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Estradiol-17β Stimulates H_2S Biosynthesis by ER-dependent CBS and CSE Transcription in Uterine Artery Smooth Muscle Cells *in vitro*

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Abstract

Endogenous hydrogen sulfide (H_2S), synthesized by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), is a potent vasodilator that can be stimulated by estradiol-17β ($E_2β$) in uterine artery (UA) smooth muscle (UASMC) *in vivo*; however, the underlying mechanisms are unknown. This study tested a hypothesis that $E_2β$ stimulates H_2S biosynthesis by upregulating CBS expression *via* specific estrogen receptor (ER). Treatment with $E_2β$ stimulated time- and concentration- dependent CBS and CSE mRNA and protein expressions and H_2S production in cultured primary UASMC isolated from late pregnant ewes, which were blocked by ICI 182, 780. Treatment with specific ERα or ERβ agonist mimicked these $E_2β$-stimulated responses, which were blocked by specific ERα or ERβ antagonist. Moreover, $E_2β$ activated both *CBS* and *CSE* promoters and ICI 182,780 blocked the $E_2β$-stimulated responses. Thus, $E_2β$ stimulates H_2S production by upregulating CBS and CSE expression *via* specific ER-dependent transcription in UASMC *in vitro*.

Keywords

Estrogens; hydrogen sulfide; CBS; CSE; uterine artery smooth muscle cells; *in vitro*

INTRODUCTION

Estrogens potently dilate selected vasculatures throughout the body, with the greatest response in the uterus as reflected by dramatic rise in uterine blood flow (UBF) following local or systemic administration of 17β-estradiol ($E_2β$) in various nonpregnant (NP) and pregnant (P) animal models (Ford et al., 1982; Magness and Rosenfeld, 1989; Rosenfeld et al., 1973). Estrogen-induced uterine vasodilation is important for pregnancy since circulating estrogens increase nearly 1000-fold, accompanied by as much as 50–80 fold rise in UBF in P *vs*. NP states (Magness and Rosenfeld, 1989; O'Leary et al., 1991). Estrogen-induced and pregnancy-associated rises in UBF facilitate the bidirectional maternal-fetal exchange of gases (e.g., O_2 and CO_2) and provide the sole nutrients to support fetal development and

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survival (Rosenfeld, 1977). Any constraints in UBF during pregnancy lead to preeclampsia and intrauterine growth restriction, threatening the health of both the mother and her fetus during pregnancy and after birth (Barker, 1995; Osol and Moore, 2014). Enhanced uterine artery (UA) endothelial production of nitric oxide (NO) has been recognized as a key player in estrogen-induced and pregnancy-associated uterine vasodilation because: 1) estrogens stimulate NO production *via* endothelial NO synthase (eNOS) in UA endothelial cells (EC) *in vivo* (Magness et al., 2001) and *in vitro* (Chen et al., 2004) and 2) local NO inhibition significantly attenuates estrogen-induced rise in UBF in NP ewes (Magness et al., 2005; Rosenfeld et al., 1996). However, local NO inhibition blocks only ~65% estrogen-induced uterine vasodilation (Magness et al., 2005; Rosenfeld et al., 1996), suggesting that mechanisms in addition to NO exist.

Hydrogen sulfide (H_2S) is a gaseous signaling molecule of the gasotransmitter family after NO and carbon monoxide (Wang, 2012) that function as potent vasodilators (Yang et al., 2008). Endogenous H_2S is primarily synthesized from L-cysteine by two enzymes: cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) (Bukovska et al., 1994; Erickson et al., 1990; Kredich et al., 1973); their expression can be tissue/cell specific as both are needed to generate H_2S in some tissues while one enzyme is sufficient in others (Gadalla and Snyder, 2010; Mustafa et al., 2009). Our recent work first shows H_2S to be a novel UA vasodilator since H_2S production and the expression of UA smooth muscle (SM) and EC CBS, but not CSE, are significantly stimulated by exogenous estrogens in ovariectomized (OVX) NP ewes (Lechuga et al., 2015) and also are significantly augmented and positively linked to endogenous estrogens during pregnancy in human UA (Sheibani et al., 2017); moreover, a slow releasing H_2S donor GYY4137 dose-dependently relaxes NP and P rat UAs, with greater potency in P state and vascular bed-specific effects (Sheibani et al., 2017). However, the mechanisms by which $E_2β$ stimulate UASM and EC H_2S biosynthesis remains to be elusive.

The large tube-shaped primary UA is composed of a thin layer of ECs covering the luminal surface that is surrounded by a thick layer of SM cells (SMCs). The role of ECs in uterine hemodynamics has been extensively studied; in comparison, the role of SMCs in estrogen-induced UA dilation is currently much less understood. Early studies show that estrogens stimulate UASMC proliferation during pregnancy-associated UA remodeling (Keyes et al., 1996). Our previous work shows that UASMCs are direct target cells of estrogens as they express ERα and ERβ (Byers et al., 2005; Liao et al., 2005). Indeed, estrogens stimulate endothelial-independent vasorelaxation (Jiang et al., 1991; Mugge et al., 1993) and various signaling pathways, including activation of extracellular signal-activated kinases (ERK½) and protein kinase C (Khan et al., 2010), cyclic GMP secretion, expression of large conductance potassium channels (BK_{Ca}) (Rosenfeld et al., 1996; Rosenfeld et al., 2002), and membrane hyperpolarization (White et al., 1995) in UASMCs. However, whether SMCs produces vasodilators is always neglected.

We hypothesize that $E_2β$ stimulates UASMC H_2S biosynthesis *via* specific ER-dependent CBS transcription. The objective of this study were to first establish an *in vitro* UASMC culture model from late pregnant ewes by which we aimed to determine in UASMC *in vitro* if: 1) $E_2β$ stimulates H_2S production via enhanced expression of CBS and/or CSE

expression; 2) $E_2\beta$-stimulated H_2S biosynthesis is mediated by specific ERs; 3) ERα and ERβ play a role in $E_2\beta$-stimulated H_2S biosynthesis; and 4) $E_2\beta$-stimulates ER-dependent *CBS* and/or *CSE* transcription.

