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Enhanced Sp1/YY1 Expression Directs CBS Transcription to Mediate VEGF-stimulated Pregnancy Dependent H₂S Production in Human Uterine Artery Endothelial Cells

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Abstract

Pregnancy and vascular endothelial growth factor (VEGF) stimulate uterine artery (UA) endothelial cell (UAEC) hydrogen sulfide (H₂S) production via selectively upregulating cystathionine β-synthase (CBS) but not cystathionine γ-lyase (CSE) expression. This study was conducted to determine the mechanisms by which VEGF utilizes to stimulate pregnancy dependent upregulation of CBS and H₂S production in human UAEC. The proximal human *CBS* promoter contains 4 specific protein 1 (Sp1a/b/c/d) sites and one YY1 site; luciferase assays using reporter genes driven by human *CBS* promoter with a series of 5'-deletions identified a promoter sequence (–574 to –394) containing Sp1d and the YY1 sites critical for basal and VEGF-stimulated *CBS* promoter activation. VEGF stimulated pregnancy-dependent recruitment of Sp1 to Sp1d and YY1 to YY1, and also recruited YY1 to Sp1c and increased Sp1/YY1 association in pregnant hUAEC, suggesting formation of a Sp1/YY1 complex at Sp1c site. Endothelial Sp1 and YY1 proteins were significantly greater in pregnant than nonpregnant human UA. VEGF stimulated pregnancy dependent Sp1 and YY1 protein expression in vitro. Treatment with Sp1 and YY1 siRNAs completely blocked Sp1/YY1-mediated pregnancy dependent CBS protein upregulation and H₂S production by VEGF in hUAEC. VEGF did not *trans*-activate *CSE* promoter nor increase CSE expression and Sp1/YY1 knockdown did not affect CSE expression in hUAEC. Thus, pregnancy augments EC Sp1 and YY1 expression and promotes the recruitment of Sp1/YY1 to their DNA binding sequences in proximal human *CBS* promoter to upregulate CBS transcription, underlying a novel mechanism to mediate VEGF-stimulated pregnancy dependent endothelial H₂S production in human UA.

Summary

Pregnancy augments human uterine artery cell (endothelium vs. smooth muscle)-specific expressions of Sp1 and YY1 and promotes their interactions with the proximal human *CBS* promoter in response to VEGF stimulation. This pathway provides a transcriptional mechanism responsible for VEGF-stimulated pregnancy dependent CBS expression and H₂S production that accounts, in part, for uterine vasodilation during pregnancy.

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Keywords

H₂S biosynthesis; CBS transcription; Sp1/YY1; UAEC; VEGF; Pregnancy

INTRODUCITON

Once conceived, the maternal cardiovascular system undergoes dynamic changes including mild decreases in systemic blood pressure and vascular resistance and significant increases in blood volume and total cardiac output¹; these changes result in profound rise in uterine blood flow (UtBF) to facilitate maternal-fetal exchanges of respiratory gases (O₂ and CO₂), to exhaust metabolic wastes, and to provide the sole source of nutrients for fetal development and survival². Constrained UtBF is a denominator of many pregnancy disorders including preeclampsia with or without intrauterine fetal growth restriction, affecting the well-beings of the mother and fetus during pregnancy and predisposing both to a higher risk in metabolic diseases such as diabetes and cardiovascular diseases later in life³. Local uterine artery (UA) production of orchestrated vasodilators, especially nitric oxide (NO) produced by UA endothelial cells (UAEC)^{4,5}, has been long recognized to be critical for pregnancy-associated rise in UtBF. However, local UA NO blockade only modestly inhibits baseline UtBF in pregnant (P) sheep⁶, clearly demonstrating that other mechanisms exist to mediate uterine hemodynamics during pregnancy.

Hydrogen sulfide (H₂S) is a potent proangiogenic vasodilator that belongs to the “gasotransmitter” family after NO and carbon monoxide⁷. Endogenous H₂S is mainly produced via metabolizing L-cysteine by two enzymes: cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE)⁸. UA H₂S production is augmented via selective endothelium and smooth muscle CBS upregulation in ovine and human pregnancy in vivo^{9,10}. H₂S donors relax pressurized rat and human UA ex vivo^{9,11}, linked to activation of the large conductance Ca²⁺-activated voltage-dependent potassium (BK_{Ca}) channels¹¹ that are crucial for pregnancy-associated rise in UtBF^{12,13}. Thus, H₂S serves as new vasodilator to mediate pregnancy-associated uterine vasodilation.

Vascular endothelial growth factor (VEGF) expression is augmented, and maternal circulating VEGF levels increase during pregnancy^{14,15}. Dysregulated VEGF signaling by increased soluble *fms*-like tyrosine kinase-1 (sFlt1) leads to preeclampsia-like conditions in animals¹⁶. Prenatal adenoviral *VEGF* gene delivery to UA reverse undernutrition-induced fetal growth restriction via upregulating UtBF^{17,18}, implicating a crucial role of VEGF signaling in pregnancy-associated rise in UtBF. VEGF augments pregnancy-dependent H₂S production via selectively upregulating CBS in primary human UAEC (hUAEC) in vitro¹⁹. Endogenous H₂S serves as an autocrine and paracrine factor for placental and endometrial angiogenesis^{20,21}. H₂S donors can partially rescue *sFlt1*-induced preeclampsia in rats, involving upregulating VEGF and angiogenesis^{22,23}. Hence, interactions between VEGF and H₂S are central for the two key mechanisms, *i.e.*, the expansion and vasodilation of uterine and placental vasculatures, for upregulating pregnancy-associated rise in UtBF²⁴.

The mechanisms by which VEGF utilizes to stimulate pregnancy dependent UAEC H₂S production via selective CBS expression are currently unknown. Simultaneous increases

in UA endothelium CBS mRNA and protein in vivo during pregnancy^{9, 10} and upon VEGF stimulation in primary hUAEC in vitro¹⁹ suggest that transcriptional mechanisms are involved. The human *CBS* gene promoter is GC-rich and lacks the classic TATA box but contains various putative *cis*-regulatory elements for binding transcription factors including specificity proteins (Sp1) and Yin Yang 1 (YY1)²⁵. Sp1 is known to function as a *trans*-activator to mediate growth factor-regulated CBS expression²⁶ and the ubiquitously expressed YY1 regulates the expression of genes important for cell invasion and EC angiogenesis^{27, 28}. Sp1 and YY1 cooperate to regulate gene expression in vascular endothelium²⁹. This study was conducted to determine the transcriptional mechanisms controlling VEGF-stimulated pregnancy-dependent CBS upregulation in human UA. We concluded that enhanced expression of Sp1/YY1 and their coordinated interactions with the Sp1 and YY1 sites in the *CBS* promoter mediate VEGF-stimulated pregnancy-dependent *CBS* transcription for regulating H₂S biosynthesis in human UAEC.

METHODS

The data supporting the findings of this study are available from the corresponding author upon reasonable request. Detailed materials and methods are available in the Data Supplement.

Human subjects, cell isolation, and culture

Primary UAEC from the main UAs of nonpregnant (NP) and P women (n = 3/group), termed NP and P hUAEC, respectively, were isolated by collagenase digestion as previously described¹⁹. The UAs were collected from patients undergoing hysterectomy with written consent at the University of California Irvine Medical Center, with ethical approval (HS #2013-9763) by the Institutional Review Board for Human Research. The NP subjects were recruited from women aged 30-45 years, without steroid treatment, and elective hysterectomy due to fibroids. The P subjects, aged 30 to 45 years, were recruited with suspected placental accrete; UAs were collected immediately after Cesarean hysterectomy between 35-36 weeks' gestation. After purification and EC determination, cells were stored in liquid N₂ at passage 2 (P2). Frozen UAEC aliquots were thawed and seeded in endothelial cell medium (ECM, ScienCell, La Jolla, CA) containing 5% fetal bovine serum (FBS), endothelial growth supplements, and 1% penicillin/streptomycin for experimental use within 5 passages. Cells at ~70% confluence were cultured in M199 medium containing 0.1% bovine serum albumin (BSA), 0.5% FBS, 1% antibiotics, and 25 mM hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) overnight, and then treated with or without VEGF₁₆₅ (VEGF) in fresh medium.

Statistical analysis

Each experiment was repeated at least three times with cells derived from different NP or P subjects. Data are presented as means ± SEM and analyzed by one-way or two-way analysis of variance (ANOVA), followed by the *Newman Keuls* test for multiple comparisons using *SigmaPlot* 14.5 (Systat Software Inc.). Student *t*-test was used for comparison of data between two groups. Significance was defined as $p < 0.05$ and higher statistical power was indicated in figure legends.

