Increased 2-Methoxyestradiol Production in Human Coronary Versus Aortic Vascular Cells

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

Abstract—Estradiol may be cardioprotective; however, the mechanisms involved remain unclear. Recent findings that estradiol attenuates neointima formation in estrogen receptor knockout mice suggest that the cardioprotective effects of estradiol may be mediated through estrogen receptor–independent mechanisms. Because 2-methoxyestradiol, an endogenous metabolite of estradiol with no affinity for estrogen receptors, is more potent than estradiol in inhibiting vascular smooth muscle cell growth, it is feasible that 2-methoxyestradiol mediates in part the cardioprotective effects of estradiol. To address this hypothesis, we examined the kinetics of 2-methoxyestradiol synthesis in vascular smooth muscle cells and endothelial cells. In human aortic smooth muscle cells, the V\textsubscript{max}, K\textsubscript{m}, and V\textsubscript{max}/K\textsubscript{m} ratio values for conversion of 2-hydroxyestradiol to 2-methoxyestradiol were 19±0.69 pmol·min\textsuperscript{-1}·10\textsuperscript{6} cells, 0.52±0.085 μmol/L, and 44±4.9 pmol·min\textsuperscript{-1}·μmol/L per 10\textsuperscript{6} cells, respectively. In human coronary artery vascular smooth muscle cells, the V\textsubscript{max}, K\textsubscript{m}, and V\textsubscript{max}/K\textsubscript{m} ratio values for conversion of 2-hydroxyestradiol to 2-methoxyestradiol were 16±0.59 pmol·min\textsuperscript{-1}·10\textsuperscript{6} cells, 0.23±0.011 μmol/L, and 69±3.6 pmol·min\textsuperscript{-1}·μmol/L per 10\textsuperscript{6} cells, respectively (all values significantly different compared with human aortic smooth muscle cells). Also, in human aortic versus coronary artery endothelial cells, the V\textsubscript{max} (33±0.24 versus 22±0.33 pmol·min\textsuperscript{-1}·10\textsuperscript{6} cells, respectively), K\textsubscript{m} (0.20±0.010 versus 0.099±0.014 μmol/L, respectively), and V\textsubscript{max}/K\textsubscript{m} (163±7.7 versus 243±41 pmol·min\textsuperscript{-1}·μmol/L per 10\textsuperscript{6} cells, respectively) values were significantly different. Our results indicate that vascular smooth muscle and endothelial cells effectively metabolize 2-hydroxyestradiol to 2-methoxyestradiol. The lower K\textsubscript{m} and higher V\textsubscript{max}/K\textsubscript{m} ratio of human coronary versus aortic cells indicate a faster rate of local metabolism of 2-hydroxyestradiol to 2-methoxyestradiol in the coronary circulation at low levels of 2-hydroxyestradiol. (Hypertension. 2001;37[part 2]:658-662.)

Key Words: catechol-O-methyltransferase ■ estrogen ■ endothelium ■ muscle, smooth, vascular ■ coronary artery disease

Estradiol may protect premenopausal women from coronary artery disease. Recent findings that estradiol prevents injury-induced neointima formation in mice lacking functional estrogen receptors (ERs), either ER\textalpha or ER\beta, suggest that the antivasoocclusive effects of estradiol may be mediated in part through ER-independent mechanisms.

Our studies demonstrate that 2-hydroxyestradiol and 2-methoxyestradiol, major endogenous metabolites of estradiol with little or no affinity for ERs, are more potent than estradiol in inhibiting vascular smooth muscle cell (VSMC) growth. Moreover, our studies indicate that methoxysteroidols mediate the antiinflammatory effects of estradiol and 2-hydroxyestradiol on VSMC growth. Compared with other vascular beds, the incidence of coronary artery disease is specifically increased in postmenopausal women. For these reasons, and because the formation of 2-methoxyestradiol involves methylation of 2-hydroxyestradiol by catechol-O-methyltransferase (COMT), we hypothesize that the cardio-protective effects of estradiol on the coronary circulation may in part be due to the efficient conversion 2-hydroxyestradiol to 2-methoxyestradiol by COMT in the coronary artery.

