

Hydrogen sulfide-mediated endothelial function and the interaction with eNOS and PDE5A activity in human internal mammary arteries

Journal of International Medical Research

2019, Vol. 47(8) 3778–3791

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DOI: 10.1177/0300060519847386

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Abstract

Objective: To investigate the role of hydrogen sulfide (H₂S) in human internal mammary arteries (IMA) and its interaction with endothelial nitric oxide synthase (eNOS) and phosphodiesterase (PDE)5A activity.

Methods: Human IMA segments from patients undergoing coronary artery bypass grafting (CABG) were studied by myography for acetylcholine and sodium hydrosulfide (NaHS)-induced relaxation. Locations of 3-mercaptopyruvate sulfurtransferase (3-MPST) and cysteine aminotransferase (CAT) were examined immunohistochemically. Levels of H₂S, eNOS, phosphorylated-eNOS^{ser1177}, and PDE5A were measured.

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Results: In IMA segments from 47 patients, acetylcholine-induced relaxation (resistant to N^G -nitro-L-arginine and indomethacin) was significantly attenuated by aminooxyacetic acid or L-aspartate (CAT inhibitors), iberiotoxin (large-conductance calcium-activated K^+ channel blocker), TRAM-34 plus apamin (intermediate- and small-conductance Ca^{2+} -activated K^+ channel blockers) or glibenclamide (ATP-sensitive K^+ channel blocker). 3-MPST and mitochondrial CAT were found in endothelial and smooth muscle cells while cytosolic CAT was located only in endothelial cells. Acetylcholine significantly increased the H_2S levels. The H_2S donor, NaHS, increased eNOS phosphorylation and down-regulated PDE5A.

Conclusions: Human conduit artery endothelium releases H_2S under basal and stimulated conditions, involving the 3-MPST/CAT pathway, eNOS phosphorylation, PDE5A activity, and potassium channels. These findings may provide new therapeutic targets for treating vasospasm in CABG grafts and facilitate the development of new vasodilator drugs.

Keywords

Endothelium, human internal mammary artery, H_2S , vasorelaxation, coronary artery bypass grafts

Date received: 4 December 2018; accepted: 9 April 2019

Introduction

Hydrogen sulfide (H_2S) plays regulatory roles in the cardiovascular, nervous, and immune systems.¹ In the brain, H_2S is mainly generated by cystathionine β -synthase, while in the cardiovascular system it is mainly produced by cystathionine γ -lyase.^{2,3} Vascular endothelium has been demonstrated to produce H_2S through metabolism by 3-mercaptopyruvate sulfurtransferase (3-MPST) and cysteine aminotransferase (CAT), and in a mouse model, H_2S was shown to be a smooth muscle relaxant released from the endothelium.⁴ Further, 3-MPST produces H_2S from 3-mercaptopyruvate, which is generated by CAT from L-cysteine and α -ketoglutarate in the presence of the cofactors thioredoxin and dihydrolipoic acid.⁵

Cyclic guanosine monophosphate (cGMP) is synthesized by soluble guanylyl cyclases (sGCs) and degraded by phosphodiesterases (PDEs), and plays an important role in nitric oxide (NO)-stimulated vasorelaxation.^{6,7} H_2S and NO are suggested to be mutually required to evoke angiogenesis

and vasodilatation, and although H_2S does not directly activate sGC, it maintains a tonic inhibitory effect on PDE5, thereby delaying the degradation of cGMP.¹ Exposure of endothelial cells to H_2S has been shown to increase intracellular cGMP in a NO-dependent manner, and activate protein kinase G (PKG) and its downstream effector, vasodilator-stimulated phosphoprotein.¹ In addition, oligopeptide DT-2 (a PKG-I inhibitor) was found to reduce the sodium hydrosulfide (NaHS)-induced vasorelaxation in both endothelium-intact or denuded rat aortic rings,⁸ suggesting that H_2S may induce relaxation by stimulating PKG-I.⁸

Hydrogen sulfide has been shown to relax rat aortic tissues via opening of ATP-sensitive K^+ (K_{ATP}) channels in the sarcolemma,^{2,9} leading to a decrease in blood pressure in rats.¹⁰ Moreover, activation of voltage-gated potassium channels may be another key mechanism in the vasorelaxation induced by H_2S ,¹¹ and co-application of Ca^{2+} -activated K^+ (K_{Ca}) channel blockers (apamin and charybdotoxin) has been reported to reduce

H₂S-induced relaxation of endothelium-intact aortic tissues.¹² Therefore, the present authors hypothesized that H₂S may induce vasorelaxation by stimulating different potassium channels. In a recent study of human mesenteric arteries, H₂S-induced relaxation was demonstrated to be endothelium-dependent and to involve mechanisms related to the stimulation of K_{ATP} channels.¹³

To date, published work regarding the release of H₂S from endothelium in the human conduit arteries, and the related mechanism of H₂S-induced relaxation, remains scarce. The present study examined the role of H₂S in human conduit arteries, using internal mammary arteries (IMA), and the possible mechanisms related to a cGMP-dependent pathway and potassium channels.

