Testosterone Alters Maternal Vascular Adaptations
Role of the Endothelial NO System

Vijayakumar Chinnathambi, Meena Balakrishnan, Jayanth Ramadoss, Chandrasekhar Yallampalli, Kunju Sathishkumar

Abstract—Sex steroid hormones estradiol and progesterone play an important role in vascular adaptations during pregnancy. However, little is known about the role of androgens. Plasma testosterone (T) levels are elevated in preeclampsia, mothers with polycystic ovary, and pregnant African American women, who have endothelial dysfunction and develop gestational hypertension. We tested whether increased T alters vascular adaptations during pregnancy and whether these alterations depend on endothelium-derived factors, such as prostacyclin, endothelium-derived hyperpolarizing factor, and NO. Pregnant Sprague Dawley rats were injected with vehicle (n=12) or T propionate [0.5 mg/Kg per day from gestation day 15–19; n=12] to increase plasma T levels 2-fold, similar to that observed in preeclampsia. Telemetric blood pressures and endothelium-dependent vascular reactivity were assessed with wire-myograph system. Phospho-endothelial NO synthase and total endothelial NO synthase were examined in mesenteric arteries. Mean arterial pressures were significantly higher starting from gestation day 19 until delivery in T-treated dams. Endothelium-dependent relaxation responses to acetylcholine were significantly lower in mesenteric arteries of T-treated dams (pD2 [-log EC50]=7.05±0.06; Emax =89.4±1.89) compared with controls (pD2=7.38±0.04; Emax=99.9±0.97). Further assessment of endothelial factors showed NO-mediated relaxations were blunted in T-treated mesenteric arteries (Emax=42.26±5.95) compared with controls (Emax=76.49±5.06); however, prostacyclin- and endothelium-derived hyperpolarizing factor-mediated relaxations were unaffected. Relaxation to sodium nitroprusside was unaffected with T-treatment. Phosphorylations of endothelial NO synthase at Ser1177 were decreased and at Thr495 increased in T-treated mesenteric arteries without changes in total endothelial NO synthase levels. In conclusion, increased maternal T, at concentrations relevant to abnormal clinical conditions, cause hypertension associated with blunting of NO-mediated vasodilation. T may induce the increased vascular resistance associated with pregnancy-induced hypertension. (Hypertension. 2013;61:647-654.) • Online Data Supplement

Key Words: blood pressure ■ endothelium-derived hyperpolarizing factor ■ eNOS phosphorylation ■ hypertension ■ mesenteric arteries ■ prostacyclin ■ vasodilation

Pregnancy is characterized by major cardiovascular adaptations, including marked decreases in systemic vascular resistance and mean arterial pressure (MAP) and an increase in maternal cardiac output and total blood volume.1,2 Studies in rats suggest that the enhanced endothelial vasodilatory actions allow peripheral vessels to accommodate these increases in blood flow and volume.3 Consistently, maternal vascular adaptations are accompanied by enhanced release of 3 major endothelium-derived vasodilatory factors, including NO, prostacyclin (PGI2), prostaglandin H synthase, and endothelium-derived hyperpolarizing factor (EDHF). Further, there are concomitant pregnancy-induced increases in mRNA and protein expression of endothelial NO synthase (eNOS), prostaglandin H synthase, and EDHF activity. Altered vascular adaptations during pregnancy are directly related to several maternal/fetal vascular pathologies, such as increased systemic vascular resistance, hypertension, proteinuria, poor placental growth, decreased nutrient transport, and low birth weight, which are characteristics of preeclampsia.

Most studies have investigated the beneficial role of sex steroid hormones, especially estradiol and progesterone on cardiovascular function during pregnancy. However, little is known about the role of androgens. The relation between testosterone and maternal cardiovascular function deserves special consideration because plasma levels of testosterone are increased 2- to 3-fold in pathological pregnancies, such as in preeclampsia, and androgen levels in preeclamptic women positively correlate with higher average systolic blood pressure. Also, pregnant hyperandrogenemia women with polycystic ovary syndrome and pregnant African American women have high maternal and cord blood testosterone levels and are at increased risk for developing preeclampsia. Previous studies have focused on the effects of increased maternal testosterone on cardiovascular...
consequences in the offspring.²⁷–²⁹ but, surprisingly, there is a paucity of literature about the effect of testosterone on the maternal cardiovascular system during pregnancy.

