Effects of the Catechol and Methoxy Metabolites of 17β -Estradiol on Nitric Oxide Production by Ovine Uterine Artery Endothelial Cells

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Abstract

Nitric oxide (NO) production is essential to facilitate rises in uterine blood flow (UBF) during pregnancy. It has been proposed that the metabolites of $E_2\beta$, 2-hydroxyestradiol (2-OHE₂), 4-hydroxyestradiol (4-OHE₂), 2-methoxyestradiol (2-ME₂), and 4-methoxyestradiol (4-ME₂) play a role in mediating vasodilation and rises in UBF during pregnancy. We previously showed that the $E_2\beta$ metabolites stimulate prostacyclin production in pregnancy-derived ovine uterine artery endothelial cells (P-UAECs); however, it is unknown whether the $E_2\beta$ metabolites also induce NO production. Herein, UAECs derived from nonpregnant and pregnant ewes were used to test the hypothesis that $E_2\beta$ metabolites stimulate NO production in a pregnancy-specific manner. Specific estrogen receptor (ER) and adrenergic receptor (AR) antagonists were used to determine the roles of ERs or ARs in $E_2\beta$ metabolite-induced NO production. $E_2\beta$ and its metabolites increased total nitric oxide metabolites (NOx) levels (NO₂ + NO₃) in P-UAECs, but not in NP-UAECs. Pretreatment with combined 1 μ mol/L 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenyl]-1H-pyrazole dihydrochloride (MPP; ER- α antagonist) and 1 μ mol/L 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP; ER- β antagonist) inhibited the rises in NOx levels stimulated by $E_2\beta$ and 2-ME₂, but had no effect on 2-OHE₂, 4-OHE₂, or 4-ME₂-stimulated rises in NOx levels. Pretreatment with yohimbine (α_2 -AR antagonist) and propranolol ($\beta_{2,3}$ -AR antagonist) inhibited the rises in NOx levels stimulated by 2-OHE₂, but not by $E_2\beta$, 4-OHE₂, 2-ME₂, or 4-ME₂. These data demonstrate that $E_2\beta$ metabolites stimulate NO synthesis via ERs or ARs in UAECs in a pregnancy-specific manner, suggesting that these metabolites contribute to rises in vasodilation and UBF during pregnancy.

Keywords

estrogen metabolites, endothelium, nitric oxide, adrenergic receptors, pregnancy

Introduction

Normal pregnancy is characterized as a physiologic state of increased vasodilation, facilitated by vasoactive substances, such as prostacyclin (PGI₂) and nitric oxide (NO), produced by the endothelium.¹⁻⁵ Increased vasodilation during normal pregnancy facilitates rises in uterine blood flow (UBF) and increased nutrient delivery to the developing fetus.⁶ Thus, compromised vasodilation during pregnancy is associated with severe pathologies, such as preeclampsia (PE) and intrauterine growth restriction (IUGR), which exhibit low uteroplacental blood flow concomitant with low levels of vasodilators (ie, PGI₂ and NO).⁷⁻⁹ Both PE and IUGR are causes of significant maternal and fetal morbidity and mortality; therefore, it is essential to understand the regulation of vasodilation via endothelial-derived NO synthesis during pregnancy.

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Synthesis of NO is catalyzed by activated endothelial nitric oxide synthase (eNOS), whose regulation is partly mediated by estrogens.¹⁰⁻¹⁴ During normal pregnancy, uterine artery eNOS expression and circulating levels of 17 β -estradiol ($E_2\beta$) are elevated in association with rises in UBF.^{6,15} In addition, in ovariectomized animals, administration of $E_2\beta$ leads to endothelium-dependent vasodilation, rises in UBF, and elevations of uterine artery eNOS expression and NO synthesis.^{12-14,16-18} Although $E_2\beta$ is thought to be the major player in the regulation of cardiovascular function and adaptations during pregnancy, several recent studies indicate a potential role for the bioactive $E_2\beta$ metabolites, the catecholestradiols and methoxyestradiols, in mediating pregnancy-specific adaptations.¹⁹⁻²¹ Within the uteroplacental compartment, $E_2\beta$ is metabolized by Cytochrome P450s (CYP450s) into 2-hydroxyestradiol (2-OHE₂) and 4-hydroxyestradiol (4-OHE₂), which are further metabolized by catechol-o-methyltransferase (COMT) into 2-methoxyestradiol (2-ME₂) and 4-methoxyestradiol (4-ME₂).²² One of the proposed functional roles of $E_2\beta$ metabolites is the regulation of vasodilation. Direct uterine arterial infusion of 2-OHE₁ and 2-OHE₂ in nonpregnant (NP) sheep causes vasodilatation,²³ whereas 4-OHE₂ was reported to also interact directly with calcium channels to locally increase UBF in gilts.^{24,25} It is noteworthy that even though clearance rates of catecholestradiols, mainly attributed to metabolism by COMT in red blood cells, were estimated to be in excess of 30,000 L/d,²³ systemic plasma catecholestradiol levels in pregnancy are 10-fold greater than in NP women.²⁶ Thus, compared to estrone and $E_2\beta$, 2-OHE₁ and 2-OHE₂ appear to be weak estrogens, requiring 63 and 500 times the dose of $E_2\beta$ to overcome this enormous metabolic clearance rate in NP and pregnant (P) sheep. $E_2\beta$ metabolites, particularly 2-OHE₂ or 2-ME₂, appear to mediate vasodilation through the production of NO^{27,28} in several systemic vascular beds, for example, pulmonary artery²⁸, coronary artery,²⁹ aorta,²⁷ and human umbilical vein endothelial cells.²⁷ In addition, we recently demonstrated that the $E_2\beta$ metabolites (2-OHE₂, 4-OHE₂, 2-ME₂, and 4-ME₂) increase the production of PGI₂ by uterine artery endothelial cells (UAECs) in a pregnancy-specific manner,³⁰ further implicating their role in mediating vasodilation during pregnancy. However, the role of these estrogen metabolites in regulating the production of NO in the uterine vasculature during normal pregnancy has not been determined.

