Role of hydrogen sulfide in sulfur dioxide production and vascular regulation

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Abstract

Both hydrogen sulfide (H\textsubscript{2}S) and sulfur dioxide (SO\textsubscript{2}) are produced endogenously from the mammalian metabolic pathway of sulfur-containing amino acids and play important roles in several vascular diseases. However, their interaction during the control of vascular function has not been fully clear. Here, we investigated the potential role of H\textsubscript{2}S in SO\textsubscript{2} production and vascular regulation \textit{in vivo} and \textit{in vitro}. Wistar rats were divided into the vehicle, SO\textsubscript{2}, DL-propargylglycine (PPG) + SO\textsubscript{2}, \beta-cyano-L-alanine (BCA) + SO\textsubscript{2} and sodium hydrosulfide (NaHS) + SO\textsubscript{2} groups. SO\textsubscript{2} donor was administered with or without pre-administration of PPG, BCA or NaHS for 30 min after blood pressure was stabilized for 1 h, and then, the change in blood pressure was detected by catheterization via the common carotid artery. Rat plasma SO\textsubscript{2} and H\textsubscript{2}S concentrations were measured by high performance liquid chromatography and sensitive sulfur electrode, respectively. The isolated aortic rings were prepared for the measurement of changes in vasorelaxation stimulated by SO\textsubscript{2} after PPG, BCA or NaHS pre-incubation. Results showed that the intravenous injection of SO\textsubscript{2} donors caused transient hypotension in rats compared with vehicle group. After PPG or BCA pre-treatment, the plasma H\textsubscript{2}S content decreased but the SO\textsubscript{2} content increased markedly, and the hypotensive effect of SO\textsubscript{2} was significantly enhanced. Conversely, NaHS pretreatment upregulated the plasma H\textsubscript{2}S content but reduced SO\textsubscript{2} content, and attenuated the hypotensive effect of SO\textsubscript{2}. After PPG or BCA pre-incubation, the vasorelaxation response to SO\textsubscript{2} was enhanced significantly. While NaHS pre-administration weakened the SO\textsubscript{2}-induced relaxation in aortic rings. In conclusion, our \textit{in vivo} and \textit{in vitro} data indicate that H\textsubscript{2}S negatively controls the plasma content of SO\textsubscript{2} and the vasorelaxant effect under physiological conditions.

Introduction

Sulfur dioxide (SO\textsubscript{2}) was previously considered a toxic gas, but it has been proven that it can be endogenously produced from the metabolism of sulfur-containing amino acids, with L-
cysteine as a substrate and catalyzed by aspartate aminotransferase (AAT) [1,2]. Our previous studies revealed the existence of the SO$_2$/AAT pathway in arteries and its vasodilator function [3]. Studies have also confirmed its effect on vascular function [4]. Supplementation with SO$_2$ donors could protect against various cardiovascular diseases. Emerging evidence indicated that endogenous SO$_2$ is a new gasotransmitter involved in cardiovascular regulation. Interestingly, in a variety of pathological models, including atherosclerosis [5], pulmonary hypertension [6], and myocardial ischemia-reperfusion injury [7], SO$_2$ was found to affect the production of another gasotransmitter, hydrogen sulfide (H$_2$S). However, the interaction between H$_2$S and SO$_2$ under physiological conditions is largely unknown.

L-Cysteine is used as a substrate to produce the endogenous H$_2$S in the cardiovascular system through cystathionine-γ-lyase (CSE) [8–11]. Endogenous H$_2$S not only has physiological functions, such as vasorelaxation, but also has pathophysiological effects, including the inhibition of hypertension [10,12–17]. Recently, we showed that in a monocrotaline-induced pulmonary hypertensive rat model, the H$_2$S pathway in pulmonary artery endothelial cells is damaged, which reduces the sulfhydration of AAT, thus enhancing AAT activity and increasing SO$_2$ production [18]. However, it still unclear whether H$_2$S affects the SO$_2$ pathway under physiological conditions.