Most investigators have studied the effect of increased testosterone in cardiovascular function of males and non-pregnant females.³⁰–³² Increased testosterone has been shown to decrease endothelial function through its influence on the production or function of NO,³²,³³–³⁵ PGI²,³⁶ and EDHF³⁷ and affect vascular reactivity³⁸,³⁹ and systemic blood pressure.³⁰–³² Thus, it is possible that the functions of endothelium in modulating the vascular adaptations associated with pregnancy are altered in response to increased testosterone. Therefore, we hypothesized that increased testosterone may decrease endothelium-mediated cardiovascular adaptations of pregnancy. We tested this hypothesis by injecting testosterone into pregnant rats, mimicking the 2-fold increase in plasma testosterone levels observed in preeclamptic women.²⁷,²⁸,⁴⁰ Blood samples were drawn from the femoral vein 2 hours after testosterone injection on GD19 and analyzed for testosterone levels in Enzo Life Sciences, Farmingdale, NY) as reported previously.²⁷,²⁸ We injected rats with testosterone instead of dihydrotestosterone because it is the levels of testosterone and not dihydrotestosterone that are increased and correlate with complicated pregnancies.³⁵–³⁶ Moreover, this model is not associated with changes in levels of maternal estradiol, progesterone, or corticosterone.⁴⁶

Results
The period of gestation and mean litter size were not significantly affected by testosterone treatment. Fetal weights (control: 2.62±0.06 g; testosterone-treated: 1.99±0.08 g), placental weights on GD 20 (control: 0.54±0.08 g; testosterone-treated: 0.43±0.13 g), and birth weight of pups (control: 6.30±0.19 g; testosterone-treated: 5.75±0.19 g) were significantly reduced (P<0.05; n=8 litters in each group) in the testosterone-treated group compared with controls, consistent with our previous reports.²⁷,²⁸,⁴⁰ The plasma testosterone levels on GD 19 (2 hours after injection) from testosterone-treated dams were 2.1±0.17 ng/mL compared with 1.0±0.11 ng/mL in vehicle-treated control dams.

MAP and Heart Rate Measurements
Rats are nocturnal animals and continuous monitoring of blood pressure by telemetry revealed a characteristic circadian pattern with higher arterial pressure and heart rate values during the dark cycle (active phase) compared with the light cycle (Figure 1). In control animals (n=7), MAP was steady up to GD 17/18 and then progressively decreased as pregnancy advanced, reaching a nadir on GD21 and then increasing up to delivery on GD22. In testosterone-treated rats (n=7), MAP was similar to control rats during the early phase of treatment; however, the MAP did not show the expected decrease that occurred in control pregnant rats. Pregnant rats on testosterone had significantly higher MAP starting from GD19 until delivery on GD22 compared with the respective time point in controls (Figure 1A; P<0.05). Changes in both systolic and diastolic blood pressures were similar to that of MAP; therefore, data are not shown. No differences in heart rate were observed between controls and testosterone-treated dams (Figure 1B; n=7 rats in each group).
differences between control (n=7) and testosterone-treated rats (n=8; Figure 3B; Table). However, inhibition of PGI2 and EDHF pathways, leaving NO as the only intact pathway, showed significant relaxation to ACh, and this relaxation response was significantly lower in the mesenteric arteries of testosterone-treated pregnant rats (E_{max}: 42.26±5.95%; n=9) compared with control pregnant rats (E_{max}: 76.49±5.06%; n=9; Figure 3C; Table). Blockade of all 3 pathways with inhibitors completely abolished ACh-induced relaxation (data not shown). Overall, these data imply that testosterone treatment does not affect PGI2 or EDHF components of relaxation, but only inhibits the NO component of relaxation responses to ACh. Sodium nitroprusside caused concentration-dependent relaxation of phenylephrine contraction that was not different in mesenteric arteries of control (n=6) and testosterone-treated (n=6) pregnant rats (Figure 4).