It has been well-documented that $E_2\beta$ mediates its actions, including regulation of NO synthesis, via the classic estrogen receptors (ERs) α and β .^{31,32} However, less is known regarding the receptors mediating the actions of the $E_2\beta$ metabolites. Evidence was also provided, albeit indirect, suggesting that the UBF responses to 2-OHE₁ and 2-OHE₂ functioned via ERs.^{23,33,34} Some studies have reported that the $E_2\beta$ metabolites have little to no affinity for the classic ERs.^{35,36} We have previously reported that some of the actions of the catechol metabolites of $E_2\beta$ in UAECs, such as stimulated mitogenesis, are mediated independent of the classic ERs and are rather mediated via β -adrenoceptors (adrenergic receptor [ARs]).³⁷ In addition, studies performed by Figueroa et al and Miller and

Vanhoutte have demonstrated that stimulation of α - and β -ARs with catecholamines and other AR agonists results in vasorelaxation of precontracted vessels, eNOS phosphorylation, and NO biosynthesis.³⁸⁻⁴⁰ This led to the hypothesis that the effects of the catecholestradiols on UAEC NO production might be mediated via ARs and independent of ERs. Given that no receptors have been identified to, or excluded from, mediating the actions of the methoxy metabolites of $E_2\beta$, we hypothesize that either ERs or ARs might mediate the effects of the methoxyestradiols on stimulating UAEC NO synthesis.

In the present study, we therefore determined (1) whether the $E_2\beta$ metabolites stimulate the production of NO in UAECs and whether this is a pregnancy-specific response and (2) whether ERs or ARs are involved in mediating the effects of $E_2\beta$ metabolites on UAEC NO production. We hypothesized that the $E_2\beta$ metabolites will induce NO production in UAECs derived from P ewes, but not from NP ewes, and that these responses are mediated via ARs, but not ERs.

Methods

Materials

2-Hydroxyestradiol, 4-OHE₂, 2-ME₂, and 4-ME₂ were obtained from Steraloids Inc (Newport, Rhode Island). Propranolol-HCl was purchased from Sigma-Aldrich (St Louis, Missouri). $E_2\beta$, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinyloxy)phenyl]-1H-pyrazole dihydrochloride (MPP), 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP), and yohimbine-HCl were obtained from Tocris Bioscience (Bristol, United Kingdom).

Cell Preparation and Culture

All procedures were approved by the University of Wisconsin-Madison Research Animal Care and Use Committees for both the Medical School and the College of Agricultural and Life Sciences, following the recommendation from the American Veterinary Medicine Association guidelines for humane treatment and euthanasia of laboratory farm animals as described by Landeros et al.⁴¹ Briefly, UAECs were obtained from P ewes (gestational age = 120-130; term = 147 days) or luteal phase NP ewes of mixed Western and Polypay breeds and euthanized under sodium pentobarbital (50-70 mg/kg) anesthesia through bilateral thoracotomy and exsanguination by cardiac laceration. Uterine artery endothelial cells were isolated from both left and right uterine arteries by collagenase digestion and cultured in growth media, endothelial basal media (EBM), with 20% fetal bovine serum, 100 mg/mL penicillin, and 100 mg/mL streptomycin.⁴² Finally, cell validations were conducted on each cell preparation for platelet endothelial cell adhesion molecule-1 (PECAM-1) and eNOS expression, acetylated low-density lipoprotein (LDL) uptake, and smooth muscle myosin expression (negative control) prior to long-term storage as described by Jobe et al.⁴³

Experimental Treatments

All experiments herein were performed in vitro using passage 4, primary cell lines. At ~70% confluence, cells were transferred to 6-well plates (4.0×10^5 cells/well) for NO measurements (see below). Experiments were performed in at least 4 different NP-UAEC and P-UAEC preparations, each representing individual animals. For all studies, cells were serum starved with EBM for 24 hours followed by 30-minute incubation in freshly modified Krebs buffer [(HEPES): 25 mmol/L, NaCl: 125 mmol/L, KCl: 5 mmol/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1 mmol/L, KH_2PO_4 : 1 mmol/L, glucose: 6 mmol/L, adjusted to pH = 7.4] to allow equilibrium. Both NP-UAECs and P-UAECs were treated with 2 concentrations (10 and 100 nmol/L) of $\text{E}_2\beta$, 2-OHE₂, 4-OHE₂, 2-ME₂, or 4-ME₂ for 10 minutes. The doses and time of agonist treatment were chosen based on data published by Pastore et al,³² indicating the effects of 10 and 100 nmol/L $\text{E}_2\beta$ in NO levels produced by P-UAECs at different time points. Treatment media were collected and stored at -80°C until measurement of NO levels was performed via high-performance liquid chromatography (HPLC).

Determination of Receptor-Mediated NO Production

Pastore et al³² reported incomplete inhibition of $\text{E}_2\beta$ -induced NOx production in P-UAECs with the nonselective ER antagonist ICI 182 780 pretreatment, but complete inhibition with the combination of selective ER- α (MPP) and ER- β (PHTPP) antagonists. Therefore, to determine the role of ER α/β in $\text{E}_2\beta$ -, 2-OHE₂-, 4-OHE₂-, 2-ME₂-, and 4-ME₂-stimulated NO production in P-UAECs, cells were pretreated for 1 hour with the combination of 1 $\mu\text{mol/L}$ MPP and 1 $\mu\text{mol/L}$ PHTPP followed by 10 nmol/L $\text{E}_2\beta$, 2-OHE₂, and 4-OHE₂ or 100 nmol/L 2-ME₂ or 4-ME₂; these concentrations were chosen based on our previous P-UAEC studies for NOx production,³² proliferation,⁴³ and PGI₂ biosynthesis,³⁰ as well as preliminary dose responses using all the metabolites (data not shown). We previously reported the complete absence of α_1 -ARs and β_1 -ARs and strong expression of α_2 -ARs and $\beta_{2,3}$ -ARs in UAECs³⁷; therefore, 1 $\mu\text{mol/L}$ yohimbine (α_2 -AR antagonist; 30 minutes pretreatment) and 1 $\mu\text{mol/L}$ propranolol ($\beta_{2,3}$ -AR antagonist; 1 hour pretreatment) were used to determine the role of α_2 -ARs and $\beta_{2,3}$ -ARs, respectively. Pregnancy-derived ovine uterine artery endothelial cells were pretreated for 30 minutes with yohimbine or for 1 hour with propranolol followed by 10-minute treatment with 10 nmol/L $\text{E}_2\beta$, 2-OHE₂, and 4-OHE₂ or 100 nmol/L 2-ME₂ and 4-ME₂. Following treatments, treatment media were collected and stored at -80°C until further analysis.

Measurement of Nitrite and Nitrate (NOx) Production

Measurement of NO production was performed through HPLC using NOx analyzer (ENO-30) and Insight autosampler (AS-700) from EICOM USA (San Diego, California), by measuring the levels of nitrite (NO_2) and nitrate (NO_3) (NOx) found

in the collected media. Total NOx production equals the sum of NO_2 and NO_3 determined against a standard curve and after subtraction of the blank to remove background peak values.

