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MPST but not CSE is the primary regulator of hydrogen sulfide production and function in the coronary artery

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[1]*Department of Biomedical Engineering, Johns Hopkins University, Baltimore, Maryland;* [2]*Department of Anesthesiology and Critical Care Medicine, Johns Hopkins University, Baltimore, Maryland;* [3]*Department of Medicine (Cardiology), Johns Hopkins University, Baltimore, Maryland; and* [4]*Department of Anethesiology and Pain Medicine, Ajou University School of Medicine, Suwon, Korea*

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Kuo MM, Kim DH, Jandu S, Bergman Y, Tan S, Wang H, Pandey DR, Abraham TP, Shoukas AA, Berkowitz DE, Santhanam L. MPST but not CSE is the primary regulator of hydrogen sulfide production and function in the coronary artery. *Am J Physiol Heart Circ Physiol* 310: H71–H79, 2016. First published October 30, 2015; doi:10.1152/ajpheart.00574.2014.—Hydrogen sulfide (H_2S) has emerged as an important gasotransmitter in the vasculature. In this study, we tested the hypothesis that H_2S contributes to coronary vasoregulation and evaluated the physiological relevance of two sources of H_2S, namely, cystathionine-γ-lyase (CSE) and 3-mercaptypyruvate sulfertransferase (MPST). MPST was detected in human coronary artery endothelial cells as well as rat and mouse coronary artery; CSE was not detected in the coronary vasculature. Rat coronary artery homogenates produced H_2S through the MPST pathway but not the CSE pathway in vitro. In vivo coronary vasorelaxation response was similar in CSE knockout mice, wild-type mice (WT), and WT mice treated with the CSE inhibitor propargylglycine, suggesting that CSE-produced H_2S does not have a significant role in coronary vasoregulation in vivo. Ex vivo, the MPST substrate 3-mercaptopyruvate (3-MP) and H_2S donor sodium hydrosulfide (NaHS) elicited similar coronary vasoreactivity responses. Pyruvate did not have any effects on vasoreactivity. The vasoactive effect of H_2S appeared to be nitric oxide (NO) dependent: H_2S induced coronary vasoconstriction in the presence of NO and vasorelaxation in its absence. Maximal endothelial-dependent relaxation was intact after 3-MP and NaHS induced an increase in preconstriction tone, suggesting that endothelial NO synthase activity was not significantly inhibited. In vitro, H_2S reacted with NO, which may, in part explain the vasoconstrictive effects of 3-MP and NaHS. Taken together, these data show that MPST rather than CSE generates H_2S in coronary artery, mediating its effects through direct modulation of NO. This has important implications for H_2S-based therapy in healthy and diseased coronary arteries.

hydrogen sulfide; coronary vasoregulation; coronary tone; cystathionine-γ-lyase; 3-mercaptopyruvate sulfurtransferase

NEW & NOTEWORTHY

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BECAUSE OXYGEN EXTRACTION in the myocardium is maximal, increased metabolic demand can only be matched by increasing coronary blood flow. The impaired vasodilatory response of the coronary vasculature results in insufficient supply of blood and oxygen, leading to myocardial ischemia, as manifested in patients with coronary artery disease (30). Therefore, identifying and characterizing mechanisms underlying coronary tone regulation are of clinical relevance.

Coronary tone is regulated locally by the endothelium predominantly through vasodilatory mediators nitric oxide (NO) and endothelial-derived hyperpolarizing factors (EDHFs). NO is produced by endothelial nitric oxide synthase (eNOS) and has been demonstrated to regulate resting coronary tone (11, 23) and metabolic demand-mediated vasodilation (21). Of the EDHFs, hydrogen peroxide (H_2O_2) has been the most widely researched. H_2O_2 has similarly been shown to be involved in coronary autoregulation (27), metabolic demand-mediated vasodilation (28) and flow-mediated vasodilation (3).

Another EDHF, hydrogen sulfide (H_2S), has recently emerged as another important gasotransmitter in the vasculature. H_2S is produced from L-cysteine by cystathionine-γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (MPST) (22). CSE converts L-cysteine into H_2S with pyridoxal-5′-phosphate (PLP) as a cofactor. L-cysteine-dependent production of H_2S by MPST is a two-step reaction. First, cysteine aminotransferase converts L-cysteine along with α-ketoglutarate into 3-mercaptopyruvate (3-MP). MPST then converts 3-MP into H_2S (20). CSE appears to have an important role in vascular tone regulation as mice lacking CSE are hypertensive and have attenuated endothelial-dependent vasodilation (29).

Vasoactive effects of H_2S vary by vascular bed. In the mesenteric bed, H_2S is a vasodilator (7), while in the pulmonary bed, it is a vasoconstrictor (17). In the aorta, the vasoactive effects of H_2S depends on dose (14): H_2S is a vasoconstrictor at low doses and a vasodilator at high doses. In the coronary vasculature, H_2S has been reported to be a vasodilator (5, 6). Exogenous H_2S induces coronary vasodilation independent of the endothelium through 4-aminopyridine-sensitive K_v, BK_{Ca}, and K_{ATP} channels (4, 5, 6, 8). The role of CSE as a producer of endogenous H_2S has also been investigated, although conclusions regarding its contribution are inconsistent. Some studies support little contribution of CSE in coronary vasoregulation (4, 6) while others support involvement (5, 8).

The role of MPST in coronary vasoregulation has not been examined. Moreover, the current literature on H_2S involvement in coronary vasoregulation lacks direct measurement of H_2S production in the coronary artery. In this study, we hypothesized that MPST is a source of endogenous H_2S in the coronary

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vasculature. We measured the protein abundance of the two H_2S-producing enzymes and measured in vitro H_2S production through both pathways. We next investigated the role of CSE-derived H_2S in vivo by measuring in vivo coronary vasorelaxation function in CSE inhibited and CSE knockout models. We then investigated the role of MPST-derived H_2S by characterizing the vasoactive effects of MPST substrate 3-MP ex vivo.

MATERIALS AND METHODS

Animals. All animal use was approved by the Animal Care and Use Committee at Johns Hopkins University. In vivo coronary vasoreactivity experiments were performed in male, 12-to 18-wk-old CSE-deficient ($CSE^{-/-}$) and Bl6/129S (Jackson) wild-type mice. Male 250- to 300-g Sprague-Dawley rats (Harlan) were used for ex vivo experiments.

Cell culture. Human coronary artery (HCAECs; Lonza), pulmonary artery (HPAECs; Invitrogen), and aortic endothelial cells (HAECs; Lonza) were cultured in endothelial basal medium supplemented with 5% fetal bovine serum, endothelial cell growth supplements, and penicillin/streptomycin (Lonza).

Protein expression. Protein expression was determined in human endothelial cells and rat vascular tissue by Western blot. Rat aorta and left coronary artery were harvested for analysis. Cells were lysed and tissue was homogenized in 1× radioimmunoprecipitation assay (RIPA) buffer (Upstate) containing protease inhibitors (Roche). Antibodies used were rabbit-polyclonal anti-MPST antibody (1:1,000; Atlas Antibody) and rabbit-polyclonal anti-CSE antibody (1:1,000; ProteinTech). Mouse-monoclonal anti-GAPDH antibody was used as loading control (1:5,000; Novus Biologicals). Horseradish peroxidase-conjugated donkey anti-rabbit or sheep anti-mouse secondary antibody (1:5,000; GE Healthcare) was used for detection. Blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Rat liver homogenates were used as positive controls.

