Effects of Gonadal Steroids and Their Antagonists on DNA Synthesis in Human Vascular Cells

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Abstract—The cardiovascular effect of estrogen is currently under intense investigation, but the role of androgens in vascular biology has attracted little attention. Because endothelial repair and vascular smooth muscle cell (VSMC) proliferation affect atherogenesis, we analyzed the effects of 17β-estradiol (E2), dihydrotestosterone (DHT), and sex hormone antagonists on DNA synthesis in human umbilical VSMCs and in E304 cells (a human umbilical endothelial cell line). In VSMCs, both E2 and DHT had a biphasic effect on [3H]thymidine incorporation into DNA: low concentrations (0.3 nmol/L for E2, 3 nmol/L for DHT) stimulated [3H]thymidine incorporation (+35% and +41%, respectively), whereas high concentrations (30 nmol/L for E2, 300 nmol/L for DHT) inhibited [3H]thymidine incorporation (−40%). In contrast, E2 (0.3 to 300 nmol/L) and DHT (3 to 3000 nmol/L) dose-dependently enhanced [3H]thymidine incorporation in E304 cells (peak, +85% for both). In VSMCs, high concentrations of E2 and DHT inhibited platelet-derived growth factor (PDGF)– or insulin-like growth factor (IGF-1)–induced DNA synthesis (−50% to 80%), whereas PDGF- or IGF-1–dependent DNA synthesis in E304 cells was further increased by E2. The antiestrogens tamoxifen and raloxifene mimicked the effects of E2 on DNA synthesis in both VSMCs and E304 cells. However, when coincubated with a stimulatory concentration of E2 (0.3 nmol/L), tamoxifen and raloxifene blocked E2-induced [3H]thymidine incorporation in E304 cells but not in VSMCs. Finally, the androgen antagonist flutamide inhibited the biphasic effects of DHT on VSMCs and blocked the increase in DNA elicited by DHT in E304 cells. The results suggest complex, dose-dependent, and cell-specific interactions of estrogens, androgens, and their respective antagonists in the control of cellular proliferation in the vascular wall. Gonadal steroid–dependent inhibition of VSMC proliferation and stimulation of endothelial replication may contribute to vascular protection and remodeling responses to vascular injury. (Hypertension. 1998;32:39-45.)

Key Words: estradiol ■ dihydrotestosterone ■ muscle, smooth, vascular ■ tamoxifen ■ raloxifene ■ flutamide

Animal and human studies indicate that estrogens are protective against coronary atherosclerosis. Multiple potential mechanisms may be involved, such as favorable effects on lipids,1 fibrinolysis,2 coronary flow,3,4 and myocardial contractility.5 Although recent evidence suggests the existence of both estrogen and androgen receptors in VSMCs and endothelial cells,6 the precise biological role of sex hormones in either cell type is currently incompletely understood. There are well-known examples of estrogenic and/or androgenic control of cell growth and proliferation in classic target organs for sex hormone action. Because endothelial repair and VSMC proliferation have defined pathophysiological roles in vascular injury and atherogenesis, the potential modulation of such processes by sex hormones is of obvious interest. In several tissues, the effects of estrogen, including growth modulation, are linked to the induction of CK activity, and this has been used as a general genomic response marker for gonadal steroids. CK is involved in cellular energy buffering and is closely related to changes in cell replication rate in various cell types.7–9

The present study was undertaken to explore the effects of estrogens and androgens on DNA synthesis and CK activity in human endothelial cells and VSMCs. The results are consistent with complex, dose-dependent, and cell-specific in vitro effects of both E2 and DHT on proliferative activity in the vascular wall.

Methods

Reagents

E2, DHT, progesterone, IGF-1, and PDGF were purchased from Sigma Chemical Co. TAM was the gift of Dr A. Wakeling (Xeneca Ltd, Macclefield, UK). TMI was obtained from Dr A. Beigon (Pharmos Ltd, Rehovot, Israel). RAL was the gift of Dr B. Fournier (Ciba-Geigy Ltd, Basel, Switzerland). The antidiotopic antiestrogen antibody 1D5 and its F(ab’)-, and Fab’- fragments were prepared by us, as previously described.10 The monoclonal antiestradiol antibody E15 was raised in mice by us and characterized as previously described.11 The Fab’ monomer of E15 was prepared and analyzed as reported elsewhere.11

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Selected Abbreviations and Acronyms

CK = creatine kinase
DHT = dihydrotestosterone
E₂ = 17β-estradiol
IGF-1 = insulin-like growth factor-1
PDGF = platelet-derived growth factor
RAL = raloxifene
TAM = tamoxifen
TMI = tamoxifen methiodide
VSMC = vascular smooth muscle cell

Cell Cultures

Umbilical Artery Smooth Muscle Cells
The use of human umbilical cords was approved by our institution’s human subject committee. Umbilical VSMCs were prepared as previously described with minor modifications. In brief, umbilical cords were collected shortly after delivery, and arteries were dissected, cleaned of blood and adventitia, and cut into tiny slices (1 to 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 to 7 days. Cells were fed twice a week and, on confluence, trypsinized and transferred to 24-well dishes. Cells were used only at passages 1 to 3 when expression of smooth muscle actin was clearly demonstrable.

