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The vasodilatory effect of testosterone on renal afferent arterioles

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Abstract

Background—Gender differences exist in a variety of cardiovascular and renal diseases, and testosterone may contribute to the discrepancy. Afferent arterioles (Af-Art) are the major resistance vessels in the kidney, and play an important role in the development of renal injury and hypertension.

Objective—The present study aimed to determine the acute effect and underlying mechanism(s) of testosterone on Af-Art.

Methods—The mRNA expression of androgen receptors (AR) in microdissected Af-Art was measured by RT-PCR. An *in vitro* microperfusion model was used to measure the diameter of Ar-Art in mice. Nitric oxide (NO) was evaluated by an NO-sensitive fluorescent dye, 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) diacetate.

Results—Testosterone had no effect on microperfused Af-Art when added into the bath. Therefore we pre-constricted the Af-Art to about 30% with norepinephrine (NE, 10^{-6}mol/L); administration of testosterone (10^{-9} to 10^{-7}mol/L) subsequently dilated the Af-Art in a dose-dependent manner ($p<0.001$; n=7). AR mRNA was expressed in microdissected Af-Art measured by RT-PCR. An AR antagonist, flutamide (10^{-5}mol/L), totally blocked testosterone (10^{-8}mol/L)-induced vasodilator effect. NO production of Af-Art wall was increased when testosterone was added into the bath solution after NE treatment, from 278.4 ± 12.1 units/min to 351.2 ± 33.1 units/min ($p<0.05$; n=3). In the presence of NO inhibition with N^G-nitro-L-arginine methyl ester (L-NAME, 3×10^{-4}mol/L), the testosterone-induced dilatation was blunted compared with NE ($p<0.05$).

Conclusions—We conclude that testosterone dilates pre-constricted mouse Af-Art in a dose-dependent manner by activation of AR and partially mediated by NO.

Keywords

kidney; testosterone; androgen receptors; nitric oxide

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Introduction

Gender differences exist in a variety of cardiovascular and renal diseases. Sex steroids are indicated to be involved in these differences. For example, hypertension and chronic coronary artery disease occur more frequently in men than in premenopausal women[1, 2]. The incidence of end-stage renal disease of all cause is higher in men than in women[3]. Renal diseases in women progress at a slower rate than they do in blood pressure-and lipid level-matched men [4]. Glomerular filtration rate (GFR) in healthy women declines more slowly compared with that in healthy men[5]. These studies are long-term observations and the underlying mechanisms are suggested to be *via* a classic pathway which is dependent on androgens binding to the nuclear receptors.

Contrary to the classic genomic effect, androgens also display rapid non-genomic potentially beneficial effects as recently reported in clinical trials and animal studies[6, 7]. In men with established coronary artery disease, short-term intracoronary administration of testosterone, at physiological concentrations, induces coronary artery dilatation and increases coronary blood flow within minutes[8]. Also, acute oral administration of testosterone increased cardiac output, and reduced systemic vascular resistance compared with baseline in male patients with stable chronic heart failure[9]. Both in heart failure patients and in healthy controls testosterone at high concentration (>10μm) dilated subcutaneous resistant arteries ex vivo[10]. The non-genomic effect usually does not depend on the binding of testosterone to the classic nuclear androgen receptors (AR) with the subsequent process of gene transcription and protein translation[11–13]. The mechanisms are still unclear. Despite of the contradictions among studies, nitric oxide (NO) seems to be involved in this response because NO synthase (NOS) inhibition has been shown to decrease the dilation effect both *in vitro*[14] and *in vivo*[15]. Membrane ion channels including Ca^{2+}[16] and K^+ channels[12] also play a role in the non-genomic effect.

Afferent arterioles (Af-Art) are the major resistance vessels in the kidney[17]. They are indispensible in regulation of both myogenic response and tubuloglomerular feedback, which are the primary mechanisms for renal autoregulation. These regulatory mechanisms are key factors for control of intraglomerular pressure and salt and water balance, thereby making them important for the development of renal injury and hypertension. Studies showed that testosterone induced relaxation of precontracted rabbit renal arteries *in vitro* in an endothelium dependent manner,[18] however, the acute effect of testosterone on renal microcirculation has not been reported, to our knowledge. In the present study, we aimed to determine the direct and acute effect of testosterone on renal afferent arterioles and the underlying mechanisms. Using an Af-Art microperfusion model in mice, we found that testosterone induced acute vasodilation of the Af-Art *via* the AR and partially mediated by NO.

Methods

All procedures and experiments were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center. All studies are consistent to the *Guide for the Care and Use of Laboratory Animals*, National Institutes of Health. All chemicals were purchased from Sigma (St. Louis, MO) except as indicated.

Isolation and microperfusion of mouse afferent arterioles

The methods are the same as we previously described to isolate and perfuse the Af-Art with intact glomerulus[19, 20]. Briefly, male C57BL/6J mice (8 weeks) were anesthetized with ketamine and xylazine, and kidneys were removed and sliced along the corticomedullary axis. Slices were placed in ice-cold minimum essential medium (MEM; Gibco, Grand

Island, NY) containing 5% bovine serum albumin and dissected under a stereomicroscope (model SMZ 1500; Nikon). A single superficial Af-Art and its intact glomerulus were microdissected, transferred to a temperature-regulated chamber mounted on an inverted microscope (Eclipse Ti; Nikon) with Hoffmann modulation using a micropipette and cannulated with an array of glass pipettes. Af-Arts were perfused with MEM from the proximal end in an orthograde direction. Microdissections and perfusions were completed within 60 minutes at 4°C, and the samples were then gradually warmed to 37°C for the rest of the experiment. Once the temperature was stable, a 30-min equilibration period was allowed before taking any measurements. The imaging system consisted of a microscope (Eclipse Ti; Nikon), digital charge-coupled device camera (CoolSnap; Photometrics), xenon light (LB-LS/30; Shutter Instruments), and optical filter changer (Lambda 10–3; Shutter Instruments). Images were displayed and analyzed with NIS-Elements imaging software (Nikon).

