Androgen-Dependent Angiotensinogen and Renin Messenger RNA Expression in Hypertensive Rats

Yiu-Fai Chen, Allen J. Naftilan, and Suzanne Oparil

Our previous studies demonstrated that the sexually dimorphic pattern of hypertension in the spontaneously hypertensive rat is androgen dependent. Gonadectomy retards the development of hypertension in young males, but not in females, and administration of testosterone propionate to gonadectomized spontaneously hypertensive rats of both sexes confers a male pattern of blood pressure development. The current study tested the hypothesis that renal and hepatic renin and angiotensinogen gene expression are also androgen dependent in the spontaneously hypertensive rat. Male and female spontaneously hypertensive rats underwent gonadectomy or a sham operation at 4 weeks of age. Subgroups of gonadectomized rats of both sexes were implanted with a 15-mm or 30-mm Silastic capsule filled with testosterone at the same time the gonadectomy was performed; a third group received an empty Silastic capsule. Northern and slot blot analyses were used to characterize and quantitate renin and angiotensinogen messenger RNA (mRNA) in the kidney and liver 18 weeks after the gonadectomy. Blood pressure, plasma renin activity, and hepatic angiotensinogen mRNA levels were higher in intact males than in females. Orchidectomy retarded the development of hypertension and lowered plasma renin and renal and hepatic angiotensinogen mRNA levels, and testosterone replacement restored the male pattern of hypertension and plasma renin and increased renal and hepatic angiotensinogen mRNA. Ovariectomy did not alter blood pressure or plasma renin but did lower renal renin and renal and hepatic angiotensinogen mRNA; testosterone increased blood pressure, plasma renin, renal renin and angiotensinogen mRNA, and hepatic angiotensinogen mRNA levels in ovariectomized females. These data suggest that the androgen-dependent development of hypertension in spontaneously hypertensive rats may be related to androgen-induced activation of the renin-angiotensin system. (Hypertension 1992;19:456-463)

KEY WORDS • sex characteristics • renin • angiotensinogen • messenger RNA • kidney • liver • essential hypertension • spontaneously hypertensive rats

A sexually dimorphic pattern of blood pressure has been observed in several forms of hypertension. The prevalence of essential hypertension is lower in premenopausal women than in age-matched men.1 In the DEA/J and CBA strains of mice, the male develops higher blood pressure than the age-matched female.2 In the Dahl salt-sensitive rat, the spontaneously hypertensive rat of the Okamoto strain (SHR), and the deoxycorticosterone acetate-NaCl hypertensive rat, hypertension develops more rapidly and becomes more severe in the male than in the female.3-8 Recent studies from our laboratory have demonstrated that the sexually dimorphic pattern of blood pressure development in SHR is androgen dependent.9 However, the mechanism by which androgen mediates the sexual dimorphism of blood pressure in SHR is unknown.

Several studies have suggested that androgenic regulation of renin-angiotensin system activity may contribute to the development of androgen-dependent hypertension. Submandibular gland (SMG) renin activity has been shown to decrease after castration and to increase after testosterone treatment, as well as during puberty, in male mice.10-14 A recent study by Wagner et al14 demonstrated that dihydrotestosterone treatment of female NMRI mice results in an increase in renin messenger RNA (mRNA) concentration in the SMG, adrenal gland, and brain, and a decrease in renin mRNA concentration in the kidney. In addition, Ellison et al15 demonstrated that male Wistar-Kyoto (WKY) rats castrated as weanlings and normal adult female WKY rats implanted with testosterone display significant increases in renal angiotensinogen mRNA levels. Based on this information, we hypothesized that androgens participate in the development of hypertension in the SHR through effects on the expression of renin, or...
angiotensinogen genes, or both. In the present study, we tested the hypothesis that renin and angiotensinogen gene expression are androgen dependent in male and female SHR. Our results demonstrated that plasma renin activity (PRA), hepatic angiotensinogen mRNA levels, and in female rats, renal renin mRNA levels were enhanced by exogenous androgen treatment, suggesting that the androgen-dependent development of hypertension in SHR may be related to androgen-induced activation of renin-angiotensin system activity.