MATERIALS & METHODS

Chemicals and antibodies

17β-estradiol, 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 182, 780 (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-piperidinylet hoxy)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-β-actin monoclonal antibody was from Ambion (Austin, TX). Fetal bovine serum (FBS) was from Lonza (Walkersville, MD). Monoclonal antibodies against ERα and ERβ were from Fisher Scientific (Pittsburgh, PA). Antibodies against CBS, CSE, α-smooth muscle actin (α-SMA), eNOS, and caveolin-1 were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). DMEM and M199, Platinum Taq DNA polymerase, and $Alexa^{488}$ and $Alexa^{588}$-labeled immunoglobulin G (IgG) were from Invitrogen (Carlsbad, CA).

Primary UASMC isolation, culture, characterization, and treatment

Primary UASMCs were isolated from late pregnant ewes (120–130 days of gestation, normal term ≈145 days). The Institutional Animal Care and Use Committee of the University of California approved the animal use protocol. Immediately after sacrifice, the main UAs were dissected; the lumen was filled with 0.1% collagenase for a 45-min digestion at 37°C to remove the endothelium. Endothelium-denude UAs were cut into ~1-cm segments; after softening the tissue by incubation with 0.1% collagenase for 20 min, the SM layer was dissected mechanically under a Stereo Microscope (10x). The SM segments were then minced (<1 mm^3) and digested with 0.1% collagenase in M-199–0.1% BSA at 37°C for 45 min. The cells were then collected and cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin. UASMCs were frozen in liquid N2. UASMCs at passage 2 were characterized by flow cytometry with SM and EC markers α-SMA and eNOS, respectively, as well as the expression of caveolin-1. Cells were used for later experiments within 7 passages.

Cells at ~80% 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. Following equilibration in fresh serum-free M-199, cells were treated with or without ER antagonists (1 μM) for 1 h, followed by treatment with or without $E_2\beta$ (10 nM) for 48 h. For time course experiments, cells were treated with $E_2\beta$ (10 nM) for up to 72 h. For dose response experiments, cells we treated with 0–1μM $E_2\beta$ for 48 h. For ER agonist experiments, cells were treated with $E_2\beta$ (10 nM) or specific ER agonists (10 nM) for 48 h. Ethanol was used to dissolve $E_2\beta$ and ER agonists and antagonists. Final

ethanol concentrations were less than 0.5% and did not alter cellular responses surveyed in this study.

Immunofluorescence microscopy and image analysis

UASMCs were grown on glass coverslips to reach ~80% confluency. Following treatments, cells were washed in cold phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde for 20 min at room temperature. After permeablized with 0.2% Triton-100 in PBS for 15 min at room temperature, the cells were labeled with 1 μg/mL of anti-α-SMA, caveolin-1, CBS, or CSE overnight at 4°C, followed by $Alex^{486}$ or $Alex^{588}$-labeled corresponding IgGs (2μg/ml) for 1 h at room temperature. Coverslips were mounted with Prolong Gold antifade reagent containing 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) for labeling cell nuclei. Samples were examined under a fluorescence microscope (Leica Corporation, Deerfield, IL) and digital images were acquired using a CCD camera and *Simple*PCI image analysis software (Hamamatsu Corporation, Sewickley, PA). The images were used to determine relative CBS and CSE proteins by averaging mean fluorescence intensity (15 cells/image and 5 images/animal) by using *Simple*PCI as described (Lechuga et al., 2015). Normalized CBS and CSE proteins were presented as fold change of vehicle (veh) treated control cells.

Flow cytometry

UASMCs were fixed with 70% ethanol for 1 h at 4°C. The cells were washed with PBS and resuspended in PBS containing 1% BSA at a concentration of 1×10^6/ml. After incubation with 10% goat serum for 30 min, the cells (2.5×10^5/ml) were incubated with PBS containing 1 μg/ml anti-α-SMA, anti-eNOS, or anti-caveolin-1 for 1 h at room temperature, followed by FITC-labeled anti-mouse (SC2010, 1:200) or anti-rabbit (SC2012, 1:200) IgG (Santa Cruz) overnight at 4°C in dark. Positive staining was verified by omitting first antibody and with only secondary antibodies. The stained cells were analyzed by FACSCalibur using CellQuest software (Becton Dickinson, Mountain View, CA).

SDS-PAGE and immunoblotting

SDS-PAGE and immunoblotting was performed as previously described (Lechuga et al., 2015; Sheibani et al., 2017).

Methylene Blue Assay

UASMCs (5×10^5/treatment in duplicate) were homogenized in 50 mM ice-cold potassium phosphate buffer pH 8.0. H_2S production was determined by using the methylene blue assay as previously described (Lechuga et al., 2015; Sheibani et al., 2017). H_2S concentration was calculated based on a calibration curve generated from NaHS solutions. For CBS and CSE inhibition experiments, their respective inhibitor CHH or BCA, were added separately or in combination (final conc. = 2 mM) to the reaction mixtures prior to initiating the assay.

Reverse transcription (RT-PCR) and quantitative real-time RT-PCR (qPCR)

Total RNAs were extracted from UASMCs using Trizol reagent (Invitrogen, Carlsbad, CA). RNA was quantified by $OD_{260/280}$ and first-strand complementary DNA (cDNA) was

synthesized by using random primers and AMV Reverse Transcriptase (Promega, Madison, WI) with 2 μg RNA template as previously described (Lechuga et al., 2015; Liao et al., 2005). RT-PCR using *ERα* and *ERβ* specific primers was conducted to assess ERα and ERβ mRNAs reference to ribosomal protein L19 as described previously (Liao et al., 2005). *q*PCR using *CBS* and *CSE* specific primers was conducted to assess CBS and CSE mRNAs reference to ribosomal protein L19 by using the comparative CT method (*ΔΔ*CT method) as described previously (Lechuga et al., 2015; Sheibani et al., 2017).

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 UASMCs by using FuGENE HD transfection reagents (1:3, μl/ng) for 24 h at 37°C. Cells transfected with an empty vector or β-actin promoter vector (S717678) were served as negative and positive transfection controls, respectively. After recovery in DMEM medium containing 10% FBS for 18–20 h, cells were serum-starved overnight and then treated with vehicle or 10 nM $E_2β$ for 24 h with or without ICI 182,780. The RenSP luciferase activity normalized by cypridina luciferase activity in both cells and supernatant were measured to assess CBS and CSE transcription as previously described (Mata-Greenwood et al., 2010).

