



Dihydrotestosterone and estradiol-17 β mutually neutralize their inhibitory effects on human vascular smooth muscle cell growth *in vitro*

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ABSTRACT

We reported previously that high concentrations of either estradiol-17 β (E₂) or dihydrotestosterone (DHT) inhibit growth of human cultured vascular smooth muscle cells (VSMC), mediated by cell membrane receptors and MAP-kinase-kinase activity (MEK). We now tested whether the presence of the opposite gender's dominant sex hormone modifies these effects. We incubated VSMC with various concentrations of E₂ and DHT or protein bound hormones (E₂-BSA or T-BSA), alone or in various combinations. High concentration of E₂ or E₂-BSA inhibited VSMC growth and stimulated MEK. In the presence of 3 nM DHT, high concentration of E₂ no longer inhibited ³[H] thymidine incorporation or increased MEK. Moreover, when high DHT concentration (300 nM) was added to VSMC exposed to high E₂, VSMC growth actually increased without change in MEK. DHT at 300 nM suppressed VSMC growth and increased MEK while 0.3 nM E₂ had only marginal effect on this interaction, and 30 nM E₂ reversed the inhibitory effect of DHT on cell growth. The inhibitory effects of both E₂ and DHT on VSMC cell growth and the stimulation of MEK was apparently mediated by cell membrane receptors, as it persisted when bovine serum albumin (BSA)-bound hormones were used. Further, inhibition of VSMC growth induced by E₂-BSA was reversed in the presence of T-BSA and vice versa. These results suggest that while female and male sex hormones affect VSMC growth similarly, they interfere in a dose-, hormone- and MEK-dependent manner with each other's effect.

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1. Introduction

Despite the fact that randomized clinical trials in post-menopausal women have found no cardiovascular benefits in the use of estrogen replacement therapy (ERT), and not withstanding the potential role of older age in the negative impact of ERT in studies such as the Women Health Initiative (1), interest in the effects of estrogens in the vasculature has not subsided. As the apparent contradiction between these studies and the gender-related protection from cardiovascular morbidity in pre-menopausal women continues to raise attention, there is also a surging interest in the interaction between androgens and cardiovascular function and disease. Recent analyses indicate that hypogonadal men may be more susceptible to atherosclerosis [1–3]. Some experimental findings may be congruous with this understanding. For example, just like estradiol, testosterone can act as a vasodilator in a variety of arterial beds, including the coronary and pulmonary

circulation, presumably by blocking calcium channels in vascular smooth muscle [4]. Further, testosterone confers symptomatic benefits in patients with coronary heart disease and heart failure [5,6] presumably through its action as a vasodilator. Much like estradiol, testosterone can also increase nitric oxide availability and improve flow-mediated dilation in hypogonadal men [7,8]. Finally, just as estradiol-17 β (E₂), testosterone was shown to exert anti-atherogenic effects in the cholesterol fed rabbit [9,10]. However, in the context of the gender-related difference in susceptibility to cardiovascular disease, these multiple potentially beneficial effects of testosterone do not simplify the understanding of the integrated role of sex hormones in vascular processes. On one hand, estrogens and androgens appear to exhibit both similar and opposing effects in the vasculature. On the other hand, neither androgen-free, nor estrogen-free environment exist in normal humans. For example, in healthy adults during the reproductive years, testosterone levels are in the range of 1–2 nmol/l in females and 9–30 nmol/l in men whereas estradiol-17 β concentrations range between 0.5 and 2 nmol/l in females and are less than 0.2 nmol/l in men [11,12]. It is therefore rather surprising that the joint effects of androgens and estrogens on vascular physiology remain entirely unstudied.

We have previously reported that both E₂ and dihydrotestosterone (DHT) exert a bimodal effect on human vascular smooth

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muscle cell proliferation, such that low concentrations of either of these hormones increase DNA synthesis, whereas high concentrations, either in their free form or as protein bound form inhibit VSMC growth [13–17]. The growth inhibitory effects, attained at high concentrations were dependent on binding to cell membrane receptors and mediated via MAP-kinase–kinase activity (MAPK or MEK) [13,14]. We have now used these findings as a model in which potential interaction between such vascular “estrogenic” and “androgenic” effects can be tested. At the center of the experiments in this study, then, is the question of whether or not the action of either androgens or estrogens is modified by the presence of the opposite gender hormone.

