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Methoxyestradiols Mediate the Antimitogenic Effects of Estradiol on Vascular Smooth Muscle Cells via Estrogen Receptor-Independent Mechanisms

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Estrogen receptors (ERs) are widely held to mediate the ability of 17β-estradiol (estradiol) to attenuate injury-induced proliferation of vascular smooth muscle cells (VSMCs) leading to vascular lesions. However, recent findings that estradiol prevents injury-induced vascular lesion formation in knockout mice lacking either ERα or ERβ seriously challenge this concept. Here we report that the local metabolism of estradiol to methoxyestradiols, endogenous metabolites of estradiol with no affinity for ERs, is responsible for the ER-independent inhibitory effects of locally applied estradiol on rat VSMC growth. These finding imply that local vascular estradiol metabolism may be an important determinant of the cardiovascular protective effects of circulating estradiol. Thus, interindividual differences, either genetic or acquired, in the vascular metabolism of estradiol may define a given female's risk of cardiovascular disease and influence the cardiovascular benefit she receives from estradiol replacement therapy in the postmenopausal state. These findings also imply that nonfeminizing estradiol metabolites may confer cardiovascular protection in both women and men.

Key Words: **hormone replacement therapy; menopause; vascular remodeling; metabolism; cardiovascular disease.**

Estradiol, an endogenous estrogen, attenuates vascular injury. For example, in animals physiological concentrations of estradiol inhibit the development of atherosclerosis (1), lessen balloon injury- and allograft-induced vascular lesions (1, 2) and reduce the proliferation of VSMCs (2), a process that importantly contributes to the development of vascular pathology in response to injury (1, 2). Because most biological effects of estrogens are mediated by ERs and blood vessels contain both ERα and ERβ (3–5), the prevailing view is that ERs mediate the vascular protective effects of estradiol. However, the recent findings that estradiol inhibits injury-induced lesion formation in arteries of mice lacking either ERα (6) or ERβ (7) seriously challenges this concept. Thus, other non-ER mechanisms may be operative.

It is conceivable that the vasculoprotective effects of estradiol are not mediated exclusively by estradiol interactions with ERs, but rather in part by local (i.e., vascular) conversion of estradiol to nonestrogenic metabolites that act to inhibit vascular lesion formation independently of ERs by exerting antimitogenic effects on VSMCs. Although VSMCs express cytochrome P450 (CYP450) enzymes that convert estradiol to 2- and 4-hydroxyestradiol (8) and catechol-*O*-methyltransferase (COMT) that metabolizes 2- and 4-hydroxyestradiol to 2- and 4-methoxyestradiol (9), the hypothesis that conversion of estradiol to metabolites occurs in VSMCs and mediates the antimitogenic effects of locally applied estradiol is untested. To test this hypothesis we investigated the inhibitory effects of estradiol on the growth of rat aortic VSMCs in the presence and absence of modulators (inhibitors or activators) of CYP450 and COMT (Fig. 1). Moreover, we investigated the capability of these cells to metabolize 2-hydroxyestradiol, a major circulating estradiol metabolite, to generate 2-methoxyestradiol.

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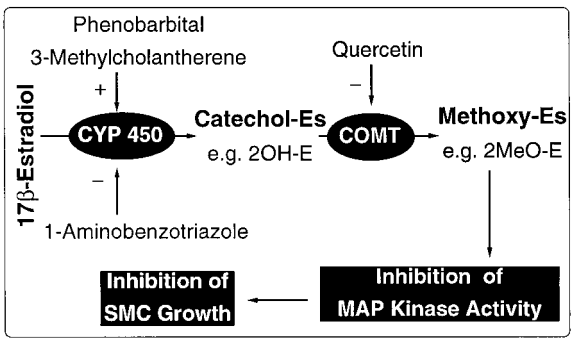

Methoxyestradiols (Methoxy-Es); 2-Methoxyestradiol (2MeO-E); Catecholestradiols (Catecol-Es); 2-Hydroxyestradiol (2-OH-E); Cytochrome P450 (CYP 450); Catechol-O-Methyltransferase (COMT); Inhibition (-); Induction (+)

FIG. 1. Schematic representation of the hypothesis and the experimental approach to demonstrate that metabolism of estradiol to methoxyestradiols is responsible for mediating the inhibitory effects of estradiol on VSMC growth.