Statistical analysis

Each experiment was repeated at least three times with cells derived from different ewes. Data were presented as mean ± SEM and analyzed by one- or two-way analysis of variance (ANOVA), followed by *Bonferroni* test for multiple comparisons using *Sigma*Plot (Systat Software Inc.). Paired Student's *t*-test was used for data comparison between two groups. Significance was defined as $P<0.05$, unless higher statistical power is indicated in the figure legends.

RESULTS

Characterization of a primary UASMC model

Primary UASMCs exhibited spindle-shaped classical smooth muscle cell morphology in culture and immunostained positively with the SM marker α-SMA and expressed high levels of the scaffolding protein caveolin-1 (Fig. 1A). Flow cytometry analysis showed that UASMCs expressed 99.9% α-SMA and caveolin-1, with low eNOS expression (Fig. 1B). UASMCs expressed ERα and ERβ mRNAs (Fig. 1C) and proteins (Fig. 1D) with levels quantitatively comparable to that of intact UAs. Immunoblot analysis revealed eNOS protein in intact UA but not in UASMCs (Fig. 1D).

$E_2β$ stimulates UASMC H_2S production in vitro: role of CBS and CSE.

Treatment with 10 nM $E_2β$ resulted in a 4.77 ± 0.34 fold increase ($P<0.01$) in H_2S production in UASMCs. Addition of CHH, BCA, or their combination did not alter baseline H_2S production in Veh-treated cells. Either CHH or BCA alone significantly inhibit $E_2β$-stimulated H_2S production, although the inhibition was partial. Their combination did not

fully enhance either CHH or BCA inhibition of $E_2\beta$-stimulated H_2S production; it did further reduce $E_2\beta$-stimulated H_2S production to baseline as seen in Veh-treated cells (Fig. 2).

$E_2\beta$ stimulates CBS and CSE in time- and concentration-dependent manners in UASMCs

$E_2\beta$ significantly stimulated CBS and CSE mRNAs in a time-dependent manner in UASMC *in vitro*. Following treatment with 10 nM $E_2\beta$, levels of both CBS and CSE mRNAs began to increase significantly at 24 h, maximized at 48 h (CBS: 5.11 ± 1.03 fold vs. control; $P<0.01$; CSE: 5.61 ± 0.93 fold vs. control, $P<0.01$), and plateaued at 72 h (Fig. 3A). $E_2\beta$ also significantly stimulated CBS and CSE proteins in a time-dependent manner. Following treatment with 10 nM $E_2\beta$, levels of both CBS and CSE proteins began to significantly increase at 24 h and maximized by 48 h (Fig. 3B).

$E_2\beta$ also significantly stimulated CBS and CSE mRNAs in a concentration-dependent manner in UASMC *in vitro*. At 48 h post-treatment, 0.1 nM $E_2\beta$ effectively stimulated both CBS and CSE mRNA expressions. CBS and CSE mRNA expression was increased with increasing doses of $E_2\beta$ and was maximized with 100 nM $E_2\beta$ and plateaued with 1 μM $E_2\beta$ (Fig. 3C). At 48 h post-treatment, 0.1 nM $E_2\beta$ effectively stimulated both CBS and CSE proteins. The responses increased with increasing concentrations of $E_2\beta$, and maximized with 1μM $E_2\beta$ for CBS (3.69 ± 0.09 fold vs. control, $P<0.01$) and 10nM $E_2\beta$ for CSE (2.51 ± 0.03 fold vs. control, $P<0.01$) (Fig. 3D).

$E_2\beta$ stimulates ER-dependent CBS and CSE transcription in UASMCs

Treatment with $E_2\beta$ provoked 4.00 ± 0.30 ($P<0.001$) and 4.21 ± 0.61 ($P<0.001$) fold increases in CBS and CSE promoter activities in UASMCs. Treatment with ICI was able to completely attenuated $E_2\beta$-stimulated CBS and CSE promoter activation (Fig. 4A). Consistent with these findings, treatment with 10 nM $E_2\beta$ resulted in 5.11 ± 1.03 ($P< 0.001$) and 5.61 ± 0.93 ($P< 0.001$) fold increases in CBS and CSE mRNAs in UASMCs, respectively. Treatment with 1 μM ICI did not alter baseline but completely attenuated $E_2\beta$-stimulated CBS and CSE mRNA expressions (Fig. 4B).

We next assessed the role of ERα and ERβ in $E_2\beta$-stimulated CBS and CSE mRNA expression. Treatment with specific agonist of ERα (PPT, 10 nM) or ERβ (DPN, 10 nM) alone, or their combination, was able to fully mimic $E_2\beta$-stimulated CBS and CSE mRNA expression (Fig. 4C). Treatment with the specific antagonist for ERα (MPP, 1 μM) or ERβ (PHTPP, 1 μM) alone partially blocked $E_2\beta$-stimulated CBS and CSE mRNA expressions; their combination was able to fully attenuate $E_2\beta$-stimulated CBS and CSE mRNA expressions (Fig. 4D). Either MPP or PHTPP alone or their combination did not alter baseline CBS and CSE mRNA levels in UASMCs.

$E_2\beta$ stimulates specific ER-dependent CBS and CSE protein expressions and activities in UASMCs

Immunocytochemistry analysis revealed that UASMCs exhibited low basal CBS and CSE proteins in culture. Both CBS and CSE proteins were mainly localized in the cytoplasm of Veh, $E_2\beta$, and ICI-treated cells (Fig. 5A). Quantitative immunocytochemistry analysis

showed that treatment with 10 nM $E_2\beta$ stimulated 3.08 ± 0.35 ($P<0.001$) and 2.29 ± 0.18 ($P<0.001$) fold increases in CBS and CSE proteins, respectively; the stimulations were completely blocked by ICI. Immunoblot analysis revealed that treatment with 10 nM $E_2\beta$ stimulated 3.17 ± 0.09 ($P< 0.001$) and 2.51 ± 0.04 ($P< 0.001$)-fold increases in CBS and CSE proteins, respectively; 1 μM ICI did not alter baseline expression but completely blocked $E_2\beta$-stimulated CBS and CSE protein expressions (Fig. 5B).