2. Experimental methods

2.1. Reagents

All reagents used were of analytical grade. Chemicals, E₂, DHT and the creatine kinase assay kit were purchased from Sigma Chemicals Co. (St. Louis, MO). All tissue culture products were obtained from Biological Industries (Beit Haemek, Israel).

2.2. Cell culture

Vascular smooth muscle cells (VSMC) were prepared from human umbilical artery as previously described with minor modifications [14,16]. In brief, umbilical cords were collected shortly after delivery and arteries were dissected, cleaned of blood and adventitia, and cut into tiny slices (1–3 mm). The segments were kept in culture in Medium 199 containing 20% fetal calf serum, glutamine, and antibiotics. Cell migration was detected within 5–7 days. Cells were fed twice a week and, on confluence, trypsinized and transferred to 24-well dishes using charcoal stripped serum. Cells were used only at passages 1–3 when expression of smooth muscle α -actin was clearly demonstrable [14,16].

2.3. Preparation of macromolecular conjugates

Estradiol-6-(O)-carboxymethyl oxime (E₂-6-CMO) [16] was conjugated to ovalbumin (Ov) via a two-step reaction as described previously [15] and the testosterone–BSA conjugate (T–BSA) was prepared as reported by us elsewhere [15]. UV analysis of the conjugates indicated that the conjugates contained 10 moles of hapten per mole of protein. The conjugates are pure with no measurable free hormones.

2.4. Hormonal treatment

At sub confluence cells were treated for 24 h with 0.3 or 30 nM E₂ and/or 3 or 300 nM DHT, after which DNA synthesis or creatine kinase specific activity (CK) was determined. MAPK activity was assessed as previously described following 15 min of exposure to the various steroid hormones [18]. Concentrations of the protein-bound hormones were 10-fold higher in most experiments, except for some experiments with E₂–BSA, since the effects of 30 and 300 nM E₂–BSA were practically identical.

2.5. Assessment of DNA synthesis

Cells were grown until sub confluence and then treated with various hormones as indicated. Twenty-two hours later, [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 of 0.3N NaOH, samples were aspirated and [³H] thymidine incorporation into DNA was determined [19].

2.6. Creatine kinase extraction and assay

To compare the effect of the various hormones on growth to the more classical effects of E₂, we measured creatine kinase brain type specific activity, an established genomic response marker of E₂. Cells were treated for 24 h with the various hormones as specified, and were then scraped off the culture dishes and homogenized by freezing and thawing three times in an extraction buffer as previously described [16]. Supernatant extracts were obtained by centrifugation of homogenates at 14,000 \times g for 5 min at 4 °C in an Eppendorf microcentrifuge. Creatine kinase activity (CK) was assayed by a coupled spectrophotometric assay described previously [16]. Protein was determined by Coomassie blue dye binding using BSA as the standard.

2.7. Europium labeled reagents

Anti-activated MAPK antibody (0.2 mg IgG in 0.9 ml PBS) was dialyzed against 50 mM carbonate/bicarbonate buffer for 2 h. Europium labeling reagent (300 nmoles in 100 μ l of water) was then added. The reaction mixture was incubated overnight at 4 °C and the labeled protein was purified by gel filtration on Sephadex G-25 M. The europium labeled antibody was eluted with 50 mM Tris–HCl buffer (pH 7.5) and stored at 4 °C until use [19,20].

2.8. Preparation of cell extracts for MAP kinase

Each treatment was performed in quadruplets. Cells were grown until sub confluence and then tested with various hormones or their conjugates as indicated. Effects on MAP kinase were tested in cells 5 days after the medium has been changed, at a time in which replacement with fresh medium would have taken place. Following 15 min exposure to the various hormones, cells were washed twice with calcium-free and magnesium-free cold phosphate buffered saline (PBS). Subsequently, 0.3 ml of 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% glycerol, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 10 mM β -glycerol phosphate, 2 mM phenylmethylsulfonyl fluoride and protease inhibitors (1 mM benzamide, 2 nM sodium vanadate, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin and 10 μ g/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 \times g, the supernatants (lysates) containing total cell extracts were removed. The cell lysate corresponding to each treatment was divided into three aliquots. One aliquot was used for protein determination with Coomassie blue using BSA as the standard; the second aliquot was used for Western immunoblotting and the third, for a two-site MAP-kinase assay [15,16].

