Methoxyestradiols Mediate the Antimitogenic Effects of Locally Applied Estradiol on Cardiac Fibroblast Growth

Raghvendra K. Dubey, Delbert G. Gillespie, Lefteris C. Zacharia, Marinella Rosselli, Bruno Imthurn, Edwin K. Jackson

Abstract—Estradiol inhibits cardiac fibroblast growth and may protect against cardiac remodeling associated with heart disease. However, the mechanisms by which estradiol attenuates cardiac fibroblast growth remain unclear. Because cardiac fibroblasts express cytochrome P450s (CYP450s) and catechol-O-methyltransferase (COMT) capable of converting estradiol to hydroxyestradiols and methoxyestradiols, respectively, and because hydroxyestradiols and methoxyestradiols (estradiol metabolites with little affinity for estrogen receptors) are potent inhibitors of cardiac fibroblast growth, we hypothesized that the antimitogenic effects of estradiol are mediated via hydroxyestradiols and/or methoxyestradiols. The inhibitory effects of estradiol (1 to 100 nmol/L) on serum-stimulated 3H-thymidine incorporation (DNA synthesis), 3H-proline incorporation (collagen synthesis), and cell number (proliferation) were enhanced (P<0.005) by CYP450 inducers 3-methylcholanthrene (10 μmol/L) and phenobarbital (10 μmol/L). Moreover, the inhibitory effects of estradiol were blocked by the CYP450 inhibitor 1-aminobenzotriazole (10 μmol/L) and the COMT inhibitors quercetin (10 μmol/L) and OR486 (10 μmol/L). In contrast to estradiol, the modulators of CYP450 and COMT were poor ligands for estrogen receptors (binding affinity ≤0.0001% versus estradiol). In cardiac fibroblasts, both quercetin and OR486 inhibited the metabolism of hydroxyestradiol to methoxyestradiol and blocked the inhibitory effects of hydroxyestradiol on cardiac fibroblast proliferation and DNA and collagen synthesis. The abrogating effects of quercetin and OR486 on the metabolism and antimitogenic effects of 2-hydroxyestradiol were mimicked by 20 μmol/L norepinephrine and isoproterenol, substrates for COMT. Our findings provide evidence that estradiol can inhibit cardiac fibroblast growth via an estrogen receptor–independent pathway that involves the local metabolism of estradiol to methoxyestradiols. (Hypertension. 2002;39[part 2]:412-417.)

Key Words: hormones ■ menopause ■ fibroblasts ■ metabolism ■ cardiovascular diseases

Epidemiological studies provide evidence that estrogens are cardioprotective; however, the mechanisms by which estrogens induce cardioprotection are not fully understood.1 Because coronary artery disease is the most frequent cause of death among women, most research has focused on evaluating the effects of estrogens on vascular cells (endothelial cells and smooth muscle cells [SMCs]) involved in the vascular remodeling process associated with vaso-occlusive disorders. Thus, much less is known about the influence of estrogens on cardiac fibroblasts (CFs).1

Similar to SMCs within the vasculature, abnormal growth of CFs also is importantly involved in the pathophysiology of cardiovascular diseases, including cardiac remodeling induced by hypertension, myocardial infarction, and myocardial reperfusion injury after ischemia.2 CFs, which compose 60% of the total heart cells, contribute to pathological structural changes in the heart by undergoing proliferation, depositing extracellular matrix proteins, and replacing myocytes with fibrotic scar tissue.2 Thus CF-induced cardiac remodeling may participate in diastolic and systolic dysfunction, leading to congestive heart failure.

Like SMCs,3 CFs possess functional estrogen receptors (ERs)4 α and β, and estradiol inhibits mitogen-induced growth in both SMCs and CFs. Because most biological effects of estrogens are mediated by ERs, the prevailing view is that ERs mediate the cardiovascular protective effects of estradiol. However, the reports that estradiol inhibits injury-induced lesion formation in arteries of mice that lack either ER-α3 or ER-β6 seriously challenges this hypothesis. Moreover, our recent findings that 2-hydroxyestradiol and 2-methoxyestradiol, major endogenous metabolites of estradiol with little or no affinity for ERs, are more potent than estradiol in inhibiting SMC7 and CF8 growth suggest that alternative non-ER mechanisms may be operative.