Methods

Animal Preparation

Male and female SHR of the Okamoto strain (IBU 3 colony) were obtained from Taconic Farms, Germantown, N.Y., at 4 weeks of age. All rats were maintained four per cage at constant humidity (60±5%), temperature (34±1°C), and light cycle (6 AM to 6 PM), and were fed a standard (1% NaCl) rat pellet diet (Ralston Purina diet, St. Louis, Mo.) ad libitum. Systolic blood pressure was measured weekly in conscious, pretrained restrained rats by the tail-cuff method using an electrophygomanometer and physiograph recorder (Narco Bio-Systems, Houston, Tex.). Body weight and heart rate were determined on the same day as the blood pressure measurement.

Two days after arrival, rats of each sex were randomly divided into four subgroups. The first group underwent a sham operation and served as intact controls. The second group underwent gonadectomy and were implanted subcutaneously with empty Silastic capsules made from Dow Corning Silastic Medical Grade Tubing (1.57 mm i.d. x 3.18 mm o.d., Dow Corning Corp, Midland, Mich.). The other two groups were implanted (subcutaneously) with 15-mm or 30-mm Silastic capsules that were filled with crystalline testosterone propionate (TP) (Sigma Chemical Co., St. Louis, Mo.) and sealed with Silastic adhesive type A (Dow Corning) at the time of the gonadectomy. All capsules were preincubated in phospate-buffered saline (0.01 M PO 4, 0.15 M NaCl, pH 7.4) at 37°C for 24 hours before insertion. A 15-mm capsule without TP raises serum testosterone one levels to the range normally found in intact adult male rats. The capsules were changed every month under light ether anesthesia. Eighteen weeks after the time of the gonadectomy, rats were killed by decapitation, and peripheral organs were immediately snap-frozen in liquid nitrogen and stored at −80°C until further measurements.

RNA Isolation

Total tissue RNA was extracted from kidney and liver by the method of Chomczynski and Sacchi. Briefly, homogenization of tissue was carried out in 4 M guanidine isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sodium-N-lauryl sarcosine, and 0.1 M β-mercaptoethanol. The protein in the homogenate was removed by phenol-chloroform extraction. The RNA was precipitated by isopropanol and washed with ethanol. The precipitated RNA was dissolved in H2O and quantitated by absorbance at 260/280 nm. Protein contamination was assessed by the ratio of 260/280 nm optical absorbance. Samples with 260/280 nm o.d. ratios less than 1.75 were subjected to further phenol chloroform extraction. The ratio of absorbance at 260 nm to 280 nm in samples used for analysis in these studies was 1.8–2.0.

Renin and Angiotensinogen Complementary DNA and 18S Ribosomal Oligonucleotide Probes

Full length (1.5 kb) rat renin complementary DNA (cDNA) and full length (1.7 kb) rat angiotensinogen cDNA probes were kindly supplied by Dr. Kevin Lynch, University of Virginia, Charlottesville, Va. To quantitate 18S ribosomal RNA, an oligonucleotide [5'-ACGTATCTGATGTCGCAGA-3'] complementary to rat 18S ribosomal RNA was used. The renin and angiotensinogen cDNA probes were labeled with [32P]dCTP by nick translation (Promega Nick Translation System, Madison, Wis.) to a specific activity of 10^6 to 10^7 cpm/μg, and the oligonucleotide was end labeled with [32P]y ATP with T4 polynucleotide kinase. The labeled probes were separated from unincorporated nucleotides by Sephadex G-50 chromatography.