Dose-response curves

Testosterone was purchased from Sigma (testosterone propionate, catalog number T1875), dissolved in ethanol at the concentration of 10mg/ml and stored as stock solution. Working solutions at different concentrations were made from the stock solution in MEM in the following experiments. The final concentration of ethanol was 3.4×10^{-4} – 3.4×10^{-8} % (v/v).

After the 30-minute equilibration period, testosterone (10^{-8}mol/L) was added into the bath to test whether it displayed a vasoconstrictor effect. To determine whether testosterone had a vasodilator effect, we pre-constricted the Af-Art with norepinephrine (NE, 10^{-6}mol/L) in the bath for 5 min. Next the bath solution was switched to NE plus testosterone and the diameter of the Af-Art was measured at 5 min thereafter. One concentration was tested firstly, and then 10 min was allowed for elution with MEM before using another concentration in the Af-Art. All the vascular responses were measured at the same time point. Ethanol was used as vehicle in control experiments at the concentration of 3.4×10^{-4} %, equal to the solution containing testosterone of 10^{-7} mol/L. To further compare the effect of different concentrations of testosterone to Af-Art, we calculated the percentage of testosterone-induced vasodilation with the following formula: Dilation of the Af−Art $\%=(Dia_{NE+TES}-Dia_{NE})/Dia_{NE}\times100\%$ (Dia, Diameter of Af-Art). To test if the rapid effect of testosterone was mediated by AR, an AR antagonist, flutamide (10^{-5}mol/L), was added in bath to pretreat Af-Art for 15min, and the above experiment was repeated. To determine the role of NO in testosterone-induced vasodilation, a NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME, 3×10^{-4}mol/L), was added to the bath for 30 min, and then testosterone was added into the bath. The percentage of Af-Art dilation in the present of flutamide or L-NAME was also calculated in the same order as above.

NO measurement with fluorescence

A NO-sensitive fluorescent dye, 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) diacetate was used to measure NO production in the Af-Art. DAF-FM (10^{-5}mol/L) was added into the arteriole perfusate and the perfusion was maintained for 40–60 minutes, and then washed off by MEM for 10 min. Thereafter, NE and NE plus testosterone (10^{-8}mol/L) were added into the bath. DAF-FM was excited at 495 nm and the emitted fluorescence was recorded at 515 nm. Square-shaped regions of interest (ROIs) were set on the wall of Af-Art and mean intensity of ROIs recorded every 5 seconds and corrected for background. The rate of increase in fluorescence intensity was calculated and NO was measured for 5 min.

Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR was used to determine whether ARs were expressed in Af-Art. Af-Arts (n=10 to 20) were microdissected in ice-cold MEM and transferred into RLT buffer (RNeasy Micro

Kit, Bio-Rad) for total RNA extraction. Total RNA were amplified by a Message Sensor RT kit. PCR was performed in a BioRad thermal cycler (Bio-Rad, Hercules, CA). Negative controls were performed by omitting cDNA template from the PCR amplification. Sequence of AR primers were 5'-GCCCGGCAAATCTCAACAACTCAA-3' (forward primer) and 5'-TTAGGGAAAGGAACGTCGTGAGCA-3' (reverse primer). β-actin served as a "housekeeping" gene. The mixed samples were heated to 95°C for 5 min and then cycled for 40 cycles at 94°C for 30sec, 59°C for 20sec, 72°C for 30sec. Final extension was 8 min at 72°C. Complimentary DNA was electrophoresed on a 1.5% agarose gel, and stained with ethidium bromide. Images were captured using a VersaDoc image analysis system (Bio-Rad, Hercules, CA).

Statistical analysis

Data were collected as repeated measures over time under different conditions. We tested only the effects of interest, using analysis of variance (ANOVA) for repeated measures and a post-hoc Fisher LSD test or a Student's paired t-test when appropriate. The changes were considered to be significant if $p<0.05$. Data were presented as mean ± SEM.

Results

Testosterone dilates Af-Art

To test the acute effect of testosterone, we added different doses of testosterone to the perfused Af-Art. In non-pre-constricted Af-Art, testosterone had no effect on the Af-Art, indicating that testosterone had no vasoconstrictive effect (from 11.0±0.9um to 12.1±1.2μm, n=4). To test if testosterone had any vasodilatory effect, we pre-constricted the Af-Art with NE and added testosterone, since the microdissected and perfused arterioles lack basal vascular tone. As shown in Figure 1, Af-Art were pre-constricted by about 30%, from 11.6±0.2 to 8.0±0.2 μm, by NE (10^{-6}mol/L) in the bath (aggregate value for all groups; n = 6–8 in each group); testosterone dilated the Af-Art at the concentrations of 10^{-7} to 10^{-9} mol/L with a maximum effect at 10^{-8} mol/L ($p<0.001$). Testosterone at 10^{-10}mol/L failed to have an effect. All the effects occurred within 5 min after testosterone administration. The vehicle had no constriction or dilatation effect on pre-constricted Af-Art. These data indicate that testosterone causes acute vasodilation of the Af-Art. Testosterone at 10^{-8}mol/L had a significant dilatory effect and is considered to be within the physiological range. Therefore, this concentration was used in the following experiments.

