Role of Methoxyestradiols in the Growth Inhibitory Effects of Estradiol on Human Glomerular Mesangial Cells


Abstract—Metabolism of locally applied 17β-estradiol (estradiol) to methoxyestradiols contributes to the growth inhibiting effects of estradiol on vascular smooth muscle cells via an estrogen receptor (ER)-independent mechanism. Because vascular smooth muscle cells are phenotypically similar to glomerular mesangial cells, it is feasible that estradiol inhibits glomerular mesangial cell growth via a similar mechanism, and this possibility was investigated. In human glomerular mesangial cells, estradiol concentration dependently (1 to 100 nmol/L) inhibited serum-induced proliferation (cell number) and DNA ([3H]-thymidine incorporation) and collagen ([3H]-proline incorporation) synthesis. The inhibitory effects of estradiol were mimicked by 2-hydroxyestradiol and 2-methoxyestradiol, metabolites of estradiol with little affinity for ERs. 2-Hydroxyestradiol and 2-methoxyestradiol were more potent growth inhibitors than estradiol. The inhibitory effects of estradiol were enhanced by CYP450 inducers 3-methylcholanthrene (10 μmol/L) and phenobarbital (10 μmol/L) and blocked by the CYP450 inhibitor 1-aminobenzotriazole (10 μmol/L). The growth inhibitory effects of estradiol were also blocked by quercetin (10 μmol/L) and OR 486 (10 μmol/L) inhibitors of catechol-O-methyltransferase (converts catecholestradiols to methoxyestradiols). ICI182780 (ER antagonist with ER binding affinity similar to estradiol) blocked the growth inhibitory effects of estradiol (1 to 100 nmol/L) only at concentrations (>50 μmol/L) that inhibited estradiol metabolism to catecholestradiols. The growth inhibitory effects of 2-hydroxyestradiol were abrogated by quercetin and OR486 (two structurally dissimilar catechol-O-methyltransferase inhibitors), but not by ICI182780. However, the growth inhibitory effects of 2-methoxyestradiol were unaltered by catechol-O-methyltransferase inhibitors and ICI182780. In conclusion, our findings provide the first evidence that methoxyestradiols mediate the growth inhibitory effects of locally applied estradiol on glomerular mesangial cell growth via an ER-independent mechanism. (Hypertension. 2002;39[part 2]:418-424.)

Key Words: metabolism ■ estrogen ■ renal disease ■ glomerulosclerosis ■ mesangium ■ menopause

Estradiol may induce protective effects on the kidney. For example, compared with age-matched men, the rate of progression of renal disease in premenopausal women is decreased.1,2 With the onset of menopause, decreased synthesis of 17β-estradiol (estradiol) is accompanied by accelerated progression of renal diseases, and estradiol replacement therapy slows this process.1,3

Although estradiol induces renoprotection, the mechanisms involved remain poorly defined. Inasmuch as coronary artery disease is the most frequent cause of death in postmenopausal women,4 most studies have focused on evaluating the effects of estradiol on vascular cells. In this context, it is well established that the inhibitory effects of estradiol on vascular smooth muscle cell growth is known to protect the vasculature against occlusive disorders.5 Analogous to the vascular remodeling process in atherosclerosis, abnormal growth of glomerular mesangial cells (GMCs) following glomerular injury contributes to the glomerular remodeling process associated with glomerulosclerosis.4 Our previous studies show that estradiol inhibits GMC growth,3 and we hypothesized that, via this inhibition action, estradiol may protect the kidney against glomerulosclerosis. However, the mechanisms by which estradiol inhibits GMC growth are unknown. The facts that GMCs are phenotypically similar to vascular smooth muscle cells6,4 and possess functional estrogen receptors1 suggest that the mechanisms by which estradiol induces its growth inhibitory effects may be similar in GMCs and vascular smooth muscle cells.

