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Estradiol-17β, and its CYP450- and COMT-Derived Metabolites Stimulate Proliferation in Uterine Artery ECs: Role of ER-α vs. ER-β

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Abstract

Estradiol-17β and its metabolites which are sequentially synthesized by cytochrome P450s (CYP450s) and catechol-*O*-methyltransferase (COMT) to form 2 and 4-Hydroxyestradiol (2-OHE_2 and 4-OHE_2) and 2- and 4-Methoxestradiol (2-ME_2, and 4-ME_2) are elevated during pregnancy. We investigated whether CYP450s and COMT are expressed in uterine artery endothelial cells (UAECs) and if $E_2β$ and its metabolites modulate cell proliferation via ER-α and/or ER-β and play roles in physiologic uterine angiogenesis during pregnancy. Cultured ovine UAECs from pregnant (P-UAECs) and nonpregnant (NP-UAECs) ewes were treated with 0.1-100 nmol/L of $E_2β$, 2-OHE_2, 4-OHE_2, 2-ME_2, and 4-ME_2. ER-α or ER-β specificity was tested using ICI 182,780, ER-α-specific MPP, ER-β –specific PHTPP antagonists and their respective agonists ER-α-specific PPT and ER-β –specific DPN. Angiogenesis was evaluated using BrdU Proliferation Assay. Utilizing confocal microscopy and Western analyses to determine enzyme location and levels, we observed CYP1A1, CYP1A2, CYP1B1, CYP3A4 and COMT expression in UAECs; however, expressions were similar between NP-UAECs and P-UAECs. $E_2β$, 2-OHE_2, 4-OHE_2, and 4-ME_2 treatments concentration-dependently stimulated proliferation in P-UAECs, but not NP-UAECs; 2-ME_2 did not stimulate proliferation in either cell type. Proliferative responses of P-UAECs to $E_2β$ were solely mediated by ER-β, whereas responses to $E_2β$ metabolites were neither ER-α nor ER-β mediated. We demonstrate an important vascular role for $E_2β$, its CYP450- and COMT-derived metabolites and ER-β in uterine angiogenesis regulation during pregnancy that may be dysfunctional in preeclampsia and other cardiovascular disorders.

Keywords

angiogenesis; hypertension; pregnancy; endothelium; estradiol metabolites; CYP450s

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Disclosures None

Introduction

Pregnancy is associated with dramatic uterine blood flow (UBF) rises resulting from vascular adaptations including vasodilatation and angiogenesis.[1] These adaptations are critical in pregnancy since their dysfunctions are implicated in pathologic pregnancies such as preeclampsia which complicate 6-8% of all pregnancies in the USA and account for 50,000 maternal deaths per year worldwide.2,3,4

Regulation of vascular adaptations during pregnancy is mediated partly by estrogens, which are elevated during gestation.[5] Estradiol-17β (E_2β) infusion in sheep markedly reduces uterine and systemic vascular resistance causing rises in uterine and systemic blood flows.6 Uterine arterial administration of the nonselective estrogen receptor (ER) antagonist ICI 182,780 in pregnant sheep lowers UBF, demonstrating that endogenous estrogen via ERs helps maintain uterine perfusion.[7] In human umbilical vein endothelial cells (HUVECs) 8 and myometrial microvascular ECs, E_2β promotes proliferation, an index of angiogenesis.[9]

The effects of estrogen on uterine vascular adaptations may be further modulated by its biologically active metabolites. E_2β metabolism catalyzed by cytochrome P450s (CYP450s) and catechol-*O*-methyltransferase (COMT) produces the catecholestradiols 2-Hydroxyestradiol (2-OHE_2) and 4-Hydroxyestradiol (4-OHE_2), and the methoxyestradiols 2-Methoxestradiol (2-ME_2) and 4-Methoxyestradiol (4-ME_2).[10,11] Evidence supports the involvement of E_2β-derived metabolites in pregnancy and in the regulation of angiogenesis; 2-ME_2- and COMT-deficient mice exhibit preeclampsia-like symptoms including impaired angiogenesis and hypertension.[12] Treatment with low concentration of 2-OHE_2, 4-OHE_2, 2-ME_2, or 4-ME_2 induces proliferation in cultured HUVECs whereas high concentration of 2-OHE_2 or 2-ME_2 inhibits proliferation.[13]

Thus, we tested the hypothesis that CYP450s and COMT may be expressed in the uterine vasculature and that E_2β, its CYP450s and COMT-derived metabolites participate in the regulation of uterine angiogenesis during pregnancy. Late pregnant and nonpregnant ovine uterine artery endothelial cells (P-UAECs and NP-UAECs) consistently express ER-α and ER-β and exhibit pregnancy-specific responses to angiogenic ligands demonstrating that they are a good model to evaluate direct receptor-mediated actions of E_2β and its metabolites.14,[15] We investigated: 1) the expression and intracellular distribution of CYP450s and COMT in P-UAECs versus NP-UAECs; 2) whether E_2β, 2-OHE_2, 4-OHE_2, 2-ME_2, and 4-ME_2 stimulate greater proliferation of P-UAECs than NP-UAECs; and 3) if E_2β and its metabolites induce proliferative responses via ER-α and/or ER-β.

