Title

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Journal

Biology of Reproduction, 100(2)

ISSN

0006-3363

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Publication Date

2019-02-01

DOI

10.1093/biolre/ioy207

Peer reviewed

Biology of Reproduction, 2019, 100(2), 514–522
doi:10.1093/biolre/ioy207
Research Article
Advance Access Publication Date: 2 October 2018

Research Article

$E_2\beta$ stimulates ovine uterine artery endothelial cell H_2S production in vitro by estrogen receptor-dependent upregulation of cystathionine β-synthase and cystathionine γ-lyase expression†

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†__Grant Support:__ This study was supported in part by National Institutes of Health (NIH) grants RO1 HL70562 and R21 HL98746 to DBC and PO1 HD38843 and RO1 HL117341 to RRM. TJL was an American Heart Association (AHA) Pre-doctoral Fellow (AHA14PRE18570033). The content is solely the responsibility of the authors and does not necessarily the official views of NIH and AHA.
Edited by Dr. Romana Nowak, PhD, University of Illinois Urbana-Champaign

Received 5 October 2017; Revised 29 August 2018; Accepted 27 September 2018

Abstract

Endogenous hydrogen sulfide (H_2S) is a potent vasodilator and proangiogenic second messenger synthesized from L-cysteine by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CTH). Estrogens are potent vasodilators that stimulate H_2S biosynthesis in uterine arteries (UA) in vivo; however, the underlying mechanisms are unknown. We hypothesized that estrogens stimulate H_2S biosynthesis in UA endothelial cells (UAEC) via specific estrogen receptor (ER)-dependent mechanisms. In cultured primary UAEC, treatment with estradiol-17β ($E_2\beta$) stimulated CBS and CTH mRNAs and proteins in a time- and concentration-dependent fashion. As little as 0.1 nM $E_2\beta$ was effective in increasing CBS and CTH expressions and these stimulatory effects maximized with 10–100 nM $E_2\beta$ at 48–72 h. $E_2\beta$ also activated CBS and CTH promoters in UAEC, leading to CBS and CTH expression. Treatment with $E_2\beta$ stimulated H_2S production, which was blocked by specific inhibitors of either CBS or CTH and their combination and the ER antagonist ICI 182780. Treatment with either specific agonist of ERα or ERβ stimulated both CBS and CTH mRNA and protein expressions and H_2S production to levels similar to that of $E_2\beta$. Specific antagonist of either ERα or ERβ blocked $E_2\beta$-stimulated CBS and CTH mRNA and protein expressions and H_2S production. Combinations of either ERα or ERβ agonists or their antagonists had no additive effects. Thus, $E_2\beta$ stimulates H_2S production by upregulating CBS and CTH mRNA and protein expressions through specific ERα or ERβ-dependent *CBS* and *CTH* transcription in UAEC in vitro.

Summary Sentence

Estradiol-17β stimulates uterine artery endothelial cell hydrogen sulfide biosynthesis.

Key words: estrogen, estrogen receptors, hydrogen sulfide, endothelium, uterine artery, vasodilation.

Introduction

Circulating estrogens are significantly elevated during the follicular phase of the ovarian cycle and pregnancy [1, 2]. Estrogens are potent vasoactive hormones that dilate selected vasculature beds in organs throughout the body with the greatest response in reproductive tissues, especially the uterus [3, 4]. The vasodilatory effect of estrogens is of major physiological significance during pregnancy because uterine vasodilation as measured by a rise in uterine blood flow (UBF) elevates more than 50- to 80-fold during pregnancy to facilitate the bidirectional mother–fetus exchanges of gases (i.e. O_2 and CO_2) and to provide the sole nutrient support to fetal development and survival [5]. Insufficient rise in UBF during pregnancy results in pregnancy disorders such as fetal growth restriction and preeclampsia [6], not only raising the risk of maternal and infant morbidity and mortality but also contributing to the susceptibility of both the mother and child to cardiovascular and other metabolic disorders later in life [7, 8].

Local uterine artery (UA) endothelium production of orchestrated vasodilators, including nitric oxide (NO) [9], endothelium-derived hyperpolarizing factor (EDHF) [10], and vascular endothelial growth factor (VEGFA) [11], etc., is crucial for mediating estrogen-induced and pregnancy-associated uterine vasodilation. Endothelial NO production via endothelial NO synthase (NOS3) activation and/or expression has been identified as a key player in uterine vasodilation as NOS3-derived NO is important for vascular remodeling, decreased vascular resistance, increased UBF, and pregnancy outcomes [12–15]. NOS3-derived NO seems to function as a focal mediator as it interacts with nearly all known vasodilators, including EDHF and VEGF [10, 11]. However, blockade of local NO production only inhibits approximately 65% estradiol-17β (E2β)-induced uterine vasodilation in nonpregnant ewes [9] and prolonged UA NOS inhibition only modestly reduces basal uteroplacental vasodilation in the last one third of ovine pregnancy [16], suggesting that mechanisms other than NO are present to maintain uterine hemodynamics.

