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Testosterone Effects on Renal Norepinephrine Content and Release in Rats With Different Y Chromosomes

Thomas J. Jones, Gail Dunphy, Amy Milsted, Daniel Ely

Abstract—The Y chromosome in spontaneously hypertensive rats (SHR) and stroke-prone rats has been shown to contain a locus that contributes to the hypertensive effect; both the sympathetic nervous system and testosterone may be involved. The objective of this study was to look at the effects of testosterone on renal norepinephrine (NE) release and content in the isolated perfused kidney in different Y chromosome backgrounds. The study involved male SHR, Wistar-Kyoto rats (WKY), and 2 consomic strains with different Y chromosomes (n=5 to 8 per group). Adult animals were castrated, and implants containing testosterone propionate were placed at the base of the neck. Blood testosterone levels were measured by radioimmunoassay 2 weeks after castration. The left kidney was isolated and perfused with oxygenated Krebs solution at a constant flow and temperature with KCl and electrical stimulation of the renal nerves. Perfusate was collected and analyzed for NE by high-performance liquid chromatography. Lactate dehydrogenase analyses were performed as a marker for potential tissue damage. Renal perfusate and renal tissue NE levels were significantly elevated by testosterone. The average NE increase with a single testosterone implant was 13.2 ng/mL, and for a double testosterone implant it was 29.8 ng/mL. The Y chromosome from the SHR produced a significant increase in renal NE release compared with the WKY Y chromosome. Significance was shown between all groups: 1 versus 2 implants, $P=0.0067$; 1 versus sham implants, $P=0.015$; 2 versus sham implants, $P<0.001$. In conclusion, testosterone caused an enhanced renal NE release that was strain-specific, with the Y chromosome raising renal NE content and release. (*Hypertension*. 1998;32:880-885.)

Key Words: androgens ■ catecholamines ■ gender ■ genetics ■ steroids

The tendency for males to have a greater incidence of hypertension than females¹⁻³ in most mammalian species, at least until menopause (humans), has led to the implication of sex steroids as a possible hormonal component in the development and maintenance of essential hypertension.⁴ The sympathetic nervous system (SNS) has also been implicated in some forms of hypertension through increased release of norepinephrine (NE) and increased tissue NE content and turnover.⁵⁻¹¹ Our laboratory is interested in testosterone, the SNS, and their interactions as major players in the evolution and development of high blood pressure. Recently, we showed that the Y chromosome from spontaneously hypertensive rats (SHR) when back-crossed into Wistar-Kyoto rats (WKY) increases blood pressure and SNS indicators.¹² The kidney appears to be a target organ for androgens. Testosterone dramatically increases kidney weight.¹³ When a female SHR kidney is transplanted to a male SHR, the kidney morphologically and physiologically responds like a male kidney.^{14,15} Steroid binding receptors have been biochemically and autoradiographically demonstrated in the rat renal proximal tubule,¹⁶ and anatomic sex differences exist in the proximal tubule that change after castration.¹⁷

Therefore, the hypothesis tested was that testosterone would increase the amount of NE released from the kidney

and that the SHR Y chromosome would be associated with a greater increase and storage of NE in the presence of testosterone than the Y chromosome from WKY.

Methods

SHR and WKY were obtained originally from Harlan Sprague-Dawley (Indianapolis, Ind) and since 1981 have been inbred at The University of Akron. Two consomic strains were developed at The University of Akron to study the role of the Y chromosome in hypertension. The SHR/y strain carries autosomes from the normotensive WKY strain and the Y chromosome from the hypertensive SHR. The SHR/a strain carries autosomes from the SHR strain and the Y chromosome from the normotensive WKY strain.¹⁸ The animals were housed in groups of 2 to 3 in a polyethylene cage (45×25×20 cm) with a stainless steel top and heat-treated wood chips for bedding (P.J. Murphy Forest Products). Rat chow (Teklad Rodent diet) and water were provided ad libitum. All animals were kept in environmentally controlled conditions of 25°C to 27°C, 40% to 50% humidity, and a 12-hour light/dark cycle. A baseline blood pressure from each animal was obtained 2 weeks before surgery by tail-cuff sphygmomanometry to check that blood pressures were in the right range for each strain: SHR (170 to 200 mm Hg), WKY (110 to 130 mm Hg), SHR/y (150 to 170 mm Hg), and SHR/a (150 to 170 mm Hg). If pressures were not in these ranges, the animals were not used. The present animals were 1 generation removed (F₁₆) from animals in which blood pressure was measured by aortic telemetry (F₁₇) and should have had comparable pressures (SHR, 195 mm Hg;