METHODS

SMC Cultures

Aortic VSMCs were cultured from the thoracic aortas obtained from ether anesthetized Sprague–Dawley rats (n = 21) and as described by us previously (10).

Growth Studies

All cell culture and growth studies were conducted using phenol-red-free culture medium and steroid-free serum (FCS).

[^{3}H]Thymidine incorporation. Subconfluent VSMCs were growth arrested for 48 h with and without 10 μM 3-methylcholantherene or phenobarbital. Growth was initiated with 2.5% FCS in DMEM containing or lacking 3-methylcholantherene or phenobarbital in the presence or absence of 0.1 nM to 10 μM of estrogens (estradiol or endogenous estradiol metabolites) or estrogens plus 0.1–100 μM of ICI-182780, quercetin, 1-aminobenzotriazole or luteolin. Treatments were repeated after 20 h with [^{3}H]thymidine. Four hours later, [^{3}H]thymidine incorporation was determined (10).

Cell number experiments. Subconfluent VSMCs were treated (as above) every 24 h, and on day 5 cells were counted (Coulter counter). In some experiments, cells were treated every 48 h and counted on days 4, 8, and 12.

[^{3}H]Proline incorporation. Confluent VSMCs were growth arrested by feeding DMEM + 0.4% BSA for 48 h with and without 10 μmol/L 3-methylcholantherene or phenobarbital. Growth was initiated by treating cells for 48 h with fresh 3-methylcholantherene or phenobarbital, 2.5% FCS and L-[^{3}H]proline with and without various treatments (as above). L-[^{3}H]Proline incorporation was then determined (5).

MAP Kinase Activity

Confluent VSMCs were growth arrested for 48 h with and without 10 μmol/L 3-methylcholantherene or phenobarbital. VSMCs were washed with PBS and pretreated for 24 h with or without various test agents (as above) in the presence or absence of fresh 3-methylcholantherene or phenobarbital. Cells were stimulated for 10 min with PDGF-BB (25 ng/ml), and MAP kinase activity was determined in 5-μl aliquots of cytosolic extracts normalized to 1 mg/ml protein (5).

Binding Studies

Cytosolic fractions were extracted from MCF-7 cells (5) and incubated for 8 h (4°C) with [^{3}H]17β-estradiol (10^{-9} mol/L) with and without various competing ligands. Dextran-coated charcoal separated free from bound radioligand. Relative binding affinity of agents were calculated as the ratio of IC_{50} values.

Metabolism of 2-Hydroxyestradiol to 2-Methoxyestradiol

Confluent VSMCs were incubated with 2-hydroxyestradiol for 4 h, 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 HPLC with UV detection using gradient elution.

Effects of ICI 182780 on Metabolism of Estradiol

Extracts of human hepG2 cells expressing CYP1A2 (11, 12) were incubated for 25 min (37°C) with estradiol with and without ICI182780 in 50 mM phosphate buffer containing NADPH (8.3 mg/ml). Subsequently, internal standard (2-fluoroestradiol; 50 pmol/10 ml)was added, the reaction was extracted with methylene chloride and metabolites were derivatized with trifluoroacetic anhydride and analyzed by negative ion GC–MS.

Statistics

All experiments were performed in triplicates or quadruplicates with 6–8 separate cultures. Data are presented as mean ± SEM. Statistical significant ($P < 0.05$) was assessed with ANOVA, Student's t test, or Fisher's LSD test.