CFs express cytochrome P450 (CYP450) enzymes that convert estradiol to 2- and 4-hydroxyestradiols and catechol-
O-methyltransferase (COMT) that metabolizes 2- and 4-hydroxyestradiol to 2- and 4-methoxyestradiol. Also, hydroxysteroid dehydrogenases and methoxysteroid dehydrogenases are potent inhibitors of CF growth. Therefore, it is conceivable that the antimitogenic effects of estradiol on CFs are not mediated exclusively by estradiol interactions with ERs, but rather in part by local (ie, cardiac) conversion of estradiol to nonestrogenic metabolites. In the present study, we tested our hypothesis that local conversion of estradiol to hydroxy and methoxy metabolites mediates the antimitogenic effects of locally applied estradiol on CFs. To test this hypothesis, we investigated the inhibitory effects of estradiol on DNA synthesis, collagen synthesis, and proliferation of rat CFs in the presence and absence of modulators (inhibitors or activators) of CYP450s and COMT.

Methods

Cardiac Fibroblast Cell Culture
Female Sprague-Dawley rats that weighed 150 to 200 g were obtained from Charles River (Wilmington, MA) and were fed a standard rat diet and tap water ad libitum. CFs were cultured from the left ventricles by our previously described method, using the enzymatic digestion and selective plating technique. Cells were cultured in steroid-free and phenol red-free medium. Purity of CFs was assessed by morphological characterization and by positive and negative immunostaining with antibodies against von Willebrand factor VIII, sarcomeric actin (striated muscle; monoclonal), desmin, and vimentin, and as described by us earlier. Our findings suggest that the purity of cultured CFs was >98%. CFs in second and third passages were used in all studies.

Growth Studies
CFs were grown to subconfluence and growth-arrested for 48 hours in the presence or absence of 3-methylcholanthrene (3-MC; 10 µmol/L) or phenobarbital (10 µmol/L). For [3H]-thymidine incorporation, growth was initiated by treating growth-arrested cells for 20 hours with DMEM supplemented with 2.5% FCS and containing or lacking fresh 3-MC or phenobarbital in the presence or absence of various treatments or vehicle. 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 0.3 N NaOH and 0.1% sodium dodecylsulfate after incubation at 50°C for 2 hours. Aliquots from 4 wells for each treatment with 10 mL of scintillation fluid were counted in a liquid scintillation counter.

For cell number experiments, CFs were plated at a density of 5×10⁴ cells/well and allowed to attach overnight, growth-arrested for 48 hours, and then 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 cells were dislodged and counted on days 4, 8, and 12.

For [3H]-proline incorporation, confluent monolayers of CFs were growth-arrested for 48 hours in the presence or absence of 10 µmol/L 3-MC or phenobarbital. Collagen synthesis was initiated by treating cells for 48 hours with fresh 3-MC, phenobarbital or vehicle, 2.5% FCS, and [3H]-proline (1 µCi/mL) in the presence or absence of various treatments. [3H]-proline incorporation was determined at the end of the experiment as previously described. Briefly, 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 CFs. To make sure that the inhibitory effects of the experimental agents on collagen synthesis were not due to changes in cell number, we conducted the experiments in confluent monolayers of cells in which changes in cell number were precluded. In addition, 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.

Metabolism of Catecholestradiols to Methoxyestradiols
Confluent CFs 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 ultraviolet detection using gradient elution.

Binding Studies
Cytosolic extracts were extracted from CFs and incubated for 8 hours (4°C) with (H)-17β-estradiol (10⁻⁵ mol/L) with and without various competing ligands. Free from bound radioligand was separated by adding dextran-coated charcoal, and the bound [3H]-estradiol was quantified by counting on a β-scintillation counter.

Statistics
Statistical significant (P<0.05) was assessed with analysis of variance, Student's t-test, or Fisher's least significant difference test.

Results
To address the potential role of endogenous estradiol metabolites in regulating CF growth, we first tested the potency of estradiol and estradiol metabolites to inhibit FCS-induced growth of CFs. Growth was assessed by determining DNA synthesis ([3H]-thymidine incorporation), proliferation (cell number), and collagen synthesis ([3H]-proline incorporation). Treatment of growth-arrested CFs with 2.5% FCS induced DNA synthesis from 3569±42 dpm/well in the presence of the 0.4% BSA to 26 414±335 dpm/well (P<0.05 versus 0.4% BSA), collagen synthesis from 10 177±136 dpm/10⁴ CFs in the presence of the 0.4% BSA to 65 137±274 dpm/10⁴ CFs (P<0.05 versus 0.4% BSA), and cell proliferation on day 6 from 5627±199 cells/well in the presence of BSA to 88 004±379 cells/well (P<0.05 versus 0.4% BSA). The 2-hydroxy- and 2-methoxy metabolites of estradiol inhibited FCS-induced DNA synthesis (Figure 1A), proliferation (Figure 1B), and collagen synthesis (Figure 1C) with (H)-17β-estradiol (10⁻⁵ mol/L) with and without various competing ligands. Free from bound radioligand was separated by adding dextran-coated charcoal, and the bound [3H]-estradiol was quantified by counting on a β-scintillation counter.

