Female Spontaneously Hypertensive Rats Have a Compensatory Increase in Renal Regulatory T Cells in Response to Elevations in Blood Pressure

Ashlee J. Tipton, Babak Baban, Jennifer C. Sullivan

Abstract—Female spontaneously hypertensive rats (SHR) have more regulatory T cells (Tregs) in their kidneys than males. The goal of this study was to determine the impact of blood pressure (BP) on the renal immune profile. We hypothesize that increases in BP promote a proinflammatory renal T cell and cytokine profile in SHR, although females will have greater hormone-dependent increases in Tregs and males will have greater increases in Th17 cells. Renal T cell and cytokine profiles were assessed in male and female Wistar-Kyoto rats and male and female SHR treated with vehicle or hydrochlorothiazide and reserpine (HCTZ) from 6 to 12 (6-HCTZ) or 11 to 13 weeks of age (2-HCTZ). Regardless of sex, SHR had a more proinflammatory renal immune profile than Wistar-Kyoto rats. 6-HCTZ attenuated age-related increases in BP and 2-HCTZ reversed hypertension compared with vehicle-treated SHR. Neither 6-HCTZ nor 2-HCTZ altered CD3+, CD4+, or CD8+ T cells in either sex. Both treatments decreased Tregs only in female SHR abolishing sex differences in Tregs. 6-HCTZ has no impact on Th17 cells in either sex and 2-HCTZ had a minimal impact on renal Th17 cells. To further assess mechanisms mediating sex differences in the renal immune profile, male and female SHR were gonadectomized to determine the impact of sex hormones. Gonadectomy increased proinflammatory markers in both sexes, suggesting that both male and female sex hormones are anti-inflammatory. In conclusion, BP contributes to sex differences in the renal T-cell profile of SHR; female SHR increase renal Tregs in response to increases in BP. (Hypertension. 2014;64:00-00.)

Key Words: cytokines ■ sex identity ■ hypertension ■ kidney ■ Th17 cells ■ T-lymphocytes

T cells play a pathogenic role in the development and progression of numerous cardiovascular diseases, including hypertension. T cells contribute to the development of hypertension in genetic, angiotensin (Ang)–II, and salt-sensitive male experimental animals. Furthermore, elevated blood pressure (BP) is associated with an increase in renal CD3+ T cells in male experimental models of hypertension, whereas decreases in BP correlate with decreased immune cell infiltration. Male spontaneously hypertensive rats (SHR) have greater renal T-cell infiltration and proinflammatory cytokine expression compared with normotensive Wistar-Kyoto rats (WKY) as early as 3 weeks of age, suggesting that SHR exhibit a proinflammatory immune profile even before BP increases. We recently demonstrated that there are sex differences in T cells in kidneys of SHR: males have more proinflammatory Th17 cells and females have more immune-suppressive regulatory T cells (Tregs). However, the mechanisms responsible for sex differences in the renal T-cell profile of SHR and the impact of BP status on the T-cell profile of females remain unknown.

Cytokines are key determinants in coordinating immune responses and have also been implicated in BP control in male experimental models of hypertension. In male mice, interleukin-6 (IL-6) contributes to the development of high salt and Ang-II–induced hypertension. Similarly, interleukin-17 (IL-17) has been suggested to be critical in promoting Ang-II–induced hypertension in male mice. In contrast, interleukin-10 (IL-10) protects against hypertension-induced vascular dysfunction and attenuates increases in BP. These studies demonstrate that cytokines also play a role in BP control, yet it is unclear whether or not sex influences the cytokine profile in experimental models of hypertension.

Historically, sex differences in hypertension have been attributed to sex hormones, and sex hormones are known to impact the immune system. In particular, both estrogen and testosterone have been shown to stimulate Treg formation in vitro and in vivo. Therefore, despite the fact that estrogens are thought to be cardioprotective and androgens are perceived to promote cardiovascular disease, both male and female sex hormones have been demonstrated to promote an anti-inflammatory immune profile.

