Testosterone modulates platelet aggregation and endothelial cell growth through nitric oxide pathway

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Abstract

The aim of the present study was to investigate the effect of testosterone on the modulation of cellular events associated with vascular homeostasis. In rat aortic strips, 5–20 min treatment with physiological concentrations of testosterone significantly increased nitric oxide (NO) production. The rapid action of the steroid was suppressed by the presence of an androgen receptor antagonist (flutamide). We obtained evidence that the enhancement in NO synthesis was dependent on the influx of calcium from extracellular medium, because in the presence of a calcium channel blocker (verapamil) the effect of testosterone was reduced. Using endothelial cell (EC) cultures, we demonstrated that androgen directly acts at the endothelial level. Chelerythrine or PD98059 compound completely suppressed the increase in NO production, suggesting that the mechanism of action of

the steroid involves protein kinase C and mitogen-activated protein kinase pathways. It is known that endothelial NO released into the vascular lumen serves as an inhibitor of platelet activation and aggregation. We showed that testosterone inhibited platelet aggregation and this effect was dependent on endothelial NO synthesis. Indeed, the enhancement of NO production elicited by androgen was associated with EC growth. The steroid significantly increased DNA synthesis after 24 h of treatment, and this mitogenic action was blunted in the presence of NO synthase inhibitor *N*-nitro-*L*-arginine methyl ester. In summary, testosterone modulates vascular EC growth and platelet aggregation through its direct action on endothelial NO production.

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Introduction

The clinical observation that premenopausal women have lower incidences of cardiovascular disease (CVD) than age-matched men and postmenopausal women suggests that ovarian hormones have a protective effect on the cardiovascular system (Farhat *et al.* 1996). A great deal of evidence from observational and experimental studies, in both animals and humans, supports the concept of a protective effect of estrogens on the cardiovascular system (Koledova & Khalil 2007). Thus, it was wholly unexpected when the Women's Health Initiative, a large-scale trial of hormone replacement therapy (HRT) in postmenopausal women, was terminated early due to a surprising increase in the incidence of cardiovascular events (Hsia *et al.* 2006). The risk/benefit of HRT for prevention of CVD is still controversial (Ouyang *et al.* 2006).

The menopausal transition involves a progressive decline in ovarian steroidogenesis capacity. Beyond menopause, although the plasmatic levels of estradiol (E₂) have a marked

drop, the ovarian synthesis of androgens and progestins is also affected (Qiao *et al.* 2008). This is why during menopause, the contribution of sex steroids in the regulation of vascular homeostasis is altered. Based on this, it is proposed that the higher incidence of CVD observed in postmenopausal women could be due not only to alterations in cellular and molecular actions of E₂, but also suitable to changes in the contribution of androgens to vascular behavior regulation. In females, the vascular effects of E₂ have been extensively investigated and characterized (Miller & Duckles 2008), whereas androgens have received less attention. Therefore, the improvement in the knowledge of testosterone vascular action would be useful to for a better understanding of postmenopausal vascular pathology.

Vascular homeostasis mainly depends on the tight control of endothelial cell (EC) and smooth muscle cell functions and the regulation of the interactions between blood cells (platelets and monocytes) and vascular cells (Villar *et al.* 2008). Vascular endothelium plays a critical role in the maintenance of antiatherogenic and antithrombotic

properties of blood vessels. Besides acting as a selective diffusion barrier, endothelium allows the integration of hormonal and nutritional signals present in blood, their transduction toward the underlying smooth muscle layer, and the regulation of vascular tone. This is achieved by the secretion of biochemical factors such as nitric oxide (NO), prostacyclin, and endothelin-1. In addition, endothelium modulates platelet aggregation, leukocyte adhesion and transendothelialization, and muscle cell migration (Moncada & Higgs 2006, Miller & Mulvagh 2007). Under vascular injury, this control function can be lost, a fact that conducs to endothelium dysfunction and development of atherosclerosis, vascular diseases, and aging (Cooke & Dzau 1997). EC proliferation is particularly important in vascular repair following injury. When the endothelial layer is damaged, vessel repair is achieved by migration and proliferation of adjacent ECs (Moreno *et al.* 2009).