Hydrogen sulfide (H_2S) is a gaseous signaling molecule that belongs to the gasotransmitter family after NO and carbon monoxide [17]. Endogenous H_2S in mammalian tissues is primarily synthesized via conversion of L-cysteine by two pyridoxal 5′-phosphate-dependent enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CTH, also called CSE) [18–20]. In mammals, H_2S potently dilates various vasculatures via activating ATP-dependent potassium (K_{ATP}) channel [21] and relaxes smooth muscle via activating large conductance calcium-activated potassium (BK_{Ca}) channel [22]. We recently reported that a slow-releasing H_2S donor GYY4137 dose-dependently relaxes UAs from nonpregnant and pregnant rats, with significantly greater potency in the pregnant state and vascular bed specificity [23]. Others have shown H_2S to be a placental vasodilator [24, 25] and dysregulated CTH/H_2S signaling in the placenta results in preeclampsia-like conditions [25]. Thus, H_2S plays an important role in uterine and placental hemodynamic regulation.

We have recently reported that both CBS and CTH are expressed in UA endothelium and smooth muscle in sheep [26] and human [23] UAs and both contribute to baseline UA H_2S biosynthesis. However, UA H_2S biosynthesis and endothelium and smooth muscle CBS, but not CTH, upregulation are significantly stimulated by exogenous $E_2\beta$ in a sheep model of estrogen replacement therapy [26] and are associated elevated endogenous estrogens during the proliferative phase of the menstrual cycle and pregnancy in women [23]. These data show that CBS is the major enzymatic source of augmented UA H_2S biosynthesis in response to both exogenous and endogenous estrogen stimulation. However, the underlying mechanism by which estrogens regulate CBS expression to stimulate H_2S biosynthesis is unknown in UA vascular cells.

Estrogens elicit diverse biological functions in target cells/tissues that possess specific ERs (i.e. ERα and ERβ) [27, 28], including vascular endothelial cells (ECs) such as uterine artery ECs (UAEC) [29]. By binding to specific transcription factor ERs, estrogens initiate the transcription of target genes that possess estrogen-responsive elements in their promoters in the nucleus [30]. Estrogen-induced uterine vasodilation is at least partially mediated by specific estrogen receptors since both exogenous and endogenous estrogen-induced rises in UBF can be inhibited by ICI 182780 (ICI) [31]. We hypothesize herein that estrogens stimulate H_2S biosynthesis by specific ERα and/or ERβ-dependent upregulation of CBS transcription. The objectives of this study were to determine in cultured UAEC whether (1) $E_2\beta$ stimulates H_2S production with enhanced mRNA and protein expression of CBS and/or CTH; (2) $E_2\beta$ stimulation of CBS and/or CTH expression is mediated by ER-dependent transcription; and (3) ERα and ERβ plays different roles in $E_2\beta$-stimulated H_2S biosynthesis.

Materials and methods

Chemicals and antibodies

$E_2\beta$, hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), fatty acid free bovine serum albumin (BSA), O-(carboxymethyl) hydroxylamine hemihydrochloride (CHH), sodium dodecyl sulfate (SDS), and all other chemicals unless specified were from Sigma (St. Louis, MO). ICI 182780 (ICI), 4,4,4-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT), diarylpropionitrile (DPN), 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride (MPP), 4-[2-Phenyl-5,7-bis (trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3yl]phenol (PHTPP) were from Tocris (Ellisville, MO). β-cyano-L-alanine (BCA) was from Cayman Chemical (Ann Arbor, MI). Anti-ACTH monoclonal antibody was from Ambion (Austin, TX). Monoclonal antibodies of CBS and CTH were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Anti-biotin antibody was from Cell Signaling Technology (Beverly, MA). Cell culture media MCDB131 and M199, prolong gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI), and Alexa^{488} goat anti-mouse IgG were from Invitrogen (Carlsbad, CA).

Cell culture and treatments

Primary UAEC were isolated by collagenase digestion from late pregnant ewes (120–130 days of gestation, normal term ≈ 145 days) as previously described [13, 32]. The Animal Care and Use Committee from the University of California approved the animal use protocol.

Frozen UAEC aliquots (passage 2) were thawed and seeded in MCDB131 containing 10% fetal bovine serum (Lonza, Walkersville, MD) and 1% antibiotics for experimental use at passages 4–5. Briefly, cells at approximately 70% confluence were cultured in serum/phenol red-free M-199 medium containing 0.1% fatty acid-free BSA, 0.5% charcoal stripped FBS, 1% penicillin/streptomycin, and 25 mM HEPES overnight for approximately 16 h. Following equilibration in fresh serum-free M-199, cells were treated with $E_2\beta$, ER agonists, or $E_2\beta$ with or without ER antagonists as previously described [32]. Ethanol was the vehicle used to dissolve $E_2\beta$ and ER agonists and antagonists. Final ethanol concentrations used were less than 0.5% and did not alter cellular responses surveyed in this study.

Methylene blue assay

UAEC (1×10^6/treatment in duplicate) were homogenized in 50 mM ice-cold potassium phosphate buffer pH 8.0. Using the methylene blue assay, H_2S production was determined as previously described [23, 26]. The H_2S concentration was calculated based on a calibration curve generated from NaHS solutions. CHH or BCA at a final concentration of 2 mM was added to the reaction mixtures prior to initiating the assay for determining specific CBS and CTH activities, respectively.

RNA extraction, polymerase chain reaction, and quantitative real-time RT-PCR

RNA extraction, polymerase chain reaction (PCR), and quantitative real-time RT-PCR was performed as previously described [23, 26]. Comparative CT method (ΔΔCT method) was used to calculate mRNA expression with L19 as the internal reference control.