RESULTS

VEGF stimulates pregnancy-dependent *trans*-activation of human *CBS* promoter

Following treatment with 10 ng/ml VEGF, *CBS* mRNA began to increase and reached its maximum (2.99 ± 0.20 fold vs control, $P < 0.001$) at 24 h, declined but remained significantly greater than baseline at 48 h (Fig. 1A). In the presence of actinomycin D, the half-life of *CBS* mRNA levels in VEGF-treated cells (9.1 ± 0.4 h) did not differ from that (8.8 ± 0.4 h) in control cells (Fig. 1B). Basal activity of wild-type (WT) human *CBS* promoter was greater in P vs. NP hUAEC. Treatment with 10 ng/ml VEGF for 24 h increased WT human *CBS* promoter activity by 1.98 ± 0.06 -fold ($p < 0.05$) in P hUAEC but failed to activate *CBS* promoter in NP hUAEC. The stimulatory effects of VEGF on *CBS* promoter activation were concentration-dependent in P hUAEC; VEGF significantly increase *CBS* promoter activity at 1 ng/ml and reached its maximum at 10-100 ng/ml. Basal human *CSE* promoter activity did not differ in NP and P hUAEC; VEGF at all concentrations did not activate *CSE* promoter in NP and P hUAEC (Fig 1 C&D). Because VEGF stimulate H_2S production in association with increased *CBS* but not *CSE* protein expression in P but not NP hUAEC¹⁹, VEGF stimulation of pregnancy-dependent UAEC H_2S production is largely mediated by selective upregulating *CBS* transcription.

Sp1 and YY1 are important for basal *CBS* promoter activity and its activation by VEGF

By using Length-Aware Site Alignment Guided by Nucleotide Association (LASAGNA) motif search tool (https://biogridlasagna.engr.uconn.edu/lasagna_search/index.php), we found 4 putative Sp1 binding sites (i.e., Sp1d, Sp1c, Sp1b, and Sp1a sites) and a putative YY1 binding site between Sp1c and Sp1d sites, in the full-length 914 bp proximal human *CBS* promoter (Fig. 2A). When Luc-constructs driven by the *CBS* promoter with a series of 5' deletions were transfected in P hUAEC, baseline full-length *CBS* promoter activity began to significantly decrease from position -753 to -486 bp. The VEGF-stimulated full-length promoter activation was significantly dropped at position -574 to -486 bp when Sp1d was deleted. Deletion to position -394 containing the YY1 site further decreased basal *CBS* promoter activity and completely abrogated VEGF-stimulated *CBS* promoter activity (Fig. 2B). A deletion from position -259 to -95 bp containing the Sp1c further decreased basal *CBS* promoter activity but further 5' deletions to delete the Sp1b and Sp1a sites did not reduce basal *CBS* promoter activity (Fig. 2B). Thus, the region (-574 to -394 bp) containing Sp1d (-532 to -523 bp) and YY1 (-483 to -467 bp) sites is critical for baseline and VEGF-stimulated human *CBS* promoter activity.

To verify the importance of Sp1d and YY1 sites in human *CBS* transcription, we compared baseline and VEGF-stimulated full-length human *CBS* promoter activity with its mutants containing mutations in either Sp1d, YY1, or both sites. Mutations in either Sp1d or YY1 site alone resulted in ~33% ($p < 0.05$) decrease in basal human *CBS* promoter activity, which was not enhanced by mutations of both sites. VEGF stimulated WT human *CBS* promoter activity (1.60 ± 0.08 -fold over baseline, $p < 0.001$) did not differ from that (1.67 ± 0.01 -fold over baseline, $p < 0.001$) of its Sp1d mutant. However, YY1 mutation (mYY1) not only decreased basal *CBS* promoter activity but also completely abrogated VEGF-stimulated promoter activation. Mutations in both Sp1d and YY1 (mSp1/YY1)

resulted in decreased basal *CBS* promoter activity similarly to that containing mYY1 and also completely abrogated VEGF-stimulated promoter activation (Fig. 2C).

Sp1 and YY1 interactions with human *CBS* promoter

In resting NP hUAEC, Sp1 was only recruited to Sp1d site (Amplicon 3) in human *CBS* promoter. In P hUAEC, in addition to Sp1d, Sp1 was also recruited to Sp1a/b site. Pregnancy increased Sp1 binding to Sp1a/b by 2.50 ± 0.26 ($p < 0.05$) and Sp1d by 1.89 ± 0.17 ($p < 0.05$) fold. Baseline Sp1 binding to Sp1c site (amplicon 2) was barely detectable in both NP and P cells. Treatment with 10 ng/ml VEGF for 24 h significantly increased Sp1 binding to Sp1a/b and Sp1d sites but did not affect Sp1 binding to Sp1c site in NP hUAEC. In P hUAEC, VEGF increased Sp1 binding to Sp1c by 2.04 ± 0.30 ($p < 0.05$) and to Sp1d by 2.04 ± 0.30 ($p < 0.05$) fold, but not to Sp1a/b. Baseline YY1 binding was only detected in amplicon 3 as expected since human *CBS* promoter only contains one YY1 site to be amplified in amplicon 3 (Fig. 3A). Baseline YY1 binding to the YY1 site did not differ between NP and P hUAEC; however, treatment with VEGF increased YY1 binding to the YY1 site by 2.44 ± 0.30 ($p < 0.05$) fold in P hUAEC and modestly ($p > 0.05$) in NP hUAEC. Moreover, VEGF also increased YY1 binding to the Sp1c site by 4.05 ± 0.25 ($P < 0.05$) fold in P hUAEC (Fig. 3A), suggesting that VEGF recruited YY1 to the Sp1c site. We then preformed Co-IP studies to determine whether VEGF promotes Sp1 association with YY1. Sp1 antibody pulled down YY1 and YY1 antibody also pulled down Sp1 in untreated P hUAEC; treatment with 10 ng/ml VEGF for 30 min increased levels of Sp1-bound YY1 by 1.60 ± 0.02 ($p < 0.05$) fold and YY1-bound Sp1 by 2.34 ± 0.06 ($p < 0.05$) fold (Fig. 3B). Thus, Sp1 and YY1 are associated with each other in resting hUAEC and the interaction can be stimulated by VEGF only in P hUAEC.

Sp1 and YY1 proteins in human UA in vivo and their expressions to VEGF in in hUAEC in vitro

Sp1 and YY1 proteins were immunolocalized in both EC and SMC of NP and P human UAs, with pregnancy-dependent cell (EC vs. SMC) specific expression patterns. Baseline Sp1 protein was detectable in EC but was only 50% of that in SMC ($p < 0.05$) in NP UA. Pregnancy upregulated Sp1 protein by 2.89 ± 0.65 ($p < 0.01$) fold in EC and significantly more by 4.52 ± 0.16 ($p < 0.001$) fold in SMC in human UA. Baseline YY1 protein was detectable in EC and was 5.06 ± 1.30 ($p < 0.001$) fold greater than that in SMC in NP UA. Pregnancy increased YY1 protein levels by 3.93 ± 0.68 ($p < 0.01$) fold in EC and significantly more by 7.42 ± 2.02 ($p < 0.05$) fold in SMC in human UA (Fig 4A). The pregnancy-dependent upregulated EC Sp1 and YY1 proteins were retained in cultured hUAEC, with 2.44 ± 0.50 ($p < 0.05$) in Sp1 and 3.83 ± 0.25 ($p < 0.001$) fold in P vs. NP hUAEC (Fig. 4B). Treatment with 10 ng/ml VEGF stimulated Sp1 and YY1 protein expressions in P hUAEC in a time-dependent manner; VEGF began to increase Sp1 and YY1 proteins at 24 h; both reached peak levels at 48 h (Sp1: 1.97 ± 0.16 fold, $p < 0.05$; YY1: 2.29 ± 0.38 fold, $p < 0.05$), and returned to baseline at 72 h. Treatment with increasing concentrations (0.1-100 ng/ml) of VEGF at 48 h also stimulated Sp1 and YY1 protein expressions in a dose-dependent manner. VEGF at 0.1 ng/ml effectively increased Sp1 and YY1 proteins; the responses peaked with 10 ng/ml VEGF stimulating Sp1 protein by $2.52 \pm$

0.24 ($p < 0.05$) and YY1 by 2.59 ± 0.42 ($p < 0.05$) fold. VEGF at 100 ng/ml did not stimulate Sp1 and YY1 protein expression (Fig. 4C).

