Identification of Differential ER-Alpha Versus ER-Beta Mediated Activation of eNOS in Ovine Uterine Artery Endothelial Cells

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ABSTRACT

Endothelial nitric oxide (NO) production is partly responsible for maintenance of uterine vasodilatation during physiologic states of high circulating estrogen levels, e.g., pregnancy. Although 3%–5% of estrogen receptors (ER-alpha/beta) localize to the endothelial plasmalemma, these receptors are responsible for the nongenomic vasodilator responses. Estradiol induces endothelial NO synthase (eNOS) activation to increase NO production; however, it is unknown if eNOS regulation is dependent on both ERs. We hypothesize that ER-alpha and/or ER-beta are capable of changing eNOS phosphorylation and increasing NO production in uterine artery endothelial cells (UAECs). UAECs were 1) treated with vehicle or increasing concentrations (0.1–100 nM) or timed treatments (0–30 min) of estradiol and 2) pretreated with the inhibitors ICI 182,780 (nonspecific ER), 1,3-Bis(4-hydroxyphenyl)-5[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride (MPP; ER-alpha specific), or 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP; ER-beta specific) followed by estradiol to analyze the changes in eNOS phosphorylation. These data demonstrate that 1) eNOS phosphorylation changes occur via ER-alpha- and ER-beta-dependent mechanisms and 2) ER-alpha and ER-beta can both increase NO levels independently from each other.

INTRODUCTION

Pregnancy-induced elevations in uterine blood flow (UBF) are temporally associated with concurrent increases in plasma estradiol-17β (E2β) levels [1], which are thought to be partly responsible for increasing the expression and activity of endothelial NO synthase (eNOS) [2–4]. Utilizing ovariectomized animal models, several laboratories have demonstrated a marked increase in UBF after exogenous administration of E2β [5–7]. Additionally, in vivo studies have established the physiologic cause-effect relationship between systemic administration of E2β and local inhibition of either estrogen receptors (ERs; with ICI 182,780) or NO synthase (with L-NAME) [8–10]. Furthermore, during pregnancy these specific inhibitors, ICI 182,780 and L-NAME, also partially reduced (25%–30%) uteroplacental blood flow from its maximal levels, thus delineating the relationship of endogenous E2β and de novo synthesis of NO through eNOS [8, 10, 11]. E2β is known to induce its estrogenic effects by binding to its two classical receptors, ERα and ERβ [12]. It is clinically important to understand the role of both ERs in the uterine and the placental vasculature because both ERs were shown to induce estrogenic effects in a tissue- and species-specific manner while also differing in their expression patterns in uterine arteries isolated from normal versus severe preeclampsia patients [10, 13–19]. Studies have detailed the E2β-ERα molecular mechanism in the cardiovascular system and to some extent in the uterine vasculature [10, 13–16, 20–22]. However, studies identifying the E2β-ERβ role or the molecular pathways involved in increasing NO bioavailability in the uterine vasculature are still lacking [10, 13–16, 20–22].

Endothelial NO synthase has a complex regulatory mechanism that correctly targets the enzyme to the caveolae and controls the NO bioavailability in the endothelium [13, 23]. It is regulated by protein-protein interactions and posttranslational modifications that can render the enzyme more or less active [13, 23–25]. For example, changes in stimulatory phosphorylation sites Ser1177 eNOS and Ser635 eNOS or the inhibitory phosphorylation site Thr495 eNOS function to tightly control the amount of NO produced by endothelial cells [13, 26–32]. Treatments of endothelial cells with E2β [14, 33], as well as the calcium-mobilizing agonists such as ATP [25, 31] and bradykinin [34], can induce the posttranslational modifications of eNOS resulting in activation. However, the
comprehensive changes in all of the three key eNOS phosphorylation sites and their overall role in NO production under the exogenous stimulation via ERα and/or ERβ in vitro are still unclear. Therefore, we hypothesize that ERα and/or ERβ will alter eNOS stimulatory and inhibitory phosphorylation sites while also increasing NO production in uterine artery endothelial cells (UAECs). The aims for this study are 1) to establish the time and dose relationship between ERα and ERβ and changes in eNOS stimulatory phosphorylation sites and 2) to identify and compare the roles of ERα and ERβ in altering the phosphorylation state of eNOS and NO production.

