Title
ERα/ERβ-directed CBS transcription mediates E2β-stimulated hUAEC H2S production.

Journal
Journal of Molecular Endocrinology, 70(2)

ISSN
0952-5041

Authors
Bai, Jin
Lechuga, Thomas J
Makhoul, Joshua
et al.

Publication Date
2023-02-01

DOI
10.1530/jme-22-0175

Peer reviewed

 Accepted Manuscript published as JME-22-0175.R1. Accepted for publication: 06-Dec-2022

ERα/ERβ-directed *CBS* Transcription Mediates E_2β-stimulated hUAEC H_2S Production

Authors: Jin Bai, PhD, *Thomas J. Lechuga, PhD, Joshua Makhoul, MD, Hao Yan, MD/PhD, Carol Major, MD, Afshan Hameed, MD, and Dong-bao Chen, PhD

Address: Department of Obstetrics and Gynecology, University of California, Irvine, CA 92697

Short title: ER-directed *CBS* transcription mediates EC H_2S production.

Current Address: *Department of Biology, San Bernardino Valley College, San Bernardino, CA 92410

Sources of funding: The present study was supported in part by American Heart Association (AHA) postdoctoral fellowships AHAPOST903757 (JB) and AHAPOST34380384 (TJL) and the National Institutes of Health (NIH) grants RO1HL70562, R01HD105699, and R21HD097498 (to D-b C). The content is solely the responsibility of the authors and does not necessarily represent the official view of AHA and NIH.

Disclosure: The authors declare no conflict of interest.

Correspondence: Dong-bao Chen, Ph.D., Department of Obstetrics and Gynecology, University of California Irvine, Irvine, CA 92697. Tel: 949-824-2409; E-mail: dongbaoc@uci.edu

Word Counts:

Abstract: **248**

Main text: **5117**

Figures: **7**

Table: **3**

Supplemental Figure: **1**

ABSTRACT

Elevated endogenous estrogens stimulate human uterine artery endothelial cell (hUAEC) hydrogen sulfide (H_2S) production by selectively upregulating the expression of H_2S synthesizing enzyme cystathionine β-synthase (CBS), but the underlying mechanisms are underdetermined. We hypothesized that *CBS* transcription mediates estrogen-stimulated pregnancy-dependent hUAEC H_2S production. Estradiol-17β ($E_2β$) stimulated CBS but not cystathionine γ-lyase (CSE) expression in pregnant human UA ex vivo, which was attenuated by the estrogen receptor (ER) antagonist ICI 182,780. $E_2β$ stimulated CBS mRNA/protein and H_2S production in primary hUAEC from nonpregnant and pregnant women, but with greater responses in pregnant state; all were blocked by ICI 182,780. Human *CBS* promoter contains multiple estrogen-responsive elements (EREs), including one ERE preferentially binding ERα (αERE) and three EREs preferentially binding ERβ (βERE), and one full ERE (α/βERE) and one half ERE (½α/βERE) binding both ERα and ERβ. Luciferase assays using reporter genes driven by human *CBS* promoter with a series of 5'-deletions identified the α/βEREs binding both ERα and ERβ (α/βERE and ½α/βERE) to be important for baseline and $E_2β$-stimulated *CBS* promoter activation. $E_2β$ stimulated ERα/ERβ heterodimerization by recruiting ERα to α/βEREs and βERE, and ERβ to βERE, α/βEREs, and αERE. ERα or ERβ agonist alone *trans*-activated *CBS* promoter, stimulated CBS mRNA/protein and H_2S production to levels comparable to that of $E_2β$-stimulated, while ERα or ERβ antagonist alone abrogated $E_2β$-stimulated responses. $E_2β$ did not change human *CSE* promoter activity and CSE mRNA/protein in hUAEC. Altogether, estrogen-stimulated pregnancy-dependent hUAEC H_2S production occurs by selectively upregulating CBS expression via ERα/ERβ-directed gene transcription.

Keywords: Estrogens, Estrogen receptor, H_2S biosynthesis; *CBS* transcription, Pregnancy

Accepted Manuscript published as JME-22-0175.R1. Accepted for publication: 06-Dec-2022

INTRODUCTION

Normal pregnancy is associated with profound uterine artery (UA) dilation exemplified by ~20-50-fold rises in uterine blood flow (UtBF) in late pregnant (P) vs. nonpregnant (NP) state, which results in a large volume of maternal blood to be delivered to the maternal-fetal interface to perfuse the placenta (Rosenfeld, 1977, Palmer et al., 1992). UtBF carries out the bidirectional maternal-fetal gas (*i.e.*, O_2 and CO_2) exchanges and provides nutrients to support fetal and placental growth and survival (Sanghavi and Rutherford, 2014, Thornburg et al., 2000). Abnormal UA Doppler flow is linked to preeclampsia (Yu et al., 2008) and constrained UtBF results in intrauterine growth restriction (Roth et al., 2017).

Pregnancy-associated UA dilation is accompanied by elevated endogenous estrogens whose total levels in the third trimester in women can reach as high as 1000-fold that of NP state (Magness et al., 1998). Daily estradiol-17β (E_2β) treatment increases baseline UtBF 30-45% for up to 10 days in intact and ovariectomized NP ewes (Magness et al., 1993); acute E_2β treatment provokes rapid (20-30 min) and even more robust up to 10-fold rise in UtBF within 90-120 min (Magness and Rosenfeld, 1989, Reynolds et al., 1998). Enhanced UA endothelial cell (EC) production of nitric oxide (NO) via increased endothelial NO synthase (eNOS) expression and activation is known to mediate estrogen-induced UA dilation (Rosenfeld et al., 1996, Magness et al., 2001, Chen et al., 2004). Estrogen receptor (ER) antagonist ICI 182,780 (ICI) inhibits exogenous and endogenous estrogen-induced rises in UtBF in sheep (Magness et al., 2005), to the extent similar to that of UA local NO blockade by L-N^G-nitro arginine methyl ester (L-NAME) (Van Buren et al., 1992, Rosenfeld et al., 1996, Magness et al., 2005, Rosenfeld and Roy, 2014), establishing proximal physiological cause-effect relationships among endogenous estrogen-mediated ER activation, local NO production, and estrogen-induced UA dilation in pregnancy. However, both ICI and L-NAME only block ~70% of E_2β-induced and ~26% of baseline

pregnancy-associated rises in UtBF (Van Buren et al., 1992, Rosenfeld et al., 1996, Magness et al., 2005, Rosenfeld and Roy, 2014), suggesting other mechanisms to mediate uterine hemodynamics.

The proangiogenic vasodilator hydrogen sulfide (H_2S) is endogenously produced mainly from L-cysteine by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) (Papapetropoulos et al., 2009, Yang et al., 2008). We have recently shown that through selective upregulation of EC and smooth muscle (SM) CBS expression (Lechuga et al., 2015, Lechuga et al., 2019b, Sheibani et al., 2017), UA H_2S production is stimulated by exogenous $E_2β$ treatment in ovariectomized sheep (Lechuga et al., 2015) and also positively correlates with endogenous estrogens in sheep (Lechuga et al., 2019c) and women (Sheibani et al., 2017). H_2S stimulates pregnancy-dependent relaxation of pressurized UA ex vivo (Sheibani et al., 2017, Li et al., 2020) via activating SM large conductance Ca^{2+}-activated and voltage-dependent potassium channels (Li et al., 2020) for estrogen-induced UA dilation in pregnancy (Rosenfeld et al., 2001, Rosenfeld and Roy, 2012). Thus, H_2S is a novel UA dilator that regulates uterine hemodynamics in pregnancy.

Mechanistically, $E_2β$ stimulates primary ovine UAEC and UASMC H_2S production in vitro by stimulating specific-ER dependent upregulation of *CBS* transcription, involving ERα and ERβ (Lechuga et al., 2019b, Lechuga et al., 2019a). However, $E_2β$ also stimulates CSE expression in these ovine UA cell models, contrasting to in vivo conditions (Sheibani et al., 2017, Lechuga et al., 2015, Lechuga et al., 2019c). Moreover, the nuclear events underlying ERα/ERβ-mediated *CBS* transcription that mediates $E_2β$ stimulation of UA H_2S biosynthesis in pregnancy are currently unknown. ERα and ERβ are nuclear transcription factors (TFs); ligated ERα and ERβ can initiate transcription via direct interactions with estrogen response elements (EREs) to stimulate the expression of target genes (Driscoll et al., 1998). We hypothesized that estrogens stimulate pregnancy-dependent UA H_2S

Accepted Manuscript published as JME-22-0175.R1. Accepted for publication: 06-Dec-2022

production through selective upregulation of *CBS* transcription via ERα and ERβ interaction with the EREs in the *CBS* promoter. By using ex vivo cultures of freshly prepared UA rings and primary NP and P human UAECs (NP and P hUAEC), we showed herein that $E_2β$ stimulates pregnancy/specific ER-dependent human UA H_2S production by selective upregulation of *CBS* mRNA and protein expression at the level of transcription involving direct interactions between ERα/ERβ and the proximal *CBS* promoter EREs.

MATERIALS AND METHODS

Antibodies and chemicals

Antibodies used in this study were summarized in Table 1. ICI 182,780, 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]-1Hpyrazoledihydrochloride (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). Estradiol-17β ($E_2β$), fatty acid free bovine serum albumin (BSA), O-(carboxymethyl) hydroxylamine hemihydrochloride (CHH), fetal bovine serum (FBS), sodium dodecyl sulfate (SDS), hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), and all other chemicals unless specified were from Sigma (St. Louis, MO).

Human subjects and UA collection

The main UAs were collected with written consent from NP and P women (n = 5/group) in the event of hysterectomy at the University of California Irvine Medical Center, with ethical approval (HS #2013-9763) from Institutional Review Board for Human Research. The NP subjects were recruited from women aged 39-76 years who were no on steroid treatment and underwent elective hysterectomy due to fibroids, including a 39 year-old in the proliferative phase and a 42 year-old in the secretory

phases of the menstrual cycle and three postmenopausal woman. The P subjects were recruited from women aged 24 to 38 years with suspected placental accrete; UAs were collected immediately after Cesarean hysterectomy between 35-36 weeks' gestation. Subjects' characteristics were summarized in Table 2. UAs were dissected from parametrium, paracervical tissues, and adjacent myometrium, placed in chilled endothelial cell medium (ECM, ScienCell, La Jolla, CA) with 1% antibiotics, and transported to the laboratory within 1 h.