Northern and Slot Blot Analyses

Both Northern and slot blot analyses were used to identify and quantitate renin and angiotensinogen mRNA. For Northern analysis, 5–50 μg RNA samples were denatured in 50% formamide and 6% formaldehyde (in 22.5 mM 3-[N-morpholino]propanesulfonic acid [MOPS] with 1.2 mM EDTA); size fractionated by electrophoresis through 1.5% agarose, 3% formaldehyde gels, in 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0; and blotted onto Nytran membrane (0.45 μm, Schleicher & Schuell, Inc., Keene, N.H.) in 20× SSC buffer (1× SSC=0.15 M NaCl, 15 mM sodium citrate, pH 7.0) according to a modification of the technique of Thomas. For slot blot analysis, duplicate samples of 10 and 20 μg RNA were denatured in 6% formaldehyde in 8.3× SSC, incubated at 60°C for 20 minutes, and applied directly onto Nytran membrane. Using a Schleicher & Schuell slot blot apparatus, the blots were baked at 80°C under a vacuum for 2 hours, prehybridized in 50°C denaturated formamide, 5× NaCl, 1% sodium dodecyl sulfate (SDS), boiled salmon sperm DNA (200 μg/ml), and yeast transfer RNA (tRNA) (25 μg/ml) at 42°C for at least 2 hours, and then hybridized in the same buffer with phosphorus-32-labeled probes (10^6 cpm/ml) at 42°C overnight. After hybridization, the blots were washed twice in 500 ml of 2× SSC, 0.1% SDS at room temperature for 15 minutes and three times in 0.2× SSC, 0.1% SDS at 65°C for 30 minutes. Blots were dried and subsequently exposed to x-ray film (Kodak X-OMAT film, Sigma). The density of autoradiographic signals was quantitated with an optical densitometer (E.C. Apparatus Corporation, St. Petersburg, Fla.).

To quantitate total RNA loaded onto Northern or slot blots, the phosphorus-32–labeled renin or angiotensinogen cDNA probes were stripped from the membrane after washing in 5 mM Tris-HCl (pH 8.0), 0.2 mM Na2EDTA, 0.05% sodium pyrophosphate, 0.002% polyvinyl-pyrolidone, 0.002% bovine serum albumin, 0.002% Ficoll at 65°C for 2 hours. The membrane was then rehybridized with the phosphorus-32–labeled 18S probe. Signals were quantitated with an optical densitometer.
oligonucleotide probe under the conditions specified above. Since ribosomal RNA is the predominant constituent of total cellular RNA and the 18S subunit represents a fixed proportion of total RNA, hybridization of this oligonucleotide can be used as a measure of the total cellular RNA loaded for each sample. To estimate the tissue renin or angiotensinogen mRNA concentration, the renin mRNA/18S RNA or angiotensinogen mRNA/18S RNA ratios were determined by dividing the absorbance corresponding to renin or angiotensinogen cDNA probe hybridization by the absorbance corresponding to 18S probe hybridization. This allows for correction for any misloading or overloading of the slot blots with RNA. When the densitometric units for the 10-μg and 20-μg samples were corrected using the densitometric units of the 18S RNA, there was no statistically significant difference between the two samples. This indicates that overloading of the slot blot did not occur, even with 20 μg RNA.

**Plasma Renin Activity Assay**

PRA was measured by a modification of the method of Haber et al. Active renin was measured by the radioimmunoassay of angiotensin I (Ang I) generated during the incubation of samples at 37°C, pH 7.4 for 3 hours in the presence of angiotensinase inhibitors. Renin activity was expressed as nanograms of Ang I generated per milliliter of sample per hour of incubation. The sensitivity of the Ang I radioimmunoassay was 5 pg/tube.

**Statistical Analysis**

Results were expressed as mean±SEM. Data were analyzed using the CRUNCH statistical package on an IBM PC/AT computer. Statistical comparisons of systolic blood pressure, body weight, PRA, tissue weight, and renin and angiotensinogen mRNA levels were initially performed using two-way analysis of variance (ANOVA) (androgen effect times sex difference). If the two-way ANOVA was significant, a one-way ANOVA was applied for each of the two variables, androgen effect or sex difference. If the one-way ANOVA was significant, a post-hoc comparison was performed using the Newman-Keuls test. Differences were reported as significant if the value of p<0.05.

