Role of putative membrane receptors in the effect of androgens on human vascular cell growth

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Abstract

We have reported previously that dihydrotestosterone (DHT) induces a biphasic effect on DNA synthesis in human vascular smooth muscle cells (VSMC), i.e. stimulation at low concentrations and inhibition at high concentrations. In contrast, DHT dose-dependently stimulated [3H]thymidine incorporation in a human endothelial cell line (ECV304). Additionally, DHT increased the specific activity of creatine kinase (CK) in both vascular cell types. In the present study, we have determined whether some of these effects are exerted via membrane-binding sites. We measured changes in DNA synthesis and CK after treatment with DHT and the membrane-impermeant testosterone-3-carboxymethyl oxime conjugated to bovine serum albumin (BSA) (T-BSA). High concentrations of either DHT or T-BSA inhibited VSMC proliferation (by 52±22% and 51±25% respectively). DHT as well as T-BSA increased DNA synthesis in ECV304 cells dose-dependently. In contrast, T-BSA did not affect CK in either cell type. In both cell types, DHT as well as T-BSA increased mitogen-activated protein kinase (MAPK) kinase activity as measured by total phosphorylated MAPK. Further, the inhibitory effect of either the free or protein-bound androgens on DNA synthesis was blocked by UO126, an inhibitor of MAPK kinase activity. T-BSA conjugate labeled with Europium showed binding to whole VSMC, which could be displaced by excess T-BSA, but not by estradiol-BSA or the free hormones. Finally, using T-BSA linked to the fluorescent dye Cy3·5, we directly demonstrated the presence of membrane-binding sites for androgen in VSMC. Hence, the inhibitory effects of testosterone on DNA synthesis in VSMC are apparently exerted by membrane-binding sites for androgen, do not require intracellular entry of the hormone and its binding to the classical nuclear receptors and are linked to MAPK activation.


Introduction

There is considerable interest in the effects of estrogens in the vasculature, but the interaction between androgens and cardiovascular function and disease has gained much less attention. Although it is generally assumed that the enhanced susceptibility of the male to atherosclerosis is somehow related to the abundance of androgen and/or to the low circulating estrogen concentrations relative to women, direct examination of specific effects of androgens in vascular cells in vitro or on cardiovascular function in vivo has often revealed potentially beneficial effects, such as vasodilation (Yue et al. 1995, Ding & Stallone 2001), induction of endothelial nitric oxide (Tatcum-Talom et al. 2002) and inhibition of vascular smooth muscle cell (VSMC) proliferation (Williams et al. 2002). Evidence suggests that hypogonadal males actually have an increased rate of cardiovascular events (Hak et al. 2002). On the other hand, androgen replacement therapy has been recently reported to impair endothelial-dependent vasodilation in hypogonadal men (Zitzman et al. 2002). As the prevalence of male hypogonadism rises steadily with age and men are increasingly subject to androgen replacement also to restore hormonal ‘normalcy’ in anticipation of improved sexual function, muscle strength, bone density, mental function and quality of life, it seems imperative to gain a better insight into the effects of androgens in human vascular tissue.

We have previously reported that dihydrotestosterone (DHT) exerts a biphasic effect on DNA synthesis in human VSMC, i.e. stimulation at a low concentration and inhibition at high concentrations (Somjen et al. 1998). A recent report suggested that the androgen precursor dehydroepiandrosterone (DHEA) inhibits VSMC via androgen receptor- as well as estrogen receptor-independent mechanisms (Williams et al. 2002). Although the effects of DHT on VSMC growth in our study were inhibited by the androgen receptor antagonist flutamide, the precise site of...
these interactions is presently unclear. There has been a recent surge of interest in the non-genomic effects of both estrogens and androgens (Benten et al. 1999, Guo et al. 2002, Simoncini et al. 2002). We have previously reported that the effects of estradiol (E2) on VSMC, but not on endothelial cell growth, were mediated, in part, by a putative membrane receptor (Somjen et al. 2002). These findings led us to examine the possibility that testosterone-dependent modulation of DNA synthesis in human vascular cells is exerted via non-genomic mechanisms, using membrane-impermeant testosterone conjugates. The results indicate that androgen complexes are incapable of entering the cell label membrane-associated binding sites and inhibit DNA synthesis in human VSMC.

