Role of Putative Membrane Receptors in the Effects of Estradiol on Human Vascular Cell Growth

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The present study was designed to determine whether some of the effects of estrogen on human vascular cell growth are exerted through membrane-binding sites, using native as well as novel protein-bound, membrane non-permeant estrogenic complexes. We measured changes in DNA synthesis and creatine kinase-specific activity (CK), after treatment with estradiol-17β (E₂), estradiol-17β-6-(O)-carboxymethyl oxime conjugated to bovine serum albumin (BSA) (E₂-BSA), 6-carboxymethyl genistein (CG) or 6-carboxymethyl genistein bound to the high molecular protein keyhole limpet hemocyanin (CG-KLH), and 7-(O)-carboxymethyl daidzein (CD) or 7-(O)-carboxymethyl daidzein linked to keyhole limpet hemocyanin (CD-KLH). High concentrations of either E₂ or E₂-BSA inhibited DNA synthesis in vascular smooth muscle cells (VSMC) (39% ± 23%, 28% ± 15%, 32% ± 16%, and 15% ± 7%, respectively). Estradiol as well as CG and CD increased DNA synthesis dose dependently in endothelial ECV-304 cells. The CG and CD, as well as CG-KLH and CD-KLH, stimulated DNA synthesis dose dependently in VSMC (66% ± 2%, 100% ± 17%, 66% ± 6%, and 41% ± 8% at 300 nmol/L, respectively). In contrast all forms of protein-bound hormones were unable to affect DNA synthesis in ECV-304 cells or CK in either cell type. In VSMC, both free and bound hormones increased mitogen-activated protein-kinase (MAPK)-kinase activity, which was blocked by UO126, an inhibitor of MAPK-kinase. Furthermore, the effects of E₂, E₂-BSA, or CG-KLH on DNA synthesis were inhibited by UO126. Using the E₂-BSA linked to the fluorescent dye Cy3.5, we directly demonstrated the presence of membrane-binding sites for E₂ in VSMC and ECV 304 cells. Hence, the effects of E₂ on DNA synthesis in human VSMC, but not in endothelial cells, are apparently exerted by membrane-binding sites for E₂ and do not require intracellular entry of E₂ through the classic nuclear receptor route. Am J Hypertens 2004;17:462–469 © 2004 American Journal of Hypertension, Ltd.

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There is growing evidence that several estrogen-dependent effects are induced through cell membrane-associated signaling rather than by the classic nuclear receptor route of steroid hormone action. In the vasculature, reported rapid effects of estrogen such as acute vasodilation in vivo and estrogen receptor (ER) α-mediated release of nitric oxide (NO) from endothelial cells in vitro represent examples of apparent nongenomic estrogenic effects as they are observed within minutes after the application of estradiol. Recent evidence suggests not only that ER-α receptors are present in the cell membrane, but that they can translocate into the nucleus in response to extracellular stimuli such as estradiol per se or ligand-independent activators of ER-α (eg, epidermal growth factor, serum).

The inhibitory effects of estrogens on vascular smooth muscle cell (VSMC) growth are well established, and presumably exert some protective vascular effects. Contrary to the effects of estadiol on vascular contractility and NO release, estrogenic modulation of VSMC growth is expressed in the course of several days and hence, cannot be considered as a rapid effect. Furthermore, although the precise mechanism of estradiol-induced inhibition of VSMC growth remains elusive; it appears that this effect persists in ER-β, but not in ER-α knockout mice. We have previously reported that estradiol and some phytoestrogens exert a biphasic effect on human VSMC growth, such that low concentrations (in the range observed in men and postmenopausal women) increase,
whereas higher concentrations, characteristic of premenopausal women, decrease DNA synthesis in cultured VSMC.10,15,16 The fact that there is an inhibitory effect of estradiol on VSMC proliferation that possibly depends on membrane-associated signaling is suggested because it requires activation of mitogen-activated protein (MAP)-kinase and is blocked by inhibition of MAP-kinase–kinase.17 However, direct evidence for the involvement of membrane receptors in the inhibitory effect of estradiol on VSMC replication has not been thus far presented. The present study was undertaken to determine whether or not estrogenic modulation of DNA synthesis in human vascular cell is exerted by putative estrogenic membrane receptors.

Methods

Regents

Enhancement solution for measuring europium fluorescence was purchased from Wallac (Turku, Finland). Steroids, chemicals, and the creatine kinase kit were from Sigma (St. Louis, MO). The labeling reagent 1-(p-thiocyanatophenyl)-diethylene-tri-N1,N2,N3,-tetraacetate chelated with europium (activated Eu-chelate) was kindly provided by Dr. I. Hemmila Wallac (Turku, Finland). Cy3.5 bis reactive dye was purchased from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom).

