Catecholamines Abrogate Antimitogenic Effects of 2-Hydroxyestradiol on Human Aortic Vascular Smooth Muscle Cells

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

Abstract—Catechol-O-methyltransferase (COMT)–mediated methylation of 2-hydroxyestradiol (endogenous estradiol metabolite) to 2-methoxyestradiol (angiogenesis inhibitor) may be responsible for the antimitogenic effects of 2-hydroxyestradiol on vascular smooth muscle cells (VSMCs). Catecholamines are also substrates for COMT, and increased levels of catecholamines are associated with vasocclusive disorders. We hypothesize that catecholamines may abrogate the vasoprotective effects of 2-hydroxyestradiol by competing for COMT and inhibiting 2-methoxyestradiol formation. To test this hypothesis, we investigated the antimitogenic effects of 0.001 to 0.1 μmol/L of 2-hydroxyestradiol on human aortic VSMC proliferation (cell number and DNA synthesis), collagen synthesis, and migration in the presence and absence of catecholamines. Norepinephrine, epinephrine, and isoproterenol concentration-dependently abrogated the inhibitory effects of 2-hydroxyestradiol on cell number, DNA synthesis, collagen synthesis, and cell migration. These modulatory/attenuating effects of catecholamines were not abrogated in the presence of the α- and β-adrenergic receptor antagonists, phentolamine mesylate and propranolol, respectively. In contrast to 2-hydroxyestradiol, the antimitogenic effects of 2-methoxyestradiol (0.1 μmol/L) were not attenuated by isoproterenol (1 μmol/L) or quercetin (competitive inhibitor of COMT, 10 μmol/L). Norepinephrine, epinephrine, and isoproterenol concentration-dependently (10 to 500 μmol/L) inhibited the metabolism of 2-hydroxyestradiol (0.25 to 2 μmol/L) to 2-methoxyestradiol, and the potency of the catecholamines to reverse 2-hydroxyestradiol–induced inhibition of VSMC proliferation, collagen synthesis, and migration was correlated with their ability to inhibit 2-methoxyestradiol formation. Our findings suggest that catecholamines within the vasculature may abrogate the anti–vaso-occlusive effects of estradiol and 2-hydroxyestradiol by blocking 2-methoxyestradiol formation. (Arterioscler Thromb Vasc Biol. 2001;21:1745-1750.)

Key Words: estradiol ■ catechol estrogens ■ methoxyestradiol ■ menopause ■ vascular remodeling

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stradiol induces anti–vaso-occlusive effects in some, but not all, women.1 Because estradiol prevents injury-induced neointimal formation in mice lacking functional estrogen receptors (ERs),2,3 the anti–vaso-occlusive effects of estradiol may in part be mediated by ER-independent mechanisms. In this regard, we have recently shown that the antimitogenic effects of estradiol on vascular smooth muscle cell (VSMC) growth are mediated via its metabolites with little or no affinity for ERs.4

We hypothesize that 2-hydroxyestradiol mediates, in part, the cardiovascular protective effects of estradiol. In support of this hypothesis, our previous findings show that 2-hydroxyestradiol, a major endogenous metabolite of estradiol with little affinity for ERs, is more potent than estradiol in inhibiting VSMC growth.4,5 We also hypothesize that the growth-inhibitory effects of 2-hydroxyestradiol are mediated mostly via 2-methoxyestradiol. Indeed, 2-methoxyestradiol, the methylated product of 2-hydroxyestradiol with no binding affinity for the ERs, is even more potent than is 2-hydroxyestradiol in inhibiting cell growth.4,6 Moreover, our prior studies have demonstrated that under physiological conditions, 2-hydroxyestradiol is metabolized to 2-methoxyestradiol via catechol-O-methyltransferase (COMT).4

Because catecholamines are physiological substrates/competitive inhibitors of COMT7 and because increased synthesis of catecholamines under pathological conditions is known to induce vaso-occlusive disorders,8 we hypothesize that increased levels of catecholamines under pathophysiological conditions may abrogate the cardiovascular protective effects of 2-hydroxyestradiol by competing for COMT and inhibiting 2-methoxyestradiol formation. The goal of the present study was to determine in human aortic VSMCs whether catecholamines abrogate the inhibitory effects of 2-hydroxyestradiol on VSMC growth by blocking the metabolism of 2-hydroxyestradiol to 2-methoxyestradiol.

