Catecholamines Block 2-Hydroxyestradiol-Induced Antimitogenesis in Mesangial Cells

Lefteris C. Zacharia, Edwin K. Jackson, Delbert G. Gillespie, Raghvendra K. Dubey

Abstract—Methylation of 2-hydroxyestradiol to 2-methoxyestradiol by catechol-O-methyl transferase (COMT) mediates the antimitogenic effects of 2-hydroxyestradiol on vascular smooth muscle cells. Moreover, 2-hydroxyestradiol inhibits growth of glomerular mesangial cells (GMCs). Because catecholamines are substrates for COMT, which is expressed in GMCs, we hypothesize that catecholamines may abrogate the antimitogenic effects of 2-hydroxyestradiol on GMCs by competing for COMT and inhibiting 2-methoxyestradiol formation. To test this hypothesis, we investigated the antimitogenic effects of 2-hydroxyestradiol on rat GMCs in the presence and absence of catecholamines. The capability of GMCs to methylate 2-hydroxyestradiol in the presence and absence of catecholamines was also evaluated. GMCs metabolized 2-hydroxyestradiol in a concentration-dependent manner with a V_{max} of 12.03 ± 0.32 pmol/10^6 cells/min and an apparent K_{m} of 0.23 ± 0.04 μmol/L. Norepinephrine (10 μmol/L) and epinephrine (10 μmol/L) significantly inhibited methylation of 0.25 μmol/L 2-hydroxyestradiol. Norepinephrine concentration-dependently abrogated the ability of 2-hydroxyestradiol to inhibit ^3H-thymidine incorporation (index of DNA synthesis). In the presence of 5, 10, and 40 μmol/L norepinephrine, the inhibitory effect of 0.1 μmol/L 2-hydroxyestradiol on ^3H-thymidine incorporation was reduced from 51 ± 0.7% to 46 ± 0.4%, 39 ± 0.3%, and 25 ± 0.7%, respectively. Similar to DNA synthesis, the inhibitory effects of 2-hydroxyestradiol on cell number and ^3H-proline incorporation (index of collagen synthesis) on GMCs were abrogated by catecholamines. Our findings provide evidence that methylation of 2-hydroxyestradiol inhibits GMC proliferation and extracellular matrix synthesis and may in part protect against renal proliferative diseases. Moreover, catecholamines may abrogate the renoprotective effects of 2-hydroxyestradiol in the glomeruli by inhibiting COMT and 2-methoxyestradiol formation. (Hypertension. 2002;39:854-859.)

Key Words: estrogen ■ catecholamines ■ metabolism ■ sympathetic nervous system ■ renal disease ■ glomerulosclerosis

Estradiol may have protective effects in the kidney. Compared with age-matched men, the rate of progression of renal disease is lower in premenopausal women. With the onset of menopause and the reduction in 17β-estradiol (estriadol) synthesis, the progression of renal disease accelerates, and estradiol replacement therapy slows the progression of renal disease.

The mechanisms by which estradiol affords renoprotection are unknown. However, it is known that estradiol inhibits pathological responses (cell proliferation and extracellular matrix production) to vascular injury in part by conversion to 2-hydroxyestradiol, which in turn is methylated by catechol-O-methyl transferase (COMT) to 2-methoxyestradiol. Our results suggest that 2-methoxyestradiol importantly contributes to estradiol-induced vascular protection via estrogen receptor (ER)-independent mechanisms. Glomerulosclerosis engages mechanisms in glomerular mesangial cells (GMCs) similar to those engaged in vascular smooth muscle cells following vascular injury, and because GMCs are phenotypically similar to vascular smooth muscle cells, estradiol may mediate renoprotection in part via conversion to 2-methoxyestradiol. In support of this hypothesis, our recent studies demonstrate that estradiol inhibits growth of GMCs via its metabolites 2-hydroxyestradiol and 2-methoxyestradiol and via ER-independent mechanisms.