Methods

For complete details on specific materials and methodology, please see http://hyper.ahajournals.org.

Cell Preparation and Culture

Cell preparations were approved by the University of Wisconsin-Madison School of Medicine Research Animal Care Committee as previously described.14,[15] UAECs were isolated and validated from late gestation (120-130 days; term= 147 days; n=6) and nonpregnant (luteal n=5 and follicular n=2) ewes.[15] At passage 5, ~ 70% confluence, cells were transferred to slides, 96 well plates, or lysed for protein extraction as needed for respective experiments.

Protein Extraction and Western Immunoblotting

Western immunoblotting was performed as previously described.[14] CYP1A1, CYP1A2, CYP1B1, CYP3A4, COMT, and ER-β expressions were detected using mouse anti-

CYP1A1and rabbit anti-CYP1A2, anti-CYP1B1, anti-CYP3A4, anti-COMT or anti-ER-β antibodies. GAPDH was utilized as a loading control.

Immunofluorescence Confocal Microscopy

Immunofluorescence confocal microscopy was performed as previous described.[14] UAECs were washed twice with ice cold PBS and fixed for 15 min with 3% paraformaldehyde. Fixed cells were rinsed with 50 mM glycine solution, permeabilized with 0.1% Triton-X for 3 min, blocked for 30 min with goat serum and incubated (20 min) with primary antibodies for CYP1A1, CYP1A2, CYP1B1, CYP3A4 and COMT. Subsequently, cells were incubated (30 min) with secondary antibodies Alexa Fluor 488 anti-mouse or anti-rabbit IgGs. Scanning was done with a radiance 2100 MP Rainbow confocal/multiphoton laser scan microscope system (Bio-Rad, Hercules, CA).

Experimental Treatments and Blockade and Activation of ER-α and ER-β

UAEC proliferation experiments were performed in quadruplicates and replicated in at least six NP-UAEC and P-UAEC preparations. For concentration response studies, UAECs in 96 well plates were serum starved (24 hrs) in endothelial basal medium (EBM) and medium was replaced with EBM or EBM containing 0.1, 1, 10 or 100 nmol/L $E_2β$, 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2 (24 hrs). ERs were blocked by pretreating UAECs for 1 hr with 1μmol/L of the ER antagonist ICI 182, 780 (ICI), or ER-α selective antagonist 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinyleth oxy)phenol]-1H-pyrazole dihydrochloride (MPP), or ER-β selective antagonist 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a] pyrim idin-3-yl]phenol (PHTPP). Additional concentration response studies were performed using 0, 0.1, 1, 10 or 100 nmol/L of the ER-α selective agonist 4,4′,4″-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) or the ER-β selective agonist 2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN). We also studied the effects of 0.1 nmol/L PPT + 0.1 nmol/L DPN and 1 μmol/L PHTPP + 0.1 nmol/L DPN to further evaluate receptor activation, additive effects, and specificity of ER-β selective agonist receptor activation.

BrdU Cell Proliferation Assays

BrdU was added for 16 hrs during the 24 hrs of steroid treatment and an *in vitro* index of proliferation was evaluated. Plates were read using Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT). Results are expressed as fold increases over untreated control after subtracting the "blank" (wells incubated without Brdu).

Statistical Analysis

Data (means ± SEM) were analyzed using a Two-way ANOVA with "Pregnancy" and "Concentration" as two "between" factors. Analyses of simple effects were performed using One-way ANOVA followed by post-hoc Student-Newman Keuls test. Pairwise comparisons were performed using Bonferroni or Student-Newman-Keuls test. Biphasic concentration response (deviation from the standard monotonic sigmoid shape) description was determined by nonlinear regression using the logarithm of agonist concentration against various responses. Level of significance was established *a priori* at $P<0.05$.

Results

CYP1A1, CYP1A2, CYP1B1, CYP3A4 and COMT are expressed in UAECs

Western analyses indicated the presence of CYP1A1, CYP1A2, CYP1B1, CYP3A4, and COMT in NP-UAECs and P-UAECs (Figure 1A). However, no differences were seen between NP-UAECs and P-UAECs in their levels of expression (Figure 1B). Confocal microscopy revealed no difference between NP-UAECs and P-UAECs in intracellular distribution patterns of these enzymes. Therefore, unless noted, P-UAECs images are shown. CYP1A1, CYP1A2, and CYP3A4 were localized in cytoplasmic and nuclear compartments of P-UAECs (Figure 2A, B and D). CYP1B1 was localized in the nuclear region, whereas COMT was localized in the cytoplasmic compartment (Figure 2C, 2E).