**Results**

Table 1 shows the effects of gonadectomy and testosterone administration on body weight, organ weight, and systolic blood pressure in male and female SHR. Body weight was significantly lower in high-dose testosterone-treated orchidectomized male rats than in the other three groups at 22 weeks of age. Systolic blood pressure in orchidectomized rats was significantly lower than in intact rats or rats with testosterone replacement at the end of the experiment. Kidney weight-to-body weight ratio was significantly lower in orchidectomized rats than in intact males. Testosterone treatment significantly increased kidney weight, which was significantly and positively correlated (r=0.473, p<0.01) with blood pressure when all animals from all four groups were included in the analysis. Liver weight-to-body weight ratios were not different among the male groups.

Body weight was significantly higher in ovariectomized rats than in the other three female groups at the end of the experiment. Ovariectomy had no effect on systolic pressure in female SHR, but administration of both doses of testosterone increased blood pressure in female rats. Kidney weight-to-body weight ratio was significantly lower in ovariectomized rats and significantly higher in testosterone-treated female rats than that in intact females (Table 1), and kidney weight was significantly and positively correlated (r=0.742, p<0.001) with blood pressure in the four female groups. Liver weight-to-body weight ratio was significantly lower in ovariectomized rats than in the other three female groups (Table 1).

PRA was significantly higher in intact male rats and in orchidectomized rats treated with high-dose testosterone (Figure 1, top panel) than in orchidectomized rats. In female SHR, PRA was significantly higher in both testosterone-treated groups than in the intact and ovariectomized groups (Figure 1, bottom panel). PRA was significantly higher (p<0.05) in intact male SHR.
Figure 1. Bar graphs show effects of gonadectomy and testosterone administration on plasma renin activity (PRA) in male and female spontaneously hypertensive rats. Results represent mean±SEM. Numbers in each column represent the number of animal per group. TP, testosterone propionate; 15 mm and 30 mm, length of the Silastic capsules; $\varnothing$, gonadectomized male; $\varnothing$, gonadectomized female.

(1.85±0.26 ng/ml/hr, $n=12$) than in intact female SHR (1.12±0.20 ng/ml/hr, $n=14$). Testosterone treatment resulted in significant increases in PRA in both sexes. These increases were significantly greater in the female than in the male groups with both doses of testosterone.

For Northern analysis, total RNA from kidney or liver was probed with either the renin or the angiotensinogen cDNA (Figure 2). These results demonstrate that a single transcript of the correct size (1.5 kb for renin and 1.7 kb for angiotensinogen) was detected with the renin and angiotensinogen probes. Angiotensinogen mRNA in liver was the same size as that in kidney (Figure 2). No renin mRNA was detectable in rat liver.

To better quantitate renal renin and renal and hepatic angiotensinogen mRNA levels, slot blot analysis was used. Renal renin mRNA transcript levels were androgen dependent in female rats only. In male SHR, renal renin mRNA levels (represented by renin mRNA to 18S RNA ratios) were not significantly different among the four experimental groups (Figure 3, top panel; one-way ANOVA). Further, renal renin mRNA levels in intact females were not different from those in intact males. Renin mRNA levels were significantly reduced by ovariectomy in female SHR, and testosterone administration increased renin mRNA to the level seen in intact females (Figure 3, bottom panel; one-way ANOVA). When all female groups were combined, there were significant androgen effects on renin mRNA levels (two-way ANOVA).