2.9. Two-site MAP-kinase assay

Microtiter strips (Lab systems, Oy, Helsinki, Finland) were coated over 70 h at 4 °C with the general anti-MAP kinase antibody (2.5 μ g/ml PBS, pH 7.4, 200 μ l/well). The antibody solution from each well was then decanted, and the microtiter strips were blocked with 200 μ l/well blocking buffer (PBS containing 2% BSA) for 2 h at room temperature. The microtiter strips were then washed twice with buffer and the cell lysates were transferred (100 μ l/well) in triplicate to microtiter wells. Assay buffer (100 μ l) was subsequently added to each well and the strips were incubated overnight at 4 °C and washed three times. Europium labeled, activated anti-MAP kinase antibody (192 ng/well in 200 μ l of assay buffer) was then added, and strips were incubated under shaking conditions for 2 h at room temperature. Strips were washed four times and processed for time-resolved fluorescence as described previously

[15,16]. This assay actually determines the net change due to the combined effects on both kinase and phosphatase activities.

2.10. Expression of mRNA of ER α and ER β by real time PCR in vascular cells

RNA was extracted from cultured VSMC and was subjected to reverse transcription as previously described [19,20]. For ER α , we used 5 μ l of cDNA in the reaction mixture with the primers 5'-AA-TTCTGACAATCGACGCCAG-3' (forward) and 5'-GTGCTTCAACATTCT-CCCTCTC-3' (reverse), for 30 cycles at 94 °C for 30 s, at 57 °C for 30 s and at 72 °C for 1 min. For ER β , the same amount of cDNA was used with the primers 5'-TGCTTTGGTTGGGTGATTGC-3' (forward) and 5'-TTTGCTTTTACTGCTCCTGCG-3' (reverse) for 30 cycles at 94 °C for 30 s, at 58 °C for 30 s and at 72 °C for 1 min. ER α and ER β cDNA will be used as standard controls.

2.11. 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.

3. Results

3.1. Effects of growth-inhibitory doses of E₂ and DHT on DNA synthesis in VSMC in the presence and absence of the other hormone

As shown in Fig. 1 both E₂ and DHT, at high concentrations (30 and 300 nM, respectively) inhibited DNA synthesis in VSMC. When E₂, at the same concentration which, in itself, elicited inhibition of [³H] thymidine incorporation of ~48% was incubated with VSMC in the presence of DHT, this growth inhibitory effect was eliminated (~5% stimulation) and even reversed in a dose-related

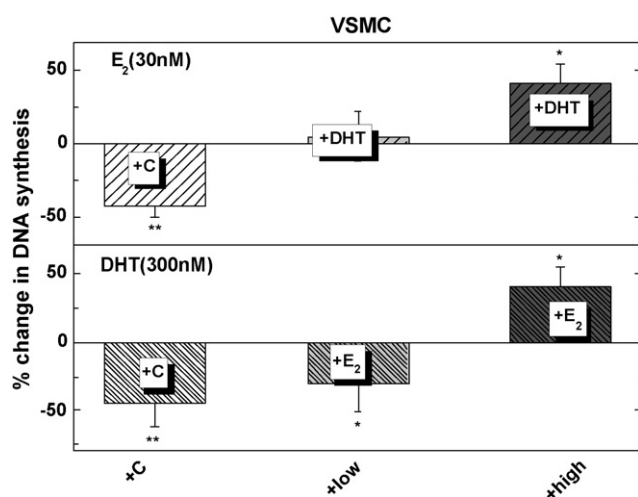


Fig. 1. The combined effect of high and low concentrations of estradiol-17 β (E₂) and DHT on DNA synthesis in cultured vascular smooth muscle cells (VSMC). Cells were treated for 24 h with vehicle or 0.3 or 30 nM E₂ as well as with 3 or 300 nM DHT, either alone or in combination with the “opposite hormone”. The upper panel depicts changes in DNA synthesis in cultured cells exposed to a high E₂ concentration (30 nM), either in the absence of DHT (left bar) or in the presence of low (3 nM; middle bar) or high (300 nM; right bar) concentration of DHT. The lower panel shows changes in DNA synthesis in cultured cells exposed to a high DHT concentration (300 nM) either in the absence of E₂ (left bar) or in the presence of low (0.3 nM; middle bar) or high (30 nM; right bar) concentration of E₂. Results are means \pm S.E.M. for *n* = 5 for each group. Experimental means compared to control means: **P* < 0.05; ***P* < 0.01. Mean basal [³H] thymidine incorporation was 7200 \pm 1080 dpm/well.