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WKY, 122 mm Hg; SHR/y, 152 mm Hg; SHR/a, 155 mm Hg¹²). All procedures and tests were in accordance with the American Physiological Society statement on the humane treatment of animals and were approved by The University of Akron Institutional Animal Care and Use Committee.

The experimental design for the study used 84 castrated rats and involved the following 3 treatments and 4 strains: sham implants in WKY (n=7), SHR (n=6), SHR/y (n=5), and SHR/a (n=7); 1 testosterone implant in WKY (n=6), SHR (n=11), SHR/y (n=8), and SHR/a (n=9); and 2 testosterone implants in WKY (n=6), SHR (n=6), SHR/y (n=7), and SHR/a (n=6).

Castration, Testosterone Implants, and Kidney Retrieval

Animals were sedated with 50 mg/kg Brevital (Eli Lilly & Co) and surgically castrated. Testosterone implants were composed of Silastic tubing (Dow Corning Midland) with a length of 19 mm (ID, 0.062 mm; OD, 0.125 mm), containing 10 mg testosterone propionate. The ends were sealed with Silastic medical-grade silicone adhesive (Type A, Dow Corning).¹⁹ Before implantation, the implants were cured overnight in 5% bovine serum albumin, 10 mmol/L sodium phosphate buffer, 0.9% NaCl, and 0.0001% merthiolate and soaked in 70% ethanol for 2 hours. Two weeks after testosterone implantation, the animals were again sedated (50 mg/kg Brevital), and a retro-orbital blood sample was taken before kidney retrieval to determine serum testosterone levels.²⁰ The serum samples were collected retro-orbitally immediately before kidney isolation and analyzed by radioimmunoassay for testosterone concentration (Bio-Rad Laboratories). The correlation with another kit was $r=0.991$, sensitivity was 0.08 ng/mL at the 95% confidence limit, and the highest cross-reactivity with potential interfering steroids was with 5 α -dihydrotestosterone (6.65%). The coefficient of variation for our sample range within-run was 7.4% to 11.6% and for between-run was 12.5% to 16.96%.

Animals were heparinized (1000 U/cc) and then sedated with Brevital for kidney removal. The body cavity was opened, and the left and right adrenal glands were isolated and removed. Adipose and connective tissues around the left kidney were removed so they would not adhere to the kidney and interfere with the stimulation procedure. The vessels branching off the aorta in the region were isolated and ligated. The main bifurcation of the aorta between the left and right kidneys, along with the placement of ligatures at the posterior end of the aorta proximal to the femoral bifurcation, were to aid in organ removal. The kidneys were removed, and a 21-gauge blunted needle was secured in the aorta for retrograde perfusion of the left kidney. The ischemic time from aortic ligation to start of isolated kidney perfusion was <1 minute. The right kidney was frozen and stored at -70°C for later NE analysis. The animal was then terminated with a bolus injection of Brevital directly into the heart. The 21-gauge needle supporting the aorta and kidney was suspended and perfused using a Gilson pump (minipuls 2) at a rate of 6 mL/min. The pressure was monitored through a Statham gauge pressure transducer (model P23 ID) attached to a Gould physiograph (model 2400S). The pressure of the unstimulated perfused kidney was an average of 50 mm Hg and during stimulation rose to an average of 100 mm Hg. Krebs solution composed of 119 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L CaCl₂, 1.2 mmol/L MgSO₄, 1.2 mmol/L KH₂PO₄, 25 mmol/L NaHCO₃, and 14 mmol/L dextrose was oxygenated with 95% O₂/5% CO₂ and warmed and maintained at 35°C on a stir plate. The kidney was allowed to equilibrate until a constant pressure was achieved.

