Estradiol-17β and its Cytochrome P450- and Catechol-O-Methyltransferase–Derived Metabolites Selectively Stimulate Production of Prostacyclin in Uterine Artery Endothelial Cells

Role of Estrogen Receptor-α Versus Estrogen Receptor-β

Sheikh O. Jobe, Jayanth Ramadoss, Andrew J. Wargin, Ronald R. Magness

Abstract—Metabolism of estradiol-17β to 2-hydroxyestradiol, 4-hydroxyestradiol, 2-methoxyestradiol, and 4-methoxyestradiol contributes importantly to the vascular effects of estradiol-17β in several vascular beds. However, little is known about the role of estradiol-17β metabolites via the different estrogen receptors (ER-α/ER-β) on de novo endothelial prostacyclin and thromboxane production. We hypothesized that estradiol-17β and its metabolites, via ER-α or ER-β, can enhance the prostacyclin/thromboxane ratio through the classic phospholipase A₂, cyclooxygenase-1, and prostacyclin synthase pathway in ovine uterine artery endothelial cells (UAECs) derived from pregnant (P-UAECs) versus nonpregnant (NP-UAECs) ewes. Western analyses showed higher expression of phospholipase A₂, cyclooxygenase-1, and prostacyclin synthase in UAECs from the pregnant state, whereas thromboxane synthase was lowered in UAECs from the nonpregnant state. In UAECs from the pregnant state, estradiol-17β, 2-hydroxyestradiol, 4-hydroxyestradiol, 2-methoxyestradiol and 4-methoxyestradiol concentration and time-dependently increased prostacyclin compared with controls. Prostacyclin increases in UAECs from the nonpregnant state were of a lower magnitude. Estradiol-17β and its metabolites stimulated higher prostacyclin/thromboxane ratios in UAECs from the pregnant state compared with UAECs from the nonpregnant state. Estradiol-17β–induced prostacyclin increases were abrogated by the antagonists SC-560 (cyclooxygenase-1), U-51605 (Prostacyclin synthase), ICI 182780 (ICI; both ER-α/β), and 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride (MPP; ER-α), but not by 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolyl]-1,5-azapyrimidin-3-ylphenol (PHTPP; ER-β). Prostacyclin increases induced by its metabolites were abolished by SC-560 and U-51605, but unaltered by ICI, MPP, or PHTPP. Our findings demonstrate that estrogen via primarily ER-α and its metabolites via ER-independent mechanisms influence the de novo endothelial biosynthesis of prostacyclin, which may be important in the regulation of vascular tone. These findings also shed light on the complexities of estrogen signaling via its metabolism and the functional heterogeneity of the ERs.

Key Words: catecholamines • endothelium • estradiol metabolites • estrogen receptors • pregnancy • prostacyclin • thromboxane

Prostacyclin (PGI₂) and thromboxane (TXA₂), 2 major vasoactive prostanoids that exert opposing effects on vascular tone, are end products of the sequential reactions catalyzed by cytosolic phospholipase A₂ (cPLA₂), cyclooxygenase-1/2 (COX-1/2), and either PGI₂ synthase (PGIS) or TXA₂ synthase (TXAS) enzymes, respectively. Classically, PGI₂, a powerful vasodilator, is produced mainly by vascular endothelial cells, whereas TXA₂, a potent vasoconstrictor, is principally produced by platelets. In this regard, little is known about whether endothelial cells can produce TXA₂ and whether this production plays a role in regulating the ratio of PGI₂ and TXA₂. PGI₂/TXA₂ ratio is considered of relevance in the regulation of physiological and clinical vascular tone, with several studies showing that an imbalance in the generation of PGI₂ relative to TXA₂ is associated with hypertension, atherosclerosis, and gestational vascular diseases such as preeclampsia.

The dramatic rises in uterine blood flow during pregnancy are temporally associated with increases in de novo uterine vascular PGI₂ secretion and are accompanied by augmented expression of uterine artery endothelial cPLA₂, COX-1, and PGIS. These uterine blood flow rises are also partly mediated by increases in the plasma levels and actions of estrogen via the classical estrogen receptors (ERs). Infusion of estradiol-17β (E₂) in sheep causes rises in uterine blood flow,
increases the uterine artery endothelial expression of cPLA₂, COX-1, and PGIS, which leads to increases of the stable PGI₂ metabolite, 6-keto-Prostaglandin F₁α (6-keto-PGF₁α).⁷,¹²,¹³ In human umbilical vein endothelial cells (HUVECs), E₂β has also been shown to selectively stimulate PGI₂ production in vitro primarily via ER-α.¹⁴ However, less is known about the influence of E₂β on endothelial TXA₂ production and on the endothelial PGI₂/TXA₂ ratio.

The stimulatory effects of estrogen on uterine vascular endothelial PGI₂ production may be further modulated by its biologically active metabolites. E₂β is sequentially metabolized by cytochrome P450s to form the catecholestadiols, 2-hydroxyestradiol (2-OHE₂) and 4-hydroxyestradiol (4-OHE₂), followed by metabolism of these catecholestadiols by catechol-O-methyltransferase to form 2-methoxyestradiol (2-ME₂) and 4-methoxyestradiol (4-ME₂).⁵⁻¹⁰ In cultured HUVECs, 2-ME₂ stimulates production of PGI₂.¹⁹ Despite this knowledge, very little is known about the effects of E₂β metabolites on endothelial PGI₂ levels. Furthermore, nothing is known about the roles of the classical ERs on E₂β metabolite-induced endothelial PGI₂ levels. It is also unclear whether E₂β metabolites can alter TXA₂ production and the PGI₂/TXA₂ ratio in endothelial cells and whether this is altered in pregnancy.

