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Analysis of Vascular Hydrogen Sulfide Biosynthesis

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Abstract

With potent vasodilatory and pro-angiogenic properties, hydrogen sulfide (H_2S) is now accepted as the third gasotransmitter after nitric oxide (NO) and carbon monoxide. Endogenous H_2S is mainly synthesized by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). Akin to previous studies showing hormonal regulation of NO biosynthesis, we first reported that uterine and systemic artery H_2S biosynthesis is regulated by exogenous estrogens in an ovariectomized sheep model of estrogen replacement therapy, specifically stimulating CBS, but not CSE, expression, in uterine (UA) and mesenteric (MA), but not carotid (CA), arteries in ovariectomized nonpregnant sheep. We have found significantly elevated H_2S biosynthesis due to CBS upregulation under estrogen-dominant physiological states, the proliferative phase of menstrual cycle and pregnancy in primary human UAs. Our studies have pioneered the role of H_2S biology in uterine hemodynamics regulation although there is still much that needs to be learned before a thorough elucidation of a role that H_2S plays in normal physiology of uterine hemodynamics and its dysregulation under pregnancy complications can be determined. In this chapter we describe a series of methods that we have optimized for analyzing vascular H_2S biosynthesis, including (1) real-time quantitative PCR (qPCR) for assessing tissue and cellular levels of CBS and CSE mRNAs, (2) immunoblotting for assessing CBS and CSE proteins, (3) semiquantitative immunofluorescence microscopy to specifically localize CBS and CSE proteins on vascular wall and to quantify their cellular expression levels, and (4) methylene blue assay for assessing H_2S production in the presence of selective CBS and CSE inhibitors.

Keywords

H_2S; qPCR; Immunoblotting; Immunofluorescence microscopy; Methylene blue assay

1 Introduction

Hydrogen sulfide (H_2S) has now been accepted as the third “gasotransmitter” after nitric oxide (NO) and carbon monoxide due to its NO-like biological properties [1–3]. Endogenous H_2S is mainly synthesized by two key enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) [4, 5]. These enzymes produce H_2S from L-cysteine, CBS via a β-replacement reaction with a variety of thiols and CSE by disulfide elimination followed by reaction with various thiols [4, 5]. H_2S can be also synthesized via 3-mercaptopyruvate sulfurtransferase (3MST), cytosolic cysteine aminotransferase (cCAT),

and mitochondrial cysteine aminotransferase (mCAT), but to a much less extent [6]. In mammals, H_2S potently dilates various vascular beds via activating ATP-dependent potassium (K_{ATP}) channel [7] and relaxes smooth muscle via activating large conductance calcium-activated potassium (BK_{Ca}) channel [8]. H_2S also promotes angiogenesis in vitro and in vivo [9]. Thus, H_2S functions as a potent vasodilator [10].

Estrogens are potent vasodilators that cause blood flow to rise in organs throughout the body with the greatest effects occurring in reproductive tissues, especially the uterus [11, 12]. In ovariectomized (OVX) nonpregnant ewes, daily estradiol-17β (E_2) increases basal uterine blood flow by 30–40% after 6–7 days in the absence of changes in arterial pressure or heart rate [13] and reduced responses to vasoconstrictors [14]. In addition, acute E_2 exposure causes even more robust up to tenfold rise in UBF within 90–120 min after a bolus intravenous injection of 1 μg/kg [15, 16]. This uterine vasodilatory effect of estrogens is of major physiological significance during the follicular phase of the ovarian cycle and pregnancy in which circulating estrogens are significantly elevated [16–19]. During normal pregnancy, estrogen levels increase substantially to upregulate UBF that provides the sole source of nutrients and oxygen supplies for the fetus and the exit of the metabolic wastes and respiratory gases of the fetus. UBF is a critical rate-limiting factor for a healthy pregnancy because (1) dramatic rise in uterine blood flow in the last one-third of gestation is directly linked to fetal growth and survival and (2) insufficient rise in uterine blood flow results in preeclampsia, intrauterine growth restriction, and many other pregnancy disorders, affecting late life after birth and the mother's well-being during pregnancy and postpartum [20]. Thus, mechanistic investigations on estrogen-induced and pregnancy-associated uterine and systemic vasodilatation will identify therapeutic targets or even preventive options for pregnancy disorders.

We have reported that exogenous estrogen replacement therapy stimulates CBS, but not CSE, mRNA and protein expression, and promotes H_2S production in uterine (UA) and mesenteric (MA) but not carotid (CA) arteries [21]. Additionally, we have found that CBS, but not CSE, is upregulated in both the endothelium and smooth muscle of human UA during the estrogen "dominant" physiological states, i.e., the proliferative phase of the menstrual cycle and pregnancy [22]. Other enzymes, i.e., 3MST, cCAT, and mCAT, are also detectable in human UA but unchanged during the menstrual cycle and pregnancy. In organ bath studies using wire myography, we also have found that a slow-releasing H_2S donor GYY4137 potently dilates primary UAs of nonpregnant and pregnant rats; however, the sensitivity (pD2) to GYY4137 is significantly greater ($p < 0.001$) in pregnant (7.43 ± 0.02) than nonpregnant (5.97 ± 0.01) UA. Moreover, GYY4137 does not dilate pregnant rat MAs [23]. Thus, these data suggest that augmented H_2S via selective upregulation of endothelial and smooth muscle CBS plays a role in estrogen-stimulated and pregnancy-augmented vasodilation in UA and selected vascular beds.

In this chapter, we described a series of methods that we have developed for analyzing vascular tissue and cell H_2S biosynthesis, including (1) real-time quantitative PCR (qPCR) for assessing steady-state levels of CBS and CSE mRNAs, (2) immunoblotting for assessing CBS and CSE proteins, (3) semiquantitative immunofluorescence microscopy for quantifying CBS and CSE proteins and their cellular (endothelium *vs.* smooth muscle)

localization, and (4) methylene blue assay for assessing H_2S production in the presence of selective CBS and CSE inhibitors.