Ex vivo UA ring studies and immunofluorescence microscopy

Isolated pregnant UAs were cut into small rings (0.5 cm long) and were cultured in fibronectin (Sigma)-coated dish with phenol red free M199 (Gibco Laboratories, Thermo Scientific) containing 0.1% fatty acid-free BSA, 0.5% charcoal-stripped FBS, 1% penicillin/streptomycin, and 25 mM HEPES. The rings were treated with 10 nM E2β in the absence or presence of 1 μM of ICI 182,78 for 24 h; the rings were fixed in 4% paraformaldehyde, paraffin embedded, and then sectioned for analyzing CBS and CSE proteins by immunofluorescence microscopy. Briefly, sections (6 μm) were blocked in non-specific binding in phosphate buffered saline (PBS) containing 1% BSA at room temperature (RMT) for 30 min, then incubated with 1 μg/ml anti-CD31 (Dako) in 0.5% BSA-PBS overnight at 4℃. Following three 5-minute washes in PBS, the sections were incubated with Alexa^{568} mouse immunoglobin G (IgG, 2 μg/ml) at RMT for 1 h. After three 20-min washes in PBS, sections were blocked with 1% BSA-PBS and then incubated with 1 μg/ml of anti-CBS or anti-CSE antibodies at 4℃ overnight, followed by Alexa^{488} rabbit IgG or Alexa^{488} mouse IgG (2 μg/ml) at RMT for 1 h. IgG was used as a negative control. The sections were washed and mounted with SlowFade gold antifade mountant containing 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen) for labeling nuclei. The sections were examined under a confocal laser scanning microscope Olympus FV3000 (Olympus

Accepted Manuscript published as JME-22-0175.R1. Accepted for publication: 06-Dec-2022

Corporation, Tokyo, Japan). Images were acquired for quantifying CBS and CSE proteins in smooth muscle cells (SMC) and EC as previously described (Lechuga et al., 2015). Briefly, the average mean gray-value of cells from negative control without primary antibody accounted for autofluorescence and nonspecific background, which was subtracted from all counts generated from specific antibody-treated samples. CBS and CSE protein levels were presented as fold changes relative to average fluorescence intensity of smooth cells in untreated controls.

Cell isolation, culture, and treatment

Human UA endothelial cells (hUAEC) were isolated as described previously (Zhang, et.al., 2017; Bai and Chen, 2021). Briefly, UAs were dissected free of connective tissues and rinsed free of blood by phosphate buffered saline (PBS). Intact UA segments (~4 cm long) were filled with PBS containing 2 mg/mL collagenase II with ends tightened and allowed for digestion at 37°C for 45 min. Endothelial cell sheets were flushed out and plated in a 100-mm culture dish and cultured in complete ECM containing 5% FBS, endothelial growth supplements, and 1% penicillin/streptomycin. Endothelial cell colonies were manually picked and separately plated in 24-well plate. After purification and EC determination, cells were stored in liquid N_2 at passage 2. Frozen UAEC aliquots were thawed and subcultured in complete ECM for experimental use within 5 passages. Subconfluent (~80% confluence) NP and P hUAECs were starved in phenol red free M199 containing 0.1% fatty acid-free BSA, 0.5% charcoal-stripped FBS, 1% penicillin/streptomycin, and 25 mM HEPES, overnight. Cells were treated with $E_2\beta$, ER agonists, or $E_2\beta$ with or without ER antagonists as previously described (Lechuga et al., 2019b). Ethanol was the vehicle for dissolving $E_2\beta$ and ER agonists and antagonists. Final ethanol concentrations used were less than 0.5% and did not alter cellular responses surveyed in this study.

RNA extraction, reverse transcription (RT), polymerase chain reaction (PCR), and quantitative real-

time PCR (qPCR)

RNA extraction, RT, PCR, and qPCR were performed as previously described (Lechuga et al., 2019b); gene-specific primers used for PCR and qPCR were listed in Table 3, Fig. 5B&6C. The relative mRNA levels by qPCR were calculated by using $^{\Delta\Delta}$CT method with L19 as the internal reference.

Cell transfection and luciferase assay

To determine the effects of estrogens on *CBS* or *CSE* transcription, a 914 bp 5′ promoter of human *CBS* gene and a 942 bp 5' promoter of human *CSE* gene were subcloned into the luciferase-expression reporter pGL3.Basic (Promega) to generate the pCBS(-753).Luc and pCSE(-942).Luc constructs and then transfected in hUAEC for reporter gene expression studies. The pGL3 firefly luciferase plasmid DNA and pRL-TK control renilla luciferase constructs were co-transfected by using GenJet in vitro DNA transfection kit (1:4, μl/ng) overnight at 37◦C. Cells transfected with a blank vector and SV-40 promoter vector were served as negative and positive transfection controls, respectively. To further delineate the promoter region(s) responsible for estrogen induction, 5′ deletion analysis of the wild-type *CBS* promoter in pCBS(-753).Luc was conducted using a Mung Bean Nuclease 5′ Deletion Kit for Kilo-Sequencing (TaKara Bio Inc., Kusatsu, Japan) as previously described (Bai et al., 2019).

The DNA fragments of the full-length *CBS* promoter containing mutated α/βERE2 (-699 - CGCGGCCTCCGGtattTG- -682) and mutated ½α/βERE1 (-338-GAAatGCAAGAAGTTAAC- -321) sites in pCBS(-753).Luc construct were synthesized from Azenta (Chelmsford, MA) and subcloned into luciferase-expression reporter pGL3.Basic (Promega). The α/βERE2/½α/βERE1 double mutated construct (mα/βERE2/½α/βERE1) was with the same mutations in both α/βERE2 and ½α/βERE1 sites. After transfection, cells were recovered for 18–20 h in ECM containing 5% FBS. Cells were serum starved overnight and treated with vehicle or E_2β (10 nM) for 24 h. Cells were harvested for measuring

Accepted Manuscript published as JME-22-0175.R1. Accepted for publication: 06-Dec-2022

firefly luciferase and renilla luciferase activities using a Dual-GloR Luciferase Assay Kit (Promega, Madison, WI), as described (Bai and Chen, 2021). Renilla luciferase activity was normalized to firefly luciferase activity and then calculated as % of control to determine promoter activity.

Chromatin immunoprecipitation (ChIP)-qPCR

To characterize the effects of estrogens on ERα or ERβ interactions with specific EREs in human *CBS* and *CSE* promoters in hUAEC, ChIP-qPCR studies were performed using a SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology, Before, MA) with specific ERα or ERβ antibodies, respectively. Briefly, pregnant hUAEC (~4×10^6 cells/group) were treated with 10 nM $E_2\beta$ for 24 h, fixed in 1% formaldehyde for 8 min, followed by quenching with 1M glycine for 5min at RMT. Cells were harvested with a cell scraper, pelleted by centrifugation, and then dissolved in 50 µl ChIP buffer with protease inhibitor complex. The samples were digested with a 0.1 µl of Micrococcal Nuclease at 37 ℃ for 20 min to shear chromatin with fragment size of 0.1–0.8 kb. After a 10 µl aliquot of the cell lysate was saved as the input control (1:100 dilution), the rest of the sample was subjected to immunoprecipitation using ChIP certified antibodies of ERα (1:200, Active Motif, Carlsbad, CA) or ERβ (1:200, Thermo), following instructions of the assay kit with minor modifications (Bai and Chen, 2021). Rabbit IgG was used as the negative control for ChIP. After decrosslinking by heating at 65°C for 2 h, qPCR was performed to amplify the amplicons containing specific EREs in the human *CBS* or *CSE* promoters as predicted in Fig. 5&6, respectively, using primers listed in Fig. 5B &6C, respectively. All amplicons were gel analyzed and ethidium bromide staining for image acquisition. Signal intensity was normalized by that amplified using the input sample and then calculated as fold of untreated control.

Immunoblotting

Total cell lysate (20 µg proteins/lane) was subjected to SDS-PAGE and immunoblotting with

CBS (1:1000; Abcam) or CSE (1:500; Santa Cruz), respectively as previously described (Lechuga et al., 2019b). Parallel blotting for β-actin was conducted to serve as the loading control using a monoclonal antibody from Ambion (1:10000; Austin, TX). Band intensity was quantified by using NIH ImageJ, normalized to β-actin, and presented as fold of corresponding controls.

Methylene blue assay

H_2S production was determined by the methylene blue assay as described (Lechuga et al., 2019b). To determine the specific contribution of CBS and CSE in H_2S production, CHH or BCA at a final concentration of 2 mM was added to the reaction mixtures prior to initiate the assay. Briefly, primary hUAEC were seeded at the density of 0.5×10^6/ml in duplicate and treated with or without $E_2β$ (10 nM) for 24 h. Cells were harvested and homogenized in 50 mM ice-cold potassium phosphate buffer with pH 8.0. Concentrations of H_2S was calculated by a calibration curve generated with NaHS solutions.

Statistical analysis

Each experiment was repeated at least three times with cells derived from different NP and P subjects. Data are presented as means ± SEM and analyzed by one-way analysis of variance (ANOVA), followed by the Newman Keuls test for multiple comparisons using GraphPad Prism (GraphPad Software). Non-paired student 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

$E_2β$ on CBS and CSE protein expression in isolated human UA ex vivo

Human UA EC and SMC CBS, but not CSE, protein increases in pregnancy in association with elevated circulating estrogens (Sheibani et al., 2017). By using semi-quantitative immunofluorescence microscopic analysis (Lechuga et al., 2019c, Lechuga et al., 2015, Sheibani et al., 2017), we determined

Accepted Manuscript published as JME-22-0175.R1. Accepted for publication: 06-Dec-2022

whether estrogens regulate cellular (EC vs. SMC) CBS and CSE protein expression in cultures of isolated P human UA rings ex vivo. In untreated UA rings, CBS and CSE proteins were immunolocalized in both EC and SMC, and baseline CBS and CSE proteins in EC were ~ 7 times higher than that in SMC. Treatment with 10 nM $E_2\beta$, a physiological relevant concentration in human pregnancy (Abbassi-Ghanavati et al., 2009), for 24 h increased CBS protein expression (fold, EC: 2.21 ± 0.11, $p<0.05$; SMC: 10.53 ± 2.41, $p<0.05$), without altering CSE protein. Treatment with ICI completely blocked $E_2\beta$-stimulated CBS protein expression in both EC and SMC in P UA cultures (Fig. 1). In addition, baseline CBS mRNA in P UA rings was 3.37 ± 0.54 fold ($p<0.05$) greater to that in NP UA rings; baseline CBS protein (measured by immunoblotting) in P UA rings was 2.34-fold higher than that in NP UA rings. $E_2\beta$ stimulated CBS mRNA by 3.04 ± 0.36 fold in NP and 2.85 ± 0.17 fold ($p<0.01$) in P UA rings ex vivo. $E_2\beta$ also stimulated CBS protein in NP and P UA rings ex vivo; ICI blocked $E_2\beta$-stimulated CBS mRNA and protein expressions in NP and P human UA rings ex vivo. UA total CSE mRNA and protein did not differ in pregnancy and were unaltered by estrogens (Fig. S1).