Statistical Analysis

Data were analyzed using SigmaPlot (version 12.5). One-way analyses of variance (post-hoc test, Student-Newman-Keuls) were used to determine the effects of hormone and inhibitors in NP-UAECs and P-UAECs. Nonparametric analysis (Kruskal-Wallis equality-of-populations rank test or Mann-Whitney rank sum test), followed by Student's *t* test were performed on data sets that did not pass normality (Shapiro-Wilk test) and variance tests (2-ME₂ and 4-ME₂ dose effects). Data are presented as means $\pm$ standard error of the mean in percentage increase from baseline NOx levels of vehicle control. Results were considered significant at $P < .05$.

Results

Effects of $\text{E}_2\beta$, 2-OHE₂, 4-OHE₂, 2-ME₂, and 4-ME₂ on NO Production by NP-UAECs and P-UAECs

Quantification of total NOx ($\text{NO}_2 + \text{NO}_3$) levels stimulated for 10 minutes with $\text{E}_2\beta$ and its metabolites showed rises in NOx production in a pregnancy-specific manner, as expected from our previous studies.³² Average unstimulated basal NOx levels were 463.2 ± 34 nmol/L in NP-UAECs and 320.7 ± 43 nmol/L in P-UAECs. Stimulation with 10 and 100 nmol/L $\text{E}_2\beta$ for 10 minutes increased NOx levels in NP-UAECs by 13.9% ($P = .39$) and 43.0% ($P = .51$), respectively; while in P-UAEC, NOx levels were increased with 10 nmol/L and 100 nmol/L $\text{E}_2\beta$ by 74.5% ($P = .006$) and 63.5% ($P = .009$), respectively (Figure 1A). Similarly, rises in NOx levels stimulated by 10 and 100 nmol/L 2-OHE₂ were only 2.6% ($P = .24$) and 15.1% ($P = .24$) in NP-UAECs and 93.7% ($P = .04$) and 34.1% ($P = .21$) in P-UAECs, respectively (Figure 1B). 4-Hydroxyestradiol at 10 nmol/L increased NOx levels by 8.3% ($P = .78$) in NP-UACs and 95.4% ($P = .006$) in P-UAEC, while 100 nmol/L raised levels by 8.22% ($P = .78$) in NP-UAECs and 29.7% ($P = .184$) in P-UAECs (Figure 1C). With 10 and 100 nmol/L 2-ME₂ stimulation, NP-UAEC total NOx levels increased by 31.3% ($P = .3$) and 10.4% ($P = .3$), while in P-UAECs, 10 and 100 nmol/L 2-ME₂ stimulation resulted in 41.0% ($P = .32$) and 100.4% ($P = .013$) rises in NOx levels, respectively (Figure 1D). Similarly, minimal rises of 17.9% ($P = .5$) and 9.2% ($P = .2$) in NOx levels were observed with 10 and 100 nmol/L 4-ME₂ in NP-UAECs, while in P-UAECs, 10 and 100 nmol/L 4-ME₂ caused an increase in NOx levels of 50.5% ($P = .21$) and 93.2% ($P = .04$), respectively (Figure 1E).

Role of Classic ERs on $\text{E}_2\beta$ -, 2-OHE₂-, 4-OHE₂-, 2-ME₂-, and 4-ME₂-Stimulated Total NOx Production

Given that all significant rises in NOx levels stimulated by $\text{E}_2\beta$ and its metabolites were observed in P-UAECs, we then

Figure 1. Effects of E₂β, 2-OHE₂, 4-OHE₂, 2-ME₂, and 4-ME₂ on rises of NOx production by NP-UAECs and P-UAECs. Total NOx (NO₂ + NO₃) levels were quantified by an HPLC-based assay. (A) Significant elevations in NOx levels after 10-minute treatment with E₂β were observed in P-UAECs, but not in NP-UAECs at both 10 and 100 nmol/L concentrations. Both (B) 2-OHE₂ and (C) 4-OHE₂ had no effect on NP-UAECs but induced significant rises in NOx levels at 10 nmol/L, but not 100 nmol/L in P-UAECs. Both (D) 2-ME₂ and (E) 4-ME₂ had no effect on NP-UAECs at 10 or 100 nmol/L; however, in P-UAECs, 100 nmol/L, but not 10 nmol/L, 2-ME₂ and 4-ME₂ induced significant rises in NOx levels. Data are presented as percentage change from basal NOx levels (vehicle control) as means ± SEM. **P* < .05 versus control, one-way ANOVA (post-hoc test, Student-Newman-Keuls). ANOVA, analysis of variance; E₂β, estradiol; 2-ME₂, 2-methoxyestradiol; 4-ME₂, 4-methoxyestradiol; NOx, nitric oxide metabolite; NP, nonpregnant; 2-OHE₂, 2-hydroxyestradiol; 4-OHE₂, 4-hydroxyestradiol; P, pregnant; SEM, standard error of the mean; UAEC, uterine artery endothelial cells.

determined the role, if any, that the ERs play in mediating the rises in NOx levels stimulated by E₂β metabolites. A combination of the specific ER-α (MPP) and ER-β (PHTPP) antagonists was used to effectively inhibit both ERs. Pretreatment with 1 μmol/L MPP + 1 μmol/L PHTPP alone did not significantly alter NOx levels, that is, only a 12.3% increase from basal levels was observed. Confirming our previous report by Pastore et al,³² the combination of MPP + PHTPP completely inhibited the rises in NOx levels induced by 10 nmol/L E₂β from 71.9% to 12.6% in P-UAECs (*P* = .004; Figure 2A). However, NOx levels stimulated by 10 nmol/L 2-OHE₂ (72.7%), 4-OHE₂ (65.4%), and 100 nmol/L 4-ME₂ (68.6%) alone were not inhibited by pretreatment with MPP + PHTPP, that is, NOx levels remained elevated at 58.0% with 2-OHE₂, 79.3% with 4-OHE₂, and 59.2% with 4-ME₂ (Figure 2B, C, and E). Unexpectedly, we observed that MPP + PHTPP significantly reduced the rises in NOx levels induced by 100 nmol/L 2-ME₂ from 64.4% to 21.9% (*P* = .005; Figure 2D).

