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Original Research

Testosterone to estrogen conversion is not responsible for the vasodilating effects of testosterone *ex vivo*

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Abstract: Men have a higher incidence of cardiovascular disease, but poorer vasodilatation than women. However, testosterone exerts vasodilating actions *ex vivo*. We aimed to determine if reactive oxygen species (ROS) produced *in vivo* could cause 'eNOS uncoupling' that accounts for the disparity between *in vivo* and *ex vivo* results. Ovariectomized SHR and WKY rats were divided into 3 groups: untreated, estradiol benzoate (EB) treated, and testosterone propionate (TP) treated. EB and TP rats were treated for 8 weeks, and blood pressure, serum estrogen, progesterone, and testosterone were measured. Rats were euthanized and aorta samples were taken for examination of nitric oxide, phosphorylated eNOS (p-eNOS), H_2O_2, gr91phos, and pAkt. Mesenteric arterial rings were used in myographic studies of endothelium dependent and independent vasorelaxation. The influence of testosterone added to the bathing solution of rings from testosterone-supplemented rats with/without an eNOS inhibitor, with/without blockade of androgen or estrogen receptors, and with/without an inhibitor of gp91phox was examined. Treatment with testosterone for 8 weeks did not change endothelium-dependent relaxation in response to acetylcholine in the presence or absence of the eNOS inhibitor L-NAME, or in the presence or absence of blockade of the androgen receptors, estrogen receptors, or gp91phox. No change in nitric oxide, p-eNOS, pAkt, or gp91phos of the aorta was noted. A significant increase in H_2O_2 was seen in testosterone-supplemented SHR rats, but this was not accompanied by eNOS uncoupling. These results suggest that conversion of testosterone to estrogen is not responsible for its vasodilating effects seen *ex vivo*.

Key words: Androgen; Endothelium-dependent vasodilation. eNOS uncoupling; NO.

Introduction

Men have a greater incidence of hypertension and cardiovascular disease (CAD) than women of a similar age (1, 2). Reproductive age women with polycystic ovary syndrome (PCOS), who often have higher androgen levels than age-matched female controls, have an increased risk of hypertension and other CAD. Male spontaneously hypertensive rats (SHR) have a higher blood pressure than female SHR rats. Castration removes the blood pressure sex difference in SHR rats, and administration of testosterone to castrated males reverses the effect of castration (3).

The above findings suggest that androgens reduce vasodilation *in vivo*. However, other *in vivo* studies have suggested that androgens promote vasodilation. In men, low plasma testosterone levels have been associated with reduced vasodilation and increased CAD risk, even after excluding other risk factors for endothelial dysfunction (4, 5). In addition, testosterone therapy in both aged men and hypogonadal men is associated with a lessening of arterial wall stiffness (6, 7). Similar vasodilating actions are seen in arterial tissues from animal models, and in cell culture experiments. Testosterone induces relaxation of the aortic rings from both Wister-Kyoto (WKY) and SHR rats in a concentration dependent manner by opening ATP-sensitive potassium channels (8). Testosterone, and its metabolite dihydrotestosterone (DHT), induces relaxation in pig prostatic small artery rings by blocking Ca^{++} channels (9). Physiological concentrations of testosterone induce a rapid (15-30 min) increase in nitric oxide (NO) in human aortic endothelial cells (HAEC) by androgen receptor (AR)-dependent activation of endothelial nitric oxide synthase (eNOS) and the phosphatidylinositol 3-kinase (PI3)/Akt signaling pathway (10). Dehydroepiandrosterone (DHEA) has also been reported to increase NO production in endothelial cells by activation of eNOS and the MAPK-ERK1/2 pathway. Furthermore, DHEA-treated endothelial cells and aortic tissue from DHEA-treated OVX Wistar rats show dose-dependent increases in both eNOS expression and activity (11). In summary, androgens have been shown to induce vasodilation through endothelium-dependent and endothelium-independent mechanisms, both *ex vivo* and *in vitro*. In general, vasorelaxation induced by physiological concentrations of androgens appears to be endothelium-dependent, and vasorelaxation induced by higher pharmacological concentrations seems to be endothelium-independent.

Thus, the effects of androgens seem contradictory, with androgens promoting both vasoconstriction and vasodilation *in vivo,* while inducing vasorelaxation *ex vivo* and *in vitro*. The *in vivo* vasoconstricting action of androgens might be due to an additional systemic action that increases the activity of a vasoconstricting

substance such as angiotensin. However, we hypothesized that there might also be a separate physiological pathway induced by androgens *in vivo* that indirectly blocks the vasorelaxing effects of androgens that are seen *ex vivo and in vitro*.