As mentioned above, the importance of H$_2$S and SO$_2$ for the modulation of blood pressure and arterial tension has been gradually revealed, but their interaction in the control of blood pressure and vascular function has not been clear. Thus, our research aimed at exploring the possible role of H$_2$S in SO$_2$ production and vascular regulation under physiological conditions.

Materials and methods

Reagents

Acetylcholine chloride (ACH) and phenylephrine (PE) were from Beijing Chemical Reagent Company and Tianjin Amino Acid Company in China, respectively. Sodium sulfite and sodium bisulfite (Na$_2$SO$_3$/NaHSO$_3$, the SO$_2$ donor), DL-propargylglycine (PPG, a selective CSE inhibitor), β-cyano-L-alanine (BCA, another CSE inhibitor), and sodium hydrosulfide (NaHS, the H$_2$S donor) were from Sigma, USA. Since SO$_2$, HSO$_3^-$ and HSO$_3^{2–}$ can be transformed into each other in biological system [1], NaHSO$_3$/Na$_2$SO$_3$ was used as the SO$_2$ donor (S1 Fig). NaHSO$_3$ and Na$_2$SO$_3$ were dissolved in deionized water at a molar ratio of 1:3. The fresh SO$_2$ donor stock solution was then diluted with bath solution to obtain a series of working solutions with different concentrations. The handling and properties of NaHS were given in a brochure by Stauffer (1974). It is produced by the absorption of H$_2$S in sodium hydroxide and shipped as a 45% solution with a specific gravity of 1.303 and a pH of 10.4 [19]. NaHS powder was rapidly dissolved in normal saline (0.9%) to obtain the desired concentration of stock solutions (pH 7.4) which was immediately injected intravenously into the right external iliac vein of rats or added into the organ bath solution of aortic rings (37°C). Previous study showed that given a physiological pH around 7.4 and temperature of 37°C, NaHS solution will yield about one-third of the undissociated H$_2$S gas and the other two-thirds remain as HS$^–$ [20]. The composition of the Krebs' solution with pH 7.2–7.4 was as follows: NaCl (120 mmol/L), KCl (5.5 mmol/L), NaHCO$_3$ (20 mmol/L), CaCl$_2$ (2.5 mmol/L), MgCl$_2$·6H$_2$O (1.2 mmol/L), NaH$_2$PO$_4$ (1.2 mmol/L), EDTA-Na$_2$ (0.03 mmol/L) and glucose (10 mmol/L).

Animal experiment

Animal care and operation procedures were carried out strictly in accordance with the Animal Management Rule of the Ministry of Health of the People’s Republic of China (Documentation
The protocol was approved by the Animal Care Committee of Peking University First Hospital (Protocol Number: J202044) and conformed to the ARRIVE guidelines (https://journals.plos.org/plosbiology/article?id=10.1371/journal.pbio.3000411). Forty male Wistar rats (body weight, 200±5 g) were purchased from the Experimental Animal Center, Peking University Health Science Center (Beijing, China). They were housed at a constant temperature of 25˚C under a 12-h light-dark cycle and maintained on ad libitum food and water. Rats were monitored twice daily for health status and husbandry conditions. In the health monitoring, we closely observed the food consumption, water intake, body weight and general assessment of rat activity, panting, and fur condition of the rats. During the whole experiment, intraperitoneal injection of sodium pentobarbital (45 mg/kg) was used for anesthesia and supplemented with an additional dose of 10 mg/kg. All efforts were made to minimize suffering. They were randomly assigned to five groups (n = 8 each) as follows: (1) vehicle group where the rats were intravenously injected with the equal volume of physiological saline; (2) SO₂ group where the rats were intravenously injected with Na₂SO₃/NaHSO₃ (40 μmol/kg, pH 7.4); (3) PPG + SO₂ group where the rats were given PPG (30 mg/kg) intravenously and then Na₂SO₃/NaHSO₃ (40 μmol/kg) 30 min later; (4) BCA + SO₂ group where the rats were given BCA (50 mg/kg) intravenously and then Na₂SO₃/NaHSO₃ (40 μmol/kg) 30 min later; (5) NaHS + SO₂ group where the rats were given NaHS (56 μmol/kg) [21,22] intravenously and then Na₂SO₃/NaHSO₃ (40 μmol/kg) [23,24] 30 min later. PPG (30 mg/ml), BCA (50 mg/ml), NaHS (56 μmol/ml) or Na₂SO₃/NaHSO₃ (40 μmol/ml) in a final volume of 200 μl was injected intravenously into the femoral vein within a minute. After the experiment, the rats were euthanized with intravenous injection of an overdose of sodium pentobarbital.

