Clinically Used Estrogens Differentially Inhibit Human Aortic Smooth Muscle Cell Growth and Mitogen-Activated Protein Kinase Activity

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**Abstract**—Some estrogenic compounds modify vascular smooth muscle cell (SMC) biology; however, whether such effects are mediated in part by estrogen receptors is unknown. The purpose of this study was to evaluate whether the actions of clinically used estrogens on human aortic SMC biology are mediated by estrogen receptors. We examined the effects of various clinically used estrogens in the presence and absence of ICI 182,780, an estrogen receptor antagonist, on cultured human aortic SMC DNA synthesis ([3H]thymidine incorporation), cellular proliferation (cell counting), cell migration (modified Boyden chamber), collagen synthesis ([3H]proline incorporation), and mitogen-activated protein kinase activity. FCS-induced DNA synthesis, cell proliferation, collagen synthesis, platelet-derived growth factor–induced SMC migration, and mitogen-activated protein kinase activity were significantly inhibited by physiological (10^(-9) mol/L) concentrations of 17β-estradiol and low concentrations (10^(-8) to 10^(-7) mol/L) of 17β-estradiol, estradiol valerate, estradiol cypionate, and estradiol benzoate but not by estrone, estriol, 17α-estradiol, or estrone sulfate. The inhibitory effects of 17β-estradiol and other inhibitory estrogens were completely reversed by 100 μmol/L ICI 182,780, and the rank-order potency of various estrogens to inhibit SMC biology matched their rank-order affinity for estrogen receptors. The inhibitory effects of estrogens on SMC biology are in part receptor-mediated. Because the cardioprotective effects of hormone replacement therapy are most likely mediated by modification of SMC biology, whether hormone replacement therapy protects a given postmenopausal woman against cardiovascular disease will depend partially on the affinity of the estrogen for estrogen receptors in vascular SMCs. (Arterioscler Thromb Vasc Biol. 2000;20:964-972.)

**Key Words:** estrogens ■ vascular smooth muscle ■ postmenopausal women ■ cardiovascular disease ■ mitogen-activated protein kinase

Some epidemiological studies provide strong evidence that hormone replacement therapy (HRT) affords cardioprotection in postmenopausal women,1 whereas other epidemiological studies and a recent clinical trial do not support this notion.2,3 Although the reasons for these discordant findings are unknown, the inconsistent results reported to date may be due to heterogeneity in the responses of postmenopausal women to HRT. Indeed, in a given cohort of postmenopausal women, HRT has been shown to provide cardioprotective effects in only 50% to 60%.4 To correctly interpret the results of completed clinical studies and to better design new clinical trials, it is critical to elucidate the independent variables that govern the cardioprotective effects of HRT.

Our working hypothesis is that the degree of cardioprotection afforded by HRT is strongly influenced by the binding affinity of the specific estrogen to estrogen receptors in vascular cells and to the level of expression of estrogen receptors in vascular cells in an individual postmenopausal woman. The rationale for this hypothesis is 2-fold. First, several different types of estrogens are used clinically,5 and estrogens differ greatly in their chemical characteristics, binding affinity to estrogen receptors, and biological effects.6 Second, it is well known that the expression of estrogen receptors in vascular cells is decreased in some postmenopausal women.

An important prediction of our working hypothesis is that at least some of the vascular effects of estrogens are mediated by estrogen receptors in vascular cells. In this regard, although it is known that vascular cells express α- and β-estrogen receptors7 and that in the vasculature of postmenopausal women the expression of estrogen receptors is decreased,8 lack of specific estrogen receptor antagonists has precluded a comprehensive evaluation of this issue. Fortunately, a pure estrogen receptor antagonist, ICI 182,780, has
recently been developed. ICI 182,780 is devoid of agonistic activity and binds to α- and β-estrogen receptors,9,10 and we have shown that ICI 182,780 blocks the binding of 17β-estradiol to estrogen receptors in human aortic smooth muscle cells (SMCs).11 Therefore, ICI 182,780 provides a useful tool to investigate whether the effects of estrogens are receptor-mediated.