Patients and methods

Study population and vessel preparation

Fresh human IMA segments were obtained from patients undergoing coronary artery bypass graft (CABG) surgery at TEDA International Cardiovascular Hospital, Chinese Academy of Medical Sciences, Tianjin, China between January 2016 and June 2017. Inclusion criteria comprised elective CABG surgery and patients receiving standard preoperative drug therapy including antiplatelet, antiangina, or anti-hypertensive drugs, as appropriate. Human IMA tissue was obtained whenever appropriate patients became available; thus, no specific study-group allocation procedure was performed and IMA segments were randomly allocated into the different groups. Patients who underwent emergency surgery, or who underwent simultaneous CABG plus other surgery, such as valve replacement, were excluded.

Approval to use the discarded vessels was obtained from the Ethics Committee

of TEDA International Cardiovascular Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College Sciences, and the patients provided written informed consent for their tissue to be included.

Following collection, the vessels were immediately immersed in 4°C Krebs solution and transferred to the laboratory within 5–10 min. The vessels were placed in a glass dish filled with Krebs solution and carefully cleaned of fat and connective tissue. In some IMA ring segments, the endothelium was denuded by gently rubbing the intimal surface with a thin polyethylene tube. In the remaining segments, great care was taken not to touch the inner surface of the blood vessels. This technique allowed the experiments to be performed with functionally intact endothelium, as shown previously,¹⁴ compared with denuded IMA.

Vessels were cut into 3 mm-long rings and mounted in an organ-bath containing Krebs solution (composition as previously described),^{9,15} maintained at 37°C and bubbled continuously with 95% oxygen/5% CO₂. In addition, protein was extracted from some vessels incubated for ≥ 60 min, to perform Western blotting and enzyme-linked immunosorbent assay (ELISA), and other vessels were used to measure H₂S concentration. Vessels harvested from three patients were immediately immersion-fixed in 4% formaldehyde for immunohistochemistry.

H₂S-induced relaxation

Myography. Human IMA ring segments were suspended on wire hooks in a 6 ml bath within a myograph system modified for large vessel studies (model 610M; DMT, Aarhus, Denmark). Each ring segment resting unstretched on the wire hooks was equilibrated in Krebs solution for ≥1 h, as previously described.^{16,17}

For inhibitor experiments, the 3-mm IMA ring segments from the same patient were allocated to different groups, including a control, so that each vascular ring had its own control from the same patient.

Normalization. The IMA ring segments were normalized as previously described.^{16,17} Briefly, each ring segment was stretched in progressive steps every minute to determine the individual length-tension curve. A computer iterative fitting program, Myodaq and Myodata, version 2.01 (Maastricht University, Maastricht, the Netherlands) was used to determine the exponential curve pressure and internal diameter by recording the internal diameter (μm) and corresponding wall tension (mN/mm^2) at the end of each step. Based on length-tension curves, stretching was stopped when the transmural pressure of the rings reached 100 mmHg. The rings were then released and set at 90% of the internal diameter at 100 mmHg.¹⁸ This degree of passive tension was then maintained throughout the experiment.

Precontraction and relaxation. Following equilibration for 45 min in the organ chamber, IMA ring segments were precontracted using a previously determined concentration ($-8 \log \text{M}$)^{15,19} of U-46619 (Cayman Chemical, Ann Arbor, MI, USA). When the U-46619-induced contraction reached a stable plateau, cumulative concentration-relaxation curves were established for acetylcholine (-10 to $-4.5 \log \text{M}$; Sigma-Aldrich, St Louis, MO, USA) and the H_2S donor, NaHS (-11 to $-2.5 \log \text{M}$; Sigma-Aldrich) ($n=8$ for each treatment). IMA relaxation was expressed as the percent reversal of U-46619-induced precontraction. One concentration-relaxation curve was obtained for each IMA ring.

All drugs used in the present study were prepared in distilled water, except for

indomethacin, glibenclamide and TRAM-34, which were dissolved in ethanol.

Procedures and measurements

Effect of CAT inhibition on endogenous H_2S -induced vasorelaxation. To investigate the effect of CAT inhibitors on the acetylcholine response in human IMA, ring segments were divided into three groups and incubated with Krebs solution containing the NO synthase inhibitor, N^G -nitro-L-arginine (L-NNA, $100 \mu\text{mol}/\text{L}$; Sigma-Aldrich) plus the cyclooxygenase inhibitor, indomethacin ($10 \mu\text{mol}/\text{L}$) (control); L-NNA plus indomethacin plus the CAT inhibitor, aminoxyacetic acid (AOAA, $100 \mu\text{mol}/\text{L}$; Sigma-Aldrich); or L-NNA plus indomethacin plus a competitive CAT substrate, L-aspartate ($1000 \mu\text{mol}/\text{L}$; Sigma-Aldrich). After equilibration for 45 min in the organ chamber, ring segments were precontracted with $-8 \log \text{M}$ of U-46619. Acetylcholine (-10 to $-4.5 \log \text{M}$)-induced relaxation curves were then established.

Acetylcholine-induced release of H_2S in IMA.