Because of the expanding literature linking T-cell infiltration and cytokines with hypertension in male hypertensive experimental models, the overall goal of this study was to determine the impact of sex, sex hormones, and BP status on
the T cell and cytokine profile of the kidney. We hypothesize that increases in BP promote a proinflammatory renal T-cell profile in both sexes of SHR, although females will have greater sex hormone-dependent increases in Tregs and males will have greater increases in Th17 cells.

Materials and Methods

Animals

Twelve- to 13-week-old male and female SHR and WKY were used in this study (Harlan Laboratories, Indianapolis, IN). A subset of male and female SHR were gonadectomized at 10 weeks of age and studied at 13 weeks of age as previously described (expanded methods are available in the online-only Data Supplement).

Hydrochlorothiazide and Reserpine

Two-week study (2-HCTZ): 9-week-old male and female SHR were implanted with telemetry devices (Data Sciences International, St Paul, MN) to record BP. Rats were allowed 1-week recovery and 1 week of stable baseline recording. At 11 weeks of age, rats were randomized to receive vehicle or hydrochlorothiazide (55 mg/kg/d) and reserpine (4.5 mg/kg/d) (HCTZ) in drinking water from 11 to 13 weeks of age.

Six-week study (6-HCTZ): 6-week-old male and female SHR were randomized to receive vehicle or hydrochlorothiazide (10–55 mg/kg/d) and reserpine (0.6–4.5 mg/kg/d) in drinking water until 12 weeks of age. BP was measured weekly via tail-cuff plethysmography as previously described.

For both studies, rats were individually housed throughout the study. Water intake and body weights were measured every 3 days and the doses of drugs titrated as needed to maintain consistent BP lowering (Tables S1 and S2).

Statistical Analysis

All data are presented as mean±SE. Flow cytometry data were compared using 2-way analysis of variance followed by a Bonferroni post hoc test. Telemetry, tail-cuff, body weight, and water intake data within each sex were analyzed using repeated-measures analysis of variance. Telemetry and tail-cuff data between sexes and between vehicle and treated rats were compared using t test. Analyses were performed using GraphPad Prism Version 5.0 software (GraphPad Software Inc, La Jolla, CA), and for all comparisons, differences were considered statistically significant with P<0.05.

Results

SHR Have Greater Renal T Cell and Cytokine Expression Than WKY, Regardless of Sex

Male SHR have a higher systolic BP than age-matched female SHR (mm Hg: males 203±8, n=6; females 170±1, n=6; effect of sex: P=0.005). Male and female WKY had lower BP than SHR (mm Hg: males 135±3, n=4; females 127±2, n=6; effect of strain: P<0.0001), although BP was comparable between male and female WKY (interaction: P=0.02).

Regardless of sex, WKY had significantly fewer renal CD3+ T cells (expressed as % of total kidney cells: male SHR: 9±0.6%, female SHR: 7±0.5%; male WKY: 2±0.3%, female WKY: 2±0.3%; effect of strain: P<0.0001; n=11–13), CD4+ T cells, and CD8+ T cells, and Th17 cells compared with same-sex SHR (Figure 1; for all effect of strain: P<0.0001). Sex differences in the renal T-cell profile of male and female SHR from our previous study were confirmed in the present study, but there were no sex differences in any of the T-cell subtypes in kidneys of WKY (Figure 1; CD4+ effect of sex and interaction, P=0.0001; CD8+ effect of sex, P=0.0007; effect of strain, P=0.0005; interaction, P=0.0007). 6-HCTZ treatment did not significantly alter renal Tregs (Figure 2A and 2B; CD4+ T cells (A), CD8+ T cells (B), Foxp3+ regulatory T cells (C), and ROR-γt+ Th17 cells (D). *P<0.05 vs same strain male; and †P<0.05 vs sex-matched SHR.