### Preparation of rat model and measurement of blood pressure

Two polyethylene catheters were inserted into the left common carotid artery (LCCA) and the right external iliac vein. The mean blood pressure (MBP) was measured via the LCCA, and the extracorporeal end of the catheter was connected to a pressure sensor and PowerLab Software for recording blood pressure and respiratory conditions. (BL-410, Chengdu TME Technology, China) [25]. The extracorporeal end of catheter in the right external iliac vein was connected to a 5 ml syringe for intravenous bolus injection of chemicals and collection of venous blood samples.

### Measurement of plasma H₂S and SO₂ content

Plasma sulfide levels were detected using a free radical analyzer TBR4100 with an H₂S-selective sensor (World Precision Instruments, China) to reflect H₂S content as previously described [26]. Briefly, the electrode was activated in deionized water for more than 2 h. The measured item of this analyzer was set to millivolt. The sensitive sulfur electrode was immersed into 1 mL of sample, so was the reference electrode. And the millivolt value was recorded after the reading was stable. Plasma SO₂ concentration was detected by HPLC [3]. Briefly, the sulfite in the sample was reduced to a sulfhydryl compound by the addition of sodium borohydride. Then, it was combined with monobromobimane, and perchloric acid was added to remove the protein in the sample, which was neutralized by use of Tris-HCl (pH 3.0). Small sulfhydryl molecules were separated from other fractions through chromatographic analysis and determined with a fluorescence detector.

### AAT activity assay

Purified AAT protein (Roche Diagnostics, Mannheim, Germany) was incubated with or without PPG (100 μmol/L) for 30 min at 37˚C in PBS buffer. After incubation, the activity of AAT
was measured using AAT Assay Kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions and was expressed in Carmen’s Unit.

**Preparation of rat aortic rings**

Thoracic aortas were rapidly isolated from anesthetized male Wistar rats (n = 8 per group) with the removal of adherent adipose and connective tissue. Each aorta was cut into 3–4 aortic rings of 3 mm in length and immersed in Krebs’ buffer at 4˚C. During the whole process, artificially overstretching blood vessels was avoided to maintain their activity.

**Measurement of the rat aortic contractility**

The aortic rings were immersed into organ baths containing oxygenated Krebs’ buffer at 37.5˚C, fixed, and connected with a tension sensor. The latter was then connected with a multi-channel physiological recorder to record the tension value and display them through PowerLab software [3]. The rings were first stretched to a tension of 1 g (international unit 9.8×10^{-3} Newton) and equilibrated for 60 min. During this period, the incubation solution was replaced every 15 min. After the tension stabilized, the aortic rings were ready for testing.