eNOS Expression and Phosphorylation
eNOS mRNA levels were significantly reduced (P=0.0251; n=7 in each group) in mesenteric arteries of testosterone-treated pregnant rats compared with control pregnant rats (Figure 5A). However, immunoblot analysis indicated that there were no significant differences in protein levels of total eNOS in mesenteric arteries between control and testosterone-treated pregnant rats (Figure 5B; n=5 in each group). Examination of phosphorylation status of eNOS, as an indicator activity state, demonstrated site-specific changes (Figure 6A). When expressed as a ratio of total eNOS, phosphorylation at Ser1177 was significantly lower

Table. The E_{max} and pD_{2} of Concentration Response Curves Induced by ACh in PE-Precontracted Resistance Mesenteric Arteries of Control and Testosterone-Treated Pregnant Rats

<table>
<thead>
<tr>
<th>Vasodilatory Component</th>
<th>Control</th>
<th>Testosterone-Treated</th>
<th>Control</th>
<th>Testosterone-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total dilation</td>
<td>7.38±0.04</td>
<td>7.05±0.06*</td>
<td>99.9±0.97</td>
<td>89.4±1.89*</td>
</tr>
<tr>
<td>PG12-mediated†</td>
<td>ND</td>
<td>ND</td>
<td>13.0±4.62</td>
<td>10.4±3.99</td>
</tr>
<tr>
<td>EDHF-mediated‡</td>
<td>6.45±0.23</td>
<td>6.26±0.30</td>
<td>77.68±5.75</td>
<td>79.08±5.32</td>
</tr>
<tr>
<td>NO-mediated§</td>
<td>4.85±2.38</td>
<td>ND</td>
<td>76.49±5.06</td>
<td>42.26±5.95*</td>
</tr>
</tbody>
</table>

Data represent the mean±SEM from 7 rats in each group. ACh indicates acetylcholine; EDHF, endothelium-derived hyperpolarizing factor; ND, not determined; PE, phenylephrine; and PG12, prostacyclin.
*P<0.05 vs corresponding measurements in control.
†Was studied after inhibition of eNOS (L-NAME, 10^{-4} mol/L) and EDHF (apamin and charybdotoxin, 10^{-7} mol/L each) pathways, leaving PG12 as the only intact pathway.
‡Was studied after inhibition of eNOS and PG12 (indomethacin, 10^{-4} mol/L), leaving EDHF as the only intact pathway.
§Was studied after inhibition of PG12 and EDHF pathways, leaving NO as the only intact pathway.
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(3.3-fold) in the mesenteric arteries of testosterone-treated pregnant rats compared with controls (Figure 6B; \(P=0.0021\); \(n=5\) in each group). Phosphorylation at Ser635 was unchanged with testosterone treatment (Figure 6C). In contrast, phosphorylation at Thr495 was significantly higher (1.3-fold) in testosterone-treated compared with control rats (Figure 6D; \(P=0.0117\); \(n=5\) in each group). Human umbilical vein endothelial cells in culture exposed to testosterone also resulted in similar alterations in eNOS phosphorylation (see online-only Data Supplement).

Plasma NO\textsubscript{x} Levels

The level of plasma NO\textsubscript{x} at GD20 was significantly lower in testosterone-treated dams compared with controls (control: 4.0±0.71 \(\mu\text{mol/L}\); testosterone-treated: 2.6±0.37 \(\mu\text{mol/L}\); \(P=0.002\); \(n=12\) in each group).