Role of Adrenoceptors on E₂β, 2-OHE₂, 4-OHE₂, 2-ME₂, and 4-ME₂-Stimulated Total NOx Production by P-UAECs

Given that previous studies published by our group indicate that ARs mediate some of the actions of 2-OHE₂ and 4-OHE₂ in P-UAECs,³⁷ we tested the involvement of both α-ARs and β-ARs in mediating NO production in P-UAECs stimulated by E₂β and its metabolites using selective inhibitors. Pretreatment with 1 μmol/L yohimbine (α-AR antagonist) and 1 μmol/L propranolol (β-AR antagonist) alone had no effect in total NOx production in P-UAECs. NOx levels with yohimbine and propranolol were 1.9% (*P* = .821) and 11.0% (*P* = .337) above baseline levels. The rises in NOx levels stimulated by 10 nmol/L E₂β (98.2%) were not inhibited by pretreatment with either yohimbine (106%) or propranolol (78.9%; Figure 3A). However, both yohimbine and propranolol significantly inhibited the rises in NOx levels stimulated by 10 nmol/L 2-OHE₂ from 69.9% to -1.99% (*P* < .001) and 11.0% (*P* = .002),

Figure 2. Effects of ER- α and ER- β inhibition on NOx rises stimulated by E₂β and its metabolites in P-UAECs. Inhibition of both ER- α and ER- β in P-UAECs was performed with combined treatment of MPP (ER- α antagonist, 1 μ mol/L) + PHTPP (ER- β antagonist, 1 μ mol/L) for 1 hour, followed by 10 nmol/L E₂β, 2-OHE₂, and 4-OHE₂, or 100 nmol/L 2-ME₂ and 4-ME₂ for 10 minutes. Pretreatment with MPP + PHTPP alone had no effect, but completely inhibited the rises in NOx stimulated by (A) E₂β, and (D) 2-ME₂, but did not inhibit the rises in NOx stimulated by (B) 2-OHE₂, (C) 4-OHE₂, or (E) 4-ME₂. Data are presented as percentage change from basal NOx levels (vehicle control) as means $\pm$ SEM. One-way ANOVA (post-hoc test, Student-Newman-Keuls) was performed for all treatments. * $P < .05$ increase versus vehicle control, ‡ $P < .05$ inhibition versus hormone treatment alone. ANOVA, analysis of variance; ER, estrogen receptor; E₂β, estradiol; 2-ME₂, 2-methoxyestradiol; 4-ME₂, 4-methoxyestradiol; MPP, 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinyloxy)phenol]-1H-pyrazole dihydrochloride; NOx, nitric oxide metabolite; 2-OHE₂, 2-hydroxyestradiol; 4-OHE₂, 4-hydroxyestradiol; P, pregnant; PHTPP, 4-[2-phenyl-5,7-bis(trifluoromethyl)-pyrazolo[1,5-a]pyrimidin-3-yl]phenol; SEM, standard error of the mean; UAEC, uterine artery endothelial cells.

respectively, demonstrating a role of both α -ARs and β -ARs in mediating the effects of 2-OHE₂ in P-UAECs NO production (Figure 3B). Inhibition of α -ARs and β -ARs did not have an effect on the rises in NOx levels stimulated by 10 nmol/L 4-OHE₂ (60.0%), as pretreatment with yohimbine followed by 4-OHE₂ remained at 50.8% ($P = .076$) above baseline levels and propranolol followed by 4-OHE₂ resulted in NOx levels at 53.2% ($P = .61$) above baseline (Figure 3C). As expected, the rises in NOx levels stimulated by 2-ME₂ (100%) were only partially, and not significantly, decreased to 70.5% ($P = .26$) by yohimbine and 47.8% ($P = .11$) by propranolol (Figure 3D). Finally, the 4-ME₂-induced rises in NOx levels up to 69.7% above baseline were unaltered by pretreatment with yohimbine, which remained at 66.5% ($P = .88$) and were partially, but not significantly, inhibited by propranolol down to 35.5% ($P = .21$; Figure 3E).

Discussion

Estrogen and its metabolites are thought to regulate critical modulators of hemodynamic changes noted during gestation, such as vasodilator production and angiogenesis.^{21,22,30,37,43} Furthermore, aberrant regulation of estrogen and its OH and ME metabolites has been associated with the development of pregnancy disorders such as PE.^{21,44} In this regard, we reported that abnormal synthesis, metabolism, and accumulation of E₂β and its bioactive metabolites 2-OHE₂ and 4-OHE₂ were seen in pregnancies complicated by PE and that this was exacerbated with the severity of the gestational disease,²¹ where abnormally low uteroplacental blood flow is observed.⁴⁵⁻⁴⁸ Further, in vivo evidence supports estrogen-derived metabolites in modulating pregnancy adaptations include 2-ME₂- deficient and COMT-deficient mice exhibiting PE-like symptoms including

Figure 3. Effects of α -AR or β -AR inhibition on NOx rises stimulated by E₂β and its metabolites in P-UAECs. Pregnancy-derived ovine uterine artery endothelial cells were pretreated with yohimbine (α_2 -AR selective antagonist; 1 μ mol/L; 30 minutes) or propranolol ($\beta_{2,3}$ -AR antagonist, 1 μ mol/L; 1 hour), followed by 10 nmol/L E₂β, 2-OHE₂, and 4-OHE₂ or 100 nmol/L 2-ME₂, and 4-ME₂ treatment for 10 minutes. Treatment with yohimbine or propranolol alone had no effect on NOx levels by themselves. In P-UAECs: (A) pretreatment with yohimbine or propranolol did not inhibit the rises in NOx levels induced by E₂β. (B) Inhibition of both α_2 -AR and $\beta_{2,3}$ -AR with yohimbine and propranolol completely inhibited the rises in NOx levels stimulated by 2-OHE₂. Neither yohimbine nor propranolol pretreatment inhibited the rises in NOx stimulated by (C) 4-OHE₂, (D) 2-ME₂, or (E) 4-ME₂. Partial decreases in the rises in NOx levels stimulated by both 2-ME₂ and 4-ME₂ were observed with propranolol. Data are presented as percentage change from basal NOx levels (vehicle control) as means $\pm$ SEM. One-way ANOVA (post-hoc test, Student-Newman-Keuls) was performed for all treatments. **P* < .05 increase versus vehicle control, ‡*P* < .05 inhibition versus hormone treatment alone. ANOVA, analysis of variance; AR, adrenergic receptor; E₂β, estradiol; 2-ME₂, 2-methoxyestradiol; 4-ME₂, 4-methoxyestradiol; NOx, nitric oxide metabolite; 2-OHE₂, 2-hydroxyestradiol; 4-OHE₂, 4-hydroxyestradiol; P, pregnant; SEM, standard error of the mean; UAEC, uterine artery endothelial cells.

impaired angiogenesis and hypertension.⁴⁴ E₂β metabolites, 2-OHE₂, 4-OHE₂, 2-ME₂, and 4-ME₂, have been shown to influence vasodilation in some systemic vascular beds^{23,27}; however, it is unknown whether they regulate the production of NO which is thought to partly mediate rises in UBF during pregnancy. In the present study, we show for the first time that (1) the E₂β catechol and methoxy metabolites stimulate the production of NO in UAECs derived from P ewes, but not from NP ewes; (2) E₂β and 2-ME₂ mediate NO synthesis via an ER-dependent mechanism, while AR-dependent mechanisms are involved in 2-OHE₂-induced NO synthesis; and (3) 4-OHE₂- and 4-ME₂-induced NO synthesis is mediated independent of either ERs or ARs. Overall, this study shows that E₂β metabolites play a role in regulating NO synthesis. Of particular importance are the pregnancy-specific effects

observed in P-UAECs which indicate an important role for these hormones during gestation. In addition, this study further affirms the ability of the catecholestradiols to “hijack” the adrenergic system to induce their vasodilator actions in P-UAECs, while at least one methoxyestradiol retains the ability to act via ERs. This indicates the existence of multiple and redundant mechanisms in the uterine artery endothelium by which estrogenic actions regulate NO synthesis and thus vasodilation during pregnancy.