Figure 1. T-cell profile in kidneys of 12- to 13-week-old male (M) and female (F) spontaneously hypertensive rats (SHR) (n=11) and Wistar-Kyoto rats (WKY) (n=13). Shown are the percent of CD4+ T cells (A), CD8+ T cells (B), Foxp3+ regulatory T cells (C), and ROR-γt+ Th17 cells (D). *P<0.05 vs same strain male; and †P<0.05 vs sex-matched SHR.

To further characterize the renal immune profile, renal cytokine levels were measured via flow cytometric analysis. Consistent with data in Figure 1, male SHR had significantly more renal cells expressing the proinflammatory cytokines IL-6 and IL-17 than female SHR (Figure 2A and 2B; IL-6 effect of sex, P<0.0001; IL-17 effect of sex, P<0.0006). In contrast, female SHR had more IL-10+ cells than male SHR (Figure 2C; effect of sex, P<0.0001). Expressions of IL-6, IL-17, and IL-10 were also greater in SHR compared with WKY, regardless of sex (Figure 2; for all effect of strain, P<0.0001). Expression of all 3 cytokines were comparable in male and female WKY (IL-6 and IL-10 effect of sex and interaction, P=0.0001; IL-17 effect of sex, P=0.0006; interaction, P=0.0012).

Attenuating Age-Related Increases in BP Prevents the Development of a Sex Difference in Renal Tregs

Male and female SHR were administered HCTZ in the drinking water from 6 to 12 weeks of age (6-HCTZ). 6-HCTZ treatment attenuated age-related increases in BP in SHR and abolished the sex difference in BP observed in 12-week-old vehicle-treated SHR (Figure 3). 6-HCTZ treatment did not change weight gain or water intake compared with vehicle-treated animals (Table S1).

6-HCTZ treatment did not significantly alter renal CD3+ (expressed as % of total kidney cells: Male SHR, 4±0.2%; Male + 6-HCTZ, 4±0.3%; Female SHR, 5±0.5%; Female + 6-HCTZ, 5±0.3%; effect of treatment: P=0.8; n=5–6), CD4+, or CD8+ T cells compared with same-sex vehicle controls (Figure 4A and 4B; CD4+ effect of treatment, P=0.7; CD8+ effect of treatment, P=0.5). However, attenuating age-related increases in BP significantly decreased the percent of renal Tregs only in female SHR, thereby abolishing the sex difference in renal Tregs (Figure 4C; effect of sex, P=0.0007; effect of treatment, P=0.004; interaction, P=0.0005). 6-HCTZ treatment did not significantly alter Th17 cell counts in either sex, and males treated with 6-HCTZ maintained more renal Th17...
cells than treated females (Figure 4D; effect of sex, $P<0.0001$; effect of treatment, $P=0.2$; interaction, $P=0.2$).

**Reversing Hypertension in Adult SHR Abolishes the Sex Difference in Tregs and Minimizes the Sex Difference in Th17 Cells**

To determine whether sex differences in renal Tregs could also be abolished by reversing hypertension, additional male and female SHR were administered HCTZ from 11 to 13 weeks of age (2-HCTZ). Baseline BP was greater in male SHR than female SHR (Figure 5; $P<0.05$). 2-HCTZ treatment lowered BP relative to same-sex vehicle controls in both males and females and abolished the sex difference in BP. Body weight and daily water intake increased in both sexes from 11 to 13 weeks of age, but there were not differences between vehicle and treated groups of the same sex (Table S2).

2-HCTZ treatment did not affect renal CD3$^+$ T cells (expressed as % of total kidney cells: M SHR, 5±0.8%; M 2-HCTZ, 5±0.7%; F SHR, 5±0.3%; F 2-HCTZ, 5±0.4%; effect of treatment, $P=0.5$; n=4–7), CD4$^+$, or CD8$^+$ T cells (Figure 6A and 6B; CD4$^+$ effect of treatment, $P=0.1$; CD8$^+$ effect of treatment, $P=0.1$). However, decreasing BP significantly lowered Tregs in female SHR relative to vehicle-treated female SHR with no effect in males and the sex differences in Tregs observed in control SHR was abolished (Figure 6C; effect of sex, $P=0.2$; effect of treatment, $P=0.003$; interaction, $P=0.002$). In contrast to the 6-HCTZ treatment, 2-HCTZ treatment also minimized the sex difference in Th17 cell counts in SHR (Figure 6D; effect of treatment, $P=0.9$; effect of sex, $P=0.09$; interaction, $P=0.06$).

