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Original Article

Hydrogen sulfide exerts antithrombotic effects and inhibits deep vein thrombosis through NOS-PECAM-1 signaling pathway

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Abstract: Deep vein thrombosis (DVT) is initiated in aggregation and adhesion of inflammatory cells to vascular endothelium mediated by adhesion molecules, thereby activating platelets and inducing thrombosis. Hydrogen sulfide (H_2S) is a gasotransmitter with anti-adhesive, anti-inflammatory and vasoactive properties. This study aimed to investigate the inhibitory effect of platelet aggregation and thrombosis by H_2S. Platelet aggregation by H_2S was measured in human washed platelets. A mouse DVT model was established by femoral vein ligation, with intraperitoneally injected sodium hydrosulfide (NaHS, 10 mmol/kg per day) or saline for 6 days before thrombosis. After 48 hours, thrombus weight was measured and the femoral vein tissue was harvested to detect the expression of endothelial NOS (eNOS), inducible NOS (iNOS) and PECAM-1 expressions. Small interfering RNA (siRNA) was applied to inhibit PECAM-1 expression in HUVECs cells to explore the role of PECAM-1 in the anti-thrombotic effect. Our results showed that NaHS inhibited aggregation of human washed platelets in a concentration dependent manner, which could be attenuated by L-NAME, a nitric oxide synthase inhibitor. NaHS significantly decreased thrombus weight and increased protein expressions of PECAM-1, eNOS and iNOS in DVT mice. Inhibition of PECAM-1 expression in HUVECs by small interfering RNA (siRNA) can significantly increase the adhesion activity of HUVECs and platelets and attenute inhibition of platelet function by hydrogen sulfide. In conclusions, H_2S demonstrates inhibitory effects on platelet activation and thrombosis involving the NOS-PECAM-1 pathway and may have preventive and therapeutic value for DVT and other clinical disorders with increased risk of thrombotic events.

Keywords: Deep vein thrombosis (DVT), hydrogen sulfide (H_2S), PECAM-1, nitric oxide (NO), platelet aggregation

Introduction

Deep vein thrombosis (DVT) is blood coagulation process in the deep veins, thereby causing the venous reflux disorder. DVT is thought to be caused by the combination of so-called virchow triad, namely blood hypercoagulable state, slow blood flow and vascular endothelial injury [1]. Thrombosis initiated at aggregation and adhesion of inflammatory cells to the vascular endothelium, and decreased blood flow velocity also promote adhesion of neutrophils and mononuclear cells to the vascular endothelium, subsequently activating platelets, tissue factor and coagulation factor XII (FXII) [2]. Platelets could perform rapid repair of endothelial lining through aggregation and adhesion at the site of injury, and excessive aggregation and adhesion of platelet cause thrombosis formation [3]. Platelet acts as the key link to promote venous thrombosis, and adhesion of platelet on endothelial cells is dependent on complex interactions between platelets and monocytes, neutrophils [4], which are regulated by a variety of cell adhesion molecules [5]. Currently, the mechanisms underlying the relationship between cell adhesion molecules and thrombus formation are not yet fully understood.

Platelet endothelial cell adhesion molecule-1 (PECAM-1, also known as CD31) is a cell surface glycoprotein, which belongs to the cell adhesion molecules of the immunoglobulin

superfamily. PECAM-1 is mainly expressed on platelet, monocyte, neutrophil and endothelial cells [6]. PECAM-1 is a regulatory signal molecule of vascular biology, including platelet function [7-10], vascular regeneration [11], angiogenesis [12], integrin regulation [13], lymphocyte activation [14], and leukocyte migration in endothelial cells [15]. PECAM-1 demonstrates strong antiplatelet function, and the main mechanism lies in the binding of its immunoreceptor tyrosine-based inhibitory motifs (ITIMs) to protein tyrosine phosphatase-2 (SHP-2), thereby inhibiting the collagen receptor glycoprotein VI (GPVI) on platelet surface [16]. The antiplatelet function of PECAM-1 can show in vivo antithrombotic effect. Experimental results showed that PECAM-1 knockout mice exhibited larger arteriovenous thrombus and longer thrombus regression time [17, 18]. PECAM-1 also can regulate inflammatory responses, and promote chemokine-mediated leukocyte migration to the site of inflammation [19]. However, PECAM-1 mainly exert anti-inflammatory effects in endothelial cells, such as enhancing endothelial barrier function [20] and protecting endothelium free from endotoxin attack [21]. Inflammatory reaction can promote hypercoagulable state, enhance vascular permeability and thrombosis [22, 23], and its mechanisms may lie in the fact that inflammatory reaction promotes interaction between inflammatory leukocytes and damaged endothelium and leads to platelet recruitment. Moreover, chronic inflammatory reaction also promotes vascular remodeling, which is regulated by platelet [24].

Hydrogen sulfide (H_2S) is one gaseous signal molecule with wide physiological and pharmacological regulatory effects [25-27]. Endogenous hydrogen sulfide are produced by three kinds of enzymes, including cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3-MST) [28, 29]. Hydrogen sulfide could inhibit inflammation [30, 31] and nuclear factor-κB (NF-κB) activation [32], enhance heme oxygenase-1 (HO-1) expression [33], remove nitrite [34], and regulate intracellular redox state [35]. Recent reports showed that hydrogen sulfide could inhibit platelet aggregation stimulated by various inducers [36], and inhibit adhesion of platelet to collagen [37]. Some drugs which can release hydrogen sulfide also showed in vivo antithrombotic effect [38, 39].