Levels of H_2S in human IMA tissue were quantified using a methylene blue assay method, as previously described.^{20,21} Human IMA was pre-incubated for 4 h (as determined by pilot experiments) in Krebs solution (control), or in Krebs containing acetylcholine ($1000 \mu\text{mol}/\text{L}$). Tissues were then homogenized in ice-cold phosphate buffered saline (PBS; pH 8.0), and the homogenate was centrifugated at $5\ 200 \times g$ for 10 min at 4°C . Next, $75 \mu\text{l}$ of the supernatant fluid was transferred to a test tube containing $450 \mu\text{l}$ of dH_2O and mixed with $250 \mu\text{l}$ of 1% acetic acid zinc (Amresco [VWR Life Science], Radnor, PA, USA) at 37°C for 10 min, before adding $250 \mu\text{l}$ of 10% trichloroacetic acid (Amresco). After centrifuging the mixture at $5\ 200 \times g$ at 4°C for 10 min, $750 \mu\text{l}$ of

the supernatant was incubated with 221 μl of 20 mmol/l N, N-dimethyl-p-phenylenediamine sulfate (Amresco) in 7.2 mol/l HCl (Sigma), immediately followed by the addition of 221 μl of 30 mmol/l FeCl_3 (Amresco) in 1.2 mol/l HCl, to generate methylene blue. The absorbance of the resulting solution at 670 nm was measured after 20 min using a microplate reader. The H_2S concentration was calculated against a linear standard curve of H_2S (measured using NaHS standard solutions of 0, 18.75, 37.5, 75, 150 and 300 $\mu\text{mol/l}$), and the results were expressed as μmol of H_2S per gram of vascular tissue ($\mu\text{mol/g}$ tissue).^{20,21}

Immunohistochemistry. Formaldehyde-fixed tissues were paraffin embedded and cut into 4- μm transverse sections onto Adhesive slides (Cat. No. SLI-20010501, MXB Biotechnologies, FuZhou, China) and then placed in an oven maintained at 60°C for 1 h. The sections were microwaved for antigen retrieval then incubated with primary antibodies against cytosolic CAT (cCAT; rabbit-anti-aspartate aminotransferase antibody [ab170950], Abcam Cambridge, MA, USA; 1:50 dilution), mitochondrial CAT (mCAT; mouse anti-MCAT [sc-100477], Santa Cruz Biotechnology Dallas, TX, USA; 1:50 dilution) and 3-MPST (rabbit-anti-MPST antibody [HPA001240-100UL], Sigma-Aldrich; 1:250 dilution) overnight at 4°C. The sections were rinsed three times in PBS followed by incubation with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody at 37°C for 30 min. The immunoreactive signal was developed using a 3,3'-diaminobenzidine colour developing substrate for 5 min, then the sections were mounted and visualized under a light microscope (BX41, Olympus Corporation, Japan), as previously described.²² Image processing and analyses were performed using CellSens Standard software (Olympus Corporation, Japan).

Effect of blocking K_{Ca} or K_{ATP} channels on acetylcholine-induced relaxation in the presence of L-NNA and indomethacin. To investigate the effect of potassium channel inhibitors in H_2S -mediated relaxation, IMA ring segments were divided into four groups and incubated with Krebs solution containing L-NNA plus indomethacin (control); L-NNA plus indomethacin plus the K_{ATP} channel blocker glibenclamide (10 $\mu\text{mol/L}$); L-NNA plus indomethacin plus the large-conductance calcium-activated K^+ [BK_{Ca}] channel blocker iberiotoxin (10⁻² $\mu\text{mol/L}$; Sigma-Aldrich); or L-NNA plus indomethacin plus the intermediate-conductance Ca^{2+} -activated K^+ [IK_{Ca}] channel blocker TRAM-34 (1 $\mu\text{mol/L}$; Sigma-Aldrich) plus the small-conductance Ca^{2+} -activated K^+ [SK_{Ca}] channel blocker apamin (10⁻¹ $\mu\text{mol/L}$; Sigma-Aldrich). Acetylcholine-induced relaxation curves were then established as mentioned above.

NaHS (exogenous H_2S donor)-induced relaxation in IMA. To demonstrate the relaxation induced by exogenous H_2S , the H_2S donor NaHS was applied in cumulative concentration (-9 to -2.5 log M) to endothelium-denuded and endothelium-intact IMA ring segments with contraction induced by U-46619.

Effect of H_2S on levels of endothelial NO synthase (eNOS), phosphorylated (p)-eNOS and PDE5A. Fresh IMA rings were incubated with Krebs solution (control), or Krebs containing 100 $\mu\text{mol/L}$ NaHS, for 24 h at 37°C. IMA segments from 15 patients were divided into 5 groups: (1) NaHS-treated (to measure total eNOS); (2) NaHS-treated (to measure p-eNOS); (3) without NaHS treatment (control for groups 1 and 2); (4) NaHS-treated (to measure PDE5A); and (5) without NaHS treatment (control for group 4). Each group was homogenized in lysis solution (KeyGEN Biotech, Nanjing, China). Supernatants were collected after

centrifugation at $12\,800 \times g$ for 10 min at 4°C , and were used for measuring eNOS and p-eNOS by Western blotting, with GAPDH used as the internal control, as previously described¹ or for measuring PDE5A levels by ELISA, using a PDE Assay Kit (Cat No. 60300; Amsbio, Lake Forest, CA, USA) according to the manufacturer's instructions.

Statistical analyses

All results are presented as mean \pm SEM. Student's *t*-test was used for comparisons between two sets of data and analysis of variance (ANOVA) was used for group comparisons, followed by post-hoc Bonferroni test to detect the individual differences. Statistical analyses were performed using SPSS software, version 20.0 (IBM, Armonk, NY, USA) and a *P* value <0.05 was considered statistically significant.