Treatment with specific agonist of ERα (PPT, 10 nM) or ERβ (DPN, 10 nM) alone fully mimicked $E_2\beta$-stimulated CSE protein expressions; however, either agonist alone only partially mimicked $E_2\beta$-stimulated CBS expression, but their combination was able to fully mimic $E_2\beta$-stimulated CBS protein expression (Fig. 5C). Treatment with specific antagonist for ERα (MPP, 1 μM) or ERβ (PHTPP, 1 μM) alone or in combination blocked $E_2\beta$-stimulated CBS and CSE protein expressions (Fig. 5D). Either MPP or PHTPP alone or their combination did not alter baseline CBS and CSE protein levels in UASMCs.

Treatment with ICI did not alter baseline but completely blocked $E_2\beta$-stimulated H_2S production (Fig. 5E). Treatment with the ERα agonist PPT or ERβ agonist DPN alone provoked 2.95 ± 0.76 and 2.91 ± 0.77 fold increases ($P<0.05$) in H_2S production, respectively. The effects of PPT or DPN alone were less effective ($P<0.05$) than $E_2\beta$; however, the combination of PPT and DPN was as effective as $E_2\beta$ to stimulate H_2S production in UASMCs (Fig. 5F).

DISSCUSSION

We have recently shown that exogenous and endogenous estrogens stimulate UA H_2S biosynthesis by selectively upregulating SMC and EC CBS, but not CSE, mRNA and protein expressions *in vivo* (Lechuga et al., 2015), raising a key question as to by what mechanisms $E_2\beta$ selectively stimulates CBS, but not CSE, expression in vascular SMCs and ECs. In this study, we chose to focus on UASMCs to address the question because: 1) in comparison to ECs, the SMC contribution to UA hemodynamics is much less understood and 2) unlike NO that is well-documented to be mainly produced by UAEC (Govers and Rabelink, 2001), UASMCs is a major source of vascular H_2S when the SMC and EC composition of UA and their cellular (SMC *vs.* EC) CBS and CSE expression patterns (Lechuga et al., 2015; Sheibani et al., 2017) are taken into consideration.

To accomplish our goal to address the mechanisms underlying estrogen selective stimulation of CBS expression in UASMCs, herein we have successfully developed a primary UASMC culture model from late pregnant ewes. The cells display typical spindle-shaped morphology in culture and highly express the SM marker α-SMA in culture. Quantitative analysis by flow cytometry revealed that they are >99.9% positive for α-SMA and caveolin-1. Flow cytometry analysis also detects low levels of eNOS protein in UASMCs; however, eNOS protein was not detected by immunoblotting, suggesting that the eNOS protein is background staining within the flow cytometry analysis. These cells express high levels of ERα and ERβ mRNAs (Fig. 1C) and proteins (Fig. 1D) in culture and levels of both retain comparable to that in intact UA *in vivo* (Liao et al., 2005), even after several passages (up to 7 tested, data not shown). Nonetheless, these findings show that UASMCs in culture possess

functional ER-dependent responsiveness to $E_2\beta$ stimulation and thus, providing us with a suitable model for delineating the cellular and molecular mechanisms by which $E_2\beta$ stimulates UA H_2S biosynthesis *in vitro*. Indeed, with this cell model we now first report that $E_2\beta$ stimulates UASMC H_2S production *in vitro*, which is mediated by specific ER as this is blocked by ICI. Moreover, we show that both CBS and CSE are involved in $E_2\beta$-stimulated UASMC H_2S production as the stimulation was sensitive to both CBS and CSE inhibition.

Our data show that $E_2\beta$ stimulates CBS and CSE mRNA and protein in time- and concentration-dependent manners in UASMCs. $E_2\beta$ stimulation of CBS mRNA and protein expressions in UASMCs *in vitro* is consistent with our recent *in vivo* findings that $E_2\beta$ stimulates CBS mRNA and protein expressions in UASMCs in ewes (Lechuga et al., 2015) and women (Sheibani et al., 2017). $E_2\beta$ also stimulates CSE mRNA and protein expressions in UASMCs *in vitro*. This observation is unexpected because it not only contrasts to our *in vivo* findings that UASMC CSE mRNA and protein are not altered by estrogens in sheep (Lechuga et al., 2015) and women (Sheibani et al., 2017), but also other reports showing that $E_2\beta$ does not stimulate CSE expression in mouse mesenteric artery SMCs *in vitro* (Li et al., 2012). Although the mechanisms underlying this discrepancy between the *in vitro* and *in vivo* findings is elusive, this raises a bell of caution when using *in vitro* cell models to explore the mechanisms underlying *in vivo* phenomena. However, estrogen stimulation of both CBS and CSE expression in UASMCs *in vitro* is further supported by the inhibition studies showing that both CBS and CSE are involved in $E_2\beta$ stimulation of H_2S biosynthesis in UASMCs *in vitro* (Fig. 2).

Our data clearly show that $E_2\beta$-stimulation of CBS and CSE mRNA and protein expressions are mediated by specific ER in UASMCs as the stimulations are completely attenuated by ICI 182, 780. UASMCs express both ERα and ERβ (Liao et al., 2005). In cells expressing both ERα and ERβ, including UAECs they can play similar or even opposite roles in mediating estrogen signaling (Zhang et al., 2012). Thus, an important question arises as to what specific roles ERα and ERβ play in mediating $E_2\beta$ stimulation of H_2S biosynthesis in UASMCs. In studies using ER isoform-specific agonists and antagonists, we have found that both ERα and ERβ are involved in $E_2\beta$ stimulation of CBS and CSE mRNA and protein expressions in UASMCs *in vitro*; however, each seems to play a unique role in stimulating either CBS and CSE mRNA and protein in the cells. For instance, activation of either ERα or ERβ is sufficient to mediate $E_2\beta$-stimulated CBS and CSE mRNA and CSE protein expressions because this stimulation can be mimicked by treatment with either specific agonist for ERα (PPT) or ERβ (DPN) alone or their combination. In contrast, $E_2\beta$-stimulated CBS protein expression requires activation of both ERα and ERβ as this is fully mimicked by PPT and DPN combination and only partially by either one agonist. These findings are strengthened by studies using the specific ERα antagonist MPP and ERβ antagonist PHTPP. Consistently, we have shown that $E_2\beta$ stimulation of UASMC H_2S production requires activation of both ERα and ERβ as this was fully mimicked by PPT and DPN combination and only partially by either agonist.

