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Research Article

Ovine uterine artery hydrogen sulfide biosynthesis in vivo: effects of ovarian cycle and pregnancy†

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Abstract

Uterine vasodilation dramatically increases during the follicular phase of the estrous cycle and pregnancy, which are estrogen-dominant physiological states. Uterine vasodilation is believed to be mainly controlled by local uterine artery (UA) production of vasodilators and angiogenic factors. The extremely potent vasodilator and proangiogenic hydrogen sulfide (H_2S) is synthesized via metabolizing L-cysteine by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CTH). This study was designed to determine if UA H_2S production increases with augmented expression and/or activity of CBS and/or CTH during the ovarian cycle and pregnancy in sheep. Uterine arteries from intact nonpregnant (NP) luteal and follicular phase and late (130–135 days, term $\approx$ 145 days) pregnant (P) ewes were collected; endothelium-enriched proteins (UAendo) and endothelium-denuded smooth muscle (UAvsm) were mechanically prepared for accessing CBS and CTH proteins by immunoblotting; their cellular localization was determined by semi-quantitative immunofluorescence microscopy. H_2S production was measured by the methylene blue assay. Immunoblotting revealed that CBS but not CTH protein was greater in $P > > >$ NP follicular $>$ luteal UAendo and UAvsm ($P < 0.001$). H_2S production was greater in $P > > >$ NP UAendo and UAvsm ($P < 0.01$). Pregnancy-augmented UAendo and UAvsm H_2S production was inhibited by the specific CBS but not CTH inhibitor. CBS and CTH proteins were localized to both endothelium and smooth muscle; however, only CBS protein was significantly greater in P vs NP UA endothelium and smooth muscle. Thus, ovine UA H_2S production is significantly augmented via selectively upregulating endothelium and smooth muscle CBS during the follicular phase and pregnancy in vivo.

Summary Sentence

Summary Sentence Ovine UA hydrogen sulfide production is significantly upregulated via selective upregulation of endothelium and smooth muscle cystathionine β-synthase expression in the follicular phase and pregnancy.

Key words: uterine artery, hydrogen sulfide, cystathionine β-synthase, ovarian cycle, pregnancy, sheep, in vivo.

Introduction

Uterine vasodilation, as measured by a rise in uterine blood flow (UBF), dramatically increases during the estrogen-dominant physiological states of the follicular phase of the estrous cycle and pregnancy [1–3]. Estrogens are potent vasodilators that cause blood flow to rise in many organs throughout the body with the greatest responses occurring in reproductive tissues, especially the uterus [3, 4]. Daily estradiol-17β ($E_2\beta$) increases baseline UBF by 30–40% after 6–7 days in ovariectomized nonpregnant (NP) ewes, without changing arterial pressure and heart rate [5]. Acute $E_2\beta$ exposure causes even more robust up to 10-fold rise in UBF within 90–120 min after a bolus intravenous injection of 1 μg/kg $E_2\beta$ [6, 7]. Total estrogen levels increase up to ~1000-fold during human pregnancy [8], which upregulate UBF that provides the sole source of nutrients and oxygen supplied via the placenta for the fetus and the removal of the metabolic wastes and respiratory gases of the fetus [9]. Dramatic rises in UBF, mainly via vasodilation and angiogenesis, in the last one-third of gestation are directly linked to fetal growth and survival [10]. Insufficient rises in UBF are seen in preeclampsia, fetal growth restriction, and many other pregnancy disorders, affecting life later after birth and the mother's well-being during pregnancy and postpartum [11]. Thus, UBF is a critical rate-limiting factor for pregnancy health.

