Impact of androgen-induced oxidative stress on hypertension in male SHR

by

Radu Iliescu *, Valeria E. Cucchiarelli *, Licy L. Yanes, Joshua W. Iles, and Jane F. Reckelhoff

* These authors contributed equally to the study

Department of Physiology and Biophysics
And The Center for Excellence in Cardiovascular-Renal Research
University of Mississippi Medical Center
2500 N. State Street
Jackson, MS

Short title: Androgens, oxidative stress and hypertension.

Corresponding author:
Jane F. Reckelhoff, Ph.D.
Department of Physiology and Biophysics
University of Mississippi Medical Center
2500 N. State Street
Jackson, MS 39216-4505
Telephone: 601-984-1819
FAX: 601-984-1817
Email: jreckelhoff@physiology.umsmed.edu
ABSTRACT

Men have higher blood pressure than women, and androgens and oxidative stress have been implicated as playing roles in this sexual dimorphism. The SHR is an animal model of both androgen- and oxidative stress-mediated hypertension. Therefore, the present studies were performed to test the hypothesis that androgens cause hypertension in SHR in part by stimulating superoxide production via NADPH oxidase. Castration of male SHR reduced blood pressure by 15% and attenuated both basal and NADPH-stimulated superoxide production in kidney cortical homogenates. Expression of p47phox and gp91phox but not p22phox subunits of NADPH oxidase were significantly lower in kidney cortex from castrated males compared to intact males. Moreover, inhibition of NADPH oxidase with apocynin caused approximately 15 mm Hg reduction in blood pressure and reduced basal and NADPH-stimulated superoxide production in intact male SHR, but had no effect on blood pressure or superoxide production in castrated males. These data support the hypothesis that androgens cause oxidative stress and thereby increase blood pressure in male SHR via an NADPH oxidase-dependent mechanism.

Key words: hypertension, oxidative stress, androgens, superoxide anion
Introduction

Men have higher blood pressure than age-matched, premenopausal women. In addition, hypertension is more prevalent and more severe in men as compared to age-matched women (6). The sexual dimorphism of blood pressure appears to involve cardiovascular effects of the sex hormones, with androgens playing a role in mediating the elevated blood pressure found in men (19). This premise is supported by clinical findings that during puberty, when sex hormone levels surge, blood pressure increases in boys at a more rapid rate than in age-matched girls (23, 25). Moreover, hyperandrogenemia in reproductive-age women is associated with increased blood pressure, independent of age and ovulatory status (3). However, the mechanisms by which androgens may increase blood pressure have not been completely elucidated.

The spontaneously hypertensive rat (SHR) is a well characterized model of hypertension, in which the males exhibit higher blood pressure than the females (21). Studies conducted in our laboratory showed that gonadectomy of male SHR reduces their blood pressure to the levels found in females and testosterone treatment of ovariectomized female SHR increases blood pressure to the level of males (21, 22). These data support a role for androgens in mediating the higher blood pressure in SHR.

Increased levels of oxygen radicals, leading to a state of oxidative stress, have also been demonstrated in hypertensive individuals (13, 17), and men have enhanced levels of oxidative stress as compared to age-matched women (11). In male SHR, oxidative stress plays a role in the development and maintenance of hypertension, since treatment with oxygen radical
scavengers can attenuate or reverse hypertension in this model (10, 24). Renal production of superoxide anion is also higher in male SHR than in normotensive rats (1) and may contribute to the renal dysfunction that results in hypertension. In support of this contention, Makino and colleagues found that increasing oxidative stress in the kidney, by inhibiting superoxide dismutase, increases blood pressure in normotensive animals (14), and we have found that increasing oxidative stress in male SHR with molsidomine further increases their blood pressure (9). However, there are sex differences in the level of oxidative stress and the depressor response to superoxide scavengers in SHR. We have previously shown that markers of oxidative stress are higher and superoxide scavenging is more effective in reducing blood pressure in male SHR than females (10). The mechanism(s) responsible for the sexual dimorphism regarding oxidative stress and blood pressure in SHR is not clear, however. The major source of superoxide anion leading to increased oxidative stress in cardiovascular tissues is thought to be the multimolecular enzyme NADPH oxidase (7). The kidneys of male SHR have been shown to overexpress the genes for the components of the NADPH oxidase enzyme even before their blood pressure increases (7), suggesting a causative role for the renal NADPH oxidase-dependent oxidative stress in the development of hypertension in SHR.