Results

Fresh IMA tissue was obtained from 47 patients who underwent CABG surgery, comprising 30 male and 17 female patients, with a mean age of 62.4 ± 10.7 years. All patients had received standard preoperative drug therapy including antiplatelet, antiangina, or antihypertensive drugs, as appropriate. It should be noted that CABG patients commonly have hypertension and diabetes, but specific clinical data for the patients who donated tissue for inclusion in the present study were not recorded.

Resting vessel parameters

The IMA ring segments were suspended at an equivalent transmural pressure of 70.4 ± 2.3 mmHg at 90% of the internal diameter at 100 mmHg after normalization.¹⁹

Effect of CAT inhibition on endogenous H_2S -induced relaxation

Acetylcholine induced almost full relaxation ($92.37\% \pm 0.77\%$) in the endothelium-intact (E+) IMA rings whereas the relaxation was abolished in the endothelium-denuded (E-) rings (Figure 1A and B). In the endothelium-intact groups treated with CAT inhibitors, acetylcholine-induced relaxation was partially inhibited by L-NNA plus indomethacin ($P < 0.01$, two-way ANOVA) and the inhibition (circa 30%) was similar to previously reported values.¹⁹ Acetylcholine-mediated relaxation was also significantly inhibited in the presence of L-NNA plus indomethacin plus AOAA ($P < 0.01$, two-way ANOVA) and L-NNA plus indomethacin plus L-aspartate ($P < 0.01$, two-way ANOVA) compared with controls (L-NNA plus indomethacin; Figure 1A and B). Acetylcholine-induced E_{max} presented no significant differences between rings treated with L-NNA plus indomethacin plus AOAA ($23.81\% \pm 0.84\%$) and rings treated with L-NNA plus indomethacin plus L-aspartate ($24.29 \pm 0.54\%$, $P > 0.05$, unpaired *t*-test). EC_{50} values in the L-NNA plus indomethacin plus AOAA group (-7.12 ± 0.01 log M) were higher than in the control group (-7.29 ± 0.04 log M; $P < 0.01$, one-way ANOVA and Bonferroni test), but there were no statistically significant differences between L-NNA plus indomethacin plus L-aspartate treatment (-7.29 ± 0.03 log M) and the control group (L-NNA plus indomethacin; $P > 0.05$, one-way ANOVA and Bonferroni test; Figure 1C).

H_2S production in IMA

A linear standard curve of H_2S was established to quantify H_2S in the IMA tissues ($R^2 = 0.995$; Figure 2A). The fresh human IMA tissue generated detectable amounts

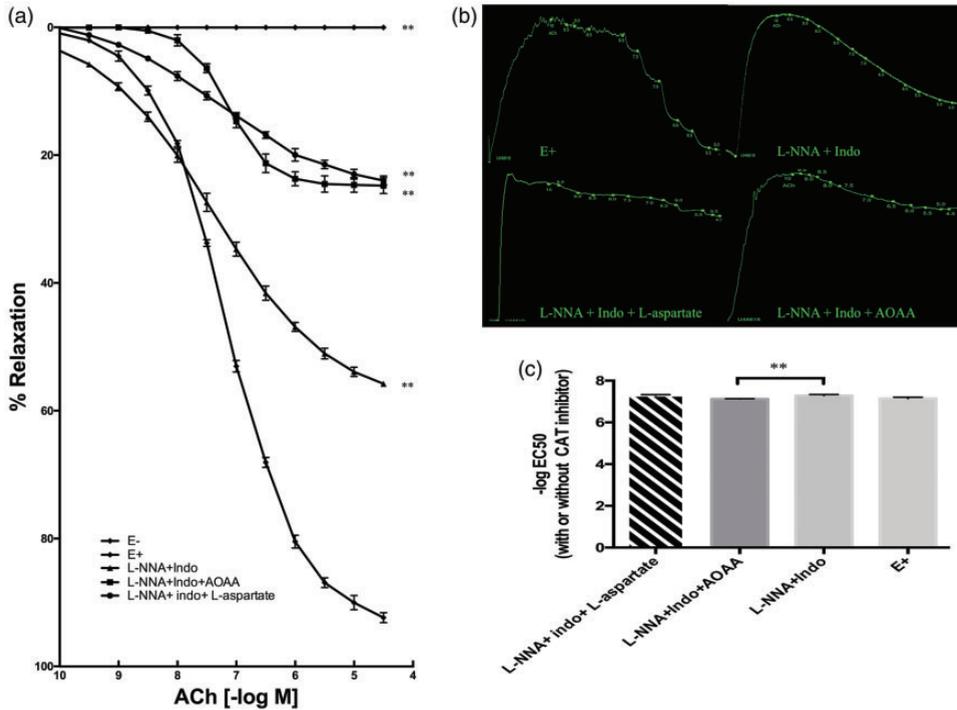


Figure 1. (A) Effect of cysteine aminotransferase (CAT) inhibitors on endogenous H₂S-induced relaxation. Acetylcholine (ACh) induced almost full relaxation in human endothelium-intact (E+) internal mammary artery (IMA) rings, and this relaxation was inhibited by N^G-nitro-L-arginine (L-NNA) plus indomethacin (Indo) (***P* < 0.01, two-way ANOVA); ACh-induced relaxation in E+ rings was further inhibited by L-NNA plus Indo plus aminooxyacetic acid (AOAA; a general inhibitor of pyridoxal 5'-phosphate-dependent enzymes), and L-NNA plus Indo plus L-aspartate (a competitive CAT substrate). In contrast, there was no ACh-induced relaxation in endothelium-denuded (E-) IMA rings (*n* = 8; ***P* < 0.01 versus E+; two-way ANOVA); (B) Original trace of Figure 1A; (C) -Log EC₅₀ for each group; L-NNA plus Indo plus AOAA was significantly lower than L-NNA plus Indo (***P* < 0.01; *n* = 8; one-way ANOVA and Bonferroni test). Data presented as mean ± SEM.

of H₂S (Figure 2B) and the detected H₂S was increased by acetylcholine (*P* < 0.01 versus control, unpaired *t*-test), demonstrating that human IMA is capable of generating H₂S when stimulated with acetylcholine.

