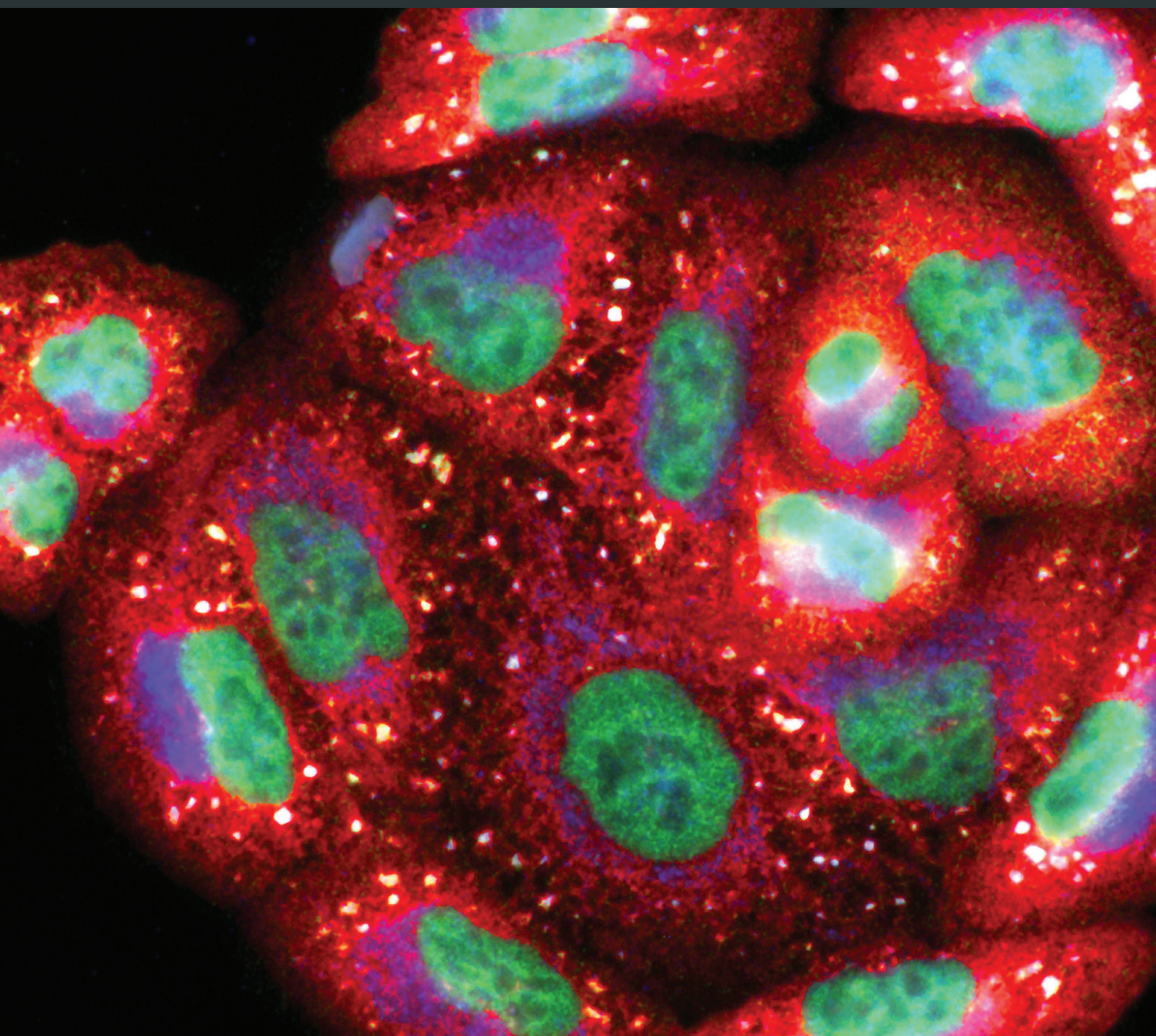
Oxidative Medicine and Cellular Longevity

Hydrogen Sulfide: Biogenesis, Physiology, and Pathology

Guest Editors: Jin-Song Bian, Kenneth R. Olson, and Yi-Chun Zhu



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Editorial

Hydrogen Sulfide: Biogenesis, Physiology, and Pathology

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The seminal studies by Kimura's group in the late 1990s [1, 2] ushered in a new era of biological signaling mediated by hydrogen sulfide (H_2S). H_2S has been described as the third "gasotransmitter" along with nitric oxide (NO) and carbon monoxide (CO; [3]). While none of these molecules actually signal as a gas, their hydrophilicity and lipophilicity enable them to readily traverse intracellular compartments where they serve in a variety of autocrine and paracrine signals capacities.

Of the three, however, H_2S is arguably the most chemically reactive and biologically diverse. As a weak acid, H_2S exists as the dissolved gas and hydrosulfide anion (HS^-) in nearly equal proportions in the circumneutral intracellular milieu. H_2S is readily oxidized to per- and polysulfides (H_2S_n) forming a highly reactive group of molecules (reactive sulfide species, RSS) that readily form reversible covalent bonds, coordinate with metal ligands, and participate in a variety of redox reactions [4, 5]. H_2S was key in the origin of life [6] and it remains as the only inorganic substrate used by eukaryotic cells to generate ATP [7]. H_2S mediates many physiological functions in various biological systems including cytoprotection, neuromodulation, ischemic responses, and oxygen sensing. Exogenous application of H_2S may protect cell function against ischemic and oxidant injuries. Despite the fact that the multiple roles of H_2S in biology have been intensely discussed over the last twenty years, many questions remain to be resolved. The eclectic nature of H_2S is featured in this special issue as a collection of original articles and reviews that examine a variety of the physiological functions of H_2S as well as its therapeutic attributes.

The role of H_2S in oxidative stress has been one of the main focuses over the last two decades. The review by Xie et al. focuses on the new understanding and mechanisms of the antioxidant effects of H_2S based on recent reports. As a poor reducing agent, H_2S may react directly with and quench superoxide, peroxynitrite, and other ROS. However, the direct antioxidant effect may not be its main function. H_2S can scavenge free radicals via activation of both nonenzymatic (e.g., glutathione and thioredoxin) and enzymatic (e.g., superoxide dismutase, catalase, and glutathione peroxidase) antioxidants. Apart from stimulation of cellular antioxidant defenses, H_2S may also inhibit the overproduction of ROS via modification of the structure of p66Shc at cysteine-59. This subsequently inhibits mitochondrial ROS generation in the mitochondria. Thus, this review provides new insights to better understand the important role of the H_2S in oxidative stress and the related diseases.

Being gaseous molecules and mediators, both H_2S and NO play important roles in various biological systems. While the individual signaling mechanisms mediated by H_2S and NO in mammals are extensively studied, our understanding about the potential relationship between these two gasotransmitters is woefully incomplete. Nagpure and Bian reviewed recent research progress in the interactions between these two gaseous mediators. H_2S may reduce oxidized NO leading to the formation of HSNO as an intermediate. Further reduction and direct displacement of HSNO by H_2S result in the formation of another intermediate product, nitroxyl (HNO). Interestingly, HNO produces chemical and physiological functions different from NO and H_2S. The role of this

interaction in heart and vascular physiology and pathology is also summarized in this review.

In the original article by D. Wu et al., the authors studied the interaction between H_2S and NO in vasculature using a novel H_2S and NO conjugated donor, ZYZ-803. The authors found that, by releasing H_2S and NO, ZYZ-803 time- and dose-dependently relaxed rat aortic rings. Mechanistic studies revealed that the effect of ZYZ-803 was mediated by cGMP. Interestingly, suppression of either H_2S or NO generation with their respective inhibitors abolished the vasorelaxant effect of ZYZ-803. These interesting data suggest that H_2S and NO generated from ZYZ-803 can cooperatively regulate vascular tone.

In the original article by P. Huang et al., the authors investigated whether H_2S can prevent high-salt diet-induced renal injury. Dahl rats on a high-salt diet for 8 weeks developed hypertension and kidney injury with excessive oxidative stress and an obvious reduction of endogenous H_2S. Exogenous application of H_2S, however, decreased blood pressure and improved renal function. The authors believe that the beneficial effects of H_2S resulted from Keap1/Nrf2 disassociation.

Sulfur dioxide (SO_2) has also long been thought to be a toxic gas. A group of scientists in China recently reported that this gas can be produced endogenously. In the review article by Y. Huang et al., the authors summarized the physiological and pathological functions of SO_2 in the cardiovascular system. These include regulation of vascular tone and heart contractility and its effects on disease status like systemic and pulmonary hypertension, atherosclerosis, and ischemic diseases. Based on these effects, they propose that endogenous SO_2 may be a potential therapeutic target for cardiovascular diseases.

In the original article by N. Li et al., the authors examined the effect of H_2S on protein expression of various H_2S generating enzymes in both ischemic heart tissue and cultured cardiomyocytes during hypoxia. They found that NaHS treatment upregulated protein expression of various enzymes with different patterns in the two pathological situations. The authors believe that CSE may serve as the most important enzyme to regulate H_2S production in myocardium. This is because when CSE was deleted, the compensatory expressions of CBS and 3-MST were not enough to rescue H_2S levels and protect the heart. These interesting data suggest that exogenous H_2S may alter the production of endogenous H_2S and therefore protect the heart with a new mechanism.

Respiratory and gastrointestinal epithelia are directly exposed to environmental- or bacterial-derived H_2S which can raise the H_2S concentration in these tissues. E. Pouokam and M. Althaus discussed in their review article how epithelial cells quickly process and maintain H_2S level to prevent excessive increases. They proposed a new concept that electrolyte and liquid transport machinery inside epithelia may serve as a fortress against potentially harmful higher level H_2S. In this interesting review article, reactions to exposed H_2S and potential physiological and pathophysiological implications were also discussed.

Vascular calcification (VC) is a refractory component of many cardiovascular diseases and is associated with hypertension (HT) and endoplasmic reticulum stress (ERS). In this original research article, R. Yang et al. induced VC and HT in rats with vitamin D_3 and observed that daily i.p. injection of H_2S (as NaSH) over 28 days prevented VC, HT, and conversion of vascular smooth muscle cells from a contractile to an osteoblast-like phenotype common in VC. H_2S also prevented VC-induced downregulation of cystathionine γ-lyase and the authors presented evidence that suggested H_2S also ameliorated ERS. This article adds yet another piece to the ever growing body of evidence that H_2S is a general cardiovascular protectant.

Unfortunately, this issue can only touch on a few select aspects of H_2S biology. However, we hope this broad-spectrum, albeit limited, approach serves to illustrate the myriad of physiological processes potentially influenced by H_2S and entice new investigators into this exciting field.

Jin-Song Bian
Kenneth R. Olson
Yi-Chun Zhu

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Research Article

Hydrogen Sulfide Improves Vascular Calcification in Rats by Inhibiting Endoplasmic Reticulum Stress

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In this study, the vitamin D_3 plus nicotine (VDN) model of rats was used to prove that H_2S alleviates vascular calcification (VC) and phenotype transformation of vascular smooth muscle cells (VSMC). Besides, H_2S can also inhibit endoplasmic reticulum stress (ERS) of calcified aortic tissues. The effect of H_2S on alleviating VC and phenotype transformation of VSMC can be blocked by TM, while PBA also alleviated VC and phenotype transformation of VSMC that was similar to the effect of H_2S. These results suggest that H_2S may alleviate rat aorta VC by inhibiting ERS, providing new target and perspective for prevention and treatment of VC.

1. Introduction

Vascular calcification (VC) refers to the abnormal deposition of calcium and phosphorus minerals on the walls of blood vessels. The phenomenon of VC is common in atherosclerosis, hypertension, diabetic vascular disease, aging, and chronic kidney disease, and an important risk factor of cardiocerebrovascular diseases and related deaths [1]. There has been no clinical therapy specifically developed to treat VC.

The deposition of calcium mineral in VC was initially considered to occur passively. However, according to recent studies VC is actually an active biological process similar to bone development that is highly regulated, preventable, and can be reversed. In particular, the development and progression of VC involve the transformation of vascular smooth muscle cells (VSMCs) from a contractile to an osteoblast-like phenotype [2–5]. An agent that could prevent this transformation would have great guiding significance in research and potential for clinical application.

Recent studies have revealed that paracrine/autocrine vasoactive factors such as cardiovascular peptides [6], cytokines, and gaseous molecules [7] have an important regulatory role in the development and progression of VC. Hydrogen sulfide (H_2S) is newly identified as a gaseous signaling molecule after discovery of nitric oxide and carbon monoxide. H_2S affects the cardiovascular system in multiple ways, by dilating blood vessels, lowering blood pressure [8], and regulating the apoptosis and proliferation of VSMCs. Recent studies have suggested that H_2S also inhibits VC and the phenotype transformation of VSMCs [9]. However, the specific mechanism is unknown. The thorough investigation of the mechanism of the ameliorative effect of H_2S on VC should contribute to the transformation from bench to bedside and be useful to discover new target and strategy for treatment of VC.

The endoplasmic reticulum is the largest intracellular organelle of the cell. A disturbance of homeostasis in the endoplasmic reticulum leads to a series of cellular responses cumulatively known as endoplasmic reticulum stress (ERS). ERS has been implicated in the development and progression of a variety of cardiovascular diseases, and the development and progression of VC. The apoptosis of VSMCs that is caused by ERS promotes VC, and also the phenotype transformation of VSMCs [10–12]. In addition, recent studies have suggested

that H_2S may exert a protective effect on the cardiovascular system by inhibiting ERS [13–15]. Therefore, we hypothesized that H_2S may alleviate VC by inhibiting ERS.

2. Materials and Methods

2.1. Rats and Reagents. All animal procedures complied with the Animal Management Rule of the Ministry of Health, People's Republic of China (documentation number 55, 2001) and the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication number 85-23, revised 1996) and approved by the Animal Care Committee of Hebei Medical University.

Male Sprague-Dawley rats (180–200 g) were supplied by the Animal Center of Hebei Medical University (Shijiazhuang, China) and housed under standard conditions (room temperature 20 ± 8°C, humidity 60 ± 10%, lights from 600 to 1800 h). They were freely supplied with standard rodent chow and water.

Tunicamycin (Tm) was from Cayman Chemical (Ann Arbor, USA). The alkaline phosphatase (ALP) and calcium detection kit was from BioSino Biotechnology and Science (Beijing, CN). Antibodies against calponin, RUNX2 (runt-related transcription factor 2), and GRP78 (78-kDa glucose-regulated protein) were from Epitomic (Burlingame, USA). Antibodies against SM22α, BMP2 (bone morphogenetic protein 2), active caspase-12, and beta-actin were from GeneTex (Irvine, USA). Antibodies against CHOP (C/EBP homologous protein) were from Affinity Biosciences (Cincinnati, USA). The secondary antibodies were from Kirkegaard & Perry Laboratories (Gaithersburg, USA). An enhanced chemiluminescence (ECL) kit was from Multi-Sciences Biotech (Hangzhou, CN). Other chemicals and reagents were of analytical grade.

2.2. The Model of VC In Vivo. Male Sprague-Dawley rats (180–200 g) were randomly divided into 7 groups ($n = 6$, each) as follows: the control; vitamin D_3 plus nicotine (VDN); VDN + sodium hydrosulfide (NaHS); VDN + tunicamycin (Tm); VDN + Tm + NaHS; VDN + 4-phenylbutyric acid (PBA); and VDN + PBA + NaHS. In these groups, VDN treatment imposed calcification and therefore the VC model [16], NaHS acted as an H_2S donor, Tm acted as an ERS agonist, and PBA as an ERS inhibitor.

The rats in the noncontrol groups were given vitamin D_3 (300 000 IU/kg, intramuscularly) simultaneously with nicotine (25 mg/kg in 5 mL peanut oil, intragastrically) at 08:00 hours on the first day. The nicotine administration was repeated at 2000 hours. On days 2 and 15, the rats were retreated with vitamin D_3.

In the rat groups that received NaHS (i.e., VDN + NaHS, VDN + Tm + NaHS, and VDN + PBA + NaHS), the NaHS was injected daily (56 μmol/kg) during the 28 days of nicotine treatment. In the rat groups that received Tm (VDN + Tm and VDN + Tm + NaHS), Tm was injected daily (intraperitoneal, 5 μg/kg) during the same period. In the rat groups that received PBA (VDN + PBA and VDN + PBA + NaHS), PBA was injected daily (intraperitoneal, 50 mg per rat) during the same period. Rats in the control group received normal saline intramuscularly (rather than vitamin D_3) and 2 gavages of peanut oil (5 mL/kg, i.e., without nicotine) during the same period. The rats of the control and VDN groups were injected with saline vehicle (0.2 mL/100 g) per day, at a volume similar to the volumes of NaHS, Tm, and/or PBA injected into the rats of the other groups, during the same period. The aortic tissues of all the rats were harvested on day 28, as described below.

2.3. Blood Pressure Measurement. At the end of the 28-day experimental period, the rats were anesthetized with urethane (intraperitoneal, 1 g/kg). A cannula was inserted into the right carotid artery to measure blood pressure. Systolic blood pressure was measured using a pressure transducer (BL420F-Powerlab, TaiMeng, Chengdu, China). After measurement, the blood and aortas were collected, and the aortas were stripped of intima and adventitia and treated as described below.

2.4. Hematoxylin and Eosin (H&E) Staining. To quantify vascular medial calcification, the aortic roots (from the aortic opening to 0.5 cm from the opening section of the aorta) were separated and stored in 4% paraformaldehyde for H&E histopathological staining.

2.5. Quantification of Calcium Content and ALP Activity in Aortas. Calcium levels were determined by colorimetry through a reaction with o-cresolphthalein complexone. ALP activity was measured using an ALP Colorimetric assay kit, in accordance with the kit's instructions.

2.6. Western Blot Analysis. Aortas were homogenized in lysis buffer. Equal amounts of protein samples were loaded on 10% or 12% sodium dodecyl sulfate gels and then transferred to a nitrocellulose membrane. Nonspecific proteins were blocked with 5% nonfat dried milk for 1 h. Membranes were incubated serially with the primary antibodies overnight at 4°C, and with secondary antibody (horseradish peroxidase-conjugated anti-goat or anti-rabbit IgG) for 1 h. The reaction was visualized by enhanced chemiluminescence, and an autoradiograph was scanned. Protein concentrations were analyzed using NIH Image software and normalized to that of β-actin. All experiments were repeated at least 3 times.

2.7. Measurement of Plasma H_2S Levels. Whole blood sample was collected, anticoagulated, and centrifuged at 3000 rpm for 15 min to obtain the plasma. H_2S levels in plasma were measured as Shen et al. reported [17]. H_2S levels were calculated using a standard curve generated from a sodium sulfide solution (0–100 μM).

2.8. Statistical Analyses. Statistical analyses were performed using GraphPad software (GraphPad Prism v5.00 for Windows; GraphPad Software, San Diego, CA, USA). Comparisons between 2 groups were conducted using the unpaired Student's t-test. Comparisons among ≥3 groups were analyzed by one-way analysis of variance and then by Newman-Keuls test. Data are expressed as mean ± standard deviation. $P < 0.05$ was considered statistically significant.

Table 1: General characteristics and vascular total calcium and plasma and aortic ALP.

	SBP	Calcium Aorta, mmol/g DW	ALP Plasma, IU/L	ALP Aorta, IU/g protein
Control	118.1 ± 3.7	6.11 ± 2.21	135.4 ± 18.2	250.20 ± 60.22
VDN	155.8 ± 5.1[a]	51.38 ± 5.12[a]	349.4 ± 31.6[a]	462.42 ± 61.17[a]
VDN + NaHS	114.3 ± 5.3[b]	13.14 ± 8.56[b]	174.4 ± 23.6[b]	193.52 ± 23.34[b]
VDN + Tm	151.7 ± 2.6[a,c]	60.56 ± 10.01[a,c]	361.2 ± 26.8[a,c]	510.46 ± 41.10[a,c]
VDN + Tm + NaHS	150.0 ± 4.9[a,c]	55.56 ± 6.34[a,c]	340.6 ± 18.0[a,c]	490.20 ± 26.48[a,c]
VDN + PBA	110.3 ± 3.3[b]	14.01 ± 5.78[b]	125.4 ± 31.2[b]	221.56 ± 22.46[b]
VDN + PBA + NaHS	108 ± 9.9[b]	13.89 ± 8.02[b]	133.0 ± 28.0[b]	216.08 ± 31.64[b]

[a] $P < 0.05$ cf. control group; [b] $P < 0.05$ cf. VDN; [c] $P < 0.05$ cf. VDN + H_2S group.
SBP, systolic blood pressure.

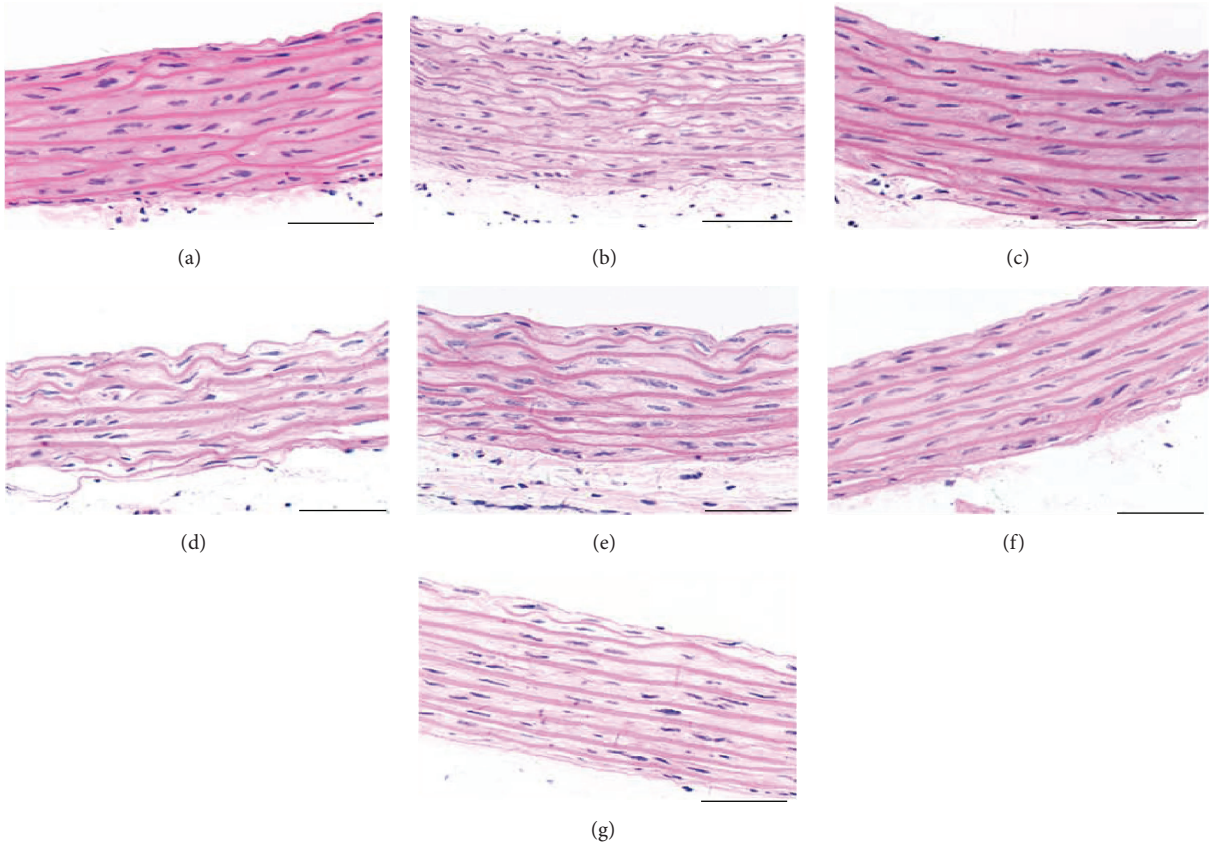

Figure 1: H&E staining of the aorta in rats (400x). (a) Control group. (b) VDN group. (c) VDN + NaHS group. (d) VDN + Tm group. (e) VDN + Tm + NaHS group. (f) VDN + PBA group. (g) VDN + PBA + NaHS group. Scale bar is 50 μm.

3. Results

3.1. Comparison of VDN and Control Rats. In the VDN (nontreated VC model) rat group, the mean systolic blood pressure, ALP activity, and calcium content of aortal tissues were significantly higher relative to that of the control rats (Table 1). Furthermore, in the VDN group, H&E staining revealed thickening and structural disorder of vascular elastic fibers (Figure 1), and the Western blot results showed that the protein levels of SM22α and calponin, that is, molecular markers of a VSMC contractile phenotype, were significantly lower than that of the controls. Conversely, in the VDN group the protein levels of BMP2 and RUNX2, which indicate a VSMC osteoblast-like phenotype, were significantly higher than that of the controls (Figure 2).

Compared with the control rats, in the VDN rats the protein levels of the ERS markers GRP78, active caspase-12, and CHOP, were all significantly higher (Figure 3).

3.2. Comparison of VDN + NaHS and VDN Rats. In the VDN + NaHS group, relative to the VDN rats the NaHS treatment was associated with higher systolic blood pressure and aortal tissue ALP activity, and calcium content was lower (Table 1). Furthermore, relative to the VDN rats, rats in

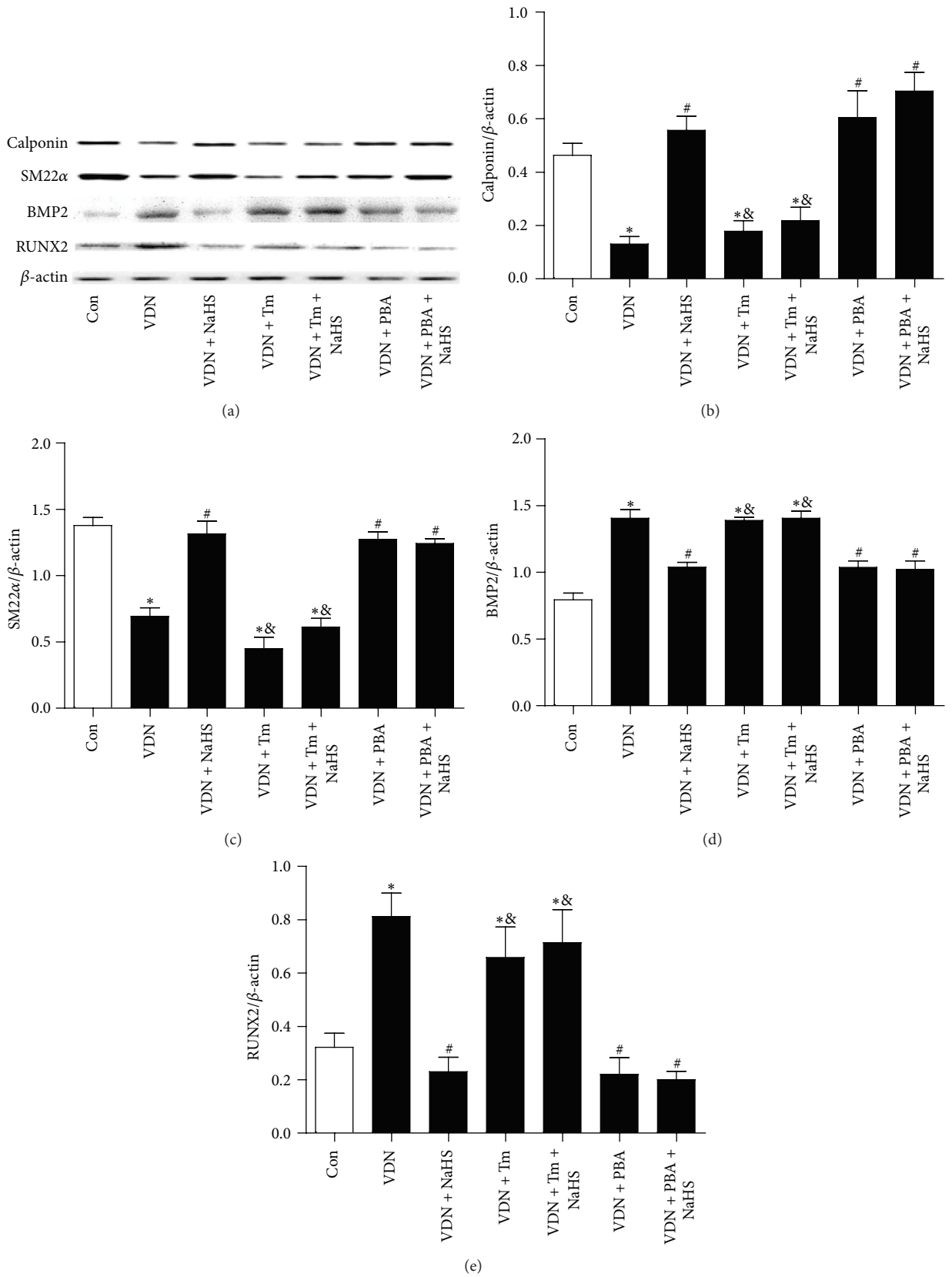

FIGURE 2: Tm blocks the effect of H_2S on transformation in VSMCs in calcified aorta. (a) Representative protein levels of calponin, SM22α, BMP2, RUNX2, and β-actin; (b–e) quantitative analysis of protein levels of calponin, SM22α, BMP2, and RUNX2. $^{*}P < 0.05$ cf. control group; $^{\#}P < 0.05$ cf. VDN group; $^{\&}P < 0.05$ cf. VDN + NaHS group.

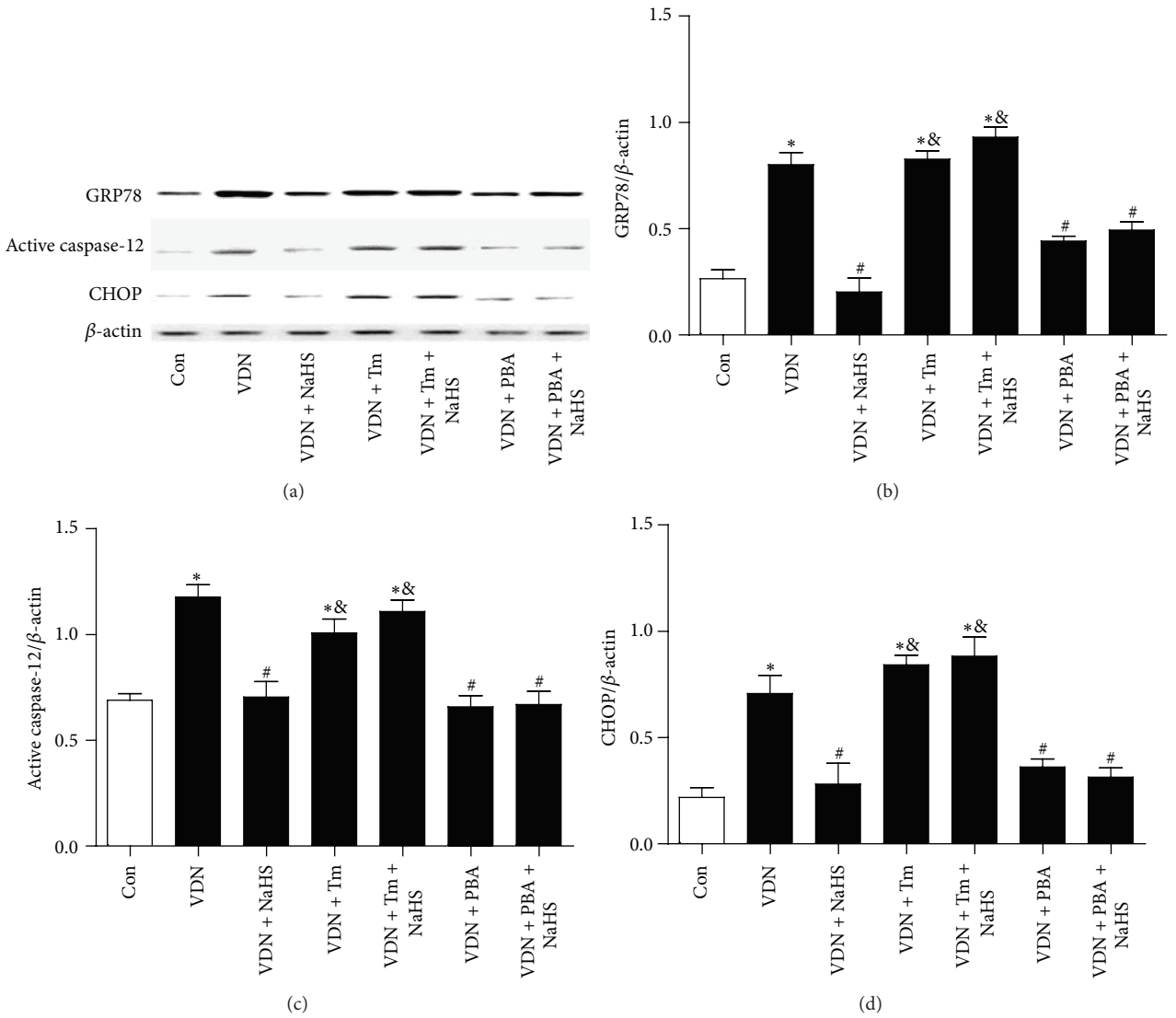

Figure 3: Tm blocks the effect of H_2S on activation of ERS in calcified aorta. (a) Representative protein levels of GRP78, active caspase-12, CHOP, and β-actin; (b–d) quantitative analysis of protein levels of GRP78, active caspase-12, and CHOP. $^{*}P < 0.05$ cf. control group; $^{\#}P < 0.05$ cf. VDN group; $^{\&}P < 0.05$ cf. VDN + H_2S group.

the VDN + NaHS group appeared to have less thickening and structural disorder of vascular elastic fibers (Figure 1), higher protein levels of SM22α and calponin, and lower protein levels of BMP2 and RUNX2 (Figure 2).

Compared with the VDN rats, the VDN + NaHS rats had lower levels of the ERS markers GRP78, active caspase-12, and CHOP (Figure 3).

3.3. ERS Involved in the Ameliorated Effect of NaHS on VC in VDN Rats. The ERS inducer Tm and the ERS inhibitor PBA were used to investigate the role of ERS in VC. Tm could block the ameliorated effect of NaHS on VDN rats. Tm increased the ALP activity and calcium content (Table 1), downregulated the protein level of SM22α and calponin, and upregulated the protein level of BMP2 and RUNX2 in aorta of VDN rats with NaHS treatment (Figure 2). The protein level of GRP78, active caspase-12, and CHOP was also induced by Tm (Figure 3). However, there was no difference between VDN rats and VDN + Tm rats.

The effect of PBA on VC in VDN rats was similar as that of NaHS. PBA treatment also could decrease the ALP activity and calcium content in calcified aorta (Table 1). In VDN plus PBA rats, the downregulation of SM22α and calponin was reversed, and the upregulation of BMP2 and RUNX2 was simultaneously decreased (Figure 2). The increased protein level of GPR78, active caspase-12, and CHOP in calcified aorta was inhibited by PBA treatment (Figure 3). There was no synergistic effect of NaHS plus PBA on VDN rats.

3.4. Regulation of Akt Signaling Pathway in VC. Compared with the control rats, the protein levels of Akt and phospho-Akt were both lower in the VDN rats. NaHS treatment significantly reversed the downregulation of Akt and phospho-Akt (Figure 4).

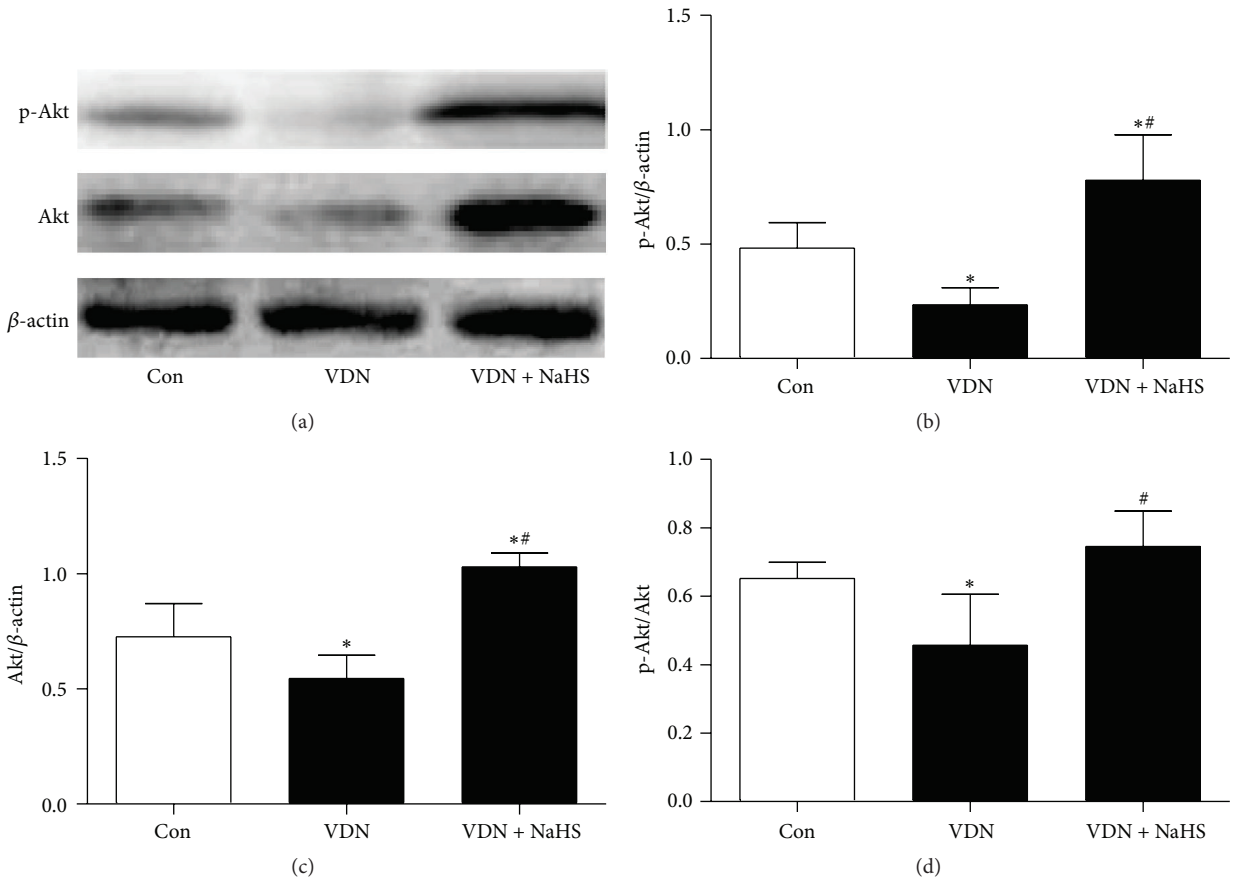

FIGURE 4: Regulation of Akt signaling pathway in aorta. (a) Representative protein levels of phospho-Akt, Akt, and β-actin; (b–d) quantitative analysis of protein levels of phospho-Akt and Akt. $^{*}P < 0.05$ cf. control group; $^{\#}P < 0.05$ cf. VDN group.

3.5. Protein Levels of Cystathionine γ-Lyase (CSE) in the Aorta and Plasma Levels of H_2S. In the VDN rats, the protein levels of CSE in the aorta and plasma levels of H_2S were lower than that in the control rats. NaHS treatment could significantly increase the protein levels of CSE and plasma levels of H_2S in VDN rats (Figure 5).

4. Discussion

In this study, a rat model of VC was established using the VDN method, to investigate the effect of H_2S on VC and the phenotype transformation of VSMCs. In addition, H_2S can also inhibit ERS of calcified aortic tissues. The effect of H_2S on alleviating VC and the phenotype transformation of VSMCs can be blocked by Tm. PBA also alleviated VC and phenotype transformation of VSMCs, similar to the effect of H_2S. Activation of Akt signaling may be involved in the ameliorated effect of H_2S on ERS and VC. Exogenous NaHS treatment upregulated the protein expressions of aortic CSE and plasma levels of H_2S in VDN rats. These results suggest that H_2S may alleviate VC of the rat aorta by inhibiting ERS. Thus, H_2S may be a new target for research of the prevention and treatment of VC (Figure 6).

