Sexual Dimorphism in Renal Production of Prostanoids in Spontaneously Hypertensive Rats

Jennifer C. Sullivan, Jennifer M. Sasser, David M. Pollock, Jennifer S. Pollock

Abstract—Male spontaneously hypertensive rats (SHR) have higher blood pressure, blunted pressure–natriuresis relationship, and accelerated progression of renal injury compared with female SHR. Renal medullary prostanoids mediate vascular tone, salt and water balance, and renin release and, as a result, are involved in the maintenance of renal blood flow and the pathogenesis of hypertension. The aim of this study was to determine whether a gender difference exists in prostanoid production in SHR and whether sex steroids influence prostaglandin (PG) production. Thirteen-week-old intact and gonadectomized male and female SHR rats were placed in metabolic cages for 24-hour urine collection. Prostanoid excretion was determined using enzyme immunoassay. Kidneys were isolated and separated into outer and inner medulla for Western blot analysis. Female SHR had enhanced urinary excretion of PG E2 (PGE2) metabolites and thromboxane B2, an indicator of renal thromboxane production, compared with male SHR. There were no gender differences in excretion of systemic thromboxane or prostacyclin. Correspondingly, female SHR had enhanced microsomal PGE2 synthase protein expression in the renal inner medulla and greater cyclooxygenase-2 (COX-2) expression in the outer medulla. Orchidectomy was associated with increased PGE2 metabolite excretion and microsomal PGE synthase protein expression. Thromboxane B2 excretion was not affected by gonadectomy in either male or female SHR. Protein expressions of COX and cytoplasmic PGE2 synthase in the renal medulla were unchanged by gonadectomy in both sexes. These results demonstrate a sexual dimorphism in renal production of prostanoids in SHR and that PGE production is testosterone sensitive and estrogen insensitive. (Hypertension. 2005;45:406-411.)

Key Words: gender ■ cyclooxygenase ■ prostaglandins ■ thromboxane ■ kidney

Male spontaneously hypertensive rats (SHR) have higher blood pressure, a blunted pressure–natriuresis relationship, and accelerated progression of renal injury compared with female SHR. Evidence suggests testosterone as an important factor in the increased blood pressure and renal damage in male SHR. Castration and chronic blockade of the androgen receptor attenuates renal injury and decreases blood pressure in male SHR to levels seen in female SHR.1–3 More specifically, the sexual dimorphism in blood pressure in SHR has been attributed, in part, to testosteron-derpendent stimulation of the renin-angiotensin system (RAS).1–3 More specifically, the sexual dimorphism in blood pressure in SHR has been attributed, in part, to testosteron-derpendent stimulation of the renin-angiotensin system (RAS).1–3

The RAS is important in regulating kidney function and interacts with a number of other systems, including prostanoids.4 Prostanoids are important in the physiological control of vascular tone, renin release, and blood pressure.5,6 In particular, there is evidence that cyclooxygenase-2 (COX-2)–derived prostanoids regulate hemodynamics and sodium and water reabsorption in the renal medulla.1,5,7 The deleterious renal effects associated with COX inhibition further suggest that COX-derived prostanoids have antihypertensive and natriuretic effects.5 Therefore, a gender difference in COX or prostanoid production may influence the development of hypertension or moderate the pressure reached in female SHR.

There are 2 primary COX isoforms: COX-1 and COX-2. COX-1 is constitutively expressed in the kidney and is localized in mesangium, endothelium, and epithelium, specifically cortical- and medullary-collecting duct cells.5,7 COX-2 is induced by inflammatory stimuli and is also constitutively localized in the macula densa, thick ascending limbs, and inner medullary interstitial and collecting duct cells.1–7 The primary COX products in the kidney are thromboxane A2 (TxA2), prostaglandin (PG) E2 (PGE2), and prostacyclin (PGI2). TxA2, primarily synthesized through COX-1 activity by thromboxane synthase, is a vasoconstrictor.6 COX-2 metabolites PGI2 and PGE2 are mediators of medullary blood flow and renal salt handling produced by vascular and tubular structures in the kidney.6,7 PGE2 is the major COX metabolite in the kidney and is critical to the pressure–natriuretic response as well as the ability of the kidney to maintain water and electrolyte balance.5,8,9