SDS-PAGE and immunoblotting

SDS-PAGE and immunoblotting with specific antibodies listed in supplemental table S1 were performed as previously described [23, 26].

Immunofluorescence microscopy

UAEC were grown on glass coverslips to reach approximately 80% confluence and treated as described above. Following treatments, cells were washed in PBS and fixed in 4% paraformaldehyde for 20 min at room temperature. Cells were permeablized by incubating in 0.2% Triton-X in PBS for 15 min at room temperature. Autofluorescence was quenched by washing with 300 mM glycine in PBS, and nonspecific binding was blocked by incubation with PBS containing 1% BSA, 0.125% saponin, and 1% gelatin. Cells were incubated with 1 μg/mL of anti-CBS or anti-CTH. Following incubation with Alexa^{488}-labeled secondary antibody (1:1000), coverslips were mounted onto slides with Prolong Gold antifade reagent (Invitrogen) containing DAPI. Slides were examined under a Leica fluorescence microscope (Leica Corporation, Deerfield, IL). Digital images were acquired using a CCD camera and *Simple*PCI image analysis software (Hamamatsu Corporation, Sewickley, PA) and used for determining relative CBS and CTH proteins by quantifying mean green fluorescence intensity (15 cells/image and 5 images/animal) using *Simple*PCI. Normalized CBS and CTH protein levels were presented as fold change in the average fluorescence intensity of vehicle control treated cells.

Cell transfection and luciferase assay

RenSP luciferase-reporter constructs containing the promoters of human CBS (S711027), CTH (S712215), β-actin (S717678), and all transfection and luciferase reagents were purchased from Switchgear Genomics (Carlsbad, CA). The RenSP luciferase plasmid DNA and cypridina luciferase TK control construct were co-transfected into UAEC by using FuGENE HD transfection reagents (1:3, μl/ng) for 24 h at 37°C. UAEC transfected with an empty vector and β-actin promoter vector were served as negative and positive transfection controls, respectively. After transfection, cells were allowed to recover for 18–20 h in DMEM containing 10% FBS. Cells were serum-starved overnight and treated with vehicle or $E_2\beta$ (10 nM) for 24 h with or without ICI 182780. The RenSP luciferase activity in both cells and supernatant was measured and normalized to cypridina luciferase activity as previously described [33].

Statistical analysis

Each experiment was repeated at least three times with cells derived from different pregnant ewes. Data are presented as means ± SEM and analyzed by one-way or two-way analysis of variance (ANOVA), followed by the Bonferroni test for multiple comparisons using *Sigma*Plot (Systat Software Inc.). Student paired *t*-test was used for comparison of data between two groups. Significance was defined as $P < 0.05$, unless higher statistical power is indicated in the figure legends.

Results

$E_2\beta$ stimulates H_2S production: role of CBS, CTH, and ER

Compared to vehicle-treated controls, treatment with $E_2\beta$ (10 nM) for 48 h stimulated a 2.43 ± 0.21-fold ($P < 0.01$) increase in H_2S production in UAEC in vitro. The stimulation was blocked by pretreatment with 1 μM ICI; incubation with ICI alone had no effect (Figure 1A). $E_2\beta$-stimulated H_2S production was significantly inhibited by the specific CBS inhibitor CHH or CSE inhibitor BCA alone; the combination of CHH and BCA completely blocked $E_2\beta$-stimulated H_2S production (Figure 1B).

$E_2\beta$ stimulates CBS and CTH mRNA/protein expressions in a time- and concentration-dependent manner

$E_2\beta$ significantly stimulated CBS and CTH mRNAs in a time-dependent manner in UAEC in vitro. Following treatment with 10 nM $E_2\beta$, both CBS and CTH mRNA levels began to increase significantly at 24 h, maximized at 48 h (CBS: 2.61 ± 0.27-fold vs control; $P < 0.01$; CTH: 3.24 ± 0.41 fold vs control, $P < 0.01$), and plateaued at 72 h (Figure 2A). $E_2\beta$ also significantly stimulated CBS and CTH proteins in a time-dependent manner. Following treatment with 10 nM $E_2\beta$, both CBS and CTH protein levels began to significantly increase at 24 h and the stimulation continued up to 3 days (Figure 2B).

$E_2\beta$ also significantly stimulated CBS and CTH mRNAs in a concentration-dependent manner in UAEC in vitro. Treatment with 0.1 nM $E_2\beta$ for 48 h effectively stimulated both CBS and CTH mRNA expressions. Within the $E_2\beta$ concentrations tested, CBS mRNA continued to increase and maximized at 1 μM (3.02 ± 0.32-fold vs control, $P < 0.01$). CTH mRNA maximized by treatment with 10 nM $E_2\beta$ (3.25 ± 0.41-fold vs control, $P < 0.01$) and plateaued