Preparation of Estradiol Macromolecular Conjugates

Estradiol-6-(O)-carboxymethyl oxime (E2-6-CMO)18 was conjugated to bovine serum albumin (BSA) and ovalbumin (Ov) through a two-step reaction, as described previously.19 Conjugates of daidzein with keyhole limpet hemocyanin (KLH) 7-(O)-carboxymethyl daidzein (CD-KLH) and 6-carboxymethyl genistein-KLH (CG-KLH) were prepared as previously described by Kohen et al.19,20 Ultraviolet analysis of the E2-BSA and E2-ovalbumin indicated that the conjugates contained 10 moles of hapten per mole of protein. The TLC analysis of these conjugates showed that the conjugates did not have traces of free E2 or E2-6-CMO.

Preparation of Cy3.5-Labeled Steroid BSA Conjugates

The membrane impermeant steroid conjugates E2-6-CMO-BSA (E2-BSA) (1 mg) and 4-chloro-androstenedione-3-(O)-carboxymethyl oxime BSA (CLAD-BSA, 1 mg, prepared in our laboratory21; used for negative control) were labeled with the reactive dye bis Cy3.5 according to the instructions of the manufacturer. The labeled protein contained 1 mole of the dye per mole of protein.

Cell Culture

Vascular smooth muscle cells were prepared from human umbilical artery as previously described with minor modifications.10,22 Cells were used only at passages 1 to 3 when expression of smooth muscle α-actin was clearly demonstrated.

The ECV 304 (E304) cells, an endothelial cell line derived from a human umbilical vein, were purchased from American Type Culture Collection (Manassas, VA) and grown as described previously.10,15,16 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, free or conjugated compounds, as indicated. Twenty-two hours after the exposure to these agents, [3H]thymidine was added for 2 h.10,15,16 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 0.3 N NaOH, samples were aspirated, and [3H]thymidine incorporation into DNA was determined by counting in a Packard Tri Carb 2100TR liquid scintillation analyzer (Meriden, CT).

Creatine Kinase Extraction and Assay

Cells were treated for 4 h, the optimal time observed from previous studies,10,15,16 with the various hormones and agents 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.10,15,16 Supernatant extracts were obtained by centrifugation of homogenates at 14,000 g for 5 min at 4°C in an Eppendorf microcentrifuge (Hamburg, Germany). Creatine kinase activity (CK) was assayed by a coupled spectrophotometric assay described previously.10,15,16 Protein was determined by Coomassie blue dye binding using BSA as the standard.

Immunofluorescence Studies of Membrane Estrogen-Binding Sites

Cells (5 × 10^3 cells/chamber) were grown on Lab-TekII chamber slide system (Nalge Nunc International, Naperville, IL). After reaching subconfluence, cells were washed with magnesium and calcium-free phosphate buffered saline (PBS) for 5 min at 37°C. The PBS was then aspirated and cells were placed at 4°C in a shaking bath for 30 min with Cy3.5-labeled E2-BSA (15 μL) or Cy3.5-labeled CLAD-BSA (15 μL). After staining, the cells were washed five times with cold PBS containing 1% BSA and fixed for 10 min at 4°C with 3% para-formaldehyde in PBS. After one washing in PBS, the stained cells were mounted with Elvanol mounting solution for fluorescent microscopic and confocal fluorescent microscopic evaluation.
Assessment of Total MAP-Kinase Activity

To evaluate the possibility that membrane-impermeant estrogenic ligands activate MAP-kinase (MAPK) activity as free E₂, we determined total MAPK in treated vascular cells. General anti-MAP kinase rabbit antibody [ERK-2 (c-14), sc-154] was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Activated MAP-kinase antibody was purchased from Sigma. Enhancement solution was purchased from Wall-lac. The europium-chelating agent was a generous gift from I. Hemmilla. The MAP-kinase-kinase inhibitor UO126 was purchased from Alexis (Lausen, Switzerland). Each treatment was performed in quadruplet. After 15 min of exposure to the various free or macroprotein-bound ligands, cells were washed twice with calcium- and magnesium-free cold PBS. Subsequently, 0.3 mL of lysis buffer was added to each plate. Lysis buffer consisted of 20 mmol/L HEPES at pH 7.5, containing 150 mmol/L NaCl, 1% triton-X 100, 10% glycerol, 1 mmol/L EDTA, 1 mmol/L NaF, 10 mmol/L β-glycerol phosphate, 2 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitors (1 mmol/L benzamidine, 2 mmol/L 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 (which was done in triplicate) was combined and divided into three aliquots. One aliquot was used for protein determination with Coomassie blue using BSA as the standard.