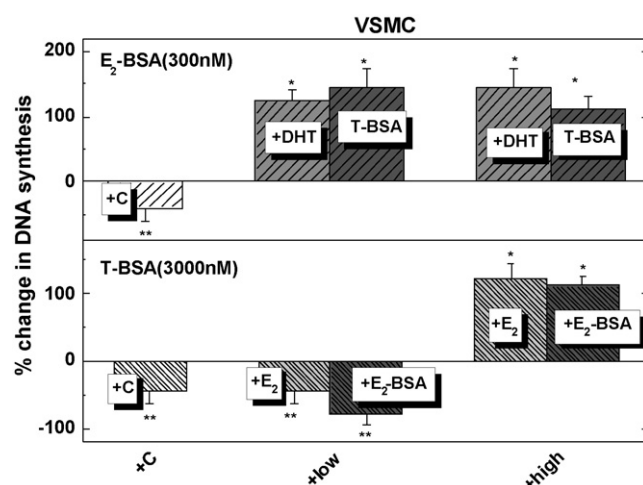


Fig. 2. The effect of various concentrations of free or protein bound-E₂, E₂-BSA and DHT or testosterone-BSA (T-BSA) on DNA synthesis cultured VSMC. Cells were treated for 24 h with the following agents alone or in combination with the “counter hormone” in its free or bound form: 3 or 300 nM E₂-BSA; 30 or 3000 nM T-BSA. Results in the upper panel are for cultured VSMC exposed to 300 nM E₂-BSA alone (left bar) or in the presence of low concentrations of free DHT (3 nM) or BSA-bound (30 nM) T (middle two bars) or a high concentration of free (300 nM) DHT or protein bound T (3000 nM) (right two bars). Results in the lower panel are for cultured VSMC exposed to 3000 nM T-BSA (left bar) only or in the presence of low concentrations of free E₂ (0.3 nM) or BSA-bound (3 nM)-E₂ (middle two bars) or a high concentration of free E₂ (30 nM) or protein bound E₂ (300 nM) (right two bars). Results are means \pm S.E.M. for *n* = 5 for each group. Experimental means are compared to control means: **P* < 0.05; ***P* < 0.01. Mean basal [³H] thymidine incorporation was 6950 \pm 880 dpm/well.

fashion: a low dose of DHT entirely neutralized the suppression of DNA synthesis; when VSMC were incubated in the presence of both a high concentration of E₂ (30 nM), and a high concentration of DHT (300 nM), the net effect was a 47% increase in [³H] thymidine incorporation. Low E₂ concentration (0.3 nM) did not appreciably affect the inhibitory effect of a high concentration of DHT on DNA synthesis (50 vs. 34% inhibition). The inhibitory effect of DHT (300 nM) was, however, entirely reversed in the presence of a high concentration of E₂, such the presence of a high concentration of both hormones, nullified the individual inhibitory effect of each of them, yielding a combined net ~50% increase in [³H] thymidine incorporation.

3.2. Effect of growth-inhibitory concentrations of macroprotein-bound estradiol-17 β and testosterone on DNA synthesis in VSMC in the presence and absence of the free and protein-bound form of the other hormone

Fig. 2 shows that when conjugated to BSA, high concentrations of either E₂ or T induce significant suppression of [³H] thymidine incorporation in VSMC (45% by both). These findings confirm, in essence, our previous reports [15–16]. The inhibitory effect of high concentrations of the conjugated hormones, however, is modified differentially in the presence of the free or BSA-bound “counter hormone”. When T-BSA was present at a concentration, which in itself inhibits DNA synthesis, the addition of a low concentration of either free- or BSA-bound E₂ did not modify its inhibitory effect (45 and 60%, respectively). However, in the presence of a high concentration of free or BSA-bound E₂, T-BSA no longer suppressed [³H] thymidine incorporation. Rather, the combined presence of high T-BSA and high concentrations of either free or BSA-bound E₂ resulted in an increase in DNA synthesis (110 and 105% respectively).