$E_2\beta$ on H_2S biosynthesis in primary NP and P human UAECs in vitro

$E_2\beta$ stimulated EC and SMC CBS mRNA and protein in both NP and P UA ex vivo (Fig. 1 and Fig. S1). We focused our well-defined primary NP and P hUAEC models (Zhang et al., 2017) to explore the mechanisms underlying estrogen regulation of UA H_2S biosynthesis in pregnancy in vitro. Baseline H_2S production in P hUAEC was 2.07 ± 0.09 fold ($p<0.05$) greater that of NP hUAEC. Treatment with 10 nM $E_2\beta$ for 48 h stimulated H_2S production in hUAEC (fold; NP, 3.22 ± 0.07 fold, $p<0.05$; P, 2.51 ± 0.09 fold, $p<0.05$). ICI blocked $E_2\beta$-stimulated H_2S production in NP and P hUAEC (Fig. 2A, left). Treatment with an CBS inhibitor *O*-(carboxymethyl) hydroxylamine hemihydrochloride (CHH, 1 mM) inhibited baseline H_2S production by ~50% in both NP and P hUAEC; treatment with an CSE inhibitor

β-cyano-L-alanine (BCA, 1 mM) did not alter baseline H_2S production and had no additive effect to CHH (Fig. 2A, right). Baseline CBS mRNA levels in P hUAEC were 3.36 ± 0.54 fold ($P<0.05$) to that of NP hUAEC. Treatment with 10 nM $E_2\beta$ for 48 h stimulated CBS mRNA by 3.04 ± 0.36 fold ($p<0.05$) in NP hUAEC and 2.84 ± 0.20-fold ($P<0.05$) in P hUAEC (Fig. 2B). Consistently, baseline CBS protein levels in P hUAEC were 4.29 ±1.25 fold to that of NP hUAEC ($p < 0.05$). $E_2\beta$ increased CBS protein by 1.66 ± 0.21 fold ($p<0.05$) in NP hUAEC and 2.27 ± 0.66 fold ($p<0.05$) in P hUAEC; (Fig. 2C). ICI completely blocked $E_2\beta$-stimulated CBS mRNA and protein in NP and P hUAEC (Fig. 2B&C). Baseline CSE mRNA and protein levels did not differ in NP and P hUAEC and $E_2\beta$ also had no effects on CSE mRNA and protein in NP and P hUAEC (Fig. 2A-C).

$E_2\beta$ on *CBS* and *CSE* promoter activation in NP and P hUAECs

Baseline *CBS* promoter activity was 1.92 ± 0.03 fold ($p< 0.01$) in P vs. NP hUAEC. Treatment with 10 nM $E_2\beta$ for 24 h increased *CBS* promoter activity by 1.64 ± 0.05 fold ($p<0.05$ vs. untreated controls) in NP hUAEC, and with more potency by 2.23 ± 0.14 fold ($p < 0.01$ vs. controls and $p <0.01$ vs. NP cells) in P hUAEC (Fig. 3A). In P hUAEC, treatment with increasing doses (0.01-100 nM) of $E_2\beta$ for 24 h stimulated *CBS* promoter activity in a dose-dependent manner; *CBS* promoter activity significantly increased with 0.1 nM $E_2\beta$ and maximized with 10 nM $E_2\beta$ (2.03 ± 0.03 fold vs. untreated controls, $p<0.001$) (Fig. 3B). Baseline *CSE* promoter activity did not differ in NP and P hUAEC; $E_2\beta$ at all concentrations did not alter *CSE* promoter activity in NP and P hUAEC (Fig. 3A&B).

ERα and ERβ in $E_2\beta$ activation of *CBS* Promoter

hUAEC expresses both ERα and ERβ (Mishra et al., 2019). ICI blockade of $E_2\beta$-stimulated CBS mRNA/protein expression (Fig. 1&2) suggest ERα and ERβ interactions with *CBS* promoter EREs in *CBS* transcription. We then searched putative EREs in human *CBS* (ID: NG_008938.1) promoter by

Accepted Manuscript published as JME-22-0175.R1. Accepted for publication: 06-Dec-2022

using the Length-Aware Site Alignment Guided by Nucleotide Association (LASAGNA) motif search tool (https://biogridlasagna.engr.uconn.edu/lasagna_search/index.php). We found that that the proximal 914 bp human *CBS* promoter contains one putative ERE that preferentially binds ERα, three putative EREs that preferentially binds ERβ, and one full and a half putative EREs that bind both ERα and ERβ, and we designated them as αERE, βERE, α/βERE, and ½α/βERE, respectively (Fig. 4A).

To determine the estrogen-responsive region(s) in proximal human *CBS* promoter, we performed luciferase reporter gene expression assays using a series of 5'-deletion constructs of pCBS(-753).Luc reporter gene construct, with decreasing lengths of human *CBS* promoter from the position -753 to +18. When transfected in P hUAEC, baseline full-length *CBS* promoter activity began to significantly decrease from position -753 to -617 bp that contains βERE3 and α/βERE2 sites. A deletion from position -617 to -486 bp further decreased basal *CBS* promoter activity, although no ERE was present. A further deletion from position -349 to -259 bp in which the ½α/βERE1 site is present abolished E_2β-stimulated *CBS* promoter activity (Fig. 4B). Thus, the region (-753 to -617 bp) that contains α/βERE2 (-699 to -682 bp) and the region (-349 to -259 bp) that contains ½α/βERE1 (-338 to -321 bp) are crucial for baseline and E_2β-stimulated *CBS* promoter activity.

We further compared baseline and E_2β-stimulated full-length *CBS* promoter activity with its mutants in which these two sites were mutated to verify the importance of the α/βERE2 and ½α/βERE1 sites in human *CBS* transcription. Mutations in either α/βERE2 or ½α/βERE1 resulted in ~40% ($p < 0.01$) reduction in baseline human *CBS* promoter activity; double mutations in both sites were not additive in decreasing baseline *CBS* promoter activity. E_2β stimulated 1.82 ± 0.11 and 1.68 ± 0.29 increases ($p < 0.01$ vs. baseline) in human full-length *CBS* promoter in which either α/βERE2 or ½α/βERE1 was mutated; these responses did not differ from that of E_2β-stimulated wild-type human

CBS promoter activity (1.80 ± 0.07 fold, $p < 0.01$ vs baseline). However, the response of $E_2\beta$-stimulated activity of *CBS* promoter with mutations in both these two sites was significantly reduced to 1.44 ± 0.10 fold ($p<0.01$) to that of baseline, which was also significantly lower than that of $E_2\beta$-stimulated wild-type *CBS* promoter activity (Fig. 4C).

ERα and ERβ Interactions with proximal human *CBS* and *CSE* Promoters

We further analyzed ERα and ERβ Interactions with *CBS* and *CSE* Promoters by ChIP-PCR. We found that in untreated P hUAEC, ERα was readily recruited to αERE1 (amplicon 2) and that ERβ was readily recruited to βERE1 (amplicon 1), βERE2, and ½α/βERE1 (amplicon 3), and βERE3 (amplicon 5) in the proximal human *CBS* promoter; treatment with 10 nM $E_2\beta$ for 24 h did not alter the binding intensities in the sites that preferentially bind ERα or ERβ, respectively, as predicted by bioinformatics analysis (Fig. 4A). In untreated P hUAEC, baseline ERα bindings to α/βERE2 (amplicon 4) and βERE2 and ½α/βERE1 (amplicon 3) were barely detectable; treatment with 10 nM $E_2\beta$ for 24 h increased ERα binding to α/βERE2 site (amplicon 4) by 2.20 ± 0.21 fold ($p<0.01$) and to βERE2 and ½α/βERE1 sites (amplicon 3) by 1.97 ± 0.06 fold ($p<0.01$), suggesting that $E_2\beta$ recruits ERα dimerization with ERβ at the βERE or α/βERE sites. As expected, treatment with $E_2\beta$ increased ERβ binding to βERE2 and ½α/βERE1 (amplicon 3) by 2.20 ± 0.32 fold ($p < 0.05$). ERβ was readily bound to αERE1 (amplicon 2) and α/βERE2 (amplicon 4) in untreated cells; however, $E_2\beta$ treatment increased ERβ binding to αERE1 (amplicon 2) and α/βERE2 (amplicon 4) by 1.89 ± 0.34 and 1.88 ± 0.16 fold ($p<0.05$), respectively, suggesting ERβ dimerization with ERα at αERE in human *CBS* promoter by $E_2\beta$.

Bioinformatics analysis using LASAGNA also revealed that the proximal 590 bp human *CSE* (ID: NG_008041.1) promoter contains two putative EREs (αERE1 and αERE2) that preferentially binds ERα and one putative half EREs (½βERE) that preferentially bind ERβ. In unstimulated P hUAEC,

Accepted Manuscript published as JME-22-0175.R1. Accepted for publication: 06-Dec-2022

ERβ was readily recruited to ½βERE, and ERα and ERβ were readily recruited to αERE1 and αERE2; $E_2β$ treatment did not alter the bindings of ERα and ERβ to these EREs (Fig. 6).

ERα and ERβ in $E_2β$-stimulated hUAEC H_2S biosynthesis

We used specific agonists and antagonists of ERα and ERβ to determine the specific roles of ERα and ERβ in $E_2β$ stimulation of H_2S biosynthesis in P hUAEC. Treatment with 10 nM $E_2β$ for 24 h stimulated *CBS* promoter activity by 2.28 ± 0.26 fold and mRNA expression by 2.41 ± 0.31 fold ($p<0.05$). These stimulations were mimicked by either ERα agonist PPT or ERβ agonist DPN alone; their combination had no additive effects. In addition, E2β-stimulated *CBS* promoter activation and mRNA expression were blocked by either ERα antagonist MPP, ERβ antagonist PHTPP, or their combination (Fig. 7A&B). PPT, DPN, or their combination significantly increased CBS protein and H_2S production to levels comparable to that of $E_2β$ (CBS protein: 2.12 ± 0.23-fold vs control, H_2S production: 1.96 ± 0.18-fold vs control). MPP but not PHTPP alone significantly inhibited $E_2β$-stimulated CBS protein expression; MPP in combination PHTPP completely blocked $E_2β$-stimulated CBS protein expression. Either MPP or PHTPP and their combination completely blocked $E_2β$-stimulated H_2S production (Fig. 7C&D).

