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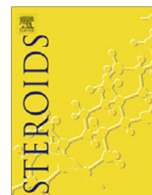

Cellular actions of testosterone in vascular cells: Mechanism independent of aromatization to estradiol

Adrián E. Campelo [a,b], Pablo H. Cutini [a,b], Virginia L. Massheimer [a,b,*]

[a] Cátedra de Bioquímica Clínica II, Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, San Juan 670, B8000ICN Bahía Blanca, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

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ABSTRACT

In this work we investigated the role of testosterone on cellular processes involved in vascular disease, and whether these effects depend on its local conversion to estradiol. Cultures of rat aortic endothelial and smooth muscle cells *in vitro* treated with physiological concentrations of testosterone were employed. Testosterone rapidly increased endothelial nitric oxide production. To evaluate whether this non genomic action was dependent on testosterone aromatization we used an aromatase inhibitor. Anastrozole compound did not modify the fast increase in nitric oxide production elicited by testosterone. The hormonal effect was completely blocked by an androgen receptor antagonist (flutamide); meanwhile it wasńt modified by the presence of an estrogen receptor antagonist (ICI182780).The possibility of intracellular estradiol synthesis was ruled out when no differences were found in estradiol measurements performed in culture incubation medium from control and testosterone treated cells. The 5α-reductase inhibitor finasteride partially suppressed the enhancement in nitric oxide production, suggesting that the effect of testosterone was partially due to dihydrotestosterone conversion. Testosterone stimulated muscle cell proliferation independent of local conversion to estradiol. When cellular events that play key roles in vascular disease development were analyzed, testosterone prevented monocyte adhesion to endothelial cells induced by a proinflammatory stimulus (bacterial lipopolysaccharides), and prompted muscle cell migration in presence of a cell motility inducer. In summary, testosterone modulates vascular behavior through its direct action on vascular cells independent of aromatization to estradiol. The cellular actions exhibited by the steroid varied whether cells were under basal or inflammatory conditions.

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1. Introduction

The incidence of cardiovascular and vascular disease is greater in men compared with age matched premenopausal women. However, during menopause this incidence increases dramatically. There is a long-standing hypothesis that estrogen levels might contribute to provide vascular protection. However, the results of clinical trials raise an important controversy about the risk/benefit of hormone replacement therapy [1]. Although the direct effects of estrogen on cardiovascular system are well recognized [2], much less is known regarding the vascular actions of testosterone. Indeed many questions arise about how testosterone affects vascular function in women as they age, and how changes in androgen status contribute to cardiovascular disease processes in postmenopausal women.

The androgen receptor (AR) has been identified in vascular cells [3], and recent reports show that this receptor mediates a variety of actions of androgens in endothelial and vascular smooth muscle cells [4]. Several studies have established that androgens induce vasorelaxation. Physiological to pharmacological concentrations (100 pM–10 μM) of testosterone promote vasodilatation, both via endothelium dependent, and endothelium independent mechanism [5]. In human umbilical vein endothelial cells (HUVEC), testosterone regulates cellular growth and apoptosis [6], and attenuates the TNF-α-induced expression of atherogenic vascular cell adhesion molecule-1 (VCAM-1) [7].

Testosterone could be metabolized to estradiol through aromatization. Since, the presence of aromatase in blood vessel, either in endothelial cells (EC) and vascular smooth muscle cells (VSMC) has been reported [7,8], it has been suggested that testosterone induced vasorelaxation might be mediated through local conversion to estrogen. However, the evidence reported in the literature is controversial. In male vasculature, it has been proposed that testosterone aromatization may have important effects on endothelial function [9]. On the other hand, several studies show that

* Corresponding author at: Cátedra de Bioquímica Clínica II, Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, San Juan 670, B8000ICN Bahía Blanca, Argentina. Tel.: +54 291 4595100; fax: +54 291 4595130.
E-mail address: massheim@uns.edu.ar (V.L. Massheimer).

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inhibition of P-450 aromatase or estrogen receptor (ER) antagonism do not prevent testosterone induced vasorelaxation [5]. Testosterone may also be reduced at the alpha position to its active metabolite dihydrotestosterone (DHT) by 5α-reductase. Two types of 5α-reductase enzyme have been identified: type I and type II. Blood vessels are capable to locally metabolize testosterone to DHT, since the presence of 5α-reductase type II has been demonstrated in EC and VSMC [10,11]. The conversion of testosterone to DHT can be prevented by steroidal and non steroidal 5α-reductase inhibitors. Finasteride is a potent steroidal inhibitor of 5α-reductase type II, which in humans reduces prostatic DHT levels by 70–90% [12].