Thus, we hypothesized that E₂β and its metabolites, 2-OHE₂, 4-OHE₂, 2-ME₂, and 4-ME₂, can augment the PGI₂/TXA₂ ratio via ER-α and ER-β, through the classical cPLA₂-COX-1-PGIS pathway in uterine artery endothelial cells (UAECs) in the pregnant (P-UAECs) compared with UAECs in the nonpregnant (NP-UAECs) state. Thus, we investigated: (1) the expression of cPLA₂, COX-1, PGIS, and TXAS in P-UAECs compared with NP-UAECs; (2) whether treatment with E₂β, 2-OHE₂, 4-OHE₂, 2-ME₂, or 4-ME₂, can increase the PGI₂/TXA₂ ratio in P-UAECs more than NP-UAECs; (3) the role of COX-1 and PGIS in regulating P-UAEC PGI₂ levels; and (4) the roles of ER-α and ER-β on PGI₂ levels induced by E₂β and its metabolites.

### Methods

#### Cell Preparation and Culture

Animal use protocols and procedures were approved by the University of Wisconsin-Madison School of Medicine Research Animal Care Committee. Ovine NP-UAECs and P-UAECs were isolated, validated, and cultured from nonpregnant (luteal n=2 and follicular n=2) and late gestation (120–130 days; term, 147 days; n=4) ewes as previously described.⁶,¹⁷,¹³ At passage 4 and >70% confluence and serum-starved (24 hours), cells were lysed for Western blotting or transferred to 6-well plates for treatments as needed for respective experiments.

#### Protein Extraction and Western Immunoblotting

Protein extraction and Western Immunoblot analyses were performed as described previously.⁶,¹⁷,¹³ cPLA₂, COX-1, PGIS, and TXAS expressions were detected using mouse anti-cPLA₂, rabbit anti-COX-1, rabbit anti-PGIS, or rabbit anti-TXAS (1:1000) and respective secondary antibodies (1:2000). β-actin was used as loading control. Positive control was only used for the expression of TXAS using human platelet lysates. Human platelet lysates were a generous donation from the Platelet and Neutrophil Immunology Laboratory, Blood Center of Wisconsin.

#### Experimental Treatments, Blockade of Enzymes and Receptors

All experiments were performed in quadruplicate and replicated in at least 4 different P-UAEC (n=4) and NP-UAEC (n=4) preparations. For time and concentration-response studies, P-UAECs in 6-well plates were serum-starved (24 hours) in Endothelial Basal Medium (EBM), washed with serum-free EBM, and the medium was replaced with EBM vehicle (Control) or EBM containing 0.1, 1, 10, or 100 nmol/L of E₂β, 2-OHE₂, 4-OHE₂, 2-ME₂, 4-ME₂, or Ca²⁺ ionophore (A23187; positive control) for 0, 2, 4, 8, 12, or 24 hours. For nonpregnant versus pregnant concentration-response studies, NP-UAECs and P-UAECs in 6-well plates were serum-starved (24 hours) in EBM, washed with serum-free EBM, and medium was replaced with EBM vehicle (Control) or EBM containing 0.1, 1, 10, or 100 nmol/L of E₂β, 2-OHE₂, 4-OHE₂, 2-ME₂, 4-ME₂, or A23187 for 12 hours based on time course studies. COX-1 or PGIS blockade was performed by pretreating P-UAECs with SC-560 and U-51605 (1 μmol/L, 1 hour), respectively, followed by E₂β, 2-OHE₂, 4-OHE₂, 2-ME₂, 4-ME₂, or A23187 treatments at optimal dose (determined from concentration-response curves). ERs were targeted with E₂β for 2, 4, 8, 12, or 24 hours and treated with E₂β, 2-OHE₂, 4-OHE₂, 2-ME₂, 4-ME₂, or A23187 for 12 hours.