P-UAEC proliferation in response to $E_2\beta$, 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2

Biphasic concentration proliferative responses were observed in P-UAECs after $E_2\beta$ treatment with maximum responses observed at a concentration of 0.1 nmol/L (Figure 3A). In contrast, $E_2\beta$ did not induce NP-UAEC proliferation at any concentration. Similarly, P-UAECs but not NP-UAECs exhibited a biphasic proliferative response to 2-OHE_2 and 4-OHE_2 (Figure 3B, 3C). The magnitude of P-UAECs proliferation at 0.1 nmol/L of $E_2\beta$, 2-OHE_2 and 4-OHE_2 were 2.07 ± 0.16, 1.79 ± 0.02 and 1.78 ± 0.02 fold of control, respectively.

2-ME_2 did not stimulate proliferation of P-UAECs or NP-UAECs (Figure 3D). 4-ME_2 at all concentrations induced proliferation in P-UAECs, but not in NP-UAECs (Figure 3E). Proliferation of P-UAECs at the physiologic concentration of 0.1 nmol/L of 4-ME_2 was 1.50 ± 0.16 fold of control (Figure 3E). However, response to 4-ME_2 was not biphasic and the maximum proliferation of 1.74 ± 0.04 fold was observed at 100 nmol/L. Additional validation of cell proliferation utilizing ViaLight Plus Kit (Lonza Inc., Rockland, ME) was performed and it confirmed increases in total viable cell numbers after treatment with $E_2\beta$ or its metabolites.

Proliferation of P-UAECs via classic ERs

Antagonism with ICI was tested at a physiologic concentration of 0.1 nmol/L $E_2\beta$ and its metabolites (Figure 4). ICI alone had no effect on P-UAEC proliferation, however, it totally abrogated proliferative responses to $E_2\beta$ indicating the requirement of ER-α and/or ER-β. In contrast, ICI did not have an effect of the proliferative responses of P-UAECs to 2-OHE_2, 4-OHE_2 and 4-ME_2. Figure 4 also illustrates that at 0.1 nmol/L, $E_2\beta$ was more potent than 2-OHE_2, 4-OHE_2 and 4-ME_2 which were equipotent in stimulating P-UAECs proliferation.

Proliferation of P-UAECs via ER-β not ER-α

In P-UAECs, ER-α blockade with 1 μmol/L MPP did not abolish the proliferative effects of $E_2\beta$, 2-OHE_2, 4-OHE_2, or 4-ME_2 (Figure 5). In contrast, $E_2\beta$-induced proliferation was completely inhibited by 1 μmol/L of the ER-β selective antagonist PHTPP (Figure 6). However, PHTPP did not alter P-UAECs proliferative responses to the estrogen metabolites.

We further evaluated if the ER-β-mediated proliferative responses in P-UAECs were due to an increase in ER-β protein levels between NP-UAECs, P-UAECs and P-UAECs treated with 0.1 nmol/L $E_2\beta$ or its metabolites. Shown in Figure S1 (please see http://hyper.ahajournals.org), ER-β expressions were not different amongst these groups (P=0.943).

Treatment of P-UAECs with ER-α selective agonist PPT did not induce proliferation (Figure 7A). In contrast, all ER-β selective agonist DPN concentrations stimulated cell proliferation 1.50 ± 0.05 fold of control (Figure 7B). Because these P-UAEC responses were less than $E_2\beta$ alone and did not exhibit a concentration-dependent response, we examined combination of PPT (0.1 nmol/L) and DPN (0.1 nmol/L) (Figure 7C). No further increases in P-UAEC proliferative responses were observed. Moreover, pretreatment with 1 μmol/L PHTPP completely inhibited DPN-induced responses in P-UAECs; (Figure 7C).

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Discussion

The key novel findings observed from this study are: 1) UAECs express CYP1A1, CYP1A2, CYP1B1, CYP3A4, and COMT; 2) $E_2\beta$, 2-OHE_2, 4-OHE_2, and 4-ME_2 stimulate P-UAEC, but not NP-UAEC, proliferation; and 3) $E_2\beta$-induced cell proliferative responses are mediated primarily via ER-β, whereas $E_2\beta$ metabolites-induced proliferative responses are independent of ER-α and ER-β.

UAECs constitutively express enzymes that may metabolize $E_2\beta$ to its hydroxy-(CYP1A1, CYP1A2, CYP3A4, CYP1B1) and subsequently methoxy- (COMT) metabolites. Consistent with our findings, are reports showing that CYP450s and COMT are expressed in aortic, coronary artery and umbilical vein ECs.[11, 16, 17,18] However, this is the first characterization of the localized intracellular expression of CYP450s in endothelial cells. Our data also confirm findings that COMT is primarily an intracellular cytosolic enzyme.[19] Although little is known about the intracellular localization of these enzymes in endothelial cells, it is possible that intracellular compartmentalization is associated with enzymatic function.