It was initially assumed that VC is a passive process, resulting from disturbance of calcium/phosphorus metabolism and consequent deposition of these minerals. However, a major breakthrough in recent studies on the mechanism of VC revealed that VC is actually an active biological process similar to bone development, which is highly regulated, preventable, and can be reversed. The transformation of VSMCs from a contractile to osteoblast-like phenotype has been observed in both the vessels of patients with hypertension, diabetes, or chronic renal failure, and in the rat VC model established with a high-calcium, high-phosphorus, and high vitamin D_3 and nicotine diet [18, 19]. In the present study, the calcium content and ALP activity of the VDN group were significantly higher than that of the control group. Western blot analysis showed that levels of the proteins that indicate a contractile phenotype (SM22α and calponin-1) were significantly lower in the VDN group relative to that of the control, while levels of the proteins associated with an osteoblast-like phenotype (RUNX2 and BMP2) were significantly higher. H&E staining revealed that, compared with the control group, the aortic elastic plate of the VDN group was significantly thicker and more disordered, with breakage of the elastic fibers. These results indicated that construction of the rat

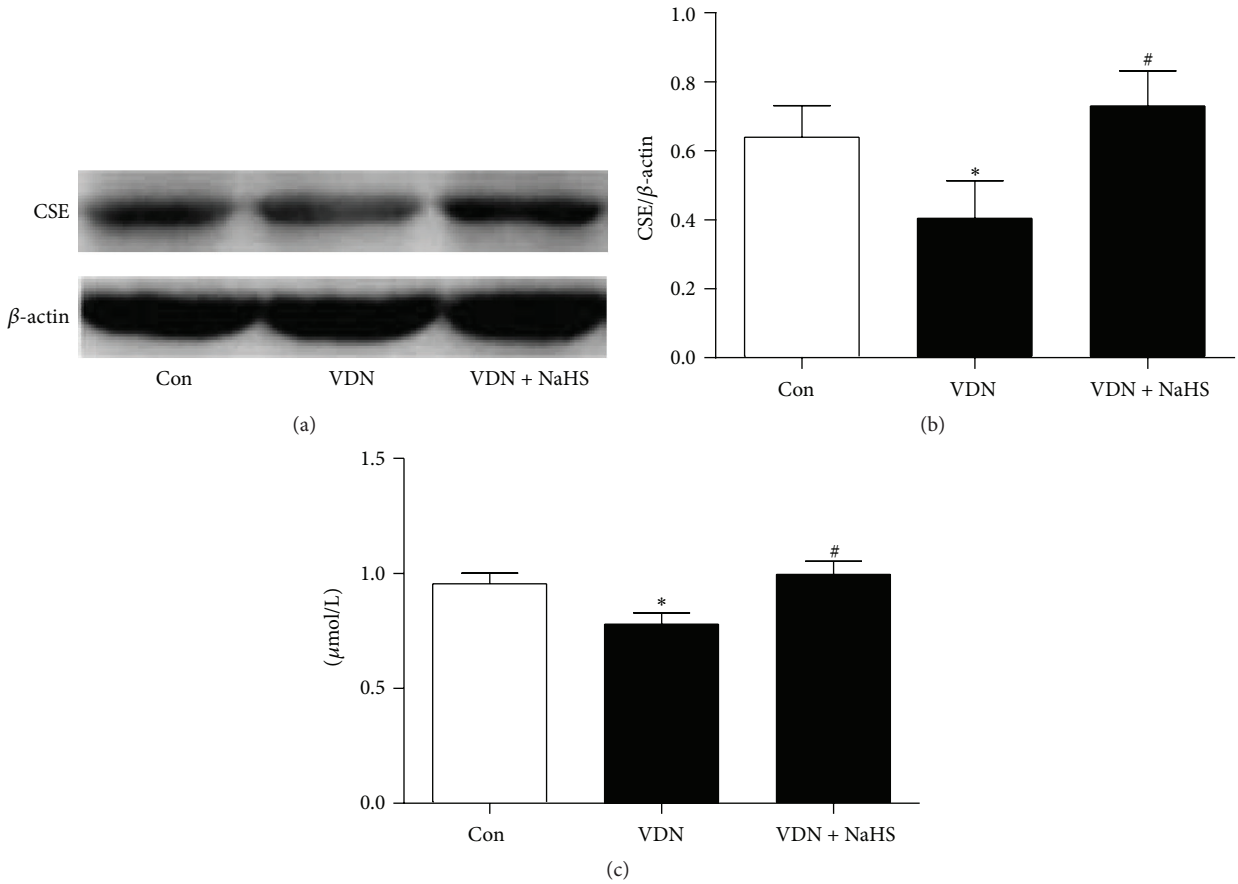

FIGURE 5: Protein levels of CSE in aorta and plasma levels of H_2S. (a) Representative protein levels of CSE and β-actin; (b) quantitative analysis of protein levels of CSE. $^{*}P < 0.05$ cf. control group; $^{\#}P < 0.05$ cf. VDN group.

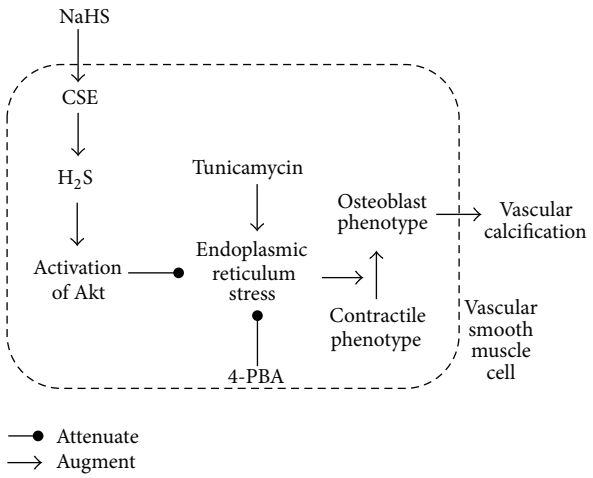

FIGURE 6: Schematic representation of the ameliorative effect of NaHS on VC.

VC model was successful and confirmed the transformation of VSMCs from a contractile to osteoblast-like phenotype during VC.

H_2S is a newly identified gaseous signaling molecule after nitric oxide and carbon monoxide. H_2S has a rotten egg-like odor and is toxic, by inhibiting cytochrome C oxidase [20]. *In vitro* studies have shown that H_2S can reduce extracellular calcium deposition and inhibit expression of the genes responsible for the osteoblast-like transformation of VSMCs. In addition, H_2S also inhibits upregulation of the sodium-phosphate cotransporter Pit-1, induced by uptake of phosphate and phosphoric acid. CSE inhibitor blocks these effects [11]. These results suggest that H_2S strongly inhibits phosphate-induced VSMC calcification and osteoblast-like differentiation.

In vivo and *in vitro* experiments both indicate that H_2S inhibits the phenotype transformation of VSMCs and alleviates VC [9]. In the present experiment, the calcium content of the aorta and ALP activity of the VDN group were both higher compared with that of the control group, while NaHS treatment significantly alleviated such increase. H&E staining revealed thickening and structural disorder of the vascular elastic fibers in the VDN group, and NaHS treatment significantly alleviated these changes. In addition, NaHS upregulated levels of protein related to the contractile phenotype of the aortic VSMCs, while inhibiting levels of proteins responsible for osteoblast-like phenotype. These results further show that H_2S inhibits phenotype transformation in VSMCs and alleviates VC.

Any stimulus that interferes with endoplasmic reticulum function (such as hypoxia, oxidative stress, nutrition deficiency, and calcium homeostasis disturbance) may lead to accumulation of unfolded or misfolded protein in the endoplasmic reticulum, leading to ERS. Studies have indicated that apoptosis of VSMCs is an important aspect of VC, while ERS-induced apoptosis is obviously active in the calcified rat aorta [20–22]. Inhibition of ERS-induced VSMC apoptosis significantly alleviates development of VC [23–25]. Bone morphogenetic protein (BMP) signaling is a key aspect of VC that has an important role in osteoblast-like differentiation of VSMCs. It has been reported that BMP2 promotes VSMC calcification through induction of oxidative stress and ERS, and subsequently production of XBP1 (X-box binding protein 1), which binds to the Runx2 (runt-related transcription factor 2) promoter and promotes Runx2 expression [26]. In the present study, we also found that GRP78, CHOP, and active caspase-12 levels were significantly elevated in the calcified rat aorta, while the ERS inhibitor significantly alleviated VC. This indicated that ERS participates in the development of VC. NaHS inhibited ERS in the calcified rat aorta, and the ERS agonist blocked this effect of NaHS. These results suggest that H_2S may alleviate VC by inhibiting ERS.

H_2S putatively activates Akt signaling [27–30], and activation of the Akt signaling pathway has a crucial role in counteracting ERS [31–33]. Several factors could inhibit ERS by activating Akt signaling, resulting in a protective effect on the cardiovascular system [34]. Our present results showed that the protein levels of phospho-Akt and Akt were both upregulated by NaHS treatment. This suggests that H_2S ameliorated ERS through activation of the Akt signaling pathway.

H_2S is synthesized endogenously by the catalysis of 3 enzymes: CSE, cystathionine-β-synthase, and 3-mercaptopyruvate sulfurtransferase. CSE has a crucial role in maintaining cardiovascular homeostasis. Wu et al. [6] and our research team have both demonstrated that the expression and activity of CSE were reduced in the calcified aorta, which resulted in reduction of endogenous H_2S. We have further shown that NaHS treatment upregulated the protein levels of CSE. The stimulation effect of exogenous H_2S treatment on the production of endogenous H_2S is in accord with other published articles [35, 36].

5. Conclusion

In this study, using a rat model of VC, we showed that H_2S significantly alleviates VC, probably by inhibiting ERS. This study provides new strategies and a target for prevention and treatment of VC.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Rui Yang and Xu Teng contributed equally to this paper.

Acknowledgments

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Review Article

Epithelial Electrolyte Transport Physiology and the Gasotransmitter Hydrogen Sulfide

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Hydrogen sulfide (H_2S) is a well-known environmental chemical threat with an unpleasant smell of rotten eggs. Aside from the established toxic effects of high-dose H_2S, research over the past decade revealed that cells endogenously produce small amounts of H_2S with physiological functions. H_2S has therefore been classified as a "gasotransmitter." A major challenge for cells and tissues is the maintenance of low physiological concentrations of H_2S in order to prevent potential toxicity. Epithelia of the respiratory and gastrointestinal tract are especially faced with this problem, since these barriers are predominantly exposed to exogenous H_2S from environmental sources or sulfur-metabolising microbiota. In this paper, we review the cellular mechanisms by which epithelial cells maintain physiological, endogenous H_2S concentrations. Furthermore, we suggest a concept by which epithelia use their electrolyte and liquid transport machinery as defence mechanisms in order to eliminate exogenous sources for potentially harmful H_2S concentrations.

1. Introduction

"All things are poisons, for there is nothing without poisonous qualities. It is only the dose which makes a thing poison." This famous quote from Paracelsus (1493–1541) is still important for research in physiology and especially for recent investigations on a toxic gas, hydrogen sulfide, and its potential role as a physiologically relevant signalling molecule.

Hydrogen sulfide (H_2S) is well known as an environmental chemical threat and even more for its unpleasant smell of rotten eggs. The odour threshold for H_2S is about 0.003–0.02 ppm and concentrations above 50 ppm have toxic effects such as irritations of the eye and respiratory tract [1]. At 150–200 ppm H_2S, the olfactory sense for this gas is lost and higher concentrations lead to the formation of pulmonary oedema, unconsciousness, and eventually death [1]. The toxic effects of H_2S are mainly based on the inhibition of the mitochondrial respiratory chain, especially cytochrome c oxidase [2, 3]. However, consistent with the principle of Paracelsus, research of the past decade has revealed that cells endogenously produce small amounts of H_2S which are not simply a metabolic by-product and play an important role in cellular signalling processes [4]. Similar to nitric oxide (NO) or carbon monoxide (CO), H_2S has therefore been classified as a "gasotransmitter," a gaseous cellular signalling molecule [4, 5]. Furthermore, a therapeutic potential for low-dose H_2S has been discovered [4] and H_2S-releasing pharmacological compounds have been designed [6] and are currently evaluated as potential therapeutics in various models of disease [7].

A major challenge for cells and tissues is the maintenance of physiological (low) concentrations of H_2S in order to prevent potential toxicity. In this review article, we describe epithelial reactions to H_2S. We focus on epithelia of the respiratory and intestinal tract since these tissues are predominantly exposed to a variety of exogenous and potentially dangerous sources for H_2S, that is, inhaled H_2S in the lung and microbiota-generated H_2S in the gut. Furthermore, epithelial cells endogenously produce low concentrations of H_2S with potential implications for cellular signalling

processes. In line with the principle of Paracelsus, epithelia therefore have to find a balance between potentially toxic exogenous and physiological, endogenous H_2S concentrations. In the following sections we will describe the chemistry as well as sources of H_2S to which epithelia are exposed, their reactions to exogenous and endogenous H_2S, and potential physiological/pathophysiological implications with respect to epithelial function.

2. Hydrogen Sulfide: Properties, Exogenous Sources, and Enzymatic Production

2.1. Chemical Properties of Hydrogen Sulfide. H_2S is a colourless and flammable gas characterized by its rotten eggs or blocked sewer smell. At 20°C, one gram of H_2S will dissolve in 242 mL water. Temperature and time influence the concentration of H_2S; temperature elevation increases the solubility of this gas. Oxidation occurring over time in solution leads to precipitation of elemental sulfur, giving a cloudy aspect to the solution (for review see [4]). Experimental work with this molecule is complicated since H_2S evaporates easily from aqueous solutions with a half-life on the minute time-scale [4, 8, 9]. In solution, H_2S is a weak acid, dissociating into the hydrosulfide anion or thiolate form HS^- and the sulfide anion S^{2-} building the following equilibrium:

$$H_2S \longleftrightarrow HS^- \longleftrightarrow S^{2-} \quad (1)$$

with p*K*a1 and p*K*a2 values of 6.9 and >12, respectively.

Consequently, at 37°C and physiological pH, there are almost equal amounts of H_2S and HS^- (but no S^{2-}) in cells and tissues and nearly a 20% H_2S/80% HS^- ratio in extracellular fluid or plasma [4]. H_2S is highly lipophilic with a dipole moment of 0.97 [10], allowing for a rapid cell membrane permeation [11] and hence potential interference with cell respiration. Diffusion of H_2S into the cells is hence a primary problem of respiratory and intestinal epithelia which are predominantly exposed to exogenous and potentially dangerous levels of H_2S.

2.2. Exogenous and Endogenous H_2S Sources

2.2.1. Exogenous H_2S Sources. There are organic and inorganic sources of H_2S (Figure 1). The pulmonary epithelium can be exposed to H_2S by inhalation of environmental H_2S gas (Figure 1(a)). H_2S may be released by volcanoes as natural gas or contained in sulfur deposits or (healing) sulfur springs. Additional significant H_2S sources are industrial processes such as petroleum refinery, rayon manufacturing, and paper, swine, and pulp mill industry [4]. 125,000 employees in 73 industries of the United States are potentially exposed to H_2S [4].

In the intestinal tract, methionine- or cysteine-rich diet may end up as H_2S production/release. The intestinal flora may produce H_2S via sulfate-reducing bacteria (SRB) (Figure 1(b), Table 1). SRBs use several substrates like short-chain fatty acids, other organic acids, or alcohol as electron donors for reduction of sulfate or other oxidized sulfur molecules to produce H_2S. Furthermore, endogenous substrates in the large intestine, such as sulfated polysaccharides (sulfomucins), can be used by the microflora for H_2S generation [12, 13]. Sulfates may also be directly delivered by food such as dried fruits, nuts, bread, wine, or brassica vegetables. The key bacterial enzyme used during these processes is dissimilatory sulfite reductase [14]. Furthermore, Shatalin and colleagues demonstrated that clinically relevant and pathogenic nonsulfur bacteria such as *Bacillus anthracis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus,* and *Escherichia coli* produce H_2S endogenously [15]. These species contain orthologues of the mammalian H_2S-generating enzymes cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3MST) and metabolize cysteine [15]. Overall, up to millimolar H_2S concentrations can occur in the content of human colon [13, 16] due to food compounds and activity of the intestinal flora.

In experimental studies, exogenous H_2S is mainly administered by "H_2S donors." Sulfur salts such as NaHS, Na_2S, and Lawesson's reagent are routinely used for H_2S delivery. However, these molecules are fast donors (in fact, these salts readily dissociate), contrasting with a continuous and more physiologically relevant production and release of H_2S which may occur in the body. Synthetic slow-releasing H_2S donors are, for example, GYY4137 or ATB-429 (for review, see [6, 17]). Natural H_2S donors are, for example, compounds in garlic extracts such as diallyl poly(di,tri)sulfides which slowly release H_2S. Also the sulfur-compounds S-allylcysteine (SAC) and allicin are found in garlic. These organic polysulfides may react with intracellular thiols like glutathione (GSH) to produce H_2S [4].

2.2.2. Endogenous, Intracellular H_2S. H_2S is endogenously produced by various cell types, including epithelial cells, as a result of L-cysteine metabolism. For the detailed biochemistry of cellular H_2S production pathways, the reader is referred to excellent recent review articles [18, 19]. In the past years, a growing body of evidence suggests a concept by which intracellular H_2S concentrations are determined by (1) its enzymatic production; (2) its intracellular storage as bound sulfane sulfur; and (3) its oxidative degradation by mitochondria.

(1) Enzymatic H_2S Production. H_2S is generated by three enzymes: CBS, CSE and 3MST. L-cysteine is the substrate for pyridoxal-5′-phosphate (vitamin B6) dependent CBS and CSE. However, it can be synthesized from L-methionine through the transsulfuration pathway catalysed by methionine adenosyltransferase (MAT) and glycine N-methyltransferase (GNMT). Serine is then transferred to homocysteine leading to cystathionine, a reaction catalysed by CBS. CSE converts cystathionine to cysteine. Cysteine may be converted to L-cystine which in turn will be converted to thiocysteine and further to H_2S. CSE and CBS together catalyse the production of H_2S from cysteine. The abovementioned processes occur within the cytosol. Cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfur transferase (MPST), both found in cytosol and mitochondria, catalyse

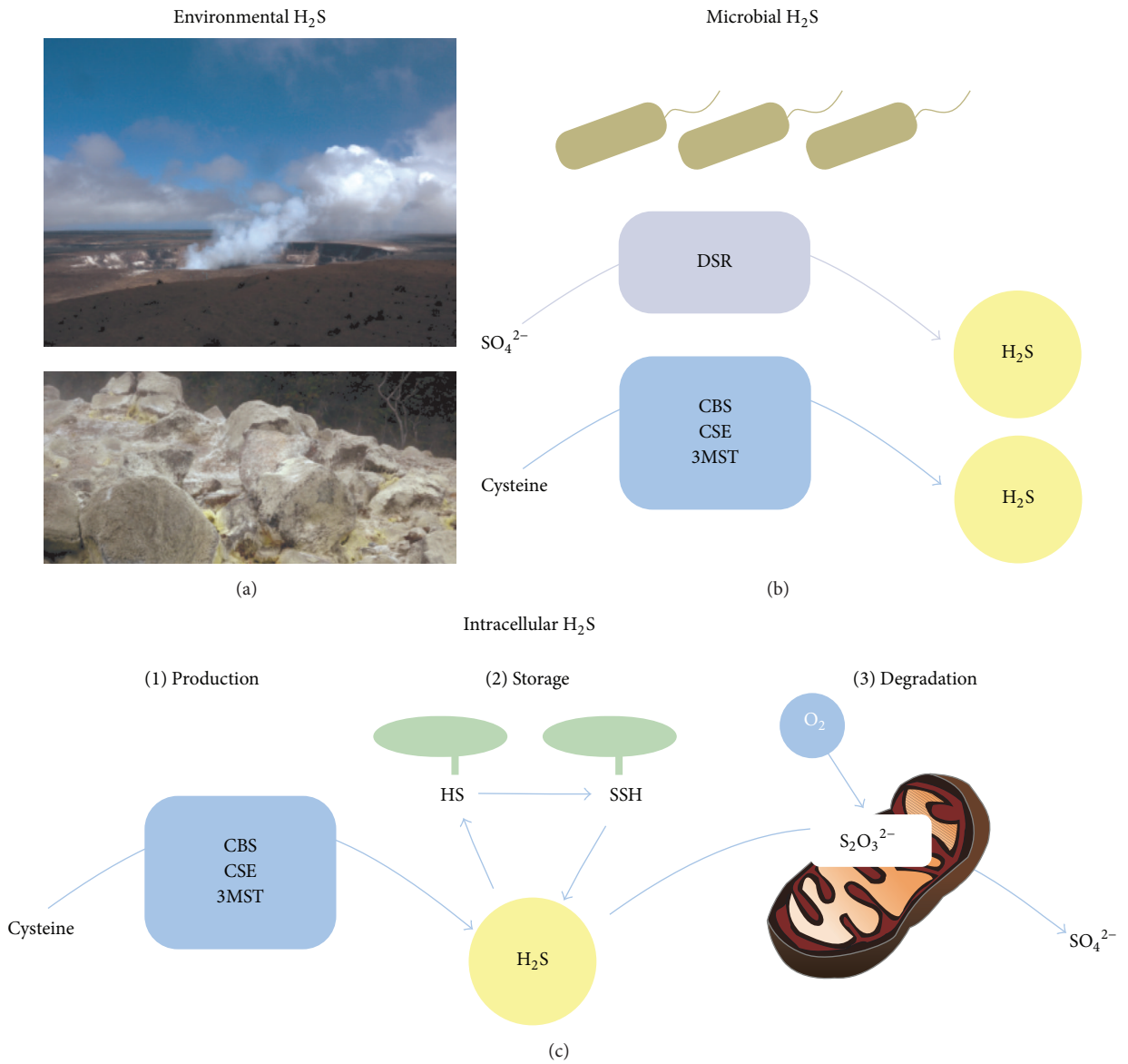

Figure 1: Sources of hydrogen sulfide. (a) Environmental hydrogen sulfide (H_2S) occurs due to geothermal activities such as volcanoes, sulfur deposits, or sulfur springs. The pictures show the Halema'uma'u crater (top) and sulfur deposit (bottom) on Hawaii Big Island. (b) Microbial production of H_2S. Sulfate-reducing bacteria dissimilate sulfate into H_2S via dissimilatory sulfite reductase (DSR). Mesophilic bacteria use orthologues of the mammalian H_2S-generating enzymes cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3MST) in order to generate H_2S from cysteine. (c) Mammalian cells produce H_2S from cysteine via CBS, CSE, and 3MST (1). H_2S can be stored in a reductant-labile intracellular pool as bound sulfane sulfur (2). The degradation of H_2S occurs via oxidative metabolic pathways in mitochondria (3).

the conversion of cysteine to 3-mercaptopyruvate and H_2S, respectively [4, 9, 20].

In addition, there is enzyme-free generation of H_2S; however, this represents only a minor source of this gasotransmitter. Here, oxidation of sulfide produces thiosulfate, which in turn interacts with intracellular thiols such as GSH to release H_2S.

(2) Intracellular H_2S-Storage. Enzymatically produced H_2S can be intracellularly stored as bound sulfane sulfur [18]. This occurs by the oxidative formation of hydrodisulfides or persulfides, for example, by modification of the sulfur of cysteine residues in proteins, which establish a reductant-labile pool of sulfane sulfur [18, 21]. A study by Shibuya and colleagues demonstrated that HEK293-F cells expressing 3MST contained larger amounts of sulfane sulfur than nontransfected cells or cells expressing enzymatically inactive 3MST [22]. This indicates that enzymatically produced H_2S is transformed into bound sulfane sulfur, thereby reducing the concentration of free intracellular H_2S.

(3) Mitochondrial H_2S-Degradation. H_2S affects mitochondrial respiration by inhibiting cytochrome c oxidase [2] with an IC_{50} value of 0.32 μM in colonic epithelial cell

Table 1: H_2S-producing bacteria of the digestive tract.

Bacteria genus	Location	Process	References
Veillonella	Oral cavity and stomach	Metabolism of carbohydrates/lactate	[23] [24]
Actinomyces	Oral cavity and small intestine	Metabolism of lactate	[23] [24]
Prevotella	Oral cavity	Metabolism of short-chain fatty acids	[23] [24]
Desulfosarcina, *Desulfotomaculum*, *Desulfonema*, *Desulfovibrio (Desulfomonas)*, *Desulfococcus*, *Desulfobacter*	Human and animal colon	Reduction of sulfate or other oxidized sulfur compounds using H_2 or lactate	[25] [26] [14]

homogenates [27]. Despite this finding, colonic epithelial cells are surprisingly resistant to even millimolar H_2S concentrations [13, 27] due to a detoxifying sulfide metabolism [13]. Remarkably, low (micromolar) concentrations of H_2S are even able to stimulate cellular respiration and represent an energy substrate for epithelial cells (for excellent review see [13]). H_2S is metabolized in mitochondria by the sulfide oxidation pathway [18, 28]. In the presence of oxygen, sulfide:quinone oxidoreductases (SQR) oxidize H_2S to persulfide which is subsequently oxidized into sulfite via dioxygenase. Sulfite is further transferred into thiosulfate by rhodanese. Thiosulfate reductase and sulfite oxidase eventually metabolize thiosulfate into sulfate which is excreted. Interestingly, epithelial tissues which are predominantly exposed to H_2S, such as colon epithelia, have a particular high metabolic, H_2S-detoxifying capacity [29, 30].

The oxidative metabolism of H_2S in mitochondria implies that concentrations of free H_2S in normoxic tissues are low. The group of Olson demonstrated with H_2S-specific amperometric sensors that endogenous H_2S production inversely correlates with oxygen concentrations [28, 31–33]. Hence, significant amounts of H_2S are only produced in anoxic environments and rapidly disappear in the presence of oxygen. This dynamic relationship between oxygen and H_2S indicates the necessity to maintain free H_2S concentrations low and, furthermore, led to the hypothesis that H_2S might be a cellular oxygen sensor [28, 34, 35].

In sum, recent evidence suggests that there is a dynamic intracellular life-cycle of H_2S. Its enzymatic production is antagonized by storage as bound sulfane sulfur and oxidative metabolism, thereby limiting the concentration of free H_2S to the submicromolar range [36, 37]. Aside from the intracellular biochemical mechanisms to maintain low H_2S concentrations, there are epithelial mechanisms which prevent the exposure to potentially dangerous, high H_2S concentrations. This will be described in the next sections.

3. Epithelial Reactions to H_2S

Similar to nervous, connective and muscle tissues, epithelia are a basic type of animal tissue. They are continuous sheets of tightly packed cells lining the surfaces and cavities of the body [38]. Epithelial cells are attached to each other by protein complexes such as tight junctions. The structure of an epithelium depends on the morphology of its cells and on the amount of cell layers it is made of. In monolayer epithelia (also called simple epithelia) the cells rest on the basal membrane. Flat and scale-like cells are characteristic of simple squamous epithelia. Cube-shaped cells constitute the simple cuboidal epithelium while simple columnar epithelia (gastrointestinal tract) are built of column-shaped cells. The cell nuclei may be disposed at different levels allowing for a pseudocellular stratification, a characteristic of the pseudostratified columnar epithelium which can be ciliated (trachea, upper respiratory tract) or not (penile urethra). When made of many layers, the epithelium is qualified as "stratified." Here, the shape of the upper most cells determines the type of epithelium. Hence, stratified squamous keratinized (skin) or not (oesophagus), stratified cuboidal (ducts of sweat glands), or stratified columnar (conjunctiva) epithelia can be distinguished. The lower most cell layers may comprise different types of cells. A further characteristic of stratified epithelia, besides keratinization, is their ability to contract and stretch. For the latter, they are called transitional epithelia (urothelium).

Epithelia are multifunctional structures which protect from mechanical forces or physical trauma, from desiccation or toxins. They act as barriers against pathogens, regulating exchanges of, for example, water and electrolytes between the *milieu intérieur* and the *milieu extérieur*. The epithelia of the lung or the digestive tract are especially faced with building a protective barrier towards the outer environment on the one hand and at the same time allowing for efficient exchange, such as the respiratory gases or food components, on the other. Vectorial transport of electrolytes and water is a fundamental feature of these epithelia, regulating the diffusion barrier for respiratory gases in the lung, or exchanging nutrients and water from intestinal content. Furthermore, these epithelia use secretory mechanisms as a defence reaction against potentially harmful substances by flushing their outer surfaces. Hence, the epithelial electrolyte and water transport machinery represents both a key feature for normal physiology of these organs and primary defence mechanism. In the following section we discuss the impact of H_2S,

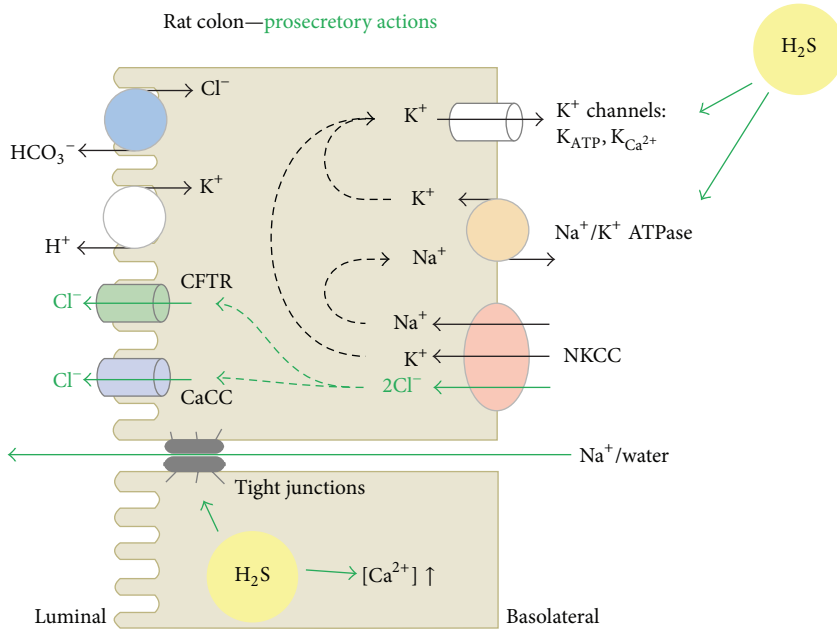

Figure 2: Epithelial ion transport responses to exogenous hydrogen sulfide in rat colon. Channels and transport mechanisms activated by H_2S in the rat colonic epithelium. Anion (Cl^-) secretion through the cystic fibrosis transmembrane conductance regulator (CFTR) or calcium-dependent chloride channels (CaCC) is enabled after building up a cytosolic anion potential, which results from the activation of basolateral transporters notably: the Na^+/K^+-ATPase maintaining the K^+ concentration gradient between the intracellular and the extracellular spaces; the Ca^{2+}-dependent ($K_{Ca^{2+}}$) and ATP-sensitive (K_{ATP}) K^+ channels, responsible for the driving force allowing for uptake of Cl^- via the basolateral Na^+-K^+-Cl^- cotransporter type 1 (NKCC1). H_2S donors induce activation of the Na^+/K^+-ATPase and basolateral K^+ conductance, enabling intracellular accumulation of Cl^-, which will be secreted via the CFTR or CaCC. Also, the apical Na^+/Ca^{2+} exchanger was activated in parallel with increase in paracellular permeability.

in both potentially harmful and physiological concentrations, on electrolyte and water transport mechanisms in intestinal and pulmonary epithelia.

3.1. H_2S and Intestinal Epithelia

3.1.1. Intestinal Epithelial Responses to Exogenous H_2S. The intestinal epithelium fulfils the above mentioned epithelial functions, with a more or less secretory or absorptive profile depending on the segment. The determination of intestinal epithelial functions finds its origins in its cellular organization. Intestinal epithelia are organized into finger-like protrusions called villi, absent in the colon, and invaginations called crypts of Lieberkühn (short: crypts). Four types of cells constitute the intestinal epithelium: enterocytes, goblet cells, Paneth cells, and enteroendocrine cells. The Paneth cells, absent in the colon, are located in the crypts and promote defence by their antimicrobial secretions. Also, pluripotent stem cells are located in the crypts; their mitosis generates the other cells, which migrate up the crypt-villus axis and differentiate. The goblet cells produce and secrete mucus, protecting against shear stress and chemical damage [38]. Enteroendocrine cells produce hormones or peptides for digestion or chemical sensors for digestive reflexes. Enterocytes are polarized cells representing the absorptive cell lineage. They carry an apical brush border which is responsible for enzymatic digestion, as well as ion, water, and nutrient uptake. These transport pathways may be transcellular passive (facilitated or not), active, or paracellular through cell junctions such as tight junctions.

Intestinal ion transport, which can be switched from absorption into secretion of water and electrolytes, is controlled by neurotransmitters and hormones, but also gasotransmitters such as CO, NO, H_2S, and nitroxyl (HNO) [39, 40].

The colonic epithelium absorbs water and electrolytes under basal conditions; however, distention of the gut wall by intestinal content induces electrolyte secretion in order to generate a fluid film which facilitates the protrusion of the content. The switch to secretion is also observed after exposure of the epithelium to bacterial products and observed in the development of secretory diarrhoea under pathophysiological conditions. Secretion by the colonic epithelium is predominantly driven by a transepithelial secretion of chloride ions into the lumen, which osmotically facilitates liquid secretion. In order to secrete chloride across the apical membrane of the epithelial cells, the basolateral membrane has to establish a driving force for anion efflux via anion channels (e.g., cystic fibrosis transmembrane conductance regulator, CFTR, or Ca^{2+}-sensitive chloride channels) located in the apical membrane (Figure 2). In the basolateral membrane, potassium channels generate the driving force for chloride secretion by maintaining the negative membrane potential which is dominated by a potassium diffusion potential [41,

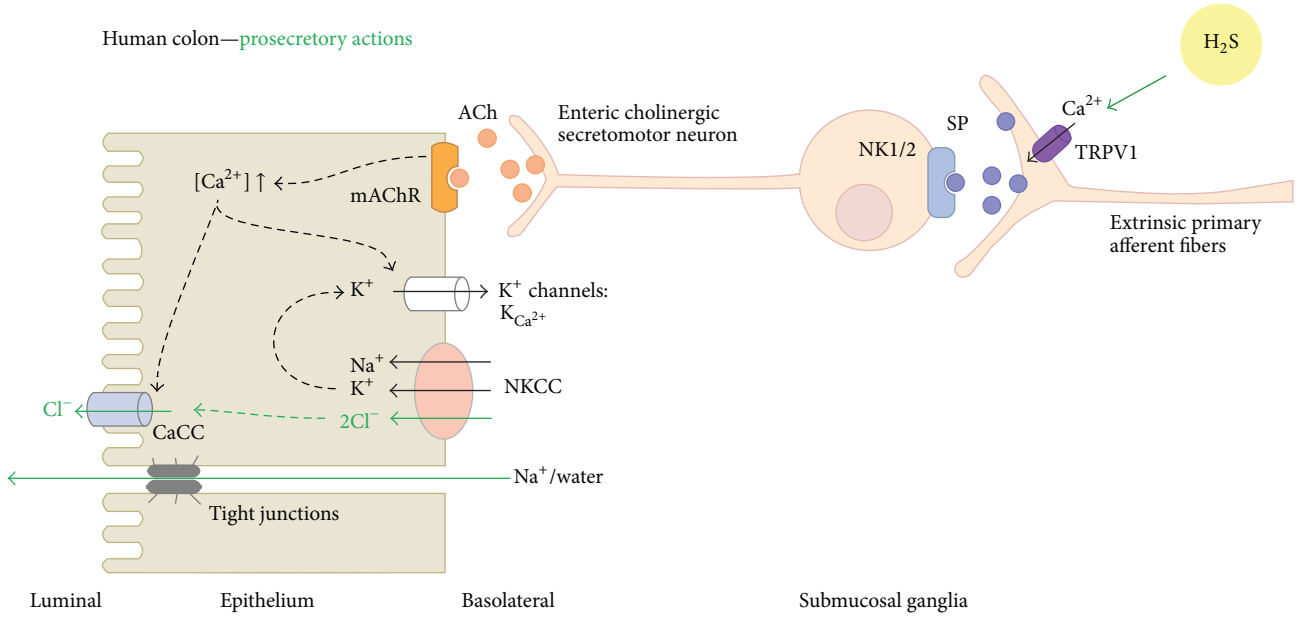

FIGURE 3: Exogenous hydrogen sulfide stimulates electrolyte secretion in human colon via activation of submucosal neurons. H_2S stimulates TRPV1 channels in extrinsic primary afferent fibres, which leads to the release of substance P (SP). SP binds to neurokinin receptors (NK) 1 and 2 of enteric cholinergic secretomotor neurons in submucosal ganglia. The subsequently released acetyl choline (ACh) binds to muscarinergic acetyl choline receptors (mAChR) in the epithelial cells. This stimulates a rise in intracellular calcium concentrations which triggers electrolyte secretion by activating calcium-dependent chloride (CaCC) and potassium ($K_{Ca^{2+}}$) channels.

42]. The Na^+/K^+-ATPase maintains the potassium concentration gradient between the intra- and the extracellular space as a prerequisite for the establishment of the potassium diffusion potential (Figure 2).

H_2S affects key ion transport processes within the colonic epithelium. In rat, guinea pig, and human colon preparations H_2S (applied by sulfur salts) elicits a secretory response [43–45]. In rat colon, H_2S activates the apical membrane Na^+/Ca^{2+} exchanger extruding Ca^{2+} (Figure 2). It also induces an increase in cytosolic Ca^{2+} concentration as a result of the release of stored Ca^{2+} from intracellular organelles via IP_3-receptors or ryanodine receptors (RyR). Cytosolic accumulation of Ca^{2+} triggers the opening of basolateral Ca^{2+}-dependent potassium channels (K_{Ca}). Furthermore, an ATP-sensitive basolateral potassium (K_{ATP}) conductance is activated in the presence of H_2S. The K_{ATP} channels carrying this conductance may consist of the following combinations: Kir6.1/SUR1, Kir6.1/SUR2B, Kir6.2/SUR1, or Kir6.2/SUR2B [46]. They also exert a protective function against energy depletion as their inhibition by glibenclamide under this condition causes a huge increase in the colonic epithelium conductance [46]. The protective characteristic of K_{ATP} channels was also demonstrated in rats with colitis as their blockade with glibenclamide worsened the disease and increased mortality [47].

Both basolateral potassium conductance (K_{ATP} and K_{Ca}) build the driving force for apical chloride efflux (Figure 2). Furthermore, the Na^+/K^+-ATPase is also activated by H_2S [45]. In addition to the secretion of chloride, potassium is also secreted and the epithelial permeability increases [43, 48]. As a consequence of these secretion processes, sodium ions move paracellularly to the lumen (for electroneutrality) and water follows osmotically.

Aside from the direct effect of H_2S on ion channels and transporters within the epithelium, exogenous H_2S indirectly stimulates chloride secretion by activating secretomotor neurons in human and guinea pig colon mucosa/submucosa preparations [44] (Figure 3). This is supported by the facts that (1) the H_2S-induced secretory response in these preparations is inhibited by tetrodotoxin and (2) it is absent in the human colon epithelial cell line T84 [44]. The reason for the different actions of H_2S in rat and human/guinea pig colon preparations is unknown but might be explained by differences in the species. In human/guinea pig colon, H_2S activates the transient receptor potential vanilloid receptor 1 (TRPV1) in extrinsic primary afferent fibres, which results in a release of substance P [49]. Substance P binds to neurokinin receptors (NK) in enteric cholinergic secretomotor neurons [49]. The subsequent release of acetylcholine by these neurons stimulates epithelial chloride secretion via a muscarinergic receptor- and calcium-mediated signalling axis (Figure 3).

In addition to the described effects of H_2S on the secretion of chloride ions, H_2S activates bicarbonate secretion which in turn neutralizes excess acid produced in the gastrointestinal lumen thereby protecting from gastric or duodenal mucosa ulcers [50, 51]. Furthermore, H_2S increases mucus production from goblet cells, reversing inflammation-associated mucus deficiency [52].

The secretory response to exogenous H_2S could be interpreted as a defence mechanism of the intestinal epithelium which prevents potentially harmful H_2S concentrations

which might exceed the oxidative capacities of the epithelial cells. Since the oxidative capacity is particularly high in intestinal epithelia [30] the secretory response would be triggered by rather high exogenous H_2S concentrations. In sum, exogenous H_2S triggers a secretory epithelial response in the intestine which prevents toxic effects of H_2S as well as onset of inflammatory processes by flushing the exogenous sources for H_2S from the epithelial wall.

3.1.2. Endogenous H_2S Production in Intestinal Epithelia. Gut tissue produces and releases H_2S [53] and the H_2S-generating enzymes CBS and CSE have been found within rat colonic epithelium [43]. The local H_2S concentration within the intestinal wall is unknown but the production rate of H_2S in rat ileum is in the range of 12 nmol/min/g tissue [54]. Consistent with the described prosecretory actions of H_2S, inhibitors of CBS and CSE dose-dependently decreased basal anion secretion across rat distal colon preparations, indicating that endogenous H_2S production might contribute to basal (secretory) ion transport mechanisms in the epithelium [43]. These observations seem to contrast the principle of oxidative degradation of H_2S under normal physiological conditions (see Section 2.2.2); however, the environment of the gut might generate local, even subcellular anoxic milieus which would allow for enzymatic H_2S production. Whether or not endogenous H_2S influences basal secretory processes via the same mechanisms and ion channels/transporters as exogenous H_2S is unknown. Furthermore, the administration of the H_2S "precursor" L-cysteine stimulated a secretory response in guinea pig colon preparations, which was sensitive to CBS and CSE inhibitors as well as the TRP channel inhibitor capsaicin [44]. This indicates that the indirect secretory epithelial response in this preparation (see Section 3.1.1) does occur not only due to exogenous H_2S, but also as a result of enhanced intracellular H_2S production in submucosal neurons. Whether such endogenous H_2S production in submucosal neurons occurs under physiological (normoxic) conditions and contributes to ion transport regulation is unknown.

3.1.3. H_2S in Intestinal Epithelia: Pharmacological Aspects. The local concentration of H_2S as well as the velocity of the H_2S-generating pathway determines whether H_2S acts as a prosecretory (as described above) or as an antisecretory agent. This was demonstrated by assessing chloride secretion by rat enterocytes using different H_2S-generating compounds: GYY4137 as a very slow releaser, L-cysteine and diallyl trisulfide as relatively slow releasers, and NaHS as a fast releaser. The last two compounds activated chloride secretion whereas L-cysteine and GYY4137 induced the opposite (antisecretory) effect [48]. Such concentration-dependent proabsorptive/prosecretory epithelial actions have already been described for NO and indicate that the kinetics of production, degradation, and even formation of reactive intermediates determines the physiological effects of gasotransmitters. These facts should be considered when evaluating (1) the contribution of (endogenous and exogenous) H_2S to the pathogenesis of diseases as well as (2) H_2S-releasing molecules for therapeutic purposes.

For example, pro- and anti-inflammatory effects are ascribed to H_2S (for review, see [4]). In rat models of colitis, inflammation was resolved by NaHS, Lawesson's reagent, and diallyl trisulfide. These H_2S donors achieved such a positive action by downregulating the expression of proinflammatory cytokines (IL-1β, TNF, INFγ, IL-12, and IL-13) and of chemokines (for review, see [7]). This is consistent with the observed therapeutic effects of, for example, the slow-release ATB-429 in a mouse model of colitis [55]. By contrast, other studies describe detrimental effects such as proinflammatory actions or impairment of gastrointestinal integrity [56–58].

In a rat model of colitis, the secretory response to H_2S was reduced in colon epithelial preparations. This reduction was prevented when the animals were fed on a S-reduced diet (which reduces microflora-mediated H_2S formation), but not by inhibitors of H_2S-generating enzymes [59]. This is consistent with an enhanced number of SRBs and faecal H_2S production in patients with ulcerative colitis [60, 61]. These findings indicate that alteration in exogenous, that is, colonic, microflora-derived H_2S, determines the efficacy of H_2S-liberating molecules in diseased tissues. This appears to be reasoned in a desensitisation of the intestinal epithelium to repeated exposure to H_2S [43]. Again, this example demonstrates that the kinetics of H_2S production and local concentration are important determinants of epithelial reactions to this molecule.