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. Pre-treatment of CFs for 48 hours with CYP450 inducers (3-, 2-methoxyestradiol, 2-hydroxyestradiol, and 4-methoxyestradiol) significantly limited potent and inhibited FCS-induced increases in DNA synthesis, cell proliferation, and collagen synthesis (data not shown) only at high concentrations (>1 µmol/L; data not shown) not attained physiologically. The lowest concentration of estradiol, 2-methoxyestradiol, and 2-hydroxyestradiol that significantly inhibited FCS-induced increases in cell number was 1 nmol/L (physiological concentration). Treatment of CFs for 4 days with a physiological concentration (1 nmol/L) of estradiol inhibited cell proliferation by 19%. At this concentration, 2-methoxyestradiol and 2-hydroxyestradiol inhibited cell number by 44% and 38%, respectively.
and phenobarbital (10 μmol/L) did not influence FCS-induced DNA synthesis, cell proliferation, and collagen synthesis. The inhibitory effects of physiological concentrations of estradiol (0.001 μmol/L) on FCS-induced growth (DNA synthesis and cell proliferation) were enhanced in the presence of CYP450 inducers 3-methylcholantherene and phenobarbital (Figures 2A and 2B). Similar modulatory effects of 3-methylcholantherene and phenobarbital were also observed on collagen synthesis (Figure 2C). The inhibitory effects of estradiol (1 nmol/L) on FCS-induced proliferation of CFs on day 4 of the growth curve was enhanced from 18 to 44% and 38% by the CYP450 inducers phenobarbital and 3-methylcholantherene, respectively (Figure 2B). In the presence of the CYP450 inducers phenobarbital and 3-methylcholantherene, the concentration-dependent inhibitory effects of estradiol on FCS-induced growth (DNA synthesis, cell number, and collagen synthesis) were completely abolished by the CYP450 inhibitor ABT (Figure 3). Moreover, the enhanced inhibitory effects of estradiol observed in the presence of CYP450 inducers 3-methylcholantherene and phenobarbital on all parameters of CF growth were blocked by ABT (data not shown). Trypan blue exclusion tests and 3-[4,5-dimethylthiazol-2-y]-2,5-diphenyl tetrazolium bromide (MTT) assay indicated no loss in viability of cells treated with various agents.

Similar to the CYP450 inhibitor ABT, the concentration-dependent inhibitory effects of estradiol on cell proliferation,
DNA synthesis, and collagen synthesis were completely abolished by the COMT inhibitors quercetin and OR486 (Figure 4). Moreover, the enhanced inhibitory effects of estradiol observed in the presence of CYP450 inducers (3-methylcholantherene and phenobarbital) on all parameters of CF growth were blocked by ABT (data not shown). Similar to quercetin, catecholamines (isoproterenol, norepinephrine), which are substrates for COMT and can act as competitive inhibitors of COMT, also reversed the effects of estradiol on CF growth.\(^1\) The inhibitory effects of 10 nmol/L 2-hydroxyestradiol on FCS-induced CF proliferation was reduced from 37±2 to 13±1.6% and 5±1.24% by 20 μmol/L of norepinephrine and isoproterenol, respectively. Treatment of CFs with isoproterenol or norepinephrine alone did not significantly alter cell proliferation in response to 2.5% FCS. In CFs treated with 2.5% FCS in the absence or presence of isoproterenol and norepinephrine cell proliferation was 100, 94±4, and 95±3%, respectively.

The growth inhibitory effects of estradiol were blocked by ICI182780, and these effects were concentration dependent (Figure 6A). The lowest concentration of ICI182780 that significantly blocked the inhibitory effects of 1 μmol/L estradiol was 10 μmol/L; at a concentration of 50 μmol/L, ICI182780 completely blocked the inhibitory effects of 1 μmol/L estradiol (Figure 6A). As compared with ICI182780, both ABT and quercetin were more potent in antagonizing the growth-inhibitory effects of estradiol (Figure 6A).