Taken together, these data prompted us to hypothesize that androgens stimulate renal NADPH oxidase-dependent oxidative stress which contributes to hypertension in male SHR. In the present study, we ascertained whether gonadectomy of male SHR alters renal superoxide production and/or renal protein levels of NADPH oxidase subunits. We determined as well whether inhibition of NADPH oxidase with apocynin would reduce oxidative stress and thereby decrease blood pressure in intact versus gonadectomized male SHR.
Methods

Rats: Intact and castrated male spontaneously hypertensive rats (SHR) were obtained from Taconic Farms (Germantown, NY) (n=8/group). Castration surgery was performed by the vendor, at 9 weeks of age. The rats were maintained on standard rat chow (Teklad, Harlan SD, Indianapolis, IN) and tap water, in an environment with 12h/12h light/dark cycle. The protocols complied with the Guidelines for the Care and Use of Laboratory Animals by National Institutes of Health, and were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center.

NADPH oxidase blockade in SHR: Intact and castrated male SHR, aged 12 weeks (n=8/group), were given apocynin (100 mg/kg/d) or vehicle (45% polyethylene glycol, 45% castor oil, 10% ethanol--total volume = 100 µl/100 g body weight) by s.c. injection for 7 days. On day 5 of treatment, rats were anesthetized with isoflurane, and catheters were placed in the femoral artery and exteriorized at the back of the neck, as we have previously described (27). On day 8, blood pressure was measured by Power Lab (AD Instruments) connected to Dell computer for 4 hr in conscious, freely moving rats in their home cages.

Kidney cortex expression of NADPH oxidase subunits: Kidneys from intact and castrated male SHR (n=5-6/group), treated with or without apocynin, were removed, weighed and renal cortex was dissected. Medullary tissue was discarded and cortical tissue was homogenized in 20 mM HEPES containing EDTA (10 mM) and a protease inhibitor cocktail (Sigma). Samples of the cortical homogenate (25 µg protein) were loaded onto a 4-20% gradient gel (Biorad), electrophoretically separated and transferred to nitrocellulose membrane. Following blocking
with a solution of 5% non-fat dry milk, the membrane was probed with anti-gp91, anti-p47, or anti-p67 antibodies, as previously described (4). Membranes were developed using enhanced chemiluminescence (KPL, Gaithersburg, MD), and the protein bands were visualized by densitometry using Scion Image software beta version 4.02 (Scion Corp, Frederick, MD). Band densities were normalized to the total amount of protein loaded in each well as determined by densitometric analyses of membranes stained with Ponceau S. Results are expressed as relative optical density representing the specific band density factored for the total protein band density.

**Lucigenin chemiluminescence for detection of superoxide and NADPH oxidase activity in kidney cortex:** To test the effect of gonadectomy and/or apocynin treatment on superoxide production by the kidney, spontaneous superoxide generation as well as NAD(P)H-stimulated superoxide production (index of NAD(P)H oxidase activity) were measured in kidney cortical homogenates, using lucigenin chemiluminescence method in a luminometer (Autolumat Plus 953, Berthold, Germany), as previously described by Park et al. (15). Lucigenin was used at a final concentration of 5µM, and for the measurement of NADPH-stimulated activity, NADPH (final concentration: 0.1 mmol/L) was added.

**Statistics:** Comparisons for multigroup and multifactorial analysis were performed by two way analysis of variance and by using the Student-Newman-Keuls method for multiple comparison procedures. The criterion for significant differences between groups of study was P<0.05.

**Results**

**Effect of gonadectomy and NADPH oxidase inhibition on body weight in intact and castrated male SHR:** Vehicle treated castrated rats had lower body weights than intact males
apocynin treatment did not affect body weight in either castrated or intact rats (283 ± 2 and 302 ± 4g). There were no differences in kidney size among groups (data not shown).

**Effect of gonadectomy and NADPH oxidase inhibition on blood pressure in intact and castrated male SHR:** Blood pressure was significantly higher in intact males than castrated rats (Figure 1), as we have shown previously (21). Intact and castrated male SHR were treated for 1 week with the NADPH oxidase inhibitor, apocynin, which reduced blood pressure by approximately 15 mm Hg in intact males, but had no effect on blood pressure in castrated rats.

**Effect of gonadectomy and apocynin on expression of NADPH oxidase subunits and superoxide production in kidney of SHR:** Expression of p47^{phox}, gp91^{phox} and p67^{phox} were measured in the kidney cortex from intact and castrated male SHR. As shown in Figure 2, castration reduced expression of p47^{phox} and gp91^{phox} in kidney cortex, but not p22^{phox}. Apocynin treatment reduced expression of all the NADPH oxidase subunits measured in intact male SHR. In castrated males, apocynin increased expression of gp91^{phox} and p47^{phox}, but had no effect on expression of p22^{phox}.