In is conceivable that increased levels of catecholamines may abrogate the renoprotective effects of 2-hydroxyestradiol by competing for COMT and inhibiting the biosynthesis of 2-hydroxyestradiol. This hypothesis is supported by the observations that patients with the nephrotic syndrome have higher levels of catecholamines, increased sympathetic activity accelerates the process of glomerulosclerosis in animal models, and increased synthesis of catecholamines under pathological conditions induces vasoocclusive disorders. Indeed, our previous studies demonstrate that catecholamines—physiological substrates/competitive inhibitors of COMT—abrogate the...
inhibitory effects of 2-hydroxyestradiol on vascular smooth muscle cell growth by blocking the metabolism of 2-hydroxyestradiol to 2-methoxyestradiol.\(^5\)

The goals of the present study were 2-fold. First, we wished to determine whether rat GMCs actually convert 2-hydroxyestradiol to 2-methoxyestradiol and, if so, to determine the kinetic characteristics of this pathway. Second, we wished to determine whether the ability of 2-hydroxyestradiol to inhibit GMC growth and collagen synthesis is abrogated by catecholamines.

**Methods**

All experiments were conducted in phenol red-free medium, and fetal calf serum (FCS) was charcoal stripped and steroid free. GMCs were grown from Sprague-Dawley rats (Charles River, Wilmington, Mass) as previously described.\(^14\)

For the metabolism studies, cells grown to subconfluence in 12-well plates were washed with PBS and subsequently incubated with 0.125 to 5 \(\mu\)mol/L 2-hydroxyestradiol in DMEM for 1 hour. The 2-methoxyestradiol formed was analyzed by high-performance liquid chromatography (HPLC) and the apparent \(K_m\) and \(V_{max}\) values calculated using GraphPad Prism version 3.0 (GraphPad Software, Inc.). To investigate the effects of catecholamines on 2-hydroxyestradiol metabolism, cells were incubated for 1 hour with 0.25 \(\mu\)mol/L 2-hydroxyestradiol in the presence and absence of 10 \(\mu\)mol/L of either epinephrine (EPI), norepinephrine (NE), or isoproterenol or 1 \(\mu\)mol/L quercetin (COMT competitive inhibitor). The concentration (10 \(\mu\)mol/L) of catecholamines selected was based on our inhibition kinetics studies conducted in vascular smooth muscle cells,\(^3\) which are phenotypically similar to GMCs. This is the lowest concentration at which NE and EPI significantly inhibit 2-methoxyestradiol formation by competing with 0.25 \(\mu\)mol/L of 2-hydroxyestradiol, for which can be quantified by HPLC. To investigate the inhibitory effects of the COMT inhibitor OR-486, 2 hydroxyestradiol (1 \(\mu\)mol/L) was incubated with 0.1 to 2.5 \(\mu\)mol/L OR-486. Ascorbic acid (1 \(\mu\)mol/L) was used in all treatments to prevent oxidation of 2-hydroxyestradiol.

At the end of the incubation period, the medium was collected, internal standard added (16 \(\mu\)mol/L 2-hydroxyestradiol), and the samples processed and analyzed with HPLC as previously described.\(^3\) \([\text{H}]\)Thymidine incorporation (index of DNA synthesis), \([\text{H}]\)proline incorporation (index of collagen synthesis), and cell proliferation were conducted as previously described.\(^4\) For \([\text{H}]\)thymidine incorporation, GMCs were plated in 24-well tissue culture dishes and allowed to grow to subconfluence. Cell growth was initiated by treating growth-arrested cells for 20 hours with DMEM containing 2.5% FCS in the presence or absence of the test agents. After 20 hours of incubation, the treatments were repeated with freshly prepared solutions but supplemented with \([\text{H}]\)thymidine (1 \(\mu\)Ci/mL, specific activity 20 Ci/mmol) for an additional 4 hours. For \([\text{H}]\)proline incorporation, confluent monolayers of growth-arrested GMCs were treated for 36 hours with DMEM supplemented with 2.5% FCS plus L-\([\text{H}]\)proline (1 \(\mu\)Ci/mL, specific activity 100 Ci/mmol) in the presence or absence of the test agents. For both \([\text{H}]\)thymidine and \([\text{H}]\)proline incorporation, experiments were terminated by washing the cells with PBS and with trichloroacetic acid, the precipitate solubilized, and aliquots counted in a liquid scintillation counter. For cell number experiments, GMCs were allowed to attach overnight, were growth-arrested (48 hours), and then were treated every 24-hours for 4 days. On day 5, the cells were dislodged and counted on a Coulter counter (Beckman Coulter, Inc.).