Our recent studies shown that in vascular smooth muscle cells, the antigrowth effects of estradiol are largely mediated via its local conversion to hydroxy and methoxy metabolites that have little affinity for estrogen receptors (ERs).6 This finding suggests that in addition to the conventional ER-dependent mechanisms, ER-independent mechanisms may
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Figure 1. Schematic representation of the hypothesis and the experimental approach to demonstrate that metabolism of estradiol and its 2-methoxy metabolites plays an important role in inducing the biological effects of estradiol. This hypothesis is supported by the recent reports that estradiol prevents injury-induced vascular lesion formation in mice lacking functional ERα or ERβ. Because GMCs express cytochrome P450 (CYP450) enzymes that can mediate the inhibitory effects of estradiol on mesangial cell growth, we evaluated the capability of GMCs to metabolize estradiol to hydroxyestradiol (CYP450); Catechol-O-Methyltransferase (COMT); 2-hydroxyestradiol (2-OH-E); Cytochrome P450 (Catechol-Es); 2-hydroxyestradiol (2-OH-E); Cytochrome P450 (Catechol-Es); 2-methoxyestradiol (2 MeO-E); Catecholestradiols (Catechol-Es); 2-hydroxyestradiol (2-OH-E); Cytochrome P450 (CYP450); Catechol-O-Methyltransferase (COMT); Inhibition (−); Induction (+). 2-Methoxyestradiol (2 MeO-E) and its 2-hydroxy metabolites, and 2-hydroxyestradiol that significantly inhibit FCS-induced growth on GMCs. The 2-hydroxy and 2-methoxy metabolites of estradiol inhibited FCS-induced growth in GMCs.

Mesangial Cell Culture

GMCs cultured from normal female donors and in 3rd passage were obtained from Clonetics Corp. (Walkersville, MD). All chemicals for cell growth (3H-thymidine incorporation, 3H-proline incorporation, cell number) studies were purchased as described before. GMCs in 3rd passage were grown under standard tissue culture conditions in phenol red free DMEM/F12 medium supplemented with 10% FCS (steroid free) and antibiotics. Confluent GMCs were dislodged by trypsinization, washed, and plated for growth studies at required densities in multiwell plates.

DNA and Collagen Synthesis

3H-Thymidine and 3H-proline incorporation studies were performed as measures of DNA and collagen synthesis, respectively. GMCs were plated at a density of 2.5×10^4 cells/well in 24-well tissue culture dishes and allowed to grow in DMEM/F12 containing 10% FCS under standard tissue culture conditions. The monolayers of GMCs were then growth arrested by feeding DMEM containing 0.4% bovine serum albumin (BSA) for 48 hours. Growth was stimulated by treating growth arrested GMCs with DMEM supplemented with 2.5% FCS and containing or lacking the various treatments. For DNA synthesis, after 20 hours of incubation, the cells were pulsed with 3H-thymidine (1 μCi/mL) for an additional 4 hours. For collagen synthesis, the cells were treated for 48 hours in the presence of 3H-L-proline (1 μCi/mL). The experiments were terminated by washing the cells twice with Dulbecco’s phosphate buffered saline and twice with ice-cold trichloroacetic acid (10%). The precipitate was solubilized in 500 μL of 0.3N NaOH and 0.1% SDS after incubation at 50°C for 2 hours. Aliquots from 4 wells for each treatment with 10 mL scintillation fluid were counted in a liquid scintillation counter, and each experiment was conducted using three to four separate cultures. The results are expressed as mean±SEM. Statistical analysis was performed with the use of Student’s unpaired t test and one way ANOVA. Values of P<0.05 are considered to be significantly different.