To confirm the vascular reactivity, the aortic rings were first treated with PE (1 μmol/L) for contraction. After reaching equilibrium, they were treated with ACH (1 μmol/L) for relaxation. Those with good reactivity were used for the following procedures. After the peak of relaxation, the rings were rinsed with Krebs’ buffer three times. When the tension of the rings became stable again and returned to the initial level, the aortic rings were incubated with vehicle, PPG (100 μmol/L), BCA (100 μmol/L) or NaHS (50 μmol/L) for 10 min, and then PE (3 μmol/L) was added to make the rings in precontraction state. After that, a dose-response to \( \text{Na}_2\text{SO}_3/\text{NaHSO}_3 \) (50–1000 μmol/L) in aortic rings was detected. Relaxation was expressed as the percentage reduction of the maximum precontraction achieved by PE.

In the experiment of measuring vasoconstriction response, the rings were first stretched to a tension of 1 g, equilibrated for 20 min, and then contracted with 60 mmol/L KCl for 15 min to test its contractility. After rinsing with Krebs’ buffer three times and the tension stabilized, the rings were incubated with \( \text{Na}_2\text{SO}_3/\text{NaHSO}_3 \) (1 mmol/L) or PPG (100 μmol/L) plus \( \text{Na}_2\text{SO}_3/\text{NaHSO}_3 \), and subsequently a dose-response to PE was detected. Contraction was expressed as the percentage of its peak contraction with 60 mmol/L KCl.

**Measurement of plasma nitric oxide (NO) content**

The plasma NO content was detected using the NO assay kit (Applygen, Beijing, China). Briefly, 50 μL of standard and plasma sample were added to a 96-well plate and 50 μl of Griess R1 solution was then added to these wells and incubated at 37˚C for 2 h. After that, 50 μl of Griess R2 solution was added and incubated in dark at 37˚C for 5 min, and the absorbance values were measured at 540 nm.

**Western blot**

The aortic tissues were homogenated with lysis buffer containing protease inhibitors and phosphatase inhibitors. Then, the supernatants were obtained by centrifuging at 12000 g for 20 min. An equal amount of aortic protein was isolated by SDS-PAG electrophoresis and transferred to nitrocellulose (NC) membranes. The NC membranes were blocked using 5% skim milk and then incubated with heme oxygenase (HO)-1 antibody (diluted 1:1000, Enzo Life Sciences, Farmingdale, NY, USA), HO-2 antibody (diluted 1:1000, Enzo Life Sciences), endothelial NO synthase (eNOS) antibody (diluted 1:1000, Cell Signaling Technology, Danvers, MA,
USA), and β-tubulin (diluted 1:5000, Beyotime Biotechnology, Beijing, China) overnight, respectively. After that, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich Corporation, St Louis, MO, USA). The bands were detected with a chemiluminescence detection system (ProteinSimple, San Francisco, CA, USA).

Statistics
SPSS 15.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. Data are expressed as the mean±SEM. A Student’s t-test was performed to compare differences between two groups. Paired-sample t-test was performed to compare the changes of plasma H\textsubscript{2}S and SO\textsubscript{2} contents in the rats before and after treatment. One-way ANOVA followed by least significant difference (LSD) test was performed to compare differences among multiple groups. P values less than 0.05 were considered significant.

Results

\textbf{H\textsubscript{2}S inhibits the plasma levels of SO\textsubscript{2} in vivo}

To investigate the effect of H\textsubscript{2}S on SO\textsubscript{2} level in vivo, the rats were intravenously injected with PPG, BCA or NaHS to downregulate or upregulate H\textsubscript{2}S level. As shown in Table 1, after intravenous injection of PPG (30 mg/kg), the rat plasma H\textsubscript{2}S concentration was decreased significantly (from 20.22±0.31 μmol/L to 11.65±0.47 μmol/L), whereas the plasma SO\textsubscript{2} content was increased notably (from 15.33±0.72 μmol/L to 21.55±2.41 μmol/L). In rats injected with BCA, another CSE inhibitor, we observed similar findings as in PPG-treated rats. Conversely, rats injected with NaHS to increase plasma H\textsubscript{2}S content (from 20.60±0.40 μmol/L to 34.75±1.47 μmol/L) exhibited a reduced plasma SO\textsubscript{2} content (from 15.16±0.65 μmol/L to 12.75±1.12 μmol/L). To explore whether PPG had any direct effect on SO\textsubscript{2} production, the purified SO\textsubscript{2} synthase AAT protein was treated with or without PPG in PBS buffer. The results showed that there was no difference in the activity of the purified AAT protein between the control group and the PPG group (Fig 1), implying that PPG had no direct effect on the SO\textsubscript{2} production. These data suggest that H\textsubscript{2}S negatively regulates the plasma levels of SO\textsubscript{2}.