Endothelial Cells
E304 cells, an endothelial cell line derived from a human umbilical vein, were obtained from American Type Culture Collection and grown in Medium 199 containing 10% fetal calf serum, glutamine, and antibiotics.

Detection of Estrogen Receptors
Cultured cells were washed with 0.5 mL cold methanol, fixed by incubating the cells with 1 mL methanol at −20°C, and then washed twice with 1 mL of cold PBS for 5 minutes at 0°C. The cells were then incubated with 250 μL FITC-labeled monoclonal antiestrogen antibody 1D6 directed against the steroid binding domain of the estrogen receptor for 1 hour at 0°C in the dark. After the incubation, cells were washed twice with cold PBS for 5 minutes. The labeled receptors were visualized by fluorescence microscopy. Control cultures were incubated in the same way with FITC-labeled IgG.

Assessment of DNA Synthesis
Cells were grown until subconfluence and then treated with various hormones or agents as indicated. Twenty-two hours later, [³H]thymidine (thymidine [methyl-³H], 50 Ci/mmol, 1 μCi/mL, ICN Pharmaceutical Inc) was added for 2 hours. Cells were then treated with 10% ice-cold trichloroacetic acid (TCA) for 5 minutes and washed twice with 5% TCA and then with cold ethanol. The cellular layer was dissolved in 0.3 mL of 0.3N NaOH, the samples were collected, and [³H]thymidine incorporation into DNA was determined. Comparisons between the control and various treatments were made by ANOVA.

Statistical Analysis

Results

Estrogen Receptors
As shown in Figure 1, incubation of VSMCs and E304 cells with the FITC-labeled 1D6, an antibody that binds to the estrogen receptor, resulted in nuclear labeling (Figure 1), whereas control cells incubated with FITC-labeled IgG showed no staining. In most cells, labeling was entirely confined to the nucleus. However, occasional cytoplasmic (but not membranous) staining was also seen.

Modulation of DNA Synthesis by Gonadal Steroids

Endothelial Cells
Both E₂ (0.3 to 300 nmol/L) and DHT (3 to 3000 nmol/L) induced dose-dependent increases in [³H]thymidine incorporation into DNA (Figure 2). As shown, cells were somewhat more sensitive to E₂ than to DHT (one order of magnitude). In contrast, progesterone (6 μmol/L) had no effect on DNA synthesis.

VSMCs
E₂ and DHT had a biphasic effect on DNA synthesis. At a low concentration of E₂ (0.3 nmol/L), [³H]thymidine incorporation was stimulated, whereas higher concentrations of E₂ (≥30 nmol/L) led to inhibition of DNA synthesis (Figure 2). Similar dose-related effects were seen with DHT, ie, stimulation with a low concentration (3 nmol/L) and inhibition with high concentrations (≥300 nmol/L). As with endothelial cells, [³H]thymidine incorporation into DNA was unaffected by progesterone.

Regardless of the variable effect on DNA synthesis, E₂ and DHT (but not progesterone) dose-dependently stimulated the specific activity of CK in both cell types (Figure 3).