RESULTS

Endogenous metabolites of estradiol differentially inhibited FCS-induced DNA synthesis (Fig. 2A), proliferation (Fig. 2B) and collagen synthesis (Fig. 2C) and in the following order of potency: 2-methoxyestradiol > 2-hydroxyestradiol > estradiol ≥ 4-methoxyestradiol. The lowest concentrations of estradiol, 2-methoxyestradiol,

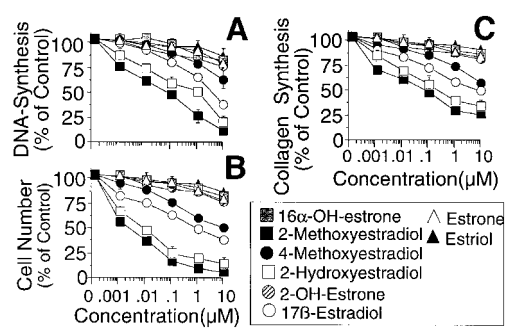

FIG. 2. Effects of increasing concentrations of endogenous estradiol metabolites on FCS-induced DNA synthesis (A), cell number (B), and collagen synthesis (C) in VSMCs. The results are presented as percentage change from control (VSMCs treated with 2.5% FCS alone). Values for each data point represent means ± SEM from 3–4 separate experiments conducted in quadruplicate.

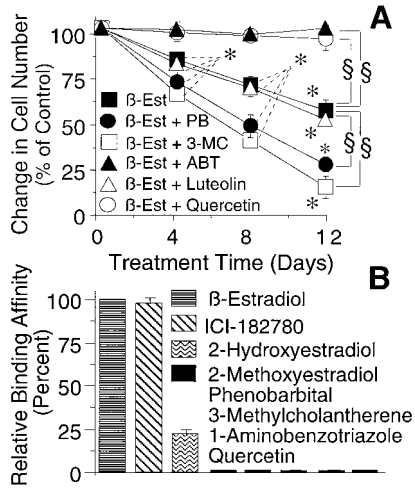

FIG. 3. Modulatory effects of various agents (inhibitors/activators of CYP450 and COMT) on the antimitogenic effects of estradiol (A) and their relative binding affinity to estrogen receptors (B; details under Results/Methods). Modulation of the time-dependent inhibitory effects of estradiol (β-Est) 1 nM (physiological concentration; A) on 2.5% FCS-induced proliferation of VSMCs by 10 μM of 3-methylcholantherene (3-MC), phenobarbital (PB), quercetin, luteolin, and 1-aminobenzotriazole (ABT). Values represent means ± SEM from 3–4 separate experiments conducted in quadruplicate. *$P < 0.05$ vs cells treated with FCS alone; §significantly ($P < 0.05$) different from β-estradiol alone.

2-hydroxyestradiol, and 4-methoxyestradiol that significantly inhibited FCS-induced increases in cell number were 1 nM (physiological concentration), 0.1 nM, 0.1 nM, and 0.1 nM, respectively. In contrast, estrone, estriol, 16α-hydroxyestrone, 2-hydroxyestrone and 4-methoxyestrone were significantly less potent and inhibited FCS-induced increases in DNA synthesis, cell proliferation and collagen synthesis only at high concentrations (>1 μM; Fig. 2) not attained physiologically. Treatment of VSMCs with 1 nM (physiological concentration) of estradiol for 4, 8, and12 days inhibited FCS-induced cell proliferation in a cumulative fashion and by 16 ± 3, 33 ± 2.8, and 47 ± 4%, respectively (Fig. 3A).

Modulatory Effects of CYP450 Stimulators and Inhibitors on 17β-Estradiol-Induced Inhibition of VSMCs

