Estradiol Metabolites Inhibit Endothelin Synthesis by an Estrogen Receptor-Independent Mechanism

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Abstract—Estradiol inhibits endothelin-1 synthesis, an effect that may contribute to the cardiovascular protective effects of estradiol. Recent findings that estradiol inhibits neointima formation in mice lacking estrogen receptors suggests that the cardiovascular protective effects of estradiol may be mediated by means of an estrogen receptor-independent mechanism. Because 2-hydroxyestradiol and 2-methoxyestradiol, metabolites of estradiol with little/no affinity for estrogen receptors, are more potent than estradiol in inhibiting vascular smooth muscle cell growth, we investigated whether these metabolites also inhibit endothelin-1 synthesis by means of an receptor-independent mechanism. Treatment of porcine coronary artery endothelial cells for 4 to 24 hours with 0.001 to 1 μmol/L of estradiol, 2-hydroxyestradiol, or 2-methoxyestradiol concentration-dependently inhibited basal as well as serum-induced (2.5%), TNFα-induced (10 ng/mL), angiotensin II–induced (100 nmol/L), and thrombin-induced (4 U/mL) endothelin-1 synthesis. Estradiol, 2-hydroxyestradiol, and 2-methoxyestradiol also inhibited serum-induced mitogen-activated protein kinase activity. As compared with estradiol, its metabolites were more potent in inhibiting endothelin-1 secretion and mitogen activated protein kinase activity. The inhibitory effects of 2-hydroxyestradiol and 2-methoxyestradiol on endothelin-1 release and mitogen-activated protein kinase activity were not blocked by ICI182780 (50 μmol/L), an estrogen receptor antagonist. Our findings indicate that the estradiol metabolites 2-hydroxyestradiol and 2-methoxyestradiol potently inhibit endothelin-1 synthesis by means of an estrogen receptor-independent mechanism. This effect of estradiol metabolites may be mediated by inhibition of mitogen activated protein kinase activity and may contribute to the cardioprotective effects of estradiol. (Hypertension. 2001;37[part 2]:640-644.)

Key Words: postmenopause ▪ endothelial cells ▪ vascular remodeling ▪ cardiovascular disease ▪ estradiol metabolites ▪ coronary artery

Increased endothelin-1 (ET-1) synthesis is associated with vaso-occlusive disorders,1,2 and estradiol inhibits ET-1 synthesis, perhaps by means of estrogen receptor (ER)-dependent pathways.3,4 Thus, inhibition of ET-1 production by estradiol may contribute to its cardioprotective effects. However, the mechanisms by which estradiol inhibits ET-1 synthesis remain unclear.

In this regard, although the biological effects of estradiol are thought to be ER mediated,1 recent findings indicate that estradiol inhibits neointima formation in mice lacking either ERα or ERβ, suggesting that the cardiovascular protective effects of estradiol may be mediated in part by means of ER-independent mechanisms.5,6 This notion is further supported by the observation that estradiol is unable to inhibit neointima formation in nongonadectomized male rats7 even though these rats express high levels of ERα and ERβ.8

2-Hydroxyestradiol and 2-methoxyestradiol are major endogenous metabolites of estradiol that have low or no affinity for ERs.1 Even so, these estradiol metabolites are more potent than estradiol in inhibiting vascular smooth muscle cell growth.9,10 Thus, we hypothesize that these metabolites play a major role in mediating the cardiovascular protective effects of estradiol by means of an ER-independent mechanism.

The main goal of the present study was to investigate whether estradiol metabolites inhibit ET-1 synthesis in coronary artery endothelial cells by means of an ER-independent mechanism. Another objective was to compare the efficacy of various clinically used estrogens in inhibiting ET-1 synthesis. Finally, we investigated whether inhibition of mitogen activated protein kinase (MAPK) activity could participate in the effects of estradiol metabolites on endothelial biology.

Methods

Isolation and Culture of Porcine Coronary Artery Endothelial Cells
Hearts from female pigs were obtained from a local slaughter house and placed in ice-cold oxygenated DMEM containing antibiotics. The epicardial coronary arteries were isolated, and the fat and
connective tissue were removed. Endothelial cells were isolated by collagenase/dispase digestion. The isolated cells were washed in DMEM/F12 medium supplemented with 10% fetal calf serum (FCS) and endothelial cell growth supplement (Clonetics), and cells were grown to confluence under standard tissue culture conditions. The purity of the cultures, which was characterized by immunostaining with factor VIII antigen and by assaying the preferential uptake of dextran blue, was 98%. Cells were passaged by trypsinization, and cells in second and third passage were used for all experiments.