Results

Treatment with FCS stimulated 3H-thymidine and 3H-proline incorporation and cell number by approximately 5- to 7-fold (P<0.05). To address the potential role of endogenous estradiol metabolites in regulating GMC growth, we first tested the potency of estradiol and estradiol metabolites to inhibit FCS-induced growth on GMCs. The 2-hydroxy and 2-methoxy metabolites of estradiol inhibited FCS-induced DNA synthesis (Figure 2A), proliferation (Figure 2B) and collagen synthesis (Figure 2C) in the following order of potency: 2-methoxyestradiol>2-hydroxyestradiol>estradiol. In contrast, estrone, estriol, 16α-hydroxyestrone, 2-hydroxyestrone, estrone sulfate, and 4-methoxyestrone were significantly less potent and did not inhibit GMC growth (DNA synthesis, collagen synthesis, cell proliferation) within the concentration range (1 to 100 nmol/L) used (data not shown). The lowest concentration of estradiol, 2-methoxyestradiol, and 2-hydroxyestradiol that significantly
inhibited FCS-induced increases in cell number was 1 nmol/L. Treatment of GMCs for 4 days with a physiological concentration (1 nmol/L) of estradiol inhibited cell proliferation by 16%. At this concentration, 2-methoxyestradiol and 2-hydroxyestradiol inhibited cell number by 38% and 28%, 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 inducers (3-methylcholantherene and phenobarbital) and an inhibitor (1-aminobenzotriazole) of CYP450s. Treatment of GMCs for 48 hours with 3-methylcholantherene (10 µmol/L), phenobarbital (10 µmol/L), or 1-aminobenzotriazole did not influence FCS-induced DNA synthesis, cell proliferation, or collagen synthesis. The inhibitory effects of physiological concentrations of estradiol (1 nmol/L) on FCS-induced DNA synthesis and cell proliferation were enhanced by 3-methylcholantherene and phenobarbital (Figures 3A and 3B). Similar modulatory effects of 3-methylcholantherene and phenobarbital were also observed on collagen synthesis (Figure 3C). The inhibitory effects of estradiol (1 nmol/L) on FCS-induced proliferation of GMCs were enhanced from 18% to 41%, and 35% by 3-methylcholantherene and phenobarbital, respectively. In the presence of phenobarbital and 3-methylcholantherene, the concentration-dependent inhibitory effects of estradiol on FCS-induced DNA synthesis, cell number, and collagen synthesis were almost doubled and the inhibitory curve shifted to the left (Figure 3). In contrast to the CYP450 inducers, the concentration-dependent inhibitory effects of estradiol on cell proliferation, DNA synthesis, and collagen synthesis were abolished by the CYP450 inhibitor 1-aminobenzotriazole (Figure 4). Moreover, the enhanced inhibitory effects of estradiol observed in the presence of 3-methylcholantherene plus phenobarbital on all parameters of GMC growth were also blocked by 1-aminobenzotriazole (Figure 4). Trypan blue exclusion tests and MTT assay indicated no loss in viability of cells treated with various agents.

The concentration-dependent inhibitory effects of estradiol on cell proliferation, DNA synthesis, and collagen synthesis were abolished by the catechol-O-methyltransferase (COMT) inhibitors quercetin and OR486 (Figure 5). Moreover, the enhanced inhibitory effects of estradiol observed in the presence of CYP450 inducers 3-methylcholantherene plus phenobarbital on all parameters of GMC growth were also blocked by quercetin and OR486 (data not shown).

The growth inhibitory effects of estradiol were blocked by ICI182780, and these effects were concentration dependent (Figure 7A). The lowest concentration of ICI182780 that significantly blocked the inhibitory effects of 100 nmol/L estradiol was 10 µmol/L, and a concentration of 50 µmol/L ICI182780 completely blocked the inhibitory effects of 100
nmol/L estradiol (Figure 7A). Compared with ICI182780, both 1-aminobenzotriazole and quercetin were more potent in antagonizing the growth inhibitory effects of estradiol (Figure 7A). Quercetin is not only a COMT substrate, but also a ligand for type II ER. 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 GMC growth in the presence of luteolin, a high affinity type II ER ligand.6 In contrast to quercetin and OR486, the inhibitory effects of estradiol on GMC growth were not blocked by luteolin (Figure 7A).

We have previously shown that at concentrations greater than 1 µmol/L, ICI182780 inhibits the metabolism of estradiol to 2- and 4-hydroxyestradiol by CYP1A2 and with apparent K_m of 45 µmol/L and 27 µmol/L, respectively.6 In the present study, 1 µmol/L ICI 182780 was unable to block the inhibitory effects of 1 nmol/L estradiol, even though the estradiol to ICI182780 ratio was 1:1000 (Figure 7B). Moreover, the inhibitory effects of physiological concentrations of estradiol on GMC growth were cumulative in nature, ie, the inhibitory effects of estradiol increased with time of exposure. Treatment of GMCs with a physiological concentration (1 nmol/L) of estradiol for 2, 4, 8, and 12 days inhibited FCS-induced cell proliferation by 7±1%, 20±2%, 32±2%, and 44±3%, respectively (Figure 7B).