**Statistics**

All experiments were conducted at least in triplicate and repeated 3 to 4 times using separate cultures. Results are presented as mean±SEM. Statistical analysis were performed using ANOVA, Fisher’s Least Significant Difference (LSD) test as appropriate. A value of \(P<0.05\) was considered statistically significant.

**Results**

GMCs efficiently metabolized 2-hydroxyestradiol (2-OHE) to 2-methoxyestradiol in a concentration-dependent manner (Figure 1A). Saturation of the metabolic conversion of 2-OHE to 2-methoxyestradiol was attained at 2.5 to 5 \(\mu\)mol/L 2-hydroxyestradiol (Figure 1A). From the relationship of concentration versus rate-of-reaction, the apparent \(K_m\) was calculated to be 0.228±0.039 \(\mu\)mol/L and the \(V_{max}\) was 12.03±0.32 pmol/min per million cells.

Norepinephrine (NE), epinephrine (EPI), isoproterenol (ISO), and quercetin inhibited methylation of 2-OHE (Figure 1B). A concentration of 10 \(\mu\)mol/L of NE, EPI, and ISO inhibited methylation of 2-OHE (0.25 \(\mu\)mol/L) by 7.2±1.3%, 8.1±1.4%, and 21.5±2.8%, respectively (\(P<0.05\)). Quercetin, at a concentration of 1 \(\mu\)mol/L, inhibited 2-OHE metabolism by 50.8±2.3%. Compared with NE and EPI, ISO was more potent in inhibiting 2-OHE metabolism, and at a
concentration of 10 μmol/L, ISO inhibited 2-OHE metabolism by 21% (P<0.05). Metabolism of 2-OHE (1 μmol/L) was also inhibited by OR-486, a selective COMT inhibitor, in a concentration-dependent manner (Figure 1C).

Treatment with 2.5% FCS stimulated [3H]thymidine incorporation by approximately 8-fold (P<0.001 versus 0.25% FCS) and [3H]proline incorporation by approximately 6-fold (P<0.001 versus 0.25% FCS). Treatment with 0.001 to 1 μmol/L of 2-OHE inhibited [3H]thymidine and [3H]proline incorporation in a concentration-dependent manner (Figures 2A and 3). The inhibitory effects of 2-OHE (0.1 μmol/L) on [3H]thymidine incorporation were abrogated in the presence of EPI, NE, ISO, and quercetin (Figure 2B). Treatment with the catecholamines alone had a slight, but significant, inhibitory effect on [3H]thymidine incorporation (<5%). At concentrations of 0.1, 1, and 5 μmol/L, ISO reversed the inhibitory effect of 2-OHE on [3H]thymidine incorporation from 50% to 48±0.56%, 34±0.6%, and 28±0.2%, respectively, and EPI reversed the inhibitory effects of 2-OHE from 47% to 39±0.5%, 25±0.8%, and 22±0.2%, respectively. Similarly, at concentrations of 5, 10, and 40 μmol/L, NE reversed the inhibitory effects of 2-OHE from 51% to 46±0.4%, 39±0.3%, and 25±0.7%, respectively. Quercetin, a potent inhibitor of COMT, at a concentration of 0.1 and 5 μmol/L, reversed the inhibitory effects of 2-OHE on [3H]thymidine incorporation from 51% to 22±0.7% and 10±0.6%, respectively. Similarly OR-486, a selective COMT inhibitor, at a concentration of 10 μmol/L reversed the inhibitory effect of 2-OHE on [3H]thymidine incorporation from 46% to 7±0.4% (data not shown).