NO is the main regulator of vascular homeostasis and protects the vasculature against thrombosis and atherosclerosis. Endothelial NO promotes blood vessel relaxation, prevents platelet aggregation and blood cell adhesion to endothelial surface, and decreases the expression of proinflammatory genes (Ignarro 1989, Harrison *et al.* 2006). NO is synthesised by the enzyme NO synthase (NOS), through the conversion of L-arginine to L-citrulline (Förstermann 2010). In vascular tissue, distinct isoforms of NOS have been identified: constitutive NOS (cNOS), inducible NOS (iNOS; Knowles & Moncada 1994), and neuronal NOS (nNOS; White *et al.* 2005). cNOS is expressed in ECs (eNOS; Liu *et al.* 1997) and its activation is calcium and calmodulin dependent (Förstermann 2010), whereas iNOS is located in vascular smooth muscle cells (VSMCs) and its expression is regulated by cytokines and endotoxins (Kanno *et al.* 1994). Type 1 nNOS has been recently identified as a predominant isoform expressed in porcine and human coronary artery smooth muscle (White *et al.* 2005), and it has been proposed that E₂ relaxes coronary artery not only by targeting eNOS but also by increasing NO production in smooth muscle cells via nNOS activation (Han *et al.* 2007).

The androgen receptor (AR) has been identified in vascular cells (Liu *et al.* 2003), and recent reports show that this receptor mediates a variety of actions of androgens in ECs and VSMCs (Li & Al-Azzawi 2009).

Several studies have established that androgens induce vasorelaxation. Physiological to pharmacological concentrations (100 pM–10 µM) of testosterone promote vasodilatation with dual mechanism. At lower concentrations the effect is endothelium dependent, whereas the vasorelaxation induced by pharmacological concentrations (>100 µM) appears to be endothelium independent (Perusquia & Stallone 2010). Indeed, it has been reported that in ECs from human umbilical vein testosterone regulates cellular growth and apoptosis (Ling *et al.* 2002) and attenuates the tumor necrosis factor α (TNF α)-induced expression of the atherogenic vascular cell adhesion molecule-1 (Mukherjee *et al.* 2002).

On the other hand, it has been reported that dehydroepiandrosteredione (DHEA) stimulates EC proliferation and angiogenesis through a rapid mechanism mediated by pertussis toxin (PTX)-sensitive G proteins and ERK1/2 pathways (Liu *et al.* 2008). Moreover, other authors demonstrated that DHEA triggers NO synthesis due to an enhanced expression of eNOS and additionally through a nongenomic stimulation of enzyme activity (Simoncini *et al.* 2003).

In this study, we investigated the effect of testosterone on the modulation of cellular events associated with vascular homeostasis such as vasoactive production, cell growth, and platelet aggregation. Moreover, the molecular mechanism of action of the steroid was also evaluated.

Materials and Methods

Materials

[³H]-Thymidine was purchased from New England Nuclear (Chicago, IL, USA). Griess reagents were purchased from Britania Laboratories (Buenos Aires, Argentina). Trypsin/EDTA (10×), L-glutamine, amphotericin B, penicillin/streptomycin, and fetal bovine serum (FBS) were obtained from PAA Laboratories (Pasching, Austria). PD98059, chelerythrine, testosterone, N-nitro-L-arginine methyl ester (L-NAME), flutamide, DMEM, and all other reagents were purchased from Sigma Chemical Co.

Animals

Sexually mature female Wistar rats (4–7 months old) that had progressed through at least three consecutive estrous cycles were fed with standard rat food, given water *ad libitum*, and maintained on a 12 h light:12 h darkness cycle. The estrous cycle activity was evaluated by optical microscopic examination of vaginal smears, and the animals chosen for the experimental design were those with similar cycle length and the same number of days spent at each stage of the cycle. All animal work was performed at the Unit of Animal Care belonging to the Biology, Biochemistry, and Pharmacy Department of the University. The Animal Care Use Committee approved the protocol used.

Rat aortic strips preparation

Rat aortic strips (RAS) were obtained as previously described (Selles *et al.* 2005). Briefly, animals were killed by cervical dislocation and the thoracic aorta was immediately removed and placed in cold Dulbecco's PBS, cleaned of adherent connective tissue, and cut into 1.5-mm strips. Special care was taken to avoid contact with luminal surface in order to preserve the endothelial integrity. The strips were placed in the incubation medium: 145 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 1 mM CaCl₂, 10 mM glucose, 10 mM HEPES, pH 7.35, and pre-equilibrated for 10 min at 37 °C in

a shaking thermostated water bath. *In vitro* treatments were performed by incubation of RAS with the hormone for short time intervals. Control groups received vehicle alone (isopropanol <0.1%, v/v). When antagonist compounds (flutamide, verapamil) were employed, they were added to the incubation medium 30 min before the treatment.