Two-Site Assay for Assessment of MAP-Kinase Activity

For the performance of the two-site MAP-kinase assay, which assesses the net accumulation of phosphorylated MAPK resulting from both MAPK-kinase and MAPK-phosphatase activity, microtiter strips (Labsystemrs, Oy, Helsinki, Finland) were coated for 70 h at 4°C with the general anti-MAP kinase antibody (2.5 μg/mL PBS at 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 wash solution, after which the cell lysates were transferred (100 μL/well) in triplicate to the microtiter wells. Assay buffer was then added to each well and the strips were incubated overnight at 4°C and washed three times. Subsequently, europium-labeled, activated anti-MAP kinase antibody (192 ng/well in 200 μL of 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.

Expression of ER-α and ER-β in VSMC

RNA was extracted from cultured VSMC cells, which were shown before to contain ER-α and ER-β by Western blot analysis, and subjected to reverse transcription as previously described. For ER-α, we used 5 μL of complementary DNA in the reaction mixture with the primers 5′-AATTCTGACAACTCGACGCAG-3′ (forward) and 5′-GTGCTTTCAACATTCTCCCCCTCCTC-3′ (reverse), for 30 cycles at 94°C for 30 sec, at 57°C for 30 sec, and at 72°C for 1 min. For ER-β, the same amount of cDNA was used with the primers 5′-TGCTTTGGTTTGCTGATTGC-3′ (forward) and 5′-TTTGCTTTTACTGTCCTCTGC-3′ (reverse) for 30 cycles at 94°C for 30 sec, at 58°C for 30 sec, and at 72°C for 1 min (Rutherford et al). The ER-α and ER-β cDNA were used as standard controls.

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 < .05 was considered significant.

Results

Effect of E₂-BSA on DNA Synthesis in VSMC and ECV 304 Cells

When cells were incubated with increasing concentrations of E₂ or E₂-BSA and DNA synthesis was measured, the following results were obtained. In VSMC, at low concentrations E₂ stimulated DNA synthesis, but high concentrations, it had an inhibitory effect. The E₂-BSA induced similar effects, although higher concentrations were required. In ECV 304 cells, the dose-dependent stimulation of DNA synthesis observed with E₂ was maximal at 300 nmol/L, with a decline at 3000 nmol/L. This effect was not mimicked by E₂-BSA, which was completely inactive (Fig. 1).

Effect of E₂-BSA on CK Specific Activity in VSMC and in ECV 304 Cells

When cells were incubated with increasing concentrations of E₂, there was a dose-dependent stimulation of CK specific activity in VSMC and ECV 304 cells, in which the maximal response was observed at 30 nmol/L with a decline at 300 and 3000 nmol/L. In both cell types, however, the dose-dependent stimulation of CK specific activity by E₂ was not mimicked by E₂-BSA, which was completely inactive (Fig. 1).
Changes for the comparison with control (vehicle treated) cells. Statistical assessment was done by ANOVA. Basal \([3H]\)thymidine incorporation into DNA in VSMC and in ECV 304 cells was 6471 ± 1340 and 64136 ± 11675 dpm/well, respectively. Basal CK specific activity in VSMC and in ECV 304 cells was 0.057 ± 0.012 and 0.065 ± 0.010 μmol/min/mg protein, respectively.

Effect of CG-KLH and CD-KLH on DNA synthesis in VSMC and in ECV 304 Cells

When cells were incubated with increasing concentrations of CG\(^{20}\) or CD\(^{19}\) and CG-KLH or CD-KLH\(^{20}\) and DNA synthesis was measured, the following results were obtained. In both VSMC and ECV 304 cells, CG and CD dose-dependently stimulated \([3H]\)thymidine incorporation. The CG-KLH and CD-KLH elicited similar effects in VSMC, although higher doses were needed. In ECV 304 cells, the dose-dependent stimulation of DNA synthesis by CG and CD was not mimicked by CG-KLH or CD-KLH, which were completely inactive, exerting no statistically significant changes (Tables 1 and 2).