This study aimed to explore whether PECAM-1 is involved in regulation of hydrogen sulfide on platelet aggregation and thrombosis. We investigated the in vitro inhibitory effect of sodium hydrosulfide (NaHS) on platelet aggregation, and in vivo antithrombotic effect in DVT mice. We applied nitric oxide (NO) synthase inhibitor NG-nitro-L-arginin-methylester (L-NAME) to investigate whether these effects are dependent on nitric oxide signaling pathway. We also explored the regulatory effect of hydrogen sulfide on PECAM-1. Small interfering RNA (siRNA) was used to investigate whether PECAM-1 is involved in inhibition of platelet aggregation and thrombosis by hydrogen sulfide. Our study provides the experimental basis for the clinical treatment of DVT.

Materials and methods

Chemicals

The H_2S donor sodium hydrosulfide (NaHS; Sigma Chemical, Deisenhofen, Germany) was dissolved in physiological saline to a concentration of 100 mmol/L. The nitric oxide synthase (NOS) inhibitor L-NAME (NG-nitro-L-arginin-methylester) (Sigma Chemical) was dissolved in physiological saline to a concentration of 100 mg/L. (FITC)-labeled fibrinogen was from Invitrogen (Carlsbad, CA, USA). The anti-eNOS-antibody, anti-iNOS-antibody, anti-PECAM-1-antibody and anti-Akt-antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other substances were obtained from Sigma Aldrich (Taufkirchen, Germany).

Blood donors and animals

Human washed platelets were collected from the fresh blood of healthy volunteers who had not taken any medications for at least 10 days, and were resuspended in modified Tyrode-HEPES (N-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid) buffer (Platelet final concentration: 4×10^8/mL. A signed written informed consent according to the Declaration of Helsinki was obtained from each volunteer. Study program was discussed and approved by the Research Ethics Committee of Shandong University.

Wide-type C57BL/6 mice were used in all animal experiments. These mice included male or female, were 6 to 8 weeks of age, weighting 22~25 g. They were fed in well ventilated and

specific pathogen free room with 20-25°C temperature, 50~60% relative humidity and 12 h light-dark cycle. The mice had access to standard laboratory chow and water ad libitum.

Platelet aggregation assay

Platelet aggregation in whole blood was measured by Multiplate impedance aggregometry (Multiple platelet function analyzer, Dynabyte, Munich, Germany) according to the manufacturer's protocol. The fresh blood was mixed with anticoagulant (3.13% sodium citrate), centrifuged at 340 g for 15 minutes to acquire platelet-rich plasma. After another centrifugation at 600 g for 10 minutes in the presence of 2 ng/mL Ilomedin, platelets were washed and resuspended in calcium-free modified Tyrode-HEPES (N-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid) buffer (138 mmol/L NaCl, 2.7 mmol/L KCl, 12 mmol/L $NaHCO_3$, 400 mmol/L Na_2HPO_4, 1 mmol/L $MgCl_2$, 5 mmol/L D-glucose, and 5 mmol/L HEPES), and adjusted to a platelet final concentration of 4×10^8/mL for further experiments. Platelet number was counted by a resistance particle counter (Coulter Z2, Beckman Coulter, Krefeld, Germany). Platelet suspension was placed in a test tube containing hirudin (13 μg/mL) to avoid fluctuation in physiological calcium concentration. Samples were kept rest for 30 minutes, and platelet aggregation test was performed 30 to 180 minutes after blood had been drawn. An aliquot 300 μl suspension was mixed with 300 μl saline solution, and was then incubated with NaHS at 37°C for 5 minutes under constant stirring (800/min). Platelet aggregation test was performed by adding different platelet agonist solutions, such as arachidonic acid (AA, 0.5 mmol/L), thrombin receptor activating peptide (TRAP, 32 μmol/L), adenosine diphosphate (ADP, 6.5 μmol/L), or collagen (3.2 μg/mL). Platelet suspension was incubated with NaHS and L-NAME (10 μmol/L) to investigate whether the effect of NaHS is nitric oxide dependent. The platelet aggregation amplitudes were determined by changes in impedance recorded over 6 minutes in triplicates and results were expressed as mean arbitrary aggregation units (AU).

Flow cytometry analysis on fibrinogen binding capability

Platelet suspension was incubated with increasing concentrations of NaHS (0, 0.1, 0.5, 1, 5, 10 mmol/L) and stimulated with ADP (6.5 μmol/L), and then was incubated with fluorescein isothiocyanate (FITC)-labeled fibrinogen (150 μg/mL) at room temperature for 20 minutes. Flow cytometry analysis was performed using a FACSCalibur from 5000 events and data were analyzed using CellQuest Pro software (Version 3.3; BD Biosciences).

DVT mice model

A total of 30 C57BL/6 mice were randomly divided into 3 groups and received normal saline (control group), NaHS (10 mmol/kg), or NaHS+L-NAME (100 mg/kg), for continuous 6 days, and then began to make DVT model.

Mice was placed in a supine position with fixed limbs, and their bilateral thigh hair was removed. Mice were anesthetized by intraperitoneal injection of ketamine (125 mg/kg) and xylazine (25 mg/kg). 75% alcohol disinfection was performed below the xiphoid to toe end. Asepsis hole towel was paved and 1 cm incision was performed around groin governor to separate subcutaneous tissue and expose femoral artery and vein and femoral nerve. Mosquito clamp was applied to clamp femoral vein in three segments, with 3 seconds for each clamp. After clamp the skin incision was suture and fixed with plaster.

After establishment of DVT model, mice were fed continually to observe and record double foot swelling and acral skin color. After 48 hours, mice were intraperitoneally injected with 3% pentobarbital sodium (1 ml/kg) and kept supine position. Skin incision was made along the bilateral femoral vein to expose and separate bilateral femoral vein, and to observe the formation, severity and relief of the femoral vein thrombosis. Thrombi were taken from femoral vein and weighed, and femoral vein tissues were separated and flushed with physiological saline solution and frozen in liquid nitrogen.