Materials and Methods

Reagents

Enhancement solution for measuring Europium fluorescence was purchased from Wallac (Turku, Finland). Steroids, chemicals, carboxymethoxylamine-hemihydrochloride and the creatine kinase (CK) kit were from Sigma (St Louis, MO, USA). The labeling reagent N-1(p-isothiocyanatophenyl)-diethylenetri-N1,N2,N3,-tetra acetate chelated with Europium (activated Eu-chelate) was kindly provided by Dr I Hemmila, Wallac. Cy3-5 bis reactive dye was purchased from Amersham Pharmacia Biotech (Amersham, Bucks, UK). The mitogen-activated protein kinase (MAPK) kinase inhibitor U0126 was purchased from Alexis. Assay buffer and wash solution for the DELFIA assay was prepared as previously described (Amir-Zaltsman et al. 2000). The coating solution for the DELFIA assay was phosphate-buffered saline (PBS), pH 7.4. Each treatment was performed four times.

Preparation of testosterone macromolecular conjugates

Testosterone-3-(O)-carboxymethyl oxime (T-3-CMO) (Kohen et al. 1975) was prepared by conjugating testosterone to carboxymethoxylamine hemihydrochloride. Briefly, testosterone (200 mg) was dissolved in ethanol (2 ml) and a solution of carboxymethoxylamine hemihydrochloride (260 mg) in 2 M KOH was added. The reaction mixture was stirred at room temperature overnight. Subsequently, ethanol was evaporated from the reaction mixture. Water was then added, and the mixture was extracted with ethyl acetate. The aqueous phase was separated and acidified to pH 2 with 10% HCl. The precipitate that formed was filtered, washed with water, dried and recrystallized from acetone–petroleum ether to give T-3-CMO. Examination of the T-3-CMO by thin layer chromatography (TLC) in the solvent system chloroform:methanol:acetic acid (94.7:5:0.3) showed a single spot of Rf 0.14. In the same solvent system, testosterone showed an Rf of 0.7. T-3-CMO was conjugated to bovine serum albumin (BSA) via a two-step reaction as described previously (Kohen et al. 1998). In the first step of the synthesis, T-3-CMO (3 mg) was dissolved in dry dioxane (200 µl), and N-hydroxy succinimid (2 mg) and carbodi-imide (2 mg) were added to the reaction mixture. After an overnight reaction at room temperature, urea was formed and the supernatant was analyzed by TLC using the solvent system of chloroform:methanol:acetic acid (94:7:5:0.3). A compound with an Rf of 0.95 was visualized, indicating that the active N-hydroxysuccinimide ester derivative of T-3-CMO had been obtained. In the second step of the synthesis, the N-hydroxy succinimide ester of T-3-CMO in dioxane (200 µl) was added drop-wise to a solution of BSA (10 mg) in 1 ml 0.13 M NaHCO3 (pH 8.5). The reaction mixture was stirred at room temperature for 2 h, and dialyzed against PBS, pH 8 at 4°C and stored at −20°C until use. Ultra violet analysis of the conjugate indicated that it contained 10 µmol steroid/mol protein. TLC analysis of this conjugate (T-BSA) indicated that T-BSA was free of T-3-CMO and of testosterone.

Preparation of Europium-labeled T-BSA conjugate

T-BSA (2 mg in 200 µl PBS) (30 nM) was dialyzed against 50 mM carbonate/bicarbonate buffer, pH 9.5, for 3 h. Double-distilled water (200 µl) was added to activated Europium chelate labeling reagent. The chelating agent (300 nM in 100 µl water) was then added to the T-BSA solution. The reaction mixture was stirred and incubated overnight at 4°C and the labeled protein was purified by gel filtration on Sephadex G-25 M. T-BSA conjugate labeled with Europium was eluted with 50 mM Tris–HCl buffer (pH 7.5) and stored at 4°C until use.

