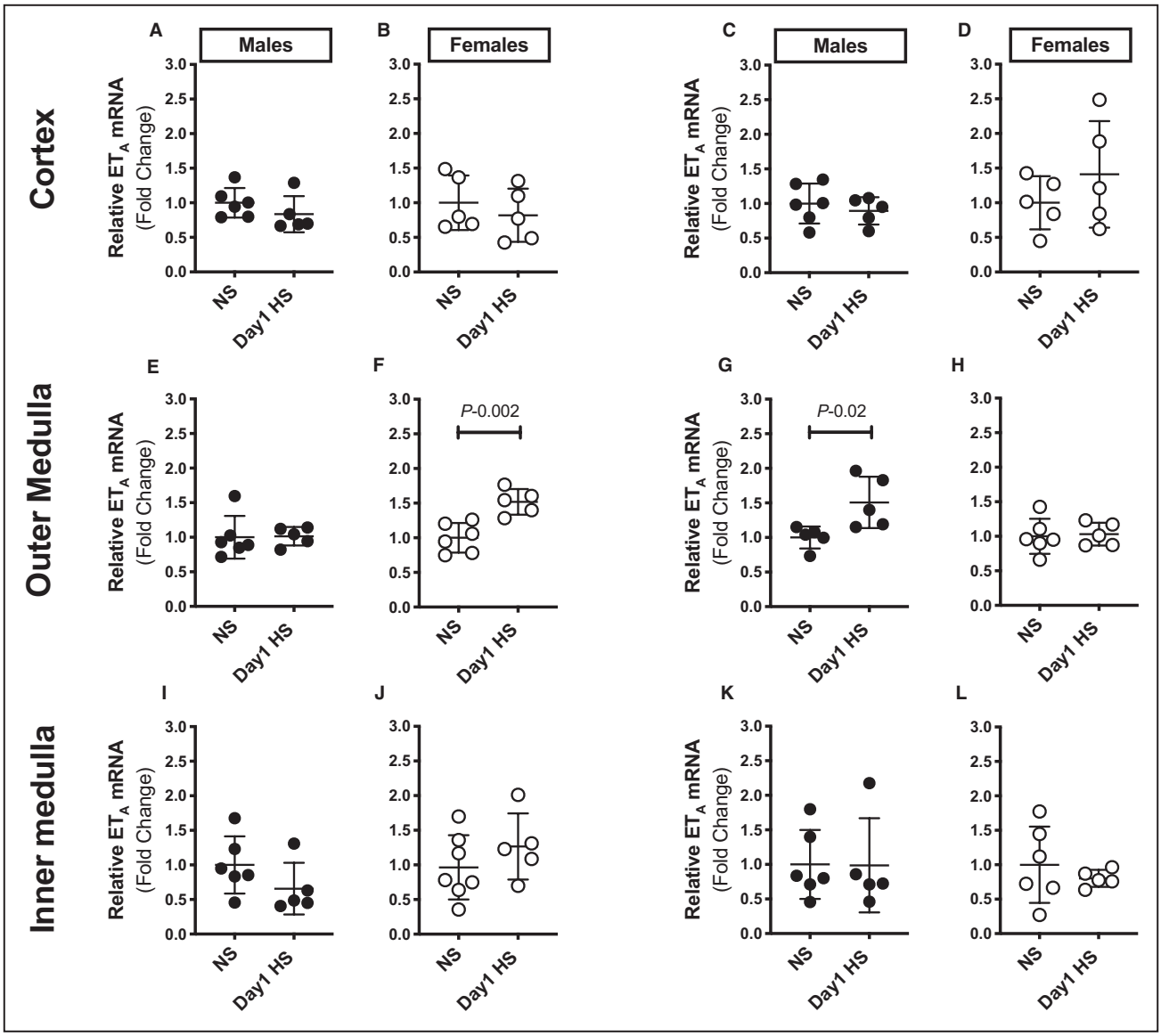


Figure 5. HS diet challenge increases renal outer medullary endothelin receptor expression in a sex-dependent manner. mRNA expression of cortical (**A** through **D**), outer medullary (**E** through **H**), and inner medullary (**I** through **L**) ET_A and ET_B receptors in male and female Sprague Dawley rats during the NS diet phase or on day 1 of the HS diet challenge (n=5–6 rats in each group). Gene expression values represent the fold change from corresponding NS levels. Statistical comparisons were performed by unpaired Student *t* test. $P<0.05$ vs corresponding NS values. ET_A indicates endothelin receptor subtype A; ET_B, endothelin receptor subtype B; HS, high salt; and NS, normal salt.

No sex differences were observed in the urinary excretion of NOx, aldosterone, or norepinephrine (Figure 7B through 7D).