In support of a higher NADPH oxidase activity in intact males, basal and NADPH-stimulated superoxide production, as measured by lucigenin chemiluminescence, was also higher in renal cortical homogenates from intact male SHR than castrated rats (Figure 3, Panels A and B). Similarly, inhibition of NADPH oxidase with apocynin reduced both basal and NADPH-stimulated superoxide production in cortical homogenates from intact males (see Figure 3A and
Discussion

In the present study we tested the hypothesis that the androgen-mediated increase in blood pressure in male SHR is in part due to androgen-stimulated oxidative stress, and determined whether NADPH oxidase plays a role in this process. The main findings of this study are: 1) Gonadectomy decreases BP while reducing basal and NADPH-stimulated superoxide anion generation and protein levels of p47phox and gp91phox in the kidney cortex of male SHR; and 2) Inhibition of NADPH oxidase with apocynin attenuates hypertension in intact male SHR, but not in castrated males. This effect is paralleled by a decrease in renal superoxide anion generation and expression of NADPH oxidase subunits in intact males.

As we have shown previously, the higher blood pressure in male SHR, compared to females, requires the presence of androgens, since gonadectomy of males reduces blood pressure to the levels found in females. Furthermore, while ovariectomy alone has no effect on blood pressure in female SHR, testosterone supplements increase blood pressure in a dose-dependent manner in ovariectomized rats (5, 10). These data show that androgens are capable of increasing the blood pressure in the SHR model, but fail to provide indications as to the mechanism(s) involved. Since oxidative stress is thought to contribute to the development of hypertension (24), and men have higher levels of oxidative stress than women (11), our present study was designed to evaluate the role of oxidative stress in mediating androgen-dependent hypertension in male SHR.
To ascertain a direct contribution of oxidative stress to the hypertension of male SHR, we investigated the effect of NADPH oxidase inhibition with apocynin on BP levels of male SHR. Apocynin treatment attenuated NADPH oxidase-dependent superoxide anion generation in kidney cortex of male SHR and led to significant reductions in BP. The mechanism by which apocynin reduces superoxide anion generation is thought to involve inhibition of the subunit assembly of NADPH oxidase (16). We have found that 1 week treatment with apocynin also decreased expression of the NADPH oxidase subunits measured. This effect has been described in previous studies (2, 12) and may further contribute to the attenuation of oxidative stress by apocynin. While these data indicate that renal oxidative stress contributes to increased BP in male SHR, we assumed that the role that androgens play in this process could be determined by studying the effect of apocynin on BP in the absence of androgens. We expected that gonadectomy would attenuate the decrease in BP induced by apocynin. Indeed, we found that apocynin treatment of castrated SHR did not reduce their BP, nor further decreased superoxide anion generation by NADPH oxidase in their kidneys. Furthermore, protein levels of gp91phox and p47phox were actually augmented by apocynin in gonadectomized male SHR. However, the expression of the regulatory subunit p22phox was not altered. Therefore, the mechanism(s) mediating the effect of apocynin on NADPH oxidase subunits expression in the absence of androgens remain elusive. However, despite the increase in one catalytic subunit and one regulatory subunit of the enzyme, superoxide production was not impacted in the castrated rats treated with apocynin. These data suggest that either the upregulation of all subunits is necessary to produce NADPH-dependent superoxide generation and increased BP, or that even in the face
of upregulation of some of the subunits of NADPH oxidase, oxidative stress only increases BP when androgens are present.