Western blotting

Protein samples were extracted from femoral vein tissues and HUVEC cells, and were lysed with RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS 50 mM Tris-Cl, pH 7.4) containing 20% (V/V) cocktail protease inhibitors (Sigma-Aldrich). After sonication, centrifugation at 12,000 g was performed on these cell lysates for 30 min at 4°C

to remove insoluble debris. Proteins (50 μg) were separated by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12% separation gels, and 50 μg protein was loaded into each well. Then proteins were electrophoretically transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA), and were blocked with 3% BSA in TBS-T buffer (3 g/L Trisbase, 8 g/L NaCl, 0.2 g/L KCl, 0.1% Tween-20, pH 7.4) at 4°C. After that, the membranes were incubated with primary mouse monoclonal antibodies against human eNOS, iNOS, PECAM-1, Akt (Ser473) or β-actin (all 1:1000 dilutions) overnight at 4°C. Membranes were then incubated with secondary antibodies (IgG) labeled with horseradish peroxidase (1:1000 dilutions) in TBS-T plus 3% BSA at room temperature for 1 h, and were visualized using enhanced chemical luminescence (ECL, Pierce® ECL Plus Western Blotting Substrate, Pierce Biotechnology, Inc., Rockford, IL, USA). Densitometric analysis was performed by normalization eNOS, iNOS, PECAM-1 against the internal loading control β-actin.

Cell culture

Human umbilical vein endothelial cells (HUVEC) cells were purchased from ATCC and cultured in RPMI-1640 medium (Invitrogen-GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Sijichun Bioengineering Materials Inc., Hangzhou, Zhejiang, China) in a humidified incubator under 5% CO_2 atmosphere and at 37°C.

SmalI interfering RNA (siRNA)

The human cDNA sequence of PECAM-1 was obtained from GenBank (accession number: BC051822), and PECAM-1 specific RNA oligonucleotides were synthesized by GenePharma Co., Ltd. (Shanghai, China). The oligonucleotides sequences were as follows: PECAM-1 (position 687-705): GGCCCCAAUACACUUCACAdTdT, PECAM-1 (position 567-585): AACCACUGCAGAGUACCAGdTdT, random control scrambled sequence: GCCAAACGAUCCCAUACCUdTdT. by annealing of The sense and antisense RNA oligonucleotides were resolved in buffer (50 mmol/L Tris/HCl; 100 mmol/L NaCl; pH 7.5), and annealing was performed by heating to 95°C and slowly cooling to room temperature, thereby generating siRNA. The subconfluent (~75-80%) cultured human HUVEC cells were transfected with duplexes (120 pmol per 3.5 cm culture dish) using the liposomal reagent Genetrans II (MoBiTec, Göttingen, Germany) according to the manufacturer's instructions. After 48 hours the cells were transferred to medium containing 2% FBS and western blpt was performed to measure PECAM-1 protein expression.

Platelet adhesion assay

HUVEC cells were seeded on a gelatin coated coverslips at a concentration of 5 × 105/ml and grown to confluence. After transfection with PECAM-1 siRNA for 48 hours, Washed platelets (50 × 106/μl) were added to the coverslips and incubated with NaHS for 20 minutes under vibrating at 37°C. By washing with PBS, non-adhered platelets were removed. Cells were fixed with 3.7% paraformaldehyde for 15 minutes at room temperature, followed by staining with crystal violet for 10 minutes at 37°C. Cells on the coverslips were mounted and imaged by microscopy. The number of HUVEC cells with platelets adhered was counted and normalized to total number of HUVEC cells.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD). The commercially available software (SPSS version 19.0) was applied in statistical analysis. The statistical significance of difference between two groups was determined by Student t test (unpaired, two tailed). The statistical significance of difference between three or more groups was determined by one-way ANOVA followed by Bonferroni's multiple comparison test. A probability values of $P<0.05$ were considered statistically significant.

Results

Hydrogen sulfide inhibits platelet function

We performed impedance aggregometry method to explore the role of hydrogen sulfide on platelet aggregation. Human washed platelets from healthy volunteers were incubated with different concentrations of NaHS (0.1, 0.5, 1, 5, 10 mmol/L) for 5 minutes. Results showed that NaHS could inhibit platelet aggregation in a concentration dependent manner, which was induced by arachidonic acid (AA, 0.5 mmol/L),