EC and VSMC are the major components of blood vessels, and play key roles in vascular health and diseases. A major consequence of vascular dysfunction is atherosclerosis, a chronic inflammatory process characterized by formation of atheromatous plaques, arterial wall thickening, and narrowing of the arterial lumen. Atherosclerosis and related cardiovascular diseases are the leading causes of death in developed countries. Atherosclerotic lesion is the result of a complex and multifactorial process that occurs at level of the arterial intima in response to endothelial dysfunction [13,14]. The early steps of this vascular disease involve impairment of vasoactive production, leukocyte and platelet adhesion to endothelium and endothelial transmigration of monocytes [15]. The inflammatory environment of the artery wall causes VSMC dedifferentiation, decrease of contractile properties, and induction of a proliferative and often motile phenotype [16,17]. VSMC exist in the normal blood vessel wall in a quiescent and differentiated state with low rate of cell proliferation and turnover. Under vascular damage, the endothelium denudation would compromise the appropriate regulation of VSMC growth, proliferation, migration, and apoptosis [18]. Overall, regulation of EC behavior and VSMC phenotype is important both at homeostasis and in the development of vascular diseases.

We have recently demonstrated that, in rat aortic tissue, testosterone at physiological concentrations stimulates EC growth and inhibits platelet aggregation through its direct action on endothelial nitric oxide (NO) production. The molecular mechanism of action elicited by testosterone involves a non-genomic stimulation of NO synthesis, which depends on calcium influx from the extracellular medium and, on MAPK and PKC pathways [19]. The present work was undertaken to investigate whether the vascular effects of the androgen represent a direct action of testosterone or depends on its local metabolization, and to evaluate the role of testosterone on the cellular processes involved in vascular disease.

2. Experimental

2.1. Materials

[^{3}H]-Thymidine was purchased from New England Nuclear (Chicago). Griess reagents were purchased from Britania Laboratories (Buenos Aires, Argentina). Trypsin/EDTA (10×), L-glutamine, amphotericin B, penicillin/streptomycin, and fetal calf serum were obtained from PAA Laboratories (Pasching, Austria), ICI 182780 from Tocris Bioscience (Park Ellisville, USA); Ficoll Paque Plus was purschased from GE Healthcare; testosterone, anastrozole, finasteride, flutamide, Dulbecco's modified Eagle's medium (DMEM), lipopolysaccharides (LPS from *Escherichia coli* 0127) and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Animals

Female Wistar rats were housed under controlled conditions (constant room temperature, 12-h light/12-h dark cycle, bred in our own colony, and fed with a standard rat chow diet and free access to water). Animals aged 3–5 weeks old and 120 g of weight were killed by cervical dislocation. All the procedures were carried out in accordance with the guidelines published in the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*. All animal work was performed at the Unit of Animal Care belonging to the Department of Biology, Biochemistry and Pharmacy at the University. The Animal Care Use Committee of this Unit approved the protocol used.

2.3. Culture of endothelial and vascular smooth muscle cells

EC and VSMC cultures were obtained from aortic rings explants isolated from young Wistar female rats (3–5 weeks old) as previously described [20]. Briefly, the full length thoracic aorta was aseptically removed and then cut into ring segments (2 mm). Ring explants were seeded on 60-mm matrix-coated petri-dishes (NUNC) containing phenol red-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% (v/v) fetal calf serum (FCS), 1.8 mg sodium bicarbonate, 100 U/mL penicillin, 10 μg/mL streptomycin, 2.5 μg/mL amphotericin-B, and 2 mM L-glutamine. Explants were incubated at 37 °C in 5% CO_2 atmosphere. In order to establish a pure EC culture, after 3 days of culture, ring explants were removed and transferred into new culture dishes with fresh DMEM supplemented with 10% (v/v) FCS. Transfer of the same rings into new culture dishes at 5-days intervals then resulted in the progressive development of mixed cell populations (EC plus VSMC). Additional transfer of the ring explants resulted in pure cultures of VSMC. At last, the rings were discarded and EC and VSMC cultures were allowed to reach confluence. EC identification was performed by: (a) by phase-contrast microscope observation of the characteristic morphology of cobblestone shape growth in confluent monolayers, (b) by positive immunocytochemistry reactivity to Factor VIII, and to anti-Vimentin, clone V9 using DakoCytomation EnVision system, and (c) by the ability to synthesize NO. The identity of the VSMC was determined by positive immunocytochemistry reactivity to smooth muscle specific α-actin, using DakoCytomation EnVision system [21,22]. Cells from passages 2–7 were used for all experiments. All hormone solutions employed in the *in vitro* cellular treatments were prepared using isopropanol as solvent. The final concentration of the vehicle was always below than 0.1%.