It is well established that VSMC growth and tone are differentially regulated in various vascular beds. The embryonic origin and development of VSMCs differ considerably, and the coronary arteries develop independent of the systemic vasculature. This raises the possibility that the protective effects of estradiol on coronary artery VSMCs may be influenced by properties intrinsic to these cells. Because metabolism of 2-hydroxyestradiol to 2-methoxyestradiol by COMT would inhibit VSMC growth, we hypothesize that coronary artery VSMCs metabolize 2-hydroxyestradiol to 2-methoxyestradiol more efficiently than do other VSMCs.

The main objective of this study was to characterize the kinetics of 2-methoxyestradiol formation in coronary and aortic VSMCs and endothelial cells (ECs). In addition, we compared the inhibitory effects of 2-hydroxyestradiol on
mitogen-induced growth (DNA synthesis and cell proliferation) of aortic versus coronary artery VSMCs.

Methods

Cell Culture

Human female thoracic aortic VSMCs and coronary artery VSMCs and ECs \( (n=3) \) were purchased from Clonetics. Rat aortic VSMCs were cultured as explants from proximal and distal aortas obtained from Sprague-Dawley rats, \( (n=12) \) as previously described. The cells were passaged by trypsinization, and cells in the third to fourth passages were used for the experiments. All experiments were conducted in phenol red–free medium. The serum used was charcoal stripped and steroid free.

Metabolism Studies

For the metabolism studies, cells were plated in tissue culture 12-well plates and grown to confluence under standard tissue culture conditions. Rat VSMCs were grown in DMEM-F12 containing 10% FCS; ECs were grown in endothelial growth medium-2; and human VSMCs were grown in smooth muscle growth medium. Before treatment, cells were washed twice with warm (37°C) PBS. For determination of \( K_m \) and \( V_{max} \), cells were incubated in DMEM with 0.05 to 2.5 or 0.25 to 2.5 \( \mu \)mol/L 2-hydroxyestradiol and 1 mmol/L ascorbic acid for 1 hour. Ascorbic acid was used in all treatments to prevent oxidation of 2-hydroxyestradiol during the incubation period. To investigate the interaction between catecholamines and 2-hydroxyestradiol metabolism, cells were incubated for 2 hours in DMEM with 2 \( \mu \)mol/L 2-hydroxyestradiol and 1 mmol/L ascorbic acid in the presence and absence of 150 \( \mu \)mol/L of either epinephrine, norepinephrine, or isoproterenol. For concentration-dependent inhibition of 2-hydroxyestradiol metabolism by isoproterenol, cells were incubated with 0.25 or 2.5 \( \mu \)mol/L 2-hydroxyestradiol in the presence and absence of 25 \( \mu \)mol/L isoproterenol. At the end of the incubation period, the supernatant was collected. 16\( \alpha \)-hydroxyestradiol was added as an internal standard, and samples were extracted with methylene chloride. The organic solvent was removed by evaporation under vacuum, and the dried sample was redissolved in water-methanol (80:20 ratio) and analyzed by high-performance liquid chromatography (HPLC), as described below. The \( V_{max} \) and apparent \( K_m \) values were determined with GraphPad Prism version 3.0. \( V_{max} \) was expressed as pmol of 2-methoxyestradiol formed per minute per million cells, and \( K_m \) was calculated in micromoles per liter.

HPLC Assay for Metabolites

The estradiol metabolites were separated with a C-18 reverse-phase column (5 \( \mu \)m) attached to a model 1050 Hewlett Packard HPLC system. 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 over 25 minutes, 30:70 to 20:80 over 5 minutes, 20:80 to 10:90 over 1 minute, 10:90 to 0:100 over 1 minute, followed by a 3-minute postrun at 80:20 for column equilibration.