MATERIALS AND METHODS

The Animal Care and Use Committee of the University of Wisconsin-Madison approved procedures for obtaining uterine arteries from pregnant ewes (UAECs) at Day 120–130; tem = 147. The collected uterine arteries were then used for endothelial cell isolation using collagenase digestion procedures [35] and fluorescent-activated cell sorting (FACS) with Alexa 488 acetylated low-density lipoprotein (LDL) (L-23380; Invitrogen).

Cell Culture Preparation

The isolated and validated UAECs were cultured in growth medium HyClone minimal essential medium with Earle with 20% fetal bovine serum (FBS), 100 mg/ml penicillin, and 100 mg/ml streptomycin and propagated [35]. For experiments, passage 3, UAECs were plated in T75 flasks containing phenol-free endothelial basal medium (EBM; Lonza) supplemented with 20% FBS and 1% penicillin-streptomycin. Cells were treated with appropriate drug treatment and lysed for Western blotting and/or the media were collected for NO metabolites (NO2− + NO3−) analysis.

Experimental Treatments and Receptor Blockade

Experiments were performed in at least three different UAEC preparations. For time and concentration-response studies, UAECs grown in six-well plates were serum starved in EBM and media was changed to modified Krebs buffer 30 min prior to treatments. For experiments, passage 3, UAECs were plated in six-well plates and serum starved in EBM and media was changed to modified Krebs buffer 30 min prior to treatments.

For time and concentration-response studies, UAECs grown in six-well plates were serum starved in EBM, and the medium was replaced with EBM vehicle (control) or EBM containing E2 (Cat. No. 2824; Tocris) at 0.01, 0.1, 1.0, 10, and 100 nmol/L for 10 min or with EBM containing 10 nmol/L for 0, 5, 10, 15, and 30 min. The 10 nmol/L E2 concentration was chosen based on the concentration-response studies. The specific agonist treatments were done using 4,4′,4′-A′-4′-propyl-[1H]-pyrazole-1,3,5-triyltri[phenyl (PPT); ERα-specific agonist with ~410-fold binding preference towards ERα over ERβ [12]; Cat. No. 1426; Tocris) at 0.01, 0.1, 1.0, and 100 nmol/L for 20 min and 2,3-bis-(4-hydroxyphenyl)-propionitrile (DPN; ERβ-specific agonist with a ~70-fold binding preference towards ERβ over ERα [36]; Cat. No. 1494; Tocris) at 0.01, 0.1, 1.0, and 50 nmol/L for 20 min. Samples were lysed and analyzed with Western immunoblotting.

For analysis of NO metabolites, NO2−, and NO3− (NO2−), UAECs were treated with vehicle modified Krebs buffer (HEPES 25 mmol/L, NaCl 125 mmol/L, KCl 5 mmol/L, MgSO4·7H2O 1 mmol/L, KH2PO4 1 mmol/L, glucose 6 mmol/L, L-glutamate 6 mmol/L, adjusted to pH = 7.4) or modified Krebs buffer containing E2 at 10 or 100 nmol/L; PPT at 0.01 nmol/L; DPN at 1.0 nmol/L, or ATP at 100 μmol/L for 0, 10, 20, and 30 min.

ER blockade was performed using the nonselective estrogen receptor ICI 182,780 (7x,17β)-[9-[4,4,5,5-pentafluoropentylsulfonyl]nonyl]estr-1,3,5(10)-triene-3,17-diol; Cat No. 1047; Tocris) at 100 nmol/L, 1-bis-(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride at 100 nmol/L (MPP dihydrochloride; ERβ-specific antagonist with a ~200-fold binding preference towards ERβ over ERα [37]; Cat. No. 1991; Tocris), or 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol at 100 nmol/L (PHTPP: ERβ-specific antagonist with a ~36-fold binding preference towards ERβ over ERα [38]; Cat. No. 2662; Tocris). ERs were blocked by pretreating UAECs with the inhibitor for 30 min.