### Table. The Effects of E₂β, 2-OHE₂, 4-OHE₂, 2-ME₂, and 4-ME₂ on the PGI₂/TXA₂ Ratio in NP-UAECs Versus P-UAECs

<table>
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<th>Steroid</th>
<th>Cell Type</th>
<th>Control</th>
<th>0.1 nmol/L</th>
<th>1 nmol/L</th>
<th>10 nmol/L</th>
<th>100 nmol/L</th>
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<td>E₂β</td>
<td>NP-UAEC</td>
<td>1.49±0.05</td>
<td>1.49±0.06</td>
<td>1.76±0.05*</td>
<td>2.03±0.13*</td>
<td>2.45±0.01*</td>
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<td>P-UAEC</td>
<td>1.75±0.03†</td>
<td>2.27±0.02†</td>
<td>4.39±0.02†</td>
<td>5.45±0.17†</td>
<td>10.06±0.20†</td>
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<td>2-OHE₂</td>
<td>NP-UAEC</td>
<td>1.46±0.06</td>
<td>1.76±0.05*</td>
<td>2.04±0.12†</td>
<td>2.52±0.08†</td>
<td>2.72±0.02*</td>
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<tr>
<td></td>
<td>P-UAEC</td>
<td>1.94±0.02†</td>
<td>2.94±0.01†</td>
<td>4.25±0.01†</td>
<td>5.75±0.17†</td>
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<td>4-OHE₂</td>
<td>NP-UAEC</td>
<td>1.49±0.05</td>
<td>1.76±0.05*</td>
<td>2.03±0.10†</td>
<td>2.45±0.04*</td>
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<td>P-UAEC</td>
<td>1.71±0.03†</td>
<td>2.78±0.02†</td>
<td>4.67±0.01†</td>
<td>5.76±0.03†</td>
<td>9.78±0.09†</td>
</tr>
<tr>
<td>2-ME₂</td>
<td>NP-UAEC</td>
<td>1.43±0.07</td>
<td>1.74±0.05*</td>
<td>1.98±0.11*</td>
<td>2.34±0.03*</td>
<td>2.50±0.04*</td>
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<td>P-UAEC</td>
<td>1.84±0.02†</td>
<td>2.83±0.01†</td>
<td>4.66±0.02†</td>
<td>6.54±0.05†</td>
<td>12.59±0.25†</td>
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<tr>
<td>4-ME₂</td>
<td>NP-UAEC</td>
<td>1.50±0.06</td>
<td>1.74±0.05*</td>
<td>1.98±0.11*</td>
<td>2.37±0.07*</td>
<td>2.30±0.03*</td>
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<tr>
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<td>P-UAEC</td>
<td>1.86±0.01†</td>
<td>2.96±0.01†</td>
<td>4.67±0.06†</td>
<td>6.72±0.03†</td>
<td>11.72±0.15†</td>
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</tbody>
</table>

*Increases (P<0.05; n=4) in UAEC PGI₂/TXA₂ ratios compared with untreated controls.
†Increases (P<0.05; n=4) in P-UAEC PGI₂/TXA₂ ratios compared with respective NP-UAECs group at specified concentration.

E₂β indicates estradiol-17β; UAEC, uterine artery endothelial cells; NP-UAEC, UAECs in the nonpregnant; P-UAEC, UAECs in the pregnant; 2-OHE₂, 2-hydroxyestriadiol; 4-OHE₂, 4-hydroxyestriadiol; 2-ME₂, 2-methoxyestriadiol; and 4-ME₂, 4-methoxyestriadiol. A higher concentration-dependent increase in the PGI₂/TXA₂ ratio was observed in P-UAECs with E₂β, 2-OHE₂, 4-OHE₂, 2-ME₂, and 4-ME₂ treatments compared with NP-UAECs.

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E₂β indicates estradiol-17β; UAEC, uterine artery endothelial cells; NP-UAEC, UAECs in the nonpregnant; P-UAEC, UAECs in the pregnant; 2-OHE₂, 2-hydroxyestriadiol; 4-OHE₂, 4-hydroxyestriadiol; 2-ME₂, 2-methoxyestriadiol; and 4-ME₂, 4-methoxyestriadiol. A higher concentration-dependent increase in the PGI₂/TXA₂ ratio was observed in P-UAECs with E₂β, 2-OHE₂, 4-OHE₂, 2-ME₂, and 4-ME₂ treatments compared with NP-UAECs.
blocked by pretreating P-UAECs for 1 hour with 1 μmol/L of the nonselective ER antagonist ICI 182 780, ER-α–selective antagonist 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinyloxy)phenol]-1H-pyrazole dihydrochloride (MPP), or the ER-β–selective antagonist 4-[2-phenyl-5,7-bis (trifluoromethyl) pyrazol[1,5-a]pyrim idin-3-yl]phenol (PHTPP) followed with treatments with EBM vehicle or EBM containing 0.1, 1, 10, or 100 nmol/L of Eβ, 2-OHE2, 4-OHE2, 2-ME, 4-ME, or A23187 for optimal time determined from time courses.

**PGE2 and TXA2 Assays**

After steroid treatments, media from individual 6-well plates were collected to measure production levels of PGE2 or TXA2 by using enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI). Indices of both PGE2 and TXA2 levels were calculated from measuring their stable nonenzymatic hydrolysis products, 6-keto-PGF1α and thromboxane B2 (TXB2), respectively, in duplicates. Productions were calculated per manufacturer’s instructions after subtracting the value of the blank wells to remove background absorbance values. The levels of PGE2 or TXA2 in NP-UAECs and P-UAECs are expressed as the fold or ratio increases over untreated control in pg/mL corrected against a standard curve, nonspecific binding, maximum binding and normalized to the amount of protein per well in mg.

**Statistical Analysis**

Data are presented as a fold change of untreated control and expressed as means±SEM. For the PGE2/TXA2 ratio, data are presented as the ratio of PGE2/TXA2 calculated from the absolute pg/mL concentrations from the same treatment wells in duplicates from all cell lines studied. Data were analyzed using a 2-way ANOVA (SigmaPlot 11 Statistical Software). When appropriate, an analysis of the simple effect was performed using 1-way ANOVA followed by post hoc Student-Newman-Keuls test. Pairwise comparisons were performed using Student-Newman-Keuls test. Level of significance was established a priori at P<0.05.

