Endothelial regulation of eNOS, PAI-1 and t-PA by testosterone and dihydrotestosterone in vitro and in vivo


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ABSTRACT: The aim of this study is the identification of direct endothelial regulation by the androgens testosterone (T) and dihydrotestosterone (DHT). We tested the effects of T and DHT on nitric oxide (NO) synthesis and on tissue plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) expression in human endothelial cells and in ovariectomized (OVX) rats. The results showed that at physiological concentrations T and DHT increase endothelial synthesis of NO. This depends on a rapid recruitment of the extracellular-related kinase (ERK) 1/2 and of the phosphatidylinositol 3-OH kinase (PI3K)/Akt cascades, resulting in endothelial nitric oxide synthase (eNOS) Ser1177-phosphorylation. In addition, a later increase of eNOS expression is found. With supra-physiological amounts of T or DHT the induction of NO synthesis is lost. A concentration-related increase of t-PA expression starting from physiological concentrations of T or DHT is found, whereas PAI-1 is augmented only with higher doses. Although DHT exerts these actions through androgen receptors (AR), T acts in part through aromatase-dependent conversion to 17β-estradiol. Ovariectomy is associated with significant changes in eNOS, t-PA and PAI-1 expression in the aorta of Wistar rats and T and DHT result in modifications on eNOS, PAI-1 and t-PA that are in line with the in vitro experiments. In conclusion, T and DHT act on endothelial cells through AR or via conversion to estradiol. Physiological, but not higher amounts are associated with enhanced NO synthesis and an increased t-PA/PAI-1 ratio. These findings are useful to understand the impact of androgens in ageing individuals.

Key words: androgens / cardiovascular disease / endothelial cells / fibrinolysis / nitric oxide

Introduction

Androgens are key regulators in men and women. They control bone density, muscle mass and strength, adipose tissue distribution, mood, energy, psychological well-being and sexual function (Burger, 2002). Moreover, some of these steroids also act as pro-hormones, being converted to estrogens by aromatase (Burger, 2002).

The vascular system is very sensitive to sex steroid hormones. Estrogen is a powerful regulator at this level and the hormonal changes associated with the menopause are established contributors to the worsening cardiovascular function of ageing women (Mendelsohn and Karas, 1999, 2005; Fu and Simoncini, 2007).

Although a considerable amount of information has been gathered on the role of androgens in human physiology and disease, the function of these hormones on the cardiovascular system is still unclear (Wu and von Eckardstein, 2003).

Deregulated androgen production is associated with a variety of clinical disorders in women. Hyperandrogenic conditions, such as the polycystic ovary syndrome, have been related to higher incidence of cardiovascular diseases (Shaw et al., 2008), mostly because of the frequent presence of some of the features of the metabolic syndrome, such as overweight, hypertension, insulin resistance and altered lipid levels (Teede et al., 2006; Cussons et al., 2007). In support of the relevance of elevated androgens for cardiovascular risk, the Study of Women across the Nation (SWAN) recently reported that around the menopausal transition the presence of lower levels of sex hormone-binding globulin (SHBG) or a higher free androgen index (FAI) are predictive of future cardiovascular events (Sutton-Tyrrell et al., 2005).

On the other side, lower androgen levels in males are associated with visceral obesity, insulin resistance, low high-density lipoprotein cholesterol and elevated triglycerides, low-density lipoprotein cholesterol and plasminogen activator Type I, as well (Wu and von Eckardstein, 2003). These changes also result in increased cardiovascular risk in ageing males (Barrett-Connor, 1995; Hak et al., 2002). Similar information comes from males receiving androgen deprivation therapy (Basaria, 2009).

Although the understanding of the potential impact of lower-than-normal androgens in women has been made difficult by
the lack of adequately sensitive hormonal assays, there is a rationale to hypothesize that there may be functional cardiovascular changes due to altered androgen levels during female ageing. The recent clinical development of androgen therapy to counteract sexual dysfunction after the menopause has raised the interest in understanding more broadly the bodily impact of re-establishing normal levels of these sex steroids in deficient women, particularly for the potential long-term cardiovascular actions (Ling et al., 2009).