In vitro amperometric H_2S measurement. Tissue H_2S production was measured by amperometry using the Apollo 4000 Free Radical Analyzer detector (WPI) and a 3-mm H_2S-selective electrode (WPI). Reactions were performed as previously described (22). For MPST-mediated H_2S production, rat liver and coronary tissue samples were homogenized in cold 10× PBS buffer pH 7.4 containing 1 mM dithiothrietol (DTT; New England BioLabs) and protease inhibitor (Roche). Total reaction volume was 200 μl and total protein amount used was 300 μg for liver and 30 μg for coronary. Homogenate was incubated with MPST substrate 3-MP (Sigma) for 50 min at 37°C in 2-ml gas-tight vials (Fisher). Liver homogenate was incubated with the listed 3-MP concentrations. Coronary homogenate was incubated with 1 mM 3-MP. After 50 min of incubation, 200 μl of 10× PBS adjusted to pH 6.0 were added to the reaction to promote release of H_2S and to stop the reaction. The reaction was incubated at 37°C for another 10 min. Two milliliters of headspace gas for liver samples and 3 ml of headspace gas for coronary samples were withdrawn from the gas-tight vial using a 10-ml syringe (BD) with a 22-G needle (BD) attached and injected into a scintillation vial containing 15 ml of 10× PBS (ThermoScientific) in which the amperometric probe was equilibrating. The amount of H_2S produced by the reaction was measured by the probe in units of pA. Data were recorded 2 min after the headspace gas was injected.

For CSE-mediated H_2S production, the tissue was homogenized in 10× PBS buffer containing protease inhibitor and incubated with 2 mM pyridoxal-5′-phosphate (PLP; Sigma) and L-cysteine (Sigma) (16). Liver homogenate was incubated with listed L-cysteine concentrations. Coronary homogenate was incubated with 50 mM L-cysteine. Reaction incubation and measurement procedures were performed as described.

Experiments were performed in triplicate. H_2S produced from liver homogenate was measured as the difference in amperometric signal between the liver homogenate incubated with the indicated substrate concentration and baseline signal of the homogenate with no substrate. H_2S produced by the coronary tissue was calculated as the difference between the substrate incubated with coronary homogenate and baseline signal of the substrate incubated with no protein homogenate. A calibration curve for the amperometric probe was generated using the H_2S donor sodium hydrosulfide (NaHS) and assuming that one-third of NaHS is in soluble H_2S form (24) to equate pA units to H_2S concentration.

To examine the interaction of H_2S with NO, increasing concentrations of the no donor sodium nitroprusside (SNP) were added to NaHS (10^{-3} M) and allowed to react for 1 min. Headspace gas was analyzed as above. In another set of experiments, increasing concentrations of the NO donor *S*-nitrosoglutathione (GSNO) were incubated with NaHS (10^{-3} M) for 1 h followed by headspace sampling as above. Glutathione (GSH) was used as a control to evaluate the effects of GSNO.

In vitro fluorometric H_2S measurement. Fluorometric measurement of H_2S was performed using 7-azido-4-methylcoumarin (AzMC; Sigma). Reactions were carried out in 1× PBS (Invitrogen) in a black 96-well plate with total reaction volume of 100 μl. To determine NO-H_2S reactivity, increasing concentration of NO donor SNP was added to 10^{-3} M NaHS and allowed to react for 1 min. Fifty micromoles of AzMC were then added and incubated for 5 min at room temperature. Fluorescence intensity was measured by SpectraMax Gemini EM plate reader (Molecular Devices) at 365-nm excitation and 450-nm emission. Reactivity of AzMC to H_2S was confirmed by adding 50 μM AzMC to increasing NaHS concentrations, as listed.

In vivo coronary vasoreactivity. Coronary tone was evaluated in vivo by measuring coronary flow velocity using transthoracic echocardiography as previously described (26). Mice were anesthetized with 1.5% isoflurane and coronary flow velocity was measured using the Vevo 2100 (Visual Sonics) and a 40 MHz transducer (Visual Sonics). The transducer was mounted on a stand (Visual Sonics) to measure flow velocity from the same location for the entire experiment. Chest hair was removed by depilatory cream. The animal was secured onto a heated platform to monitor ECG, heart rate, and body temperature.

The left main coronary artery was visualized by color Doppler flow from a modified long axis view of the left ventricle. Flow velocity profile of the blood flow through the coronary artery was captured by pulse wave velocity. The difference in angle between blood flow and ultrasound beam was accounted for with angle correction done on the Vevo mainframe.

Coronary vasorelaxation was induced by intravenous transfusion of adenosine triphosphate (ATP; Sigma) or the β-adrenergic agonist dobutamine (Dob; Hospira). ATP was administered to evaluate agonist-induced coronary vasorelaxation and dobutamine to evaluate metabolic demand-mediated vasorelaxation. Increase in diameter corresponded with an increase in flow velocity. Drugs were infused through a catheter inserted into the lateral tail vein. Catheters were custom-made by attaching a 30-G needle (BD) to size PE-10 polyethylene tubing (BD). The dose of drug administered was controlled with a syringe pump (Harvard Apparatus) by adjusting infusion rate.

Working concentration of ATP was 0.5 mg/ml. ATP was infused at 20, 40, 80, and 160 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 1 min/dose. Coronary flow velocity was allowed to return to baseline between each dose. Working concentration of dobutamine was 0.2 mg/ml. Dobutamine was infused at 5, 10, 20, and 30 $\mu g \cdot kg^{-1} \cdot min^{-1}$ at 2 min per dose. The CSE inhibitor propargylglycine (PPG; Sigma) dissolved in saline was administered intravenously at 50 mg/kg 30 min before flow velocity measurements. All drugs were prepared in a heparinized 0.9% saline solution. Peak diastolic flow velocities of three cardiac cycles were measured for data analysis.

Wire myography. Rats were euthanized by isoflurane overdose, and the left main and descending coronary artery was dissected and sectioned into ~1-mm rings. Precise vessel section length was measured and vessel sections were mounted onto a wire myograph (DMT). Vessels were bathed in 95%-5% O_2-CO_2 oxygenated Krebs physiological solution containing the following (in mM): 118 NaCl, 4.7 KCl, 25.0 $NaHCO_3$, 1.2 $MgSO_4$, 1.1 KH_2PO_4, 11 glucose, and 2.5 $CaCl_2$-$2H_2O$ maintained at 37°C. Vessels were equilibrated for 30 min and then stretched in 0.3-mN increments to the tension equivalent to 50 mmHg (6.7 kPa), as determined by the DMT normalization module for LabChart. After being stretched, vessels were equilibrated for 15 min, and then subjected to two rounds of 60 mM KCl bolus administration into the bath. Vessels were preconstricted at 10^{-7} M or $10^{-6.5}$ M U46619 (Cayman) for 15 min. Vessels were then subjected to cumulative dose response of NaHS (10^{-5} M, 10^{-4} M, and 10^{-3} M; Sigma) or 3-MP (10^{-6} M, 10^{-5} M, 10^{-4} M; Sigma).

To test the involvement of arachidonic acid, rings were next incubated with 10 μM phospholipase A2 inhibitor 4-4(-octadecylphenyl)-4-oxobutenoic acid (OBAA; Tocris) for 30 min prior to U46619 preconstriction, and NaHS and 3-MP dose responses were carried out. Rings were then incubated with 100 μM N^G-nitro-L-arginine methyl ester (L-NAME; Caymen) for 15 min before U46619 preconstriction to test the involvement of NOS-dependent NO. In a separate experiment, coronary artery rings were mechanically denuded to examine the role of the endothelium in the H_2S- and 3-MP-mediated response.