AR are expressed in the Af-Art and involved in the rapid effect of testosterone

To determine whether AR was involved in the acute effect of testosterone, we first tested whether AR was expressed in mouse Af-Art by RT-PCR. As shown in Figure 2, AR mRNA was found to be present in Af-Art. Next, we tested the effect of testosterone on the Af-Art in the presence of AR antagonist, flutamide, in perfused and pre-constricted Af-Art. Flutamide (10^{-5}mol/L) was added into the bath 15 min before NE administration and was present in the following measurement. As shown in Figure 3A, the diameter of the perfused Af-Art was decreased from 11.7 ± 0.4 to 9.1±0.5 μm by NE. Testosterone (10^{-8}mol/L) caused a vasodilation that was abolished when flutamide was added to the bath (8.9±0.4 μm, n=9). Without flutamide, NE constricted the Af-Art from 12.1 ± 0.5 to 8.3 ± 0.6 μm, and testosterone (10^{-8} mol/L) dilated it to 10.6 ± 0.5μm (n=6). The dilation percentage was further compared in Figure 3B. These data indicate that the acute dilatory effect of testosterone is mediated by AR that is expressed in the Af-Art.

Testosterone enhances NO generation in the Af-Art

To determine the role of NO in testosterone-induced dilation of the Af-Art, we used fluorescent dye DAF-FM to measure NO production. As shown in Figure 4, after being corrected by background, the basal production of NO was 244.4 ± 10.7 units/min. When NE was added in, NO production was 278.4 ± 12.1 units/min. In the presence of both NE and testosterone, NO production was 351.2 ± 33.1 units/min ($p<0.05$ vs NE alone; n=3), which increased by 25.7 ± 8.7% compared with NE alone. These data indicated that testosterone rapidly enhanced NO generation in the Af-Art.

We then tested whether blockade of NO by inhibition of NOS diminished the effect of testosterone. As shown in Figure 3A, in the presence of L-NAME (3×10^{-4} mol/L) in the bath, the diameter of Af-Art was decreased from 11.3±1.1 μm to 8.6±0.3 μm. After testosterone was added into bath in the presence of L-NAME, the diameter of Af-Art was 10.0±0.4μm ($p<0.05$; n=8). The dose of L-NAME has been shown to block the effect of NO in isolated perfused arterioles. [21] Although testosterone still dilated the Af-Art in the presence of L-NAME, the percentage of the dilatation was significantly less compared with NE ($p<0.05$, Figure 3B), indicating that testosterone induces Af-Art vasodilation, at least, partially *via* NO.

Discussion

The major finding of the present study is that testosterone acutely dilated the Af-Art. This effect was mediated by the ARs expressed in the Af-Art. Testosterone enhanced NO generation in the Af-Art, while inhibition of NOS attenuated the testosterone-induced vasorelaxation.

The action of testosterone is classically known to be mediated by an intracellular protein, the AR, which belongs to the steroid hormone superfamily. In target tissue testosterone directly binds to the nuclear AR, or is converted to dihydrotestosterone, which also binds the AR. The hormone-receptor complex binds to the androgen response elements, either inducing or suppressing the androgen responsive genes [22], thereby inducing a genomic effect. Testosterone contributed to the exacerbation of hypertension in SHR by reducing pressure-natriuresis,[23] and augmented renal vascular responses to angiotensin II in New Zealand genetically hypertensive rats.[24] Absence of testosterone prevented endothelial dysfunction and increases in blood pressure secondary to insulin resistance, which involved the Cyp4A/20-HETE system.[25] ARs are widely expressed in many tissues besides the reproductive organs. In the kidney, ARs are highly expressed in proximal tubule cells[22],cortical collecting ducts[26], glomerular endothelial cells and podocytes[27]. In Af-Art, ARs were found in rats by immunohistochemistry[27]. In our study, we microdissected the mouse Arf-Art of mice and found the mRNA expression of AR.

In addition to the genomic effects, androgens have rapid, non-genomic effects which occur within minutes in vasculature. Most studies showed a vasodilator effect of testosterone, such as in human umbilical artery[28], coronary artery[29], rat aorta[12], human radial artery[30], and internal mammary artery[31]. Blocking transcription with actinomycin D and translation with cycloheximide does not inhibit testosterone-induced vasodilation[13], indicating a non-genomic effect. In contrast, in isolated, perfused rat heart, testosterone (10^{-10}–10^{-7} mol/L) rapidly blocked the vasodilator effect of adenosine and increased vascular resistance[32]. The Af-Art is responsible for the predominant pre-glomerular vascular resistance[17] which largely determines intraglomerular pressure, glomerular filtration rate, and as such, controls renal hemodynamics and blood pressure. The rapid, direct effect of testosterone on the Af-Art, to our knowledge, has not been investigated. We found testosterone dilated Af-Art within 5 min, which is consistent with other findings on large non-resistant arteries[12, 28] and small

resistant vessels[14, 33]. In the present experiments, we performed dose-response studies in the range from 10^{-10} to 10^{-7} mol/L, with the lowest effective concentration of 10^{-9} mol/L, which is considered to be within the physiological range[6].