Discussion

To our knowledge, this is the first study evaluating the effect of increased testosterone levels on maternal vascular adaptations in pregnant animals together with a detailed investigation of the underlying mechanisms. Major findings are that increased testosterone levels in pregnant rats lead to (1) increases in MAP, (2) inhibition of endothelium-dependent mesenteric arterial relaxation, and (3) decreases in vascular mesenteric relaxations that are mediated by alterations in the endothelium-dependent NO-pathway, but not EDHF- or PGI\textsubscript{2}-mediated pathways. Moreover, testosterone-induced reductions in endothelial NOS activity are associated with decreased phosphorylation of excitatory eNOS at Ser1177 and increased phosphorylation of inhibitory eNOS at Thr495. Importantly, these testosterone effects in pregnant rats are observed at an in vivo concentration similar to that observed in pathological pregnancies, such as in preeclampsia.

The arterial pressure in normal pregnant rats is stable until GD17 or 18 and then gradually decreases until GD21. Similarly, in pregnant women, arterial pressure is stable during early stages of first trimester and then gradually decreases reaching a nadir during the second trimester.\textsuperscript{2,41} Interestingly, increased testosterone prevented the decrease in blood pressure observed in normal pregnancy such that the testosterone-treated pregnant rats have higher blood pressure compared with control pregnant rats. The lack of a pregnancy-related fall in blood pressure indicates a failure in normal cardiovascular adaptations and is considered to be a cardinal feature of preeclampsia.\textsuperscript{32} This failure suggests that the mechanisms controlling blood pressure during pregnancy are perturbed by increased testosterone. This effect on arterial pressure increase is without any changes in heart rate, indicating that testosterone does not affect the sympathetic activity. In the search for
the possible mechanisms involved in the testosterone-induced increases in systemic arterial pressure, we found that increased testosterone caused inhibition of ACh-induced relaxation of resistance mesenteric arteries, suggesting that increased testosterone inhibits an endothelium-dependent relaxation pathway.

In the systemic circulation, the principal endothelium-dependent vasodilators are NO, PGI2, and EDHF. Studies have shown that PGI2 plays an important role in mediating vascular relaxation in uterine arteries42; however, its contribution in the mesenteric arterial relaxation, and in general to systemic blood pressure, is minimal, which is consistent with our findings and previous data illustrating that infusion of indomethacin into pregnant rats does not induce hypertension.44 Although a previous study reported that PGI2 production in cultured aortic vascular smooth muscle cells in vitro,46 we find that the PGI2-mediated relaxation was not affected by the presence of increased testosterone. This observation is also supported by similar levels of prostaglandin H synthase mRNA in the mesenteric arteries of testosterone-treated and control rats (data not shown). The current study shows that EDHF contributes substantially to mesenteric arterial relaxation during pregnancy, supporting earlier findings,43 but the presence of increased testosterone does not alter EDHF-mediated relaxation. However, in cerebral arteries of male rats, testosterone inhibited EDHF-mediated relaxation.43 The differences between our study and that of Gonzales et al.47 may be attributable to the sex, pregnancy status, or vascular bed examined. Indeed, sex and, in particular, pregnancy status can play a major role in many aspects of vascular function.

The most striking finding of our study is that NO-mediated mesenteric vasodilation was significantly decreased in testosterone-treated pregnant rats compared with controls, suggesting that increased testosterone selectively blunts endothelial NO function. It has been suggested that during pregnancy there is a relative predominance of NO-dependent vasodilation48 that may increase its susceptibility to the effect of increased testosterone. The decreased NO-mediated arterial relaxation in testosterone-treated pregnant rats is not because of decreased vascular smooth muscle sensitivity to NO, as relaxation of mesenteric rings to sodium nitroprusside, an exogenous NO donor, was not different between control and testosterone-treated rats. This suggests that the decreased relaxation in the testosterone-treated rats is more likely a result of changes in the synthesis/release of NO. The current study demonstrates a significant decrease in basal eNOS mRNA expression, but a similar difference was not noted at the level of the protein. Similar differences in eNOS mRNA but not protein expression levels were reported in pigs and sheep.33,46,47