The inhibitory effects of physiological concentrations of estradiol on CF growth were cumulative in nature, ie, the inhibitory effects of estradiol increased with time of exposure. Treatment of CFs with a physiological concentration (0.001 μmol/L) of estradiol for 4, 8, and 12 days inhibited FCS-induced cell proliferation by 17±1.6, 31±3.2, and 43±2.4%, respectively (Figure 6B). We have previously shown that at concentrations >1 μmol/L, ICI182780 inhibits the metabolism of estradiol to 2- and 4-hydroxyestradiol by CYP1A2 and with apparent inhibition constants (Ks) of 45 μmol/L and 27 μmol/L, respectively.\(^7\) In the present study, 1 μmol/L ICI182780 was unable to block the inhibitory effects growth-inhibitory effects of either 2-hydroxyestradiol or 2-methoxyestradiol (Figure 5). Similar to quercetin and OR486, the inhibitory effects of 2-hydroxyestradiol but not 2-methoxyestradiol were blocked by catecholamines (isoproterenol, norepinephrine), which are also competitive inhibitors of COMT.\(^1,1\) The inhibitory effects of 10 nmol/L 2-hydroxyestradiol on FCS-induced CF proliferation was reduced from 37±2 to 13±1.6% and 5±1.24% by 20 μmol/L of norepinephrine and isoproterenol, respectively. Treatment of CFs with isoproterenol or norepinephrine alone did not significantly alter cell proliferation in response to 2.5% FCS. In CFs treated with 2.5% FCS in the absence or presence of isoproterenol and norepinephrine cell proliferation was 100, 94±4, and 95±3%, respectively.

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of 1 nmol/L estradiol, even though the estradiol to ICI182780 ratio was 1:1000 (Figure 6B).

CFs incubated with 0.25 μmol/L 2-hydroxyestradiol for 60 minutes effectively metabolized 2-hydroxyestradiol to 2-methoxyestradiol, and this metabolism was inhibited by 10 μmol/L quercetin and OR486 but not by ICI182780 (Figure 6C). Similar to the COMT inhibitors, the conversion of 2-hydroxyestradiol to 2-methoxyestradiol was inhibited by 11.4±0.8 and 39±0.6% in presence of norepinephrine and isoproterenol, respectively.

In cytosolic extracts of CFs, the binding of ³H-17β-estradiol was blocked by 10 μmol/L of nonlabeled 17β-estradiol and ICI182780. In contrast, the binding of ³H-17β-estradiol was not significantly altered by 3-methylcholantherene, phenobarbital, ABT, quercetin, and OR486 (Figure 6D).

**Discussion**

Our previous studies demonstrated that estradiol inhibits CF growth and that the inhibitory effects of estradiol on CF growth are not reversed by the ER antagonist 4-hydroxytamoxifen. Thus, we hypothesized that the antimitogenic effects of estradiol are ER independent. Our previous studies also show that 2-hydroxyestradiol and 2-methoxyestradiol, major endogenous metabolites of estradiol with little affinity for ERα or ERβ, are more potent than estradiol in inhibiting CF growth. Because CFs express CYP450s and COMT, enzymes responsible for metabolizing estradiol to hydroxyestradiols and methoxyestradiols, we postulated that local conversion of estradiol to methoxyestradiols is, in part, responsible for the antimitogenic effects of estradiol on CF growth. Our hypothesis is strongly supported by the present findings that the inhibitory effects of estradiol on CF growth are enhanced by CYP450 inducers (3-methylcholantherene and phenobarbital) and abolished by a CYP450 inhibitor (ABT).

Our hypothesis is also supported by the observation that the inhibitory effects of 2-hydroxyestradiol but not 2-methoxyestradiol on CF growth are prevented by COMT inhibitors quercetin or OR486, drugs that have no binding affinity for ERα or ERβ. Moreover, quercetin and OR486 block the growth-inhibitory effects of estradiol, in either the absence or presence of CYP450 inducers. In contrast, ICI182780, an ER antagonist, does not block the growth-inhibitory effects of either 2-hydroxyestradiol or 2-methoxyestradiol. These findings provide evidence that the conversion of 2-hydroxyestradiol to 2-methoxyestradiol by COMT is responsible for the inhibitory effects of 2-hydroxyestradiol and that the effects of 2-methoxyestradiol are ER independent, as would be expected by the low affinity of 2-hydroxyestradiol and 2-methoxyestradiol for ERs. The hypothesis that the inhibitory effects of estradiol are mediated via generation of methoxyestradiols is further supported by our observation that CFs metabolize 2-hydroxyestradiol to 2-methoxyestradiol and this metabolic conversion is blocked by the COMT inhibitors quercetin and OR486.