Preparation of Cy3-5-labeled steroid–BSA conjugates

The membrane-impermeant steroid conjugates T-6-CMO–BSA (1 mg) and 4-chloro-androstenedione-3-(O)-carboxymethyl oxime BSA (CLAD–BSA; 1 mg, prepared in our laboratory (Kohen et al. 1999), used for negative control) were labeled with the reactive dye bis Cy3-5 according to the manufacturer’s instructions. The labeled protein contained 1 mol dye/mol protein.

Cell culture

VSMC were prepared from human umbilical artery as previously described with minor modifications (Somjen et al. 1998, 2000). Cells were used only at passages 1–3 when expression of smooth muscle actin was clearly demonstrable. ECV304 cells, an endothelial cell line derived from a human umbilical vein, were purchased from American
Type Culture Collection, Manassas, VA, USA and grown in medium 199 containing 10% fetal calf serum, glutamine and antibiotics.

Assessment of DNA synthesis
Cells were grown until subconfluence and then treated with various hormones or agents as indicated. Twenty-two hours following exposure to these agents, [3H]thymidine was added for 2 h. Cells were then treated with 10% ice-cold trichloroacetic acid (TCA) for 5 min and washed twice with 5% TCA and then with cold ethanol. The cellular layer was dissolved in 0.3 ml 0.3 M NaOH, samples were aspirated and [3H]thymidine incorporation into DNA was determined by counting in a Packard Tri-Carb 2100TR liquid scintillation analyser (Somjen et al. 1998, 2000).

CK extraction and assay
Cells were treated for 4 h with the various hormones and agents as specified, and then were scraped off the culture dishes and homogenized by freezing and thawing three times in an extraction buffer as previously described (Somjen et al. 1998, 2000). Supernatant extracts were obtained by centrifugation of homogenates at 14 000 g for 5 min at 4 °C in an Eppendorf microcentrifuge. CK activity was assayed by a coupled spectrophotometric assay described previously (Somjen et al. 1998, 2000). Protein was determined by Coomassie blue dye binding using BSA as the standard. The results are given as CK specific activity, which is defined as CK activity/mg protein.

Association of the membrane-impermeant T-BSA conjugate labeled with Europium with vascular cells
Cells (4 × 10^5 cells/well) cultured in 24-well plates for 48 h were washed once with ice-cold binding medium (Dulbecco’s modified Eagles’ medium +0.1% BSA and HEPES 25 mM, pH 7-4) using reaction conditions as described previously (Mazor et al. 2002). Subsequently, cells were incubated for 90 min at 4 °C with the steroid protein conjugates (E_2-6-CMO ovalbumin, T-3-CMO-BSA; Kohen et al. 1975; 10 µM/well). T-BSA conjugate labeled with Europium (1:1000, 200 µl in binding medium) was then added and the incubation was continued for another 60 min at 4 °C. Binding was terminated by four successive washes with ice-cold binding medium. Enhancement solution (300 µl/well) was then added to the cells, and the samples (200 µl) were collected for time-resolved fluorescence determination using an Arcus time-resolved fluorometer (Wallac).

Immunofluorescence studies of membrane androgen-binding sites
Cells (5 × 10^3 cells/chamber) were grown on Lab-TekII chamber slide system (Nalge Nunc International, Naperville, IL, USA). After reaching subconfluence, cells were washed with magnesium- and calcium-free PBS for 5 min at 37 °C. PBS was then aspirated and cells were placed at 4 °C in a shaking bath for 30 min with T-BSA (15 µl) or CLAD-BSA (15 µl) labeled with Cy3·5. After washing, the cells were washed five times with cold PBS containing 1% BSA and fixed for 10 min at 4 °C with 3% paraformaldehyde in PBS. Following one washing with PBS, the stained cells were mounted with Elvanol mounting solution for microscopic evaluation.