DISCUSSION

Our present study delineated the transcriptional mechanism underlying estrogen-stimulated uterine artery H_2S biosynthesis in pregnancy. Here we show that $E_2β$ stimulates specific ER-dependent EC and SMC CBS (but not CSE) mRNA/protein expression in human UA ex vivo. $E_2β$ also stimulates ER-dependent CBS (but not CSE) mRNA/protein and H_2S production in both NP and P hUAECs in vitro; however, the estrogen responses are significantly greater in P state, demonstrating pregnancy dependence of estrogen-stimulated hUAEC H_2S biosynthesis. ERα and ERβ readily occupy their

respective α and β EREs in proximal human *CBS* promoter in the NP state, while $E_2β$ stimulates ERα heterodimerization with ERβ and vice versa, to occupy these EREs in pregnant state. Activation of either ERα or ERβ mimics $E_2β$-induced H_2S biosynthesis, including *CBS* promoter activation, CBS mRNA/protein expression, and H_2S production. Moreover, $E_2β$ does not activate human *CSE* promoter containing EREs which are readily occupied by ERα and ERβ and nor alter ER interactions with human *CSE* promoter EREs. Thus, estrogens stimulate pregnancy dependent hUA H_2S biosynthesis via selective activation of *CBS* gene transcription via heterodimerization of ERα and ERβ to interact with the proximal *CBS* promoter EREs.

The human *CBS* gene contains five transcription starting sites, i.e., *1a, 1b, 1c, 1d, and 1e*, respectively, to encode multiple transcripts with different 5′-untranslated regions, among which the ones containing exon *1a* and *1b* are the most abundant (Gaustadnes et al., 1998). Previously, we have shown that $E_2β$ activates human *CBS-1b* promoter in ovine UAEC and UASMC (Lechuga et al., 2019b, Lechuga et al., 2019a), suggesting that human *CBS-1b* promoter contains EREs. Here, we first confirmed the specific ER-dependent stimulatory effect of $E_2β$ on human *CBS-1b* promoter in hUAEC by using luciferase reporter gene expression studies using luciferase reporter construct driven by the human *CBS-1b* promoter. We then used luciferase reporter construct driven by human *CBS-1b* promoter with a series of 5′ deletions identified the promoter sequence (−753 to -259 bp) to be the major region responsible for *trans*-activating *CBS* promoter by $E_2β$ in hUAEC.

The proximal human *CBS* promoter contains multiple putative EREs, including one αERE and three βEREs that preferentially bind ERα and ERβ, respectively. There are also one full α/βERE and one half ERE (½α/βERE) that bind both ERα and ERβ. They are presented in the region (−753 to -259 bp) pivotal for mediating $E_2β$-induced *trans*-activation of *CBS* promoter in hUAEC. Mutation of either

Accepted Manuscript published as JME-22-0175.R1. Accepted for publication: 06-Dec-2022

α/βERE2 or ½α/βERE1 site resulted in lower baseline *CBS* promoter activity, further supporting the importance of these sites in basal *CBS* expression. However, a single mutation in either α/βERE2 or ½α/βERE1 site did not affect *CBS* promoter *trans*-activation by $E_2β$. Double mutation in both sites not only reduces basal *CBS* promoter activity, but also results in decreased response to $E_2β$ stimulation. Hence, the α/βERE2 and ½α/βERE1 sites are needed to maintain optimal baseline CBS expression and more importantly both sites are required for *CBS* promoter *trans*-activation by $E_2β$ in hUAEC.

In resting P hUAEC, ERα binding to αERE1 and βERE1&3 sites and ERβ binding to βERE1 and βERE3 sites are high; these ER/ERE interactions are not altered by $E_2β$, suggesting that these baseline ER/ERE interactions may contribute to baseline EC CBS expression in pregnancy (Sheibani et al., 2017). ERα and ERβ binding to α/βERE2 or ½α/βERE1 sites are very low and ERβ barely binds to αERE1 in NP state. However, treatment with $E_2β$ significantly stimulates the recruitment of ERα to α/βERE2 ½α/βERE1 and βERE2 sites, and ERβ to βERE2-α/βERE2, ½α/βERE1, and αERE1 sites. These data show that estrogens differentially regulate ERα and ERβ interactions with specific EREs and that ERα and ERβ heterodimerize onto the α/βERE2 and ½α/βERE1 sites in the major estrogen-responsive region (−753 to -259 bp) in the human *CBS* promoter in hUAEC.

Human UAEC express ERα and ERβ; pregnancy augments ERβ expression to mediate estrogen-stimulated hUAEC angiotensin type-2 receptor expression (Mishra et al., 2019). Our current study shows that both ERα and ERβ are required for *trans*-activating human *CBS* promoter by $E_2β$. Consistently, we show that either PPT or DPN alone can activate *CBS* promoter and CBS mRNA/protein expression and H_2S production to levels comparable to that of $E_2β$-stimulated. Co-treatment with either MPP or PHTPP alone can effectively attenuate $E_2β$-stimulated *CBS* promoter activity, CBS mRNA expression, and H_2S production, showing either ERα or ERβ suffices to mediate $E_2β$-stimulated H_2S biosynthesis in hUAEC.

Of note, deletion of -753 to -617 bp in human *CBS* promoter results in decreases baseline activity, suggesting other TFs to be involved since this region does not contain ERE. Human *CBS* promoter contains Sp1 and YY1 sites to mediate enhanced H_2S production via *CBS* transcription upon stimulation with vascular endothelial growth factor (Bai and Chen, 2021). ERα and ERβ can interact with Sp1 (Safe, 2001). Further studies are needed to explore whether other ER-interacting TFs are involved in *trans*-activating *CBS* promoter to participate in estrogen-stimulated H_2S biosynthesis in pregnancy.

Unlike our previous ovine UAEC studies in which $E_2β$ stimulates both CBS and CSE expression in vitro (Lechuga et al., 2019b), our current study shows that $E_2β$ selectively stimulates CBS expression without altering CSE in NP and P hUAECs with greater responses in P state. Thus, our human UAEC models provide physiologically relevant data closely mimicking our in vivo findings (Sheibani et al., 2017, Lechuga et al., 2015, Lechuga et al., 2019c), together consistently supporting a conclusion that estrogens stimulate pregnancy-dependent hUAEC H_2S biosynthesis by selectively upregulating CBS expression via direct ERα and ERβ interactions with EREs in the proximal *CBS* promoter.

Conclusion

Our current study demonstrates that estrogens stimulate pregnancy-dependent H_2S production by specific nuclear ER-dependent upregulation of *CBS* transcription via direct ERα and ERβ interactions with the proximal *CBS* promoter EREs in hUAEC, thereby informing a novel mechanism for mediating estrogen-induced uterine vasodilation in pregnancy (Fig. 8). Pregnancy is a physiological state of elevated endogenous estrogens whose major role is to stimulate maternal-fetal interface vasodilation and angiogenesis and collectively these mechanisms raise UtBF to execute the bidirectional exchanges obligatory for fetal growth (Berkane et al., 2017, Thornburg et al., 2000, Magness, 1998). Constrained UtBF and dysregulated estrogen biosynthesis and metabolism in pregnancy contribute to the

Accepted Manuscript published as JME-22-0175.R1. Accepted for publication: 06-Dec-2022

pathogenesis of preeclampsia (Kanasaki et al., 2008, Berkane et al., 2017, Jobe et al., 2013); yet, how estrogens regulate UtBF remains incompletely understood. UA CBS/H_2S production is upregulated by estrogen replacement therapy (Lechuga et al., 2015) and pregnancy (Sheibani et al., 2017). Enhanced local CBS/H_2S stimulates maternal-fetal interface vasodilation and angiogenesis (Li et al., 2020, Qi et al., 2020, Sheibani et al., 2017, Chen et al., 2017), thus emerging as a novel pathway for regulating estrogen-induced uterine hemodynamics in pregnancy. Administration of H_2S donors rescue animal models of preeclampsia (Wang et al., 2013, Holwerda et al., 2014). Thus, data from this study implicate that targeting CBS/H_2S pathway may provide a novel strategy for developing therapeutic interventions for hypertension-related pregnancy disorders such as preeclampsia with intrauterine growth restriction.

Acknowledgments: The authors thank all the volunteers for donating the tissue samples.

REFERENCES

Abbassi-Ghanavati M, Greer LG & Cunningham FG 2009 Pregnancy and laboratory studies: a reference table for clinicians. *Obstet Gynecol* 114 1326-1331. DOI: 10.1097/AOG.0b013e3181c2bde8.

Bai J & Chen DB 2021 Enhanced Sp1/YY1 expression directs *CBS* transcription to mediate VEGF-stimulated pregnancy-dependent H_2S production in human uterine artery endothelial cells. *Hypertension* 78 1902-1913. DOI: 10.1161/HYPERTENSIONAHA.121.18190.

Bai J, Jiang X, He M, Chan BCB & Wong AOL 2019 Novel mechanisms for IGF-I regulation by glucagon in carp hepatocytes: up-regulation of HNF1alpha and CREB expression via signaling crosstalk for IGF-I gene transcription. *Front Endocrinol (Lausanne)* 10 605. DOI: 10.3389/fendo.2019.00605.

Berkane N, Liere P, Oudinet JP, Hertig A, Lefevre G, Pluchino N, Schumacher M & Chabbert-Buffet N 2017 From pregnancy to preeclampsia: a key role for estrogens. *Endocr Rev* 38 123-144. DOI: 10.1210/er.2016-1065.

Chen DB, Bird IM, Zheng J & Magness RR 2004 Membrane estrogen receptor-dependent extracellular signal-regulated kinase pathway mediates acute activation of endothelial nitric oxide synthase by estrogen in uterine artery endothelial cells. *Endocrinology* 145 113-25. DOI: 10.1210/en.2003-0547.

Chen DB, Feng L, Hodges JK, Lechuga TJ & Zhang H 2017 Human trophoblast-derived hydrogen sulfide stimulates placental artery endothelial cell angiogenesis. *Biol Reprod* 97 478-489. DOI: 10.1093/biolre/iox105.

Driscoll MD, Sathya G, Muyan M, Klinge CM, Hilf R & Bambara RA 1998 Sequence requirements for estrogen receptor binding to estrogen response elements. *J Biol Chem* 273 29321-30. DOI: 10.1074/jbc.273.45.29321.

Gaustadnes M, Kluijtmans LA, Jensen OK, Rasmussen K, Heil SG, Kraus JP, Blom HJ, Ingerslev J & Rudiger N 1998 Detection of a novel deletion in the cystathionine beta-synthase (CBS) gene using an improved genomic DNA based method. *FEBS Lett* 431 175-9. DOI: 10.1016/s0014-5793(98)00743-1.

Holwerda KM, Burke SD, Faas MM, Zsengeller Z, Stillman IE, Kang PM, van Goor H, McCurley A, Jaffe IZ, Karumanchi SA & Lely AT 2014 Hydrogen sulfide attenuates sFlt1-induced hypertension and renal damage by upregulating vascular endothelial growth factor. *J Am Soc Nephrol* 25 717-25. DOI: 10.1681/ASN.2013030291.

Jobe SO, Tyler CT & Magness RR 2013 Aberrant synthesis, metabolism, and plasma accumulation of circulating estrogens and estrogen metabolites in preeclampsia implications for vascular dysfunction. *Hypertension* 61 480-7. DOI: 10.1161/HYPERTENSIONAHA.111.201624.