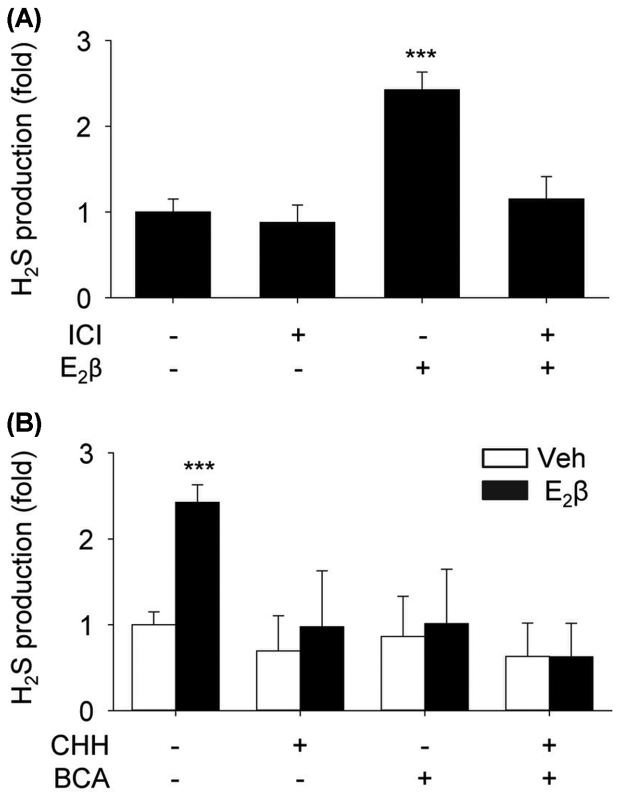

Figure 1. Effects of $E_2\beta$ on H_2S production—role of CBS, CTH, and ER. (A) Primary uterine artery endothelial cells (UAEC) were treated with vehicle or estradiol-17β ($E_2\beta$, 10 nM) with or without the estrogen receptor (ER) antagonist ICI 182780 (ICI, 1 μM) for 48 h. Protein extracts (1×10^6 cells) were used to determine hydrogen sulfide (H_2S) production. (B) UAEC were treated with vehicle or 10 nM $E_2\beta$ for 48 h, and protein extracts were used to determine H_2S production in the presence or absence of an inhibitor of cystathionine β-synthase (CBS, cystathionine γ-lyase (CTH), or CHH and BCA, respectively. Data (means ± SEM) were collected from different cell preparations cells prepared from three to five ewes. *** $P < 0.001$ vs control.

with 1 μM $E_2\beta$ (Figure 2C). Treatment with 0.1 nM $E_2\beta$ for 48 h effectively stimulated both CBS and CTH proteins. The responses increased with increasing concentrations of $E_2\beta$, and maximized with 100 nM $E_2\beta$ (CBS: 3.04 ± 0.16-fold vs control, $P < 0.01$; CTH: 3.17 ± 0.63 fold vs control, $P < 0.01$) (Figure 2D).

$E_2\beta$ stimulation of CBS and CTH expressions is mediated by specific ERs

In the presence of the specific ER antagonist ICI (1 μM), $E_2\beta$-stimulated CBS and CTH mRNAs (Figure 3A) were completely abrogated. Treatment with 10 nM $E_2\beta$ for 48 h also significantly stimulated CBS (2.46 ± 0.17-fold vs control, $P < 0.01$) and CTH (2.39 ± 0.10-fold vs control, $P < 0.01$) protein expressions, which were completely blocked by ICI (Figure 3B). Immunofluorescence analysis with specific anti-CBS and anti-CTH antibodies showed relative low baseline CBS and high CTH proteins in UAEC and confirmed ICI blockade of $E_2\beta$ stimulation of CBS and CTH protein expressions (Figure 3C). Treatments with vehicle or ICI alone did not alter CBS and CTH mRNA and protein expressions.

Either ERα or ERβ is sufficient to mediate $E_2\beta$ stimulation of CBS and CTH expressions

UAEC expresses both ERα and ERβ [29] and they may play common or even opposite roles in mediating estrogen regulation of gene expression in a cell [34]; we determined the specific roles of ERα and ERβ in estrogen stimulation of CBS and CTH expressions by using ER isoform-specific agonists or antagonists. Compared to vehicle-treated controls, treatment with the ERα agonist PPT alone induced 2.55 ± 0.26-fold ($P < 0.01$) and 2.98 ± 0.91-fold ($P < 0.01$) increases in CBS and CTH mRNAs, respectively. Treatment with the ERβ agonist DPN alone induced 2.45 ± 0.15-fold ($P < 0.01$) and 2.39 ± 0.41-fold ($P < 0.01$) increases in CBS and CTH mRNAs, respectively (Figure 4A). These increases were comparable to that of $E_2\beta$-stimulated fold increases in CBS (2.61 ± 0.27 vs vehicle, $P < 0.01$) and CTH (3.25 ± 0.41 vs vehicle, $P < 0.01$) mRNAs, respectively. PPT alone induced 2.02 ± 0.12-fold ($P < 0.01$) and 2.70 ± 0.39-fold ($P < 0.01$) increases in CBS and CTH proteins, respectively; DPN alone induced 2.69 ± 0.12-fold ($P < 0.01$) and 2.80 ± 0.17-fold ($P < 0.01$) increases in CBS and CTH proteins, respectively (Figure 4B). These changes were also comparable to that of $E_2\beta$-stimulated fold increases in CBS (2.46 ± 0.17 vs vehicle, $P < 0.01$) and CTH (2.39 ± 0.10 vs vehicle, $P < 0.01$) proteins, respectively. The combination of PPT and DPN had no additive effects on either CBS or CTH mRNA and protein expressions (Figure 4). Treatment with either ERα antagonist MPP or ERβ antagonist PHTPP alone or their combination was sufficient to completely attenuate $E_2\beta$-stimulated CBS and CTH mRNA (Figure 4C) and protein (Figure 4D) expressions.