EC cultures

EC cultures were obtained from aortic ring explants isolated from young Wistar female rats (3–5 weeks old) as previously described (Cutini *et al.* 2009). Briefly, the full length thoracic aorta was aseptically removed and then cut into ring segments (1 mm). Ring explants were seeded in 60-mm matrix-coated Petri dishes (NUNC, Rochester, NY, USA) containing phenol red-free DMEM supplemented with 10% (v/v) FBS, 3.7 mg sodium bicarbonate, 60 µg/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B, and 2 mM L-glutamine and were incubated at 37 °C in 5% CO₂ atmosphere. After 5 days of culture, ring explants were removed and the remaining cells were allowed to reach confluence. The identity of the ECs was determined by a) phase contrast microscopic observation of the characteristic morphology of cobblestone shape growth in confluent monolayer, b) the positive immunocytochemistry reactivity to factor VIII and to anti-Vimentin, clone V9 using DakoCytomation EnVision system, and c) the bioability to synthesize NO (Bachetti & Morbidelli 2000). Cells from passages 2–7 were used for all experiments.

Measurement of NO production

RAS were treated as indicated previously and nitrites (NO₂⁻) were measured in the incubation media as a stable and nonvolatile breakdown product of the NO released, employing the spectrometric Griess reaction (Cutini *et al.* 2009). Briefly, aliquots of incubation medium were mixed with Griess reagent (1% sulfanilamide and 0.1% naphthalene-diamine dihydrochloride in 2.5% (v/v) phosphoric acid) and were incubated for 10 min at room temperature. When ECs were used, cells were seeded on 24-multiwell culture plates (NUNC) at a density of 3.5×10^4 cells/well and allowed to grow to 90% of confluence in DMEM containing 10% (v/v) FBS. When compounds chelerythrine, PD98059 and cycloheximide were used, they were added 1 h before the hormonal treatment. After respective hormonal treatment in fresh DMEM containing 1% (v/v) FBS, aliquots of culture medium supernatant were mixed with Griess reagent and incubated for 10 min at room temperature. Absorbance was measured at 548 nm in a Hitachi U-1000 spectrophotometer. The concentration of nitrite in the samples was determined with reference to a sodium nitrite standard curve performed in the same matrix. The aortic strips and cells were then dissolved in 1 M NaOH and aliquots were taken for protein determination by Lowry's method (Lowry *et al.* 1951). Results were expressed as nanomolar of NO per milligram of protein.

[³H]-Thymidine incorporation assay

ECs were seeded on 24-multiwell culture plates (NUNC) at a density of 3×10^4 cells/well in DMEM supplemented with 10% (v/v) FBS and allowed to grow to 60–70% of confluence. The cells were made quiescent by placing in serum-free DMEM for 24 h and further exposed to different concentrations of testosterone or vehicle control (isopropanol <0.1%, v/v), for 24 h in fresh DMEM containing 1% (v/v) FBS. The cells were pulsed with 1 µCi/ml of [³H]-thymidine during the last 2 h of treatment. Cells were rinsed twice with PBS to remove the unincorporated [³H]-thymidine. Ice-cold trichloroacetic acid (10%, v/v) was added and the acid-insoluble material was dissolved with 1 M NaOH. Radioactivity was measured by liquid scintillation using a Wallac1414 counter. The protein concentrations were determined by Lowry's method and the results were expressed as counts per minute per milligram of protein (Cutini *et al.* 2009).

Platelet aggregation assay

Platelet aggregation was measured using a turbidimetric assay as previously described (Polini *et al.* 2007). ECs were seeded on 24-multiwell culture plates (NUNC) at a density of 3×10^4 cells/well in DMEM supplemented with 10% (v/v) FBS and allowed to grow to 60–70% of confluence. Culture medium was replaced by 400 µl of platelet-rich plasma (PRP, 3×10^8 platelets/ml) and exposed to testosterone for 1–10 min. Immediately after the treatment, 285 µl of PRP was taken and set in a CronoLog 430 aggregometer cubette with continuous stirring. Aggregation was initiated by the addition of 2×10^{-5} M ADP. Control group containing ECs in PRP was treated with vehicle alone (isopropanol <0.1%, v/v); 60 units/ml of super oxide dismutase was added to the incubation medium before the hormonal treatment in order to prevent NO degradation (Gryglewski *et al.* 1986). When L-NAME compound was used, it was added to the incubation medium 30 min prior to hormonal treatment. Changes in light transmission were recorded for 5 min after ADP addition. The signal generated in platelet-poor plasma was taken as 100% transparent control (Kikuta *et al.* 1998). ECs were dissolved in 1 M NaOH and aliquots were taken for protein determination by Lowry's method. Results were expressed as percent inhibition of platelet aggregation per milligram of protein. The maximal platelet aggregation was considered to be that induced by the control samples. Two or three animals were used for each experiment.

