CYP450- and COMT-Derived Estradiol Metabolites Inhibit Activity of Human Coronary Artery SMCs


Abstract—The purpose of this study is to test the hypothesis that the inhibitory effects of estradiol in human coronary vascular smooth muscle cells are mediated via local conversion to methoxyestradiols via specific cytochrome P$_{450s}$ (CYP450s) and catechol-O-methyltransferase (COMT). The inhibitory effects of estradiol on serum-induced cell activity (DNA synthesis, cell number, collagen synthesis, and cell migration) were enhanced by 3-methylcholangantherene, phenobarbital (broad-spectrum CYP450 inducers), and β-naphthoflavone (CYP1A1/1A2 inducer) and were blocked by 1-aminobenzotriazol (broad-spectrum CYP450 inhibitor). Ellipticine, α-naphthoflavone (selective CYP1A1 inhibitors), and pyrene (selective CYP1B1 inhibitor), but not ketoconazole (selective CYP3A4 inhibitor) or furafylline (selective CYP1A2 inhibitor), abrogated the inhibitory effects of estradiol on cell activity, a profile consistent with a CYP1A1/CYP1B1-mediated mechanism. The inhibitory effects of estradiol were blocked by the COMT inhibitors OR486 and quercetin. The estrogen receptor antagonist ICI 182,780 blocked the inhibitory effects of estradiol, but only at concentrations that also blocked the metabolism of estradiol to hydroxyestradiols (precursors of methoxyestradiols). Western blot analysis revealed that coronary smooth muscle cells expressed CYP1A1 and CYP1B1. Moreover, these cells metabolized estradiol to hydroxyestradiols and methoxyestradiols, and the conversion of 2-hydroxyestradiol to 2-methoxyestradiol was blocked by OR486 and quercetin. These findings provide evidence that the inhibitory effects of estradiol on coronary smooth muscle cells are largely mediated via CYP1A1- and CYP1B1-derived hydroxyestradiols that are converted to methoxyestradiols by COMT. (Hypertension. 2003;41[part 2]:807-813.)

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

 Estradiol protects the blood vessels against vasoocclusive disorders. 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 (SMCs), 2 a process that contributes to vascular pathology after vascular injury. Because the biological effects of estrogens are mediated by estrogen receptors (ERs), and arteries express both ER$_{a}$ and ER$_{b}, 1-3$ the antivasoocclusive actions of estradiol are thought to be ER mediated. However, the recent findings that estradiol inhibits injury-induced lesion formation in arteries of mice lacking either ER$_{a}$ or ER$_{b}, 3$ and inhibits injury-induced SMC proliferation in double knockout mice lacking both ER$_{a}$ and ER$_{b}, 4$ challenge this concept. Thus, other mechanisms that do not involve ERs may participate in the vasculoprotective actions of estradiol.

We have recently shown that catecholestradiols and methoxyestradiols, endogenous metabolites of estradiol with little or no affinity for ERs, are potent inhibitors of SMC growth. 5 Moreover, production of estradiol metabolites plays a key role in regulating growth of cancer cells. 7 Therefore, it is possible that the vasculoprotective 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 inhibitory effects on SMC migration, growth, and extracellular matrix production.

The metabolism of estradiol to catecholestradiols and methoxyestradiols is catalyzed by the sequential actions of cytochrome P$_{450s}$ (CYP450s) 8 and catechol-O-methyltransferase (COMT). 9 Multiple isoforms of CYP450 that are capable of metabolizing estradiol to hydroxyestradiols have been identified; however, it is not known which CYP450s mediate the ER-independent inhibitory effects of locally applied estradiol. In the present study, we investigated the enzymes involved in mediating the inhibitory effects of estradiol on human coronary artery SMCs.
SMC Cultures and Growth Studies

Human female coronary artery SMCs were purchased from Clonetics and cultured under standard tissue culture conditions as described previously. Studies were conducted using phenol red-free culture medium. Subconfluent SMCs were growth-arrested for 48 hours in the presence or absence of 10 μmol/L 3-methylcholantherene (3-MC), phenobarbital, or β-naphtoflavone. For 3H-thymidine incorporation, growth was initiated by treating growth-arrested cells for 20 hours with Dulbecco’s modified Eagle’s medium supplemented with steroid-free fetal calf serum (FCS; 2.5%) and containing or lacking fresh 3-MC, phenobarbital, or β-naphtoflavone in the presence or absence of various treatments or vehicle. After 20 hours of incubation, treatments were repeated with freshly prepared solutions but supplemented with 3H-thymidine (1 μCi/mL) for an additional 4 hours. Incorporation of 3H-thymidine in the acid-insoluble fraction was subsequently measured on a scintillation counter by our previously described method.