Protein Extraction and Western Immunoblot Analysis

The total cell extracts were collected using a disposable cell scraper, vortexed, and clarified by centrifugation (13000 × g, 5 min). The protein content of the samples was measured by a Bio-Rad procedure using bovine serum albumin as the standard. Aliquots of the extracts were frozen at −80°C until Western blot analysis could be performed. Equal amounts of total cell lysates were heated to denatured (95°C, 10 min) in Laemmli buffer, separated on precast 4–20% SDS-PAGE, and electrophoretically (100 V, 55 min) transferred to polyvinylidene fluoride membranes [20, 39]. Membranes were used to identify Ser1177eNOS (1:3000 dilution; product no. 9571; Cell Signaling Technologies), Ser635eNOS and Ser415eNOS and the inhibitory phosphorylation site Thr495eNOS and/or eNOS starting at the physiological concentration of 0.1 nmol/L (Fig. 1A). Ser1177eNOS increased to 6.1-fold at 0.1 nmol/L, 5.3-fold at 1.0 nmol/L, 7.8-fold at 10 nmol/L, and 2.34-fold at 100 nmol/L E2. Ser635eNOS also increased 9.8-fold at 0.1 nmol/L, 1.2-fold at 1.0 nmol/L, 7.8-fold at 10 nmol/L, and 6.98-fold at 100 nmol/L E2. In addition, the inhibitory phosphorylation site Thr495eNOS significantly decreased to 0.24-fold with 10 nmol/L and 100 nmol/L E2 treatments, with an increase of 4.1-fold with 0.1 and no change observed with 1.0 nmol/L (Fig. 1A). We thus selected 10 nmol/L E2 as the optimal concentration to quantify the change in eNOS phosphorylation pattern from 0 to 30 min (Fig. 1C).

We observed an increase in Ser1177eNOS starting at 5 min and lasting up to 30 min E2 treatment (2.4-, 4.1-, 1.5-, and 3.8-fold, respectively; Fig. 1C). We also observed an E2-induced increase in Ser635eNOS that remained elevated for the same period of time when compared to Ser1177eNOS, 5–30 min (4.2-, 4.1-, 4.3-, and 6.4-fold, respectively). The reduction in Thr495eNOS phosphorylation reached statistical significance after the 15- and 30-min E2 treatments (0.8- and 0.2-fold).

Nitrate and Nitrite (NO2− and NO3−) Analysis

NO levels were measured using NOx Analyzer (ENO-30) and Insight autoanalyzer (AS-700) from Eicom Corporation. NOx values were calculated per manufacturer’s instructions after subtracting the value of the blank well to remove background peak values.

The UAEC preparations (n = 4) used for agonists and antagonist treatments were plated in six-well plates and serum starved in EBM and media was changed to modified Krebs buffer 30 min prior to treatments.

Statistical Analysis

Data are representative of n = 3 or n = 4 separate experiments and presented as means ± SEM; n = UAECs isolated from different animals. ANOVA followed by post hoc Bonferroni (vs. corresponding control) multiple-comparison test or Student t-test when appropriate was used for statistical analysis. Results were considered significant at P < 0.05. Statistical analyses were performed with GraphPad Prism 5.0b software.

RESULTS

Estrogen-Induced Dose-Response and Time-Course Curves Identifying Changes in eNOS Phosphorylation in UAECs

Dose-response curves were designed to identify the estrogen dosage that can both increase stimulatory phosphorylation sites and also decrease the inhibitory site. When compared to control samples, E2 increased stimulatory phosphorylation sites at sites Ser1177eNOS and Ser635eNOS starting at the physiological concentration of 0.1 nmol/L (Fig. 1A). Ser1177eNOS increased to 6.1-fold at 0.1 nmol/L, 5.3-fold at 1.0 nmol/L, 7.8-fold at 10 nmol/L, and 2.34-fold at 100 nmol/L E2. Ser635eNOS also increased 9.8-fold at 0.1 nmol/L, 1.2-fold at 1.0 nmol/L, 2.82-fold at 10 nmol/L, and 6.98-fold at 100 nmol/L E2. In addition, the inhibitory phosphorylation site Thr495eNOS significantly decreased to 0.24-fold with 10 nmol/L and 100 nmol/L E2 treatments, with an increase of 4.1-fold with 0.1 and no change observed with 1.0 nmol/L (Fig. 1A). We thus selected 10 nmol/L E2 as the optimal concentration to quantify the change in eNOS phosphorylation pattern from 0 to 30 min (Fig. 1C). We observed an increase in Ser1177eNOS starting at 5 min and lasting up to 30 min E2 treatment (2.4-, 4.1-, 1.5-, and 3.8-fold, respectively; Fig. 1C). We also observed an E2-induced increase in Ser635eNOS that remained elevated for the same period of time when compared to Ser1177eNOS, 5–30 min (4.2-, 4.1-, 4.3-, and 6.4-fold, respectively). The reduction in Thr495eNOS phosphorylation reached statistical significance after the 15- and 30-min E2 treatments (0.8- and 0.2-fold).