Perfusate Assay for NE and Lactate Dehydrogenase

Two samples of renal perfused fluid were collected at 5 time points: the first collection (1 mL) was for lactate dehydrogenase (Sigma Chemical Co) to determine the level of tissue damage obtained through the removal and perfusion of the kidney, and the second collection (6 mL) was to determine the levels of NE present using high-performance liquid chromatography with electrochemical de-

tection (HPLC-ELD).²¹ The first collection was a steady-state control with normal Krebs solution (4.7 mmol/L KCl) 7 minutes after pressure equilibrium had been achieved. The kidney was then perfused with Krebs solution containing 15 mmol/L KCl to potentiate the potassium channels and facilitate vasoconstriction. The kidney was stimulated at the base of the renal artery (Grass Stimulator, model 5D9) with a frequency of 4 cps, duration of 4 milliseconds, and intensity of 18 V. The stimulus was applied for 1 minute. Therefore, 5 collections for NE were taken: the first sample was for the control, the middle sample was used for the representative stimulated sample, and the final sample was another control. The second and fourth samples were not used. Each collection for NE was stabilized in a solution of mobile phase and immediately frozen in liquid nitrogen; the NE samples were stored until analysis at -70°C . The first perfusate collection was a control sample without stimulation, followed by 3 stimulation periods with a 7-minute equilibration period between each, during perfusion with normal Krebs solution; a final control perfusate was collected with no electrical or potassium stimulation. The average length of time for sample collection was about 70 seconds for 7 mL, and the average total time for a kidney experiment once perfusion was complete was ≈ 35 minutes.

Kidney Homogenization

After the perfusion experiments, the left kidney was weighed and adipose tissue was removed. Each kidney was placed in a glass specimen jar with 2 mL of mobile phase (0.035 mol/L citric acid monohydrate, 0.09 mol/L sodium acetate, 130 $\mu\text{mol/L}$ octylsulfate, sodium salt, and 11% methanol) used in the HPLC assay. The kidneys were homogenized (model m133/1281-0, Biospec Products Inc) at 4°C to 6°C, transferred to a 12 \times 75-mm test tube, and centrifuged (Damon/IEC division centrifuge model IE HN-SII) at 4°C to 6°C and 10 000 rpm for 10 minutes. The supernatant was removed and stored in a microcentrifuge tube at -70°C for NE analysis. Values for NE content and release are presented as absolute values and fractional overflow ratios because the right kidney was used for content analyses (total NE) and the left kidney for the isolation perfusion studies.

The statistical analysis included 1- and 2-way ANOVAs and appropriate follow-up *t* tests using Sigma Stat (Jandel Scientific). Significance was assumed if $P<0.05$.

Results

Serum testosterone levels were significantly different among implant groups: sham, single implants, and double implants

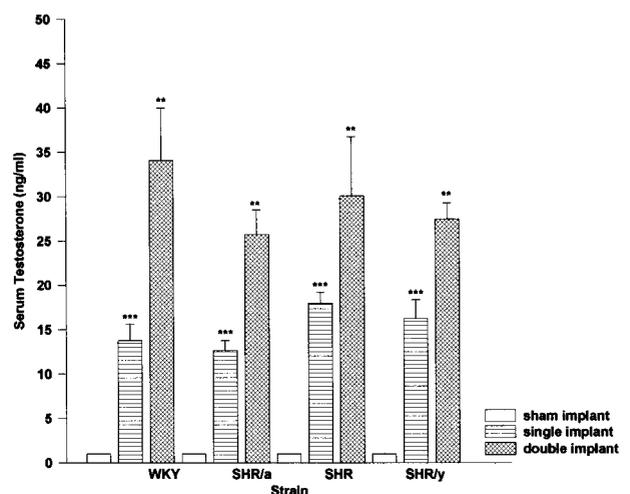


Figure 1. Blood serum testosterone levels by strain 2 weeks after implantation (means and bars indicate standard error, 2-way ANOVA): strain, $F=1.10$, NS; implant, $F=119.9$, $P<0.001$; interaction, $F=0.9$, NS. ** $P<0.01$, *** $P<0.001$ compared with previous bar, *t* test.