To measure cell number, SMCs were plated (5×10⁵ cells/well) and allowed to attach overnight. Cells were growth-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, cells were treated every 48 hours with estradiol, and then cells were dislodged and counted on days 4, 8, 12, and 16.

For 3H-proline incorporation, confluent monolayers of SMCs were growth arrested for 48 hours in the presence or absence of 10 μmol/L 3-MC or phenobarbital. Collagen synthesis was stimulated by treating cells for 48 hours with 2.5% FCS in the presence of 3H-l-proline (1 μCi/mL) and 3-MC, phenobarbital, or β-naphtoflavone and with various other treatments. Incorporation of 3H-proline in the acid-insoluble fraction was subsequently measured on a scintillation counter by our previously described method. Moreover, confluent monolayers of SMCs were used to preclude the influence of changes in cell number.

Modified Boyden chambers were used to evaluate the effects of various treatments on 2.5% FCS–induced SMC migration, as previously described. To investigate the effects of CYP450 and COMT modulators, SMCs were pretreated with the respective modulators as described above under DNA synthesis.

Metabolism Studies

To assess whether SMCs can metabolize estradiol, SMCs grown to confluence in 75-cm² flasks and pretreated for 24 hours with or without 3-MC (10 μmol/L) or phenobarbital (10 μmol/L) were incubated with Dulbecco’s modified Eagle’s medium/F12 containing 1 μmol/L estradiol for 24 hours. After incubation, the medium and the cells were collected, and the samples were treated with acetone containing 1 mmol/L ascorbic acid. Subsequently, 2-fluoroestradiol (50 pmol/10 mL; internal standard) was added to each sample, and the metabolites, together with the internal standard, were extracted with dichloromethane. The dichloromethane was evaporated, and the residue was derivatized with trifluoroacetyl anhydride and analyzed by negative-ion gas chromatography–mass spectrometry (GC-MS). Standards of the metabolites were extracted similarly along with the internal standards and were used to generate a standard curve.

To assess metabolism of catecholestradiols to methoxyestradiols, confluent monolayers of SMCs 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.

CYP1A1 and CYP1B1 Expression Studies

To investigate whether the human coronary vascular SMCs express CYP1A1 and CYP1B1, cell lysates from cultured SMCs were analyzed by Western blotting and probed with antibodies to CYP1A1 (rabbit antihuman polyclonal antibodies; Chemicon International Inc) and CYP1B1 (rabbit antihuman polyclonal antibodies; Gentest Corp).

Statistics

Statistical significance (P<0.05) was assessed with ANOVA, Student t test, or Fisher least significant difference test.

Results

Trypan blue exclusion tests and MTT assay indicated no loss in viability of cells treated with various agents. Treatment of growth-arrested SMCs with 2.5% FCS induced DNA synthesis (3H-thymidine incorporation), collagen synthesis (3H-proline incorporation), proliferation (cell number), and cell migration.

 Estradiol, 2-hydroxyestradiol, and 2-methoxyestradiol inhibited FCS-induced DNA synthesis (3H-thymidine incorporation), collagen synthesis (3H-proline incorporation), proliferation (cell number), and cell migration.

Estradiol, 2-hydroxyestradiol, and 2-methoxyestradiol inhibited FCS-induced DNA synthesis (Figure 1A), proliferation (Figure 1B), collagen synthesis (Figure 1C), and cell migration (Figure 1D) in the following order of potency (from most to least): 2-methoxyestradiol, 2-hydroxyestradiol, and estradiol. Physiological concentrations (1 mmol/L) of estradiol significantly inhibited FCS-induced increases in cell number. The inhibitory effects of estradiol increased with time of exposure. Treatment of SMCs with 1 mmol/L of estradiol for 4, 8, 12, and 16 days inhibited FCS-induced cell proliferation by 17%, 31%, 45%, and 68%, respectively.