eNOS Phosphorylation Changes in Response to ERα or ERβ Activation in UAECs

To determine if ERα and ERβ can alter eNOS phosphorylation state, UAECs were treated with E2 (10 nmol/L) or
ERα/β-specific agonists, E2β and PPT (ER-α-specific agonist) similarly increased eNOS stimulatory phosphorylation at sites Ser1177eNOS and Ser635eNOS (Fig. 2, A and B). For eNOS, E2β induced a 1.49-fold increase; PPT increased eNOS to 1.12-fold at 0.1 nmol/L, 1.086-fold at 1.0 nmol/L, 0.95-fold at 10 nmol/L, and 1.27-fold at 100 nmol/L. E2β induced a 1.93-fold increase in Ser635eNOS whereas PPT increased 1.21-fold at 0.1 nmol/L, 1.82-fold at 1.0 nmol/L, 1.4-fold at 10 nmol/L, and 2.0-fold at 100 nmol/L. E2β induced a decrease in the inhibitory phosphorylation at site Thr495eNOS to 0.71-fold, and PPT decreased phosphorylation at this site to 0.71-fold at 0.1 nmol/L, 0.66-fold at 1.0 nmol/L, 0.64 at 10 nmol/L, and 0.65-fold at 100 nmol/L (Fig. 2C). Dose-response curves for the ERβ-specific agonist DPN were also constructed. We observed no change in the stimulatory phosphorylation sites Ser1177eNOS or Ser635eNOS (Fig. 3A, A and B) under any DPN treatment dose tested. E2β induced a decrease in the inhibitory phosphorylation at site Thr495eNOS to 0.84-fold. DPN also decreased the inhibitory phosphorylation of Thr495eNOS to 0.82-fold at 0.1 nmol/L, 0.82-fold at 1.0 nmol/L, 0.84-fold at 10 nmol/L, and 0.85-fold at 50 nmol/L (Fig. 3C).

**Effects of Nonspecific ER Inhibitor ICI 182,780 on E2β-Induced Changes in eNOS Phosphorylation Sites in UAECs**

UAECs were pretreated with ICI 182,780 to determine if ERα and ERβ alter eNOS phosphorylation state. ICI 182,780 alone had no effect on stimulatory phosphorylation sites Ser1177eNOS and Ser635eNOS (Fig. 4, A and B), whereas the observed E2β-induced increases in Ser1177eNOS (2.01-fold) and Ser635eNOS (1.48-fold) were blunted by ICI 182,780 pretreatment (Fig. 4, A and B). The inhibitory phosphorylation at site Thr495eNOS was removed with E2β treatment (0.86-fold); however, the reduction in Thr495eNOS was also observed with ICI 182,780 alone (0.93-fold at 0.1 μmol/L, 0.88-fold at 1 μmol/L, and 0.83-fold at 10 μmol/L) or when followed with E2β treatment (0.84-fold at 0.1 μmol/L, 0.85-fold at 1 μmol/L, and 0.81-fold at 10 μmol/L) (Fig. 4C).

**Effects of ERα- and ERβ-Mediated Increase in NOx Levels (NO2 and NO3) in UAECs**

We quantified the total, basal, and stimulated NOx levels (NO2 + NO3) using an HPLC-based assay by using E2β, ERα-specific agonist (PPT), ERβ-specific agonist (DPN), and ATP (positive control) treatments (Fig. 5, A and B). Basal percentage changes of total NOx levels of UAECs were quantified as 16%, 0.7%, and 9% at 10, 20, and 30 min, respectively (Fig. 5A). Concentrations of 10 and 100 nmol/L E2β increased NOx levels by 32% and 62% at 10 min, 64% and 39% at 20 min, and 26% and 20% at 30 min. The 1.0 nmol/L...
PPT and DPN increased NOx levels by 40% and 47% at 10 min, 52% and 34% at 20 min, and 28% and 31% at 30 min. The 100 μmol/L ATP increased NOx levels by 23% at 10 min, 28% at 20 min, and 43% at 30 min.