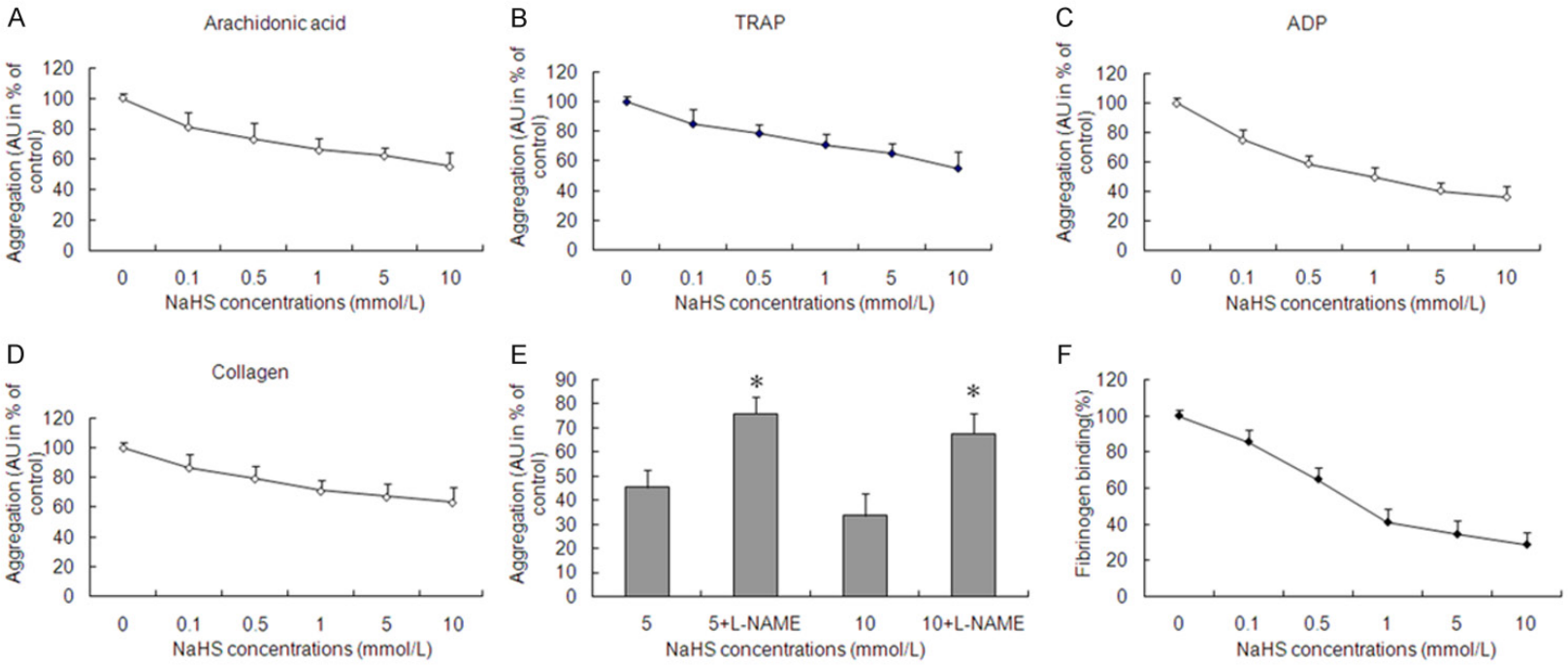

Figure 1. NaHS showed inhibitiory effects on platelet aggregation. Human whole blood was incubated with different concentrations of NaHS (0.1, 0.5, 1, 5 and 10 mmol/L) 37 °C for 5 minutes, then was added with different inducer for platelet aggregation test, such as (A) arachidonic acid (AA, 0.5 mmol/L), (B) thrombin receptor activating peptides (TRAP, 32 μmol/L), (C) adenosine diphosphate (ADP, 6.5 μmol/L), or (D) collagen (3.2 μg/mL). Data of each group were expressed relative to the number of control group (Without NaHS). n = 5. (E) Platelet inhibition effect of NaHS can be suppressed by nitric oxide synthase inhibitor L-NAME (10 μmol/L). Significant difference was marked with '*' compared with the non L-NAME group ($P<0.05$). (F) NaHS can decrease ADP-stimulated fibrinogen binding force in a concentration dependent manner. Human whole blood was incubated with different concentrations of NaHS (0.1, 0.5, 1, 5, 10 mmol/L), followed by ADP (6.5 μmol/L) stimulating, and fibrinogen binding force was determined by flow cytometry.