Reactive oxygen species (ROS) are increased in a number of conditions, including hypertension. In the presence of ROS, eNOS is altered from a protective enzyme to a contributor to oxidative stress, a change resulting in reduced NO bioavailability and endothelial dysfunction; this alteration is referred to as "eNOS uncoupling" (12-14). Hyperhomocysteinemia is associated with increased risk of atherosclerosis and thrombosis, and impaired endothelial-dependent vasodilatation which may be due to alterations of eNOS (15). Other study has suggested that an increase in ROS may inhibit 5-hydroxytryptamine- induced eNOS phosphorylation, thus contributing to endothelial dysfunction associated with cardiovascular disease (16). Interestingly, insulin has been shown to improve the endothelium-independent relaxation and contractile response in the aorta of hypertensive diabetic rats (17).

Although previous studies have shown that eNOS uncoupling is involved in changes that contribute to vascular endothelial dysfunction, little is known about whether eNOS uncoupling and the associated endothelial dysfunction is related to the vasoconstrictive effect of androgens seen *in vivo*, but not *ex vivo*. In the current study, we investigated the long-term effects of testosterone on endothelium-dependent relaxation *in vivo*, and the mechanisms involved.

Materials and Methods

Animal treatment

Experiments were carried out at the Animal Experimental Center in Shanghai Jiaotong University Affiliated Sixth People's Hospital. The Institutional Animal Care and Use Committee of the hospital reviewed and approved all animal procedures. Eight-week-old female WKY and SHR rats (18 each) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All rats were fed a standard rat chow diet, with free access to water, and experiments were begun after 2 weeks. The WKY and SHR rats were each randomly divided into 3 groups (6 in each group) according to weight using a random digits table, and all were ovariectomized. The 6 groups were: 1) untreated ovariectomized WKY (WKY+OVX); 2) estradiol benzoate (EB) treated ovariectomized WKY (WKY+OVX+EB); 3) testosterone propionate (TP) treated ovariectomized WKY (WKY+OVX+TP); 4) untreated ovariectomized SHR (SHR+OVX); 5) estradiol benzoate (EB) treated ovariectomized SHR (SHR+OVX+EB); and 6) testosterone propionate (TP) treated ovariectomized SHR (SHR+OVX+TP). The WKY+OVX+EB and SHR+OVX+EB groups, which served as positive controls, were given EB dissolved in tea oil (National Institutes for Food and Drug Control, Shanghai, China) via intramuscular injection at a dose of 0.25 mg/kg every other day, and the WKY+OVX+TP and SHR+OVX+TP groups were treated with TP (GM Pharmaceutical Co, Shanghai, China) at a dose of 3 mg/kg every other day. The TP was also dissolved in tea oil and given via intramuscular injection (18). The WKY+OVX and SHR+OVX groups were only injected with tea oil of same volume every other day, and served as blank controls. The overall experimental design is summarized in the Supplemental data.

Blood pressure

Systolic blood pressure (SBP) was measured at the start of the experiment, and at the end of the 4th and 8th weeks via the tail-cuff method, with the rats restrained and the tails warmed for 20 min prior to measurement. SBP was recorded in triplicate using a rat arteria caudalis BP measuring device (BP-98A, Softron Biotechnology, Beijing, China), with the average of the 3 values used for analysis.

Measurement of sex hormone levels

At the end of the study, all rats were anesthetized, and blood samples were collected from their hearts into anticoagulation tubes that were sealed, mixed, and placed at 4°C for 2 h. The samples were then centrifuged at 4000 *g* for 10 min at 4°C, and the supernatant collected and stored at 80°C. Testosterone, estrogen, and progesterone concentrations were assayed using a radioimmunoassay kit (Northern Institute of Biotechnology, Beijing, China) according to the manufacturer's protocols. Radioactivity was measured using a gamma counter (GC-120, Zhongjia Photoelectric Instrument Co. Hefei, China).

Vascular function studies

Mesenteric artery ring preparation.