DNA Synthesis and Cell Proliferation

As described before,2 \([^{3}H] \) thymidine incorporation (index of DNA synthesis) and cell number (cell proliferation) studies were conducted to investigate the physiological relevance of differential 2-methoxyestradiol formation on the growth of human coronary and aortic VSMCs. Briefly, VSMCs were plated at a density of \( 5 \times 10^4 \) cells per well in 24-well tissue culture dishes and allowed to grow to subconfluence in DMEM/F-12 (phenol red–free) medium containing 10% FCS under standard tissue culture conditions. The cells were then growth-arrested by feeding DMEM (phenol red–free) containing 0.4% albumin for 48 hours. For DNA synthesis, growth-arrested VSMCs were treated with 0.1 \( \mu \)mol/L 2-hydroxyestradiol in DMEM containing 2.5% FCS. After 20 hours of incubation, the treatments were repeated as above with freshly prepared solutions but supplemented with \([^{3}H] \) thymidine (1 \( \mu Ci/mL \)) for an additional 4 hours.

Data are presented as mean±SEM, and groups were compared by means of a Mann-Whitney U test. The criterion of significance was set at \( P<0.05 \).

Results

As shown in Figure 1, the apparent \( K_m \) in human coronary VSMCs \( (n=7) \) was calculated to be 0.23±0.011 \( \mu \)mol/L. In contrast, human aortic VSMCs \( (n=12) \) had a significantly \( (P<0.05) \) higher apparent \( K_m \) of 0.52±0.085 \( \mu \)mol/L. Although the \( V_{max} \) in human coronary VSMCs was slightly smaller than the \( V_{max} \) in human aortic vascular smooth muscle cells (16±0.59 versus 19±0.69 pmol⋅min\(^{-1}\) per 10⁶ cells, respectively; \( P<0.05 \)), the \( V_{max}/K_m \) ratio was significantly \( (P<0.05) \) greater in human coronary VSMCs compared with human aortic vascular smooth muscle cells (69±3.6 versus 44±4.9 pmol⋅min\(^{-1}\)⋅\( \mu \)mol/L per 10⁶ cells (Figure 1).

Figure 1. Line graphs showing conversion of 2-hydroxyestradiol to 2-methoxyestradiol by cultured human coronary VSMCs (HCASMC; A) and aortic VSMCs (HASMC; B) in representative experiment. C compares apparent \( K_m \), \( V_{max} \), and \( V_{max}/K_m \) ratio values for metabolism of 2-hydroxyestradiol in coronary and aortic VSMCs (values represent mean±SEM; \( n=12 \) for HASMCs, \( n=7 \) for HCASMCs). * \( P<0.05 \), HCASMCs vs HASMCs.

The experiments were terminated by washing the cells twice with Dulbecco’s PBS and twice with ice-cold trichloroacetic acid (10%). The precipitate was solubilized in 500 \( \mu \)L of 0.3N NaOH and 0.1% SDS (50°C for 2 hours). Aliquots from 4 wells for each treatment with 10 mL of scintillation fluid were counted in a liquid scintillation counter. For cell number experiments, VSMCs were allowed to attach overnight, were growth-arrested for 48 hours, and were treated with 0.1 \( \mu \)mol/L 2-hydroxyestradiol in DMEM containing 2.5% FCS. This treatment regimen was repeated every 48 hours for 4 days. On day 5, cells were dislodged and counted on a Coulter counter.

Similar findings were observed in human aortic versus coronary artery endothelial cells. In human aortic \( (n=4) \) versus coronary artery \( (n=4) \) endothelial cells, the \( V_{max} \) (33±0.24...
versus 22 ± 0.33 pmol · min⁻¹ per 10⁶ cells, respectively), $K_m$ (0.20 ± 0.010 versus 0.099 ± 0.014 μmol/L, respectively), and $V_{max}/K_m$ (163 ± 7.7 versus 243 ± 41 pmol · min⁻¹ · μmol/L per 10⁶ cells, respectively) were significantly ($P < 0.05$) different (Figure 2).