The physiologic plasma $E_2\beta$ concentration in women ranges from 0.1-2.2 nmol/L and increase dramatically during pregnancy.[5] We demonstrate that a physiologic concentration of $E_2\beta$ stimulates P-UAEC, but not NP-UAEC proliferation. The P-UAEC proliferative responses are similar to estrogenic stimulation of HUVECs and retinal microvascular ECs.[8,13,20] Moreover, $E_2\beta$ promotes murine endometrial endothelial proliferation *in vivo*.[21,22] These current data are also consistent with our previous findings[23] that $E_2\beta$ increases P-UAEC [H^3]-thymidine incorporation and tube formation; however maximum P-UAEC responses to $E_2\beta$ were seen at 1 nmol/L[23] and not 0.1 nmol/L; NP-UAECs were not evaluated. These results demonstrate that P-UAEC proliferative responses are induced by gestational programming at the level of endothelial cell signaling, supporting reports that pregnancy-induced programming in P-UAECs leads to increased responsiveness to agonists and these effects are retained in cultured primary cell lines.[15] Furthermore, the complete lack of mitogenic response of NP-UAECs may be specific to $E_2\beta$ since NP-UAECs show proliferation in response to ATP,VEGF, bFGF, and high (≥5%) serum.[15, 24, 25,26] The mechanistic significance of pregnancy-induced estrogenic programming on physiologic angiogenesis in P-UAECs remains to be elucidated.

Plasma catecholestrogens levels in pregnancy are 10-fold greater than in nonpregnant women.[27] Our finding that low levels of 2-OHE_2 and 4-OHE_2 stimulate P-UAEC, but not NP-UAEC proliferation, supports the proposal that CYP450s- and COMT-derived metabolites of $E_2\beta$ may play roles in the regulation of uterine angiogenesis during pregnancy. Low concentrations of 2-OHE_2 and 4-OHE_2 stimulate HUVEC proliferation and direct uterine arterial infusion of 2-OHE_2 in nonpregnant sheep causes vasodilatation, whereas 4-OHE_2 interacts directly with calcium channels to locally increase blood flow in gilts.[13,28,29] These findings suggest that catecholestradiols play roles in pregnancy-induced vascular adaptations.

O-methylation of catecholestradiols produces less potent and antiproliferative metabolites of $E_2\beta$.[10] Interestingly, we demonstrate that 4-ME_2 stimulated P-UAEC, but not NP-UAEC proliferation, consistent with observations that low 4-ME_2concentrations stimulate HUVEC proliferation.[13] However, 2-ME_2 was not mitogenic on UAECs supporting numerous reports of its antiproliferative effects.[30,31,32] The reason for divergent proliferative patterns between 2-ME_2 and 4-ME_2 is unclear. However, 2-ME_2 disrupts tubulin polymeration and induces cell-cycle arrest in the mitotic phase in endothelial and smooth muscle cells.[33,34,35] Thus, differences in association of 2-ME_2 and 4-ME_2 with regulators of mitosis, may likely account for their divergent responses.

Demonstrating a role for ER-α and ER-β, ICI abrogated $E_2\beta$-induced P-UAEC proliferation, supporting previous observations that ICI blocks $E_2\beta$-induced P-UAEC [H^3]-thymidine incorporation[23] and $E_2\beta$-induced VEGF-mediated proliferation.[9] Antagonism of ER-β with PHTPP abrogated $E_2\beta$-induced P-UAEC proliferation and ER-β activation with DPN-induced proliferation demonstrating an ER-β only effect. However, although activation of ER-α with PPT did not alter P-UAEC proliferation, PPT stimulates proliferation of human myometrial microvascular endothelial cells.[36] Therefore, the differences in P-UAECs proliferation in response to DPN, PPT, or $E_2\beta$ may be due to their distinct differences in affinity for ERs in association with the complex nature of ER-ligand complexes.[37,38] Nevertheless, PHTPP inhibition of $E_2\beta$- and DPN-induced P-UAEC proliferation validates that these $E_2\beta$ effects are solely ER-β mediated and independent of ER-α. Equally importantly, NP-UAECs, P-UAECs, and P-UAECs treated with $E_2\beta$ express similar levels of ER-β (Figure S1, please see http://hyper.ahajournals.org.) demonstrating that the ER-β-mediated $E_2\beta$ effects are not dependent on ER-β expression levels, but rather on other gestational-programming factors at the level of P-UAECs signaling.

$E_2\beta$ metabolites possess little affinity for classical ERs[10,39] and unlike $E_2\beta$, the effects of its metabolites on P-UAECs proliferation are not mediated via ER-α or ER-β. These results also indirectly suggest that CYP450s and COMT expressed in the UAECs do not possess high enough enzymatic activity under these conditions to metabolize $E_2\beta$. However, $E_2\beta$ metabolites may induce proliferative effects via other estrogen associated receptors like GPR30 found in endothelial cells.[40,41] Nonetheless, the exact mechanism of action of estrogen metabolites on uterine vascular ECs remains to be determined.