Vascular function studies were performed on mesenteric artery rings with a multiwire myography system (DMT 620M, Danish Myo Technology A/S, Denmark). Second-order mesenteric arteries were isolated from every WKY and SHR rat. The arteries were dissected free of adventitia, cut into 3-mm ring segments, mounted on wire, cannulated in a vessel chamber for myographic studies, and perfused with a physiological salt solution (PSS; 130 NaCl, 4.7 KCl, 1.6 $CaCl_2$, 1.17 $MgSO_4.7H_2O$, 1.18 KH_2PO_4, 14.9 $NaHCO_3$, 5.5 glucose, 0.026 EDTA [values in mM]) at 37°C, and continuously aerated with a 95% O_2/ 5% CO_2 gas mixture. Isometric tension changes were measured with a digital force isometric transducer connected to a data acquisition system (AD Instruments). Each arterial ring was equilibrated in 5 mL of PSS for 60 min prior to the experiment. Based on preliminary studies, the resting tension was determined to be 0.25 g for the optimal preload for force development. PSS buffer containing 60 mM KCl was added to the chambers, the contraction allowed to plateau, and the samples washed twice with PSS. When the tissue relaxed to a basal tension level, buffer containing 60 mM KCl was added and the above steps repeated 3 - 4 times until the contractile response to KCl differed by no more than 20%. After this confirmation, the testing proceeded.

Endothelium dependent and independent relaxation.

For relaxation studies, maximal tone was induced with phenylephrine (PE; 10^{-4} M, Sigma-Aldrich, USA), and then either acetylcholine (Ach; 10^{-9}~3×10^{-7} M, Sig-

ma-Aldrich) or sodium nitroprusside (SNP; 10^{-10}~10^{-6} M, Sigma-Aldrich) were added cumulatively.

Androgen protocol.

To explore the effect of androgens on vasorelaxation *ex vivo*, TP (100 nM) was added to the bathing fluid 30 min before endothelium dependent or independent relaxation testing. Then, eNOS inhibitor L-NAME (10^{-4} M, Sigma-Aldrich), AR inhibitor flutamide (Flu; 100 nM, National Institutes for Food and Drug Control, Shanghai, China), estrogen receptor inhibitor ICI 182780 (10 μM, Sigma-Aldrich), and phosphotidylinsitol-3-kinase (PI3K) inhibitor LY 294002 (30 μM, Beyotime Institute of Biotechnology, Shanghai, China) were independently added 30 min before initiating treatment with TP. Samples with TP were incubated with these selective inhibitors for another 30 min. Subsequently, phenylephrine and acetylcholine were added to arterial rings one after another to investigate whether the vascular effect induced by testosterone *ex vivo* was endothelium-dependent or not, and to determine the involvement of related receptors and signaling pathways. Responses were expressed as a percentage contraction of PE-induced maximum tone, with contraction in the absence of any intervention considered 100%. The half maximal effective concentration (EC50) and pD2 (−Log EC50) were also recorded to assess the sensitivity of vascular reactivity.

Nitrite/nitrate levels

Total NO production in the aorta was determined by measuring the concentration of nitrate and nitrite, stable NO metabolites, using the modified Griess reaction method. The nitrite/nitrate levels were determined using a nitrite/nitrate assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. The samples were lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology), and protein quantification performed with a BCA protein assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocols, with nitrite/nitrate levels normalized with protein content.

Hydrogen peroxide assay

Hydrogen peroxide content in aortic tissue was measured using a hydrogen peroxide assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. In brief, the lysis buffer solution was added to the aortic tissue at a ratio of 100 mL per 5 mg, the mixture was centrifuged at 12 000 *g* for 5 min at 4°C, and the supernatants were collected. Finally, 96-well plates containing 50 uL of the supernatants and 100 uL of test solutions were mixed and rested at room temperature for 30 min. The absorbance at 560 nm was measured with a microplate reader (BioTek, USA). The content of H_2O_2 in aortic tissue was calculated according to a standard concentration curve, and normalized with protein content.

Western blotting

Rat aorta protein extracts were separated by SDS-PAGE, with the following antibodies (Ab): eNOS (1:1000, Sigma-Aldrich), phospho-eNOS (Ser1177) (1:500, Abcam, Cambridge, UK), gp91phox (1:500, Abcam), Akt (1:1000, Cell Signaling Technology, USA), phospho-Akt (Ser473) (1:1000, Cell Signaling Technology), and GAPDH (1:4,000, Santa Cruz, CA, USA), with GAPDH used as a loading control. Membranes were incubated with primary Ab overnight at 4°C, washed with PBS, and incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. Blots were imaged with an enhanced chemiluminescence reagent (Thermo Scientific), and quantified using Quantity One software (BioRad, Hercules, CA, USA).