The goal of the present study was to investigate whether the vascular effects of estrogens are estrogen receptor-mediated. Our strategy was 2-fold: (1) to determine the relation between estrogen receptor affinity and vascular effects of clinically used estrogens and (2) to determine whether the vascular effects of clinical used estrogens are blocked by ICI 182,780. Estrogens inhibit mitogen-induced proliferation of SMCs, migration of SMCs from the media to the intima, and deposition of extracellular matrix proteins, such as collagen.12,13 Moreover, numerous in vivo studies in various female animal models have shown that neointimal formation in atherosclerosis and after balloon catheter-induced injury is increased in the absence of estrogen and inhibited in the presence of estrogen.12,14-15 Therefore, in the present investigation, we examined the effects of clinically used estrogens in the absence and presence of ICI 182,780 on human SMC growth (DNA synthesis and proliferation), directed migration, collagen synthesis, and mitogen-activated protein (MAP) kinase activity.

Methods

Tissue culture reagents and culture ware were purchased from Gibco Laboratories, except for FCS, which was obtained from HyClone Laboratories Inc. 17β-Estradiol, estradiol benzoate, estradiol cypionate, estradiol valerate, estrone, estrone sulfate, estriol, myelin basic protein (MBP), Triton X-100, β-glycerophosphate, EGTA, dithiothreitol, Na3VO4, aprotinin, pepstatin, leupeptin, and benzamidine were purchased from Sigma Chemical Co. The estrogen receptor antagonist ICI 182,780 was a gift from Tocris (Langford, Bristol). 4-Hydroxytamoxifen was purchased from Research Biochemicals International. [3H]Thymidine (specific activity 11.8 Ci/mmol) was purchased from ICN Biomedicals. L-[3H]Proline (specific activity 23 Ci/ml), [γ-32P]ATP (specific activity 3 Ci/ml), and [1H]17β-estradiol (specific activity 72 Ci/mmol) were purchased from NEN.

Arterial SMCs cultured from adult thoracic aortas were obtained from female Sprague-Dawley rat or male donor heart transplants. The cells were cultured by the explant method and cultured as described by us previously.14 SMC purity was characterized by immunofluorescence staining with smooth muscle specific anti-smooth muscle α-actin monoclonal antibodies and by morphological criteria specific for SMCs, as described in detail previously.14 SMCs in the third and fifth passages were used for all the studies. Immunohistochemistry was used to ascertain the presence of estrogen receptors α and β in the cultured SMCs, as previously described.17 Briefly, SMCs grown to confluence in chamber slides were fixed in 3.7% formaldehyde, incubated for 1 hour with monoclonal antibodies to human estrogen receptor α or β (1:10 dilution Alexis Biochemicals), washed, exposed to FITC-labeled goat anti-rabbit IgG secondary antibody (dilution 1:50, Sigma), and examined by fluorescence microscopy. Control studies were conducted in parallel in which primary antibody was omitted or neutralized with blocking peptides to estrogen receptors α and β (Alexis). These controls were consistently negative (data not shown).

[3H]Thymidine incorporation (index of DNA synthesis) and cell number (cell proliferation) studies were conducted to investigate the effects of various test agents on cell growth. SMCs were plated at a density of 5×103 cells per well in 24-well tissue culture dishes and allowed to grow to confluence in DMEM/F12 (phenol red-free) medium containing 10% FCS (steroid free and delipidated) under standard tissue culture conditions. The cells were then growth-arrested by feeding DMEM (phenol red free) containing 0.4% albumin for 48 hours. For DNA synthesis, growth was initiated by treating growth-arrested cells for 20 hours with DMEM containing 2.5% FCS in the presence or absence of the test agents. To evaluate the effects of estrogen receptor antagonists, cells were pretreated for 1 hour with ICI 182,780 before the treatment with the test agents. After 20 hours of incubation, the treatments were repeated with freshly prepared solutions but supplemented with [3H]Thymidine (1 μCi/mL) for an additional 4 hours. The experiments were terminated by washing the cells twice with Dulbecco’s PBS and twice with ice-cold trichloroacetic acid (10%). The precipitate was solubilized in 500 μL of 0.3N NaOH and 0.1% SDS (50°C for 2 hours). Aliquots from 4 wells for each treatment with 10 mL scintillation fluid were counted in a liquid scintillation counter. For cell number experiments, SMCs were allowed to attach overnight, were growth-arrested for 48 hours, and were then treated every 24 hours for 4 days; on day 5, the cells were dislodged and counted on a Coulter counter.