$E_2\beta$ stimulates ER-dependent activation of *CBS* and *CTH* promoters

Compared to vehicle-treated controls, treatment with 10 nM $E_2\beta$ for 24 h significantly stimulated the promoter activities of *CBS* (3.71 ± 0.65-fold, $P < 0.001$) and *CTH* (3.77 ± 0.31-fold, $P < 0.001$) in UAEC. Co-treatment with ICI completely attenuated $E_2\beta$-stimulated activation of *CBS* and *CTH* promoters (Figure 5).

Discussion

Consistent with our most recent in vivo findings that UA H_2S production and endothelium and smooth muscle CBS but not CSE expressions are significantly greater in the proliferative phase and pregnancy, positively linked to elevated endogenous estrogens in women [23] and that exogenous estrogens significantly stimulate UA H_2S biosynthesis via selective upregulation of endothelium and smooth muscle CBS, but not CTH, mRNA, and protein expressions in OVX ewes [26], we now report that $E_2\beta$ stimulates UAEC H_2S production in association with upregulation of not only CBS but also CSE expression in vitro. The stimulatory effect of $E_2\beta$ on CTH mRNA and protein expression in UAEC in vitro is unexpected; however, the increased CBS and CTH protein expressions contribute to the increased H_2S production upon $E_2\beta$ stimulation in UAEC in vitro because UAEC H_2S production can be significantly inhibited by the specific inhibitor of either CBS (CHH) or CTH (BCA) alone and completely by their combination.

Our current data shown that $E_2\beta$ stimulates CBS mRNA and protein expressions in a time- and concentration-dependent manner, with as little as 0.1 nM $E_2\beta$ being effective to stimulate both CBS mRNA and protein expressions in UAEC. This low-dose $E_2\beta$ is physiologically relevant because 0.1–10 nM $E_2\beta$ is in the range

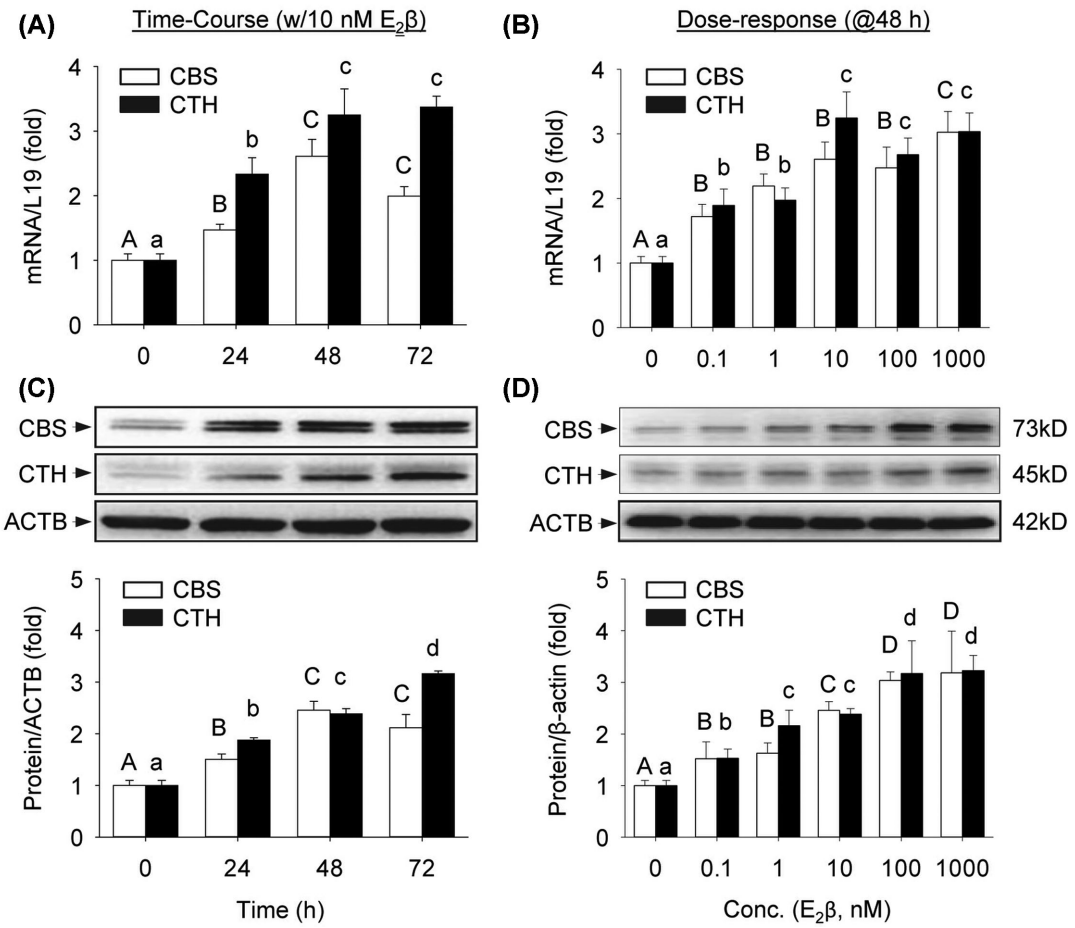

Figure 2. Time course and concentration response of $E_2\beta$ on mRNA and protein expressions of CBS and CTH. Primary uterine artery endothelial cells (UAEC) were treated with 10 nM estradiol-17β ($E_2\beta$) for up to 3 days to assess cystathionine β-synthase (CBS) and cystathionine γ-lyase (CTH) mRNA (A) and protein (B), or with increasing concentrations of $E_2\beta$ (0–1 μM) for 48 h to assess CBS and CTH mRNA (C) and protein (D). Data (means ± SEM) were collected from different cell preparations cells prepared from three to five ewes. Bars with different letters differ significantly ($P < 0.05$); capital and lower case letters pertain to CBS and CTH, respectively.