Immunohistochemistry

Immunohistochemical staining showed that 3-MPST was located in both the endothelium and the smooth muscle (Figure 3 left), cCAT was located only in the endothelial

cells (Figure 3 middle), and mCAT was located in both the endothelium and the smooth muscle (Figure 3 right).

Effect of K_{Ca} or K_{ATP} channel blockers on acetylcholine-induced relaxation in the presence of L-NNA plus indomethacin

The addition of L-NNA plus indomethacin inhibited acetylcholine-mediated relaxation. The presence of L-NNA plus indomethacin plus iberiotoxin, or L-NNA plus indomethacin plus glibenclamide, further inhibited

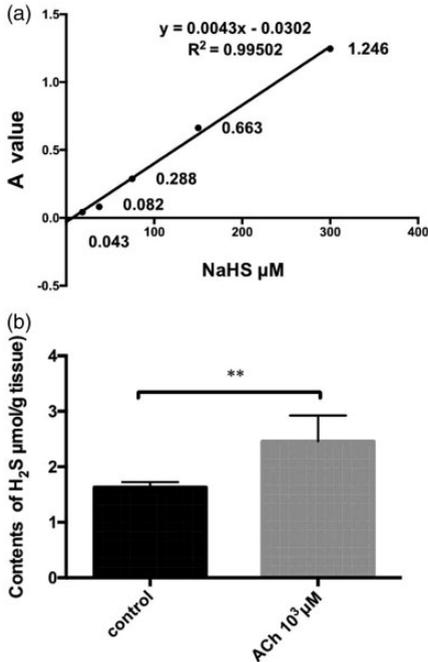


Figure 2. (A) Standard curve of NaHS gradient solutions that was produced to quantify the concentration of H_2S in fresh human internal mammary artery (IMA) tissue; (B) The concentrations of H_2S in human IMA were increased by incubating with acetylcholine (ACh) for 4 h (** $P < 0.01$ versus control; $n = 5$; unpaired t -test). Data presented as mean \pm SEM.

the acetylcholine-induced relaxation versus L-NNA plus indomethacin (both $P < 0.01$, two-way ANOVA). The greatest inhibition of acetylcholine-mediated vasorelaxation was observed in vessels treated with L-NNA plus indomethacin plus TRAM-34 plus apamin, and this inhibition was significantly higher versus vessels treated with L-NNA plus indomethacin plus iberiotoxin ($P < 0.01$, two-way ANOVA). E_{max} inhibition of vessels treated with L-NNA plus indomethacin plus TRAM-34 plus apamin ($12.42\% \pm 0.29\%$) was also significantly higher ($P < 0.01$, unpaired t -test) than in vessels treated with L-NNA plus indomethacin plus iberiotoxin ($23.12\% \pm 0.52\%$; Figure 4A). The EC_{50} values in the group treated with L-NNA plus indomethacin plus iberiotoxin ($-7.56 \pm 0.07 \log \text{M}$) were significantly lower than in the control group treated with L-NNA plus indomethacin ($-7.29 \pm 0.04 \log \text{M}$), and EC_{50} values in the groups treated with L-NNA plus indomethacin plus TRAM-34 plus apamin ($-6.49 \pm 0.02 \log \text{M}$) and L-NNA plus indomethacin plus glibenclamide ($-6.09 \pm 0.09 \log \text{M}$) were significantly higher versus the control group (all $P < 0.01$; one-way ANOVA and Bonferroni test; Figure 4B).

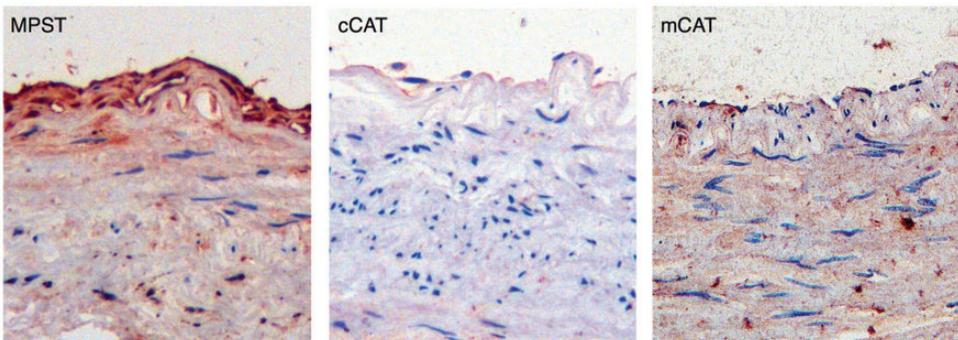


Figure 3. Representative photomicrographs showing immunohistochemistry staining of 3-mercaptopyruvate sulfurtransferase (3-MPST) and cysteine aminotransferase (CAT) in human internal mammary artery tissue; 3-MPST was shown to be located in both the endothelium and smooth muscle (left); cytosolic CAT (cCAT) was shown to be located only in the endothelial cells (middle) and mitochondrial CAT (mCAT) was found to be located in both the endothelium and smooth muscle (right); original magnification $\times 200$.