Acetylcholine (ACh; Sigma) 10^{-5} M was added after NaHS 10^{-3} M and 3-MP 10^{-4} M to determine if endothelial-mediated vasorelaxation function remained intact. In separate challenges, vessels were preconstricted with U46619, and after steady constriction was reached, 100 μM L-NAME were added. After a steady L-NAME-induced constriction was reached, ACh 10^{-5} M was added to evaluate endothelial-mediated vasorelaxation after NOS inhibition.

Data analysis and statistics. Statistical significance was determined by two-way analysis of variance with Bonferroni posttest or Student's *t*-test (GraphPad). Data are reported as means ± SE.

RESULTS

CSE and MPST expression in cells and tissue. CSE expression varied between human endothelial cell lines: expression was robust in HAECs and HPAECs and minimal in HCAECs in comparison (Fig. 1*A*). MPST and eNOS abundances were in similar in all the cell types. Rat liver was used as a positive control.

In rats (Fig. 1*B*) MPST was expressed in similar abundance in coronary artery and aorta. In mice, MPST expression was more robust in the coronary artery compared with the aorta (Fig. 1*C*). CSE expression was more robust in aortic tissue in both rats and mice compared with the coronary artery (Fig. 1, *B* and *C*). GAPDH was used as the loading control.

MPST- and CSE-mediated H_2S production in tissue and cells. Enzymatic kinetics of MPST and CSE were characterized in rat liver homogenate, where both enzymes are robustly expressed (Fig. 1*A*) (10, 19). The K_m value of MPST for 3-MP in the reaction conditions was 0.46 ± 0.08 mM 3-MP and V_{max} was 254.1 ± 9.6 nM H_2S/h (Fig. 2*A*). For the CSE reaction with L-cysteine as substrate, K_m was 21.38 ± 4.31 mM L-cysteine, and V_{max} was 1,777 nM H_2S/h (Fig. 2*B*).

To measure enzyme activity, the coronary artery homogenate was incubated with 1 mM 3-MP or 50 mM L-cysteine. H_2S produced in these conditions was 32.4 ± 6.1 nM for 3-MP incubation and 3.6 ± 6.5 nM for L-cysteine incubation. MPST-mediated production was significantly higher than CSE-mediated production (Fig. 2*C*; $P = 0.0056$).

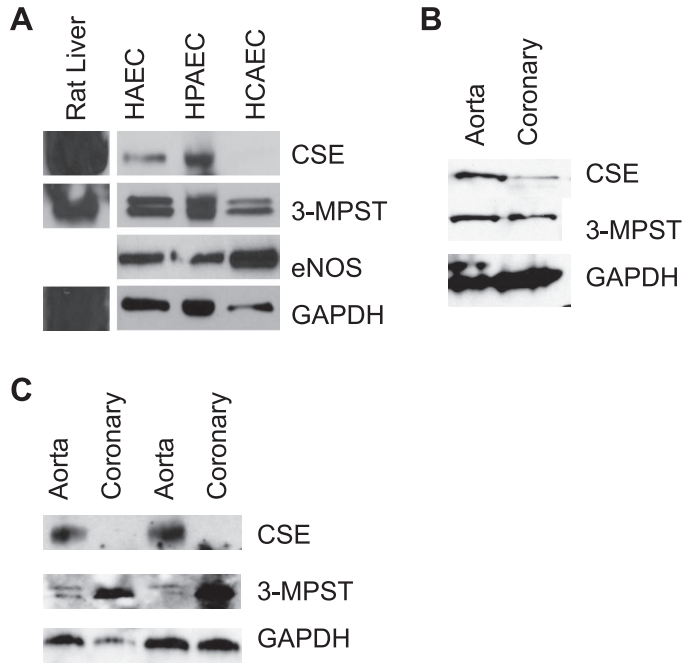

Fig. 1. Cystathionine-γ-lyase (CSE) and 3-mercaptypyruvate sulfertransferase (MPST) expression in cells and tissue. Protein expression was measured in human endothelial cells from the aorta (HAECs), pulmonary artery (HPAECs), and coronary artery (HCAECs) (*A*; with rat liver used as positive control) and aorta and coronary artery from mice (*B*) and coronary artery and aorta from rat (*C*) (with GAPDH used as loading control).

Similarly, in HCAECs, 3-MPST-dependent H_2S production (1.38 ± 0.12 nM) was significantly higher than CSE-mediated production (0.25 ± 0.1 nM; $P = 0.0017$; Fig. 2*D*).

Role of CSE-derived H_2S in coronary vasorelaxation in vivo. ATP-mediated vasorelaxation (Fig. 3*A*) was not different among WT, WT treated with PPG, and $CSE^{-/-}$ mice ($P = 0.33$ for WT vs. PPG treated and $P = 0.31$ for WT vs. $CSE^{-/-}$). Metabolic demand-mediated vasorelaxation induced by dobutamine (Fig. 3*B*) was also not different between cohorts ($P = 0.66$ for WT vs. PPG-treated and $P = 0.23$ for WT vs. $CSE^{-/-}$).

Vasoactive effects of exogenous H_2S and 3-MP. 3-MP caused dose-dependent vasoconstriction in coronary rings (Fig. 4*A*): 10^{-6} M decreased preconstriction tone by 3.6 ± 2.2% while 10^{-5} M significantly increased preconstriction tone by 19.7 ± 9.5% ($P = 0.022$ compared with 3-MP 10^{-6} M), and 10^{-4} M increased preconstriction tone by 68.6 ± 26%. ($P = 0.059$ compared with 3-MP 10^{-5} M). Pyruvate did not have any effects on vasoconstriction (Fig. 5*E*). The vasoconstrictive response to 3-MP was not significantly altered following incubation with the PLA2 inhibitor OBAA ($P = 0.29$ OBAA vs. untreated) but was dramatically reduced following NOS inhibition with L-NAME. A dose of 10^{-4} M 3-MP produced an 8.2 ± 4.3% increase in preconstriction tone with L-NAME incubation compared with 68.6 ± 26.2% increase in tone in untreated vessels ($P = 0.026$). Removal of the endothelium similarly abolished vasoconstrictive effect of 3-MP and unmasked its vasodilatory effect: 10^{-4} M produced a 7.9 ± 6.5% decrease in tone in denuded vessels compared with the 68.6 ± 26% increase in tone in endothelial-intact vessels ($P = 0.034$).