of circulating estrogen levels in nonpregnant and pregnant states [1, 35, 36]. Together with our recent in vivo findings [23, 26], these observations further strengthened *CBS* as an estrogen-responsive gene in ECs. $E_2\beta$ stimulates both mRNA and protein expressions of CBS in UAEC in vitro, suggesting that the stimulation occurs mainly at the level of transcription. Indeed, $E_2\beta$ activates the *CBS* promoter in UAEC in vitro. ICI completely attenuates $E_2\beta$ stimulation of CBS mRNA and protein expressions as well as its promoter activation, indicating the involvement of specific ERs.

We have previously shown that UAEC are direct targets of estrogens as both ERα and ERβ are expressed in UAEC [29, 37]. In target cells expressing ERα and ERβ, ligated receptors function as transcription factors to regulate gene expression via interaction with estrogen response elements (ERE) in the promoter of target genes [28]. In this mode, estrogens also regulate the expression of genes without classical EREs via crosstalk between ligated ERs with other ERE-interacting transcription factors such as AP-1 or Sp1 [27, 38]. The genomic effects of estrogens regulate the expression of various genes that are key enzymes such as NOS3 [39] and prostaglandin synthase [40] for synthesizing NO and prostacyclin, respectively. In addition, ERα and ERβ may play different and even opposite roles in regulating cellular responses to estrogens [34]. We show herein that either PPT or DPN stimulates CBS mRNA and protein expressions to levels comparable to that of $E_2\beta$-stimulated. Co-treatment with either MPP or PHTPP attenuates $E_2\beta$-stimulated CBS mRNA and protein expressions and neither the combination of the agonists nor the antagonists have additive effects. Thus, activation of either ERα or ERβ is sufficient to mediate $E_2\beta$ stimulation of CBS expression in UAEC in vitro.

$E_2\beta$ also stimulates CTH mRNA and protein expressions in UAEC in vitro, similar to its effects on CBS expression. Also similar is that $E_2\beta$ stimulation of CTH mRNA and protein expressions is mediated by ER-dependent *CTH* transcription, which can be activated by either ERα or ERβ because $E_2\beta$ stimulates CTH mRNA and protein expressions as well as *CTH* promoter activation and all responses can be blocked by ICI. Thus, these data demonstrate that *CTH* is also an estrogen-responsive gene in UAEC in vitro. Nonetheless, estrogen stimulation of CTH expression is unexpected because it contradicts not only to our in vivo studies showing that exogenous or endogenous $E_2\beta$ does not alter CTH mRNA and protein in UA endothelium in women [23] and sheep [26], but also to other studies showing that $E_2\beta$ does not stimulate CTH expression in mouse mesenteric smooth muscle cells in vitro [41]. The mechanism underlying the discrepancy between the effect of $E_2\beta$ on CTH expression