Angiotensinogen mRNA levels in both kidney and liver were androgen dependent in male and female rats. Renal angiotensinogen mRNA levels were significantly reduced by orchidectomy (Figure 4, top panel). Testosterone replacement increased renal angiotensinogen mRNA to levels significantly higher than those in intact male controls (one-way ANOVA). Renal angiotensinogen mRNA levels in intact female SHR were significantly lower than those in intact males. Ovariectomy reduced hepatic angiotensinogen mRNA levels further, and testosterone administration restored renal angiotensinogen mRNA levels toward normal intact female control levels (Figure 4, bottom panel). There were significant androgen times sex effects on renal angiotensinogen mRNA levels (two-way ANOVA). Hepatic angiotensinogen mRNA levels were significantly reduced by orchidectomy (Figure 5, top panel). Testosterone replacement partially restored hepatic angiotensinogen mRNA levels in male SHR. Hepatic angiotensinogen mRNA levels in intact female SHR were significantly lower than those in intact males. Ovariectomy

Figure 2. Northern blot of kidney (left panel) renin and angiotensinogen messenger RNA (mRNA) and hepatic (right panel) angiotensinogen mRNA of representative spontaneously hypertensive rats hybridized to $[^{32}P]a$-dCTP-labeled full length (1.5 kb) rat renin complementary DNA (cDNA) and full length (1.7 kb) rat angiotensinogen cDNA probes, respectively. The times listed below each panel refer to the time of exposure of the x-ray film.
reduced hepatic angiotensinogen mRNA levels further, and low-dose testosterone administration restored hepatic angiotensinogen mRNA levels toward normal intact female control levels (Figure 5, bottom panel). High-dose testosterone administration increased hepatic angiotensinogen mRNA levels in ovariectomized female SHR to the levels of intact male controls. There were significant androgen times sex effects on hepatic angiotensinogen mRNA levels (two-way ANOVA).

**Discussion**

The major findings of the present study were 1) that systolic blood pressure and PRA in SHR were androgen dependent, being significantly higher in intact males than in intact females and in testosterone-treated gonadectomized rats; 2) that renal and hepatic angiotensinogen mRNA levels were androgen dependent in SHR of both sexes; but 3) that renal renin mRNA levels were androgen dependent in female rats only. These data suggest that the androgen-dependent development of hypertension in SHR may be related to androgen-induced activation of the renin-angiotensin system activity in this model.

We have previously reported that the sexually dimorphic pattern of blood pressure development in SHR is androgen dependent rather than estrogen dependent. The current study confirms and extends these and previous observations that blood pressure in young (9-week-old) male stroke-prone SHR can be reduced by surgical castration, treatment with the testosterone receptor antagonist cyproterone acetate, which does not elevate testosterone, or with the testosterone receptor antagonist flutamide, which leads to a feedback elevation of gonadotrophic hormones and plasma testosterone. Both androgen receptor antagonists attenuated the subsequent development of hypertension when given to male stroke-prone SHR for the first 10 days after birth. These treatments had no effect on blood pressure in male stroke-prone SHR with established hypertension (25 weeks old). Similarly, surgical castration of male rats and androgenization of female rats by administration of testosterone in the neonatal period caused reversal of the sexually dimorphic pattern of hypertension development in SHR. These data and the results of the current study indicate that testosterone contributes to the development of hypertension in young male SHR but not to the maintenance of established hypertension in the mature animal.