Androgen receptors (AR) have been identified in vascular cells nearly 20 years ago (Kimura et al., 1993), and later research has shown that these receptors mediate a variety of actions of androgens in endothelial and smooth muscle cells. Endothelial cells are key regulators of cardiovascular function. Deranged production of vasodilating agents and of enzymes that control coagulation and fibrinolysis are some of the main consequences of endothelial dysfunction, and play a central role in the phenomena that lead to vascular diseases (Pober et al., 2009). It is thus interesting to understand whether endogenous or exogenous androgens may regulate the cardiovascular system through direct actions on endothelial cells.

Nitric oxide (NO) is a potent anti-inflammatory and antiatherogenic factor, which is also of paramount importance for the regulation of vascular tone and blood flow as well as for the haemostatic process (Harrison et al., 2006). Parenteral testosterone (T) administration enhances vasodilatation in men and women in part via endothelial-dependent actions that are likely to involve the release of NO (Rosano et al., 1999; Worboys et al., 2001).

An imbalanced fibrinolytic activity is also an established risk factor for cardiovascular disease. The fibrinolytic system is under the control of the tissue plasminogen activator (t-PA) and of its physiological inhibitor plasminogen activator inhibitor-1 (PAI-1). Increased plasma PAI-1 and decreased t-PA are suggested to represent a risk factor for cardiovascular disease (Ridker, 1994), but may have a different relevance across genders and hormonal status (Asselbergs et al., 2007). Some of these differences might be related to T, that has been shown to increase the release of t-PA and to reduce the synthesis of PAI-1 in endothelial cells (Jin et al., 2007).

In the present study, we tested the effects of T and dihydrotestosterone (DHT), on endothelial NO, t-PA and PAI-1 production in human cultured endothelial cells and in rodents. Moreover, we characterized some of the mechanistic basis of the actions of these hormones in endothelial cells.

### Materials and Methods

#### Cell cultures and treatments

Human umbilical vein endothelial cells (HUVECs), from both male as well as female fetuses, were kept 48 h in Dulbecco’s modified Eagle’s medium (DMEM) containing steroid-deprived FBS. Whenever an inhibitor was used, we added the compound 30 min before the active treatments. Before experiments investigating non-transcriptional effects, HUVECs used, we added the compound 30 min before the active treatments.

Before experiments investigating non-transcriptional effects, HUVECs were kept in DMEM containing no FBS for 8 h. T and DHT were from Sigma-Aldrich Corp (Saint Louis, MO, USA).

#### Animal treatments

Fertile female Wistar rats, weighing 175–199 g (Harlan, S. Pietro al Natisone, Italy), were kept under 14 h of illumination per day (0600–2000 hours) and had free access to standard rat chow and tap water. After 14 days the rats were either sham-operated or OVX in the same estrous cycle stage. OVX rats were treated with T and DHT for 14 days (10 or 100 μg/kg/day). T and DHT were dissolved in pure ethanol and administered orally after dilution in 0.9% NaCl. Animals were euthanized by decapitation under pentobarbital anesthesia (30 mg/kg), and the abdominal aorta was obtained. Aortas were snap-frozen in dry ice and stored at −80°C. Animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (www.nap.edu/readingroom/books/labrats).

#### Hormone assays

Circulating plasma levels of T, DHT and estradiol were determined using ELISA kits (DHT assay: DRG Instruments Marburg, Germany; estradiol assay: DSL, Webster, TX, USA; T assay: radim Spa, Pomezia, Italy) following passage of sera through C-18 Sep-Pak cartridges previously equilibrated with absolute methanol (Waters Corporation, Milford, MA, USA). Values are expressed as nmol/l for T and pmol/l for DHT and estradiol. For each hormone, all samples were analyzed in the same assay; the intra-assay coefficients of variation were 5.3% for DHT, 4.5% for T and 3.9% for estradiol.

