Sex and sex hormones influence the development of albuminuria and renal macrophage infiltration in spontaneously hypertensive rats

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MANY CARDIOVASCULAR diseases, including hypertension, are associated with renal injury. There is a sex difference in renal injury, with men experiencing greater severity and a more rapid progression of renal disease than women; however, the molecular mechanisms protecting against renal injury in women are unknown. The goal of this study was to determine whether sex hormones modulate blood pressure and the progression of albuminuria during the developmental phase of hypertension in male and female spontaneously hypertensive rats (SHR). Studies were also performed to examine how sex and sex hormones influence two major risk factors for albuminuria, overactivation of the renin-angiotensin system and oxidative stress. Blood pressure was measured by telemetry in gonad-intact and gonadectomized male and female SHR. Microalbumin excretion, measured over time, and macrophage infiltration were used to assess renal health. Male SHR had significantly higher blood pressures than female SHR, and gonadectomy decreased blood pressures in males with no effect in females. Male SHR displayed a gonad-sensitive increase in albuminuria over time, and female SHR had a gonad-sensitive suppression in macrophage infiltration. Female SHR had higher plasma ANG II levels and similar levels of renal cortical ANG II vs. levels shown in males but less AT1-receptor protein expression in the renal cortex. Female SHR also had a gonad-sensitive decrease in renal oxidative stress. Therefore, the renal protection afforded to female SHR is associated with lower blood pressure, decreased macrophage infiltration, and decreased levels of oxidative stress.

Sex and sex hormones influence the development of albuminuria and renal injury in male SHR to a greater extent than females. The mechanisms protecting against renal injury in females are not well characterized. Two risk factors for the progression and development of renal injury are overactivation of the renin-angiotensin system (RAS) and oxidative stress. Activation of AT1 receptors mediates most of the well-known biological functions of ANG II, including vasoconstriction, inflammation, and production of reactive oxygen species (3). Treatment with either an AT1-receptor blocker and/or an angiotensin-converting enzyme (ACE) inhibitor blocks the development of renal injury in male SHR (19, 23). Oxidative stress contributes to the development and progression of a variety of renal pathologies, and antioxidant treatment improves renal function and decreases markers of renal injury in male SHR (31, 34). Renal superoxide levels are elevated in male SHR compared with levels shown in male Wistar-Kyoto rats (11), and the renal cortex from male SHR expresses high levels of immunoreactive nitrotyrosine and glomerulosclerosis (10). ANG II has been shown to contribute to oxidative stress-induced renal injury via AT1-receptor activation of NADPH oxidase (14). Treatment of male SHR with ACE inhibitors or angiotensin-receptor blockers decreases renal indexes of oxidative stress and improves renal function (19).

There are sex differences in both the RAS and oxidative stress; male SHR have a more activated RAS and greater levels of oxidative stress than female SHR (7, 12, 29, 45). Renal injury and the RAS are also sensitive to regulation by sex hormones. Estrogens decrease mesangial cell proliferation, collagen synthesis, and activity of the RAS (25, 32); therefore, estrogen is thought to protect the kidney from renal injury. Conversely, testosterone increases mesangial cell proliferation and activity of the RAS (25, 36) and is considered to promote renal injury. Relatively little, however, is known regarding the role of estrogen in the development of hypertension and renal injury. Ovariectomy has been reported either to have no effect (5, 29) or to increase blood pressure in female SHR (35). Similarly, estrogens have been reported to either decrease (1, 8, 15) or increase indexes of renal injury (17) in experimental animals, whereas postmenopausal hemodialysis-dependent women tend to have low serum estrogen levels (18). Although the majority of evidence suggests a protective role for estrogen in the development of renal injury, the mechanisms responsible are unclear. The goals of our study were to determine 1) whether sex hormones modulate blood pressure and the early development of albuminuria over time and macrophage infiltration in young male and female SHR and 2) the effect of gonadectomy on two major risk factors for renal injury, the RAS and oxidative stress. We hypothesize that female sex hormones contribute to maintaining renal health, in part by suppressing renal ANG II and oxidative stress levels.