**Sex Hormones Are Anti-Inflammatory in SHR Regardless of Sex**

To gain additional mechanistic insight into sex differences in the immune profile of SHR, renal T-cell and cytokine profiles
were measured in gonad-intact and gonadectomized male (orchiectomized) and female (ovariectomized) SHR. Sex differences observed in the renal T-cell profile of gonad-intact SHR were verified. Gonadectomy had no effect on renal CD3+ T-cell counts in either sex (CD3+ expressed as % of total kidney cells: intact male, 9±1%; orchiectomized, 8±1%; intact females, 7±1%; orchiectomized, 9±1%; n=5–11; effect of sex hormones, P=0.4). Gonadectomy increased renal CD4+ T-cells and Th17 cells relative to gonad-intact males and females; however, the increase was comparable in both sexes such that orchiectomized SHR maintained more renal CD4+ T-cells and Th17 cells than orchiectomized SHR (Figure 7A and 7D; CD4+ effect of sex, P=0.0003; Th17 effect of sex, P<0.0001; for both effect of sex hormones, P<0.0001; CD4+ interaction, P=0.5; Th17 interaction, P=0.2). Gonadectomy did not change renal CD8+ T-cells in either sex; therefore, ovariectomized SHR maintained more CD8+ T-cells than orchiectomized SHR (Figure 7B; effect of sex, P=0.008; effect of sex hormones, P=0.5; interaction, P=0.5). Tregs were decreased by gonadectomy in both sexes, although ovariectomy resulted in a greater decrease in Tregs than orchidectomy (Figure 7C; effect of sex, P<0.001; effect of sex hormones, P<0.0001; interaction, P=0.01). Gonadectomy also increased the number of IL-6+ and IL-17+ cells to a similar degree in both sexes thereby maintaining the sex difference observed in gonad-intact SHR (Figure 8; for both effect of sex, P<0.0001; for both effect of sex hormones, P<0.0001; IL-6 interaction, P=0.5; IL-17 interaction, P=0.4). In contrast, gonadectomy decreased IL-10+ renal cells in both sexes (Figure 8; effect of sex, P<0.0001; effect of sex hormones, P<0.0001); however, the decrease in renal IL-10 was greater in orchiectomized SHR than in orchiectomized SHR (interaction, P=0.004).

Discussion

The majority of studies showing increased immune cell infiltration in experimental models of hypertension have been conducted in males, despite the fact that females account for ~50% of all hypertensive cases in the United States. We recently published that there are sex differences in the immune profile of SHR; female SHR have more renal Tregs, whereas males have greater Th17 cells counts. The major novel finding of the current study is that the sex differences in renal Tregs in young, adult SHR is BP dependent. There are no apparent sex differences in the renal immune profile of normotensive WKY; and lowering BP in SHR abolished the sex difference in Tregs by decreasing counts in females. In addition, both male and female sex hormones are anti-inflammatory. Therefore, although female sex hormones likely contribute to the higher incidence of Tregs in females relative to males, sex hormones alone cannot account for sex differences in the immune profile of SHR. Taken together, the current studies suggest that Tregs serve as an important feedback mechanism which may account for the consistently lower BP in female SHR relative to males.