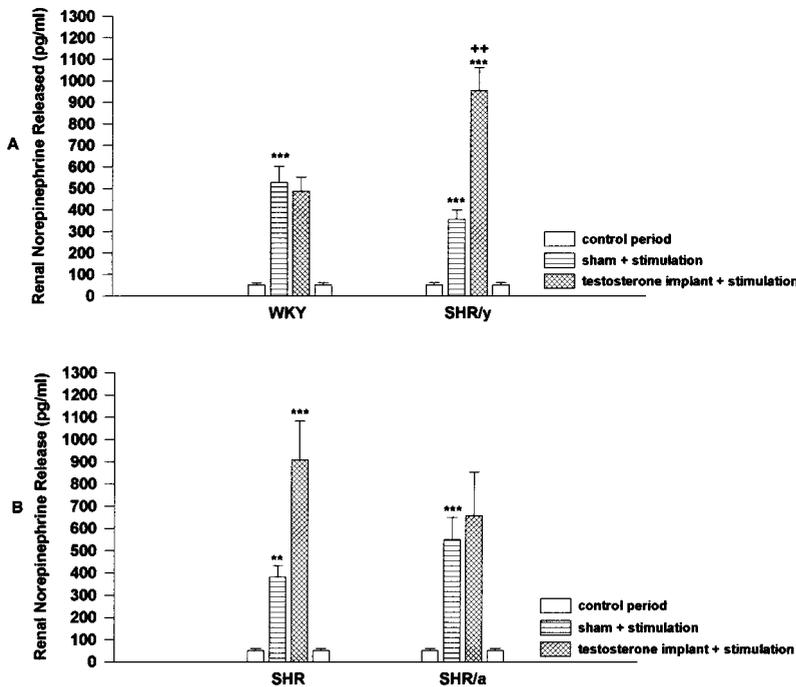


Figure 2. Renal NE after castration and 2-week implantation with testosterone propionate or sham implants. A, WKY and SHR/y: control vs sham, *** $P < 0.001$; sham vs testosterone SHR/y, *** $P < 0.0001$; WKY vs SHR/y testosterone, ++ $P < 0.01$. B, SHR and SHR/a: control vs sham, ** $P < 0.05$, *** $P < 0.001$; sham vs testosterone in SHR, *** $P < 0.001$. Values are mean \pm SE (control periods are before and after stimulation).

($F = 120$, $P < 0.001$), and the t tests showed significant differences ($P < 0.05$) within strains among treatment groups (Figure 1). There were no significant differences among strains and no interaction between treatment and strain. Lactate dehydrogenase levels in the kidney perfusate were low, with an average of 7 U/L, which indicates minimal cellular damage.

Figure 2 shows an increase in the release of NE in the perfusate from the left kidney. The NE release data are pooled across all 4 strains to illustrate the general effect of the testosterone. This increase in NE release was associated directly with the level of testosterone present (sham, 450 pg/mL; single implant, 580 pg/mL; or double implant, 750 pg/mL) and demonstrated a significant increase in the release of NE between implants (ANOVA, $F = 130$, $P < 0.0001$).

In WKY, electrical stimulation increased renal NE overflow significantly compared with the control values, but testosterone did not further increase it. In contrast, in SHR/y rats there was a significant enhancement of renal NE overflow with testosterone treatment during stimulation (Figure 2). There were similar increases in renal NE overflow during stimulation without significant testosterone enhancement in SHR/a, whereas the SHR group showed testosterone enhancement.

Kidney NE content in the right nonperfused control was examined in all 4 strains of rats (Figure 3). The comparison of strains, treatments, and the NE content for the right kidney showed that the SHR/y was the highest compared with the WKY group.

The comparison of all strains, implant type, and NE content showed a significant difference between the WKY and SHR/y right control kidney ($P = 0.004$). There were no significant differences found among the other strains. The Table shows the percentage of stimulus-induced fractional overflow of renal NE, which is based on the amount released compared with the total content of the right kidney that was not stimulated. This assumes that both kidneys have approximately the same NE content. From a previous study in our laboratory, we found there were no differences in NE content