2 Materials

Prepare all solutions without sodium azide using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ-cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature unless indicated. Diligently follow all waste disposal regulations when disposing waste materials.

2.1 CBS/CSE mRNA Analysis

2.1.1 RNA Purification and cDNA Synthesis

1. TRIzol reagent (Life Technologies, Carlsbad, CA, USA) or similar acid-guanidinium-phenol reagent.
2. Fine microdissection scissors.
3. DNase/RNase-free 1.7 mL microcentrifuge tubes.
4. Benchtop microcentrifuge.
5. Direct-zol RNA Mini Prep (Zymo Research, Irvine, CA, USA).
6. Biowave DNA (Biochrom, Cambridge, UK).
7. Random primers (Promega, Madison, WI, USA).
8. Nuclease free water.
9. Thermocycler.
10. 10 mM dNTP (Promega).
11. Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Promega).
12. M-MLV 5× reaction buffer (Promega).

2.1.2 qPCR

1. Primers (*see* Table 1).
2. RT^2 SYBR Green/ROX qPCR master mix (Qiagen, Germantown, MD, USA).
3. 96-well optical PCR plate.
4. PCR optical adhesive.
5. StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA).

2.2 CBS/CSE Protein Analysis

2.2.1 Sample Lysate Preparation

1. Fine microdissection scissors.

2. Nondenaturing lysis buffer containing protease inhibitor: 10 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% NP-40, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 1% protease inhibitor cocktail.
3. Sonicator.
4. Benchtop microcentrifuge.
5. Pierce BCA Protein Assay Kit (Life Technologies, Grand Island, NY, USA).
6. 5 SDS-PAGE sample buffer: 250 mM Tris–HCl pH 6.8, 10% (w/v) SDS, 30% (v/v) glycerol, 0.02% (w/v) bromophenol blue, 5% (v/v) β-mercaptoethanol.

2.2.2 SDS-PAGE and Transfer

1. Acrylamide gel casting apparatus and combs.
2. Distilled water.
3. 4× Resolving Gel buffer: 1.5 M Tris, 0.4% SDS, pH 8.8.
4. Acrylamide/Bis (30%/29:1).
5. 10% (w/v) ammonium persulfate (APS).
6. 50% glycerol.
7. *N*,*N*,*N*′,*N*′-tetraethylmethylenediamine (TEMED).
8. Resolving gel: 6.4 mL H_2O, 5 mL 4 resolving gel buffer, 6.6 mL 30% (v/v) acrylamide/bis, 5 mL, 2 mL 50% glycerol, 10 μL of TEMED, and 100 μL of 10% (w/v) APS (*see* Note 1).
9. 4× Stacking Gel buffer: 0.5 M Tris, 0.4% SDS, pH 6.8.
10. Stacking gel: 12 mL H_2O, 5 mL 4 resolving gel buffer, 3 mL 30% (v/v) acrylamide/bis, 10 μL of TEMED, and 400 μL of 10% (w/v) APS.
11. 20 × 10 cm gel system containing loading rig, glass plates, and buffer tank.
12. SDS-PAGE pre-stained polypeptide markers (Thermo Scientific, Waltham, MA, USA).
13. SDS-PAGE running buffer: 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS.
14. Nitrocellulose membrane.
15. Methanol.
16. Blot Absorbent Filter Paper.
17. Transfer buffer.
18. Semidry transfer system.

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1. Due to limited quantities of arteries, we have chosen to finely mince the tissue. For RNA extraction it is more desirable to freeze the tissue in liquid N_2 and grind with a mortar and pestle. This technique can result in greater tissue loss; use only if excess tissue is available. Alternatively, total RNA can be harvested from in vitro studies using cell culture models (minimum 0.5×10^6 smooth muscle or endothelial cells).

2.2.3 Immunoblotting

1. Benchtop rocker.
2. Blocking solution.
3. Mouse anti-CSE primary antibody (S-374249, Santa Cruz Biotech, Dallas, TX, USA).
4. Rabbit anti-CBS primary antibody (ab131155, Abcam, Cambridge, UK).
5. TBS-T: 20 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.5.
6. Anti-rabbit HRP-conjugated secondary antibody (Cell Signaling, Beverly, MA, USA).
7. Anti-mouse HRP-conjugated secondary antibody (Cell Signaling).
8. TBS: 20 mM Tris, 150 mM NaCl, pH 7.5.
9. Clear film (such as standard kitchen plastic wrap).
10. SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA).
11. ChemiImager Imaging System (Alpha Innotech, San Leandro, CA, USA).
12. Mild stripping buffer: 15 g glycine, 0.1% SDS, 1% Tween-20, 1 L H_2O, pH 2.2.
13. PBS (1×): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4.
14. Rabbit anti-β-actin (Ambion, Austin, TX, USA).
15. NIH Image J (https://imagej.nih.gov/ij/).
16. Microsoft Excel (Microsoft Corp, Redmond, WA, USA).

2.3 Semiquantitative Fluorescence Microscopy

1. Slides containing tissue sections (*see* Note 2).
2. Xylenes.
3. Ethanol (100%, 90%, 70%).
4. Deionized water (diH_2O).
5. 1× PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4.
6. 0.05% Trypsin (Thermo Fisher Scientific).
7. 300 mM Glycine-PBS: 1 L diH_2O, 22.56 g glycine, filtered (*see* Note 3).
8. Blocking solution: 50 mL 1 PBS, 50 μL gelatin, 0.5 g BSA, 0.0625 g saponin.
9. PAP pen (Invitrogen, Carlsbad, CA, USA).