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Methods

Study Design and Procedures

All experiments were conducted in phenol red–free medium, and FCS was charcoal-stripped and steroid free. Human female thoracic aortic VSMCs were purchased from Clonetics and cultured under standard tissue culture conditions as previously described.2

[3H]Thymidine incorporation (index of DNA synthesis) and cell proliferation were conducted as previously described.4,5,9,10 For [3H]thymidine incorporation, VSMCs were plated at a density of 5 × 10^4 cells per well in 24-well tissue culture dishes and allowed to grow to confluence under standard tissue culture conditions. 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 [3H]thymidine (1 µCi/mL) for an additional 4 hours. The [3H]thymidine experiments were terminated by washing the cells twice with PBS and twice with ice-cold trichloroacetic acid (10%). The precipitate was solubilized, and aliquots from 4 wells for each treatment were counted in a liquid scintillation counter. For cell number experiments, VSMCs were allowed to attach overnight, were growth-arrested for 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. All experiments were conducted in at least triplicate.

For collagen synthesis studies, determination of [3H]proline incorporation (nonspecific index of collagen synthesis) and quantification of collagen type I production by VSMCs, cells were plated as previously described.6,7,9,10 For [3H]proline incorporation, confluent monolayers of VSMCs grown in 24-well plates were growth-arrested for 48 hours in 0.25% FCS and then treated for 36 hours with DMEM supplemented with 2.5% FCS plus [3H]proline (1 µCi/mL) in the presence or absence of the test agents. Subsequently, [3H]proline incorporation into the acid-precipitable cellular fraction was assayed as described for [3H]thymidine incorporation. For assaying collagen type I production, VSMCs grown to confluence in 6-well plates were treated as described above but in the absence of [3H]proline. After the treatment, the supernatant and the scraped and solubilized monolayers of VSMCs were collected and pooled together. Total collagen type I (soluble and insoluble fractions) was subsequently assayed by ELISA with the use of primary antibody against collagen (rabbit anti-human type I collagen, Biodesign International) and as described in detail previously.10 For [3H]proline and collagen type I production experiments, confluent monolayers of VSMCs were treated in parallel, and [3H]thymidine incorporation and cell-counting experiments were performed to ensure that the changes in [3H]proline incorporation and collagen production were not due to changes in cell proliferation. All experiments were conducted in at least triplicate.

For cell migration studies, we used 6.5-mm-diameter Transwell plates (Costar) with an 8-µm polycarbonate membrane pore size. VSMCs were starved overnight in 0.25% FCS and trypsinized, and 30,000 cells were placed on each membrane. The treatments were prepared, and 0.5 mL was placed in the lower well chamber. After an incubation of 5 hours at 37°C, the media were removed, and cells from the upper phase of the membrane were washed away. The membranes were then placed in methanol for fixation for 30 minutes, followed by a 30-minute incubation in Hoechst stain solution (0.5 µg/mL). The membranes were then separated from the wells and mounted on glass slides, and the labeled nuclei of the migrated cells were visualized by fluorescence microscopy. Cells that migrated were determined by counting 12 different spots on each slide and taking the average.