Whether ARs are involved in the non-genomic effect of testosterone is still controversial[34]. In the present study, AR antagonist flutamide diminished the testosterone induced vasodilation, indicating testosterone's effect was mediated by AR. In agreement with our findings, Gorczynska and Handelsman found testosterone at higher concentrations (0.3–3×10^{-3} mol/L) induced a rapid Ca^{2+} increase, which was blocked by flutaminde in Sertoli cells[35]; in human prostate cancer cells, dimethylnortestosterone, or 5α-dihydrotestosterone, was shown to increase Ca^{2+} rapidly, and the effect was blocked by pre-incubation with hydroxyflutamide, suggesting an involvement of the AR[36]. However, there is some evidence that the testosterone-induced rapid vasodilation was not attenuated by pre-treatment with the AR blocker flutamide[11, 12]. In addition, in vessels isolated from testicular feminized mice, which lack a functional AR, a testosterone-mediated vasodilation was found [37]. These studies do not support the functional role for AR in testosterone's vasodilating effect. The reasons for above discrepancies are not clear, but may be strain and cell-specific.

Mechanism responsible for the testosterone-induced vasorelaxation have been studied and intensively reviewed[6, 7, 34], but the precise mechanism has yet been fully understood. Briefly, the vasodilator effect of Tes is associated with the cell membrane ion channel function in vascular smooth muscle, including the inactivation of L-type voltage-operated $Ca2^{+}$ channels and the activation of K^{+} channels. In addition, roles of prostanoids and endothelium derived nitric oxide were also studied. A number of studies showed inhibition of prostanoids by indomethacin did not reduce the vasodilator effect[15,38], while Marachelli and colleagues found indomethacin enhanced the relaxant action of testosterone in endothelium-denuded renal arteries[18]. In endothelium-denuded vessels testosterone (>10 μM) induced vasodilation, but at lower physiological concentrations NO appears to be involved in the vasodilator effect. For example, in human pulmonary arteries from males, only vessels with intact endothelium developed testosterone induced vasodilation within the physiological range (10^{-9} mol/L), while endothelium-denuded vessels required a concentration as high as 3×10^{-5} mol/L to show a dilatory response[33]. Inhibition of NOS with L-NAME significantly inhibited maximal relaxations to testosterone in the rat mesenteric arterial bed[14]. In our study, we first employed a NO sensitive fluorescent dye to specifically measure the NO production, which showed testosterone enhanced NO generation in the Af-Art. Then we used L-NAME to inhibit NOS activity and found an attenuated effect of testosterone-induced vasorelaxation. However, these data indicate that the acute effect of testosterone is partially NO dependent, since the vasodilatory effect of testosterone was not completely abolished by L-NAME. The other mechanism that mediates testosterone-induced vasodilatation of the Af-Art is to be determined. We feel that metabolites of arachidonic acid are the most possible candidates. Testosterone may either increase dilatory factors like prostaglandins and EETs or decrease constrictive factors like 20-HETE and TXA_2.

Conclusions

We found a rapid vasorelaxation effect of testosterone on renal Af-Art, medicated by the AR, and partially involving activation of NOS. The acute effect of testosterone might be beneficial in acute renal injury, when an acute increase in GFR is necessary to abrogate renal ischemia.

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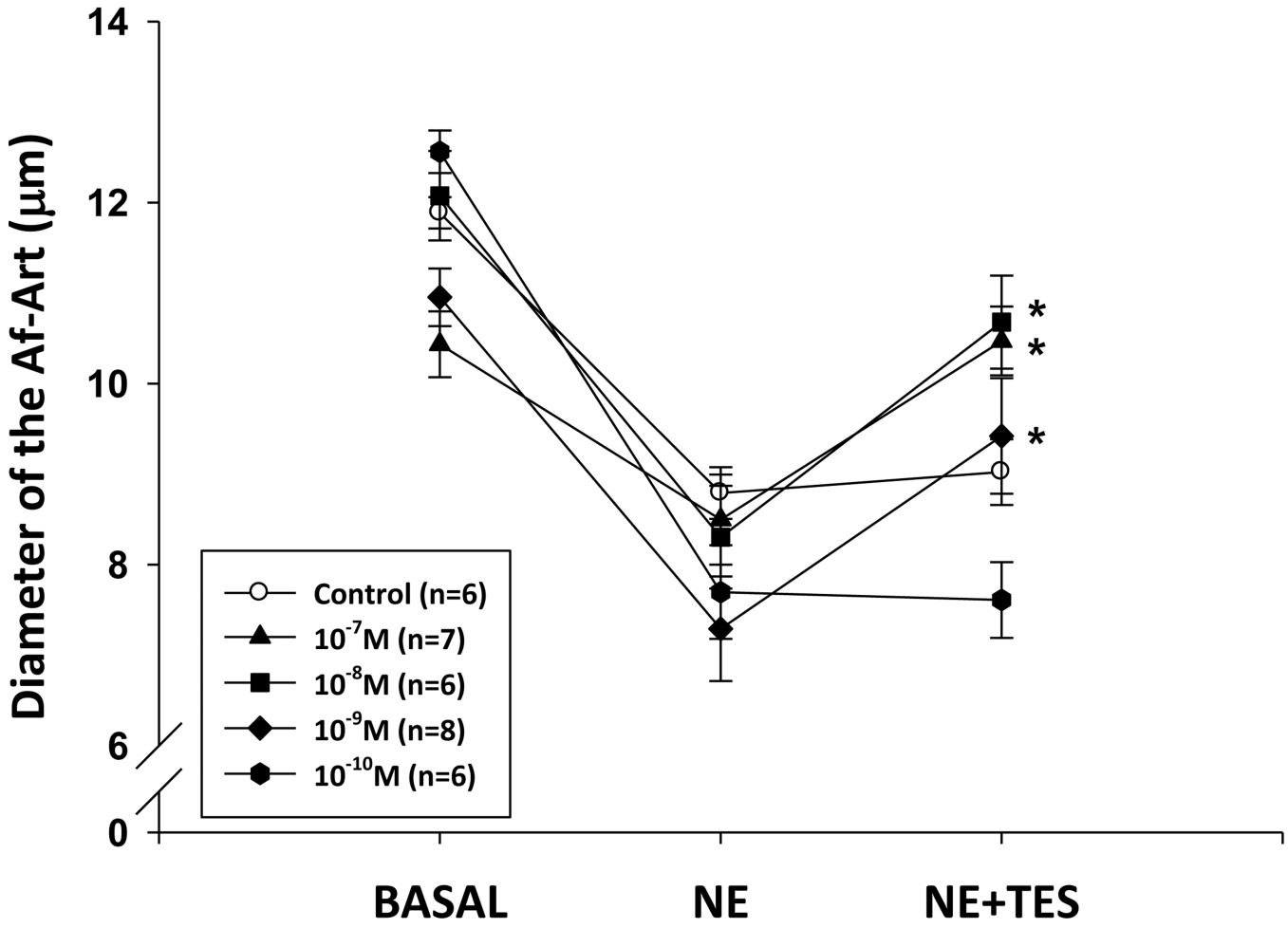
1A