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2. Use the equation "=POWER(2,-X)" in Microsoft Excel, where X is each value generated in **step 10**.
3. Due to limited quantities of arteries, we have chosen to finely mince the tissue. For protein extraction it is more desirable to use a tissue homogenizer. This technique can result in greater tissue loss; use only if excess tissue is available. Alternatively, protein can be harvested from in vitro studies using cell culture models (minimum 0.5×10^6 smooth muscle or endothelial cells).

10. Mouse anti-CD31 primary antibody (Dako, Carpinteria, CA, USA).
11. Antibody dilution buffer: 50% blocking solution, 50% 1× PBS.
12. Alexa 568 conjugated goat anti-mouse IgG secondary antibody.
13. Mouse anti-CSE primary antibody (Santa Cruz Biotech).
14. Rabbit anti-CBS primary antibody (Abcam).
15. Alexa 488 conjugated goat anti-mouse IgG secondary antibody (Invitrogen).
16. Alexa 488 conjugated donkey anti-rabbit IgG secondary antibody (Invitrogen).
17. ProLong Gold Antifade Mountant with DAPI (Invitrogen).
18. Coverslips.
19. Leica fluorescence microscope (Leica Corp, Deerfield, IL, USA).
20. Charge-coupled device camera with the SimplePCI image analysis software (Hamamatsu Corp, Sewickley, PA, USA).
21. Microsoft Excel (Microsoft Corp).

2.4 H_2S Activity Assay

1. Fine microdissecting scissors.
2. Sonicator.
3. Benchtop microcentrifuge.
4. Pierce BCA Protein Assay Kit (Life Technologies, Grand Island, NY, USA).
5. 37 °C water shaking water bath.
6. 12 mL outer tubes.
7. 2 mL inner tubes.
8. 0.5 × 1.5 cm cut filter paper.
9. 2.5 × 2.5 cm cut parafilm squares.
10. 50 mM potassium phosphate buffer, pH 8.0.
11. 200 mM L-cysteine.
12. 80 mM pyridoxal 5′-phosphate.
13. β-cyano-L-alanine (BCA).
14. *O*-(carboxymethyl)hydroxylamine hemihydrochloride (CHH).
15. 1% zinc acetate.
16. 50% trichloroacetic acid (TCA).
17. *N*,*N*-dimethyl-*p*-phenylenediamine sulfate, 20 mM in 7.2 M HCl.
18. $FeCl_3$, 30 mM in 1.2 M HCl.

19. Sodium hydrosulfide (NaHS).
20. Deionized water.

3 Methods

3.1 Analysis of CBS and CSE mRNAs

3.1.1 RNA Extraction

1. Finely mince ~1 cm length of snap-frozen artery tissues (~100 mg) in 600 μL ice-cold TRIzol reagent (Life Technologies, Carlsbad, CA, USA, #15596) with fine microdissecting scissors (Fisher Scientific, Hampton, NH) in a 1.7 mL microcentrifuge tube (Fisher) on ice (*see* Note 1).
2. Remove tissue debris by benchtop centrifugation at 12,000 × *g* for 1 min; transfer supernatant to a fresh RNase-free tube (Fisher).
3. Proceed to RNA purification using Direct-zol RNA Mini Prep (Zymo Research, Irvine, CA, USA, R2052), following manufacturer's protocol and elute purified RNA in 30 μL of nuclease-free water.
4. Dilute 2 μL (1:100) of RNA in 198 of nuclease-free water to quantify RNA by $OD_{260/280}$.

3.1.2 cDNA Synthesis: Reverse Transcription

1. Add 1 μg purified RNA, 1 μL random primers (Promega, Madison, WI, USA), and nuclease-free water up to 14 μL in RNase-free microcentrifuge tube.
2. Heat the tube for 5 min at 70 °C on a thermocycler to melt template secondary structure.
3. Immediately return on ice to prevent template secondary structure from reforming.
4. Add 4 μL of 5× RT reaction buffer (Promega), 1 μL of 10 mM dNTPs (Promega), and 200 units (1 μL) of Moloney Murine Leukemia Virus Reverse Transcriptase (Promega) for 20 μL total reaction volume. Gently mix.
5. Incubate the tube for 1 h at 37 °C.
6. Return to ice and dilute cDNA template with 80 μL of nuclease-free water (final volume = 100 μL).

3.1.3 qPCR

1. Prepare 10 μM stocks for each set of primers of CBS, CSE, and L-19 (Table 1).
2. Prepare qPCR master mix. For each reaction, mix 7.5 μL of 2 × RT^2 SYBR Green/ROX qPCR master mix (Life Technologies, #4367660), 3.6 μL nuclease-free H_2O, and 0.45 μL of 10 μM primer sets for each target.

3. Add 3 μL of cDNA template into a well of an optical 96-well qPCR plate (Life Technologies, 4346906). Run each sample in triplicate. Seal the plate with PCR optical adhesive film.
4. Quick-spin plate to sediment the cDNA-master mix reaction to the bottom of the well.
5. StepOnePlus real-time PCR system (Applied Biosystems, #4376592). Set up computer to run plate with SYBR Green for quantitative comparative ΔΔCt.
6. Assign targets for appropriate primer pair (CBS, CSE, or L19) and sample designation.
7. Set up the following real-time thermal cycler program: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s.
8. Program the StepOnePlus real-time PCR system to turn on optics to detect SYBR green signal after the 72 °C extension step of each cycle.
9. Set the StepOnePlus real-time PCR system to run the following melting curve program immediately after completion of the above program: 95 °C for 15 s, 60 °C for 1 min (optics off), and 60 °C to 95 °C at 2 °C per min (optics on).
10. Calculate by subtracting the average L19 Ct for a triplicate from each Ct for CBS or CSE of that sample and average in Microsoft excel.
11. Calculate the $2^{-\Delta\Delta Ct}$ relative mRNA levels of each triplicate measurement for each sample by taking the log base −2 of each Ct (*see* Note 2).
12. Average the triplicate and calculate fold changes vs. the average of the control group (Fig. 1).