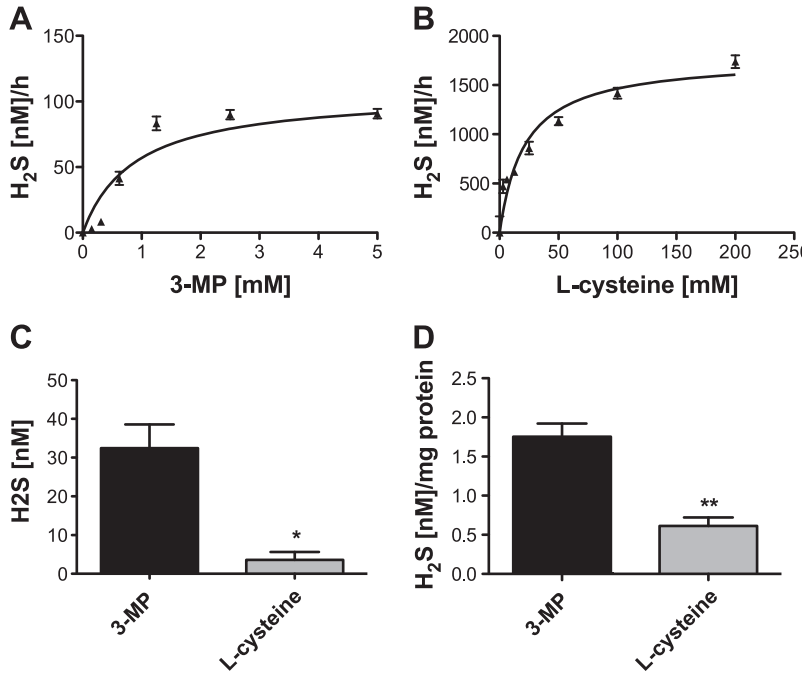

Fig. 2. In vitro amperometric measurement of tissue H_2S production. Michaelis-Menten kinetics of MPST (*A*) and CSE (*B*) were characterized in rat liver homogenate. For coronary H_2S production, rat coronary homogenate was incubated with 1 mM -mercaptopyruvate (3-MP) to measure MPST-mediated H_2S production or with 50 mM L-cysteine to measure CSE-mediated H_2S production (*C*). For cellular H_2S production, lysates of HCAEC were incubated with 3-MP (1 mM) to measure MPST-mediated H_2S production or L-cysteine (50 mM) to measure CSE-mediated H_2S production (*D*). Statistical significance was evaluated by Students' *t*-test (*$P < 0.05$; **$P < 0.01$).

NaHS induced a dose-dependent vasoconstriction in the coronary arteries (Fig. 4*B*): a 4.9 ± 3.7% increase in preconstriction tone occurred with 10^{-5} M, 18.3 ± 4.7% increase occurred with 10^{-4} M ($P = 0.029$ vs. 10^{-5} M), and 218.5 ± 52.0% occurred with 10^{-3} M ($P = 0.0025$ vs. NaHS 10^{-4} M). Following PLA2 inhibition with OBAA, NaHS also produced dose-dependent vasoconstriction but to a lesser degree: 10^{-5} M produced a 95.9 ± 30.9% increase in preconstriction tone with OBAA incubation compared with 218.0 ± 52.0% increase in preconstriction tone in untreated vessels ($P = 0.039$). Following L-NAME incubation, NaHS induced dose-dependent vasodilation: 10^{-5} M NaHS increased preconstriction tone by 2.8 ± 0.9% while 10^{-4} M NaHS decreased preconstriction tone by 10.0 ± 7.5% (P = ns compared with NaHS 10^{-5} M). 10^{-3} M NaHS further decreased tone to 76.9 ± 12.9% of preconstriction tone ($P = 0.001$ compared with NaHS 10^{-4} M). In endothelial-denuded coronary arteries, NaHS also had little vasoconstrictive effect at 10^{-5} M and 10^{-4} M and induced vasodilation at 10^{-3} M: 10^{-3} M NaHS reduced preconstriction tone by 76.5 ± 7.9% in denuded vessels compared with 218.5 ± 52.0% increase in tone in endothelial-intact vessels ($P = 0.00081$).

Representative traces of 3-MP dose responses are shown in Fig. 5 for untreated (Fig. 5*A*) and with OBAA incubation (Fig. 5*B*), L-NAME incubation (Fig. 5*C*), and endothelial denuding (Fig. 5*D*). Representative trace of pyruvate dose response is shown in Fig. 5*E*. Representative traces of NaHS dose responses are shown in Fig. 6 for untreated (Fig. 6*A*), with OBAA incubation (Fig. 6*B*), L-NAME incubation (Fig. 6*C*), and endothelial denuding (Fig. 6*D*).

NO-H_2S interaction. Since enhanced preconstriction tone could be explained by decreased NO bioavailability, we tested whether H_2S had a direct effect on NOS activity and NO production. Maximum endothelial-mediated vasorelax-

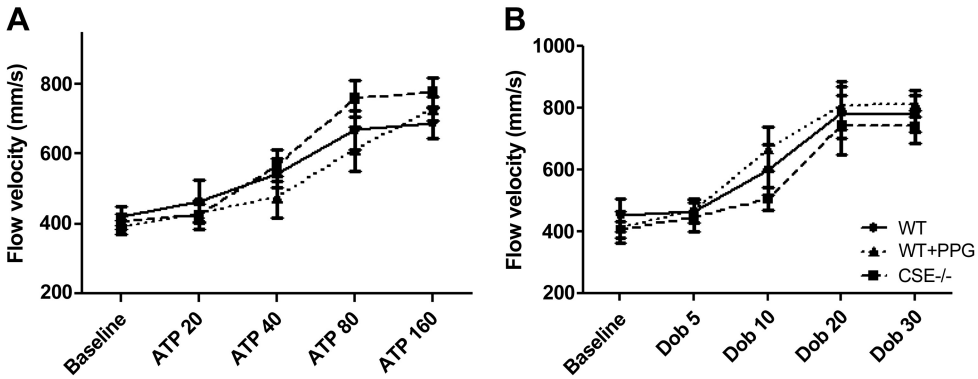

Fig. 3. In vivo coronary vasorelaxation in CSE knockout and pharmacologically-inhibited mice. Coronary vasorelaxation function was evaluated in vivo by measuring increase in flow velocity using high-resolution ultrasound. Dose-dependent increases in flow velocity to ATP (*A*), representing agonist-induced vasorelaxation, and dobutamine (*B*), representing metabolic demand-induced vasorelaxation, were obtained. CSE activity was inhibited by intravenous administration of 50 mg/kg propargylglycine (PPG) 30 min before flow velocity measurement. ATP-induced vasorelaxation response was not different among wild-type (WT), PPG-treated wild-type, and CSE knockout mice (*A*). Vasorelaxation response to dobutamine-induced increase in cardiac metabolic demand was also not different between cohorts (*B*).

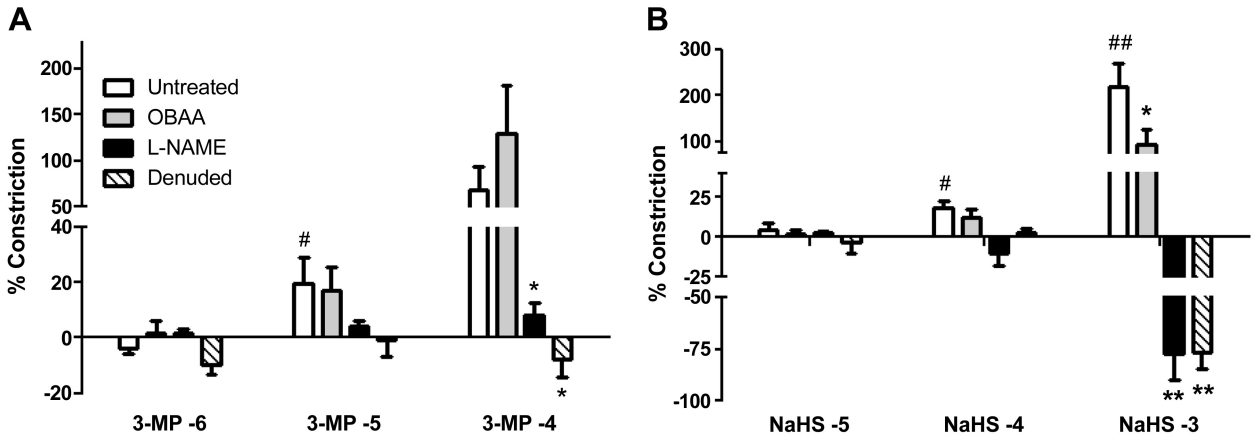

Fig. 4. Vasoactive effects of 3-MP and NaHS. Physiological role of MPST in coronary vasoregulation was determined by wire myography in the rat left coronary artery. Data are displayed as percent change from preconstriction tone. 3-MP induced vasoconstriction in preconstricted arteries in a dose-dependent manner (*A*). Incubation with 10 μM phospholipase 2A inhibitor 4-4(-octadecylphenyl)-4-oxobutenoic acid (OBAA) did not alter 3-MP vasoconstrictive effects. 100 μM N^G-nitro-L-arginine methyle ester (L-NAME) incubation significantly reduced 3-MP-mediated vasoconstriction. Endothelial removal resulted in 3-MP-mediated vasodilation. Exogenous H_2S donor NaHS also induced vasoconstriction in preconstricted arteries in a dose-dependent manner (*B*). OBAA incubation attenuated NaHS-induced vasoconstriction. L-NAME and endothelial denuding removed NaHS-induced vasoconstriction and only presented NaHS-induced vasodilation. #$P < 0.05$ and ##$P < 0.01$, comparing between previous dose within the same treatment group; *$P < 0.05$ and **$P < 0.01$, when comparing treated groups with untreated within a dose.