Pretreatment of VSMCs for 48 h with CYP450 inducers (3-methylcholantherene [10 μM] and phenobarbital [10 μM]; 11, 12) and a CYP450 inhibitor (1-aminobenzotriazole; ABT [0.1–10 μM]; 13) did not influence FCS-induced cell proliferation, DNA synthesis and collagen-synthesis. The time-dependent inhibitory effects of physiological concentrations of estradiol (1 nM) on FCS-induced proliferation of VSMCs was enhanced by ≈100% by the CYP450 inducers 3-methylcholantherene and phenobarbital, respectively (Fig. 3A). Moreover, the inhibitory effects of estradiol were completely abolished by the CYP450 inhibitor ABT (Fig. 3A). ABT also blocked the enhanced inhibitory effects of estradiol observed in the presence of CYP450 inducers, 3-methylcholantherene and phenobarbital (data not shown). The time-dependent inhibitory effects of estradiol on cell proliferation were also blocked in presence of the COMT inhibitor, quercetin (Fig. 3A). The modulatory effects of CYP450 and COMT inhibitors and inducers did not correlate to the binding affinity of these agents to ERs. As shown in Fig. 3B, the relative binding affinity for ER was 100% and 98 ± 2% for estradiol and ICI-182780, and less than 0.001% for 3-MC, PB, ABT, quercetin. Similar modulatory effects were also observed on DNA synthesis and collagen synthesis (Figs. 4A and 4B). Trypan blue exclusion tests and MTT assay indicated no loss in viability of cells treated with various agents.

Effects of COMT Inhibitor (Quercetin), ER antagonist (ICI182780) and Type-II ER Antagonist (Luteolin) on Inhibition of VSMC Growth by Estradiol, 2-Hydroxyestradiol and 2-Methoxyestradiol

The growth inhibitory effects of estradiol were blocked by ICI-182780 (Figs. 5A–5C; top panels). Similar to our previous finding (5), the lowest concentration of ICI-182780 that significantly blocked the inhibitory effects of 1 μM estradiol was 10 μM (reversed the inhibitory effects by approximately 9%), moreover, at a concentration of 50 μM ICI-182780 completely blocked the inhibitory effects of 1 μM estradiol (data not shown).

In contrast to quercetin, ICI-182780 and ABT the inhibitory effects of estradiol were not blocked by lu-

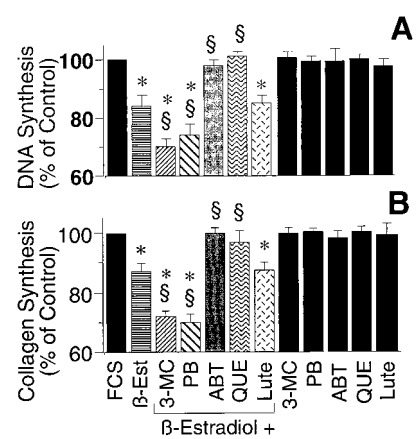

FIG. 4. Modulatory effects of various agents (inhibitors/activators of CYP450 and COMT) on the antimitogenic effects of estradiol. Modulation of the time-dependent inhibitory effects of estradiol (β-Est) 1 nM **(**physiological concentration; A) on 2.5% FCS-induced DNA synthesis (A) and collagen synthesis (B) in VSMCs by 10 μM of 3-methylcholantherene (3-MC), phenobarbital (PB), quercetin (QUE), luteolin (Lute), and 1-aminobenzotriazole (ABT). Values represent means ± SEM from 3–4 separate experiments conducted in quadruplicate. *$P < 0.05$ vs cells treated with FCS alone; §significantly ($P < 0.05$) different from β-estradiol alone.

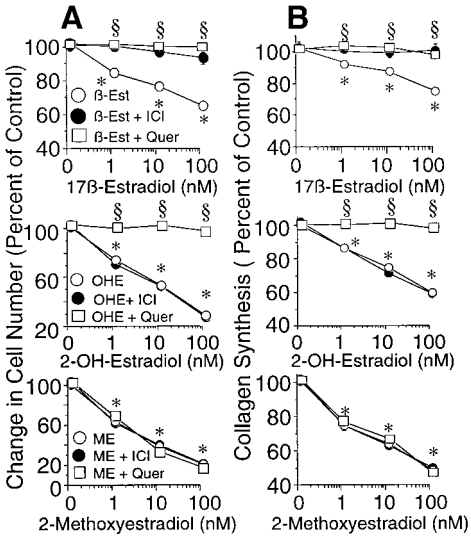

FIG. 5. Inhibitory effects of 1–100 nM 17β-estradiol (β-Est; top A and B), 2-hydroxyestradiol (OHE; middle A and B) and 2-methoxyestradiol (ME; bottom A and B) on FCS-induced growth (cell number [A] and collagen synthesis [B]) of VSMCs in the presence and absence of the estrogen receptor antagonist ICI-182780 (ICI; 100 μM) or quercetin (Quer; 10 μM). Values are mean ± SEM from 3 separate experiments conducted in quadruplicate. *$P < 0.05$ vs control; §significant reversal of inhibitory effect. Similar effects were observed on DNA synthesis.

teolin (Figs. 3A and 4A), a high affinity type-II ER-ligand (14).