To evaluate the effects of catecholamines on the metabolism of 2-hydroxyestradiol (2OHE) and 2-methoxyestradiol (2-MeOE), VSMCs were plated in 12-well plates and grown to confluence in DMEM/F-12 and 10% FCS. Before the treatments, the cells were washed twice with PBS and treated in DMEM for 2 hours with (2 µmol/L) 2-hydroxyestradiol in the presence or absence of 0 to 500 µmol/L catecholamines. Quercetin (5 µmol/L, a known COMT inhibitor) was used as a positive control. To prevent oxidative breakdown of 2-hydroxyestradiol, all experiments were conducted in the presence of 1 mmol/L ascorbic acid. After the treatments, the supernatants were collected, and 2-hydroxyestradiol and 2-methoxyestradiol were extracted 3 times with a total of 6 mL methylene chloride. The organic solvent was evaporated under vacuum, and the dried sample was reconstituted in water and methanol (80:20) and analyzed by high-performance liquid chromatography (HPLC).11 Briefly, using 1µm-hydroxyestradiol as an internal standard, we separated estradiol metabolites by using a C-18 reverse-phase column (5 µm) attached to a Hewlett-Packard HPLC system (HP model 1050). The metabolites were detected with a UV detector set at 280 nm. The mobile phase consisted of water and methanol in the following gradient: from 80:20 (water:methanol) to 30:70 for 25 minutes, from 30:70 to 20:80 for 5 minutes, from 20:80 to 10:90 for 1 minute, from 10:90 to 0:100 for 1 minute, and at 80:20 for column equilibration 3 minutes after the run.

Statistical Analysis

All experiments were conducted in triplicate or quadruplicate and repeated 3 or 4 times with the use of separate cultures. Results are presented as mean±SEM. Statistical analyses were performed by using ANOVA, paired Student t test, Fisher least significant difference test, or Bonferroni t test as appropriate. A value of P<0.05 was considered statistically significant.

Results

Norepinephrine (NE), epinephrine (EPI), and isoproterenol (ISO) inhibited methylation of 2-hydroxyestradiol in a concentration-dependent manner (Figure 1). A concentration of 150 µmol/L of ISO, EPI, and NE inhibited the metabolism of 2-hydroxyestradiol (2 µmol/L) by 62%, 34%, and 15%, respectively. Quercetin (5 µmol/L) inhibited 2-hydroxyestradiol
methylation by 85% (Figure 1A). At concentrations of 300 and 500 μmol/L, NE inhibited 2-hydroxyestradiol metabolism by 23% and 25%, respectively, whereas EPI inhibited metabolism by 47% and 58%, respectively (*P<0.05). Compared with NE and EPI, ISO was more potent in inhibiting 2-hydroxyestradiol metabolism, and at the lowest concentration (50 μmol/L), ISO inhibited 2-hydroxyestradiol by 37% (*P<0.05).

Because catecholamines act as competitive inhibitors of COMT,7 we examined the inhibition kinetics of ISO by assaying the effects of 25, 100, and 200 μmol/L ISO on the concentration-dependent metabolism of 2-hydroxyestradiol (0.25 to 10 μmol/L). As expected, the curve for the concentration-dependent metabolism of 2-hydroxyestradiol was shifted to the right in the presence of increasing concentrations of ISO (Figure 1B). The K_i for ISO was calculated to be 60.5 μmol/L (see Dixon plot, Figure 1C).

Treatment with 2.5% FCS stimulated [3H]thymidine incorporation by ~8-fold (*P<0.001 versus 0.25% FCS). Treatment with 0.001 to 1 μmol/L of 2-hydroxyestradiol inhibited DNA synthesis in a concentration-dependent manner (Figure 2A), and at 0.1 μmol/L, 2-hydroxyestradiol inhibited FCS-induced [3H]thymidine incorporation by 60% (Figure 2B). EPI, NE, ISO, and quercetin abrogated the antimitogenic effects of 2-hydroxyestradiol in a concentration-dependent manner, as determined by DNA synthesis (Figure 2B). Treatment with the catecholamines (EPI, NE, and ISO) or quercetin alone had no significant effect on DNA synthesis and modulated DNA synthesis <3±2%. ISO at concentrations of 0.1, 1, and 5 μmol/L reversed the inhibitory effect of 2-hydroxyestradiol from 60% to 44%, 29%, and 21%, respectively. EPI at concentrations of 0.1, 1, and 5 μmol/L reversed the inhibitory effect from 60% to 49%, 31%, and 27%, respectively. NE at concentrations of 5, 10, and 40 μmol/L reversed the inhibitory effect from 60% to 54%, 49%, and 35%, respectively. Quercetin, a potent COMT inhibitor, at a concentration of 0.1 μmol/L reversed the 2-hydroxyestradiol–mediated DNA synthesis inhibition from 60% to 13%, and at a concentration of 1 μmol/L completely reversed the inhibitory effect.