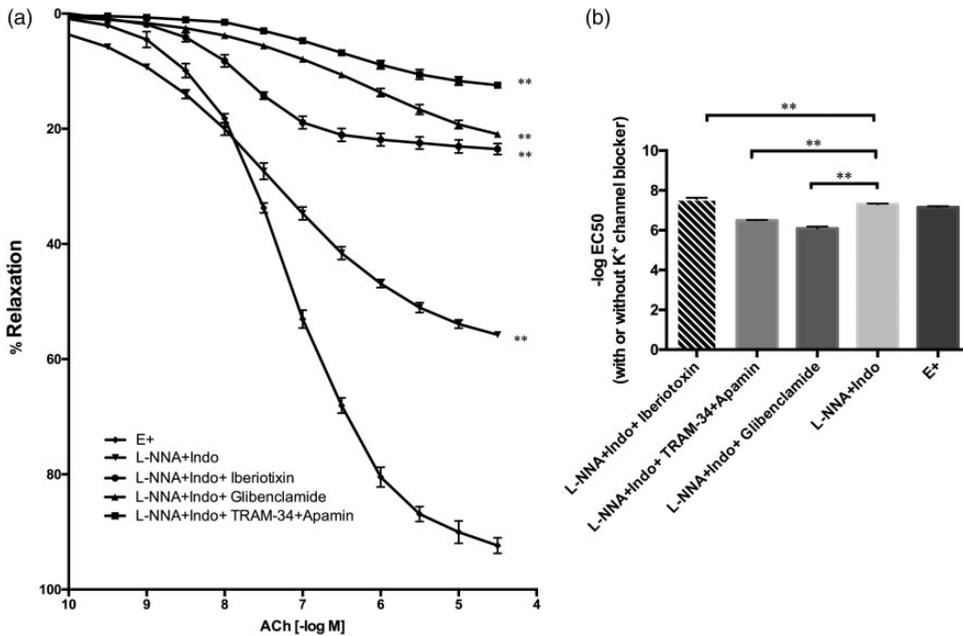


Figure 4. Effect of different K^+ channel blockers on acetylcholine (ACh)-induced relaxation in human internal mammary artery rings. (A) ACh-induced relaxation was significantly reduced in the presence of N^G -nitro-L-arginine (L-NNA) plus indomethacin (Indo). The K^+ channel blockers iberiotoxin, glibenclamide and TRAM-34 plus apamin further inhibited the residual ACh-induced relaxation that was resistant to L-NNA plus Indo ($**P < 0.01$ versus L-NNA plus Indo [control]; two-way ANOVA); (B) Regarding $-\log EC_{50}$ values, each experimental group had significantly higher EC_{50} values versus the control group (L-NNA plus Indo) except L-NNA plus Indo plus iberiotoxin, in which the EC_{50} was lower ($**P < 0.01$ versus control; $n = 8$; one-way ANOVA and Bonferroni test). Data presented as mean \pm SEM.

Effect of endothelium in exogenous H_2S -induced relaxation

There were no statistically significant differences in NaHS-mediated relaxation between endothelium-denuded and endothelium-intact IMA (Figure 5), suggesting that exogenous H_2S -induced relaxation in the present experiments was endothelium-independent.

Effect of H_2S on eNOS, p-eNOS and PDE5A levels

Western blots showed that there was no difference in levels of eNOS protein between IMA rings exposed to NaHS (H_2S donor; $-4 \log M$) for 24 h at $37^\circ C$, and IMA rings

incubated in Krebs only (controls; Figure 6A), however, NaHS was associated with significantly increased levels of eNOS phosphorylation at its activation site, ser1177 (increased p-eNOS^{ser1177} levels) versus controls ($P < 0.01$, unpaired t -test; Figure 6A).

The ELISA experiments showed that stimulation of IMA with NaHS ($-4 \log M$) for 24 h at $37^\circ C$ significantly down-regulated the PDE5A levels compared with control IMA tissue exposed to Krebs only ($P < 0.01$, unpaired t -test; Figure 6B).

Discussion

The present study revealed that in human IMA conduit arteries (1) the endothelium

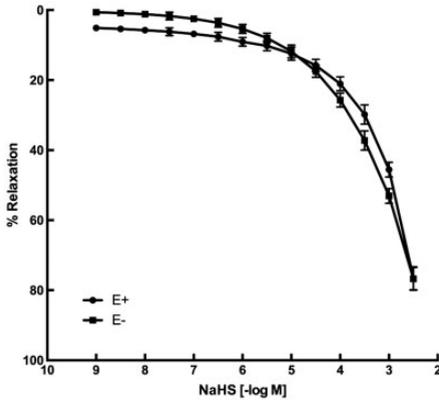


Figure 5. The NaHS-mediated relaxation in human internal mammary artery (IMA) rings was not affected by denudation of the endothelium ($P > 0.05$, endothelium-intact IMA versus endothelium-denuded IMA; $n = 8$; two-way ANOVA). Data presented as mean \pm SEM.

releases H_2S under both basal and acetylcholine-stimulated conditions; (2) the release of H_2S involves 3-MPST coupled with CAT pathway; and (3) the H_2S -induced relaxation involves stimulating the phosphorylation of eNOS and inhibiting PDE5A activity, as well as opening of potassium channels.