It is well established that hypertension is associated with an increase in renal T cells in male experimental models of hypertension. The current study expands our understanding of the immune system in hypertension by demonstrating that hypertensive females also exhibit greater renal T-cell counts than normotensive females, which is consistent with our previous publication demonstrating that lymphocyte suppression decreases BP in female SHR. Consistent with reports showing greater CD3+ renal T-cell infiltration in male SHR relative to male WKY, SHR of both sexes also have more renal CD4+ and CD8+ T cells, Tregs, and Th17 cells than same-sex WKY. Expanding on our previous results and consistent with a sex difference in the T-cell profile of male and female SHR, the current study also demonstrates that male SHR also have higher levels of the proinflammatory cytokines, IL-6 and IL-17, whereas females had greater numbers of kidney cells expressing the anti-inflammatory cytokine IL-10. In contrast to our findings, male SHR have been reported to have lower levels of circulating IL-6 than female SHR; however, the authors did not report tissue levels and the local cytokine environment of the kidney may be different from the circulation. Indeed, we published that male SHR have fewer circulating Th17 cells and more Tregs compared with female SHR, underscoring the importance of measuring local levels of cytokines and immune cells at the tissue level. It should be noted that cytokines in the current study were assessed by flow cytometry and reflect the percent of total kidney cells that are positive for the individual cytokines. This is a potential limitation of the current study; however, multiple commercially available enzyme-linked immunosorbent assays were tested and levels in the rat were below the sensitivity threshold of the individual assays. Based on the key role, these cytokines have been shown to play in BP control in other studies; future studies will determine the impact of these cytokines on BP and renal function in male and female SHR.

Although the current study clearly demonstrates greater numbers of renal T cells in SHR compared with WKY, they do not conclusively link greater T cells in SHR to higher BP. Indeed, strain differences in renal T cells between male SHR and WKY have been reported to be evident as early as 3 weeks of age, well before BP increases in SHR. These data suggest that male SHR inherently have more renal T cells than male WKY. Therefore, additional experiments were designed to directly address the impact of elevated BP on the renal T-cell profile in SHR. In addition, because male SHR exhibit a
more rapid age-related increase in BP compared with females, sex differences in the renal T-cell profile in young, adult SHR may simply reflect the higher BP in the male. Attenuating the degree of hypertension and abolishing the sex difference in BP in SHR did not alter total T-cell counts in the kidney of either male or female SHR, although there were sex-specific effects on T-cell subtypes following BP lowering. Female SHR exhibited a decrease in renal Tregs following an attenuation of BP, whereas male SHR maintained more Th17 cells than female SHR even though BP was comparable in both sexes. This result suggests that the greater number of Th17 cells in the male SHR does not simply reflect them having a higher BP. In contrast, reversing established hypertension narrowed the sex difference in Th17 cells (P<0.06), as a result of a nonsignificant decrease in Th17 cell counts in male SHR with treatment. It is of interest that Th17 cells were potentially differentially altered in SHR depending on whether their BP was allowed to reach hypertensive levels. These data may suggest that although the development of hypertension is not dependent on Th17 cells, the maintenance of hypertension in male SHR may be. There is controversy in the literature about the role of the Th17 cell pathway in hypertension. It was initially reported that male IL-17 knockout mice fail to develop Ang-II hypertension, although a more recent study demonstrated that knockout of key Th17 cell effector cytokines, IL-17 and interleukin-23, does not alter increases in BP to deoxycorticosterone acetate–salt+Ang-II administration in male mice despite a decrease in Th17 cells. A separate study also showed that although triple therapy lowers BP in male uninephrectomized Sprague-Dawley rats on a deoxycorticosterone acetate–salt diet, renal levels of Th17 cells were comparable to control. When taken together, these studies call into question the role of Th17 cells in hypertension in either sex. Future studies are needed to directly assess the contribution of the different T-cell subtypes to BP control in both sexes.

In addition to increases in BP, aging of SHR from 6 to 12 weeks of age is also associated with sexual maturation and increases in sex hormones. To gain additional mechanistic insight potentially driving sex differences in the renal immune profile and to determine the contribution of sex hormones to the immune profile, we assessed the impact of gonadectomy on the renal T cell and cytokine expression in SHR. Despite a decrease in Th17 cells,38 a separate study also showed that although triple therapy lowers BP in male uninephrectomized Sprague-Dawley rats on a deoxycorticosterone acetate–salt diet, renal levels of Th17 cells were comparable to control. When taken together, these studies call into question the role of Th17 cells in hypertension in either sex. Future studies are needed to directly assess the contribution of the different T-cell subtypes to BP control in both sexes.