#### eNOS activity assay

eNOS activity was determined as conversion of [3H]arginine to [3H]citrulline in endothelial cell lysates. Briefly, HUVECs were harvested in ice-cold PBS containing 1 mM EDTA. The cell lysates were pelleted in a microfuge (2 min, 16 110 × g, 4°C) and subsequently homogenized in a buffer containing 25 mM Tris–HCl, pH 7.4, 1 mM EDTA and 1 mM EGTA. eNOS activity was detected by measuring the conversion of [3H]-arginine to [3H]-citrulline with the NO synthase assay kit (Calbiochem, La Jolla, CA, USA), according to the manufacturer’s instructions. About 0.001 mCi (0.037 MBq) of [3H]-arginine were added to each sample well for the reaction. Rat cerebellum extracts, containing elevated amounts of NOS, were used as positive controls, whereas endothelial cell extracts incubated in the presence of the competitive NOS inhibitor L-NAME (1 mM) served were used to subtract the non-specific activity.

#### Nitrite assay

NO production was determined by a nitrite assay using 2,3-diaminonaphthalene. Fluorescence of 1-(H)-naphtotriazole was measured with excitation and emission wavelengths of 365 and 450 nm. Standard curves were constructed with sodium nitrite. Non-specific fluorescence was determined in the presence of the eNOS inhibitor N(G)-monomethyl-l-arginine L-NMA (3 mM).

#### Immunoblottings

Cell lysates were separated by 10% SDS–PAGE. Antibodies used were: eNOS (Transduction Laboratories, Lexington, KY, USA), Ser1177-P-eNOS (Upstate Biotechnology, Lake Placid, NY, USA), wild type or Tyr204-P-ERK 1/2 (Calbiochem, San Diego, CA, USA), wild type and Thr308-P-Akt (Upstate Biotechnology, Lake Placid, NY, USA), PAI-1 (sc-8979, Santa Cruz Biotechnology, Santa Cruz, CA, USA), t-PA (sc-5241, Santa Cruz Biotechnology). These last two antibodies do not recognize t-PA-PAI-1 complexes, they only identify t-PA or PAI-1. Primary and secondary antibodies were incubated with the membranes with standard technique.

#### Statistical analysis

All values are expressed as mean ± SD. Statistical differences between mean values were determined by ANOVA, followed by the Fisher’s protected least significance difference.
Results

Effects of T and DHT on NO synthesis in human endothelial cells

To test the effects of T and DHT on endothelial NO we measured NO release in the cell culture medium along with the cellular expression and enzymatic activity of the endothelial nitric oxide synthase (eNOS). Steroid-deprived human umbilical vein endothelial cells (HUVECs) were exposed to a range of concentrations of T and DHT (0.1–1000 nM) for 48 h. Both T and DHT increased the synthesis of NO and this was related to the concentration of the steroids, with maximal effects being observed in the presence of lower concentrations of the hormones (that correspond to the physiological range) and lesser increases with higher doses (Fig. 1A and B). The enzymatic activity of eNOS followed a similar pattern in the presence of T or DHT (Fig. 1A and B). Similarly, cellular contents of immunoreactive eNOS were increased by exposure to physiological concentrations of T or DHT, but not to higher concentrations of the hormones (Fig. 1C and D).

The enhanced NO release and eNOS activity observed in the presence of T and DHT ensued in a time-dependent manner. Both androgens, provided at the concentration of 1 nM, increased NO release and eNOS activity as early as 30 min, with a maximal action

Figure 1 Dynamics of NO and eNOS regulation by T and DHT in human endothelial cells. Steroid-deprived HUVECs were treated for 48 h with increasing T or DHT concentrations (A–D) or for different times (E–H). (A, B, E and F) The amounts of NO released in the cell culture medium along with the enzymatic activity of eNOS. (C, D, G and H) Cellular amount of eNOS. White bars represent nitrites levels in HUVEC and the black bars represent the enzymatic activity of eNOS. The experiments were repeated three times with comparable results. *P < 0.05 versus control.
after 48 h. A significant time-related increase of NO release and eNOS activity could also be found after treatment with DHT (Fig. 1E and F). Visible increases of eNOS protein expression were found in the presence of both steroid hormones after at least 12 h of treatment (Fig. 1G and H).