ation with 10^{-5} M ACh was not different between vessels preconstricted only with U46619 and vessels that exhibited increased preconstriction tension from the addition of 10^{-3} M NaHS or 10^{-4} M 3-MP (Fig. 7*A*). ACh-induced vasorelaxation was 86.0 ± 2.4% for U46619 only preconstricted vessels compared with 81.0 ± 7.2% relaxation for vessels with enhanced preconstriction tone from NaHS and 72.7 ± 8.6% relaxation for vessels with increased preconstriction tone from 3-MP. ACh-induced vasorelaxation was also significantly reduced with L-NAME incubation compared with untreated vessels: vasorelaxation was 20 ± 5% with L-NAME incubation compared with 86.0 ± 2.4% in untreated U46619 only vessels. We next tested whether NO could directly interact with H_2S. Fluorescence intensity increased with increasing dose of NaHS, demonstrating that AzMC reacted with NaHS (Fig. 7*B*). Increasing SNP concentration caused decreasing fluorescence intensity and therefore H_2S availability (Fig. 7*C*). In a complementary approach, the NO-H_2S interaction was examined amperometrically. In these experiments, increasing doses of SNP as well as GSNO caused decreased H_2S availability, while GSH did not affect H_2S (Fig. 7*D*).

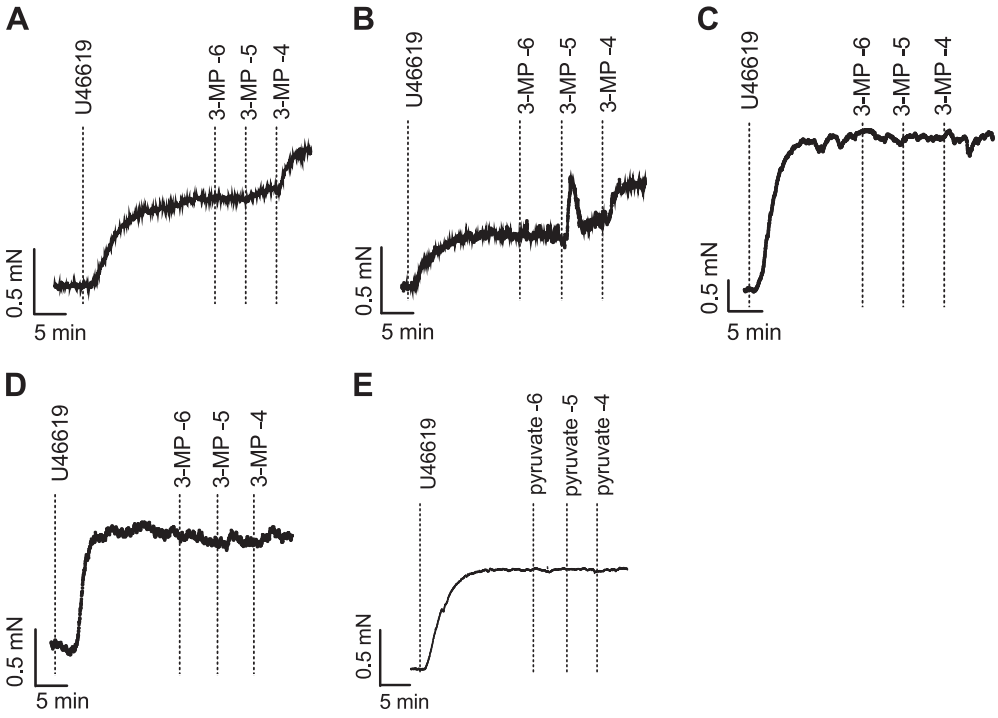

Fig. 5. 3-MP dose response traces. Representative vasotension traces of 3-MP dose response in untreated coronary artery (*A*), after incubation with 10 μM OBAA (*B*), 100 μM L-NAME (*C*), and after endothelial denudation (*D*). Pyruvate (*E*) was used as a control for 3-MP.

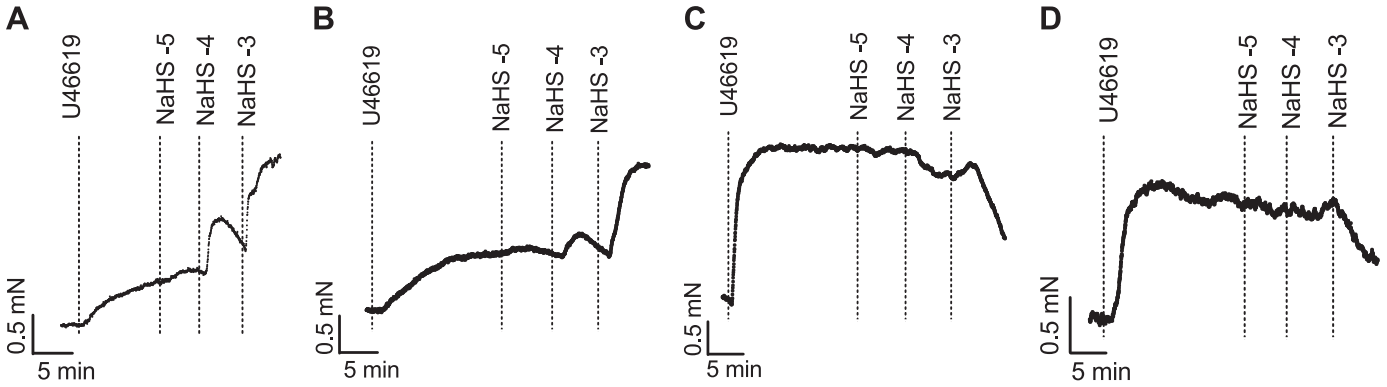

Fig. 6. NaHS dose response traces. Representative vasotension traces of NaHS in untreated coronary artery (*A*), after OBAA incubation (*B*), after L-NAME incubation (*C*), and after endothelial denudation (*D*).

DISCUSSION

We investigated the role of H_2S and the contribution of vascular H_2S producers CSE and MPST in coronary tone vasoregulation. The new findings of this study are as follows: *1*) CSE is not a significant contributor to coronary vasoregulation; the data shown here provide evidence that supports the involvement of MPST in coronary vasoregulation; *2*) in HCAECs and in rat and mouse coronary arteries, MPST is the predominant source of H_2S; and *3*) this study supports the role of H_2S as a coronary vasoconstrictor when NO bioavailability is physiological and vasodilator in the absence of NO.