Statistical analysis

Mean and standard deviation (SD) were presented for all variables. Degree of relaxation was quantified by maximum contraction (%). To examine group differences, and effect of time or of Ach concentration, generalized estimation equation (GEE) analysis, including an interaction term consisting of time (or concentration) and group was performed. Once the interaction term became significant, strata analyses by time (or concentration) as well as group was performed. Analysis of variance (ANOVA) or independent sample t-test was used to determine differences between groups at a given time point or a given Ach concentration. When a significant result was identified with ANOVA, Bonferroni's correction method for parameters with homogeneous SD, or Dunnett's T3 test for those with heterogeneous SD, across groups were performed. All statistical analyses were 2-sided and a value of $P < 0.05$ was considered statistical significance. Statistical analyses were performed with PASW (version 22.0, IBM Corp., Armonk, NY, USA).

Results

Serum estrogen, testosterone, and progesterone concentrations

Sex hormone levels in the 6 groups of rats are summarized in Table 1. Untreated WKY rats had similar levels of testosterone, estradiol, and progesterone. Progesterone levels were similar in treated and untreated WKY rats. WKY rats receiving testosterone had higher

Table 1. Sex hormone levels.

	WKY			SHR			
	OVX (n=6)	**OVX+EB (n=6)**	**OVX+TP (n=6)**	**OVX (n=6)**	**OVX+EB (n=6)**	**OVX+TP (n=6)**	***P***
Testosterone, pmol/L	0.7 ± 0.5	0.6 ± 0.4	11.7 ± 7.9	0.7 ± 0.4	0.8 ± 0.5	12.6 ± 8.4	**0.017**
Estradiol, pmol/L	40.8 ± 21.6	326.8 ± 86.7 [a]	53.2 ± 25.2 [b]	52.5 ± 20.6	284.9 ± 75.4 [c]	66.4 ± 24.2 [d]	**<0.001**
Progesterone, pmol/L	6.5 ± 5.1	10.4 ± 6.9	8.6 ± 7.6	7.8 ± 6.4	9.8 ± 9.3	9.2 ± 7.9	0.949

Data are presented as mean ± standard deviation, and tested by analysis of variance. Letters denote significantly different from WKY+OVX group [a], WKY+OVX +EB group [b], SHR+OVX group [c], or OVX+EB group [d], $P < 0.05$. Bold value indicates significant differences among groups, $P < 0.05$.

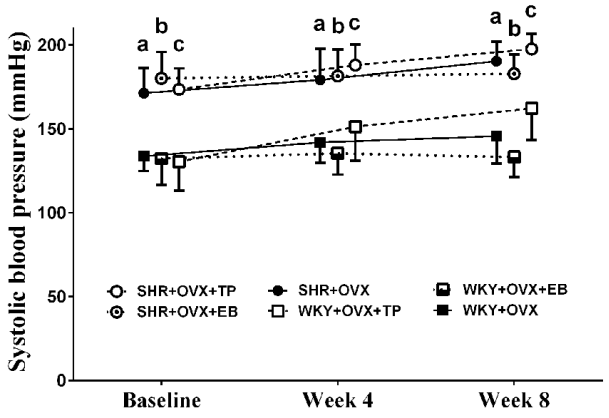

Figure 1. Change in systolic blood pressure in the 6 groups. Data are presented as mean ± standard deviation. Generalized estimation equation (GEE) analysis was used to test the time effect of each group, and analysis of variance (ANOVA) was performed to test group differences at each time point (n = 6 per group). Letters denote significant differences from the WKY+OVX group [a], WKY+OVX+EB group [b], or WKY+OVX+TP group [c], $P < 0.05$.

testosterone levels than WKY rats receiving no testosterone, but the difference did not reach statistical significance. WKY rats receiving estradiol had significantly higher estrogen levels than WKY rats receiving no treatment ($P = 0.003$) or testosterone ($P = 0.004$). Similar results were seen in WKY rats.

SBP

Changes in SBP in response to hormone treatment are shown in Figure 1. As expected, the SHR subgroups had higher SBP levels than the WKY subgroups at all time points. In untreated and testosterone-treated WKY rats, SBP increased gradually over time. There was no significant time trend in the WKY+OVX+EB group. Similar results were observed in SHR rats.