Preparation of cell extracts for MAPK
Each treatment was performed four times. Following 15-min exposure to the various hormones, cells were washed twice with calcium- and magnesium-free cold PBS. Subsequently, 0·3 ml lysis buffer was added to each plate. Lysis buffer consisted of 20 mM HEPES, pH 7·5, containing 150 mM NaCl, 1% triton-X 100, 10% glyc- erol, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 10 mM β-glycerol phosphate, 2 mM phenylmethylsulfonyl fluoride and protease inhibitors (1 mM benzamidine, 2 mM sodium vanadate, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 10 µg/ml pepstatin). The plates were gently agitated at 4 °C for 10 min. The cells were then scraped from each plate, and transferred to Eppendorf tubes. After centrifugation of the tubes at 4 °C for 10 min at 14 000 g, the supernatants (lysates) containing total cell extracts were removed. The cell lysate corresponding to each treatment was combined and divided into three aliquots. One aliquot was used for protein determination with Coomassie blue using BSA as the standard. Another aliquot of the cell lysate was used for Western immunoblotting while the third aliquot was used for a two-site MAPK assay.

Two-site MAPK assay
Microtiter strips (Labsystems; Oy, Helsinki, Finland) were coated over 70 h at 4 °C with the general anti-MAPK antibody (2·5 µg/ml PBS, pH 7-4, 200 µl/well). The antibody solution from each well was then decanted, and the microtiter strips were blocked with 200 µl/well blocking buffer (PBS containing 2% BSA) for 2 h at room temperature. The microtiter strips were then washed twice with buffer after which the cell lysates were then transferred (100 µl/well) in triplicate to the microtiter wells. Assay buffer (100 µl) was then added to each well and the strips were incubated overnight at 4 °C and washed three times. Subsequently, Europium-labeled, activated anti-MAPK antibody (192 ng/well in 200µl assay buffer) was added, and the strips were incubated under shaking conditions for 2 h at room temperature. The strips were then washed four times and processed for time-resolved fluorescence as described previously (Somjen et al. 2002). This assay actually determines the net change due to the combined effects on both MAPK kinase.
and phosphatase activities. For the performance of the two-site MAPK assay, which assesses the net accumulation of phosphorylated MAPK resulting from both MAPK kinase and MAPK phosphatase activity, microtiter strips were coated over 70 h at 4 °C with the general anti-MAPK antibody (2·5 µg/ml PBS, pH 7·4, 200 µl/well). The antibody solution from each well was then decanted, and the microtiter strips were blocked with 200 µl/well blocking buffer (PBS containing 2% BSA) for 2 h at room temperature. The microtiter strips were then washed twice with buffer after which the cell lysates were then transferred (100 µl/well) in triplicate to the microtiter wells. Assay buffer (100 µl) was then added to each well and the strips were incubated overnight at 4 °C and washed three times. Subsequently, Europium-labeled, activated anti-MAPK antibody (192 ng/well in 200 µl assay buffer) was added, and the strips were incubated under shaking conditions for 2 h at room temperature. The strips were then washed four times and processed for time-resolved fluorescence as described previously (Amir-Zaltsman et al. 2000).

Western blot analysis

To evaluate the possibility that membrane-impermeant androgenic ligands activate MAPK activity just like free DHT per se, we determined total MAPK in treated vascular cells. General anti-MAPK rabbit antibody (ERK-2 (c-14), sc-154) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The 3,3',5,5'-tetramethyl-benzoidine (TMB) substrate kits for peroxidase and horse anti-mouse peroxidase were from Vector Laboratories (Burlingame, CA, USA).

For Western blot immunoblotting, equal amounts (30 µg) of cell extracts and molecular weight markers were subjected to SDS-PAGE as previously described (Somjen et al. 2002). Proteins were transferred to nitrocellulose membranes and stained with Ponceau Red to verify equal protein loading and transfer. After blocking with tris buffered saline (TBS) containing 1% goat serum or 1% BSA and washed three times, membranes were incubated overnight with the general anti-MAPK antibody (1 g/ml TBS containing 1% horse serum and 1% BSA) or TBS alone. Membranes were subsequently washed three times in Tween-TBS (T-TBS) and incubated with secondary antibodies for 2 h (horse anti-mouse peroxidase at 1:10 000 dilution) in T-TBS containing 1% horse serum and 1% BSA for membranes probed with the activated anti-MAPK antibody or the goat anti-rabbit peroxidase at 1:10 000 dilution in T-TBS containing 1% goat serum or 1% BSA for membranes probed with the general anti-MAPK antibody. Membranes were washed three times with T-TBS and processed for staining with TMB substrate for peroxidase. Signals were then quantified by densitometry using the Quantity 1 program of Bio-Rad (Hercules, CA, USA).