Interaction Between Gonadal Steroids and PDGF or IGF-1

Endothelial Cells
PDGF or IGF-1 alone, as well as the combination of either growth factor with E₂ or DHT, increased [³H]thymidine incorporation in endothelial cells (Figure 4). At the low concentrations of gonadal steroids, no synergistic or additive effect was seen between E₂ or DHT and PDGF or IGF-1. However, [³H]thymidine incorporation was significantly increased (P<0.05) when E304 cells were exposed to the higher concentration of E₂ or DHT in the presence of either PDGF or IGF-1 alone compared with the effects of PDGF or IGF-1 alone (Figures 4 and 5). E₂ increased CK activity (0.3 nmol/L: 30±3%; 30 nmol/L: 90±11%). CK activity was likewise stimulated by PDGF (1 ng/mL: 30±5%; 5 ng/mL: 52±11%), and IGF-1 (12.5 ng/mL: 43±13%; 25 ng/mL: 52±3%). In the presence of E₂ (30 nmol/L), both PDGF (5 ng/mL) and IGF-1 (25 ng/mL) elicited even larger increments in CK (107±10% and 93±2%, respectively). DHT also increased CK activity (at 3 nmol/L: 38±5%; at 300 nmol/L: 56±4%). However, the effect of DHT (300 nmol/L) was not modified in the presence of PDGF (1 or 5 ng/mL, 53±9% and 70±11%, respectively).
The separate effects of E₂ (0.3 and 30 nmol/L), PDGF (1 and 5 ng/mL), or IGF-1 (12.5 and 25 ng/mL) on DNA synthesis in VSMCs are shown in Figure 4. Stimulation of [³H]thymidine incorporation was induced by either of these growth factors and by the low dose of E₂. However, when E₂ (30 nmol/L) was present, the stimulatory effects of IGF-1 and PDGF were entirely inhibited. Furthermore, growth factor–induced DNA synthesis was suppressed to below basal levels. CK-specific activity was increased by E₂ (0.3 nmol/L: 20±4%; 30 nmol/L: 74±13%), IGF-1 (12.5 ng/mL: 25±7%; 25 ng/mL: 72±9%), or PDGF (1 ng/mL: 44±10%; 5 ng/mL: 133±29%), as well as by the combinations of E₂ (30 nmol/L) with PDGF (43±13% and 52±3% at the two concentrations applied) or IGF-1 (84±7% and 107±10% at the two concentrations applied). DHT (at 300 nmol/L but not at 3 nmol/L) inhibited PDGF-dependent DNA synthesis (Figure 5). In contrast,
CK activity was stimulated by DHT alone (3 nmol/L: 54±11%; 300 nmol/L: 83±9%), PDGF alone (1 ng/mL: 41±12%; 5 ng/mL: 48±9%), or by their combination (300 nmol/L DHT+PDGF: 68±9% and 122±19%, respectively), although no additive effect was observed.

**Effect of Antiestrogens and Flutamide on DNA Synthesis**

**Endothelial Cells**
In E304 cells, both TAM (30 nmol/L and 3 μmol/L) alone or RAL (30 nmol/L and 3 μmol/L) alone stimulated DNA synthesis, whereas TMI was ineffective (Figure 6). When E₂ was also added to antiestrogen-containing dishes (TAM, RAL, or TMI at 100-fold excess), this agonist-like effect of antiestrogens on DNA synthesis was not discernible (Figure 6). Whereas the antiandrogen flutamide was ineffective by itself, it inhibited the DHT-dependent increase in DNA synthesis (Figure 7).

**VSMCs**
In VSMCs, the antiestrogen TMI had no effect, whereas both TAM and RAL mimicked the effect of E₂, exhibiting a stimulatory effect at a low dose (30 nmol/L) and an inhibitory effect at a higher dose (3 μmol/L). Notably, in the presence of a low concentration of E₂, the antiestrogens (at 100-fold excess) produced an agonist-like effect of E₂ on DNA synthesis in VSMCs.
DNA synthesis (Figure 9) or CK-specific activity in either cell type. However, its (Fab') proteolytic fragment, which was likewise inactive on its own, reversed E2-dependent suppression of [3H]thymidine incorporation in VSMCs and E2-induced stimulation of DNA synthesis in E304 cells (Figure 9). CK-specific activity was enhanced by 30 nmol/L of E2 (76±12%). E15 (2.5 µg/mL) had no effect on basal (−11±13%) or E2-stimulated CK activity (68±12%). Whereas its Fab′ fragment (2.5 µg/mL) likewise had no independent effect (14±7%), it entirely blocked the effect of E2 (2±4%).

**Discussion**

In the present report, we focus on one aspect of the interaction of gonadal steroids with the vasculature, ie, the effects of sex hormones and their antagonists on DNA synthesis in endothelial and smooth muscle cells derived from human umbilical vessels. Previous investigations indicated that the myointimal proliferative response to balloon injury in the rat carotid artery was inhibited by estrogens but not by androgens, thus suggesting a differential effect on the response to injury.14,15 The effects of estradiol on DNA synthesis in our study are consistent with the concept that estrogen might influence vascular wall remodeling by inhibition of VSMC proliferation and increasing the rate of reendothelialization. The inhibition of DNA synthesis in VSMCs by higher E2 concentrations is in accordance with previous reports in porcine and human cells.16,17