Testosterone-mediated decreases in the mesenteric vascular function via the NO-pathway were accompanied by concomitant decreases in the eNOS activity state. Immunoblotting demonstrated that the excitatory Ser1177eNOS was decreased whereas no differences were observed at the excitatory Ser315 levels. Ser1177eNOS is the most widely investigated excitatory phosphorylation site in the systemic vasculature and is regulated by numerous kinases including acutely transforming retrovirus AKT8 in rodent T cell lymphoma (AKT) and adenosine monophosphate-activated protein kinase (AMPK) and our studies suggest that testosterone specially affects the excitatory Ser1177 site. Our studies also show that testosterone increases phosphorylation at Thr495eNOS, Thr495eNOS, an inhibitory site in the Ca2+-calmodulin binding region of eNOS is reported to be significantly phosphorylated in the caveolar subcellular domain of endothelial cells and its dephosphorylation leads to increased enzyme activity.48 Expressing as a ratio of excitatory Ser1177 to inhibitory Thr495 eNOS would better suggest eNOS activity state. Testosterone treatment significantly decreased the Ser1177/Thr495 ratio (0.97±0.06; n=5; P<0.05) in mesenteric vessels compared with controls (3.6±0.7; n=5). However, the ratio of Ser457/Thr495 in mesenteric arteries of testosterone-treated rats (2.6±0.2) was not significantly different compared with controls (3.0±0.1). Thus, these findings suggest that chronic testosterone administration during gestation not only leads to decreased activity state of eNOS (as measured by decreased Ser1177 phosphorylation), but also produces decreased availability of eNOS (due to increased Thr495 phosphorylation) that is essential for eNOS activation. Endothelial cells in culture
that cause pregnancy-associated vascular adaptations such as consequently decrease production and activity of other factors it is possible that the decreased endothelial NO function may tion that may lead to hypertensive diseases of pregnancy. Also, role for increased testosterone in causing endothelial dysfunc-

tion during pregnancy; our study for the first time demonstrates a potential tension, proteinuria, and intrauterine growth retardation. 54, 55 from day 17 to 22 of gestation resulted in sustained hyper-

vascular endothelial growth factor 57-58 and placental growth factor. 59 Ultimately, placental blood flow and transfer of nutrients may be adversely affected, and this may contribute to abnormal fetal growth and development. 40 In the current study, placental weights and pups born to testosterone-treated dams were significantly smaller than those born to control dams.

In conclusion, increased testosterone is associated with increased arterial pressure and selectively inhibits the endo-

thelium-dependent NO-mediated vascular relaxation path-

way in resistance vessels of pregnant rats. These results have important implications in determining the underlying factors for the adverse effects of testosterone on fetal growth and development.

**Perspectives**

Several studies show that the plasma levels of testosterone are 2-fold higher in preeclamptic pregnancies compared with normal pregnancies. Additionally, increased testosterone during pregnancy is associated with abnormal fetal growth and development leading to adult-life diseases, yet the mechanisms underlying the detrimental effects of testosterone remains unknown. In the current study, clinically relevant concentrations of testosterone produced increases in arterial pressure with significant effects on the mesenteric vasculature of pregnant rats. The endothelium-dependent relaxation pathway involving the NO production in endothelial cells is inhibited in systemic vessels of pregnant rats with elevated testosterone. The results suggest a role for testosterone as a possible media-

tor of increased vascular resistance and elevated blood pres-

sure during pregnancy. Therefore, some of the vascular effects observed during preeclampsia may indeed be androgen-medi-

ated. The ability of increased testosterone to influence cardio-

vascular function during pregnancy may contribute to some of the negative effects of testosterone on fetal growth and develop-

ment. Understanding testosterone’s influences on the cardio-

vascular system could lead to new therapeutic approaches.

**Figure 6.** Phosphorylation of endothelial NO synthase (eNOS) in mesenterial arteries isolated from control and testosterone-treated pregnant rats.

Tissue lysates were immunoblotted with antibodies recognizing Ser1177, Ser635, or Thr495-phosphorylated eNOS, and blots were reprobed with anti-eNOS antibody. A, Representative Western blots of phospho-eNOS and total eNOS. The level of (B) Ser1177, (C) Ser635, and (D) Thr495-phosphorylation of eNOS was quantified by scanning densitometry and normalized to total eNOS. Values are means±SE; n=5 for each group. *P≤0.05 vs control.
to antagonize some hypertensive effects during pregnancy. Furthermore, these results provide a novel approach to understanding the underlying factors that contribute to the pathogenesis of fetal origins of adult diseases.