**Treatment Protocols for Endothelin-1 Synthesis Studies**

To study the effects of estradiol and its metabolites on ET-1 synthesis in porcine coronary artery endothelial cells (PCAEcs), confluent monolayers of PCAEs were washed twice with HBSS and treated with DMEM/Ham’s F12 supplemented with 0.4% bovine serum albumin (BSA) containing or lacking 0.001 to 1 μmol/L of estradiol, 2-hydroxyestradiol, or 2-methoxyestradiol. After 24 hours of treatment, the monolayers were treated for an additional 4 hours with fresh treatments in the presence of vehicle, angiotensin II (100 nmol/L), thrombin-stimulated (Thr; 4 U/mL), and TNF-α-stimulated (10 ng/mL), respectively (Figure 2). Physiological concentrations (1 nmol/L) of estradiol significantly inhibited basal synthesis of ET-1 by 19% (percentage increase), respectively (Figure 2). Treatment of PCAECs for 8 hours with physiological concentrations (2 nmol/L) of estradiol inhibited basal synthesis of ET-1 by 19±2% (P<0.05). Significant reductions in ET-1 levels were also evident in PCAECs treated under basal conditions for 12 and 24 hours (Figure 1).

**Protocols for MAPK Activity Measurement**

PCAEcs were grown to confluence in 35-mm2 culture dishes and were made quiescent by feeding DMEM containing 0.4% BSA for 48 hours. Growth arrested PCAEs washed with PBS and pretreated for 24 hours with or without various test agents were stimulated with FCS (2.5%) for 10 minutes. Some cells were pretreated for 1 hour with IC182780 before treatment with the test agents. After stimulation, the cells were washed with ice-cold PBS and extraction buffer (50 mmol/L β-glycerophosphate, 1.5 mmol/L EGTA, 1 mmol/L dithiothreitol, 100 μmol/L NaVO4, 10 μg/mL aprotinin, 5 μg/mL pepstatin, 20 g/mL leupeptin, and 1 mmol/L benzamidine), scraped off the plates, and sonicated for 20 seconds in 0.5 mL of extraction buffer. The extracts were collected, and the cytosolic fraction was separated by centrifuging the extracts at 100,000 g for 1 hour. The supernatants were collected for MAPK activity assays. The MAPK activity in the cytosolic extracts was quantified as previously described by us.

**Results**

ET-1 levels were increased in a time-dependent manner in the medium collected from PCAECs incubated for 4 to 24 hours under basal conditions (Figure 1). Preliminary studies showed that treatment of PCAECs with FCS stimulated ET-1 secretion maximally at 4 hours, and this time point was selected for all subsequent studies. As compared with vehicle-treated controls, treatment of PCAECs for 4 hours with FCS, angiotensin II, thrombin, and TNFα induced ET-1 secretion by 138±14%, 116±7%, 98±5%, and 120±8% (percentage increase), respectively (Figure 2). Treatment of PCAECs for 8 hours with physiological concentrations (2 nmol/L) of estradiol inhibited basal synthesis of ET-1 by 19±2% (P<0.05). Significant reductions in ET-1 levels were also evident in PCAECs treated under basal conditions for 12 and 24 hours (Figure 1).

Estradiol inhibited FCS-, angiotensin II-, thrombin-, and TNFα-induced ET-1 secretion in a concentration-dependent manner (Figure 2). Physiological concentrations (1 nmol/L) of estradiol significantly (P<0.05) decreased ET-1 secretion induced in response to FCS (from 138±16% to 82±8%),

![Figure 1](image1.png)  
*Figure 1. Time-dependent inhibitory effects of 2 nmol/L of estradiol, 2-hydroxyestradiol (2-OH), and 2-methoxyestradiol (2-MeOE) on the release of ET-1 in the medium by PCAECs treated for various times. Values represent mean±SEM from a representative experiment conducted in triplicate, and similar results were obtained in three independent experiments. *P<0.05 versus control cells treated with vehicle alone.*