Statistical analysis

Differences between the mean values obtained from the experimental and the control groups were evaluated by analysis of variance (ANOVA). A P value less than 0·05 was considered significant. Values are means ± S.E.M.

Results

Effect of T-BSA on DNA synthesis in VSMC and ECV304 cells

When cells were incubated with increasing concentrations of DHT or T-BSA and DNA synthesis was measured, the following results were obtained. In VSMC, DHT at a low concentration stimulated DNA synthesis whereas a high concentration of DHT had an inhibitory effect. T-BSA induced similar, though somewhat smaller, stimulatory effects (200 ± 36% compared with 372 ± 36%), but comparable inhibitory effects with regard to DHT (Fig. 1). In ECV304 cells, DHT-induced a dose-dependent stimulation of DNA synthesis. This effect was not mimicked by T-BSA, which was completely inactive (Fig. 2).

Effect of T-BSA on CK-specific activity in VSMC and in ECV304 cells

When cells were incubated with increasing concentrations of DHT, there was a dose-dependent stimulation of CK-specific activity in both VSMC and ECV304 cells. In contrast, T-BSA was unable to affect CK activity in either VSMC or ECV304 cells (Figs 1 and 2).

Association of T-BSA with VSMC

The binding of Europium-labeled T-BSA conjugate to intact VSMC could be displaced by T-BSA but not by E2-BSA. (Table 1). Additionally, excess free DHT or testosterone had no effect on Europium-labeled T-BSA binding (Table 1).

Localization of membrane androgen-binding sites with the use of the membrane-impermeant T-BSA conjugate labeled with Cy3·5

Figure 3 depicts membranal staining generated by the association of T-BSA-Cy3·5 with membrane-binding sites for testosterone in VSMC cells. No staining was seen with the core CLAD-BSA-Cy3·5 complex lacking the testosterone molecule (data not shown).

Effect of androgenic ligands on MAPK in VSMC

The effects of DHT and T-BSA on total activated MAPK kinase in VSMC are summarized in Fig. 4, showing dose-dependent stimulation ranging from 0·03 to...
3000 nM. As analyzed by Western blot, DHT (at 300 nM) also stimulated p44 (ERK-2), but not p42 (ERK-1) (Fig. 5). In addition, the MAPK kinase inhibitor UO126 reduced basal $[^{3}H]$thymidine incorporation significantly (by 48 ± 15%) but entirely blocked the suppression of DNA synthesis induced by high concentrations of either DHT or T-BSA (Fig. 6). MAPK kinase inhibition also significantly attenuated the increase in DNA elicited by a low concentration of DHT (from 218 ± 11% to 125 ± 16%). Notably, MAPK kinase inhibition had no effect on basal CK-specific activity (−3 ± 3%) but significantly reduced DHT-induced CK activity (Fig. 6).

**Effect of androgenic ligands on MAPK in ECV304**

The effects of DHT on total activated MAPK kinase activity in ECV304 are summarized in Fig. 4, showing dose-dependent stimulation ranging from 0·03 to 3000 nM. As analyzed by Western blot, DHT (at 300 nM) also stimulated p44 (ERK-2) but not p42 (ERK-1) (Fig. 5). In addition, the MAPK kinase inhibitor UO126 reduced basal $[^{3}H]$thymidine incorporation slightly (by 50 ± 20%) but did not significantly inhibit DHT-induced stimulation of DNA synthesis, compared with the slightly reduced basal proliferation rate (Fig. 7). MAPK kinase inhibition slightly reduced basal CK-specific activity (by 23 ± 10%) but had no significant effect on the increase in CK-specific activity induced by DHT (Fig. 7).