2.4. Measurement of NO production

Nitric oxide production was measured by Griess reaction as previously described [23]. EC 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) FCS. When the compounds anastrozole, flutamide, ICI182780 or finasteride were used, they were added 1 h before hormonal treatment. After respective hormonal treatment in fresh DMEM containing 1% (v/v) FCS, aliquots of culture medium supernatant were mixed with Griess reagent (1% sulphanilamide and 0.1% naphthylenediamine dihydrochloride in 2.5% (v/v) phosphoric acid) and incubated 10 min at room temperature. Nitrites (NO_2^-) were measured in the incubation media as a stable and non-volatile breakdown product of the NO released. The concentration of nitrite in the samples was determined with reference to a sodium nitrite standard curve performed in the same matrix, using a microplate reader Synergy HT-Biotek. Cells were then dissolved in 1 M NaOH and aliquots were taken for protein determination. Results were expressed as nmol of NO per mg of protein.

2.5. [^{3}H]-Thymidine incorporation assay

VSMC were seeded on 24 multi-well plates (NUNC) at a density of 3×10^4 cells/well in DMEM supplemented with 10% (v/v) FCS and allowed to grow to 60–70% 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) FCS. The cells were pulsed with 1 μCi/mL of [^{3}H]-thymidine during the last 2 h of treatment. Cells were rinsed twice with phosphate-buffered saline (PBS) to remove the unincorporated [^{3}H]-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 method and the results were expressed as cpm per mg of protein [19].

2.6. Cell migration assay

VSMC were seeded at a density of 5×10^5 cells/cm^2 in 60-mm NUNC dishes with DMEM containing 10% FCS, and grown to 90% confluence. Cells were starved for 24 h with 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 after, the detached cells were washed twice with PBS, and cultured in fresh DMEM containing 1% FCS plus testosterone or vehicle control. After 24 h of culture, cells were fixed in paraformaldehyde 4%, and stained with Giemsa. Migration was quantified by counting the number of cells present in the scratched area in ten different microscopic fields representative of each culture plate. EC migration was recorded using a Nikon EclipseTS100–Nikon D3100 optical microscope system. Results are expressed as mean ± SD of the number of migrated cells/ field [20].

2.7. Monocyte adhesion assay

Peripheral blood mononuclear cells isolation was performed using density gradient (Ficoll-Paque Plus) and monocytes were isolated by adherence to plastic dishes [24]. Subsequently, these monocytes were cultured in DMEM with 10% FCS. In parallel, EC were starved for 24 h with serum free medium and then exposed to hormonal treatment in presence or absence of 1 μg/mL bacterial LPS as previously described [24]. An exact number of monocytes was seeded on pretreated EC and incubated for 2 h at 37 °C in a humidified 5% CO_2 atmosphere. In the experiments where testosterone treated monocytes were employed, cultured monocytes were incubated with 1 nM testosterone in DMEM (1% FCS) and afterwards they were seeded on EC. EC and adhered monocytes were dyed using Giemsa stain. Bound monocytes were counted in ten fields. Images (400×) were obtained using a Nikon EclipseTS100–Nikon D3100 optical microscope system. Results are expressed as mean ± SD of the number of counted cells.

2.8. Measurement of estradiol concentration

EC and VSMC were treated with testosterone for 30 min. The incubation medium was collected, and estradiol concentration was measured by competitive chemiluminescent enzyme immunoassay, using a commercially available kit (Immulite 2000, Siemens).

2.9. Statistical analysis

Each experimental condition was reproduced in at least three independent experiments performed by quadruplicate. All data are presented as mean ± SD. Comparisons between two means were made using Student's *t*-test, and multiple comparisons with one or two ways ANOVA, followed by Fisher least significant difference test. Differences at $p < 0.05$ were considered significant.

3. Results

3.1. Testosterone induced NO production in EC

Endothelial NO production was measured in EC monolayers exposed to different concentrations of testosterone (0.1–100 nM). As can be observed in Table 1, the steroid significantly enhanced NO production at all doses tested, with maximal effect between 1–10 nM. We chose 1 nM Testosterone as appropriate concentration for the subsequent assays. In order to evaluate whether the rapid non genomic action of testosterone on NO production depends on local conversion to estradiol we employed the compound anastrozole, a nonsteroidal aromatase inhibitor. Fig. 1 shows that the fast increase in NO production elicited by 5 min treatment with testosterone, was not modified by the presence of 100 nM anastrozole, which was added 1 h prior to hormone treatment. Similar results were obtained with anastrazole 10–200 nM (data not shown). To further investigate the possibility of an indirect action of testosterone mediated by local estradiol synthesis, we used an estradiol receptor antagonist. In presence of ICI 182780 compound (5 μM), no changes in the stimulatory action of testosterone were observed (Fig. 2). In contrast, the presence of an androgen receptor antagonist (flutamide) before testosterone treatment completely suppressed the enhancement in NO synthesis induced by the steroid (Fig. 2). Moreover, to assess whether testosterone treatment promotes cellular estradiol synthesis, estradiol concentration was measured in the incubation medium of EC and VSMC. Estradiol production was measured in the incubation medium of EC and VSMC exposed to testosterone for 30 min (Table 2). No significant differences were observed between control and testosterone treated groups, either in EC or VSMC. Altogether these results suggest that testosterone conversion to estradiol was not required for the stimulatory action of the androgen on the vasoactive production. We also evaluated the contribution of testosterone metabolization to DHT on endothelial NO production. For this purpose, finasteride was selected as 5α-reductase inhibitor. EC were treated with 10 nM testosterone for 5 min in presence or absence of finasteride. Fig. 3 shows that the enhancement in NO production elicited by testosterone (75% above control) was partially suppressed by pretreatment of EC with 1 or 5 μM finasteride. However, statistically significant increase remained detectable in the presence of the inhibitor (39; 42% above control).