3.2 Immunoblot Analysis of CBS and CSE Proteins

3.2.1 Protein Extraction and Sample Preparation

1. Finely mince ~1.5 cm length of artery tissues in 500 μL ice-cold nondenaturing lysis buffer [24] with protease inhibitor cocktail (Fisher) with fine microdissecting scissors in a 1.7 mL microcentrifuge tube on ice (*see* Note 3).
2. Sonicate samples for 30 s.
3. Spin samples in a benchtop microcentrifuge at 12,000 RPM (~13,500 × *g*) at 4 °C for 10 min.
4. Transfer supernatant to a fresh microcentrifuge tube and repeat **step 3**.
5. Determine protein concentration of lysates using the Pierce BCA Protein Assay Kit (Life Technologies) following the manufacturers protocol.
6. Mix the protein samples with 5 SDS-PAGE sample buffer [250 mM Tris–HCl pH 6.8, 10% (w/v) SDS, 30% (v/v) glycerol, 0.02% (w/v) bromophenol blue,

5% (v/v) β-mercaptoethanol] and use 1× sample buffer to bring up the protein concentration to 1 μg/μL. Boil the samples for 5 min at 95 °C on a heat block.

7. Quick spin samples and keep on ice until use or store at −20 °C for later use.

3.2.2 SDS-PAGE and Transfer

1. Load 20 μg (e.g., 20 μL of 1 μg/μL) of protein samples into the wells of a 10% SDS-PAGE mini-gel; load 5 μL of pre-stained polypeptide marker (Thermo Scientific, Waltham, MA, USA, 26616) into the first well and 2.5 μL in the last well (*see* Note 4).
2. Secure the gel in an vertical mini-gel system (C.B.S. Scientific, San Diego, CA, USA, #MGV-102) and initiate electrophoresis at 80 V until the blue dye in the samples have passed the interface of the stacking/resolving gels, and then run at 120 V until the blue dye in the samples has nearly ran to the bottom of the gel. Check occasionally and add running buffer to top reservoir if needed.
3. Remove gel from glass plates and trim the stacking gel and any unnecessary gel (*see* Note 5).
4. Soak gel in transfer buffer (25 mM Tris 190 mM glycine 20% methanol, pH 7.5) for 15 min on a benchtop rocker.
5. Activate a nitrocellulose membrane (Thermo Scientific, #88518) with methanol, and incubate in transfer buffer for 15 min with gentle rocking.
6. In a large trey with transfer buffer, sandwich the inverted gel with a nitrocellulose membrane on top with two filter papers. Complete the sandwich with two additional filter papers. Avoid any air bubbles in the sandwich.
7. Moisten the surface of the top and bottom plates of a semidry transfer system (Thermo Scientific, #HEP-3) with transfer buffer.
8. Place sandwich on semidry transfer system and carefully cover with the top plate. Lock and tighten the system evenly.
9. Run transfer at 200 mA per sandwich for 1.5 h.
10. Open the semidry transfer system. Remove top filter paper. Remove and invert the membrane and then orient it by cutting the top right corner. Soak the membrane in TBS buffer (20 mM Tris, 150 mM NaCl, pH 7.5) (*see* Note 6).

3.2.3 Immunoblotting

1. Gently remove gel, invert, and stain with Coomassie blue if desired (*see* Note 7).

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4. To help designate the order of the samples, load more protein marker (5 μL) on the left side of the gel and less volume (2.5 μL) on the right side.
5. Cutting the right corner translates further designates the samples on the gel. The cut right side of the gel corresponds to the lesser volume of protein ladder loaded.
6. Cutting the right corner translates the order and designation of your samples on the gel to the blot. The cut right side of the gel corresponds to cut right side of the membrane.
7. Follow any standard Coomassie blue destain protocol to confirm proteins in the gel, and quantify if desired.

2. Transfer membrane to 5% BSA in TBS-T (TBS + 0.1% Tween-20) blocking solution for 1 h.
3. Seal the membrane in a plastic pouch with primary antibodies diluted in 2 mL of 5% BSA in TBS-T. Store at 4 °C overnight with agitation.
4. Rabbit ant-CBS polyclonal antibody (Abcam ab131155, 1:200 for 0.2 μg/mL final concentration). CBS will be detected at approximately 61 kDa. Mouse anti-CSE monoclonal antibody (Santa Cruz S-374249, 1:500 for 0.4 μg/mL final concentration). CSE will be detected at approximately 45 kDa.
5. Wash the membrane with TBS-T for 3 × 5 min on a rocker.
6. Incubate the blot with HRP-linked anti-rabbit IgG (Cell Signaling, Beverly, MA, USA #7074) for CBS or HRP-linked anti-mouse IgG (Cell Signaling #7076) at 1:1000 in TBS-T for 1 h at room temperature.
7. Wash the membrane with TBS-T for 3 × 5 min on a rocker.
8. Incubate the membrane with the SuperSignal® West Femto Maximum Sensitivity Substrate (Fisher #34095); typically less than 1 mL is needed per blot.
9. Visualize and capture chemiluminescence signals in Alpha Innotech ChemiImager Imaging System. Save the images at 8-bit Tagged Image File Format (TIFF) files for analysis.
10. Strip the membrane by incubation in a mild stripping buffer (0.1% SDS, 0.15% glycine, 1% Tween20, pH 2.2) at 50 °C for 45 min with some agitation.
11. Re-probe the membrane with rabbit anti-β-actin (Ambion AM4302, 1:10,000 for 0.1 μg/mL final concentration) at 4 °C overnight with agitation. β-Actin will be detected at approximately 42 kDa.
12. Wash the membrane with TBS-T for 3 × 5 min on a rocker.
13. Incubate the blot with HRP-linked anti-rabbit IgG (Cell Signaling #7074, 1:1000).
14. Repeat **steps 7–9** to detect β-actin signals and save the images.
15. Use NIH Image J to conduct densitometry analysis of CBS, CSE, and β-actin.
16. Normalize each CBS/CSE band to corresponding β-actin signals (Fig. 2).