FCS increased the cell number in growth-arrested VSMCs by ~8-fold (data not shown). 2-Hydroxyestradiol at 0.1 μmol/L inhibited FCS-induced increases in cell number by 55%, and catecholamines reversed this inhibitory effect (Figure 2C). At a concentration of 5 μmol/L, ISO and EPI reversed the inhibitory effects of 2-hydroxyestradiol from 55% to 24% and 22%, respectively. Treatment with catecholamines alone also induced a mild inhibitory effect on VSMC growth. ISO, EPI, and NE at the concentrations used caused a significant inhibitory effect of 7%, 8%, and 15%, respectively.

FCS (2.5%) stimulated [3H]proline incorporation in VSMCs by ~6-fold (*P<0.001 versus 0.25% FCS). Treatment with 0.1 μmol/L of 2-hydroxyestradiol inhibited FCS-induced [3H]proline incorporation by 62%, and these inhibitory effects of 2-hydroxyestradiol were abrogated by catecholamines (Figure 3A). At concentrations of 5 μmol/L, ISO and EPI reversed the inhibitory effects of 2-hydroxyestradiol on proline incorporation from 62% to 17% and 20%, respectively. Moreover, at 10 μmol/L, NE reversed the inhibition from 62% to 38%, whereas 0.1 μmol/L quercetin reversed the inhibition from 60% to 4%.

Similar to the effects on proline incorporation, 0.1 μmol/L of 2-hydroxyestradiol inhibited collagen type I production by VSMCs by 54.6% (Figure 3B). Compared with the catecholamines (NE, EPI, and ISO), quercetin was more potent in abrogating the inhibitory effects of 2-hydroxyestradiol on collagen type I production (Figure 3B). At concentrations of 5 μmol/L, ISO and EPI reversed the inhibitory effects of 2-hydroxyestradiol on collagen type I production from 54.6% to 32% and 25.2%, respectively. Moreover, at 10 μmol/L, NE reversed the inhibition from 54.6% to 42%, whereas 0.1 μmol/L quercetin reversed the inhibition from 54.6% to 6%.

FCS stimulated VSMC migration, and this effect was strongly inhibited by 2-hydroxyestradiol (Figure 4). At 0.1 μmol/L, 2-hydroxyestradiol inhibited FCS-induced VSMC migration by 85%. As shown in Figure 4, the inhibitory effect of 2-hydroxyestradiol on cell migration was abrogated by NE (10 μmol/L) and quercetin (0.1 μmol/L). Compared with quercetin, NE was less potent in reversing the inhibitory effects of 2-hydroxyestradiol on VSMC migration (Figure 4).
At equimolar concentrations of 0.1 μmol/L, quercetin and NE reversed the inhibitory effects of 2-hydroxyestradiol on VSMC migration by 86% and 5%, respectively.

To confirm that the concentrations of catecholamines used to maximally modulate the growth effects of 2-hydroxyestradiol (0.1 μmol/L) were associated with the inhibition of 2-methoxyestradiol formation, we evaluated the effects of 5 to 10 μmol/L ISO and ISO on the metabolism of 0.25 μmol/L of 2-hydroxyestradiol to 2-methoxyestradiol. In the presence of 5 μmol/L ISO and 5 and 10 μmol/L EPI, the formation of 2-methoxyestradiol in cells treated for 2 hours was inhibited by 17.4±4% (P<0.05 versus metabolism in absence of ISO) and by 7.09±0.35% and 14±0.4%, respectively (P<0.05 versus metabolism in the absence of EPI).