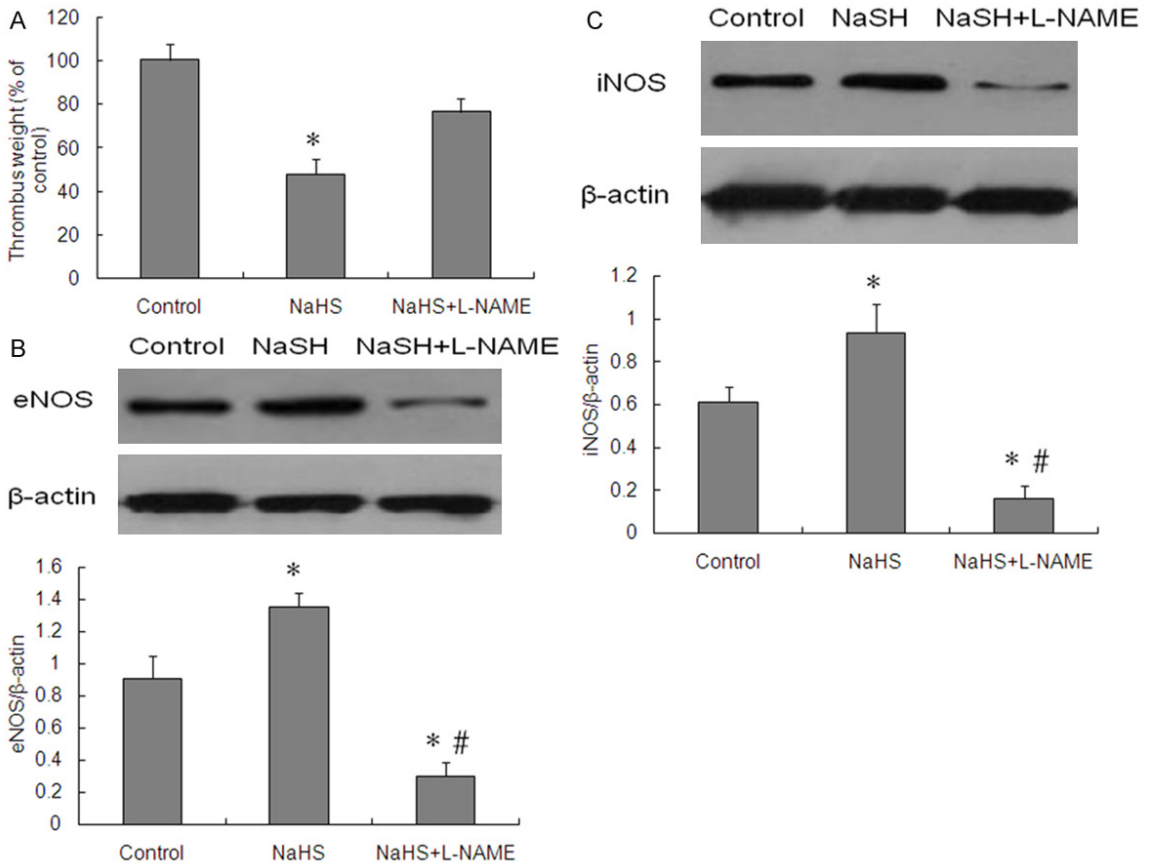

Figure 2. NaHS showed in vivo antithrombotic effects. (A) NaHS inhibited mice thrombus formation in a nitric oxide dependent manner. C57BL/6 mice were given intraperitoneal injection of NaHS solution (10 mmol/kg body weight), once a day, in the sixth day 2~4 hours after NaHS administration DVT model was established by clamp method. L-NAME (10 mg/kg body weight) was given DVT mice to investigate wthether antithrombotic effect is dependent on NO. At 48 h after clamping, the thrombus was taken out and weighed. The data for each group was thrombus volume relative to the control group. NaHS enhanced mouse endothelial expression of eNOS (B) and iNOS (C) proteins. Western blot was used to analysis the endothelial eNOS and iNOS protein expression in DVT mice. One representative figure was shown from three independent experiments. The density of protein bands were converted into grayscale values and normalized to that of the internal control β-actin. Results are expressed as mean ± SD. Compared with the control group, NaHS group showed higher eNOS and iNOS protein expression. L-NAME treatment significantly reduced eNOS and iNOS protein levels. *Compared with the control group, the difference was significant ($P<0.05$). #Compared with the NaHS group, the difference was significant ($P<0.05$). n = 10.

thrombin receptor activating peptide (TRAP, 32 μmol/L), adenosine diphosphate (ADP, 6.5 μmol/L) and collagen (3.2 μg/mL) (**Figure 1A-D**). Among all four inducing agents, NaHS showed the strongest inhibitory effect on ADP-induced platelet aggregation. So we chose ADP as the main inducer in the later experiments.

We then investigated the effect of hydrogen sulfide on platelet aggregation in the presence of nitric oxide synthase L-NAME (10 μmol/L). L-NAME could significantly weaken inhibitory effect of NaHS on ADP-induced platelet aggregation, but could not restore to platelet aggregation function without NaHS (**Figure 1E**). This suggests that nitric oxide signaling pathway might be involved in the inhibitory effect of hydrogen sulfide (H_2S) on platelet aggregation.

Platelet aggregation is dependent on conformational changes of integrin αIIb/β3 caused by enhanced inside-outside signal, and then increase fibrinogen binding force with platelet. Therefore, we used flow cytometry to measure platelet fibrinogen binding capability. Washed human platelets containing citrate were incubated with different concentrations of NaHS (0.1, 0.5, 1, 5 and 10 mmol/L), and then incubated with ADP (6.5 μmol/L). NaHS could decrease ADP-stimulated fibrinogen binding ca-

pability, with was consistent with decreased platelet aggregation by NaHS (**Figure 1F**). This suggests that hydrogen sulfide can interfere with inside-outside signal of integrin αIIb/β3 on the platelets.

Hydrogen sulfide demonstrates antithrombotic actions

Since hydrogen sulfide showed strong in vitro antiplatelet aggregation activity, we then explored the potential role of hydrogen sulfide on in vivo thrombus formation in a mouse DVT model. C57BL/6 mice were given intraperitoneal injection of NaHS solution (10 mmol/kg body weight) once a day, and DVT model was established using clamp method on the sixth day 2 to 4 hours after administration. We observed and analyzed the development of thrombosis after clamp, and found compared with control group, NaHS group showed slower development of venous thrombosis, with significantly smaller thrombus volume 48 hours after clamp ($P<0.05$). The decreased thrombus volume by NaHS could be partly attenuated by L-NAME (**Figure 2A**).

We further performed western blot to measure the protein expression of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) in endothelium of the DVT mice. NaHS group showed significantly higher eNOS and iNOS protein expression (about 50% higher expression level) than those of the control group. However, L-NAME treatment could significantly decrease eNOS and iNOS protein expressions compared with the control group and NaHS group (**Figure 2B**, **2C**).

Hydrogen sulfide enhances PECAM-1 expression in platelet and endothelium

In order to explore whether hydrogen sulfide can regulate PECAM-1 while inhibiting platelet function, we detected PECAM-1 protein contents in the platelets and endothelial cells. Western blot analysis showed that NaHS significantly increased PECAM-1 protein content of platelets in a concentration-dependent manner (**Figure 3A**). We also found that NaHS increased PECAM-1 protein expression in vein endothelium of DVT mice compared with the control group, which could be attenuated by L-NAME co-incubation (**Figure 3B**). This suggests that PECAM-1 might participate in the antiplatelet and antithrombotic effects of hydrogen sulfide. Meanwhile, up-regulation of PECAM-1 protein by hydrogen sulfide is dependent on the presence of nitric oxide.

In order to explore whether hydrogen sulfide regulate downstream signaling pathways of PECAM-1 protein, we analyzed effect of NaHS on platelet Akt (ser473) phosphorylation stimulated by ADP. NaHS inhibited the platelet phosphorylation of Akt (ser473) in a concentration dependent manner (**Figure 3C**). This suggests that PECAM-1 may be involved in antiplatelet and antithrombotic effects of hydrogen sulfide, and there is a potential H_2S-NO-PECAM-1-Akt signaling axis.

PECAM-1 is involved in antithrombotic function of hydrogen sulfide

In order to explore the role of PECAM-1 in antithrombotic effect of hydrogen sulfide, we applied siRNA oligonucleotides to inhibit the PECAM-1 expression in HUVEC cells. PECAM-1 protein expression was significantly decreased 48 hours after transfection (about 5% the expression level of control group) (**Figure 4A**). The results showed that ADP could significantly decrease HUVECs and platelets adhesion activity. Further study showed that siRNA-PECAM-1 can significantly weaken hydrogen sulfide platelet inhibition function in HUVEC cells (**Figure 4B**). This suggests that PECAM-1 is essential for platelet adhesion inhibition effect of hydrogen sulfide.