**Effects of T and DHT on t-PA and PAI-1 in human endothelial cells**

The administration to steroid-deprived HUVECs of T and DHT also resulted in changes in t-PA and PAI-1 protein expression. Both T and DHT up-regulated the expression of t-PA when used in the lower range of concentrations, whereas no increase was seen with higher doses (Fig. 2A and B). On the contrary, PAI-1 cell amounts were increased by the administration of T or DHT in a concentration-related fashion (Fig. 2A and B).

**Effect of T and DHT on t-PA and PAI-1 in human endothelial cells**

The effects of T on the expression of the three target proteins were partially inhibited by blockade of either ER or AR (Fig. 3A and B), as well as by inhibition of aromatase (Fig. 3C). The modulatory actions of DHT were instead abrogated only by flutamide (Fig. 3A and B), consistent with the non-aromatizable chemical structure of this steroid. This suggests that the effects of T on eNOS, t-PA and PAI-1 are mediated in part by direct actions on the AR, but in part via conversion to estrogens.

**Rapid actions of T and DHT on NO release and eNOS activity**

Steroid hormones, including androgens, exert their signaling actions through both genomic and non-genomic mechanisms, and eNOS is expressed...
an established target of such actions (Fu and Simoncini, 2008). When endothelial cells were treated for 30 min with T and DHT at different concentrations, significant increases of NO synthesis and eNOS activity were observed at lower concentrations, with reduced actions when the doses of the hormones were further raised (Fig. 4A and B). Maximal activation was found within the physiological range of concentration for both steroids. Rapid activation of eNOS by T and DHT was non-transcriptional, as shown by the lack of an increase in eNOS protein expression. In support of this, we found a marked increase in phosphorylation of eNOS on Ser^{1177} (Fig. 4C and D), which followed the same pattern of concentration-related activation.

**Signaling pathways implicated in T- and DHT-stimulated NO release and eNOS activity**

Phosphorylation on Ser^{1177} results in eNOS activation and is targeted by a number of mediators of extra-nuclear signaling pathways recruited by sex steroid hormones, such as the phosphatidylinositol 3-OH kinase (PI3K)/Akt pathway or the extracellular-related protein kinase (ERK) 1/2 cascade (Dimmeler et al., 1999; Simoncini et al., 2000). HUVECs exposed rapidly (30 min) to T or DHT displayed a quick recruitment of both pathways, with enhanced phosphorylation of ERK 1/2 on Tyr^{204}, as well as of Akt on Thr^{308} (Fig. 5A and B). In support of the relevance of these cascades for the currently explored effects of androgens, the blockade of MEK 1/2 (which activates ERK 1/2) with PD98059, or of PI3K with wortmannin resulted in impaired activation of NO synthesis or of eNOS by T and DHT (Fig. 5C and D). In addition, similar to what found for the longer-term actions of T and DHT, the enhanced NO synthesis and eNOS activity linked to T administration were in part abrogated by blocking both ER (with ICI 182,780) and AR (with flutamide), although the effect of DHT was only blocked by flutamide (Fig. 5C and D), confirming that even in this rapid time frame, some of the actions of T can be mediated by conversion to estrogens.

**Effects of T and DHT on rat aortic expression of eNOS, PAI-1 and t-PA**

Finally, in an effort to check whether the in vitro actions of T and DHT were found also in an in vivo setting, we administered two different doses of T and DHT (10 and 100 μg/kg/day) to OVX female rats, with fertile animals as controls and studied the protein contents of eNOS, PAI-1 and t-PA in abdominal aortas. As can be seen in Fig. 6A, ovariectomy is associated with a marked decline in the plasma levels of T, DHT and estradiol in these animals. The two doses of T result in a significant increase in all three plasma steroids, with the 10 μg/kg/day dose resulting in T levels that are very similar to those found in fertile animals, whereas the 100 μg/kg/day dose results in T levels that are nearly five times higher than in intact animals. In parallel, the two doses of DHT did not turn into modifications of circulating levels of T nor of estradiol, but only of DHT, as expected.