GMCs metabolized 2-hydroxyestradiol (2 µmol/L for 1 hour) to 2-methoxyestradiol, and this metabolism was inhibited by 10 µmol/L of quercetin (96±0.8%, P<0.05) or OR486 (95±0.5%, P<0.05) but not by ICI182780 (Figure 7C).

**Discussion**

Our findings strongly suggest that the inhibitory effects of estradiol on GMC growth are mediated via CYP450-derived metabolites. Treatment of GMCs with estradiol, 2-hydroxyestradiol, or 2-methoxyestradiol, but not estrone, estriol, 16α-hydroxyestradiol, estrone sulfate, hydroxyestrone, or methoxyestrone, inhibits serum-induced GMC growth, and 2-hydroxyestradiol or 2-methoxyestradiol are more potent than estradiol in this regard. Importantly,
affinity for ERs, 6 attenuate the inhibitory effects of inhibitors quercetin and OR486,14 which have no binding 2-hydroxyestradiol in inhibiting GMC growth lead us to hypothesize that methoxyestradiols are the ultimate mediators of estradiol. Moreover, quercetin, as well as OR486, block the growth inhibitory effects of estradiol, either in the absence or presence of CPY450 inducers. In contrast, ICI182780 (50 μmol/L), an ER antagonist, 6 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 GMCs metabolize 2-hydroxyestradiol to 2-methoxyestradiol and that this metabolic conversion is blocked by the COMT inhibitors quercetin and OR486. Quercetin not only blocks the conversion of catecholestrogens to methoxyestrogens,11 but also binds to the type II ER15 that has been implicated in regulating cell growth.13 The finding that the inhibitory effects of estradiol are not blocked by luteolin, a high affinity type II ER ligand,15 rules out the participation of type II ER. Moreover, it 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, blocks the antigrowth effects of both estradiol and 2-hydroxyestradiol, but not 2-methoxyestradiol. The growth inhibitory effects of estradiol are blocked by ICI182780, an ER antagonist that binds with equal affinity to both ERα and ERβ,16 a finding seemingly inconsistent 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 CPY450s and inhibits estradiol metabolism. This notion is supported by our recent finding that ICI182780 inhibits the metabolism of estradiol to catecholestrogens in extracts of human hepg2 cells expressing the CYP1A2 isozyme, which is responsible for metabolizing estradiol to catecholestrogens.6 The potential that ICI182780 may block the antigrowth effects of estradiol by inhibiting COMT can also be ruled out because ICI182780 blocks the antigrowth effects of estradiol, but not 2-hydroxyestradiol and 2-methoxyestradiol. Moreover, in contrast to quercetin and OR486, ICI182780 fails 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, the fact 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,6 suggests that ICI182780 blocks the inhibitory effects of estradiol by blocking estradiol metabolism to catecholestrogens, the precursors of methoxyestradiols.

Our contention that local conversion of estradiol to methoxyestradiols is responsible for its ER-independent antigrowth effects in GMCs is supported by our recent finding

![Figure 7. A, Concentration-response curve comparing the abrogatory effects of 1-aminobenzotriazole (ABT), quercetin, ICI182780 (ICI), and luteolin on the inhibitory effects of estradiol (0.1 μmol/L) on 2.5% FCS-induced DNA synthesis in GMCs treated for 24 hours. *P<0.05 versus GMCs treated with FCS alone. B, Antagonistic effects of concentrations of ICI182780 (ICI) that inhibit estradiol (E) metabolism (50 μmol/L) and do not inhibit estradiol metabolism (1 μmol/L) on the inhibitory effects of 1 and 50 nmol/L estradiol, respectively, on FCS-induced growth (cell number) of GMCs treated for 2, 4, 8, or 12 days. The ratio of estradiol and ICI182780 was 1:1000 under both treatment conditions. The data are presented as percent of control where 100% is defined as the increase in cell number in response to 2.5% FCS alone. *P<0.05 versus GMCs treated with FCS alone; §significant (P<0.05) reversal of the inhibitory effects of estradiol. C) Inhibitory effects of quercetin (Que; 10 μmol/L), ICI182780 (ICI; 50 μmol/L), and OR486 (OR; 10 μmol/L) on the metabolism of 2-hydroxyestradiol (2-ME) formation by cultured GMCs. *P<0.05 versus methoxyestradiol (2-ME) formation by GMCs alone.](http://hyper.ahajournals.org/)