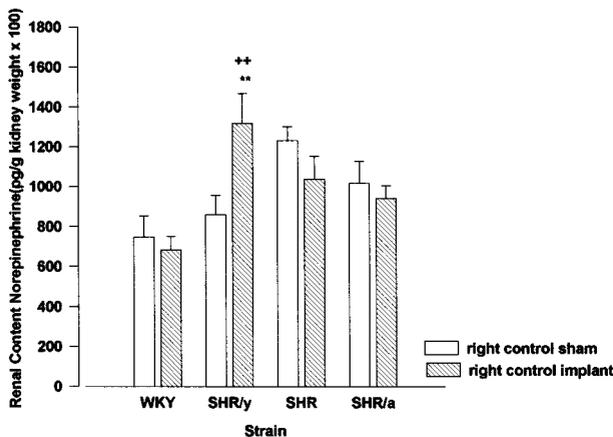


Figure 3. Kidney NE content by strain and treatment in the control nonperfused right kidney after castration and 2-week implantation with testosterone propionate or sham implants. Two-way ANOVA: strain, $F = 6.437$, $P < 0.01$; treatment, $F = 0.178$, NS; interaction, $F = 3.818$, $P = 0.01$. ** $P = 0.004$ WKY vs SHR/y right control implant; ++ $P < 0.01$ for SHR/y sham vs implant (mean \pm SE).

Stimulus-Induced Fractional Overflow Percentage of Renal Norepinephrine

Treatment	WKY	SHR/y	SHR	SHR/a
Sham castrated control	0.74 \pm 0.15	0.48 \pm 0.07	0.31 \pm 0.04	0.60 \pm 0.15
Testosterone implant	0.70 \pm 0.10	0.75 \pm 0.09*	0.75 \pm 0.13*	0.66 \pm 0.38
Increase, %	-5	56	142	10

Values are mean \pm SEM.

* $P < 0.05$ testosterone implant compared with castrated sham control.

or release from the left versus right kidney (L. Eveleth, unpublished results, 1996). Testosterone increased the fractional NE release in SHR and SHR/y ($P < 0.05$) but not in SHR/a or WKY. SHR/y showed a greater fractional release than SHR/a but not as high as that of SHR.

Discussion

The SHR Y chromosome (present in the SHR and SHR/y groups) was associated with increased renal NE release in the presence of testosterone compared with the response associated with the WKY Y chromosome (present in the WKY and SHR/a groups). Previously, we showed that the presence of androgens and their receptors was necessary for the maximum effect of the Y chromosome on blood pressure.²² Testosterone most likely was the main steroid responsible for the blood pressure effect because castration reduced blood pressure, and testosterone replacement after castration restored blood pressure to control levels.^{19,22,23} However, not as much is known about the interaction of NE and testosterone. Testosterone can influence NE metabolism, storage, and release.²⁴ For instance, long-term castration decreases the density of adrenergic neurons and produces morphological changes that are reversed with testosterone replacement.²⁵ Androgenic hormones can induce and/or maintain the sensitivity of the α_2 -adrenergic receptor system in the Wistar rat and may be involved in the maturation of the system.²⁶ However, this would seem contradictory to our results because this would reduce NE overflow. Different tissues and different rat strains may respond differently. Inhibition of NE uptake by testosterone has been shown in the isolated rat heart.²⁷ Testosterone has been shown to stimulate the increased expression of α_1 -receptors in myocytes grown in tissue culture after 48 to 96 hours because of synthesis of new proteins.²⁸ In the central nervous system, peripheral testosterone also modulates NE metabolism. For instance, testosterone implants similar to those used in our study reduced NE turnover in the basal hypothalamus.²⁹ Also, the presence of testosterone significantly potentiates the vasopressor action of NE,³⁰ and in castrated rats potassium-stimulated NE release was reduced in olfactory bulbs³¹ compared with that in intact animals. One of the primary interactions of testosterone and NE is testosterone regulation of tyrosine hydroxylase, the rate-limiting step in the biosynthesis of catecholamines found in the superior cervical ganglia.³² Activation of the renal adrenergic receptor increases sodium reabsorption by promoting $\text{Na}^+ - \text{H}^+$ exchange at the brush border of renal tubules.³³ Because the presence of the Y chromosome in the SHR/y animals produced an early rise in testosterone levels and was associated with a rise in blood pressure³⁴ and elevated sympathetic nervous system indices,¹² which approach those of SHR, it appears possible that the Y chromosome, through both enhanced SNS activity and an earlier testosterone rise, may cause more NE to be released from the kidney.