The inhibitory effects of 2-hydroxyestradiol (Fig. 5, middle panels), but not 2-methoxyestradiol (Fig. 5; bottom panels), on VSMC proliferation were completely prevented by quercetin, a competitive inhibitor of COMT (15). Moreover, quercetin blocked the growth inhibitory effects of estradiol (Fig. 5, top panels). In contrast, ICI-182780 (100 μM), an estrogen receptor antagonist (18), did not block the growth inhibitory effects of either hydroxyestradiol (Fig. 5, middle panel) or 2-methoxyestradiol (Fig. 5, bottom panels).

Metabolism of 2-Hydroxyestradiol to Methoxyestradiol(s) by VSMCs

Incubation of VSMCs with 2-hydroxyestradiol, effectively metabolized 2-hydroxyestradiol to 2-methoxyestradiol (apparent k_m of 0.48 ± 0.1 μmol/L and V_{max} of 17.1 ± 0.8 pmol/min/10^6 cells in rat VSMCs) and this metabolism was inhibited by querectin (Fig. 6A).

Effects of ICI 182780 on Estradiol Metabolism

ICI182780 inhibited the metabolism of estradiol to catecholestradiol (4-hydroxyestradiol) by extracts of human hepG2 cells expressing CYP1A2 and these inhibitory effects were concentration dependent (Fig. 6B). The inhibitory effects of ICI-182780 were observed at concentrations greater than 1 μM. The apparent K_i of ICI-182780 for inhibition of estradiol metabolism to 4-hydroxyestradiol by 1A2 was 26.8 μmol/L.

Compared to either quercetin or ABT, ICI-182780 was less potent in blocking the inhibitory effects of estradiol on VSMC growth. In this regard, the lowest concentrations of quercetin, ABT, ICI-182780 of estradiol were 1, 1 and 10 μM, respectively. Moreover, 1 μM ICI-182780 was unable to block the inhibitory effects of 1 nM estradiol, even though the estradiol to ICI-182780 ratio was 1:1000 (Fig. 6C).

Effects of Estradiol and Its Metabolites on MAP Kinase Activity

Treatment of growth arrested SMCs with PDGF (25 ng/ml) increased MAP kinase activity from 0.05 ± 0.013 pmol/min/mg protein to 13.12 ± 0.07 pmol/min/mg protein, and the stimulatory effects of PDGF were inhibited by the MAP kinase inhibitor PD98059 (10 μM) to 0.83 ± 0.05 pmol/min/mg protein. In VSMCs pretreated for 60 min with estradiol, 2-hydroxyestradiol and 2-methoxyestradiol the stimulatory effects of PDGF on MAP kinase activity were inhibited in a concentration-dependent manner and in the following order of potency: 2-methoxyestradiol > 2-hydroxyestradiol > estradiol (Fig. 7A).

