Methoxyestradiols Mediate Estradiol-Induced Antimitogenesis in Human Aortic SMCs

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Abstract—Estrogen receptors (ERs) are considered to mediate the ability of 17β-estradiol (estradiol) to reduce injury-induced proliferation of vascular smooth muscle cells (VSMCs), leading to vascular lesions. However, the finding that estradiol attenuates formation of vascular lesions in response to vascular injury in knockout mice that lack either ER-α or ER-β challenges this concept. Our hypothesis is that the local metabolism of estradiol to methoxyestradiols, metabolites of estradiol with little affinity for ERs, mediates the ER-independent antimitogenic effects of estradiol on VSMCs. In human VSMCs, 2-methoxyestradiol and 2-hydroxyestradiol were more potent than was estradiol in inhibiting DNA synthesis ([3H]-thymidine incorporation), collagen synthesis ([3H]-proline incorporation), cell proliferation (cell number), and cell migration (movement of cells across a polycarbonate membrane). The inhibitory effects of estradiol on VSMCs were enhanced by cytochrome-P450 (CYP450) inducers 3-methylcholanthrene and phenobarbital. Moreover, the inhibitory effects of estradiol were blocked in the presence of the CYP450 inhibitor 1-aminobenzotriazole and the catechol-O-methyltransferase inhibitors quercetin and OR486. Both OR486 and quercetin blocked the conversion of 2-hydroxyestradiol to 2-methoxyestradiol; moreover, they blocked the antimitogenic effects of 2-hydroxyestradiol but not of 2-methoxyestradiol. The ER antagonist ICI182780 blocked the inhibitor effects of estradiol on VSMCs, but only at concentrations (>50 µmol/L) that also inhibit the metabolism of estradiol to hydroxyestradiols (precursors of methoxyestradiols). In conclusion, the inhibitory effects of locally applied estradiol on human VSMCs are mediated via a novel ER-independent mechanism involving estradiol metabolism. These findings imply that vascular estradiol metabolism may be an important determinant of the cardiovascular protective effects of estradiol and that nonfeminizing estradiol metabolites may confer cardiovascular protection regardless of gender.

Key Words: hormones □ menopause □ estradiol □ metabolism □ coronary artery disease □ remodeling □ cardiovascular diseases

Estradiol reduces vascular injury. In this regard, physiological concentrations of estradiol attenuate the development of atherosclerosis, decrease balloon injury–induced and allograft–induced vascular lesions, and inhibit the proliferation of vascular smooth muscle cells (VSMCs), a process that contributes to vascular pathology after vascular injury. Inasmuch as many of the biological effects of estrogens are mediated by estrogen receptors (ERs), and arteries express both ER-α and ER-β, the current dogma is that ERs mediate the vascular-protective effects of estradiol. However, the finding that estradiol inhibits injury-induced VSMC proliferation in arteries of mice that lack either ER-α or ER-β as well as in double knockout mice that lack both ER-α and ER-β (ER-α–ER-β–/–) challenges this concept. Thus, other mechanisms that do not involve ERs may participate in the vascular-protective actions of estradiol. It is possible that the vascular-protective actions of estradiol are mediated in part by local (vascular) conversion of estradiol to metabolites that inhibit vascular lesion formation independently of ERs by exerting antigrowth effects on VSMCs. VSMCs produce cytochrome P450 (CYP450) enzymes that metabolize estradiol to 2- and 4-hydroxyestradiol and catechol-O-methyltransferase (COMT) that converts 2- and 4-hydroxyestradiol to 2- and 4-methoxyestradiol. However, the hypothesis that conversion of estradiol to hydroxyestradiols and methoxyestradiols occurs in human VSMCs and mediates the antigrowth effects of locally applied estradiol is not well tested. In the present study, we tested this hypothesis by examining the inhibitory effects of estradiol on the growth of human aortic VSMCs in the presence and absence of modulators (activators or inhibitors) of CYP450 and COMT.
Methods

SMC Cultures and Growth Studies

Human female aortic VSMCs were purchased from Clonetics (San Diego, Calif) and cultured under standard tissue culture conditions as described previously. Studies were conducted using phenol red-free culture medium. Subconfluent VSMCs were grown arrested for 48 hours in the presence or absence of 10μmol/L of 3-MC or phenobarbital, and the effects of various treatments or vehicle on steroid-free fetal calf serum (FCS; 2.5%)-induced [3H]-thymidine incorporation into DNA were assessed as previously described. Aliquots from 4 wells for each treatment with 10 mL scintillation fluid were counted in a liquid scintillation counter.