Unlike T-BSA, the inhibitory effect of E₂-BSA on DNA synthesis (45%) was eliminated by either free DHT or BSA-bound T at either low (110 and 125%, respectively) or high concentrations (125 and

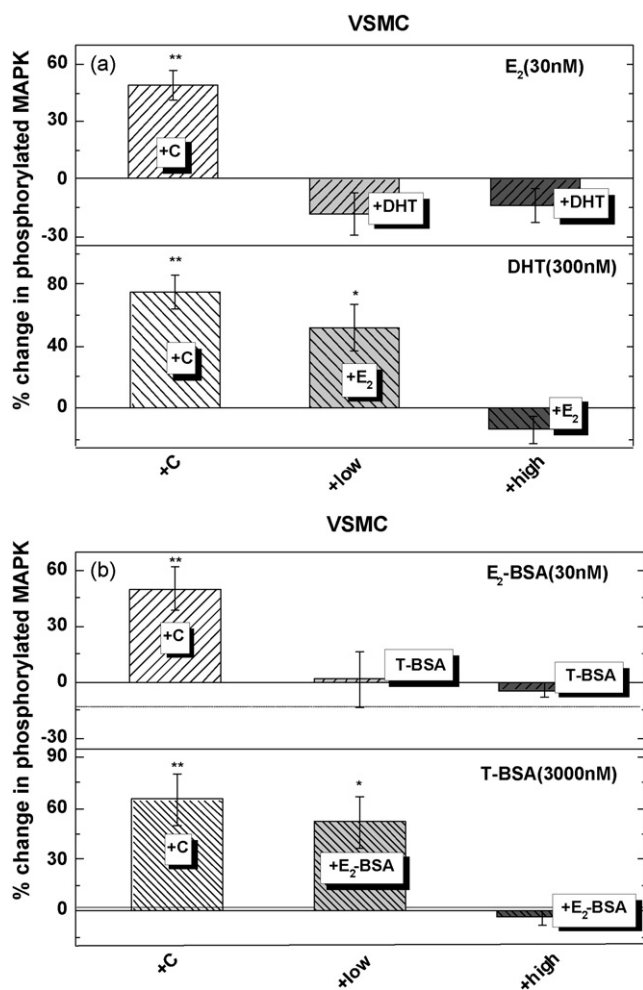


Fig. 3. (a) The effect of E₂ and DHT on the “opposite hormone”-induced phosphorylated-MAPK in cultured VSMC. Cells were treated for 15 min with one of the following: vehicle, 0.3 nM E₂, 30 nM E₂, 3 nM DHT, 300 nM DHT. Combinations of “opposite hormones” were also tested. The upper panel represents the effect on phosphorylated-MAPK of 30 nM E₂ in the absence (left bar) or presence of low (3 nM; middle bar) or high (300 nM; right bar) concentrations of DHT. The lower panel represents the effect on phosphorylated-MAPK of 300 nmol/l of DHT in the absence (left bar) or presence of low (0.3 nM; middle bar) or high (30 nM; right bar) concentrations of E₂. Results are means ± S.E.M. for n = 4 for each group. Experimental means are compared to control means: *P < 0.05; **P < 0.01. (b) The effect of protein bound E₂ (E₂-BSA) and testosterone-BSA (T-BSA) on phosphorylated-MAPK in the absence or presence of the bound form of the “opposite hormone” in cultured VSMC. Cells were treated for 15 min with one of the following: vehicle, 0.3 nM E₂-BSA, 30 nM E₂-BSA, 30 nM T-BSA and 300 nM T-BSA. Combinations of “opposite hormones” were also tested. The upper panel represents the effect on phosphorylated-MAPK of 30 nM E₂-BSA in the absence (left bar) or presence of low (30 nM; middle bar) or high (3000 nM; right bar) concentrations of T-BSA. The lower panel represents the effect on phosphorylated-MAPK of 3000 nM of T-BSA in the absence (left bar) or presence of low (3 nM; middle bar) or high (30 nM; right bar) concentrations of E₂-BSA. Results are means ± S.E.M. for n = 4 for each group. Experimental means are compared to control means: *P < 0.05; **P < 0.01.