![Figure 2](image2.png)  
*Figure 2. Concentration-dependent inhibitory effects of estradiol (β-Estradiol), 2-hydroxyestradiol (2-OH), and 2-methoxyestradiol (2-MeOE) on fetal calf serum (FCS; 2.5%), angiotensin II-stimulated (Ang II; 100 nmol/L), thrombin-stimulated (Thr; 4 U/mL), and TNFα-stimulated (10 ng/mL) release of ET-1 in the medium of PCAECs treated for 4 hours. Values represent mean±SEM from a representative experiment conducted in triplicate, and similar results were obtained in three independent experiments. *P<0.05 versus control cells treated with vehicle or the stimulatory agents alone; §P<0.05 versus inhibitory effects of estradiol.*
estradiol, and various clinically used estrogens (17β-estradiol, βE; 17α-estradiol, αE; estradiol cypionate, EC; estradiol valerate, EV; estradiol benzoate, EB; estrone, E2; and estriol, E3) in the presence and absence of IC182780 (50 μmol/L) on 2.5% fetal calf serum–induced ET-1 release into the medium of cells stimulated for 4 hours. Values represent mean±SEM from three separate experiments, each conducted in triplicate. *P<0.05 versus control cells treated with vehicle alone; §P<0.05, significant reversal of the inhibitory effects.

Figure 3. Comparison of the inhibitory effects of 10 nmol/L of estradiol and various clinically used estrogens (17β-estradiol, βE; 17α-estradiol, αE; estradiol cypionate, EC; estradiol valerate, EV; estradiol benzoate, EB; estrone, E2; and estriol, E3) on 2.5% fetal calf serum–induced ET-1 release into the medium of cells stimulated for 4 hours. Values represent mean±SEM from three separate experiments, each conducted in triplicate. *P<0.05 versus control cells treated with vehicle alone.

2-hydroxyestradiol and 2-methoxyestradiol, on FCS-stimulated ET-1 secretion (Figure 4), as well as on basal ET-1 release (data not shown). Similar to estradiol the inhibitory effects of estradiol valerate, estradiol cypionate, and estradiol benzoate were reversed by IC182780 (Figure 3).

Genistein, an ERβ ligand at nanomolar concentrations, inhibited basal (data not shown) as well as FCS-stimulated (Figure 5) ET-1 secretion by PCAECs in a concentration-dependent manner. The inhibitory effects of genistein were observed at concentrations of 1 μmol/L and higher, moreover the inhibitory effects of genistein were not reversed by IC182780 (Figure 5).

Treatment of growth-arrested cells with FCS (2.5%) increased MAPK activity from 0.187 to 7.25 pmol/min/mg protein, and the stimulatory effects of FCS were inhibited by the MEK inhibitor PD98059 (10 μmol/L) to 0.7 pmol/min/mg protein. In cells pretreated for 24 hours with estradiol, 2-hydroxyestradiol, or 2-methoxyestradiol, the stimulatory effects of FCS on MAPK activity were inhibited in a concentration-dependent manner (Figure 6). Compared with estradiol, 2-hydroxyestradiol and 2-methoxyestradiol were more potent in inhibiting FCS-induced MAPK activity. In

Figure 4. Effects estradiol of IC182780 (50 μmol/L) on the inhibitory effects of estradiol (β-Est; 10 nmol/L), 2-hydroxyestradiol (2-OHE; 10 nmol/L), and 2-methoxyestradiol (2-MeOE; 10 nmol/L) on 2.5% FCS-induced ET-1 release into the medium of cells incubated for 4 hours. Values represent mean±SEM from three separate experiments, each conducted in triplicate. *P<0.05 versus control cells treated with vehicle alone; §P<0.05, significant reversal of the inhibitory effects.

Figure 5. Concentration-dependent effects of genistein on 2.5% fetal calf serum–stimulated release of ET-1 in the medium of PCAECs treated for 4 hours in the presence and absence of IC182780 (50 μmol/L). Values represent mean±SEM from three separate experiments, each conducted in triplicate. *P<0.05 versus control cells treated with vehicle alone.