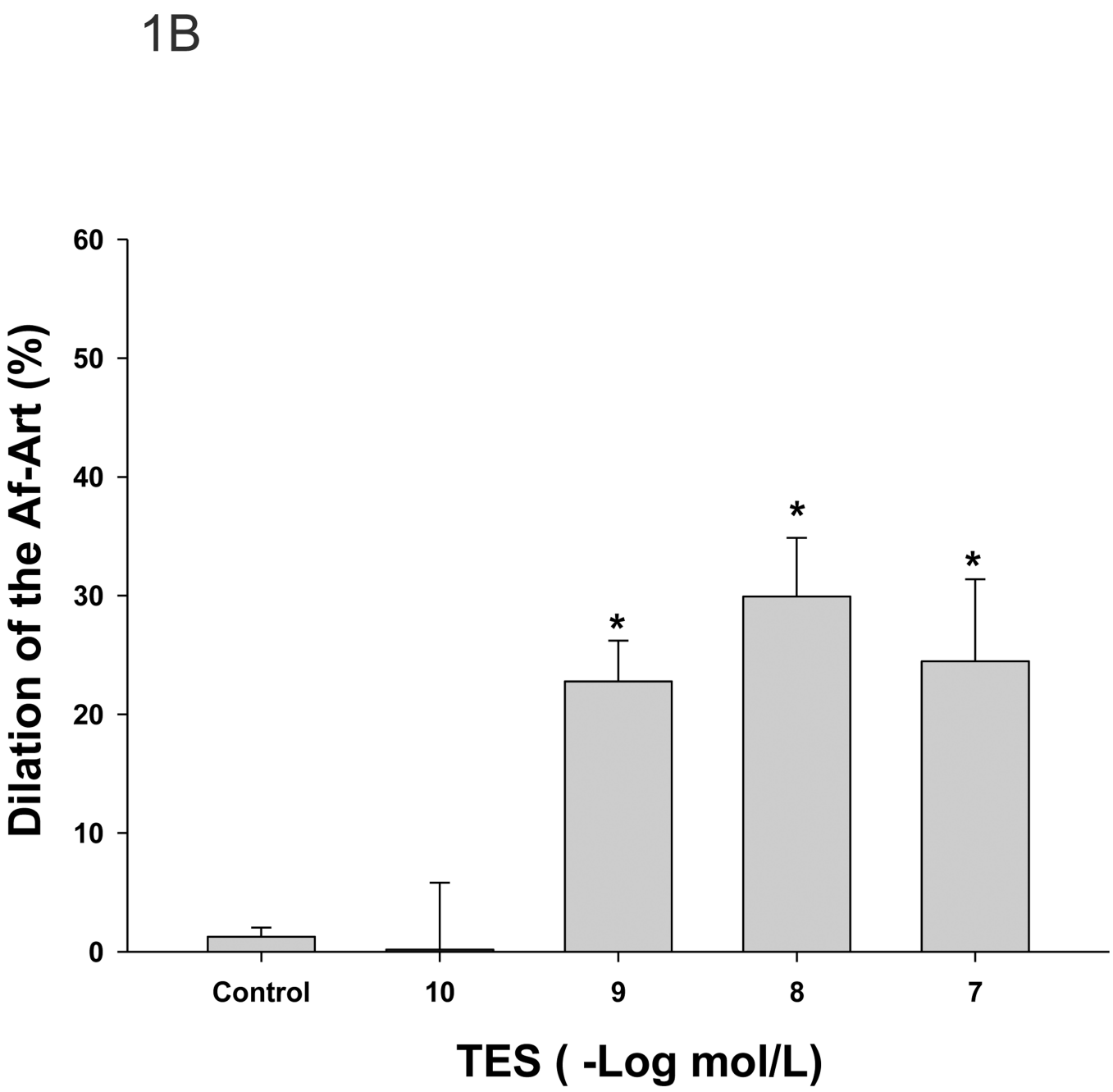


Figure 1. Testosterone dilates the Af-Art

A. Testosterone (TES) induced a dose-responsive relaxation of preconstricted Af-Art by norepinephrine (NE). Af-Art was dilated 2.1±0.5 μm at 10^{-7} mol/L; 2.3±0.4 μm at 10^{-8} mol/L; and 1.9± 0.6 μm at 10^{-9} mol/L (*p<0.001 *v.s.* NE). TES at 10^{-10} mol/L did not induce changes in diameter of the Af-Art. B. The dilatory effect of testosterone expressed in percentage (*p<0.05 *v.s.* control).