3.2. Testosterone stimulated VSMC proliferation

Afterwards, the effect of the androgen on VSMC growth was evaluated. Fig. 4 shows the results obtained from time course effect

Table 1
Effect of testosterone on nitric oxide in endothelial cells: dose response study.

Treatment	nmol NO/mg protein
Control	4.82 ± 0.46
0.1 nM testosterone	5.94 ± 0.44**
1 nM testosterone	7.41 ± 0.71**
10 nM testosterone	7.49 ± 0.83**
100 nM testosterone	6.91 ± 0.75**

EC were incubated with testosterone for 10 min at the indicated concentrations. NO production was measured as described under methods. Results are expressed as mean ± SD of three independent experiments (n = 5).
** $p < 0.01$ respect to control.

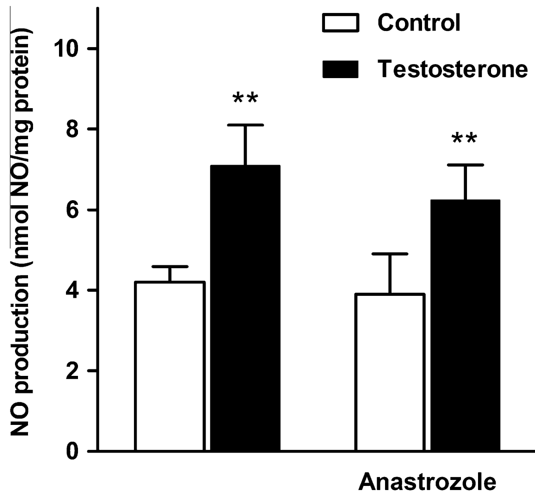

Fig. 1. Effect of anastrozole on NO synthesis induced by testosterone. EC were pre-incubated in absence or presence 100 nM anastrozole for 60 min, and then exposed to 1 nM testosterone for 5 min. NO production was measured as described in Experimental section. Results are the mean ± SD of three independent experiments performed by quadruplicate. $^{**}p < 0.01$ with respect to control.

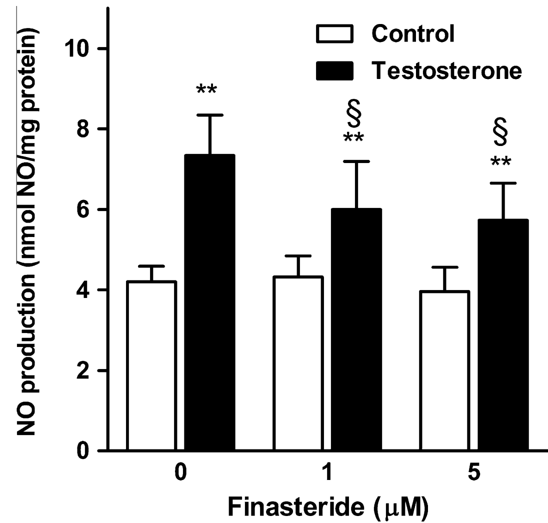

Fig. 3. Effect of 5α-reductase inhibitor on NO production induced by testosterone. EC were pre-incubated in absence or presence 1 or 5 μM finasteride for 60 min, and then exposed to 1 nM testosterone for 5 min. NO production was measured as described in Experimental section. Results are the mean ± SD of three independent experiments performed by quadruplicate. $^{**}p < 0.01$ with respect to each control; $^{‡}P < 0.05$ vs testosterone alone.

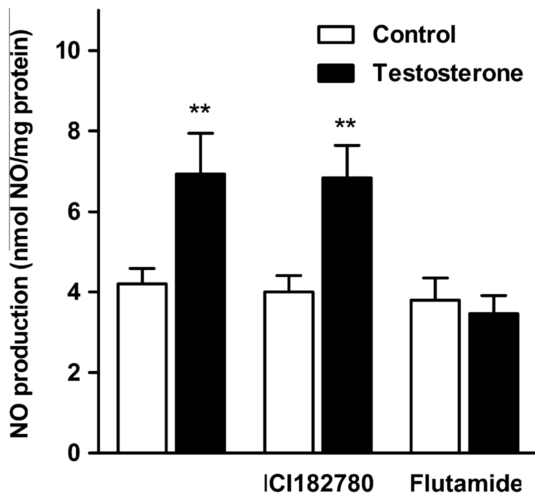

Fig. 2. Effect of AR antagonist flutamide and ER antagonist ICI182780 on NO production induced by testosterone. EC were pre-incubated in absence or presence 50 nM flutamide or 5 μM ICI 182780 for 60 min, and then exposed to 1 nM testosterone for 5 min. NO production was measured as described in Experimental section. Results are the mean ± SD of three independent experiments performed by quadruplicate. $^{**}p < 0.01$ with respect to control.