Sp1 and YY1 in baseline and VEGF-stimulated CBS expression and H₂S production in hUAEC

Treatment with 10 ng/ml VEGF for 48h stimulated Sp1, YY1, and CBS, but not CSE proteins in P hUAEC. Treatment with specific Sp1 siRNA downregulated Sp1 by ~50% ($p < 0.01$) and treatment with YY1 siRNA downregulated YY1 by ~53% ($p < 0.01$) in P hUAEC. Sp1 knockdown did not alter baseline CBS protein; YY1 knockdown decreased baseline CBS protein by 49% ($P < 0.05$). VEGF-stimulated CBS protein expression was unchanged by knockdown of Sp1 or YY1 alone but completely blocked by knockdown of both Sp1 and YY1. Knockdown of Sp1 or YY1 alone and both did not alter baseline and VEGF-stimulated CSE protein expression in P hUAEC (Fig. 5). Baseline H₂S production in P hUAEC (0.31 pmol/cell/h) was significantly greater than that in NP hUAEC (0.06 pmol/cell/h), consistent with our previous report¹⁹. VEGF stimulated H₂S production in both NP and P hUAEC. Knockdown of either Sp1 or YY1 alone or their combination did not alter baseline H₂S production but abrogated VEGF-stimulated H₂S production in both NP and P hUAEC (Fig. 6).

DISCUSSION

Our recent studies have shown that enhanced local EC and smooth muscle (SMC) cell H₂S production contributes to UA relaxation in pregnancy⁹⁻¹¹ and that endometrial stroma and trophoblast derived H₂S functions as a paracrine factor to stimulate endometrial and placental angiogenesis^{9, 20, 21}. We have also reported that VEGF stimulates pregnancy dependent H₂S production via selectively upregulating CBS protein expression in hUAEC¹⁹. Therefore, augmented CBS/H₂S signaling mediates the proangiogenic and vasodilatory effects of VEGF to upregulate UtBF during pregnancy. However, how VEGF-stimulates pregnancy-dependent uterine artery CBS/H₂S signaling is yet to be determined. Herein we show that VEGF stimulates pregnancy-dependent CBS mRNA expression via upregulating *CBS* transcription via enhanced Sp1 and YY1 expression and their interactions with the corresponding binding sites in the proximal *CBS* promoter, without altering mRNA stability. Sp1 and YY1 knockdown completely abolished VEGF-stimulated CBS protein expression and H₂S production in hUAEC. VEGF does not activate *CSE* transcription and Sp1 and YY1 knockdown also does not affect CSE expression in hUAEC. Thus, VEGF stimulation of pregnancy dependent UAEC H₂S production primarily occurs via selective upregulation of CBS transcription involving enhanced expression of Sp1 and YY1 and their interactions with the *CBS* promoter.

The human *CBS* gene contains five transcription starting sites designated as -1a, -1b, -1c, -1d and -1e, respectively²⁵, leading to multiple transcripts with different 5'-untranslated regions, and the ones containing exon -1a and -1b are most abundant²⁵. Serum or fibroblasts growth factor stimulate human *CBS* transcription via the -1b promoter²⁵, suggesting that VEGF may activate this promoter. In the present study, we have first used luciferase reporter constructs driven by a series of 5' deletion *CBS* promoter to determine

the VEGF-responsive regions of the human *CBS*-1b promoter and found that the promoter sequence (-574 to -394) is primarily responsible for *trans*-activating *CBS* promoter by VEGF in hUAEC. Next, by using the LASAGNA motif search tool we found that the human *CBS*-1b promoter contains four Sp1 (a, b, c, and d) sites and one YY1 site adjacent to Sp1d. Interestingly, the Sp1d and YY1 sites are present in the promoter sequence (-574 to -394) critical for VEGF-stimulated *trans*-activation of *CBS*-1b promoter. We have therefore further determined the role of Sp1d and YY1 in VEGF-stimulated *trans*-activation of *CBS* promoter by luciferase reporter expression studies using full-length *CBS* promoter in which Sp1d and YY1 sites are mutated. The promoter with mutated Sp1d is with lower baseline activity but still can be *trans*-activated by VEGF. The promoter with mutated YY1 is not only with lower baseline activity but also a complete loss of response to VEGF stimulation and so does the one with Sp1/YY1 double mutations. Thus, Sp1 is needed to maintain baseline *CBS*-1b promoter activity and YY1 is critically important for VEGF-stimulated *CBS* transcription in hUAEC.

Herein we have also performed ChIP-PCR studies to further determine Sp1 and YY1 interactions with the human *CBS* promoter. In resting NP hUAEC, only one specific DNA sequence (amplicon 3) is amplified from the ChIP samples obtained by using Sp1 or YY1 antibodies, suggesting that recruitment of Sp1 to Sp1d site and YY1 to YY1 site is required for basal *CBS* expression in NP state. Of note, both sites are pocketed in the promoter sequence (-574 to -394) needed for basal and VEGF-stimulated *trans*-activation of *CBS*-1b promoter, as determined by promoter mapping luciferase reporter gene expression studies. In untreated P hUAEC, Sp1 is recruited to the Sp1d site with increased binding activity compared to that in NP cells and also to the Sp1a/b site. In addition to be recruited to the YY1 site with increasing binding activity, YY1 is also recruited to the Sp1c site, suggesting formation of a Sp1/YY1 complex at this site since Sp1c does not contain the core YY1 binding sequence. Thus, in addition to increasing interactions of Sp1 with Sp1d and YY1 with YY1, recruitments of Sp1 to Sp1a/b and YY1 to Sp1c seem to be needed for pregnancy-augmented EC *CBS* expression in human UA in vivo as we recently reported ^{9, 11}.

In P hUAEC, VEGF stimulates the recruitment of Sp1 to Sp1d site and YY1 to YY1 site. Interestingly, VEGF also recruits Sp1 and YY1 to the Sp1c site. Recruitment of YY1 to Sp1c suggests formation of a YY1/Sp1 complex, which is supported by our Co-IP studies showing that VEGF increases Sp1 association with YY1 in P hUAEC. Thus, increased binding of Sp1 to Sp1d/c, YY1/YY1, and formation of a Sp1/YY1 complex on Sp1c, are important for VEGF-stimulated pregnancy dependent *CBS* transcription. In NP hUAEC, VEGF increases Sp1 recruitment to Sp1d site; however, without increased YY1 binding to YY1 and Sp1c sites in NP cells, this does not suffice to initiate *CBS* transcription by VEGF. VEGF also increases Sp1 binding to Sp1a/b site; however, this seems not be functionally important due to the inability of the promoter sequence (> -184) that contains Sp1a/b to initiate *CBS* transcription as determined by luciferase assay.

In human UA, Sp1 and YY1 proteins express in a pregnancy dependent cell (EC vs. SMC) specific manner. In vitro, VEGF stimulates Sp1 and YY1 protein expressions in P hUAEC. Increased EC Sp1 and YY1 expression adds another layer to the regulatory mechanism via

Sp1/YY1-mediated upregulation of *CBS* transcription for explaining pregnancy-augmented VEGF stimulation of CBS expression in human UA. Both Sp1 and YY1 are also upregulated even more in SMC than EC in human UA. This study does not explore the functional sequelae of increased SMC Sp1 and YY1 in human UA. Sp1 promotes a contractile to synthetic phenotypic switch of vascular SMC³⁰ and YY1 inhibits vascular SMC differentiation³¹. Thus, increased SMC Sp1 and YY1 may play a role in pregnancy-induced UA SMC remodeling involving a phenotypic switch that favors vasodilation.

Lastly, we used lentiviral delivery of specific siRNAs to knock down Sp1 or YY1 for demonstrating a role of Sp1/YY1 in CBS expression and H₂S production in hUAEC. Knockdown of YY1 but not Sp1 decreases baseline CBS protein and knockdown of both Sp1 and YY1 but not alone blocks VEGF-stimulated CBS protein expression, without altering CSE protein expression. Consistent with our previous report¹⁹, basal H₂S production is lower in NP vs. P cells and VEGF stimulates H₂S production in both NP and P hUAEC. The reason that VEGF stimulates H₂S production in NP cells without affecting CBS and CSE protein expression can be attributed to posttranslational modifications of both enzymes such as phosphorylation of CBS⁸ and CSE³² and CSE interaction with Ca²⁺/calmodulin³³, complexing the mechanism of H₂S biosynthesis. Nonetheless, knockdown of either Sp1 or YY1 completely attenuates VEGF-stimulated H₂S production in NP and P hUAEC, demonstrating a critical role of Sp1 or YY1 in basal and VEGF-stimulated H₂S production in NP and P hUAEC.