Our results are consistent with previous reports in the literature showing that inclusion of the BP-lowering drug hydralazine does not alter Ang-II–induced increases in renal CD4+ or CD8+ T-cell counts.33 In contrast, we previously published that triple antihypertensive therapy including hydralazine, hydrochlorothiazide, and reserpine attenuated NO-Nitro-L-arginine methyl ester–mediated increases in renal T-cell counts in both sexes of SHR.34 However, hydralazine has been shown to reduce the number of adherent and migrating leukocytes in the vasculature of male SHR independent on an effect on BP;35 therefore, the current study did not include hydralazine to lower BP. HCTZ was used in the current study to lower BP to minimize nonspecific-drug effects on immune cells. To our knowledge, there are no studies that have reported an effect of hydrochlorothiazide or reserpine on T-cell differentiation or tissue infiltration, although reserpine has been shown to block T-cell activation and proliferation in vitro. Although we cannot rule out an effect of HCTZ treatments on the immune profile independent of BP, it seems unlikely that this would impact only 1 T-cell subtype in 1 sex. However, this is a potential limitation of the current study. As Ang-II has been demonstrated to impact immune cells, pharmacological drugs that alter the renin–angiotensin system were not used.

Attenuating age-related increases in BP had no effect on the renal T-cell profile in male SHR, although male SHR maintained more Th17 cells than female SHR even though BP was comparable in both sexes. This result suggests that the greater number of Th17 cells in the male SHR does not simply reflect them having a higher BP. In contrast, reversing established hypertension narrowed the sex difference in Th17 cells (P<0.06), as a result of a nonsignificant decrease in Th17 cell counts in male SHR with treatment. It is of interest that Th17 cells were potentially differentially altered in SHR depending on whether their BP was allowed to reach hypertensive levels. These data may suggest that although the development of hypertension is not dependent on Th17 cells, the maintenance of hypertension in male SHR may be. There is controversy in the literature about the role of the Th17 cell pathway in hypertension. It was initially reported that male IL-17 knockout mice fail to develop Ang-II hypertension, although a more recent study demonstrated that knockout of key Th17 cell effector cytokines, IL-17 and interleukin-23, does not alter increases in BP to deoxycorticosterone acetate–salt+Ang-II administration in male mice despite a decrease in Th17 cells. A separate study also showed that although triple therapy lowers BP in male uninephrectomized Sprague-Dawley rats on a deoxycorticosterone acetate–salt diet, renal levels of Th17 cells were comparable to control. When taken together, these studies call into question the role of Th17 cells in hypertension in either sex. Future studies are needed to directly assess the contribution of the different T-cell subtypes to BP control in both sexes.
expression in the testes.\textsuperscript{22} Similarly, estrogen stimulates Treg production in vitro and in vivo in CB57BL/6 mice, and estrogen-stimulated conversion of CD4\textsuperscript{+} T cells into Tregs can be blocked by an estrogen receptor antagonist.\textsuperscript{19} Regardless, sex hormones cannot account for all of the observed sex differences in the renal T-cell profile in SHR. However, gonadectomy of female SHR resulted in a greater decrease in Tregs than in males suggesting that female sex hormones contribute to greater Tregs in female SHR. Future studies will continue to pursue additional mechanisms driving sex differences in the immune profile of SHR.

Perspectives

Despite all of the current therapeutics available for the treatment of hypertension, there is a critical need for new treatment options to increase the percentage of individuals with controlled BP. This is a challenge because of our lack of knowledge about the molecular mechanism(s) driving essential hypertension in either sex. In this study, we identify that female SHR have a compensatory increase in renal Tregs in response to elevated BP. We propose that this is 1 way in which female SHR maintain a lower BP than male SHR.\textsuperscript{26} Future studies are needed to determine the molecular mechanism(s) responsible for the compensatory increase in renal Tregs in female SHR because it might be a potential therapeutic target for the treatment of hypertension in both sexes.