Of note, same conclusions for the roles of ERα and ERβ in $E_2\beta$ stimulation of CSE mRNA and protein are drawn based on findings using ERα and ERβ agonists and antagonists;

however, different conclusions are obtained for the roles of ERα and ERβ in E2β stimulation of CBS mRNA and protein. These results suggest that ERα and ERβ regulate E_2β stimulation of CBS and CSE expression similarly at the mRNA level but differently at the protein level. Although this study does not offer the underlying mechanisms, our current findings, nonetheless, suggest that ERα and ERβ play different roles in mediating enhanced uterine artery production of H_2S in uterine hemodynamic regulation, consistent with our previous studies showing different roles of ERα and ERβ in estrogen signaling in UAEC (Zhang et al., 2012).

E_2β stimulates both mRNA and protein expressions of CBS and CSE *in vitro* (current study) and CBS *in vivo* (Lechuga et al., 2015; Sheibani et al., 2017), suggesting that these stimulations occur at the level of transcription. Indeed, E_2β stimulates >4-fold increases in *CBS* and *CSE* promoter activity in UASMCs *in vitro* (Fig. 4A). The human *CBS* promoter is >4k bp and encodes five distinct 5' non-coding exons (Kraus et al., 1998), containing putative estrogen response elements (ERE) for binding ERα and ERβ, as well as other transcription factors including Sp1, Sp3 and AP-1 (Ge et al., 2001; Renga, 2011). The human *CSE* promoter is >1.5k bp and contains binding sites for ER, Sp1 and AP-1 (Renga, 2011; Yin et al., 2012). Thus, E_2β stimulation of CBS and CSE transcription may occur through direct interactions between ERs and EREs in UASMCs. To this end, further studies are needed to delineate the specific roles of ERα and ERβ and other transcription factors, such as Sp1 and AP1, that can interact with ERα and ERβ to activate gene transcription (Schultz et al., 2005; Yang et al., 2011) during E_2β stimulation of CBS and CSE transcription in UASMCs.

Altogether, our current study demonstrates that E_2β stimulates UASMC H_2S production *in vitro* by upregulating CBS and CSE mRNA and protein expression *via* specific ERα and ERβ-dependent activation of *CBS* and *CSE* transcription in UASMC *in vitro* (Fig. 6).

PERSPECTIVES

Estrogen-induced and pregnancy-associated uterine vasodilation as measured by a rise in UBF is believed to be primarily mediated by orchestrated vasodilator networks locally produced by UA endothelium, including NO (Rosenfeld et al., 2002; Valdes et al., 2009). Endothelial NO mediates less than 65% estrogen-induced uterine vasodilation (Magness et al., 2005; Rosenfeld et al., 1996); however, the mechanisms in addition to endothelial NO have not been defined and whether UA SMCs also produces vasodilators to mediate estrogen-induced uterine vasodilation is neglected. Our current findings, together with our recent *in vivo* work showing H_2S as a novel UA vasodilator (Lechuga et al., 2015; Sheibani et al., 2017), provide a novel mechanism for estrogen-induced uterine vasodilation *via* SM-derived H_2S. Understanding the effects of estrogens and the mechanisms by which estrogens regulate the expression of vasodilator producing enzymes in UASM provides fundamental understanding of normal pregnancy-associated vascular adaptations and clues for the pathophysiology of preeclampsia and other cardiovascular disorders. Nonetheless, our findings necessitate the evaluation of SM H_2S in the cardiovascular protective effects of estrogens as it pertains to the regulation of vascular tone in normal physiology and

dysregulation in the pathophysiology of vascular diseases, such as hypertension, atherosclerosis, and gestational vascular diseases such as preeclampsia and IUGR.

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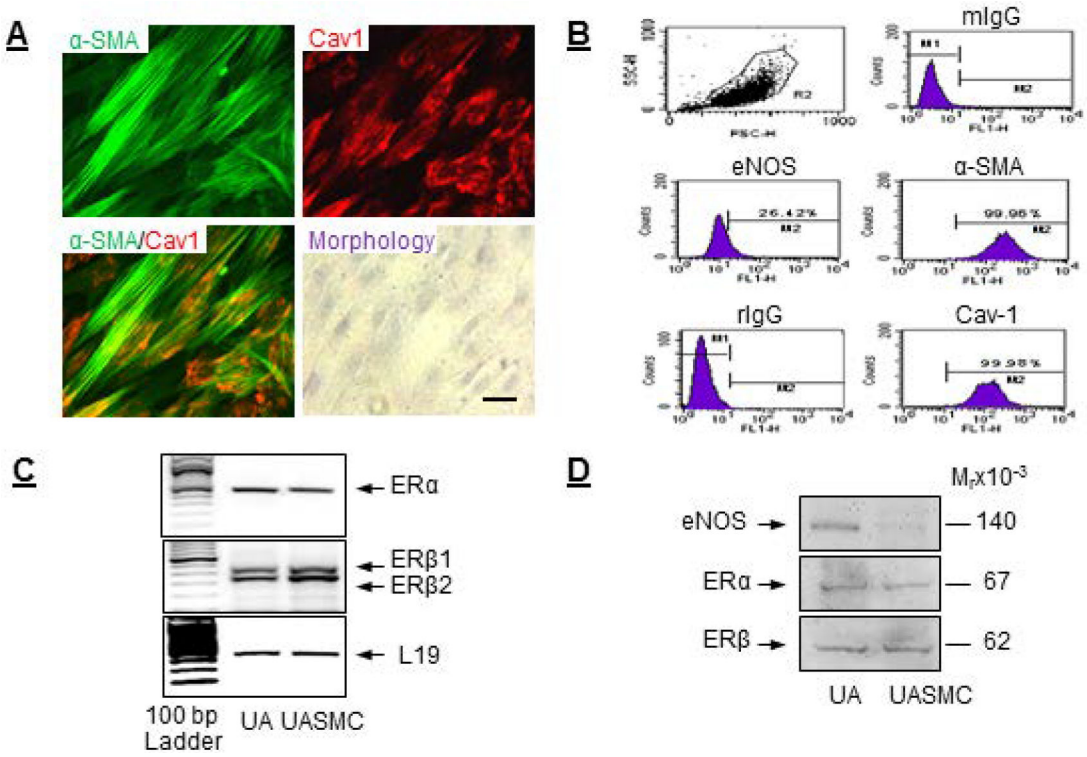