\textbf{H\textsubscript{2}S attenuated the hypotensive effect of SO\textsubscript{2} in vivo}

To explore the significance of SO\textsubscript{2} level inhibition by H\textsubscript{2}S, the rats were intravenously injected with physiological saline, PPG, BCA or NaHS for 30 minutes, followed by SO\textsubscript{2} derivatives (Na\textsubscript{2}SO\textsubscript{3}/NaHSO\textsubscript{3}) injection, and the changes of their blood pressure were monitored in real time (Fig 2A). Injection of physiological saline did not affect MBP of rats in vehicle group (Fig 2B). However, the MBP of rats in the SO\textsubscript{2} group dropped by 22.55% within 1.5 minutes

Table 1. Plasma contents of H\textsubscript{2}S and SO\textsubscript{2} in rats before and after treatment with PPG, BCA or NaHS.

| Groups | H\textsubscript{2}S content (μmol/L) | SO\textsubscript{2} content (μmol/L) |
|--------|---------------------------------|---------------------------------|
|        | 0 min                           | 5 min                           | 30 min                           | 0 min                           | 30 min                           |
| PPG    | 20.22±0.31                      | –                               | 11.65±0.47*                      | 15.33±0.72                      | 21.55±2.41*                      |
| BCA    | 20.63±0.34                      | –                               | 11.00±0.83*                      | 12.48±0.30                      | 20.58±1.52*                      |
| NaHS   | 20.60±0.40                      | 34.75±1.47*                     | 20.94±0.31                      | 15.16±0.65                      | 12.75±1.12*                      |

Data expressed as mean ± SEM; n = 8 in each group; paired-sample t-test
*P<0.05 compared with the plasma H\textsubscript{2}S content of rats before treatment in the corresponding group
*P<0.05 compared with the plasma SO\textsubscript{2} content of rats before treatment in the corresponding group. PPG, DL-propargylglycine; BCA, β-cyano-L-alanine; NaHS, sodium hydrosulfide.

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after the intravenous injection of SO$_2$ derivatives; then, the MBP began to rise, and returned to the basal level 10 minutes after injection of SO$_2$ derivatives (Fig 2B). The rat MBP in the SO$_2$ group was significantly lower than that in the vehicle group within 1.5 minutes after the injection of SO$_2$ derivatives (Fig 2C). In the PPG+SO$_2$ group, there was no significant difference in MBP between the baseline and 30 minutes after administration of PPG; the MBP dropped by 32.19% within 1.5 min after SO$_2$ derivatives injection; then it began to rise, and returned to baseline 10 minutes after SO$_2$ derivatives injection (Fig 2B). The decrease in MBP of the PPG +SO$_2$ group was markedly greater than that of the SO$_2$ group within 1.5 minutes after SO$_2$ derivatives injection (Fig 2C). Similarly, the administration of rats with BCA, another CSE inhibitor, also exacerbated the hypotensive effect of SO$_2$. The MBP was significantly decreased

Fig 1. PPG had no direct effect on AAT activity. Purified AAT protein was incubated with or without PPG (100 μmol/L) for 30 min at 37˚C. Data are presented as mean ± SEM; n = 9 in each group; Student’s t-test; ns, not significant.
within 1 minute after H_2S donor NaHS injection (S2 Fig) and then returned to the basal level within 30 minutes (Fig 2B). Although pretreatment of rats with NaHS caused a decrease in MBP within 1–3 minutes after the injection of SO_2 derivatives, the decrease in MBP was significantly smaller than that of the SO_2 group (Fig 2). These data suggest that H_2S could inhibit the hypotensive effect of SO_2.