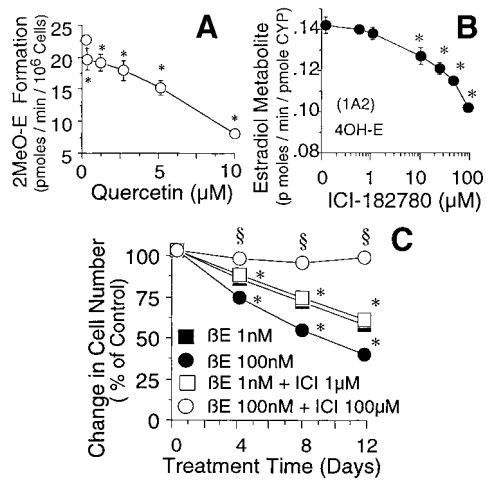

FIG. 6. (A) Inhibitory effects of quercetin on the metabolism of 2-hydroxyestradiol (10 μmol/L) to 2-methoxyestradiol (2MeO-E) by rat (○)VSMCs. (B) Concentration-dependent inhibitory effects of ICI-182780 on the metabolism of estradiol to 4-hydroxyestradiol (4-OH-E; B) by extracts of human cells expressing hepG2 cells expressing the CYP450-isozyme 1A2. Values represent mean ± SEM from a representative experiment conducted in triplicate. *Significant ($P < 0.05$) inhibition of estradiol or 2-hydroxyestradiol metabolism; (C) antagonistic effects of concentrations of ICI-182780 (ICI) that inhibit estradiol (β-E) metabolism (100 μM; ○) and do not inhibit estradiol metabolism (1 μM; □) on the inhibitory effects of 1 nM (■) and 100 nM (●) estradiol, respectively, on FCS-induced growth of VSMCs. The ratio of estradiol and ICI-182780 was 1:1000 under both treatment conditions. The data are presented as % of control where 100% is defined as the increase in cell number in response to FCS alone. *$P < 0.05$ vs VSMCs treated with FCS alone; §significant ($P < 0.05$) reversal of the inhibitory effects of estradiol.

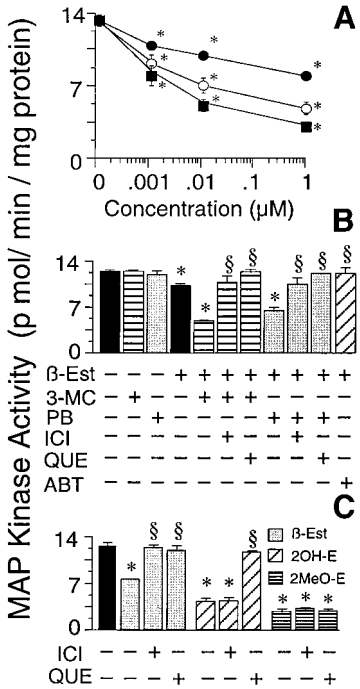

FIG. 7. Role of estradiol metabolism in mediating the inhibitory effects of estradiol on PDGF-induced MAP kinase activity in aortic VSMCs. (A) Concentration-dependent inhibitory effects of estradiol (β-Est; ●), 2-hydroxyestradiol (2OH-E; ○) and 2-methoxyestradiol (2MeO-E; ■) on PDGF-BB-induced (25 ng/ml) MAP kinase activity in VSMCs. (B) Inhibitory effect of physiological concentrations of (1 nM) estradiol (β-Est) on MAP kinase activity measured in VSMCs pretreated for 48 h with or without 10 μL 3-MC or phenobarbital (PB) and in the presence or absence of quercetin (10 μM; QUE); ICI-182780 (100 μM; ICI) or 1-aminobenzotriazole (10 μM; ABT). (C) Modulatory effects of ICI-182780 (100 μM; ICI) and quercetin (10 μM; QUE) on the inhibitory effects of 1 μM of β-Estr, 2OH-E, and 2MeO-E) in VSMCs treated for 24 h in the presence or absence of ICI-182780 or quercetin. Treatment with 3-MC, phenobarbital, ICI-182780, and quercetin alone did not influence FCS-induced MAP kinase activity significantly and the variations were <2%. Values are mean ± from 3 separate experiments. *$P < 0.05$ vs control (cells treated with PDGF-BB alone) and §$P < 0.05$ vs cells treated with estradiol in the presence or absence of 3-MC or phenobarbital.