Endothelium-dependent and endothelium-independent relaxation

Nitroprusside-induced endothelium-independent relaxation was similar in untreated, estradiol-treated, and testosterone-treated rats (Figure 2). In both WKY and SHR rats, Ach-induced, endothelium-dependent vasodilation was more pronounced in estradiol-treated, and less pronounced in testosterone-treated rats than in untreated rats. However, statistical significance between groups was only seen in testosterone-treated groups, and only at the 2 highest Ach concentrations; that is, a significantly lower degree of relaxation was found in SHR+OVX+TP compared to WKY+OVX+TP rats at log(Ach) = -7 and -6.5 (Figure 2A). The pD2 value (log of the 50% of maximal effect concentration) for Ach-induced relaxation was 7.7 ± 0.4 in the WKY+OVX group, 8.0 ± 0.5 in the WKY+OVX+EB group, 7.5 ± 0.6 in the WKY+OVX+TP group, 7.2 ± 1.0 in the SHR+OVX group, 7.6 ± 0.8 in the SHR+OVX+EB group, and 6.8 ± 1.0 in the SHR+OVX+TP group.

eNOS inhibition in the arteries of testosterone-treated rats

The results of eNOS inhibition in the arteries of testosterone-treated rats are summarized in Figure 3. Addition of testosterone to the tissue bath had no effect on

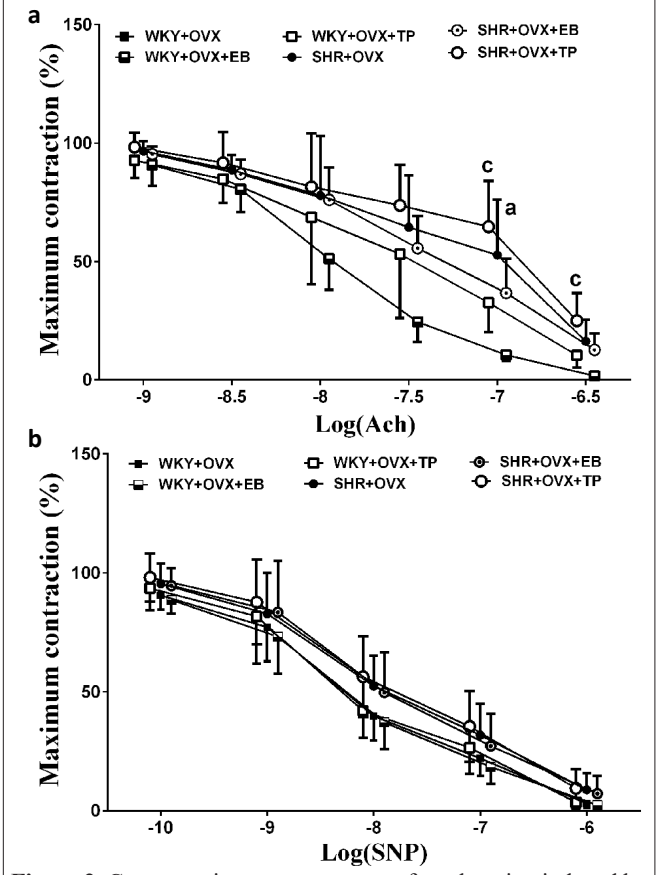

Figure 2. Concentration response curves for relaxation induced by Ach (A) and SNP (B) in mesenteric arteries. Mesenteric arteries were precontracted with phenylephrine (100 μmol/L). Relaxation is expressed as a percentage maximum contraction. Data are presented as mean ± standard deviation. Generalized estimation equation analysis was used to test for differences between various concentrations for each group, and analysis of variance (ANOVA) was performed to test group differences at each concentration (n = 6 per group). Letters denote significant differences from the WKY+OVX group [a] or WKY+OVX+TP group [c], $P < 0.05$.

Ach-induced vasorelaxation, although a trend to lesser relaxation was seen when testosterone was added to the bath solution of the SHR+OVX+TP group. The pD2 values for each group were: WKY+OVX+TP-PSS, 7.4 ± 0.7; WKY+OVX+TP-TP, 8.0 ± 0.5; SHR+OVX+TP-PSS, 6.7 ± 0.6; SHR+OVX+TP-TP, 7.1 ± 0.9 (Figure 3A).

Addition of the eNOS inhibitor did not change the response to Ach at lower Ach concentrations. However, at higher concentrations the eNOS inhibitor caused significantly more inhibition of vasodilation in SHR rats than WKY rats.