Similar to the effects on [3H]thymidine incorporation, the inhibitory effects of 2-OHE on FCS-induced [3H]proline incorporation were abrogated by catecholamines (ISO, EPI, and NE; Figure 3A) and by the COMT inhibitor OR-486 (Figure 3B).

FCS increased cell number in growth-arrested GMCs by approximately 8-fold (data not shown). 2-OHE at 0.1 μmol/L inhibited FCS-induced increases in cell number by 44%, and this inhibitory effect was abrogated by 5 μmol/L of catecholamines (ISO, EPI, NE) as well as by COMT inhibitor OR-486 (Figure 4). Treatment with catecholamines or OR-486 alone had no effect on GMC proliferation.

To rule out the participation of ERs in mediating the antimitogenic effects of 2-OHE and 2 methoxyestradiol, we also studied the antimitogenic effects of these estradiol metabolites in the presence and absence of ICI 182780 (50 μmol/L), a pure ER antagonist. The inhibitory effects of 0.1 μmol/L of 2-OHE or 2 methoxyestradiol on 2.5% FCS-induced [3H]thymidine incorporation were not blocked by ICI 182780 (data not shown).

To rule out the potential involvement of catecholamine-induced calcium overload in contributing to its abrogatory effects, we assessed the vitality and viability of cells following catecholamine treatment. In cells treated with or without catecholamines, FCS induced DNA synthesis to a similar extent (growth induced by 10-fold in cells treated with FCS, and by 8.2-, 9.0-, and 8.6-fold in cells treated with NE, ISO,
and EPI, respectively [10 μmol/L], suggesting that cell vitality as measured by growth response was not significantly altered. Similarly, trypan blue exclusion studies showed no significant change in cell viability after treatment with catecholamine (number of dead cells <0.1% in each case). Finally, the abrogatory effects of the catecholamines on 2-OHE–induced inhibition of GMC growth were not blocked by the adrenergic receptor blockers (phenolamine, propranolol). EPI reversed the inhibitory effects of 2-OHE to 87±1.9%, and in the presence of phenolamine, the reversal was 91±1.6% (100% represents cells treated with FCS alone). Similarly, ISO reversed the inhibitory effects of 2-OHE to 78±1.1%, and in the presence of propranolol, the reversal was 89±2.0% (100% represents cells treated with FCS alone). Our findings suggest that the modulatory effects of catecholamines are independent of their effects on adrenergic receptors, including the stimulatory effects of catecholamines on intracellular calcium.

Discussion
Abnormal growth of vascular smooth muscle cells contributes to vascular disease following vascular injury, and estradiol attenuates vascular injury–induced neointimal hyperplasia. Similarly to vascular smooth muscle cells, in the wake of glomerular injury, GMCs contribute significantly to the development of glomerulosclerosis by proliferating and producing excessive amounts of extracellular matrix (eg, collagen). Because GMCs are phenotypically similar to vascular smooth muscle cells, by analogy it is likely that estradiol inhibits GMC growth and attenuates the development of glomerulosclerosis. This line of reasoning is supported by our recent finding that estradiol inhibits the growth of GMCs and by the observation of a lower progression rate of renal disease in women compared with men.

Although the received view is that the biologic effects of estradiol on vascular smooth muscle cell growth are receptor-mediated, the recent findings that estradiol attenuates the vascular response to vascular injury in mice lacking ER-α or ER-β suggest that ER-independent mechanisms may also participate in mediating the protective effects of estradiol on the vessel wall. Indeed, our recent findings in rat aortic vascular smooth muscle cells indicate that the vascular protective effects of estradiol are mediated in part by conversion of estradiol to 2-OHE, which in turn is methylated by COMT to yield the highly antiproliferative estradiol metabolite, 2-methoxyestradiol. We hypothesize that a similar mechanism may apply for the renoprotective effects of estradiol, and indeed our recent studies demonstrate that estradiol inhibits the growth of GMCs via its metabolites 2-OHE and 2-methoxyestradiol. Because 2-OHE and 2-methoxyestradiol have little or no affinity for ERs, it is likely that the antimitogenic effects of estradiol in GMCs are ER-independent. However, the potential participation of other, yet undiscovered ERs cannot be ruled out.