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METHODS

Animals. Male and female SHR (Harlan Laboratories, Indianapolis, IN) were studied during the developmental phase of hypertension (from 8 to 16 wk of age). 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 Medical College of Georgia Institutional Animal Care and Use Committee. Rats were housed in temperature- and humidity-controlled, light-cycled quarters and maintained on standard rat chow (Harlan Teklad). Rats were periodically placed in metabolic cages to facilitate 24-h urine collection at 8, 9, 11, 12, 14, and 16 wk of age. At 8 wk of age, a subset of animals was implanted with telemetry transmitters (Data Sciences, St. Paul, MN) for the continuous monitoring of blood pressure. At 9 wk of age, rats were placed on telemetry receivers for measurement of blood pressure. For gonadectomized animals, telemetry devices were implanted at 9 wk of age, and gonadectomy surgeries were performed at 11 wk of age as previously described (38). Ovariectomy was confirmed as previously described (4). At 16 wk of age, rats were anesthetized with pentobarbital sodium (Nembutal, 65 mg/kg ip; Abbott Laboratories, North Chicago, IL), a terminal blood sample was taken, kidneys were removed, and the renal cortex was isolated and snap frozen in liquid nitrogen. In a separate set of animals, kidneys were prepared for immunohistochemical analysis as previously described (20).

Assays and chemicals. Microalbumin excretion was measured by ELISA (Cayman Chemicals, Ann Arbor, MI). ANG II levels were measured by enzyme immunoassay directly in plasma and after methanol extraction of the renal cortex as previously described (26) (Cayman Chemicals). According to the kit manufacturer, quality control testing revealed that there was 4% cross-reactivity with ANG I, 100% cross-reactivity with ANG II, 36% cross-reactivity with ANG III, 33% cross-reactivity with ANG 3–8, and <0.01% cross-reactivity with ANG 1–7 for the angiotensin enzyme immunoassay. Plasma renin activity (PRA) was measured by radioimmunoassay (DiaSorin, Stillwater, MN).

Immunohistochemical analysis. Briefly, kidneys were perfusion fixed with formalin, paraffin embedded, and sectioned at a thickness of 4 μm onto Superfrost plus slides. Slides were incubated overnight in the absence or presence of primary antibody to ED-1 (CD58; Serotec, Kidlington, Oxford, UK) in humidity chambers at 4°C, followed by incubation for 30 min with peroxidase-conjugated goat anti-mouse IgG (Serotec) at room temperature. ED-1 staining was followed by incubation for 30 min with diaminobenzidine (DakoCytomation), counterstained with Mayer’s hematoxylin, and coverslipped. To assess renal injury, kidney sections were stained with a periodic acid-Schiff stain kit, Gomori’s trichrome stain kit, or picro Sirius red according to kit, Gomori’s trichrome stain kit, or picro Sirius red according to

RESULTS

Mean arterial pressure. Sex differences in blood pressure were verified by continuously monitoring pressure for 24 h per day using telemetry from 9 to 16 wk of age in male and female SHR. By 12 wk of age, blood pressure was significantly greater in male SHR than in female SHR (Fig. 1A). Blood pressure was also monitored in a subset of male and female SHR that were gonadectomized at 11 wk of age (Fig. 1B). Immediately after gonadectomy surgery, blood pressure was increased and more variable in both ovariectomized (OVX) female and orchidectomized (ORX) male SHR. However, by 16 wk of age, gonadectomy significantly decreased blood pressure in male SHR, whereas blood pressure was not altered by gonadectomy in females (Fig. 1C).