In summary, our current study demonstrates that pregnancy augments EC Sp1 and YY1 expression in human UA and promotes the recruitment of Sp1/YY1 to their DNA binding sequences in proximal human *CBS* promoter; this pathway underlies a novel mechanism by which VEGF utilizes to stimulate endothelial CBS upregulation for producing H₂S in human UA during pregnancy.

Perspectives

Our recent work has shown that enhanced H₂S production via selective CBS upregulation contributes to pregnancy-associated uterine vasodilation and endometrial/placental angiogenesis^{9, 11, 20, 21}, as well as immune tolerance at the maternal-fetal interface³⁴, and that pregnancy augments VEGF-stimulated human artery endothelial cell H₂S production via selective CBS upregulation¹⁹. Adenoviral delivery of *VEGF* gene abelites undernutrition-induced fetal growth restriction via upregulating UtBF^{17, 18}. Administration of H₂S donors rescue animal models of preeclampsia induced by sFlt1, involving upregulating VEGF^{22, 23}. Thus, the CBS/H₂S and VEGF systems interact to play a key role in uterine hemodynamic regulation in pregnancy. Our current study further elaborates that upregulated EC Sp1 and YY1 and enhanced interactions of these transcription factors with their DNA binding sequences in the *CBS* promoter, underling the specific pathways by which VEGF utilizes to *trans*-activate *CBS* transcription for mediating VEGF-stimulated human UAEC H₂S production in pregnancy. This pathway provides a novel strategy for potential therapeutic development for hypertension-related pregnancy disorders such as preeclampsia with fetal growth restriction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Sanghavi M, Rutherford JD. Cardiovascular physiology of pregnancy. *Circulation*. 2014;130:1003–1008. doi: 10.1161/CIRCULATIONAHA.114.0090292. [PubMed: 25223771]
2. Thaler I, Manor D, Itskovitz J, Rottem S, Levit N, Timor-Tritsch I, Brandes JM. Changes in uterine blood flow during human pregnancy. *Am J Obstet Gynecol*. 1990;162:121–5. doi: 10.1016/0002-9378(90)90834-t. [PubMed: 2301480]
3. Gluckman PD, Hanson MA, Cooper C, Thornburg KL. Effect of in utero and early-life conditions on adult health and disease. *N Engl J Med*. 2008;359:61–73. doi: 10.1056/NEJMra0708473 [PubMed: 18596274]
4. Magness RR, Shaw CE, Phernetton TM, Zheng J, Bird IM. Endothelial vasodilator production by uterine and systemic arteries. II. Pregnancy effects on NO synthase expression. *Am J Physiol*. 1997;272:H1730–1740. doi: 10.1152/ajpheart.1997.272.4.H1730 [PubMed: 9139957]
5. Nelson SH, Steinsland OS, Suresh MS, Lee NM. Pregnancy augments nitric oxide-dependent dilator response to acetylcholine in the human uterine artery. *Hum Reprod*. 1998;13:1361–1367 [PubMed: 9647573]
6. Rosenfeld CR, Roy T. 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*. 2014;307:H1196–1203. doi: 10.1152/ajpheart.00996.2013 [PubMed: 25128169]
7. Wang R. Physiological implications of hydrogen sulfide: A whiff exploration that blossomed. *Physiol Rev*. 2012;92:791–896. doi: 10.1152/physrev.00017.2011 [PubMed: 22535897]
8. Filipovic MR, Zivanovic J, Alvarez B, Banerjee R. Chemical biology of H₂S signaling through persulfidation. *Chem Rev*. 2018;118:1253–1337. doi: 10.1021/acs.chemrev.7b00205 [PubMed: 29112440]
9. Sheibani L, Lechuga TJ, Zhang HH, Hameed A, Wing DA, Kumar S, Rosenfeld CR, Chen DB. Augmented H₂S production via cbs upregulation plays a role in pregnancy-associated uterine vasodilation. *Biol Reprod*. 2017;96:664–672. doi: 10.1095/biolreprod.116.143834 [PubMed: 28339573]
10. Lechuga TJ, Qi QR, Magness RR, Chen DB. Ovine uterine artery hydrogen sulfide biosynthesis in vivo: Effects of ovarian cycle and pregnancy. *Biol Reprod*. 2019;100:1630–1636. doi: 10.1093/biolre/iox027 [PubMed: 30772913]
11. Li Y, Bai J, Yang YH, Hoshi N, Chen DB. Hydrogen sulfide relaxes human uterine artery via activating smooth muscle BKCa channels. *Antioxidants*. 2020;9:274–279. doi: 10.3390/antiox9111127
12. Rosenfeld CR, Cornfield DN, Roy T. Ca²⁺-activated K⁺ channels modulate basal and E₂β-induced rises in uterine blood flow in ovine pregnancy. *Am J Physiol Heart Circ Physiol*. 2001;281:H422–431. doi: 10.1152/ajpheart.2001.281.1.H422 [PubMed: 11406511]
13. Hu XQ, Dasgupta C, Chen M, Xiao D, Huang X, Han L, Yang S, Xu Z, Zhang L. Pregnancy reprograms large-conductance Ca²⁺-activated K⁺ channel in uterine arteries: Roles of ten-

- eleven translocation methylcytosine dioxygenase 1-mediated active demethylation. *Hypertension*. 2017;69:1181–1191. doi: 10.1161/HYPERTENSIONAHA.117.09059 [PubMed: 28396535]
14. Ni Y, May V, Braas K, Osol G. Pregnancy augments uteroplacental vascular endothelial growth factor gene expression and vasodilator effects. *Am J Physiol*. 1997;273:H938–944. doi: 10.1152/ajpheart.1997.273.2.H938 [PubMed: 9277513]
 15. Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, Schisterman EF, Thadhani R, Sachs BP, Epstein FH, Sibai BM, Sukhatme VP, Karumanchi SA. Circulating angiogenic factors and the risk of preeclampsia. *N Engl J Med*. 2004;350:672–683. doi: 10.1056/NEJMoa031884 [PubMed: 14764923]
 16. Bergmann A, Ahmad S, Cudmore M, Gruber AD, Wittschen P, Lindenmaier W, Christofori G, Gross V, Gonzalves A, Grone HJ, Ahmed A, Weich HA. Reduction of circulating soluble flt-1 alleviates preeclampsia-like symptoms in a mouse model. *J Cell Mol Med*. 2010;14:1857–1867. doi: 10.1111/j.1582-4934.2009.00820.x [PubMed: 19538465]
 17. Carr DJ, Wallace JM, Aitken RP, Milne JS, Mehta V, Martin JF, Zachary IC, Peebles DM, David AL. Uteroplacental adenovirus vascular endothelial growth factor gene therapy increases fetal growth velocity in growth-restricted sheep pregnancies. *Hum Gene Ther*. 2014;25:375–384. doi: 10.1089/hum.2013.214 [PubMed: 24593228]
 18. Carr DJ, Wallace JM, Aitken RP, Milne JS, Martin JF, Zachary IC, Peebles DM, David AL. Peri- and postnatal effects of prenatal adenoviral vegf gene therapy in growth-restricted sheep. *Biol Reprod*. 2016;94:142. doi: 10.1095/biolreprod.115.133744 [PubMed: 27103444]
 19. Zhang HH, Chen JC, Sheibani L, Lechuga TJ, Chen DB. Pregnancy augments vegf-stimulated in vitro angiogenesis and vasodilator (NO and H₂S) production in human uterine artery endothelial cells. *J Clin Endocrinol Metab*. 2017;102:2382–2393. doi: 10.1210/jc.2017-00437 [PubMed: 28398541]
 20. Chen DB, Feng L, Hodges JK, Lechuga TJ, Zhang H. Human trophoblast-derived hydrogen sulfide stimulates placental artery endothelial cell angiogenesis. *Biol Reprod*. 2017;97:478–489. doi: 10.1093/biolre/iox105 [PubMed: 29024947]
 21. Qi QR, Lechuga TJ, Patel B, Nguyen NA, Yang YH, Li Y, Sarnthiyakul S, Zhang QW, Bai J, Makhoul J, Chen DB. Enhanced stromal cell CBS-H₂S production promotes estrogen-stimulated human endometrial angiogenesis. *Endocrinology*. 2020;161:bqaa176. doi: 10.1210/endo/bqaa176. [PubMed: 32987401]
 22. 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. Dysregulation of hydrogen sulfide producing enzyme cystathionine gamma-lyase contributes to maternal hypertension and placental abnormalities in preeclampsia. *Circulation*. 2013;127:2514–2522. doi: 10.1161/CIRCULATIONAHA.113.001631 [PubMed: 23704251]
 23. Holwerda KM, Burke SD, Faas MM, Zsengeller Z, Stillman IE, Kang PM, van Goor H, McCurley A, Jaffe IZ, Karumanchi SA, Lely AT. Hydrogen sulfide attenuates sflt1-induced hypertension and renal damage by upregulating vascular endothelial growth factor. *J Am Soc Nephrol*. 2014;25:717–725. doi: 10.1681/ASN.2013030291 [PubMed: 24335973]
 24. Magness RR. Maternal cardiovascular and other physiologic responses to the endocrinology of pregnancy. In: Bazer FW (eds) *Endocrinology of Pregnancy*. Contemporary Endocrinology, Vol 9. 1998;Humana Press, Totowa, NJ.:pp 507–539. doi:10.1007/978-1-4612-1804-3_18
 25. Kraus JP, Oliveriusova J, Sokolova J, Kraus E, Vlcek C, de Franchis R, Maclean KN, Bao L, Bukovsk, Patterson D, Paces V, Ansoorge W, Kozich V. The human cystathionine beta-synthase (cbs) gene: Complete sequence, alternative splicing, and polymorphisms. *Genomics*. 1998;52:312–324. doi: 10.1006/geno.1998.5437 [PubMed: 9790750]
 26. Maclean KN, Kraus E, Kraus JP. The dominant role of Sp1 in regulating the cystathionine beta-synthase –1a and –1b promoters facilitates potential tissue-specific regulation by kruppel-like factors. *J Biol Chem*. 2004;279:8558–8566. doi: 10.1074/jbc.M310211200 [PubMed: 14670973]
 27. Natesan S, Gilman MZ. DNA bending and orientation-dependent function of YY1 in the c-fos promoter. *Genes & Dev*. 1993;7:2497–2509. doi:10.1101/gad.7.12b.2497 [PubMed: 8276234]
 28. Zhang S, Kim JY, Xu S, Liu H, Yin M, Koroleva M, Guo J, Pei X, Jin ZG. Endothelial-specific YY1 governs sprouting angiogenesis through directly interacting with RBPJ. *Proc Natl Acad Sci U S A*. 2020;117:4792–4801. doi: 10.1073/pnas.1916198117 [PubMed: 32075915]