Significantly enhanced local uterine artery (UA) production of orchestrated vasodilator networks, including prostacyclin [12, 13], nitric oxide (NO) [14–16], endothelium-derived hyperpolarizing factor (EDHF) [17], and vascular endothelial growth factor (VEGF) [18], has been shown to play a role in mediating estrogen-induced and pregnancy-associated rises in UBF. Estrogens and pregnancy significantly stimulate endothelial NO synthase (NOS3) expression and activation as well as NO production by UA endothelium in vivo and in vitro [15, 19–21]. In hemochorial placentation, NOS3 deficiency impairs spiral artery remodeling, increases vascular resistance, decreases placenta oxygen consumption, and results in significantly decreased pregnancy outcome in mice [22, 23]. NOS inhibition during pregnancy results in preeclampsia-like symptoms and fetal growth restriction that are directly linked to reduced UBF [24]. In addition, NO seems to interact with nearly all known UA vasodilators, including EDHF, VEGF, and estrogens [7, 14, 17, 25]. Mounting evidence has shown that local UA endothelium NOS3/NO is important for mediating basal, estrogen-induced, and pregnancy-associated rises in UBF using the well-established sheep model [7, 14, 15, 26, 27]. However, blockade of UA NO production only inhibits ~65% the $E_2\beta$-induced rise in UBF [7, 14] and UA NO inhibition only modestly alters baseline UBF in the last one third of ovine pregnancy [28, 29]. Thus, other vasodilators derived from UA endothelium and/or the smooth muscle in addition to endothelium/NO must be present to mediate the UA dilation during the follicular phase and pregnancy.

Hydrogen sulfide (H_2S) is a signaling molecule of the "gasotransmitter" family, which was described after NO and carbon monoxide [30, 31]. Akin to NO, H_2S potently dilates various vascular beds and possesses potent angiogenic activity, thus functioning as a potent vasodilator [32]. Endogenous H_2S is mainly synthesized from L-cysteine by two key enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CTH). CBS and CTH expression is highly tissue/cell specific as both are required to produce H_2S in some tissue/cells while one is sufficient in some others [31]. CTH/H_2S signaling is important in placental development and function as well as pregnancy because (1) H_2S potently dilates placental vasculature [33] and (2) dysregulation of CTH results in maternal hypertension and placental abnormalities in preeclampsia [34] and fetal growth restriction [35] in humans.

We recently reported in ovariectomized NP ewes that the H_2S biosynthesis system is present in the UA, which is significantly stimulated by exogenous estrogen replacement therapy via selectively upregulating CBS expression [36]. We also showed augmented UA H_2S production in women linked to endogenous estrogens during the menstrual cycle and pregnancy in women; importantly, H_2S functions as a new UA vasodilator system since augmented H_2S production contributes to pregnancy-associated rat UA dilation [37]. Barcroft et al. first quantitated UBF in pregnant rabbits [38]; experimental studies for mechanistic understanding uterine hemodynamics are mostly done with the sheep model. However, it is unknown whether ovine UA H_2S biosynthesis is regulated during the ovarian cycle and pregnancy. In this study, we utilized the well-established NP vs. P sheep models to study the hypothesis that UA H_2S biosynthesis is augmented in the estrogen-dominant physiological states of the ovarian cycle and pregnancy.

Materials and methods

Chemicals and antibodies

Monoclonal antibody against human CBS antibody was from Abcam (Cambridge, MA, Supplementary Table S1). Monoclonal antibody against human CTH was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PECAM (also called CD31) antibody was from Dako (Carpinteria, CA). Anti-ACTB (also called β-actin) monoclonal antibody was from Ambion (Austin, TX). Prolong Gold antifade reagent with 4, 6-diamidino-2-phenylindole (DAPI), Alexa^{488} and Alexa^{568} conjugated goat anti-mouse immunoglobulin G (IgG) were from Invitrogen (Carlsbad, CA). Horseradish peroxidase-conjugated goat anti-mouse IgG was from Cell Signaling (Beverly, MA). β-cyano-L-alanine (BCA) were from Cayman Chemical (Ann Arbor, MI). O-(carboxymethyl)hydroxylamine hemihydrochloride (CHH) and all other chemicals unless specified were from Sigma (St. Louis, MO).

Isolation and preparation of arteries from sheep during the ovarian cycle and pregnancy

Mixed Western breed ovary-intact NP (luteal n = 3 and follicular n = 4) and P (n = 5, gestational age = 125 ± 4) ewes were sacrificed with intravenous pentobarbital sodium (~50 mg kg^{-1}). Procedures for animal handling and experimental protocols were approved by the University of Wisconsin–Madison Research Animal Care Committees of both the Medical School and the College of Agriculture and Life Sciences, and followed the recommended American Veterinary Medicine Association guidelines for euthanization of laboratory farm animals. The NP sheep were synchronized to the luteal (day 10–11) and follicular (day –1 to 0) phases as described [16].