Fig. 1: Establishment and characterization of an ovine uterine artery smooth muscle cells (UASMC) model.

Primary UASMCs from late pregnant ewes were isolated and characterized. (A) Cellular expression and co-localization of α-SMA and caveolin-1 by immunocytochemistry and assessment of cell morphology. (B) Flow cytometry for cellular expression of α-SMA, caveolin-1, and eNOS. ERα and ERβ (C) mRNA and (D) protein expressions in UASMCs and intact UAs. Scale bar is 50 μm.

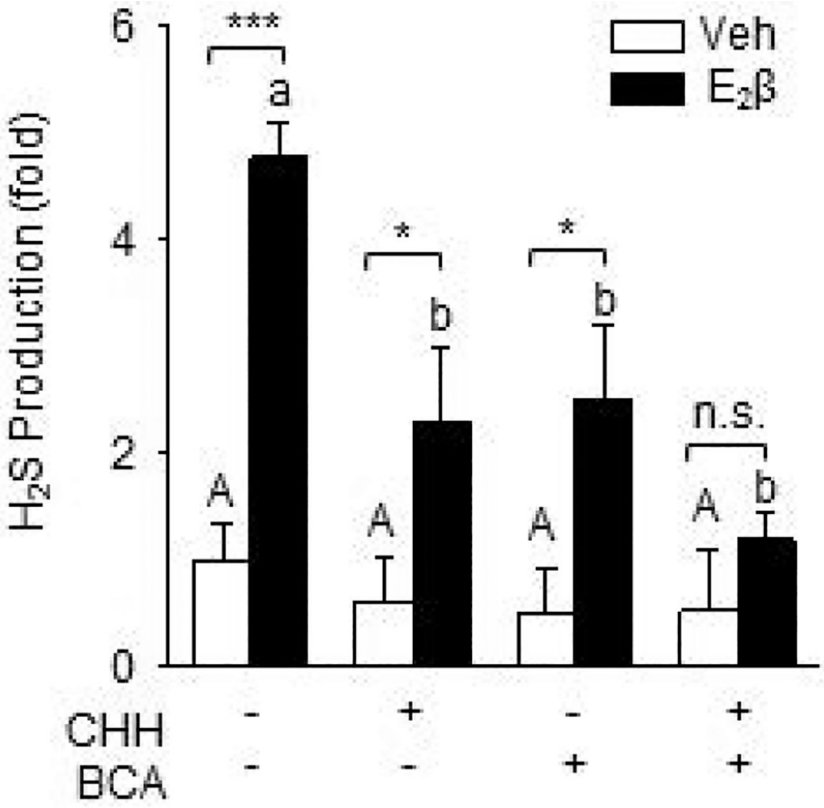

Fig. 2: $E_2\beta$ stimulates UASMC H_2S production *in vitro*.

Cells were treated with vehicle or 10 nM $E_2\beta$ for 48 h. Cell lysates were subjected to the methylene blue assay to determine H_2S production with CHH and/or BCA. Data (mean ± SEM) were collected from cells prepared from 3–5 different ewes. Bars with different letters differ significantly ($p < 0.05$), capital letters pertain to Veh and lowercase to $E_2\beta$, * $P<0.05$, *** $P<0.001$.