EC migration assay

ECs were seeded at a density of 5×10^5 cells/cm² in 60-mm NUNC dishes with DMEM containing 10% FBS and grown to 90% of confluence. Cells were starved for 24 h in serum-free medium. In order to evaluate EC migration a wound was made by pressing a razor blade down on the dish to cut the cell

layer. The blade was then gently moved to one side to remove part of the monolayer. Immediately, the detached cells were washed twice with PBS and cultured in fresh DMEM containing 1% FBS plus testosterone or vehicle control. After 24 h of culture, cells were fixed in 4% paraformaldehyde and stained with Giemsa. Migration was quantified by counting the number of cell nuclei that migrated at the scratched area in at least seven different microscopic fields representative of each culture plate. EC migration was recorded using an OLYMPUS C7070WZ optical microscope system. Results were expressed as means $\pm$ S.D. of number of migrated cells/field (Sandoval *et al.* 2010).

Statistical analysis

Each experimental condition has been reproduced in at least three independent experiments performed by quadruplicate. All data are presented as mean $\pm$ S.D. Comparisons between two means were made using Student's *t*-test and multiple comparisons with one-way or two-way ANOVA, followed by Fisher least significant difference test. Differences at $P < 0.05$ were considered significant.

Results

Using RAS, we studied the effect of testosterone on NO production. Figure 1 shows the time course of NO synthesis induced by treatment with 1 nM testosterone.

Figure 1 Time course profile of the effect of testosterone on NO production in RAS. RAS were exposed to 1 nM testosterone at the time indicated. Controls to each time point were also performed. NO production was measured as described in Materials and Methods section. Results are the average $\pm$ S.D. of three independent experiments performed by quadruplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ with respect to each control, ^a $P < 0.05$ (3 vs 5 min testosterone treatment), and ^b $P < 0.05$ (15 vs 20 min testosterone treatment).

Figure 2 Effect of flutamide on NO synthesis induced by testosterone. RAS were preincubated in the absence or presence of 10 nM flutamide for 30 min and then exposed to 1 nM testosterone for 5 min. NO production was measured as described in Materials and Methods section. Results are the average $\pm$ S.D. of three independent experiments performed by quadruplicate. *** $P < 0.001$ with respect to control.

NO production was markedly enhanced after 5 min of exposure to the hormone (79% above control, $P < 0.01$). This incremental increase was sustained for 15 min and returned to baseline at 20 min of hormonal treatment.

To test whether the enhancement of NO production induced by testosterone involved the participation of the AR, RAS were preincubated with the AR antagonist flutamide for 30 min and then exposed to 1 nM testosterone for an additional 5 min. As can be observed in Fig. 2, the presence of the antagonist (10 nM) completely suppressed the testosterone-induced stimulation in NO synthesis. Similar results were obtained with 100 nM flutamide (data not shown).

Considering that the activity of some members of the NOS family is regulated by calcium, we studied the role of extracellular calcium in the stimulation of NO production elicited by testosterone treatment. For this purpose, RAS were treated with the hormone in a calcium-free medium (plus 1 mM EGTA). Figure 3 shows that the increase in NO production induced by 1 nM testosterone was reduced in the absence of calcium in the incubation medium (80 vs 26% above control, testosterone vs testosterone + EGTA). Furthermore, the role of extracellular calcium was evaluated using a calcium channel blocker. When the influx of calcium was blocked by the presence of 10^{-4} M verapamil, similar results were obtained (80 vs 28% above control, testosterone vs testosterone + verapamil). These data suggest that the stimulatory action of testosterone on NOS activity is dependent in part on the influx of calcium from the extracellular medium.

RAS represent an experimental system that contains ECs, VSMCs and fibroblasts. To study whether androgen exerts a

Figure 3 Study of the effect of extracellular calcium on testosterone-induced NO synthesis. (A and B) RAS were placed in the incubation medium containing 1 mM CaCl_2 in the presence or absence of 100 μM verapamil and then exposed to 1 nM testosterone for 5 min. (C) RAS were incubated in a calcium-free medium plus 1 mM EGTA and treated with 1 nM testosterone or vehicle for 5 min. NO production was measured as described in Materials and Methods section. Results are the average $\pm$ s.d. of three independent experiments ($n=5$). *** $P<0.001$ with respect to control and $^\dagger P<0.05$ with respect to testosterone.

direct action at the endothelial level, we performed EC cultures and measured the effect of the steroid on endothelial NO production. These data are presented in Table 1. As can be observed, the hormone induced a very fast stimulation of endothelial NO synthesis. The time course profile, elicited by testosterone in isolated EC, does not exhibit the drop in NO production observed in RAS after 20 min treatment. The enhancement in the vasoactive production was sustained along the whole time interval. Figure 4 shows that this stimulatory action was exhibited at physiological concentrations (0.1–1 nM).