Effect of CG-KLH and CD-KLH on CK Specific Activity Synthesis in VSMC and in ECV 304 Cells

When cells were incubated with increasing concentrations of CG or CD and CG-KLH or CD-KLH and CK specific activity was measured, the following results were obtained. In both VSMC and ECV 304 cells, CG and CD dose-dependently stimulated CK specific activity. The

Table 1. The effect of 6-carboxymethyl genistein (CG) or CG-KLH (CG conjugated to the macroprotein keyhole limpet hemocyanin) or carboxymethyl daidzein (CD) or CD-KLH (CD conjugated to the macroprotein keyhole limpet hemocyanin) at 0.03 to 3000 nmol/L on DNA synthesis in VSMC and ECV 304 cells

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<tr>
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<th>VSMC</th>
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<tr>
<td></td>
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<tr>
<td>0.0</td>
<td>0 ± 14</td>
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<tr>
<td>0.3</td>
<td>109 ± 24†</td>
<td>15 ± 10†</td>
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<td>74 ± 6†</td>
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<td>3000</td>
<td>86 ± 10†</td>
<td>−35 ± 24‡</td>
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Cells were hormonally treated for 24 h for DNA, as described in the Methods section. Results are means ± SEM of 4 to 9 incubates from 2 to 3 experiments and are expressed as percent change from basal \([3H]\)thymidine incorporation. * \(P < .05\); † \(P < .01\); ‡ \(P = \) nonsignificant changes for the comparison with control (vehicle treated) cells. Statistical assessment was done by ANOVA. Basal \([3H]\)thymidine incorporation into DNA in VSMC and in ECV 304 cells was 6471 ± 1340 and 64136 ± 11675 dpm/well, respectively.

VSMC and ECV 304 in percentage change in DNA synthesis. CG, CG-KLH, CD, and CD-KLH in nanomoles per liter.
CG-KLH and CD-KLH were not able to mimic the effects of CG or CD, and were completely inactive in both cell types (Tables 1 and 2).

**Expression of ER-α and ER-β in VSMC**

Fig. 2 depicts reverse transcriptase–polymerase chain reaction (RT–PCR) products of mRNA extracted from cultured VSMC cells with specific primers for either ER-α or ER-β. As shown, both estrogen receptor isoforms are expressed in cultured VSMC.

**Localization of Membrane E2-Binding Sites with the Use of the Membrane Impermeant Cy3.5-Labeled E2-BSA Conjugate**

Fig. 3 depicts membrane staining generated by the association of E2-BSA-Cy3.5 with membrane-binding sites for E2 in VSMC and ECV 304 cells. No staining was seen with CLAD-BSA-Cy3.5. Confocal microscopy depicts (Fig. 4) non-nuclear staining, probably membrane estrogen binding in both cells.

**Effect of Macroprotein-Bound Estrogenic Ligands on Total MAPK in VSMC and the effect of MAPK-Kinase Inhibition on Estrogenic Modulation of VSMC Growth**

The effects of E2, E2-BSA, and CG-KLH on total activated MAPK activity in VSMC are summarized in Fig. 5. As shown, free E2 as well as either one of the estrogenic compounds conjugated with a membrane impermeable macroprotein, E2-BSA and CG-KLH, induced a significant stimulation of MAPK activity. Basal MAPK activity

**Table 2.** The effect of 6-carboxymethyl genistein (CG) or CG-KLH (CG conjugated to the macroprotein keyhole limpet hemocyanin), or carboxymethyl daidzein (CD), or CD-KLH (CD conjugated to the macroprotein keyhole limpet hemocyanin) at 0.03-3000nM on CK specific activity in VSMC and ECV 304 cells

| VSMC                | ECV 304               |
|---------|----------------|----------------|
| CG      | CG-KLH | CD    | CD-KLH | CG      | CG-KLH | CD    | CD-KLH |
| 0.0     | 0 ± 16  | 0 ± 16 | 0 ± 16  | 0 ± 16  | 0 ± 17  | 0 ± 17 | 0 ± 17  | 0 ± 17  |
| 30      | 75 ± 10* | 40 ± 22† | 66 ± 18* | 23 ± 18‡ | 58 ± 12* | -17 ± 15‡ | 64 ± 18* | 0 ± 11‡ |
| 300     | 154 ± 35† | 3 ± 18‡ | 100 ± 11† | 15 ± 12‡ | 133 ± 25† | 38 ± 25‡ | 100 ± 18† | 43 ± 32‡ |
| 3000    | 153 ± 22† | 12 ± 9‡ | 113 ± 28† | -6 ± 16‡ | 100 ± 29† | -25 ± 13‡ | 107 ± 21† | 43 ± 21‡ |