3.2. H_2S and Pulmonary Epithelia

3.2.1. Pulmonary Epithelial Responses to Exogenous H_2S. In contrast to secretory actions of H_2S in the intestine, a growing body of evidence suggests that H_2S inhibits epithelial electrolyte absorption in a variety of tissues and species [33, 62–68], including epithelia of the lung. Electrolyte absorption by pulmonary epithelial cells is facilitated by the activity of basolaterally localised Na^+/K^+-ATPases which establish an electrochemical driving force for the entry of sodium ions through cation channels and/or transporters in the apical epithelial membrane [69]. The concerted action of these apical entry pathways and basolateral Na^+/K^+-ATPases generates a vectorial absorption of sodium ions across the epithelium. Consequently, sodium absorption facilitates the paracellular absorption of chloride ions and water (Figure 4).

The rate-limiting step for sodium absorption in many vertebrate epithelia including those of the lungs is the epithelial sodium channel (ENaC) [70–73]. Experimental evidence from lung epithelial preparations suggests that exogenous H_2S reduces ENaC-mediated epithelial electrolyte absorption (Figure 4). H_2S-releasing compounds such as the sulfur salts NaHS and Na_2S decreased basal ENaC-mediated sodium absorption in airway epithelial preparations from pigs and mice [62], as well as distal lung tissues from rats [33] and amphibians [63]. Consistent with the concept that lung liquid clearance largely depends on ENaC-mediated sodium transport [74–76], the sulfur salts decreased the basal rate of transepithelial liquid absorption in rat lungs [33, 68]. A decreased capacity to absorb excess lung liquid would facilitate the formation of liquid accumulation in lungs. Mice

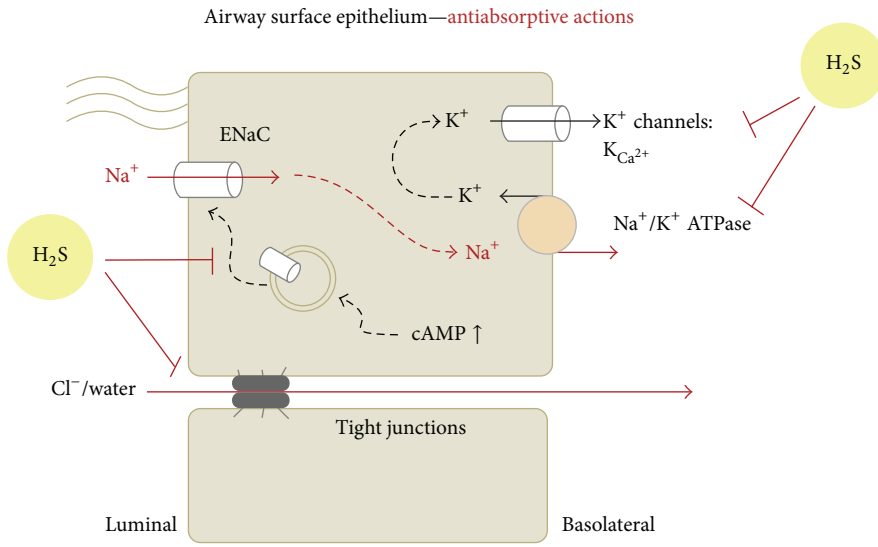

FIGURE 4: Airway epithelial ion transport responses to exogenous hydrogen sulfide. In airway surface epithelia, H_2S decreases basal absorption of Na^+ and eventually water by decreasing the electrochemical driving forces for apical entry of Na^+ through epithelial sodium channels (ENaC). This occurs by inhibition of the basolateral Na^+/K^+-ATPase as well as $K_{Ca^{2+}}$ channels. Furthermore, exogenous H_2S prevents the translocation of subapical vesicles containing ENaCs to the membrane thereby abrogating the action of proabsorptive, cAMP-dependent stimuli.

and rats develop such pulmonary oedema after exposure to exogenous H_2S [68, 77, 78]. Furthermore, pulmonary oedema formation is characteristic for patients who suffer from acute H_2S poisoning [79], thus providing further evidence that exogenous H_2S reduces epithelial sodium and liquid absorption in lungs.

The precise mechanism how ENaC-mediated epithelial sodium absorption is impaired by H_2S has not been completely clarified. Patch-Clamp and heterologous expression experiments revealed that there is no direct effect of acute H_2S exposure on ENaC activity [62, 66, 67]. By contrast, long-term exposure to H_2S (NaHS) reduces the expression of the ENaC α-subunit by ERK1/2 mediated signalling [68]. However, the observed inhibition of ENaC-mediated epithelial sodium absorption by H_2S occurs too fast (within minutes) [33, 62, 63] to be explained by changes in gene expression of sodium transporting molecules. As mentioned above, the electrochemical driving force for apical sodium entry through ENaC is generated by the basolateral Na^+/K^+-ATPase. Apical membrane permeabilisation studies revealed that the activity of the Na^+/K^+-ATPase is reduced by H_2S [62, 63] (Figure 4). As described, the Na^+/K^+-ATPase is also influenced by H_2S in rat distal colon epithelia [45]. Furthermore, it is speculated that H_2S-induced inhibition of sodium-uptake in larval zebrafish might be due to impaired Na^+/K^+-ATPase activity [65], further indicating that this enzyme is a molecular target for H_2S.

Aside from the Na^+/K^+-ATPase, basolateral potassium channels contribute to the electrochemical driving force for sodium absorption by maintaining a negative membrane potential which facilitates cation influx at the apical epithelial plasma membrane. It was demonstrated that H_2S inhibits basolateral potassium channels in lung epithelia [62, 63] (Figure 4). Inhibitor studies suggest that K_{Ca} channels are likely targeted by H_2S; however, the precise type of basolateral potassium channel has yet to be identified. Inhibition of potassium channels as well as the Na^+/K^+-ATPase impairs the electrochemical gradient which is necessary for ENaC-mediated sodium influx and thereby reduces overall transepithelial sodium and, consequently, liquid absorption.

How H_2S leads to a decreased activity of these basolateral transporting molecules remains unknown. As mentioned above, the rapid effects of H_2S suggest changes in the activity of the transporting molecules rather than changes in their expression levels. This might be achieved by either changing their transport activity rates or, alternatively, their abundance in the basolateral plasma membrane.

In rat renal tubular epithelial cells, NaHS induces endocytosis of the Na^+/K^+-ATPase thereby inhibiting renal sodium absorption [64]. This occurs via a signalling cascade which is initiated by activation of the epidermal growth factor receptor by H_2S. In H441 lung epithelial cells, there is no change in membrane abundance of the Na^+/K^+-ATPase within the time course of sodium transport inhibition by H_2S [62], indicating that in lung epithelia H_2S rather interferes with the transport activity of this enzyme.

Activity changes of transport proteins might be achieved by a posttranslational modification due to H_2S. Initially it was suggested that H_2S is able to directly modify thiol groups in proteins (including K_{ATP} channels), a mechanism which was referred to as S-sulfhydration [80, 81], although persulfidation is the more correct term [82]. Recently it became clear that H_2S alone is not able to modify thiol groups, but derivatives of H_2S such as polysulfides [83] or reaction products of H_2S and NO such as nitroxyl (HNO) are able to do so [82]. Elegant work by Eberhardt and colleagues recently dissected

a mechanism underlying the activation of a member of the TRP channel family (TRPA1) by H_2S. These authors observed that HNO, which is formed by a redox reaction between H_2S and NO, induced calcium influx into neurons from dorsal root ganglia of wild type, but not TRPA1-*knock-out* mice [84]. The primary targets for HNO are thiols [85] and the N-terminal region of TRPA1 contains cysteine residues which are necessary for activation of the channel by sulfhydryl-reactive agents [86, 87]. Mutation of these residues to lysine prevented the activation of human TRPA1 by HNO [84]. Furthermore, the authors demonstrated that HNO induces a formation of disulfide bonds and suggest a model by which disulfide bond formation between two cysteine pairs induces a conformational change which leads to channel opening [84].

Whether a similar mechanism would also account for the observed inhibition of the Na^+/K^+-ATPase and basolateral potassium channels in lung epithelia remains to be investigated. Interestingly, NO also inhibits basolateral transporting molecules in H441 lung epithelial cells, with similar kinetics to H_2S [88], and posttranslational thiol-modification modulates the activity of the Na^+/K^+-ATPase [89]. Furthermore, HNO was recently shown to influence the Na^+/K^+-ATPase and basolateral potassium channels in distal rat colon epithelia [40], suggesting that these molecules represent molecular targets for reactive derivatives of H_2S. However, it has to be noted that H_2S stimulates the Na^+/K^+-ATPase in the rat colon, whereas an inhibition of the Na^+/K^+-ATPase was observed in airway surface epithelia. This discrepancy might be explained by variations in the Na^+/K^+-ATPase subunit composition. The Na^+/K^+-ATPase consists of core α- and β-subunit, of which there are four and three isoforms, respectively [90]. In addition, these core α/β-complexes assemble with an additional subunit of the FXYD protein family [91]. Tissue-dependent differences in the molecular Na^+/K^+-ATPase compositions might account for the different reactions to H_2S. Furthermore, basolateral potassium channels indirectly influence Na^+/K^+-ATPase activity by either enhancing or diminishing basolateral potassium-recycling and hence activity of the Na^+/K^+-ATPase. It is possible that the potassium channel repertoire of the basolateral membrane indirectly contributes to the observed variety in responses to H_2S.

Aside from the influence of H_2S on basal epithelial sodium transport processes, H_2S reduces the efficacy of proabsorptive stimuli. In lung epithelia, H_2S abrogated the stimulation of ENaC-mediated sodium transport and lung liquid clearance by β-adrenergic receptor agonists [33]. This is the result of an impairment of the cAMP/protein kinase A signalling axis, although the molecular target for H_2S remains unknown. In amphibian A6 (renal) cells, H_2S prevented the activation of ENaC by reactive oxygen species [66] and advanced glycation end-products [67], likely due to antioxidative mechanisms.

In sum, the currently available data indicate antiabsorptive actions of exogenous H_2S on electrolyte absorbing pulmonary epithelia.

3.2.2. Endogenous H_2S Production in Pulmonary Epithelia. In the human respiratory system, CBS/CSE have been detected in nasal mucosa [92, 93], submucosal glands [92, 93], and human H441 airway epithelial cells [33]. 3MST has only been detected at the mRNA level in human H441 cells [33]. In rodents, CBS/CSE are present in mouse airway epithelium [94], whereas CSE/3MST but not CBS were detected in rat lung homogenates [95]. All three proteins are present in airways and alveoli of cow and sea lion lungs [31]. These data indicate that pulmonary epithelia contain H_2S-generating pathways. Consistently, using H_2S-specific amperometric sensors it was shown that rat lung homogenates [95] as well as H441 epithelial cell lysates [33] produce detectable amounts of H_2S.

However, H_2S production is only detectable in the absence of oxygen (see Section 2.2.2). Consistently, inhibition of H_2S-generating enzymes under normoxic conditions does not alter baseline sodium absorption by these cells [62]. This indicates that H_2S is not a basal regulator of pulmonary epithelial sodium transport processes and the observed inhibition of sodium and water absorption rather represents an epithelial reaction to either exogenous H_2S or deregulated endogenous H_2S generation under pathophysiological conditions.

3.2.3. H_2S in Lung Epithelia: Physiological and Pathophysiological Aspects. How can these findings be integrated into a physiological context? The antiabsorptive and prosecretory epithelial responses can be interpreted as an epithelial defence mechanism against potentially harmful H_2S concentrations which exceed the oxidative H_2S-degrading capacities of the epithelial cells. Liquid accumulation at the luminal side of the epithelia would eliminate the potential sources of H_2S (such as microbiota) by "flushing" of epithelia-covered compartments.

The main epithelial defence mechanism in the lung is airway mucociliary clearance [96, 97], whereas in the distal, alveolar regions of the lungs, primary defence against potential pathogens is maintained by alveolar macrophages. The entire epithelial surface of the airways is covered by a thin film of liquid, the airway surface liquid (ASL). This ASL is composed of a liquid phase surrounding the cilia of the airway epithelial cells, the periciliary liquid (PCL), and a gel-like mucus layer on top of the PCL [98]. The mucus layer is a trap for inhaled particles and potential pathogens. Due to ciliary movement, the PCL as well as the mucus layer moves towards the larynx, thereby clearing the trapped particles/pathogens from the lungs. Mucus clearance depends on the degree of hydration of the mucus gel: an increase in ASL volume enhances mucociliary clearance [96, 97, 99]. Hence, mechanisms which result in hydration of the ASL will eventually strengthen mucociliary clearance and clear the pulmonary epithelial surface from potential pathogens. The ASL volume and, consequently, the degree of mucus hydration are regulated by liquid secretion and absorption across the pulmonary epithelium.

The liquid feeding the PCL/ASL is produced by submucosal glands [96], a process which depends on vectorial

anion secretion by serous cells of the glands [97]. A recent study demonstrated that submucosal glands of pig tracheal epithelia secrete liquid upon exposure to bacteria such as *Pseudomonas aeruginosa* [100], thereby showing that liquid secretion and augmented mucociliary clearance are a primary defence mechanism in the airways. Whether or not H_2S elicits anion secretion in submucosal glands is currently unknown. In experiments addressing liquid secretion/absorption rates in fluid-filled rat lungs, there was no effect of H_2S over that of the ENaC-inhibitor amiloride, indicating that H_2S does not induce a detectable liquid secretion [33]. However, in this preparation the major liquid movements occur across the large surface of the alveolar regions. A potential contribution of gland secretion of the upper airways might be difficult to detect with this approach.

Aside from liquid secretion by submucosal glands, the surface epithelium of the airways is considered to absorb excess liquid [96]. This concept is supported by an anatomical view of the epithelia-covered lung structures. The alveolar surface of the human lung is by a magnitude of 10^5 larger than that of the trachea [101]. Given that the epithelial surface liquid (alveolar liquid and ASL) is continuously transported towards the larynx, the volume of liquid lining the airway epithelia should also increase by the same magnitude. However, the height of the liquid layer lining the epithelia is relatively constant, indicating that excess volume is absorbed by the surface epithelial cells of the airways [101]. A reduction in epithelial sodium and liquid absorption would therefore result in a volume increase of the ASL. An *in vitro* approach using cultured human airway epithelia demonstrated that volume increase of the ASL results in a higher rate of mucus transport [102]. Furthermore, patients with the hereditary disease Pseudohypoaldosteronism Type 1 have a reduced ENaC activity in the airways and thus an increased ASL volume [99]. Consistently, these patients display a mucus transport rate which is profoundly above that of normal subjects [99]. This example demonstrates that a reduction in ENaC-mediated sodium absorption eventually results in an increased mucociliary clearance and would therefore be consistent with the idea of an H_2S-induced "defence-flushing" of epithelial surfaces. The occurrence of rhinorrhoea during exposure to H_2S [103] is consistent with an increased amount of surface liquid in the respiratory system. Furthermore, the formation of rhinorrhoea or, in the distal lung, pulmonary oedema due to H_2S poisoning might be interpreted as a hyperresponsive epithelial defence reaction to exogenous H_2S.

4. Conclusion and Perspective

The herein reviewed findings teach a concept by which physiological effects elicited by H_2S critically depend on its concentration as well as kinetics of its production and metabolism. In accordance with Paracelsus' principle, the local concentration is the critical factor which allows the separation of physiology and toxicology of H_2S. The precise determination of local H_2S concentrations is still a major technical challenge (for review see [36, 37]) and hampers the classification of toxic, physiological, and beneficial roles for this molecule.

The currently available data on (1) the biochemistry of endogenous H_2S production; (2) its correlation with oxygen availability; and (3) epithelial reactions to exogenous H_2S suggest that normal physiology aims to maintain a low endogenous H_2S concentration which is likely in the submicromolar range. The future challenges will be to understand pathophysiological conditions, in which these systems are impaired, and the precise determination of concentration windows which allow for pharmacological interference. Furthermore, the contribution of exogenous (i.e., microbiota-derived) H_2S in experimental studies should be taken into consideration. At this stage it appears most suitable to close with the introductory quote: *"All things are poisons, for there is nothing without poisonous qualities. It is only the dose which makes a thing poison."*

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

The H_2S Donor NaHS Changes the Expression Pattern of H_2S-Producing Enzymes after Myocardial Infarction

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Aims. To examine the expression patterns of hydrogen sulphide- (H_2S-) producing enzymes in ischaemic heart tissue and plasma levels of H_2S after 2 weeks of NaHS treatment after myocardial infarction (MI) and to clarify the role of endogenous H_2S in the MI process. *Results*. After MI surgery, 2 weeks of treatment with the H_2S donor NaHS alleviated ischaemic injury. Meanwhile, in ischemia myocardium, three H_2S-producing enzymes, cystathionine γ-lyase (CSE), cystathionine-β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3-MST) significantly increased. Plasma H_2S levels were also elevated. *In vitro*, NaHS treatment protected cardiomyocytes from hypoxic injury and raised CBS levels in a concentration-dependent manner. Different from *in vivo* results, however, CSE or 3-MST expression did not change. NaHS treatment increased the activity of CSE/CBS but not of 3-MST. When CSE was either knocked down (*in vitro*) or knocked out (*in vivo*), H_2S levels significantly decreased, which subsequently exacerbated the ischaemic injury. Meanwhile, the expressions of CBS and 3-MST increased due to compensation. *Conclusions*. Exogenous H_2S treatment changed the expressions of three H_2S-producing enzymes and H_2S levels after MI, suggesting a new and indirect regulatory mechanism for H_2S production and its contribution to cardiac protection. Endogenous H_2S plays an important role in protecting ischaemic tissue after MI.

1. Introduction

Hydrogen sulphide (H_2S) has been recognized as the third gasotransmitter, following nitric oxide and carbon monoxide. It is synthesized endogenously by the catalysis of two pyridoxal-5-phosphate-dependent enzymes, namely, cystathionine-β-synthase (CBS) and cystathionine γ-lyase (CSE), both utilizing L-cysteine as substrate [1, 2]. CBS is mainly localized in the nervous system, whereas CSE has a crucial role in maintaining cardiovascular homeostasis [3, 4]. The third H_2S-producing enzyme, 3-mercaptopyruvate sulfurtransferase (3-MST), has recently been discovered; this enzyme generates H_2S in both the nervous and cardiovascular systems [5]. Accumulating evidence indicates that H_2S is vital in regulating cardiovascular functions [6]. H_2S can relax smooth muscle cells by activating ATP-dependent potassium (K^+) channels (K_{ATP}), leading to a subsequent decrease in blood pressure [7]. It also has a substantial role in regulating cellular metabolism and alleviating heart fibrosis and inflammation [8]. H_2S can reduce infarct size and preserve left ventricular function after MI or myocardial ischaemia/reperfusion *in vivo* [9–11]. It also regulates the outward K^+ current (Ito) channels, extends action potential duration in epicardial myocytes, and shows efficacious protection against fatal arrhythmias in a rat model of myocardial infarction (MI) [12].

Qipshidze et al. reported that H_2S promotes angiogenesis and significantly limits the extent of MI injury. They also observed a decrease in CSE expression after MI. NaHS treatment for 4 weeks upregulates CSE but not CBS expression [13]. Zhu et al. also reported the reduction of CSE mRNA in rats 48 h after MI [9]. However, less is known about the expression patterns of the three H_2S-producing enzymes between 48 h and 4 weeks after MI, during which the fate of ischaemic cardiomyocytes is determined by many endogenous and exogenous signals. In this study, we tested the expressions of three H_2S-producing enzymes in ischaemic heart tissues 2 weeks after MI and measured plasma H_2S levels. We also clarified the role of endogenous H_2S after MI.

2. Materials and Methods

2.1. The Mouse MI Model and NaHS Administration In Vivo. Adult male C57BL/6 mice (8 weeks) were purchased from the Institute of Laboratory Animal Science of the Chinese Academy of Medical Sciences (Shanghai, China). Experimental protocols were approved by the ethics committee for experimental research, Shanghai Medical College, Fudan University.

Mice were anaesthetized and ventilated with a rodent ventilator (Kent Scientific Corporation, USA). A parasternal incision was made with surgical scissors by cutting the skin and intercostal muscles between the left third and fourth ribs; MI was induced by occluding the left anterior descending coronary artery (LAD) using a 7-0 silk suture beneath the LAD, 1 mm inferior to the left auricle. The sham group experienced the same procedure without coronary artery occlusion.

After MI surgery, different concentrations of NaHS solution (0, 25, 50, and 100 μmol/kg/day) were injected intraperitoneally for 2 weeks (first administration was shortly after MI on day 1). Normal saline was used as control. The sham group received same NaHS or normal saline treatment as MI groups. On the 15th day (no NaHS treatment on that day), mice were sent to get echocardiographic assessment. After that mice were anesthetized and killed for plasma to measure H_2S levels and for protein from ischemic area used for western blot to test the changes of three enzymes.

For acute infarct size determination, NaHS solution (0, 25, 50, and 100 μmol/kg) was administered 15 min before MI surgery. The infarct size is determined on day 2.

2.2. Echocardiographic Assessment. Echocardiography was performed as previously described [14]. Briefly, the mice were mildly anaesthetized with isoflurane (1%), and two-dimensional guided M-mode tracings were recorded using a Vevo 770 high-resolution system (Visualsonics, Toronto, Canada). Left ventricular internal dimension systole (LVIDs), left ventricular internal dimension diastole (LVIDd), left ventricular ejection fraction (LVEF), and left ventricular fractional shortening (LVFS) were measured to evaluate left ventricular function. All parameters were the average values from three cardiac cycles.

2.3. Determination of Infarct Size. Evans' Blue-2,3,5-triphenyltetrazolium chloride (TTC) double staining was introduced to measure the infarct size and the areas at risk as previously described [10]. Mice were injected with NaHS solution (0, 25, 50, and 100 μmol/kg) for 15 min before MI surgery. Twenty-four hours later, these mice were anaesthetized and their chests were opened again to expose the hearts. Evans' Blue (1.0%, Sigma-Aldrich, St. Louis, MO) solution was injected intravenously into the femoral vein to distinguish ischaemic from nonischaemic areas. The hearts were immediately excised and sliced into 1-2 mm thick slices parallel to the atrioventricular groove and immersed in 1% TTC (Sigma-Aldrich, St. Louis, MO) at 37°C for 15 min. The size of the area at risk (AAR) and the infarct area (INF) were quantified using ImageJ software by an observer blinded to the study.

2.4. Western Blot. Proteins were extracted from mouse left ventricles 2 weeks after MI or from cardiomyocytes pretreated with NaHS or vehicle 30 min before the 6 h exposure to hypoxic or normoxic conditions. Protein concentrations were determined by the BCA method (Shen Neng Bo Cai Corporation, Shanghai, China). Protein samples (50 μg) were subjected to 10% SDS-PAGE, transferred to polyvinylidene fluoride membranes (Millipore-Upstate, Billerica, MA, USA), and blocked with 5% nonfat milk. The membranes were then probed with primary antibodies against CSE, CBS (Abcam, Cambridge, UK), or 3-MST (Santa Cruz Biotechnology, CA). GAPDH was used as the internal control (Cell Signaling Technology). These experiments were performed at 4°C overnight. After washing with TBST 3X, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 h. SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific-Pierce, Waltham, MA, USA) was then added and detected on radiographic films. Gray values of bands were quantified with the SmartViewer software.

2.5. Measurement of H_2S Levels and the Activity of H_2S-Producing Enzymes. Whole blood sample was collected, anticoagulated, and centrifuged at 3000 rpm for 15 min to obtain the plasma. The medium from cardiomyocyte cultures was collected. H_2S levels in plasma or culture medium were measured as previously described [15]. H_2S levels were calculated using a standard curve generated from a sodium sulphide solution (0–100 μM). The activities of CSE/CBS and 3-MST were measured according to the methods described by Tao et al. [16]. Briefly, cardiomyocytes were incubated with the substrate, L-cysteine, in the presence of pyridoxal-5′-phosphate for CSE/CBS or α-ketoglutarate for 3-MST. H_2S levels were measured, and the amount of H_2S produced by 1 μg protein/min was calculated, representing the activity of H_2S-producing enzymes. However, because the detection of CSE and CBS activity shared the same reaction system, their activities could not be separated using this method.

2.6. Cell Culture and Treatment. Primary cultures of neonatal cardiomyocytes were established according to previously described methods [10]. The experimental protocol was approved by the ethics committee for experimental research,

Shanghai Medical College, Fudan University. In brief, the hearts dissected from 1-day-old Sprague-Dawley rats were washed in cold phosphate-buffered saline (PBS) containing 130 mM NaCl, 3 mM KCl, 1 mM NaH_2PO_4, 4 mM glucose, and 20 mM HEPES (pH adjusted to 7.4 with NaOH) for three times to remove blood. The hearts were then minced in a drop of 0.125% trypsin (Sigma-Aldrich, St. Louis, MO, USA) to as small a size as possible and then subjected to a series of incubations at 37°C in a normal saline-buffered trypsin solution. After centrifugation, cell pellets were resuspended in Dulbecco's modified Eagle medium/F-12 (Gibco, Grand Island, NY) containing 10% heat-inactivated fatal bovine serum (Gibco, Grand Island, NY), 100 U/mL penicillin, and 100 μg/mL streptomycin. To increase the purity of cardiomyocytes, dissociated cells were plated in 100 mm culture dishes and kept in a 5% CO_2 incubator at 37°C for 1 h. Nonmyocytes readily attached to the bottom of the dish, whereas most cardiomyocytes remained suspended in the medium. The resulting suspensions of cardiomyocytes were diluted to a density of 1×10^6 cells/mL and plated in 60 mm dishes or 6-well plates for protein extraction or 96-well plates for the Cell Counting Kit 8 (CCK8) and lactic dehydrogenase (LDH) tests and then cultured in a humidified 5% CO_2 incubator at 37°C. 5-Bromo-2′-deoxyuridine (100 μM) was added during the first 48 h to prevent proliferation of residual nonmyocytes.

To establish the hypoxia model, cardiomyocytes were cultured in DMEM/F-12 without serum and exposed to hypoxic conditions (95% N_2 + 5% CO_2) for 6 h. For the NaHS treatment groups, 25, 50, and 100 μM of NaHS solution were added 30 min before hypoxia.

CSE small interfering RNAs (CSE siRNA) were purchased from Ambion (Austin, TX, USA). Cardiomyocytes were transfected with 25 nM CSE siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's guidelines.

2.7. CCK8 and LDH Activity. Cardiomyocytes were seeded on 96-well plate and treated with vehicle or NaHS for 30 min, followed by 6 h exposure to hypoxic or normoxic conditions. Culture medium was collected and LDH activity was tested according to manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Cardiomyocytes were used for CCK8 activity measurement according to manufacturer's instructions (Dojindo Molecular Technologies, Japan).

2.8. CSE Knock-Out Mice Identification. PCR-genotyping of CSE KO mice was identified according to the methods before [17]. Briefly, genomic DNA was extracted from heart tissues and subjected to PCR with paired primers: CSE knock-out mice (CSE KO) forward, 5′-CCTGGATATAAGCGCCAAAG-3′, and reverse, 5′-AGGAACCAGGGCGTATCTCT-3′, wild type (WT) forward, 5′-CCTGGATATAAGCGCCAAAG-3′, and reverse, 5′-CGAGAATTCCATTGCTCAGG. The amplified DNAs were then identified by DNA electrophoresis, from the representative photo of which we could separate CSE KO mice (single band, 309 bp) from WT mice (single band, 167 bp).

2.9. Statistical Analysis. Data are expressed as means ± SEM. Statistical analysis was performed using an SPSS software package, version 17.0 (SPSS, Inc., Chicago, IL, USA). The statistical comparisons among groups were performed by one-way ANOVA. Paired data were evaluated by two-tailed Student's t-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. The H_2S Donor (NaHS) Protected Ischaemic Myocardium from Myocardial Infarction Injury. We used echocardiography to evaluate systolic and diastolic function. Echocardiography revealed that 2 weeks of NaHS treatment at 50 and 100 μmol/kg/day significantly increased cardiac function compared with cardiac function after treatment with normal saline. LVEF and LVFS increased, whereas LVIDs and LVIDd decreased (Figures 1(a) and 1(b)). Further, the infarct size was measured by Evans' Blue-TTC staining. NaHS pretreatment at 50 and 100 μmol/kg for 15 min significantly decreased the INF compared with the normal saline group (Figure 1(c)).

The above results showed that NaHS treatment after MI surgery could efficiently protect the ischaemic heart compared with the untreated group.

3.2. H_2S Levels and Expressions of H_2S-Producing Enzymes Increased with NaHS Treatments. Ischaemic heart tissues from MI mice showed decreased CSE and 3-MST levels after 2 weeks; in contrast, CBS expression significantly increased. Two weeks of NaHS treatments in the range of 25–100 μmol/kg/d significantly increased the protein expression of all three enzymes in ischemia myocardium (Figures 2(a) and 2(c)). In noninfarcted control hearts, NaHS treatment for 2 weeks increased the expression of CSE (50 μmol/kg/day) and 3-MST (25 μmol/kg/day), while NaHS (25–100 μmol/kg/day) did not change expression of CBS (Figure 2(b)). Plasma H_2S levels decreased after MI surgery, whereas 2 weeks of NaHS treatments alleviated the reduction. NaHS treatment (50 and 100 μmol/kg/day) significantly raised the plasma H_2S levels after MI (Figure 2(d)).

3.3. The H_2S Donor NaHS Protected Cardiomyocytes from Hypoxia-Induced Injury In Vitro. Cardiomyocytes are the major heart cells, and more importantly, they are highly terminated and cannot proliferate after birth. Protecting cardiomyocytes from ischaemic injury is essential. LDH and CCK8 tests are classic methods to detect cell viability and toxicity. Compared with the normoxic group, LDH activity in the culture medium of cardiomyocytes exposed to hypoxic conditions was much higher, whereas NaHS treatment in the range of 25–100 μM significantly decreased LDH activity. NaHS treatment at 50 and 100 μM also increased CCK8 values, indicating improved cardiomyocyte activity (Figure 3(a)). Cells produced less H_2S under hypoxic conditions, whereas NaHS treatment significantly increased H_2S production in a concentration-dependent manner (Figure 3(b)). Different from the enzyme expression patterns in ischaemic heart tissues, hypoxia did not change CSE or 3-MST expressions, whereas an increase in CBS expression still occurred

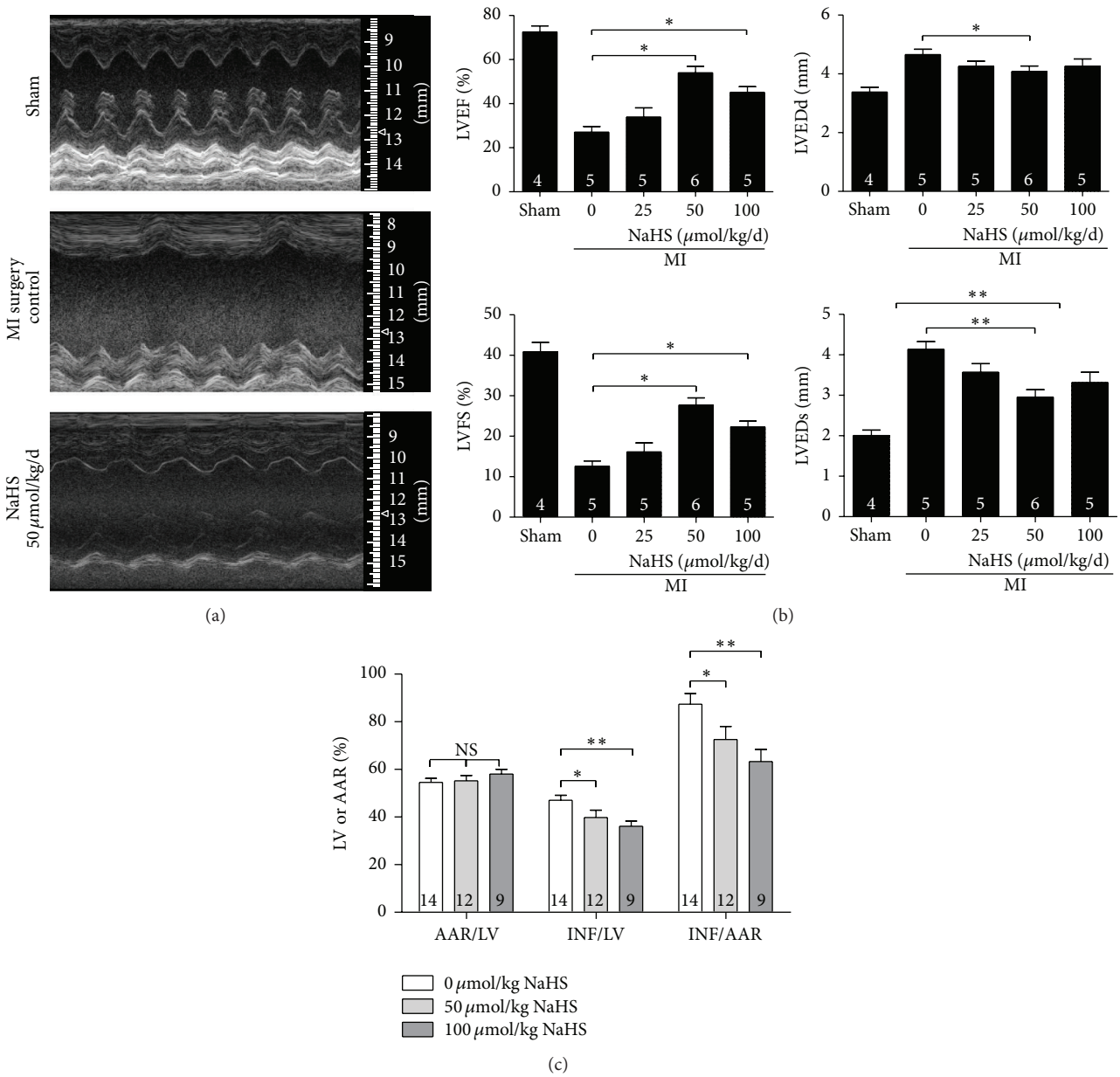


FIGURE 1: The H_2S donor NaHS protects ischaemic myocardium from myocardial infarction injury. (a) Representative echocardiogram from the sham, MI surgery control, and NaHS 50 μmol/kg/day treatment groups; (b) Echocardiographic parameter analysis. LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; LVIDs, left ventricular internal dimension systole; LVIDd, left ventricular internal dimension diastole. (c) Statistics of Evans' Blue-TTC staining to show the percentage of area at risk or infarct area. LV, left ventricle; AAR, area at risk; and INF, infarct area. Numbers inside bars denote the number of animals per group. Values are mean ± SE. $^{*}P < 0.05$ and $^{**}P < 0.01$.

in vitro. NaHS treatments did not change CSE or 3-MST expressions under hypoxic conditions but caused an increase in CBS levels in a concentration-dependent manner (Figures 3(c) and 3(d)). Hypoxia decreased the activity of CSE/CBS, and 50 μM NaHS treatment significantly diminished this decrease (Figure 3(e)). 3-MST activity was not influenced (Figure 3(f)).

3.4. Endogenous H_2S Protected Ischaemic Myocardium from Myocardial Infarction Injury. CSE is the main H_2S-producing enzyme in the cardiovascular system, catalyzing the synthesis of endogenous H_2S from L-cysteine; we used CSE KO mice to study whether endogenous H_2S plays a role in cardiac protection after MI. CSE KO mice were characterized by analysing genomic DNA, CSE mRNA, and protein levels, which were compared with WT mice (Figure 4(a)). Plasma H_2S levels in four groups were then tested: (1) CSE KO without MI, (2) CSE KO with MI, (3) WT without MI, and (4) WT with MI. As expected, plasma H_2S levels were significantly lower in mice from the CSE KO without MI group

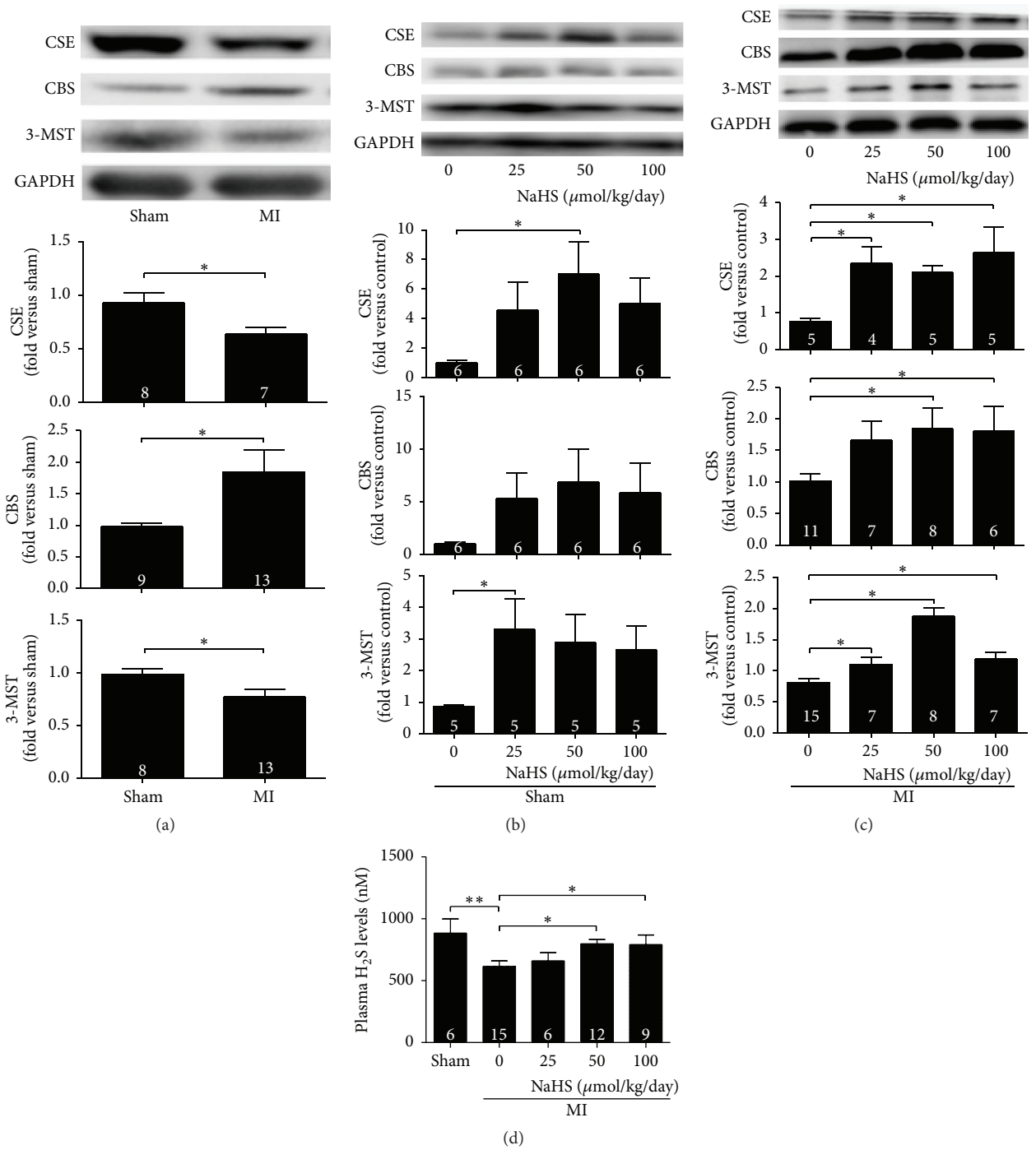
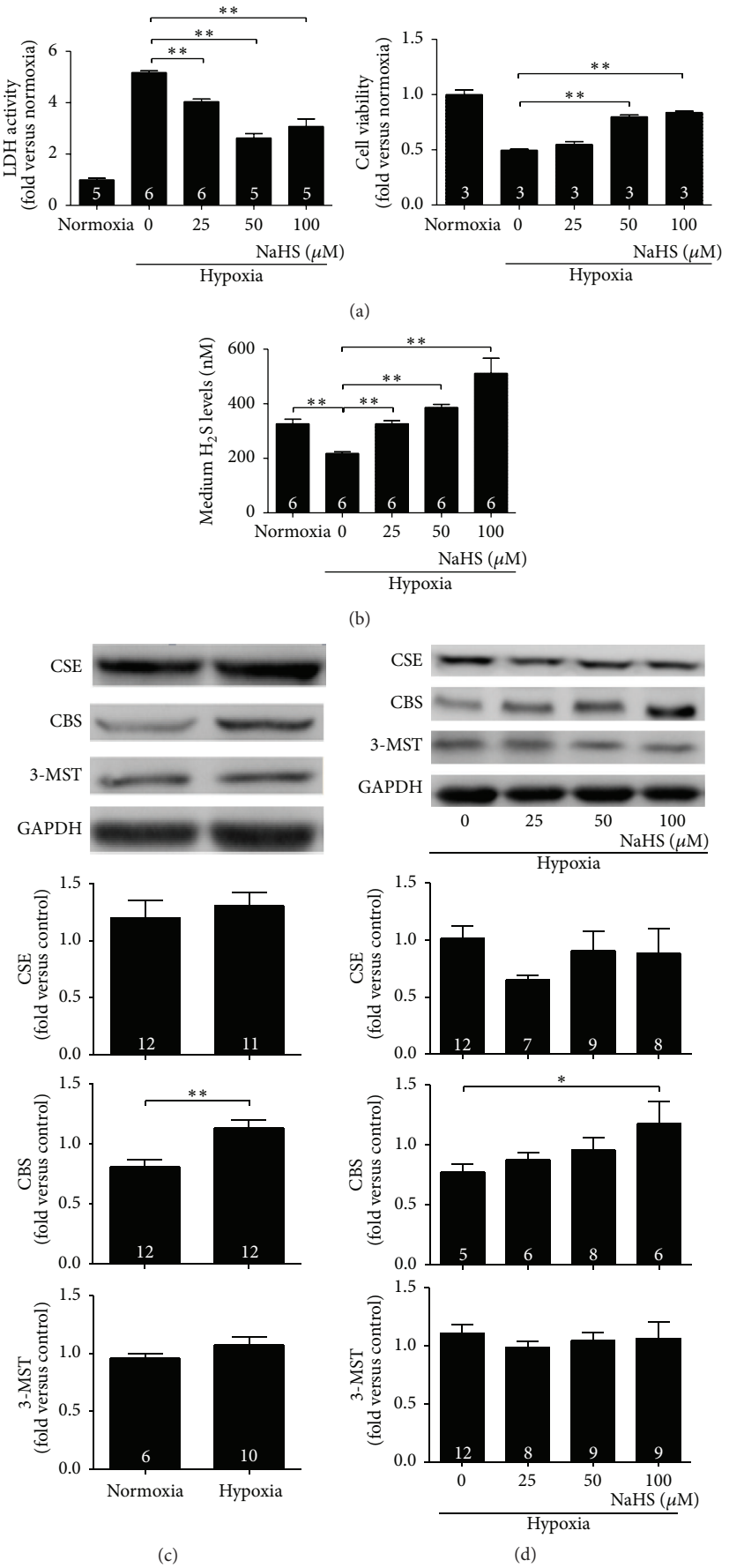


Figure 2: Exogenous H_2S increased plasma H_2S levels and the three H_2S-producing enzymes expression in ischaemic myocardium. (a) The expressions of CSE and 3-MST significantly decreased, whereas CBS expression increased 2 weeks after MI surgery. (b) In noninfarcted myocardium, NaHS treatment for 2 weeks increased the expression of CSE (50 μmol/kg/day) and 3-MST (25 μmol/kg/day), while NaHS (25–100 μmol/kg/day) did not change expression of CBS; (c) H_2S donor NaHS (25–100 μmol/kg/day) increased the expression of the three enzymes in ischaemic myocardium 2 weeks after MI. (d) Changes in plasma H_2S levels in sham, MI surgery control, and NaHS treatment groups. Plasma H_2S levels decreased 2 weeks after MI, whereas 50 and 100 μmol/kg/day NaHS treatment ameliorated this trend. Numbers inside bars denote the number of animals per group. Values are mean ± SE. $^*P < 0.05$.