Compared with sham-operated fertile rats, the level of aortic eNOS expression markedly decreased in OVX animals, whereas the amounts of t-PA and PAI-1 increased (Fig. 6B). The administration of a low dose of T (10 μg/kg/day) to OVX rats was associated with a restored aortic expression of eNOS and with a down-regulation of both t-PA and PAI-1, with an expression profile comparable to that of fertile animals (Fig. 6B). However, these actions were largely lost when the higher dose of T (100 μg/kg/day) was used (Fig. 6B). The administration of DHT did not affect the aortic expression of eNOS in OVX animals, at either dose (Fig. 6B). In parallel, no reduction of t-PA or PAI-1 expression were seen with either lower or higher doses of DHT, differently from what found with T, supporting the concept that the two androgens act differently.

![Figure 4](https://academic.oup.com/molehr/article-abstract/16/10/761/1064557)

**Figure 4** Rapid activation of NO synthesis by T and DHT in endothelial cells. Steroid-deprived HUVECs were treated for 30 min with increasing T and DHT concentrations. (A and B) The amounts of NO released in the cell culture medium and display the activity of eNOS. (C and D) Display the cellular amount of eNOS and Ser^{1177}-P-eNOS. White bars represent nitrates levels in HUVEC and the black bars represent the enzymatic activity of eNOS. The experiments were repeated three times with comparable results. *P ≤ 0.05 versus control.
The main finding of our paper is the identification of direct endothelial regulation by the androgens T and DHT. We find that these steroids alter the synthesis of NO, PAI-1 and t-PA in vitro and in vivo, and that these actions are tightly related to the concentration of the hormones used. These findings are in line with the growing awareness of the relevance of androgen levels in ageing individuals for cardiovascular risk, and support the hypothesis that some of the actions of these hormones are played via direct vascular actions.

The interaction between androgens and cardiovascular disease is unclear. Clinical studies show a correlation between low endogenous T and cardiovascular events in ageing males (Barrett-Connor, 1995; Hak et al., 2002; Wu and von Eckardstein, 2003). In hypogonadal males, administration of T results in a cytokine shift to a state of reduced inflammation, with reduced plasma tumor-necrosis factor (TNF)-α and interleukin (IL)-1β and increased IL-10, along with a reduction in cholesterol (Malkin et al., 2004). In the presence of established coronary heart disease (CHD) in ageing males, exogenous T increases vasodilatation in the coronary district (English et al., 2000; Harrison et al., 2006), such that it has been proposed as a therapeutic strategy for men with heart disease (Pugh et al., 2004).

The relevance of low T in ageing women has been the object of intense debate (Ling et al., 2009). Recent data shows that T in post-menopausal women is positively correlated to flow-mediated dilatation in the brachial artery (Montalcini et al., 2007). In parallel, physiological T replacement in post-menopausal women using long-term estrogen therapy improves both endothelial-dependent and -independent brachial artery vasodilatation (Worboys et al., 2001). On the same line, in women with hypopituitarism, T administration improves insulin resistance (Miller et al., 2007). However, whether a role for preventing CHD with T replacement to physiological levels in androgen-deficient women exists is still to be proven with clinical studies.

The available evidence on the actions of T on the vessels is somewhat conflicting, but overall suggests that some actions of this steroid may be protective for vascular cells. For instance, T administration to castrated animals results in a reduction of the progression of atherosclerosis (Nathan et al., 2001). These actions are likely related to the anti-inflammatory and anti-atherogenic actions of T on the expression of endothelial-leukocyte adhesion molecules (Hatakeyama et al., 2002; Zhang et al., 2002a, b), that play a role in driving accumulation of white blood cells into the vascular wall, and thus in the development of atherosclerosis. Consistent with the hypothesis that the cardiovascular actions of androgens are dependent on their concentration, a similar study using supra-physiological amounts of DHT (40–400-fold higher than those used in our experiments) showed an opposite effect, with enhanced adhesion of monocytes to endothelial cells (McCrohon et al., 1999). These studies may offer a background to the reported inverse association between T levels and aortic atherosclerosis in elderly males (Hak et al., 2002).