[L-3H]Proline incorporation studies were performed to investigate the effects of various test agents on collagen synthesis. Confluent monolayers of SMCs were made quiescent by feeding DMEM containing 0.4% BSA for 48 hours. Growth-arrested SMCs were treated for 36 hours with DMEM supplemented with 2.5% FCS plus L-[3H]Proline (1 μCi/mL) in the presence or absence of the test agents. To evaluate the effects of estrogen receptor antagonists, cells were pretreated for 1 hour with ICI 182,780 before treatment with the test agents. The experiments were terminated by washing the cells twice with PBS and twice with ice-cold trichloroacetic acid (10%). The precipitate was solubilized as described above, and aliquots from 4 wells for each treatment were counted in a liquid scintillation counter. Each experiment was conducted in triplicate and with 3 separate cultures of SMCs. To ensure that the inhibitory effects of the experimental agents on collagen synthesis were not due to changes in cell number, the experiments were conducted in confluent monolayers of cells in which changes in cell number were precluded. Additionally, cell counting was performed in cells treated in parallel with the cells used for the collagen synthesis studies, and the data were normalized to cell number.

Modified Boyden chambers (Neuro Probe Inc) were used to evaluate the effects of various estrogens on platelet-derived growth factor (PDGF)-BB–induced SMC migration as previously described.18 To evaluate the effects of estrogen receptor antagonists, cells were pretreated for 1 hour with ICI 182,780 before treatment with the test agents.

The effects of the test agents on MAP kinase were also assessed because MAP kinase is an important mediator of cell growth. SMCs grown to confluence in 35-mm² culture dishes were made quiescent by feeding DMEM containing 0.4% BSA for 48 hours. Growth-arrested SMCs were washed with PBS and incubated for either 1 hour or 24 hours with or without the various test agents. Some cells were stimulated with PDGF-BB (25 ng/mL) for 10 minutes, whereas others were not. To evaluate the effects of estrogen receptor antagonists, cells were pretreated for 1 hour with ICI 182,780 before treatment with the test agents. After stimulation with PDGF-BB, cells were washed with ice-cold PBS and extraction buffer (50 mMol/L β-glycerophosphate, 1.5 mMol/L EGTA, 1 mMol/L diethiothreitol, 100 μMol/L Na3VO4, 10 μMol/L aprotinin, 5 μMol/L pepstatin, 20 μMol/L leupeptin, and 1 mMol/L benzamidine), scraped off the plates, and sonicated for 20 seconds in 0.5 mL of extraction buffer. The extracts were collected, and the cytosolic fraction was separated by centrifuging the extracts at 100 000g for 20 minutes at 4°C. The supernatants were diluted to a concentration of 1 mg protein per milliliter and stored at −70°C for MAP kinase activity assays. The MAP kinase activity in the cytosolic fractions was quantified by the method of Bornfeldt et al,18 with minor modifications. Briefly, cytosolic extracts (5 μL) were added to 30 μL of MAP kinase assay buffer containing 25 mMol/L β-glycerophosphate, 1.25 mMol/L EGTA, 0.5 mMol/L dithiothreitol, 150 μMol/L Na3VO4, 2 μMol/L peptide inhibitor for cAMP-dependent protein kinase (H-7TATAPASGTGAAAI-NH2, Bachem Bioscience Inc.), 1 mg/mL BSA, 10 μMol/L calmidazolium, 0.33 mmol/mL MBP, and 100 μMol/L [γ-32P]ATP. After incubation for 15 minutes at 30°C, 25 μL aliquots of the reaction mixture were spotted onto...
Results