We used the previously described and validated rapid isolation procedure to obtain endothelium-derived proteins devoid of smooth muscle [39]. Briefly, UAs were excised, placed in ice-cold phosphate-buffered saline (PBS: 8 mM sodium phosphate, 2 mM potassium phosphate, 0.15 M NaCl, pH 7.4), dissected free of connective tissue, and rinsed free of blood and snap frozen in liquid nitrogen.

Portions of UA were opened longitudinally, and the endothelium/tunica intima was gently scraped (3–6 times) from the UA and placed into lysis buffer (50 mM Tris, 0.15 M NaCl and 10 mM EDTA, pH 7.4, 0.1% Tween 20, 0.1%β-mercaptoethanol, 0.1 M phenylmethylsulphonyl floride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin) using a curved-end spatula, as previously described [19, 39]. The remaining "scraped" vessel was rubbed with a wet cotton swab and any remaining adventitia was completely removed before the denuded artery was placed in lysis buffer. The endothelium-isolated proteins (UAendo) and denuded arteries (UAvsm) were snap frozen in liquid nitrogen immediately upon collection and were stored at −20°C. Thus, UAendo represents an endothelium-enriched preparation that was mechanically scraped from the tunica media; UAvsm denote denuded vessels that have had their endothelium removed leaving the basal lamina and SM. Additional intact artery segments were collected for immunofluorescence microscopy; they were fixed in 4% formaldehyde in sodium cacodylate buffer (0.1 M, pH 7.4) for 24 h, and were then stored at 4°C in sodium cacodylate buffer containing 0.01% sodium azide until dehydration and embedding into paraffin blocks.

SDS-PAGE and immunoblotting

SDS-PAGE and immunoblotting were performed as previously described [36, 37]. Final concentrations of ACTB, CBS, and CTH antibodies used were at 1:10,000, 1:200, and 1:500, respectively.

Methylene blue assay

UAendo or UAvsm lysates were pooled (40 μg pooled lysate/reaction mixture in duplicate) and homogenized in 50 mM ice-cold potassium phosphate buffer (pH 8.0). Using the methylene blue assay, H_2S production was determined as previously described [36, 37]. The H_2S concentration was calculated based on a calibration curve generated from NaHS solutions. CHH and/or BCA at a final concentration of 2 mM were added to the reaction mixtures prior to initiating the assay for determining specific CBS and CTH activities, respectively.

Semi-quantitative immunofluorescence microscopy and image analysis

The cellular-specific expression of CBS and CTH proteins in ovine UA was determined by immunofluorescence microscopy. Sections were deparaffinized in xylene, and rehydrated by passing through graded ethanol. Sections were incubated in 0.05% trypsin to unmask antigens at room temperature for 30 min. Autofluorescence was quenched by washing the sections with 300 mM glycine in PBS (3 × 20 min); nonspecific binding was blocked by incubating with PBS containing 1% BSA, 0.125% saponin, and 1% gelatin at room temperature for 30 min. Sections were then incubated with anti-PECAM (5 μg/ml) overnight at 4°C. Following three 5-min washes in PBS, the sections were then incubated with Alexa^{568} conjugated goat anti-mouse IgG (2 μg/ml) at room temperature for 1 h. Following three 20-min washes in PBS, sections were then blocked and incubated with 1 μg/ml of anti-CBS or anti-CTH antibodies at room temperature for 2 h, followed by incubation with Alexa^{488} conjugated goat anti-mouse IgG (2 μg/ml; Invitrogen, Carlsbad, CA) at room temperature for 1 h. Following three 20-min PBS washes, the sections were mounted with Prolong Gold antifade reagent (Invitrogen) containing DAPI for labeling cell nuclei. Samples were examined under a Leica fluorescence microscope (Leica Corporation, Deerfield, IL), and digital images were acquired using a charge-coupled device camera with the *Simple*PCI image analysis software (Hamamatsu Corporation, Sewickley, PA). The images were used to determine relative levels of CBS and CTH proteins by quantifying mean green fluorescence intensity using *Simple*PCI image analysis software. For all groups, CBS and CTH levels were averaged from data collected from 5–6 images per subject, and 3–4 subjects per group. Intima and media areas were outlined using the "Region of Interest" selection tool, and "Mean Green Value" was recorded for a cell. The average "Mean Green Value" from negative control sections without primary antibody accounted for autofluorescence and nonspecific background, which was subtracted from all counts generated from specific antibody-treated samples. CBS and CTH protein levels were presented as fold change in the average fluorescence intensity.