Table 2
Measurement of estradiol concentrations in the incubation medium of EC or VSMC treated with testosterone.

	Estradiol level (pg/ml)	
	EC	VSMC
Control	17.3 ± 0.86	17.6 ± 1.10
1 nM testosterone	16.1 ± 0.85	16.0 ± 0.88
10 nM testosterone	16.3 ± 0.98	16.3 ± 0.84

EC or VSMC were exposed to testosterone at the indicated concentrations for 30 min. Incubation medium was collected and estradiol concentrations were determined as described under methods. Results are expressed as mean ± SD of 3 independent experiments.

of testosterone on muscle cell proliferation. As can be observed synchronized control cells exhibited their higher rate of proliferation at 24 h of culture. At this time, the steroid significantly enhanced ^{3}H-thymidine incorporation (40% above control). The mitogenic action of the steroid remained for 36–48 h of hormonal treatment. In order to check whether the mitogenic action of testosterone was an indirect effect mediated by local conversion to estradiol, the aromatase inhibitor anastrozole was employed. Fig. 5 shows that the presence of anastrozole does not alter the stimulatory action of testosterone on DNA synthesis. The participation of AR on this genomic action of the steroid was studied using flutamide. Preincubation of VSMC with the AR antagonist completely suppressed the enhancement in cell proliferation induced by 24 h treatment with 10 nM testosterone.

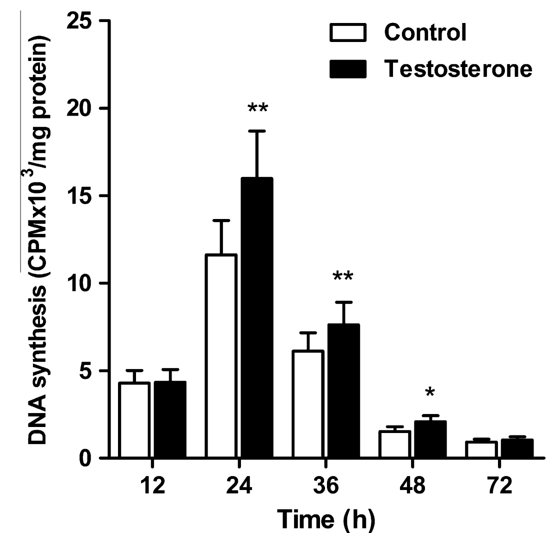

Fig. 4. Effect of testosterone on [^{3}H]-thymidine incorporation. Sub-confluent VSMC were incubated in serum-free medium for 12 h and then treated with 1 nM testosterone at the time indicated. 1 μCi/mL of [^{3}H]-thymidine was added during the last 2 h of treatment. [^{3}H]-Thymidine incorporation was measured as described in Experimental section. Results are the mean ± SD of three independent experiments performed by quadruplicate. $^{**}p < 0.01$; $^{*}p < 0.05$ with respect to each control.

3.3. Testosterone stimulated VSMC migration

Bearing in mind the pivotal role of SMCV migration and EC-monocyte adhesion in the progression of vascular lesions, we evaluated the effect of testosterone on these cellular events either under basal or stress conditions. The evidence obtained from cell migration assays shows that, under basal conditions the androgen does not modify muscular cell mobility (Fig. 6). Norepinephrine (NE), a well known stimulatory agent of vascular cell motility increased cell migration. When VSMC were treated with testosterone prior to NE addition, the number of cell that crossed to the denuded area was significantly increased (1.5-fold with respect to NE alone).

3.4. Testosterone inhibited monocyte adhesion to EC

Fig. 7 shows the data obtained from monocyte adhesion experiments. We found that under basal conditions, the number of adhered monocytes was similar either in control or testosterone group. When EC were exposed to the proinflammatory agent LPS, monocyte adhesion was significantly enhanced, but the presence of testosterone previous to LPS addition completely prevented the LPS effect (Fig. 7A). Furthermore, when monocytes were exposed to testosterone before their addition to testosterone treated EC, a significant inhibition in monocyte adhesion was observed (Fig. 7B, black bar)

4. Discussion

Vascular health is modulated by a plethora of agonists and hormones including androgens. At molecular level, we provided evidence that testosterone regulates vascular function "per se" and not through its conversion to estradiol. The steroid action is independent of testosterone aromatization on both, the non genomic effect on NO synthesis, and the genomic control of cellular growth. At cellular level, the data presented in this study shows that testosterone is able to modulate events that play key roles in vascular disease development. The hormone prevents monocyte adhesion to EC, and prompts VSMC proliferation and migration. The cellular