Discussion

In this study we have shown that hydrogen sulfide has inhibitory effects on in vitro platelet aggregation and in vivo thrombosis. The sodium hydrosulfide (NaHS), which releases hydrogen sulfide, could inhibit platelet aggregation stimulated by arachidonic acid (AA), thrombin receptor activating peptides (TRAP), adenosine phosphate (ADP) and collagen in a concentration-dependent manner. We found that NaHS could reduce ADP-stimulated fibrinogen binding capacity in a concentration-dependent manner, which is consistent with the inhibitory effect of NaHS on platelet aggregation. We establised a DVT mice model using clamp method, and found intraperitoneal injection of NaHS could delay the development of venous thrombosis and significantly reduced thrombus

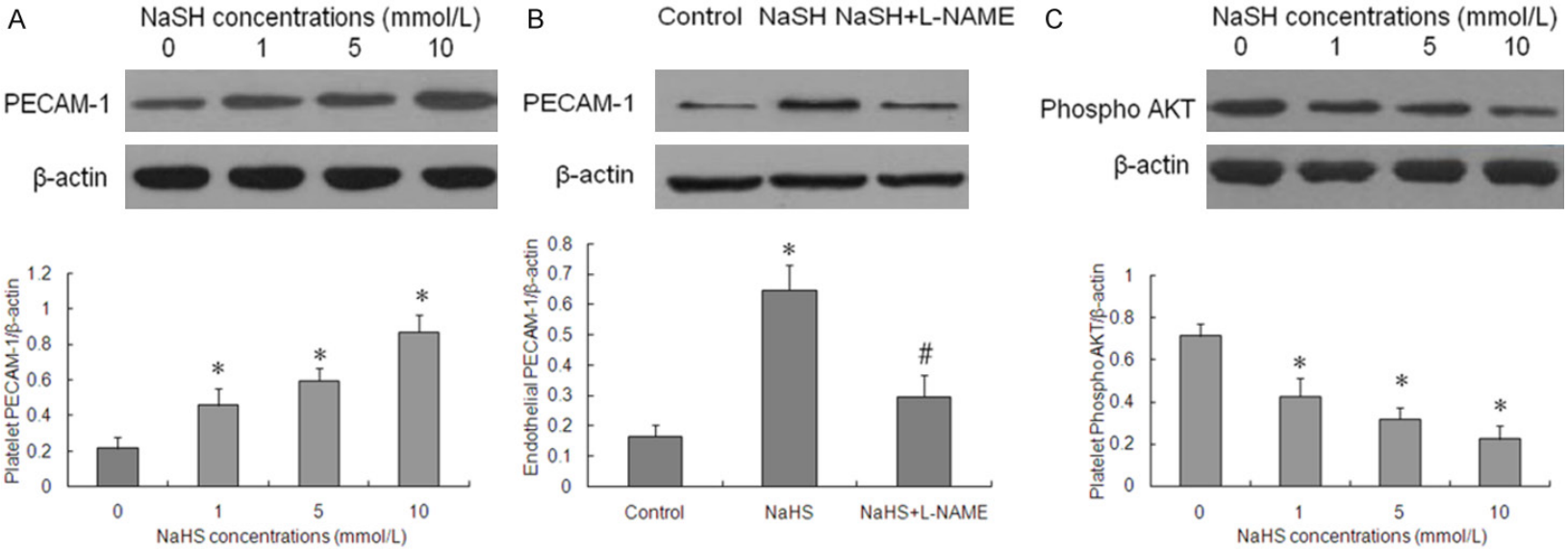

Figure 3. NaHS enhanced PECAM-1 expression in platelet and endothelium. A. NaHS enhanced platelet PECAM-1 protein expression. Washed human platelets were incubated with NaHS (1, 5 and 10 mmol/L) for 1 hour, and western blot analysis was performed to determin PECAM-1 protein content. NaHS significantly increased PECAM-1 protein expression in a concentration-dependent manner. B. NaHS enhanced the expression of PECAM-1 protein in mouse vein endothelial cells. Compared with the control group, NaHS group showed higher expression of PECAM-1 protein. The PECAM-1 level was significantly decreased after L-NAME treatment, but it was still higher than that in the control group. C. NaHS inhibited the expression of phosphorylated (Ser473) Akt protein in human platelets in a concentration dependent manner. *Compared with the control group, the difference was significant ($P<0.05$). #Compared with the NaHS group, the difference was significant ($P<0.05$). n = 10.