3.3 Semiquantitative Immunofluorescence Microscopy

3.3.1 Immunofluorescence Microscopy

1. Fix 2 cm freshly collected artery rings in 4% paraformaldehyde overnight and embedded in paraffin, and then cut 5 μm sections and place on glass slides.
2. Place slides in a slide rack and de-paraffinize sections in 100% xylene 2 × 10 min.
3. Hydrate sections in graded ethanol (100%, 95%, 70%), each grade for 2 × 5 min.

4. Wash slides 1 × 5 min in diH_2O and then 1 × 5 min in 1× PBS.

5. Antigen retrieval: incubate slides with pre-warmed (37 °C) 0.05% Trypsin (Fisher # 25300054) at RT for 30 min, and then wash slide in deionized water (diH_2O) for 5 min.

6. Incubate the slides in 300 mM glycine in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.45 μM filtered; *see* Note 8) for 3 × 20 min at RT to quench autofluorescence.

7. Block non-specific proteins in PBS blocking solution containing 0.01% gelatin, 1% BSA, and 0.001% saponin at RT for 1 h.

8. Incubate the slides with anti-CD31 antibody (Dako #M0823, 1:40) in antibody dilution buffer (50% blocking solution and 50% 1 PBS) in humidifying chamber at 4 °C overnight (*see* Note 9).

9. Wash slides 3 × 5 min in 1× PBS.

10. Incubate the slides with Alexa^{568}-labeled goat anti-mouse IgG (Fisher A-11004, 1:1000) in humidifying chamber at RT for 1 h in dark (*see* Note 10).

11. Wash the slides 3 × 20 min in 1× PBS. Keep slides in dark (*see* Note 11).

12. Repeat **step 7**.

13. Incubate the slides with anti-CBS or CSE antibody (1 μg/mL) in antibody dilution buffer in humidifying chamber at 4 °C overnight.

14. Wash slides 3 × 5 min in 1× PBS.

15. Incubate the slides with Alexa^{488} donkey anti-rabbit IgG (Thermo Scientific # A-21206 1:1000 for CBS) or Alexa^{488}-labeled goat anti-mouse IgG (Thermo Scientific # A-11001, 1:1000 for CSE) in humidifying chamber at RT for 1 h in dark.

16. Repeat **step 11**.

17. Mount the slides with ProLong Gold Antifade Mountant with DAPI (Fisher P36930), and keep the slides overnight at RT in dark.

18. Visualize the slides with a fluorescence microscopy (20×) and capture the images in TIFF files (Fig. 3).

3.3.2 Image Analysis

1. Open image in Simple PCI Image analysis software (Hamamatsu Corp, Sewickley, PA, USA).

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8. 300 mM Glycine-PBS should be filtered prior to use to remove residual glycine crystal and other debris that may interfere later with image acquisition.

9. Make a humidifying chamber by lining the bottom of a flat container with wet paper towels, such as an empty slide box with a lid. If the container does not have a lid, carefully cover with plastic wrap to prevent slides from drying during long incubations.

10. It is best to protect fluorescent secondary antibodies from direct light. Close the lid of the humidifying chamber or cover with foil while the sections incubate with antibodies.

11. Cover slides with foil during all washes after this point to protect sections bound with fluorescent secondary antibodies.

2. Calibrate image; we use 20× with 1.5× magnification settings.
3. Right click image and select “measurement ROI.”
4. Select ROI tool and draw ROI on specific cell structures or cell types being assessed.
5. Select “measurements”; choose “area” and “mean green.”
6. Choose “measure to spreadsheet.”
7. Copy and paste the data to Excel.
8. Subtract average background fluorescence (from negative control images) from all images analyzed.
9. Report florescence as RFI/μm^2 (fold of control).

3.4 H_2S Activity Assay

3.4.1 Methylene Blue Assay

1. Finely mince artery segment (1–1.5 cm in length, ~100 mg) in ice-cold 50 mM potassium phosphate buffer, pH 8.0, with fine microdissecting scissors in 1.7 mL microcentrifuge tube on ice (*see* Note 3).
2. Sonicate samples for 30 s.
3. Spin samples in a benchtop microcentrifuge at 12,000 RPM for 10 min at 4 °C.
4. Transfer supernatant to a fresh microcentrifuge tube and repeat **step 3**.
5. Determine protein concentration of lysates using the Pierce BCA Protein Assay Kit.
6. Add the following reagents to a 12 mL outer tube:
   a. Protein lysate (at least 40 μg protein) in 0.925 mL ice-cold potassium phosphate buffer.
   b. 50 μL of L-cysteine (200 mM) to get 10 mM final concentration.
   c. 25 μL of pyridoxal 5′-phosphate (80 mM) to get 2 mM final concentration.
7. Add *O*-(carboxymethyl)hydroxylamine hemihydrochloride (CHH, Sigma Aldrich #C13408) and/or β-cyano-L-alanine (BCA, Caymen Chemical, Ann Arbor, MI, USA, #10010947) for a final concentration of 2 mM to designated reaction tubes.
8. Soak a piece of filter paper (0.5 × 1.5 cm) with 0.3 mL of 1% zinc acetate in a 2 mL inner tube, and put the uncapped tube in each 12 mL outer tube.
9. Flush the tube with a slow stream of nitrogen gas (N_2) for 20 s and then seal with double layer of parafilm.
10. Transfer tubes to a 37 °C shaking water bath and incubate for 90 min.