To rule out the participation of α- and β-adrenergic receptors in mediating the attenuating effects of catecholamines on the antimitogenic effects of 2-hydroxyestradiol, the modulatory effects of EPI and ISO were tested in the presence and absence of the α- and β-adrenergic receptor antagonists, respectively. As shown in Figure 5, the antimitogenic effects of 0.1 μmol/L of 2-hydroxyestradiol on VSMC proliferation (DNA synthesis and cell number) were significantly attenuated in the presence of 1 μmol/L EPI and ISO, and these effects were not altered in the presence of the α- and β-adrenergic receptor antagonists, respectively. As shown in Figure 5, the antimitogenic effects of 0.1 μmol/L of 2-hydroxyestradiol on VSMC proliferation (DNA synthesis and cell number) were significantly attenuated in the presence of 1 μmol/L EPI and ISO, and these effects were not altered in the presence of the α- and β-adrenergic receptor antagonists, respectively. 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Discussion
Abnormal growth of VSMCs contributes to the vascular remodeling process associated with neointimal formation and involves 3 distinct processes: (1) migration of VSMCs from the media into the intima, (2) proliferation of VSMCs, and (3) increased synthesis and deposition of extracellular matrix proteins, such as collagen.

The inhibitory effects of estradiol on injury-induced neointimal formation is a key mechanism via which estradiol is thought to mediate its protective effects on the vasculature of postmenopausal women receiving hormone replacement therapy. Although the biological effects of estradiol on VSMCs are thought to be receptor-mediated, the recent findings that estradiol prevents neointimal formation in mice lacking functional ER-α and ER-β suggest that ER-independent mechanisms may also participate in mediating the vascular protective effects of estradiol on the vessel wall.

The present study demonstrates that 2-hydroxyestradiol, an endogenous estradiol metabolite, inhibits human VSMC DNA and collagen synthesis, as well as proliferation and migration of human VSMCs, consistent with our previous findings in rat VSMCs. The present study also shows that the inhibitory effects of 2-hydroxyestradiol on VSMC proliferation, migration, and collagen synthesis and the conversion of 2-hydroxyestradiol to 2-methoxyestradiol are completely blocked by quercetin, a COMT inhibitor with no affinity for ERs. These findings provide evidence that human VSMCs express COMT activity and that the inhibitory effects of 2-hydroxyestradiol on VSMC proliferation and migration, and collagen synthesis are mediated via the local conversion of 2-hydroxyestradiol to 2-methoxyestradiol. This contention is further supported by our previous findings that compared with estradiol, 2-methoxyestradiol and 2-hydroxyestradiol are more potent inhibitors of rat VSMC and cardiac fibroblast proliferation.

Because the generation of 2-methoxyestradiol is COMT dependent, endogenous inhibitors of COMT may abrogate the inhibitory effects of estradiol on VSMCs. We hypothesize that catecholamines, which are COMT substrates and are known to promote vaso-occlusive disorders, could abrogate the inhibitory effects of 2-hydroxyestradiol on VSMCs. The present study demonstrates that increasing concentrations of NE, EPI, and ISO competitively inhibit the conversion of 2-hydroxyestradiol to 2-methoxyestradiol and abrogate the inhibitory effects of 2-hydroxyestradiol on VSMC proliferation, migration, and collagen synthesis. The relative potencies of catecholamines to block the inhibitory effects of 2-hydroxyestradiol matched their relative abilities to block COMT. Our findings provide evidence that catecholamines can abrogate the vascular protective effects of catecholestradiols by inhibiting COMT activity and thereby blocking the formation of methoxyestradiols. Thus, the overall vascular protective effects of estradiol in any individual may in part depend on the expression of COMT activity as well as the levels of catecholamines.

Catecholamines induce and inhibit VSMC proliferation via α1-adrenergic receptors and β2-adrenergic receptors, respectively. The abrogation of the inhibitory effects of 2-hydroxyestradiol by catecholamines could be due to their proliferation-promoting effects. However, as shown in the present study, the concentrations of catecholamines that abrogate the antimitogenic effects of 2-hydroxyestradiol have little or no effect on VSMC proliferation. Moreover, the abrogating effects of EPI and ISO on the antimitogenic effects of 2-hydroxyestradiol are not blocked by the α or β-adrenergic receptor antagonists, phentolamine and propranolol, respectively. This suggests that catecholamines block the antimitogenic effects of 2-hydroxyestradiol by inhibiting 2-methoxyestradiol formation. This notion is further strengthened by our finding that the antimitogenic effects of 2-hydroxyestradiol, but not 2-methoxyestradiol, are abrogated by ISO.