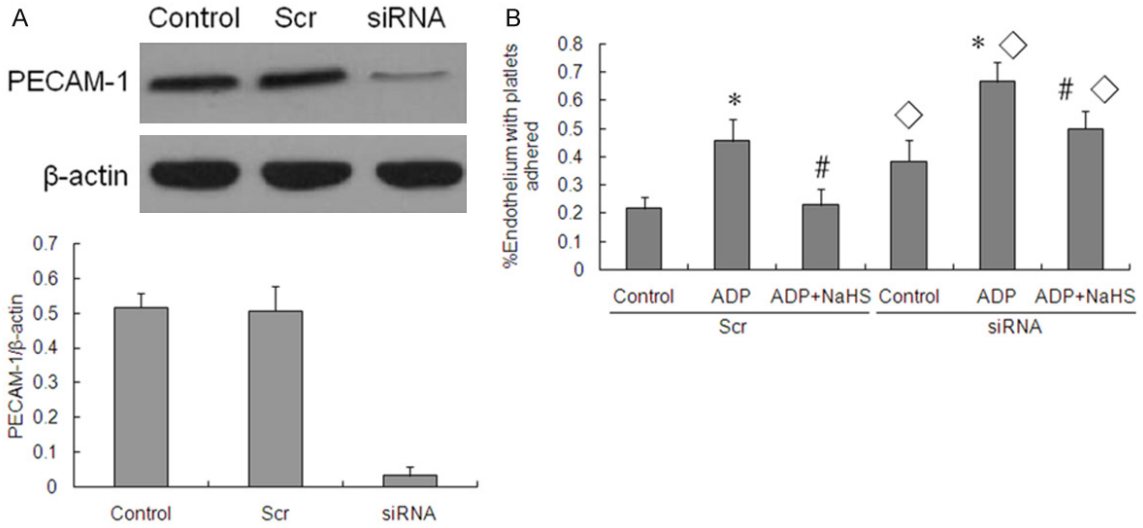

Figure 4. PECAM-1 is involved in antithrombotic function of NaHS. A. siRNA-PECAM-1 significantly inhibited the expression PECAM-1 protein in HUVEC cells. Western blot analysis was performed to measure PECAM-1 protein content 48 hours after transfection. PECAM-1 protein expression was significantly inhibited, with approximately 5% expression level of control group. Transfection with random sequence (Scr) had no effect on PECAM-1 protein expression. B. PECAM-1 gene silencing increased platelet adhesion with HUVEC cells. ADP can significantly increase the adhesion activity of HUVEC with platelet, and this effect can be weakened by NaHS treatment. siRNA-PECAM-1 can significantly suppress the inhibitory platelet adhesion with HUVEC cells by NaSH. Note: *Compared with the control group, the difference was significant ($P<0.05$). #Compared with the ADP group, the difference was significant ($P<0.05$)]. ◇Compared with the HUVEC transfected with Scr), the difference was significant ($P<0.05$). n = 5.

volume 48 hours after clamp. Inhibitory effects of hydrogen sulfide on platelet aggregation and thrombosis depend on the presence of nitric oxide, which is evidenced by the fact that L-NAME, a nitric oxide synthase inhibitor, could atenuate reduced platelet aggregation and thrombus formation by hydrogen sulfide. In DVT mice, NaHS enhanced the expression of two nitric oxide synthase eNOS and iNOS. NaHS enhanced human platelet PECAM-1 expression, and inhibited the phosphorylation of Akt (Ser473). NaHS also enhanced mice vein endothelial expression of PECAM-1 protein in a NO-dependent manner. In order to explore whether PECAM-1 is essential for platelet aggregation and thrombosis by hydrogen sulfide, we used siRNA technology to inhibit the expression of PECAM-1 in HUVEC. PECAM-1 gene silencing could enhance adhesion of activated platelets with HUVECs cells by NaHS. Our study suggests that there may be a H_2S-NO-PECAM-1 signal pathway, which is involved in inhibition of in vitro platelet aggregation and in vivo anti-thrombosis by hydrogen sulfide.

Our study showed that hydrogen sulfide has wide inhibitory effect on platelet aggregation, which is consistent with other researcher's reports [36, 38]. One important mechanism for the inhibition of platelet aggregation depends on inhibition of gap junction channels inside platelet [40]. The inside-outside signal of integrin αIIb/β3 leads to conformational change of platelet, and increases fibrinogen binding capacity with platelet, which is essential for platelet aggregation, thereby activating fibrinogen on platelets and forming thrombi [41]. The gap junction channels play an important role in the communication between platelets within thrombolus, and enhance fibrinogen binding capability, produce platelet secretory granules and promote clot retraction, thereby becoming a target for the treatment of thrombosis [42]. Our study showed that hydrogen sulfide inhibits fibrinogen binding capacity with platelet, which supports other experimental results that hydrogen sulfide inhibits platelet gap junction channels [40]. In fact, platelets treated wth gap junction channels blocker showed similar results compared with hydrogen sulfide, such as suppressed platelet aggregation and reduced fibrinogen binding [42, 43]. Due to the fact that the gap junction channels can maintain homostasis of platelet and thrombolus, we speculate that inhibition of gap junction channels by hydrogen sulfide may also

contribute to the inhibition of progression from platelet aggregation to thrombosis, thereby play a role in in vivo anti-thrombosis. Our study found that hydrogen sulfide also showed antithrombotic effect in vivo, which is consistant with the in vitro platelet inhibition effect. Since most deep vein thrombosis occurs in large vessels, we chose mouse femoral vein to establish DVT model by clamp method. NaHS-treated mice showed smaller size of thrombus in the femoral vein, and this suggests potential clinical application value.

We then explored the downstream pathway of hydrogen sulfide on platelet aggregation and thrombosis, and found that inhibition of in vitro platelet aggregation and in vivo thrombosis by hydrogen sulfide is nitric oxide dependent. In DVT mice, hydrogen sulfide could also upregulate the protein expression of two kinds of nitric oxide synthase, eNOS and iNOS. As a physiological signal molecule, nitric oxide has wide antithrombotic effects. Endogenous and exogenous nitric oxide can both play important role in anti-thrombosis by directly inhibiting platelet aggregation [44]. In DVT rats, veneous nitric oxide released from lipid microbubbles can accelerate the dissolution of thrombosis [45]. Many experimental results confirmed that there exist complicated interactions between hydrogen sulfide and nitric oxide in the regulation of cellular function, cardiovascular function and inflammatory reaction [46, 47]. Furthermore, in the regulation of angiogenesis and endothelial dependent vasodilation, there is mutual dependence and synergy between hydrogen sulfide and nitric oxide, so blocking any one gas molecules can inhibit the vascular biological function produced by the other gas molecule [48]. The mechanism underlying mutual dependence between hydrogen sulfide and nitric oxide may lie in cystathionine γ-lyase (CSE), an endogeneous synthetase of hydrogen sulfide. The cysteine residues of CSE enzyme is a potential target of nitric oxide S-nitrosylation, thereby enhancing the activity of CSE [49]. In addition to the direct effect on CSE protein, nitric oxide also can stimulate CSE activity through enhancing cGMP-dependent kinase activity [50]. In contrast, hydrogen sulfide can promote the Akt kinase-dependent phosphorylation of eNOS (Ser1177) and increase nitric oxide production in endothelial cells [51]. Hydrogen sulfide can also enhance the interleukin-1β (IL-1β) induced nuclear factor (NF κB) activation, and increase iNOS protein expression and nitric oxide production in rat vascular smooth muscle cells [52]. Our results showed that hydrogen sulfide could upregulate the expression of vascular endothelial eNOS and iNOS proteins in DVT mice, which is consistant with the resutls from Na2S, another hydrogen sulfide donor [53]. This suggests that nitric oxide signaling pathway lie in the downstream of hydrogen sulfide. In our experiments the hydrogen sulfide is exogenous, and L-NAME inhibited the generation of nitric oxide and inhibited antithrombotic effect of hydrogen sulfide. However, it is unable to determine whether nitric oxide has effect on endogenous hydrogen sulfide which needs to be confirmed by further experiments, such as detection of CSE expression in mice vascular endothelial cells.