Protein abundance measurement by Western blot showed that CSE and MPST are expressed in human endothelial cells and rat and mouse vascular tissue, although the relative expression between vascular beds varies between the species. CSE expression was robust in the aorta compared with the coronary artery in rats and mice. In contrast, MPST expression was similar in the aorta and coronary in rats and MPST

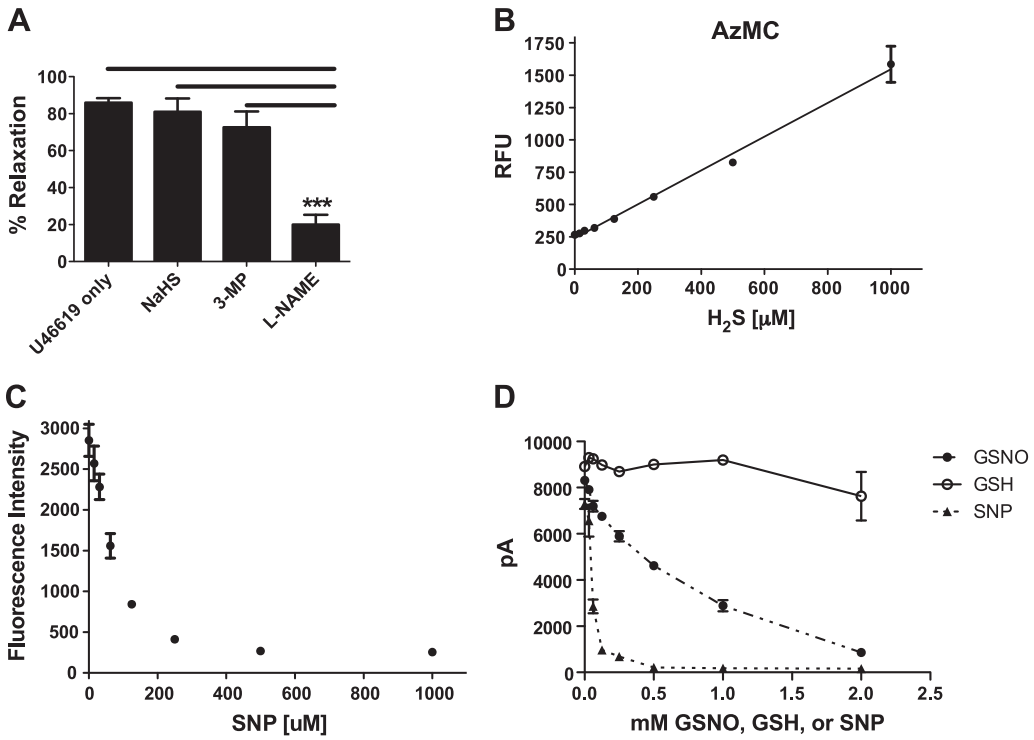

Fig. 7. H_2S-mediated vasoconstriction via reduced endothelial nitric oxide synthase (eNOS) activity and decreased nitric oxide (NO) bioavailability. eNOS activity was measured by percent relaxation with addition of 10^{-5} M acetylcholine (ACh) bolus after maximum increase in preconstriction tension induced by NaHS, 3-MP, or L-NAME (*A*). ACh relaxation response was compared with vessels preconstricted with U46619 only. Maximum ACh-induced endothelial-mediated relaxation was not different among U46619 only and NaHS or 3-MP constricted vessels; L-NAME significantly attenuated ACh-induced relaxation. Statistical significance was evaluated with 1-way ANOVA, followed by Tukey's post hoc analysis (***$P < 0.001$). H_2S effect on NO bioavailability was measured in vitro by fluorometric probe 7-azido methylcoumarin (AzMC). Fluorescence intensity of AzCM increased with H_2S dose (*B*). Addition of increasing dose of NO donor sodium nitroprusside (SNP) to 10^{-3} M NaHS resulted in a dose-dependent decrease in fluorescence intensity (*C*). H_2S-NO interaction was monitored amperometrically. Addition of increasing doses of the NO donors *S*-nitrosoglutathione (GSNO) or SNP resulted in a dose-dependent decrease in H_2S in the headspace of an air-tight vial as measured amperometrically. Glutathione (GSH) did not affect H_2S concentration (*D*).

expression was much higher in the mouse coronary artery compared with the aorta. The differences in the relative abundance of the proteins in the conduit arteries and arterioles could be due to the distinct properties and functions of these vessels. Conduit vessels are highly compliant and offer little resistance to flow. The arterioles on the other hand are resistance vessels and regulate organ perfusion and blood pressure by vasoconstriction and relaxation in response to various stimuli. The presence of H_2S-producing enzymes can provide an alternative pathway to NO-dependent vasoregulation and thus organ perfusion in conditions where NO bioavailability is compromised.

Alternatively, these differences could be due to differences in vascular health. CSE and MPST expression have been shown to be influenced by pathological conditions. In a mouse heart failure model, mice with heart failure induced by thoracic aortic constriction had elevated CSE expression and reduced MPST expression in the cardiac tissue (13). While the rats from which the tissue was harvested can be considered healthy, we did not ascertain the vascular health status of the donors of the human cell lines. Nevertheless, the expression data demonstrate that MPST is expressed in the coronary artery and support the possibility of endogenous H_2S production in the coronary vasculature.

The Michaelis-Menten kinetics of CSE and MPST were examined in vitro using rat liver homogenate. For 3-MP, the K_m value was 0.46 ± 0.14 mM 3-MP. For CSE, the K_m value was 21.38 ± 4.31 mM L-cysteine, a concentration far exceeding physiologically relevant L-cysteine values. The previously reported K_m value is 1.2 ± 0.1 mM 3-MP for wild-type rat liver-derived MPST (19), which is comparable to our findings. On the other hand, for CSE the previously reported K_m value is 1.9 mM L-cysteine for wild-type human CSE (9). Differences in reported values can be attributed to the use of a liver homogenate in our study compared with purified enzyme preparations in previous studies. In the homogenate, other proteins may compete for both PLP cofactor and L-cysteine substrate, markedly shifting the observed K_m for CSE. Moreover, in the previous studies, both reactions were performed at basic pH, pH 9.55 for the MPST reaction and pH 8.2 for the CSE reaction. The reactions in this study were performed at pH 7.4, which is also the pH at which the vasoreactivity studies were performed. The difference in reaction pH may also account for the discrepancy in the observed K_m value. The K_m value of human CSE for L-cysteine has also been shown to vary significantly with polymorphic variants (31).

In vitro amperometric H_2S measurement in rat coronary artery homogenate and HCAEC lysates showed that H_2S produced through the MPST pathway was significantly greater than through the CSE pathway. These reactions were performed at 2-mM 3-MP or 50-mM L-cysteine, concentrations greater than twofold the observed K_m value in this study, to ensure maximal (specific) activity of the enzymes. The higher expression of the 3-MPST in the coronary artery coincides with higher rates of 3-MP-dependent H_2S production in coronary artery homogenates, further supporting the idea that 3-MPST is the primary source of H_2S in the coronary vasculature.

The in vivo coronary vasorelaxation responses in CSE-deficient and CSE-inhibited mice were not different from untreated wild-type mice. In conjunction with the lack of CSE-mediated H_2S production in vitro, these in vivo data further support the notion that CSE-derived H_2S does not significantly contribute to coronary vasoregulation. Our findings are confirmed by previous studies that reported lack of coronary vasodilation upon addition of L-cysteine, suggesting minimal involvement of CSE in coronary tone modulation (4, 6). The insignificant role of CSE-produced H_2S in the coronary vasculature seems counterintuitive given the significant role of CSE-produced H_2S in regulating tone of the resistance arteries (29). However, endothelial protein expression and associated phenotypic traits have been shown to vary by vascular bed. For instance, in the heart, endocardial endothelial cells express connexin (CX) 43, CX40, and CX37 while myocardial capillary endothelial cells do not, consistent with the observation that endocardial endothelial cells possess a larger number of gap junctions than myocardial endothelial cells (1). It is therefore not unreasonable that the coronary arteries, which are controlled locally, have different main vasoactive mediators than the resistance arteries, which are controlled neurohumorally.