The above findings, together with our observation that the inhibitory effects of 2-hydroxyestradiol and 2-methoxyestradiol on FCS-induced VSMC proliferation are not blocked by the ER antagonist ICI 182780, suggest that the conversion of 2-hydroxyestradiol to 2-methoxyestradiol by COMT is responsible for mediating the antimitogenic effects of 2-hydroxyestradiol on VSMCs and that these antimitogenic effects are mediated via an ER-independent mechanism.

Could our findings be of pathophysiological significance? In vivo metabolism of estradiol to 2-hydroxyestradiol accounts for 50% of the estradiol metabolites formed, and the levels of catecholestradiols range between 0.12 and 0.3 μmol/L in peripheral blood. These findings suggest that substantial amounts of 2-hydroxyestradiol are available to be converted to 2-methoxyestradiol. Accurate data on the levels of 2-methoxyestradiol are not available; however, the serum levels of 2-methoxyestradiol in pregnant women are 30 nmol/L, and rough estimates suggest that its levels may be several-fold higher than the levels of estradiol. The fact that VSMCs and endothelial cells are well endowed with COMT would ensure pharmacologically active steady-state levels of methoxyestradiol in the blood vessel wall.

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 ~4 times greater than its plasma levels, i.e., ~50 nmol/L. However, the level of NE in any given neuroeffector junction during sympathetic activation will depend on such factors as the width of the junction and the efficiency of uptake and metabolism of NE. Thus, NE levels in some neuroeffector junctions would be much greater than 50 nmol/L during sympathetic activation. In the present study, concentrations of catecholamines as low as 100 nmol/L, significantly attenuated the growth inhibitory effects of 100 nmol/L of 2-hydroxyestradiol. Because the COMT interaction between catecholamines and 2-hydroxyestradiol is competitive, even lower levels of catecholamines would be expected to attenuate the antimitogenic effects of lower levels of 2-hydroxyestradiol. These considerations imply that increased synthesis of catecholamines under pathological conditions could effectively attenuate the inhibitory effects of 2-hydroxyestradiol on VSMC proliferation.
Similar to VSMCs, 2-hydroxyestradiol inhibits the growth of cardiac fibroblasts. Because catecholamines are known to play a key role in the cardiac remodeling process associated with ventricular hypertrophy in myocardial infarction and heart failure, it is possible that the interactions between catechol estrogens and catecholamines may participate in the pathophysiology of cardiac disorders.

Estrogen replacement therapy is not beneficial in all postmenopausal women. Our previous studies show that estrogen replacement therapy in postmenopausal women differentially increases NO synthesis, and recent studies demonstrate that estradiol must be metabolized to prevent LDL oxidation. On the basis of these findings, it is possible that the decreased cardioprotective effects of estrogen that are observed in some postmenopausal women are due to increased catecholamine synthesis and lack of metabolism of 2-hydroxyestradiol to 2-methoxyestradiol. Indeed, differences in the metabolism of estradiol to 2-hydroxyestradiol are associated with the carcinogenic effects of estradiol in women.

In conclusion, we provide the first evidence that human aortic VSMCs effectively metabolize 2-hydroxyestradiol to 2-methoxyestradiol and that COMT-mediated conversion of 2-hydroxyestradiol to 2-methoxyestradiol is essential for mediating the ER-independent inhibitory effects of 2-hydroxyestradiol on VSMC growth proliferation, migration, and collagen synthesis. Catecholamines can abrogate the antimitogenic effects of 2-hydroxyestradiol by inhibiting the local conversion of 2-hydroxyestradiol 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 on the cardiovascular system.

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References
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