29. Sade H, Holloway K, Romero IA, Male D. Transcriptional control of occludin expression in vascular endothelia: Regulation by Sp3 and YY1. *Biochim Biophys Acta*. 2009;1789:175–184. doi: 10.1016/j.bbarm.2009.01.006 [PubMed: 19437620]
30. Tang Y, Yu S, Liu Y, Zhang J, Han L, Xu Z. MicroRNA-124 controls human vascular smooth muscle cell phenotypic switch via Sp1. *Am J Physiol Heart Circ Physiol*. 2017;313:H641–H649. doi: 10.1152/ajpheart.00660.2016. [PubMed: 28667053]
31. Jin M, Wu Y, Wang Y, Yu D, Yang M, Yang F, Feng C, Chen T. MicroRNA-29a promotes smooth muscle cell differentiation from stem cells by targeting YY1. *Stem Cell Res*. 2016;17:277–284. doi: 10.1016/j.scr.2016.07.011 [PubMed: 27591939]
32. Xu X, Yan Q, Liu X, Li P, Li X, Chen Y, Simoncini T, Liu J, Zhu D, Fu X. 17beta-estradiol nongenomically induces vascular endothelial H2S release by promoting phosphorylation of cystathionine gamma-lyase. *J Biol Chem*. 2019;294:15577–15592. doi: 10.1074/jbc.RA119.008597 [PubMed: 31439665]
33. Yang G, Wu L, Jiang B, Yang W, Qi J, Cao K, Meng Q, Mustafa AK, Mu W, Zhang S, Snyder SH, Wang R. H2S as a physiologic vasorelaxant: Hypertension in mice with deletion of cystathionine gamma-lyase. *Science*. 2008;322:587–590. doi: 10.1126/science.1162667 [PubMed: 18948540]
34. Wang B, Xu T, Li Y, Wang W, Lyu C, Luo D, Yang Q, Ning N, Chen ZJ, Yan J, Chen DB, Li J. Trophoblast H2S maintains early pregnancy via regulating maternal-fetal interface immune hemostasis. *J Clin Endocrin & Metab*. 105:e4275–e4289. doi: 10.1210/clinem/dgaa357

Novelty and Significance

What Is New?

- Vascular endothelial growth factor (VEGF) stimulates pregnancy-dependent mRNA expression of the hydrogen sulfide (H₂S) synthesizing enzyme cystathionine β-synthase (CBS) via upregulating *CBS* transcription without altering its mRNA stability. Pregnancy augments Sp1 and YY1 expressions in a cell (endothelial vs. smooth muscle)-specific manner in human uterine artery in vivo. VEGF stimulates pregnancy dependent Sp1 and YY1 expression in human uterine artery endothelial cells (hUAEC) in vitro. Enhanced interactions of Sp1 and YY1 with their corresponding binding sites and formation of a Sp1/YY1 complex in a Sp1 site in human *CBS* promoter are required for VEGF-stimulated pregnancy dependent *CBS* transcription in hUAEC. Furthermore, Sp1 or YY1 inhibition completely attenuates VEGF-stimulated pregnancy dependent CBS expression and H₂S production in hUAEC.

What Is Relevant?

- Interactions between VEGF signaling and CBS/H₂S pathway play a critical role in the two key mechanisms (i.e., vasodilation and angiogenesis) for upregulating uterine blood flow that is critical for pregnancy health. The present study demonstrates that VEGF stimulates pregnancy dependent H₂S production via upregulating *CBS* transcription by enhanced expressions of Sp1 and YY1 and coordinated interactions of Sp1 and YY1 with the proximal *CBS* promoter in human uterine artery endothelial cells. These findings provide a novel mechanism for controlling uterine artery H₂S production pertaining to uterine hemodynamic regulation during pregnancy.