We demonstrated in vitro that the coronary artery can produce H_2S by the MPST pathway. Because direct MPST inhibitors are currently unavailable and transgenic deletion model was not readily accessible, we determined if MPST was important physiologically using ex vivo wire myography. The vasoactive response to MPST substrate 3-MP was similar to the vasoactive response to H_2S donor NaHS. There was no response to pyruvate, suggesting that the effects of 3-MP are due to H_2S synthesis rather than pyruvate. The vasoconstrictive effects were not significantly altered with PLA2 inhibition but disappeared with NOS inhibition and endothelial denudation. These data support that the effects of 3-MP are mediated through MPST production of H_2S as opposed to direct vascular effects of 3-MP. Although 3-MP-induced vasoconstriction was removed by L-NAME incubation and endothelial removal, 3-MP was not observed to induce vasodilation to the same degree as NaHS. Lack of vasodilatory effects of 3-MP could be due to insufficient substrate amount to produce the amount of H_2S necessary for vasodilation. Coronary vasodilation was observed in this study and has been previously reported to occur at 1-mM NaHS. This concentration of H_2S may not have been possible to produce in the tissue with the 3-MP concentration used.

Interestingly, we observed the vasoconstrictive effects of NaHS in untreated coronary arteries, which contradicts previous studies that reported vasodilatory effects of NaHS (4, 5, 6, 8). We demonstrated, however, that the vasoconstrictive effect of NaHS is eNOS and therefore NO dependent. NOS inhibition with L-NAME yielded no vasoconstriction and only vasodilation. A similar response observed with endothelial denuding identified the involvement of eNOS and eNOS-derived NO. Such NO dependence has also been observed in CO-mediated vasoactivity, where NO plays a "permissive" role in CO-induced dilatation in arteries (15).

Reducing NO bioavailability through eNOS inhibition has been shown to further increase preconstriction tension (18). The enhanced vasoconstriction with addition of NaHS observed in this study could therefore occur through eNOS inhibition or NO scavenging. Both H_2S interference with eNOS activity as well as H_2S reaction with NO have been previously described. H_2S was shown to cause a dose-dependent decrease in activity of recombinant bovine eNOS. The resulting reduction in NO bioavailability was associated with

the observed vasoconstrictive effects of NaHS in rat and mouse aorta (14). Aortic vasoconstriction observed at low NaHS doses was attributed to the vasoconstrictive effects of reduced NO bioavailability overriding the vasodilatory effects of H_2S. Aortic vasodilation observed at high NaHS doses was attributed to the vasodilatory effects of H_2S overriding the vasoconstrictive effects of decreased NO bioavailability. The reported H_2S inhibition of eNOS appears to contradict other studies that demonstrated that exogenous H_2S promoted eNOS function and eNOS production of NO (12), (2). However, the duration of the exogenous H_2S administration was different between the studies. H_2S donor treatment was chronic in the studies showing cooperative effects of H_2S on eNOS function and acute in the study that showed dose-dependent decrease in eNOS activity as well as this study. H_2S has also been shown to react with NO to form HNO or a nitrosothiol, demonstrating the scavenging effect of H_2S. Mixing NaHS with SNP reduced the vasodilatory effects of SNP in preconstricted rat aortic rings, further supporting that H_2S reacted with NO and reduced NO bioavailability through scavenging (2, 25).

Our ex vivo vasoreactivity data showed that endothelial-mediated relaxation was maximal after NaHS-induced vasoconstriction. NOS inhibition with L-NAME significantly attenuated endothelial-mediated relaxation. In addition, 10^{-5} M ACh consistently induced complete relaxation between ring sections and animals for both NaHS and 3-MP, supporting that eNOS activity was truly unimpaired. These ex vivo vasoreactivity data therefore support that H_2S-induced vasoconstriction is not through inhibition of eNOS activity.

Our fluorometric data demonstrated the scavenging interaction of H_2S and NO. Whether the reaction product of H_2S and NO is also a vasoconstrictor was not tested in this study. However, as noted earlier, this reaction product was shown previously to not have vasoactive effects (2, 25). Vasoconstriction observed in this study can therefore be attributed directly to decreased NO bioavailability. Because only the vasodilatory effects of NaHS were observed in the absence of NO, either by L-NAME inhibition or by endothelial denuding, we agree that H_2S can act a vasodilator in the coronary vasculature. We further conclude that its vasoconstrictive effects are due to its scavenging interaction with NO. The NO dependency of the vasoactive effects of H_2S suggests a change in role from regulatory to vasodilatory with development of coronary artery disease. Under healthy conditions with physiological NO bioavailability, H_2S appears to primarily regulate NO. In disease conditions with diminished NO bioavailability, H_2S could serve as a compensatory vasomediator. By extension, these findings support that exogenous H_2S induces vasoconstriction in healthy coronary arteries and vasodilation in diseased coronary arteries. Therapies involving administration of exogenous H_2S would therefore benefit coronary artery disease patients but would be counterproductive as a supplement for patients with healthy coronary arteries.

Limitations of this study. The absence of H_2S measurement in the bath during the wire myograph experiments to confirm that the effects of 3-MP are due to MPST-mediated H_2S production is a limitation of this study. This detection was limited by the sensitivity of the amperometric probe in a noisy environment. Nevertheless, similar effects and mechanism of action between 3-MP and NaHS support the conclusion that 3-MP is acting on the coronary artery through H_2S production by MPST. In vivo H_2S concentrations are estimated to range from nanomolar to low micromolar levels. Thus the concentrations of exogenous H_2S from NaHS used in the vasoreactivity experiments in this study are supraphysiological. While micromolar to millimolar concentrations of 3-MP were used in the bath, it is unclear how much intracellular H_2S this translates to. Thus, the responses described are more likely to be relevant during sulfide-based therapies.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.M.K., D.H.K., D.R.P., T.P.A., A.A.S., D.E.B., and L.S. conception and design of research; M.M.K., D.H.K., S.S.J., Y.B., S.T., H.W., and D.R.P. performed experiments; M.M.K., D.H.K., S.S.J., and L.S. analyzed data; M.M.K., D.H.K., and L.S. interpreted results of experiments; M.M.K., D.H.K., and L.S. prepared figures; M.M.K. and D.H.K. drafted manuscript; M.M.K., D.H.K., D.E.B., and L.S. edited and revised manuscript; M.M.K., D.E.B., and L.S. approved final version of manuscript.