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 CYP450s. Exposure of SMCs for 48 hours to CYP450 inducers (10 μmol/L 3-MC, 10 μmol/L phenobarbital, or 10 μmol/L β-naphtoflavone) and to a CYP450 inhibitor (0.1 to 10 μmol/L 1-aminobenzotriazole; ABT) did not influence FCS-induced DNA synthesis, cell proliferation, or collagen synthesis. However, the concentration-dependent effects of estradiol (1 to 100 nmol/L) on cell proliferation were significantly enhanced by 3-MC, phenobarbital (CYP450 inducers), and β-naphtoflavone (selective CYP1A1/
1A2 inducer). For example, the inhibitory effect of estradiol (1 nmol/L) on FCS-induced proliferation of SMCs on day 8 of the growth curve was enhanced from 29% to 51%, 56%, and 41% by the CYP450 inducers 3-MC, phenobarbital, and β-naphthoflavone, respectively (Figure 2). In contrast, the broad-spectrum CYP450 inhibitor ABT blocked the inhibitory effects of estradiol on cell proliferation, DNA synthesis, collagen synthesis, and cell migration (Figure 2).

Similar to ABT, the growth inhibitory effects of estradiol on DNA synthesis, cell proliferation, collagen synthesis, and cell migration were blocked in presence of ellipticine (selective CYP1A1 inhibitor), 10 μmol/L, pyrene (selective CYP1B1 inhibitor), 5 nmol/L, and α-naphthoflavone (selective CYP1A1 inhibitor), 10 μmol/L, but not ketoconazole (selective CYP3A4 inhibitor), 10 μmol/L or furafylline (selective CYP1A2 inhibitor), 10 μmol/L (Figure 3). Ellipticine, pyrene, and α-naphthoflavone abrogated the inhibitory effects of 100 nmol/L of estradiol on cell proliferation from 49% to 14%, 9%, and 19%, respectively.

To test further the hypothesis that metabolism of estradiol by CYP1A1 and CYP1B1 is responsible for mediating the inhibitory effects of estradiol in SMCs, we assayed the expression of CYP1A1 and CYP1B1. Human coronary artery SMCs expressed both CYP1A1 and CYP1B1 (Figure 4). Moreover, SMCs metabolized estradiol to 2- and 4-methoxyestradiol and to 2- and 4-hydroxyestradiol (Figure 4). Compared with SMCs grown in medium containing FCS alone, the expression of both CYP1A1 and CYP1B1 was increased in SMCs treated with 10 μmol/L of 3-MC and phenobarbital. Based on the densitometric analysis of the bands, the expression of CYP1A1 and CYP1B1 in response to 3-MC and phenobarbital was increased from 23 arbitrary units (AU) in controls to 34 AU (48% increase) and 35 AU (52% increase), respectively, for CYP1A1; and from 11 to 16.5 AU (50% increase) and 17 AU (54.5% increase), respectively, for CYP1B1. In SMCs pretreated with 3-MC and phenobarbital, the formation of 2- and 4-hydroxyestradiol and 2- and 4-methoxyestradiol was significantly induced. Compared with SMCs treated with phenobarbital, the formation of 2-methoxyestradiol was significantly higher in SMCs stimulated with 3-MC. Moreover, compared with 4-methoxyestradiol, the conversion of estradiol to 2-methoxyestradiol was significantly greater in presence of both 3-MC and phenobarbital.

Similar to the CYP450 inhibitors, the inhibitory effects of estradiol on DNA synthesis, cell proliferation, collagen synthesis, and cell migration were blocked by the COMT inhibitors quercetin and OR486 (Figure 5). The CYP450 inhibitor ABT and the COMT inhibitors quercetin and OR486 also blocked the enhanced inhibitory effects of estradiol observed in the presence of CYP450 inducers, 3-MC and phenobarbital, on DNA synthesis, cell proliferation, and collagen synthesis (data not shown). ICI 182,780, an ER antagonist, blocked the inhibitory effects of estradiol on DNA synthesis in a concentration-dependent manner (Figure 5). The lowest concentration of ICI 182,780 that significantly attenuated the inhibitory effects of 1 μmol/L estradiol was 10 μmol/L.
μmol/L, and at a concentration of 50 μmol/L, ICI 182,780 completely blocked the inhibitory effects of 1 μmol/L estradiol (Figure 5). Compared with ICI 182,780, ABT, quercetin, and OR486 were more potent in antagonizing the inhibitory effects of estradiol (Figure 5). Quercetin and OR486 also blocked the inhibitory effects of estradiol on collagen synthesis, cell number, and cell migration (Figure 5).