DISCUSSION

A central dogma in kidney physiology is that it takes the body ≈3 days to reach steady-state urinary Na^+ excretion, where Na^+ intake matches excretion.[1] Consistent with this idea, we observed that urinary Na^+ excretion in male rats increases gradually in response to the induction of an HS diet, reaching a new steady state in 3 to 5 days. In striking contrast, female rats elicited robust increases in urinary Na^+ excretion on day 1 of HS. This observation indicates that females are primed to handle salt challenges more efficiently than males, which is in line with recent data.[3] Similar to our findings in rats, a previous clinical study found that the magnitude of the increase in urinary Na^+ excretion during hypertonic saline infusion is lower in men than in women.[4]

In the current rat study, HS-induced water intake and urine output was more pronounced in females. Dickinson et al[22] reported similar results in spiny mice fed an HS diet for 7 days. Supporting these results,

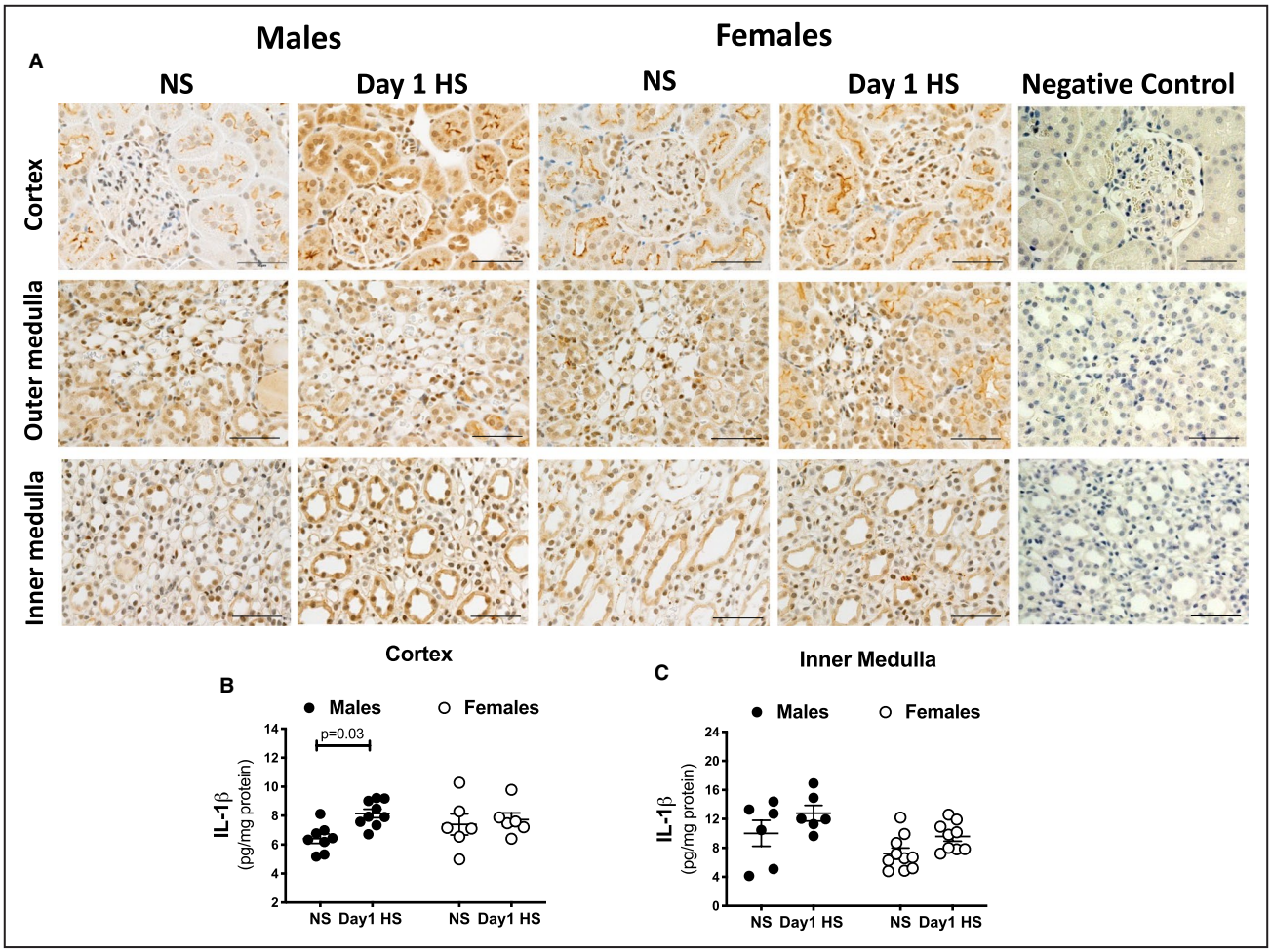


Figure 6. Renal cortical interleukin-1β expression during the HS diet challenge.
Representative images of IL-1β protein expression in the renal cortex and outer and inner medulla of male and female Sprague Dawley rats during the NS diet phase or on day 1 of the HS challenge (n=5–6 rats in each group; scale bar, 20 μm). Negative control images are represented (**A**). Protein levels of IL-1β measured by ELISA in renal cortex (**B**) and inner medulla (**C**) of male and female Sprague Dawley rats during the NS diet phase or on day 1 of the HS challenge (n=6–10 rats in each group). Brown color corresponds to IL-1β-positive staining. Statistical comparisons were performed by two-way ANOVA with Sidak's post hoc test for multiple comparisons. ANOVA results: cortex: $P_{interaction}$=0.1, P_{diet}=0.03, P_{sex}=0.5; inner medulla: $P_{interaction}$=0.8, P_{diet}=0.02, P_{sex}=0.008. HS indicates high salt; IL, interleukin; and NS, normal salt.

sex and sex hormones have been shown to regulate osmoreceptors that control thirst.[4,23–26] It has also been shown that estrogen supplementation to ovariectomized rats increases water intake and urine flow.[27] Notably, pretreatment with angiotensin II decreased water intake in male but not female rats.[28] Central interactions between estrogen and angiotensin II control the drinking behavior in rats.[29,30] The specific mechanism underlying the sex difference in water intake in the present studies remains to be determined, but sex hormonal–induced differences in the dipsogenic potential of angiotensin II is a reasonable target for future studies.