Possible eNOS uncoupling in testosterone-treated subgroup

Examination of eNOS uncoupling is presented in Figure 4. Estrogen-treated groups had higher NO and p-eNOS levels than other groups, but the differences did not reach significance. No significant differences were seen between groups in gp91phox expression, although higher levels were found in the 2 testosterone-treated groups. H_2O_2 content was higher in the testosterone-treated SHR group than in the other groups; a difference that reached significance in testosterone-treated SHR rats compared to testosterone-treated WKY rats ($P <$

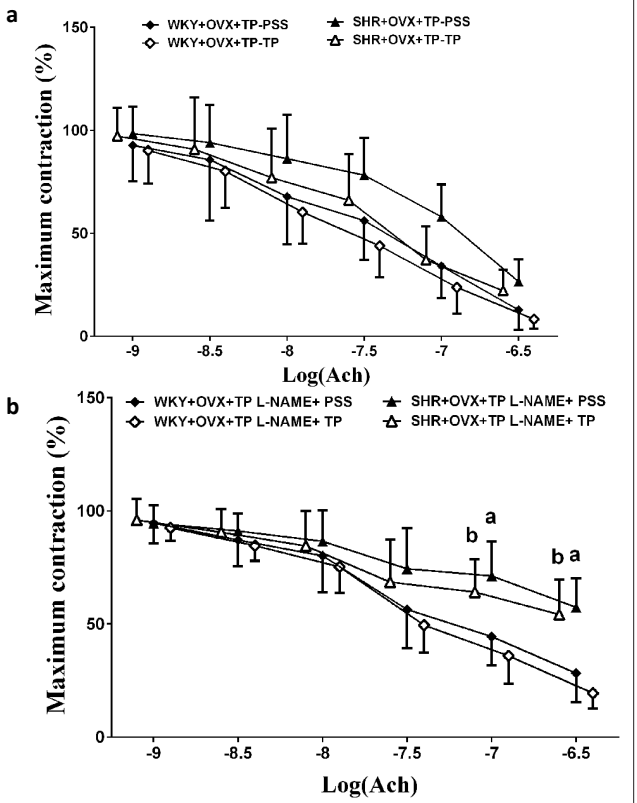

Figure 3. Concentration-response curves for relaxation induced by Ach in mesenteric arteries in the TP replacement group. Mesenteric arteries were preincubated with PSS or TP with NOS (A) or the NOS inhibitor L-NAME (B). Data are presented as mean ± standard deviation. Generalized estimation equation analysis was used to test for differences between various concentrations of Ach for each group, and independent sample t-test was performed to test group differences at each concentration (n = 6 per group). Letters denote significant differences from the WKY+OVX+TP L-NAME+PSS group [a] or WKY+OVX+TP L-NAME+TP group [b], $P < 0.05$.

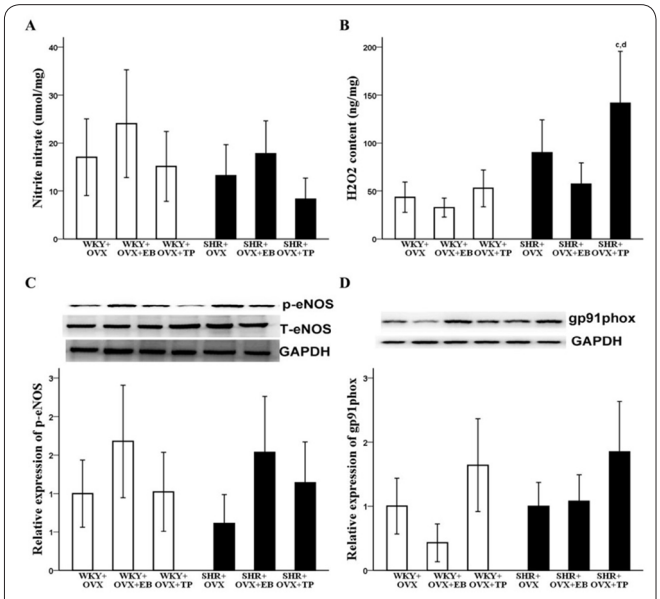

Figure 4. Mechanisms of impaired relaxation examined by nitrite/nitrate content in the aorta (A) (n = 6 per group), hydrogen peroxide content in aorta (B) (n = 6 per group), expression and phosphorylation of eNOS in the aorta (C) (n = 4 per group), and expression of gp91phox in the aorta (D) (n = 4 per group) in the TP replacement group. GADPH was used as the loading control. Data are presented as mean ± standard deviation. Generalized estimation equation analysis was used to test for differences between various concentrations of Ach for each group, and independent sample t-test was performed to test group differences at each concentration. Letters denote significant differences from the WKY+OVX+TP group [c] or SHR+OVX+EB group [d], $P < 0.05$.

0.001).