**Discussion**

Recent reports indicate that the cardiovascular system is a target for direct effects of androgen hormones, and that some of these effects take place rapidly, thus suggesting non-genomic interactions. For example, testosterone rapidly increased coronary resistance and blocked adenosine-mediated vasodilation in the perfused rat heart (Figueroa-Valverde et al. 2002) and acutely inhibited bradykinin-induced relaxation in porcine coronary artery rings (Teoh et al. 2000). In contrast, androgens were reported to elicit vasodilation in the human and canine coronary arteries (Yue et al. 1995, Chou et al. 1996, Webb et al. 1999, Ding & Stallone 2001) and induce relaxation in the rat and rabbit aorta (Yue et al. 1995, Tep-areenan et al. 2003). These dichotomous results may reflect variation in the experimental setting and the type of vascular bed under study, interspecies differences and concentration-dependent effects (Teoh et al. 2000). Several different mechanisms appear to be involved in the rapid vasorelaxant effects of testosterone: inhibition of calcium transients and voltage-dependent calcium channels (Murphy &
Khalil 1999, Perusquia & Villalon 1999, Tep-areenan et al. 2003), activation of potassium channels (Chou et al. 1996, Tep-areenan et al. 2003) and activation of endothelial nitric oxide synthesis (e-NOS) (Chou et al. 1996, Tactum-Talom et al. 2002), all of which are consistent with non-genomic mechanisms. Notably, acute effects of testosterone in the vasculature are insensitive to androgen receptor antagonists (Teoh et al. 2000, Tep-areenan et al. 2003). Rapid activation of eNOS can also be induced by DHEA in endothelial cells, which cannot convert DHEA to either androgens or estrogens (Simoncini et al. 2003). Collectively, these reports strongly support the presence of functional membrane-type androgen receptors in the vasculature, which differ from the classical androgen nuclear receptors in several aspects. Such a difference might explain why rapid testosterone-induced vasodilation is preserved in the testicular feminized Tfm mice which express mutant, non-functional androgen receptors (Jones et al. 2003).

The results of this study suggest that, in parallel to the non-genomic rapid effects of testosterone in the cardiovascular system, androgens also inhibit human VSMC growth via non-genomic interaction with membranal binding sites distinct from putative E₂ membrane receptors. Both testosterone and T-BSA suppressed DNA synthesis in VSMC, although the cell membrane-impermeant ligand T-BSA induced a significantly smaller inhibitory effect on DNA synthesis in VSMC compared with DHT per se (200 ± 36% vs 372 ± 36% respectively) (Fig. 1). This may be due to the lower affinity of T-3-CMO present in T-BSA, compared with DHT per se.

In contrast, the same ligand, apparently incapable of entering the cell following its association with the cell membrane receptor, was ineffective in inducing CK specific activity, presumably a genomic marker of androgenic effect. Of note is also the finding that, while T-BSA

![Figure 2](https://www.endocrinology.org/)

**Figure 2** The effects of DHT and T-BSA at 0.03–30 000 nM on DNA synthesis and CK activity in ECV304 cells. Cells were hormonally treated as described in the text. Results are means ± S.E.M. of 4–12 incubates from two to four experiments and are expressed as % change from basal [³H]thymidine incorporation or CK activity. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control (vehicle-treated) cells (ANOVA). Basal [³H]thymidine incorporation into DNA in ECV304 cells was 28 471 ± 3050 d.p.m./well. Basal CK specific activity in ECV304 cells was 0.066 ± 0.010 µmol/min per mg protein.

### Table 1 Total Europium-labeled BSA-T binding to VSMC cells.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>% binding</th>
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<tr>
<td>None</td>
<td>100 ± 8</td>
</tr>
<tr>
<td>T-BSA</td>
<td>48 ± 7**</td>
</tr>
<tr>
<td>Testosterone</td>
<td>98 ± 11</td>
</tr>
<tr>
<td>DHT</td>
<td>96 ± 14</td>
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<tr>
<td>E₂-BSA</td>
<td>77 ± 15</td>
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<tr>
<td>E₂</td>
<td>102 ± 8</td>
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Values are means ± S.E.M. of three experiments, each performed in triplicate.