Our finding that T and DHT enhance the production of NO in endothelial cells is in line with the previous finding of increased release of this mediator in the penis circulation during T replacement.
in castrated rats (Marin et al., 1999), as well as of the identified role of NO in mediating the actions of androgens in Leydig cells (Pomerantz and Pitelka, 1998). In addition, animals with a defect in the androgen receptor have an impaired NO-mediated attenuation of aortic reactivity to the hormone vasopressin (VP), likely because of a modulation of endothelial VP signal transduction pathways and NO release (Stallone et al., 2001), which supports the idea that androgens and their receptors are important regulators of the NO system. Similar information comes from studies with the adrenal steroid, dehydroepiandrosterone (DHEA; Simoncini et al., 2003) or with the synthetic androgenic steroid, tibolone (Simoncini et al., 2004), both of which enhance the synthesis of NO through direct regulatory actions on endothelial cells.

NO synthesis by endothelial cells is of paramount importance for the regulation of vascular tone and blood flow and for the control of the haemostatic process (Harrison et al., 2006; Pober et al., 2009). Furthermore, endothelium-derived NO is a potent anti-inflammatory and anti-atherogenic factor, being able to prevent endothelial cell dysfunction and vascular degenerative processes (Harrison et al., 2006; Pober et al., 2009). Sex steroids, particularly estrogens, are established regulators of NO release in endothelial cells via nuclear and extra-nuclear mechanisms (Fu and Simoncini, 2007). Our findings show that during exposure of endothelial cells to T or DHT NO release is enhanced both rapidly, and more consistently, via longer-term effects. The long-term actions on NO release induced by T and DHT are due to increased eNOS protein expression, which turns into enhanced overall activity in human endothelial cells. At least for T, a part of the biological effect depends on the conversion to estradiol by aromatase. This is shown by the partial blockade of T action by both ER and AR antagonists, as well as by inhibition of aromatase activity. In agreement, aromatase is expressed in vascular tissues, including endothelial cells (Harada et al., 1999) and some actions of T at this level, such as the interference with the expression of adhesion molecules, are mediated by aromatase-dependent conversion to estradiol (Mukherjee et al., 2002). However, DHT, which is not converted to estrogens, is also active in stimulating NO, which shows that the AR is per se able to regulate the eNOS gene. Indeed, the eNOS gene promoter contains both an estrogen-response element as well as an androgen-response element, which fits with this model (Kiang et al., 2007). In addition, in our results DHT is consistently effective at a 10-fold lower