The HS-induced augmentation in total body water observed only in male rats may appear paradoxical with respect to their relatively lower water intake. However, the difference between water intake and urine output being ≈4.6 mL higher in males versus females on day 1 of the HS would provide a proper explanation for the observed ≈1% increase in total body water relative to body weight in males (body weight average, 406 g). Consistent with our findings, previous work showed that total body water does not increase in female rats in response to 1% NaCl in drinking water.[31]

Our observation that serum Na^+ concentration in female rats remained unchanged while males had an increase during acclimation to HS suggests that females have a greater capacity for maintaining circulating levels of Na^+. Of note, postprandial increases in

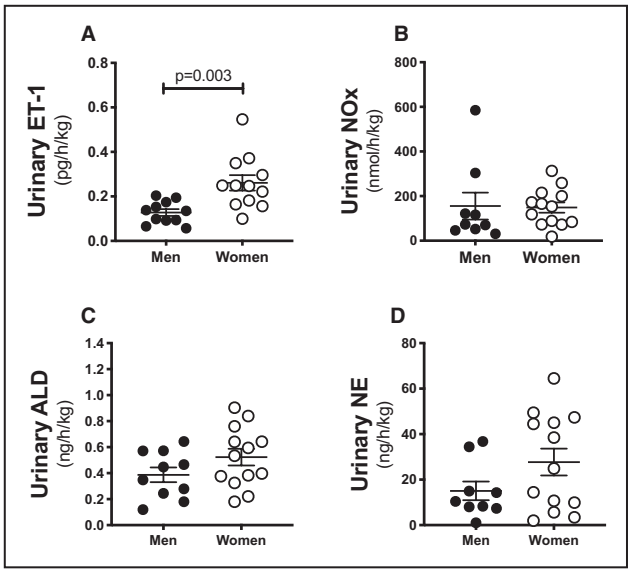


Figure 7. Women have higher urinary endothelin-1 levels than men.
Urinary excretion of endothelin-1 (**A**) nitrite/nitrate (NOx) (**B**), ALD (**C**), and NE (**D**) in healthy men and women (n=9–13 subjects in each group). Statistical comparison was performed by unpaired Student *t* test. ET-1 indicates endothelin-1; ALD indicates aldosterone; and NE, norepinephrine.

circulating levels of Na^+ impair NO-dependent vasodilation in healthy subjects.[32] The elevation in circulating Na^+ that we observed in males only suggests that males may be more susceptible to salt-related complications during acclimation to salt challenges. Earlier reports demonstrated that plasma obtained from salt-loaded subjects inhibited erythrocyte $Na^+/K^+/Cl^-$ cotransport,[33] which may contribute to alterations in plasma electrolytes.

We did not observe any effect on Na^+ transporter expression in the kidney cortex on day 1 of HS. However, the abundance of total and phosphorylated NCC was higher in the kidneys of females than in those of males, which is consistent with recently published findings.[3] Assuming that abundance is proportional to activity, these sex differences appear to be opposite to what would be presumed. However, control of transporter activity is complex and has many levels of regulation. Additional studies are warranted to determine whether sex differences in the regional distribution of Na^+ transporters[3] and transporter trafficking may contribute to acclimation to HS. Recently, Tahaei and colleagues[34] revealed that the distal convoluted tubule of female mice expresses a greater density of NCC in a shorter distal convoluted tubule structure. Interestingly, the female distal convoluted tubule has greater structural remodeling capacity to elongate in response to loop diuretics.[34] Altogether, it is clear that the female kidney is equipped with unique mechanisms to adapt to unique physiological challenges.