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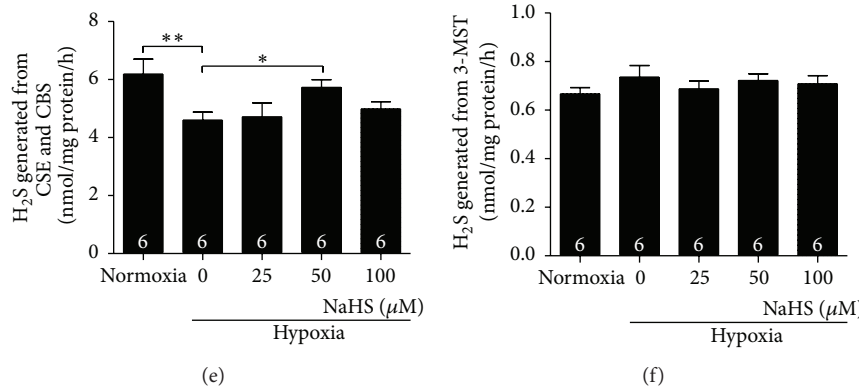

FIGURE 3: H_2S donor NaHS protects cardiomyocytes from hypoxia-induced injury. (a) Cell viability was detected with LDH and CCK8 kits. Hypoxia exposure decreased cell viability, as evidenced by high LDH activity and low CCK8 values; 30 min of NaHS pretreatment alleviated the above injury, as demonstrated by the significant drop in LDH activity and increase in CCK8 values; (b) H_2S level in the culture medium decreased after exposure to hypoxia, and 25–100 μM NaHS pretreatment in a concentration-dependent manner caused elevation of H_2S levels; (c) 6 h hypoxia exposure did not change CSE or 3-MST expressions but significantly increased CBS expression; (d) NaHS pretreatment did not change the expression of either CSE or 3-MST but increased CBS expression at 100 μM. (e) The activity of CSE/CBS declined after exposure to hypoxia, whereas 50 μM NaHS treatment caused a significant increase in their activity; (f) Hypoxia with or without NaHS treatment did not change the activity of 3-MST. Numbers inside bars denote the number of animals per group. Values are mean ± SE. $^*P < 0.05$ and $^{**}P < 0.01$.

than from the WT without MI group. Plasma H_2S levels decreased significantly after MI both in WT and in CSE KO groups (Figure 4(b)). Furthermore, as shown in Figure 4(c), INFs in CSE KO mice were more extensive than those in WT mice. Protein expression of the other two enzymes, CBS and 3-MST, increased in CSE KO mice, most likely due to compensation (Figure 4(d)).

3.5. Endogenous H_2S Protected Cardiomyocytes from Hypoxia-Induced Injury. We used CSE siRNA to knockdown CSE expression and then monitored H_2S levels in the culture medium. Compared with the hypoxia control, addition of CSE siRNA caused a significant decrease in H_2S levels in culture medium and CSE expression in cardiomyocytes. The expressions of CBS and 3-MST in cardiomyocytes significantly increased after adding CSE siRNA (Figures 5(a) and 5(b)). The activity of the three H_2S-producing enzymes did not change after the addition of CSE siRNA (Figure 5(c)). Cardiomyocyte injury was also evaluated after the addition of CSE siRNA. LDH activity was increased and CCK8 values were decreased, suggesting that knockdown of CSE expression exacerbated hypoxic injury (Figure 5(d)).

4. Discussion

In this study, we used an *in vivo* MI model and an *in vitro* hypoxic cardiomyocyte model to systemically investigate the regulation of exogenous H_2S on the expression of the three H_2S-producing enzymes. CSE KO mice were also used to study the effect of endogenous H_2S on the ischaemic hearts. Cardiac expressions of the three enzymes were quantified 2 weeks after MI surgery. Our experiments indicated four important findings: (1) exogenous H_2S significantly increased the expression of the three enzymes *in vivo*, (2) 25–100 μM NaHS pretreatment increased CBS levels in a concentration-dependent manner, and 50 μM NaHS treatment increased CSE/CBS activity *in vitro*, (3) endogenous H_2S played an important role in protecting the ischaemic heart after MI, and (4) when CSE was either knocked down (*in vitro*) or knocked out (*in vivo*), the expressions of CBS and 3-MST increased due to compensation.

Accumulating evidence has confirmed that H_2S is vital in regulating cardiovascular functions. H_2S can relax smooth muscle cells by activating ATP-dependent K^+ channels (K_{ATP}), leading to a subsequent decrease in blood pressure [7]. H_2S reduces infarct size and preserves left ventricular function after MI or myocardial ischemia/reperfusion [9–11]. In this study, we used echocardiography and Evans' Blue-TTC double staining to detect the protective role of 2-week H_2S treatment to mice suffering from MI injury. NaHS treatment at 50 and 100 μmol/kg after MI surgery effectively protected the heart from ischaemic damage. This protection was accompanied by greater CSE and 3-MST expression. From our results in Figure 3(c) those NaHS treatments did not change CSE or 3-MST expression under hypoxic condition *in vitro*; the elevated CSE and 3-MST expression after myocardial infarction by NaHS treatment *in vivo* is likely resulting from the improvement of cardiac function (e.g., less overload of noninfarcted area in NaHS-treated animals), but not from direct effect of H_2S on these enzymes.

The mechanisms in which exogenous H_2S participates in cardiac protection have been studied for years but still are not completely clear. H_2S promotes the secretion of proangiogenic factors, such as VEGF, which appears to enhance angiogenesis in the ischaemic heart and improve blood supply [13, 18]. Inhibition of cardiomyocyte apoptosis also appears to play an important role in H_2S-mediated cardiac protection [10, 11]. Recently, King reported eNOS-dependent cytoprotection by H_2S in the setting of I/R injury [19]. The manner in which exogenous H_2S affects another gaseous signalling molecule suggests that we should examine whether exogenous H_2S influences endogenous H_2S production to protect

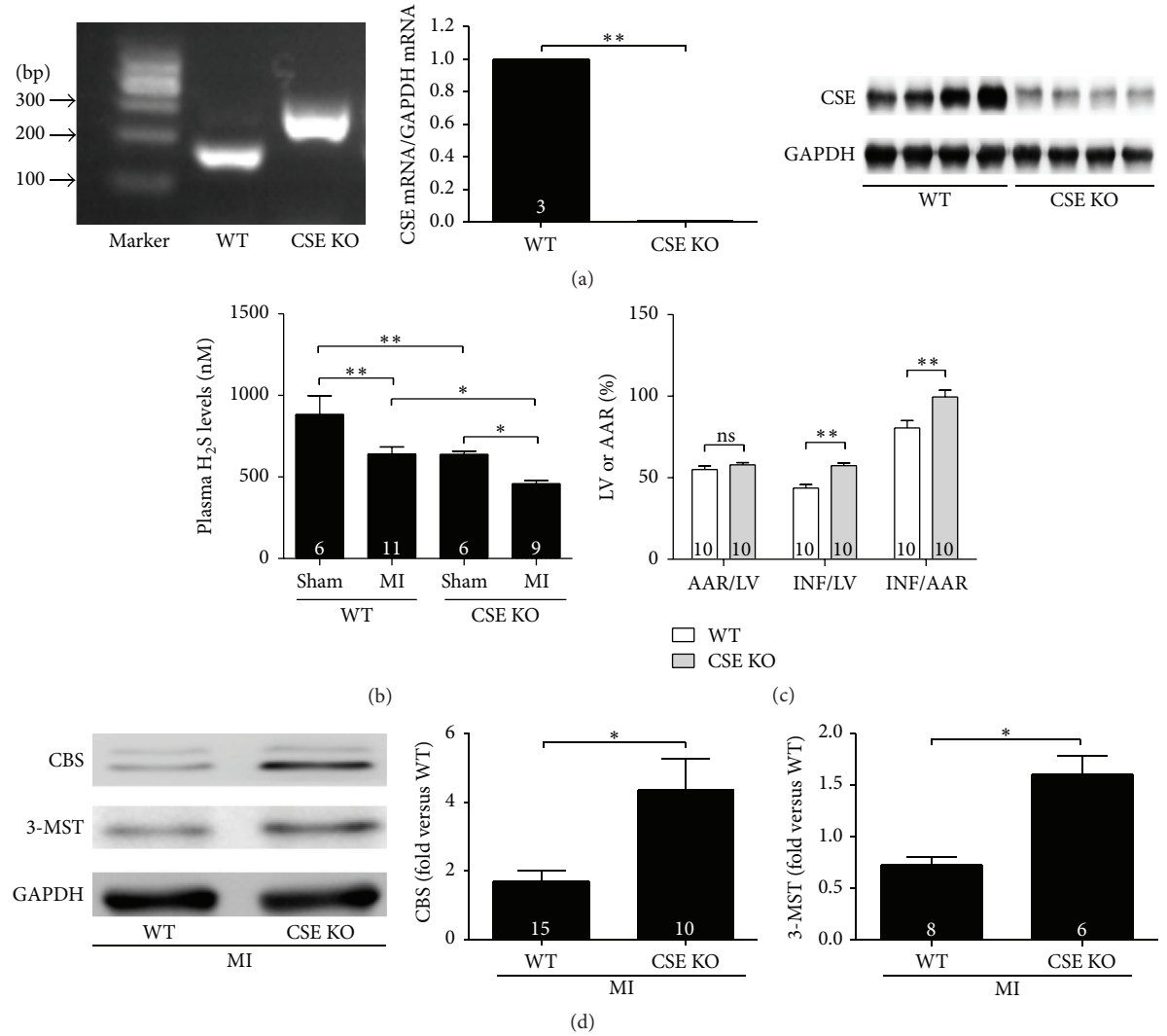

FIGURE 4: Endogenous H_2S protected ischaemic myocardium from myocardial infarction injury. (a) Characterization of CSE knock-out mice (CSE KO). Left panel: PCR analysis of genomic DNA from CSE KO mice and wide type mice (WT). Middle panel: real-time PCR analysis of CSE mRNA expression in the hearts from WT and CSE KO mice; right panel: representative western blot of CSE protein expression. (b) Plasma H_2S levels in WT and CSE KO mice with (MI) or without (sham) surgery. Plasma H_2S levels from the highest to lowest were as follows: WT without surgery, CSE KO without surgery, WT with MI surgery, and CSE KO with MI surgery; (c) infarct area in CSE KO group was larger than that in WT group detected with Evans' Blue-TTC staining; (d) expression of CBS and 3-MST increased in CSE KO mice. Numbers inside bars denote the number of animals per group. Values are mean ± SE. $^*P < 0.05$ and $^{**}P < 0.01$.

the ischaemic heart. In this study, we detected H_2S levels in plasma and H_2S-producing enzyme expression in the hearts.

After 2 weeks of intraperitoneal injection of NaHS (25–100 μmol/kg/day), the expressions of the three enzymes in ischemia myocardium increased but to different levels. In noninfarcted myocardium, NaHS treatment for 2 weeks increased the expression of CSE (50 μmol/kg/day) and 3-MST (25 μmol/kg/day), while NaHS (25–100 μmol/kg/day) did not change expression of CBS. Our results suggested that both myocardial infarction surgery (Figure 2(a)) and exogenous NaHS administration (Figures 2(b) and 2(c)) influenced the expression of H_2S-synthesizing enzymes in infarcted hearts. ① Myocardial infarction decreased the CSE expression, while exogenous NaHS administration increased its expression. The overall effect of exogenous NaHS administration on CSE in infarcted heart was elevation. ② Myocardial infarction increased the CBS expression, while exogenous NaHS administration did not change its expression. The overall effect of exogenous NaHS administration on CBS in infarcted heart was elevation. ③ Myocardial infarction decreased the 3-MST expression, while exogenous NaHS administration increased its expression. The overall effect of exogenous NaHS administration on 3-MST in infarcted heart was elevation. The complicated *in vivo* situations that caused the effect of NaHS on CBS and CSE was not ideally dose-dependent and effect on 3-MST was maximal at 50 μmol/kg. NaHS treatment at 50 and 100 μmol/kg/day significantly increased plasma H_2S levels. Qipshidze et al. previously reported that the expression of

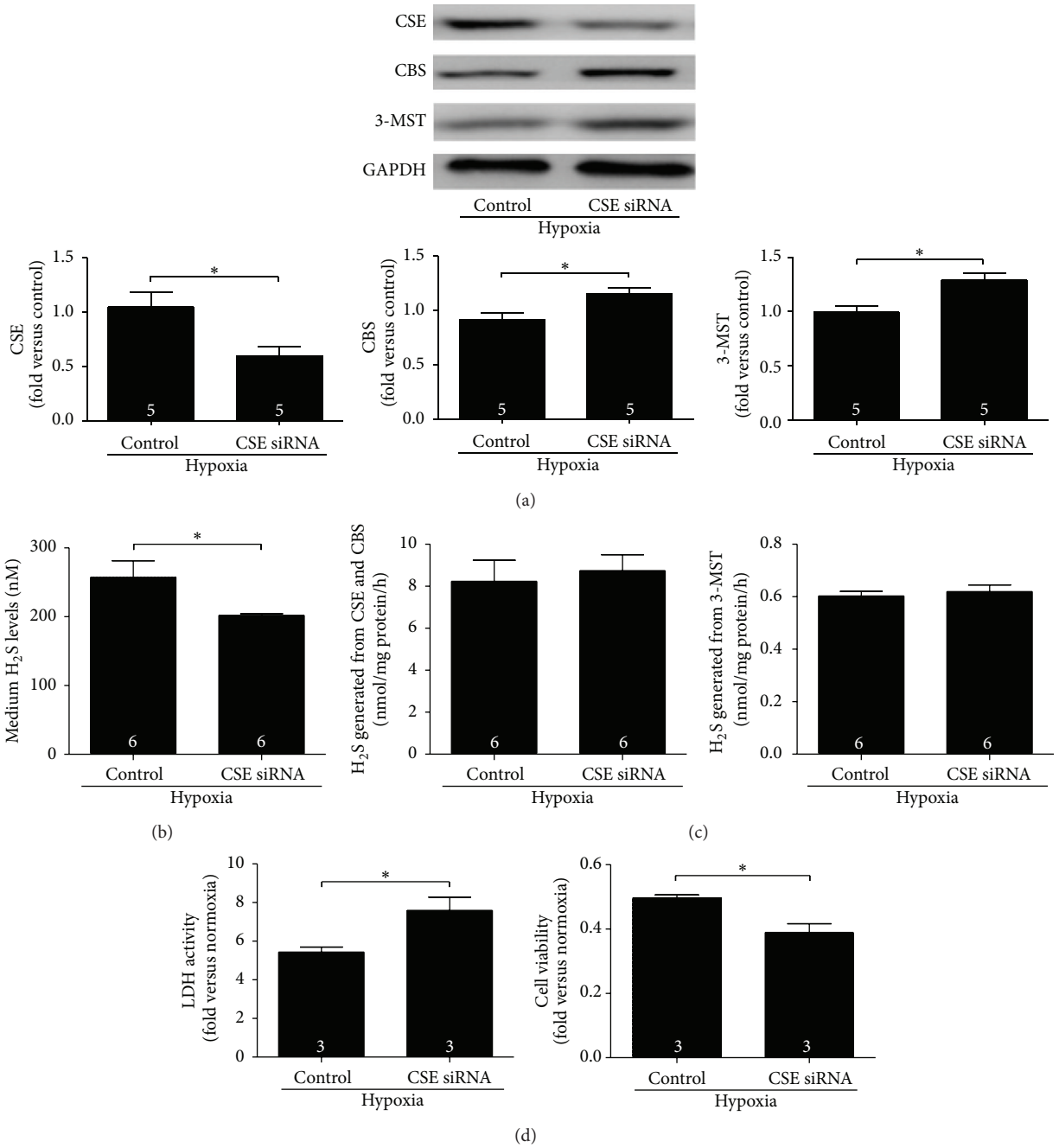

FIGURE 5: Endogenous H_2S protected cardiomyocytes from hypoxia-induced injury. (a and b) Compared with the hypoxia control, CSE siRNA significantly decreased CSE expression and H_2S levels in the culture medium, whereas CBS and 3-MST expressions increased; (c) the activity of the three H_2S-producing enzymes did not change after CSE siRNA treatment. (d) CSE siRNA exacerbated hypoxia injury, demonstrated by an increase in LDH activity and a decrease in CCK8 values. Numbers inside bars denote the number of animals per group. Values are mean ± SE. $^{*}P < 0.05$ and $^{**}P < 0.01$.

CSE increased and CBS decreased after drinking an H_2S-releasing aqueous solution for 4 weeks [13]. The possible explanation for the different results between Qipshidze et al.'s study and ours could be the different duration of NaHS treatment; 2 weeks was used in the present study, whereas Qipshidze et al.'s research extended to 4 weeks. Changes in plasma H_2S levels might also affect the expressions of the three enzymes.

Cardiomyocytes are the major cell type in the heart. We next investigated whether H_2S could protect cardiomyocytes from hypoxia-induced injury *in vitro*. Similar to the *in vivo* results, H_2S protected cardiomyocytes from hypoxic injury, as

was evidenced by lower LDH activity in the culture medium and higher cellular CCK8 values. Simultaneously, H_2S levels in NaHS-treated groups significantly increased. Because the expression and activity of both enzymes contribute to H_2S production, we next tested the expression as well as the activity of the three enzymes. While the expressions of CSE or 3-MST did not change, CBS expression increased in a concentration-dependent manner. Hypoxia decreased the activity of CSE/CBS without affecting 3-MST, which may have contributed to the decrease in H_2S levels under hypoxic conditions. Similarly, NaHS treatment may increase the activity of CSE/CBS to produce more H_2S.

The difference between *in vivo* (H_2S enhanced the expression of all three enzymes) and *in vitro* (H_2S only enhanced the expression of CBS) studies may be due to the fact that the heart contains several kinds of cells and that H_2S can be synthesized in cells other than cardiomyocytes. CSE, the major H_2S-producing enzyme in cardiovascular systems, is also expressed in smooth muscle cells [7, 19], and 3-MST also exists in endothelial cells [5]. Cells surrounding cardiomyocytes can release H_2S to maintain local H_2S concentrations. Exogenous H_2S can change the expressions of H_2S-producing enzymes in other cell types. For example, Na_2S (another H_2S donor) also enhances CSE expression in ischemia/reperfusion-stimulated brain endothelial cells [20]. Results from tissue samples revealed overall H_2S production, not just that from cardiomyocytes.

The mechanisms of upregulation of CBS by exogenous H_2S need to be studied in the future. Exogenous H_2S could specifically increase CBS expression with no effect on CSE or 3-MST, which might elevate local H_2S concentrations and thus result in cardiac protection for different reasons. Wang et al. reported that exogenous H_2S (10–80 μM) downregulates CSE expression, whereas hypoxia upregulates CSE expression in mammalian cell lines [21, 22]; these results differed from those obtained in the study. The probable explanation is that they used cell lines and NaHS treatment or hypoxia was administered separately. In our experimental conditions, we used primary cardiomyocytes and studied H_2S effects under hypoxic conditions (a combination of hypoxia and NaHS treatment), which better mimicked post-MI conditions; this may reveal the mechanism of cardiac protection of H_2S.

Finally, we used CSE KO mice or CSE siRNA to study the protective role of endogenous H_2S. When CSE was either knocked out *in vivo* or knocked down *in vitro*, the ischaemic heart or cardiomyocytes displayed more damage; at the same time, although CBS and 3-MST expression increased due to compensation, H_2S concentration still significantly decreased, confirming that CSE is the main H_2S-producing enzyme in the heart. In cardiomyocytes, CSE siRNA significantly decreased CSE protein levels, but the activity of the three enzymes did not change. Low H_2S levels in the medium may be the result of decreased CSE protein expression. In another study, King et al. also showed that CSE KO mice had large INFs, and the infarct size became less extensive when exogenous H_2S was added [19]. King's experiments also confirmed that sufficient local H_2S production around ischaemic tissues appears to be important for cardioprotection. When the major H_2S-producing enzyme did not function adequately, H_2S levels dropped and the protective effects of H_2S were impaired.

5. Conclusion

In summary, we demonstrated for the first time that 2 weeks of exogenous H_2S treatment can increase the expression of the three H_2S-producing enzymes in ischaemic heart tissue and alleviate ischaemic damage. Endogenous H_2S also appears to have a major role in protecting the ischaemic heart. This study suggested a new and indirect regulatory pathway for cardiac protection; however, the underlying mechanism needs further investigation.

Conflict of Interests

The authors declare no conflict of interests on the publication of this paper.

Authors' Contribution

Na Li and Ming-Jie Wang contributed equally to this work.

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Review Article

Hydrogen Sulfide and Cellular Redox Homeostasis

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Intracellular redox imbalance is mainly caused by overproduction of reactive oxygen species (ROS) or weakness of the natural antioxidant defense system. It is involved in the pathophysiology of a wide array of human diseases. Hydrogen sulfide (H_2S) is now recognized as the third "gasotransmitters" and proved to exert a wide range of physiological and cytoprotective functions in the biological systems. Among these functions, the role of H_2S in oxidative stress has been one of the main focuses over years. However, the underlying mechanisms for the antioxidant effect of H_2S are still poorly comprehended. This review presents an overview of the current understanding of H_2S specially focusing on the new understanding and mechanisms of the antioxidant effects of H_2S based on recent reports. Both inhibition of ROS generation and stimulation of antioxidants are discussed. H_2S-induced S-sulfhydration of key proteins (e.g., p66Shc and Keap1) is also one of the focuses of this review.

1. Introduction

In 1777, a young Swedish apothecary, Carl Wilhelm Scheele, treated ferrous sulfide with a mineral acid and noted a colorless gas with a characteristic odor of rotten eggs. He described it as "sulfuretted hydrogen." The notoriety of hydrogen sulfide (H_2S) had been considered as a toxic gas for several hundreds of years. The Permissible Exposure Limit (PEL) of H_2S is 10 ppm and sudden exposure to >400 ppm can cause rapid death. The biological effects of H_2S in physiological condition began around the turn of the 20th century. H_2S is now recognized as the third "gasotransmitter" along with nitric oxide (NO) and carbon monoxide (CO) [1]. The desulfhydration of cysteine is considered as the major source of H_2S in mammals. This process is catalyzed by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), two pyridoxal-5′-phosphate- (PLP-) dependent enzymes. CBS is primarily expressed in various regions of the brain and is essential to the production of H_2S in the central nervous system [2–4], whereas CSE is mainly expressed in the cardiovascular system [5, 6]. Recently, 3-mercaptopyruvate sulfurtransferase (3-MST) was reported as the third enzyme for H_2S production, which is localized to mitochondria and nerve endings [7, 8]. We and others proved that H_2S exerted a wide range of biological functions including neuroprotection [9, 10], cardioprotection [11, 12], antihypertension [13], and osteoblastic protection [14]. The antioxidant effect of H_2S has been most extensively investigated and was thought as the major mechanism underlying the effects of H_2S. Here, we summarize the existing knowledge about the antioxidant effect of H_2S, highlighting recent advances in our understanding of the ability of H_2S to neutralize reactive oxygen species (ROS) in vivo.

2. Free Radical, Oxidative Stress, and Cellular Antioxidant Defenses

2.1. Free Radical and Oxidative Stress. A free radical is an unstable chemical species that contains one or more unpaired electrons in its outer orbital. In organisms, the highly reactive free radicals formed from metabolism might donate their unpaired electron to another molecule or pull an electron off a neighboring molecule. The term oxidative stress has been proposed indicating a disturbance in the equilibrium status of oxidant/antioxidant systems with a progressive accumulation of ROS in intact cells. ROS are short-lived

and highly chemically reactive. At low concentrations, ROS serve as cellular signaling molecules [15]. However, at high concentrations, ROS may cause both beneficial and unbeneficial effects. In the late case, ROS may not only kill invading pathogens and microbes but also damage the components of the cell, including proteins, lipids, carbohydrates, and DNA [16]. Overwhelming evidence indicates that oxidative stress is involved in the pathophysiology of the wide array of human diseases, including cancer [17], cardiovascular disease [18], AIDS [19], diabetes mellitus [20], and neurodegenerative disorders such as aging, Parkinson's disease, and Alzheimer's disease [21, 22].

In human body, more than 95% free radicals belong to oxygen free radicals. Recent studies suggest that oxygen-free radicals play an essential role in the control of cell functions and signal transmission [23, 24]. The common oxygen free radicals consist of superoxide anion ($^{\bullet}O_2^-$), hydroxyl radical ($HO^{\bullet}$), perhydroxyl radical ($HO_2^{\bullet}$), alkoxyl radical ($RO^{\bullet}$), alkyl peroxide radical ($ROO^{\bullet}$), and so on (Table 1). Among them, $^{\bullet}O_2^-$ is very unstable and able to react spontaneously with itself producing hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) [25]. $^{\bullet}O_2^-$ is the starter of chain reaction of oxygen free radicals. $HO^{\bullet}$ is the most reactive oxygen free radical and can react with any biological molecule [26]. $HO_2^{\bullet}$ is the protonated form of superoxide anion and exhibits higher reactivity than superoxide anion [27]. In addition, other reactive oxygen metabolites such as H_2O_2 and the singlet oxygen ($^1O_2$) can also be regarded as oxygen free radicals, although they are not true free radical species. H_2O_2 may cross the biological membranes and is one of the origins of highly reactive $HO^{\bullet}$ [28]. The singlet oxygen ($^1O_2$) also has higher reactivity [29] and can be formed directly by illumination (*hv*) from molecular oxygen.

Table 1: The types of common oxygen-free radicals.

Radicals	Chemical formulas	Electron structures
Superoxide anion	$^{\bullet}O_2^-$	$[\ddot{O}-\ddot{O}]^{\bullet}$
Hydroxyl radical	$HO^{\bullet}$	$H-\ddot{O}^{\bullet}$
Perhydroxyl radical	$HO_2^{\bullet}$	$H-\ddot{O}-\ddot{O}^{\bullet}$
Alkoxyl radical	$RO^{\bullet}$	$R-\ddot{O}^{\bullet}$
Alkyl peroxide radical	$ROO^{\bullet}$	$R-\ddot{O}-\ddot{O}^{\bullet}$
Hydrogen peroxide	H_2O_2	$H-\ddot{O}-\ddot{O}-H$
Singlet oxygen	$^1O_2$	$\cdot\ddot{O}-\ddot{O}\cdot$

2.2. The Sources of ROS. ROS are widespread in living organisms. Actually, they are being continuously produced in vivo and many of them are necessary to carry out certain cellular and biological reactions [23]. When they were overproduced, cellular damage may happen [30, 31]. The origin of oxygen-free radicals may be generated exogenously or endogenously.

Exogenous sources are mainly generated by some stimulating factors. These include smoking, alcohol, certain drugs, air pollution, ionizing radiation, and hyperbaric oxygen poisoning. Compared with exogenous sources, endogenous sources play more important roles in the form of oxygen free radicals. Endogenous activities are the main sources of oxygen-free radicals in living organisms. The main endogenous sources are listed below (Figure 1, solid line arrows).

(i) Mitochondrial Electron Transport. The oxygen-free radical is the by-products of cellular metabolism. Under normal physiological conditions, most oxygen in organisms will acquire four electrons and four protons and reduce to form water by the cytochrome c oxidase from electron transport system of mitochondrial. In this procedure, no oxygen-free radical will form at last. However, if the molecular oxygen undergos sequential univalent reduction, highly reactive $^{\bullet}O_2^-$, $HO^{\bullet}$, and H_2O_2 would be formed [32]. Mitochondria are the major source of intracellular ROS. As the terminal electron acceptor of respiration, more than 90% oxygen is used to produce ATP in mitochondria and about 2% of the oxygen is transformed into ROS as respiratory chain by-products [33].

(ii) The Increase of Xanthine Oxidase (XO). There are about 10% of xanthine oxidases (XO) and 90% of xanthine dehydrogenase (XD) in endothelial cells. The xanthine dehydrogenase (XD) will be converted into xanthine oxidase (XO) during ischaemia [34]. In this condition, the adenosine triphosphate (ATP) could not release energy. Instead, it will be degraded into adenosine diphosphate (ADP), adenosine monophosphate (AMP), and hypoxanthine gradually. Upon reperfusion of the ischemic tissue, increased xanthine oxidase (XO) will convert the increased hypoxanthine to xanthine and then convert the formed xanthine to uric acid by using oxygen as electron acceptor. Oxygen is reduced and produced $^{\bullet}O_2^-$, $HO^{\bullet}$, and H_2O_2 finally [35] (Figure 2).

(iii) The Increase of Catecholamine. Sympathetic adrenomedullary system is an important stress regulation system in our bodies. Catecholamine produced by this system under an external stimulus has an important role in the adjustment of metabolism. Catecholamine can also be converted to oxygen-free radicals by autooxidation [36] (Figure 2). It is worth noting that too much catecholamine and their oxidation products, especially the superoxide anion radicals, will cause damage to the body.

(iv) NADPH Oxidase. Nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase (NOX) is another important enzyme for intracellular ROS generation. It is mainly distributed in the plasma membrane surface of phagocyte and catalyzes the one-electron reduction of oxygen to produce superoxide-free radical by utilizing NADPH as an electron donor (Figure 2). The NOX system is dormant in normal, but it can be activated by some stimulating factors, such as leukotriene, endotoxin, complement, and calcium ion [37, 38]. Thereby, more oxygen (O_2) will be quickly reduced to $^{\bullet}O_2^-$ and H_2O_2. $HO^{\bullet}$ will be also formed by further metabolism [39].

(v) Catalysis of Transitional Metals. The transitional metals, such as iron and copper, can change their valence by donating

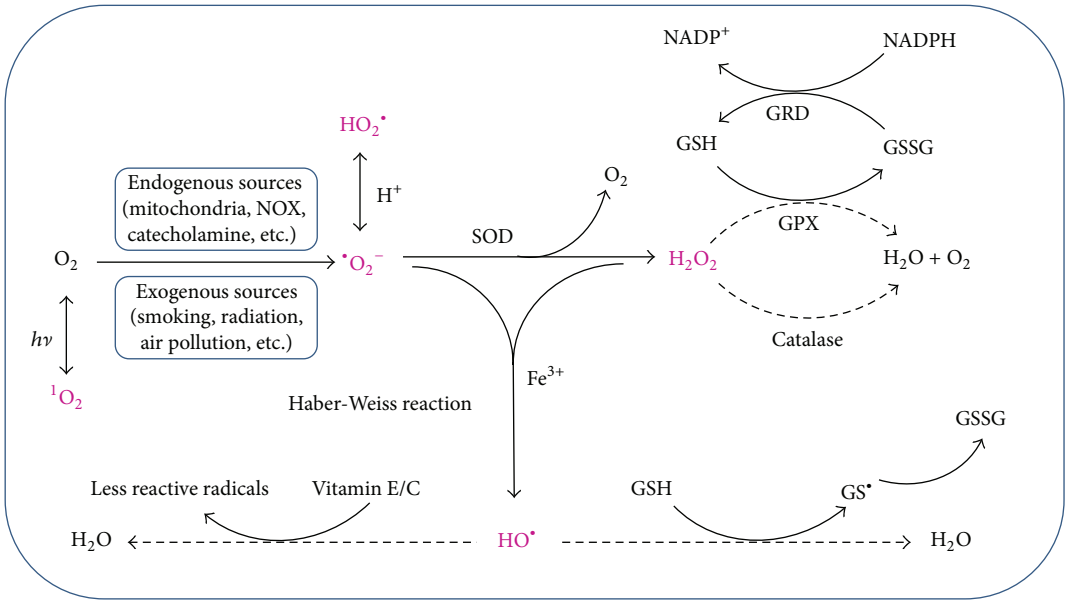

Figure 1: The main ROS generation and elimination pathways. (1) ROS (including $^{\bullet}O_2^-$, $HO_2^{\bullet}$, $HO^{\bullet}$, $^1O_2$, and H_2O_2) may be generated by exogenous (like smoking, *hν*, air pollution, etc.) and endogenous (like mitochondria, catecholamine, NOX, etc.) stimulating factors. $^{\bullet}O_2^-$ can further react with H_2O_2 to generate $HO^{\bullet}$ through the Haber-Weiss reaction in the presence of ferric irons (shown as solid line arrows). (2) Excessive $^{\bullet}O_2^-$ is eliminated by SOD by catalyzing the dismutation of $^{\bullet}O_2^-$ to H_2O_2 and O_2. H_2O_2 can be further removed by the catalysis of CAT or GPx. The catalysis of GPx needs GSH as its cosubstrate and GSH is oxidized to GSSG. GSSG can be reduced to GSH again by GR utilizing NADPH. GSH can also react with oxygen free radical directly and form the thiyl radical ($GS^{\bullet}$) and later GSSG. Vitamin E and vitamin C may react with oxygen free radical and form less reactive radicals (shown as dotted line arrows). NADPH: nicotinamide-adenine dinucleotide phosphate; NOX: NADPH oxidase; XO: xanthine oxidase; SOD: superoxide dismutase; GSH: glutathione; GSSG: glutathione disulfide; GPx: glutathione peroxidase; GR: glutathione reductase.

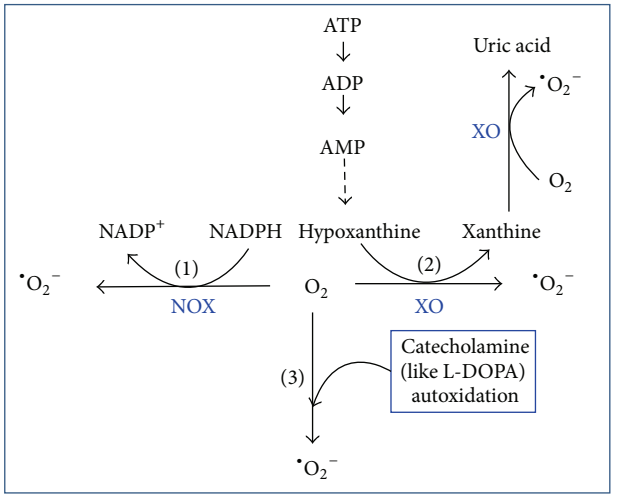

Figure 2: Endogenous superoxide anion ($^{\bullet}O_2^-$) formation pathways. (1) NOX catalyzes the one-electron reduction of oxygen to produce $^{\bullet}O_2^-$ by utilizing NADPH as an electron donor. (2) $^{\bullet}O_2^-$ formation in the process of the XO-catalyzed conversion of hypoxanthine into xanthine or xanthine into uric acid. (3) $^{\bullet}O_2^-$ formation within catecholamine autooxidation.

an electron and thus catalyze the Haber-Weiss reaction [28] as shown in (1). In the presence of superoxide anions, ferritin-bound ferric iron in cells usually can be liberated as ferrous form, thereby increasing the amount of iron [40]. The generated iron can form $HO^{\bullet}$ in the presence of hydrogen peroxide. This is the main source of hydroxyl radicals.

$$^{\bullet}O_2^- + H_2O_2 \xrightarrow{Fe^{3+}} O_2 + HO^- + HO^{\bullet} \quad (1)$$

In addition, the metabolism of arachidonic acid by cyclooxygenase [41, 42] or lipoxygenases [43, 44], cytochromes P450 of the microsomal electron transport system [45, 46], may also produce oxygen-free radicals.

2.3. Cellular Defenses of ROS. In living organisms, ROS are continuously produced because of the reduction of molecular oxygen. Although free radicals play an important role in some physiological reactions, such as cell signal transduction and regulation of muscle tone [23, 40], excessive free radicals would cause damage to the lipids, proteins, and DNA and give rise to cellular and metabolic disturbance [30]. There are enzymes and chemical scavengers that could be used to remove excessive oxygen-free radicals formed in a living body [47].

Superoxide dismutase (SOD) is a common antioxidant enzyme which contains copper, zinc, and manganese as cofactors [48]. SOD can catalyze the dismutation of $^{\bullet}O_2^-$ to molecular oxygen (O_2) and the lesser active species H_2O_2 at a higher rate than the spontaneous dismutation of $^{\bullet}O_2^-$. The formed H_2O_2 will be further decomposed to H_2O and O_2 or

be used to form $HO^{\bullet}$ through the Haber-Weiss reaction, as shown in (1), and reduced to H_2O finally (Figure 1, dotted line arrows).

Catalase (CAT) is another antioxidant enzyme that is widely distributed in tissues [49]. It could catalyze the degradation of H_2O_2 directly to water and prevent the secondary generation of other intermediate radicals. In addition, selenium-containing glutathione peroxidase (GPx) could also catalyze the reduction of H_2O_2 [50]. This reaction needs reduced glutathione (GSH) as cosubstrate and GSH will be oxidized to oxidized glutathione (GSSG). GSSG could also be reduced to GSH again by glutathione reductase (GR) utilizing NADPH.

There are also some nonenzymatic chemical antioxidants that play an important role in antioxidant, included glutathione (GSH), α-tocopherol (vitamin E), and ascorbic acid (vitamin C) [51]. As mentioned before, GSH can act as a cosubstrate in the reduction of H_2O_2 by GPx. GSH could also react with oxygen-free radical directly and form the thiyl radical and later GSSG [52]. Like GSH, vitamins E and C could also reduce oxygen-free radicals [53]. They would trap hydroxyl radicals and other reactive radicals and thus break radical chain reactions and form new less reactive radicals. These new formed radicals themselves could not be removed or make further conversion. Only with the help of GPx and other biological molecules, these new radicals can be transformed to nonreactive substances. So, excess supplement of vitamins and other chemical antioxidants is not necessary. Excess levels of vitamins cannot replace the position of enzymes in organisms [54]. On the contrary, excess chemical antioxidants may produce excess less reactive radicals by reacting with oxygen radicals. These excess radicals may cause damage to the body. Actually, chemical antioxidants usually go into effect with the cooperation of antioxidant enzymes [55].

3. Mechanisms for the Regulatory Effect of H_2S on ROS In Vivo

3.1. Quenching Free Radicals as a Chemical Reductant. At 37°C and pH 7.4, more than 80% of H_2S molecules dissolve in surface waters and dissociate into H^+, HS^-, and S^{2-} ions. HS^- is powerful one-electron chemical reluctant and presents a remarkable capacity to scavenge ROS. In addition, H_2S itself has also been recognized to be a poor reducing agent, which can react directly with and quenches the superoxide anion (O_2^-) [56, 57] and NO-free radicals like peroxynitrite [58] as well as other ROS in vitro. However, it should be noted that the physiological concentration of H_2S in vivo is believed to be at the submicromolar range [59, 60] and such low concentration of H_2S is not paralleled with its antioxidant effect. Moreover, in our previous work, NaHS pretreatment significantly inhibited H_2O_2-induced (50 μM, 2 h) mitochondrial ROS generation and protected human neuroblastoma SH-SY5Y cells against H_2O_2-induced injury even when it had been washed out before H_2O_2 administration. Similar effects were also found in MC3T3-E1 osteoblastic cells, and this antioxidant effect of H_2S lasted for at least 18 h [14]. These results indicated that other mechanisms besides chemical reductant exist in the antioxidant effect of H_2S. We speculate that H_2S might act as a trigger which will be retired after starting the process of antioxidant action.

3.2. Scavenging Free Radicals In Vivo via Nonenzymatic Antioxidants. As we mentioned before, ROS is counterbalanced in the body by a net of antioxidants, including enzymatic and nonenzymatic antioxidants. GSH and thioredoxin (Trx-1) are two biologically important nonenzymatic antioxidants in animal cells and attracted increasing attention as cellular protectants against oxidative stress in vivo.

3.2.1. H_2S Increases Intracellular Reduced Glutathione (GSH). GSH, a tripeptide consisting of cysteine, glutamate, and glycine, is a major antioxidant in the cellular defense against oxidative stress and a decreased GSH/GSSG ratio is usually taken as indicating oxidative stress. In cells, GSH is synthesized from cysteine. There are 2 cysteine forms, oxidized form cystine and reductive form cysteine. Because of its redox instability, extracellular cysteine is mostly present in cystine, which can be transported into cells through cystine/glutamate antiport system X_c^-, then reduced to cysteine, and used for GSH synthesis [61]. Glutamate is the key inhibitor of the system X_c^-. Our previous study showed that NaHS at 100 μM promoted [^{3}H]glutamate uptake in astrocytes via enhancing the trafficking of glial glutamate transporter GLT-1 (also named the excitatory amino acid transporters-2, EAAT2), enhanced cystine transport, and increased intracellular GSH synthesis finally [62] (Figure 3).