Figure 6. (Above) Concentration-dependent effects of estradiol (β-Est), 2-hydroxyestradiol (2-OHE), and 2-methoxyestradiol (2-MeOE) on 2.5% fetal calf serum (FCS)–induced MAPK activity in PCAECs. (Below) Effects of IC182780 (50 μmol/L) on the inhibitory effects of 10 nmol/L estradiol (β-Est), 2-hydroxyestradiol (2-OHE), and 2-methoxyestradiol (2-MeOE) on 2.5% FCS-induced mitogen-activated protein kinase activity. Results are mean±SEM (expressed as pmol/min/mg protein). *P<0.05 versus control cells treated with vehicle (Veh) alone; §P<0.05, significant reversal of the inhibitory effects.
cells pretreated with ICI182780 (50 μmol/L), the inhibitory effects of estradiol, but not 2-hydroxyestradiol or 2-methoxyestradiol, on MAPK activity were completely reversed (Figure 6). In PCAECs pretreated for 24 hours with physiological concentrations (1 nmol/L) of estradiol, 2-hydroxyestradiol, and 2-methoxyestradiol, FCS-induced MAPK activity was inhibited by 19±2.6%, 34±4%, and 46±4.7%, respectively.

Discussion

ET-1 is a potent vasoconstrictor peptide and enhances the mitogenic effects of mitogens on smooth muscle cell growth. Moreover, increases in ET-1 synthesis/release are associated with vaso-occlusive disorders. Because estradiol inhibits ET-1 synthesis, it is hypothesized that the protective effects of estradiol on the vessel wall are in part mediated by means of this mechanism. Many of the biologic effects of estradiol are mediated by means of ERs, and some studies suggest that the inhibitory effect of estradiol on ET-1 synthesis by endothelial cells is ER mediated. Consistent with the previous report, we also observe that estradiol inhibits the release of ET-1 and these effects are inhibited by ICI182780, an ER antagonist. However, recent data from our laboratory indicate that ICI182780 is not only an ER antagonist, but also inhibits the metabolism of estradiol to 2-hydroxyestradiol. Therefore, it is possible that blockade of the effects of estradiol on ET-1 production by ICI182780 is due in part to inhibition of metabolism of estradiol to 2-hydroxyestradiol, as well as antagonism of ERs.

Although the inhibitory effects of estradiol on ET-1 release/secretion are inhibited by ICI182780, the findings of two recent studies that estradiol prevents balloon injury–induced neointima formation in mice lacking functional ERα and ERβ suggest that ER-independent mechanisms may also participate in mediating the cardiovascular protective effects of estradiol. Under in vivo conditions, estradiol is efficiently metabolized by means of multiple CYP450 enzymes. Our previous studies indicate that 2-hydroxyestradiol and 2-methoxyestradiol, major endogenous metabolites of estradiol, possess biological activity and are more potent than estradiol in inhibiting smooth muscle cell and cardiac fibroblast proliferation. Because these metabolites possess minimal binding affinity for the ERs, we hypothesize that the in vivo cardiovascular protective effects of estradiol may be mediated in part by means of estradiol metabolites and by means of ER-independent mechanisms. This notion is supported by our present findings that basal as well as stimulated release of ET-1 by PCAECs are inhibited by 2-hydroxyestradiol and 2-methoxyestradiol, and this effect is not blocked by ICI182780.

Genistein is a phytoestrogen with a high affinity for the ERβ. In the present study, we used this agent to study the role of ERβ in regulating ET-1 synthesis. Our observation that genistein inhibits ET-1 synthesis in micromolar, but not nanomolar, concentrations suggests that the inhibitory effects of estradiol are not mediated by ERβ. This contention is supported by the fact that genistein acts as an ERβ ligand at nanomolar concentrations and inhibits tyrosine kinase activity at higher concentrations. Because inhibition of tyrosine kinase inhibits ET-1 synthesis, the observed inhibitory effects of genistein on ET-1 synthesis could be attributed to its inhibitory effects on tyrosine kinase. This conclusion is further supported by the fact that the inhibitory effect of genistein on ET-1 synthesis is not blocked by ICI182780. Taken together, these findings provide evidence that the inhibitory effects of estradiol on ET-1 synthesis may be mediated in part by means of ERα. This notion is supported by the recent observation that overexpression of ERα in endothelial cells results in a dramatic decrease in ET-1 secretion.

Although the inhibitory effects of estradiol on stimulated ET-1 release are well documented, its inhibitory effects on basal synthesis of endothelin-1 remain controversial. Our finding that estradiol metabolites inhibit basal ET-1 synthesis suggests that, under in vivo conditions, estradiol would down-regulate ET-1 release. This contention is indirectly supported by the findings that the expression of preproendothelin-1 is significantly upregulated in ovariectomized pigs.