Sex steroids and gender can regulate prostanoids in the vasculature. Estrogen increases PGI2 synthesis and release from mouse cerebral arteries, ovine fetal pulmonary artery,
porcine aortic endothelial cells, and human umbilical vein endothelial cells.\textsuperscript{9} Additionally, Dahl salt-sensitive female rats have lower blood pressure compared with males and higher plasma levels of PGE\textsubscript{2} and PGL\textsubscript{2}.\textsuperscript{10} These data suggest that estrogen and the female gender are associated with increased dilator prostanooids. In contrast, ex vivo testosterone treatment of aortas from female rats results in decreased PGI\textsubscript{2},\textsuperscript{11} and treatment of cultured rat aortic smooth muscle cells and guinea pig coronary artery smooth muscle cells increases TxA\textsubscript{2} receptor density.\textsuperscript{12} TxA\textsubscript{2} release is also more pronounced in mesenteric arteries from male SHR compared with females, suggesting that testosterone and the male gender are associated with enhanced constrictor prostanooid release.\textsuperscript{13} The aim of this study was to determine whether there is a gender difference in the renal prostanooid system in SHR and whether sex steroids influence these pathways. Gender differences in prostanooid synthesis may contribute to the elevated blood pressures in male SHR compared with females.

\textbf{Methods}

\textbf{Animals}
Male and female SHR were all studied at 12 to 13 weeks of age (Harlan Laboratories, Indianapolis, Ind) in accordance with the National Institutes of Health \textit{Guide for the Care and Use of Laboratory Animals} and approved and monitored by the Medical College of Georgia committee for animal use in research and education. Rats were housed in temperature- and humidity-controlled, light-cycled quarters. Blood pressures were confirmed using the tail-cuff method.\textsuperscript{14} Rats were placed in metabolic cages to facilitate 24-hour urine collection.

Subsets of animals were gonadectomized at 10 weeks of age and studied at 13 weeks of age. For orchidectomy surgeries, rats were anesthetized with sodium pentobarbital (50 mg/kg), and the testes were exteriorized, the spermatic cord was tied with silk suture, and testes cut free. The cut spermatic cord was returned to the inguinal canal and the wound was closed. For ovarioectomy surgeries, rats were anesthetized with sodium pentobarbital (50 mg/kg) IP and the ovaries were exteriorized, tied off with silk suture, cut free, and the incision was closed. Ovarioectomy was confirmed by uterine and body weight.\textsuperscript{15}

\textbf{Assays and Chemicals}
Urinary concentrations of electrolytes were determined by ion-selective electrodes (Synchron EL-ISE; Beckman Instruments). Urinary protein was determined using a Bradford assay (Bio-Rad). Urinary TxB\textsubscript{2}, 2,3 dinor TxB\textsubscript{2}, 6-keto PG F\textsubscript{1\alpha} (PGF\textsubscript{1\alpha}), and PGE metabolite levels were determined by enzyme immunoassay according to manufacturer instructions (Cayman Chemical). The detection levels for TxB\textsubscript{2} and 2,3 dinor TxB\textsubscript{2} are 7.8 to 1000 pg/mL; TxB\textsubscript{2} metabolite, 6-keto PGF\textsubscript{1\alpha}, and PGE metabolite, 6-keto PG F\textsubscript{1\alpha} (PGF\textsubscript{1\alpha}), and PGE metabolite levels were determined by enzyme immunoassay according to manufacturer instructions (Cayman Chemical). The detection levels for TxB\textsubscript{2} and 2,3 dinor TxB\textsubscript{2} are 7.8 to 1000 pg/mL; TxB\textsubscript{2} detection level was 0.39 to 50 pg/mL, and intra-assay and interassay CVs were <17%. The 6-keto PGF\textsubscript{1\alpha} detection level was 3.9 to 500 pg/mL, and intra-assay and interassay CVs were <10%. PGE metabolite detection level was 0.39 to 50 pg/mL, and intra-assay and interassay CVs were <10%. Plasma interleukin-6 (IL-6) and tumor necrosis factor-\alpha (TNF-\alpha) were assayed according to manufacturer instructions using an ELISA (R & D Systems); however, values were below the detection level of both assays. Plasma IL-6 detection level is 62.5 to 4000 pg/mL, intra-assay CV was 8.8%, and interassay CV was 8.1%. TNF-\alpha detection level is 12.5 to 800 pg/mL, intra-assay CV was 2.2%, and interassay CV was 9.6%.