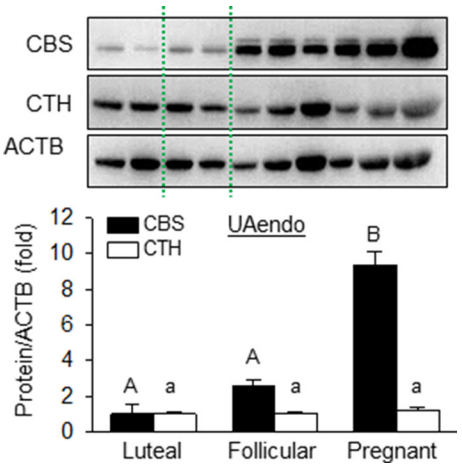

Figure 1. Uterine artery (UA) endothelial (endo) CBS/CTH expression in nonpregnant (luteal and follicular) and late pregnant ewes. CBS and CTH proteins in mechanically purified UA endothelium samples were determined by immunoblotting. Data (means ± SEM) are from 2–6 ewes/group. Bars with different letters differ significantly among the groups ($P < 0.05$).

Statistical analysis

Data are presented as means ± SEM and analyzed by one-way or two-way analysis of variance (ANOVA), followed by Bonferroni test for multiple comparisons using *Sigma*Stat (Systat Software Inc.). Significance was defined a priori as $P < 0.05$, unless indicated in the figure legends.

Results

CBS and CTH protein expression and H_2S production in UA endothelium

Immunoblotting analysis showed that UAendo CBS protein in NP follicular and P ewes were 2.61 ± 0.32-fold and 9.33 ± 0.79-fold higher than that in the NP luteal ewes, respectively, while only CBS protein in P ewes was significantly greater ($P < 0.001$). In contrast, UAendo CTH protein did not differ ($P > 0.05$) significantly among NP luteal and follicular as well as P ewes (Figure 1). Consistent with these observations, UAendo H_2S production was 2.48 ± 0.05-fold greater in P than NP luteal ewes ($P < 0.001$). Addition of the specific CBS inhibitor CHH, but not the specific CTH inhibitor BCA, inhibited ($P < 0.05$) NP luteal UAendo baseline H_2S production and abrogated ($P < 0.01$) the pregnancy-augmented UAendo H_2S production. The combination of CHH and BCA inhibited NP luteal UAendo baseline H_2S production and completely inhibited ($P < 0.01$) pregnancy-augmented UAendo H_2S production.

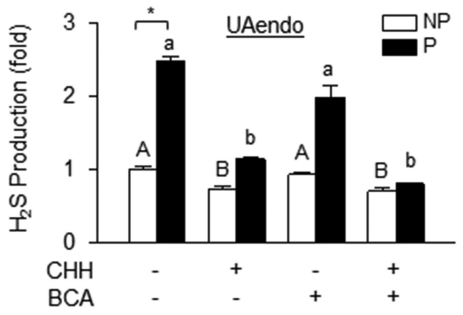

Figure 2. Uterine artery (UA) endothelial (endo) H_2S production in nonpregnant and late pregnant ewes. Uterine artery endothelium (UAendo) protein lysates from nonpregnant luteal or pregnant ewes were pooled and subjected to the methylene blue assay for measuring H_2S production in the presence or absence of the specific inhibitors of CBS (CHH), CTH (BCA), or their combination. Data (means ± SEM) are presented as fold of NP luteal without inhibitors and are pooled from 3–5 ewes per group. Bars with different letters differ significantly among the groups ($P < 0.05$). * $P < 0.01$.

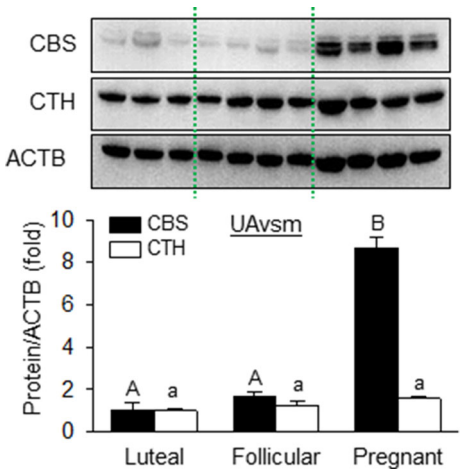

Figure 3. Uterine artery (UA) vascular smooth muscle (vsm) CBS/CTH expression in nonpregnant (luteal and follicular) and late pregnant ewes. CBS and CTH proteins were determined by immunoblotting. Data (means ± SEM) are from 3–6 ewes/group. Bars with different letters differ significantly among the groups ($P < 0.05$).