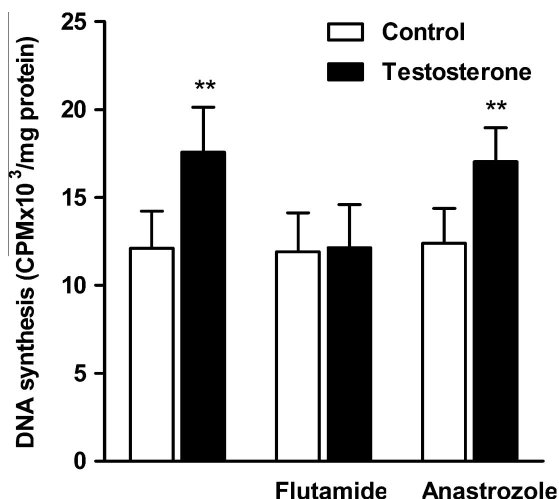

Fig. 5. Effect of flutamide and anastrozole on DNA synthesis induced by testosterone. Sub confluent VSMC were incubated for 12 h in serum-free media, pre-incubated for 1 h in DMEM (1% FCS) with or without flutamide (10 nM) or anastrozole (100 nM), and then treated with testosterone for additional 24 h. [^{3}H]-Thymidine incorporation was measured as described in Experimental section. Results are the mean ± SD of three independent experiments ($n = 4$) $^{**}p < 0.01$ with respect to control.

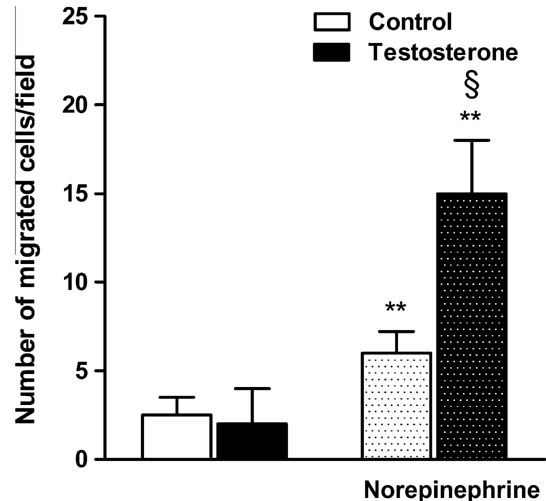

Fig. 6. Effect of testosterone on muscle cells migration. Confluent VSMC cultures were serum starved for 24 h, and cells from half of the monolayer were removed by scraping. Detached cells were washed with PBS, and the remaining monolayer was treated during 48 h with 10 nM testosterone or vehicle in the presence or absence of NE 0.1 μM. Cells presented in the scratched area were counted. Results are the mean ± SD of number of migrated cells/field from three independent experiments performed by quadruplicate. $^{**}p < 0.01$ vs control; $^{§}P < 0.05$ vs NE.

action exhibited by the steroid depends on the environment, whether the cells are under basal or proinflammatory conditions.

There is substantial evidence in the literature about the role of NO production on the regulation of vascular tone and homesotasis. In this context, estradiol vascular effects have been extensively studied [25]. It has been reported that estradiol has a dramatic impact on the response to vascular injury mediated by an enhancement in NO production due to increases both in eNOS expression and level of activation [26]. The non genomic action of estradiol on eNOS activity involves the participation of a subpopulation of ER localized in EC caveolae [27] and downstream activation of MAPK and Akt/protein kinase B signaling pathways [28].

In contrast, the contribution of androgens to vascular homeostasis has been lesser investigated. It is still unclear whether the biochemical vascular actions of testosterone are specific of the androgen through its binding to AR and activation of signaling pathways, or are estrogen dependent actions mediated by the conversion to estradiol. Data reported in literature demonstrate that P450 aromatase is expressed in endothelial and smooth muscle vascular cells [7,8]. On view of this, and since it is widespread known that estradiol exerts vasodilator and antiatherosclerotic properties, it has been suggested that vascular action of testosterone might be an indirect effect mediated by the local conversion to estradiol [9]. However in testosterone vasodilation studies, several reports exclude this possibility because the inhibition of P-450 aromatase does not prevent testosterone induced vasorelaxation [29] and, since non-aromatizable metabolites of testosterone also cause vasorelaxation [30]. In this work we demonstrated that neither the inhibition of testosterone aromatization nor the ER antagonism affected the rapid (NO production) or the long term (cellular growth) actions of testosterone on vascular wall. In the presence of an aromatase inhibitor the stimulation of NO production and the enhancement of vascular muscle cell proliferation induced by testosterone remain unchanged. Furthermore, the antagonism of ER with ICI182780 compound does not alter the hormonal effect, meanwhile the presence of flutamide completely blocks the NO production and DNA synthesis suggesting that AR but not ER is involved in the molecular mechanism of action of testosterone in rat aortic cells. The possibility of local conversion to estradiol in our