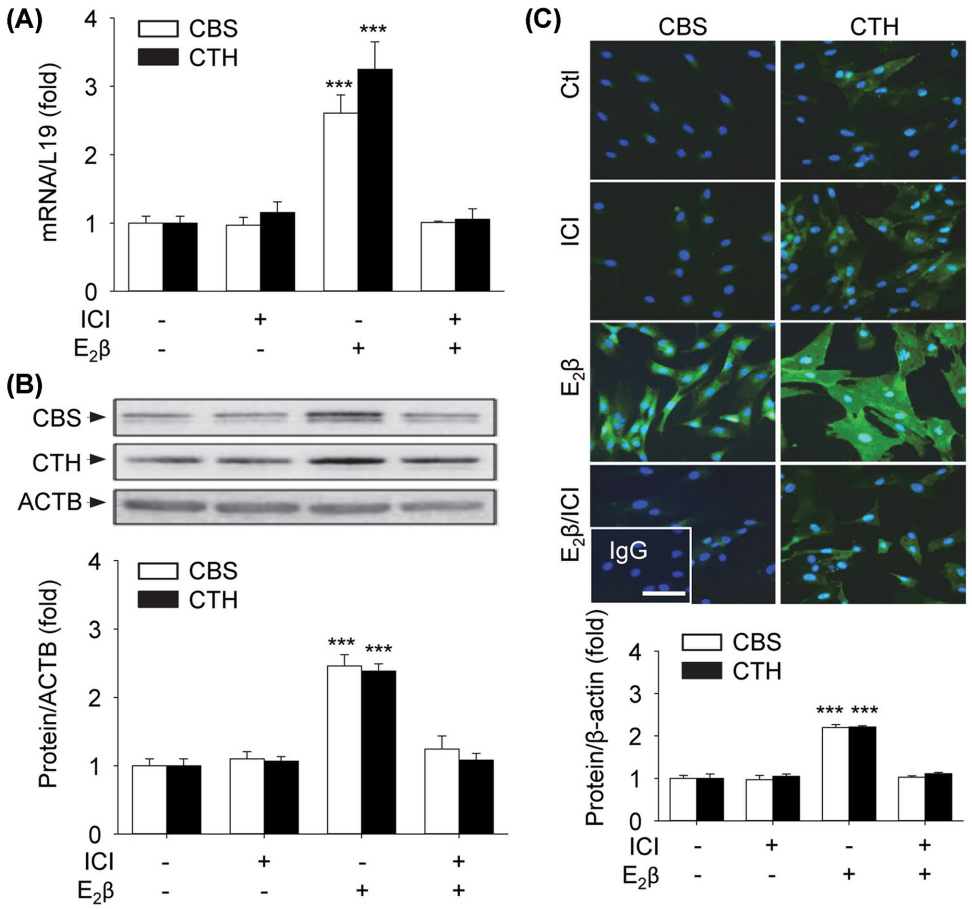

Figure 3. ER-dependency of $E_2\beta$ effects on mRNA and protein expressions of CBS and CTH. Primary uterine artery endothelial cells (UAECs) were treated with vehicle, 10 nM estradiol-17β ($E_2\beta$), 1 μM ICI 182 780 (ICI), or both for 48 h. Cystathionine β-synthase (CBS) and cystathionine γ-lyase (CTH) mRNA (A) and protein (B) were determined. (C) Immunofluorescence labeling of CBS and CTH proteins. Nuclei were stained with DAPI (blue). Protein expression was determined by relative green fluorescence intensity as fold change of control. Data (means ± SEM) were collected from different cell preparations from three to five ewes. *** $P < 0.001$ vs. Control. Scale bar = 50 μm.

in UAEC in vitro and in vivo is currently unknown. However, it is not uncommon that in vitro experiments do not always agree with in vivo findings. Nonetheless, this discrepancy is at least, in part, due to loss of cell–cell interactions and the microenvironment that ECs reside in in vivo. Regardless, caution should be exercised when interpreting in vitro findings as they pertain to in vivo conditions because in vitro and in vivo studies can sometimes produce different outcomes.

Although our current study shows that $E_2\beta$ activates CBS and CTH transcription that is linked to ER activation in UAEC, it does not provide direct evidence as to how ER interacts with the regulatory *cis*-elements in *CBS* and *CTH* promoters. The human *CBS* promoter spans over >4K bp [42] and the *CTH* promoter is approximately 1000 bp in length [43]. Both contain multiple putative *cis*-elements for binding various transcription factors, including ERE, Sp1, AP-1, and AP-3 [43, 44]. AP-1 and Sp1 have been implicated in stimulating CTH expression in vascular smooth muscle cells [43] as well as CTH [45] and CBS [42] expression in hepatocellular carcinoma cells. Since $E_2\beta$ stimulation of CBS and CTH expression in UAEC and *trans*-activation of their promoters by $E_2\beta$ is blocked by ICI, it is reasonable to infer that estrogen stimulation of CBS and CTH expression is mediated mainly by ER (ERα and/or ERβ) interaction with ERE. However, others transcription factors, such as Sp1 and AP1, cannot be excluded because $E_2\beta$ can stimulate gene expression through interactions of ER with other transcription factors [38].

Of note, in addition to ERα and ERβ, estrogens also signal via G protein-coupled estrogen receptor 1 (GPER) located on the plasma membrane [46, 47]. In this "extranuclear" nongenomic mode of estrogen signaling, binding to membrane ERα, ERβ, or GPER activates multiple protein kinase cascades within seconds to minutes to activate downstream target proteins to elicit biological functions or nuclear transcription factors to regulate latent gene expression [47]. ERα and ERβ [29] and GPER [48] are expressed in UA. The nongenomic action of estrogens is also of critical importance to the vascular effects of estrogens. For instance, activation of extracellular signal-activated protein kinases [13] and protein kinase B/Akt1 [49] via membrane ER-mediated mechanisms mediates rapid activation of NOS3 to produce NO by $E_2\beta$ in ECs, which contributes greatly to the endothelium-dependent mechanisms for the vascular effects of estrogens [50], including estrogen-induced uterine vasodilation [48]. To this end, whether estrogens activate the H_2S system via nongenomic pathway awaits to be determined.

In summary, our present study demonstrates that $E_2\beta$ stimulates H_2S production by upregulating CBS and CTH mRNA and protein expressions through specific ER-dependent *CBS* and *CTH*