Treatment with 2.5% FCS stimulated [3H]thymidine incorporation by ∼8-fold (P<0.05 versus 0.4% BSA) and [3H]proline incorporation by ∼6-fold (P<0.05 versus 0.4% BSA). Treatment with 17β-estradiol as well as progesterone inhibited FCS-induced [3H]thymidine incorporation in a concentration-dependent manner (Figure 1). Physiological concentrations of 17β-estradiol (10⁻⁹ mol/L) and progesterone (10⁻⁸ mol/L) inhibited FCS-induced [3H]thymidine incorporation by 12% (P<0.05) and 24% (P<0.05), respectively (Figure 1). A 50% decrease in FCS-induced [3H]thymidine incorporation was observed at ∼10⁻⁶ mol/L of 17β-estradiol and 2×10⁻⁶ mol/L of progesterone (Figure 1). Similar to the effects on [3H]thymidine incorporation, 17β-estradiol and progesterone inhibited 2.5% FCS–induced [3H]proline incorporation (Figure 2). Physiological concentrations (10⁻⁹ mol/L) of 17β-estradiol significantly inhibited [3H]proline incorporation, and progesterone and 17β-estradiol decreased proline incorporation by 50% at 3×10⁻⁶ mol/L.

FCS increased cell number in growth-arrested SMCs by ∼8-fold (data not shown). 17β-Estradiol and progesterone inhibited FCS-induced increases in cell number in a concentration-dependent manner (Figure 3). The lowest concentrations of 17β-estradiol and progesterone that significantly inhibited FCS-induced increases in cell number were concentrations of 10⁻⁹ mol/L, and these concentrations, which are physiological, inhibited cell number by 18±2% (P<0.05) and 14±2% (P<0.05), respectively. Progesterone and 17β-estradiol decreased cell number by 50% at 3×10⁻⁶ mol/L. Trypan blue exclusion tests and MTT assay indicated no loss in viability of cells treated with 17β-estradiol and progesterone (data not shown).

Similar to 17β-estradiol, FCS-induced [3H]thymidine incorporation, [3H]proline incorporation, and cell number were
The inhibitory effects of 17β-estradiol on PDGF-BB-induced SMC migration were mimicked by estradiol valerate, estradiol cypionate, estradiol benzoate, estrone, estriol, estrone sulfate, and 17α-estradiol. In contrast, estrone, estrone sulfate, estriol, and 17α-estradiol were significantly less potent and inhibited FCS-induced increases in [3H]thymidine incorporation, [3H]proline incorporation, and cell number only at high concentrations (>10^−6 mol/L; Figures 1 to 3) not attained therapeutically. For all tested estrogens, the potency order for inhibition of [3H]thymidine incorporation, [3H]proline incorporation, and cell number was as follows: 17β-estradiol > estradiol valerate > estradiol cypionate > estradiol benzoate > estrone > estril > estrone sulfate = 17α-estradiol.

Treatment of SMCs with PDGF-BB stimulated the migration of SMCs (P<0.05 versus cells treated with 0.4% BSA, Figure 4). PDGF-BB–induced SMC migration was inhibited in a concentration-dependent manner in SMCs pretreated with 17β-estradiol and progesterone (Figure 4, top panel). The inhibitory effects of 17β-estradiol on PDGF-BB–induced SMC migration were mimicked by estradiol valerate, estradiol cypionate, and estradiol benzoate (Figures 1 to 3). In contrast, estrone, estrone sulfate, estril, and 17α-estradiol were significantly less potent and inhibited FCS-induced increases in [3H]thymidine incorporation, [3H]proline incorporation, and cell number only at high concentrations (>10^−6 mol/L; Figures 1 to 3) not attained therapeutically. For all tested estrogens, the potency order for inhibition of [3H]thymidine incorporation, [3H]proline incorporation, and cell number was as follows: 17β-estradiol > estradiol valerate > estradiol cypionate > estradiol benzoate > estrone > estril > estrone sulfate = 17α-estradiol.