Assessment of the effects of E₂β, 2-OHE₂, 4-OHE₂, 2-ME₂, and 4-ME₂ on NO synthesis by NP-UAECs and P-UAECs has provided us with novel evidence that E₂β and its metabolites induce rises in NO synthesis in a pregnancy-specific manner. This is consistent with our previous reports showing pregnancy-specific effects of E₂β and its metabolites in

stimulating PGI₂ synthesis and proliferation in P-UAECs.^{30,37} We observed that although both 10 and 100 nmol/L E₂β increased NO levels, only 10 nmol/L 2-OHE₂ and 4-OHE₂ induced rises in NO levels, while 2-ME₂ and 4-ME₂ only at 100 nmol/L induced rises in NO synthesis. This indicates that the parent steroid E₂β and its catechol metabolites are at least one order of magnitude more potent than the methoxyestradiols in regulating NO synthesis. The relative potency of the E₂β metabolites may reflect the relative levels in which these metabolites are found during normal pregnancy; for example, the levels of 2-OHE₂ (~500 pg/mL) are much lower than the levels of 2-ME₂ (~2000 pg/mL),²¹ showing that higher concentrations of the methoxyestradiols might be needed to induce effects in vivo. Furthermore, the pregnancy-specific effects that we observed represent the importance of estrogen metabolites in the regulation of NO synthesis in the uterine vasculature during pregnancy, potentially implicating a role for these metabolites to influence vasodilation and rises in UBF during pregnancy. However, further analysis in ex vivo intact vessels and in vivo models is warranted to effectively determine the ability of E₂β metabolites to induce NO-mediated vasodilation in the uterine vasculature during pregnancy.

Past studies performed in UAECs show that expression of ER-α and/or ER-β or AR-β in NP-UAECs versus P-UAECs is not different at passage 4, thus revealing that the elevated pregnancy-specific vasodilator production responses that we observed in P-UAECs versus NP-UAECs are independent of the expression of ER-α and/or ER-β or AR-β levels, demonstrating greater receptor coupling and signaling retention even at passage 4.^{30,37,42,43} Signaling through ER-α and/or ER-β has been shown to mediate the effects of E₂β on NO synthesis.^{31,32,49,50} Furthermore, Pastore et al³² showed that inhibition of both receptors was necessary to inhibit NO production stimulated by E₂β in P-UAECs; therefore, in the present study, a combination of ER-α and ER-β antagonists was used to effectively inhibit NO production stimulated by E₂β (positive control) and its metabolites. Herein, we also show that E₂β-induced rises in NO synthesis in P-UAECs are dependent on both ER-α and ER-β, consistent with those previous reports. In addition, we observed that ER-α and ER-β are not involved in mediating the effects of 2-OHE₂, 4-OHE₂, and 4-ME₂ on NO synthesis by P-UAECs. However, inhibition of both ER-α and ER-β with MPP + PHTPP completely inhibited the 2-ME₂-mediated rises in NO synthesis, indicating the involvement of ERs in mediating the effects of 2-ME₂ in P-UAECs. This finding is contrary to our hypothesis, given that several studies have indicated that the E₂β metabolites have little, to no, affinity for the classic ERs. However, a few studies have determined that some actions of 2-ME₂ require functional ERs, such as egg transport in rats and tumor growth in breast cancer.^{51,52} Moreover, with the very substantial red blood cell COMT metabolic clearance rate noted previously,²³ 2-OHE₂ conversion to 2-ME₂ might partly account for the suggested ER-mediated rises in UBF. These current unexpected findings warrant further investigation on the mechanism of action of 2-ME₂ in regulating NO synthesis. To this end, it has been shown that

peroxisome proliferator-activated receptor γ (PPARγ) plays a role in eNOS activation and NO synthesis.^{27,53,54} Also, 2-ME₂ appears to behave as a PPARγ agonist, although it does not directly bind to PPARγ. Thus, it is entirely possible that signaling of 2-ME₂ through PPARγ-mediated mechanisms is involved in mediating NO production stimulated by 2-ME₂ in UAECs, and future studies should be performed to test this hypothesis.

In addition to testing the role of ERs in mediating the effects of E₂β metabolites on NO synthesis, we investigated whether α-ARs or β-ARs mediate the effects of catecholestradiols and methoxyestradiols in NO synthesis. We previously reported that P-UAECs express only α₂-ARs and β_{2,3}-ARs, but not α₁-ARs or β₁-ARs, and compared to NP-UAECs, β_{2,3}-ARs are unaltered, but α₂-ARs are downregulated in P-UAECs.³⁷ Jobe et al also showed that both catecholestradiols (2-OHE₂ and 4-OHE₂) mediate proliferation of P-UAECs through β_{2,3}-AR, but not α₂-AR-dependent mechanisms.³⁷ Herein, we observed that only 2-OHE₂-, but not 4-OHE₂-, 2-ME₂-, and 4-ME₂-induced rises in NOx synthesis are mediated via an AR-dependent mechanism, as blockade of either α₂-ARs and β_{2,3}-ARs completely inhibited the effects of 2-OHE₂, but not the effects of the other metabolites. However, unlike the previous report by Jobe et al,³⁷ which showed an involvement of only β-ARs in both 2-OHE₂- and 4-OHE₂-induced P-UAEC proliferation, our experiments herein show that both α-ARs and β-ARs are involved in 2-OHE₂- but not 4-OHE₂-mediated NOx production. These differences can be explained by a possible difference in the specific receptor–ligand interaction of 2-OHE₂ and 4-OHE₂ due to the placement of their specific catechol moieties. Studies indicate that ligands with different structures can cause different intermediate receptor conformational states leading to different signaling patterns and effects.^{55–57} It is possible that the exact interaction of 2-OHE₂ and 4-OHE₂ with ARs could explain why 2-OHE₂, but not 4-OHE₂, stimulates NO production through the ARs. Therefore, inhibition of ARs results in blockade of one catechol, but not the other, especially since a specific receptor–ligand interaction is needed to very rapidly stimulate NO synthesis, which might be different from that needed for more prolonged genomic signaling in cell proliferation. With regard to the methoxy metabolites, we confirmed that while these metabolites are biologically active, they do not signal through AR-dependent mechanisms; therefore, further studies are warranted to determine the specific mechanism of action in UAECs. Some studies have demonstrated that allosteric binding of agonists and antagonists to ERs and G-Protein-Coupled Receptors (GPCRs) can alter the transcriptional activity of the receptors by altering protein–protein interactions or protein–DNA interactions.^{58,59} Although currently there are no data supporting allosteric binding of 4-OHE₂ or 4-ME₂ to ERs or ARs, future studies should be performed to assess this possibility and its potential role in mediating NO synthesis. In addition, several studies have identified expression of the membrane-bound ER, G Protein-Coupled Estrogen Receptor-1 (GPER-1) in several vascular beds^{60–62}; such studies have also suggested a role for