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<th>T (nmol/L)</th>
<th>DHT (pmol/L)</th>
<th>E2 (pmol/L)</th>
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<tbody>
<tr>
<td>Fertile</td>
<td>1.35 ± 0.035</td>
<td>221.4 ± 21.7</td>
<td>335.9 ± 15.05</td>
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<tr>
<td>OVX</td>
<td>0.73 ± 0.7*</td>
<td>55.43 ± 4.5*</td>
<td>109.41 ± 7.7*</td>
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<tr>
<td>OVX + T (10 μg/kg/day)</td>
<td>1.7 ±1.04**</td>
<td>117.1± 10.7**</td>
<td>173.3± 11.4**</td>
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<td>OVX + T (100 μg/kg/day)</td>
<td>6.6± 0.7**</td>
<td>186.6± 21.7**</td>
<td>224.7± 12.5**</td>
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<tr>
<td>OVX + DHT (30 μg/kg/day)</td>
<td>0.62 ± 0.07</td>
<td>103.3 ± 9.3**</td>
<td>121.9 ± 15.5</td>
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<tr>
<td>OVX + DHT (100 μg/kg/day)</td>
<td>0.66 ± 0.7</td>
<td>155.6 ± 17.9**</td>
<td>100.6 ± 13.9</td>
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**Figure 6** *In vivo* regulation of rat aortic eNOS expression by castration and T replacement. OVX Wistar rats were treated orally for 14 days with vehicle or different T and DHT amounts (10 or 100 μg/kg/day), and abdominal aorta tissue extracts were assayed for eNOS, PAI-1 and t-PA contents. Sham-operated fertile animals served as controls. (A) Plasma levels (mean ± SD) of T, DHT and E2 in fertile or OVX female rats, treated with vehicle or different amounts of T or DHT. *P ≤ 0.01 versus fertile; **P ≤ 0.01 versus OVX. (B and C) Each band represents the blotted protein extract from a different animal’s aorta. A total of 12 animals for each experimental condition were assayed with consistent results, and representative blots are shown. The experiments were repeated three times with comparable results.
concentration as compared with T, which may also stand for a primary role of AR recruitment, given the higher binding affinity and transactivation efficiency of DHT as compared with T.

A growing amount of evidence has been gathered in the past 20 years showing that steroid hormone receptors also elicit their biological functions through extra-nuclear mechanisms (Fu and Simoncini, 2008). In the present study, we find that beyond increasing eNOS expression throughout several hours, T and DHT also increase eNOS activity and the synthesis of NO in a matter of minutes. This non-transcriptional action of the two androgens requires the recruitment of AR. In the case of T, a part of the action is also played through ER. The rapid activation of eNOS is explained by Ser1177 phosphorylation, which turns into enhanced enzyme activity (Dimmeler et al., 1999; Simoncini et al., 2000). The androgen-dependent eNOS phosphorylation is obtained through recruitment of the ERK 1/2 MAPK and of the PI3K/Akt cascades.

PAI-1 and t-PA are endothelial-derived factors that control fibrinolytic activity. The activity of the fibrinolytic system depends on the balance between t-PA and its inhibitor PAI-1. Diminished endogenous fibrinolytic activity has been implicated in the etiology of coronary artery disease and thrombosis (Ridker, 1994; Juhan-Vague et al., 1996; Ganti et al., 2002). There is evidence that PAI-1 also contributes to pathological remodeling of the vascular wall and arteriosclerosis. The direct effect of T and DHT, on the coagulatory and fibrinolytic system is still debated. Similar to what shown by other investigators (Jin et al., 2007), our data shows that concentrations of T or DHT which are similar to those present in young men and in premenopausal women result in increased t-PA and in decreased PAI-1 expression in endothelial cells. In addition, our results also show that higher doses of the same hormones result in an attenuation of these potentially beneficial effects.

Our animal data supports the in vitro studies, showing that the modifications of aortic expression of eNOS, t-PA and PAI-1 associated with castration are reverted by the administration of a physiological amount of T, but not with a pharmacological dose of the same hormone. In addition, androgen supplementation with DHT results in partially different results, possibly due to the absence of conversion to estrogens.

In conclusion, we show that T and DHT directly regulate human endothelial cells in vitro and in vivo by activating eNOS through both nuclear and extra-nuclear mechanisms and by regulating t-PA and PAI-1 expression. However, the effects of T and DHT are related to the concentration used, with physiological amounts of androgens being associated with endothelial-protective effects that are lost with higher amounts of the same hormones. Our results contribute new information on the vascular actions of androgens that may be useful for the clinical development of androgen replacement therapies for ageing individuals.

**Authors’ roles**

L.G. carried out the majority of the experiments and drafted the manuscript. V.T. performed eNOS activity assays. A.M.S. performed hormone assays. M.I.F. performed Nitrite assays. X.D.F. and S.Z. performed cell isolation, culture and treatments. A.R.G. was instrumental in funding the study and participated to the writing of the MS. T.S. planned and funded the project, supervised the experiments, wrote the paper.

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**References**