The SHR has increased SNS activity^{5,6,9,19} that can lead to increased circulating NE,^{12,19} which has been implicated in the genesis of hypertension. The testosterone-induced increase in renal NE release (125% to 167%) was seen in

both groups with an SHR Y chromosome (SHR and SHR/y animals), possibly due to increased NE stores and more release per stimulation. The percentage of increase of NE release after testosterone treatment was highest in SHR, followed by SHR/y and then SHR/a, which supports the idea that the Y chromosome influences NE release. The WKY group and the SHR/a strain without the SHR Y chromosome did not show significant testosterone enhancement of renal NE release. Our data suggest that the increased NE release is probably due to increased NE stores in SHR/y because they had an 86% increase in NE content compared with WKY, which is about half the total increase in NE release (167%). We expected the SHR group to also have elevated renal NE stores, which the castrated group did, but testosterone treatment did not significantly raise the NE content in any group except SHR/y, which appears to be due to increased NE release. Because testosterone treatment did not increase renal NE content of SHR but did increase the NE overflow, this suggests a different additional mechanism operating in the SHR/y, perhaps functioning at the biosynthetic enzyme level.

The testosterone enhancement of renal NE release is supported by our recent findings that the Y chromosome produces enhanced SNS activity with increased adrenal gland chromogranin A, which represents a long-term SNS influence.¹² The precise mechanism for this NE elevation in adrenal storage is not known but could be due to an increase in synthesis; future studies we are planning will examine the rate-limiting enzyme in the catecholamine pathway, tyrosine hydroxylase. There is also evidence supporting the idea that NE can significantly stimulate testicular testosterone in a concentration-dependent manner.³⁵ Even metabolic enzymes such as ornithine decarboxylase are regulated by steroids and catecholamines,³⁶ and recently testosterone regulation of renal proximal tubule organic anion-transporting polypeptide (oatp) has been shown in the Sprague-Dawley rat.³⁷ Their results suggest that oatp may play a role in the metabolic clearance and elimination of endogenous sex steroid hormones. Androgen receptors have been identified in the kidney proximal tubules³⁸; their function is not yet known but may involve electrolyte regulation.

The role of the kidney in hypertension is complex, involving physical, neural, and neuroendocrine regulation.³⁹ An excellent review by Di Bona and Kopp⁴⁰ details the complex involvement of the SNS and kidney function. The increased activation of the SNS could contribute to renal hypertension via several mechanisms, including increased catecholamine biosynthesis and release, and/or via central actions of angiotensin II, which can increase sympathetic nerve activity and elevate blood pressure.⁴¹ Sympathetic nerve endings possess prejunctional receptors that can be activated by chemical factors to increase or inhibit NE release.⁴² Also, α -adrenoceptor blockade-resistant pressor responses in the rat kidney are due to the sympathetic cotransmitter ATP.⁴³ There is an increased α_2 -adrenoceptor-mediated autoregulation of NE release in SHR kidneys caused by increased intrasynaptic NE.⁴⁴

The increase in SNS activity in SHR and SHR/y animals also could be reflected in changes in gene expression that occur through receptor-mediated events involving both NE and its effect on other hormones. It has been proposed that angiotensin II interacts with the SNS to facilitate NE release in the kidney.⁴⁵ Renal denervation has been shown to delay the onset or decrease the severity of renal hypertension^{46,47} and results in a major reduction in renal NE content in SHR and WKY.⁴⁷ Dihydrotestosterone also has been shown to have a tissue-specific regulation of renin mRNA in several organs important for blood pressure control, eg, the kidney, adrenal gland, and brain in female mice.⁴⁸ There also appears to be a testosterone-renin-angiotensin interaction that shows a direct relationship between androgens and renal and hepatic, angiotensinogen, and renin mRNA in hypertensive rats.⁴⁹

In conclusion, testosterone significantly increased renal NE release in the 2 strains with the SHR Y chromosome (SHR and SHR/y) but not in the 2 strains with the WKY Y chromosome (WKY and SHR/a). Because the SHR/y group treated with testosterone also had increased NE renal storage, the data support the hypothesis that the Y chromosome effect facilitates the action of testosterone on NE release. This influence on renal function may account for a portion of the blood pressure rise associated with the SHR Y chromosome.

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