The rat aorta is known to contain VSMC populations that express diverse phenotypes and originate from diverse embryonic lineages.⁷ In this regard, the proximal and distal parts of the aorta develop from cells with a distinctively different embryonic lineage.⁷,⁹ Therefore, we also investigated whether 2-hydroxyestradiol metabolism differs kinetically in proximal versus the distal aortic VSMCs. As shown in Figure 3, in proximal aortic (n = 7) versus distal (n = 4) aortic VSMCs, the $V_{max}$ (15 ± 0.57 versus 19 ± 0.36 pmol · min⁻¹ per 10⁶ cells, respectively), $K_m$ (0.44 ± 0.070 versus 0.13 ± 0.059 μmol/L, respectively), and $V_{max}/K_m$ (40 ± 7.1 versus 150 ± 63 pmol · min⁻¹ · μmol/L per 10⁶ cells, respectively) were significantly ($P < 0.05$) different (Figure 2).

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We also investigated whether the metabolism of 2-hydroxyestradiol to 2-methoxyestradiol is differentially inhibited by catecholamines, which are endogenous substrates for COMT.¹⁰ As shown in Figure 4, in the presence of 150 μmol/L norepinephrine, epinephrine, or isoproterenol, the metabolism of 2-hydroxyestradiol to 2-methoxyestradiol in human aortic VSMCs versus human coronary VSMCs was inhibited by 14.6 ± 2.2% versus 6.3 ± 1.9% ($P < 0.05$), 34.7 ± 2.7% versus 19.8 ± 2.9% ($P < 0.05$), and 62 ± 1.8% versus 37 ± 1.7% ($P < 0.05$), respectively. As shown in Figure 5, isoproterenol was also more effective in inhibiting 2-hydroxyestradiol methylation in rat upper aortic VSMCs compared with human coronary VSMCs.

As shown in Figure 6, treatment with 2-hydroxyestradiol (0.1 μmol/L) inhibited FCS-induced DNA synthesis and cell proliferation by 53 ± 0.87% and 70 ± 0.02%, respectively, in human coronary VSMCs compared with a 45 ± 0.28% inhibition in DNA synthesis and a 64 ± 0.1% inhibition of cell proliferation in human aortic VSMCs. In this regard, the inhibition of DNA synthesis and cell proliferation by 2-hydroxyestradiol was significantly ($P < 0.05$) greater in human coronary versus aortic VSMCs.

**Discussion**

The results of this study indicate that methylation of 2-hydroxyestradiol to 2-methoxyestradiol occurs in coronary and aortic VSMCs and ECs. In this regard, human coronary VSMCs and ECs methylate 2-hydroxyestradiol with an apparent $K_m$ that is one-half the $K_m$ observed in human aortic VSMCs versus human coronary VSMCs. In this regard, the proximal and distal parts of the aorta develop from cells with a distinctively different embryonic lineage.⁷,⁹ Therefore, we also investigated whether 2-hydroxyestradiol metabolism differs kinetically in proximal versus the distal aortic VSMCs. As shown in Figure 3, in proximal aortic (n = 7) versus distal (n = 4) aortic VSMCs, the $V_{max}$ (15 ± 0.57 versus 19 ± 0.36 pmol · min⁻¹ per 10⁶ cells, respectively), $K_m$ (0.44 ± 0.070 versus 0.13 ± 0.059 μmol/L, respectively), and $V_{max}/K_m$ (40 ± 7.1 versus 150 ± 63 pmol · min⁻¹ · μmol/L per 10⁶ cells, respectively) were significantly ($P < 0.05$) different (Figure 2).