ICI 182,780 alone did not alter basal NOx levels in UAECs and unexpectedly did not reduce the E2β-induced increase in NOx levels (Fig. 5C). Antagonism with 100 nmol/L ERα-specific antagonist (MPP) and ERβ-specific antagonist (PHTPP) were also studied. ERα blockade did not abolish the NOx increase stimulated by the 10 nmol/L E2β treatment (70%) demonstrating residual ERβ actions. The ERβ blockade also did not abolish the NOx increase stimulated by the 10 nmol/L E2β treatment (20%) demonstrating residual ERα actions. However, combining MPP + PHTPP antagonism, we observed the complete abrogation of basal (−16%) and E2β-induced increase in NOx levels (−54%) (Fig. 5C), demonstrating that both receptors must be blocked in order to observe a significant reduction in basal and stimulated NOx levels. Finally, these data also validate those observation obtained from ERα/ERβ-specific agonists shown in Figure 5B, where both receptors were capable of increasing NOx levels when activated.

**DISCUSSION**

The first aim for this study was to establish the relationship between dose/time and the E2β-induced changes in eNOS phosphorylation. The findings herein demonstrated that E2β induced changes in the three key phosphorylation sites of eNOS, indicating that eNOS was rendered more active, i.e., there were higher detected levels of Ser1177 eNOS and Ser635 eNOS and lower detected levels of Thr495 eNOS. Additionally, we observed the impact on
basal NOx levels only when both receptors were blocked with their specific agonists. In UAECs, we have previously identified that E2b and E2b-bovine serum albumin conjugate induces an overall increase in eNOS phosphorylation and an increase in the total NOx production, suggesting a role for membrane ERs [14]. However, it was not until this current study that we were able to test the roles for each ER in altering eNOS phosphorylation patterns and the increase in NOx levels in UAECs. Herein, we identified that E2b can alter three key eNOS phosphorylation sites (Fig. 1), rendering the enzyme more active, thus increasing NOx levels (Fig. 5). The activation of ERα (via PPT) increased Ser1177eNOS and Ser635eNOS while also decreasing Thr495eNOS when using 1 nmol/L PPT, a concentration close to its half-maximal response and shown to have ~70-fold preference for ERβ over ERα [36]. These observations identified the differences in eNOS posttranslational regulation that occur under the activation of each receptor. Additionally, it is noteworthy that Ser635eNOS is largely influential over the sustained NO production in UAECs (>30 min) versus the immediate short-term increase in Ser1177eNOS [25, 40] and was also reported to be decreased when protein kinase A (PKA) [40] or mitogen-activated protein kinase kinase (MEK) [13] pathways were blocked. Moreover, we observed the decrease in Thr495eNOS with the activation of either ER. Surprisingly, we also observed a decrease in Thr495eNOS with ICI 182,780, which may point to an additional ER or ER regulatory protein in UAECs yet to be explored in the uterine vasculature. In this regard, GPER-1 is a seven-transmembrane-spanning G-protein-coupled receptor that is activated by ICI 182,780 [41]. Several investigators have shown that GPER-1 can induce rapid estrogenic effects in ERα-positive cancer cells, human endothelial cells, and B-lymphocytes, and it has been implicated in having physiological functions in reproductive

FIG. 3. Changes in eNOS phosphorylation in response to ERβ activation in UAECs. A) At 20 min, E2β increased Ser1177eNOS stimulatory phosphorylation by 1.35-fold whereas DPN had no effect on this site. B) E2β increased Ser635eNOS stimulatory phosphorylation by 1.48-fold whereas DPN did not increase phosphorylation at this site. C) E2β induced a decrease in the inhibitory phosphorylation at site Thr495eNOS by 0.84-fold. DPN also decreased the inhibitory phosphorylation of Thr495eNOS to 0.82-fold at 0.1 nmol/L, 0.82-fold at 1.0 nmol/L, 0.84-fold at 10 nmol/L, and 0.85-fold at 50 nmol/L. D) Representative Western blots for phosphorylated eNOS, total eNOS, and β-actin. Cnt, control. *P < 0.05.