Cells were hormonally treated for 4 h for CK, as described in the Methods section. Results are means ± SEM of 4 to 9 incubates from 2 to 3 experiments and are expressed as percentage change from basal CK activity. * P < .05; † P < .01; ‡ P = nonsignificant changes for the comparison with control (vehicle treated) cells. Statistical assessment was done by ANOVA. Basal CK specific activity in VSMC and in ECV 304 cells was 0.077 ± 0.018 and 0.055 ± 0.007 μmol/min/mg protein, respectively. Other abbreviations as in Table 1.
was reduced in the presence of the MAPK-kinase inhibitor U0126 by 28% ± 24%, but the inhibitor blocked completely the hormonal stimulation of MAPK activity (Fig. 5). The MAPK-kinase inhibitor U0126 reduced baseline [3H]thymidine incorporation (−38% ± 17%) and inhibited both E2- and E2-BSA-induced suppression of DNA synthesis. In addition, U0126 inhibited the increase in [3H]thymidine incorporation elicited by CG-KLH (Fig. 5). In contrast, MAPK-kinase inhibition had no effect on the increase in CK specific activity induced by E2, E2-BSA, or CG-KLH, with no effect on CK activity by itself (36% ± 25%; Fig. 5).

Discussion

The results of this study suggest that E2 inhibits human VSMC cell growth through an interaction with membrane-binding sites, which recognize, in addition to E2, several phytoestrogens, but do not interact with dihydrotestosterone.

First, the cell membrane impermeant ligand E2-BSA induced the same inhibitory effect on DNA synthesis in VSMC as E2 per se, albeit at 10-fold higher concentrations (Fig. 1). This may be due to the lower affinity of E2-6-CMO, the E2 derivative present in E2-BSA, compared with E2 per se. In addition, association with the macroprotein may somewhat hinder the binding of E2 to its putative membrane receptor, while still allowing receptor activation in terms of the effects on VSMC growth. In contrast, the same ligand, apparently incapable of entering the cell after its association with the cell membrane receptor, was entirely ineffective in inducing CK activity, a classic genomic marker of estradiol’s effect (formerly termed estradiol-induced protein). Of note is also the finding that although E2-BSA inhibited VSMC cell growth, it had no effect on cell proliferation in the endothelial ECV 304 cells, in which E2 itself induced a substantial and dose-dependent increase in DNA synthesis. Further heterogeneity in what is routinely presumed as “estrogenic effects” is exemplified by the effects of two phytoestrogen derivatives, carboxymethyl genistein and carboxymethyl daidzein and their respective macroprotein conjugates CG-
KLH and CD-KLH on cell growth. The CG and CD alone had a stimulatory role on [3H]thymidine incorporation in VSMC and ECV 304 cells. In their macroprotein conjugate forms, however, the stimulatory effect on [3H]thymidine incorporation was retained only in VSMC, but not in ECV 304 cells (Tables 1 and 2).

Although we provide evidence that both ER-α and ER-β, as analyzed by PCR, are expressed in VSMC, we cannot determine based on our studies whether or not E2 and the phytoestrogen derivatives interact with the same binding sites. However, to the extent that all listed effects are estrogen mimetic, both inhibitory (E2) and stimulatory (E2, CG, CD) effects on growth in VSMC appear to be membrane related, that is, they do not depend on translocation of the ligand from the membrane into the cell. Furthermore, the finding that not only E2, but also macroprotein-bound estrogenic compounds (ie, E2-BSA and CG-KLH) increase total activated MAPK, and that these effects were inhibited by the MAPK-kinase inhibitor UO126 in VSMC, is consistent with membrane-associated signaling. The observation that the effects of E2, E2-BSA, and CG-KLH on DNA synthesis (but not on CK activity) in VSMC could be blocked by the MAPK-kinase inhibitor UO126, further suggests that this membrane-dependent transduction pathway is involved in the effects of the various estrogen derivatives on VSMC growth.

In contrast to these apparently membrane-related effects in VSMC, acceleration of ECV 304 replication by E2 itself or by phytoestrogen derivatives requires the entry of the active ligand into the cell as it cannot be reproduced when these ligands are linked to a membrane impermeant dye. Furthermore, observations in preliminary experiments show that europium-ovalbumin-E2 binding to both types of human vascular cells examined can be significantly blocked by excess of either E2-ovalbumin or daidzein-ovalbumin, as well as by an estrogen antagonist (ICI 182780), but not by testosterone-BSA (data not shown). This suggests receptor specificity of the membrane-binding site for E2 and at least some related compounds, such as the phytoestrogen daidzein.

In conclusion, membrane receptors for E2 mediate E2-dependent inhibition of human VSMC growth. Although the same or similar binding sites for E2 can be visualized in endothelial cells, they do not appear to be involved in the effects of E2 on DNA synthesis in these cells. Elucidation of the precise subtype of the membrane receptors for E2 affecting VSMC growth awaits further investigation.

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