The hypothesis that estradiol induces renoprotection by conversion to 2-OHE followed by methylation to 2-methoxyestradiol is supported further by the findings of the current study. In this regard, the present study demonstrates that 2-OHE inhibits DNA synthesis, collagen synthesis, and proliferation and that the inhibitory effects of 2-OHE on GMCs are completely blocked by OR-486 and quercetin, selective COMT inhibitors, but are not blocked by the ER antagonist, ICI 182780. This suggests that the antimitogenic effects of 2-OHE are mediated via an ER-independent mechanism. However, the role of other unknown ERs with no binding affinity for ICI 182780 cannot be ruled out.

The current study also demonstrates that GMCs metabolize 2-OHE to 2-methoxyestradiol via an enzyme with a low Kₘ for 2-OHE and a high Vₘₙₙₙ for the formation of 2-methoxyestradiol. Moreover, this study establishes that inhibitors of COMT block the metabolism of 2-OHE to 2-methoxyestradiol, a finding that strongly implies that COMT catalyzes the methylation of 2-OHE. Inasmuch as 2-methoxyestradiol formation is catalyzed by COMT, it is conceivable that endogenous substrates for COMT would abrogate the renoprotective effects of estradiol and 2-OHE. In this regard, catecholamines, which are well-known substrates for COMT, are implicated in glomerulosclerosis. It is possible, therefore, that catecholamines may worsen renal injury by decreasing the conversion of 2-OHE to 2-methoxyestradiol by GMCs.

In support of the above hypothesis, in the present study we show that the catecholamines NE, EPI, and ISO inhibit the conversion of 2-OHE to 2-methoxyestradiol and abrogate the inhibitory effects of 2-OHE on GMC growth. Our findings provide strong evidence that catecholamines can abrogate the renoprotective effects of 2-OHE by inhibiting COMT activity and thereby reducing the biosynthesis of 2-methoxyestradiol. Our results also imply that the renoprotective effects of estradiol in any individual may depend in part on the levels of catecholamines in the glomeruli.
Catecholamines are known to affect cell growth, and it is possible that the reversal of 2-OHE by catecholamines was mediated by adrenergic receptors rather than by inhibition of COMT. In this regard, in vascular smooth muscle cells, catecholamines induce and inhibit growth via α-1 adrenergic receptors and β-2 adrenergic receptors, respectively. However, our previous studies in human vascular smooth muscle cells demonstrate that inhibition of growth by 2-OHE is abrogated by catecholamines, and this effect of catecholamines is not blocked by antagonists of either α or β adrenergic receptors. In the present study, a direct effect of catecholamines on GMC growth was also ruled out because catecholamines did not markedly affect GMC proliferation in the absence of 2-OHE. This suggests that catecholamines block the antimitogenic effects of 2-OHE by inhibiting COMT and 2-methoxyestriadiol formation.

In vivo metabolism of estradiol to 2-OHE accounts for 50% of the estradiol metabolites formed, and the levels of catecholestradiols range between 0.12 to 0.3 µmol/L in peripheral blood. Substantial amounts of 2-OHE are thus available to be converted to 2-methoxyestradiol. Because of the rapid conversion of 2-OHE to 2-methoxyestradiol, accurate data on the levels of 2-OHE are not available. Nonetheless, the serum levels of 2-methoxyestradiol in pregnant women are 30 nmol/L, and rough estimates suggest that 2-methoxyestradiol levels may be several-fold higher than the levels of estradiol, GMCs and intact kidneys are well endowed with COMT, ensuring pharmacologically active steady state levels of methoxyestradiol.