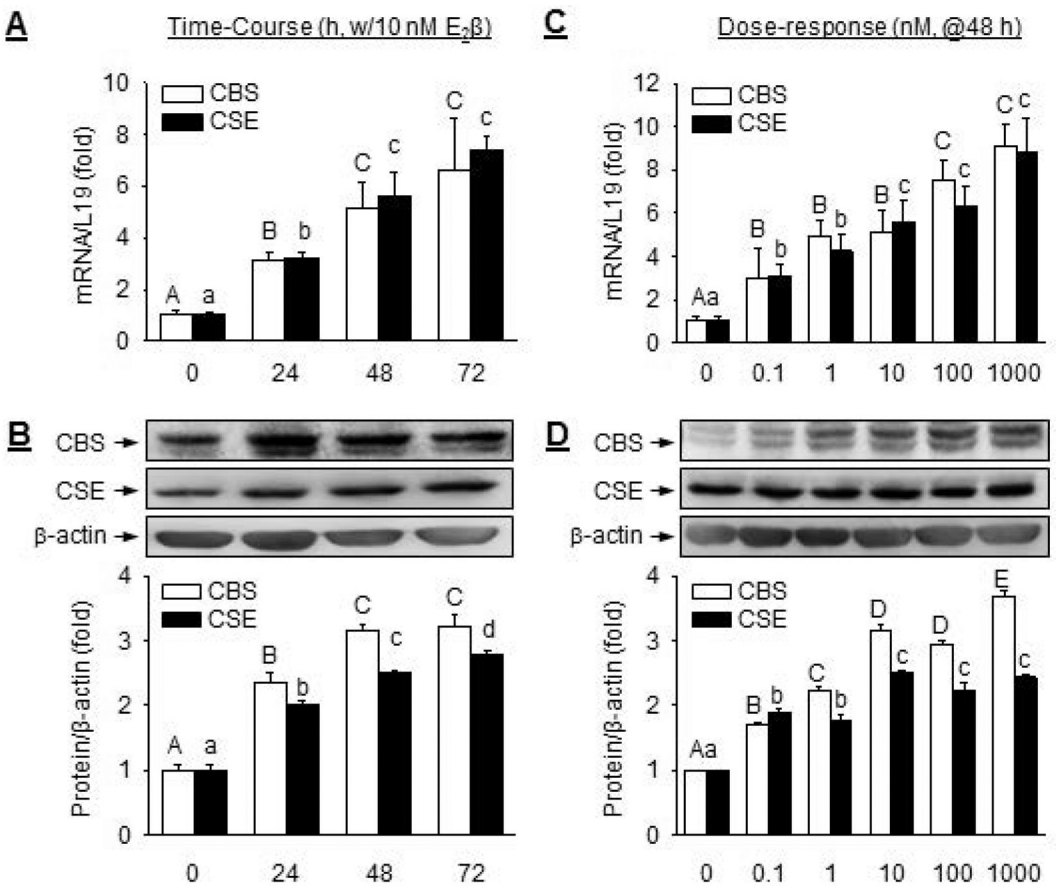

Fig. 3: $E_2\beta$ stimulates CBS and CSE mRNA and protein expression in a time- and concentration-dependent manner.

UASMCs were treated with 10 nM $E_2\beta$ for up to 3 days (time-course) to asses CBS and CSE (A) mRNA and protein (B), or with increasing concentrations of $E_2\beta$ (0–1 μM) for 48 h (dose-response) to asses CBS and CSE mRNAs (C) and proteins (D). Data (mean ± SEM) were collected from cells prepared from 3–5 different ewes. Bars with different letters differ significantly ($p < 0.05$), capital letters pertain to CBS and lowercase to CSE.

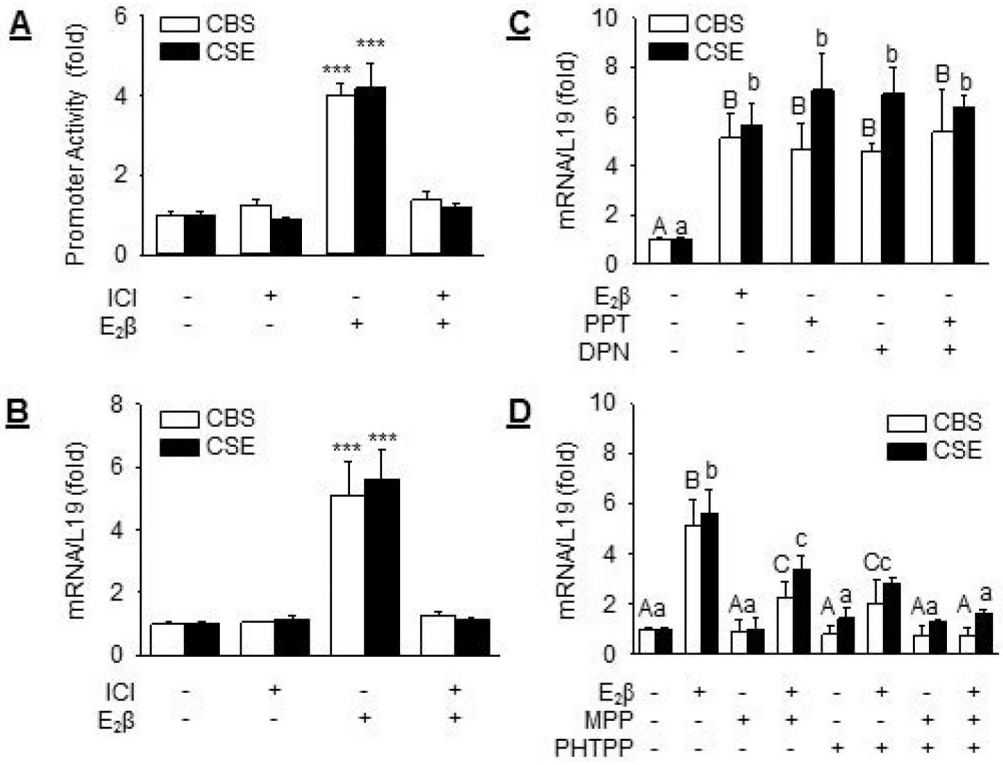

Fig. 4: Role of ER in $E_2\beta$ stimulation of CBS and CSE transcription.

(A) Human CBS and CSE promoter luciferase-reporter constructs were transfected into UASMCs. Twenty-four hours later cells were allowed to recover before treatment with vehicle or 10 nM $E_2\beta$ with or without 1 μM ICI for 24 h. Promoter activity was determined. (B) UASMCs were treated with vehicle or 10 nM $E_2\beta$ in the presence or absence of 1 μM ICI for 48 h. CBS and CSE mRNAs were determined by qPCR. (C) Cells were treated with vehicle, 10 nM $E_2\beta$, 10 nM agonist of ERα (PPT) or ERβ (DPN), or PPT+DPN to assess the role of ERα and ERβ agonists on CBS and CSE mRNA expressions. (D) Cells were treated with vehicle or 10nM $E_2\beta$ in the presence or absence of 1 μM antagonist of ERα (MPP) or ERβ (PHTPP), or MPP+PHTPP to assess the role of ERα and ERβ antagonists on CBS and CSE mRNA expressions. Data (mean ± SEM) were collected from cells prepared from 3–5 different ewes. Bars with different letters differ significantly ($P < 0.05$), capital letters pertain to CBS and lowercase to CSE. *** $P<0.001$.