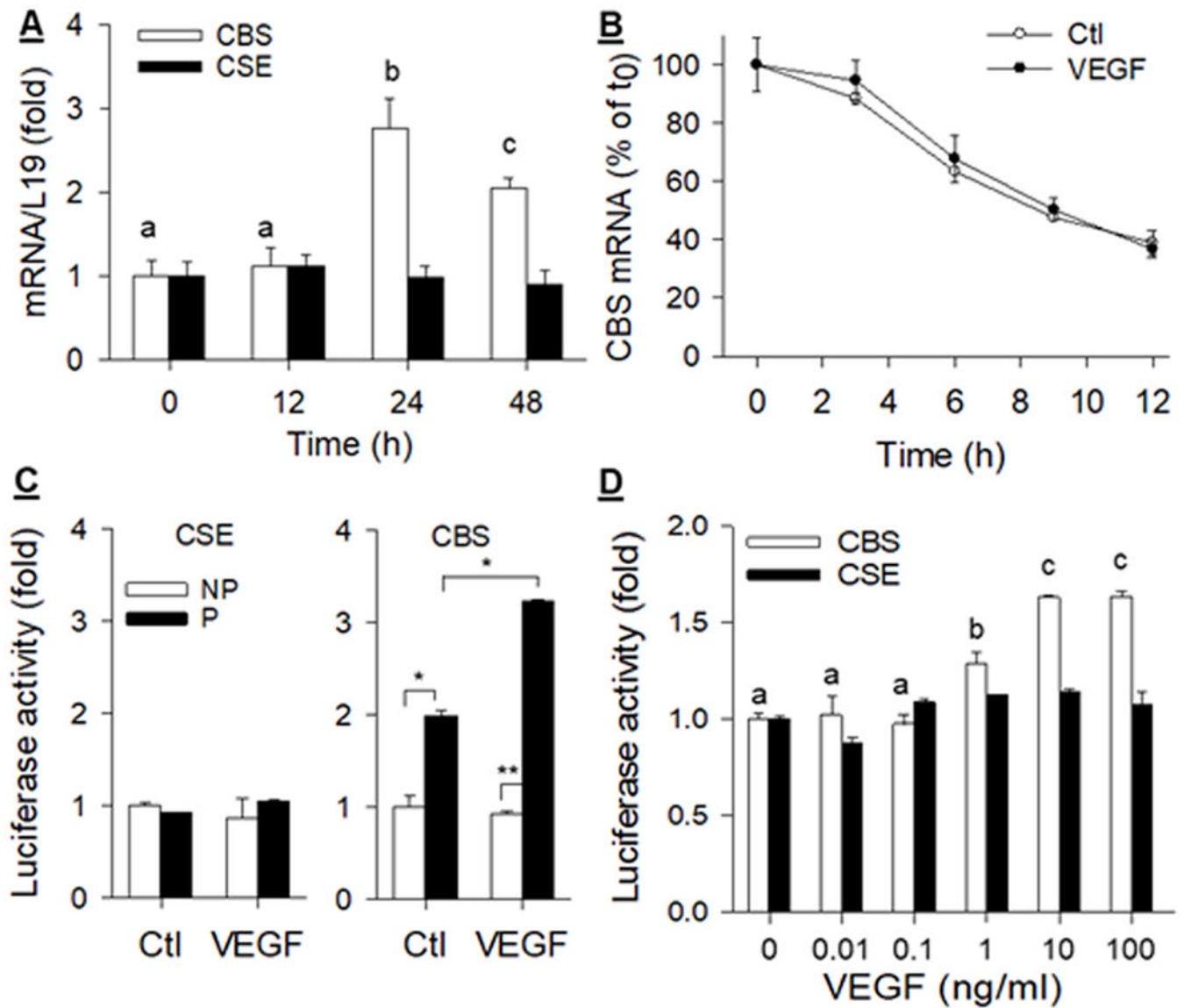


Fig. 1: VEGF stimulates pregnancy dependent human uterine artery endothelial CBS expression.

A: Primary human uterine artery endothelial cells (hUAEC) were treated with 10 ng/mL vascular endothelial growth factor (VEGF) for up to 2 days to assess cystathionine β -synthase (CBS) and cystathionine γ -lyase (CSE) mRNA. Bars with different letters differ significantly ($p < 0.05$). Data (means \pm SEM) were collected from different hUAEC preparations from different nonpregnant (NP) and pregnant (P) women. **B:** P hUAEC were treated with or without actinomycin D (5 μ g/ml). Total RNA samples were extracted at 0, 3, 6, 9 and 12 h post actinomycin D. CBS and L19 were analyzed using real time quantitative reverse transcription-PCR (RT-qPCR). Data (means \pm SEM, $n=3$) were presented as a percentage of relative CBS expression at time 0. **C:** Both non-pregnant (NP) and P 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 VEGF (10 ng/ml) treatment for 24 h, *trans*-activation

of *CBS* or *CSE* promoter was measured by luciferase reporter gene expressions as a ratio of firefly/Renilla luciferase activities. Data (mean \pm SEM. N=3) were presented as fold of controls. * $p < 0.05$ and ** $p < 0.01$. D: P hUAEC were transfected with luciferase constructs driven by wild-type human *CBS* promoter or *CSE* promoter and co-transfected with the thymidine kinase-Renilla luciferase vector, treated with increasing doses (0-100 ng/ml) of VEGF for 24 h. Data were summarized as mean \pm SEM from three independent experiments; Bars with different letters differ significantly ($p < 0.05$).

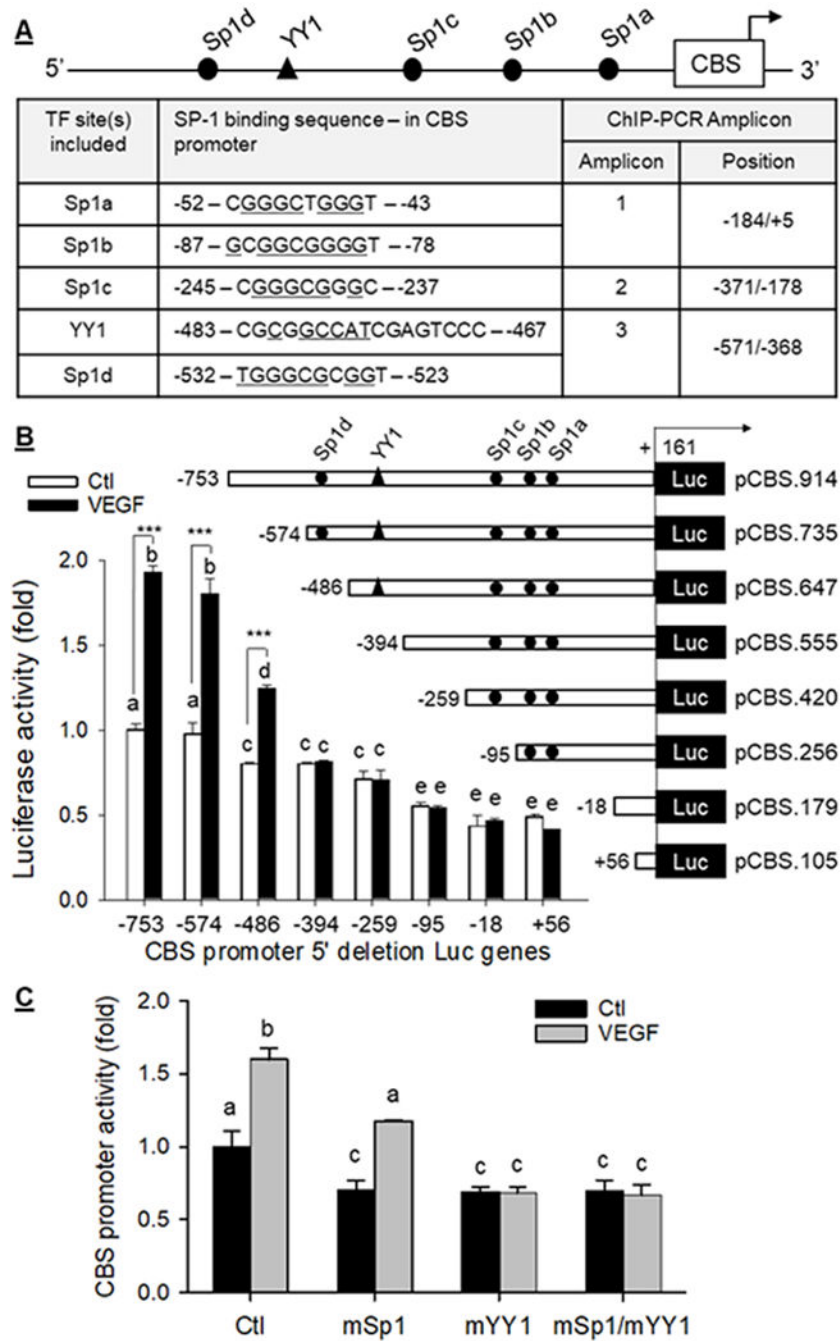


Fig. 2: Sp1/YY1 in VEGF-stimulated CBS transcription.

A: Putative Sp1 and YY1 binding sites in human *CBS* promoter and primers designed to amplify amplicons containing specific Sp1/YY1 sites for chromatin immunoprecipitation-PCR. P hUAEC were transfected with luciferase reporter constructs driven by wild-type human *CBS* promoter (-753/+161) or its 5'-deletions (**B**), and wild type (WT) pCBS(-574).Luc construct or its mutations in the Sp1d, YY1, or both sites (**C**), and with thymidine kinase-Renilla luciferase vector as internal control. After treatment with VEGF (10 ng/ml) for 24 h, *trans*-activation of *CBS* promoter was measured. Data (means ± SEM,

n=3) were expressed as fold of baseline WT *CBS* promoter activity. Bars with different letters differ significantly ($p < 0.05$). ***, $p < 0.001$ vs controls.