this receptor in mediating $E_2\beta$ -stimulated vasodilation.⁶²⁻⁶⁵ However, previous attempts in our laboratory to identify expression of GPER-1 in NP- and P-UAECs have not provided conclusive data to demonstrate their expression or function. Thus, it is unlikely that GPER-1 is involved in mediating the effects of $E_2\beta$ or any of its metabolites in UAECs.

Overall, the pregnancy-specific synthesis of NO stimulated by $E_2\beta$ metabolites in P-UAECs is indicative of an important mechanism by which estrogens increase vasodilation to mediate rises in UBF during pregnancy. These data also offer further evidence of a potential role for $E_2\beta$ metabolites in the development of pathologic pregnancies complicated by low vasodilation. Several studies have demonstrated the development of hypertension, PE-like phenotypes, and intrauterine fetal growth restriction upon inhibition of NO synthesis during pregnancy in animal models.⁶⁶⁻⁶⁸ Recent studies have shown that in COMT-knockout (KO) mice, where conversion of catecholestradiols to methoxyestradiols would be abrogated, hypertension concomitant with fetal growth retardation is present.^{44,69} In this COMT-KO model, the deleterious fetal responses are reversed by treatment with methoxyestradiols and the cyclic guanosine monophosphate (c-GMP) -specific phosphodiesterase-5 inhibitor, sildenafil.⁶⁹ Sildenafil presumably allows for greater NO-mediated cGMP production to maintain vasorelaxation⁷⁰ and has been shown to augment the vasodilatory effects of $E_2\beta$,⁷⁰ which, based on the current observations, would likely be a result of the combined action of $E_2\beta$ and its metabolites in the uterine vasculature. Overall, the present study provides strong evidence for the role of CYP450- and COMT-synthesized $E_2\beta$ metabolites in the regulation of NO synthesis and provides clues of the possible role these metabolites play in regulating vasodilation during normal pregnancy. Furthermore, it is important to recognize the complexity of estrogen signaling, especially when concerning the uterine vasculature and pregnancy, as knowledge of the effects of $E_2\beta$ metabolites in vascular homeostasis during pregnancy is critical for future studies seeking to establish possible avenues for treatment of hypertensive diseases of pregnancy.

Authors' Note

The work reported in this article was performed at the University of Wisconsin–Madison. R.V.L. contributed to the conception and design of the study, acquisition of data, analysis and interpretation of data, and writing of the manuscript. M.B.P. contributed to the design of the study and acquisition of data and interpretation and revised the article for intellectual content. R.R.M. contributed to the conception and design of the study, interpretation of data and discussion, and revised manuscript for intellectual content. All authors approved the final version to be published.

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References

1. Conrad KP, Joffe GM, Kruszyna H, et al. Identification of increased nitric oxide biosynthesis during pregnancy in rats. *FASEB J*. 1993;7(6):566-571.
2. Resnik R, Killam AP, Barton MD, Battaglia FC, Makowski EL, Meschia G. The effect of various vasoactive compounds upon the uterine vascular bed. *Am J Obstet Gynecol*. 1976;125(2):201-206.
3. Sladek SM, Magness RR, Conrad KP. Nitric oxide and pregnancy. *Am J Physiol*. 1997;272(2 Pt 2):R441-R463.
4. Weiner CP, Thompson LP. Nitric oxide and pregnancy. *Semin Perinatol*. 1997;21(5):367-380.
5. Yang D, Lang U, Greenberg SG, Myatt L, Clark KE. Elevation of nitrate levels in pregnant ewes and their fetuses. *Am J Obstet Gynecol*. 1996;174(2):573-577.
6. Magness RR. Maternal cardiovascular and other physiologic responses to the endocrinology of pregnancy. In: Bazer FW, ed. *The Endocrinology of Pregnancy*. Totowa, NJ: Humana Press Inc; 1998:507-539.
7. Tranquilli AL, Bezzeccheri V, Giannubilo SR, Scagnoli C, Mazzanti L, Garzetti GG. Amniotic levels of nitric oxide in women with fetal intrauterine growth restriction. *The J Matern Fetal Neonatal Med*. 2003;13(2):115-118.
8. Remuzzi G, Marchesi D, Zoja C, et al. Reduced umbilical and placental vascular prostacyclin in severe pre-eclampsia. *Prostaglandins*. 1980;20(1):105-110.
9. Seligman SP, Buyon JP, Clancy RM, Young BK, Abramson SB. The role of nitric oxide in the pathogenesis of preeclampsia. *Am J Obstet Gynecol*. 1994;171(4):944-948.
10. White RE. Estrogen and vascular function. *Vascul Pharmacol*. 2002;38(2):73-80.
11. Stefano GB, Prevot V, Beauvillain JC, et al. Cell-surface estrogen receptors mediate calcium-dependent nitric oxide release in human endothelia. *Circulation*. 2000;101(13):1594-1597.
12. Lantin-Hermoso RL, Rosenfeld CR, Yuhanna IS, German Z, Chen Z, Shaul PW. Estrogen acutely stimulates nitric oxide synthase activity in fetal pulmonary artery endothelium. *Am J Physiol Renal Physiol*. 1997;273(1 pt 1):L119-L126.
13. Chang K, Lubo Z. Review article: steroid hormones and uterine vascular adaptation to pregnancy. *Reprod Sci*. 2008;15(4):336-348.