The inhibitory effects of physiological concentrations (1 nM) of estradiol were enhanced from 17 ± 2% to 63 ± 4% and 52 ± 3% by 3-methylcholantherene and phenobarbital, respectively, and blocked by the CYP450 inhibitor ABT (Fig. 7B). Quercetin (COMT inhibitor) selectively blocked the inhibitory effects of estradiol and 2-hydroxyestradiol, but not of 2-methoxyestradiol (Fig. 7C). Querectin also completely blocked the inhibitory effects of estradiol (1 nM) observed in the presence of 3-methylcholantherene and phenobarbital (Fig. 7B). The inhibitory effects of estradiol, but not 2-hydroxyestradiol and 2-methoxyestradiol, were blocked by 100 μM ICI-182780 (Figs. 7B and 7C).Treatment of VSMCs with phenobarbital, 3-methylcholantherene and quercetin alone did not significantly alter PDGF-BB-induced MAP kinase activity (Fig. 7B).

Binding Studies

In extracts of estrogen receptor positive MCF-7 cells ^{3}H-17β-estradiol showed a high binding affinity. Relative binding affinity of various modulatory agents used in the present study was 98 ± 2 for ICI-182780; 22 ± 3 for 2-hydroxyestradiol; 0.04 ± .02 for 2-methoxyestradiol; 0.001 ± .0.001 for quercetin and 0 (no binding) for phenobarbital, 3-methylcholantherene and 1-aminobenzotriazole (Fig. 3B).

DISCUSSION

We hypothesize that conversion of estradiol to methoxyestradiols occurs in VSMCs and mediates in part the antimitogenic effects of estradiol on VSMC growth. This hypothesis is supported by our findings that methoxyestradiols and their precursors hydroxyestradiols are more potent than estradiol in inhibiting VSMC growth, the inhibitory effects of estradiol on VSMC growth are enhanced by CYP450 inducers and the inhibitory effects of estradiol, both in presence and absence of CYP450 inducers, are abolished by the CYP450 inhibitor ABT.

Our hypothesis is also supported by the observation that the inhibitory effects of 2-hydroxyestradiol, but not 2-methoxyestradiol, on VSMC growth are prevented by quercetin, a competitive inhibitor of COMT (15) which has no binding affinity for ERs (16). Moreover, quercetin blocks the growth inhibitory effects of estradiol, either in the absence or presence of CPY450 inducers. In contrast, ICI-182780 (100 μM), an ER antagonist (16), 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.

Although quercetin blocks the conversion of catecholestrogens to methoxyestrogens (15), it also binds to the type-II ER (14) which may regulate cell growth (14). However, in contrast to quercetin, the inhibitory effects of estradiol are not blocked by luteolin, a high affinity type-II ER ligand (14). This finding 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.

The hypothesis that the inhibitory effects of estradiol are mediated via generation of methoxyestradiols is further supported by our observation that VSMCs metabolized 2-hydroxyestradiol, a major endogenous estradiol metabolite, to 2-methoxyestradiol. Moreover, the metabolism of 2-hydroxyestradiol to 2-methoxy-

estradiol is inhibited by the COMT inhibitor querectin (15).

The growth inhibitory effects of estradiol are blocked by ICI-182780, an ER antagonist that binds with equal affinity to both ERα and ERβ (16), a finding seemingly at odds with the hypothesis that methoxyestradiols mediate the growth inhibitory effects of estradiol. However, because ICI-182780 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 finding that ICI-182780 inhibits the metabolism of estradiol to the catecholestrogen (4-hydroxyestradiol) in extracts of human hepG2 cells expressing CYP1A2 isozyme. This CYP450 isozyme is responsible for metabolizing estradiol to catecholestrogen (11, 12, 15).

The above findings suggest that the inhibitory effects of ICI-182780 may be mediated either via antagonism of ER or via inhibition of estradiol metabolism. However, the fact that the antagonistic effects of ICI-182780 are not dependent on the estradiol to ICI-182780 ratio, but rather on concentrations of ICI-182780 that inhibit estradiol metabolism, suggests that ICI-182780 blocks the inhibitory effects of estradiol by blocking estradiol metabolism to catecholestrogens, the precursors of methoxyestradiols. The notion that metabolism of estradiol is responsible for inhibiting VSMC growth is also supported by our observation that the inhibitory effects of physiologic concentrations of estradiol on VSMC growth are cumulative in nature and that the inhibitory effects of estradiol increase with time of exposure.