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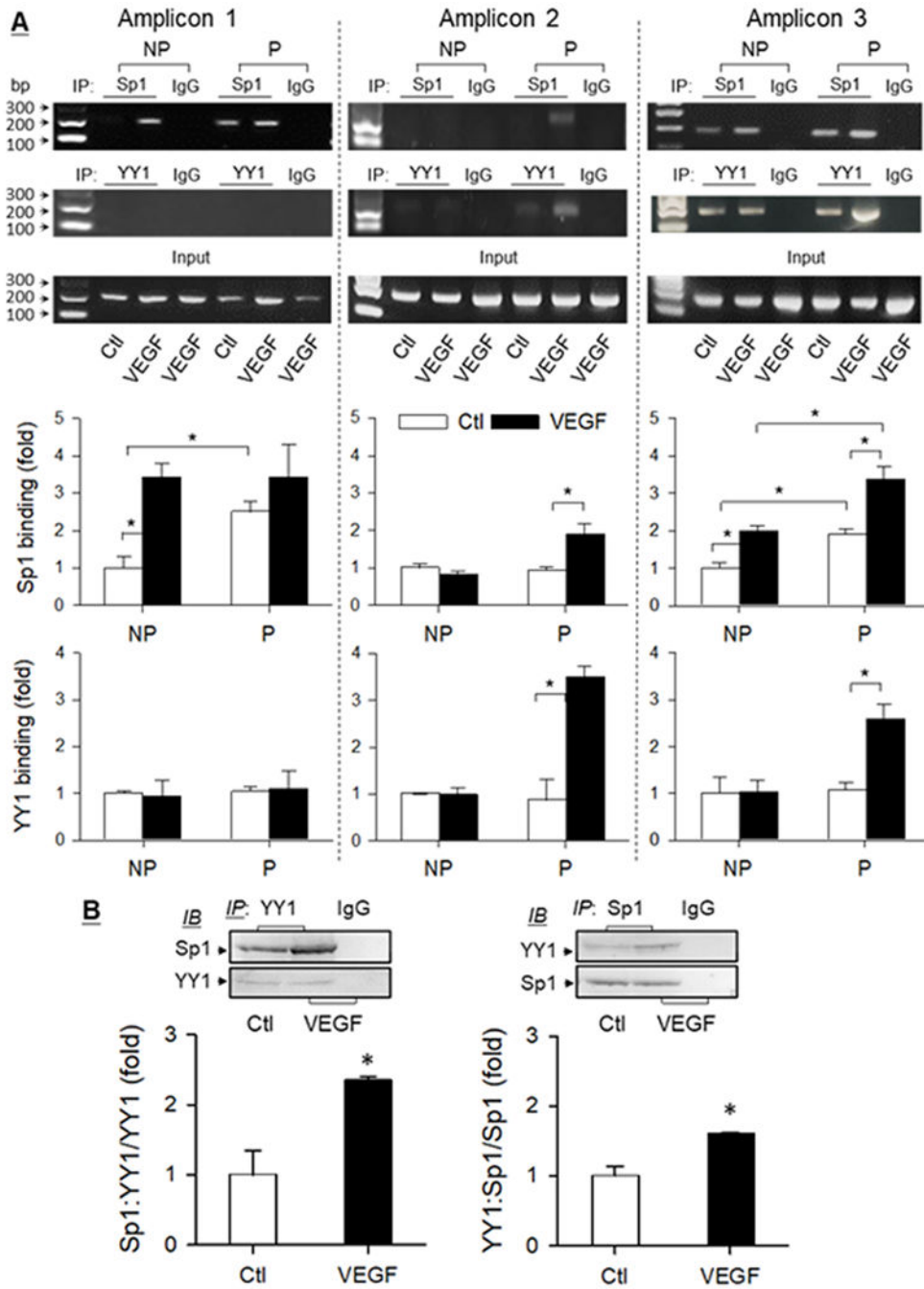


Fig. 3: Sp1/YY1 interactions with CBS promoter.

A: Binding of Sp1 and YY1 to human CBS promoter. Nonpregnant (NP) and pregnant (P) hUAEC were treated with or without VEGF (10 ng/ml, 24 h). Sheared DNA from the cells were subjected to chromatin immunoprecipitation (ChIP) using Sp1 or YY1 antibodies; IgG was used as negative controls. Sp1 and YY1 binding with specific sites were amplified by PCR using primers as designed in Fig. 2A. Data (means \pm SEM) were summarized as fold of control from three experiments using cells from different women. *, $p < 0.05$ vs control. **B.** Sp1 and YY1 association. P hUAEC were treated with or without VEGF (10 ng/ml, 30 min).

Proteins (1 mg/sample) were used immunoprecipitation (IP) with Sp1 or YY1 antibodies and IgG was used as control. The IP samples were immunoblotted with Sp1 or YY1. Levels of Sp1 bound YY1 and YY1 bound Sp1 were summarized as means \pm SEM from NP or P hUAEC from three different women. *, $p < 0.05$ vs control.

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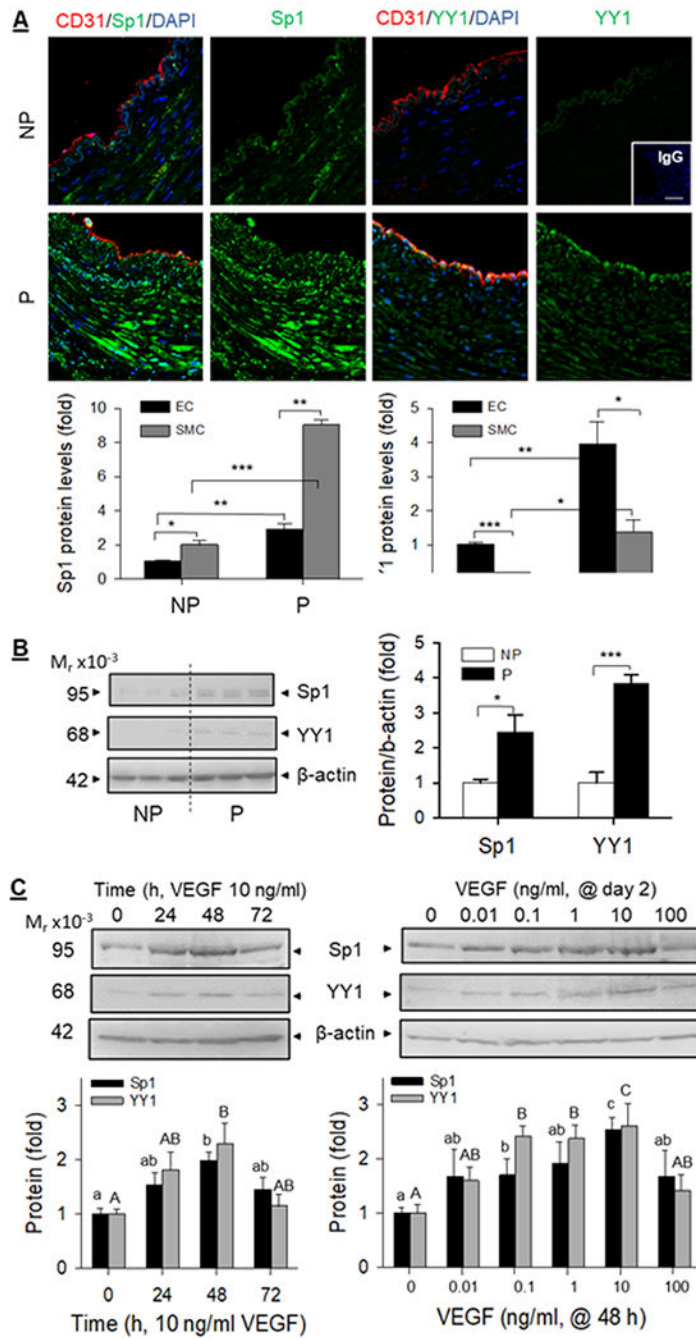


Fig. 4: Sp1 and YY1 proteins in human uterine artery and effects of VEGF on hUAEC Sp1/YY1 expression.

A: Paraffin-embedded human uterine artery rings were immunolabeled by specific Sp1 or YY1 antibodies and CD31 antibody for labeling endothelial cells. Sections were mounted with DAPI to label nuclei and examined under confocal microscopy. Images were taken to determine Sp1 and YY1 proteins (relative green fluorescence intensity) in endothelium (EC) and smooth muscle (SM) using Image J and summarized in graphs. *, p < 0.05, **, p < 0.01, ***, p < 0.001 vs NP controls. **B:** Proteins (20 μg/lane) from NP and P hUAEC (n=3/group) were used for immunoblotting with Sp1 and YY1 antibodies and β-actin was measured as

loading control. Data were summarized as means \pm SEM and presented as fold of NP. *, $p < 0.05$, ***, $p < 0.01$ vs NP. C: P hUAEC were treated with VEGF (10 ng/ml, 3 days) or with increasing concentrations (0–100 ng/ml) of VEGF for 2 days to assess Sp1 and YY1 proteins by immunoblotting. Data were obtained from three experiments using cells from different women and calculated as means \pm SEM (fold of baseline). Small and capital letter on bars represent differences in Sp1 and YY1, respectively, and bars with different letter differ significantly ($p < 0.05$).