Having found that testosterone stimulates NO synthesis via its direct action on ECs, we continued with the study of the mechanism of steroid action. Owing to the fact that the hormonal effect on NO synthesis occurred in a very short time of treatment, we evaluated whether the steroid produces its biochemical action in a nongenomic manner, independent of gene transcription. To that end, we measured NO

production in ECs in the presence of cycloheximide, an inhibitor of protein synthesis. Figure 5 shows that the increase in NO production induced by testosterone was not affected by the presence of cycloheximide, suggesting the non-genomic feature of this effect. Bearing in mind that eNOS is regulated by phosphorylation, we evaluated whether the protein kinase C (PKC) system would modulate the hormonal action of testosterone on NO production. For this purpose, we chose chelerythrine as the PKC inhibitor. Preincubation of ECs with 0.5 μM chelerythrine completely suppressed the enhancement in NO synthesis induced by 5 min of treatment with 1 nM testosterone (Fig. 5). Similar results were obtained using 0.2 or 1 μM chelerythrine. We also tested the effect of the mitogen-activated protein kinase (MAPK) signaling cascade on the hormonal regulation of the vasoactive synthesis. As can be seen in Fig. 5, the hormonal stimulation of NO production was blunted by the presence of the MEK inhibitor, PD98059 (1 μM).

It is known that when endothelial NO is released into the vascular lumen it serves as a modulator of platelet activation, inducing an inhibition of platelet aggregation. We tested the effect of androgen on vascular-dependent platelet aggregation. ECs were incubated with PRP and subsequently exposed to testosterone treatment for 1–10 min. Immediately, aliquots of PRP were transferred to the aggregometer and ADP-induced platelet aggregation was registered. Figure 6A shows a representative profile of the aggregation process corresponding to basal (PRP without ECs), control (ECs in PRP with vehicle alone), and testosterone (ECs plus PRP plus testosterone). Maximal aggregation was determined under basal conditions, and the aggregation process decreased progressively in control and hormonal groups. These data showed that the steroid exhibits an antiaggregatory effect compared to control group. Table 2 shows the quantitative data of the dose–response study (0.01–10 nM). As can be observed, androgen exhibited a significant antiaggregatory action at all doses tested. Table 2 also shows that a direct effect of testosterone on platelet aggregation was ruled out, since the addition of testosterone to PRP in the absence of ECs induced maximal aggregation similar to the basal group. Afterward, we tested whether NO mediates the antiaggregatory effect of the steroid. To that end, ECs were preincubated with L-NAME, a NOS-specific inhibitor, followed by the addition of PRP and steroid treatment. The inhibition of platelet aggregation induced by the hormone was partially

Table 1 Rapid action of testosterone on endothelial nitric oxide production

Treatment	NO production (nmol/mg protein) ^a				
	3 min	5 min	10 min	15 min	20 min
Control	5.17 $\pm$ 0.97	4.25 $\pm$ 1.10	4.50 $\pm$ 0.83	5.05 $\pm$ 0.91	4.97 $\pm$ 0.99
1 nM testosterone	8.37 $\pm$ 0.88 [†]	6.90 $\pm$ 0.60 [†]	7.38 $\pm$ 0.70 [†]	7.92 $\pm$ 1.20*	7.25 $\pm$ 1.11*

* $P<0.05$ with respect to each control; [†] $P<0.01$ with respect to each control.

^aEC were incubated with 1 nM testosterone at the indicated times. NO production was measured as described. Results represent the average $\pm$ s.d. of three independent experiments ($n=5$).

Figure 4 Stimulation of endothelial NO production by testosterone: dose-response profile. ECs cultured in the presence of FBS 1% (v/v) were incubated with testosterone at the indicated concentrations for 5 min. NO production was measured as described in Materials and Methods section. Results are the average $\pm$ s.d. of four independent experiments ($n=4$). *** $P<0.001$ with respect to control.

suppressed in the presence of NOS inhibitor, suggesting that the antiaggregatory action of androgen is dependent on the release of NO from ECs (Fig. 6B).