Kanasaki K, Palmsten K, Sugimoto H, Ahmad S, Hamano Y, Xie L, Parry S, Augustin HG, Gattone VH, Folkman J, Strauss JF & Kalluri R 2008 Deficiency in catechol-O-methyltransferase and 2-methoxyoestradiol is associated with pre-eclampsia. *Nature* 453 1117-21. DOI:

Accepted Manuscript published as JME-22-0175.R1. Accepted for publication: 06-Dec-2022

10.1038/nature06951.

Lechuga TJ, Bilg AK, Patel BA, Nguyen NA, Qi QR & Chen DB 2019a Estradiol-17beta stimulates H_2S biosynthesis by ER-dependent CBS and CSE transcription in uterine artery smooth muscle cells in vitro. *J Cell Physiol* 234 9264-9273. DOI: 10.1002/jcp.27606.

Lechuga TJ, Qi QR, Kim T, Magness RR & Chen DB 2019b E2beta stimulates ovine uterine artery endothelial cell H2S production in vitro by estrogen receptor-dependent upregulation of cystathionine beta-synthase and cystathionine gamma-lyase expression. *Biol Reprod* 100 514-522. DOI: 10.1093/biolre/ioy207.

Lechuga TJ, Qi QR, Magness RR & Chen DB 2019c Ovine uterine artery hydrogen sulfide biosynthesis in vivo: effects of ovarian cycle and pregnancy. *Biol Reprod* 100 1630-1636. DOI: 10.1093/biolre/ioz027.

Lechuga T J, Zhang HH, Sheibani L, Karim M, Jia J, Magness RR, Rosenfeld CR & Chen DB 2015 Estrogen replacement therapy in ovariectomized nonpregnant ewes stimulates uterine artery hydrogen sulfide biosynthesis by selectively up-regulating cystathionine beta-synthase expression. *Endocrinology* 156 2288-98. DOI: 10.1210/en.2015-1086.

Li Y, Bai J, Yang YH, Hoshi N & Chen DB 2020 Hydrogen sulfide relaxes human uterine artery via activating smooth muscle BKCa channels. *Antioxidants (Basel)* 9. DOI: 10.3390/antiox9111127.

Magness RR 1998 Maternal cardiovascular and other physiologic responses to the endocrinology of pregnancy., *In: Bazer F.W. (eds) Endocrinology of Pregnancy. Contemporary Endocrinology, Vol 9,* Humana Press, Totowa, NJ.(Chapter 2) pp 507-539. DOI: 10.1007/978-1-4612-1804-3_18.

Magness RR, Parker CR, Jr & Rosenfeld CR 1993 Systemic and uterine responses to chronic infusion of estradiol-17 beta., *Am J Physiol* 265 E690-8. DOI: 10.1152/ajpendo.1993.265.5.E690.

Magness RR, Phernetton TM, Gibson TC & Chen DB 2005 Uterine blood flow responses to ICI 182 780 in ovariectomized oestradiol-17beta-treated, intact follicular and pregnant sheep. *J Physiol* 565 71-83. DOI: 10.1113/jphysiol.2005.086439.

Magness RR, Phernetton TM & Zheng J 1998 Systemic and uterine blood flow distribution during prolonged infusion of 17beta-estradiol. *Am J Physiol* 275 H731-43. DOI: 10.1152/ajpheart.1998.275.3.H731.

Magness RR & Rosenfeld CR 1989 Local and systemic estradiol-17 beta: effects on uterine and systemic vasodilation. *Am J Physiol* 256 E536-42. DOI: 10.1152/ajpendo.1989.256.4.E536.

Magness RR, Sullivan JA, Li Y, Phernetton TM & Bird IM 2001 Endothelial vasodilator production by uterine and systemic arteries. VI. Ovarian and pregnancy effects on eNOS and NOx. *Am J Physiol Heart Circ Physiol* 280 H1692-8. DOI: 10.1152/ajpheart.2001.280.4.H1692.

Mishra JS, Te Riele GM, Qi QR, Lechuga TJ, Gopalakrishnan K, Chen DB & Kumar S (2019) Estrogen receptor-beta mediates estradiol-induced pregnancy-specific uterine artery

endothelial cell angiotensin type-2 receptor expression. *Hypertension* 74 967-974. DOI: 10.1161/HYPERTENSIONAHA.119.13429.

Palmer S. K, Zamudio S, Coffin C, Parker S, Stamm E & Moore LG 1992 Quantitative estimation of human uterine artery blood flow and pelvic blood flow redistribution in pregnancy. *Obstet Gynecol* 80 1000-6. Available at: https://www.ncbi.nlm.nih.gov/pubmed/1448242.

Papapetropoulos A, Pyriochou A, Altaany Z, Yang G, Marazioti A, Zhou Z, Jeschke MG, Branski LK, Herndon DN, Wang R & Szabo C 2009 Hydrogen sulfide is an endogenous stimulator of angiogenesis. *Proc Natl Acad Sci U S A* 106 21972-7. DOI: 10.1073/pnas.0908047106.

Qi QR, Lechuga TJ, Patel B, Nguyen NA, Yang YH, Li Y, Sarnthiyakul S, Zhang QW, Bai J, Makhoul J & Chen DB 2020 Enhanced stromal cell CBS-H_2S production promotes estrogen-stimulated human endometrial angiogenesis. *Endocrinology* 161. DOI: 10.1210/endocr/bqaa176.

Reynolds LP, Kirsch JD, Kraft KC, Knutson DL, McClaflin WJ & Redmer DA 1998 Time-course of the uterine response to estradiol-17beta in ovariectomized ewes: uterine growth and microvascular development. *Biol Reprod* 59 606-12. DOI: 10.1095/biolreprod59.3.606.

Rosenfeld CR 1977 Distribution of cardiac output in ovine pregnancy. *Am J Physiol* 232 H231-5. DOI: 10.1152/ajpheart.1977.232.3.H231.

Rosenfeld CR, Cornfield DN & Roy T 2001 Ca2+-activated K+ channels modulate basal and E2beta-induced rises in uterine blood flow in ovine pregnancy. *Am J Physiol Heart Circ Physiol* 281 H422-31. DOI: 10.1152/ajpheart.2001.281.1.H422.

Rosenfeld CR, Cox BE, Roy T & Magness RR 1996 Nitric oxide contributes to estrogen-induced vasodilation of the ovine uterine circulation. *J Clin Invest* 98 2158-66. DOI: 10.1172/JCI119022.

Rosenfeld CR & Roy T 2012 Large conductance Ca2+-activated and voltage-activated K+ channels contribute to the rise and maintenance of estrogen-induced uterine vasodilation and maintenance of blood pressure. *Endocrinology* 153 6012-20. DOI: 10.1210/en.2012-1717.

Rosenfeld CR & Roy T 2014 Prolonged uterine artery nitric oxide synthase inhibition modestly alters basal uteroplacental vasodilation in the last third of ovine pregnancy. *Am J Physiol Heart Circ Physiol* 307 H1196-203. DOI: 10.1152/ajpheart.00996.2013.

Roth CJ, Haeussner E, Ruebelmann T, Koch FV, Schmitz C, Frank HG & Wall WA 2017 Dynamic modeling of uteroplacental blood flow in IUGR indicates vortices and elevated pressure in the intervillous space - a pilot study. *Sci Rep* 7 40771. DOI: 10.1038/srep40771.

Safe S 2001 Transcriptional activation of genes by 17 beta-estradiol through estrogen receptor-Sp1 interactions. *Vitam Horm* 62 231-52. DOI: 10.1016/s0083-6729(01)62006-5.

Sanghavi M & Rutherford JD 2014 Cardiovascular physiology of pregnancy. *Circulation* 130 1003-8. DOI: 10.1161/CIRCULATIONAHA.114.009029.

Sheibani L, Lechuga TJ, Zhang H, Hameed A, Wing DA, Kumar S, Rosenfeld CR & Chen DB 2017

Accepted Manuscript published as JME-22-0175.R1. Accepted for publication: 06-Dec-2022

Augmented H2S production via cystathionine-beta-synthase upregulation plays a role in pregnancy-associated uterine vasodilation. *Biol Reprod* 96 664-672. DOI: 10.1095/biolreprod.116.143834.

Thornburg KL, Jacobson SL, Giraud GD & Morton MJ 2000 Hemodynamic changes in pregnancy. *Semin Perinatol* 24 11-4. DOI: 10.1016/s0146-0005(00)80047-6.

Van Buren GA, Yang DS & Clark KE 1992 Estrogen-induced uterine vasodilatation is antagonized by L-nitroarginine methyl ester, an inhibitor of nitric oxide synthesis. *Am J Obstet Gynecol* 167 828-33. DOI: 10.1016/s0002-9378(11)91597-x.

Wang K, Ahmad S, Cai M, Rennie J, Fujisawa T, Crispi F, Baily J, Miller MR, Cudmore M, Hadoke PW, Wang R, Gratacos E, Buhimschi IA, Buhimschi CS & Ahmed A 2013 Dysregulation of hydrogen sulfide producing enzyme cystathionine gamma-lyase contributes to maternal hypertension and placental abnormalities in preeclampsia. *Circulation* 127 2514-22. DOI: 10.1161/CIRCULATIONAHA.113.001631.

Yang G, Wu L, Jiang B, Yang W, Qi J, Cao K, Meng Q, Mustafa AK, Mu W, Zhang S, Snyder SH & Wang R 2008 H_2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine gamma-lyase. *Science* 322 587-90. DOI: 10.1126/science.1162667.

Yu CK, Khouri O, Onwudiwe N, Spiliopoulos Y, Nicolaides KH & Fetal Medicine Foundation Second-Trimester Screening G 2008 Prediction of pre-eclampsia by uterine artery Doppler imaging: relationship to gestational age at delivery and small-for-gestational age. *Ultrasound Obstet Gynecol,* 31(3), pp. 310-3. DOI: 10.1002/uog.5252.

Zhang HH, Chen JC, Sheibani L, Lechuga TJ & Chen DB 2017 Pregnancy augments VEGF-stimulated in vitro angiogenesis and vasodilator (NO and H_2S) production in human uterine artery endothelial cells. *J Clin Endocrinol Metab* 102 2382–2393. DOI: 10.1210/jc.2017-00437.