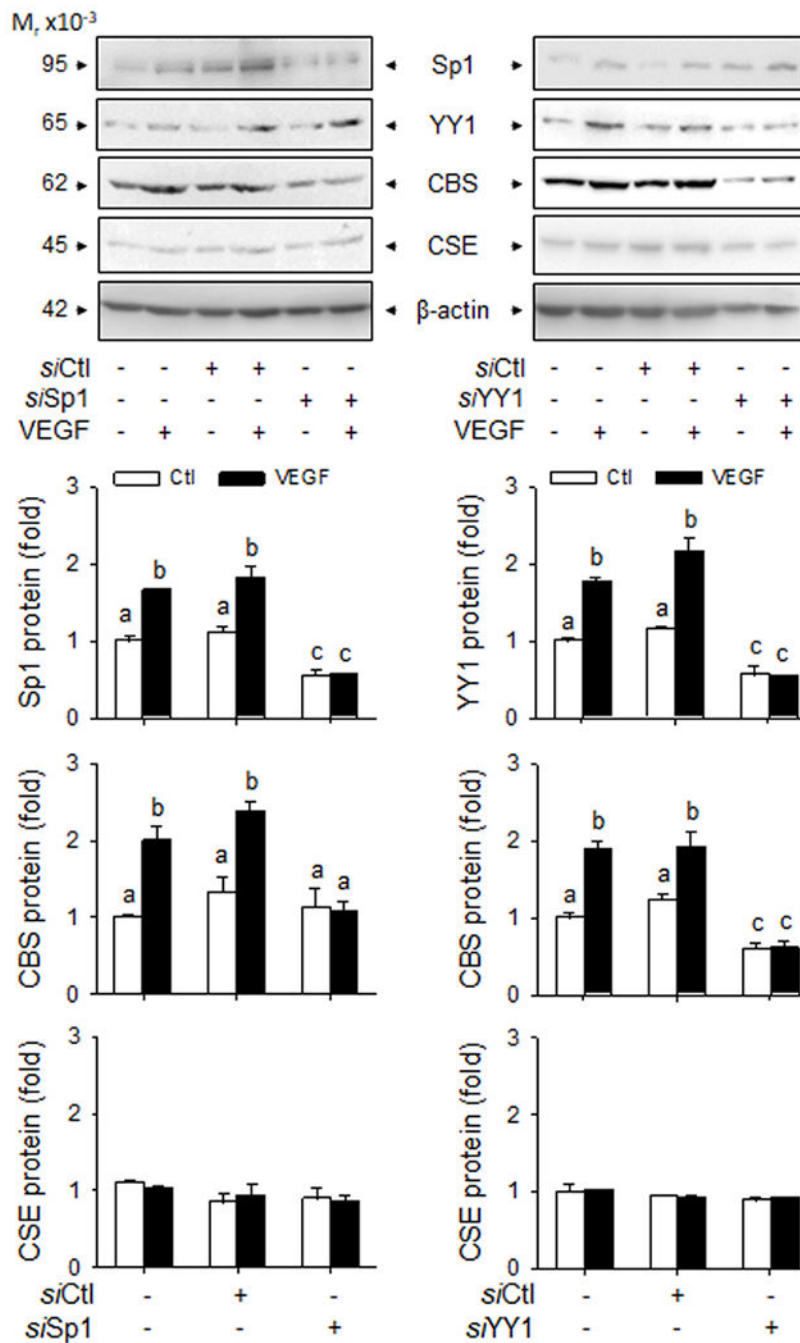


Fig. 5: Sp1/YY1 knockdown on VEGF-stimulated CBS/CSE expression. P hUAEC were infected with or without lentiviruses (10 MOI) of Sp1 (siSp1) or YY1 (siYY1) siRNAs for 72 h; lentivirus of scramble siRNA (siCtl) as control. The cells were then treated with or without VEGF (10 ng/ml) for 48 h. Cell lysates (20 μ g/lane) were immunoblotted for Sp1, YY1, CBS, and CSE; β -actin was measured as loading control. Data were obtained from three experiments using cells from different women and calculated as means \pm SEM (fold of baseline). Bars with different letters differ significantly (p < 0.05).

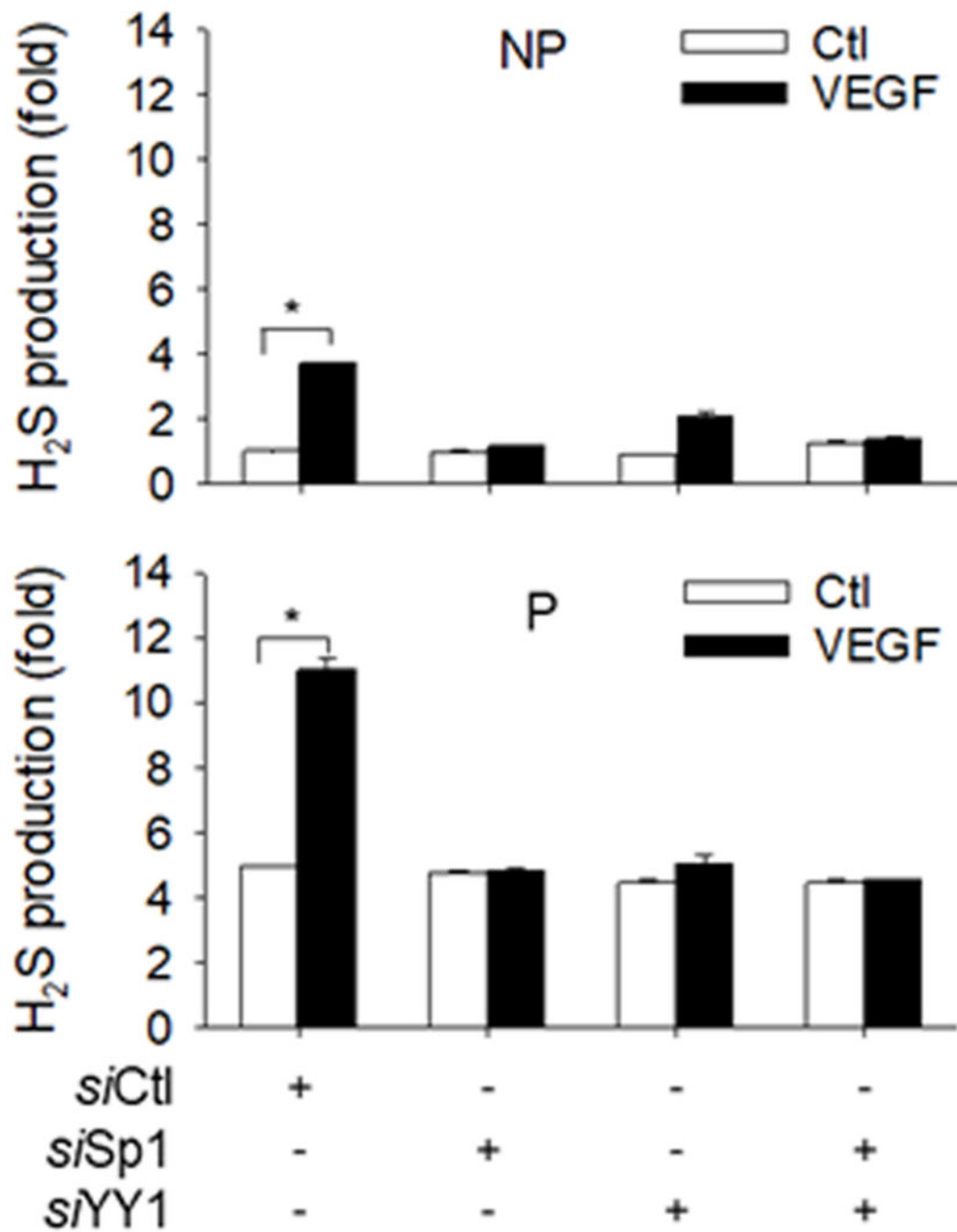


Fig. 6: Sp1/YY1 knockdown on VEGF-stimulated H₂S production.

NP and P hUAEC were infected with or without lentiviruses (10 MOI) of Sp1 (siSp1) or YY1 (siYY1) siRNAs for 72 h; lentivirus of scramble siRNA (siCtl) as control. The cells were then treated with or without VEGF (10 ng/ml) for 48 h. Protein extracts (1×10^6 cells) were used to determine hydrogen sulfide (H₂S) production by methylene blue assay. Data (means \pm SEM) were collected from NP or P hUAEC (n=3/group) from different women. ***, $p < 0.001$ vs control.