Studies from other laboratories have also proven that H_2S preserves the cellular GSH status and provides protection against oxidative damage in brain [63, 64], spinal cord [65], heart [66, 67], lung [68], kidney [69, 70], liver [71], gastrointestinal tract [72, 73], and so forth. Recently, Kimura et al. showed a different mechanism for H_2S on intracellular GSH production. They reported that H_2S produced in cells may be released into extracellular space and reduces cystine into cysteine, which thereby would be efficiently imported into cells through a cysteine transporter distinct from system X_c^- and used for GSH synthesis [74] (Figure 3). Meanwhile, Jain et al. also demonstrated that H_2S increased intracellular GSH production by upregulating the glutamate-cysteine ligase catalytic subunit (GCLC) and glutamate-cysteine ligase modifier subunit (GCLM) [75] (Figure 3).

3.2.2. H_2S Increases Intracellular Trx-1. Classic thioredoxin (Trx-1) is a small (12 kDa) ubiquitous molecule containing a characteristic Cys-Gly-Pro-Cys motif and the oxidation-reduction of Trx-1 occurs at its two cysteine residues. It was reported that Trx-1 exerts extracellular and intracellular multifunctions in cell proliferation [76], apoptosis [77], and gene expressions [78]. Moreover, Trx-1 was also shown to scavenge ROS and protect cells against oxidative stress. Trx-1 reduces hydrogen peroxide via peroxiredoxin (Prx) and oxidized Trx-1 is reduced by thioredoxin reductase [79]. Antioxidant effects of Trx-1 can also be mediated indirectly (for more details, see [79]).

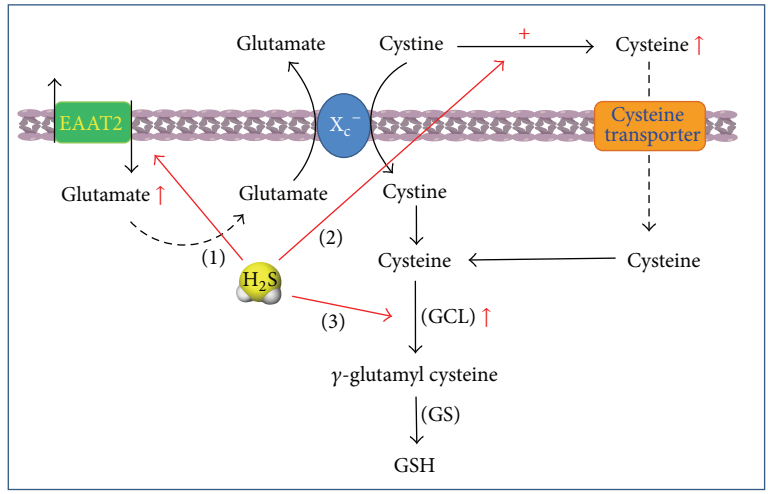

FIGURE 3: H_2S increases intracellular GSH synthesis. Cellular GSH is mainly synthesized from cysteine. (1) H_2S increases EAAT2-mediated glutamate uptake, which thereby increases cystine transportation through cystine/glutamate antiport system (X_c^-). (2) Intracellular H_2S is released into extracellular space and reduces cystine into cysteine, which would be efficiently imported into cells through a cysteine transporter distinct from system X_c^-. These two pathways provide more substrate to produce GSH. (3) H_2S increases glutamate cysteine ligase (GCL) expression and promotes GSH synthesis.

In 2008, Jha et al. reported that H_2S protected murine liver against ischemia-reperfusion (I/R) injury through upregulation of intracellular Trx-1 along with an increase in hepatic tissue GSH/GSSG ratio [71]. Trx-1 was also proved to mediate the cardioprotective effects of H_2S in the setting of ischemic-induced heart failure by Nicholson et al. [80]. They demonstrated that Na_2S treatment not only significantly increased the gene and protein expression of Trx-1 but also efficiently improved cardiac dilatation, dysfunction, and hypertrophy in the ischemic heart failure mice. Moreover, they generated transgenic mice with a cardiac-specific overexpression of a dominant negative mutant of Trx-1 and found the cardioprotective effects of Na_2S were Trx-1 dependent.

3.2.3. Potential Mechanisms of H_2S on Nonenzymatic Antioxidants Production. Despite the potential role of H_2S in the cellular antioxidant defense, studies on its antioxidant mechanism have been exceptionally limited. Recently, increasing evidence revealed that Nrf2 participated in the antioxidant effect of H_2S by promoting cellular antioxidant gene expression.

Nuclear factor (erythroid-derived 2)-like 2, also known as nuclear factor-erythroid 2 (NF-E2) related factor 2 (Nrf2), is a transcription factor that regulates a wide variety gene expression. Nrf2 is found mostly in the cytoplasm as an inactive complex with Kelch-like ECH-associated protein 1 (Keap1) [81]. Under oxidative stressed conditions, Keap1 undergoes ubiquitination and promotes Nrf2 translocation to the nucleus, in which Nrf2 binds to promoters containing the antioxidant response element (ARE) sequence and inducing ARE-dependent gene expression [82]. ARE is a *cis*-acting regulatory element, which is found in promoter region of certain genes, such as Trx-1 [83], glutathione reductase [84], and thioredoxin-interacting protein (Txnip) [85]. Nrf2 can suppress the basal expression of Txnip, which binds redox-active cysteine residues of Trx-1 and inhibit its antioxidant function [85]. Nrf2 can also increase both expression and activity of glutathione reductase, which, as we mentioned above, promotes oxidized GSH recycle back to reduced GSH and increases GSH/GSSG ratio [86]. On the other hand, it was reported that H_2S can S-sulfhydrated Keap1 at cysteine-151, which causes a conformational change in Keap1 and thereby leads to Nrf2 dissociation from Keap1. The activated Nrf2 nuclear finally translocates to nuclear and promotes antioxidant gene transcription, such as GCLM, GCLC, and glutathione reductase (GR) [87]. In addition, Calvert et al. also demonstrated that H_2S increased the expression of Trx-1 and mediated cardioprotection through Nrf2 signaling [83]. Taken together, these results demonstrate that Nrf2 is the potential endogenous cardioprotective signal in the process of cellular nonenzymatic antioxidant generation induced by H_2S (Figure 4).

3.3. Scavenging Free Radicals In Vivo via Enzymatic Antioxidants. Another major mechanism for cells to maintain redox equilibrium is based on the clearance ability processed by cellular antioxidant enzymes. Superoxide dismutase (SOD), CAT, and GPx are three main antioxidant enzymes that defend against oxidative damage in vivo. There are three isoforms of mammalian SOD: the cytosolic copper/zinc-containing SOD (Cu/ZnSOD, SOD-1), the mitochondrial manganese-containing SOD (MnSOD, SOD-2), and the extracellular SOD (ecSOD, SOD-3). SOD catalyzes the dismutation of $^{\bullet}O_2^-$ into H_2O_2, while CAT reacts with H_2O_2 to form water and molecular oxygen, and GPx detoxifies H_2O_2 in the presence of GSH, producing H_2O and GSSG which is recycled to GSH by glutathione reductase in an NADPH-consuming process [88] (Figure 1, dotted line arrows). In 1995, Searcy et al. reported that H_2S is a genuine substrate of SOD and can bind at the catalytic Cu center of SOD [89].

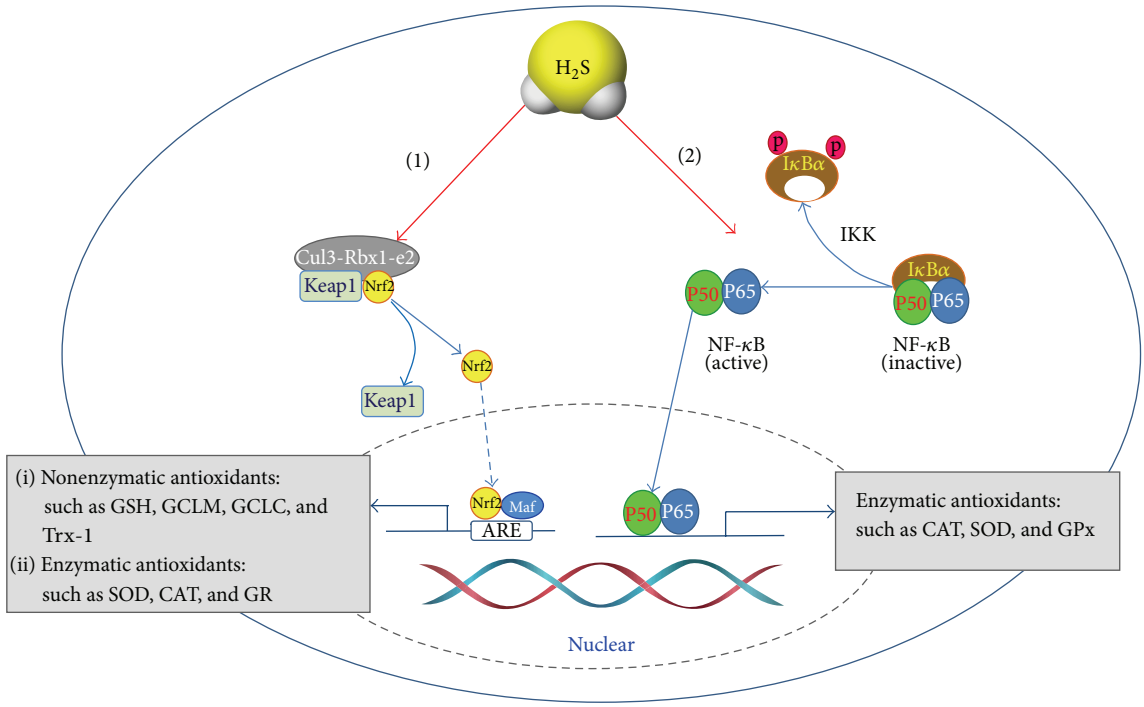

Figure 4: Effect of H_2S on intracellular enzymatic and nonenzymatic antioxidant production. (1) H_2S activates Nrf2, which translocates to nuclear, binds to ARE, and upregulates enzymatic and nonenzymatic antioxidant production. (2) H_2S stimulates NF-κB signaling, which further upregulates the expression of numerous genes including SOD, CAT, and GPx.

The binding of HS^- to SOD is very quick and the rate constant for binding is $>10^7\ M^{-1}\ S^{-1}$. When sulfide combined with SOD, there was a synergistic increase in the rate of superoxide anion scavenging. The K_m measured by the pyrogallol technique is ~80 μM HS^- [89]. Recent studies also demonstrated that H_2S could ameliorate cellular oxidative stress by improving activities of CAT [66, 90–92] and GPx [92–95].

The signal transduction pathways for H_2S to promote endogenous enzymatic antioxidant defense are much less understood. NF-κB is a family of transcription factors and plays a pivotal role in inflammation. H_2S was reported to attenuate inflammation via inhibition of NF-κB activation, which is associated with an array of diseases, such as hypoxia-induced neurotoxicity [96], cerebral ischemia [97], kidney injury [98], pulmonary fibrosis [99], and acute pancreatitis [100]. However, as a redox-sensitive transcription factor, NF-κB has also been considered as the most important factor on regulation of cellular antioxidant enzymes and was reported to be upregulated by H_2S via substance P [101, 102]. Analyzing the gene sequences of mouse GPx and CAT, Zhou et al. [103] revealed the existence of binding sites for NF-κB at position -283 in the GPx gene and at the -227 and -242 in the CAT gene. Additionally, SOD was also proved to have binding site for NF-κB in its 5′-flanking region and the DNA binding activity of NF-κB was induced in response to oxidative stress [104]. Taken together, these observations suggest that NF-κB mediated signaling pathway is most likely attributable to the augmentation of endogenous antioxidant capacity of H_2S in response to oxidative stress (Figure 4).

In addition to the activation of NF-κB, Nrf2 signaling cascade maybe another rational that accounts for the antioxidant effect of H_2S. Dreger et al. [105] identified that an ARE element existed in the SOD1 and CAT promoter, which is not only essential but also sufficient for transcriptional regulation. In their study, antioxidative enzymes in cardiac myocytes were induced via Nrf2-dependent transcriptional activation of ARE sites. On the other hand, diallyl sulfide (DAS), a kind of sulfur-containing compound, was demonstrated to cause a significant increase in the activities of SOD, CAT, GPx, GR, glutathione-S-transferase (GST), and quinone reductase (QR) in rat kidney through the activation of Nrf2 to protect the cell against oxidative stress [106]. This indicates a possible role of H_2S in ROS-interacting enzymes synthesis. However, there is no direct report to link the effect of H_2S on Nrf2 signal pathway to Nrf2-induced antioxidative enzymes synthesis at present and further investigations are needed in future.

3.4. Inhibitory Effect on Mitochondrial Free Radicals Production. Besides the capacity of cellular antioxidant defense, sequential overproduction of ROS is another vital factor in response to oxidative stress. Mitochondria is the major source of intracellular ROS and leak from the electron transfer chain is thought to be the main route [107]. Mounting evidence shows that p66Shc plays predominant roles in mitochondrial redox signaling and its phosphorylation at serine-36 acts as a switch on mitochondrial ROS production [108, 109].

p66Shc is a 66 kD Src homologous-collagen homologue (Shc) adaptor protein, which is encoded by the *shc1* gene

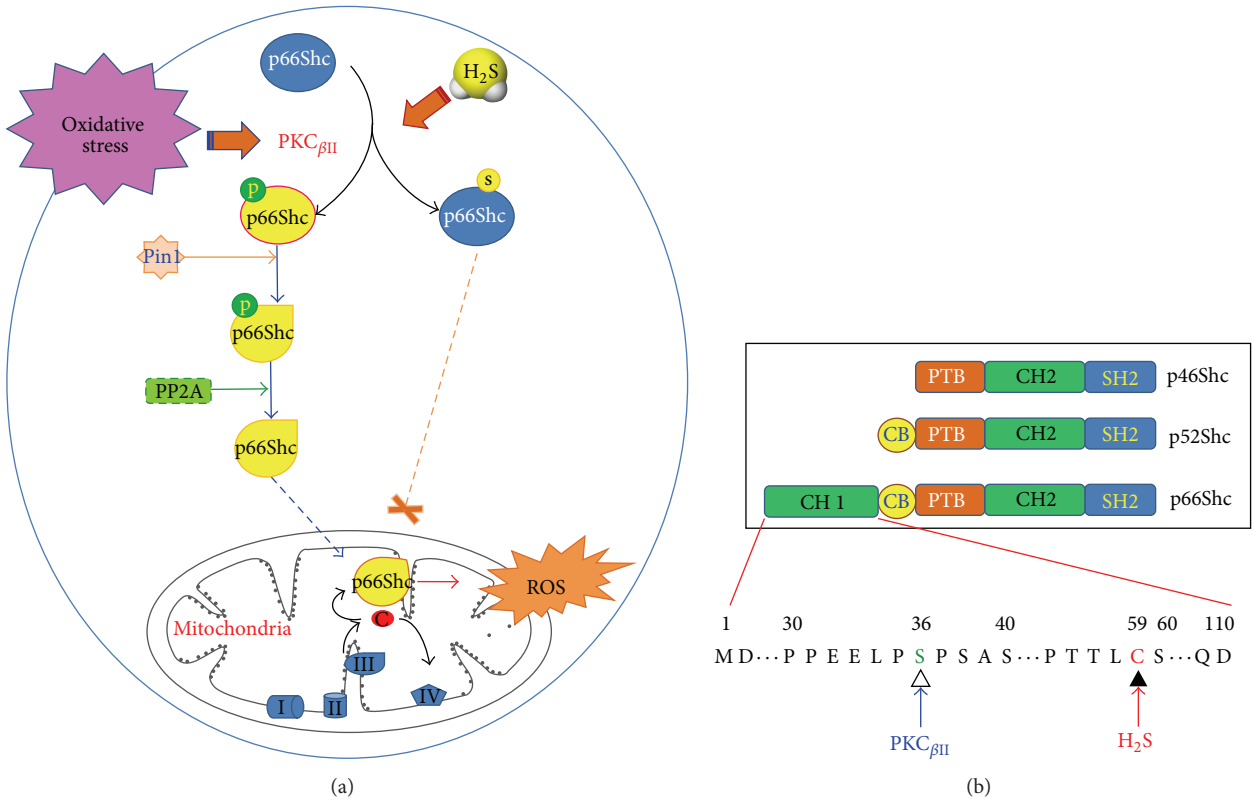

FIGURE 5: Proposed model for the effect of H_2S on p66Shc mediated mitochondrial ROS generation. (a) Showing the effect of H_2S. p66Shc is activated by $PKC_{\beta II}$-dependent phosphorylation at serine-36 in the N-terminal CH2 domain. The phosphorylated p66Shc can be isomerized by Pin1 and dephosphorylated by PP2A. This, in turn, induces mitochondrial translocation of p66Shc and ROS production. H_2S sulfhydrates p66Shc at cysteine-59, which locates in the same CH2 domain besides serine-36, disrupts the association between $PKC_{\beta II}$ and p66Shc, inhibits $PKC_{\beta II}$-mediated p66Shc phosphorylation, and decreases mitochondria ROS generation finally. (b) Showing the structure of p66Shc and the conserved serine (Ser-36) and cysteine (Cys-59) residues in the CH2 domain.

and belongs to the ShcA family. There are two other Shc family members, p46Shc and p52Shc, and all these 3 isoforms share three common functionally identical domains: the C-terminal Src homology 2 domain (SH2), the central collagen homology domain (CH1), and the N-terminal phosphor-tyrosine-binding domain (PTB) [110]. Different from the other two isoforms, p66Shc has an additional N-terminal CH2 domain which contains a critical serine residue at the position 36 (Ser-36) and shows different functions from p46Shc and p52Shc. It was proved that p66Shc has a negative influence on the Ras-mediated signaling pathway [111] but is involved in mitochondrial redox signaling. In response to oxidative stress (UV exposure or H_2O_2 treatment), p66Shc is phosphorylated by protein kinase C-βII ($PKC_{\beta II}$) at Ser-36. The activated p66Shc is then isomerized by the prolyl isomerase Pin1 and dephosphorylated by phosphatase A2 (PP2A) and finally translocates to mitochondria, where it binds to cytochrome c and transfers electrons from cytochrome c to molecular oxygen to product ROS [112, 113] (Figure 5). Migliaccio et al. reported that $p66Shc^{-/-}$ mice have a 30% increase in the life span [114]. Consistent with this report, Tomilov et al. also demonstrated that macrophages from $p66Shc^{-/-}$ mice appeared to have defect in the activation of the NADPH oxidase and therefore less superoxide production was observed [115].

Recently, our group demonstrated for the first time that H_2S may inhibit mitochondrial ROS production via a p66Shc-dependent signal transduction. Protein S-sulfhydration had been proposed to emerge as a major functional alteration of proteins, such as the potassium channels (like KATP, IKca, and SKca) [116], PTP1B [117], NF-κB [118], and Keap1 [87]. We proved that H_2S sulfhydrated p66Shc at cysteine-59, which resides in the proximity to the phosphorylation sites serine-36. S-sulfhydration of p66Shc further impaired the association of $PKC_{\beta II}$ and p66Shc, attenuated H_2O_2-induced p66Shc phosphorylation, and reduced mitochondrial ROS generation [119]. This new finding provides new insights and clues to better understand the important role of the H_2S in oxidative stress and oxidative stress related disease (Figure 5).

4. Challenges and Conclusions

The antioxidant activity of H_2S discussed in this review illuminated the biochemical mechanisms of H_2S on cellular redox homeostasis. However, the effects of H_2S on redox

status are highly divergent. H_2S was also reported as a powerful prooxidant, which kills cancer cells in a ROS-dependent manner [57]. It was believed that the Janus-faced molecule serves as an antioxidant or a prooxidant depending on its local concentrations. At lower concentrations, H_2S exerts beneficial effects like protective effects in the cardiovascular system as we mentioned before, while at higher concentrations, H_2S exhibits a variety of deleterious/cytotoxic effects (for more details, see [120]).

It should also be noted that the concentration- and time-dependent effects of H_2S are very complicated. H_2S was reported to display opposite effects at different concentrations/periods. GYY4137, a slow-releasing H_2S donor, yielded very low concentrations of H_2S and was proved to kill cancer cells. NaHS, which releases higher concentrations of H_2S in short period, however, only exhibited weaker anticanner effect [121]. This may imply that both H_2S releasing speed and amount are important for its therapeutic effects. Therefore, the biological functions of H_2S should be studied in different pathological situations with varied concentrations and treatment periods. Endogenous H_2S generating enzyme activities should also be taken into consideration, as they may be activated/inhibited upon cellular oxidative stress.

In summary, we discussed the current understanding of the antioxidant effect of H_2S in this paper. Obviously, H_2S does not produce antioxidant effect via a single/simple mechanism. Multiple targets and signaling pathways are involved. H_2S can stimulate cellular enzymatic or nonenzymatic antioxidants to scavenge free radicals. This may be secondary to a direct effect on antioxidants or an indirect action through activation of various signaling proteins. H_2S may also inhibit mitochondria ROS production through sulfhydration of p66Shc or membrane/cytosol ROS generation via inhibition of NADPH. To a weak extent, H_2S also quenches free radicals directly due to its chemical reducing property. Future studies to explore more action sites of H_2S in different signaling proteins and mechanisms underlying concentration- and time-dependent effects of H_2S are still warranted.

Abbreviations

3-MST: 3-Mercaptopyruvate sulfurtransferase
ADP: Adenosine diphosphate
AMP: Adenosine monophosphate
ARE: Antioxidant response element
ATP: Adenosine triphosphate
CAT: Catalase
CBS: Cystathionine β-synthase
CH1: collagen homology domain 1
CO: carbon monoxide
CSE: Cystathionine γ-lyase
DAS: Diallyl sulfide
GCLC: Glutamate-cysteine ligase catalytic subunit
GCLM: Glutamate-cysteine ligase modifier subunit
GPx: Glutathione peroxidase
GR: Glutathione reductase
GSH: Reduced glutathione
GSSG: Oxidized glutathione
GST: Glutathione-S-transferase
I/R: Ischemia-reperfusion
Keap1: Kelch-like ECH-associated protein 1
NO: Nitric oxide
Nrf2: Nuclear factor-erythroid 2 (NF-E2) related factor 2
P.E.L: Permissible Exposure Limit
Pin1: Prolyl isomerase
PP2A: Dephosphorylated by phosphatase A2
Prx: Peroxiredoxin
PTB: The phosphor-tyrosine-binding domain
QR: Quinone reductase
ROS: Reactive oxygen species
Shc: Src homologous-collagen
SOD: Superoxide dismutase
Trx-1: Thioredoxin 1
XD: Xanthine dehydrogenase
XO: Xanthine oxidase.

Conflict of Interests

The authors declare no conflict of interests to this work.

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Review Article

Endogenous Sulfur Dioxide: A New Member of Gasotransmitter Family in the Cardiovascular System

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Sulfur dioxide (SO_2) was previously regarded as a toxic gas in atmospheric pollutants. But it has been found to be endogenously generated from metabolism of sulfur-containing amino acids in mammals through transamination by aspartate aminotransferase (AAT). SO_2 could be produced in cardiovascular tissues catalyzed by its synthase AAT. In recent years, studies revealed that SO_2 had physiological effects on the cardiovascular system, including vasorelaxation and cardiac function regulation. In addition, the pathophysiological effects of SO_2 were also determined. For example, SO_2 ameliorated systemic hypertension and pulmonary hypertension, prevented the development of atherosclerosis, and protected against myocardial ischemia-reperfusion (I/R) injury and isoproterenol-induced myocardial injury. These findings suggested that endogenous SO_2 was a novel gasotransmitter in the cardiovascular system and provided a new therapy target for cardiovascular diseases.

1. Introduction

Sulfur dioxide (SO_2) was regarded as a toxic gas and environmental pollutant. It is colorless, transparent, odorous, and water-soluble. The harmful effects of SO_2 on human, animals, and plants have been extensively investigated [1, 2]. However, SO_2 can be endogenously generated from metabolism of the sulfur-containing amino acid L-cysteine in mammals [3]. It has features of low molecular weight, continuous production, and fast diffusion and plays extensive biological action independent of membrane receptors [4, 5]. In neutral fluid or mammal plasma, SO_2 is broken down to its derivatives, bisulfite and sulfite ($NaHSO_3/Na_2SO_3$, 1 : 3 M/M), maintaining organism homeostasis [6]. The sulfite is the physiological form of SO_2 *in vivo* [7, 8]. The reference range for total serum sulfite in healthy human beings was 0–9.85 μmol/L detected by high-performance liquid chromatography with fluorescence detection [9]. Serum sulfite was obviously increased in patients suffering from acute pneumonia and chronic renal failure, as well as pediatric acute lymphoblastic leukemia with bacterial inflammation [10–12]. Of note, Balazy et al. found that SO_2 could be produced in the porcine coronary arterial rings after incubation with calcium ionophore by gas chromatography-mass spectrometry [13]. Du et al. firstly found that endogenous SO_2/aspartate aminotransferase (AAT) pathway existed in the cardiovascular system [14]. SO_2 not only has important physiological effects on vascular tone and cardiac function but also exerts pathophysiological effects in the cardiovascular system, including regulation of hypertension, pulmonary hypertension, atherosclerosis, and cardiac ischemia-reperfusion (I/R) injury [15–18]. The abovementioned evidence suggests that the endogenous SO_2 may be a novel gasotransmitter in mammals, similar to nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S). The physiological significance of SO_2, particularly its regulatory role in the cardiovascular system, has attracted a great deal of interest in the field [19–21].

Therefore, the objective of this review was to elaborate on the generation and metabolism of endogenous SO_2 and give a summary of the physiological and pathophysiological effects of SO_2 on the cardiovascular system.

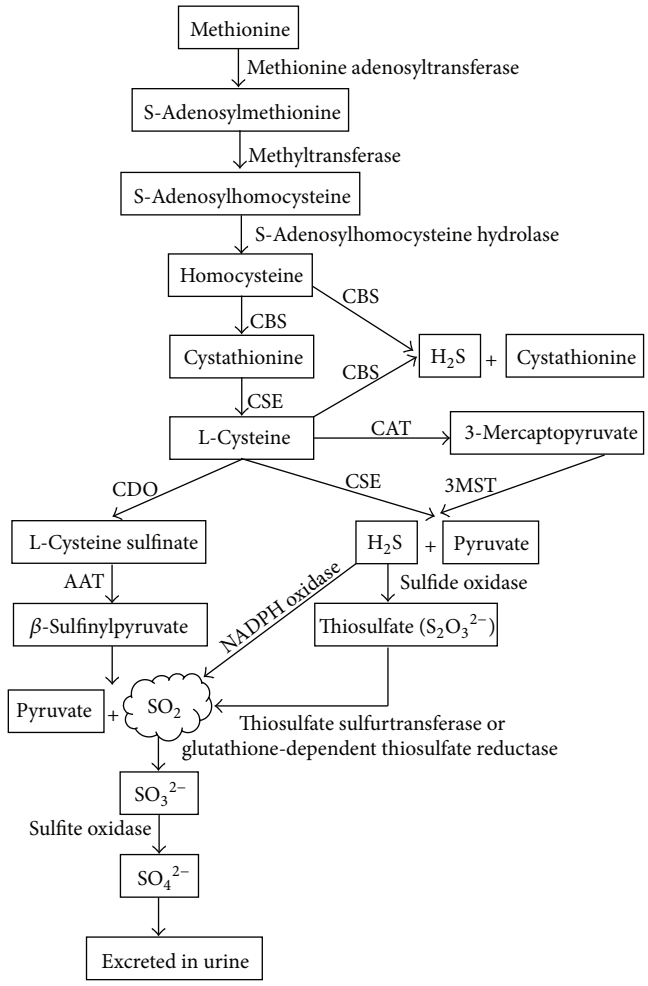

Figure 1: Illustration of production and metabolism of endogenous SO_2 in mammals. SO_2: sulfur dioxide; H_2S: hydrogen sulfide; CBS: cystathionine β-synthase; CSE: cystathionine γ-lyase; CDO: cysteine dioxygenase; AAT: aspartate aminotransferase; CAT: 2-cysteine aminotransferase; 3MST: 3-mercaptopyruvate sulfurtransferase.

2. Generation and Distribution of Endogenous SO_2 in the Cardiovascular System

SO_2 can be generated from the metabolism of L-cysteine which is converted from methionine via the transmethylation-transsulfuration pathway (Figure 1) [3, 22]. Firstly, L-cysteine is oxidized to form L-cysteine sulfinate by cysteine dioxygenase (CDO), and then the latter is transaminated to form β-sulfinylpyruvate by AAT. The β-sulfinylpyruvate spontaneously decomposes to pyruvate and SO_2 (Figure 1) [3]. Additionally, H_2S which shares the same substrate L-cysteine with SO_2 can be transferred to SO_2 *in vivo* through other pathways. Mitsuhashi et al. reported that H_2S could be converted to sulfite or SO_2 by NADPH oxidase in activated neutrophils [23]. Besides, H_2S can be first oxidized to thiosulfate by sulfide oxidase and then converted to SO_2 catalyzed by thiosulfate sulfurtransferase or glutathione-dependent thiosulfate reductase (Figure 1) [6, 24, 25]. SO_2 can exist in the gaseous form or be hydrated to sulfite, which is subsequently oxidized to sulfate by sulfite oxidase, and then the sulfate is excreted into the urine by the kidney (Figure 1) [3, 22].

Du et al. first measured endogenous SO_2/AAT pathway in the cardiovascular system of Wistar rats and found that SO_2 concentration in rat plasma was 15.54 ± 1.68 μmol/L [14]. Li and Meng reported a similar sulfite level of 12.59 ± 9.03 μmol/L in rat plasma [26]. The content of SO_2 in aortic tissue was highest, up to 5.55 ± 0.35 μmol/g protein, followed by pulmonary arteries (3.27 ± 0.21 μmol/g protein), mesenteric arteries (2.67 ± 0.17 μmol/g protein), tail arteries (2.50 ± 0.20 μmol/g protein), and renal arteries (2.23 ± 0.19 μmol/g protein), respectively [14]. Moreover, plasma AAT activity was 87 ± 18 U/L. Unlike SO_2 content, the activity of AAT in the renal arteries was higher than that in other vascular tissues mentioned above [14]. Furthermore, AAT mRNA expression was rich in endothelial cells and in vascular smooth muscle cell (VSMC) beneath the endothelial layer [14].

3. Physiological Effects of SO_2 on the Cardiovascular System

3.1. Vasorelaxant Effect of SO_2. SO_2 derivatives (mixture of sodium bisulfite and sodium sulfite, 1 : 3 M/M in neutral

solution) could induce a concentration-dependent relaxation of isolated rat aortic rings, whereas L-aspartate-β-hydroxamate (HDX), an inhibitor of SO_2 synthase AAT, caused greater vasoconstriction than that of the control group [14]. And the vasorelaxing effects of SO_2 gas and SO_2 gas solution were similar [27, 28]. Therefore, SO_2 might act as a vasoactive molecule. It had a vital vasodilating function required for maintaining normal vascular tone.

The mechanisms of this physiological vasorelaxation by SO_2 were complex. Nicardipine eliminated the vasorelaxing effect induced by SO_2 derivatives, indicating that the L-type calcium (L-Ca^{2+}) channel participated in the role of SO_2 [14]. Additionally, at low concentration (<450 μmol/L), the vasorelaxing effect of SO_2 was related to the big-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel, while at a high concentration (>500 μmol/L) the vasorelaxation induced by SO_2 was associated to adenosine triphosphate-sensitive potassium (K_{ATP}) channel activation and the L-Ca^{2+} channel [29]. Mechanistically, SO_2 and its derivatives induced the K_{ATP} and BK_{Ca} channels activation through increasing the expressions of Kir6.1, Kir6.2, SUR2B, and BK_{Ca} channel subunits α and β1 in rat aortic rings, while SO_2 and its derivatives inhibited the L-type calcium channel through decreasing the expressions of Cav1.2 and Cav1.3 [30]. Besides, SO_2 derivatives increased levels of 3′-5′-cyclic adenosine monophosphate (cAMP), prostacyclin (PGI_2), adenylyl cyclase (AC) activity, and protein kinase A (PKA) activity in rat aortic rings, indicating that the relaxing effect of SO_2 was related to the PGI_2-AC-cAMP-PKA signal pathway [31, 32]. Moreover, the endothelial nitric oxide synthase- (eNOS-) nitric oxide- (NO-) 3′-5′-cyclic guanosine monophosphate (cGMP) pathway and BK_{Ca} channel partially mediated the vasorelaxing effect of SO_2 and sodium bisulfite in an endothelium-dependent manner at low concentration (<450 μM), while at high concentration (≥1000 μM) the vasorelaxation induced by SO_2 was endothelium independent and relied on the K_{ATP} and L-Ca^{2+} channels [26, 33, 34]. Hence, ion channels, such as L-Ca^{2+}, K_{ATP}, and BK_{Ca} channels, as well as cGMP and cAMP pathways play important roles in the effects of SO_2 on vasodilation.

3.2. Negative Inotropic Effect of SO_2. In isolated perfused rat heart, gaseous SO_2 and its derivatives ($NaHSO_3$/Na_2SO_3, 1 : 3 M/M, 0–2000 μmol/L) elicited a dose-dependent negative inotropic effect, which affected the heart rate, left ventricular developed pressure (LVDP), and the first derivatives of LVDP (±LV dp/dt_{max}) [35, 36]. And the gaseous SO_2 induced a server negative effect compared to SO_2 derivatives. The mechanisms for this inotropic effect are different between high concentration and low concentrations of SO_2. At low concentrations, SO_2 produced negative inotropic effects through upregulating the activities of protein kinase C (PKC), cyclooxygenase, and cGMP, while, at high concentrations, the inotropic effects induced by SO_2 were associated with the activation of K_{ATP} channel by increasing the expressions of Kir6.2 and SUR2A and the inhibition of calcium influx via the L-type calcium channel by decreasing the expressions of Cav1.2 and Cav1.3 in rat hearts [36, 37]. Moreover, SO_2 could depress L-type calcium channel current in isolated rat cardiomyocytes [38]. These data indicated that SO_2 had a negative inotropic effect on myocardial contractility and hemodynamic parameters, which might help to explain some cardiovascular effects induced by SO_2.

4. Pathophysiological Effects of SO_2 on the Cardiovascular System

4.1. SO_2 and Hypertension. Hypertension is a major risk factor for many cardiovascular disorders. However, the pathogenesis of hypertension has not been fully elucidated. Exposure to SO_2 (50 ppm, 6 hr/d, 5 d/wk for 31 weeks) was reported to cause a slight but consistent decrease in blood pressure in susceptible to salt-induced hypertension rats [39], implying that SO_2 might regulate blood pressure. Moreover, spontaneously hypertensive rats (SHRs) exhibited a significant decrease in the plasma SO_2 content and AAT activity in both serum and aorta [15]. And SO_2 derivatives administration markedly inhibited the upregulated tail artery pressure of SHRs [15, 40], which suggested that SO_2 played a role in the progress of hypertension. Arterial remodeling predominates in severe hypertension [41]. As well, SO_2 alleviated the pressure to media, decreased the ratio of media to lumen radius, and reduced the proliferative index of smooth muscle cells in the thoracic aorta of SHRs compared to those of sterile water-treated rats [15]. These findings further verified that the inhibited endogenous SO_2/AAT pathway might participate in the development of hypertension. Vasorelaxation dysfunction is the main component of the pathogenesis of hypertension. SO_2 could increase vasorelaxation in SHR arteries by enhancing the vasodilating response to NO in isolated aortic rings and promoting NO production of aortic tissues [40]. The interaction between SO_2 and NO is involved in the mechanisms by which SO_2 regulates hypertension.

The abnormally increased proliferation of VSMCs induces vascular remodeling and accelerates the development of hypertension [42]. Both exogenous SO_2 derivatives and endogenous-derived SO_2 by AAT overexpression significantly inhibited serum-stimulated VSMC proliferation through preventing cell cycle progression from G1 to S phase and inhibiting DNA synthesis [43]. Further study demonstrated that SO_2 elevated cellular cyclic adenosine monophosphate (cAMP) production to activate the PKA signaling, subsequently phosphorylated c-Raf on Ser259 site to block its activation, and then inhibited the extracellular regulated protein kinase (Erk)/mitogen-activated protein kinase (MAPK) signaling transduction, which finally prevented cell cycle progression and led to the suppression of VSMC proliferation [43]. The inhibition of VSMC proliferation might also be involved in SO_2-mediated antihypertensive mechanisms.

4.2. SO_2 and Pulmonary Hypertension

4.2.1. SO_2 and Hypoxic Pulmonary Hypertension. Pulmonary hypertension, characterized by high pressure in pulmonary artery, is a common complication of congenital heart disease (CHD), ultimately inducing right ventricular failure and even death. A prospective cohort study showed that the serum

SO_2 levels of children were, respectively, (10.6 ± 2.4), (8.9 ± 2.3), (7.3 ± 2.9), and (4.3 ± 2.1) μM, in the control group, CHD without pulmonary hypertension group, CHD with mild pulmonary hypertension group, and CHD with moderate or severe pulmonary hypertension group [44], suggesting that a negative correlation existed between SO_2 and pulmonary hypertension. Consistent with this, a downregulated SO_2 level and AAT expression in lung tissue, accompanied with significant pulmonary hypertension, pulmonary vascular remodeling, and increased vascular inflammation, were found in rats under hypoxic condition [16, 45]. Most importantly, SO_2 derivatives could markedly lower mean pulmonary artery pressure (mPAP) of hypoxic pulmonary hypertensive rats, whereas HDX advanced pulmonary hypertension [16, 45], indicating that decreased SO_2/AAT pathway was involved in the development on hypoxic pulmonary hypertension. The hallmark pathological feature of hypoxic pulmonary hypertension is the pulmonary vascular structural remodeling including extracellular matrix accumulation, vascular smooth muscle proliferation, and inflammatory cells infiltrates [46]. SO_2 derivatives prevented pulmonary vascular remodeling in hypoxic pulmonary hypertension through promoting collagen I and III degradation, suppressing abnormal collagen deposition in pulmonary vascular walls and through inhibiting pulmonary arterial SMC proliferation by downregulating Raf-1, MEK-1, and phosphorylating ERK under hypoxia [16]. Inflammation is important in the pathogenesis of hypoxic pulmonary hypertension. In addition, SO_2 could inhibit pulmonary inflammation by suppressing expressions of nuclear factor-kappa B (NF-κB) and intercellular adhesion molecule-1 (ICAM-1) [16], indicating the inhibitory effects of SO_2 on inflammation may also be involved in the mechanism by which SO_2 protects against hypoxic pulmonary hypertension.

4.2.2. SO_2 and Monocrotaline-Induced Pulmonary Hypertension. Monocrotaline (MCT), a pyrrolizidine alkaloid, increased mPAP and the ratio of right ventricle to left ventricle plus septum, coincident with the elevated SO_2 content, AAT activity, and expression in rats [47]. SO_2 derivatives injection significantly lowered mPAP and alleviated small and median pulmonary artery structural remodeling, whereas HDX which inhibited the activity of AAT and the production of endogenous SO_2 further augmented mPAP, promoted right ventricular hypertrophy, and worsened pulmonary arteries structural remodeling [47]. These findings implied that the upregulation of endogenous SO_2/AAT pathway might play a protective role in the development of MCT-induced pulmonary hypertension. The enhancement of oxidative stress is one of the main pathogenesis of MCT-induced pulmonary hypertension [48]. SO_2 could upregulate the activities of antioxidative enzymes, including superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) in lung tissues and plasma from MCT-induced pulmonary hypertensive rats, whereas HDX decreased the activities of antioxidative enzymes [47]. These data suggested that the promotion of endogenous antioxidative capacities might be responsible for the protective role of SO_2 in MCT-induced pulmonary hypertension.

4.2.3. SO_2 and High Pulmonary Blood Flow-Induced Pulmonary Hypertension. Severe pulmonary hypertension develops secondary to high pulmonary blood flow in patients with left-to-right shunt congenital heart defects or systemic arteriovenous shunt [49, 50]. However, the underlying mechanisms for flow-induced pulmonary hypertension remain poorly understood. The endogenous SO_2/AAT2 pathway in pulmonary tissues was also inhibited in rats with pulmonary hypertension induced by high pulmonary blood flow [51]. SO_2 reduced systolic pulmonary arterial pressure and improved pulmonary arterial structural remodeling, exhibiting decreased ratio of muscularized arteries to small pulmonary arteries and increased percentage of nonmuscularized arteries in the development of high pulmonary blood flow-induced pulmonary hypertension [51]. The mechanism was unclear, however. Both SO_2 and H_2S were derived from the methionine metabolism and they could convert to each other in mammals. Moreover, the endogenous H_2S pathway exerted obvious mitigation effect on pulmonary hypertension induced by high pulmonary blood flow and H_2S had strong vasodilating effect. Therefore, the researchers investigated the impact of SO_2 on the endogenous H_2S generating pathway in the pathogenesis of high blood flow-induced pulmonary hypertension. And they found that SO_2 derivatives could upregulate the concentration of H_2S in lung tissues, as well as the expressions of the key generating enzymes of H_2S, including cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3MST) [51]. Furthermore, SO_2 increased the protein expression of these H_2S producing enzymes probably through upregulating their gene transcription. These data suggested that SO_2 alleviated pulmonary hypertension induced by high pulmonary blood flow in association with upregulating the reduced endogenous H_2S pathway.

4.3. SO_2 and Atherosclerosis. Atherosclerosis, a chronic and progressive pathological process in arteries, is a crucial pathological manifestation of cardiovascular diseases. Vascular inflammation, oxidative stress, VSMC proliferation, endothelial injury, and foam cell accumulation contribute to the formation of atherosclerotic plaque. Environmental toxicological study showed that the chronic exposure to gaseous air pollution such as SO_2, NO, and CO might lead to the promotion of atherosclerosis [52, 53]. Growing evidence demonstrated that endogenous NO, CO, and H_2S were beneficial in alleviating atherosclerosis [54–56]. They exerted significant anti-inflammation effect in the development of atherosclerosis, especially endothelium-derived NO which played a notably protective role in the early stage of atherosclerosis. However, the role of SO_2 at physiological concentration in the development of atherosclerosis was unclear. Li et al. found that plasma and aortic SO_2 contents were downregulated with the reduced aortic AAT activity in atherosclerosis rats [17], implying that the inhibition of SO_2/AAT pathway might be involved in the pathogenesis of atherosclerosis. SO_2 derivatives treatment diminished the size of atherosclerotic plaques in the coronary artery, not only by increasing H_2S/CSE pathway and the NO/nitric oxide

synthase (NOS) pathway, but also by elevating the antioxidative capacities through increasing plasma GSH-Px and SOD activities and decreasing MDA level [17]. Additionally, suppression of VSMC proliferation via cAMP/PKA signaling-mediated Erk/MAPK pathway might also contribute to the antiatherosclerotic effects of SO_2 [43].