It has been proposed that NO modulates vascular cell growth (Villalobo 2006). Using [3 H]-thymidine incorporation assay, we studied the effect of testosterone on EC proliferation. Synchronized cultures were exposed to 1 nM testosterone for different times of treatment. The steroid significantly increased DNA synthesis after 24 h of treatment (44% above control, $P<0.05$); in contrast, no statistical differences among control and treated groups were observed after 12, 36, or 48 h of treatment (Fig. 7). The mitogenic action of testosterone was blunted by the presence of the NOS inhibitor L-NAME, suggesting that the action of testosterone depends on NO generation (Fig. 8).

Finally, in order to assess the role of testosterone on cell migration, wounding assays were performed. Figure 9 shows a microphotography representative of these assays. We found that 10 nM testosterone enhances cell migration compared to nontreated cells. As can be observed, the number of cells/field in the denuded area in the monolayers exposed to 24 h treatment with the steroid was higher with respect to control group.

Discussion

The results presented in this work provide evidence linking testosterone's effect on endothelial NO production with the hormonal modulation of platelet aggregation and EC growth. Physiological concentrations of testosterone stimulate EC growth and inhibit platelet aggregation, through its direct

action on endothelial NO production. The molecular mechanism of action elicited by testosterone involves a nongenomic stimulation of NO synthesis, which depends on calcium influx from the extracellular medium and on MAPK and PKC pathways.

Endothelial NO bioavailability can be modified by a plethora of hormones and factors (Duckles & Miller 2010). eNOS activity is regulated by changes in intracellular calcium and phosphorylation. After agonist stimulation, the enhancement in cytosolic calcium levels leads to calmodulin binding to eNOS and subsequent phosphorylation. The enzymes responsible for phosphorylation depend on the primary stimulus. Several intracellular transduction cascades, such as PI3K/AKT, AC/PKA, CaMKII, PLC/PKC, and MAPK, have been involved in the hormonal regulation of eNOS activity (Förstermann 2010).

In this work we found that in rat aortic tissue, NO production is suitable to be regulated by testosterone. Physiological concentrations of testosterone stimulate NO production, with marked increases after a few minutes of treatment falling to control values at 20 min. This temporal profile would be the result of the participation of diverse signal transduction pathways that conduct to activation or inhibition of NOS. Nevertheless, when isolated ECs were used, the stimulatory action of the steroid was sustained, with a slight decrease in NO production at 20 min. In view of this fact, we suppose the difference in the temporal profile exhibited by testosterone in RAS and ECs would be due the presence of smooth muscle cells in intact aortic tissue. Moreover, the results obtained using isolated ECs support the

Figure 5 Characterization of the rapid action of testosterone on endothelial NO production. ECs cultured in the presence of FBS 1% (v/v) were preincubated with 100 μ M cycloheximide, 1 μ M chelerythrine, or 1 μ M PD98059 for 1 h and then treated with 1 nM testosterone for 5 min. NO production was measured as described in Materials and Methods section. Results are the average $\pm$ s.d. of three independent experiments ($n=5$). *** $P<0.001$ with respect to each control.

Figure 6 Effect of testosterone on ADP-induced platelet aggregation. (A) Representative profile of aggregation process: ECs were incubated for 1 min in PRP with the addition of testosterone (1–10 nM) or vehicle alone (control). PRP was quickly removed and platelet aggregation was initiated by the addition of ADP (arrow). (B) ECs were preincubated with or without L-NAME and then exposed to testosterone. Results are expressed as percentage of inhibition of platelet aggregation with respect to control group and represents the mean $\pm$ s.d. of three independent experiments ($n=5$). *** $P<0.001$ with respect to control, $^{\dagger}P<0.05$ with respect to testosterone.

hypothesis of a direct action of androgen at the endothelial level. Evidence reported in the literature shows that testosterone induces vasorelaxation from physiological to pharmacological concentrations. The vasorelaxation induced by low concentrations of androgen appears to be dependent on endothelial NO synthesis (Rowell *et al.* 2009), whereas higher concentrations are associated with a direct action on

vascular muscle cells. Our results provide evidence of a direct action of a low concentration of testosterone on endothelial NO production. When the molecular basis of this effect was studied, we found that the stimulatory action of androgen on NO generation was dependent, at least in part, on the influx of calcium from the extracellular medium. Furthermore, we obtained evidence of the participation of AR in the rapid action of the steroid in NO production. When AR was antagonized by the presence of flutamide, the effect of androgen was blunted. These findings agree with those reported by other authors that involve the participation of AR in the nongenomic action of testosterone on eNOS activation in ECs of umbilical vein (Goglia *et al.* 2010). However, several reports show that the vasorelaxation action of testosterone in male arteries is AR independent (Tep-areenan *et al.* 2002). In fact, some of these studies have used supraphysiological concentrations of testosterone. In male coronary and pulmonary arteries, 1–1000 μ M testosterone induced vasodilatation independent of AR and NO participation (Jones *et al.* 2002, 2004). On the other hand, and in agreement with our observations, Yu *et al.* (2010) recently showed that in male human aortic ECs, physiological concentrations of testosterone (1–100 nM) rapidly increase NO production via AR activation of eNOS. Perhaps, the discrepancies between the past and the present findings would be attributed to gender differences, cellular selectivity of androgen action, and/or steroid concentrations.