110%, respectively), such that the combination of high BSA-bound E₂ with either low or high concentrations of T-BSA or free DHT, resulted in stimulation of [³H] thymidine incorporation.

3.3. Interaction of free and BSA-bound E₂ and DHT on MAPK in cultured VSMC

As shown in Fig. 3a, E₂ as well as DHT, at doses that inhibited DNA synthesis in cultured VSMC, also increased phosphorylated but not total MAPK. The stimulatory effect of E₂ on phosphorylated MAPK was inhibited by both low and high concentrations of DHT.

In contrast, the stimulatory effect of DHT on phosphorylated MAPK was inhibited by a high, but not by a low concentration of E₂.

Effects of protein bound sex hormones alone and in the present of the “opposite” BSA-bound hormone on phosphorylated MAPK are partly similar to the interactions observed with the free hormones (Fig. 3b). First, both E₂-BSA and T-BSA increase MAPK. Second, the stimulatory effect of E₂-BSA on phosphorylated MAPK was inhibited by both a low and a high concentration of T-BSA. Finally, the stimulatory effect of T-BSA on phosphorylated MAPK was inhibited by a high, but not by a low concentration of E₂-BSA.

3.4. Separate and combined effects of E₂ and DHT on brain type creatine kinase activity in cultured VSMC

The inhibitory effects of both E₂ and DHT on DNA synthesis in VSMC, which comprise the model in which the combined influence of both hormones is tested in this study, are “non-classical” in that they operate via the cell membrane. We therefore also tested the interaction of E₂ and DHT in the context of one established genomic effect, i.e., the induction of the brain type creatine kinase specific activity (CK). As shown in Fig. 4a and b, low concentrations of both E₂ and DHT elicit, each in itself, a ~35–40% increase in basal CK activity, which is markedly augmented by the presence of the other hormone: DHT, 3 nM increases the ~40% rise in CK induced by 0.3 nM E₂ to nearly 300% over baseline activity, which is not further increased with a higher (300 nM) DHT concentration. Similarly, DHT induces a small increase in CK activity, which is further increased 3–4-folds in the presence of 0.3 or 30 nM of E₂. When a high concentration of either E₂ or DHT is used by itself, the induction of CK activity is substantial (~200% for E₂, ~170% for DHT); is not modified by a low concentration of the other hormone; but is further markedly increased by a high dose of the “opposite” sex hormone.

3.5. Effects of E₂ and DHT on the mRNA expression of ERα and ERβ in cultured VSMC

One potential mechanism by which androgens could modify some estrogenic effects is via modulation of estrogen receptors (ERs). Further, estrogen-induced inhibition of VSMC growth may be mediated through ERβ [19,20]. As shown in Fig. 5, DHT but not E₂, decreased ERβ mRNA expression by 35%. Further, DHT also increased ERα mRNA expression by 28%. Estradiol-17β had no effect on ER expression in this setting.

4. Discussion

Very few studies have assessed the effect of estrogens in the presence of androgens in the vascular compartment. Wagner et al. reported that esterified estrogens with and without methyl testosterone decreased arterial LDL metabolism in cynomolgus monkeys *in vivo* [20,21]. In baby chicks, dietary E₂ produced accumulations of lipid vacuoles and precipitated death in smooth muscle cells whereas combined T and E₂ supplementation resulted in increased vascular smooth muscle cell mitosis and degeneration [22].

The inhibitory effect of E₂ on VSMC growth is of potential significant importance as it might minimize myointimal proliferation, an important process in the formation of atherosclerotic lesions which can be mimicked experimentally by balloon injury. Under the latter circumstances, estrogens were shown to alleviate the VSMC proliferative response which follows endothelial injury [23–25].

In VSMC derived from the human umbilical artery, inhibition of cell growth can be elicited by both E₂ and DHT in a concentration-related manner [14–17]. Indeed, low concentrations of both classes of sex hormones increase DNA synthesis, an effect which gradually wanes with increasing concentrations, reversing its direction to a