The inhibitory effects of 2-hydroxyestradiol, but not 2-methoxyestradiol, on SMC proliferation, DNA synthesis, collagen synthesis, and cell migration (Figure 6) were completely prevented by quercetin and OR486, competitive inhibitors of COMT.\(^3,9\) In contrast to quercetin and OR486, ICI 182,780 (50 μmol/L) did not block the growth inhibitory effects of either 2-hydroxyestradiol or 2-methoxyestradiol (Figure 6).

Human coronary artery SMCs metabolized 2-hydroxyestradiol to 2-methoxyestradiol, and this metabolism was blocked by quercetin and OR486, but not ICI 182,780 (Figure 7A). Concentrations of ICI 182,780 greater than 1 μmol/L ICI 182,780 inhibited the metabolism of estradiol to 2- and 4-hydroxyestradiol by human lymphoblastoid cells expressing CYP1A1 and by supersomes expressing human CYP1B1 (Figure 7B). Compared with quercetin, OR486, ABT, or pyrene, much higher concentrations (>10 μmol/L) of ICI 182,780 were required to block the inhibitory effects of estradiol on SMC growth (Figure 5). Moreover, 1 μmol/L ICI 182,780 was unable to block the inhibitory effects of 1 nmol/L estradiol; yet, 50 μmol/L ICI 182,780 was able to block the inhibitory effects of 50 nmol/L estradiol, even though the estradiol-to–ICI 182,780 ratio was 1:1000 in both cases (Figure 7C).

**Discussion**

The purpose of the present study was to test the hypothesis that in human coronary artery SMCs, sequential conversion of estradiol to methoxyestradiols by CYP450s and COMT, respectively, mediates in part the inhibitory effects of estradiol on human coronary SMC activity (DNA synthesis, cell number, collagen synthesis, and cell migration). In support of this hypothesis, we observed that (1) 2-methoxyestradiol and its precursor, 2- hydroxyestradiol, are more potent than estradiol in inhibiting SMC activity; (2) the inhibitory effects of estradiol on SMC activity are enhanced by CYP450
hydroxylated by CYP3A4 and CYP1A2; therefore, it is possible that these CYP450 isozymes also contribute to conversion of estradiol to inhibitory metabolites. However, our finding that the inhibitory effects of estradiol are not blocked by ketoconazole, a CYP3A4 inhibitor, or by furafylline, a CYP1A2 inhibitor, suggests that these isozymes do not play a major role in mediating the inhibitory effects of estradiol.

The hypothesis that conversion of estradiol to methoxyestradiols mediates in part the inhibitory effects of estradiol on SMC growth is also supported by the observation that the inhibitory actions of 2-hydroxyestradiol, but not 2-methoxyestradiol, on SMCs are attenuated by the COMT inhibitors quercetin and OR486,10 drugs that have no binding affinity for ERs.1 In this regard, both quercetin and OR486 also decrease the inhibitory effects of estradiol on human coronary SMCs. On the other hand, even high concentrations of the ER antagonist ICI 182,780 do not block the 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 inhibitory effects of 2-hydroxyestradiol. Moreover, these results indicate that the inhibitory effects of 2-hydroxyestradiol and 2-methoxyestradiol are ER-independent, as would be anticipated because of the low affinity of hydroxyestradiols and methoxyestradiols for ERs. The hypothesis that the inhibitory effects of estradiol are due to its conversion to methoxyestradiols is further supported by our observation that human coronary SMCs metabolize 2-hydroxyestradiol to 2-methoxyestradiol, and this metabolic step is blocked by quercitin and by OR486. Equally importantly, human vascular SMCs metabolize estradiol to 2- and 4-methoxyestradiol, and the formation of these metabolites is enhanced by CYP450 inducers 3-MC and phenobarbital.