3-methylcholanaltherene and phenobarbital, CYP450 inducers12 with no affinity for ERs, enhanced the inhibitory effects of estradiol. Moreover, 1-aminobenzotriazole, a broad spectrum CYP450 inhibitor13 with no affinity for ERs,6 abrogated the antigrowth effects of estradiol in both the presence and absence of CYP450 inducers (see schematic representation in Figure 1). Our conclusion that the inhibitory effects of estradiol on GMC growth are mediated via CYP450-derived metabolites is further supported by the well-established finding that the CYP1A2 isozyme, which is responsible for metabolizing estradiol to catecholestrogens, are highly expressed in GMCs.9

The facts that catecholestrogens are rapidly metabolized to methoxyestradiols by COMT,14 GMCs express COMT activity10, and 2-methoxyestradiol is more potent than 2-hydroxyestradiol in inhibiting GMC growth lead us to hypothesize that methoxyestradiols are the ultimate mediators of the ER-independent antigrowth effects of estradiol. Our hypothesis is supported by the observations that the COMT inhibitors quercetin and OR486,14 which have no binding affinity for ERs,6 attenuate the inhibitory effects of 2-hydroxyestradiol, but not 2-methoxyestradiol, on GMC growth.
that local metabolism of estradiol to methoxyestradiol inhibits the growth of vascular smooth muscle cells, which are phenotypically similar to GMCs.6 Moreover, our recent studies support the participation of this mechanism in inducing the antigrowth effects of estradiol in cardiac fibroblasts.17 Taken together, our findings suggest that the conversion of estradiol to 2-methoxyestradiol may be a physiologically relevant and a prominent pathway via which estradiol regulates cell growth. In this context, it is important to note that metabolism of estradiol to methoxyestradiols plays a role in regulating growth of tumor/cancer cells (mammary tumors, kidney tumors in Syrian hamsters, and endometrial cancer).18 In vivo studies provide evidence that decreased formation of 2-methoxyestradiol and its precursor, 2-hydroxyestradiol, is associated with mammary cancer, endometrial cancer, and renal tumors in Syrian hamsters.16

In contrast to our findings, an earlier study showed that physiological concentrations of estradiol induced DNA synthesis and proliferation in GMCs that were not growth arrested.19 The disparate effects of estradiol in the two studies may be due to the culture conditions. In this regard, estradiol induces MAP kinase activity in mesangial cells that are not growth arrested, whereas in growth arrested (serum starved) GMCs, estradiol has no effect on basal MAP kinase activity and inhibits mitogen (PDGF and Ang II)-induced MAP kinase activity.20 Because in growth arrested GMCs mitogens induce cell proliferation via activation of MAP kinase activity21, and because estradiol inhibits these effects, it is feasible that the growth inhibitory effects of estradiol on GMCs may depend on whether a growth stimulus is present and whether the GMCs are synchronized in G0 phase of the cell cycle. Additional studies are required to resolve the discrepancy between these two studies.

With regard to the renal system, our finding that estradiol metabolism to methoxyestradiols is responsible for mediating the growth inhibiting effects of locally applied estradiol on GMCs may have clinical implications. Since increased proliferation of GMCs plays a key role in glomerulosclerosis,1 estradiol metabolites may protect against glomerular remodeling by inhibiting cell growth. Thus, the protective effects of estradiol on the progression of renal disease in postmenopausal women may not only be dependent on estradiol levels, but also on the capability of the individual to metabolize estradiol to these metabolites. However, future studies are required to investigate this possibility in more detail.

The finding that conversion of 2-hydroxyestradiol to 2-methoxyestradiol by COMT is essential for mediating the antigrowth effects of locally applied estradiol on GMCs may have clinical implications. Since increased pro-

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