FIGURE LEGENDS

Fig. 1: **$E_2\beta$ on pregnant human uterine artery CBS/CSE expression ex vivo.** (A) Freshly prepared pregnant (P) human UA rings were treated with vehicle, 10 nM estradiol-17β ($E_2\beta$), 1 μM ICI 182 780 (ICI), or both for 24 h. The rings were paraffin-embedded and then subjected to immunofluorescence labeling of cystathionine β-synthase (CBS) and cystathionine γ -lyase (CSE) proteins by specific CBS or CSE antibodies, with CD31 antibody for co-labeling endothelial cells (EC) distinct from smooth muscle cells (SMC). After incubation with corresponding fluorescently labeled secondary antibodies, sections were mounted with DAPI to label nuclei and examined under confocal microscopy. Negative control treated with IgG is shown with scale bar at 100 μm. lu: lumen. (B) Graphs summarizing levels of EC and SMC CBS and CSE proteins. Images were taken to determine CBS and CSE proteins (relative green fluorescence intensity; RFI) using Image J and summarized as fold changes relative to untreated smooth muscles in the graphs. Data (means ± SEM) were collected from sections of different cultured P hUA rings from three different subjects. *, $p<0.05$, **, $p<0.01$ vs control. Bars with different superscripts differ significantly ($p< 0.05$).

Fig. 2: **$E_2\beta$ on human uterine artery endothelial cell H_2S biosynthesis in vitro.** Primary uterine artery endothelial cells from nonpregnant (NP) and pregnant (P) women (NP and P hUAEC, respectively) 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. The cells were used for measuring H_2S production (A) by the methylene blue assay, CBS and CSE mRNA (B) by RT-qPCR, and protein (C) by immunoblotting. In the right panel of A, protein extracts from NP and P hUAEC cells were used for H_2S production in an inhibitor of CBS (CHH, 2 mM), CSE (BCA, 2 mM), or both. RT-qPCR assay of mRNA was performed with specific primers listed in Table 1, by using L-19 as an internal control for quantitation.

Accepted Manuscript published as JME-22-0175.R1. Accepted for publication: 06-Dec-2022

Immunoblotting of CBS or CSE proteins was performed with β-actin as the loading control for quantitation. Data were from cells of three different subjects and calculated as means ± SEM. Bars with different superscripts differ significantly ($p< 0.05$). *, $p<0.05$, vs. control; $, $p<0.05$ for P vs. NP.

Fig. 3. **$E_2β$ on human *CBS/CSE* promoter activation.** Primary human uterine artery endothelial cells (hUAEC) were transfected with luciferase constructs driven by wild-type human *CBS* promoter (-753/+161) or human *CSE* promoter (-942/+98) and co-transfected with the thymidine kinase renilla luciferase vector. After treatments, cells were harvested for determining firefly/renilla luciferase activities. Promoter activation was calculated as a ratio of firefly/renilla luciferase activities. A: Cells from nonpregnant (NP) and pregnant (P) women, i.e., NP and P hUAECs, were treated with $E_2β$ (10 nM) for 24 h. Data were expressed as mean ± SEM from three independent experiments. *, $p<0.05$ and ** $p<0.01$, vs. control; ## and $$, $p<0.01$ for P vs. NP; *n.s.*, not different. B: Pregnant hUAEC were treated with increasing concentrations (0-100 nM) of $E_2β$ for 24 h. Data (means ± SEM, n=3) were expressed as fold of baseline in resting cells transfected with *CBS* or *CSE* promoter constructs. Bars with different superscripts differ significantly ($p < 0.05$).

Fig. 4. **Characterization of estrogen-responsive elements (ERRs) in human *CBS* promoter.** A: Bioinformatics analysis of human *CBS* promoter revealed putative EREs that preferentially bind ERα ERβ, or both, designated as one αERE, three 3 βEREs, and a full α/βERE and a half ½α/βERE. In the table, EREs were annotated in amplicons designed for chromatin immunoprecipitation (ChIP)-qPCR assay in Fig. 5. Pregnant hUAEC were transfected with luciferase reporter constructs driven by the wild-type (wt) human *CBS* promoter [-753/+161, pCBS(-753).Luc] or a series of its 5' deletions (B) or by pCBS(-753).Luc construct (wt) or its mutations in α/βERE2, ½α/βERE1, or both. Cells were co-transfected with a thymidine kinase-renilla luciferase vector as internal control. After treatment with 10

nM $E_2\beta$ for 24 h, cells were harvested for determining firefly/renilla luciferase activities. *CBS* promoter activation was calculated as a ratio of firefly/renilla luciferase activities. Data (means ± SEM, n=3) were expressed as fold of baseline in resting cells transfected with wt *CBS* promoter construct. Bars with different letters differ significantly ($p < 0.05$). *, $p<0.05$, **, $p<0.01$.

Fig. 5. **E2β on ERα and ERβ recruitments to estrogen-responsive elements (EREs) in human *CBS* promoter.** A: Pregnant human uterine artery cells ($4x10^6$ cells/treatment) were treated with or without 10 nM $E_2\beta$ for 24 h. Cells were harvested and DNA was crosslinked to protein by 1% formaldehyde and sheared by digestion. Chromatin was immunoprecipitated with specific ERα and ERβ antibodies and IgG was used for negative control. The ChIP samples were used for amplifying the five amplicons containing specific human *CBS* promoter EREs as illustrated in Fig. 4A using specific primers listed in B. Gel images shown represent one experiment of each amplicon from three studies with similar results using cells from different subjects. Input was amplified from 1% of the ChIP sample. C: Signal intensity of each amplicon was calculated as a ratio to that of input and expressed as fold of control. Data (means ± SEM) were from cells of three different subjects. *, $p<0.05$, **, $p<0.01$.

Fig. 6. **$E_2\beta$ on ERα and ERβ recruitments to estrogen-responsive elements (EREs) in human *CSE* promoter.** A: Bioinformatics analysis of human *CSE* promoter revealed two EREs (αERE1 and αERE2) that preferentially bind ERα and a half ERE (½α/βERE) that preferentially bind ERβ. The promoter regions containing αERE1/αERE2 and ½α/βERE were annotated in amplicon 1 and 2, which were designed for chromatin immunoprecipitation (ChIP)-qPCR assay. B: Pregnant human uterine artery endothelial cells (hUAEC, 4×10^6 cells/group) were treated with or without 10 nM $E_2\beta$ for 24 h. Cells were harvested and DNA was crosslinked to protein by 1% formaldehyde and sheared by digestion. Chromatin was immunoprecipitated with specific ERα and ERβ antibodies and IgG was used for

Accepted Manuscript published as JME-22-0175.R1. Accepted for publication: 06-Dec-2022

negative control. The ChIP samples were used for amplifying the two amplicons containing specific human *CSE* promoter EREs indicated in A, using specific primers listed in C. Gel images in B showing one experiment represent similar results using cells from 2 different subjects. Input was amplified from 1% of the ChIP sample. There was no difference in all groups ($p > 0.05$).

Fig. 7: **Specific roles of ERα and ERβ in E_2β-stimulated H_2S biosynthesis.** ERα or ERβ activation: Pregnant human uterine artery endothelial cells were treated with vehicle, 10 nM of E_2β, PPT (ERα agonist), DPN (ERβ agonist), or PPT + DPN for 24 h (A) or 48 h (B, C). ERα or ERβ inhibition: P hUAEC were treated with vehicle or estradiol-17β (E_2β) (10 nM) with or without 1 μM MPP (ERα antagonist), PHTPP (ERβ antagonist), or MPP + PHTPP. *CBS* promoter activity (A), mRNA (B) and protein (C) were determined. Data (means ± SEM) were from cells of three different subjects. Bars with different letters differ significantly ($p < 0.05$).

Fig. 8: **Mechanism underlying estrogen-induced uterine artery endothelial cell hydrogen sulfide (H_2S) biosynthesis in pregnancy**. Elevated endogenous estrogens stimulate pregnancy-dependent recruitment of estrogen receptors ERα and ERβ to their corresponding estrogen response elements (EREs) in the proximal promoter of human cystathionine β-synthase (*CBS*) gene to upregulate CBS expression and H_2S production in human uterine artery.

Table 1: Antibodies used in this study			
Target antigen	**Vendor/Source**	**Catalog#**	**Working Conc.**
human β-actin	Life Technology	AM4302	1:2000 for WB
human CD31	DAKO	M0823	1:50 for IF
human CBS	Abcam	140600	1:1000 for WB
human CSE	Santa Cruz	S374249	1:500 for WB
rabbit IgG	Cell Signaling	2729	1:100 for ChIP 1:1000 for IF
Alexa568-labeled goat anti-mouse IgG	Fisher	A-11004	1:1000 for IF
Alexa488-labeled goat anti-mouse IgG	Thermo Scientific	A-11001	1:1000 for IF
Alexa488-labeled donkey anti-rabbit IgG	Thermo Scientific	A-21206	1:1000 for IF
goat anti-mouse IgG (H+L), HRP	Invitrogen	32430	1:1000 for WB
goat anti-rabbit IgG (H+L), HRP	Invitrogen	32460	1:1000 for WB

 Accepted Manuscript published as JME-22-0175.R1. Accepted for publication: 06-Dec-2022

Table 2: Clinical characteristics of human subjects (n = 5/group)			
	Nonpregnant	**Pregnant**	**P-value**
Maternal age (years)	55.2 ± 15.55	33 ± 5.29	0.016
Heart rate (bpm)	73.2 ± 13.10	83.0 ± 8.80	0.202
Gestation age (weeks)	n/a	34.9 ± 0.78	n/a
BMI (kg/m2)	22.4 ± 1.72	28.14 ± 4.94	0.041
SBP (mmHg)	117.4 ± 10.4	114.6 ± 6.0	0.616
DBP (mmHg)	73.2 ± 13.1	68.8 ± 7.22	0.529
Birth Weight (g)	n/a	2639 ± 190.34	n/a

Table 3: Primers used for RT-qPCR of CBS and CSE mRNAs			
Gene	**Forward**	**Reverse**	**Product size**
CBS	TGAGATTGTGAGGACGCCCAC	TCACACTGCTGCAGGATCTC	177 bp
CSE	TTGTATGGATGATGTGTATGGAAGG	CCAAACAAGCTTGGTTTCTGGTG	141 bp
L19	AGACCCCAATGAGACCAATG	GTGTTTTTCCGGCATCGAGC	129 bp

Accepted Manuscript published as JME-22-0175.R1. Accepted for publication: 06-Dec-2022