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Disclosures

None.

References


**Novelty and Significance**

**What Is New?**
- The current study supports that there is an increase in proinflammatory mediators in both hypertensive males and females. However, female spontaneously hypertensive rats have a compensatory increase in renal regulatory T cells following increases in blood pressure which is not observed in males. This is an important finding that advances the field because it suggests that regulatory T cells, which have been shown to protect against hypertension in various experimental models, serve as an important feedback mechanism which may account for the consistently lower blood pressure in female spontaneously hypertensive rat relative to males.

**What Is Relevant?**
- Understanding the factors that lead to differential regulation of the cardiovascular system between males and females is an important endeavor that will ultimately promote better and more individualized treatment strategies. Although it is now widely accepted that the immune system plays an important role in the pathogenesis of hypertension in males, little is known with regard to how the immune system contributes to disparate cardiovascular responses between males and females.

**Summary**
This study provides an inclusive analysis of the renal immune cell profile in spontaneously hypertensive rat by further categorizing T cells based on subtype and cellular cytokine expression. The major finding of the current study is that regulatory T cells in the kidney are directly influenced by blood pressure in hypertensive females.
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Female SHR Have a Compensatory Increase in Renal Regulatory T Cells in Response to Elevations in Blood Pressure

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Running Head: Blood Pressure status impacts renal T cells

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Materials and Methods

Animals. 12-13 week old male and female SHR and WKY were used in this study (Harlan Laboratories, Indianapolis, IN). All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved and monitored by the Georgia Regents University IACUC. Rats were housed in temperature- and humidity-controlled, light-cycled quarters and maintained on standard chow (Harlan Teklad). At the end of all studies, rats were anesthetized with ketamine/xylazine (48 mg/kg and 6.4 mg/kg, respectively, i.p.; Phoenix Pharmaceuticals, St. Joseph, MO) and kidneys were isolated for analysis.

Analytical flow cytometry. Single cell suspensions of kidneys in phosphate buffered saline were prepared as previously described 1 for intracellular analyses and phenotypic characterization of the following T cells: CD3+, CD4+, CD8+, Tregs (Foxp3+CD4+CD3+) and Th17 cells (ROR-γt+CD4+CD3+; BD Biosciences San Jose, CA; eBiosciences, San Diego, CA; R and D Systems, Minneapolis, MN) 29,30. Prepared samples were run on a four-color flow cytometer (FACS Calibur, BD Biosciences) and data were collected using CellQuestTM software as previously described 1.

References:

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<td>146±3$</td>
<td>14±2</td>
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<tr>
<td>10 weeks</td>
<td>Male SHR</td>
<td>252±10$</td>
<td>27±2$</td>
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<tr>
<td></td>
<td>Male 6-HCTZ</td>
<td>238±7$</td>
<td>24±1$</td>
</tr>
<tr>
<td></td>
<td>Female SHR</td>
<td>161±3$</td>
<td>21±3$</td>
</tr>
<tr>
<td></td>
<td>Female 6-HCTZ</td>
<td>156±3$</td>
<td>18±1$</td>
</tr>
<tr>
<td>11 weeks</td>
<td>Male SHR</td>
<td>270±10$</td>
<td>27±1$</td>
</tr>
<tr>
<td></td>
<td>Male 6-HCTZ</td>
<td>256±4$</td>
<td>29±2$</td>
</tr>
<tr>
<td></td>
<td>Female SHR</td>
<td>170±2$</td>
<td>24±3$</td>
</tr>
<tr>
<td></td>
<td>Female 6-HCTZ</td>
<td>166±3$</td>
<td>19±1$</td>
</tr>
<tr>
<td>12 weeks</td>
<td>Male SHR</td>
<td>268±3$</td>
<td>25±3$</td>
</tr>
<tr>
<td></td>
<td>Male 6-HCTZ</td>
<td>275±7$</td>
<td>21±0$</td>
</tr>
<tr>
<td></td>
<td>Female SHR</td>
<td>171±3$</td>
<td>21±1$</td>
</tr>
<tr>
<td></td>
<td>Female 6-HCTZ</td>
<td>173±3$</td>
<td>18±2$</td>
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Table 1. Metabolic parameters from 6-HCTZ treatment. $ p<0.05$ vs. same sex at 6 weeks of age; % $ p<0.05$ vs. age-matched vehicle.
Table S2.