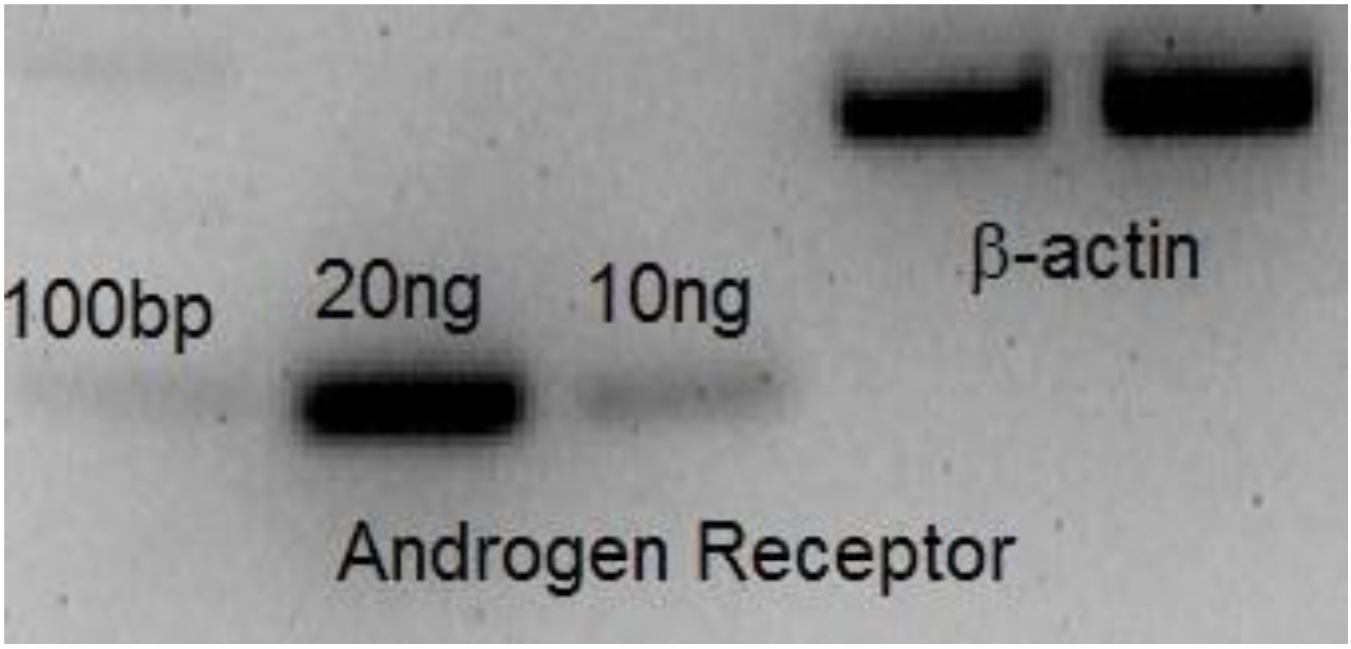
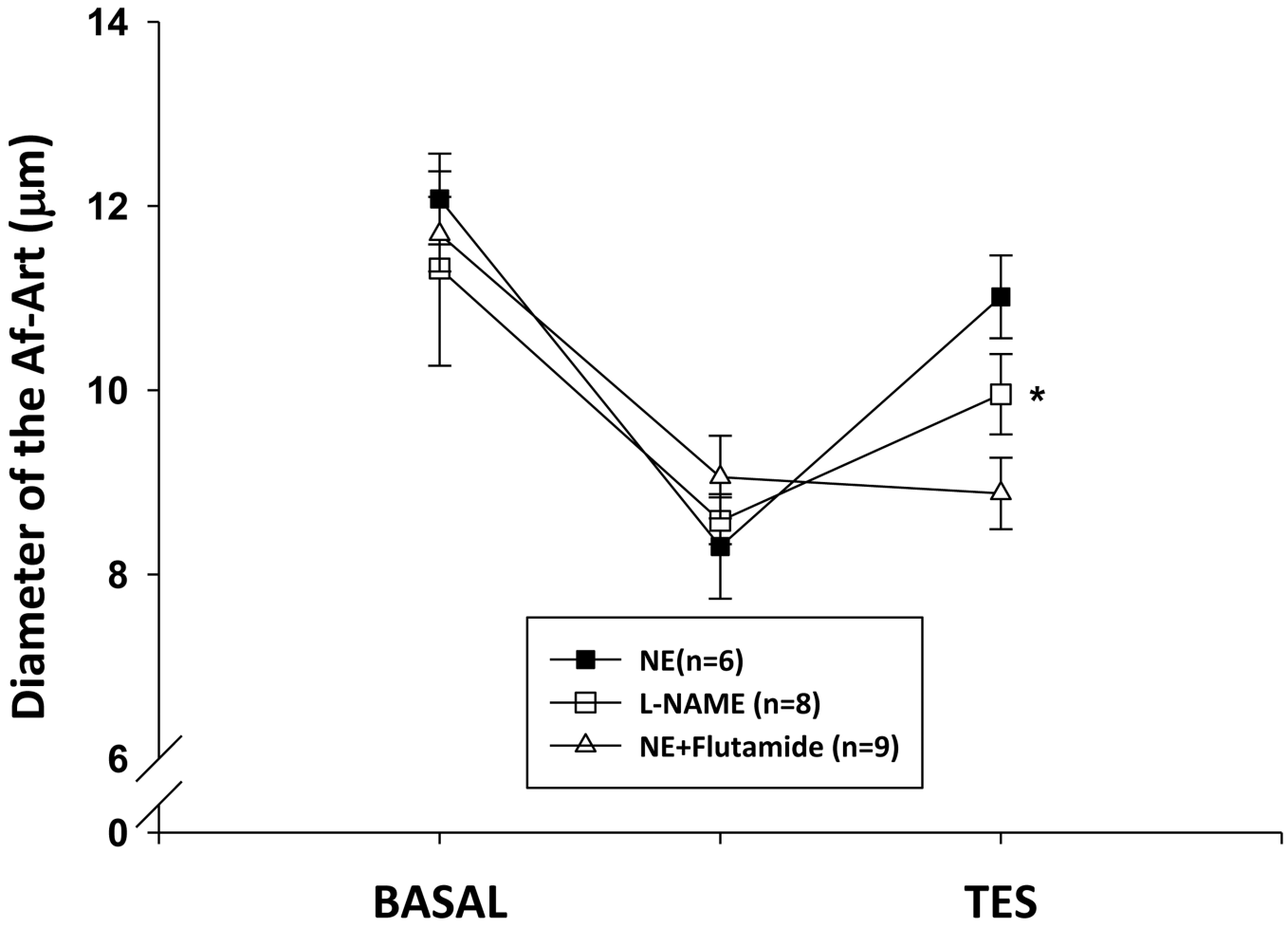