4.4. SO_2 and Myocardial Ischemia Reperfusion. Myocardial ischemia-reperfusion (I/R) injury is an important cause of tissue and cell injury and often leads to heart failure. The main mechanisms involve inflammation, oxidative damage, and intracellular and mitochondrial calcium overload [57]. In rat myocardial I/R models made by ligating the left coronary artery for 30 min and reperfusion for 120 min, AAT1 protein expression was significantly decreased compared to sham operation group [18]. And SO_2 derivatives preconditioning for 10 min before ischemia (with a low concentration of sulfur dioxide of 1–10 μmol/kg) significantly decreased myocardial infract size and lowered levels of myocardial enzymes creatine kinase (CK) and lactate dehydrogenase (LDH) in plasma of rats with I/R injury *in vivo* [18]. SO_2 preconditioning also increased cardiac function and attenuated myocardium apoptosis induced by I/R [18]. Ischemic preconditioning-induced endoplasmic reticulum stress (ERS) plays a protective role in the ischemia injury. Glucose-regulated protein 78 (GRP78), C/EBP homologous protein (CHOP), and phosphorylated eukaryotic initiation of the factor 2α-subunit (p-eIF2α) are the markers of myocardial ERS. SO_2 pretreatment induced myocardial GRP78 expression and eIF2α phosphorylation prior to myocardial I/R, while inhibiting expressions of myocardial GRP78, CHOP, and p-eIF2α in rats with myocardial I/R [18]. Dithiothreitol, an ERS activator [58], mimicked the cardioprotective effect of SO_2, whereas ERS inhibitor 4-phenylbutyrate abolished the cardioprotection of SO_2 preconditioning [18, 59]. The above data suggested that augmentation of ERS by SO_2 preconditioning before myocardial I/R contributed to cardioprotection against lethal ischemia. Moreover, SO_2 preconditioning significantly elevated the phosphorylation of Akt and PI3K p85 and attenuated the myocardial damage in rats with I/R injury [60]. LY294002, a PI3K inhibitor, prevented the protective function of SO_2 preconditioning as well as SO_2-induced GRP78 and p-eIF2α expression [18, 60], indicating that PI3K/Akt signaling pathway likely mediated the activation of ERS by SO_2 pretreatment in rats subjected to myocardial I/R. In addition, oxidative stress is involved in the pathogenesis of myocardial I/R. SO_2 preconditioning with low dose of SO_2 (1 and 5 μmol/kg) prior to ischemia could significantly elevate plasma levels of SOD, GSH, and GSH-Px and reduce the MDA level [61], indicating that SO_2 preconditioning enhanced the antioxidative capacity in rats with myocardial I/R. MAPK signaling, one of the most important pathways in cell signal transduction, is crucial to myocardial I/R. SO_2 preconditioning significantly improved cardiac function and reduced myocardial expression of phosphorylated ERK1/2 protein in isolated rat heart with I/R [62]. Pretreated with PD98059, the ERK1/2 inhibitor abolished the above functions of SO_2 [62]. These data indicated that inhibition of ERK1/2 signal pathway activation mediated the cardioprotection of SO_2 preconditioning in isolated rat heart subjected to I/R. Taken together, elevation of PI3K/AKT signaling, suppression of ERK-MAPK pathway, augmentation of ERS, enhancement of antioxidative capacity, and attenuation of cardiomyocyte apoptosis might be involved in SO_2-mediated cardiac protective mechanisms.

4.5. SO_2 and Myocardial Injury. Myocardial injury is a common feature in various cardiac diseases. The underlying mechanisms include hypoxia, overactive oxidative stress, and calcium overload. A previous study found that endogenous SO_2/AAT pathway was downregulated in isoproterenol-(ISO-) induced myocardial injury in rats [63]. Administration of SO_2 (85 mg/(kg day)) could alleviate cardiac dysfunction and myocardial damage induced by ISO [63]. These data demonstrated that endogenous SO_2 might be an important regulator in the pathophysiological process of myocardial injury. The molecular mechanisms underlying the cardioprotective effects of SO_2 were still unknown. Oxidative stress was involved in the pathogenesis for ISO-induced myocardial injury. ISO produced oxygen free radicals caused membrane lipid peroxidation, injured the structure of cardiomyocytes, and finally resulted in myocardial damage [64]. But SO_2 could increase myocardial antioxidant capacity in rats with myocardial injury by increasing the myocardial activity of SOD and GSH, upregulating the mRNA expression of SOD2 and GSH-Px1, and decreasing products of oxidative stress such as H_2O_2 and $O_2^{\bullet-}$ [63]. Oxidative stress could cause ERS in rat cardiomyocytes [65]. And the overactivated ERS would contribute to the development of myocardial injury. SO_2 significantly inhibited the excessive activation of ERS, which might be involved in the mechanism by which SO_2 derivatives protected against myocardial injury induced by ISO [66]. In addition, the products of oxidative stress cause the cardiomyocyte membrane damage and morphological mitochondrial injury. SO_2 also attenuated ISO-induced mitochondrial swelling and deformation, which was important feature in apoptosis [63]. And cardiomyocyte apoptosis is a key pathological change in myocardial injury. Of note, supplementation of SO_2 derivatives alleviated ISO-induced myocardial injury partly through reducing cardiomyocyte apoptosis [67]. The antiapoptotic function of SO_2 was mediated by promoting bcl-2 expression, suppressing bax expression, enhancing mitochondrial membrane potential, inhibiting mitochondrion MPTP opening, reducing the release of cytochrome c from mitochondrion into cytoplasm, and decreasing the activation of caspase-9 and caspase-3 [67]. Therefore, the bcl-2/cytc/caspase-9/caspase-3 pathway was involved in the ISO-induced myocardial injury in rats. Intracellular calcium homeostasis exerts a fundamental effect on myocardial physiology and pathology. And calcium overload is an important mechanism involved in myocardial injury. SO_2 treatment could inhibit the increased intracellular free Ca^{2+} concentration induced by ISO in H9C2 cells [68], indicating that the protective effect of SO_2 in myocardial injury might be related to the calcium homeostasis regulated by SO_2 in cardiomyocytes. Moreover, SO_2 derivatives

could modulate L-type calcium current and voltage-gated potassium channels in rat cardiomyocytes, indicating that ion channels might also be involved in the effect of SO_2 on cardiomyocyte injury [69, 70].

5. Interaction among SO_2 and Other Gasotransmitters

SO_2 and H_2S share the same endogenous substrate L-cysteine, and they can transform into each other under some biochemical condition [6, 23, 71]. Moreover, they share similar regulatory roles including vasorelaxation, antioxidative action, and inhibition of inflammation and apoptosis. Chen et al. found that SO_2 upregulated the concentration and production of H_2S in hypoxic rats. And SO_2 increased the expression of CSE and 3MST in pulmonary arteries of hypoxic pulmonary hypertensive rats [72]. In addition, SO_2 alleviated pulmonary hypertension and improved the pulmonary vascular pathological injury induced by high pulmonary blood flow in association with upregulating the endogenous H_2S pathway [51]. Furthermore, SO_2 derivatives have a marked antiatherogenic effect with an increased aortic H_2S/CSE pathway in atherosclerotic rats [17]. In rats with myocardial I/R injury, SO_2 preconditioning markedly upregulated the myocardial H_2S level and CSE expression [61]. The above findings provide some evidence that there is a crosstalk between SO_2 and H_2S. Moreover, NO also shares a variety of the similar biological effects of H_2S and SO_2, including vasodilation, antioxidation, and anti-inflammatory actions. Li and Meng found that a low concentration (3 or 5 nM) of a NO donor sodium-nitroprusside enhanced the vasodilating effect of SO_2 by nearly sixfold [26], suggesting that SO_2 and NO have a synergistic effect on vasodilation. In contrast, the NOS inhibitor L-NAME could abolish the vasorelaxing effect of SO_2 derivatives (0.5 and 1 mM) in endothelium-intact rings, indicating that endothelium-dependent vasorelaxation induced by SO_2 was partially mediated by a NOS pathway [73]. Additionally, both acute and prolonged SO_2 exposure upregulated the eNOS-NO-cGMP pathway, which might be involved in the vasodilation induced by SO_2 [34]. Moreover, SO_2 increased vasorelaxation in SHRs by enhancing the vasorelaxing response to NO and upregulating NO production in aortic tissues [40]. And SO_2 also increased NO/NOS pathway in rats with atherosclerosis [17]. By contrast, SO_2 pretreatment reduced the myocardial tissue levels of NO and expression of iNOS in rats with I/R [61]. These data suggest that there is also an interaction between SO_2 and NO. Hence, endogenous SO_2 participates in crosstalk with H_2S and NO and an endogenous gaseous messenger molecule network might exist in mammals. However, there are still many questions to be answered about the interactions among these gases. For example, the exact pertinence among these gases in the various pathways of cardiovascular protection has not been fully explored. It is also not known if a combination of these gases will provide synergistic effects in the therapy of cardiovascular diseases. Therefore, additional studies are needed to further investigate interactions among the gasotransmitter pathways.

6. Conclusions

In summary, SO_2 can be generated in the cardiovascular system of mammals and the SO_2/AAT pathway participates in many biological functions [22, 74]. Endogenously derived SO_2 or SO_2 derivatives at physiological concentrations play a crucial role in normal physiological process including regulation of vascular tone and cardiac function. In addition, SO_2/AAT pathway has important pathophysiological significance in many cardiovascular diseases, such as hypertension, pulmonary hypertension, atherosclerosis, ischemia-reperfusion injury, and myocardial injury. Just as NO, carbon monoxide (CO), and H_2S, SO_2 is also an endogenous gaseous signaling molecule in the cardiovascular system [71, 75]. However, the biological mechanisms by which endogenous SO_2 regulates different cardiovascular diseases and the further cardiovascular effects of SO_2 still need to be deeply investigated.

Clarifying the interactions among SO_2 and other endogenous gasotransmitters could improve clinical translation. SO_2 could upregulate endogenous level of H_2S or NO in several cardiovascular diseases such as atherosclerosis, systemic hypertension, or pulmonary hypertension [17, 40, 51]. These lines of evidence imply a crosstalk among SO_2 and other gasotransmitters (NO, CO, and H_2S) in the cardiovascular system, which requires further exploration.

An understanding of the cardiovascular protective function of SO_2 may lead to a new therapeutic strategy based on the modulation of SO_2 production. Thus, the function and signaling pathway relating to AAT in the cardiovascular system are worthy of further investigation. Additionally, the design of SO_2-controlled releasing agents under physiological condition is extremely urgent, because the stable and reliable SO_2 donors are not only the useful research tools, but also potential therapeutic agents to treat cardiovascular diseases. Nowadays, the majority of cardiovascular studies on SO_2 have been performed in rats and mice, which lack clinical evidence. Exploring the role of SO_2 in large animal models with similar cardiovascular features as human suffering from cardiovascular diseases would help a transition to clinical trials.

Abbreviations

SO_2: Sulfur dioxide
AAT: Aspartate aminotransferase
I/R: Ischemia-reperfusion
CDO: Cysteine dioxygenase
H_2S: Hydrogen sulfide
NO: Nitric oxide
CO: Carbon monoxide
HDX: L-Aspartate-β-hydroxamate
L-Ca^{2+}: L-type calcium
BK_{Ca}: Big-conductance Ca^{2+}-activated K^+
K_{ATP}: Adenosine triphosphate-sensitive potassium
cAMP: 3′-5′-Cyclic adenosine monophosphate
PGI_2: Prostacyclin
AC: Adenylyl cyclase
PKA: Protein kinase A
cGMP: 3′-5′-Cyclic guanosine monophosphate

LVDP: Left ventricular developed pressure
PKC: Protein kinase C
SHR: Spontaneously hypertensive rats
eNOS: Nitric oxide synthase
VSMCs: Vascular smooth muscle cells
PDGF-BB: Platelet-derived growth factor-BB
Erk 1/2: Extracellular regulated protein kinase 1/2
MAPK: Mitogen-activated protein kinase
CHD: Congenital heart defects
mPAP: Mean pulmonary artery pressure
NF-κB: Nuclear factor-kappa B
ICAM-1: Intercellular adhesion molecule-1
MCT: Monocrotaline
SOD: Superoxide dismutase
GSH-Px: Glutathione peroxidase
CAT: Catalase
CSE: Cystathionine γ-lyase
CBS: Cystathionine β-synthase
3MST: 3-Mercaptopyruvate sulfurtransferase
NOS: Nitric oxide synthase
CK: Creatine kinase
LDH: Lactate dehydrogenase
GRP78: Glucose-regulated protein 78
CHOP: C/EBP homologous protein
eIF2α: Eukaryotic initiation of the factor 2α-subunit
p-eIF2α: Phosphorylated eIF2α
ERS: Endoplasmic reticulum stress
ISO: Isoproterenol.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Hydrogen Sulfide Inhibits High-Salt Diet-Induced Renal Oxidative Stress and Kidney Injury in Dahl Rats

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Background. The study was designed to investigate if H_2S could inhibit high-salt diet-induced renal excessive oxidative stress and kidney injury in Dahl rats. *Methods*. Male salt-sensitive Dahl and SD rats were used. Blood pressure (BP), serum creatinine, urea, creatinine clearance rate, and 24-hour urine protein were measured. Renal ultra- and microstructures were observed. Collagen-I and -III contents the oxidants and antioxidants levels in renal tissue were detected. Keap1/Nrf2 association and Keap1 s-sulfhydration were detected. *Results*. After 8 weeks of high-salt diet, BP was significantly increased, renal function and structure were impaired, and collagen deposition was abundant in renal tissues with increased renal MPO activity, H_2O_2, MDA, GSSG, and $^{\bullet}OH$ contents, reduced renal T-AOC and GSH contents, CAT, GSH-PX and SOD activity, and SOD expressions in Dahl rats. Furthermore, endogenous H_2S in renal tissues was decreased in Dahl rats. H_2S donor, however, decreased BP, improved renal function and structure, and inhibited collagen excessive deposition in kidney, in association with increased antioxidative activity and reduced oxidative stress in renal tissues. H_2S activated Nrf2 by inducing Keap1 s-sulfhydration and subsequent Keap1/Nrf2 disassociation. *Conclusions*. H_2S protected against high-salt diet-induced renal injury associated with enhanced antioxidant capacity and inhibited renal oxidative stress.

1. Introduction

Numerous studies have demonstrated that high salt not only caused hypertension, but also resulted in kidney injury [1–8]. The kidney injury and fibrosis of Dahl rats induced by high salt were reported to be closely related to oxidative stress [8, 9]. However, up to now, the mechanisms responsible for high-salt-induced kidney injury have been unclear.

As the third gaseous signal molecule after nitric oxide (NO) and carbon monoxide (CO), hydrogen sulfide (H_2S) is produced by the metabolism of sulfur-containing amino acid. The key enzymes of synthesis, cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and mercaptopyruvate transsulphurase (MPST), are abundantly expressed in the kidney [10, 11]. H_2S has various pathophysiological roles including relaxing blood vessels, lowering blood pressure [12, 13], anti-inflammatory response [14], antioxidative stress [15], and inhibiting proliferation of smooth muscle cells [16]. Studies showed that H_2S might protect neurons against oxidative injury by promoting the generation of antioxidants—glutamine [17]—and participated in the regulation on hypertension induced by Ang II through antioxidative stress [18]. In hypoxia pulmonary hypertension, H_2S could inhibit oxidative stress of lung tissue [15] and collagen deposition caused by inhibiting oxidative stress [19]. Similarly, Otunctemur et al. found that H_2S had a protective effect on gentamicin-induced kidney injury [20]. Further study found that, in a mouse model of unilateral ureteral obstruction, H_2S could alleviate the oxidative stress by upregulating catalase (CAT), Superoxide dismutase (SOD), and glutathione (GSH), thereby

improving renal fibrosis [21]. However, whether H_2S could protect against high-salt-induced kidney injury and what are the possible mechanisms remain unclear. Therefore, the present study was undertaken to explore the protective effects of H_2S on high-salt-induced kidney injury in Dahl rats and its possible mechanisms.

2. Materials and Methods

2.1. Animal Grouping. Thirty 5-week-old healthy male salt-sensitive (Dahl) rats and 40 male SD rats (Charles River Laboratory Animal Technology Co., Ltd., China; License number: SCXK 2012-0001) were fed in Animal Center of Peking University First Hospital. They were randomly divided into three groups after adapting to the environment for one week. Dahl rats were divided into control group (Dahl + NS), high-salt group (Dahl + HS), and high-salt + NaHS group (Dahl + HS + NaHS), while SD rats were divided into control group (SD + NS), high-salt group (SD + HS), high-salt + NaHS group (SD + HS + NaHS), and high-salt + hydroxylamine group (SD + HS + HA), with 10 rats in each group. The rats in Dahl + NS and SD + NS groups were fed with normal diet and 0.9% normal saline was given via intraperitoneal injection daily; the rats in Dahl + HS and SD + HS groups were fed with the diet containing 8% salt and intraperitoneally injected with 0.9% normal saline every day; the rats in Dahl + HS + NaHS and SD + HS + NaHS group were fed with high-salt diet while 90 μmol/kg NaHS was given by intraperitoneal injection every day [22]; and the rats in SD + HS + HA group were fed with high-salt diet while 12.5 mg/kg HA, an inhibitor of CBS, was given by intraperitoneal injection every day [23]. The NaHS and HA were prepared daily with 0.9% normal saline. All rats drank water freely throughout the experiment in 25°C constant temperature environment, maintaining 12 h/12 h circadian rhythms. This experiment strictly followed the laboratory animal welfare and the operating guide of laboratory institutions and was licensed by Peking University First Hospital Experiment and Ethics Committee.

2.2. Detection of Arterial Blood Pressure and Biochemical Indices in Rats. After eight weeks of experiment, 25% urethane (0.5 mL/100 g) was given to rats through intraperitoneal injection for anesthesia. Physiological multilead recorder (BL-420F, Chengdu TME, Chengdu, China) was used to record arterial blood pressure. Automatic biochemical analyzer (Hitachi 7600, Japan) was used to detect serum creatinine, creatinine clearance rate, serum urea, and 24-hour urine protein.

2.3. Observation of Kidney Ultramicrostructure. 2 mm × 2 mm × 2 mm of renal cortical tissue was put in 3% glutaraldehyde to fix, making electron microscope specimens and observing the changes in kidney ultrastructure.

2.4. Observation of Renal Pathological Structure. 0.2 cm of tissue specimens from kidney transverse section was taken and fixed in 4% paraformaldehyde, paraffin embedding, taking 0.5 μm of section for Periodic acid-Schiff staining (Shanghai Genmed Pharmaceutical Co., Ltd., China) and Masson staining (Beijing Rocchi Biotechnology Co., Ltd., China) in strict accordance with the kit experimental steps. Leica Q550CW Image Processing and Analysis System was used for image acquisition and semiquantitative analysis of the results of kidney PAS and Masson staining. The semiquantitative analysis of kidney PAS staining was used to evaluate glomerulosclerosis. Depending on the severity of glomerulosclerosis, it is divided into four grades: (0) normal; (1) glomerulosclerosis area <25%; (2) 25%–50%; (3) 50%–75%; and (4) >75%. Ten visual fields were randomly selected for each rat to calculate the proportion of sclerotic glomeruli. Five visual fields of renal cortex were randomly selected for each rat to calculate the average index number of renal fibrosis [24].

2.5. Detection of Renal Collagen Content. The appropriate renal tissue was taken, adding PBS buffer (pH 7.2, 0.05 M) according to the mass volume ratio of 1 : 10 (mg/μL), fully ground to renal tissue homogenate, and centrifuged at 12000 g/min for 10 min, taking the supernatant. Double antibody sandwich enzyme-linked immunosorbent assay (ELISA) was used to detect the content of collagen-I and collagen-III (American R&D Co., Ltd., USA) and hydroxyproline content was measured by colorimetry (Nanjing Jiancheng Bioengineering Institute, China). The experimental procedures were in strict accordance with the kit instructions.

2.6. Determination of H_2S Content in Renal Tissue

Preparation of Renal Tissue Homogenate. The renal tissue of rats was taken from −70°C freezer into 1.5 mL of EP tube. 0.01 mol/L PBS solution was added according to the mass volume ratio (1/10), ground on the ice at 4°C, and centrifuged at 12000 g/min for 10 min, taking the supernatant. Free Radical Detection Analyzer TBR4100 (World Precision Instruments, Shanghai, China) was used to measure H_2S content in the supernatant of renal tissue homogenates [25]. Firstly, 2 mm of ISO-H_2S-100 sensor (ISO-H_2S-2, WPI, Shanghai, China) was placed in PBS buffer solution (pH 7.2, 0.05 M) for polarization. When the stable reference current appeared (usually as 100–2000 PA), the sensor was calibrated. Approximate 10–15 mm of the top of the sensor was immersed in 20 mL of PBS buffer solution (pH 7.2, 0.05 M), until the stable current appeared on the display. Then, the detecting sensors were successively inserted in six kinds of different concentrations of Na_2S solution formulated by PBS buffer (5 μL, 10 μL, 20 μL, 40 μL, 80 μL, and 160 μL). The current output would rise rapidly to a plateau after each sample was added. As long as the current reached a plateau, the next sample was detected. Then, the calibration curve was made based on the output signal (pA) and the corresponding H_2S concentration (μmol/L). There was about 10–15 mm of the sensor that was immersed in each sample. H_2S content of each sample was calculated according to pA- H_2S concentration calibration curve.

2.7. Detection of Oxidative Stress Indices of Renal Tissue. The right amount of renal tissue was taken; PBS buffer (pH 7.2,

0.05 M) was added according to the mass volume ratio of 1 : 10 (mg/μL), fully ground to renal tissue homogenate, and centrifuged at 12000 g/min for 10 min, taking the supernatant. Biochemical colorimetry was used to detect the following indices: SOD, myeloperoxidase (MPO), CAT, glutathione peroxidase (GSH-PX) activity and oxidized glutathione (GSSG), GSH, hydrogen peroxide (H_2O_2), malondialdehyde (MDA), hydroxyl radical (OH) content, and total antioxidant capacity (T-AOC). The experimental procedures were in strict accordance with the kit instructions (Nanjing Jiancheng Bioengineering Institute, China).

2.8. Protein Levels of SOD2 and SOD1 of Renal Tissue Detected by Western Blot. The renal tissues were homogenized at 4°C by adding 1x tissue lysates according to the mass volume ratio of 1 : 10 (mg/μL) and then centrifuged at 12000 g/min for 10 min, taking the supernatant. The equal amounts of protein mixture samples were added into each channel for polyacrylamide gel electrophoresis and then transferred onto a membrane. After closing by milk at room temperature for 1 h, primary antibodies SOD1 (Stressgen, USA), SOD2 (Stressgen, USA), and β-actin (Santa Cruz Biotechnology) were added, incubated at 4°C overnight, and washed by TTBS, 10 min/times, for a total of four times. Then, HRP-labeled secondary antibody (Sigma, USA) was added, incubated at room temperature for 1 h, washed by TTBS, 10 min/times, for a total of four times, and then incubated with chemiluminescent reagents for 1 min before being exposed, developed, and fixed. AlphaImager gel imaging system was used to scan protein bands and measure optical density of protein bands, and β-actin was used as interior reference to correct [26].

2.9. The Association of Keap1 and Nrf2 Detected with Coimmunoprecipitation. The coimmunoprecipitation was performed as described previously [27]. Briefly, the renal tissues were fully ground on ice with RIPA lysis buffer (20 mm Tris, 150 mm NaCl, 1% Triton X-100, EDTA, and proteinase and phosphatase inhibitors) and then centrifuged at 12000 g/min for 20 min at 4°C. The equal amount of protein supernatant after protein quantization was incubated overnight at 4°C with Keap1 or Nrf2 antibody. Protein A/G-magnetic beads were added at 4°C and incubated for 4 h and then at 4°C, centrifuged at 10000 g/min for 10 min. Protein A/G-magnetic beads were washed with PBS buffer (pH 7.2, 0.05 M) for 3 times and then boiled for 10 min at 100°C mixed with 2x loading buffer. The proteins were separated in 10% SDS-PAGE and detected with Nrf2 (Enzolife, USA) or Keap1 antibody (Cell Signaling Technology, USA) by Western blotting.

2.10. s-Sulfhydration Assay of Keap1. Keap1 s-sulfhydration was performed by the modified biotin switch assay as described previously [28]. The renal tissues were homogenized at 4°C with tissue lysis buffer and centrifuged at 12000 g/min for 10 min. The tissue lysates were incubated with blocking buffer (lysis buffer supplemented with 2.5% SDS and 20 mM S-methyl methanethiosulfonate) at 50°C for 20 min with frequent vortexing; then acetone was added for removing S-methyl methanethiosulfonate and proteins were precipitated at −20°C for 2 h. After acetone removal, the proteins were resuspended in tissue lysis buffer and incubated with 25 μL of EZ-Link iodoacetyl-PEG2 biotin (10 mg/mL) for 12 h at 4°C. Biotinylated proteins were precipitated by 30 μL of ultralink immobilized NeutrAvidin for 4 h at 4°C. After supernatant removal, the protein beads were washed with PBS buffer (pH 7.2, 0.05 M) for 3 times and boiled for 10 min at 100°C mixed with loading buffer without β-mercaptoethanol. Then, the biotinylated proteins were detected with Keap1 antibody by Western blotting [28].

2.11. Statistical Analysis. SPSS13.0 software was used for statistical analysis. The results are expressed as mean ± standard error, the means between the two groups were compared by independent sample t-test, and the means among many groups were compared by one-way ANOVA. $P < 0.05$ was considered significant.

3. Results

3.1. High-Salt Diet Induced the Significant Increase of Blood Pressure and Renal Impairment in Dahl Rats. Compared with the rats of Dahl + NS, blood pressure of rats in Dahl + HS group was significantly increased ($P < 0.01$, Figure 1(a)); renal function was decreased, as reflected by the reduced creatinine clearance rate ($P < 0.01$, Figure 1(b)) and the increased content of serum creatinine and serum urea ($P < 0.01$, Figures 1(c) and 1(d)); however, compared with the rats of SD + NS group, blood pressure and renal function of SD rats did not change in SD + HS group. There was no significant change in creatinine clearance rate, serum creatinine, and serum urea between rats of SD + NS group and SD + HS group ($P > 0.05$, Figures 1(a)–1(d)).

3.2. High-Salt Diet Caused the Increase in Urine Protein and Renal Structural Damage in Dahl Rats. Compared with the rats of Dahl + NS group, 24 h urine protein of rats in Dahl + HS group was significantly increased ($P < 0.01$, Figure 2(a)), and the electron microscopic result of renal tissue showed that the foot process of glomerular podocytes had an extensive fusion or even disappeared (Figure 2(b)). PAS staining of renal tissue showed that there was an obvious glomerular sclerosis in rats of Dahl + HS group ($P < 0.01$, Figures 3(a) and 3(b)). Masson staining of renal tissue showed that there was renal fibrosis in rats of Dahl + HS group ($P < 0.01$, Figures 3(c) and 3(d)).

3.3. High-Salt Diet Induced Decrease in H_2S Content of Renal Tissue in Dahl Rats. Compared with rats in Dahl + NS group, H_2S content of renal tissue of rats in Dahl + HS group was significantly decreased ($P < 0.05$, Figure 4).

3.4. H_2S Improved Blood Pressure and Renal Function of Dahl Rats Induced by High Salt. Compared with Dahl + HS rats, blood pressure of rats in Dahl + HS + NaHS group was significantly decreased ($P < 0.01$, Figure 1(a)); renal function was significantly improved as demonstrated by the obviously increased creatinine clearance rate and

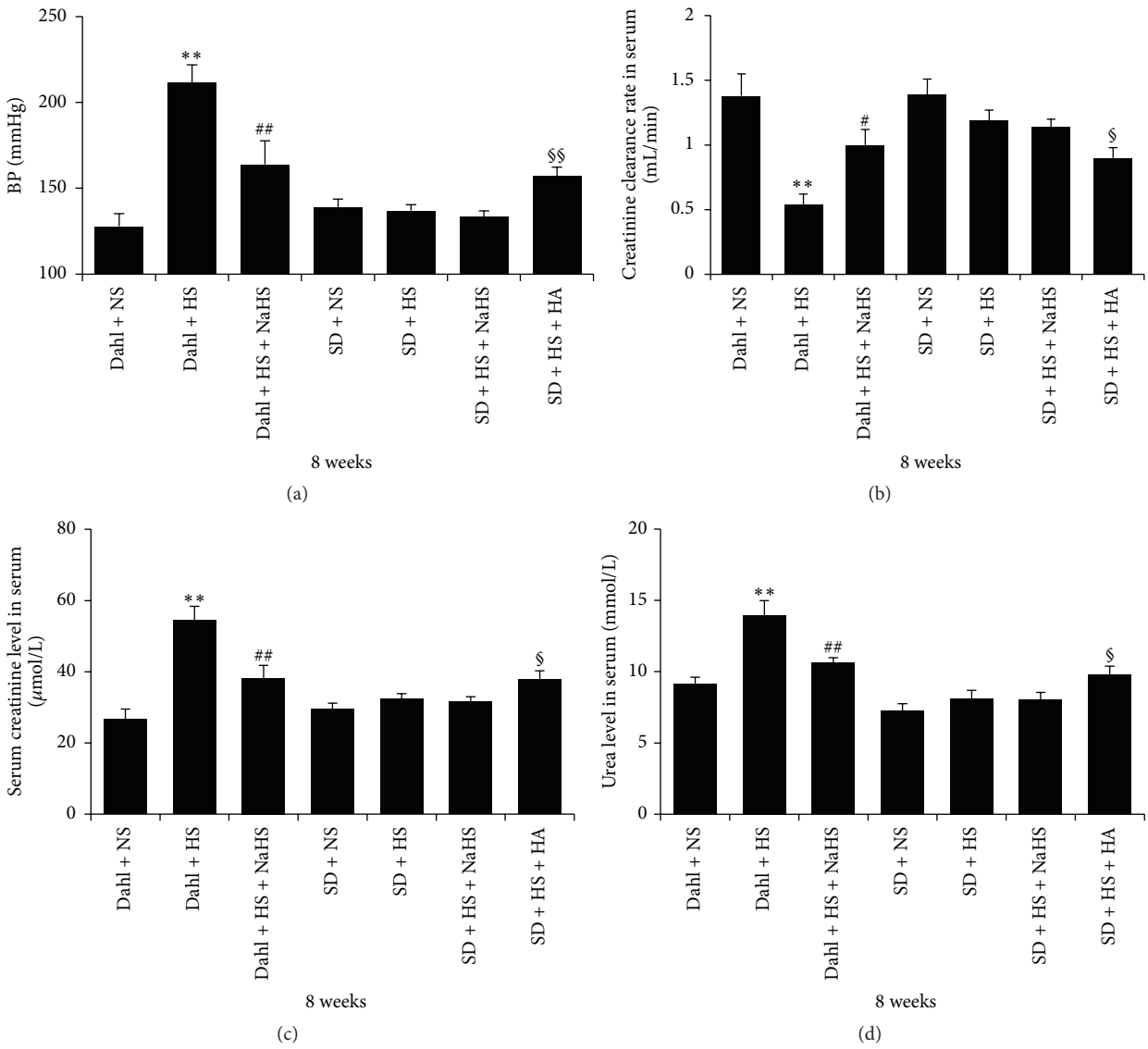

FIGURE 1: H_2S improved blood pressure and renal function of Dahl rats induced by high salt. (a) BP and (b and d) renal function indices (mean ± SE, $n = 10$). $^{**}P < 0.01$ versus Dahl + NS; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus Dahl + HS; $^{\$}P < 0.05$ versus SD + HS; and $^{\$\$}P < 0.01$ versus SD + HS.

the significantly decreased content of serum creatinine and serum urea ($P < 0.05$, $P < 0.01$, Figures 1(b)–1(d)) after the treatment with NaHS. However, blood pressure and renal function did not differ between rats of SD + HS group and SD + HS + NaHS group ($P > 0.05$, Figures 1(a)–1(d)). While, compared with SD + HS rats, blood pressure of SD + HS + HA rats was significantly increased ($P < 0.01$, Figure 1(a)), renal function was significantly damaged with the obviously decreased creatinine clearance rate and the significantly increased content of serum creatinine and serum urea ($P < 0.05$, Figures 1(b)–1(d)) after the treatment with HA.

3.5. H_2S Improved 24 h Urine Protein and Renal Structural Damage of Dahl Rats Induced by High Salt. Compared with Dahl + HS rats, 24 h urine protein of Dahl rats in high-salt group was decreased after the treatment with NaHS ($P < 0.01$, Figure 2(a)), and the electron microscope result of renal tissue showed that the foot process fusion of glomerular podocytes had a significant relief (Figure 2(b)). PAS staining and semiquantitative analysis showed that there was a significant relief in glomerular sclerosis ($P < 0.01$, Figures 3(a) and 3(b)). Masson staining and semiquantitative analysis showed that there was a significant relief in renal fibrosis ($P < 0.01$, Figures 3(c) and 3(d)). However, in SD rats, NaHS had no significant effect on 24 h urine protein and renal structure, but, compared with SD + HS rats, HA significantly increased 24 h urine protein ($P < 0.05$, Figure 2(a)) and damaged renal structure (Figure 2(b)).

3.6. H_2S Reduced the Renal Collagen Content in Dahl Rats Induced by High Salt. Compared with Dahl + NS group, the contents of collagen-I, collagen-III, and hydroxyproline of

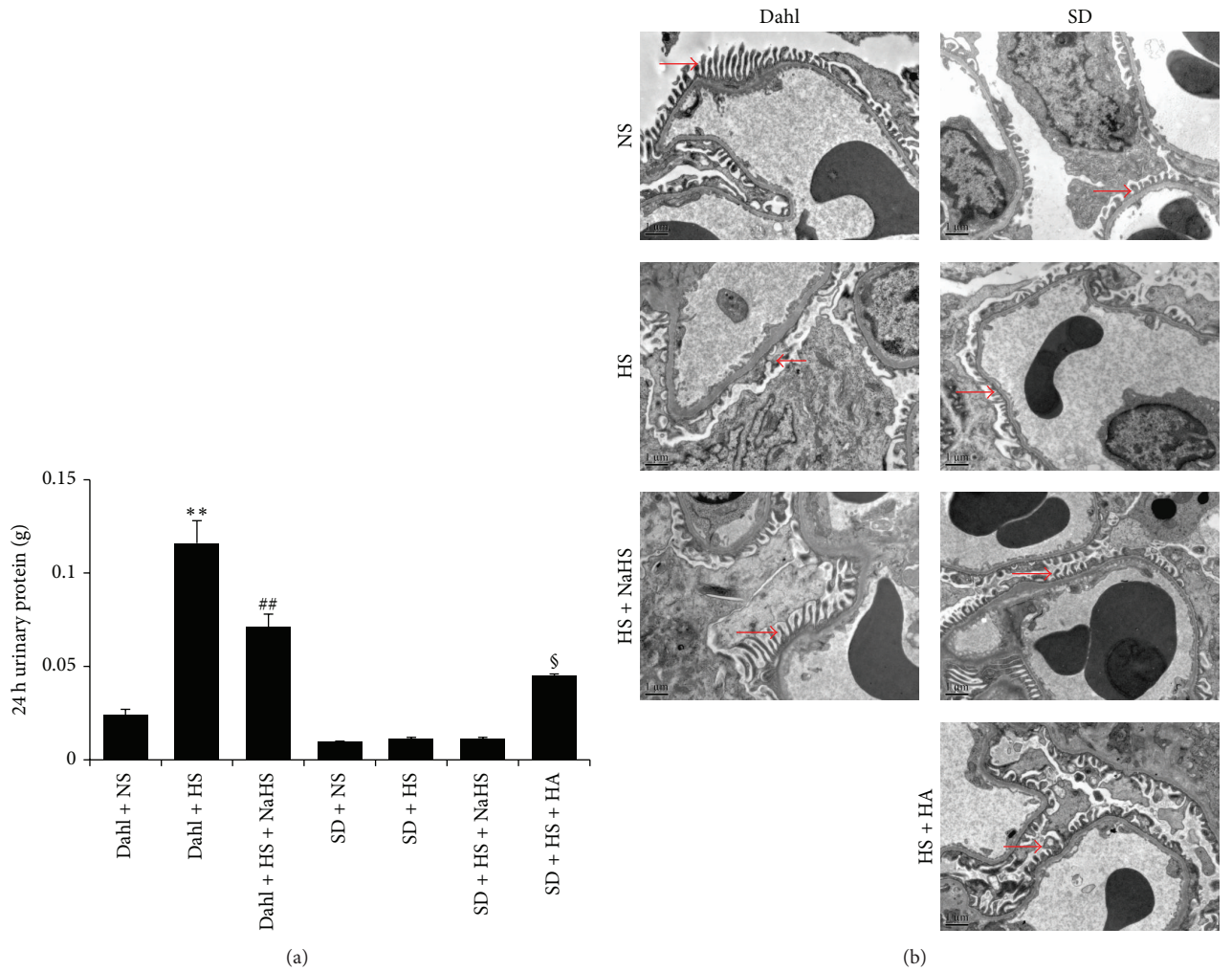

FIGURE 2: H_2S improved proteinuria and renal structural damage of Dahl rats induced by high salt. (a) 24 h urinary protein (mean ± SE, $n = 10$). $^{**}P < 0.01$ versus Dahl + NS; $^{\#\#}P < 0.01$ versus Dahl + HS; and $^{\S}P < 0.05$ versus SD + HS. (b) Electron microscope of kidney changes in kidney ultrastructure (TEM 8000x). →: foot process of glomerular podocytes.

renal tissue in Dahl + HS group were significantly increased ($P < 0.01$, Figures 5(a)–5(c)). Compared with Dahl + HS group, however, the contents of collagen-I, collagen-III, and hydroxyproline of renal tissue in Dahl + HS + NaHS group were significantly reduced ($P < 0.05$, $P < 0.01$, Figures 7(a)–7(c)). Compared with rats of SD + HS group, NaHS had no significant effect on the contents of collagen-I, collagen-III, and hydroxyproline in rat renal tissue of SD + HS + NaHS group ($P > 0.05$, Figures 5(a)–5(c)), but HA significantly increased the contents of collagen-I, collagen-III, and hydroxyproline in rat renal tissue of SD + HS + NaHS group ($P < 0.05$, $P < 0.01$, Figures 5(a)–5(c)).

3.7. H_2S Reduced Renal MDA, H_2O_2, $^{\bullet}OH$, and GSSG Content and MPO Activity in Dahl Rats with High-Salt Diet. Compared with Dahl + NS group, the contents of MDA, H_2O_2, $^{\bullet}OH$, and GSSG of renal tissues in Dahl + HS group were significantly increased ($P < 0.01$, $P < 0.05$, Figures 6(a)–6(d)), and MPO activity was increased ($P < 0.01$, Figure 6(e)). However, compared with Dahl + HS group, the contents of MDA, H_2O_2, $^{\bullet}OH$, and GSSG of renal tissue in Dahl + HS + NaHS group were significantly decreased ($P < 0.05$, $P < 0.01$, Figures 6(a)–6(d)), and MPO activity was decreased ($P < 0.01$, Figure 6(e)). Compared with SD + NS group, however, the contents of MDA, H_2O_2, $^{\bullet}OH$, and GSSG of renal tissue in SD + HS group did not alter ($P > 0.05$, Figures 6(a)–6(d)), nor the MPO activity ($P > 0.05$, Figure 6(e)). Compared with SD + HS group, the contents of MDA, H_2O_2, $^{\bullet}OH$, and GSSG of renal tissue did not change in SD + HS + NaHS group ($P > 0.05$, Figures 6(a)–6(d)), and there was no increase in MPO activity ($P > 0.05$, Figure 6(e)), while the contents of MDA, H_2O_2, $^{\bullet}OH$, and GSSG of renal tissue were increased in SD + HS + HA group ($P < 0.05$, Figures 6(a)–6(d)), and there was a significant increase in renal MPO activity ($P < 0.05$, Figure 6(e)).