**Results**

**Expression of cPLA2, COX-1, PGIS, and TXAS in NP-UAECs Versus P-UAECs**

Western immunoblotting revealed the protein expression of cPLA2, COX-1, PGIS, and TXAS in both NP-UAECs and P-UAECs (Figure 1A). Densitometric analyses showed that the protein expressions of cPLA2, COX-1, and PGIS were significantly higher in P-UAECs compared with NP-UAECs (Figure 1B). In contrast, the expression of TXAS was significantly reduced in P-UAECs compared with NP-UAECs (Figure 1B).

**Basal and Calcium Ionophore Stimulated PGI2 and TXA2 Production in NP-UAECs and P-UAECs**

Basal production of PGI2 was higher by P-UAECs compared with NP-UAECs (Figure 2A). Unstimulated basal production of PGI2 by P-UAECs at 8, 12, and 24 hours was 1.31±0.23-, 1.43±0.23-, and 1.53±0.23-fold, respectively, corresponding to 13±1.0, 20±1.1, and 23±0.22 pg/mg of protein, respectively, at the same time points. A23187 stimulated significantly lower concentration-dependent production of TXA2 in P-UAECs compared with NP-UAECs (Figure 2D). The maximum production by P-UAECs at 12 hours was 1.49±0.09-fold of control compared with maximum production by NP-UAECs at 1.93±0.64-fold of control.

**Eβ, 2-OHE2, 4-OHE2, 2-ME, and 4-ME, Stimulated a Time- and Concentration-Dependent PGI2 Increase in P-UAECs**

Time- and concentration-dependent PGI2 increases were observed in P-UAECs after Eβ treatment with highest responses observed at a concentration of 100 nmol/L at 12 hours treatment time (Figure 3A). Increases of PGI2 were noted after 4 hours in response to almost all concentration of Eβ studied with the exception of 0.1 nmol/L. However, at 4, 8, 12, 24 hours, there were significant differences in PGI2 levels in response to 10 and 100 nmol/L Eβ which were higher compared with 0.1 or 1 nmol/L concentrations.
Similarly, exposure of P-UAECs to 2-OHE₂ and 4-OHE₂ treatments also stimulated a time- and concentration-dependent PGI₂ increases by P-UAECs with maximum responses observed at a concentration of 100 nmol/L at 12 hours of treatment (Figure 3B and 3C). Nevertheless, PGI₂ increases were noted after 4 hours in response to all concentration of 2-OHE₂ and 4-OHE₂ studied, and this increased in a time- and concentration-dependent manner compared with untreated control. No further increases in 2-OHE₂ and 4-OHE₂ -induced PGI₂ responses by P-UAECs were seen after 12 hours at all concentrations studied. There were significant differences in PGI₂ increases in response to 10 and 100 nmol/L of 2-OHE₂ and 4-OHE₂ which were higher compared with 0.1 or 1 nmol/L concentrations at 2, 4, 8, 12, and 24 hours. However, 0.1 or 1 nmol/L concentrations stimulated significant increases of PGI₂ compared with untreated control at 2, 4, 8, 12, and 24 hours.

The time course and concentration responses of PGI₂ levels in P-UAECs induced by 2-ME₂ and 4-ME₂ are shown in Figure 3D and 3E. Both methoxyestradiols increased PGI₂ in a time- and concentration-dependent manner, with maximal effects observed at a concentration of 100 nmol/L at 12 hours of treatment. However, increases of PGI₂ by P-UAECs were seen at all concentrations studied at 2 hours of treatment or greater. There were significant differences in PGI₂ increases in response to 1, 10, and 100 nmol/L of 2-ME₂ and 4-ME₂, which were higher compared with 0.1 nmol/L concentration at 4 hours of treatment or greater.

**Eβ, 2-OHE₂, 4-OHE₂, 2-ME₂, and 4-ME₂ Stimulated a Greater Concentration-Dependent PGI₂ Increase by P-UAECs More Than NP-UAECs**

A concentration-dependent increase in production of PGI₂ was observed by P-UAECs after E₂β treatment with highest responses observed at a concentration of 100 nmol/L (Figure 4A). On the contrary, E₂β also induced PGI₂ increases by NP-UAECs; however the increase by NP-UAECs was significantly less compared with P-UAECs and no further concentration response was observed after the 10 nmol/L concentration (Figure 4A). Differences in PGI₂ increases between NP-UAECs and P-UAECs were observed at 1 nmol/L, 10 nmol/L, 100 nmol/L, and 1 μmol/L concentrations.

Similar to E₂β, 2-OHE₂, and 4-OHE₂ treatments also stimulated greater concentration-dependent PGI₂ production increases by P-UAECs compared with NP-UAECs (Figure 4B and 4C). Maximum responses were observed at a concentration of 100 nmol/L, with no further increases seen with higher concentrations. 2-OHE₂ and 4-OHE₂ treatments did stimulate elevations of the production of PGI₂ in NP-UAECs; however, these responses were significantly lower compared with the P-UAEC responses, with no further increases seen after the 10 nmol/L concentration.