In the cell number experiments, VSMCs were plated (5 × 10^4 cells/well) and allowed to attach overnight. Cells were grown arrested for 48 hours and subsequently treated every 24 hours for 4 days. On day 5, cells were dislodged by trypsinization and counted on a Coulter counter. In some experiments, the cells were treated every 48 hours with estradiol, and then cells were dislodged and counted on days 4, 8, 12, and 16.

For collagen synthesis, confluent monolayers of VSMCs were grown arrested for 48 hours in the presence or absence of 10μmol/L 3-MC or phenobarbital, and the effects of various treatments or vehicle on 2.5% FCS-induced [3H]-proline incorporation into collagen were assessed as previously described.6 Cell counting was performed in cells treated in parallel to the cells used for the collagen synthesis studies, and the data were normalized to cell number.

For cell migration studies, we used 6.5-mm diameter Transwell plates (Costar) with an 8-micron polycarbonate membrane pore size. VSMCs were serum-starved overnight in 0.25% FCS and then trypsinized. Approximately 30 000 cells were plated on each polycarbonate membrane. Treatments were in 0.5 mL and were placed in the lower well chamber of the Transwell plates. After an incubation of 5 hours at 37°C, the media were removed. Next, cells from the upper surface of the membrane were removed, whereas cells on the lower surface were fixed in methanol and stained with Hoechst (0.5 μg/mL). The membranes were then mounted on glass slides, and the labeled nuclei of the migrated cells were visualized by fluorescent microscopy. Cells that migrated were determined by counting 12 different spots on each slide and taking the average.

Metabolism of Catecholestradiols to Methoxyestradiols

Confluent VSMCs were incubated with 2-hydroxyestradiol for 4 hours, internal standard (16α-hydroxyestradiol) was added, samples were extracted with methylene chloride, extracts were dried under vacuum, residues were reconstituted in mobile phase, and samples were analyzed by high performance liquid chromatography with UV detection using gradient elution.

ER Expression Studies

To investigate whether the SMCs used express ER-α and ER-β, cell lysates from cultured SMCs were analyzed by Western blots and probed with antibodies to ER-α (purified antisem to human ER-α; Alexis Corp, Lausen, Switzerland) and ER-β (purified antisem to human ER-β; Alexis Corp).

Statistics

Statistical significant (P<0.05) was assessed with ANOVA. Student’s t test, or Fisher’s least significant difference test.

Results

To address the potential role of estradiol metabolites in regulating VSMC growth, we first tested the potency of estradiol and estradiol metabolites to inhibit FCS-induced growth of human aortic VSMCs. Treatment of growth arrested VSMCs with 2.5% FCS induced DNA synthesis ([3H]-thymidine incorporation), collagen synthesis ([3H]-proline incorporation), and proliferation (cell number) by 7-fold (P<0.05 versus 0.4% BSA), 6-fold (P<0.05 versus 0.4% BSA) and 12- to 14-fold (P<0.05 versus 0.4% BSA), respectively. Estradiol metabolites differentially inhibited FCS-induced DNA synthesis (Figure 1A), proliferation (Figure 1B), and collagen synthesis (data not shown) in the following order of potency: 2-methoxyestradiol > 2-hydroxyestradiol > 4-methoxyestradiol > estradiol. Only high concentrations (>1 μmol/L) (Figure 1) of estrone, estriol, 16α-hydroxyestrone, 2-hydroxyestrone, and 4-methoxyestrone inhibited FCS-induced increases in DNA synthesis, cell proliferation, and collagen synthesis (data not shown). Concentrations of estradiol as low as 1 nmol/L (physiological concentration) and concentrations of 2-methoxyestradiol, 2-hydroxyestradiol, and 4-methoxyestradiol as low as 0.1 nmol/L significantly inhibited FCS-induced increases in cell number. Estradiol inhibited DNA synthesis and cell proliferation by 44% and 50%, respectively, at a concentration of 1 μmol/L. At this concentration, 2-methoxyestradiol, 2-hydroxyestradiol, and 4-methoxyestradiol inhibited DNA synthesis by 72%, 62%, and 58%, respectively, and cell number by 86%, 73%, and 64%, respectively. The inhibitory effects of estradiol increased with time of exposure. Treatment of human aortic VSMCs with a physiological concentration (1 nmol/L) of estradiol for 4, 8, 12, and 16 days inhibited FCS-induced cell proliferation by 17%, 31%, 45%, and 68%, respectively (Figure 2A).