In mammals, H_2S is enzymatically produced through pyridoxal 5'-phosphate (PLP)-dependent pathways.²⁰ CAT is a PLP-dependent enzyme and endogenous H_2S is generated by 3-MPST coupled with CAT in rat thoracic aortic endothelial cells.⁴ CAT is found in both cytosolic and mitochondrial forms in the rat liver.²³ H_2S is reported to be a potent vasodilator of human pulmonary arteries²⁴ and mesenteric arteries.¹³ Further, it has been previously suggested that cystathionine α -lyase, and the H_2S it generates, together play a physiological role in regulating the diameter of arteries in human IMA.²⁵

The present study demonstrated release of H_2S from the human conduit artery under both basal and acetylcholine-stimulated

conditions, by acetylcholine-induced relaxation and by direct measurement of H_2S levels in IMA tissue. In addition, to the best of the authors' knowledge, this is the first time that L-NNA and indomethacin-resistant relaxation induced by acetylcholine is shown to be further inhibited by both 3-MPST/CAT inhibitor AOAA (a general inhibitor of PLP-dependent enzymes)²⁰ and the competitive CAT substrate L-aspartate,²⁰ suggesting that the release of H_2S from the human conduit artery involves the 3-MPST/CAT pathway. Direct immunohistochemistry evidence revealed that 3-MPST and mCAT are located in both endothelial and smooth muscle cells and cCAT is located in endothelial cells only. Although the H_2S producing enzymes, MPST and CAT, were detected in both endothelium and smooth muscle cells, the acetylcholine-induced relaxation was abolished when the endothelium was removed. These results may be due to a number of factors: First, the present study showed that cCAT is located in the endothelial cells only and mCAT is located in both the endothelium and smooth muscle. The role of endothelium for endothelium-dependent relaxation is mainly due to endothelium-derived relaxing factors, such as NO, endothelium-dependent hyperpolarizing factor (EDHF), and H_2S . Therefore, when the endothelium is removed, endothelium-dependent relaxation is actually abolished. This is true for NO, as has been repeatedly reported previously, although eNOS is present in the smooth muscle. Similarly, when the endothelium is removed, the H_2S -mediated relaxation is also abolished. These observations suggest that endothelium-dependent relaxation related to endothelium-derived NO or endothelium-derived H_2S is a dominant mechanism in acetylcholine-mediated relaxation. Secondly, the above facts may suggest that cCAT is the major enzyme, since it is located only in the endothelium. It appears that any mCAT-derived H_2S , if any, would not be

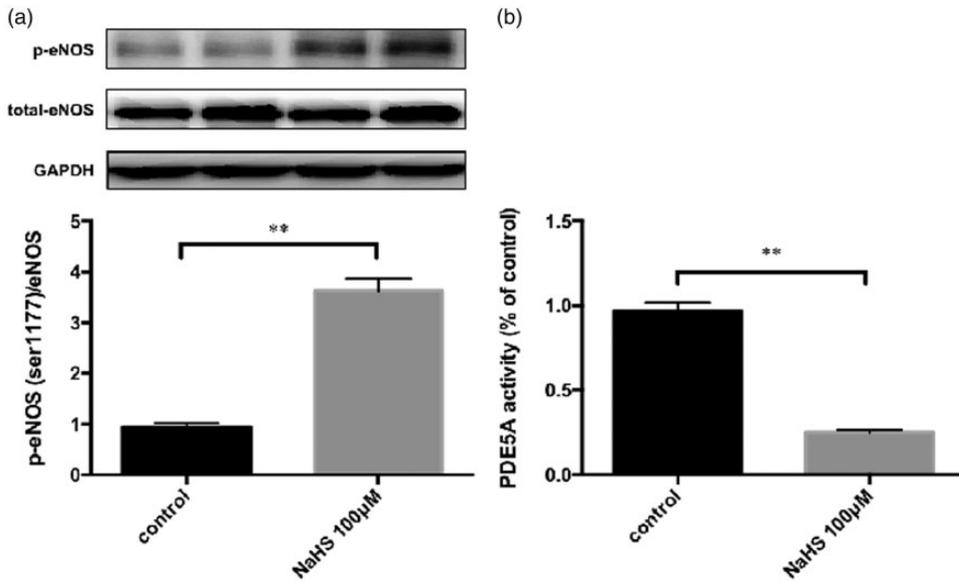


Figure 6. Levels of endothelial nitric oxide synthase (eNOS), phosphorylated (p)-eNOS, and phosphodiesterase (PDE)5A in human internal mammary artery (IMA) rings. (A) Top panel shows representative Western blots of IMA protein extracts from IMA rings that had been incubated with Krebs alone (control; two left lanes) or Krebs with $-4 \log M$ NaHS (two right lanes) for 24 h. Results normalised to GAPDH (bottom figure) showed that exogenous H_2S did not affect eNOS levels but increased p-eNOS^{ser1177} levels in human IMA (** $P < 0.01$ versus control; $n = 5$; unpaired t-test). (B) Results from enzyme-linked immunosorbent assay showing that stimulation of IMA with NaHS ($-4 \log M$) significantly down-regulated PDE5A levels compared with the control (** $P < 0.01$ versus control; $n = 5$; unpaired t-test). Data presented as mean \pm SEM.

enough to induce significant smooth muscle CAT-mediated relaxation. Taken together, the authors believe that in the present study, L-NNA and indomethacin-resistant relaxation induced by acetylcholine is mediated by endothelium-derived H_2S .