The current study demonstrated that PRA was significantly higher in intact male SHR than in intact female SHR and that testosterone treatment increased PRA in both sexes. The effect of exogenous testosterone on PRA was greater in the female for unclear reasons. This finding suggests that the androgen-dependent development of hypertension in SHR may be related to activation of the renin-angiotensin system. Renal and hepatic angiotensinogen mRNA levels were androgen dependent in both sexes; they decreased after gonadectomy and increased after testosterone replacement. Thus, androgens can be added to the list of hormones and local modulators that regulate angiotensinogen synthesis and release. These include estrogen, insulin, glucocorticoids, angiotensin II, volume depletion, and...
In contrast to angiotensinogen, renal renin mRNA levels were androgen dependent in female SHR only; they decreased after ovariectomy and increased to male levels after testosterone replacement. However, neither castration nor testosterone replacement altered renal renin mRNA levels in male SHR. The latter finding is consistent with previous reports in normotensive mice and rats. Mouse renal renin levels are similar in both sexes, and neither castration of adult male mice nor administration of androgen to adult female mice has a significant stimulatory effect on renal renin activity or mRNA levels.\textsuperscript{14,30} Further, mouse renal renin levels and renin mRNA levels remain stable throughout ontogeny in both sexes.\textsuperscript{13} In contrast, renin expression in the submaxillary gland, testis, adrenal gland, and brain of the mouse are androgen dependent.\textsuperscript{14,30} The mechanisms of this tissue-specific differential regulation of renin gene expression in response to androgen treatment and of the apparent unresponsiveness of renal renin mRNA levels to androgens remain to be elucidated. Despite the unresponsiveness of renal renin mRNA to androgen, a sexual dimorphism (male higher) of active plasma renin in SHR has been found (current study), and one of inactive plasma renin has been described in Wistar rats,\textsuperscript{31} in mouse strains with one or two renin genes,\textsuperscript{32} and although less pronounced, in humans.\textsuperscript{33} In male mice and rats, inactive plasma renin seems to originate mainly from the kidney.\textsuperscript{31-33} Thus, renin release appears to increase in response to testosterone in the absence of increased renal renin mRNA concentrations. This lack of correlation between PRA and renal renin mRNA is similar to that reported by Pratt et al,\textsuperscript{34} who found that sodium restriction significantly increased plasma renin concentration but had no effect on renal renin mRNA concentrations in male SHR and WKY rats. In the present study, testosterone-induced increases in renal size may have been responsible for the testosterone-induced increase in PRA, but it is clear that regulation of PRA occurs at the posttranscriptional level in response to a number of stimuli in other models.

These findings, coupled with our observations that testosterone enhances angiotensinogen mRNA levels in liver and kidney, suggest that the blood pressure increases seen in intact male SHR or testosterone-replaced SHR of either sex may be angiotensin dependent. Unpublished data from the laboratory of Dr. Kathleen Berecek indicate that lifetime treatment with the angiotensin converting enzyme inhibitor captopril abolishes the sexual dimorphism of hypertension in SHR, lending support to this concept.

A second mechanism that has been proposed to explain the effects of testosterone on blood pressure in the SHR is enhanced sympathetic nervous system activity. Sympathetic tone is markedly increased during the developmental phase of hypertension in male SHR\textsuperscript{34-37} but returns toward normotensive control levels in the established phase of hypertension in this model. Careful studies of sympathetic neural function in female or castrated male SHR during the developmental phase of hypertension have not been carried out. Testosterone has been shown to modulate norepinephrine storage and release in sympathetic fibers innervating the rat vas deferens,\textsuperscript{38} raising the possibility that similar mechanisms could operate at noradrenergic nerve terminals on resistance vessels and in the brain.\textsuperscript{39} Previous studies have shown that testosterone has an effect on the development of noradrenergic neu-
tions in the hypothalamus. Further, our own laboratory has shown that posterior hypothalamic norepinephrine stores increase in response to an androgen stimulus in developing SHR. Levels of norepinephrine in the posterior hypothalamic region were significantly greater in intact male SHR and in testosterone-treated SHR of both sexes than in intact ovariectomized female SHR. The posterior hypothalamic area is a sympathoexcitatory region: electrical stimulation of discrete neuronal groups within the posterior hypothalamic area increases arterial pressure, heart rate, and vascular resistance, and this effect is greater in SHR than in WKY rats. Even before the onset of hypertension, the noradrenergic innervation of the posterior hypothalamic area is greater in male SHR than in normotensive male rats, suggesting that exposure to androgen at a young age in SHR of either sex may accelerate the differentiation of the noradrenergic system in this brain region. These findings suggest the possibility that intact male and testosterone-treated gonadectomized SHR of both sexes may have increased androgen-dependent Dopadependent outflow during the developmental phase of hypertension and that this may contribute to the increased blood pressure seen in the male of this strain. Further study is needed to test this hypothesis.

References


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Y F Chen, A J Naftilan and S Oparil

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