To investigate whether the local metabolism of estradiol to metabolites by CYP450s is responsible for the growth inhibitory effects of estradiol, we studied the effects of estradiol in the presence and absence of modulators of CYP450. Exposure of human VSMCs for 48 hours to CYP450 inducers (3-MC [10 μmol/L] and phenobarbital [10 μmol/L])13,14 and to a CYP450 inhibitor (1-aminobenzotriazole [ABT], 0.1 to 10 μmol/L)15 did not influence FCS-induced DNA synthesis, cell proliferation, or collagen synthesis. However, the time-dependent effects of estradiol (1 nmol/L) on cell proliferation
were accentuated by the CYP inducers 3-MC and phenobarbital. For example, the inhibitory effect of estradiol (1 nmol/L) on FCS-induced proliferation of human aortic VSMCs on day 8 of the growth curve was enhanced from 31% to 58% and 69% by the CYP450 inducers 3-MC and phenobarbital, respectively (Figure 2A). In contrast, the CYP450 inhibitor ABT blocked the inhibitory effects of estradiol on cell proliferation (Figure 2A). Similar to the CYP450 inhibitor ABT, the inhibitory effects of estradiol on cell proliferation were blocked in presence of COMT inhibitors quercetin and OR486 (Figure 2B). The inhibitory effects of estradiol on DNA synthesis were completely abolished by the CYP450 inhibitor ABT and the COMT inhibitors quercetin and OR486 (Figure 2C); moreover, these effects of ABT, quercetin, and OR486 were concentration dependent (Figure 2C). Apart from augmenting the effects of estradiol on cell number (Figure 2A), 3-MC and phenobarbital also enhanced the inhibitory effects of estradiol on collagen synthesis (Figure 3A) and cell migration (Figure 3B). The CYP450 inhibitor ABT and the COMT inhibitors quercetin and OR486 also blocked the enhanced inhibitory effects of estradiol observed in the presence of the CYP450 inducers 3-MC and phenobarbital on collagen synthesis, SMC migration (Figure 3A and 3B), and cell proliferation (data not shown). Trypan blue exclusion tests and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay indicated no loss in viability of cells treated with various agents.

ICI182780 blocked the growth inhibitory effects of estradiol in a concentration-dependent manner (Figure 2C). The lowest concentration of ICI182780 that significantly attenuated the inhibitory effects of 1 μmol/L estradiol was 10 μmol/L, and at a concentration of 50 μmol/L, ICI182780 completely blocked the inhibitory effects of 1 μmol/L estradiol (Figure 2B). Compared with ICI182780, ABT, quercetin, and OR486 were more potent in antagonizing the growth inhibitory effects of estradiol (Figure 2C).

To rule out the participation of the type II ER in mediating the modulatory effects of quercetin on the growth effects of estradiol, we evaluated the effects of estradiol on VSMC growth in the presence of luteolin, a high-affinity type II ER ligand.16 In contrast to the effects of quercetin and OR486, ICI182780 (50 μmol/L), an ER antag-

Figure 2. Effects of various treatments on the inhibitory effects of estradiol (β-E) on FCS-induced proliferation of VSMCs (A and B) and DNA synthesis (C) by VSMCs. A, The modulatory actions of CYP450 inhibitors and activators. B, The modulatory actions of COMT inhibitors. The concentration of estradiol was either 1 nmol/L (A and B) or 1 μmol/L (C). For 3-methylcholantherene (3-MC), phenobarbital (PB), quercetin (Quer), OR486 (OR), and 1-aminobenzotriazole (ABT), the concentration was 10 μmol/L or as indicated in Figure. For luteolin, the concentration was 25 μmol/L or as indicated. Values for each data point represent mean±SEM from 3 separate experiments conducted in quadruplicate. *P<0.05 vs cells treated with FCS alone; §P<0.05 vs estradiol alone.