Fig. 1: $E_2\beta$ on Pregnant human UA CBS/CSE ex vivo – IF analysis

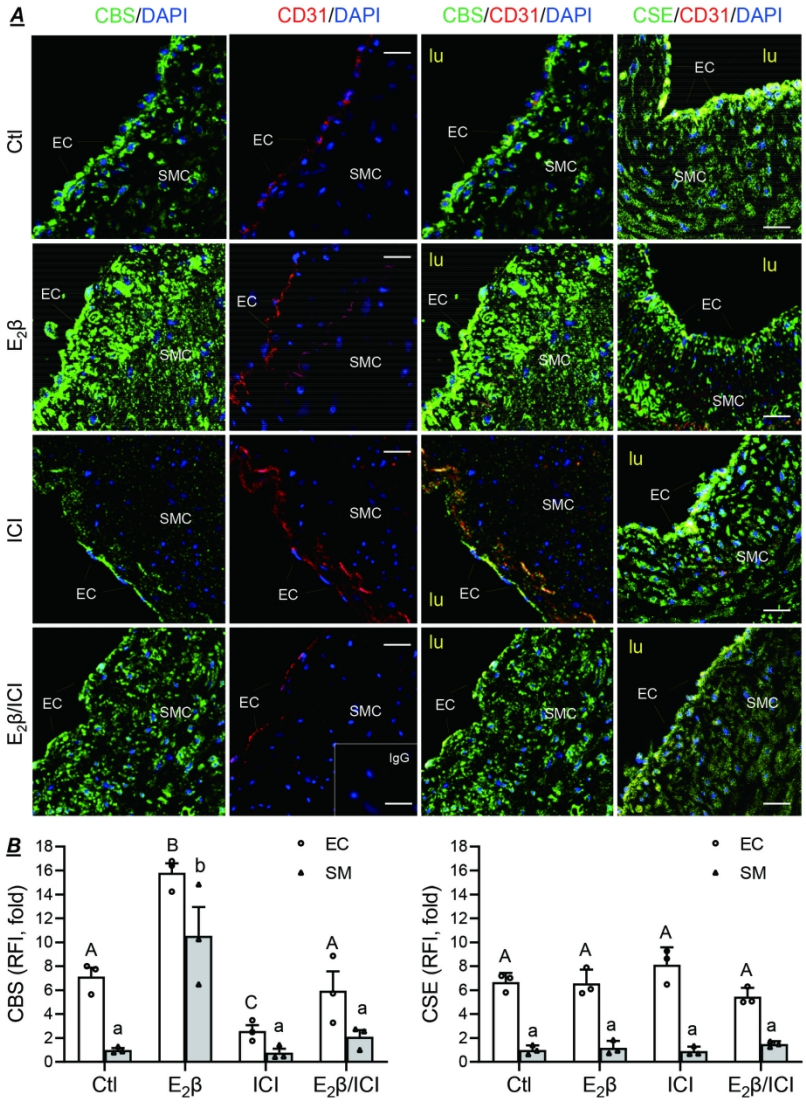


Fig. 1

179x256mm (300 x 300 DPI)

Fig. 2: $E_2\beta$ on NP and P hUAEC H_2S biosynthesis in vitro

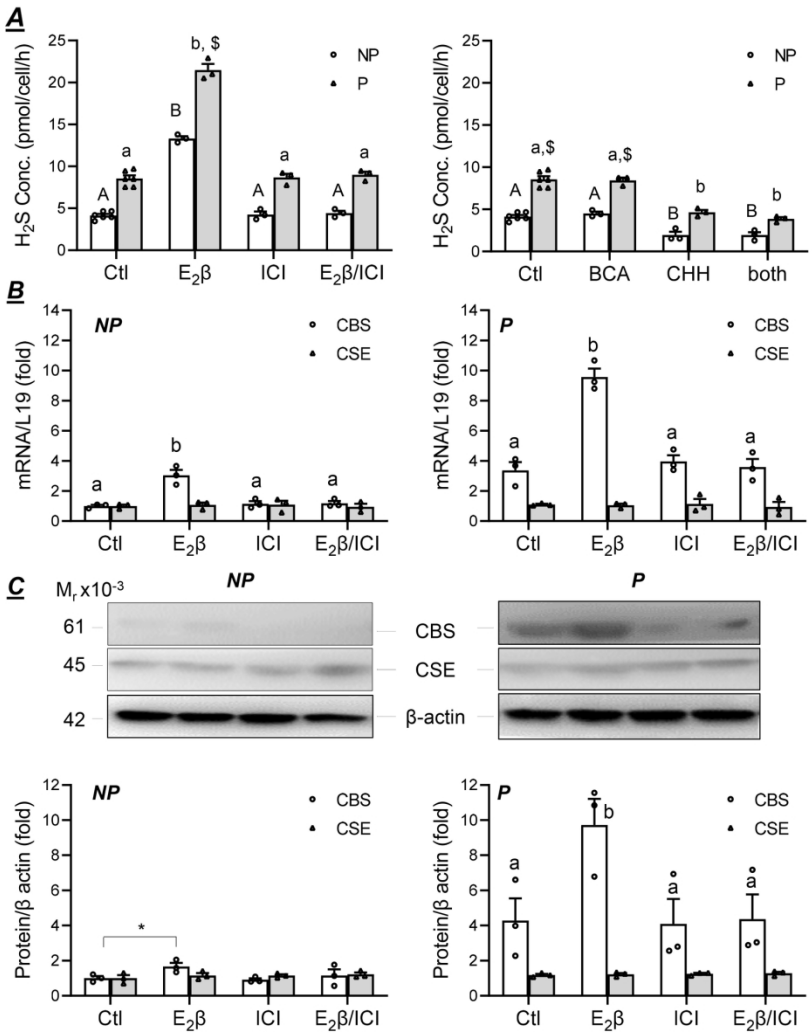


Fig. 2

156x212mm (300 x 300 DPI)

Accepted Manuscript published as JME-22-0175.R1. Accepted for publication: 06-Dec-2022

Fig. 3: $E_2\beta$ on CBS/CSE promoter activation in NP and P hUAEC

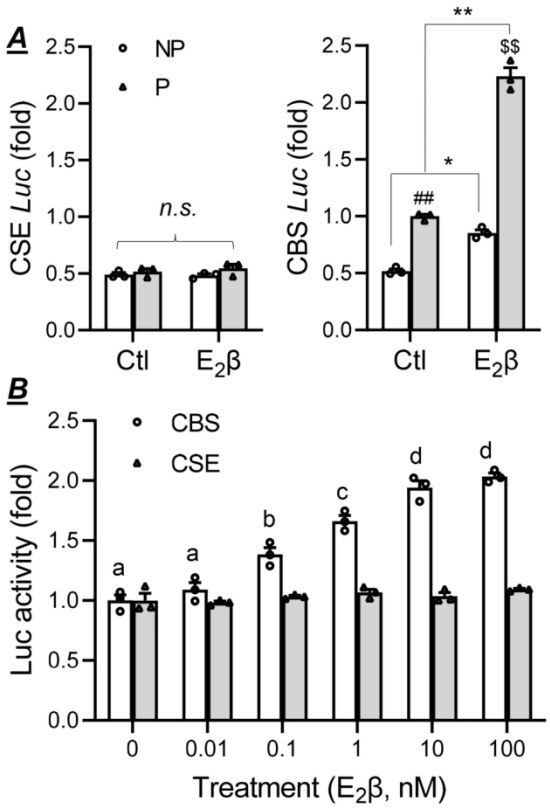
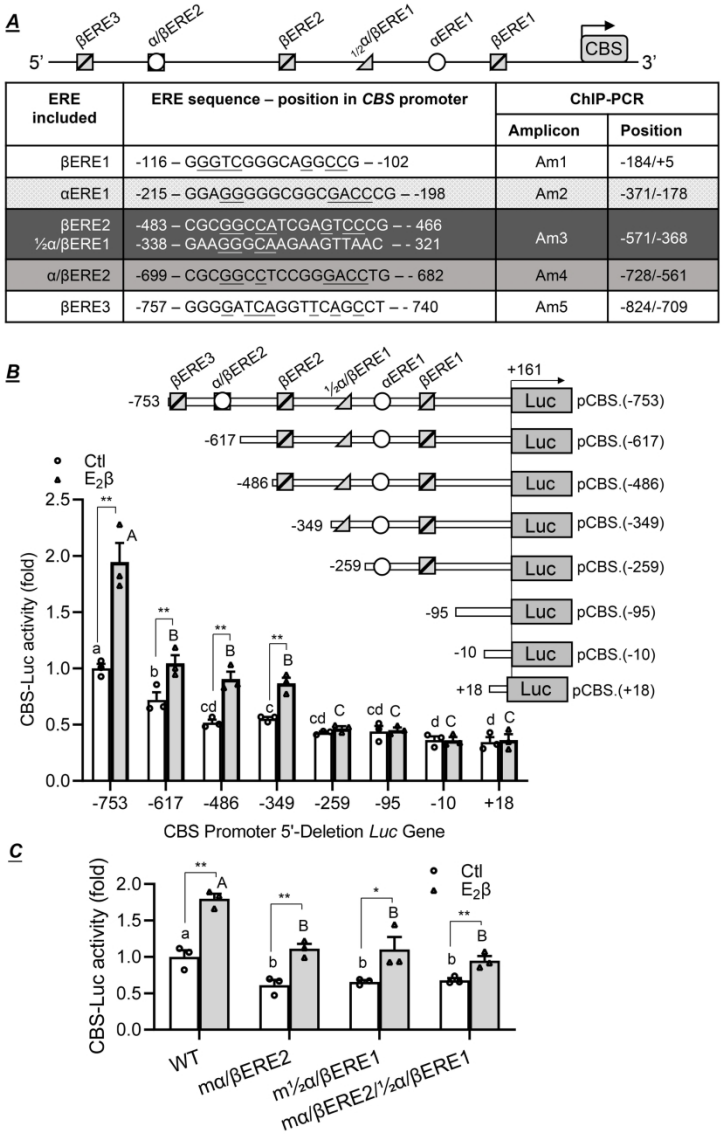


Fig. 3

146x144mm (300 x 300 DPI)

Fig. 4: $E_2\beta$ on CBS/CSE promoter activation in P hUAEC



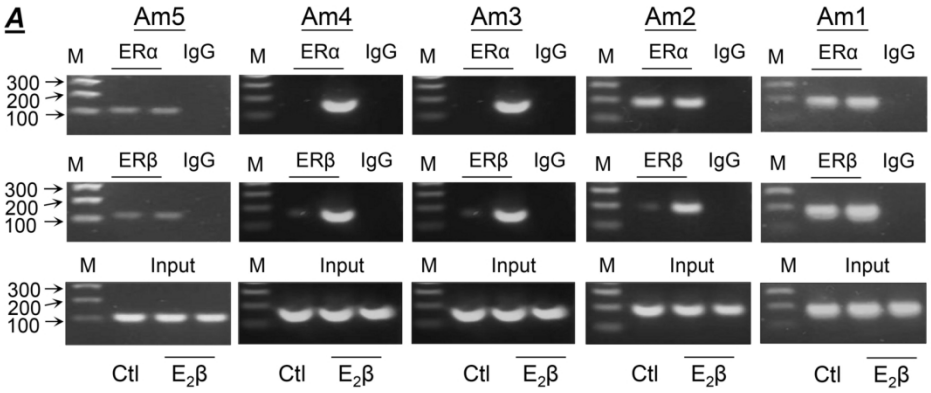
ERE included	ERE sequence – position in *CBS* promoter	ChIP-PCR	
		Amplicon	Position
βERE1	-116 – GGGTCGGGCAGGCCG – -102	Am1	-184/+5
αERE1	-215 – GGAGGGGGCGGCGACCCG – -198	Am2	-371/-178
βERE2 ½α/βERE1	-483 – CGCGGCCATCGAGTCCCG – - 466 -338 – GAAGGGCAAGAAGTTAAC – - 321	Am3	-571/-368
α/βERE2	-699 – CGCGGCCTCCGGGACCTG – - 682	Am4	-728/-561
βERE3	-757 – GGGGATCAGGTTCAGCCT – - 740	Am5	-824/-709