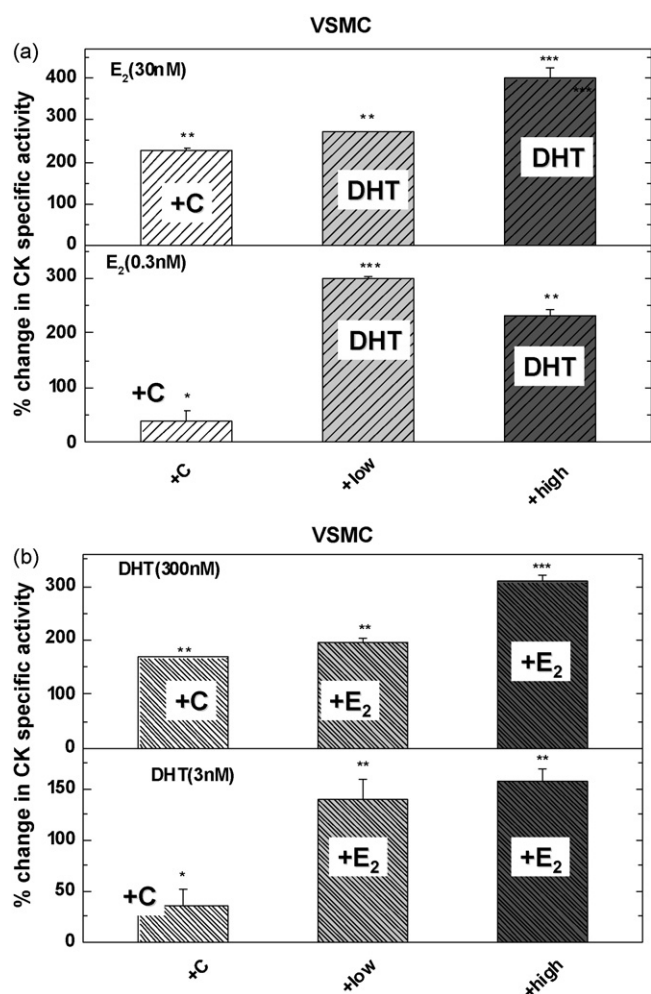


Fig. 4. Effects of E₂ and DHT on creatine kinase (CK) specific activity in the absence or presence of low or high concentrations of the “opposite hormone” in cultured VSMC. (a) Effect on CK of 30 nM E₂ (upper panel) or 0.3 nM E₂ (lower panel) in the absence (left bar) or presence of low (3 nM; middle bar) or high (300 nM; right bar) concentrations of DHT. Cells were treated for 24 h with vehicle or 0.3 or 30 nM of E₂ as well as with 3 or 300 nM of DHT either alone or in combination with the “opposite hormone”. (b) Effect on CK of 300 nM DHT (upper panel) or 3 nM DHT (lower panel) in the absence (left bar) or presence of low (0.3 nM; middle bar) or high (30 nM; right bar) concentrations of E₂. Cells were treated for 24 h with vehicle or 0.3 or 30 nM of E₂ as well as with 3 or 300 nM of DHT either alone or in combination with the “opposite hormone”. C–control; Results are means ± S.E.M. for n = 6 for each group. Experimental means compared to control means: *P < 0.05; **P < 0.01; ***P < 0.001. Basal specific activity was 25 ± 4 nmol/(min mg) protein.

growth inhibitory effect when high concentrations of either DHT or E₂ alone are present. In humans, circulating levels of testosterone are higher than those of estradiol-17β (~100-fold in men; 1–3-fold in reproductive females), and hence, “high” and “low” concentrations of each are used herein relative to physiologic concentrations (for testosterone, 1–2 nmol/l in females; 9–30 nmol/l in men; for estradiol-17β, 0.5–2 nmol/l in females; less than 0.2 nmol/l in men) [11,12].

The interaction between estrogens and androgens in VSMC is function-specific, yielding a dichotomous outcome when brain specific CK activity and cell growth are under investigation. In the case of the induction of brain specific CK activity, a marker of the genomic action of E₂, formerly known an “estradiol-inducible protein”, which is also induced by androgens, the effects of E₂ and DHT were not only consistently stimulatory across hormone type, hormone combination and concentrations used, but also additive or even synergistic. However, with respect to growth, E₂ and DHT