In UAECs, we have previously identified that E2β and E2β-bovine serum albumin conjugate induces an overall increase in eNOS phosphorylation and an increase in the total NOx production, suggesting a role for membrane ERs [14]. However, it was not until this current study that we were able to test the roles for each ER in altering eNOS phosphorylation patterns and the increase in NOx levels in UAECs. Herein, we identified that E2b can alter three key eNOS phosphorylation sites (Fig. 1), rendering the enzyme more active, thus increasing NOx levels (Fig. 5). The activation of ERα (via PPT) increased Ser1177eNOS and Ser635eNOS while also decreasing Thr495eNOS when using 1 nmol/L PPT, a concentration close to its half-maximal response and shown to have ~70-fold preference for ERβ over ERα [36]. These observations identified the differences in eNOS posttranslational regulation that occur under the activation of each receptor. Additionally, it is noteworthy that Ser635eNOS is largely influential over the sustained NO production in UAECs (>30 min) versus the immediate short-term increase in Ser1177eNOS [25, 40] and was also reported to be decreased when protein kinase A (PKA) [40] or mitogen-activated protein kinase kinase (MEK) [13] pathways were blocked. Moreover, we observed the decrease in Thr495eNOS with the activation of either ER. Surprisingly, we also observed a decrease in Thr495eNOS with ICI 182,780, which may point to an additional ER or ER regulatory protein in UAECs yet to be explored in the uterine vasculature. In this regard, GPER-1 is a seven-transmembrane-spanning G-protein-coupled receptor that is activated by ICI 182,780 [41]. Several investigators have shown that GPER-1 can induce rapid estrogenic effects in ERα-positive cancer cells, human endothelial cells, and B-lymphocytes, and it has been implicated in having physiological functions in reproductive
and cardiovascular systems [41, 42]. However, GPER-1 expression or cellular location is unknown in UAECs. But it is important to note that Thr495eNOS is a key regulatory site because of its location within the Ca$^{2+}$/calmodulin-binding domain of eNOS; thus, the removal of this phospho group facilitates eNOS binding to Ca$^{2+}$/calmodulin after an increase of intracellular Ca$^{2+}$ and increasing enzymatic activity and NO bioavailability [13, 40].

The changes in eNOS phosphorylation patterns also validate the functional increase in NOx levels observed under PPT and, to some extent, DPN stimulation observed during our current study of UAECs (Fig. 5B). Our reported increase in NOx levels is in agreement with the relaxation studies conducted in human uterine, placental [19], and rat pulmonary artery [43] studies that also tested vasodilation with ER-specific agonists (PPT and DPN). However, our results differed from aortic artery studies of mice containing a whole-body ER$^a$ or ER$^b$ knockout (KO) phenotype [44]. Darblade et al. identified ER$^a$ to be the sole ER responsible for acute vasodilatory effects in mouse thoracic aortas isolated from ER$^a^{−/−}$ or ER$^b^{−/−}$ KO versus wild-type mice [44]. The effects of a global receptor KO versus the tissue-specific KO responses may explain the difference observed between these studies. Additionally, the circulating estrogens of female versus male subjects can also influence the ER protein levels in a tissue-specific manner, which, in turn, can influence vasodilatory responses via a specific ER [7, 23]. Lastly, our published work highlights the effects of the circulating estrogen levels over the expression pattern of the ERs in reproductive versus nonreproductive vasculature observed during the ovarian cycle and pregnancy [5, 23, 45]. ER$^a$ maintained the same level of expression in almost all the vasculatures tested and isolated from animals in the ovarian cycle or pregnant state [23]. In contrast, ER$^b$ expression levels increased significantly in all the reproductive vasculature isolated from pregnant animals, suggesting a pregnancy-specific adaptation [23]. In addition to changes in ER expression levels, other pregnancy-specific adaptations are observed in freshly isolated UAECs and include increases in
eNOS expression, sustained Ca\(^{2+}\) signaling, and NO production [46]. We also reported enhanced UAEC proliferation orchestrated via Er\(\alpha\)/Er\(\beta\) mechanisms; proliferation was used as a marker for physiological uterine angiogenesis necessary during pregnancy [20].