\textbf{Western Blot Analysis}
Outer and inner medullary sections were homogenized and Western blotting was performed as described previously.\textsuperscript{16} The primary antibodies were polyclonal anti-COX-1, anti-COX-2, anti-TxA\textsubscript{2} (TP) receptor, antithromboxane synthase, anticytopylamasic PGE synthase, and a monoclonal antimicrosomal PGE synthase (Cayman Chemical). All primary antibodies were used at a dilution of 1:1000. The appropriate secondary antibody (horseradish peroxidase–conjugated anti-mouse or rabbit; Upstate Biotechnology) was applied. To verify equal loading, the above primary antibodies were stripped using Restore Western blot Stripping Buffer (Pierce), and a secondary antibody to \beta-actin (1:5000; Upstate Biotechnology) was applied, followed by a secondary antibody (anti-rabbit; Amersham). Specific bands were detected with enhanced chemiluminescence (SuperSignal Chemiluminescent Substrate; Pierce), and densitometry was performed using a digital imaging system (Alpha Innotech). All densitometric results are reported normalized to actin.

\textbf{Statistical Analysis}
Body weight, blood pressure, and food and water consumption were compared using ANOVA (Statistica). Urinary excretion data for male/female and intact/gonadectomized comparisons were analyzed using a Student \( t \) test (Statistica). Relative densitometric units for male/female and intact/gonadectomized comparisons were analyzed using an unpaired \( t \) test with Welch’s correction (Prism). For all comparisons, \( P<0.05 \) was considered statistically significant.

\textbf{Results}

\textbf{Intact Animals}
Age-matched male SHR were larger and had higher blood pressures compared with female SHR (males 295\pm3 g, 195\pm3 mm Hg; females 181\pm5 g, 177\pm2 mm Hg; \( P<0.05 \)). Urinary protein excretion was also greater in males compared with females, suggesting a higher degree of renal injury (55\pm8 and 25\pm6 mg per day, respectively; \( P<0.05 \)). In addition, the males ingested more food and water compared with females (data not shown).

\textbf{Urinary Analysis}
Urinary excretion rates were determined for PG E (PGE) metabolite, 6-keto PG F\textsubscript{1\alpha} (PGF\textsubscript{1\alpha}), 2,3-dinor thromboxane B\textsubscript{2} (dinorB\textsubscript{2}), and TxB\textsubscript{2}. PGE metabolite is a measure of PGE\textsubscript{2} production. PGF is formed by hydration of PGI\textsubscript{2} and is measured as an estimate of PGI\textsubscript{2} production. TxA\textsubscript{2} is rapidly hydrolyzed to TxB\textsubscript{2} and further metabolized to dinorB\textsubscript{2}. Because of its transient nature, TxB\textsubscript{2} measurements reflect renal TxA\textsubscript{2} production, and dinorB\textsubscript{2} is an indicator of systemic TxA\textsubscript{2} production. Urinary excretion of PGE metabolite and TxB\textsubscript{2} were greater in females compared with males (Figure 1; see legend for \( P \) values). There were no differences in urinary excretion of PGF or dinorB\textsubscript{2} between male and female SHR.

\textbf{Western Blot Analysis}
Western blot analysis was performed to determine whether the observed gender differences in PGE metabolite and TxB\textsubscript{2} excretion were attributable to differences in protein expression of key enzymes involved in the synthesis of these mediators in the renal inner or outer medulla. COX-1– and COX-2–representative Western blots and the relative densitometric units are shown in Figure 2. There were no differences with gender in COX-1 expression (see figure legend for \( P \) values). There was a trend for COX-2 expression to be increased in the inner medulla from female SHR compared with males, and COX-2 expression was significantly greater.
in the outer medulla of female SHR. Figure 3 shows representative Western blots and the relative densitometric units for cytoplasmic PGE synthase and microsomal PGE synthase. The inner medulla of male SHR had significantly greater cytoplasmic PGE synthase protein expression compared with females, whereas inner medullary microsomal PGE synthase expression was greater in female SHR. There were no differences in protein expression of thromboxane synthase or the TP receptor between male and female SHR in either the outer or inner medulla (data not shown).