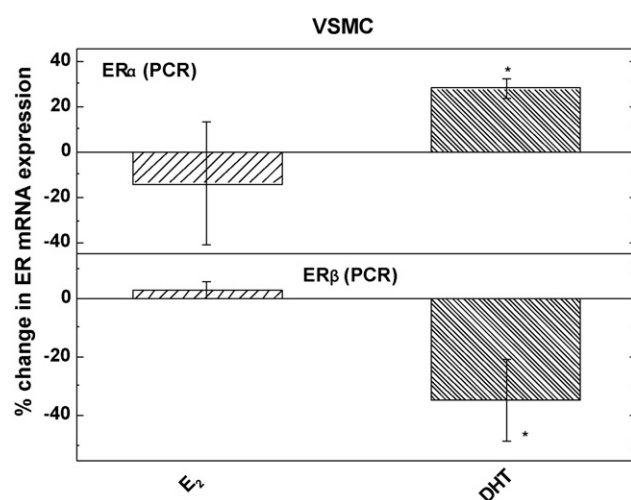


Fig. 5. The effect of E₂ or DHT on the mRNA expression of ERα and ERβ in cultured VSMC. RNA was extracted from cells following daily treatments for 3 days of 30 nM E₂ or 300 nM DHT, and was subjected to reverse transcription as described in Section 2. Results are expressed as % stimulation for six extracts. Experimental means compared to control means: *P < 0.05. The levels of ERα are 0.0032 × E - 4 and ERβ is 0.0032 × E - 4 2^{-Δct} (as measured for mRNA expression by realtime PCR) and the ratio of ERα: ERβ is 2.7:1.

mutually neutralized each other’s inhibitory effect on DNA synthesis in VSMC.

The neutralizing effect of E₂ and DHT on each other’s inhibitory effect on VSMC growth can be assessed in light of previous information on the mechanisms by which this inhibitory influence is exerted [14–17]. Although both E₂ and DHT activate MAPK to inhibit DNA synthesis in umbilical artery VSMC [15,16], concomitant effects of these hormones on MAPK in the present study are not fully concordant with the effects on [³H] thymidine incorporation. Parallel effects are seen when a low concentration of DHT added to a high, growth-inhibitory concentration of E₂, resulting in elimination of both MAPK phosphorylation and loss of inhibitory effect on DNA synthesis. Another example of similar effects is the joint presence of a low concentration of E₂ and a high DHT concentration, in which neither MAPK activation, nor the inhibition of [³H] thymidine incorporation is affected. However, the induction of VSMC growth by high E₂/high DHT is not accompanied by further changes in MAPK activation.

In terms actual hormone concentration, sex hormone-dependent inhibition on VSMC growth appears more sensitive to the presence of the counter-hormone in a predominantly estrogenic environment (“female type”) than in an androgenic (“male type”) milieu, because relatively low androgen concentrations interfere with estradiol-17β’s effects whereas only high estrogen levels can lead to the loss of the androgenic growth inhibitory influence. In this sense, it matters little whether the dominant growth inhibitory effect is exerted by free or bound hormones, again suggesting that inhibition of DNA synthesis under these circumstances is at least initiated at the membrane interface.

When DHT reverses the growth inhibitory effect of E₂, one additional effect may be involved, since DHT alters the relative abundance of ERβ/ERα mRNA expression in VSMC, by increasing ERα and suppressing ERβ expression (Fig. 5). Although Nakamura et al. reported that E₂ was able to inhibit proliferation of ERα positive VSMC but not ERβ positive VSMC [13], other studies in a different type of VSMC indicate that the inhibitory effect of E₂ on vascular smooth muscle cell proliferation is mediated through ERβ [24]. Thus, DHT-induced alteration in ER isoforms expression could play some ancillary role in our studies. Nevertheless, these changes are modest and their actual contribution to the response to estradiol in

the presence of DHT is presently uncertain. Finally, the possibility that androgen receptor expression or activity could be affected by estrogens was not examined by us.

In summary, although the “estrogen to androgen ratio” has been often utilized to explain tissue responses in classical target organs for sex hormones, the combined effects of estrogens and androgens in VSMC has not been properly explored. Our results suggest that such combined effects in VSMC are function-specific. In the case of one of the most extensively studied and presumably beneficial effects of estradiol, i.e., inhibition of VSMC proliferation, this effect is reversed by DHT in a concentration-related manner, such that stimulation – and not inhibition – of DNA synthesis is elicited in high estrogen-high androgen environment. Because neither androgen-free, nor estrogen-free milieu exist in genetically normal humans, the interaction between estrogenic and androgenic effects in vascular cells merits further studies.

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