REFERENCES

1. **Aird WC.** Phenotypic heterogeneity of the endothelium: II. Representative vascular beds. *Circ Res* 100: 174–190, 2007.
2. **Ali MY, Ping CY, Mok YY, Ling L, Whiteman M, Bhatia M, Moore PK.** Regulation of vascular nitric oxide in vitro and in vivo; a new role for endogenous hydrogen sulphide? *Br J Pharmacol* 149: 625–634, 2006.
3. **Canty JM, Schwartz JS.** Nitric-oxide mediates flow-dependent epicardial coronary vasodilation to changes in pulse frequency but not mean flow in conscious dogs. *Circulation* 89: 375–384, 1994.
4. **Casalini ED, Goodwill AG, Owen MK, Moberly SP, Berwick ZC, Tune JD.** Contribution of hydrogen sulfide to the control of coronary blood flow. *Microcirculation* 20: 12083, 2013.
5. **Chai Q, Lu T, Wang XL, Lee HC.** Hydrogen sulfide impairs shear stress-induced vasodilation in mouse coronary arteries. *Pflügers Arch* 467: 329–340, 2014.
6. **Cheang WS, Wong WT, Shen B, Lau CW, Tian XY, Tsang SY, Yao XQ, Chen ZY, Huang Y.** 4-Aminopyridine-sensitive K^+ channels contributes to NaHS-induced membrane hyperpolarization and relaxation in the rat coronary artery. *Vascul Pharmacol* 53: 94–98, 2010.
7. **Cheng YQ, Ndisang JF, Tang GH, Cao K, Wang R.** Hydrogen sulfide-induced relaxation of resistance mesenteric artery beds of rats. *Am J Physiol Heart Circ Physiol* 287: H2316–H2323, 2004.
8. **Hedegaard ER, Nielsen BD, Kun A, Hughes AD, Kroigaard C, Mogensen S, Matchkov VV, Frobert O, Simonsen U.** K(V)7 channels are involved in hypoxia-induced vasodilatation of porcine coronary arteries. *Br J Pharmacol* 171: 69–82, 2014.
9. **Huang S, Chua JH, Yew WS, Sivaraman J, Moore PK, Tan CH, Deng LW.** Site-directed mutagenesis on human cystathionine-gamma-lyase reveals insights into the modulation of H2S production. *J Mol Biol* 396: 708–718, 2010.
10. **Ishii I, Akahoshi N, Yu XN, Kobayashi Y, Namekata K, Komaki G, Kimura H.** Murine cystathionine gamma-lyase: complete cDNA and genomic sequences, promoter activity, tissue distribution and developmental expression. *Biochem J* 381: 113–123, 2004.
11. **Jones LF, Brody MJ.** Coronary blood-flow in rats is dependent on the release of vascular nitric-oxide. *J Pharmacol Exp Ther* 260: 627–631, 1992.
12. **King AL, Polhemus DJ, Bhushan S, Otsuka H, Kondo K, Nicholson CK, Bradley JM, Islam KN, Calvert JW, Tao YX, Dugas TR, Kelley EE, Elrod JW, Huang PL, Wang R, Lefer DJ.** Hydrogen sulfide cytoprotective signaling is endothelial nitric oxide synthase-nitric oxide dependent. *Proc Natl Acad Sci USA* 111: 3182–3187, 2014.
13. **Kondo K, Bhushan S, King AL, Prabhu SD, Hamid T, Koenig S, Murohara T, Predmore BL, Gojon G, Gojon G, Wang R, Karusula N, Nicholson CK, Calvert JW, Lefer DJ.** H2S protects against pressure

overload-induced heart failure via upregulation of endothelial nitric oxide synthase. *Circulation* 127: 1116–1127, 2013.
14. **Kubo S, Doe I, Kurokawa Y, Nishikawa H, Kawabata A.** Direct inhibition of endothelial nitric oxide synthase by hydrogen sulfide: contribution to dual modulation of vascular tension. *Toxicology* 232: 138–146, 2007.
15. **Leffler CW, Parfenova H, Jaggar JH.** Carbon monoxide as an endogenous vascular modulator. *Am J Physiol Heart Circ Physiol* 301: H1–H11, 2011.
16. **Li L, Bhatia M, Zhu YZ, Zhu YC, Ramnath RD, Wang ZJ, Anuar FB, Whiteman M, Salto-Tellez M, Moore PK.** Hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. *FASEB J* 19: 1196–1198, 2005.
17. **Madden JA, Ahlf SB, Dantuma MW, Olson KR, Roerig DL.** Precursors and inhibitors of hydrogen sulfide synthesis affect acute hypoxic pulmonary vasoconstriction in the intact lung. *J Appl Physiol* 112: 411–418, 2014.
18. **Moore PK, Alswayeh OA, Chong NW, Evans RA, Gibson A.** L-NG-nitro arginine (L-NOARG), a novel, L-arginine-reversible inhibitor of endothelium-dependent vasodilatation invitro. *Br J Pharmacol* 99: 408–412, 1990.
19. **Nagahara N, Nishino T.** Role of amino acid residues in the active site of rat liver mercaptopyruvate sulfurtransferase–cDNA cloning, overexpression, and site-directed mutagenesis. *J Biol Chem* 271: 27395–27401, 1996.
20. **Olson KR.** The therapeutic potential of hydrogen sulfide: separating hype from hope. *Am J Physiol Regul Integr Comp Physiol* 301: R297–R312, 2011.
21. **Quyyumi AA, Dakak N, Andrews NP, Gilligan DM, Panza JA, Cannon RO.** Contribution of nitric-oxide to metabolic coronary vasodilation in the human heart. *Circulation* 92: 320–326, 1995.
22. **Shibuya N, Mikami Y, Kimura Y, Nagahara N, Kimura H.** Vascular endothelium expresses 3-mercaptopyruvate sulfurtransferase and produces hydrogen sulfide. *J Biochem* 146: 623–626, 2009.
23. **Shiode N, Morishima N, Nakayama K, Yamagata T, Matsuura H, Kajiyama G.** Flow-mediated vasodilation of human epicardial coronary arteries: effect of inhibition of nitric oxide synthesis. *J Am Coll Cardiol* 27: 304–310, 1996.
24. **Wang YF, Zhao X, Jin HF, Wei HL, Li W, Bu DF, Tang XY, Ren Y, Tang CS, Du JB.** Role of hydrogen sulfide in the development of atherosclerotic lesions in apolipoprotein e knockout mice. *Arterioscler Thromb Vasc Biol* 29: 173–179, 2009.
25. **Whiteman M, Li L, Kostetski I, Chu SH, Siau JL, Bhatia M, Moore PK.** Evidence for the formation of a novel nitrosothiol from the gaseous mediators nitric oxide and hydrogen sulphide. *Biochem Biophys Res Commun* 343: 303–310, 2006.
26. **Wikstrom J, Gronros J, Gan LM.** Adenosine induces dilation of epicardial coronary arteries in mice - Relationship between coronary flow velocity reserve and coronary flow reserve in vivo using transthoracic echocardiography. *Ultrasound Med Biol* 34: 1053–1062, 2008.
27. **Yada T, Shimokawa H, Hiramatsu O, Kajita T, Shigeto F, Goto M, Ogasawara Y, Kajiya F.** Hydrogen peroxide, an endogenous endothelium-derived hyperpolarizing factor, plays an important role in coronary autoregulation in vivo. *Circulation* 107: 1040–1045, 2003.
28. **Yada T, Shimokawa H, Hiramatsu O, Shinozaki Y, Mori H, Goto M, Ogasawara Y, Kajiya F.** Important role of endogenous hydrogen peroxide in pacing-induced metabolic coronary vasodilation in dogs in vivo. *J Am Coll Cardiol* 50: 1272–1278, 2007.
29. **Yang GD, Wu LY, Jiang B, Yang W, Qi JS, Cao K, Meng QH, Mustafa AK, Mu WT, Zhang SM, Snyder SH, Wang R.** H(2)S as a physiologic vasorelaxant: Hypertension in mice with deletion of cystathionine gamma-lyase. *Science* 322: 587–590, 2008.
30. **Zeiher AM, Krause T, Schachinger V, Minners J, Moser E.** Impaired endothelium-dependent vasodilation of coronary resistance vessels is associated with exercise-induced myocardial-ischemia. *Circulation* 91: 2345–2352, 1995.
31. **Zhu WD, Lin A, Banerjee R.** Kinetic properties of polymorphic variants and pathogenic mutants in human cystathionine gamma-lyase. *Biochemistry* 47: 6226–6232, 2008.