2-ME₂ and 4-ME₂ treatments also stimulated greater concentration-dependent PGI₂ production increases by P-UAECs compared with NP-UAECs (Figure 4D and 4E). Maximum responses of P-UAECs to 2-ME₂ were seen at 100 nmol/L, with no further increases at greater doses. On the contrary, 4-ME₂ induced maximum PGI₂ production increases by P-UAECs at a concentration of 10 nmol/L, with no significant further increases with higher concentrations. Interestingly, 4-ME₂ was the only estrogen metabolite that stimulated a difference in PGI₂ production responses between NP-UAECs and P-UAECs at a concentration as low as 0.1 nmol/L. Elevated PGI₂ production was also noted by NP-UAECs in response to 2-ME₂ and 4-ME₂; however, these responses were of lower magnitude compared with P-UAEC responses, with no further increases seen after the 10 nmol/L concentration.

**Figure 2.** Basal unstimulated and calcium ionophore stimulated prostacyclin (PGI₂) and thromboxane (TXA₂) production by uterine artery endothelial cells derived from the nonpregnant state (NP-UAECs) and UAECs derived from the pregnant state (P-UAECs). A, Basal production of PGI₂ by P-UAECs was higher compared with NP-UAECs. B, The calcium ionophore (A23187) stimulated more production of PGI₂ in P-UAECs compared with NP-UAECs. C, Basal production of TXA₂ was not different in P-UAECs compared with NP-UAECs. D, A23187 stimulated more production of TXA₂ in NP-UAECs compared with P-UAECs. *Increases in TXA₂ production (P<0.05; n=4) compared with untreated control and time 0. †Increases in TXA₂ production (P<0.05; n=4) compared with respective P-UAEC group at specified concentrations.
The levels of TXA2 production stimulated by Eβ, 2-OHE2, 4-OHE2, 2-ME2, and 4-ME2 increased linearly but did not reach statistical significance compared with untreated control in either NP-UAECs or P-UAECs. Therefore, these TXA2 productions are discussed within the context of the PGI2/TXA2 ratio.

The PGI2/TXA2 Ratio: E2 and Its Metabolites Induced Time- and Concentration-Dependent Increases in Prostacyclin Production by Uterine Artery Endothelial Cells Derived from the Pregnant State (P-UAECs). Time- and concentration-dependent prostacyclin (PGI2) production by P-UAECs in response to E2, 2-OHE2, 4-OHE2, 2-ME2, and 4-ME2 increased linearly but did not reach statistical significance compared with respective 0.1 and 1 nmol/L (P<0.05; n=4) group at specified times.

Antagonism with ICI 182 780 was tested at 0.1, 1, 10, and 100 nmol/L of Eβ and its metabolites studied; however, because all concentrations yielded similar results, only the responses from the optimal concentration of 100 nmol/L are shown. Furthermore, because the NP-UAECs exhibited lower nonsignificant responses, antagonism studies were only carried out in P-UAECs. ICI 182 780 alone did not affect basal PGI2 production in P-UAECs; however, it totally abrogated PGI2 increases in response to Eβ, indicating possible involvement of either ER-α and ER-β (Figure 6). In contrast, ICI 182 780 did not have an effect on P-UAEC PGI2 increases in response to 2-OHE2, 4-OHE2, 2-ME2, or 4-ME2, indicating ER-independent mechanisms. ICI 182 780 did not have an effect on PGI2 production by P-UAECs in response to nonreceptor stimulation with A23187 (data not shown), validating specific ER-mediated Eβ responses (Figure 6).

**Figure 3.** Estradiol-17β (Eβ) and its metabolites-induced time- and concentration-dependent increases in prostacyclin production by uterine artery endothelial cells derived from the pregnant state (P-UAECs). Time- and concentration-dependent prostacyclin (PGI2) production by P-UAECs in response to (A) Eβ, (B) 2-hydroxyestradiol (2-OHE2), (C) 4-hydroxyestradiol (4-OHE2), (D) 2-methoxyestradiol (2-ME2), and (E) 4-methoxyestradiol (4-ME2). Time- and concentration-dependent PGI2 production responses were observed in P-UAECs in response to Eβ, 2-OHE2, 4-OHE2, 2-ME2, and 4-ME2. * Increases (P<0.05; n=4) in P-UAEC PGI2 production compared with respective 0.1 and 1 nmol/L (P<0.05; n=4) group at specified times.

**Table.** Basal PGI2 production (fold of control) in NP-UAECs and P-UAECs after 24 hours of treatment with various concentrations of 2-OHE2, 4-OHE2, 2-ME2, and 4-ME2. * Increases (P<0.05) in P-UAEC PGI2 production compared with respective 0.1 and 1 nmol/L (P<0.05; n=4) group at specified times.