Figure 3. Effects of various treatments on the inhibitory effects of estradiol (β-Est) on FCS-induced collagen synthesis (A) and PDGF-BB (25 ng/mL)-induced VSMC migration (B). For estradiol, the concentration was of 1 μmol/L. For 3-methylcholantherene (3-MC), phenobarbital (PB), quercetin (QUE), OR486 (OR), and 1-aminobenzotriazole (ABT), the concentration was 10 μmol/L. Values for each data point represent mean±SEM from 3 separate experiments conducted in quadruplicate. *P<0.05 vs cells treated with FCS alone; §P<0.05 vs estradiol alone.

and OR486 also blocked the enhanced inhibitory effects of estradiol observed in the presence of the CYP450 inducers 3-MC and phenobarbital on collagen synthesis, SMC migration (Figure 3A and 3B), and cell proliferation (data not shown). Trypan blue exclusion tests and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay indicated no loss in viability of cells treated with various agents.
and 4-hydroxyestradiol by CYP1A2 and with apparent K, 27 
\( \mu \text{mol/L} \).\(^{17}\) Compared with either quercetin, OR486, or ABT, 
ICI182780 was less potent in blocking the inhibitory effects 
of estradiol on VSMC growth (Figure 2B). Moreover, 1 
\( \mu \text{mol/L} \) ICI182780 was unable to block the inhibitory effects 
of 1 \( \mu \text{mol/L} \) estradiol, even though the estradiol-to-ICI182780 
ratio was 1:1000 (Figure 5B). To further reaffirm that the 
decreased potency of ICI182780 was not caused by lack of 
expression of ERs in the SMCs, we assayed the expression of 
ER-\( \alpha \) and ER-\( \beta \) in the cells used. As shown in Figure 6, the 
human aortic SMCs expressed both ER-\( \alpha \) and ER-\( \beta \).

Discussion

The purpose of this study was to test the hypothesis that in 
human VSMCs, conversion of estradiol to methoxyestradiols 
mediates in part the inhibitory effects of estradiol on VSMC 
growth. In support of this hypothesis, we observed that 
(1) methoxyestradiols and their precursors, hydroxyestradi-
ols, are more potent than estradiol in inhibiting VSMC 
growth; (2) the inhibitory effects of estradiol on VSMC 
growth are enhanced by CYP450 inducers; and (3) and the 
inhibitory effects of estradiol, both in presence and absence 
of CYP450 inducers, are abolished by CYP450 and COMT 
inhibitors.

The hypothesis that conversion of estradiol to me-
thoxyestradiols mediates in part the inhibitory effects of 
estradiol on VSMC growth is also supported by the observa-
tion that the inhibitory actions of 2-hydroxyestradiol, but not 
of 2-methoxyestradiol, on VSMCs are attenuated by COMT 
inducers quercetin and OR486,\(^{12}\) drugs that have no binding 
affinity for ERs.\(^{17,18}\) In this regard, both quercetin and OR486 
decrease the inhibitory effects of estradiol on human VSMCs 
regardless of the absence or presence of CPY450 inducers. 
On the other hand, even high concentrations of the ER 
antagonist ICI182780 do not block the growth inhibitory 
effects of either 2-hydroxyestradiol or 2-methoxyestradiol. 
This is strong evidence that the metabolism by COMT of 
2-hydroxyestradiol to 2-methoxyestradiol mediates the inhibi-
tory effects of 2-hydroxyestradiol. Moreover, these results 
indicate that the inhibitory effects of 2-hydroxyestradiol and 
2-methoxyestradiol are ER-independent, as would be anticip-
ted because of the low affinity of hydroxyestradiols and 
methoxyestradiols for ERs. The hypothesis that the inhibitory 
effects of estradiol are caused by its conversion to methoxyesta-
diols is further supported by our observation that human VSMCs 
metabolize 2-hydroxyestradiol to 2-methoxyestradiol and that 
this metabolic step is blocked by quercetin and OR486.\(^{12}\)
Quercetin not only blocks the conversion of catecholestragens to methoxyestrogens but also binds to the type II ER, which has been implicated in regulating cell growth. The finding that in contrast to the effects of quercetin, the inhibitory effects of estradiol are not blocked by luteolin, a high-affinity type II ER ligand, rules out the participation of type II ER and supports the conclusion that quercetin blocks the inhibitory effects of estradiol by inhibiting COMT and blocking the formation of methoxyestradiols. This contention is directly supported by the observations that OR486, an established COMT inhibitor, blocked the metabolic conversion of 2-hydroxyestradiol to 2-methoxyestradiol by SMCs, as well as the antimitogenic effects of both estradiol and 2-hydroxyestradiol, but not of 2-methoxyestradiol.