Figure 3. Inhibition of FCS-induced cell number by 17β-estradiol, progesterone, 17β-estradiol (10^−7 mol/L) + progesterone (10^−7 mol/L), estradiol valerate, estradiol cypionate, estradiol benzoate, estrone, estriol, 17α-estradiol, and estrone sulfate in human aortic SMCs. Values represent mean±SEM from 3 experiments, each in triplicate. *P<0.05 vs 17β-estradiol or progesterone.

Inhibition of FCS-induced increases in [3H]thymidine incorporation, [3H]proline incorporation, cell number, and SMC migration were significantly enhanced in the presence of progesterone (10^−7 mol/L; Figures 1 to 4, top panels). Similar to the effects of 17β-estradiol, progesterone enhanced the inhibitory effects of estradiol valerate, estradiol cypionate, and estradiol benzoate (data not shown). In contrast, the inhibitory effects of estrone, estril, 17α-estradiol, and estrone sulfate were not significantly influenced by progesterone (data not shown).

To investigate whether the inhibitory effects of 17β-estradiol on SMC growth were receptor-mediated, the effects of 17β-estradiol in the presence and absence of ICI 182,780, a potent estrogen receptor antagonist,9,10 were examined. The inhibitory effects of 17β-estradiol on FCS-induced [3H]thymidine incorporation, [3H]proline incorporation, cell migration, and cell number were fully reversed (Figure 5) in SMCs pretreated with ICI 182,780 (100 µmol/L). Moreover, the antagonistic effects ICI 182,780 were concentration dependent (Figure 5). The concentrations of ICI 182,780 that completely blocked the effects of 17β-estradiol (1 µmol/L) did not influence thymidine incorporation, proline incorporation, cell number, or cell migration. Pretreatment of SMCs with ICI 182,780 (100 µmol/L) also blocked the effects of all the inhibitory estrogens on [3H]thymidine incorporation (Figure 6) but did not block the inhibitory effects of progesterone in this regard (Figure 6). In contrast to ICI 182,780, the effects of 17β-estradiol (0.1 µmol/L) on SMCs were enhanced in the presence of other inhibitory estrogens (estradiol valerate, estradiol cypionate, and estradiol benzoate; 1 µmol/L) and reduced in the presence of noninhibitory estrogens (estrone, 1 µmol/L; estril, 1 µmol/L). Figure 6
showed the modulatory effects of various estrogens on 17β-estradiol (0.1 μmol/L)-induced inhibition of thymidine incorporation, and similar effects were observed on proline incorporation, cell number, and cell migration (data not shown).

Treatment of growth-arrested SMCs with PDGF (25 ng/mL) increased MAP kinase activity from 0.09 to 7.06 pmol/min/mg protein, and the stimulatory effects of PDGF were inhibited by the MAP kinase inhibitor PD98059 (30 μmol/L) to 0.9 pmol/min/mg protein. In SMCs pretreated for 1 hour with 17β-estradiol and progesterone, the effects of various estrogens on MAP kinase activity were in the following order of potency: 17β-estradiol (1 μmol/L), estradiol cypionate (EC), estradiol benzoate (EB), estrone (E1), estradiol sulfate (ES), progesterone (P), estrone sulfate, estriol, and estradiol benzoate, whereas estrone, estriol, estrone sulfate, and 17α-estradiol inhibited MAP kinase activity by only 4% to 10% (Figure 7).

In growth-arrested SMCs treated with 1 to 100 nmol/L of 17β-estradiol for 1 and 24 hours, no change in the basal MAP kinase activity was observed. The MAP kinase activity in SMCs pretreated with ICI 182,780 (100 μmol/L) and progesterone (10 nmol/L), PDGF-BB–induced MAP kinase activity was inhibited by estradiol valerate, estradiol cypionate, and estradiol benzoate, whereas estrone, estriol, estrone sulfate, and 17α-estradiol inhibited MAP kinase activity by only 4% to 10% (Figure 7).