Perspectives

It is well established that $E_2\beta$ and its metabolites possess vascular protective effects on the cardiovascular system,[42,43,44] but, little is known about estrogen metabolism and regulation of uterine angiogenesis during pregnancy. Therefore, understanding the biochemistry of $E_2\beta$ metabolism and the vascular physiology of $E_2\beta$ and its metabolites on the uterine endothelium may provide clues for understanding normal pregnancy-associated vascular adaptations and the dysfunction of endothelia in the pathophysiology of preeclampsia and other cardiovascular disorders.[3,4]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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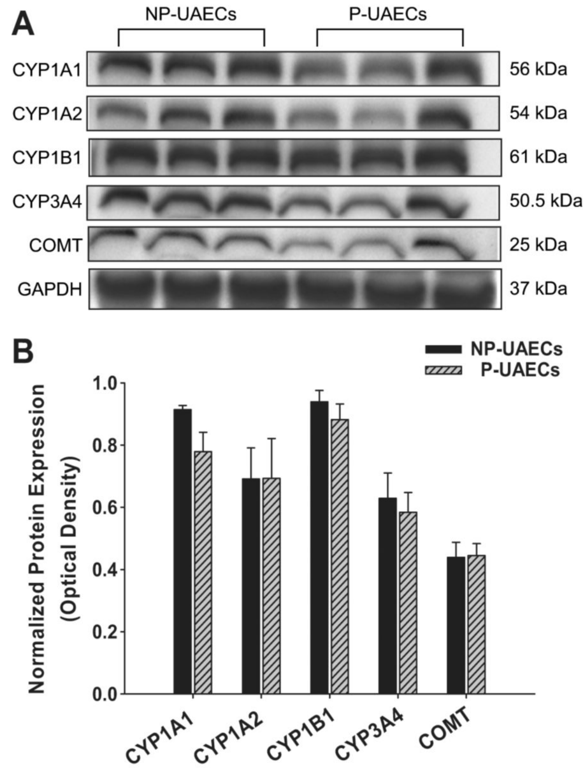

Figure 1.

(A) Immunoblots showing expression of CYP1A1, CYP1A2, CYP1B1, CYP3A4, COMT, and GAPDH in NP-UAECs and P-UAECs. (B) Densitometric analyses (Relative protein expression = enzyme expression OD /GAPDH OD) showed no difference between NP-UAECs (n=6) and P-UAECs (n=6); (P=0.949, One-Way ANOVA).