Our finding that estradiol is effective in inhibiting ET-1 synthesis, whereas 17α-estradiol, estrone, estrone sulfate, and estriol are much less active in this regard, indicates that the effects of estrogens on ET-1 production vary considerably. This conclusion is also supported by our finding that estradiol benzoate is significantly less potent than estradiol in inhibiting ET-1 synthesis. Our previous studies reveal similar differential antimitogenic effects of these clinically used estrogens on human vascular smooth muscle cells and cardiac fibroblasts. Thus, the inconsistent and confusing effects of hormone replacement therapy on cardiovascular disease risk may be due in part to the differential effects of clinically used estrogens on ET-1 synthesis by endothelial cells.

ET-1 synthesis is known to be regulated by the MAPK pathway. Inhibition of MEK by PD98059 in endothelial cells is associated with inhibition of ET-1 synthesis; whereas, stimulation of MAPK activity by mitogens such as angiotensin II and FCS is known to stimulate ET-1 synthesis. Because estradiol and its metabolites inhibit mitogen stimulated MAPK activity in vascular smooth muscle cells, we hypothesize that inhibition of MAPK activity in endothelial cells may provide a common pathway by which estradiol and its metabolites inhibit ET-1 synthesis. Indeed, the findings of the present study indicate that FCS-stimulated MAPK activity is inhibited by estradiol and its metabolites. That TNFα, thrombin, angiotensin II, and FCS induce MAPK activity, together with our finding that estradiol and its metabolites inhibit MAPK activity, suggests that the inhibitory effects of estradiol and its metabolites on ET-1 synthesis are due to their inhibitory effects on MAPK activity.

Could our findings be of physiological and clinical significance? Metabolism of estradiol to catecholestrogens is one of the most prominent pathways for estradiol metabolism, and catecholestrogens are precursors for methoxyestradiols. Thus, substantial amounts of estradiol metabolites would be available in vivo. Although the physiological levels of methoxyestradiol in humans are not known with accuracy, the serum levels of methoxyestrogens in pregnant women are 30 nmol/L. Moreover, rough estimates suggest that 2-methoxyestradiol levels may be several-fold higher than the levels of estradiol. With regard to catecholestrogens, the
levels range between 0.12 to 0.3 μmol/L in peripheral blood and the rate of urinary excretion of 2-hydroxyestradiol is 20 to 180 μg/24 hours in urine. Both VSMCs and endothelial cells are well endowed with COMT, the enzyme responsible for metabolizing catecholestrogens to methoxyestrrogens, thus likely ensuring pharmacologically active steady state levels of methoxyestradiol in the blood vessel wall. That 2-hydroxyestradiol and 2-methoxyestradiol are more potent than estradiol in inhibiting ET-1 synthesis suggests that metabolism of estradiol may play an important role in mediating the overall cardiovascular protective effects of estradiol. This suggests that the cardioprotective effects of estradiol may vary and be dependent on the metabolic capability of the individual. Notably, estrogen replacement therapy is not beneficial in all postmenopausal women, and previous results indicate that estrogen replacement therapy in postmenopausal women differentially increases nitric oxide synthesis and decreases LDL levels. Moreover, recent studies show that estradiol must be metabolized to prevent LDL oxidation. On the basis of these findings, it is possible that the variable cardioprotective effects of estrogen that are observed in postmenopausal women may be due to lack of metabolism of estradiol to 2-hydroxyestradiol and 2-methoxyestradiol. This contention is further strengthened by our recent findings that methoxyestradiols and catecholestadiols mediate the antimitogenic effects of estradiol on vascular smooth muscle cell growth by means of an ER-independent mechanism.

With regard to clinical relevance of the present findings, the overall protective effects of hormone replacement therapy may depend on the type of estrogen used. That estradiol and its metabolites inhibit ET-1 synthesis, whereas some clinically used estrogens are ineffective, suggests that estrogens may have differential protective effects on vascular biology.

In conclusion, we provide the first evidence that 2-hydroxyestradiol and 2-methoxyestradiol, the major endogenous metabolites of estradiol with no affinity for ERs, inhibit ET-1 secretion by PCAECs by an ER-independent mechanism. Moreover, we demonstrate that the inhibitory effects of estradiol on ET-1 synthesis are not mediated by ERβ. Finally, estradiol and its metabolites may attenuate ET-1 synthesis by inhibiting MAPK activity.

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