<table>
<thead>
<tr>
<th>Compound</th>
<th>NP-UAECs</th>
<th>P-UAECs</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
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<td>1</td>
</tr>
<tr>
<td>0.1 nmol/L</td>
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<td>1.1</td>
</tr>
<tr>
<td>1 nmol/L</td>
<td>1.2</td>
<td>1.2</td>
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**Note:** The levels of TXA2 production stimulated by Eβ, 2-OHE2, 4-OHE2, 2-ME2, and 4-ME2 increased linearly but did not reach statistical significance compared with untreated control in either NP-UAECs or P-UAECs. Therefore, these TXA2 productions are discussed within the context of the PGI2/TXA2 ratio.
Figure 4. Estradiol-17β and its metabolites induced concentration-dependent increases in prostacyclin production by uterine artery endothelial cells derived from the pregnant state (P-UAECs) compared with UAECs derived from the nonpregnant state (NP-UAECs). Concentration-dependent cell prostacyclin (PGI2) production by NP-UAECs and P-UAECs to responses (A) E2β, (B) 2-hydroxyestradiol (2-OHE2), (C) 4-hydroxyestradiol (4-OHE2), (D) 2-methoxyestradiol (2-ME2), and (E) 4-methoxyestradiol (4-ME2). Concentration-dependent responses at 12 hours were observed in P-UAECs in response to E2β, 2-OHE2, 4-OHE2, 2-ME2, and 4-ME2; NP-UAECs exhibited lower PGI2 production. *Increases (P<0.05; n=4) in UAEC PGI2 production compared with untreated control. †Increases in P-UAEC PGI2 production with inhibitor.

E2β, but Not its Metabolites, Stimulated PGI2 Production in P-UAECs Selectively via ER-α and Independent of ER-β

Similar to ICI 182 780 studies, antagonism with MPP and PHTPP were tested at all concentrations (0.1, 1, 10, and 100 nmol/L) of E2β and its metabolites studied. All concentrations examined yielded similar results; therefore, only the data from the optimal concentration of 100 nmol/L are shown. In P-UAECs, ER-α blockade with 1 µmol/L of MPP completely abolished the PGI2 increases stimulated by 100 nmol/L of E2β indicating a role for ER-α (Figure 7A). In contrast, MPP did not abrogate the PGI2 increases stimulated by 100 nmol/L of 2-OHE2, 4-OHE2, 2-ME2, or 4-ME2 similar to the above ICI 182 780 (Figure 7A). Moreover, E2β-induced PGI2

Figure 5. Estradiol-17β and its metabolites stimulated de novo prostacyclin (PGI2) production by uterine artery endothelial cells derived from the pregnant state (P-UAECs) via cyclooxygenase-1 (COX-1) and prostacyclin synthase (PGIS). A, The effects of the COX-1 antagonist SC-560 (1 µmol/L) on P-UAEC PGI2 increases in response to 100 nmol/L of E2β, 2-hydroxyestradiol (2-OHE2), 4-hydroxyestradiol (4-OHE2), 2-methoxyestradiol (2-ME2), and 4-methoxyestradiol (4-ME2). SC-560 abrogated PGI2 increases by P-UAECs in response to E2β and its metabolites, respectively. B, The effects of the PGIS antagonist U-51605 (1 µmol/L) on P-UAEC PGI2 increases to 100 nmol/L of E2β, 2-OHE2, 4-OHE2, 2-ME2, or 4-ME2. U-51605 abrogated PGI2 increases by P-UAECs in response to E2β and its metabolites, respectively. * Increases (P<0.05; n=4) in P-UAEC PGI2 production compared with untreated control. † Indicates inhibition (P<0.05; n=4) of P-UAEC PGI2 production with inhibitor.
production responses were not inhibited by 1 µmol/L of the ER-β-selective antagonist PHTPP, demonstrating a lack of requirement for ER-β in these responses (Figure 7B). Similar to MPP, PHTPP did not inhibit the PGI₂ production stimulated by 100 nmol/L of 2-OHE₂, 4-OHE₂, 2-ME₂, or 4-ME₂ (Figure 7B). Both MPP and PHTPP did not inhibit PGI₂ production by P-UAECs induced by the nonreceptor stimulation using Ca²⁺ Ionophore (A23187; data not shown), further validating specific ER-α mediated E₂β responses.

The key findings observed from this study are as follows: (1) Pregnancy induces higher protein expression of cPLA₂, COX-1, and PGIS in P-UAECs compared with NP-UAECs as well as a lower protein expression of TXAS in P-UAECs compared with NP-UAECs; (2) E₂β and its metabolites, 2-OHE₂, 4-OHE₂, 2-ME₂, and 4-ME₂, stimulate time- and concentration-dependent increases in PGI₂ production by P-UAECs; (3) E₂β and its metabolites stimulate a concentration-dependent increase in the PGI₂/TXA₂ ratio more in P-UAECs compared with NP-UAECs; (4) E₂β and its metabolites stimulate de novo PGI₂ production by P-UAECs via activities of COX-1 and PGIS; and (5) E₂β-induced PGI₂ production by P-UAECs is mediated primarily via ER-α and independent of ER-β, whereas 2-OHE₂, 4-OHE₂, 2-ME₂, and 4-ME₂ stimulate PGI₂ production in P-UAECs independent of either ER-α or ER-β.

We demonstrate herein that P-UAECs highly express the prostanooid system enzymes including cPLA₂, COX-1, and PGIS. These data are consistent with reports of increased expression of these enzymes in ovine uterine arteries in vivo and ex vivo and shows that the elevated expression during gestation is maintained to a great extent even through passing in culture. However, in the present study we demonstrate, for the first time, that TXAS is expressed in UAECs and that expression is lower in P-UAECs compared with NP-UAECs. Taken together, these data from our studies suggest that during pregnancy, the prostanooid enzyme system shifts its expression pattern in favor of more PGI₂ production and less TXA₂ production in support of increases in PGI₂ production by P-UAECs independent of either ER-α or ER-β.