Following with the study of the mechanism of action of testosterone, using cycloheximide as a protein synthesis inhibitor, we confirmed the nongenomic feature of steroid action. The rapid stimulation of NO production was not affected by the presence of the inhibitor. Nongenomic action of steroid hormones has been extensively associated with hormonal activation of signal transduction pathways (Falkenstein *et al.* 2000). Therefore, we evaluated the participation of signaling cascades in the effect of testosterone on endothelial NO generation. The results obtained using the compounds PD98059 and chelerythrine as inhibitors of MEK and PKC, respectively, suggest that MAPK and PKC might be

Table 2 Effect of testosterone on platelet aggregation

Treatment	Percentage of inhibition of platelet aggregation with respect to control ^a	Percentage of platelet aggregation with respect to basal ^b
10^{-11} M testosterone	$21.2 \pm 5.3^*$	98 ± 4.9
10^{-10} M testosterone	$20.5 \pm 5.0^*$	99 ± 6.9
10^{-9} M testosterone	$35.7 \pm 7.6^{\dagger}$	97 ± 7.7
10^{-8} M testosterone	$26.6 \pm 4.3^{\dagger}$	95 ± 8.9

* $P<0.05$ and $^{\dagger}P<0.01$ with respect to control.

^aECs were incubated for 1 min in PRP with testosterone. PRP was quickly removed and platelet aggregation was measured as described in Material and Methods section. Results are expressed as percentage of inhibition of platelet aggregation with respect to control (EC plus vehicle) and represent the means of three independent experiments ($n=3$).

^bAliquots of PRP (without ECs) were incubated with the indicated concentrations of testosterone. Basal group received vehicle alone. Immediately after ADP was added, platelet aggregation was measured. Results are expressed as percentage with respect to basal aggregation and represent the average $\pm$ s.d. of three independent experiments.

Figure 7 Effect of testosterone on [³H]-thymidine incorporation. Subconfluent ECs were incubated in serum-free medium for 24 h and then treated with 1 nM testosterone at the time indicated; 1 μ Ci/ml of [³H]-thymidine was added during the last 2 h of treatment. [³H]-thymidine incorporation was measured as described in Materials and Methods section. Results are the average $\pm$ S.D. of three independent experiments performed by quadruplicate. *** P < 0.001 with respect to each control, ^a P < 0.05 vs all control groups, ^b P < 0.05 vs all testosterone groups.

involved in the hormonal effect. The participation of MAPK and PKC pathways in the nongenomic action of sex steroids on NOS activity has been reported (Rauschemberger *et al.* 2008, Cutini *et al.* 2009). It has been reported that PKC mediates testosterone and dihydrotestosterone relaxation in smooth muscle gallbladder (Kline & Karpinski 2008). Indeed, it has also been described that DHEA directly regulates human eNOS activity through a non-transcriptional mechanism that depends on ERK/MAPK pathway (Simoncini *et al.* 2003). Taking in account that Raf kinase, the upstream activator of MEK kinase, might be phosphorylated by PKC, we can hypothesize that the rapid action of testosterone on NO production requires the subsequent activation of PKC and MAPK systems. MAPK and PKC cascades have been involved in antiatherogenic and anti-inflammatory effects. Although some studies demonstrated that testosterone induces vasodilatation in an endothelium-dependent manner, several reports have shown that this nongenomic vasorelaxant action of androgen is endothelium independent (Perusquía & Stallone 2010), mainly due to enhanced NO synthesis through the activation of nNOS in vascular smooth muscle. Therefore, at present we do not know whether the rapid effect of testosterone on signal transduction pathway activation, and endothelial NO production reported here, could be associated with any functional action on vascular wall. This will be the focus of future studies. However, we showed that the stimulation of NO synthesis elicited by testosterone is linked to a systemic action, the regulation of platelet activation, since NO release from the ECs modulates platelet aggregation.