11. Inject 0.5 mL of 50% trichloroacetic acid into the reaction mixture through the parafilm.
12. Incubate the tube in the shaking water bath for 60 min to complete the trapping of H_2S by the 1% zinc acetate solution to form zinc sulfide.
13. Remove the parafilm and add 50 μL of *N,N*-dimethyl-*p*-phenylenediamine sulfate (20 mM in 7.2 M HCl) and 50 μL of $FeCl_3$ (30 mM in 1.2 M HCl) to the inner 2 mL tube.
14. Measure OD_{630} after 20 min incubation at RT.

3.4.2 Calibration Curve for Each Measurement

1. Freshly prepare sodium hydrosulfide (NaHS) stock solution (1 M) by dissolving 280 mg of NaHS into 5 mL of diH_2O.
2. Serial dilute NaHS stock solution into the following concentrations: 0, 7.8, 31.25, 62.5, 125, 250, 500, and 1000 μM (*see* Note 12).
3. Measure H_2S level as stated in 6.1 simultaneously.
4. Generate a H_2S calibration curve based on OD_{630}.
5. Use the calibration curve (Fig. 4) to determine H_2S in all unknown samples.

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References

1. Wang R (2012) Physiological implications of hydrogen sulfide: a whiff exploration that blossomed. Physiol Rev 92:791–896 [PubMed: 22535897]
2. Gadalla MM, Snyder SH (2010) Hydrogen sulfide as a gasotransmitter. J Neurochem 113:14–26 [PubMed: 20067586]
3. Mustafa AK, Gadalla MM, Sen N, Kim S, Mu W, Gazi SK, Barrow RK, Yang G, Wang R, Snyder SH (2009) H2S signals through protein S-sulfhydration. Sci Signal 2:ra72 [PubMed: 19903941]
4. Leffler CW, Parfenova H, Basuroy S, Jaggar JH, Umstot ES, Fedinec AL (2011) Hydrogen sulfide and cerebral microvascular tone in newborn pigs. Am J Physiol Heart Circ Physiol 300:H440–H447 [PubMed: 21131483]
5. Bhatia M (2005) Hydrogen sulfide as a vasodilator. IUBMB Life 57:603–606 [PubMed: 16203678]
6. Shibuya N, Mikami Y, Kimura Y, Nagahara N, Kimura H (2009) Vascular endothelium expresses 3-mercaptopyruvate sulfurtransferase and produces hydrogen sulfide. J Biochem 146:623–626 [PubMed: 19605461]
7. Zhao W, Zhang J, Lu Y, Wang R (2001) The vasorelaxant effect of H(2)S as a novel endogenous gaseous K(ATP) channel opener. EMBO J 20:6008–6016 [PubMed: 11689441]
8. Li Y, Zang Y, Fu S, Zhang H, Gao L, Li J (2012) H2S relaxes vas deferens smooth muscle by modulating the large conductance Ca2+ –activated K+ (BKCa) channels via a redox mechanism. J Sex Med 9:2806–2813 [PubMed: 22906137]

12. Actual H_2S concentration is taken as 30% of the NaHS concentration in the calculation (0, 2.3, 9.38, 18.75, 37.5, 75, 150, 300 μM).

9. Papapetropoulos A, Pyriochou A, Altaany Z, Yang G, Marazioti A, Zhou Z, Jeschke MG, Branski LK, Herndon DN, Wang R, Szabo C (2009) Hydrogen sulfide is an endogenous stimulator of angiogenesis. Proc Natl Acad Sci U S A 106:21972–21977 [PubMed: 19955410]
10. Yang G, Wu L, Jiang B, Yang W, Qi J, Cao K, Meng Q, Mustafa AK, Mu W, Zhang S, Snyder SH, Wang R (2008) H2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine gamma-lyase. Science 322:587–590 [PubMed: 18948540]
11. Magness RR, Rosenfeld CR (1986) Systemic and uterine responses to alpha-adrenergic stimulation in pregnant and nonpregnant ewes. Am J Obstet Gynecol 155:897–904 [PubMed: 3766646]
12. Magness RR, Rosenfeld CR (1989) Local and systemic estradiol-17 beta: effects on uterine and systemic vasodilation. Am J Phys 256:E536–E542
13. Rosenfeld CR, Chen C, Roy T, Liu X (2003) Estrogen selectively up-regulates eNOS and nNOS in reproductive arteries by transcriptional mechanisms. J Soc Gynecol Investig 10:205–215
14. Naden RP, Rosenfeld CR (1985) Systemic and uterine responsiveness to angiotensin II and norepinephrine in estrogen-treated nonpregnant sheep. Am J Obstet Gynecol 153:417–425 [PubMed: 4050915]
15. Killam AP, Rosenfeld CR, Battaglia FC, Makowski EL, Meschia G (1973) Effect of estrogens on the uterine blood flow of oophorectomized ewes. Am J Obstet Gynecol 115:1045–1052 [PubMed: 4734886]
16. Magness RR, Phernetton TM, Gibson TC, Chen DB (2005) Uterine blood flow responses to ICI 182 780 in ovariectomized oestradiol-17beta-treated, intact follicular and pregnant sheep. J Physiol 565:71–83 [PubMed: 15774510]
17. Ford SP (1982) Control of uterine and ovarian blood flow throughout the estrous cycle and pregnancy of ewes, sows and cows. J Anim Sci 55(Suppl 2):32–42 [PubMed: 6765316]
18. Gibson TC, Phernetton TM, Wiltbank MC, Magness RR (2004) Development and use of an ovarian synchronization model to study the effects of endogenous estrogen and nitric oxide on uterine blood flow during ovarian cycles in sheep. Biol Reprod 70:1886–1894 [PubMed: 14985241]
19. Nelson SH, Steinsland OS, Suresh MS, Lee NM (1998) Pregnancy augments nitric oxide-dependent dilator response to acetylcholine in the human uterine artery. Hum Reprod 13:1361–1367 [PubMed: 9647573]
20. Lang U, Baker RS, Braems G, Zygmunt M, Kunzel W, Clark KE (2003) Uterine blood flow—a determinant of fetal growth. Eur J Obstet Gynecol Reprod Biol 110(Suppl 1):S55–S61 [PubMed: 12965091]
21. Lechuga TJ, Zhang H, Sheibani L, Karim M, Jia J, Magness RR, Rosenfeld CR, Chen DB (2015) Estrogen replacement therapy in ovariectomized nonpregnant ewes stimulates uterine artery hydrogen sulfide biosynthesis by selectively upregulating cystathionine beta synthase expression. Endocrinology 156:2288–2298 [PubMed: 25825818]
22. O'Leary P, Boyne P, Flett P, Beilby J, James I (1991) Longitudinal assessment of changes in reproductive hormones during normal pregnancy. Clin Chem 37:667–672 [PubMed: 1827758]
23. Sheibani L, Lechuga TJ, Zhang H, Hameed A, Wing DA, Kumar S, Rosenfeld CR, Chen DB (2017) Augmented H2S production via cystathionine-beta-synthase upregulationplays a role in pregnancy-associated uterine vasodilation. Biol Reprod 96:664–672. [PubMed: 28339573]
24. Chen DB, Westfall SD, Fong HW, Roberson MS, Davis JS (1998) Prostaglandin F2alpha stimulates the Raf/MEK1/mitogen-activated protein kinase signaling cascade in bovine luteal cells. Endocrinology 139:3876–3885 [PubMed: 9724043]