**H_2S negatively controls the vasorelaxant effect of SO_2 in vitro**

Vasorelaxation of arteries could result in a reduction in blood pressure. SO_2 derivatives (50 to 1000 μM) exerted a concentration-dependent dilatory effect in the aortic rings precontracted...
with 3 μmol/L PE (Fig 3). SO₂ derivatives-elicited vasodilation was significantly increased in the aortic rings preincubated with either PPG or BCA but decreased in aortic rings pretreated with NaHS (Fig 3). In addition, there was no significant difference in PE-elicited constriction in aortic rings between SO₂ group and PPG+SO₂ group (Fig 4). These results demonstrate that
H₂S might inhibit the decrease of vascular tone caused by SO₂ through weakening the SO₂-induced vasodilation, thus attenuating the transient hypotensive effect of SO₂.

**NO/eNOS and CO/HO pathway did not change during the regulation of H₂S on SO₂**

To investigate whether NO and CO are involved in the regulation of H₂S on SO₂ action, we detected the plasma NO content and the expressions of NO producing enzyme eNOS and CO producing enzymes HO-1 and HO-2 in the rat aortic tissues. The data showed that the plasma NO content (Fig 5A) and the protein expressions of aortic eNOS (Fig 5B), HO-1 (Fig 5C) and HO-2 (Fig 5D) had no significant change at 1.5 minute after SO₂ derivatives injection.

![Fig 5](https://doi.org/10.1371/journal.pone.0264891.g005)

**Fig 5. NO/eNOS and CO/HO pathway did not change during the regulation of H₂S on SO₂.** Rats were intravenously injected with PPG (30 mg/kg), BCA (50 mg/kg), NaHS (56 μmol/kg) or equal volume of physiological saline. After 30 minutes, the rats were given an intravenous injection of Na₂SO₃/NaHSO₃ (40 μmol/kg). Blood samples and aortic tissues were collected after 1.5 minutes. (A) Plasma NO content in each group. (B-D) Representative western blots and quantitative analysis of eNOS (B), HO-1 (C) and HO-2 (D) in the aortic tissues of each group. Data are presented as mean±SEM; n = 6–7 in each group; One-way ANOVA followed by LSD post hoc test.

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compared with the vehicle group. PPG, BCA or NaHS pretreatment for 30 minutes also did not change the effect of SO\(_2\) on these indexes (Fig 5). These results suggest that NO and CO pathway might not be involved in the regulation of H\(_2\)S on SO\(_2\) action.

**Discussion**

Our study, for the first time, confirmed that the interaction between H\(_2\)S and SO\(_2\) controls the basal blood pressure and vascular tone *in vivo* and *in vitro*. H\(_2\)S negatively controls the plasma contents of SO\(_2\) and its vasorelaxant effect.

Both H\(_2\)S and SO\(_2\) can be generated endogenously through the metabolic pathway of sulfur-containing amino acids in mammals [2,27–31]. Under certain biochemical conditions, H\(_2\)S and SO\(_2\) can be converted to each other in cells. For example, neutrophils convert H\(_2\)S to SO\(_2\) through oxidative stress [32]. H\(_2\)S is oxidized by sulfide oxidase to generate thiosulfate, which is further converted to SO\(_2\) in the presence of thiosulfate sulfurtransferase [1]. In addition to metabolic pathways, crosstalk between these two gasotransmitters has also been observed in monocrotaline-induced pulmonary hypertensive rat model, where H\(_2\)S was found to inhibit SO\(_2\) generation from lung tissues [18]. However, it was not clear whether there is a possible interaction between H\(_2\)S and SO\(_2\) under physiological conditions. In this study, after the intravenous injection of PPG or BCA to inhibit endogenous H\(_2\)S generation, rat plasma H\(_2\)S levels decreased but SO\(_2\) levels increased. Conversely, injection of H\(_2\)S donor NaHS to upregulate H\(_2\)S levels could downregulate plasma SO\(_2\) contents. These results indicate that H\(_2\)S inhibits the plasma levels of SO\(_2\), so as to maintain a low concentration of SO\(_2\) under physiological conditions. As for the mechanism by which H\(_2\)S inhibits SO\(_2\) levels, a previous study showed that H\(_2\)S could sulfhydrate AAT, a key enzyme catalyzing SO\(_2\) generation, to suppress its activity, thus inhibiting endogenous SO\(_2\) production and content [18].