Blockade of androgen or estrogen receptors in arteries from testosterone-treated rats

Because some of testosterone's rapid effect on vascular smooth muscle is mediated by androgen receptors, and because some testosterone is converted to estradiol and exerts a portion of its effect through estrogen receptors, we investigated Ach-induced vasodilation in the presence of testosterone in the bathing solution when an androgen or estrogen receptor blocker was also present (Figure 5). No significant effect on relaxation was seen, except for a small effect in SHR rats receiving the highest dose of Ach.

Inhibition of the P13K/Akt pathway

Activation of P13K/Akt, and phosphorylation of Akt is an intracellular pathway by which androgen bound to androgen receptors causes phosphorylation and activation of eNOS. Akt phosphorylation was similar in aortic tissue from untreated, estrogen-treated, and testosterone-treated WKY and SHR rats. Addition of the P13K inhibitor had no effect Ach-mediated vasorelaxation in the presence of testosterone (Figure 6).

Discussion

We hypothesized that elevated ROS production resulting from chronic *in vivo* testosterone treatment would cause eNOS uncoupling leading to the attenuation of endothelium-dependent relaxation. The results of this study indicated that the conversion of testosterone to estrogen was not responsible for the vasodilatory effects of testosterone seen *ex vivo*. *In vivo* testosterone is converted to estrogen to some degree, and this could result in vasodilation through both androgen and estrogen receptors (19). However, blockade of either androgen or estrogen receptors had no effect on Ach-mediated vasodilation in the presence of testosterone, except at the highest Ach concentration. Blockade of the P13K/Akt pathway, an intracellular pathway through which androgen receptor activation causes eNOS activation, also had no effect on Ach-mediated vasorelaxation in the presence of testosterone.

We found that data from SHR and WKY rats were similar except that testosterone supplementation significantly increased aortic H_2O_2 in SHR, but not WKY rats, and at the highest Ach concentrations inhibition of eNOS and inhibition of androgen and estrogen receptors had a significant (although not large) effect in SHR, but not WKY rats. Direct relaxation induced by high doses of testosterone has been reported to be significantly reduced by denudation of the endothelium in SHR, but not WKY artery specimens (8). Previous studies have suggested that the vasodilating effect of testosterone in WKY rats may be related to actions on vascular smooth muscle ion channels (9, 20). Our results showing no

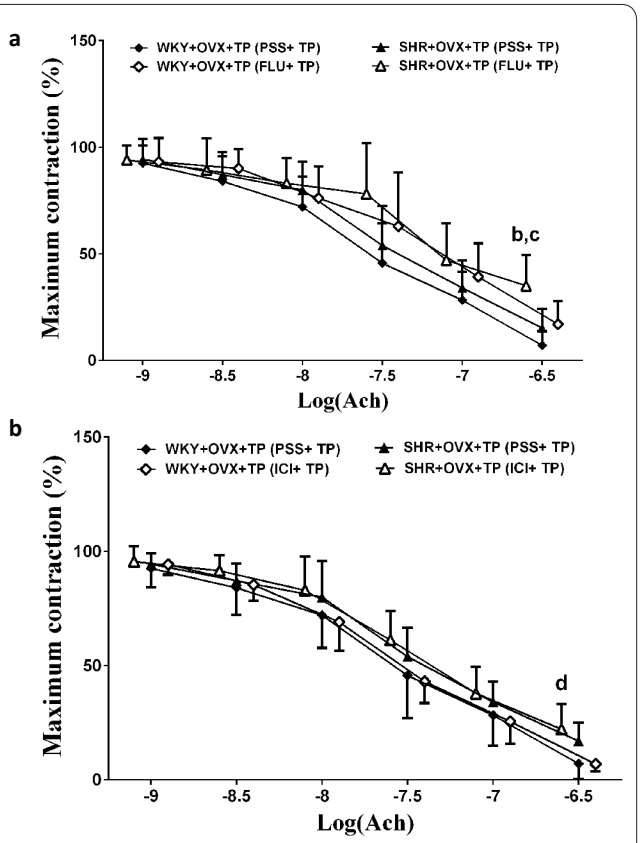

Figure 5. Impact of the androgen inhibitor flutamide (FLU) (A) and estrogen inhibitor ICI 182780 (ICI) (B) on relaxation induced by Ach in mesenteric arteries in the TP replacement groups. Mesenteric arteries were preincubated with PSS or TP. Relaxation is expressed as a percentage maximum contraction. Data are presented as mean ± standard deviation. Generalized estimation equation analysis was used to test for differences between various concentrations of Ach for each group, and independent sample t-test was performed to test group differences at each concentration (n = 6 per group). Letters denote significant differences from the WKY+OVX+TP (FLU+TP) group [b], SHR+OVX+TP (PSS+TP) group [c], or WKY+OVX+TP (ICI+TP) group [d], $P < 0.05$.