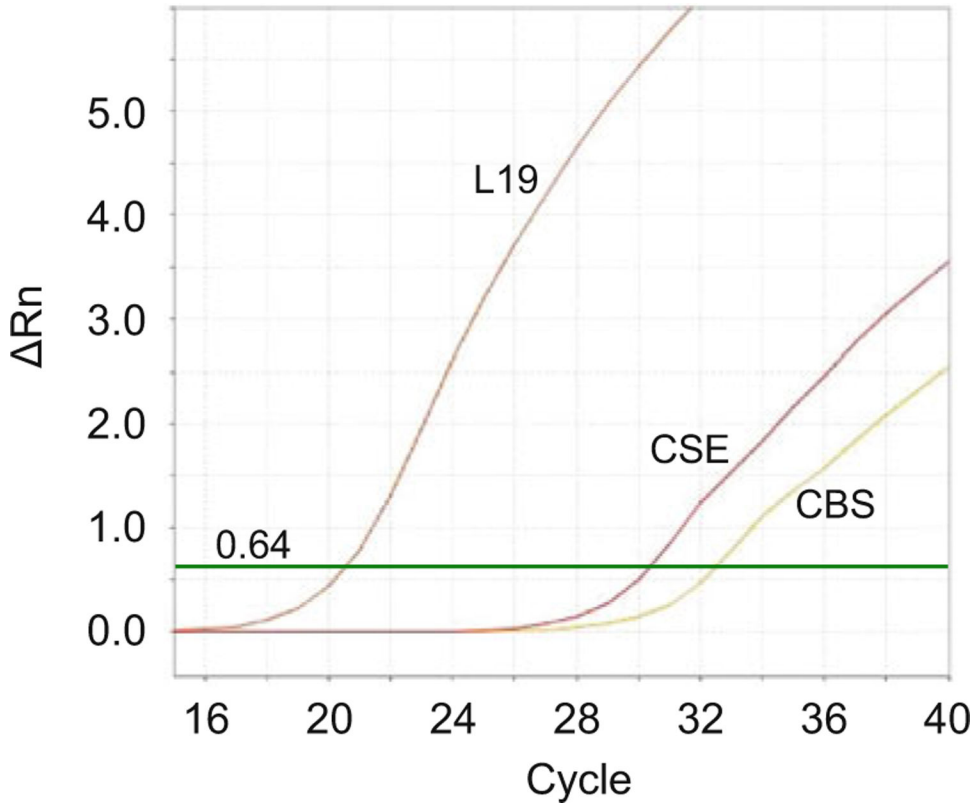

Fig. 1.

qPCR amplification plots for CBS, CSE, and L19 in arteries. Basal levels of CBS, CSE, and L19 mRNAs were determined by real-time qPCR in uterine (UA), mesenteric (MA), and carotid (CA) arteries. A typical mRNA amplification plot for each mRNA is shown. Ct value was determined by the default Ct threshold (0.64) using a StepOnePlus real-time PCR system (Applied Biosystems) and accompanied software. The average Ct value for CBS was approximately 33, 30 for CSE, and 33 for L19

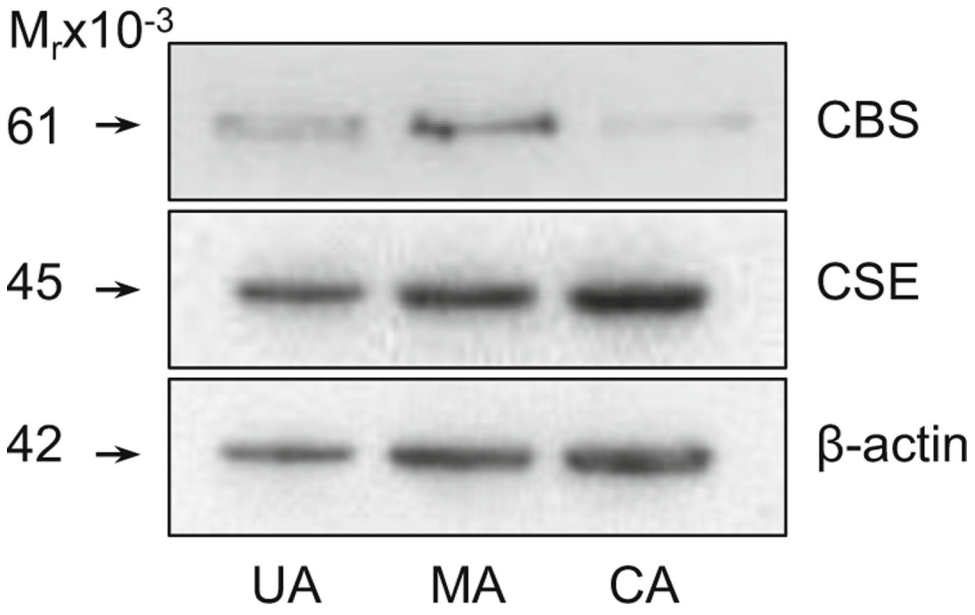

Fig. 2.