Both H\(_2\)S and SO\(_2\) are important gasotransmitters and play important roles in cardiovascular system [33,34]. Studies have shown that the administration of H\(_2\)S or SO\(_2\) donors antagonizes hypertension in a variety of hypertensive animal models including spontaneously hypertensive rats and angiotensin II-induced hypertensive mice [35–37]. In this study, MBP of rats was decreased rapidly after the intravenous injection of the SO\(_2\) donor (40 \(\mu\)mol/kg), and then returned to normal levels. This suggests that SO\(_2\) reduces blood pressure under physiological conditions, and this hypotensive effect is rapid and transient. However, the role of H\(_2\)S in the regulation of basal blood pressure by SO\(_2\) is still unclear. In this study, after the intravenous injection of PPG or BCA to inhibit endogenous H\(_2\)S generation, the plasma H\(_2\)S level was decreased, the SO\(_2\) level increased, and the hypotensive effect induced by SO\(_2\) was markedly promoted. Conversely, injection of NaHS to increase H\(_2\)S level and decrease SO\(_2\) level could attenuate the hypotensive effect of SO\(_2\). These results suggest that the interaction between H\(_2\)S and SO\(_2\) is important for the maintenance of physiological blood pressure.

It is known that the vasorelaxant effect of SO\(_2\) is one of the important mechanisms by which this compound exerts antihypertensive effect. The present study also showed that the treatment with SO\(_2\) donor could relax aortic rings. However, the role of H\(_2\)S in the regulation of vascular tension by SO\(_2\) remains unclear. In this study, the pretreatment of aortic rings with PPG or BCA to suppress endogenous H\(_2\)S generation could promote the vasorelaxation of SO\(_2\). While, pre-incubation of aortic rings with NaHS to increase H\(_2\)S level could attenuate the vasorelaxation of SO\(_2\). These results suggest that and H\(_2\)S negatively controls the vasorelaxant effect of SO\(_2\). Previous studies have reported that the K\(_{ATP}\) channel, calcium channel, and cGMP signaling are all involved in the vasorelaxant effect of H\(_2\)S and SO\(_2\) [38–43]. These targets and signaling pathways might be responsible for the biological effects of sulfur-containing gasotransmitter networks. For example, both H\(_2\)S and SO\(_2\) increase cGMP content. Although
H$_2$S does not directly activate soluble guanylate cyclase (sGC), it strongly inhibits phosphodiesterase (PDE) 5A to delay cGMP degradation [39]. SO$_2$ not only promotes the formation of a heterodimer of sGC α and β subunits to activate sGC and promote cGMP synthesis but also inhibits PDE activity to suppress cGMP degradation, thus increasing the cGMP content [44]. The promotion of these two gasotransmitters at the cGMP level activates PKG signaling and induces vasodilatory effect. However, SO$_2$ and H$_2$S would not work alone, but rather H$_2$S controls SO$_2$ action, which might prevent excessive cGMP production. However, the possible comprehensive effects of the interaction between H$_2$S and SO$_2$ on these targets remain unknown, which merits further studies.