Gonadectomy

The effectiveness of ovariectomy surgery was verified by an increase in body weight and a decrease in uterine weight (body weight ovariectomized [OVX] 205 ± 6 g; uterine weight in grams, intact, 0.43 ± 0.03; OVX 0.13 ± 0.01; \( P < 0.05 \)). Gonadectomy resulted in a decrease in blood pressure in males with no alteration in pressure among females (orchidectomized [ORX] 177 ± 3 mm Hg; OVX 175 ± 3 mm Hg). Protein excretion and food and water consumption were decreased in males after gonadectomy; ovari-

Figure 1. Urinary excretion rates. Female SHR had significantly greater excretion rates for PGE metabolite (\( P = 0.00002 \); A) and TxB\(_2\) (\( P = 0.03 \); D) compared with males. There was no difference with respect to gender in excretion rates for PGF (\( P = 0.5 \); B) or dinorB\(_2\) (\( P = 0.4 \); C). Numbers in parentheses refer to n values. Asterisk indicates significant difference from males by Student t test.

Figure 2. COX-1 and COX-2 protein expression in renal inner and outer medulla from male (M) and female (F) SHR. A shows representative Western blots, and B shows the relative densitometric units. COX-1 expression was comparable between males and females in the inner and outer medulla. COX-2 expression was greater in the inner \( (P = 0.07) \) and outer medulla \( (P = 0.03) \) of female SHR compared with males. Asterisk indicates significant difference from males by unpaired t test with Welch’s correction; \( n = 7 \) to \( 8 \); 100 \( \mu \)g protein loaded per well.
ectomy did not affect any of these parameters (ORX 30±6 mg per day; OVX 25±6). Excretion of PGE metabolite was increased by gonadectomy in males. PGE metabolite excretion was comparable in females and OVX (values in ng per day; males 19±2, ORX 27±3; P<0.05; females 47±4, OVX 53±3). Correspondingly, there was an increase in inner medullary microsomal PGE synthase protein expression in ORX compared with males, and microsomal PGE synthase expression was not affected by gonadectomy in females (relative densitometric units; males 1.1±0.1, ORX 1.4±0.13; P=0.05; females 1.5±0.2, OVX 1.3±0.12). TxB2 excretion was not affected by gonadectomy in either male or female SHR (values in ng per day; ORX 35±5; OVX 44±4). There were no changes in protein expression of COX-1, COX-2, cytoplasmic PGE synthase, or thromboxane synthase in either the inner or outer medulla (data not shown). Expression of the TP receptor was significantly decreased by ORX in the inner medulla (relative densitometric units; males 0.67±0.1, ORX 0.36±0.07; P<0.05; females 0.57±0.07, OVX 0.37±0.1).

**Discussion**

Although evidence supports a role for COX-derived prostanooids to act as antihypertensive and natriuretic factors, this study is novel in examining the influence of gender and sex steroids on prostanoid pathways in hypertension. Our experiments revealed a sexual dimorphism in the urinary excretion of prostanoids; female SHR have enhanced PGE2 and TxA2 production compared with males. The increase in TxA2 levels is attributable to enhanced renal production because there was no difference in systemic TxA2 levels between male and female SHR. Furthermore, we observed that PGE metabolite excretion and microsomal PGE synthase protein expression were enhanced by orchidectomy but unchanged by ovariectomy, suggesting that PGE production is testosterone sensitive and estrogen insensitive. Renal thromboxane production was not influenced by sex steroids. We confirmed that male SHR had greater blood pressure and proteinuria compared with age-matched females. The sexual dimorphism in blood pressure and protein excretion was abolished by orchidectomy. Together, our data support the hypothesis that testosterone drives the elevation in blood pressure and renal injury in male SHR, in part through decreasing PGE2 production.

The PG pathway is altered in SHR and proposed to contribute to the development and maintenance of hypertension. One role of dilator prostanoids is to oppose renal vasoconstriction, therefore, decreased PGE2 production may result in increased blood pressure. In support of this theory, PGE2 levels are reduced in the medulla of male SHR compared with Wistar-Kyoto rats, and the renal medulla from normotensive female rats has higher basal levels of vasodilator PGs compared with males. Whereas this is the first study to examine the effect of gender on PGE2 levels in SHR, female Dahl salt-sensitive rats and wild-type mice have higher plasma levels of PGE2 and PG12 compared with males. As expected, we found PGE2 excretion to be greater in female SHR compared with males; however excretion of PG12 metabolites was comparable. Surprisingly, PGE2 production was not sensitive to estrogen but does appear to be sensitive to testosterone. Whereas the ability of sex steroids to modulate PGE2 levels has not been examined previously, PG12 has been shown to be positively regulated by estrogen in mouse cerebral arteries and aortic smooth muscle cells, ovine fetal pulmonary artery, porcine aortic endothelial cells, and human umbilical vein endothelial cells.