CHH alone did not alter NP luteal baseline or pregnancy-augmented UAendo H_2S production (Figure 2). Thus, CBS is the major enzyme responsible for pregnancy-augmented H_2S biosynthesis in ovine UA endothelium.

CBS and CTH expression and H_2S production in UA smooth muscle

Levels of CBS protein in NP follicular and P UAvsm ewes were 1.69 ± 0.23-fold and 8.65 ± 0.65-fold higher than that in the NP luteal NP ewes, respectively, while only CBS protein in P ewes was significant ($P < 0.001$). In contrast, levels of UAvsm CTH protein did not differ ($P > 0.05$) significantly among NP luteal and follicular as well as P ewes (Figure 3). H_2S production in P UAvsm was 1.56 ± 0.05-fold greater than that in NP luteal UAvsm ($P < 0.01$). Incubation with CHH or the combination of CHH and BCA inhibited the NP luteal baseline H_2S production and the pregnancy-augmented P UAvsm H_2S production ($P < 0.01$); however, BCA alone did not alter ($P > 0.05$) either the NP luteal baseline or the pregnancy-augmented UAvsm H_2S production (Figure 4). Thus, CBS

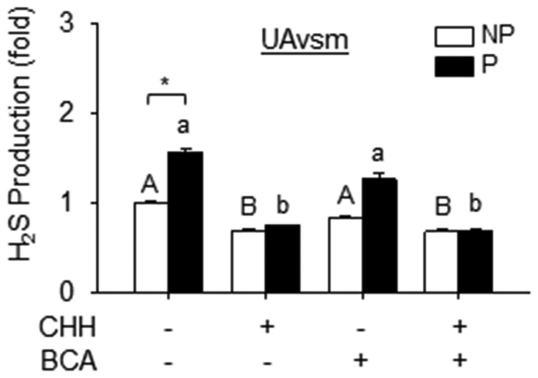

Figure 4. Uterine artery (UA) vascular smooth muscle (vsm) H_2S production in nonpregnant and late pregnant ewes. Protein lysates from nonpregnant luteal or pregnant or UAvsm were pooled and subjected to the methylene blue assay for measuring H_2S production in the presence or absence of the specific inhibitors of CBS (CHH), CTH (BCA), or their combination. Data (means ± SEM) are presented as fold of NP luteal without inhibitors and are pooled from 3–5 ewes per group. Bars with different letters differ significantly among the groups ($P < 0.05$). * $P < 0.05$.

is also the major enzyme responsible for pregnancy-augmented H_2S biosynthesis in ovine UAvsm.

Semi-quantitative immunofluorescence localization of UA CBS and CTH proteins

Immunofluorescence microscopy analysis revealed that both CBS and CTH proteins are expressed and localized in the endothelial cells at the luminal surface and in the smooth muscle cells of the UA (Figure 5A). CD31 labeling was seen to mainly stain the tunic intima especially along the internal elastic lamia. CBS protein was expressed at low levels in the CD31-labeled endothelial and but also smooth muscle in the NP luteal UA; pregnancy enhanced CBS protein expression in that levels of endothelium and smooth muscle CBS proteins were 3.31 ± 0.17-fold and 3.24 ± 0.68-fold higher ($P < 0.01$) than that of NP luteal UA, respectively ($P < 0.001$, Figure 5B). CTH immunofluorescence labeling was also observed in both UA endothelium and smooth muscle of NP luteal and pregnant ewes, and levels of CTH protein did not differ ($P > 0.05$) between either cell layers in NP vs P states (Figure 5A and B).