Fig. 4

170x266mm (300 x 300 DPI)

Accepted Manuscript published as JME-22-0175.R1. Accepted for publication: 06-Dec-2022

Fig. 5: $E_2\beta$ on ER recruitment to human *CBS* EREs (ChIP-PCR)



B Primers used for ChIP-qPCR analysis of human *CBS* promoter

Amplicon # (bp)	Forward	Reverse
Am 1 (190)	ATCAACAGGGCGTGGGAAT	CAATGTCTCGTGGCAGAGC
Am 2 (194)	TCAAGCTCATCAGTAAAGGTTCC	TGTTGATTCAACCGCAGACC
Am 3 (204)	GACTGTTCTGCGCCTCGGCTCG	TTGAAACGTGCGTCCTGGTGGGTG
Am 4 (168)	GAGCACGATGTTTGGGAAACGGC	GCAGAACAGTCGCCTTGC
Am 5 (216)	GTGTCTTTCGCTGCAGGTC	GTTTCCCAAACATCGTGCTC

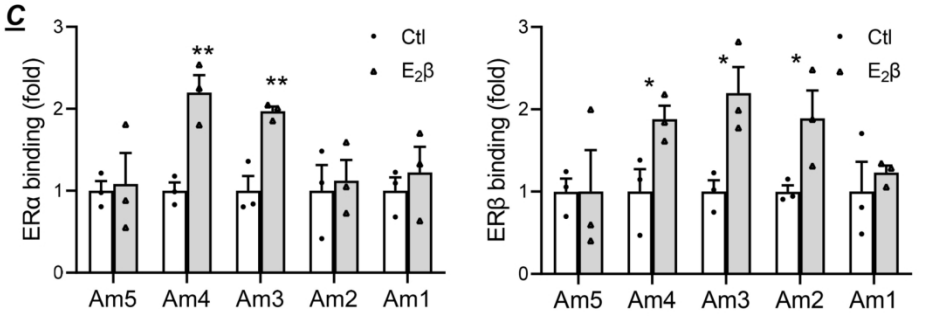
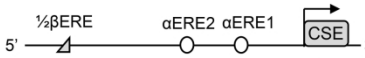


Fig. 5

154x185mm (300 x 300 DPI)

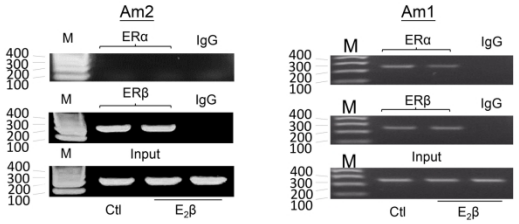
Fig. 6: $E_2\beta$ on ER recruitment to human *CSE* EREs (ChIP-PCR)

A



ERE included	Sequence – position in *CSE* promoter	ChIP-PCR	
		Amplicon	Position
αERE1	-105 – GGTCGCGGTGACGT – -92	Am1	-336/-56
αERE2	-248 – GGTCACAGAGACGC – -235		
½βERE	-565 – TACGAACAATTTAACCTT – -548	Am2	-593/-350

B



C Primers used for ChIP-qPCR analysis of human *CSE* promoter

Amplicon # (bp)	Forward	Reverse
Am1 (244)	TCCTCACTACAGTAGCTTCGTTAT	GTTACCTCTGACCACCATCCTTTG
Am2 (281)	GAAGGCTATTTATGTGGTCTGTTTACA	GCTCCTTATTGGCTGACAGCA

Fig. 6

209x279mm (300 x 300 DPI)

Accepted Manuscript published as JME-22-0175.R1. Accepted for publication: 06-Dec-2022

Fig. 7: ERα and ERβ on hUAEC CBS/H_2S production

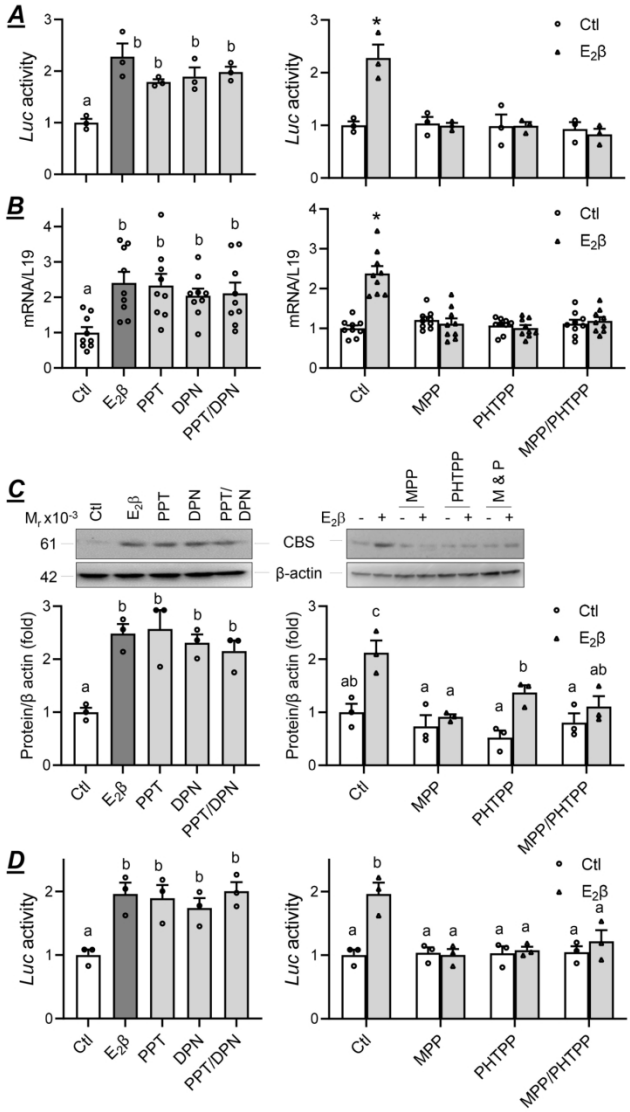


Fig. 7

118x210mm (300 x 300 DPI)

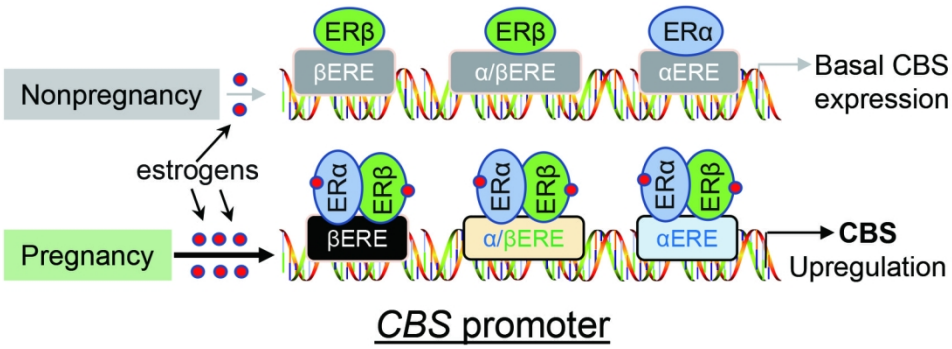


Fig. 8

151x57mm (300 x 300 DPI)

Accepted Manuscript published as JME-22-0175.R1. Accepted for publication: 06-Dec-2022

Fig. S1: $E_2\beta$ on pregnant human UA CBS/CSE mRNA/protein ex vivo

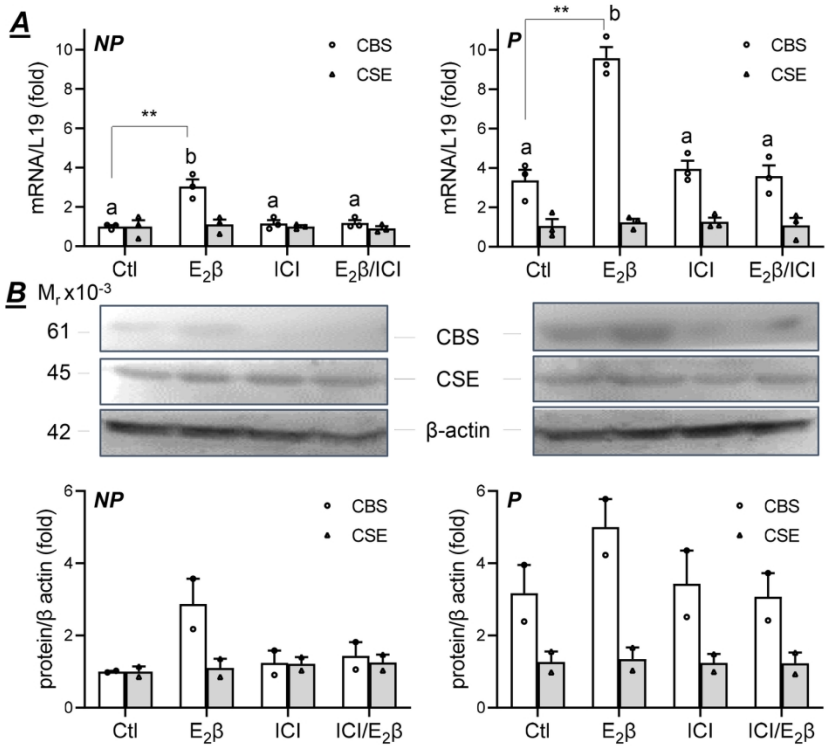


Fig. S1. E2β on human UA CBS/CSE expression ex vivo. Freshly cut human uterine artery (UA) rings from nonpregnant (NP) and pregnant (P) women were treated with vehicle, 10 nM E2β, 1 μM ICI 182 780 (ICI), or both for 24 h. A: CBS and CSE mRNAs were analyzed using real time quantitative reverse transcription-PCR (RT-qPCR) using L19 mRNA as the internal control with specific primers as listed in Table S1. Data were obtained from UAs from NP and P women (n = 3/group) and calculated as means ± SEM (fold of baseline). Bars with different letters differ significantly ($p< 0.05$). **, $p<0.01$ vs control. B: Proteins (20 μg/lane) from NP and P hUA were used for measuring CBS and CSE proteins by immunoblotting with CBS or CSE antibodies and β-actin was measured as loading control. Data from NP and P women (n=2/group) were averaged.

Fig. S1

153x196mm (300 x 300 DPI)