High concentrations of the ER antagonist ICI 182,780 block the inhibitory effects of estradiol on SMCs. This result is at odds with our hypothesis that methoxyestradiols mediate the growth inhibitory actions of estradiol. However, because the molecular structure of ICI 182,780 resembles estradiol, it is likely that ICI 182,780 competes with estradiol for CYP450s and blocks the metabolism of estradiol. This contention is supported by our finding that in human lymphoblastoid cells expressing CYP1A1, ICI 182,780 inhibited the metabolism of estradiol to catecholestradiols. Thus, the inhibitory effects of ICI 182,780 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 inhibition by ICI 182,780 is independent of the estradiol-to-ICI 182,780 ratio, but rather is dependent on whether the concentration of ICI 182,780 inhibits estradiol metabolism. The potential that ICI 182,780 may block the inhibitory effects of estradiol by inhibiting COMT can also be ruled out, as it blocked the inhibitory effects of estradiol, but not 2-hydroxyestradiol or 2-methoxyestradiol. Moreover, in contrast to quercetin and OR486, ICI 182,780 failed to inhibit the conversion of 2-hydroxyestradiol to 2-methoxyestradiol. These findings support the conclusion that ICI 182,780 blocks the inhibitory effects of estradiol on coronary artery SMCs by preventing the metabolism of estradiol to catecholestradiols, the precursors of methoxyestradiols.
Although our findings provide evidence that the inhibitory effects of estradiol are mediated by methoxyestradiols, the relative role of 2- and 4-methoxyestradiols in mediating these effects still remains unresolved. CYP1A1 largely converts estradiol to 2-hydroxyestradiol, and CYP1B1 largely metabolizes estradiol to 4-hydroxyestradiol.8,11 Thus, the inhibitory effects of estradiol are likely mediated via both 2-and 4-methoxyestradiol. However, the finding that the conversion of estradiol to 2-methoxyestradiol was significantly greater than its conversion to 4-hydroxyestradiol suggests that the antimitogenic effects of estradiol are largely 2-methoxyestradiol-mediated. This contention is further supported by our previous finding that 2-methoxyestradiol is more potent than is 4-methoxyestradiol in inhibiting SMC growth3,12 and by the finding that estradiol is metabolized endogenously to 2-methoxyestradiol,7,13,14 whereas the levels of 4-methoxyestradiol are low.15 It is unclear why the inhibitory effects of estradiol are enhanced to similar extent by 3-MC and phenobarbital, even though the formation of 2-methoxyestradiol is far greater in SMCs treated with 3-MC compared with phenobarbital. In this regard, it is possible that maximal inhibition of SMC growth is achieved at concentrations of 2-methoxyestradiol attained in response to phenobarbital; therefore, any increase beyond such levels by 3-MC cannot further enhance the effects of estradiol. Alternatively, 3-MC may inhibit the catabolism of 2-methoxyestradiol, which may result in a greater accumulation of 2-methoxyestradiol by 3-MC compared with phenobarbital. It is also plausible that 4-methoxyestradiol, which is induced to a similar extent by 3-MC and phenobarbital, is the important metabolite that mediates the antimitogenic effects of estradiol. Finally, differences in the experimental conditions (cell number, cell density, time of treatment, concentration of estradiol, presence or absence of serum) for the metabolism and growth studies may not permit an accurate and quantitative comparison between the antimitogenic effects of estradiol and the rate of metabolite formation. Thus, additional studies are required to elucidate the exact contribution of 2- and 4-methoxyestradiol in mediating the antimitogenic effects of estradiol.

Our hypothesis that estradiol metabolism to methoxyestradiols is responsible for mediating the inhibitory effects of locally applied estradiol on coronary artery SMCs has several important clinical implications. Hormone replacement therapy provides protection against coronary artery disease in only some postmenopausal women,1 a finding that may be explained by differential metabolism of estradiol to methoxyestradiols in SMCs 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 vasculoprotective effects of estradiol. This contention is further supported by the fact that compared with other vascular beds, the coronary cells have increased capacity to produce 2-methoxyestradiol.10 Our findings may also be important in explaining the recent observations that hormone replacement therapy induced adverse cardiovascular events in normal postmenopausal women. In this context, the formation of methoxyestradiols can be influenced by genetic and lifestyle factors, including diet and stress.16,17

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,7 and a reduced synthesis of 2-hydroxyestradiol, a precursor of 2-methoxyestradiol, is associated with an increased risk of cancer.7 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 nonfeminizing,13 it could also be of therapeutic benefit in men.

**Perspectives**

The present study provides evidence that estradiol inhibits the activity of human coronary artery SMCs via the local metabolism of estradiol to methoxyestradiols by CYP1A1/CYP1B1 and COMT. Our results suggest that estradiol metabolism within the coronary artery 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 coronary artery 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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**References**


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