Renal health. Microalbumin excretion, histological analysis, and macrophage infiltration were measured to assess renal health. Microalbumin excretion was measured at 8, 9, 11, 12, 14, and 16 wk of age to assess the development and progression of albuminuria. Microalbumin excretion was significantly greater in male SHR at all time points, beginning as early as 8 wk of age (Fig. 2A). Because of fluctuations in hormone levels and blood pressure after gonadectomy surgery, microalbumin in the loss of the 43-kDa band, verifying the specificity of the antibody. Specific bands were detected with the Odyssey infrared imager (LI-COR Biosciences, Lincoln, NE); AlexaFluor 680 was used for the detection of anti-AT1 antibody (Molecular Probes, Eugene, OR), and IRDye800 (Rockland, Gilbertsville, PA) was used for the detection of anti-actin antibody. Actin was used to verify equal protein loading, and all densitometric results are reported normalized to actin. Protein concentrations were determined by standard Bradford assay (Bio-Rad, Hercules, CA) using BSA as the standard.

Superoxide detection assay. Basal superoxide detection experiments were conducted as previously described with minor modifications (33). Briefly, 35 μg of cortex homogenate or buffer control were added to sample or background wells, respectively, in 96-well microplates (OptiPlate-96, Perkin-Elmer) and incubated at 37°C for 30 min in the presence of lucigenin (5 μmol/l). After a 30-min dark-adapt period, luminescence was quantified with a TopCount microplate scintillation and luminescence counter (Perkin Elmer) set to single-photon counting mode. NADPH oxidase enzymatic activity was measured similarly by lucigenin chemiluminescence (5 μmol/l) in the presence of 100 μmol/l NADPH (Alexis Biochemicals). Enzyme activity was normalized to the amount of protein such that the level of superoxide generated is expressed as counts per minute per microgram of protein. In addition, there is a linear relationship between NADPH oxidase activity and the amount of protein in the assay at the dose that we used to measure NADPH oxidase activity. Total protein was measured by standard Bradford assay (Bio-Rad, Hercules, CA) using BSA as the standard. Specificity of the assay for superoxide was confirmed by the inclusion of the superoxide dismutase mimic tempol (10 mmol/l).

Statistical analysis. All data are expressed as means ± SE. Telemetry data are expressed as the 24-h mean (12:00 AM to 11:59 PM). For ease of viewing, error bars have only been included weekly, although these error bars are representative of the level of error across the entire time course. ED-1, ANG II measurements, PRA, AT1, and superoxide data were compared with ANOVA followed by a least significant difference post hoc comparison (Statistica, Tulsa, OK). Telemetry and microalbumin excretion data from male and female SHR were analyzed with a repeated-measures ANOVA with the Greenhouse-Geisser correction applied. Blood pressure and microalbumin excretion data at 16 wk in all four groups were compared with ANOVA. For all comparisons, P < 0.05 was considered statistically significant.
ANG II and AT1 protein expression. Overactivation of the RAS is a risk factor for albuminuria. Surprisingly, plasma or the renal cortex. PRA was also measured and found to be comparable between all groups (Fig. 3C).

A number of the pathological effects of ANG II are mediated by stimulation of the AT1 receptor; therefore, we examined AT1-receptor protein expression in the renal cortex. Male SHR had significantly greater AT1-receptor protein expression than female SHR (Fig. 4). AT1-receptor protein expression was not significantly altered by ORX or OVX.

Oxidative stress. Oxidative stress is associated with the development and progression of albuminuria. Basal superoxide levels in the renal cortex were measured by lucigenin chemiluminescence. There were significantly greater levels of superoxide in the renal cortex of male SHR than in renal cortex of female SHR (Fig. 5). ORX did not alter superoxide levels; whereas OVX females had increased basal superoxide levels compared with levels in gonad-intact female SHR. Basal superoxide levels were blocked by incubation with the superoxide dismutase mimetic tempol (10 mmol/l) (data not shown).

The AT1 receptor stimulates NADPH oxidase to increase the production of superoxide; therefore, NADPH oxidase enzymatic activity was measured in the renal cortex of male and female SHR to determine whether the observed sex differences in AT1-receptor expression may result in a differential activation of NADPH oxidase. NADPH oxidase enzymatic activity was measured with exogenous substrate and therefore reflects the potential of the enzyme to make superoxide and is not an actual measurement of NADPH oxidase-derived superoxide. NADPH oxidase activity was comparable in the renal cortex of male (1,728 ± 162 cpm/µg) and female (1,850 ± 172 cpm/µg) SHR.