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Disclosures

None.

References

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TESTOSTERONE ALTERS MATERNAL VASCULAR ADAPTATIONS:
ROLE OF THE ENDOTHELIAL NITRIC OXIDE SYSTEM

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Short Title: Elevated Testosterone Alters Maternal Vascular Function
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Methods:

**Measurement of blood pressure by radiotelemetry**

Dams were anesthetized on GD 6 with 2.5% isoflurane, and a flexible catheter attached to a radio transmitter (TA11PA-C10, Data Sciences, and Minneapolis, MN) was inserted into the left femoral artery. After surgery, rats were given housed in individual cages and allowed to recover for a week. Blood pressures were recorded continuously from GD 14 (1 day prior to start of treatment) until delivery of pups. Blood pressure measurements obtained with a 10-s sampling period were averaged and recorded every 10 minutes, 24 hours a day using the software (Dataquest 4.0) provided by the manufacturer. All acquired blood pressure and heart rate data were averaged into 12-hour blocks paralleling the light-dark cycle.

**Vascular reactivity studies**

Rats were sacrificed by CO2 asphyxiation on GD20. Because resistance arteries are actual determinants of blood pressure and pregnancy induces increases in vasodilatory responses in isolated mesenteric artery in rats, we assessed the effects of pregnancy and elevated testosterone on resistance-sized mesenteric arteries. The mesenteric arcade was quickly removed and immersed in an ice-cold, oxygenated (95% O2–5% CO2) Krebs physiological saline solution (in mM: NaCl, 119; KCl, 4.7; CaCl2, 2.5; MgSO4, 1.17; NaHCO3, 25; KH2PO4, 1.18; EDTA, 0.026; and d-glucose, 5.5; pH 7.4). Arteries (3rd order branches) were cleaned, cut, and mounted on a wire myograph (DMT, Copenhagen, Denmark), then stretched to optimal tension using the normalization procedure (Powerlab, ADInstruments, Colorado Springs, CO). After equilibration, arterial rings were exposed twice to a single dose of phenylephrine (PE; 10⁻⁵ mol/l; Sigma, St. Louis, MO) followed by a single dose of acetylcholine (ACh; 10⁻⁵ mol/l; Sigma) to check functional endothelial and smooth muscle integrity. A cumulative concentration-response curve (CRC) to PE was performed to determine the EC₈₀ of the maximum response (Eₘₐₓ) for the vasoconstrictor. To investigate vascular responses to ACh (10⁻⁹ to 10⁻⁵ mol/l), a CRC was performed following preconstriction with the EC₈₀ concentration of PE. The relaxation response to ACh was investigated in the absence and presence of inhibitors. For ACh relaxation response in presence of specific inhibitors, 3 experimental protocols were used to examine: 1) The PGI₂-mediated vasodilation after inhibiting NO production with L-NAME (10⁻⁴ mol/l; Sigma) and EDHF activity with apamin and charbdotoxin (both 10⁻⁷ mol/l; Sigma), 2) The EDHF-mediated vasodilation after inhibiting NO production with L-NAME and PGI₂ formation with indomethacin (10⁻⁵ mol/l; Sigma), and 3) NO-mediated vasodilation after inhibiting PGI₂ formation with indomethacin and EDHF activity with apamin and charbdotoxin. These preparations were preincubated with inhibitors for 30 minutes before the relaxation experiments were conducted. More than 1 EDHF candidate exists; however, in general, the hyperpolarizing mechanism of EDHF is considered to be mediated by SKCa and IKCa channels (blocked by apamin and charbdotoxin, respectively) on vascular endothelium. Sodium nitroprusside (SNP, 10⁻⁹ to 10⁻⁶ mol/l) was also used to assess the endothelium-independent relaxation response in endothelium-denuded arterial rings precontracted with PE.