The specificity of estradiol’s interaction with VSMCs and E304 cells in the present work is supported by several findings. First, both cell types contained nuclear estradiol receptors. Second, under various experimental conditions the combined effects of estradiol and antiestrogens were consistent with competitive inhibition. For example, both estradiol and TAM (or RAL) increased DNA synthesis, but in accordance with observations in other estrogen responsive tissues,14 this effect was not
apparent in the presence of both the agonist and the antagonist. Similarly, both estradiol and estrogen antagonists (TAM and RAL) increased CK activity in E304 cells and VSMCs, but these actions did not persist when estradiol and these antagonists were added simultaneously to the same cultures. Third, antiidiotypic antiestrogen antibodies shown in previous studies to possess estrogen-mimetic activity9 elicited a stimulatory effect on DNA synthesis and CK activity in E304 cells and inhibited [3H]thymidine incorporation in VSMCs, thus exerting the same cell-specific effects induced by estradiol itself. Fourth, the Fab fragment of the antiidiotypic antiestrogen antibody 1D3 inhibited the effects of estradiol on DNA synthesis in E304 cells as well as in VSMCs. Finally, the Fab′ proteolytic fragment of the high-affinity antiestrogen antibody E15 blocked estradiol-dependent effects on DNA synthesis and CK-specific activity in both E304 cells and VSMCs as observed in other cell types.11

Although we did not establish which type of estrogen receptor is involved, recent observations indicating that estradiol inhibits VSMC proliferation after balloon injury in transgenic mice lacking the α form of the estrogen receptor28 raise the possibility that at least some of the effects reported herein are related to the β-type receptor.

Our results indicate that overall, estradiol induces opposing effects on DNA synthesis in endothelial cells and VSMCs. However, in VSMCs, estradiol acts in a bimodal fashion, enhancing [3H]thymidine incorporation at very low concentrations (prepubertal and postmenopausal range) while inhibiting DNA synthesis at high concentrations such as those attained during the ovulatory phase. Whether this bimodal dose dependency is unique to cells derived from umbilical vessels or rather represents a general phenomenon in human arterial tissue must be addressed in further studies. Of note is the finding that regardless of whether DNA synthesis was stimulated or inhibited, CK activity was stimulated in a dose-dependent fashion in both cell types, thus suggesting energy-requiring responses.

The interaction between estradiol and two different growth factors acting in the vasculature, PDGF and IGF-1, further underscores the differential effect of estrogen in endothelial cells and VSMCs. Estradiol entirely blocked the increase in [3H]thymidine incorporation into DNA induced by PDGF and IGF-1, whereas in E304 cells these effects were further enhanced in the presence of estradiol. Because IGF-1 and PDGF are likely involved in the proliferative response of VSMCs to various stimuli such as angiotensin II, endothelial injury, and platelet aggregation, the potent antiproliferative effect of estradiol, reducing PDGF- and IGF-1–dependent DNA synthesis to below basal levels, may have considerable pathophysiological significance.

Although some antiestrogens may confer cardiovascular protection,21 little is known with respect to their mode of action in the vasculature. The finding that TAM and RAL indeed mimic the effect of estradiol on DNA synthesis in both endothelial cells and VSMCs suggests one potential mechanism by which their vascular effect might take place. Notably, however, not all estrogen-mimetic effects of antiestrogens are necessarily conserved in the presence of estradiol, as is exemplified by the loss of stimulation of [3H]thymidine incorporation when both agonists and antagonists are present.

To our knowledge, this is the first report of an androgenic inhibition of DNA synthesis in human VSMCs and stimulation in endothelial cells. The antiandrogen flutamide effectively blocked these effects while exerting no effect on its own. The observation that DHT modulates DNA synthesis in both VSMCs and endothelial cells in a manner that closely resembles the action of estrogen, albeit at a higher concentration range, was somewhat unexpected. Given that testosterone concentrations in vivo are 10 to 100 times higher than those of estradiol, DHT modulation of DNA synthesis in this study is operative at physiological levels. However, attempts to discern the effects of estrogens and androgens in the vasculature in vivo are inevitably complicated by the fact that androgen-free or estrogen-free environments practically do not exist in adult human subjects.

In summary, estradiol and DHT modulate DNA synthesis in human vascular cells in a parallel fashion. Their effects in VSMCs are bimodal, inducing stimulation at low concentrations and inhibition at high concentrations. In contrast, [3H]thymidine incorporation into DNA by cells of endothelial origin is positively influenced by both estradiol and DHT. Antiestrogens alone exert estrogen-mimetic effects, but their mode of action is more complex in the presence of estradiol. The interactions between estrogens, androgens, PDGF, and IGF-1 may be important for vascular wall repair and remodeling.

References


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