In growth-arrested SMCs treated with 1 to 100 nmol/L of 17β-estradiol for 1 and 24 hours, no change in the basal MAP kinase activity was observed. The MAP kinase activity in control SMCs and 100 nmol/L 17β-estradiol–treated SMCs was 0.07 ± 0.03 and 0.067 ± 0.02 pmol/min/mg protein, respectively, after 1 hour of treatment and 0.066 ± 0.025 and 0.069 ± 0.01 pmol/min/mg protein, respectively, after 24 hours of treatment. These observations are consistent with the findings of Morey et al.,20 who showed that estradiol inhibits mitogen-induced but not basal MAP kinase activity in serum-starved human umbilical vein SMCs and bovine aortic endothelial cells. In contrast to our observation, Kim-
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by Hodges et al.22 expression of heterogeneous forms of estrogen cognate re-

tors were expressed in the cytosol and the nucleus of the cultured SMCs. Schulze et al21 reported delayed receptor-mediated increases in MAP kinase activity in endothelial cells grown in 2% serum before the treatment. It is feasible that cells were not completely growth-arrested and that serum may have contributed to the differences in the results obtained. Alternatively, the differences in the effects may also be due to the expression of heterogeneous forms of estrogen cognate receptors, as suggested by Hodges et al.22 Inhibition of MAP kinase activity with PD98059 (10 μmol/L) inhibited FCS-induced DNA synthesis and increases in cell number in SMCs by 29±3% and 43±4%, respectively. Moreover, the inhibitory effects of 17β-estradiol (10−9 and 10−7 mol/L) on FCS-induced DNA synthesis and cell proliferation were enhanced in an additive fashion by PD98059 (Figure 8). Similar effects were also observed on proline incorporation and cell migration (data not shown).

Binding studies revealed that [3H]17β-estradiol binds with high affinity and specificity to cultured human aortic SMCs. The binding isotherm showed a saturable binding process, and Scatchard analyses revealed that the number of binding sites in the 3 separate cultures did not vary and were 14.6, 15.3, and 15.6 fmol/mg protein, respectively (Figure 9). Competitive binding studies with various clinically used estrogens showed that the potency of these agents to bind to the estrogen receptor varied considerably and that the potency of the various estrogens to inhibit [3H]estradiol binding was as follows: 17β-estradiol≈ICI 182,780>estradiol valerate≈estradiol cypionate>estradiol benzoate>estrone>17α-estradiol≈estradiol sulfate (Figure 9). Immunostaining with monoclonal antibodies to human estrogen receptor α and with antiserum to human estrogen receptor β showed that human aortic SMCs express the α- and β-estrogen receptors (Figure 10). As shown in the photomicrographs in Figure 10, the α- and β-estrogen receptors were expressed in the cytosol and the nucleus of the cultured SMCs.

Discussion

The present study demonstrates that 17β-estradiol inhibits SMC DNA and collagen synthesis and SMC proliferation and migration. The inhibitory effects of 17β-estradiol on SMCs were mimicked by estradiol valerate, estradiol cypionate, and estradiol benzoate but not by estrone, estrone sulfate, estriol, and 17β-estradiol. The relative potencies of estrogens to inhibit SMCs matched their relative affinities for estrogen receptors. Progesterone also inhibited SMC DNA and collagen synthesis and SMC proliferation and migration and facilitated the inhibitory effects of estrogens. The inhibitory effects of 17β-estradiol on SMCs were blocked by ICI 182,780, a specific estrogen receptor antagonist.6 17β-Estradiol also inhibited MAP kinase activity in SMCs, and this effect was blocked by ICI 182,780. MAP kinase activity in SMCs was attenuated by clinically used estrogens that also inhibited SMCs but not by estrogens that had no inhibitory effects on SMCs. These findings demonstrate that in human SMCs, clinically used estrogens differentially inhibit SMC DNA and collagen synthesis and SMC proliferation and migration. Moreover, progesterone enhances the inhibitory effects of estrogens on SMCs. Finally, our findings provide evidence that estrogens and progesterone mediate their inhibitory effects on SMCs by reducing MAP kinase activity and that the inhibitory effects of estrogens are, in part, estrogen receptor–mediated.