DISCUSSION

Evidence of albuminuria was present in both male and female SHR as early as 8 wk of age, before any overt structural changes are apparent in the kidney, and renal health was influenced by both sex and sex hormones. The primary novel findings of this study are 1) gonadectomy of female SHR did not alter blood pressure, although macrophage infiltration and renal cortical oxidative stress levels were increased, and 2) renal cortical ANG II levels were comparable between male and female SHR and were unaffected by gonadectomy, although reduced renal AT1-receptor protein expression may afford some protection to female rats. Therefore, lower blood pressure and albuminuria in female and gonadectomized male SHR are independent of tissue ANG II levels.

These results support the hypothesis that sex and sex hormones influence the physiological and/or pathological actions of ANG II signal transduction in the kidneys of SHR.

In humans, as well as in experimental animals, males demonstrate a more rapid progression of renal injury and deterioration of renal function independent of blood pressure and differences in kidney size and structure vs. results shown in females (24, 27, 28, 32). Although male SHR displayed greater microalbumin excretion than female SHR, there were also detectable levels of microalbumin excretion in females, indicative of compromised renal function. However, in females, levels of albumin excretion were constant over time despite blood pressure rising. Blood pressures in 9-wk-old males and 16-wk-old females were comparable, yet levels of albumin excretion at these ages were significantly different, suggesting
that sex differences in blood pressure are not the sole factor responsible for sex differences in albuminuria.

Male sex hormones have been linked with the progression of renal injury (2, 24, 36), and female sex hormones have been postulated to be renal protective (15, 32). Our data support a role for male sex hormones to promote albuminuria, although it does not appear to be via alterations in ANG II levels, AT1-receptor protein expression, or oxidative stress. It is important to note, however, that sex hormones are unlikely to be the sole factor responsible for sex differences in albuminuria since the sex differences in albumin excretion were present as early as 8 wk of age, an age at which hormone levels are still increasing to postpubescent levels. Macrophage infiltration and renal inflammation are present in the kidneys of male SHR as early as 3 wk of age, and renal inflammation has been shown to play a role in the development of hypertension and renal injury in male animals (22, 30). Consistent with macrophage infiltration decreasing with ORX, gonadectomy of male mice has been shown to decrease ischemia-reperfusion injury and the resultant inflammation compared with that shown in gonad-intact mice (27).

Our data also support a role for female sex hormones to be renal protective, potentially by decreasing renal inflammation and levels of oxidative stress. In line with our result, tamoxifen has been shown to reduce renal inflammation in female NZB/W mice (43). Although OVX increased macrophage infiltration, this was not associated with an increase in blood pressure. It is possible that a threshold level of inflammation must be present before blood pressure is influenced, and with time there would be an increase in pressure after the loss of ovarian hormones.

Interestingly, in the present study, female SHR had higher plasma ANG II levels, yet similar ANG II levels in the renal cortex compared with male SHR. In line with our findings, Yanes et al. (45) recently reported that aged (16-mo-old) male and female SHR have comparable levels of plasma ANG II, although renal ANG II levels were higher in females. Although the RAS has been shown to contribute to the development of
renal injury in SHR, little is known regarding the function of ANG II in renal injury in SHR (23, 29, 46). In aged SHR, despite females having high renal ANG II, the AT1-receptor blocker losartan decreased blood pressure to a greater extent in males than in females, leading the authors to suggest that hypertension in aging male SHR is more dependent on RAS than that shown in females. This is supported by clinical trial data suggesting that ACE inhibition is not as effective in treating hypertension in women compared with men, although, in healthy men and women, women were found to be more sensitive to angiotensin-receptor blockade (21, 47). These data suggest that both sex and sex hormones may influence the physiological response to ANG II. This hypothesis is corroborated by studies in which chronic infusion of ANG II selectively increased blood pressure in male rats but not in age-matched females and by animal data showing that females are less responsive to AT1-receptor blockade (41, 44). We should note that there is some cross-reactivity of the antibody used in the measurement of ANG II with ANG III and ANG 3–8 (see METHODS), raising the possibility that there are differences in angiotensin peptides that we could not detect with the methods employed.