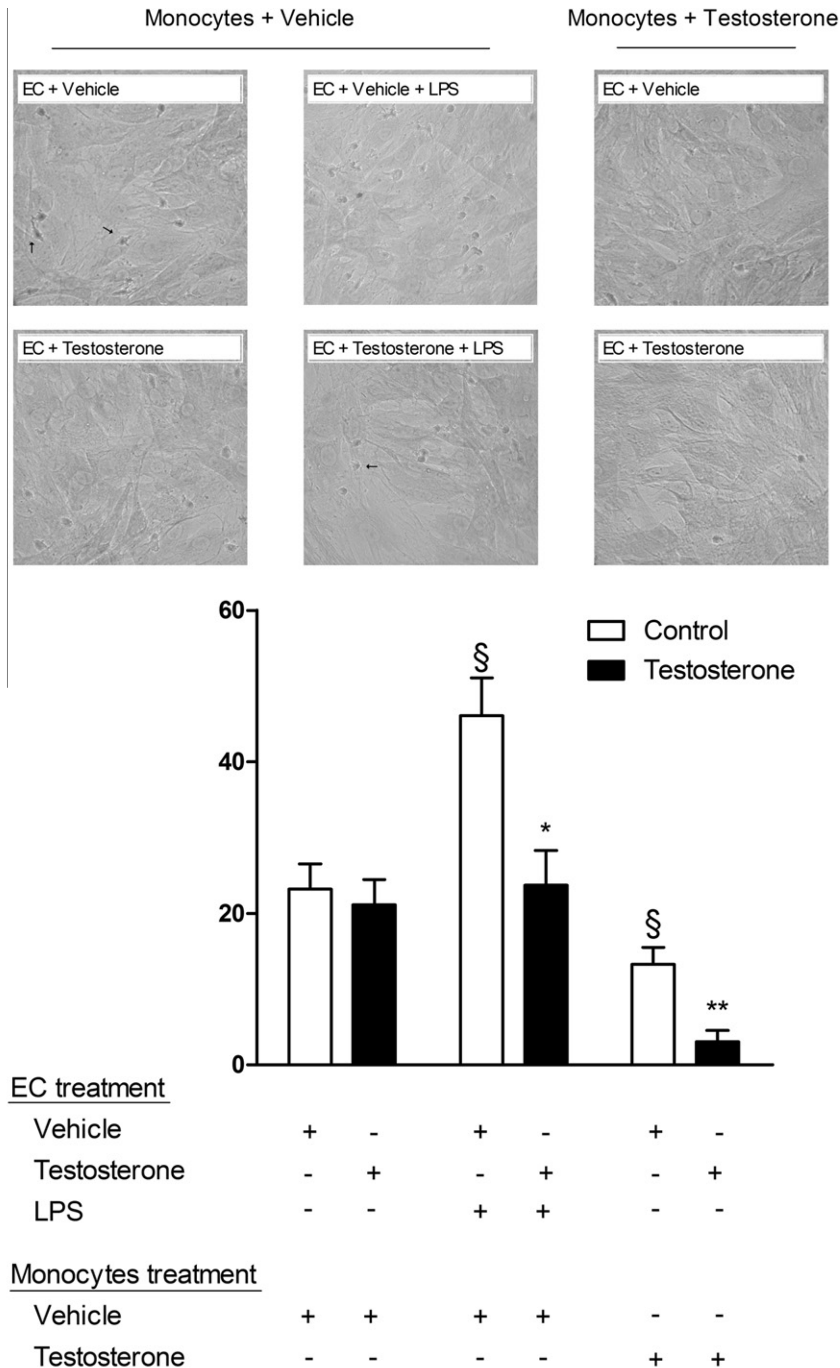

Fig. 7. Effect of testosterone on monocyte adhesion to endothelial cells. ECs cultures were treated with 1 nM T testosterone or vehicle alone (control) for 24 h in the presence of 1 μg/mL LPS, added during the last 21 h of hormonal treatment. Monocytes cultures were incubated with vehicle or 1 nM testosterone for 15 h and then seeded on control or testosterone treated EC for 2 h, as indicated. Upper panel: representative microphotographs of each experimental condition (400×). Arrows show monocyte adhered to EC Bottom (Lower) panel: bars represent the means ± SD (mean ± SD) of the number of monocytes adhered to ECs/field of three independent experiments ($n = 4$). $^{*}P < 0.05$ vs LPS; $^{§}P < 0.01$ vs control, $^{**}P < 0.01$ vs control EC with testosterone treated monocytes.