Studies by Quan and colleagues have shown that androgens stimulate proximal reabsorption in the kidney (18), and this is one mechanism by which androgens could influence blood pressure in SHR. The present study provides another mechanism via evidence that links androgens to the increased oxidative stress in mediating blood pressure regulation in SHR, since the effect of apocynin on blood pressure was abolished by castration. How androgens may stimulate oxidative stress is not clear from our present data, however. It has been shown previously by ourselves and others that oxidative stress contributes to hypertension in SHR (1, 10, 24), and NADPH oxidase subunits are overexpressed both at the mRNA and protein level in kidneys of SHR (7), suggesting that NADPH oxidase may be the enzyme responsible for the increased oxidative stress in this model. Activation of NADPH oxidase requires the translocation of the cytoplasmic subunits to the plasma membrane, where the catalytic subunit becomes activated (5). Although seven isoforms of the catalytic subunit have been identified (5), we measured protein levels of the gp91phox catalytic subunit, and two of the regulatory subunits (p47phox and p22phox) which have been shown to be expressed in the kidney (7). We found that gonadectomy reduced the expression of gp91phox and p47phox in the renal cortex of male SHR. In keeping with this, basal and NADPH-stimulated superoxide production was also decreased with gonadectomy in kidneys of male SHR. Since gonadectomy significantly decreased BP of male SHR, these data suggest that increased oxidative stress generated by NADPH oxidase may contribute to androgen-induced elevation of BP in this model. While the decrease in gp91phox and p47phox expression after gonadectomy might account for the decrease in superoxide generation, an inhibitory effect
of testosterone removal on subunit assembly and subsequent activation of NADPH oxidase cannot be excluded. Furthermore, androgens may have indirect effects on oxidative stress via other systems, such as the renin-angiotensin system or the endothelin systems. Androgens have been shown in SHR to upregulate angiotensinogen expression by us and others (8), and androgen supplements increase plasma endothelin levels in humans (26). Alternatively, androgens may differentially impact the other NOX isoforms in the kidney contributing to differences in NADPH oxidase subunit expression and superoxide production. Future studies will be necessary to sort out all these potential effects of androgens on NADPH oxidase enzyme and activity.

Several lines of evidence indicate that androgens promote cardiovascular and renal disease by activating hormonal and autacoid systems involved in the regulation of cardiovascular function (20). The data from the present study reveal the contribution of oxidative stress to the mechanisms by which androgens may alter blood pressure and mediate hypertension. Cross-sectional and longitudinal studies typically show a decline in total and bioavailable testosterone levels in men with aging and chronic disease, such as hypertension and renal disease. The reduction in androgens in the face of the increased incidence of cardiovascular disease has led investigators and physicians to presume that androgens do not play a causative role in mediating cardiovascular diseases and their complication. However, in the light of these apparently divergent findings, a different hypothesis appears legitimate and warrants further investigation: it is conceivable that the reduction in testosterone may be a mechanism of protection against further deterioration of cardiovascular function that commonly occurs with aging and chronic cardiovascular disease. Further studies are necessary to determine whether the reduction in
testosterone is the mechanism contributing to progression of cardiovascular and renal diseases or whether androgens play a causative role in mediating cardiovascular disease and thus a compensatory mechanism seeks to reduce the levels of androgens to provide protection against further damage.

In summary, gonadectomy reduces blood pressure and superoxide production and decreases the expression of p47phox and gp91phox subunits of NADPH oxidase in the kidney cortex of male SHR. Inhibition of the NADPH oxidase enzyme with apocynin reduces blood pressure in intact males to the level found in castrated males, and also reduces NADPH oxidase subunit expression and superoxide production in intact males. In contrast, apocynin has no effect on blood pressure or superoxide production in castrated rats. These data support the notion that androgens cause hypertension in male SHR in part by increasing oxidative stress via an NADPH oxidase-mediated mechanism.

Acknowledgements

These studies were supported by National Institutes of Health HL69194, HL66072 and HL51971 (JFR), and AHA# 0525320B (RI) and AHA# 0425461B (LLY). The authors would like to thank Dr. Christine Maric, Georgetown University, for technical assistance with western blots for NADPH oxidase subunits, and Dr. Mark T. Quinn, University of Montana, for antibodies to NADPH oxidase subunits.
Figure legends

Figure 1: Effect of gonadectomy and apocynin treatment on mean arterial pressure in SHR. Intact and castrated male SHR were treated with apocynin, the NADPH oxidase inhibitor, (or vehicle) for 1 week, as described in Methods (n=8/group). Blood pressure was measured in conscious, chronically instrumented rats. “Control” refers to treatment with vehicle for apocynin. *P<0.01, compared to intact male control; †, p<0.01, apocynin-treated compared to control.

Figure 2: Effect of gonadectomy and apocynin treatment on expression of gp91phox, p22phox and p47phox subunits of NADPH oxidase in kidney cortex from SHR. NADPH oxidase subunit expression was measured by western analyses as described in Methods (n=8/group). “Control” refers to treatment with vehicle for apocynin. *, P<0.01 compared to intact males; †, p<0.01, apocynin-treated compared to control.

Figure 3: Effect of gonadectomy and apocynin treatment on basal (A) and NADPH-stimulated (B) superoxide production in kidney cortex of SHR. Intact and castrated male SHR were treated with apocynin, the NADPH oxidase inhibitor, (or vehicle) for 1 week, as described in Methods (n=8/group). “Control” refers to treatment with vehicle for apocynin. *, P<0.01, for comparison of intact and castrated male controls; †, p<0.01, apocynin-treated compared to control.
References


Figure 1
Figure 2
Figure 3