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VSMCs and ECs. Moreover, the \( V_{\text{max}}/K_m \) ratio for conversion of 2-hydroxyestradiol to 2-methoxyestradiol is significantly greater in coronary versus aortic VSMCs and ECs. These kinetic findings indicate that at low physiological levels of 2-hydroxyestradiol, the rate of formation of 2-methoxyestradiol would indeed be faster in the coronary circulation versus the aorta. Consistent with this conclusion is our finding that a low concentration of 2-hydroxyestradiol more effectively inhibits DNA synthesis and cell proliferation of coronary VSMCs compared with aortic VSMCs. This observation suggests that the increased \( V_{\text{max}}/K_m \) of coronary VSMCs compared with aortic VSMCs to convert 2-hydroxyestradiol to 2-methoxyestradiol has biological consequences; for example, low concentrations of catecholestrogens more effectively inhibit growth in coronary cells. Thus, coronary VSMCs and ECs have increased ability to form 2-methoxyestradiol from low concentrations of 2-hydroxyestradiol, and increased local formation of 2-methoxyestradiol in the coronary circulation may account in part for the protective effects of estradiol against coronary artery disease.

The finding that conversion of 2-hydroxyestradiol to 2-methoxyestradiol occurs with different \( V_{\text{max}}/K_m \) ratios in human coronary cells versus human aortic cells is not surprising. Differential effects of vasoactive factors on the growth and function in various vascular beds are well established.\(^7\) Moreover, VSMCs cultured from different vascular beds as well as from different sections of a common vessel grow differentially in response to a common stimulus.\(^7\) One of the main factors contributing to these different effects is the phenotype of the cells and its embryonic lineage.\(^11\)

Studies with mammalian embryos show that different arteries and even different segments of the same artery are composed of VSMCs that differ greatly in their embryonic lineage and developmental history,\(^12\) and VSMCs from different lineage backgrounds may not have identical functional and growth-regulatory mechanisms.\(^7,12\) Therefore, our observation of increased methylation of low concentrations of 2-hydroxyestradiol in coronary VSMCs and ECs compared with aortic cells may be explained by the fact that coronary VSMCs originate from mesothelial progenitors in the proepicardial organ and that these vessels develop completely independent of the systemic vasculature.\(^7,8\)

We also observe that 2-hydroxyestradiol methylation is differentially efficient in VSMCs from the proximal and distal rat aorta. In this regard, the proximal aorta is made up of VSMCs that originate from the neural ectoderm through the cardiac neural crest, whereas the muscular distal aorta is composed of VSMCs that are recruited primarily from splanchic mesoderm.\(^9\) These two VSMC types are not equally distributed within the aorta but exhibit sharp boundaries and transition zones.\(^13\) Because a mixture of different VSMC types with diverse embryonic origins coexist within a common vessel wall, distinct VSMC populations may respond differently to 2-hydroxyestradiol.

The formation of 2-methoxyestradiol is inhibited by catecholamines (endogenous substrates for COMT\(^10\)), and the inhibitory efficacy of catecholamines differs between coronary and aortic cells. In aortic VSMCs, catecholamines are 2-fold more effective in blocking 2-methoxyestradiol formation as compared with coronary VSMCs. This suggests that in addition to more efficient methylation of low concentrations of 2-hydroxyestradiol in the coronary circulation, this biochemical step is more resistant to inhibition by catecholamines in the coronary circulation. COMT is not only responsible for the formation of 2-methoxyestradiol but also for the catabolism of catecholamines.\(^10\) Therefore, a more efficient COMT would have dual protective effects on the coronary circulation because it would be more effective in generating 2-methoxyestradiol and lowering catecholamines.

In addition to generating 2-methoxyestradiol and catabolizing catecholamines, COMT may also attenuate coronary artery disease by preventing free radical–induced deleterious effects on the vessel wall. Catalyzed by the cytochrome P450, the catecholestrogens can undergo metabolic redox cycling to produce secondary free radicals (quinones or semiquinones), which can initiate lipid peroxidation and contribute to the process of atherosclerosis.\(^14\) Redox cycling of 2- and 4-hydroxyestradiol and free radical formation depends on the availability of the hydroxy metabolites. If hydroxyestradiols are not inactivated by methylation or conjugation, the probability of undergoing redox cycling increases. Because COMT is the key enzyme responsible for methylating catecholestrogens to methoxyestrogens,\(^10\) COMT may play a critical role in protecting against free radical–induced vasooclusive disorders. Finally, apart from the above mechanisms, generation of 2-methoxyestradiol by COMT may also protect against vasooclusive disorders by acting as an antioxidant, lowering LDL, inhibiting endothelin-1 secretion, inducing