3.8. H_2S Enhanced T-AOC and Increased GSH Content and the Activity of CAT, GSH-PX, and SOD of Dahl Rat Renal Tissue Induced by High Salt. Compared with Dahl + NS group, T-AOC of renal tissue in rats of Dahl + HS group was decreased

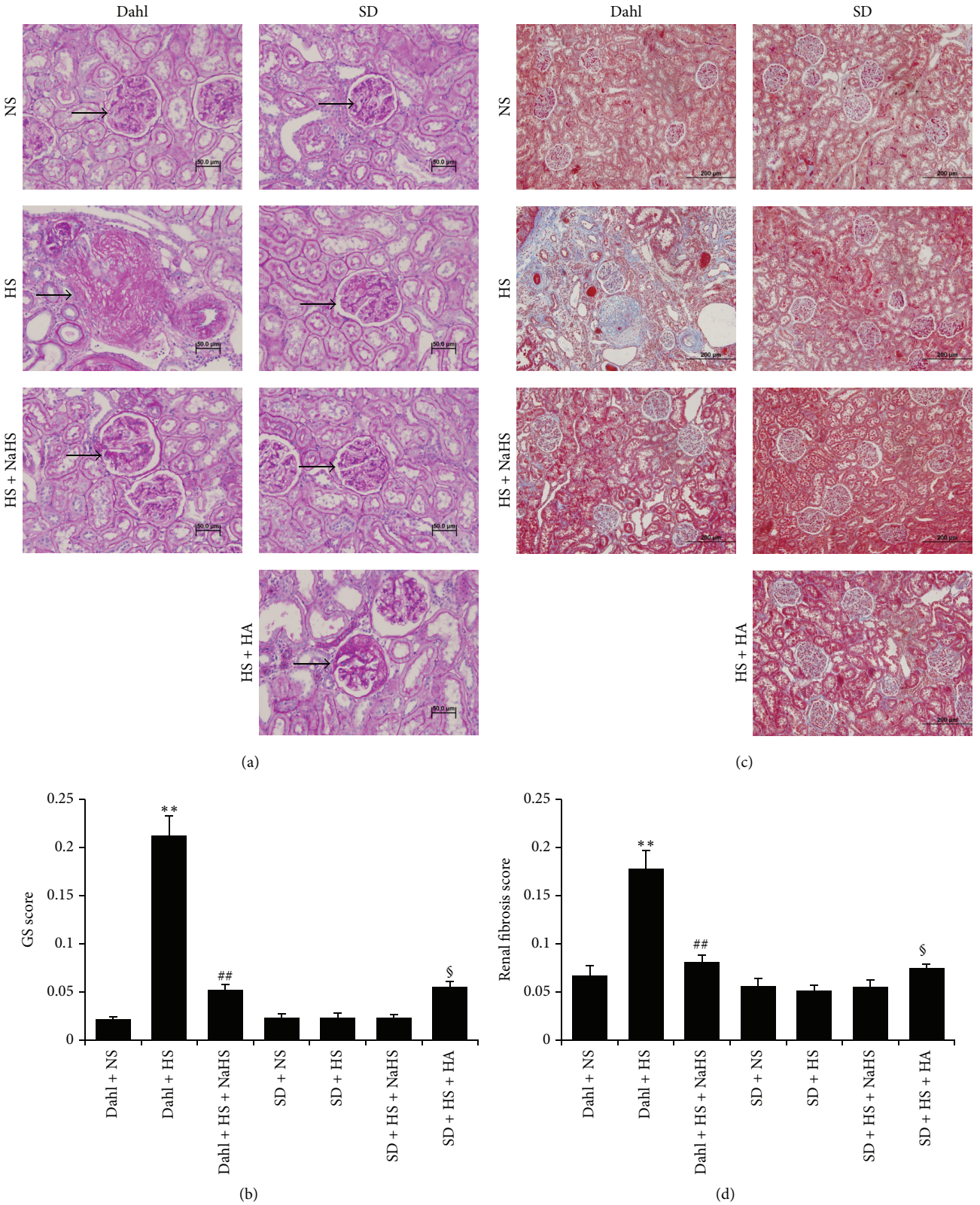

Figure 3: PAS and Masson staining showed the pathological structural changes in rat kidney in each group. (a) PAS staining of kidney (400x). (b) Semiquantitative measurement of glomerular sclerosis by PAS staining (mean ± SE, $n = 10$). (c) Kidney Masson staining (200x). (d) Semiquantitative measurement of renal fibrosis by Masson staining. $^{**}P < 0.01$ versus Dahl + NS; $^{\#\#}P < 0.01$ versus Dahl + HS; and $^{\S}P < 0.05$ versus SD + HS. →: glomerulus.

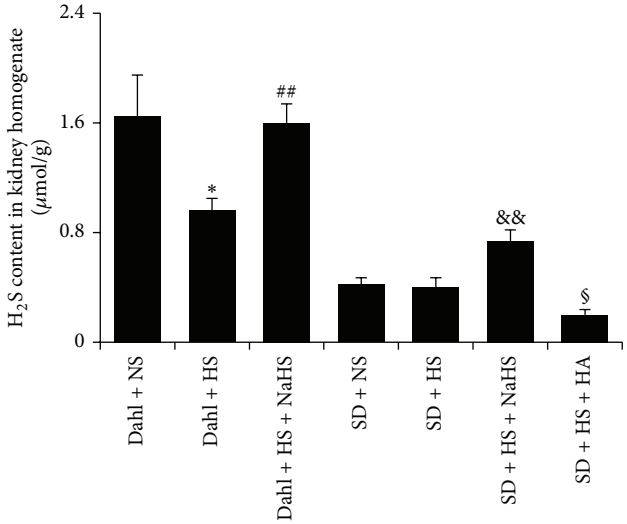

FIGURE 4: Changes in H_2S content of rat renal tissue in each group (mean ± SE, $n = 10$). $^{*}P < 0.01$ versus Dahl + NS; $^{\#\#}P < 0.01$ versus Dahl + HS; $^{\&\&}P < 0.01$ versus SD + HS; and $^{\S}P < 0.05$ versus SD + HS.

($P < 0.01$, Figure 7(a)), and antioxidant enzymes CAT, GSH-PX, and SOD activities and GSH content were significantly reduced ($P < 0.05$, $P < 0.01$, Figures 7(b)–7(e)). But compared with Dahl + HS group, T-AOC of renal tissue in Dahl + HS + NaHS group was potently enhanced ($P < 0.05$, Figure 7(a)) and renal CAT, GSH-PX, and SOD activities and GSH content were significantly increased ($P < 0.05$, Figures 7(b)–7(e)). However, compared with SD + NS group, there was no significant change in T-AOC, GSH content and the activity of CAT, GSH-PX, and SOD of renal tissue in SD + HS group ($P > 0.05$, Figure 7), and renal tissue T-AOC, GSH content and the activity of CAT, GSH-PX, and SOD did not differ between SD + HS group and SD + HS + NaHS group ($P > 0.05$, Figure 7), but renal tissue T-AOC, GSH content and the activity of CAT, GSH-PX, and SOD in SD + HS + HA group were significantly decreased as compared with SD + HS group ($P < 0.05$, $P < 0.01$, Figure 7).

3.9. H_2S Upregulated the Protein Expression of SOD1 and SOD2 of Dahl Rat Renal Tissue Induced by High Salt. Compared with Dahl + NS group, the expression of SOD1 and SOD2 protein in renal tissue of Dahl + HS group was reduced ($P < 0.01$, Figures 8(a) and 8(b)). However, compared with Dahl + HS group, the expression of SOD1 and SOD2 protein in renal tissue of Dahl + HS + NaHS group was enhanced ($P < 0.05$, Figures 8(a) and 8(b)). Among the rats of SD + NS, SD + HS, and SD + HS + NaHS groups, there was no significant change in the expression of SOD protein of renal tissue ($P > 0.05$, Figures 8(c) and 8(d)). However, compared with SD + HS group, the expression of SOD1 and SOD2 protein of renal tissue in SD + HS + HA group was decreased ($P < 0.01$, Figures 8(c) and 8(d)).

3.10. H_2S Reduced Association of Keap1 with Nrf2 and Enhanced Keap1 s-Sulfhydration in Renal Tissue of Dahl Rats with High-Salt Diet. Compared with Dahl + NS group, the association of Keap1 with Nrf2 in the renal tissue of rats was increased in Dahl + HS group ($P < 0.01$, Figure 9(a)), but Keap1 s-sulfhydration in the renal tissue of rats in Dahl + HS group was reduced ($P < 0.05$, Figure 10(a)). However, NaHS treatment significantly reduced the association of Keap1 with Nrf2 ($P < 0.01$, Figure 9(a)) and enhanced Keap1 s-sulfhydration in the renal tissues of rats ($P < 0.01$, Figure 10(a)) in Dahl + HS group. There were no significant differences in the associated Keap1 with Nrf2 and Keap1 s-sulfhydration in the renal tissues of rats among SD + NS, SD + HS, and SD + HS + NaHS groups ($P > 0.05$, Figures 9(b) and 10(b)). However, compared with SD + HS group, the association of Keap1 with Nrf2 in the renal tissues of rats in SD + HS + HA group was increased ($P < 0.01$, Figure 9(b)) and Keap1 s-sulfhydration in renal tissue was reduced ($P < 0.01$, Figure 10(b)).

4. Discussion

In recent years, studies on the effect of excessive intake of salt in diet on human health have received considerable attentions. Previous studies showed that the high-salt diet was closely associated with hypertension and could cause kidney damage [29]. As an important organ, the kidney is involved in the regulation of water-electrolyte metabolism and blood pressure. Hence, its functional and structural damage will exacerbate the process of hypertension and cause serious complications. A large number of renal transplantation experiments of animal and human have confirmed that kidney is a key factor for hypertension caused by high salt [30–36]. Therefore, the study on kidney injury caused by high salt is crucial to preventing the occurrence of hypertension due to high salt.

In the previous experiment on Dahl rats induced by high salt, it was reported that antioxidants vitamin E and vitamin C could effectively reverse the kidney damage caused by high-salt diet. Studies have shown that, as a strong reducing agent, H_2S has antioxidative stress effect [19, 20, 37]. Therefore, this experiment was undertaken to study the protective mechanisms by which H_2S regulated kidney damage of Dahl rats caused by high salt.

In the present study, we used high-salt diet to stimulate Dahl and SD rats for eight weeks, and blood pressure of Dahl rat in high-salt group was significantly increased with proteinuria. There was an obvious renal structural damage. Interestingly, the study showed that H_2S content of Dahl rat renal tissue in high-salt group was decreased while there was no change in the above indices between SD rats with and without high-salt diet. The results suggested that the decreased H_2S content of renal tissue might be involved in the mechanism for high-salt-induced hypertension and kidney damage in Dahl rats. To confirm this point, the high-salt diet was given to Dahl and SD rats with NaHS treatment daily by intraperitoneal injection and to SD rats with HA treatment daily by intraperitoneal injection. The results showed that blood pressure in rats of Dahl + HS + NaHS group was decreased significantly; proteinuria and renal structural damage were significantly reduced, but these

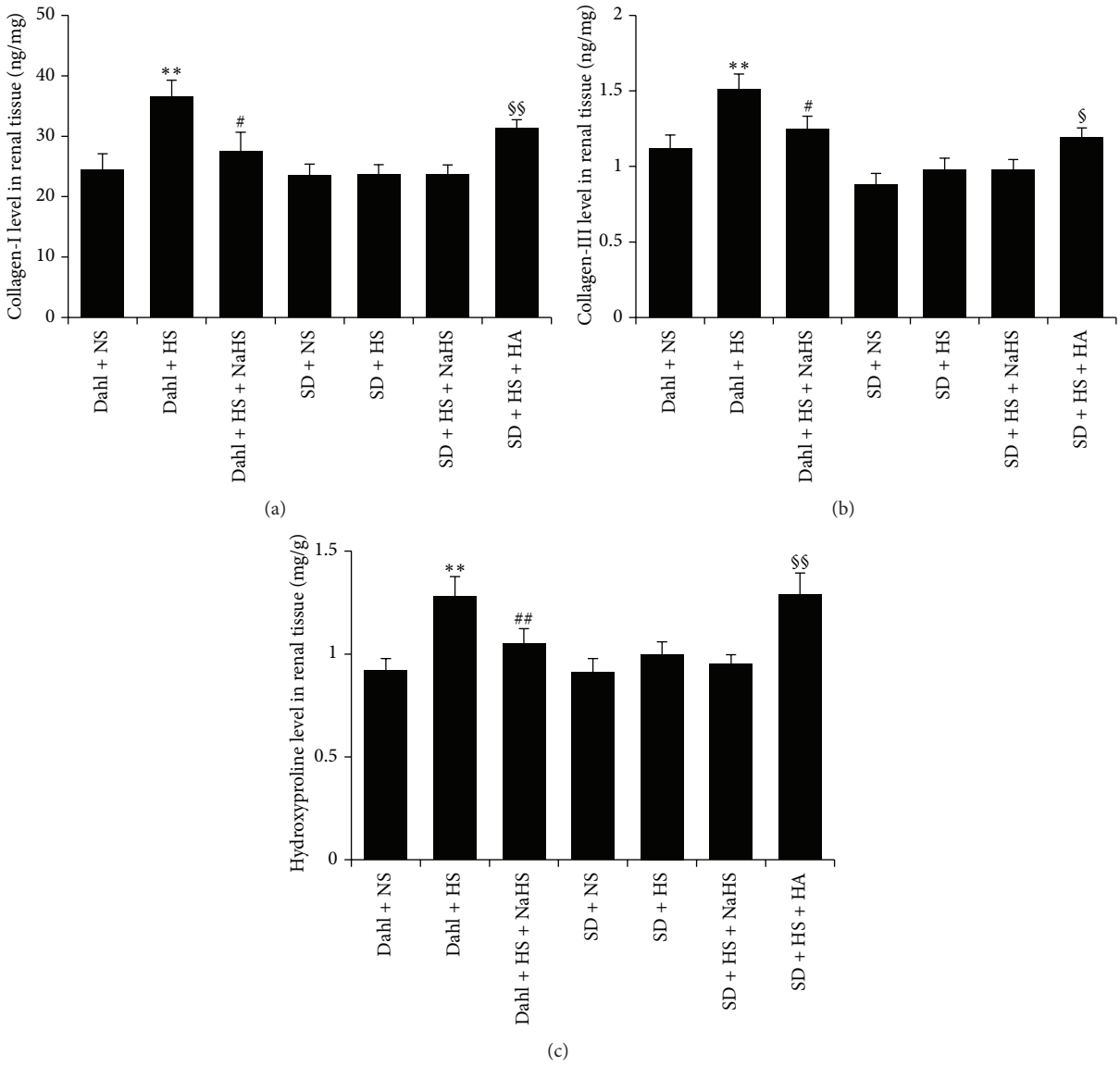

FIGURE 5: H_2S reduced the content of collagen of renal tissue in Dahl rats induced by high salt. (a) Collagen-I content of rat renal tissue in each group. (b) Collagen-III content of rat renal tissue in each group. (c) Hydroxyproline content of rat renal tissue in each group (mean ± SE, $n = 10$). $^{**}P < 0.01$ versus Dahl + NS; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus Dahl + HS; $^{\S}P < 0.05$ versus SD + HS; and $^{\S\S}P < 0.01$ versus SD + HS.

indices of SD rats did not change no matter NaHS was used or not while the blood pressure and proteinuria of SD + HS + HA group were increased significantly with renal structural damage. Previous studies showed that H_2S/CBS pathway of renal tissue was downregulated in the formation of hypertension [11]. Studies also showed that H_2S could effectively improve renal function and kidney damage in kidney transplant experiments [38–40]. Our study firstly confirmed that H_2S played a protective role in high-salt-induced kidney injury in Dahl rats.

Up to now, the mechanism by which H_2S protected against the high-salt-induced kidney function and structure has not been clear. Oxidative stress refers to a process of the imbalance between oxidation and antioxidation which can lead to the excessive accumulation of reactive oxygen species and reactive nitrogen *in vivo* or in the cell, causing the oxidative damage. ROS includes superoxide anion (O_2^-), $^{\bullet}OH^-$, and H_2O_2, and RNS includes NO, nitrogen dioxide, and peroxynitrite. There are two types of enzymatic and nonenzymatic antioxidant systems in the body, and the former includes SOD, CAT, and GSH-PX, while the latter includes vitamin C, vitamin E, and glutathione peptide [41, 42]. Studies have shown that H_2S has antioxidative stress effect [21, 43, 44]. We further detected kidney oxidative stress indices in high-salt-induced kidney animal model and found that high salt caused oxidative stress of Dahl rat renal tissue, as demonstrated by the decreased T-AOC, GSH and the decreased activity of CAT, GSH-PX, and SOD, while the levels of MDA, H_2O_2, $^{\bullet}OH$, and GSSG were increased, and MPO activity was also increased. The results suggested that high

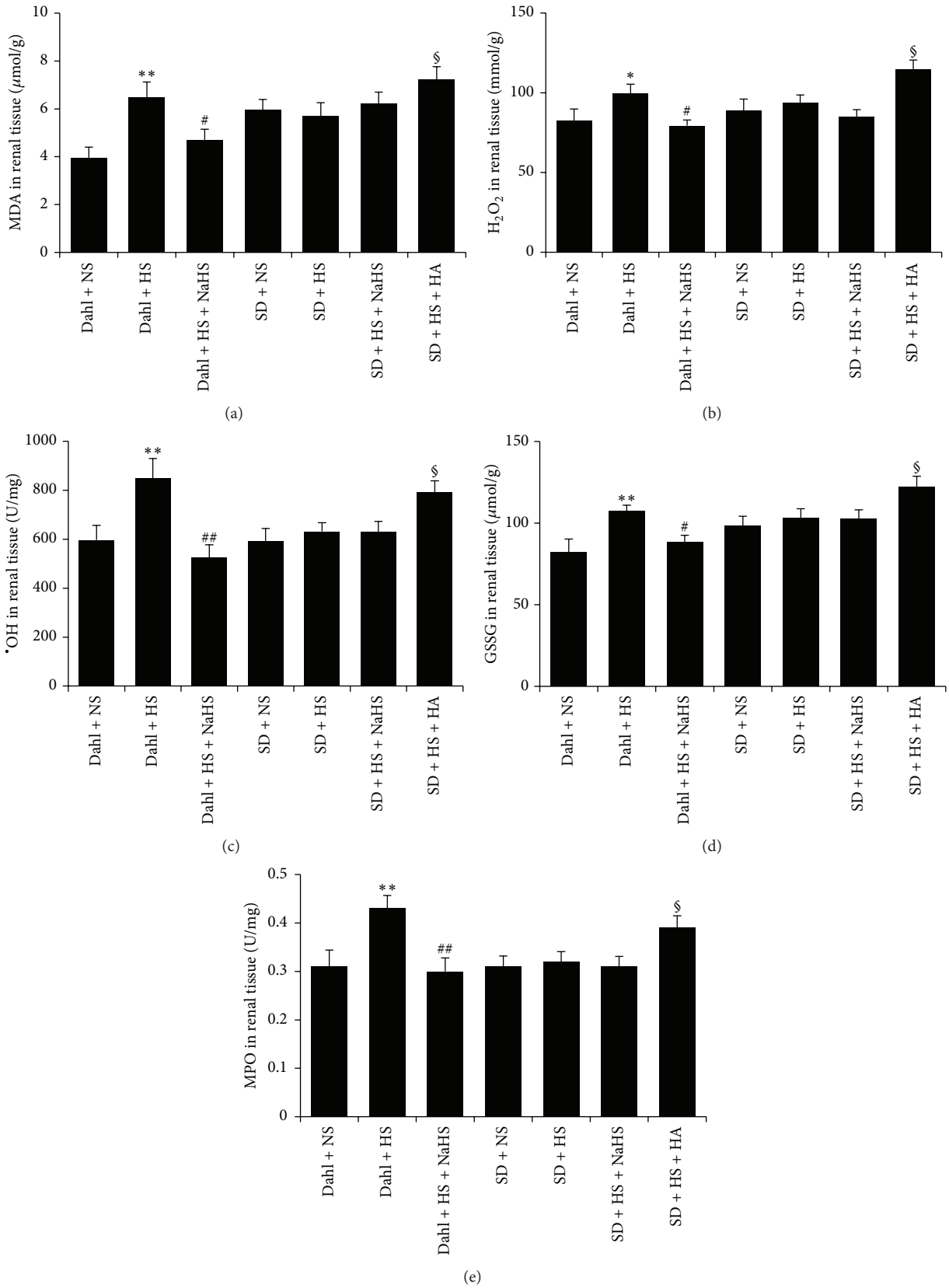

FIGURE 6: H_2S inhibited oxidative stress of Dahl rat renal tissue induced by high salt. (a) MDA content of rat renal tissue in each group. (b) H_2O_2 content of rat renal tissue in each group. (c) $^{\bullet}OH$ content of rat renal tissue in each group. (d) GSSG content of rat renal tissue in each group. (e) MPO activity of rat renal tissue in each group (mean ± SE, $n = 10$). $^{*}P < 0.05$, $^{**}P < 0.01$ versus Dahl + NS; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus Dahl + HS; and $^{\S}P < 0.05$ versus SD + HS.

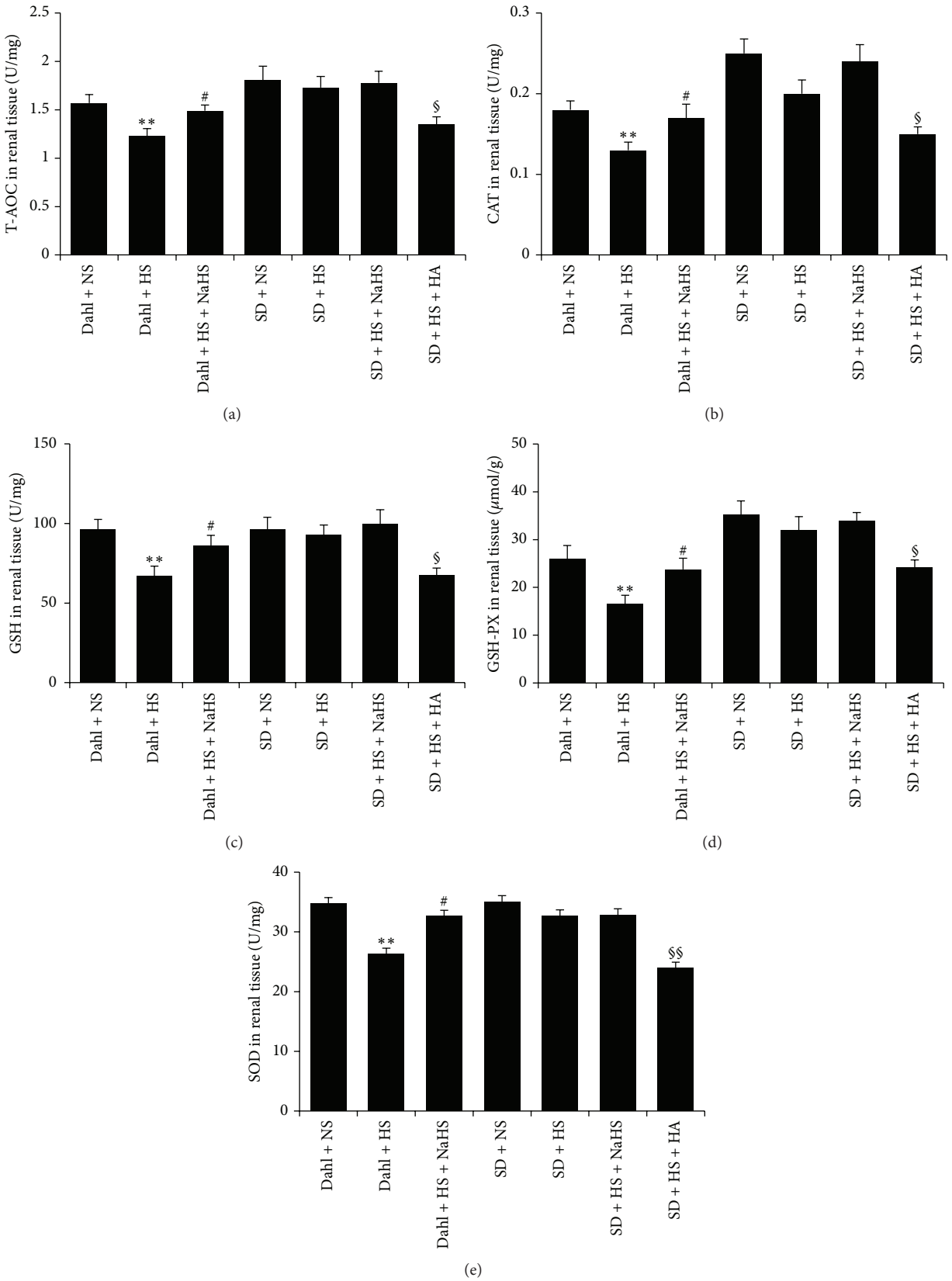

FIGURE 7: H_2S enhanced the antioxidant capacity of Dahl rat renal tissue induced by high salt. (a) T-AOC of rat renal tissue in each group. (b) CAT activity of rat renal tissue in each group. (c) GSH activity of rat renal tissue in each group. (d) GSH-Px activity of rat renal tissue in each group. (e) SOD activity of rat renal tissue in each group (mean ± SE, $n = 10$). $^{**}P < 0.01$ versus Dahl + NS; $^{\#}P < 0.05$ versus Dahl + HS; $^{\S}P < 0.05$ versus SD + HS; and $^{\S\S}P < 0.01$ versus SD + HS.

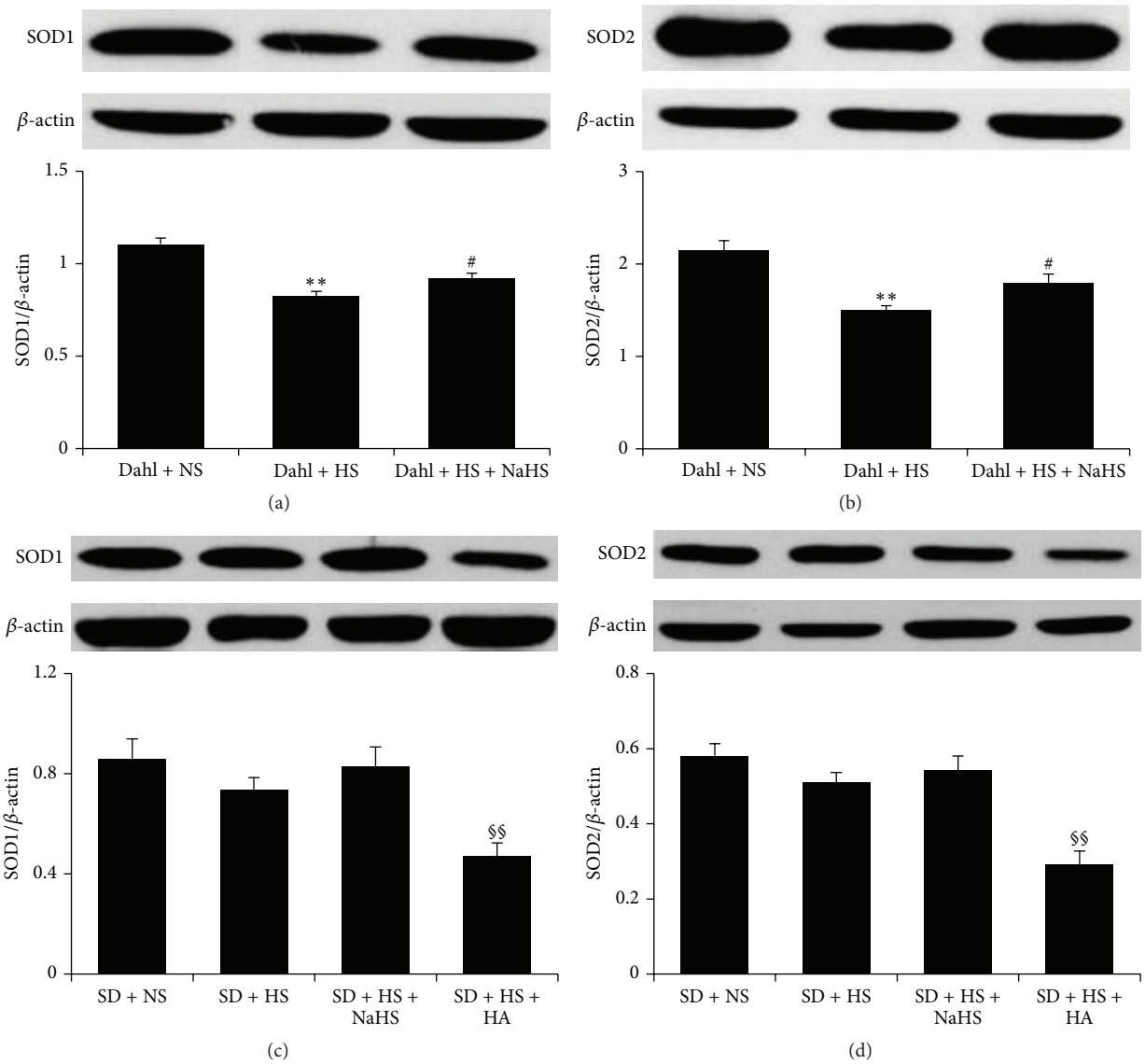

Figure 8: Protein expression of SOD1 and SOD2 in renal tissues of Dahl rats and SD rats. (a) SOD1 and (b) SOD2 protein expressions in renal tissues of Dahl rats. (c) SOD1 and (d) SOD2 protein expressions in renal tissues of SD rats (mean ± SE, $n = 10$). $^{**}P < 0.01$ versus Dahl + NS; $^{\#}P < 0.05$ versus Dahl + HS; $^{\S}P < 0.05$ versus SD + HS; and $^{\S\S}P < 0.01$ versus SD + HS.

salt could lead to oxidative stress of Dahl rat renal tissue. After giving NaHS, an H_2S donor, into Dahl rats, the renal T-AOC and GSH content were enhanced, the renal activity of CAT, GSH-PX, and SOD increased, and expression of SOD1 and SOD2 protein was upregulated, while the content of MDA, H_2O_2, $^{\bullet}OH$, GSSG, and MPO activity decreased. The results suggested that H_2S could increase the renal antioxidative ability and reduce the oxidative injury.

As well known, Keap1/Nrf2 is one of the main pathways for antioxidative system. It regulates expression of many antioxidant enzymes and plays a pivot role in the homeostasis of oxidative and antioxidative responses [27, 45–47]. Keap1 is an inhibitor of the transcription factor Nrf2 and located in the cytoplasm, binding with Nrf2 to prevent its translocation to the nucleus Keap1/Nrf2 dissociation, which results in Nrf2 nuclear translocation and increases the expression of target antioxidant enzymes such as SOD, further playing the role of antioxidation. In the present study, we found that the increase in association of Keap1 with Nrf2 in the Dahl rats with high-salt diet was reversed by treatment with H_2S donor. Furthermore, the increase in combination of Keap1 with Nrf2 in the renal tissues of Dahl + HS rats was mimicked by giving HA, an inhibitor of endogenous H_2S generation, in the renal tissues of SD rats with high-salt diet. The above results suggested that H_2S might decrease the combination of Keap1 with Nrf2, thereby releasing the free Nrf2 and subsequently activating Nrf2. Regarding the mechanism by which H_2S reduced the association of Keap1 with Nrf2, previous studies have reported that Keap1 s-sulfhydration could destroy the combination of Keap1 with Nrf2 and then

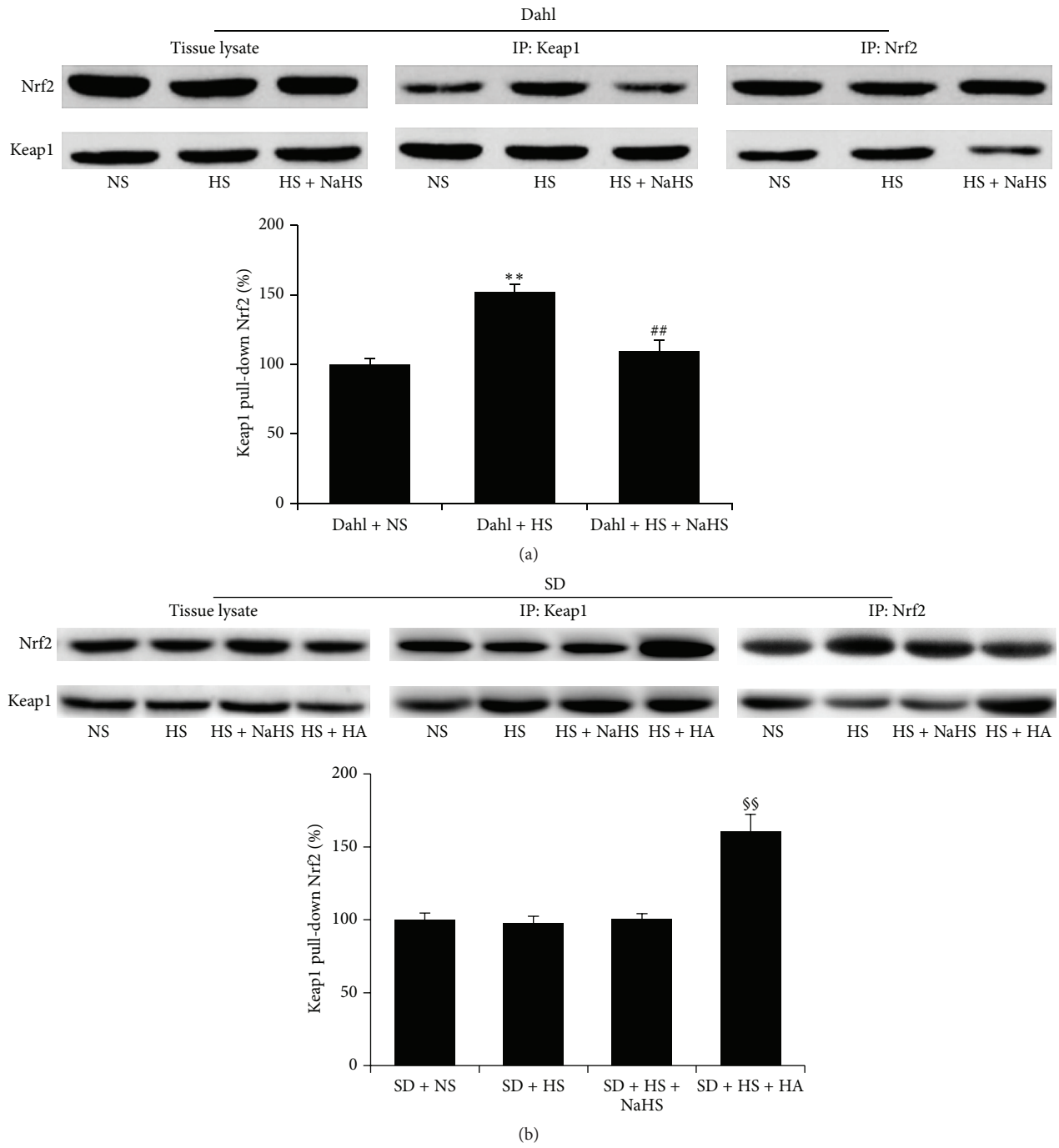

FIGURE 9: The effect of H_2S on the association of Keap1 with Nrf2 in renal tissues of Dahl rats and SD rats. (a) The association of Keap1 with Nrf2 in renal tissues of Dahl rats. (b) The association of Keap1 with Nrf2 in renal tissues of SD rats (mean ± SE, $n = 10$). $^{**}P < 0.01$ versus Dahl + NS; $^{\#\#}P < 0.05$ versus Dahl + HS; and $^{\S\S}P < 0.01$ versus SD + HS.

enhance the Nrf2 nuclear translocation [27, 46]. While our results showed that chronic supplement H_2S donor enhanced Keap1 s-sulfhydration in the renal tissues of Dahl + HS rats, CBS inhibitor HA increased Keap1 s-sulfhydration in the renal tissues of SD + HS rats. However, further studies are needed to deepen the understanding of the mechanism by which H_2S plays an important role in the antioxidation.

5. Conclusions

Renal oxidative injury and collagen deposition were observed in Dahl rats with hypertension induced by high salt. H_2S upregulated the expression of SOD protein, improved antioxidant capacity, removed ROS, and thereby improved renal function and renal structural injury. s-Sulfhydration of

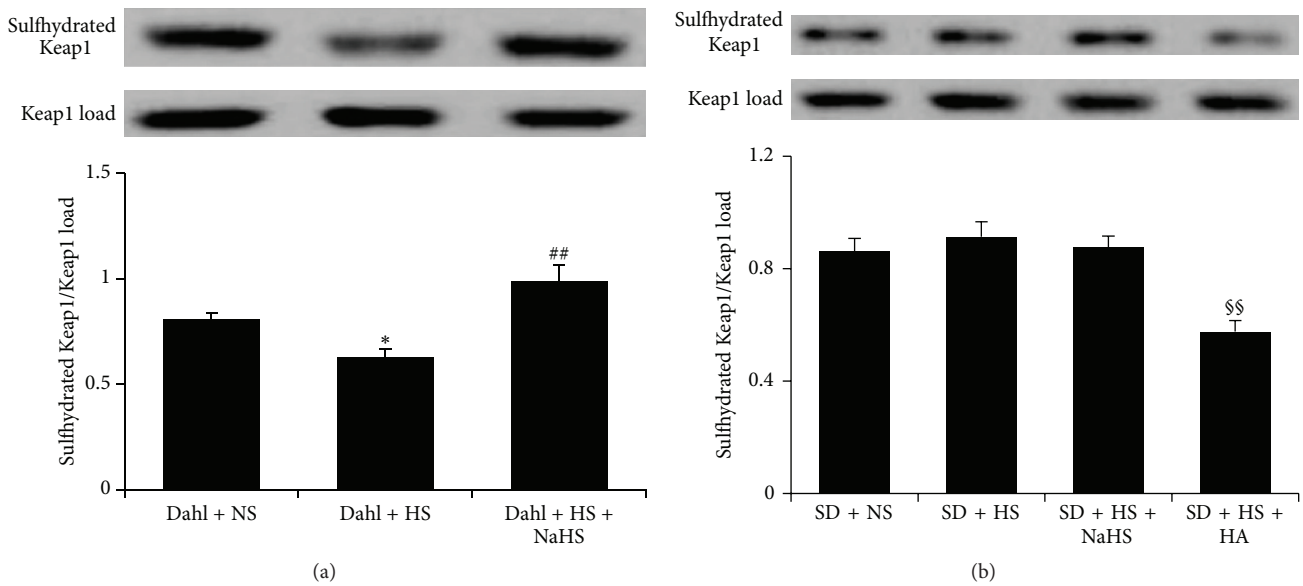

FIGURE 10: The effect of H_2S on Keap1 s-sulfhydration in renal tissues of Dahl rats and SD rats. (a) Keap1 s-sulfhydration in renal tissues of Dahl rats. (b) Keap1 s-sulfhydration in renal tissues of SD rats (mean ± SE, $n = 10$). $^{*}P < 0.05$ versus Dahl + NS; $^{\#\#}P < 0.01$ versus Dahl + HS; and $^{\S\S}P < 0.01$ versus SD + HS.

Keap1 and thereby dissociation of Keap1 with Nrf2 might be involved, the mechanisms by which H_2S enhanced the expression of antioxidant enzyme. In conclusion, H_2S could reduce the high-salt-induced renal injury in association with inhibiting renal oxidative stress in Dahl rats. Keap1/Nrf2 pathway might mediate the antioxidant role of H_2S.

Conflict of Interests

The authors report no conflict of interests with this study.

Acknowledgments

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Review Article

Interaction of Hydrogen Sulfide with Nitric Oxide in the Cardiovascular System

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Historically acknowledged as toxic gases, hydrogen sulfide (H_2S) and nitric oxide (NO) are now recognized as the predominant members of a new family of signaling molecules, "gasotransmitters" in mammals. While H_2S is biosynthesized by three constitutively expressed enzymes (CBS, CSE, and 3-MST) from L-cysteine and homocysteine, NO is generated endogenously from L-arginine by the action of various isoforms of NOS. Both gases have been transpired as the key and independent regulators of many physiological functions in mammalian cardiovascular, nervous, gastrointestinal, respiratory, and immune systems. The analogy between these two gasotransmitters is evident not only from their paracrine mode of signaling, but also from the identical and/or shared signaling transduction pathways. With the plethora of research in the pathophysiological role of gasotransmitters in various systems, the existence of interplay between these gases is being widely accepted. Chemical interaction between NO and H_2S may generate nitroxyl (HNO), which plays a specific effective role within the cardiovascular system. In this review article, we have attempted to provide current understanding of the individual and interactive roles of H_2S and NO signaling in mammalian cardiovascular system, focusing particularly on heart contractility, cardioprotection, vascular tone, angiogenesis, and oxidative stress.

1. Introduction

Endogenously produced hydrogen sulfide (H_2S) is responsible for inducing variety of physiologically favorable effects in different mammalian body systems. It is the youngest member of "gasotransmitter" family, along with nitric oxide (NO) and carbon monoxide (CO) [1]. Considered as toxic and potentially lethal gases for centuries, they are now recognized by many researchers as the important cytoprotective endogenous modulators of many physiological functions.

Although NO was identified as a gas in late eighteenth century, its role as a biological agent was confirmed only in 1980 [2]. Its generation from NO synthase (NOS) and its action as a vasodilator were discovered a few years later in 1987 [3]. NO is formed from guanidine nitrogen of L-arginine by the action of 3 isoforms of NOS, namely, endothelial (eNOS), inducible (iNOS), and neuronal (nNOS) [4]. The identification of H_2S as a toxic gas dates back even further than NO. The measurement of H_2S revealed its existence in the brain [5]. This suggests its probable physiological importance. The gradual discoveries of cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) as critical enzymes producing H_2S [6] shed more light upon its signaling pathways and widespread physiological functions.

Being gaseous molecules and mediators, H_2S and NO exhibit many common traits like the unique ability of free diffusion through cell membranes without the need of specific membrane receptors. Their endogenous enzymatic production is deftly regulated at many levels. Furthermore, they are also involved in modulation of many physiological processes in cardiovascular system (CVS) and central nervous system (CNS) [1]. While the individual signaling mechanisms mediated by H_2S and NO in mammals are extensively studied, our understanding about the potential relationship between these two gasotransmitters is woefully incomplete. In 2009, first few definitive experimental evidences began to emerge voicing the probable "crosstalk" between H_2S and NO [7]. Since then, it is now an established fact that

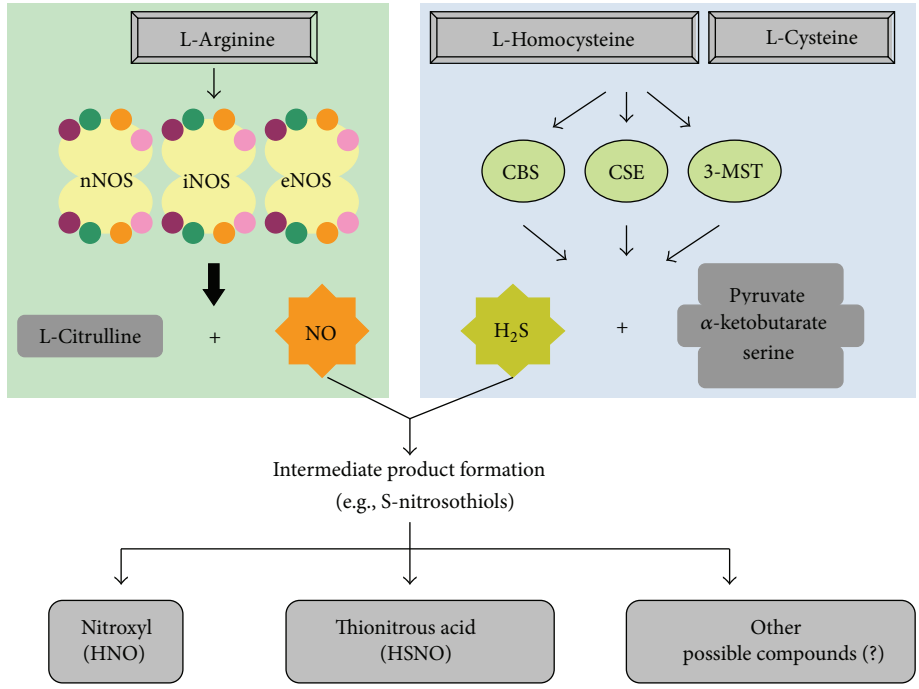

Figure 1: Biosynthesis of NO and H_2S. NO is synthesized by three different isoforms of NOS, namely, nNOS, eNOS, and iNOS. These isoforms are almost identical in structure and functions. A functional NOS is made up of two identical monomers, each with four cosubstrates (NADPH, FAD, FMN, and BH_4). L-Arginine is oxidized into L-citrulline along with the production of NO. H_2S is produced by the catalytic action of 3 different enzymes, CBS, CSE, and 3-MST. Each enzyme is expressed in organ-specific manner and catalyses the production of H_2S by oxidizing L-homocysteine and L-cysteine. Pyruvate, α-ketobutyrate and serine are produced as bi-products. The interaction between these two gases can give rise to production of few intermediate products (e.g., S-nitrosothiols). As explained in this review article later, nitroxyl (HNO), thionitrous acid (HSNO), and other possible unknown compounds have been shown to induce some interesting effect in cardiovascular system.

these two gases influence each other at many levels from their biosynthesis to the various biological responses within cellular targets [8, 9]. The therapeutic potential of these gases is immense and thus being explored via many preclinical and clinical studies [10]. In this review article, we will focus on physiological and cellular functions mediated by H_2S and NO in mammalian cardiovascular system. A prevailing understanding of the known and complex interplay between these gases and their signaling mechanisms would also be provided in the respective sections of the paper.