High concentrations of the ER antagonist ICI182780 block the inhibitory effects of estradiol on human VSMCs. This result is at odds with our hypothesis that methoxyestradiols mediate the growth inhibitory actions of estradiol. However, because the molecular structure of ICI182780 resembles estradiol, it is likely that ICI182780 competes with estradiol for CYP450s and blocks the metabolism of estradiol. In support of this idea, we recently reported that ICI182780 inhibits the metabolism of estradiol to catecholestradiols in human hepG2 cells expressing CYP1A2, an enzyme responsible in part for metabolizing estradiol to catecholestradiols. Thus, the inhibitory effects of ICI182780 may be mediated either via antagonism of ERs or by inhibition of estradiol metabolism. In this regard, it is important to note that the blockade of estradiol-induced growth inhibition by ICI182780 is independent of the estradiol-to-ICI182780 ratio, but rather is dependent on whether the concentration of ICI182780 inhibits estradiol metabolism. The potential that ICI182780 may block the antimitogenic effects of estradiol by inhibiting COMT can also be ruled out, as it blocked the antimitogenic effects of estradiol but not of 2-hydroxyestradiol and 2-methoxyestradiol. Moreover, in contrast to quercetin and OR486, ICI182780 failed to inhibit the conversion of 2-hydroxyestradiol to 2-methoxyestradiol. These findings support the conclusion that ICI182780 blocks the inhibitory effects of estradiol on VSMCs by preventing the metabolism of estradiol to catecholestradiols, the precursors of methoxyestradiols.

The findings that the inhibitory effects of estradiol are mediated by methoxyestradiols, which have little or no binding affinity for ERs, suggest that the inhibitory effects of estradiol may be ER-independent. However, the role of other unidentified ERs cannot be ruled out, as some recent studies have identified ER-γ, which is possibly a receptor for catecholestragens.

Our hypothesis that estradiol metabolism to methoxyestradiols is responsible for mediating the growth inhibitory effects of locally applied estradiol on vascular VSMCs has several important clinical implications. Hormone replacement therapy provides cardiovascular protection in only some postmenopausal women, a finding that may be explained by differential metabolism of estradiol to methoxyestradiols in VSMCs in postmenopausal women receiving estradiol replacement therapy. In particular, genetic differences in CYP450s and COMT and the presence of endogenous or exogenous molecules that inhibit CYP450s or COMT may influence the vascular-protective effects of estradiol.

Another implication of our hypothesis relates to the increased risk of cancer induced by hormone replacement therapy. 2-Methoxyestradiol decreases tumor growth, angiogenesis, and growth of cancer cells, and a reduced synthesis of 2-hydroxyestradiol, a precursor of 2-methoxyestradiol, is associated with an increased risk of cancer. Therefore, 2-methoxyestradiol may prevent both cancer and cardiovascular disease. Inasmuch as cancer (mammary and endometrial) is one of the main risks of hormone replacement therapy, it is possible that 2-methoxyestradiol could be used clinically to prevent cardiovascular disease in women without increasing the risk of cancer. In addition, because 2-methoxyestradiol is non-feminizing, it could also be of therapeutic benefit in men.

**Perspectives**

The findings of this study provide evidence in human VSMCs that the local metabolism of estradiol to methoxyestradiols, metabolites of estradiol with little or no affinity for ERs, mediates in part the ER-independent inhibitory effects of locally applied estradiol. Our results suggest that estradiol metabolism in the vascular wall may be an important determinant of the cardiovascular protective effects of circulating estradiol and that individual differences, both genetic and acquired, in the local vascular metabolism of estradiol could determine a particular woman’s risk of cardiovascular disease. Moreover, genetic or acquired differences in estradiol metabolism may determine the cardiovascular benefits a woman receives from estradiol replacement therapy in the postmenopausal state. Finally, our results also imply that nonfeminizing estradiol metabolites may afford cardiovascular protection regardless of gender.

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