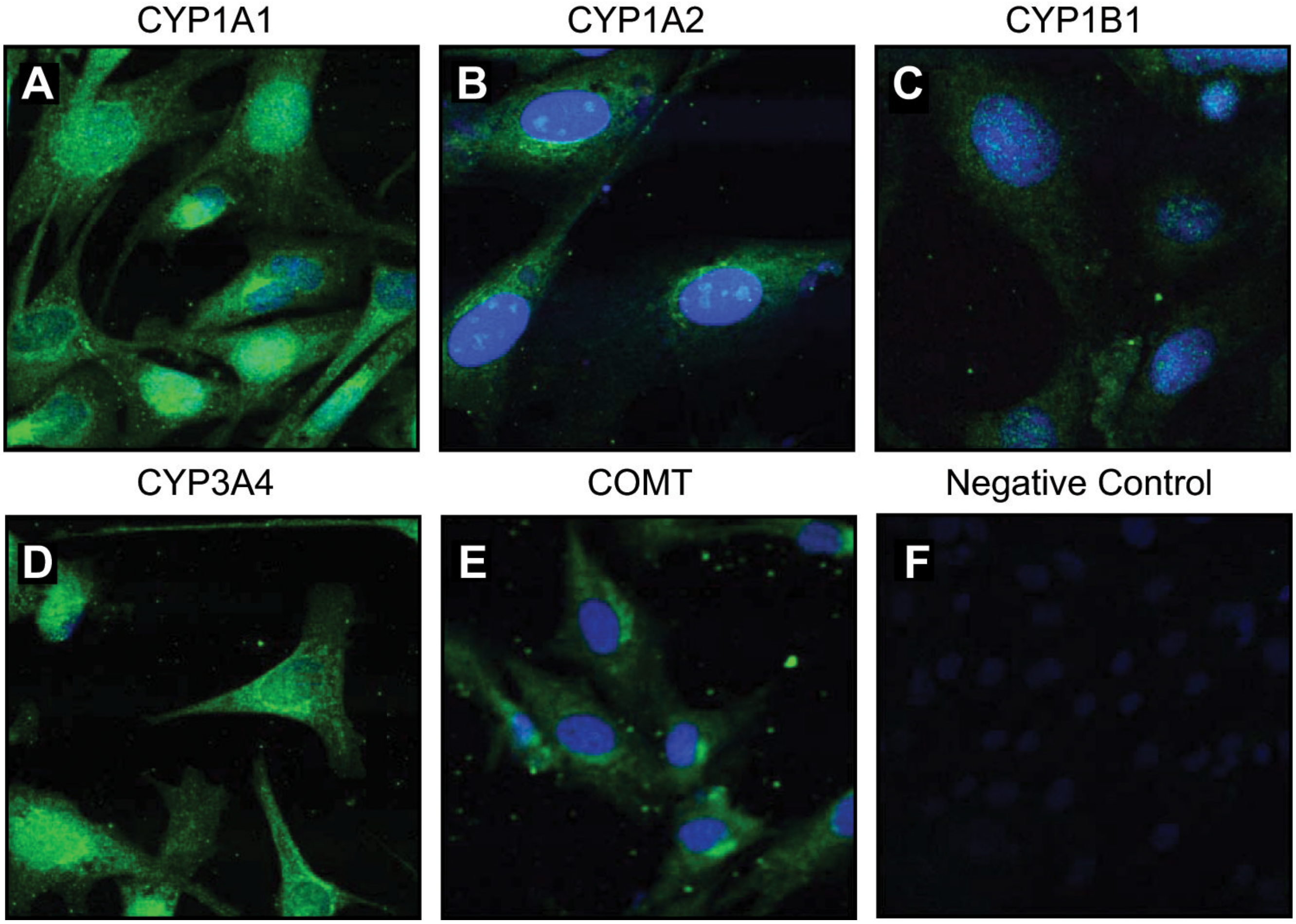

Figure 2.

Immunofluorescence microscopy showing intracellular localization of (A) CYP1A1, (B) CYP1A2, (C) CYP1B1, (D) CYP3A4, (E) COMT and (F) Negative Control in P-UAECs. Positive staining is green fluorescence with nuclei depicted in blue (DAPI). Pictures are representative of three experiments.

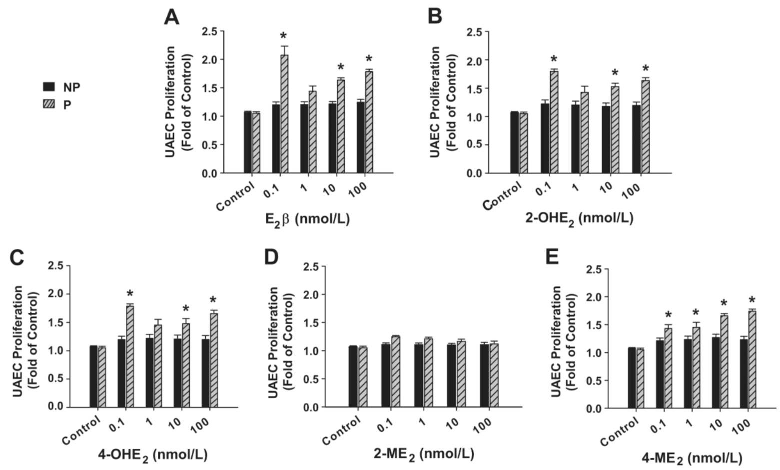

Figure 3.

Concentration-dependent cell proliferation responses of NP-UAECs and P-UAECs to (A) $E_2\beta$, (B) 2-OHE_2, (C) 4-OHE_2, (D) 2-ME_2 and (E) 4-ME_2. A biphasic proliferative response was observed in P-UAECs in response to $E_2\beta$, 2-OHE_2, and 4-OHE_2 but not 4-ME_2 compared to control with maximum responses at a physiologic concentration of 0.1 nmol/L (Two-Way ANOVA; Pregnancy × Concentration effect; $E_2\beta$, $F_{4,40}$=8.16, P<0.0001; 2-OHE_2, $F_{4,40}$=4.07, P=0.0073;, 4-OHE_2, $F_{4,40}$=3.69, P=0.0119; and 4-ME_2, $F_{4,40}$=5.05, P=0.002). NP-UAECs did not respond to $E_2\beta$ or its metabolites. No proliferation effect was observed with $2ME_2$. *Indicates an increase (P<0.05; n=6) in P-UAEC proliferation compared with both the respective NP-UAEC (n=7) group and untreated control.

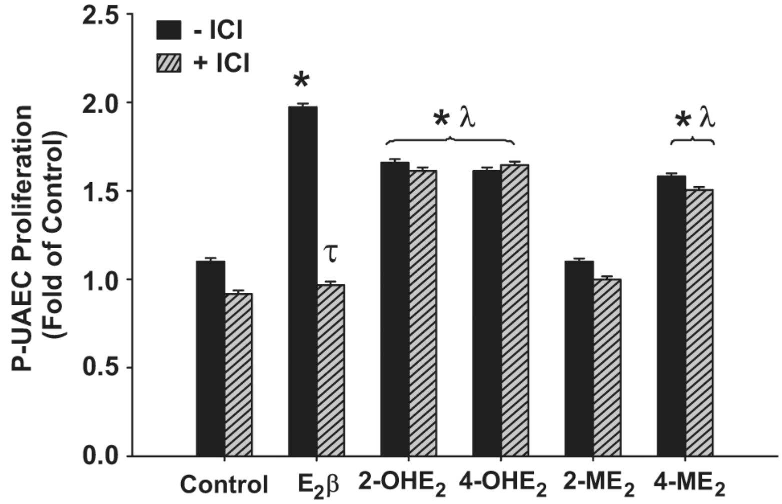

Figure 4.