Cells were incubated as described in Materials and Methods in the absence or presence of 500-fold excess of one of the following agents: none, T-BSA, E₂-BSA, testosterone, DHT or E₂. Results are expressed as Europium-labeled BSA-T binding in the presence of a defined competitor as a fraction (%) of Europium-labeled BSA-T binding in the absence of any competitor. **P < 0.01 in comparison with total binding (no competitors) (ANOVA).
inhibited VSMC growth, it had no effect on cell proliferation in the endothelial ECV304 cells, in which DHT

Figure 3  Fluorescent microscopy of VSMC cells incubated with a T-BSA-Cy3·5 conjugate. Diffuse staining of the external contour of the cells can be seen in the presence of the T-BSA-Cy3·5 complex; (a–d) different cells from four different cultures.

Figure 4  The effect of DHT (0.03–3000 nM) on total activated MAPK kinase in VSMC and ECV304 cells and of T-BSA (3–300 nM) on total activated MAPK kinase in VSMC. Results are the means ± S.E.M. of time-resolving fluorometric measurements of extracts from two to three incubates and are expressed as % stimulation in hormone-treated compared with control cells. *P<0.05, **P<0.01 compared with control values (ANOVA).
itself induced a substantial and dose-dependent increase in DNA synthesis. The finding that the inhibitory effects of DHT and T-BSA on DNA synthesis in VSMC could be completely blocked by the MAPK kinase inhibitor UO126 further suggests that this membrane-associated transduction pathway is involved in the inhibition of VSMC growth. In contrast to these apparently membrane-related effects in VSMC, acceleration of ECV304 replication by DHT requires the entry of the active ligand into the cell, as it cannot be reproduced when testosterone is linked to a membrane-impermeant protein. The possibility that endothelial cells from other sources may respond to androgens differently compared with the response pattern that we describe cannot be excluded. However, our results suggest that androgenic modulation of human vascular cell growth may be exerted in a cell-specific manner, apparently involving membrane receptors which mediate growth inhibition in VSMC, but utilizing the classical nuclear receptor route to accelerate growth in endothelial cells. DHT-induced inhibition of

Figure 5  (Upper panel) Western blot analysis of MAPK in control (C) and DHT (300 nM)-treated VSMC and ECV304 cells. (Lower panel) Densitometric measurements of the blots from three different experiments, expressed as % change in hormone-treated compared with vehicle-treated control cells of the level of p42 (ERK-1) and p44 (ERK-2). **P<0.05 compared with control values (ANOVA).

Figure 6  Effects of the MAPK kinase inhibitor UO126 (1 µM) on [3H]thymidine incorporation and CK-specific activity in the absence and presence of DHT (30 (shaded bars) or 300 nM (solid bars)) or T-BSA (3000 nM; cross-hatched bars) in VSMC. Results are expressed as % change in [3H]thymidine incorporation and CK-specific activity compared with vehicle-treated control cells (open bars). Results are the means ± S.E.M. of measurements from two to three incubates from three different experiments. Basal level of [3H]thymidine incorporation into DNA was 8471 ± 1050 d.p.m./well. Basal CK-specific activity was 0.067 ± 0.018 μmol/min per mg protein. UO126 decreased DNA synthesis by 48 ± 15% (P<0.05) and CK specific activity by 3 ± 3% (non-significant change). *P<0.05, **P<0.01 compared with control values (ANOVA).
VSMC growth is, to our knowledge, the first example of a non-rapid, sustained effect mediated via membranal androgen-binding sites in vascular tissue.

Our study has provided direct evidence for the presence of membrane androgen-binding sites in human VSMC, using two unrelated experimental approaches, i.e. the use of Europium-labeled T-BSA in a competitive binding assay in intact VSMC, and visualization of membrane binding with a membrane-impermeant, fluorescent dye-labeled testosterone conjugate. The observations that Europium-labeled T-BSA binding to human vascular cells examined in the present study can be significantly blocked by excess of T-BSA but not by E2-BSA, suggest receptor specificity of the membrane-binding site for testosterone. Of note is the fact that the free steroid hormones, either estradiol-17β, or testosterone or DHT, cannot block this binding.

In conclusion, membrane receptors for androgens apparently mediate testosterone-dependent inhibition of human VSMC growth. Elucidation of the membrane-related signaling linking the occupation of these putative receptors to the activation of MAPK-dependent pathways awaits further investigation.

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