<table>
<thead>
<tr>
<th>Age</th>
<th>Group</th>
<th>Body Weight (g)</th>
<th>24 hour Water Intake (ml)</th>
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<td>Male SHR</td>
<td>295±4</td>
<td>26±2</td>
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<td>Male 2-HCTZ</td>
<td>298±4</td>
<td>22±1</td>
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<tr>
<td></td>
<td>Female SHR</td>
<td>185±2</td>
<td>17±1</td>
</tr>
<tr>
<td></td>
<td>Female 2-HCTZ</td>
<td>179±4</td>
<td>18±2</td>
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<tr>
<td><strong>11 weeks</strong></td>
<td>Male SHR</td>
<td>315±4$</td>
<td>30±1</td>
</tr>
<tr>
<td></td>
<td>Male 2-HCTZ</td>
<td>310±7</td>
<td>27±7</td>
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<tr>
<td></td>
<td>Female SHR</td>
<td>192±2$</td>
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<td>Female 2-HCTZ</td>
<td>185±4$</td>
<td>22±4</td>
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<tr>
<td><strong>12 weeks (End of Treatment)</strong></td>
<td>Male SHR</td>
<td>328±6$</td>
<td>25±2</td>
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<td>Male 2-HCTZ</td>
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<td>22±2</td>
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<tr>
<td></td>
<td>Female 2-HCTZ</td>
<td>188±4$</td>
<td>18±1</td>
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</tbody>
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Table 2. Metabolic parameters from 2-HCTZ treatment. $ p<0.05$ vs. baseline group.
Figure S1. Representative dot plots of the T cell profile in kidneys of 12-13 week old male and female SHR and WKY. Shown are plots for CD4^+ and CD8^+ T cells as a subset of total renal cells (panel A) and Foxp3^+ Tregs and ROR-γt^+ Th17 cells gated from total CD3^+CD4^+ T cells (panel B).
Figure S2. Representative scatter plots for the cytokine profile in kidneys of 12-13 week old male and female SHR and WKY. Shown are the plots for IL-6⁺ renal cells (panel A), IL-17⁺ renal cells (panel B), and IL-10⁺ renal cells (panel C) as a subset of total renal cells.
**Figure S3.** Representative dot plots of the T cell profile in kidneys of male and female SHR treated from 6 to 12 weeks of age with HCTZ and reserpine. Shown are plots for CD4+ and CD8+ T cells as a subset of total renal cells (panel A) and Foxp3+ Tregs and ROR-γt+ Th17 cells gated from total CD3+CD4+ T cells (panel B).
Figure S4. Representative dot plots of the T cell profile in kidneys of male and female SHR treated from 11 to 13 weeks of age with HCTZ and reserpine. Shown are plots for CD4⁺ and CD8⁺ T cells as a subset of total renal cells (panel A) and Foxp3⁺ Tregs and ROR-γt⁺ Th17 cells gated from total CD3⁺CD4⁺ T cells (panel B).
Figure S5. Representative dot plots of the T cell profile in kidney of 12-13 week old intact and gonadectomized male and female SHR. Shown are plots for CD4+ and CD8+ T cells as a subset of total renal cells (panel A) and Foxp3+ Tregs and ROR-γt+ Th17 cells gated from total CD3+CD4+ T cells (panel B).
Figure S6. Representative scatter plots of the cytokine profile in kidneys of 12-13 week old intact and gonadectomized male and female SHR. Shown are the plots for IL-6⁺ renal cells (panel A), IL-17⁺ renal cells (panel B), and IL-10⁺ renal cells (panel C) as a subset of total renal cells.