The current study demonstrated the increase in NO levels under Er\(\beta\) in an Er-specific manner. The blockade studies using MPP or PHTPP (Er\(\alpha\)- and Er\(\beta\)-specific antagonist, respectively) identified that when Er\(\alpha\) or Er\(\beta\) are blocked, the increase in NOx levels observed with Er\(\beta\) treatment was not blunted; on the contrary, the blockade of Er\(\alpha\) showed NOx levels that were greater than Er\(\beta\) alone, which binds to both Er\(\alpha\) and Er\(\beta\) (Fig. 5B). The mechanisms required for Er\(\alpha\) and Er\(\beta\) for translocation to the plasma membrane have been extensively studied [47]. However, the exact location of Er\(\alpha\) and Er\(\beta\) at the plasma membrane is not completely understood. Plasma membrane fractionation studies published by Chambliss and Shaul have identified Er\(\alpha\) within the specialized signaling domains of the plasma membrane called caveolae microdomains [33, 48]. Er\(\alpha\) localizes within the caveolae “signalsomes complexes” along with Cav-1 and eNOS to facilitate the signaling mechanism for rapid and controlled NO production [33, 48]. The location of Er\(\beta\) at the plasma membrane and/or within the caveolae is a topic that requires further study; however, it is also possible that differences in Er location may provide an unappreciated alternative signaling mechanism for endogenous Er\(\beta\) to activate eNOS in UAECs. The alternative signaling mechanism may explain the increased level of total NOx observed after the blockade of Er\(\alpha\) in our studies (Fig. 5B). Alternatively, differences in NOx levels observed during this study may also point to pregnancy-specific adaptations in the redundant signaling machinery documented in UAECs, which affect the complex eNOS regulation [13, 20, 35].

Basal NO levels are important in the homeostatic maintenance of the systemic and uterine vascular tone. Several investigators have shown, using animal models, that the decrease in basal NO bioavailability is involved in the development of hypertension [49, 50]. Our data show that when both ERs are blocked with their specific antagonists (MPP + PHTPP) there is a reduction in total basal NOx levels to lower levels than those of controls (Fig. 5C). These results are in line with studies that analyzed the effects on vasodilation, both the basal and stimulated NO production, in mouse aorta rings [51] and the endothelial-dependent relaxation of the forearm from offspring of parents with essential hypertension [52]. However, the present study is the first to show that the concurrent blockade of endogenous Er\(\alpha\) and Er\(\beta\) can lead to diminished basal NOx levels, which are further exacerbated by estrogen treatment (Fig. 5C). Thus, we observed a link between ER dysregulation and the basal NOx levels in UAECs (Fig. 5A). However, the paradigm that estrogen treatment further decreases NOx levels may be explained by the dysregulation of ERs leading to altered eNOS posttranslational modifications, protein-protein interactions, and/or intracellular Ca\(^{2+}\) that are required for the enzyme’s activity in UAECs [13, 14]. The basal NO regulation may point to a critical step in vasodilatory dysfunction observed in pregnancy-related hypertensive disorders given that estrogens and estrogen metabolite synthesis become aberrant in patients with severe preeclampsia [53] and ER levels change during the ovarian cycle and pregnancy [39].

In summary, the present study demonstrates that estrogen via the activation of both ERs (Er\(\alpha\)/Er\(\beta\)) induced an increase in eNOS stimulatory phosphorylation sites Ser\(^{1177}\)eNOS and Ser\(^{635}\)eNOS, a decrease in the inhibitory phosphorylation site Thr\(^{305}\)eNOS, and an increase in total NOx levels in UAECs. We also provide evidence that the activation of Er\(\alpha\) mirrors the eNOS phosphorylation pattern observed with estrogen and increased total NOx levels. In contrast, Er\(\beta\) activation only induced a reduction in the inhibitory phosphorylation site, Thr\(^{305}\)eNOS, but Er\(\beta\) activation still induced an increase in total NOx levels, thus identifying an important regulatory site for eNOS in UAECs. These data demonstrate that activation of either Er\(\alpha\) or Er\(\beta\) leads to the increase in NO bioavailability. Lastly, another important finding from this study is the observed dysregulation of basal NOx levels that occurs only when Er\(\alpha\) and Er\(\beta\) are concurrently antagonized with their respective inhibitors. Basal NO levels are an important mechanism for vascular tone. Therefore, future studies may...
need to test the basal dysregulation we observed in vitro in an ex vivo and/or in vivo setting and whether this dysregulation is involved in the progression of hypertensive disorders such as preeclampsia.

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