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The retention of programming in P-UAECs, perhaps via epigenetic mechanisms, may also be responsible...
for elevated responses to steroids in subsequent pregnancies in vivo. We also show herein that the calcium ionophore (A23187) induces significant increases of PGI₂ production by P-UAECs and TXA₂ by NP-UAECs demonstrating that the prostanoid enzymes expressed in these endothelial cells are functional and capable of eliciting calcium-dependent and receptor-independent PGI₁ and TXA₁ production. In this study, we demonstrate that E₁β induces concentration-dependent increases of PGI₁ and PGI/TXA₁ ratios more in P-UAECs than in NP-UAECs. Although maximum responses are noted at a high concentration of 100 nmol/L, low physiological concentrations of 1 and 10 nmol/L stimulated significant concentration-dependent increases of PGI₁ and PGI/TXA₁ ratios more in P-UAECs than in NP-UAECs. In support of these findings are reports that E₁β induces production of PGI₁ in other cultured endothelial cells such as HUVECs, bovine pulmonary artery, and aortic endothelial cells. We have previously reported that the infusion of E₁β in sheep significantly increases the ex vivo uterine arterial production of the stable PGI₁ metabolite 6-keto-PGF₁α. However, to the best of our knowledge, this is the first study to evaluate the in vitro comparison with E₁β-induced uterine endothelial PGI₁ production and elevated PGI/TXA₁ ratios in the pregnant versus the nonpregnant states. In this regard, these data are also consistent with our previous findings that P-UAECs exhibit pregnancy-specific PGI₁ production in response to ATP, basic fibroblast growth factor, and epidermal growth factor.

Our findings that 2-OHE₂ and 4-OHE₂ stimulate increases of PGI₁ and PGI₁/TXA₁ ratios more in P-UAECs than in NP-UAECs support our hypothesis that cytochrome P450-derived metabolites of E₁β may play a role in the regulation of vascular responsiveness during pregnancy. It has been previously demonstrated that the uterine arterial infusion of 2-OHE₂ and 4-OHE₂ in sheep and gilts causes vasodilation and increases in uterine blood flow. 2-OHE₂ and 4-OHE₂ also significantly augment endothelial-dependent vasodilation of preconstricted vascular beds in ZSF1 rats, an animal model for hypertension, type 2 diabetes mellitus, hyperlipidemia, nephropathy, and metabolic syndrome. Because 2-OHE₂ and 4-OHE₂ are rapidly converted to their methoxy derivatives in the presence of catechol-O-methyltransferase, it is likely that the actions of the catecholestradiols on endothelial PGI₁/TXA₁ ratios maybe partly modulated by catechol-O-methyltransferase expression and activity in these endothelial cells. Nevertheless, these observations suggest that the metabolism of E₁β to the catecholestradiols, 2-OHE₂ and 4-OHE₂, may also play an essential role in the regulation of physiological vascular responsiveness via production of endothelial-derived vasodilatory factors.

The observation that 2-ME₂ and 4-ME₂ enhance PGI₁ levels and PGI₁/TXA₁ ratios in P-UAECs more than in NP-UAECs supports evidence that methoxyestradiols may positively influence vascular responsiveness during pregnancy. Consistent with these findings is the report that 2-ME₂ induces PGI₁ production in HUVECs. Low 2-ME₂ level has been implicated in preeclampsia, a disease characterized by low plasma and urinary PGI₁ and impaired uterine blood flow. These observations suggest that 2-ME₂ may be a promising physiological as well as pharmacological agent capable of clinically improving vascular responsiveness. We demonstrate for the first time that 4-ME₂ also stimulates in vitro PGI₁ increases greater in the pregnant compared with the nonpregnant state and indeed may play a role in positive pregnancy-induced uterine vascular responsiveness. Because 2-ME₂ and 4-ME₂ induced higher PGI₁ increases at very low physiologic concentrations compared to E₁β and the catecholestradiols, this suggests that the methoxyestradiols maybe more potent under these conditions and points to the notion that the vascular effects of locally produced or even circulating estrogen metabolites may be more critical than previously thought compared to the effects of the parent substrate.

Demonstrating a role for ER-α and ER-β, ICI 182,780, which nonspecifically blocks both ER-α and ER-β, completely abrogated the E₁β-induced PGI₁ production in P-UAECs. Previous studies have demonstrated that ICI 182,780 also inhibits E₁β-induced production of PGI₁ in other cultured endothelial cells, including HUVECs and ovine fetal pulmonary artery endothelial cells. However, because of the potential relevance of ER subtype selectivity in vascular function, there is considerable interest in investigating whether the classical ERs exhibit functional heterogeneity in the regulation of E₁β-induced endothelial functions. Our data show that E₁β-induced PGI₁ increases in P-UAECs are completely inhibited by the ER-α-specific MPP and unaffected by ER-β-specific PHTPP, demonstrating that E₁β-induced PGI₁ production in P-UAECs is primarily mediated by ER-α. These data are in agreement with previous observations that PGI₁ production by HUVECs was seen when these cells were treated with an ER-α-selective agonist 4,4',5'-[4-Propyl-[1H]-pyrazole-1,3,5-trihydrophen]. These data also support the notion that whereas E₁β binds and activates both ER-α and ER-β, it is the molecular and structural-based differences in these receptors that allow for a wide range of functional heterogeneity, which may partly explain the selective actions of E₁β.