Few studies have measured ANG II levels in males and females. It is much more common to measure angiotensinogen levels and PRA to assess the level of activation of the RAS. PRA and angiotensinogen levels have been reported to be greater in males and positively regulated by testosterone, whereas estrogen has been shown to reduce ACE activity and ACE mRNA in the kidney and to suppress AT1-receptor protein expression (6, 13, 29, 35). The discrepancy between renal ANG II levels and the resultant albuminuria in female SHR in this study may be related to the finding that female SHR have less AT1-receptor protein expression in the renal cortex than male SHR. Because AT1-receptor activation is linked with the progression and development of renal injury, a sex difference in AT1-receptor protein expression may contribute to a sex difference in albuminuria. Our finding is consistent with other reports that AT1 mRNA levels are higher in the aorta, mesenteric arteries, and kidneys of young male SHR than in those of females (35). In contrast to our findings, AT1-receptor protein expression has been shown to be comparable in aged male and female SHR and OVX has been shown to increase AT1-receptor mRNA expression in whole kidneys compared with young gonad-intact female SHR (35, 45). Therefore, the age of the animal, the region of the kidney, and the examination of protein vs. RNA may influence results of AT1-receptor expression examination. Renal cortical AT1-receptor expression cannot, however, explain the decrease in
albuminuria after gonadectomy in male SHR. Alternatively, there is another axis of the RAS system that may offer renal protection, including ACE2-mediated production of ANG 1–7. In gonadectomized male and female SHR, this “alternative” RAS pathway may be the predominant RAS pathway. Future studies are needed to test this hypothesis.

Oxidative stress is a risk factor for renal injury, and males have greater levels of oxidative stress than females (7, 12). Therefore, a sex difference in oxidative stress may contribute to the sexual dimorphism in albuminuria. Confirming previous reports, the renal cortex of male SHR had significantly more superoxide production than that of female SHR (12, 42). OVX increased superoxide production, supporting a role for ovarian hormones to suppress oxidative stress (9, 16). These results agree with our group’s (39) recent publication that female SHR have a sex hormone-sensitive suppression in hydrogen peroxide excretion compared with that shown in male SHR. ANG II contributes to oxidative stress-induced renal injury by AT1 receptor activation of NADPH oxidase (14, 19). Male SHR have increased protein expression of NADPH oxidase subunits in the kidney compared with male Wistar Kyoto rats, and male SHR have more AT1/NADPH oxidase-dependent superoxide generation in mesenteric arteries than female SHR (7). NADPH oxidase enzymatic activity, however, was comparable in the renal cortex of male and female SHR in our study, suggesting that NADPH oxidase is not the source of the superoxide responsible for the sex difference observed.

In conclusion, there is evidence of compromised renal function, as indicated by the presence of albuminuria early in the development of hypertension in both male and female SHR, although it is more severe in males, with both the RAS and oxidative stress likely contributing to sex differences in albuminuria. Changes in blood pressure alone cannot account for sex and sex hormone differences in albuminuria. In male SHR, there was a sex hormone-dependent elevation in blood pressure and albuminuria independent of renal cortical ANG II levels or oxidative stress. However, in female SHR, there was a sex hormone-dependent suppression of macrophage infiltration and superoxide production. Finally, the kidneys of female SHR have comparable levels of ANG II compared with kidneys from males, leading us to hypothesize that the “classical” RAS pathway contributes more to albuminuria in male SHR than in female SHR. Together, these data raise the possibility that men and women may respond differently to perturbations of the RAS and highlights the need for further studies on sex differences in the RAS. These data may have important implications for the treatment of cardiovascular and renal disease.

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