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 catecholestadiols. 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.1,3,5 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,1 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.1 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,5 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 cells,5,4 and possess functional estrogen receptors,1 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 show 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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Glomerular Mesangial Cell

Figure 1. Schematic representation of the hypothesis and the experimental approach to demonstrate that metabolism of estradiol to methoxyestradiols (Methoxy-Es) is responsible for mediating the inhibitory effects of estradiol on mesangial cell growth. 2-Methoxyestradiol (2-MeO-E); Catecholestriadiols (Catechol-Es); 2-hydroxyestradiol (2-OH-E); Cytochrome P450 (CYP450); Catechol-O-Methyltransferase (COMT); Inhibition (-); Induction (+).

Methods

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 from suppliers as described before.5 GMCs in 3rd passage were grown under standard tissue culture conditions in phenol red free DMEM/F12 containing 10% FCS (steroid free) and antibiotics. GMCs were then growth arrested by feeding DMEM containing 0.4% BSA for 48 hours. GMCs were then treated every 24 hours for 2 to 12 days with DMEM supplemented with 2.5% FCS and containing or lacking various treatments. The treatments were terminated on day 2, 4, 8, or 12 and cells were dislodged with trypsin-EDTA, diluted in Isoton-II, and counted with a Coulter counter. Aliquots from three wells were counted for each group using three separate cultures.

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. 3H-Thymidine incorporation studies were conducted in subconfluent monolayers. However, to ensure that changes in collagen synthesis were not due to a decreases in cell number, H-proline incorporation studies were conducted in confluent monolayers of cells in which changes in cell number were precluded. Cell counting was performed in cells treated in parallel to the cells used for the collagen synthesis, and the data were normalized to cell number.

Cell Proliferation

Cell counting was performed as a direct measure of cell proliferation. Trypsinized GMCs were suspended in DMEM/F12 containing 10% FCS and plated in a 24-well culture dish at a density of 1×10^4 cells/well. After incubation for 24 hours, cells were growth arrested by feeding DMEM containing 0.4% BSA for 48 hours. GMCs were then treated every 24 hours for 2 to 12 days with DMEM supplemented with 2.5% FCS and containing or lacking various treatments. The treatments were terminated on day 2, 4, 8, or 12 and cells were dislodged with trypsin-EDTA, diluted in Isoton-II, and counted with a Coulter counter. Aliquots from three wells were counted for each group using three separate cultures.

Protein Determination

Total cellular protein was determined by the Bio-Rad detergent method, which uses a modification of the Lowry assay with BSA as a standard.

Metabolism of Catechol Estradiols to Methoxyestradiols

Confluent GMCs were incubated with 2-hydroxyestradiol for 2 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, as previously described.11

Statistics

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 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 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 concentation-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 inhibitory effects of 2-hydroxyestradiol, but not 2-methoxyestradiol, on DNA synthesis (Figure 6A), collagen synthesis (Figure 6B), and proliferation (Figure 6C) were completely prevented by 10 μmol/L quercetin and OR486. In contrast to quercetin and OR486, ICI182780 (50 μmol/L), an estrogen receptor antagonist, did not block the growth inhibitory effects of either 2-hydroxyestradiol or 2-methoxyestradiol (Figures 6A to 6C).

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.
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_{iso} 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 (0.1 μmol/L) to 2-methoxyestradiol, and this metabolism was inhibited by 10 μmol/L of quercetin (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, attenuate the inhibitory effects of inhibitors quercetin and OR486, which have no binding to ERs. The hypothesis is supported by the observations that the COMT metabolites is further supported by the well-established finding 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 estradiol by inhibiting COMT and blocking the formation of methoxyestradiols. The growth inhibitory effects of estradiol are blocked by ICI182780, an ER antagonist that binds with equal affinity to both ERα and ERβ, a finding seemingly inconsistent 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 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. 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, 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 findings of 2-hydroxyestradiol, but not 2-methoxyestradiol, on GMC growth. Moreover, quercetin, as well as OR486, block the growth inhibitory effects of estradiol, either in the absence or presence of CYP450 inducers. In contrast, ICI182780 (50 μmol/L), 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 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, but also binds to the type II ER, which has been implicated in regulating cell growth. The finding that the inhibitory effects of estradiol are blocked by quercetin, a high affinity type II ER ligand, 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.
that local metabolism of estradiol to methoxyestradiol inhibits the growth of vascular smooth muscle cells, which are phenotypically similar to GMCs.

Moreover, our recent studies support the participation of this mechanism in inducing the antigrowth effects of estradiol in cardiac fibroblasts. 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).

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.

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. 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.

Because in growth arrested GMCs mitogens induce cell proliferation via activation of MAP kinase activity, 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 the 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, 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 estradiol has additional implications. Apart from metabolizing catecholestradiols, COMT, which is highly expressed in both cardiovascular and renal cells, is also involved in the metabolism of catecholamines, which are known to induce deleterious effects on the renal 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 renal system. This contention is supported by our recent findings that catecholamines block the antigrowth effects of estradiol and 2-hydroxyestradiol on rat GMCs, and that catecholamines inhibit the conversion of 2-hydroxyestradiol to 2-methoxyestradiol by rat GMCs. In the same context, we have recently reported that in vascular smooth muscle cells, norepinephrine, epinephrine, and isoproterenol inhibit the metabolism of 2-hydroxyestradiol to 2-methoxyestradiol and abrogate the antigrowth effects of 2-hydroxyestradiol on vascular smooth muscle cells. Therefore, interactions between catecholestradiols and catecholamines may play an important role in determining the effects of estradiol on the kidney.

In summary, our findings provide the first evidence that the antigrowth effects of estradiol on human GMCs are mediated via an ER-independent pathway that involves the local conversion of estradiol to methoxyestradiols (Figure 1), that estradiol may protect against progression of renal disease by inhibiting GMC growth, and that estradiol metabolism may be an important determinant of the renal protective effects of estradiol. Thus, interindividual differences, either genetic or acquired, in estradiol metabolism may define a given female’s risk of renal disease and influence the renal benefit she receives from estradiol replacement therapy in the postmenopausal state. These findings also imply that nonfeminizing estradiol metabolites may confer renal protection in both women and men.

Acknowledgments

This study was supported by Swiss National Science Foundation grants 32–54172.98 and 32–640.00 and by the National Institutes of Health grant HL55314.

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*Hypertension*. 2002;39:418-424
doi: 10.1161/01.HYP.0000020311.77209.D9

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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