In the present study, unlike E₁β, the effects of its metabolites on PGI₁ production by P-UAECs are not inhibited by the ER antagonists used in this study and thus are not mediated via ER-α and ER-β. This is in agreement with several reports that the vascular physiology and pharmacology of E₁β metabolites on many cell types, including endothelial cells, occur via ER-independent mechanisms. Thus, other receptor-mediated mechanisms not involving the classical ERs may mediate E₁β metabolites-induced PGI₁ production in P-UAECs. Indeed we and others have reported that the genomic effects of E₁β metabolites, including proliferation of P-UAECs and suppression of pancreatic islet insulin release, are mediated via the adrenergic receptors. Nevertheless, the exact mechanism of action of E₁β metabolites on nongenomic PGI₁ increases in P-UAECs remains to be determined and may likely involve adrenergic receptors and other estrogen-associated receptors like G protein–coupled receptor-30.

In conclusion, the findings from this current study demonstrate that the uterine endothelium exhibits pregnancy-specific increases in cPLA₂, COX-1, and PGIS and decreases in TXAS shifting in the endothelial PGI₁/TXA₁ ratios toward more PGI₁ production in association with rises and maintenance of uterine blood flow during pregnancy. Furthermore, we provide
evidence that Eβ primarily via ER-α and its metabolites via ER-independent mechanisms stimulate a higher de novo increases of the endothelial-derived vasodilator PGI₂ and PGI₂/TXA₃ ratios in the pregnant compared with the non-pregnant state. Although maximum production of PGI₂ and the PGI₂/TXA₃ ratio was noted with the high concentration of 100 nmol/L, low physiologically relevant concentrations of 1 and 10 nmol/L of Eβ and its metabolites also stimulated significant synthesis of these prostanooids more in P-UAECs compared with NP-UAECs. Collectively, the selective responses of P-UAECs further illustrate pregnancy-specific programming at the level of the uterine artery endothelium signaling, resulting in enhanced Eβ and its metabolites-mediated induction of PGI₂ synthesis in P-UAECs without significantly affecting TXA₃ production.

Perspectives

The mechanisms by which estrogens regulate vascular tone and vascular responsiveness during pregnancy are not well understood. However, studies have shown that it likely involves ER-mediated stimulation of endothelial-derived vasodilatory factors including NO and PGI₂. Herein, we demonstrate novel and compelling evidence that the vasoactive/vasoprotective effects of Eβ during pregnancy may also involve its sequential conversion to catecholestradiols and methoxyestradiols, which are capable of stimulating ER-independent endothelial PGI₂ synthesis. Additional studies are required to understand whether the ER-independent induction of endothelial PGI₂ synthesis by estrogen metabolites within uterine vasculature represents unappreciated signaling complexity of estrogens or just simply an evolutionary functional redundancy to maintain uterine blood flow and thus oxygen and nutrient delivery during gestation. Nevertheless, our findings necessitate the evaluation of catecholestradiols and methoxyestradiols in the regulation of vascular tone in physiology via endothelial-derived relaxing factors, as well as dysregulation in the pathophysiology of vascular diseases, such as hypertension, atherosclerosis, and gestational vascular diseases, such as preeclampsia.

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Disclosures

None.

References

What Is New?

- The uterine artery endothelium exhibits pregnancy-specific increases in phospholipase A<sub>2</sub>, cyclooxygenase-1, and prostacyclin synthase and decreases in endothelial thromboxane synthase expression.
- Estradiol-17β primarily acting via estrogen receptor-α and its metabolites 2-hydroxyestradiol, 4-hydroxyestradiol, 2-methoxyestradiol, and 4-methoxyestradiol via estrogen receptor-independent mechanisms to stimulate higher de novo increases of the endothelial-derived prostacyclin/thromboxane ratios in the pregnant compared with the nonpregnant state.

What Is Relevant?

- The selective pregnancy-specific endothelial prostanoid enzyme expressions and increases in the prostacyclin/thromboxane ratio demonstrate roles for catecholestradiols and methoxyestradiols in the regulation of vascular responsiveness in physiology via endothelial-derived relaxing factors as well as in the pathophysiology of vascular diseases characterized by blunted endothelium-dependent vasodilator responses such as hypertension, atherosclerosis, and preeclampsia.

Summary

These findings demonstrate novel and compelling evidence that the vasoactive/vasoprotective effects of estradiol-17β may involve its sequential conversion to catecholestradiols and methoxyestradiols which are capable of stimulating estrogen receptor-independent endothelial prostacyclin synthesis.
Estradiol-17β and its Cytochrome P450- and Catechol-O-Methyltransferase–Derived Metabolites Selectively Stimulate Production of Prostacyclin in Uterine Artery Endothelial Cells: Role of Estrogen Receptor-α Versus Estrogen Receptor-β
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