EC proliferation and growth is critical to maintain vascular homeostasis, wound healing, and development. We found

that testosterone increases rat aortic EC proliferation after 24 h of hormonal treatment and the mitogenic action declines substantially at 48 h. The temporal profile of EC proliferation elicited by androgen exhibits a similar shape to control. Since ECs were obtained by primary culture, the decline in the mitogenic action of the steroid could be associated with the possibility that after 36–48 h of culture, the cells undergo differentiation and maturation, and under a low rate of proliferation, the hormone is unable to exert its mitogenic action. In future studies the effect of testosterone on EC differentiation markers will be assessed. Outside this, our findings are consistent with those reported by other authors who showed that testosterone increases EC growth in bovine aortic and in vein ECs (Williams *et al.* 2004). Indeed the adrenal androgen DHEA also promotes EC proliferation (Liu *et al.* 2008). NO has been proposed to be a physiological modulator of cell proliferation in several cells of different tissue origin. Although inhibition of cell growth appears to be the major effect exerted by NO in many cell types, mitogenic action of NO has also been described (Villalobo 2006). Here, we provide evidence that the proliferative effect of testosterone depends on NO production since the preincubation with the selective NOS inhibitor completely suppressed the action of androgen.

Using wound healing assays, we also demonstrated that testosterone stimulates EC migration. It is known that EC proliferation and migration contribute to the development of new capillaries, event that requires the participation of vascular endothelial growth factor (VEGF). It has been reported that testosterone stimulates VEGF and NOS expression and promotes capillary formation in the cerebral

Figure 8 Effect of L-NAME on DNA synthesis induced by testosterone. Subconfluent ECs were incubated for 24 h in serum-free media, preincubated for 1 h with or without L-NAME (1 μ M), and then treated with testosterone for additional 24 h. [³H]-thymidine incorporation was measured as described in Materials and Methods section. Results are the average $\pm$ S.D. of three independent experiments (n = 4). *** P < 0.001 with respect to control.

Figure 9 Effect of testosterone on EC migration. Confluent EC cultures were serum starved for 24 h and cells were removed by scraping. Detached cells were washed with PBS and the remaining monolayer was treated for 24 h with 10 nM testosterone or vehicle. Dotted lines indicate the boundary between the unscratched and the scratched areas. (A) Images of representative fields of each condition after Giemsa staining (40 $\times$). The scale line represents 350 μ m. (B) Bars show mean $\pm$ S.D. of number of migrated cells/field from three independent experiments performed by quadruplicate. * $P < 0.01$.

vascular system (Jesmin *et al.* 2004). In fact, in bovine aortic ECs, testosterone and DHEA promote EC proliferation, migration, and vascular tube formation, effects dependent on PTX-sensitive G protein and ERK1/2 activation (Williams *et al.* 2004, Liu *et al.* 2008). Future studies will be conducted in order to address the possibility that the mitogenic action of testosterone could be associated with a putative androgen promotion of angiogenesis.

Immediately after synthesis, endothelial NO triggers two different effects: it translocates to smooth muscle tunica and induces GMPc-dependent vasorelaxation and may be released into the lumen and inhibited platelet aggregation (Ignarro *et al.* 1999, Förstermann 2010). We observed that the treatment of ECs with different concentrations of testosterone significantly inhibits platelet aggregation over control (ECs without hormonal treatment). The ability of the steroid to inhibited platelet aggregation depends on the presence of the endothelium. When PRP was incubated in the absence of ECs, there was no direct effect of testosterone on ADP-induced platelet aggregation. We showed that the antiplatelet activity of testosterone was related in part to the stimulation of NO production. In ECs that are unable to synthesize NO due to the presence of L-NAME, the antiaggregatory

activity of the hormone was reduced. Altogether these data suggest that at the endothelial level, testosterone acutely increases NO liberation and consequently contributes to inhibit platelet aggregation.

In summary, the data presented contribute to the understanding of the transduction mechanism of testosterone on vascular tissue. The hormonal action not only implies the modulation of the synthesis of vasodilators, but also involves the vascular regulation of platelet aggregation process, both events are tightly associated with the prevention of atherosclerotic degeneration. The evidence provided support the hypothesis that EC behavior could be modulated not only by E₂ but also by other sex steroids. Since our results were obtained mainly using *in vitro* assays with isolated cells, the whole contribution of testosterone to vascular homeostasis cannot be addressed. Vascular health depends on the integration of cellular responses from blood cells (leukocytes and platelets), ECs, and muscle cells, so that at present it is difficult to draw a parallel between our results and the *in vivo* situation. Moreover, cross-talk between the biochemical action of testosterone and of other vascular agonists may also occur. The role of testosterone in female vascular beds and its physiological relevance will be focused in future investigations.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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