Figure 2. Androgen receptor mRNA expressed in the Af-Art

RNA samples from mouse isolated Af-Art were subjected to RT-PCR using the primers to either the AR or β-actin.

3A



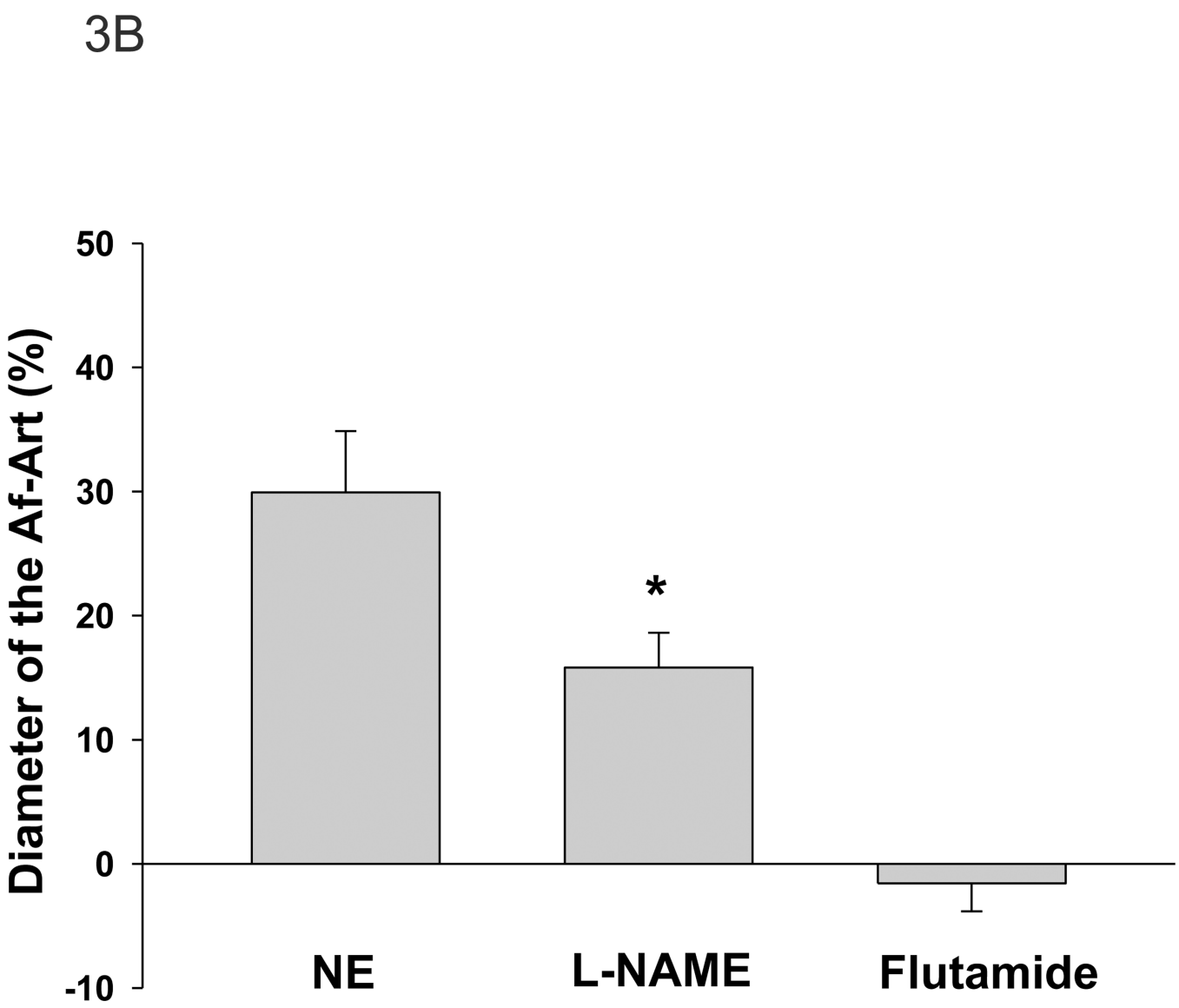


Figure 3. Inhibition of androgen receptors or NO reduces testosterone-induced vasorelaxation