Immunoblots of CBS, CSE, and β-actin in arteries. CBS, CSE, and β-actin proteins were determined by immunoblotting in uterine (UA), mesenteric (MA), and carotid (CA) arteries. 20 μg of lysates were ran on a 10% acrylamide gel. The antibodies detected a band of approximately 61 kDa for CBS, 45 kDa for CSE, and 42 kDa for β-actin

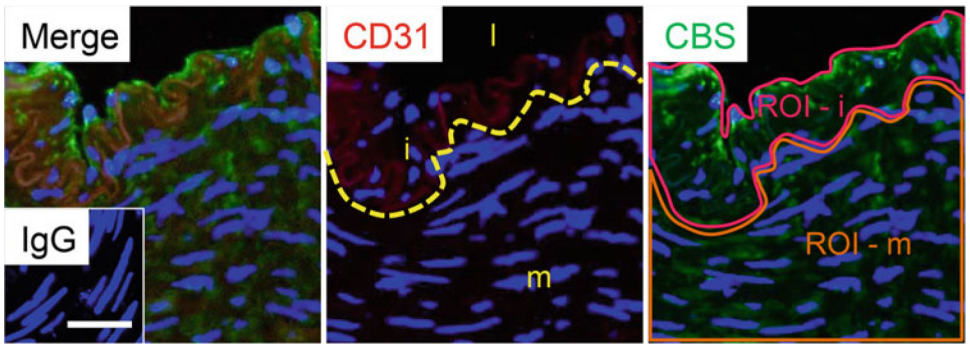

Fig. 3.

Semiquantitative assessment and localization of CBS and CSE. UA sections were labeled with antibodies against CBS, or CSE (not shown), followed by secondary Alexa^{488} (green)-labeled secondary antibody. Endothelial cells were labeled with CD31 followed by Alexa^{568} (red)-labeled secondary antibody. Cell nuclei were stained with DAPI. Negative control treated with IgG or with primary antibody omitted is shown in first panel. Representative outline of border between UA intima and media was indicated, and UA lumen (l), intima (i), and media (m) were denoted (second panel). Regions of interest for intima (ROI – i) and media (ROI – m) used for analysis in Simple PCI software are illustrated (third panel). Scale bar is 25 μm

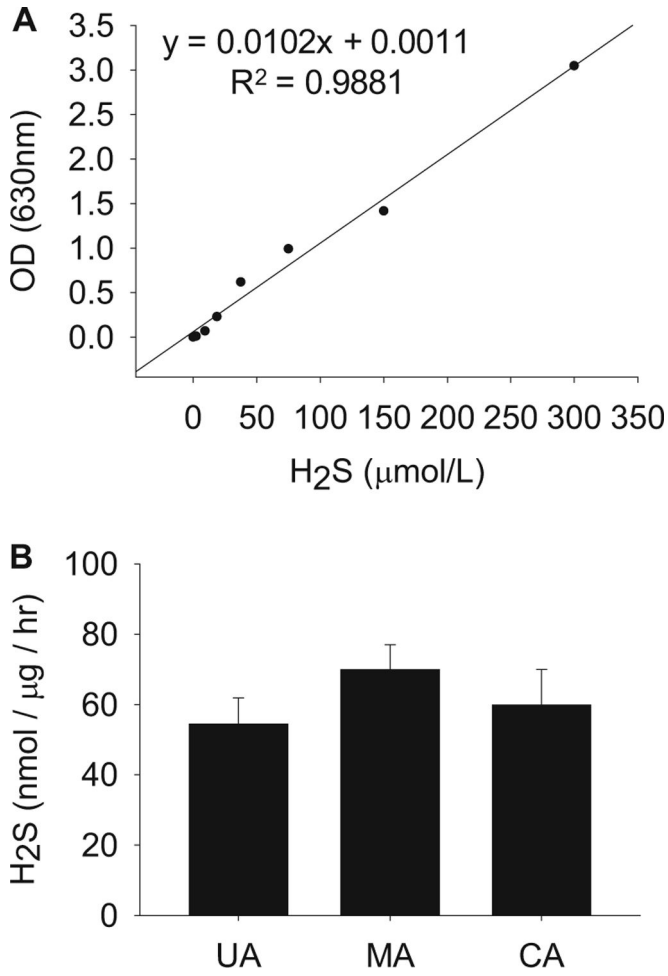

Fig. 4.

Methylene blue assay to assess H_2S production in arteries. (**a**) Example of assay standard curve generated by serial dilutions of NaHS is shown. The linear equation formed by the curve should be used to determine the H_2S levels with the OD values produced from the unknown samples. (**b**) Basal levels of H_2S were determined by using the methylene blue assay in uterine (UA), mesenteric (MA), and carotid (CA) arteries

Table 1

Primers used for qPCR analysis of CBS and CSE mRNAs

Gene	Forward	Reverse	Amplicon size
CBS	5′-TGAGATTGTGAGGACGCCCAC-3′	5′-TCACACTGCTGCAGGATCTC-3′	177 bp
CSE	5′-TTGTATGGATGATGTGTATGGAAGG-3′	5′-CCAAACAAGCTTGGTTTCTGGTG-3′	141 bp
L-19	5′-AGACCCCAATGAGACCAATG-3′	5′-GTGTTTTTCCGGCATCGAGC-3′	129 bp