Upon HS challenge, the rate of urinary endothelin-1 excretion increased in male rats only, consistent with previous reports of HS diets increasing renal endothelin-1 production and activity.[35–37] Our RNA sequencing data demonstrated enrichment in the endothelin-1 signaling pathway in female rats. In addition, the rate of urinary endothelin-1 excretion was higher in female rats than in males. Our study does not conclusively demonstrate that kidney endothelin-1 signaling is the primary cause for the sex differences in HS-induced natriuresis but does show that the kidney endothelin-1 system is highly activated under basal conditions in females. The differential HS-induced overexpression of outer medullary ET_B in males and ET_A in females is interesting and suggests a female-specific contribution of ET_A in the natriuretic response to salt, which aligns with previous studies.[8] Furthermore, ET_A and ET_B can work cooperatively to facilitate Na^+ and water excretion.[8,36,38,39] Future studies are required to determine the effect of salt on renal ET receptor protein abundance and localization.

Since the NO pathway is known to contribute to endothelin-1–induced natriuresis, we also measured the excretion of urinary NOx as a measure of NO production. The increase in urinary NOx excretion in female rats on day 1 of HS may provide an explanation for the more rapid HS-induced natriuresis observed in females. IPA also revealed an enrichment of NO and nitric oxide synthase 3 signaling pathways in inner medullae from female rats. Importantly, it has been shown that high levels of urinary NOx occur upon collecting duct endothelin-1 production.[37] This may suggest that endothelin-1 and NO signaling pathways work cooperatively to prime efficient natriuresis in females.

Control of natriuresis is complex and involves numerous signaling pathways in several systems. We postulate that sex steroids, possibly ovarian hormones, facilitate HS-induced natriuresis. Indeed, salt sensitivity is reported to be increased after menopause.[40] Ovariectomy has been shown to exacerbate salt sensitivity in Dahl salt-sensitive rats.[41] This is relevant to our recent study demonstrating a female-specific natriuretic response to activation of G protein–coupled estrogen receptors.[38,42] Therefore, it is possible, and perhaps likely, that additional pathways are involved in the enhanced efficiency of females to handle dietary Na^+ challenges. Although we did not detect sex-specific differences in aldosterone, norepinephrine, or NO excretion when rats were maintained on a NS diet, the contribution of sex differences in the downstream signaling cascades for these Na^+ regulatory factors in sex differences in acclimation to HS remains to be determined. Earlier studies have reported changes in urinary excretion of aldosterone and norepinephrine during the estrus cycle in sheep and rats, respectively.[43–45] In addition, ET_A and ET_B mRNA expression and

nitric oxide synthase activity in female rat kidneys are regulated by estradiol.[46,47] Accordingly, we anticipate that the increased variability in the urinary excretion levels of Na^+-regulatory factors in female rats would be related to different phases of the estrus cycle.

The inflammatory cytokine interleukin-1β has been shown to provoke endothelin-1 production in various cell types, including kidney epithelial cells,[20] and treatment of mouse IMCD-3 cells with interleukin-1β has been shown to promote endothelin-1 release, at least partially, via activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells).[21] Furthermore, Boesen et al[21] demonstrated that systemic infusion of interleukin-1β promotes urinary endothelin-1 excretion in male rats. We did not observe a greater interleukin-1β expression in the female kidney of NS-fed rats, suggesting that the interleukin-1β/endothelin-1 axis may not be as important in females as in males. This may be related to potential sex differences in other factors that regulate endothelin-1 such as the transcription factor tonicity-responsive enhancer-binding protein.[48,49] The increase in cortical interleukin-1β on day 1 of the HS in male rats is well poised to mediate the male-specific increase in urinary endothelin-1 excretion observed at this time point.

Data from human studies have indicated a significant correlation between urinary endothelin-1, NOx, and natriuresis.[50] Further studies suggested that impairments in the kidney endothelin-1 system may contribute to the development of essential hypertension and salt sensitivity.[51–53] Interestingly, it has been shown that patients with essential hypertension have reduced urinary endothelin-1 excretion.[52] An early study revealed that salt-sensitive men and women have lower urinary endothelin-1 excretion than their salt-resistant counterparts.[52] Despite extensive evidence of sex differences in the kidney endothelin-1 pathway in animal studies, little is known about the role of kidney endothelin-1 in regulating renal Na^+ handling in men and women. Correlating with results from our animal studies, we discovered that women excrete more endothelin-1 than men. This is consistent with the hypothesis that women are primed to better handle acute challenges to dietary sodium intake.