**eNOS expression and phosphorylation**

The levels of eNOS and its phosphorylation were examined in mesenteric arterial tissue with intact endothelium. After removing a section of mesenteric artery for vascular reactivity studies, the remaining vessels were quickly flash frozen in liquid nitrogen and homogenized for RNA
and protein isolation. Expression of eNOS was determined by quantitative real-time PCR (qRT-PCR) as well as Western blotting using standard procedures as described below. We examined eNOS and specific phosphorylation sites that have been linked to altered eNOS activity and NO production: inhibitory Thr$^{495}$ and excitatory Ser$^{635}$ and excitatory Ser$^{1177}$.

**RNA isolation and Quantitative real-time PCR (qRT-PCR)**

Total RNA was isolated from mesenteric arteries using TRIzol reagent (Invitrogen, Carlsbad, CA). All RNA isolates were made DNA free by treatment with DNase and further purification with RNeasy Clean Up Kit (QIAGEN Inc, Valencia, CA). Total RNA concentration and purity were determined using an ND-1000 Nanodrop spectrophotometer (Thermo Fisher Scientific, Newark, DE). Two micrograms of total RNA were reverse transcribed using a modified Maloney murine leukemia virus-derived reverse transcriptase (New England Biolabs Inc, Ipswich, MA) and a blend of oligo (dT) and random hexamer primers (Invitrogen, Grand Island, NY). The reaction was carried out at 28°C for 15 minutes, then 42°C for 50 minutes, and stopped by heating at 94°C for 5 minutes followed by 4°C before storage at -20°C until further analysis. One micro liter of the resulting cDNA was used for qRT-PCR. The mRNA levels of eNOS were quantified by using the CFX96 real-time thermal cycler (Bio-Rad). Primers and probe for eNOS (Rn02132634-s1, Assays-on-Demand) were ordered as kits from TaqMan® Gene Expression Assay, Applied Biosystems (Carlsbad, CA). Results were calculated using the $2^{-\Delta\Delta CT}$ method and expressed in folds increase/decrease of the gene of interest in testosterone-treated vs control rats. All reactions were performed in duplicate, and β-actin was used as an internal control.

**Western blotting**

The arteries were quickly frozen in liquid nitrogen and stored at -80°C. Arteries were homogenized in ice-cold RIPA buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM b-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin; Cell signaling Technology, Danvers, MA) containing a protease inhibitor tablet (Roche, Indianapolis, IN) and phosphatase inhibitor cocktail-2 and -3 (Sigma, St Louis, MO). Tissue lysates were centrifuged (14,000 g for 10 min at 4°C), and the protein content was measured by using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). The supernatant was resuspended in the NuPAGE® LDS sample buffer and reducing agent (Invitrogen). Proteins (30 μg) alongside of Precision Plus Standard (Kaleidoscope, Bio-Rad Laboratories, Hercules, CA) were resolved on 4% to 12% gradient NuPAGE® Bis-Tris Gels (Invitrogen) at 100 V for 2.0 hours at room temperature and then transferred onto Immobilon-P membranes (Millipore Inc., Billerica, MA) at 100 V for 1.5 hours. The membranes were blocked with 5% bovine serum albumin (BSA) for 1 hour and then incubated overnight at 4°C with primary antibodies. The primary antibodies were mouse monoclonal eNOS (1:500; BD Transduction Labs, San Diego, CA) and mouse monoclonal β-actin (1:5000; Cell Signaling, Danvers, MA). After washing, the membranes were incubated with secondary antibodies (anti-rabbit or -mouse conjugated with horseradish peroxidase) at 1:10000 dilution and detected with the Pierce ECL detection kits (Thermo Scientific, Waltham, MA). Densitometric measurement was done using AlphaEase Fluorochrom 8000 software (Alpha Innotech, Santa Clara, CA). Results were expressed as ratios of band intensity to that of β-actin. In experiments to detect the phospho-eNOS protein, mesenteric arteries were cut into small pieces, incubated in Krebs physiological saline for 60 minutes, and stimulated with PE (3x10$^{-6}$ mol/l) for 5 minutes, then ACh (10$^{-7}$ mol/l) was added and incubated for 1 minute. At the end,
vessels were flash frozen in liquid nitrogen and homogenized to evaluate eNOS phosphorylation by immunoblotting using antibodies against phosphorylated Serine \textsuperscript{1177} eNOS, phosphorylated therionine \textsuperscript{495} eNOS (1:500; Cell Signaling), and phosphorylated Serine \textsuperscript{635} eNOS (1:500; Upstate, Lake Placid, NY). The band intensities of phospho-eNOS were normalized to that of total eNOS.