Inasmuch as estradiol inhibits MAP kinase (5), it is conceivable that estradiol metabolites also mediate the inhibitory effects of estradiol on MAP kinase. This hypothesis is supported by our findings that 2-methoxyestradiol and its precursor 2-hydroxyestradiol are more potent than estradiol in inhibiting PDGF-BB-induced MAP kinase activity in human VSMCs. Moreover, the inhibitory effects of *physiological concentrations* of estradiol on MAP kinase are enhanced by CYP450 inducers and blocked by the CYP inhibitor ABT. Also, quercetin selectively blocks the inhibitory effects of estradiol and 2-hydroxyestradiol, but not of 2-methoxyestradiol, and the inhibitory effects of estradiol, but not 2-hydroxyestradiol and 2-methoxyestradiol, are blocked by 100 μM of ICI-182780.

There are multiple implications of our findings that estradiol metabolism to methoxyestradiols is responsible for mediating the growth inhibiting effects of locally applied estradiol on vascular VSMCs. Clinical data suggests that hormone replacement therapy (HRT) provides cardiovascular protection in some, but not all postmenopausal women (1). It is feasible that the differential metabolism of estradiol to methoxyestradiols in VSMCs may determine the cardiovascular protective effects of HRT in postmenopausal women. In this regard, genetic differences in P450s, COMT and the presence of endogenous or exogenous molecules that inhibit P450s or COMT may influence the vasculoprotective effects of estradiol in postmenopausal women.

2-Methoxyestradiol also inhibits tumour growth, angiogenesis and growth of cancer cells (12), and the lack of synthesis of 2-hydroxyestradiol, a precursor of 2-methoxyestradiol, is associated with increased incidence of cancer (12). Thus, 2-methoxyestradiol may be of pharmacological importance in preventing both cancer and cardiovascular disease. Since one of the disadvantages of HRT is the risk of inducing cancer (mammary, endometrial), it is feasible that 2-methoxyestradiol could be employed for prevention of cardiovascular disease in women without increasing the risk of cancer. Moreover, because 2-methoxyestradiol is nonfeminizing (17), it could be of therapeutic use in men.

Although our findings provide evidence that the antimitogenic effects of locally applied estradiol on VSMC growth are mediated via an ER-independent mechanism involving metabolism of estradiol to methoxyestradiols, other mechanisms likely participate in the vasculoprotective effects of estradiol *in vivo.* First it is possible that systemic metabolism of estradiol to hydroxyestradiols and/or methoxyestradiols may be important. Second, estradiol stimulates the rapid synthesis of NO, cAMP and adenosine (1, 18), all of which may provide vasculoprotection. Third, estradiol regulates ion-channels such as Maxi-K channels, L-type calcium channels and calcium-activated K^+ channels (1). Fourth, via ER-dependent mechanisms, estradiol inhibits apoptosis of endothelial cells, increases endothelial survival and induces endothelial cell growth (1). Together these findings provide evidence that the vasculoprotective effect of estradiol is multifactorial and mediated via ER-dependent (both genomic and non-genomic) and ER-independent (mostly due to estradiol metabolites) mechanisms.

In summary, our findings provide the first evidence that the local metabolism of estradiol to methoxyestradiols, endogenous metabolites of estradiol with no affinity for ERs, is responsible in part for the ER-independent inhibitory effects of locally applied estradiol on human and rat vascular VSMC growth. These finding imply that local vascular estradiol metabolism may be an important determinant of the cardiovascular protective effects of circulating estradiol. Thus, inter-individual differences, either genetic or acquired, in the vascular metabolism of estradiol may define a given female's risk of cardiovascular disease and influence the cardiovascular benefit she receives from estradiol replacement therapy in the postmenopausal state. These findings also imply that nonfeminizing estradiol metabolites may confer cardiovascular protection in both women and men.

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