CONCLUSIONS

In conclusion, there are clear sex differences in the mechanisms involved in maintaining fluid and electrolyte homeostasis, and women have an efficient capacity to manage dietary Na^+ challenges. Defining of these mechanistic pathways may lead to the development of sex-specific therapies for salt-sensitive hypertension and other salt-related health complications. Given that an HS diet is one of the major risk factors for the development of hypertension, the enhanced capacity of women to handle salt challenges may contribute to the female protection against hypertension during their premenopausal age, compared with postmenopausal women.

ARTICLE INFORMATION

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Affiliations

Section of Cardio-Renal Physiology & Medicine, Division of Nephrology, Department of Medicine, University of Alabama at Birmingham, AL (E.Y.G., C.D.M., I.E.O., E.M.D., K.A.H., B.K.B., C.J., R.S., J.G.J., J.S.P., D.M.P.); Department of Physiology, Medical College of Wisconsin, Milwaukee, WI (P.L., A.J.K.); Department of Physiology, University of Mississippi Medical Center, Jackson, MS (J.S.S.); Department of Urology, University of Alabama at Birmingham, AL (T.M.); and Now with Division of Nephrology and Hypertension, Vanderbilt University Medical Center, Nashville, TN (E.Y.G.).

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Disclosures

Dr Gohar is also affiliated with the Department of Pharmacology and Toxicology, Faculty of Pharmacy, Alexandria University, Egypt. The remaining authors have no disclosures to report.

Supplemental Material

Data S1
Tables S1–S3
Figures S1–S3
References 54–71

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SUPPLEMENTAL MATERIAL

Data S1.

Supplemental Methods

Metabolic cages. Animals were placed into metabolic cages 48 h before daily quantitative urine collections and measurements of food and water intake were performed. After basal 24-h urine collection, a nutrient-matched HS diet was introduced, and 24-h urine samples were collected daily for 5 consecutive days. Urine was centrifuged at 1000 × *g* for 5 min, aliquoted, and stored at −80°C until further analysis. At the end of the fifth day on the HS diet, rats (16-18 weeks old) were euthanized, and the blood, skin, and kidney tissues were harvested. Separate sets of animals were euthanized while maintained on a NS diet or on the first day of the HS diet to harvest blood, skin, gastrocnemius muscles, and kidney tissues. The kidney cortex, outer and inner medulla, whole body skin and gastrocnemius muscles were frozen in liquid nitrogen and stored at −80°C until molecular assays were performed.

Telemetry. Blood pressure measurements were made in a different cohort of animals using DSI PA-C40 transmitters (Data Sciences International, Duluth, MN) as previously detailed.[54] At the beginning of the 2-week NS diet, rats were anesthetized with 2% isoflurane, and telemetry transmitters were implanted with catheters inserted into the abdominal aorta. The catheter was secured in place with VetBond tissue glue (3M corporation, St. Paul, MN), the transmitter body was secured in place along the incision line, and the muscle layer was closed with sutures. Staples were used to close the skin. Rats were allowed to recover for at least 10 days after surgery before the basal 24-h urine collection and subsequent HS challenge. Blood pressure was recorded for 10 sec once every 10 min throughout the study.

Body water measurement. In vivo body composition of rats was determined using an 2MHz Whole Body Composition Analyzer quantitative magnetic resonance (QMR) machine (Echo Medical Systems, Houston, TX), as previously validated.[55] Rats were weighed and then placed into a clear Perspex tube, allowing constant airflow, and room for the rat to turn around. Total body water measurements were obtained at different time points in the same animals. Data were calculated relative to body weight and presented as percentage.

Ashing. As previously described,[56] whole body skin and gastrocnemius muscles were frozen at −80°C until processed. Samples were then weighed and placed in a drying oven at 80°C for 96 h. Water content was determined by subtracting the dry weight from the wet weight. Samples were ashed by heating to 150°C, 250°C, and 350°C in 6-h increments, followed by 24 h at 450°C. Finally, samples were held at 600°C for 20 h, and the ash was then dissolved in 5% nitric acid.

Measurement of electrolytes. Urinary and serum electrolytes were measured using an EasyLyte $Na^+/K^+/Cl^-$ analyzer (Medica, Bedford, MA). Skin and muscle electrolytes were measured using atomic absorption spectrometry (ICE 3000; Thermo Fisher Scientific, Waltham, MA).

Creatinine measurement. Plasma and urine creatinine levels were determined by non-derivitized, stable isotope dilution LC-MS/MS as previously described. [57] An Agilent (Wilmington, DE) Infinity 1260 LC and Infinity 1290 autosampler with a 6460 Triple Quad mass spectrometer were used.