**Plasma NO\textsubscript{x} analysis.**
Plasma was ultrafiltered through a 30-kDa molecular mass cutoff filter (Ultrafree-MC centrifugal filter units, Millipore, Bedford, MA). NO\textsubscript{x} levels in the plasma were determined using a commercially available colorimetric assay kit (Cayman Chemical, Ann Arbor, MI) on the basis of the Griess reaction.\textsuperscript{5}

**Cell cultures and treatments.**
Human umbilical vein endothelial cells (HUVECs) obtained from Lonza (Walkersville, MD, USA) were cultured in phenol red free M199 medium supplemented with 20% steroid-deprived FBS (Hyclone, Utah, USA), 30µg/ml endothelial cell growth supplement (Sigma, Saint Louis, MO, USA), 100µg/ml heparin (Sigma) and antibiotics as described.\textsuperscript{6} The cells were treated with testosterone (0.1 to 100 nM) for five days with fresh medium containing testosterone replaced every day. At the end of treatment, the culture medium was collected for measurement of NO production and the cells were lysed with cell lysis buffer (cell signaling, Danvers, MA) containing preotease and phosphatase inhibitors (Sigma) and processed for Western blotting as described above. NO\textsubscript{x} accumulation was used as an indicator of NO production and was assayed in the medium (last 24 h of treatment) by Griess reagent (Nitrate/Nitrite Colorimetric Assay Kit, Cayman chemicals, Ann Arbor, Michigan). Briefly, 100 µL of Gries reagent was added to 100 µL of each supernatant in triplicate wells of 96-well plates. The plates were read in a microplate reader (FLUOstar Galaxy, BMG Labtechnologies, Duraham, NC) at 550 nm against a standard curve of NaNO\textsubscript{2} in culture medium.

**Results**
Testosterone treatment did not significantly alter the expression of eNOS protein levels (Fig S1) but affected its phosphorylation status. Testosterone treatment caused a dose-dependent decrease in phosphorylation of eNOS at the excitatory Ser\textsuperscript{1177} and increase in phosphorylation at the inhibitory Thr\textsuperscript{495} (Fig S1). However, testosterone did not affect the phosphorylation levels at Ser\textsuperscript{635} (Fig S1). Testosterone treatment caused a dose-dependent decrease in nitrite production by endothelial cells (Fig S2).
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Figure S1. Effect of testosterone on eNOS expression and activity state in HUVEC. HUVECs were treated with testosterone for five days with fresh medium containing testosterone replaced every day. Representative Western blots for total and phosphorylated eNOS and β-actin are shown at *top*; blot density obtained from densitometric scanning of eNOS normalized to actin and phosphorylated eNOS normalized to total eNOS is shown at *bottom*. Values are given as means ± SEM of 3 to 4 independent experiments. *P ≤ 0.05 vs control.
Figure S2. Effect of testosterone treatment on nitrate/nitrite production in HUVECs. HUVECs were treated with testosterone for five days with fresh medium containing testosterone replaced every day. The cell culture medium (last 24 h) was assayed for $\text{NO}_x$ accumulation using Cayman’s nitrite/nitrate assay kit. The $\text{NO}_x$ values were calculated against NaNO$_2$ standard curve. Data points represent mean±SEM of measurements in 6-8 independent experiments. *$p<0.05$ compared with control.