Some epidemiological studies provide evidence that HRT induces cardioprotection in postmenopausal women; however, these findings have not been universal and are at present controversial.1–3 We hypothesize that chemical differences in the various clinically used estrogens result in different biological effects, which contribute to the disparate findings in the reported studies. Our finding that 17β-estradiol is effective in inhibiting SMC DNA and collagen synthesis, cell
number and migration, and MAP kinase activity but that 17α-estradiol, estrone, estrone sulfate, and estriol are much less active in this regard indicates that the effects of estrogens on vascular SMCs do vary considerably. This conclusion is also supported by our finding that estradiol benzoate is half as potent as 17β-estradiol. We have observed similar differential effects of these estrogens on rat cardiac fibroblasts.23

In the present study, the inhibitory effects of 17β-estradiol on SMCs were blocked by ICI 182,780, a pure estrogen receptor antagonist,9 suggesting that the inhibitory effects of 17β-estradiol are estrogen receptor–mediated. This hypothesis is further supported by the observation that the potency of various estrogens in inhibiting SMCs matched their affinity for estrogen receptors.6,24 Our conclusion that the inhibitory effects of estradiol may be receptor-mediated is further supported by recent findings that the effects of estradiol on endothelial cell growth and endothelin synthesis are blocked by ICI 182,780.25,26 Although the above findings provide evidence that the inhibitory effects of estradiol are receptor-mediated, the participation of other mechanisms cannot be ruled out, inasmuch as estradiol metabolites that bind to estrogen receptor with low affinity have been shown to be more potent than estradiol in inhibiting SMC27 and cardiac fibroblast23 growth.

In contrast to our finding, Iafrati et al7 have reported that 17β-estradiol inhibits balloon injury–induced neointimal formation equally in wild-type and α-estrogen receptor–deficient mice. Moreover, estrogen receptor antagonists such as tamoxifen and 4-hydroxytamoxifen are also potent cardioprotective agents.28 Because 2 isoforms of estrogen receptors, α and β, are known to mediate many effects of estradiol,8–10 a reasonable explanation for these disparate results is that 17β-estradiol mediates its inhibitory effects via β-estrogen receptors and not α-estrogen receptors. Indeed, the SMCs used in the present study express both α- and β-estrogen receptors, and ICI 182,780 blocks both the α- and β-estrogen receptor effects of 17β-estradiol.10 With regard to the effects of tamoxifen and 4-OH-tamoxifen, these agents express partial agonist activity, which may contribute to their protective effects. Alternatively, 17β-estradiol may also mediate its inhibitory effects in part via mechanisms independent of estrogen receptors.

Compared with 17β-estradiol, estrone, estrone sulfate, and estriol have significantly lower affinity for estrogen receptors and are estrogens with weak feminizing effects; thus, their use in men to protect against cardiovascular disease has been proposed.6 However, our finding that estrone, estrone sulfate, and estriol are unable to inhibit SMC function suggests that these agents may not induce cardioprotective effects. Indeed, in women with functional ovaries, the levels of 17β-estradiol are higher than the levels of estrone;5 however, in postmenopausal women, the levels of 17β-estradiol are significantly lower than the levels of estrone.5 Because estrone is synthesized in fat tissue, its levels are not dramatically reduced in menopause,9 and men also have substantial levels of estrone.5 Our findings suggest that the cardiovascular complications in postmenopausal women are due largely to a decrease in 17β-estradiol and that the use of estrone or estriol may not induce cardioprotection.