TxA2 levels are known to be increased in male SHR compared with normotensive controls, and a role for TxA2 in the determination of blood pressure in SHR has been demonstrated by the ability of thromboxane synthase inhibitors
and TP receptor blockade to decrease blood pressure in male rats. The role of thromboxane antagonism has yet to be examined in female SHR. Our finding confirms reports in the literature that female SHR excrete more TxB2 compared with males. The consequences of greater renal TxA2 production in female SHR are unknown, although our data would predict that thromboxane receptor antagonism may have a larger impact on blood pressures in female SHR. Alternatively, the increase in PGE2 may offset any impact of increased renal TxA2 on blood pressure. Future studies will address this question.

Renal medullary prostanoids are important in the control of salt and water balance and, as a result, are involved in the pathogenesis of hypertension. This was nicely illustrated in a recent article by Zewde and Mattson, in which infusion of a COX-2 inhibitor into the renal medulla increased blood pressure in normotensive male animals on a high-salt diet. PGE2 is the major COX metabolite of arachidonic acid in the kidney and is critical to the pressure–natriuresis response. Two enzymes catalyze the synthesis of PGE2: microsomal PGE synthase and cytoplasmic PGE synthase. COX-2 and microsomal PGE synthase protein expression were greater in the inner medulla from female SHR. Furthermore, COX-2 and microsomal PGE synthase colocalize in medullary interstitial cells in rats and rabbit kidneys. Accordingly, it is likely that the enhanced PGE metabolite excretion in female SHR results from a COX–2–driven increase in inner medullary microsomal PGE synthase expression. Microsomal PGE synthase has only recently been cloned, and this is the first study to examine the effects of gender and sex steroids on microsomal PGE synthase protein expression levels. Although male SHR had greater cytoplasmic PGE synthase protein expression comparable to females, levels of PGE metabolite excretion were greater in females. It is possible that despite an increase in protein expression, PGE enzymatic activity is impaired. It is not known whether the enzyme is regulated by post-translational modifications such as phosphorylation or how this would impact enzymatic activity.

Although COX–2 protein expression can be induced by inflammatory agents, COX-2 is also found constitutively localized in the medulla. It is possible that the increase in COX–2 expression in females is the result of greater inflammation; however, that seems unlikely. The kidneys of female SHR have been shown to have a slower progression of renal injury compared with males, and in our experiments, proteinuria was less in females. Furthermore, a recent article by Egan et al reported that COX-2 protein expression was increased in healthy mouse aortic smooth muscle cells by treatment with estrogen, supporting our data of enhanced COX–2 protein expression in tissue from females.

Alterations in protein expression of thromboxane synthase and TP receptor could not account for the increased TxB2 excretion in females. Increased TxB2 may be the result of enhanced thromboxane synthase in the cortex; it is unknown what region of the kidney is responsible for urinary TxB2 excretion. Alternatively, thromboxane may be handled differently in male and female SHR. As mentioned above for cytoplasmic PGE synthase, protein expression does not always equate with enzymatic activity. There may be a gender difference in the metabolism and clearance of TxA2 or post-translational modifications of the enzyme-altering activity. As expected, TP receptor protein expression was decreased by orchidectomy. Testosterone has been shown to increase TP receptor density in aortic vascular smooth muscle cells. Interestingly, there was a trend for ovariectomy to decrease TP receptor density as well, suggesting that estrogen may also increase TP receptor density.

Perspectives

Our data suggest that prostanoid production may be regulated by male sex steroids but not female sex steroids in SHR. Castration and chronic blockade of the androgen receptor attenuate renal injury and decrease blood pressure in SHR, suggesting that testosterone is an important factor in the increase in blood pressure and renal damage in SHR. Our data support these findings and suggest that one of the mechanisms by which gender mediates increases in blood pressure and kidney injury is by decreasing inner medullary microsomal PGE synthase and PGE2 production. This is an important finding because COX-2 inhibitors have been widely used clinically, with a primary side effect being renal insufficiency. Epidemiological studies involving COX-2 inhibition do not currently differentiate between men and women; our data suggest that gender and blood pressure should be considered. If hypertensive males have a lower capacity to produce PGE2 compared with hypertensive females, an inhibitor could rapidly exacerbate an already compromised prostanoid system, resulting in severe renal injury.

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