Quercetin not only blocks the conversion of catecholestrogens to methoxyestradiols but also has many other effects, including binding to the type II ER, which has been implicated in regulating cell growth. The finding that, in contrast to 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 observation that OR486, an established COMT inhibitor, blocked the antimitogenic effects of both estradiol and 2-hydroxyestradiol but not 2-methoxyestradiol.

The growth inhibitory effects of estradiol are blocked by high concentrations of ICI182780, an ER antagonist that binds with equal affinity to both ERα and ERβ, a finding seemingly at odds with the hypothesis that methoxyestradiols mediate the growth-inhibitory effects of estradiol. However, because ICI182780 is chemically similar to estradiol, it is feasible that it not only binds to ERs but also competes with estradiol for CYP450s and inhibits estradiol metabolism. This notion is supported by our recent findings that ICI182780 inhibits the metabolism of estradiol to catecholestrogens in extracts of human hepG2 cells expressing CYP1A2 isozyme, which is one of the CYP450s responsible for metabolizing estradiol to catecholestrogens. The potential that ICI182780 may block the antimitogenic effects of estradiol by inhibiting COMT can be ruled out as it blocked the antimitogenic effects of estradiol but not 2-hydroxyestradiol and 2-methoxyestradiol. Moreover, in contrast to quercetin and...
OR486, ICI182780 failed to inhibit the conversion of 2-hydroxyestradiol to 2-methoxyestradiol.

The above findings suggest that the inhibitory effects of ICI182780 may be mediated either via antagonism of ER or via inhibition of estradiol metabolism. However, that the antagonistic effects of ICI182780 are not dependent on the estradiol to ICI182780 ratio but rather on concentrations of ICI182780 that inhibit estradiol metabolism suggests that ICI182780 blocks the inhibitory effects of estradiol by blocking estradiol metabolism to catecholestradiols, the precursors of methoxyestradiols.

COMT is highly expressed in both vascular and cardiac cells and is involved in the metabolism of catecholamines, which are known to induce deleterious effects on the cardiovascular system. Because both catecholestradiols and catecholamines share COMT for their metabolism, interactions of these compounds at COMT may play an important role in determining the effects of these molecules on the cardiovascular system. This contention is supported by our findings that the antimitogenic effects of 2-hydroxyestradiol and estradiol on CF growth are blocked by catecholestradiols and that catecholestradiols inhibited the conversion of 2-hydroxyestradiol to 2-methoxyestradiol by CFs. In this regard, we recently reported that in vascular SMCs, norepinephrine, epinephrine, and isoproterenol inhibit the metabolism of 2-hydroxyestradiol to 2-methoxyestradiol and abrogate the antimitogenic effects of 2-hydroxyestradiol on vascular SMC growth. Therefore, interactions between catecholestradiols and catecholamines may play a critical role in defining the effects of estradiol/catecholestradiols on the heart. A corollary of this hypothesis is that pathological increases in catecholamines would abrogate the protective effects of estradiol/catecholestradiols.

2-Methoxyestradiol is a potent anti-angiogenic agent that inhibits the growth of cancer cells. The lack of synthesis of 2-hydroxyestradiol, a precursor of 2-methoxyestradiol, is associated with an increased incidence of cancer. Thus, 2-methoxyestradiol may be of pharmacological importance in preventing both cancer and cardiovascular disease. Because one of the disadvantages of hormone replacement therapy is the risk of inducing cancer (mammary, endometrial), it is feasible that 2-methoxyestradiol could be used for prevention of cardiovascular disease in women without increasing the risk of cancer.

In summary, our findings provide the first evidence that the local metabolism of estradiol to methoxyestradiols, endogenous metabolites of estradiol with little affinity for ERs, is responsible in part for the ER-independent inhibitory effects of locally applied estradiol on CF growth. These findings suggest that local cardiac estradiol metabolism may be an important determinant of the cardioprotective effects of circulating estradiol. Thus, interindividual differences, either genetic or acquired, in the cardiac metabolism of estradiol may define a given woman’s risk of cardiac disease and influence the cardiac benefit that she receives from estradiol replacement therapy in the postmenopausal state. These findings also suggest that nonfeminizing estradiol metabolites may confer cardioprotection in both women and men.

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