Discussion

We report here for the first time that UAendo and UAvsm H_2S biosynthesis is significantly greater in the proliferative phase (compared to luteal phase) during the ovarian cycle and further increased during pregnancy in sheep. Our current study using a well-defined NP and P sheep model and purified ex vivo UAendo and UAvsm samples are in agreement, in essence, with our recent human study showing that UA H_2S production is augmented, in association with elevated endogenous estrogens during the estrogen "dominant" physiological states of the follicular phase and pregnancy [37]. Together with our recent reports [36, 37], our current study has further strengthened that H_2S is produced by not only endothelial cells but also smooth muscle on the UA wall. UAendo and UAvsm-derived H_2S is a new UA dilator system because H_2S donor stimulates dilation of freshly isolated primary UA rings from both NP and P rats but with significantly greater potency in pregnant state and is vascular-specific [37]. Obviously, these studies have changed the view regarding how locally produced vasodilators by the UA regulate

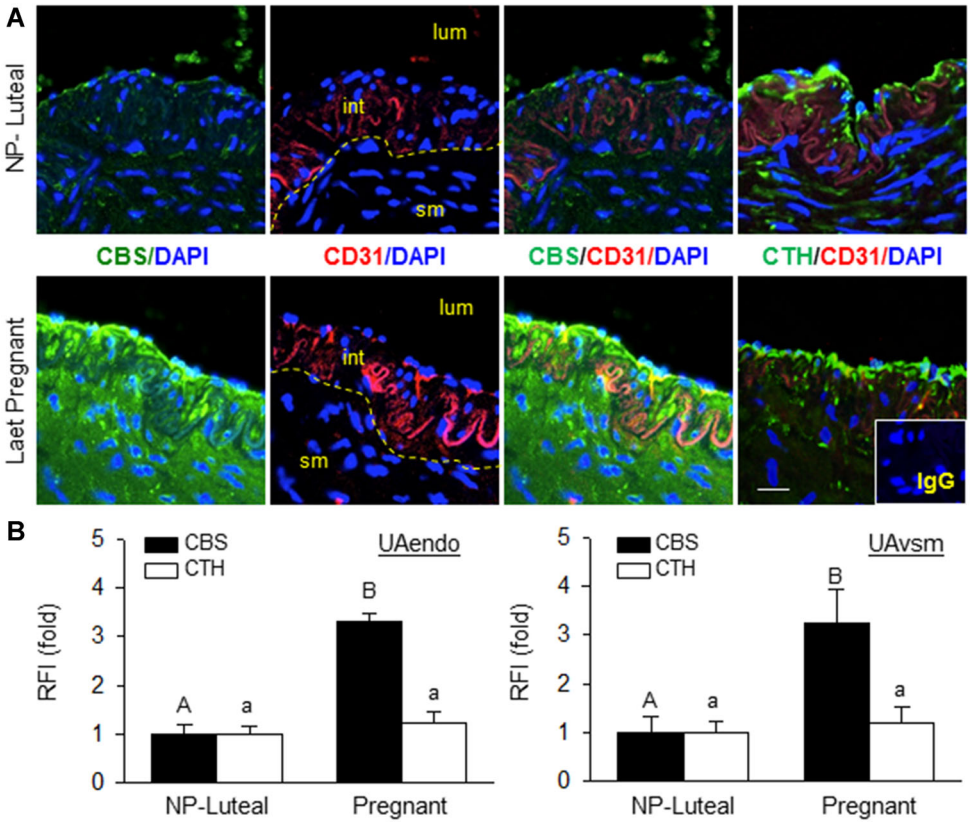

Figure 5. Uterine artery (UA) CBS and CTH protein localization and expression in endothelium and smooth muscle of nonpregnant (NP) luteal and late pregnant ewes. (A) UA sections were labeled with primary antibodies against CBS or CTH, followed by secondary Alex^{488} (Green)-labeled secondary antibody. Endothelial cells were labeled with marker PECAM/CD31 followed by Alex^{555} (Red)-labeled secondary antibody and cell nuclei were stained with DAPI (blue); IgG control bottom right insert panel. Representative outlines of borders between UA intima (int) and media (SM) were indicated and UA lumen (lum) and tunic intima (int) were denoted in the 2nd CD31/DAPI panel. (B) Fluorescence images were captured for analyzing relative green fluorescence intensity (RFI) to quantify CBS or CTH proteins. Data (means ± SEM) are from 3–4 different ewes/group. Bars with different letters differ significantly among the groups ($P < 0.05$). Scale bar = 25 μm.

uterine hemodynamics since hitherto UA vasodilation is dominantly considered to be predominately regulated by endothelium-derived vasodilators especially NO via NOS3 [18, 40–42] and the question of whether UAvsm also produces vasodilators has been for the most part neglected.