Aldosterone (ALD) measurement. Urinary ALD was measured by immunoassay as previously described.[58]

Norepinephrine (NE) measurement. Urinary NE concentrations were measured using a Norepinephrine ELISA Fast Track kit (BA E-6200; Rocky Mountain Diagnostics, Colorado Springs, CO) according to the manufacturer's directions.

Nitrite and nitrate (**NOx) measurement.** Urinary NOx concentrations were analyzed using the E-NO 30 HPLC system (Eicom, Kyoto, Japan) as previously described.[59, 60]

ET-1 measurement. ET-1 concentrations were measured using the Quantiglo ET-1 ELISA kit (QET00B; R&D Systems, Minneapolis, MN) according to the manufacturer's directions.

Atrial natriuretic peptide (ANP) measurement. Serum ANP concentrations were measured using the Rat ANP ELISA Kit (ab108797, Abcam, Cambridge, MA) according to the manufacturer's directions.

Tissue RNA isolation, cDNA synthesis, and real time PCR. RNA was isolated from kidney tissue using the Purelink Mini RNA Extraction kit (12183018A, Thermo Fisher Scientific) according to the

manufacturer's instructions. RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Then, the isolated RNA was reverse transcribed using the QuantiTect Reverse Transcription kit (Qiagen). Finally, the resulting cDNA was used to quantify mRNA by RT-PCR (CFX96 Real-Time System, Bio-Rad, Hercules, CA using TaqMan primer gene expression assays with ET-1 (Rn00561129_m1), ET_A receptor (Rn00561137_m1), ET_B receptor (Rn00569139_m1) and β-Actin (Rn00667869_m1) primers. Gene expression was quantified relative to β-actin as a housekeeping gene using the $2^{\Delta\Delta Ct}$ method.

RNA sequencing and IPA. Total RNA was extracted from inner medullary tissues collected from NS-fed male and female rats (n=8/sex) following the Trizol method (Ambion), pooled (n=4 rats/pool) by sex and quantified using a NanoDrop2000 spectrometer (Thermo Fisher Scientific). Thus, we sequenced two pools of four samples per sex. RNA library construction for sequencing was performed using the Illumina TruSeq Stranded Total RNA Kit. Samples were multiplexed 8 per lane on a 400 Gb flow cell, pair-end sequenced with an Illumina HiSeq 2500. Before the analysis, adapter sequences were removed from the output sequence reads using cutadapt (http://code.google.com/p/cutadapt/). Reads with low base quality (<13) were further trimmed and, if less than 25 base pairs, removed by FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The trimmed sequence reads were then aligned to the rat reference genome and gene annotation database (rn5) using TopHat2 (v2.1.1) with mammalian default parameters.[61] Transcripts in all of the sequencing libraries were assembled by Stringtie.[62] Differential expression analysis was performed using Cufflinks (v2.2.1).[63] The Benjamini-Hochberg procedure was used for controlling false discovery rates in multiple comparisons. A total of 26,085 unique transcripts were identified through sequencing. A list of transcripts that met the following criteria were uploaded to IPA (Qiagen) for core analysis: 1) detected in both male and female samples, and 2) identified to be differentially expressed by Cufflinks or at least a 1.5-fold different between the

sexes. Transcripts meeting these criteria are represented by color-coded points in Supplemental Figure 3. A total of 2345 transcripts mapped to genes represented in the IPA for subsequent pathway analysis.

Interleukin-1β (IL-1β) measurement. Cortical and inner medullary regions of the kidney were homogenized, sonicated and centrifuged as previously described.[64] Protein concentration in the supernatant extract was determined using Quick Start Bradford 1× Dye Reagent (RLB00, Bio-Rad). IL-1β protein level in the supernatant extract was measured using rat-specific IL-1β Quantikine ELISA kits (RLB00, R&D Systems) according to the manufacturer's instructions. Measurements were normalized to total protein measured by Bradford assay.

Immunohistochemical staining. Kidneys were fixed and immunohistochemically stained as previously described [64]. Primary antibodies against IL-1β (ab9787, Abcam, Cambridge, MA) were used at 1:3000 dilution. DAB substrate (DAKO) was used to visualize the primary antibody.

Western blotting. Kidney cortical lysates were separated by SDS-PAGE and processed for Western blot analysis as previously described.[65] Primary antibodies against the sodium transporters and channels have previously been validated.[66-71] Supplemental Table 1 lists all primary antibodies used for Western blotting in the present study. Alexa Fluor 680 (A21109, Invitrogen, Waltham, MA) and Alexa Fluor 800 (A32730, Invitrogen) secondary antibodies were used at 1:1000 dilution except for those used to detect β-actin (1:10000). To quantify protein bands, densitometry was performed with a LI-COR Odyssey Image Studio (v5.2.5), and all values were normalized to the corresponding values in the male NS group.