Our study showed that hydrogen sulfide can elevate the expression of PECAM-1 protein in human platelet and DVT mouse endothelium. As a kind of cell adhesion molecule, PECAM-1 is mainly expressed in leukocytes, platelets and endothelial cells, and involved in the maintenance of endothelial cell integrity and platelet function. In the process of thrombosis, PECAM-1 can inhibit collagen and platelet reactivity stimulated by ADP and laminin [54, 55]. PECAM-1 gene knockout mice showed greater and more stable thrombus, suggesting that PECAM-1 also has a role in inhibition of in vivo thrombosis [17]. Our results also showed enhanced expression of vein endothelial PECAM-1 protein by hydrogen sulfide, and this suggests PECAM-1 may be involved in the anti-thrombosis effect of hydrogen sulfide. Mealwhile, L-NAME treatment can partially inhibit the increase of PECAM-1 protein by hydrogen sulfide, and this suggests that in addition to direct anti-thrombosis role, nitric oxide signaling pathway is also essential for PECAM-1 activation and its antithrombotic effect. Therefore, there might be a H_2S-NO-PECAM-1 signal axis in antithrombotic effects of hydrogen sulfide. Nitric oxide signaling molecules is necessary for PECAM-1 expression and is involved in LPS-induced endothelial PECAM-1 expression [56]. Blocking calcium-dependent nitric oxide synthase could affect the expression of endothelial PECAM-1 [57]. Nitric oxide also parcitipates in elevated PECAM-1 expression in human endothelial cells by fluvastatin, and its mechanism lies in the fact that fluvastatin can upregulate eNOS protein phosphorylation (Ser1177) and activation

[58]. Recently many reports have demonstrated that statins can inhibit thrombosis and shown significant clinical effects in DVT patients [59-61], and its antithrombotic mechanism is related to enhanced PECAM-1 expression [62]. Considering the same molecular target (eNOS (Ser1177) phosphorylation) between hydrogen sulfide and statins, we speculate that hydrogen sulfide and statins share the same signal pathway in activating PECAM-1 protein, thereby playing antithrombotic effect. Our study has shown that hydrogen sulfide can increase endothelial eNOS protein expression in DVT mice and inhibit thrombosis in a NO-dependent manner, but whether there is ser1177 phosphorylation of eNOS protein needs further investigation. We also explored whether hydrogen sulfide could regulate the downstream signaling pathways of PECAM-1 protein. Results showed that NaHS can inhibit platelet phosphorylation of Akt (Ser473). Activation of PI3K/Akt signal pathway is involved in platelet aggregation stimilated by a variety of factors [63-65]. In platelets, activated PECAM-1 can lead to its tyrosine phosphorylation, and recruit protein tyrosine phosphatase SHP-2, thereby inhibiting downstream Akt kinase activity [66, 67].

We have applied siRNA technology to inhibit PECAM-1 expression to explore whether PECAM-1 is essential for antithrombotic effects by hydrogen sulfide. HUVEC cells with silenced PECAM-1 showed significantly increased adhesion activity with platelet, and attenuated the platelet inhibitory effect by NaHS. Our results support the report that PECAM-1 knockout mice have greater volume of thrombus [17]. Mealwhile, in HUVEC cells transfected with siRNA-PECAM-1, hydrogen sulfide can also inhibit ADP-induced platelet adhesion, and this suggests there are pathways in adition to PECAM-1 in inhibition of platelet adhesion, such as nitric oxide.

In conclusions, our results show that hydrogen sulfide has inhibitory role in vitro platelet aggregation and in vivo thrombosis, and the mechanism may be related to enhanced expression of eNOS, iNOS and PECAM-1 proteins. PECAM-1 protein is esential for platelet inhibitory effect and antithrombosis by hydrogen sulfide, and may be a downstream therapeutic target for DVT. Our study suggests that there may be a H_2S-NO-PECAM-1 signal pathway, which may link up in vitro platelet inhibitory effect with in vivo antithrombotic effect.

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Disclosure of conflict of interest

None.

Authors' contribution

Gang Li analyzed the data and wrote the manuscript; Tao Xia and Xin Liu performed all the experiments; Xiang-Qian Kong analyzed the data; Yang Liu designed the study and revised the manuscript.

Abbreviations

ADP, adenosine diphosphate; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; DVT, deep vein thrombosis; eNOS, endothelial nitric oxide synthase; FITC, fluorescein isothiocyanate; GPVI, glycoprotein VI; H_2S, hydrogen sulfide; HO-1, heme oxygenase-1; iNOS, inducible nitric oxide synthase; ITIMs, immunoreceptor tyrosine-based inhibitory motifs; L-NAME, NG-nitro-L-arginin-methylester; 3-MST, 3-mercaptopyruvate sulfurtransferase; NaHS, sodium hydrosulfide; NF-κB, nuclear factor-κB; NO, nitric oxide; PECAM-1, platelet endothelial cell adhesion molecule-1; SHP-2, protein tyrosine phosphatase-2; siRNA, small interfering RNA; TRAP, thrombin receptor activating peptide.

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