A. After the Af-Art were treated with the androgen receptor antagonist, flutamide (10^{-5}mol/L), testosterone (TES)-induced relaxation of the Af-Art was blocked (n=9). After L-NAME (3×10^{-4}mol/L) was added in the bath, the diameter of Af-Art was decreased from 11.3±1.1 μm to 8.6±0.3 μm. Then testosterone (TES) was added into bath in the presence of L-NAME, the diameter of Af-Art was dilated to 10.0±0.4μm (*$p<0.05$, v.s. L-NAME; n=8). B. The dilatory effect expressed in percentage. Compared with norepinephrine (NE), in the presence of L-NAME, TES's dilatory effect was significantly blunted ($p<0.05$ v.s. TES).

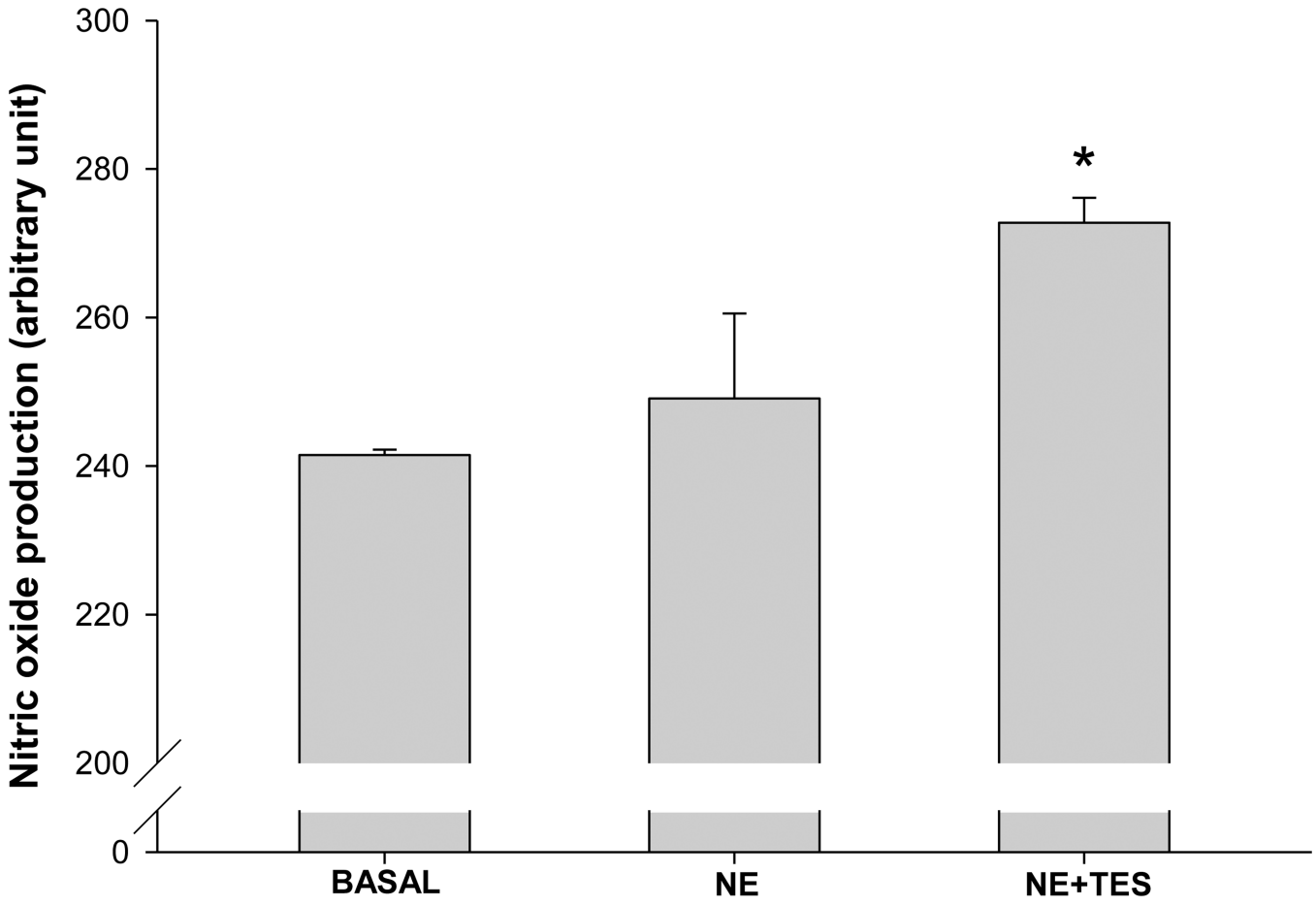


Figure 4. Testosterone enhances NO production in the Af-Art measured with DAF-FM

Comparison of the fluorescent intensity of DAF-FM in isolated perfused Af-Art in basal, norepinephrine (NE) and NE with testosterone (TES). The NE (10^{-6}mol/L) had no significant effect on the NO production. TES (10^{-8}mol/L) increased the NO production significantly (*$p<0.05$ v.s.NE; n=3).