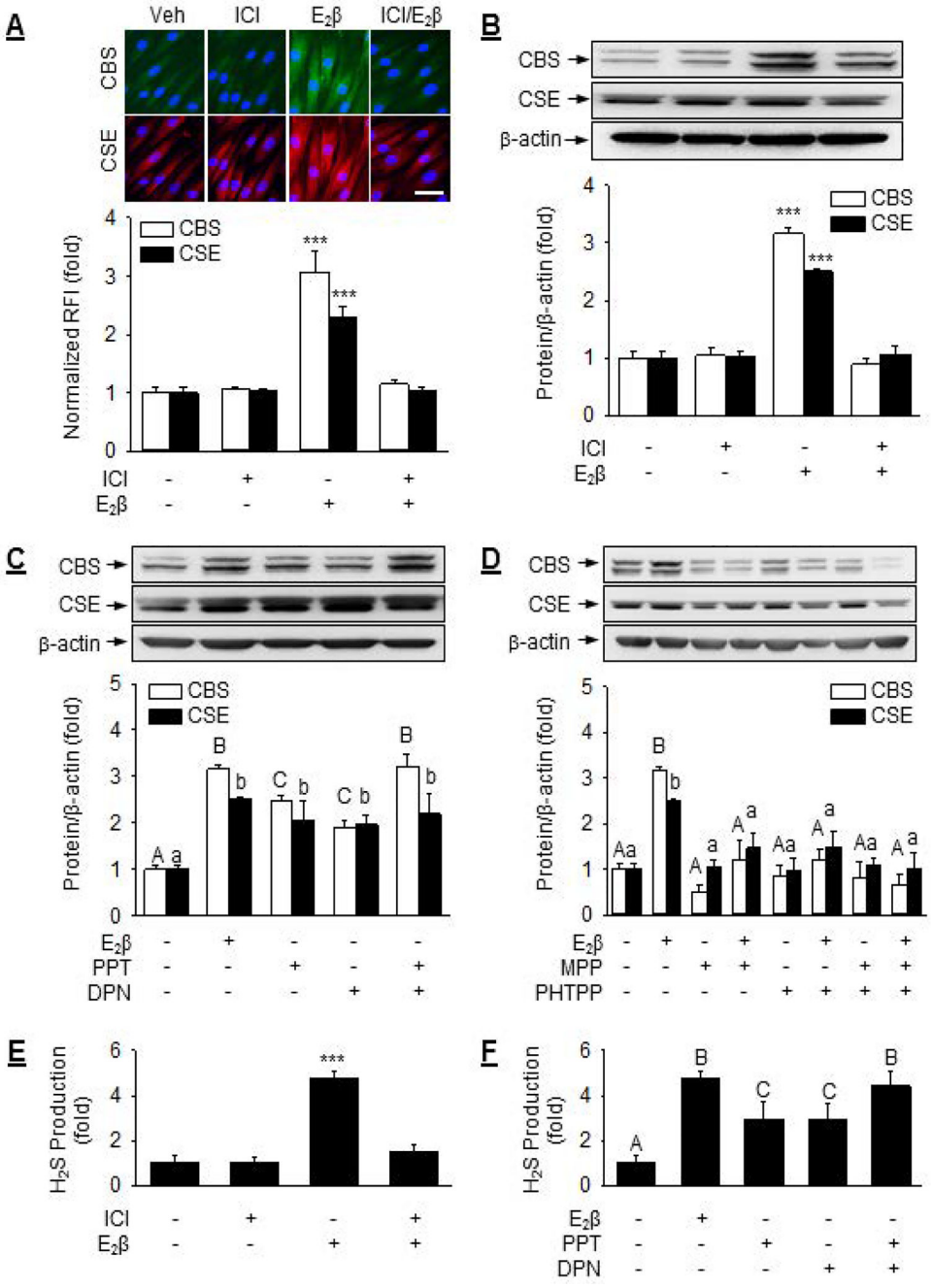

Fig. 5: Role of ER in $E_2\beta$ stimulation of CBS and CSE protein expression and activity.

(A) UASMCs were treated with vehicle, 1 μM ICI 182,780, 10 nM $E_2\beta$, or ICI + $E_2\beta$ for 48 h. Cells were then subjected to immunocytochemistry for microscopic analysis of CBS (labeled green) and CSE (labeled red) protein expressions and their cellular localization. Nuclei were labeled with DAPI (blue). Protein expression was determined by relative green or red fluorescence intensity as fold change to vehicle control (Veh). (B) UASMCs were treated with vehicle or 10 nM $E_2\beta$ with or without 1 μM ICI for 48 h. CBS/CSE protein was determined by immunoblotting. (C) Cells were treated with vehicle, 10 nM $E_2\beta$, 10 nM of agonist of PPT ERα (PPT) or ERβ (DPN), or PPT+DPN to assess the role of ER agonists

on CBS and CSE protein expressions. (D) Cells were treated with vehicle or 10nM $E_2\beta$ in with or without 1 μM antagonist of ERα (MPP) or ERβ (PHTPP), or MPP+PHTPP to assess the role of ER antagonists on CBS and CSE protein expressions. Cells were treated with vehicle or (E) 1 μM ICI with or without 10 nM of $E_2\beta$ or (F) 10 nM of PPT, DPN, or PPT+DPN to assess the role of ERα and ERβ in $E_2\beta$ stimulation of UASMC H_2S production. Data (mean ± SEM) were collected from cells prepared from 3–5 different ewes. Bars with different letters differ significantly ($P<0.05$), capital letters pertain to CBS and lowercase to CSE. *** $P<0.001$, n.s. not statistical different. Scale bar is 50 μm.

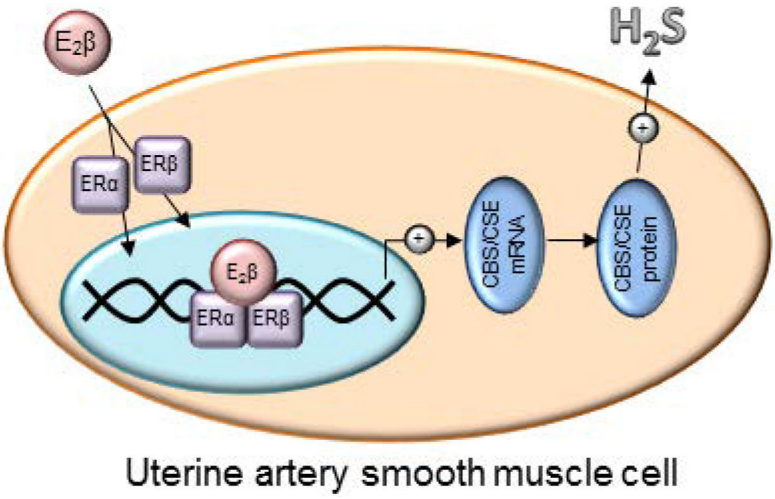

Fig. 6: ER-dependent UASMC H_2S production