Endogenous H_2S in mammalian tissues is primarily synthesized from L-cysteine by two pyridoxal 5'-phosphate-dependent enzymes CBS and CTH [30]. The expression of CBS and CTH is tissue and cell specific because in some tissues both CBS and CTH are needed to generate H_2S, while in others one enzyme is sufficient [30, 31]. CTH is closely associated with the vasculature tissue [43], whereas CBS is reported to be mainly expressed in neuronal tissue [31] and it has also been found to be expressed in the cerebral arteries of newborn pigs [44] and pulmonary arteries of mice with decreased expression in a rat model of pulmonary hypertension [45]. Since the expression of CBS and CTH is tissue and cell specific, it is necessary to understand the cellular localization of these enzymes in the UA of NP and P ewes to comprehend the role of H_2S in pregnancy-associated uterine vasodilation. We observed that pregnancy-augmented H_2S, in both UAendo and UAvsm, is associated with CBS, but not CTH protein expression, which were measured by immunoblotting with specific antibodies and confirmed by immunofluorescence microscopy. Moreover, pregnancy-augmented UAendo and UAvsm H_2S production was inhibited by CBS, but not CTH inhibition. Thus, CBS is the primary enzyme responsible for UA H_2S production during ovine pregnancy, which is consistent with our recent human study [37]. In contrast, although constitutively expressed in the UA endothelium and smooth muscle in sheep and human, CTH is not regulated upon stimulation with chronic treatment with exogenous $E_2\beta$ in NP ewes [36] or pregnancy in women [37] and ewes (current study) ex vivo and VEGF in vitro [46]. However, CTH is involved in baseline H_2S production because CTH inhibition lowers baseline but does not alter agonist- or pregnancy-stimulated UA H_2S production [36, 37] (Figures 2 and 4).

CBS is an estrogen-responsive gene on the uterine vascular wall as estrogen replacement therapy stimulates UA endothelial and smooth muscle CBS, but not CTH, expression in ovariectomized NP ewes ex vivo [36] and cultured ovine uterine artery endothelial cells (oUAEC) [47] and uterine artery smooth muscle cells (oUASMC) [48] in vitro. Consistent with dramatic increases in circulating estrogen levels during the follicular phase of the estrous cycle and pregnancy in sheep [3, 16] and during the proliferative phase of the menstrual cycle and pregnancy in women [8], augmented UAendo and UAvsm CBS protein expressions during these estrogen-dominant physiological states in ewes (Figures 1 and 3) and women [37] ex vivo show that *CBS* is a pregnancy-responsive gene in both the endothelium and smooth muscle on the vascular wall under the influence of endogenous estrogens.

How pregnancy specifically stimulates UAendo and UAvsm CBS expression and H_2S production is currently unknown. However, elevated endogenous estrogens during pregnancy are expected to play a role because our recent in vitro studies using cultured primary oUAEC and oUASMC cell models have shown that exogenous $E_2\beta$ stimulates CBS mRNA and protein expressions in primary oUAEC [47] and oUASMC [48] via estrogen receptor (ER)-dependent *CBS* transcription involving both ERα and ERβ. UAEC and UASMC are both estrogen-target cells as both cell types express both ERα and ERβ [49, 50]. However, pregnancy stimulates UAEC and UAvsm ERβ, but not ERα expression in ewes [50]. Thus, it is yet to be determined if indeed elevated endogenous estrogens stimulate UAendo and UAvsm H_2S biosynthesis via upregulating *CBS* transcription by enhanced ERβ signaling during pregnancy. In addition, other factors such as pregnancy-augmented VEGF expression may also play a role because pregnancy augments VEGF-stimulated H_2S biosynthesis via upregulating CBS expression in human UAEC in vitro [46].

In summary, the present study reveals for the first time that H_2S biosynthesis in UA endothelium and smooth muscle is augmented via selective upregulation of CBS, in association with elevated endogenous estrogens, in the proliferative phase and pregnancy in sheep. Although much is still to be learned as to how H_2S in conjunction with other vasodilators (e.g. PGI_2, NO, EDHF, etc.) specifically regulates uterine hemodynamics, this study sheds new light on the mechanisms that local vasodilators play in uterine hemodynamics because unlikely the eNOS-NO system that is exclusively expressed in the endothelium [19, 21, 51, 52], the new UA vasodilator H_2S is produced not only by the endothelium, but also the smooth muscle.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplementary Table S1. Antibodies Table.

Disclosure

The authors have no financial interests to disclose.

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