**Figure 5.** Bar graph comparing percentage inhibition of 2-methoxyestradiol formation (2-MeOE) from 2-hydroxyestradiol (2-OHE) by isoproterenol (25 \( \mu \text{mol/L} \)) in rat aortic (RASMC) and human coronary (HCASMC) VSMCs. Values represent mean±SEM from representative experiment conducted in triplicate; similar results were obtained in 3 independent experiments. *\( P<0.05 \), RASMCs vs HCASMCs.

**Figure 6.** Bar graph comparing inhibitory effects of 2-hydroxyestradiol on FCS-induced (2.5%) DNA synthesis (A) and cell number (B) in human coronary (HCASMC) and aortic (HASMC) VSMCs. Values represent mean±SEM from 3 separate experiments, each conducted in triplicate. #\( P<0.05 \) vs control cells treated with FCS alone; *\( P<0.05 \) vs coronary VSMCs treated similarly.
cAMP and prostaglandin synthesis, and inhibiting increases in intracellular calcium-calmodulin levels (reviewed in Reference 6).

The finding that estradiol metabolites inhibit VSMC growth and the fact that COMT activity is expressed in coronary artery cells suggest that metabolism of estradiol to 2-methoxyestradiol through 2-hydroxyestradiol may play an important role in mediating the overall protective effects of estradiol on the coronary circulation. Moreover, our findings imply that the cardioprotective effects of estradiol may vary and may be dependent on the metabolic capability of the individual. For example, estrogen replacement therapy is not beneficial in all postmenopausal women.1,6 Indeed, estrogen replacement therapy in postmenopausal women increases nitric oxide synthesis and lowers LDL levels in some (≈50%) but not all postmenopausal women.6 On the basis of these findings, it is possible that the decreased cardioprotective effects of estrogen that are observed in some postmenopausal women may be due to multiple factors, including decreased production of 2-hydroxyestradiol, increased synthesis of catecholamines locally in the vessel wall, and lack of metabolism of 2-hydroxyestradiol to 2-methoxyestradiol as the result of the decreased COMT activity. Indeed, differences in the metabolism of estradiol to 2-hydroxyestradiol are associated with carcinogenic effects of estradiol in women.15

We have previously shown that methoxyestradiols mediate the antimitogenic effects of estradiol on VSMC growth; however, 2-methoxyestradiol is the ultimate mediator for the antimitogenic effects of 2-hydroxyestradiol.4 The above findings together with our finding that COMT activity is highly expressed in the vascular cells may be of considerable clinical significance. In this regard, 2-methoxyestradiol also inhibits tumor growth, angiogenesis, and growth of cancer cells,15 and the lack of synthesis of 2-hydroxyestradiol is associated with increased incidence of cancer.15 Thus, 2-methoxyestradiol may be of pharmacological importance in preventing both cancer and cardiovascular disease. Because one of the disadvantages of estrogen replacement therapy is the risk of inducing cancer (mammary, endometrial), it is feasible that 2-methoxyestradiol could be used for prevention of cardiovascular disease in women without increasing the risk of cancer. Moreover, because 2-methoxyestradiol is nonfeminizing,1 it could be of therapeutic use in men.

Conclusions

We provide the first evidence that human coronary and aortic VSMCs and ECs effectively metabolize 2-hydroxyestradiol to 2-methoxyestradiol, and, as compared with the aorta, the \( V_{\text{max}} / K_m \) ratio of COMT is significantly higher in coronary VSMCs and ECs. Our findings suggest but do not prove that within the coronary artery, increased 2-methoxyestradiol formation may be responsible in part for protecting women against coronary artery disease. Thus, COMT activity and the generation of 2-methoxyestradiol may play a critical role in mediating the antivasoocclusive effects of estradiol on the cardiovascular system. Further studies are required to investigate this possibility.

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References

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