Previous studies showed that SO$_2$ supplementation for 8 weeks upregulated plasma NO/eNOS pathway in the atherosclerotic rats [5], while H$_2$S treatment for 2 h suppressed this pathway in the rat aortic tissues [45]. SO$_2$ treatment for 24 h elevated the level of HO-1, producer of the gasotransmitter CO, in human skin keratinocytes [46], while blocking H$_2$S production with PPG for 1 week induced HO-1 in rats [47]. In the present study, we found that that the plasma NO content and the expressions of aortic eNOS, HO-1 and HO-2 had no significant change at 1.5 minute after SO$_2$ injection. PPG, BCA or NaHS pretreatment for 30 minutes also did not change the effect of SO$_2$ on these indexes, suggesting that NO and CO pathway might not be involved in the regulation of H$_2$S on SO$_2$ action. Differences under experimental conditions might contribute to the discrepancy between our results and those reported in the literatures.

Although PPG is usually used as an inhibitor of H$_2$S production, it is unspecific [48]. It also inhibits other pyridoxal phosphate (PLP) enzymes. In the present study, we also used another CSE inhibitor BCA to inhibit H$_2$S production and observed that the effect of PPG could be reproduced. The potential off-target effects of these compounds must be kept in mind when interpreting the data. Therefore, genetic methods are needed to study the complex interaction between H$_2$S and SO$_2$ in the future studies.

The limitation of this study was that the effect of H$_2$S on the SO$_2$-induced vasodilation was measured in the aorta. Although the change of vascular reactivity of this conductance vessel could be used as an early indicator of the development of diseases related to abnormal blood pressure, the resistance artery is more closely correlated to blood pressure control. Therefore, it is necessary to further confirm the effect of the interaction between H$_2$S and SO$_2$ on vasodilation in arteries such as mesenteric arteries in the future. Factors such as pH, trace metals and oxygen content in buffers, material of measuring container, oxidant of sulfide and reaction of sulfide with many different species (including superoxide radical, hydrogen peroxide and peroxynitrite) were found to affect the determination of H$_2$S, resulting in the highly variable absolute H$_2$S concentration in biological samples measured by different methods [49]. In fact, measuring H$_2$S concentrations is still a matter of debate. There is no unified method for the determination of H$_2$S in plasma or tissue samples. Some studies show that plasma sulfide is well below 1 μM, and still many studies indicate that the plasma sulfide levels determined by different methods including liquid chromatography-mass spectrometry, methylene blue method, H$_2$S-selective sensor and H$_2$S-specific fluorescent probes were tens of micromoles [21,49–56], which was consistent with our results. In the future, highly selective, sensitive and accurate H$_2$S measurement methods are still needed to improve the unresolved limitations of the current methods.

**Conclusion**

Our *in vivo* and *in vitro* findings provide new clues for the integrated regulation of H$_2$S and SO$_2$ to control blood pressure and vascular tension. Our results suggest that H$_2$S negatively
controls the plasma content of SO$_2$, the vasorelaxant effect and the transient hypotensive response (Fig 6). The findings indicate that SO$_2$ acts as a compensatory defense gaseous molecule for H$_2$S when it is disturbed, playing a vascular protective role. A better understanding of the integrated regulation of these two sulfur-containing gasotransmitters in vascular function would provide new ideas to further explore the pathogenesis of cardiovascular diseases and reveal new targets for their treatment in the future.

Supporting information

S1 Fig. The interconversion of SO$_3^{2-}$, HSO$_3^-$ and SO$_2$.
(TIF)

S2 Fig. The effects of pre-administrated PPG, BCA or NaHS on rat blood pressure.
Changes in mean blood pressure of rats within 1 minute after injected with PPG (30 mg/kg), BCA (50 mg/kg) or NaHS (56 μmol/kg). Data are mean±SEM; n = 8 in each group; Student’s t-test; *P<0.05 compared with zero time point.
(TIF)

S1 Data. Raw data.
(RAR)

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