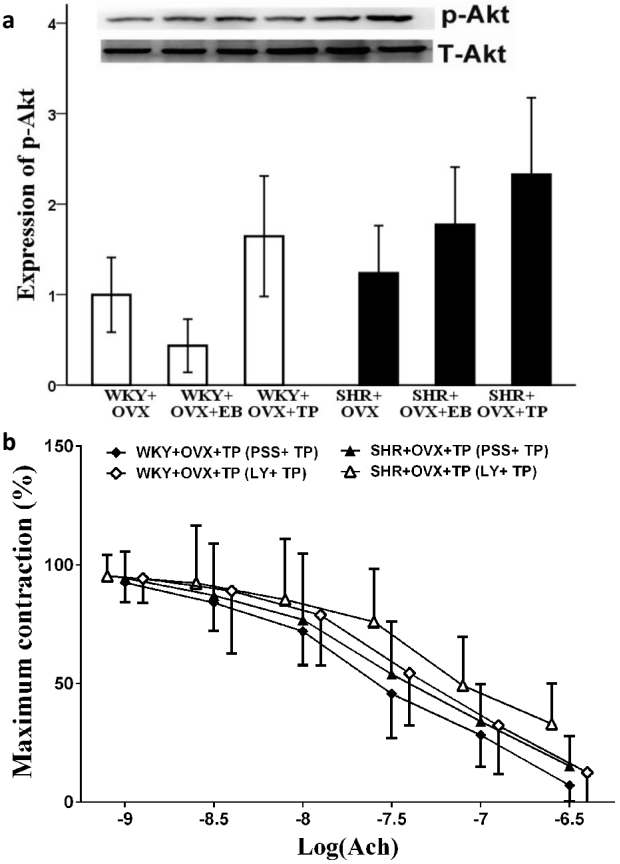

Figure 5. Impact of the androgen inhibitor flutamide (FLU) (A) and estrogen inhibitor ICI 182780 (ICI) (B) on relaxation induced by Ach in mesenteric arteries in the TP replacement groups. Mesenteric arteries were preincubated with PSS or TP. Relaxation is expressed as a percentage maximum contraction. Data are presented as mean ± standard deviation. Generalized estimation equation analysis was used to test for differences between various concentrations of Ach for each group, and independent sample t-test was performed to test group differences at each concentration (n = 6 per group). Letters denote significant differences from the WKY+OVX+TP (FLU+TP) group [b], SHR+OVX+TP (PSS+TP) group [c], or WKY+OVX+TP (ICI+TP) group [d], $P < 0.05$.

effect of testosterone on endothelium-dependent relaxation except at high Ach doses in SHR rats also suggests that the vasodilating or vasoconstrictive effect of testosterone is achieved by other mechanisms *in vivo*.

The results of estradiol-treated rats were not significantly different from those of untreated or testosterone-treated rats in this study. However, some trends seen in the data suggested that estrogen supplementation increased vasodilation. Estrogen supplementation seemed to block the increase in SBP seen in untreated and testosterone-treated rats, and increased the vasodilatory response to Ach and aortic NO and p-eNOS as compared to other groups of the same rat strain (WKY or SHR).

The signaling pathways involved in eNOS regulation include PI3K/Akt, AMP-activated protein kinase (AMPK), protein kinase A (PKA), and protein kinase C (21), of which the PI3K/Akt signaling pathway is the most common. In the current study, preincubation with a PI3K inhibitor had no effect on testosterone-induced relaxation. However, we did not study AMPK, PKA, or protein kinase C inhibition.

A unique part of this study was the use of the androgen receptor inhibitor flutamide, estrogen receptor antagonist ICI 182780, and phosphotidylinsitol-3-kinase (PI3K) inhibitor LY 294002. These antagonists are used extensively for study of receptor and signaling pathways in cell experiments, but are rarely used in *in vivo* and *ex vivo* studies. However, the primary limitation of this study is that experiments were conducted using a rat model and may not be applicable to human functions.

In summary, the current data suggest that the conversion of testosterone to estrogen is not responsible for the vasodilatory effects of testosterone seen *ex vivo*.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Author's contributions

Shijin Tan: study design / manuscript review / experi-

ments.
Dong Yi: study design / manuscript editing / experiments.
Wei Zhu: definition of intellectual content / statistical analysis.
Rong Sun: literature research / manuscript preparation / data acquisition.
Meng Wei: guarantor of integrity of the entire study / manuscript review.

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