experimental system was further analyzed by measuring estradiol concentrations in the culture medium after testosterone treatment. Our hypothesis that testosterone does not convert to estradiol was reinforced by the evidence that no differences between the control and treated groups were observed. Taking together all the facts propose that, in female rat aortic cells the stimulation of endothelial NO production and smooth muscle cell proliferation would be due to a direct action of testosterone on the isolated aortic cells. However, in HUVECs it has been reported that testosterone inhibits VCAM-1 expression. In male mice testosterone attenuates early atherogenesis by its local conversion to estradiol [7,31]. In male aromatase knockout mice the lack of aromatization impairs relaxation to acetylcholine compared to wild type mice [32]. These discrepancies between aromatization and not aromatization dependence could be due to differences in the cells type (vein or artery cells) employed as experimental systems, or in protocols conditions. Since our data belong from female rats a gender factor could also be another fact that contributes to explain the differences between whether the hormonal action requires aromatization or not. Studies in healthy young men support the

hypothesis that aromatization of testosterone to estradiol is necessary for the maintenance of normal endothelial function [33]. Indeed individual differences in enzyme expression and regulation could also occur. In this context, it has been suggested that during menopause, the varying degrees of symptoms and the different cardiovascular risk that experience postmenopausal women could be attributed to individual genotypes features [34]. On this line of thought, it has been proposed that polygenomics and pharmacogenomics approaches arise as an alternative in the future for the identification of optimal pharmacological strategies for an individual; instead of therapeutic interventions for large populations [35].

Furthermore, we provided evidence that the stimulatory action of testosterone on NO production would be partially due to DHT conversion. Using finasteride as 5α-reductase inhibitor, we found that enhancement in NO synthesis was reduced. However in the presence of finasteride a significant increase in the vasoactive production still remains, suggesting that both testosterone and DHT are responsible of the enhancement in NO. It has been demonstrated that the synthesis of the vasoactive thromboxane induced by testosterone appears to be via DHT conversion [36]. Indeed, physiological concentrations of DHT increase NO synthesis in human vein endothelial cells [37].

VSMC proliferation and migration within the vascular wall play a crucial role in the formation and subsequent progression of atherosclerotic lesions. We showed that testosterone regulates VSMC growth by the stimulation of cell proliferation. The mitogenic action of the steroid involves the participation of AR, and exhibits a temporal profile of similar shape than control cells. VSMC behavior within artery wall depends on the microenvironment created by surrounding cells and factors. We found that under basal conditions the androgen does not affect VSMC motility, but potentiates the effect of a migratory inducer. Norepinephrine induces migration of vascular smooth muscle cells and of several non vascular cells [38] by increasing reactive oxygen species production [39]. In agreement with our observations in human mammary artery VSMC, testosterone stimulates cell proliferation, effect that was abolished by flutamide [40]. Indeed, a mitogenic action of testosterone on mammary artery muscle cells, but independent of AR participation has been reported [41]. In contrast, in male porcine coronary muscle cells, an antiproliferative action of testosterone was demonstrated [42]. These discrepancies on testosterone regulation of vascular muscle cell growth would be due to differences in cell type or cell culture conditions, or to a selective androgen action on subpopulations of smooth muscle cells that exist in the arteries of several species including human [43]. Another consideration that could be taken into account is that although the influence of sex on cardiovascular disease is still under debate, it has been proposed a sexual dimorphism in the susceptibility to cardiovascular disease, suggesting a negative correlation between testosterone and cardiovascular risk in men, but positive in women [44]. The stimulation of muscle cell growth and migration induced by testosterone reported here, might be considered a negative effect on vascular health. Since vascular disease progression is in part determined by muscle cells proliferation and migration, our *in vitro* observations would be one of the factors that shape this sexual dimorphism.

EC activation by proinflammatory cytokines or bacteria endotoxins predisposes to leukocyte recruitment. Monocytes adhesion is the first critical step in vascular lesion. EC injury induces synthesis or release of surface adhesion molecules [45]. We showed that testosterone does not alter basal monocyte adhesion to EC, however if monocyte were exposed to testosterone for 24 h prior to seed on EC, leukocyte adhesion diminished in control and treated groups. Moreover, in a proinflammatory environment testosterone prevents monocyte adhesion induced by LPS stimulus. Consistent with this, it has been reported that in rats *in vivo* treated with testosterone, aortic macrophage infiltration diminished compared to placebo group [46]. The effect of testosterone on monocyte adhesion reported here could be associated with the regulation of the expression of cell adhesion molecules involved in leukocyte rolling, firm adhesion and endothelial transmigration [47].

In summary, this work demonstrated that testosterone per se modulates vascular behavior through its direct action on EC and VSMC, and that these effects do not required testosterone aromatization to estradiol. Some of the effects reported here such as improvement of NO production and inhibition of EC monocyte adhesion, could be considered a potential beneficial action to avoid vascular damage. In contrast, the stimulation of VSMC growth and migration would be an adverse action that allows atherosclerotic plaque development and progression. These *in vitro* study results, however, might not completely reflect all hormonal actions under physiological situation and to further elucidate the hormonal actions *in vivo* studies are required.

5. Conflict 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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