The effects of 1 μmol/L ICI on P-UAEC proliferative responses to 0.1 nmol/L of $E_2\beta$, 2-OHE_2, 4-OHE_2, 2-ME_2, and 4-ME_2. ICI abrogated the response of P-UAECs to $E_2\beta$ but not in response to 2-OHE_2, 4-OHE_2 and 4-ME_2 respectively (Two-Way ANOVA; Antagonist × Group effect; $F_{5,60}$=25.272, P<0.001.*Indicates an increase (P<0.05, n=6) in P-UAEC proliferation compared to untreated control. τ Indicates inhibition (P<0.05) of P-UAEC proliferation with ICI; λ indicates lower P-UAEC proliferation (P<0.05) compared to $E_2\beta$ responses alone.

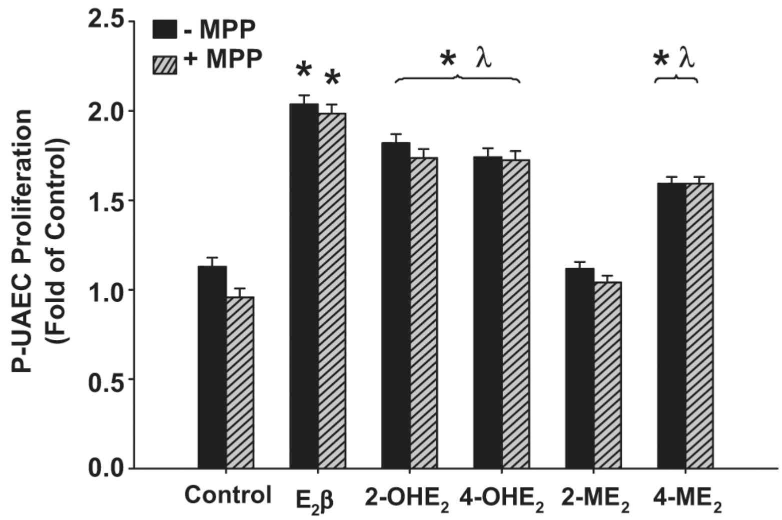

Figure 5.

The effects of the ER-α antagonist MPP (1μmol/L) on P-UAEC proliferation responses to 0.1 nmol/L of $E_2\beta$, 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2. MPP had no effect on the proliferation responses of P-UAECs to 0.1 nmol/L of $E_2\beta$, 2-OHE_2, 4-OHE_2, 2-ME_2 and 4-ME_2 (Two-Way ANOVA; Group effect, $F_{5,60}$=14.315, P<0.001). Neither a main effect of MPP nor an interaction was noted. *Indicates an increase (P<0.05; n=6) in P-UAEC proliferation compared to untreated control; λ indicates lower P-UAEC proliferation (P<0.05) compared to $E_2\beta$ responses alone.