In the present study, the role of cGMP was explored in H_2S -induced, endothelium-dependent relaxation in human IMA. p-eNOS was found to be increased and PDE5A was decreased in response to NaHS. There are few possibilities to explain this: (1) NaHS (H_2S donor) may increase p-eNOS at its activation site, Ser1177 in endothelial cells, leading to NO production and induced cGMP expression, subsequently inhibiting PDE5A; (2) NaHS (H_2S) directly inhibits PDE5A activity; (3) there may be a

combination of the above two mechanisms; and (4) other possibilities may also exist, for example, NaHS (H_2S) may stimulate other molecules in this pathway. Without measuring all the intermediate molecules (such as cGMP), it is difficult to determine which is the real mechanism. Regardless, the present experiments demonstrated that the H_2S donor, NaHS, decreased purified PDE5A levels. As reported previously,^{1,26} PDE5A is involved with cGMP degradation and therefore inhibition of PDE5A enhances cGMP pathway-mediated relaxation.

To investigate the role of potassium channels in H_2S -induced relaxation, the effect of various K_{Ca} and K_{ATP} channel inhibitors were tested. First, the role of IK_{Ca} and SK_{Ca} channels was explored in

the endothelial cells. The inhibitory role of apamin (inhibitor of SK_{Ca}) plus TRAM-34 (inhibitor of IK_{Ca}) was demonstrated in the residual component of acetylcholine-induced vasorelaxation that was resistant to L-NNA plus indomethacin. Further, to assess whether acetylcholine-induced relaxation involves activation of the BK_{Ca} and K_{ATP} channels in the vascular smooth muscle, the effects of the BK_{Ca} channel blocker iberiotoxin and K_{ATP} channel blocker glibenclamide on the acetylcholine-induced responses were tested. Relaxation was found to be significantly blocked, suggesting a role for BK_{Ca} and K_{ATP} in acetylcholine-induced relaxation in the smooth muscle of human IMA. In fact, H_2S opens BK_{Ca} , and induces $[K^+]_i$ efflux²⁷ that inhibits the capacitative $[Ca^{2+}]_i$ influx through inhibition of L-type voltage-gated Ca^{2+} channels.²⁸ In the present study, inhibition of the BK_{Ca} channel by iberiotoxin reduced the H_2S -mediated relaxation, suggesting that H_2S -induced relaxation is related to opening of the BK_{Ca} channel. The present study therefore provides evidence that H_2S released from the endothelium may induce vasorelaxation via opening of vascular smooth muscle K_{Ca} channels in human IMA.

In previous studies, the present authors have demonstrated that acetylcholine-induced, L-NNA and indomethacin-resistant relaxation in human IMA is mediated by EDHF²⁹ and K_{Ca} channels are involved in the EDHF-mediated relaxation^{29,30} and hyperpolarization.^{31,32} Further, the authors hypothesized that H_2S is a candidate of EDHF in rat cerebral vessels.^{31,32} The present study supports this notion in human arteries and the findings are consistent with observations in small mesenteric arteries, or aortae in male animal models.^{33,34}

The possible role of K_{ATP} channels in H_2S -mediated relaxation was also assessed. K_{ATP} channel opening is known to mediate

many of the biological activities of H_2S .³⁵ Sarcolemmal K_{ATP} channels are involved in intracellular $[Ca^{2+}]_i$ handling and their opening results in resistance to apoptosis during myocardial ischaemia/reperfusion injury.³⁶ Interestingly, administration of glibenclamide *in vivo* reduced the infarct-limiting effects of NaHS,¹² indicating that sarcolemmal K_{ATP} channel activation participated in the cardioprotective signalling of H_2S .¹² Further, potent vasodilatory activity by H_2S both *in vitro* and *in vivo* is assumed to be realized by opening of vascular smooth muscle K_{ATP} channels.³⁷ The present study also demonstrated that K_{ATP} channel is involved in H_2S -induced relaxation in human IMA.

In conclusion, the results of the present study demonstrated that in the human IMA conduit artery, the endothelium releases H_2S under both basal and acetylcholine-stimulated conditions and the release of H_2S involves 3-MPST coupled with CAT pathway. Further, H_2S -induced relaxation involves stimulating the phosphorylation of eNOS and inhibiting PDE5A activity. In addition, potassium channels are involved in H_2S -induced vasorelaxation in this human artery, involving two-fold actions: opening of the endothelial IK_{Ca} and SK_{Ca} channels and opening of the smooth muscle BK_{Ca} channels; both producing hyperpolarization and relaxation. These findings may provide new therapeutic targets for the treatment of vasospasm in coronary artery bypass graft vessels, and may provide a pharmacological basis for the development of new vasodilator drugs.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

Funding

This work was fully supported by grants from the National Natural Science Foundation of

China (81870288, 81870227), the Non-profit Central Research Institute Fund of Chinese Academy of Medical Sciences (2018TX31002), Binhai New Area Health Bureau (2016BWKY007, 2016BWKZ003), and China-Serbia Exchange Program (2-13, 4-12).

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