14. Rosenfeld CR, Cox BE, Roy T, Magness RR. Nitric oxide contributes to estrogen-induced vasodilation of the ovine uterine circulation. *J Clin Invest.* 1996;98(9):2158-2166.
15. Dotsch J, Hogen N, Nyul Z, et al. Increase of endothelial nitric oxide synthase and endothelin-1 mRNA expression in human placenta during gestation. *Eur J Obstet Gynecol Reprod Biol.* 2001;97(2):163-167.
16. Collins P, Shay J, Jiang C, Moss J. Nitric oxide accounts for dose-dependent estrogen-mediated coronary relaxation after acute estrogen withdrawal. *Circulation.* 1994;90(4):1964-1968.
17. Vedernikov YP, Liao QP, Jain V, Saade GR, Chwalisz K, Garfield RE. Effect of chronic treatment with 17beta-estradiol and progesterone on endothelium-dependent and endothelium-independent relaxation in isolated aortic rings from ovariectomized rats. *Am J Obstet Gynecol.* 1997;176(3):603-608.
18. Magness RR, Parker CR Jr, Rosenfeld CR. Systemic and uterine responses to chronic infusion of estradiol-17 beta. *Am J Physiol.* 1993;265(5 pt 1):E690-E698.
19. Dubey RK, Jackson EK. Cardiovascular protective effects of 17beta-estradiol metabolites. *J Appl Physiol (1985).* 2001;91(4):1868-1883.
20. Mendelsohn ME, Karas RH. The protective effects of estrogen on the cardiovascular system. *N Engl J Med.* 1999;340(23):1801-1811.
21. Jobe SO, Tyler CT, Magness RR. Aberrant synthesis, metabolism, and plasma accumulation of circulating estrogens and estrogen metabolites in preeclampsia implications for vascular dysfunction. *Hypertension.* 2013;61(2):480-487.
22. Dubey RK, Tofovic SP, Jackson EK. Cardiovascular pharmacology of estradiol metabolites. *J Pharmacol Exp Ther.* 2004;308(2):403-409.
23. Rosenfeld CR, Jackson GM. Induction and inhibition of uterine vasodilation by catechol estrogen in oophorectomized, nonpregnant ewes. *Endocrinology.* 1982;110(4):1333-1339.
24. Stice SL, Ford SP, Rosazza JP, Van Orden DE. Interaction of 4-hydroxylated estradiol and potential-sensitive Ca²⁺ channels in altering uterine blood flow during the estrous cycle and early pregnancy in gilts. *Biol Reprod.* 1987;36(2):369-375.
25. Ford SP. Factors controlling uterine blood flow during estrous and early pregnancy. In: *Rosenfeld CR, (ed.)*
26. Ball P, Knuppen R. Catecholestrogens (2-and 4-hydroxyoestrogens): chemistry, biogenesis, metabolism, occurrence and physiological significance. *Acta Endocrinol Suppl (Copenh).* 1980;232:1-127.
27. Chen W, Cui Y, Zheng S, et al. 2-Methoxyestradiol induces vasodilation by stimulating NO release via PPAR γ /PI3K/Akt pathway. *PLoS One.* 2015;10(3):e0118902.
28. Fenoy FJ, Hernandez ME, Hernandez M, Quesada T, Salom MG, Hernandez I. Acute effects of 2-methoxyestradiol on endothelial aortic NO release in male and ovariectomized female rats. *Nitric Oxide.* 2010;23(1):12-19.
29. Hill BJ, Gebre S, Schlicker B, Jordan R, Necessary S. Nongenomic inhibition of coronary constriction by 17ss-estradiol, 2-hydroxyestradiol, and 2-methoxyestradiol. *Can J Physiol Pharmacol.* 88(2):147-152.
30. Jobe SO, Ramadoss J, Wargin AJ, Magness RR. Estradiol-17beta and its cytochrome P450- and catechol-O-methyltransferase-derived metabolites selectively stimulate production of prostacyclin in uterine artery endothelial cells: role of estrogen receptor-alpha versus estrogen receptor-beta. *Hypertension.* 2013;61(2):509-518.
31. Chen Z, Yuhanna IS, Galcheva-Gargova Z, Karas RH, Mendelsohn ME, Shaul PW. Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. *J Clin Invest.* 1999;103(3):401-406.
32. Pastore MB, Talwar S, Conley MR, Magness RR. Identification of differential ER-Alpha versus ER-Beta mediated activation of eNOS in ovine uterine artery endothelial cells. *Biol Reprod.* 2016;94(6):139.
33. Magness RR, Rosenfeld CR. Local and systemic estradiol-17 beta: effects on uterine and systemic vasodilation. *Am J Physiol.* 1989;256(4 Pt 1):E536-E542.
34. Magness RR, Rosenfeld CR. The role of steroid hormones in control of uterine blood flow. In: *The Uterine Circulation (Rosenfeld CR, ed.):* Perinatology Press, Ithaca NY; 1989:239-271.
35. LaVallee TM, Zhan XH, Herbstritt CJ, Kough EC, Green SJ, Pribluda VS. 2-Methoxyestradiol inhibits proliferation and induces apoptosis independently of estrogen receptors alpha and beta. *Cancer Res.* 2002;62(13):3691-3697.
36. Dubey RK, Jackson EK. Estrogen-induced cardiorenal protection: potential cellular, biochemical, and molecular mechanisms. *Am J Physiol Renal Physiol.* 2001;280(3):F365-F388.
37. Jobe SO, Fling SN, Ramadoss J, Magness RR. A novel role for an endothelial adrenergic receptor system in mediating catecholestradiol-induced proliferation of uterine artery endothelial cells. *Hypertension.* 2011;58(5):874-881.
38. Miller VM, Vanhoutte PM. Endothelial alpha 2-adrenoceptors in canine pulmonary and systemic blood vessels. *Eur J Pharmacol.* 1985;118(1-2):123-129.
39. Figueroa XF, Poblete MI, Boric MP, Mendizabal VE, Adler-Graschinsky E, Huidobro-Toro JP. Clonidine-induced nitric oxide-dependent vasorelaxation mediated by endothelial alpha(2)-adrenoceptor activation. *Br J Pharmacol.* 2001;134(5):957-968.
40. Figueroa XF, Poblete I, Fernandez R, Pedemonte C, Cortes V, Huidobro-Toro JP. NO production and eNOS phosphorylation induced by epinephrine through the activation of beta-adrenoceptors. *Am J Physiol Heart Circ Physiol.* 2009;297(1):H134-H143.
41. Landeros RV, Jobe SO, Aranda-Pino G, Lopez GE, Zheng J, Magness RR. Convergent ERK1/2, p38 and JNK mitogen activated protein kinases (MAPKs) signalling mediate catecholestradiol-induced proliferation of ovine uterine artery endothelial cells. *J Physiol.* 2017;595(14):4663-4676.
42. Bird IM, Sullivan JA, Di T, et al. Pregnancy-dependent changes in cell signaling underlie changes in differential control of vasodilator production in uterine artery endothelial cells *Endocrinology.* 2000;141(3):1107-1117.
43. Jobe SO, Ramadoss J, Koch JM, Jiang Y, Zheng J, Magness RR. Estradiol-17beta and its cytochrome P450- and catechol-O-methyltransferase-derived metabolites stimulate proliferation in uterine artery endothelial cells: role of estrogen receptor-alpha