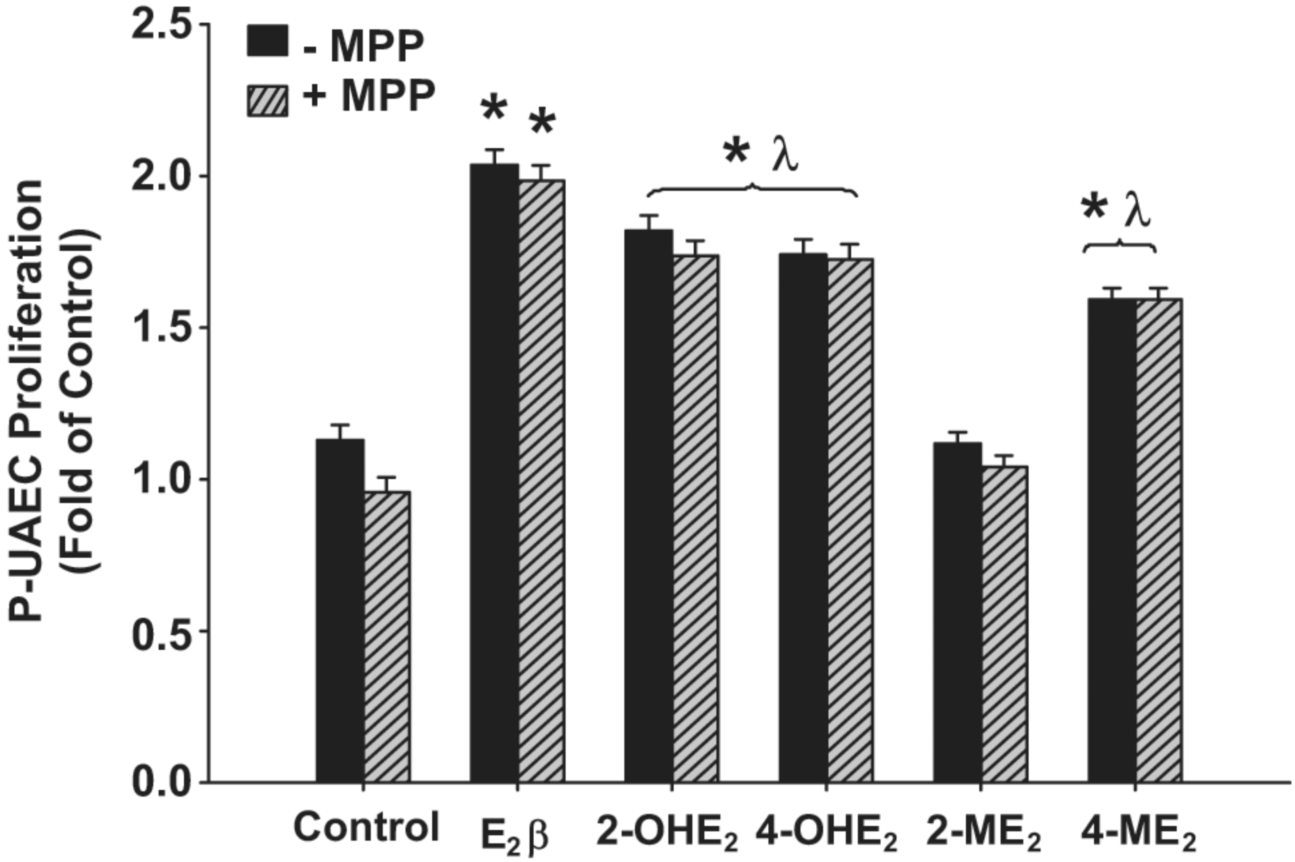

Figure 6.

The effects of the ER-β antagonist PHTPP (1μmol/L) on P-UAEC proliferative responses to 0.1 nmol/L of $E_2\beta$, 2-OHE_2, 4-OHE_2, 2-ME_2, and 4-ME_2 (Two-Way ANOVA; Antagonist × Group effect; $F_{5,60}$=17.517, P<0.001. *Indicates an increase (P<0.05; n=6) in P-UAEC proliferation compared to untreated control. τ Indicates inhibition (P<0.05) of P-UAEC proliferation with PHTPP. λ Indicates lower P-UAEC proliferation (P<0.05) compared to $E_2\beta$ responses alone.

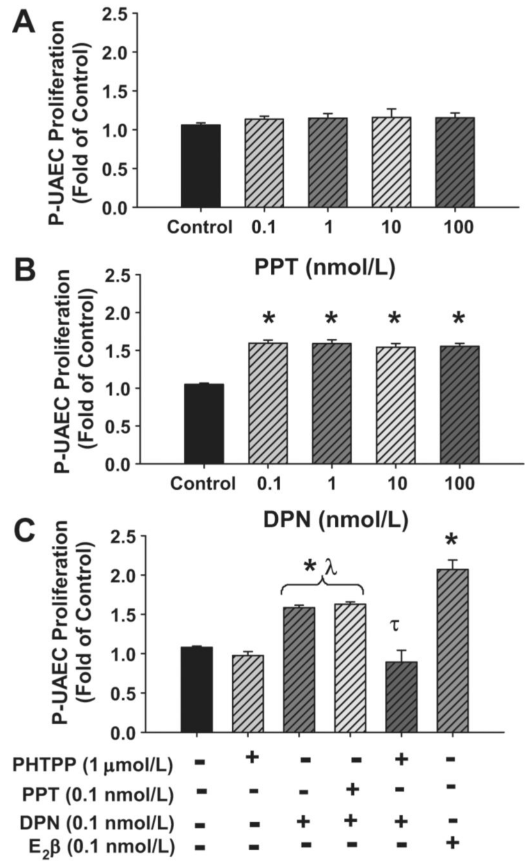

Figure 7.

Concentration-dependent effects of (A) ER-α agonist PPT (B) ER-β agonist DPN and (C) their combination on cell proliferation responses of P-UAECs. Blockade of ER-β with PHTPP (1μmol/L) prior to treatment with ER-β agonist DNP is shown in (C). *Indicates an increase ($P<0.05$; n=7) in P-UAEC proliferation compared to untreated controls. λ Indicates a difference ($P< 0.05$) in P-UAEC proliferation in response to DPN or the combination of DNP and PPT compared to $E_2\beta$ only responses. τ Indicates inhibition ($P<0.05$) of P-UAEC proliferation with PHTPP.