Figure 10. Photomicrographs of immunofluorescent identification of estrogen receptor α (A) and estrogen receptor β (B) in cultured human aortic SMCs. The photomicrographs show immunofluorescent staining of SMCs passaged 3 times, grown to subconfluence, and stained with monoclonal antibody to estrogen receptor α and anti-serum purified to human estrogen receptor β. Estrogen receptors α and β were expressed in the cytoplasm and in the nucleus of the cells. Magnification ×100. Bar=5 μm.
In the present study, the inhibitory effects of estrogens were enhanced, rather than inhibited, by progesterone. To reduce the risk of endometrial cancer, combined administration of a progestin with an estrogen is currently the preferred method of HRT in nonhysterectomized postmenopausal women. Our findings suggest that treatment with 17β-estradiol plus progesterone may be more protective against cardiovascular disease in postmenopausal women. Moreover, progesterone may also induce cardioprotective effects when administered with weaker estrogens, which are unable to inhibit SMC growth. Coadministration of progestins with estrogen have been shown to both increase and decrease the cardioprotective effects of estrogen, and progesterone has been shown to enhance the protective effects of 17β-estradiol on neointimal formation. In contrast to progesterone, synthetic progestins such as medroxyprogesterone acetate, cyproterone acetate, and norethisterone acetate, which are used clinically, have been shown to reduce the various cardioprotective effects of 17β-estradiol. Moreover, medroxyprogesterone acetate has been shown to abrogate the inhibitory effects of 17β-estradiol on neointimal formation. Progesterone is the naturally occurring progestin without any androgenic effects. In contrast, norethisterone acetate is a testosterone-derived progestin with both gestagenic and androgenic properties, and medroxyprogesterone acetate and cyproterone acetate are derivatives of 17α-hydroxyprogesterone. Hence, it is feasible that progestins used clinically have differential effects that are governed by their chemical properties. Thus, it will be important to evaluate the effects of various clinically used progestins on estrogen-induced cardioprotective effects.

Migration and proliferation of vascular SMCs is inhibited by the MAP kinase kinase inhibitor PD98059, and the MAP kinase pathway is activated at sites of balloon injury–induced neointimal formation. Our observation that 17β-estradiol inhibits MAP kinase activity and that these effects are blocked by ICI 182,780 suggests that inhibition of the MAP kinase pathway via estrogen receptors contributes to the inhibitory effects of 17β-estradiol on SMCs. This idea is further supported by our observation that MAP kinase activity is attenuated only by estrogens that also inhibit SMC growth. The finding that progesterone inhibits MAP kinase activity and that this effect is not blocked by ICI 182,780 suggests that the effects of progesterone are mediated via a mechanism separate from estrogens.

Although our findings provide evidence that estrogens inhibit MAP kinase activity via estrogen receptors, the participation of other mechanisms in the cardioprotective effect of estrogens also must be considered. Other mechanisms include reducing apoptosis and inducing endothelial cell recovery and growth, improving endothelium-mediated degradation of LDL cholesterol, suppressing collagen and elastin synthesis, restoring endothelium-dependent vasodilator mechanisms after injury, reducing LDL levels, increasing HDL levels, preventing oxidation of LDL, releasing nitric oxide and prostaglandins from vascular endothelial cells, and reducing the adhesion of activated monocytes to the endothelium by inhibiting the expression of adhesion molecules. Our finding that 17β-estradiol inhibits MAP kinase activity provides evidence of yet another mechanism by which estrogens may induce their cardioprotective effects.

Our finding that estradiol inhibits SMC growth suggests that it may protect the vasculature by inhibiting neointimal formation. Consistent with our findings, using high resolution ultrasound, Baron et al found decreases in the intimal layer of the carotid artery in postmenopausal women receiving estrogen. However, in contrast to the effects on the intima, they observed an increase in medial thickness, although the authors speculate that the medial thickness was due to increased deposition of extracellular matrix proteins. It is feasible that estradiol induces matrix protein degradation, which may result in the deposition of these proteins and influence SMC growth. Indeed, generation of certain matrix proteins, such as heparan sulfate, can inhibit SMC growth. Moreover, decreased medial thickness and increased intimal thickness has been observed in aging and atherosclerotic vessels. Therefore, the observation by Baron et al that estrogen replacement reduces the intima and increases the media may suggest that estrogen reinstates the healthy balance of connective tissue content in the arterial wall that this, in turn, acts as a feeding bed for regulating the intima and inhibiting neointimal formation. Taken together, the above findings reaffirm the notion that estradiol protects the vasculature by reducing intimal thickening, which is consistent with our findings.

We conclude that estrogens inhibit SMCs partly by reducing MAP kinase activity and that these effects of estrogens on SMCs are, in part, estrogen receptor–mediated, although participation of other mechanisms cannot be ruled out. Also, the present study indicates that clinically used estrogens vary considerably in their ability to inhibit SMCs and that the effects of estrogens on SMCs are enhanced by progesterone.

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