Circulating levels of NE and EPI are generally 1 to 2 nmol/L, increasing to as high as 12 nmol/L during moderate sympathetic nerve stimulation. However, the concentration of NE in the average neuroeffector junction is approximately 4 times greater than its plasma levels, ie, approximately 50 nmol/L. The fact that 100 nmol/L of catecholamines significantly attenuated the growth-inhibitory effects of 100 nmol/L of 2-OHE suggests that lower concentrations of catecholamines, well within the endogenous range, may effectively block the antimitogenic effects of 2-OHE by competing with COMT. Moreover, increased levels of catecholamines under pathological conditions, such as in patients with the nephrotic syndrome, may attenuate the inhibitory effects of 2-OHE on GMC growth and abrogate the protective effects of 2-OHE against renal disease.

Estrogen replacement therapy is not beneficial in all postmenopausal women. Our previous studies show that estrogen replacement therapy in postmenopausal women differentially increases nitric oxide synthesis, and recent studies demonstrate that estradiol must be metabolized to prevent low-density lipoprotein (LDL) oxidation. On the basis of these findings, it is possible that the decreased cardiorenal protective effects of estrogen that are observed in some postmenopausal women are due to increased catecholamine synthesis and lack of metabolism of 2-OHE to 2-methoxyestradiol.

In conclusion, we provide the first evidence that GMCs metabolize 2-OHE to 2-methoxyestradiol and that catechol-O-methyl transferase-mediated conversion of 2-OHE to 2-methoxyestradiol is essential for the ER-independent inhibitory effects of 2-OHE on GMC growth. Catecholamines can abrogate the antimitogenic effects of 2-OHE by inhibiting the local conversion of 2-OHE to 2-methoxyestradiol. Our findings suggest that interactions between catecholamines and endogenous catecholestradiols may play an important role in defining the overall protective effects of estradiol in the kidney.

**Perspectives**

Our findings indicate that the local conversion of 2-OHE to 2-methoxyestradiol by COMT inhibits growth of glomerular mesangial cells and that the growth-inhibitory effects of 2-OHE are attenuated by catecholamines, which are competitive substrates of COMT. Our results suggest that the conversion of estradiol to 2-OHE and then to 2-methoxyestradiol within the glomerulus/kidney may be an important determinant of the renal-protective effects of circulating estradiol and that individual differences in the local formation of 2-methoxyestradiol could influence a woman’s risk of renal disease. Thus, genetic or acquired differences in renal COMT activity may determine the renal benefits a woman receives from either endogenous estradiol in the premenopausal state or exogenous estradiol from replacement therapy in the postmenopausal state. The fact that catecholamines inhibit the conversion of 2-OHE to 2-methoxyestradiol by competing for COMT suggests that pathological increases in catecholamine release may also influence an individual woman’s risk of renal disease. Specifically, chronic stress or other disease states that elevate catecholamines may limit methylation of 2-OHE, and this may worsen the progression of renal proliferative diseases. Importantly, although use of estradiol is linked to cancer, 2-methoxyestradiol inhibits growth of cancer cells and tumors. Therefore, 2-methoxyestradiol could be used clinically to attenuate progression of renal disease in women without increasing the risk of cancer. Additionally, use of 2-methoxyestradiol would bypass the competition with catecholamines for COMT and confer renal protection independent of COMT activity and catecholamine levels. Finally, because 2-methoxyestradiol is nonfeminizing, it could also be of therapeutic benefit in men.

**Acknowledgments**

This work was supported by Swiss National Science Foundation, grant 32-64040.00 and by the United States National Institutes of Health, grant HL55314.

**References**

5. Zacharia LC, Jackson EK, Gillespie DG, Dubey RK. Catecholamines abrogate the anti-mitogenic effects of 2-hydroxyestradiol on human aortic arterial smooth muscle cells.* Hypertension* April 2002
Catecholamines Block 2-Hydroxyestradiol-Induced Antimitogenesis in Mesangial Cells
Lefteris C. Zacharia, Edwin K. Jackson, Delbert G. Gillespie and Raghvendra K. Dubey

Hypertension. 2002;39:854-859
doi: 10.1161/01.HYP.000014502.44988.39

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/39/4/854

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Questions and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/