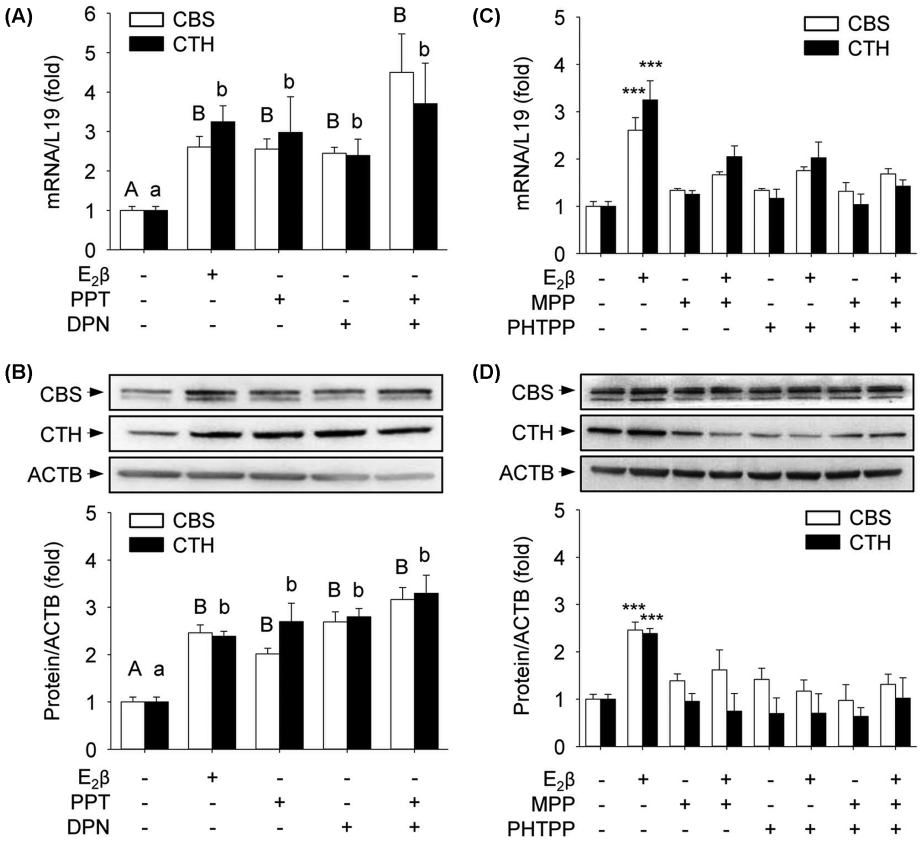

Figure 4. Specific role of ERα or ERβ in mediating $E_2\beta$ stimulation of mRNA and protein expressions of CBS and CTH. (A, B) ERα or ERβ activation: primary uterine artery endothelial cells (UAECs) were treated with vehicle, 10 nM of estradiol-17β ($E_2\beta$), PPT (ERα agonist), DPN (ERβ agonist), or PPT + DPN for 48 h. (C, D) ERα or ERβ inhibition: primary uterine artery endothelial cells (UAECs) were treated with vehicle or estradiol-17β ($E_2\beta$) (10 nM) with or without 1 μM MPP (ERα antagonist), PHTPP (ERβ antagonist), or MPP + PHTPP. Cystathionine β-synthase (CBS) and cystathionine γ-lyase (CTH) mRNA (A, C) and protein (B, D) were determined. Data (means ± SEM) were collected from different cell preparations from three to five ewes. Bars with different letters differ significantly; capital and lower case letters pertain to CBS and CTH, respectively. *** $P < 0.001$ vs Control.

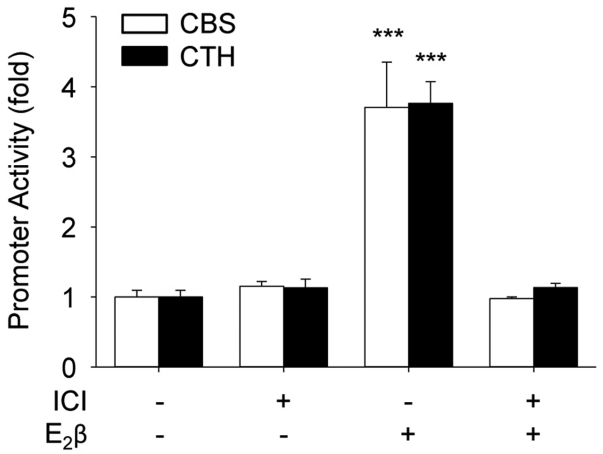

Figure 5. ER-dependency of $E_2\beta$ activation of *CBS* and *CTH* promoters. Human cystathionine β-synthase (*CBS*) and cystathionine γ-lyase (*CTH*) promoter luciferase-reporter constructs were transfected into primary uterine artery endothelial cells (UAECs). Twenty-four hours later, the cells were treated with vehicle or estradiol-17β ($E_2\beta$, 10 nM) with or without estrogen receptor (ER) antagonist ICI 182780 (ICI, 1 μM) for 24 h. Luciferase activity was determined as an index for promoter action. Data (means ± SEM) were collected from cells prepared from three to five ewes. *** $P < 0.001$ vs Control.

transcription in UAEC in vitro. Estrogen stimulation of H_2S biosynthesis in UAEC in vivo [23, 26] and in vitro (current study) obviously raises a question as to what role H_2S plays in the UA. We believe that our findings underline a physiological role of H_2S in mediating estrogen-induced and pregnancy-associated rises in UBF due to the potent vasodilatory properties of H_2S [21]. This idea is supported by observations that exogenous H_2S donor dilates rat UA more effectively in P vs NP states [23], although a role of endogenous H_2S in mediating uterine vasodilation is waiting to be determined.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplementary Table S1. Antibody Table.

Sources of funding

TJL was an American Heart Association (AHA) Pre-doctoral Fellow (AHA14PRE18570033). This study was supported in part by National Institutes of Health (NIH) grants RO1HL70562 and R21HL98746 to DBC and PO1HD38843, RO1HL87144, and RO1HL117341 to RRM. The content is solely the responsibility of

the authors and does not necessarily the official views of NIH and AHA.

Conflict of Interest/Disclosure Statement: The authors have no financial interests to disclose.

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