Table S1. Western blotting antibody details.

Antibody Target	Source	Host	Clonality	Dilution factor	Expected molecular weight (kDa)	Validated and/or citation
ENaCα	AB3530P, Chemicon, Temecula, California	Rabbit	Polyclonal	1:1000	~100	[71]
NKAα	Developmental Studies Hybridoma Bank at the University of Iowa	Rouse	Monoclonal	1:1000	112	[66]
NCC	a kind gift from Dr. McDonough, University of Southern California	Rabbit	Polyclonal	1:1000	150	[67]
pNCCT53	a kind gift from Dr. Loffing, University of Zurich	Rabbit	Polyclonal	1:1000	150	[68]
NHE3	a kind gift from Dr. McDonough, University of Southern California	Rabbit	Polyclonal	1:1000	83	[69]
pNHE3S552	SC-53962, Santa Cruz Biotechnology, Dallas, TX	Mouse	Monoclonal	0.2 μg/ml	83	[70]
Actin	A1978, Sigma, St. Louis, MO	Mouse	Monoclonal	1:10000	42	

NHE3, Na^+/H^+ exchanger isoform 3; pNHE3, phosphorylated Na^+/H^+ exchanger isoform 3; αENaC, epithelial Na^+ channel α subunit; NKAα, Na^+, K^+-ATPase α.

Table S2. Pathways identified by Ingenuity Pathway Analysis to be enriched in transcripts with differential expression between males and females.

See Excel file.

Table S3. Top 10 highly differentially expressed genes between sexes in the renal inner medullary transcriptome.

Gene	Fold change (female over male)	P values
Adh1	53.3	5.00E-05
Slc22a7	39.6	5.00E-05
Mmp13	15.1	0.00045
Acsm1	12.8	0.00095
Car15	9.9	5.00E-05
Slc5a10	9.6	5.00E-05
Slc7a12	9.6	5.00E-05
Tmigd1	8.7	5.00E-05
Akr1c1	8.2	5.00E-05
Kynu	7.7	5.00E-05

Figure S1. Urine and serum electrolytes during the HS diet challenge. Food intake (A), urinary excretion of K^+ (U_KV) (B) and Cl^- ($U_{Cl}V$) (C), and serum K^+ (D, E) and Cl^- (F, G) in male and female SD rats during the NS diet phase or the HS challenge (n= 4-9 rats in each group). Statistical comparisons were performed by repeated measures two-way ANOVA with Sidak's post-hoc test for multiple comparisons (A-C) or two-way ANOVA with Sidak's post-hoc test for multiple comparisons (D-G). *P < 0.05 vs. corresponding NS values. ANOVA results: food intake: $P_{interaction} = 0.8$, $P_{time} = 0.9$, $P_{sex} = 0.8$; U_KV: $P_{interaction} = 0.03$, $P_{time} = 0.007$, $P_{sex} = 0.4$; $U_{Cl}V$: $P_{interaction} = 0.6$, $P_{time} < 0.0001$, $P_{sex} = 0.3$; serum K^+: $P_{interaction} = 0.9$, $P_{time} = 0.0003$, $P_{sex} = 0.09$; serum Cl^-: $P_{interaction} = 0.02$, $P_{time} = 0.7$, $P_{sex} = 0.006$. NS, normal salt; HS, high salt; SD, Sprague Dawley.