- versus estrogen receptor-beta. *Hypertension*. 2010;55(4):1005-1011.
44. Kanasaki K, Palmsten K, Sugimoto H, et al. Deficiency in catechol-O-methyltransferase and 2-methoxyestradiol is associated with pre-eclampsia. *Nature*. 2008;453(7198):1117-1121.
 45. Lai KB, Sanderson JE, Yu CM. The regulatory effect of norepinephrine on connective tissue growth factor (CTGF) and vascular endothelial growth factor (VEGF) expression in cultured cardiac fibroblasts. *Int J Cardiol*. 2013;163(2):183-189.
 46. Lunell NO, Nylund LE, Lewander R, Sarby B. Uteroplacental blood flow in pre-eclampsia measurements with indium-113m and a computer-linked gamma camera. *Clin Exp Hypertens B*. 1982;1(1):105-117.
 47. Palmer SK, Moore LG, Young D, Cregger B, Berman JC, Zamudio S. Altered blood pressure course during normal pregnancy and increased preeclampsia at high altitude (3100 meters) in Colorado. *Am J Obstet Gynecol*. 1999;180(5):1161-1168.
 48. Palmer SK, Zamudio S, Coffin C, Parker S, Stamm E, Moore LG. Quantitative estimation of human uterine artery blood flow and pelvic blood flow redistribution in pregnancy. *Obstet Gynecol*. 1992;80(6):1000-1006.
 49. Chambliss KL, Shaul PW. Estrogen modulation of endothelial nitric oxide synthase. *Endocr Rev*. 2002;23(5):665-686.
 50. Chambliss KL, Yuhanna IS, Anderson RG, Mendelsohn ME, Shaul PW. ERbeta has nongenomic action in caveolae. *Mol Endocrinol*. 2002;16(5):938-946.
 51. Parada-Bustamante A, Orihuela PA, Rios M, et al. Catechol-o-methyltransferase and methoxyestradiols participate in the intraoviductal nongenomic pathway through which estradiol accelerates egg transport in cycling rats. *Biol Reprod*. 2007;77(6):934-941.
 52. Sutherland TE, Schuliga M, Harris T, et al. 2-methoxyestradiol is an estrogen receptor agonist that supports tumor growth in murine xenograft models of breast cancer. *Clin Cancer Res*. 2005;11(5):1722-1732.
 53. Barchiesi F, Jackson EK, Fingerle J, Gillespie DG, Odermatt B, Dubey RK. 2-Methoxyestradiol, an estradiol metabolite, inhibits neointima formation and smooth muscle cell growth via double blockade of the cell cycle. *Circ Res*. 2006;99(3):266-274.
 54. Barchiesi F, Lucchinetti E, Zaugg M, et al. Candidate genes and mechanisms for 2-methoxyestradiol-mediated vasoprotection. *Hypertension*. 2010;56(5):964-972.
 55. Kim J, Ahn S, Ren XR, et al. Functional antagonism of different G protein-coupled receptor kinases for beta-arrestin-mediated angiotensin II receptor signaling. *Proc Natl Acad Sci U S A*. 2005;102(5):1442-1447.
 56. Kobilka BK, Deupi X. Conformational complexity of G-protein-coupled receptors. *Trends Pharmacol Sci*. 2007;28(8):397-406.
 57. Zidar DA, Violin JD, Whalen EJ, Lefkowitz RJ. Selective engagement of G protein coupled receptor kinases (GRKs) encodes distinct functions of biased ligands. *Proc Natl Acad Sci U S A*. 2009;106(24):9649-9654.
 58. Kumar R, McEwan IJ. Allosteric modulators of steroid hormone receptors: structural dynamics and gene regulation. *Endocr Rev*. 2012;33(2):271-299.
 59. Wang L, Martin B, Brenneman R, Luttrell LM, Maudsley S. Allosteric modulators of G protein-coupled receptors: future therapeutics for complex physiological disorders. *J Pharmacol Exp Ther*. 2009;331(2):340-348.
 60. Brailoiu E, Dun SL, Brailoiu GC, et al. Distribution and characterization of estrogen receptor G protein-coupled receptor 30 in the rat central nervous system. *J Endocrinol*. 2007;193(2):311-321.
 61. Filardo EJ, Thomas P. Minireview: G protein-coupled estrogen receptor-1, GPER-1: its mechanism of action and role in female reproductive cancer, renal and vascular physiology. *Endocrinology*. 2012;153(7):2953-2962.
 62. Lindsey SH, da Silva AS, Silva MS, Chappell MC. Reduced vasorelaxation to estradiol and G-1 in aged female and adult male rats is associated with GPR30 downregulation. *Am J Physiol Endocrinol Metab*. 2013;305(1):E113-E118.
 63. Broughton BR, Miller AA, Sobey CG. Endothelium-dependent relaxation by G protein-coupled receptor 30 agonists in rat carotid arteries. *Am J Physiol Heart Circ Physiol*. 2010;298(3):H1055-H1061.
 64. Chakrabarti S, Davidge ST. G-protein coupled receptor 30 (GPR30): a novel regulator of endothelial inflammation. *PLoS One*. 2012;7(12): e52357.
 65. Rowlands DJ, Chapple S, Siow RC, Mann GE. Equol-stimulated mitochondrial reactive oxygen species activate endothelial nitric oxide synthase and redox signaling in endothelial cells: roles for F-actin and GPR30. *Hypertension*. 2011;57(4):833-840.
 66. Buhimschi I, Yallampalli C, Chwalisz K, Garfield RE. Pre-eclampsia-like conditions produced by nitric oxide inhibition: effects of L-arginine, D-arginine and steroid hormones. *Hum Reprod*. 1995;10(10):2723-2730.
 67. Edwards DL, Arora CP, Bui DT, Castro LC. Long-term nitric oxide blockade in the pregnant rat: effects on blood pressure and plasma levels of endothelin-1. *Am J Obstet Gynecol*. 1996;175(2):484-488.
 68. Yallampalli C, Garfield RE. Inhibition of nitric oxide synthesis in rats during pregnancy produces signs similar to those of pre-eclampsia. *Am J Obstet Gynecol*. 1993;169(5):1316-1320.
 69. Stanley JL, Andersson IJ, Poudel R, et al. Sildenafil citrate rescues fetal growth in the catechol-O-methyl transferase knockout mouse model. *Hypertension*. 2012;59(5):1021-1028.
 70. Zoma WD, Baker RS, Clark KE. Effects of combined use of sildenafil citrate (Viagra) and 17beta-estradiol on ovine coronary and uterine hemodynamics. *Am J Obstet Gynecol*. 2004;190(5):1291-1297.