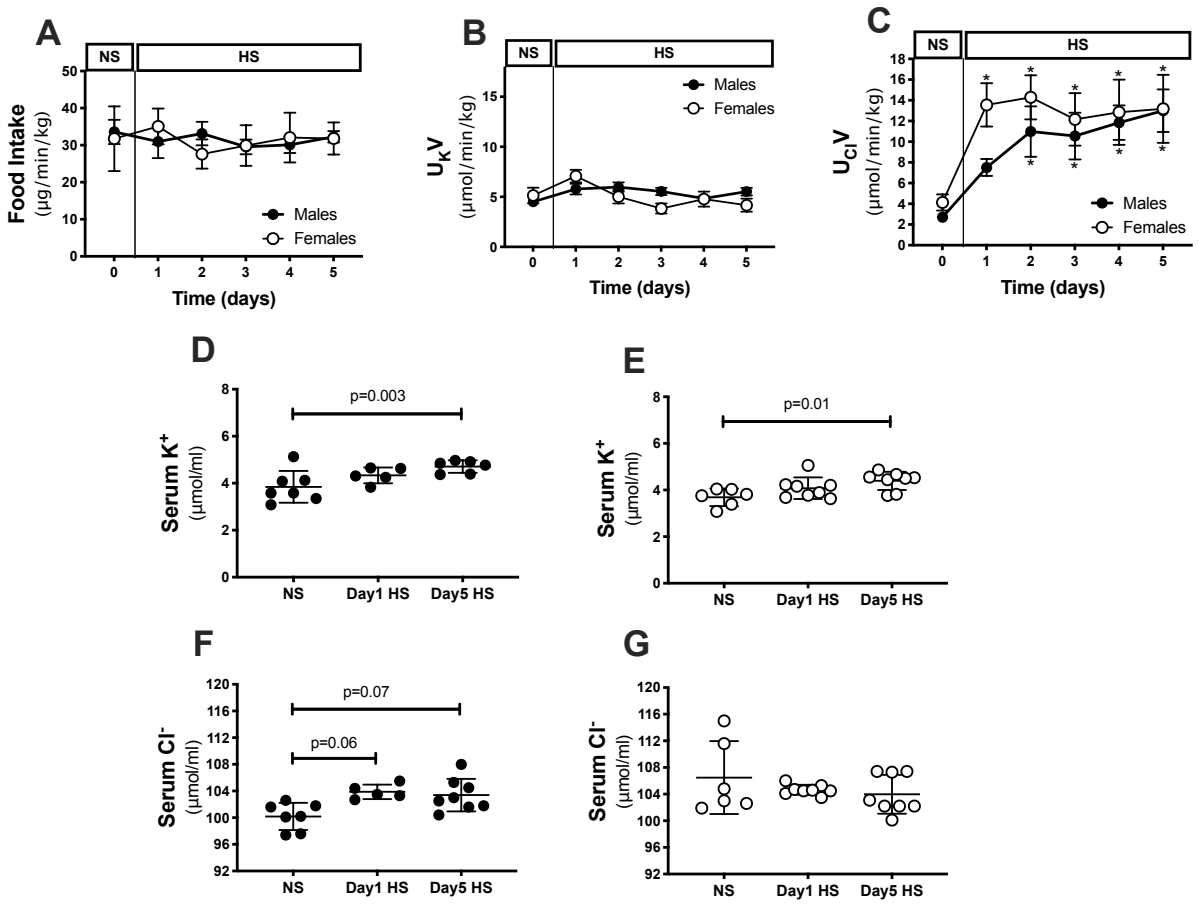

Figure S2. Blood pressure during the HS diet challenge. 24-h measurements of mean arterial pressure (MAP) in male and female SD rats during the NS diet phase or the HS diet challenge (n=4-6 rats in each group). Statistical comparisons were performed by repeated measures two-way ANOVA with Sidak's post-hoc test for multiple comparisons. *P < 0.05 vs. corresponding NS values. #P < 0.05 vs. corresponding male values. ANOVA results: $P_{interaction} = 0.1$, $P_{time} = 0.008$, $P_{sex} = 0.01$. NS, normal salt; HS, high salt; SD, Sprague Dawley.

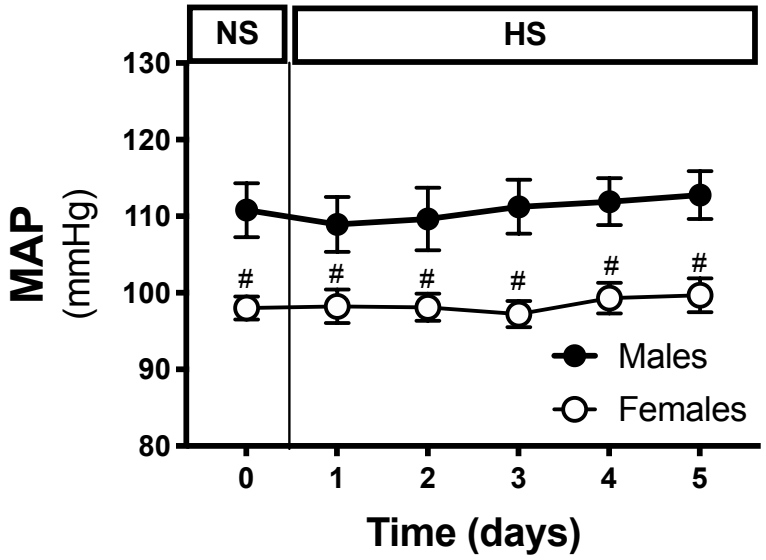

Figure S3. RNA-sequencing results plotted as A) Scatter plot comparing the mean FPKM from male and female pools and B) Volcano plot of log fold difference in gene expression (females over males) against q-value of the comparisons. Criteria for transcripts inclusion in pathway analysis were >+/- 1.5-fold difference in expression and/or q < 0.05. Transcripts meeting these criteria are represented by color-coded points, with all other transcripts in black.