2. Synthesis and Metabolism of H_2S in Mammalian Cells

A major contribution in the endogenous production of H_2S is offered by two pyridoxal-5′-phosphate- (PLP-) dependent enzymes, namely, CSE and CBS. They utilize L-cysteine or homocysteine as substrates [11]. Recently, however, a study reported that 3-mercaptopyruvate sulfurtransferase (3-MST) acts together with cysteine aminotransferase (CAT) to generate H_2S in the brain (Figure 1). They also suggested that 3-MST and CAT are primarily involved in the neuronal production of H_2S [12]. While CBS and CSE are mainly cytosolic, 3-MST is preferably expressed in mitochondria [13]. Furthermore, their distribution is highly tissue specific. CBS is primarily detected in neurons and astrocytes of CNS [14], whereas CSE is located in the CVS, especially the myocardial cells [15] and vascular smooth muscle cells [16]. The localization of CSE in endothelial cells (EC) is a bit controversial. A few research groups have detected its expression in the ECs [17, 18] while others have not reported as such [19, 20].

H_2S production by CBS involves the condensation reaction between homocysteine with L-cysteine to produce cystathionine and H_2S [21]. CSE catalyzes the conversion reaction of L-cysteine into thiocysteine and pyruvate. The thiocysteine thus generated is lysed to form cysteine and H_2S [22]. CAT, on the other hand, catalyzes the synthesis of 3-mercaptopyruvate from L-cysteine and α-ketoglutarate. 3-MST then desulfurates 3-mercaptopyruvate to generate thiosulfate. Later, H_2S is generated by reduction of thiosulfate [23]. Recently, Shibuya et al. identified a novel pathway of H_2S production specifically in kidney and the cerebellum region of the brain. H_2S can be generated from D-cysteine by activation of 3-MST and D-amino acid oxidase [24]. The intracellular storage forms of H_2S have also been identified. Acid-labile sulfur is mainly located in the iron-sulfur cluster of mitochondria. Measured in the form of sulfide, acid-labile sulfur has been detected in brains of rats, bovines, and humans. It releases H_2S only in acidic microenvironment (pH = 5.4) [19]. Due to the highly unstable nature of iron-sulfur clusters, H_2S is readily released when needed [20]. Bound sulfane sulfur,

which is presented in cytosolic region, contains divalent sulfur bond (e.g., persulfide form). It releases H_2S under basic conditions (pH 8.4) [20]. It is speculated that H_2S produced by 3-MST/CAT enzymatic pathway is stored in the bound sulfane sulfur form as lesser amount of bound sulfane sulfur has been detected in cells without 3-MST/CAT [15].

Under the physiological conditions, H_2S is quickly eliminated by various routes. Mitochondrial oxidation of deprotonated HS^- results into thiosulphate, which is further converted into sulfite and eventually sulfate. Sulfate production is the primary fate of H_2S metabolism [25]. H_2S also undergoes cytosolic methylation by thiol S-methyltransferase to produce dimethylsulfide and methanethiol. H_2S has high affinity towards hemoglobin. Thus, H_2S binds to hemoglobin producing sulfhemoglobin [19].

3. Synthesis and Metabolism of NO in Mammalian Cells

Three different isoforms of the enzyme NOS produce NO in mammals. They are commonly known as neuronal nNOS (NOS I), inducible iNOS (NOS II), and endothelial eNOS (NOS III). Although genetically distinct, all three isoforms form NO from L-arginine with the help of two cosubstrates, namely, molecular O_2 and nicotinamide-adenine-dinucleotide phosphate (NADPH). The biosynthesis also requires various cofactors like flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and tetrahydrobiopterin (BH_4) [4]. In a typical biosynthesis reaction, catalytically active NOS transfers electrons from NADPH to the heme, via FAD and FMS [26]. Calmodulin is bound to the reductase domains of monomers and it helps in facilitating the transfer of electrons [27]. These transferred electrons facilitate binding of molecular O_2 to the ferrous form by reducing iron in the heme. The ferrous form is then combined to L-arginine to synthesize L-citrulline and NO [27, 28] (Figure 1). NO, thus generated, activates number of downstream secondary signaling pathways like soluble guanylyl cyclase activation which results into cGMP formation [29].

Besides CNS, nNOS is also identified in autonomic nerves of smooth muscles in blood vessels, gastrointestinal tract, respiratory tract, and genitourinary tract [30]. The expression of iNOS is identified in many immunological cell types such as macrophages [31] and neutrophils [32]. The third form, eNOS, is mainly expressed in endothelial cells but also present in other cell types such as cardiomyocytes, hepatocytes, intestinal cells, platelets, neurons, and astrocytes [33].

nNOS is primarily activated by glutamate acting on NMDA receptors. The enzyme activity is regulated by glutamate-induced rise in intracellular calcium ($[Ca^{2+}]_i$) level and its interaction with calmodulin. Unlike nNOS, iNOS is neither affected by $[Ca^{2+}]_i$ levels nor dependent on the presence of cosubstrate NADPH and cofactor BH_4 [34]. Its activity is stimulated by exposure to pathological insults, especially bacterial endotoxins and proinflammatory cytokines such as TNF-α and interleukins. The activation of eNOS is triggered by increased $[Ca^{2+}]_i$, which in turn is elevated by phosphoinositide secondary signaling pathway. Similar to nNOS, eNOS activity is Ca^{2+} dependent and is regulated by calmodulin [30].

The nitrite (NO_2^-) and nitrate (NO_3^-), collectively known as NO_x, are the end products of endogenous NO metabolism in the mammalian cells [35]. They are also recycled physiologically to generate NO and other nitrogen oxides [36]. Recently, they have been acknowledged as "storage pools" of NO in mammalian tissues, complementing the NOS-dependent pathway of NO biosynthesis [35]. In another proposed mechanism of NO storage, reduced glutathione (GSH) is nitrosylised to generate S-nitroso-L-glutathione (GSNO) [37]. NO stored in the form of GSNO can be released by the action of many enzymes such as GSH peroxidase [38] and thioredoxin reductase [39].

Physiologically, endogenously generated NO is rapidly metabolized. It diffuses through lumen of blood vessels and intracellular compartments to react with hemoglobin. This elimination pathway leading to the formation of nitrates is considered as the major mechanism of NO catabolism [40]. In another mechanism, NO is oxidized in blood forming nitrites which react with hemoglobin producing nitrates [37]. As discussed before in this paper, excessive production of NO can be detrimental to the cells. This excessive NO reacts with bicarbonate to produce nitrosoperoxycarbonate ($ONOOCO_2^-$) and thus scavenged from the body [41].

4. Biochemistry of NO-H_2S Interaction

The biological and chemical reactivities of H_2S have been discussed thoroughly in some excellent review papers previously [42, 43]. After dissolving in water, H_2S dissociates in H^+, HS^-, and S^{2-}. The anionic form HS^- contributes to the major share while S^{2-} exists in a very small amount at the physiological pH [44]. The H_2S/HS^- form is a strong reducing agent, which is capable of reducing many organic substrates including NO and its oxidized forms. H_2S can form chemical complexes with nitrate, nitrite, S-nitrosothiols, and peroxinitrates [45]. In a previous work, Whiteman et al. demonstrated that mixture of various NO donors and NaHS (H_2S donor) forms a novel species known as nitrosothiols [46]. The study revealed that the addition of H_2S to various NO donors not only inhibits the release of NO, but also alters the expected NO-based biological function. Their mechanism of formation is elusive but the direct reaction between H_2S and NO can be ruled out as H_2S/HS^- exists in diamagnetic acid/base pair while NO exhibits paramagnetic nature at the physiological pH. The aerobic conditions maintained during these experiments might be responsible for NO oxidation leading to the formation of nitrosating species.

H_2S can reduce oxidized NO forms leading to the formation of HSNO as an intermediate. Further reduction and direct displacement of HSNO by H_2S results in the formation of yet another intermediate product, nitroxyl (HNO) [47] (Figure 2). HNO produces chemical and physiological functions different from NO [48] and H_2S [9, 49]. HNO is highly redox-sensitive and therefore regulates protein functions through "redox switches" [50, 51]. HNO can react with thiol groups in the cysteine residues to form

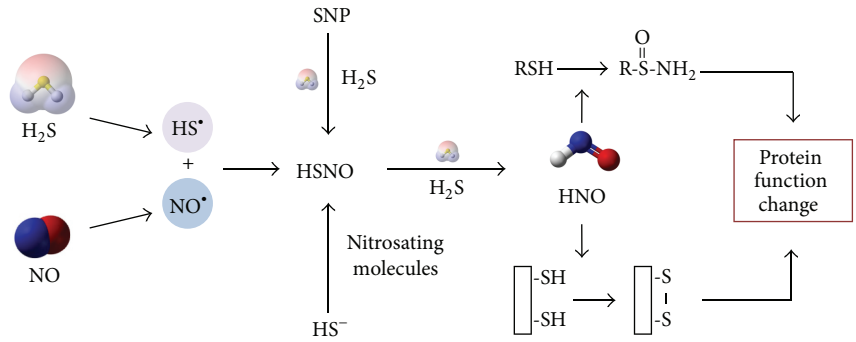

Figure 2: Simplifying depiction of chemical interaction between H_2S and NO. The radical of H_2S ($HS^{\bullet}$) reacts with that of NO ($NO^{\bullet}$) to generate thionitrous acid (HSNO). In an alternate way, HSNO can be produced by hydrosulfide ion (HS^-), an anionic form of H_2S, after its reaction with different nitrosating biomolecules. Sodium nitroprusside can also produce HSNO by reacting with H_2S. In a further step of reaction, HSNO is acted upon by H_2S to produce HNO. HNO may modify the functions of proteins by converting their reactive thiols (thiolates) in cysteine residues to N-hydroxysulfenamide (RSNHOH) or forming disulfide bond between two thiol groups in the near vicinity.

N-hydroxysulfenamide (RSNHOH) [52] or helps to form a reversible disulfide bond if there are two thiols residing in the near vicinity [53]. These modifications may induce conformational change and therefore the functions of the targeted proteins (Figure 2). The pharmacological effects of HNO donors have already brought attention of many research groups towards their potential therapeutic value against many cardiac ailments such as congestive cardiac failure. There are several types of HNO releasing compounds. The most commonly used one is Angeli's salt (Na_2 N_2O_3). Other donors include Piloty's acid ($PhSO_2NHOH$) and its derivatives, isopropylamine-$NO^{\bullet}$ (IPA/NO), and acyloxy nitroso compounds such as 1-nitrosocyclohexyl acetate (NCA, also known as the "blue compound") [54]. In the upcoming sections of this paper, we have discussed the effects of HNO on various aspects of cardiovascular physiology.

5. H_2S-NO Interaction in Cardiovascular System

5.1. Role of H_2S-NO Interaction in the Regulation of Heart Contractility. NO (both exogenous and endogenous) has concentration-dependent bimodal action on basal contractile state of cardiomyocytes. At low concentrations, NO exerts positive inotropic action [55]. The low NO levels activate adenylyl cyclase (AC) and downstream cAMP dependent signaling pathway [56]. Thus activated protein kinase A (PKA) phosphorylates voltage-dependent calcium channels and opens sarcoplasmic ryanodine receptors (Ry/R) [57]. The resultant increase in $[Ca^{2+}]_i$ is mainly responsible for positive inotropic action. On the other hand, the negative inotropic effect by higher NO concentration is mediated chiefly through cGMP dependent pathway. The increased intracellular cGMP is further shown to downregulate myofilament calcium sensitivity increasing cardiac relaxation [58]. The cGMP regulator phosphodiesterase 5A (PDE5A) is shown to modulate cardiac β-adrenergic stimulation in an eNOS dependent manner [59]. It is interesting to know that the mechanisms of action of NO depend upon the origin of endogenous NO as well. nNOS-derived NO has been demonstrated to upregulate cardiac contractility by direct protein S-nitrosylation of Ry/R receptors [60].

NaHS also produces a negative inotropic effect on cardiomyocytes by suppression of opening of K_{ATP} channels [15, 61], blockade of L-type calcium channels [62], and suppression of cAMP/PKA pathway [63]. Accumulating evidences suggest that there is a cross-talk between H_2S and NO in the heart. H_2S may directly interact with NO during pathological situations like oxidative stress and alter cardiac functions. We were among the first groups to observe that H_2S reversed the negative inotropic and lusitropic effects of NO. Mixing NO donors (SNP, SIN-1, or SNAP) with NaHS produces an opposing effect on heart contractility as compared with either gas alone. To explain this phenomenon, we proposed the formation of new thiol sensitive molecule as they found that thiols abolished the effects of the mixture of NO and H_2S in their experimental setup [9, 49]. It is also possible that H_2S reacts with either oxidized forms of NO (e.g., $NO^{\bullet}$) or nitrogen species ($ONOO^-$) through HS^- in the presence of cellular oxidants for example, molecular oxygen, ROS (e.g., H_2O_2), and oxidases. This process may generate new molecules like nitrosothiol, thionitrous acid (HSNO), or HNO [64]. Due to the strong reducing capability of H_2S [1, 65, 66], Yong et al. proposed that HNO could be one of the possible candidates [49]. This hypothesis was further confirmed by another group who studied the production of intracellular HNO in cells treated with nitrite/H_2S reaction mixture with an HNO sensor (CuBOT1) [67]. The similar results were observed when sodium nitroprusside (SNP) was used as a NO donor [68, 69]. The interaction of H_2S with NO and the resultant synthesis of thiol-sensitive compounds may also provide the justification behind the elusive bimodal effect of NO on cardiac contractility as mentioned in the beginning of this section.

Although the mechanisms for the positive inotropic effect of HNO are still not well understood, it is now believed that it is mediated by a β-adrenoceptor independent pathway [70, 71]. Inhibition of cAMP/PKA and cGMP/PKG had no significant impact on its inotropic effect [72]. In fact, the redox dependent mechanism is important for the positive inotropic

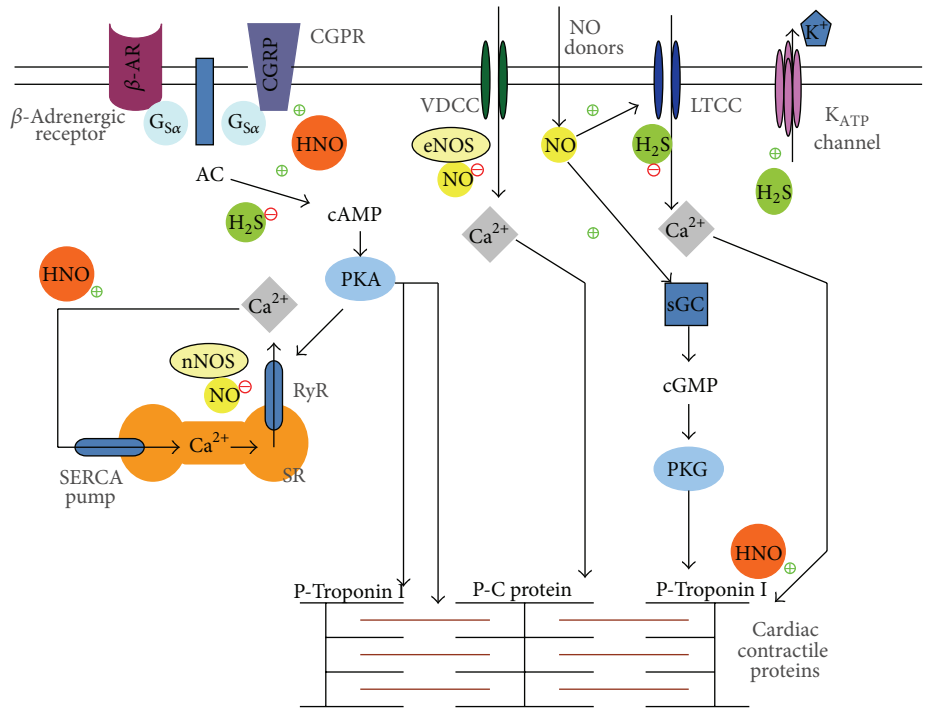

FIGURE 3: Effects of NO, H_2S, and HNO on heart contractile function. The negative inotropic effect of NO is mediated mainly by cGMP-PKG pathway in CVS. Exogenous NO is believed to act via direct phosphorylation of LTCC and cardiac contractile proteins such as troponin 1. The effect of endogenous NO depends on the source. eNOS-generated NO acts via cGMP dependent pathway. nNOS-generated NO S-nitrosylates ryanodine receptors of sarcoplasmic reticulum. H_2S also exerts negative effect on cardiac contractility via (1) opening of K_{ATP} channels, (2) blockade of LTCC, and (3) inhibition of cAMP signaling pathway. Interestingly, the intermediate product, nitroxyl (HNO), produces positive inotropic effect. The possible underlying mechanisms of action include stimulation of calcitonin gene-related peptide signaling and enhancing cardiac sarcoplasmic reticulum Ca^{2+} cycling.

effect of HNO. HNO can enhance the myofilament calcium sensitivity through formation of an actin–TM heterodimer. With mass spectrometry (MS) and a modified biotin switch assay, Gao et al. even found out the four cysteine residues in myofilament modified by HNO [8]. HNO can also modulate the thiol groups in EC-coupling proteins and regulate the functions of these proteins. For instance, HNO modulates SERCA2a/phospholamban (PLN) interaction and therefore stimulates SR function [57]. More experiments revealed PLN is important in the HNO inotropy/lusitropy, as mutation of the three cysteine residues in PLN transmembrane domain abolished the effect of HNO [73]. Tocchetti et al. showed that the effect of HNO was from a direct interaction of HNO with the sarcoplasmic reticulum Ca^{2+} pump and the ryanodine receptor 2, leading to increased Ca^{2+} uptake and release from the sarcoplasmic reticulum [72].

In addition, Paolocci et al. reported that the positive inotropic signaling was mediated by calcitonin gene-related peptide (CGRP), as treatment with the selective CGRP-receptor antagonist CGRP (8–37) prevented this effect [71]. However, this finding was later disproved as positive inotropic effects of CGRP were found to be mere sympathostimulatory in nature and downregulated by β-adrenoceptor blockers [74]. Nonetheless, the positive inotropic/lusitropic action of HNO render it to be an attractive addition to the current therapeutic armamentarium for treating patients with acutely decompensated congestive heart failure [75] (Figure 3).

5.2. Role of H_2S-NO Interaction in the Cardioprotection. Myocardial ischemia occurs when cardiac myocytes are insufficiently provided with the oxygenated blood via coronary arteries, resulting in cardiovascular morbidity and mortality [76]. Ischemic injury is a complex process involving the action and interaction of many factors. NO is one of these factors to protect heart against ischemic injury. The studies conducted in eNOS deficient ($eNOS^{-/-}$) mice [77] and eNOS overexpressing mice [78, 79] have concluded that eNOS-derived NO is a strong endogenous cardioprotective agent against cardiovascular pathologies including ischemia-reperfusion (I/R) injury and congestive cardiac failure. The administration of NO donors also has similar protective effects in I/R injury and other heart diseases in humans and other mammals [80–82]. The studies have revealed different possible underlying mechanisms including activation of sGC/cGMP/PKG signaling pathway [83], activation of subcellular K_{ATP} channels [84, 85], and Ca^{2+} influx inhibition [86].

Similarly, the cardioprotective effects of H_2S also involve multiple mechanisms (Figure 3). This was described in detail in our previous review article [64]. Downregulation of endogenous H_2S production was found to increase

myocardial infarct size, suggesting an important role of endogenous H_2S in maintaining the normal heart function [87]. In different animal models, H_2S was shown to protect heart against I/R injury via diverse mechanisms. Zhang et al. reported that H_2S stimulated opening of K_{ATP} channels in cardiomyocytes [88]. The contribution of antiapoptotic signaling activation was demonstrated by the modulation of proteins expression including Beclin-1 [89], Bcl-2, Bax, caspase 3 [90], and HSP-90 [91]. H_2S is also known to preserve mitochondrial functions by modulating cellular respiration [92]. We and other groups revealed that the cardioprotective effect of H_2S preconditioning involves the activation of PKC and sarcolemmal K_{ATP} channels, Akt, and eNOS pathways [93–96].

H_2S and NO may act in concert to protect the heart against ischemic injury. Inhibition of NO production with L-NAME, a nonselective inhibitor of NO synthases, significantly attenuated the cardioprotective effects of H_2S preconditioning [97]. Administration of NaHS alleviated isoproterenol-induced toxic cardiomyopathy through elevation of myocardial and serum NO levels [98]. H_2S may regulate NO production through modulation of eNOS and iNOS expression and activity. We showed previously that H_2S pretreatment activates eNOS pathway to confer protective effect against ischemic injury [93]. In an interesting study conducted in human umbilical vein endothelial cells (HUVECs-926), both eNOS phosphorylation and NO production were upregulated upon treatment with NaHS [99]. Moreover, malfunction of eNOS and reduced NO level were also found in CSE knockout mice. This contributes to the impaired heart function during I/R injury [100]. However, some conflicting effects were also reported. The data collected from rat and mouse aortic rings demonstrated that H_2S directly inhibited recombinant bovine eNOS activity [101]. In yet another study, both exogenous and endogenous H_2S inhibited eNOS transcription and activity [102]. Thus it is highly possible that the nature of effect of H_2S on eNOS is dependent on many factors including H_2S concentration and experimental setup.

Overexpression of iNOS and the subsequent excessive formation of NO may cause cytotoxic effects and exacerbate myocardial injury [103]. Inhibition of iNOS may produce beneficial effects in heart [104]. Apart from regulation of eNOS, H_2S also modulates iNOS expression. Hua et al. found that H_2S protected heart against CVB3-induced mice myocarditis through suppression of iNOS expression and the subsequent HO-1 pathway [105]. Taken together, NO is an important player in the cardioprotection induced by H_2S, despite different mechanisms that may be involved in various pathological situations.

In contrast to the intensive investigation on the effect of H_2S on NO generation, little is known about the effect of NO on H_2S production. A previous study showed that exogenous application of an NO donor, sodium nitroprusside, and upregulated the expression of CBS and CSE, culminating in augmented H_2S production in rat tissues [106]. These data suggest that H_2S and NO may influence the production of each other by altering their generating abilities during ischemic situations.

However, the role of HNO, the direct interaction product from these two gases, in ischemic reperfusion injury is still debated. Preconditioning with HNO also grants a protection similar to that afforded by classical ischemic preconditioning [107]. This protective effect was not from NO, as it cannot be achieved with equimolar amounts of the NO donors. The mechanisms underlying HNO-induced cardioprotection may involve mitochondrial K_{ATP} channel (mK_{ATP}) [108] (Figure 4). However, it is also worth noting that higher concentration perfusion of HNO may also produce detrimental effects during ischemic reperfusion caused by recruitment of neutrophils [109].

5.3. Role of H_2S-NO Interaction in the Maintenance of Vascular Tone. The identification of NO as an endothelium derived relaxing factor [3] is a milestone in the field of gasotransmitters biology research. NO is now established as an important regulator of vascular tone. Physiologically, NO is a powerful vasodilator exerting its effect on various arteries, resistance vessels, and veins. The underlying signaling pathway is mainly cGMP dependent [110]. NO can also mediate vasodilation in a cGMP independent manner [111, 112]. S-Nitrosohemoglobin formed by S-nitrosylation of Cys93 of the hemoglobin β subunit has been demonstrated to moderate hypoxic vasodilation [113, 114].

H_2S has a biphasic effect on vascular tone in the cardiovascular system by mediating both vasorelaxation and vasoconstriction (Figure 5). Exogenously applied H_2S in higher concentrations (NaHS $> 100\,\mu$M) relaxes vascular smooth muscles. It is suggested that the vasodilatory effect of endogenous H_2S is mainly responsible for the maintenance of basal tone in vasculature which in turn controls physiological blood pressure [115]. H_2S targets K_{ATP} channels to produce its vasodilatory effect [16, 115]. Additional mechanisms such as involvement of the Ca^{2+} channels [116], Cl^-/HCO_3^- exchanger [117], and metabolic inhibition [118] are required for the vasorelaxant effects of H_2S. Interestingly, Ali et al. demonstrated the reversal of relaxant effect of endothelium/NO-dependent vasodilators (ACh and Histamine) by the treatment of H_2S in lower concentration (NaHS $< 100\,\mu$M) [119]. This finding is in accordance with the previous results, where NaHS at concentration of $30\,\mu$M induced a strong vasoconstrictive effect by itself. The mechanisms underlying the vasoconstrictive effects of low concentration of H_2S involve downregulation of endothelial NOS, decrease of intracellular cAMP level in smooth muscle cells, and production of ROS. This was discussed in details in our previous review [64].

Various experimental studies provided evidence for the interaction between H_2S and NO and the vasoregulatory role of this interaction. The first report of summation effect between H_2S and NO on vasorelaxation came from the findings of Hosoki et al. which demonstrated that H_2S can induce stronger relaxation effect in the presence of a NO donor [120]. Furthermore, pharmacological blockade of endogenous NO production or physical removal of the endothelium, attenuated H_2S-induced relaxation [16]. These data suggest that the vasorelaxant effect of H_2S is mediated

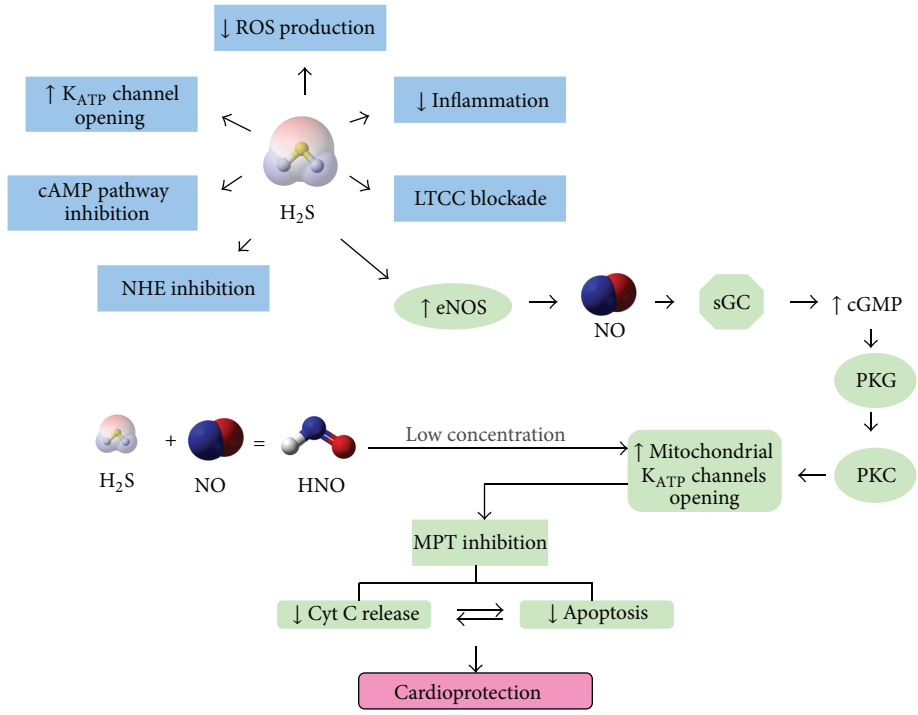

Figure 4: Cardioprotective effects of NO, H_2S, and HNO. There are multiple underlying mechanisms for cardioprotective effect of H_2S. It has been demonstrated to induce K_{ATP} channel opening, abolish inflammation and oxidative stress by inhibiting ROS production, block LTCC and intracellular cAMP signaling pathway, and inhibit Na^+/H^+ exchanger activity. Furthermore, NO also plays an important role in the cardioprotective effect of H_2S. Endogenous NO generated from eNOS in endothelial cells initiates the sGC-cGMP-PKG cascade to increase openings of subcellular (mitochondrial) K_{ATP} channels. The resultant inhibition of mitochondrial permeability transition (MPT) is responsible for decreased cytochrome C release and apoptosis in cardiomyocytes. HNO, depending on the concentration, can be either cardioprotective or cardiotoxic. At low concentration, just like NO, HNO also stimulates K_{ATP} channels opening in mitochondria.

by NO. The interplay between these two gases is different for the observed effect of vasoconstriction. Zhao and Wang found that H_2S inhibited SNP-induced vasorelaxation [116]. In line with this finding, Ali et al. found that a mixture of NO and H_2S reduced the extent of vasorelaxation compared to the relaxation with NO alone, implying the regulation of availability of NO by H_2S. Interestingly, H_2S only induced vasoconstriction in endothelium-intact vessels but not in endothelium-denuded vessels.

The contractile effect of H_2S is therefore not a direct action on vascular smooth muscle cells but an indirect effect involving endothelial cells. Furthermore, they demonstrated that NaHS, in a dose-dependent manner, significantly down-regulated vasorelaxant effect induced by chemically different NO donor molecules (e.g., SNP, SNAP). Similarly, NaHS reversed vasorelaxation induced by endogenous NO (from vascular endothelial cells) in a concentration dependent manner. This indicates that H_2S may induce vasoconstriction via direct quenching of NO. Interestingly, this group also hypothesized the formation of a new compound, nitrosothiol. Since copper sulfate, which converts nitrosothiol to nitrite and nitrates, prevented the contractile of aortic rings without influencing the vasorelaxant effect of NaHS, the generation of nitrosothiols was proved. This nitrosothiol molecule might have contributed to the modulatory effect of H_2S on vascular tone [119]. Similarly, we found that H_2S may also stimulate anion exchanger-2 activity which transports HCO_3^- in exchange of O_2^- to inactivate NO and thus inducing stronger vasoconstriction. In extracellular space, O_2^- reacts with NO to form $ONOO^-$ [121]. Since NO uptake by SMC is positively dependent on the level of intracellular O_2^- in SMC [122], the depletion of intracellular O_2^- may further inhibit NO uptake in SMC. These findings indicate that H_2S may induce vasoconstriction via inactivation of NO.

Recently, Berenyiova et al. found that of the interaction of sodium sulfide (Na_2S) and S-nitrosoglutathione (GSNO) relaxed precontracted isolated rings of rat thoracic aorta and mesenteric artery with a much stronger potency than any of these two chemicals alone. They claimed that the formation of nitroxyl (HNO) is responsible for the pronounced relaxation induced by the sulfide/GSNO cross-talk [123].

HNO is produced endogenously in vascular tissue [124–126]. It induces vasodilatory effect via multiple mechanisms. Previous reports showed that HNO may dilate vascular vessels as an endothelium-derived relaxing and hyperpolarizing factor [127, 128], via activation of a cGMP-dependent pathway [129] and via activation of TRPA1 receptor channels of trigeminal fibres inducing CGRP release [130]. Interestingly, not like NO, HNO does not develop tolerance in human blood vessels [129].

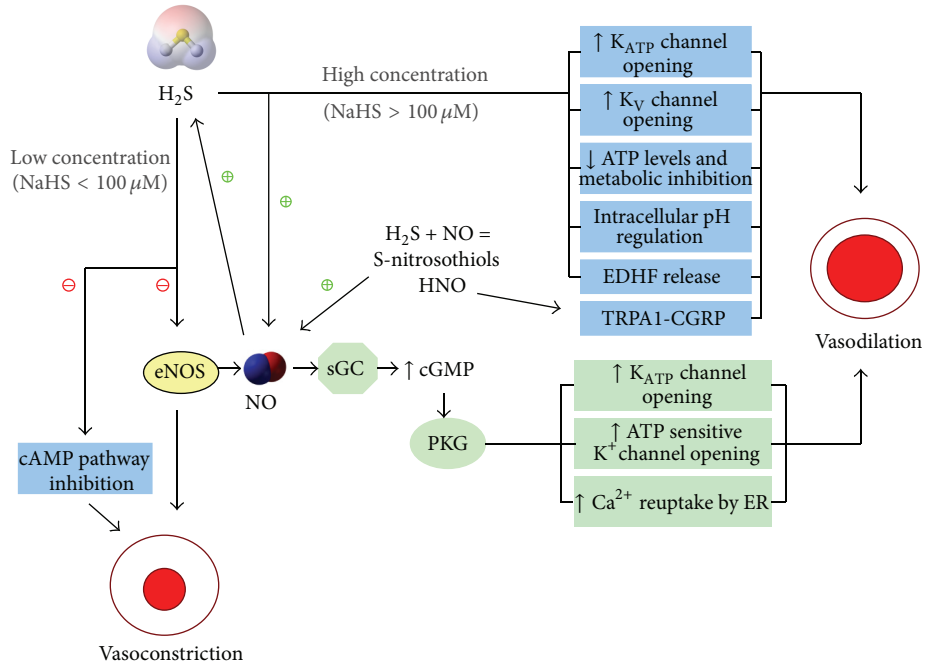

Figure 5: Regulatory effect of NO, H_2S, and S-nitrosothiols on vascular tone. Depending upon the concentration, H_2S exerts biphasic response on vascular tone. At high concentration (NaHS > 100 μM), H_2S acts as a potent vasodilator by opening of K_{ATP} and K_V channels, downregulating ATP levels and cellular metabolism, regulating intracellular pH and release of endothelium-derived hyperpolarizing factor (EDHF). At low concentration (NaHS < 100 μM), however, H_2S acts as a vasoconstrictor by inhibiting eNOS-derived NO production and intracellular cAMP pathway. NO, by itself, is a strong vasodilator. Acting via cGMP-PKG pathway, it stimulates opening of K_{ATP} and ATP-sensitive potassium channels. It also increases Ca^{2+} reuptake by endoplasmic reticulum and thus decreases intracellular Ca^{2+} levels resulting in less contraction. NO signaling is also stimulated by high concentration of H_2S, contributing to its vasodilatory effect. S-nitrothiols, derived from interplay between NO and H_2S, act as vasodilators mainly via NO signaling.

In addition to the direct interaction, H_2S and NO are also known to affect mutual production. NO can increase H_2S production in the normal vascular tissues. Incubation with NO donors increased H_2S production rate in the rat vascular tissues [16, 106]. In pulmonary hypertension, higher H_2S production and upregulated CSE level were found in the presence of L-arginine [131]. On the other hand, H_2S may downregulate the aortic L-arginine/NO pathway [101, 102, 121]. H_2S inhibited recombinant eNOS activity and thus reduced NO synthesis in the endothelium [101]. In aortic tissues, Geng et al. also reported that H_2S suppressed NO production by inhibition of eNOS transcription, abundance. and activity [102]. Coletta et al. determined the cooperative effect of H_2S and NO by silencing CSE. It attenuated the NO donor induced cGMP accumulation and vasodilator-stimulated phosphoprotein (VASP) [132]. In a recent study, Eberhardt et al. showed that HNO formed from H_2S and NO activated transient receptor potential channel A1 (TRPA1). The sensory chemoreceptor channel TRPA1 was activated via formation of amino-terminal disulphide bonds, which resulted in sustained Ca^{2+} influx. Consequently, calcitonin gene-related peptide (CGRP) was released inducing potent local and systemic vasodilation [133]. Thus it can be proposed that the H_2S and NO homeostasis is of the prime importance in maintaining vascular tone.

Short term application of exogenous H_2S reduced NO formation in cultured human umbilical vein endothelial cells through suppression of protein expression of eNOS but not those of nNOS and iNOS [102]. However, Huang et al. found that treatment with NaHS or H_2S releasing donor, ACS14, for 24 h attenuated the increase in iNOS expression caused by high glucose (25 mM). This is similar to the inhibitory effect of H_2S on iNOS expression in heart [134]. These data suggest that H_2S may regulate iNOS expression in a time-dependent manner.

5.4. Role of H_2S-NO Interaction in Angiogenesis. The formation of new blood vessels from preexisting vasculature through process of angiogenesis is the means by which cells can meet an elevated need of metabolites and in pathological conditions such as ischemia. Endothelial cells (ECs) play a pivotal role in the process by migrating towards and proliferating at the site of angiogenesis [135, 136].

Accumulating evidences suggest that gasotransmitters NO and H_2S are important factors to influence ECs and angiogenesis [8]. The relationship between NO and neovascularization is very well established [137] and found to involve cGMP transduction pathway [8]. Many angiogenic growth factors such as VEGF and basic fibroblast growth factor enhance eNOS expression and stimulate its activity to

produce NO [138]. Cai et al. observed that NaHS stimulated the *in vitro* parameters of angiogenesis such as cell growth, migration, scratched wound healing, and tube-like structure formation in cultured RF/6A endothelial cells [139]. It was speculated that H_2S exerts its effects on ECs through K_{ATP} channels that in turn facilitate activation of MAPK pathways, leading to new blood vessel formation [140].

The signaling mechanisms of H_2S and NO are not mutually exclusive for angiogenesis. In an exhaustive study conducted by Coletta et al., PKG was concluded to be a converging point for the secondary signaling mechanisms of H_2S and NO [132]. In accordance with the previous results [141], this group found that the exogenous application of H_2S decreased cGMP degradation by inhibiting PDE5A. This effect on intracellular cGMP is aided and abetted by NO which activated sGC to stimulate the production of intracellular cGMP. As mentioned previously, H_2S stimulates Akt to induce its angiogenic effect. The stimulation of Akt in turn induces eNOS phosphorylation [142]. This particular response suggests that H_2S influences eNOS activity. Very few studies have addressed the role of HNO in angiogenesis. The first strong indication for the probable antiangiogenic role of HNO came from the studies conducted in animal models of neointimal hyperplasia. It was observed that inhibition of EC proliferation was partly responsible for inhibitory effects of IPA/NO on neointimal hyperplasia. It should be noted that either IPA/NO itself or products of IPA/NO decomposition could have caused these effects [143]. While working on *in vitro* and *in vivo* models of breast cancer, Norris et al. found that HNO treatment not only reduced blood vessel density but also downregulated angiogenesis. They observed lower levels of circulating serum VEGF and HIF-1α, both of which are potent proangiogenic factors [144].

6. Role of H_2S-NO Interaction in Oxidative Stress in CVS

Obesity, hypertension, and aging are few distinct causative factor for cardiovascular diseases. They are accompanied by oxidative stress, which is the result of imbalance between ROS generating and ROS-scavenging systems [145–147]. It is now a well-established fact that ROS generation is ramped up in heart [134] and blood vessels [135] during cardiovascular pathologies. Oxidative stress is a result of excessive production of ROS like O_2^-, $^{\bullet}HO$, H_2O_2, NO, $ONOO^-$, and HClO, mainly as a byproducts of cellular aerobic metabolism. The action of certain enzymes like NADPH oxidase and NOS is of also crucial [137]. NADPH oxidase activity and mitochondrial electron transport chain are mainly responsible for ROS production in aging heart [138] and vasculature [139]. Increased ROS generation has many harmful consequences like stimulation of inflammatory response, apoptosis, and ER stress culminating into cellular damage [140].

H_2S is a well-known antioxidant [141] and it has been shown to protect vascular endothelial function under conditions of acute oxidative stress by directly scavenging O_2^- and downregulating vascular NADPH oxidase-derived O_2^- production [142]. It has been reported that NO downregulates NADPH oxidase-dependent superoxide production in human endothelial cells by S-nitrosylation of p47phox subunit [148]. The chemical properties of HNO suggest that it can act as a potent antioxidant [149] as well. The low dissociation energy of H-NO bond [150] makes HNO a strong reducing agent. Thus, HNO is speculated to quench reactive intermediate products produced during radical oxidation processes like lipid peroxidation [149]. It should also be noted that oxidation of HNO leads to production of NO, which itself is an antioxidant in nature [151]. Furthermore, HNO is demonstrated to have an effect on cGMP-dependent signaling pathway, which incidentally is a potent ROS-suppressing mechanism in the heart. The results of a study conducted by Lin et al. show that HNO suppresses NADPH oxidase by upregulating sGC and cGMP signaling in neonatal rat cardiomyocytes [152]. Interestingly, Miller et al. latest work revealed the sGC-cGMP-independent mechanism of action of HNO. They observed that HNO donors directly inhibited the activity of NADPH oxidase (vascular Nox2) in mouse cerebral arteries. They also proposed that HNO modifies reactive cysteine thiols in the subunits of vascular Nox2, thus reducing its activity [153]. HNO is also known to potentiate heme oxygenase-1 mRNA and protein expression leading to a significant elevation in its antioxidant and cytoprotective activities. It should also be noted that HNO, by downregulating O_2^- production, can increase the bioavailability of NO in oxidative stress. Impaired NO bioavailability is one of the most deleterious effect of aging on vascular well-being. Thus, HNO helps in maintaining proper functioning of CVS.

Both H_2S and NO have been shown to exhibit beneficial effect against oxidative stress in many biological systems including CVS. In last few years, the role of intermediate products released during the interaction between H_2S and NO has also been studied in oxidative stress pathology (Figure 6). Now it is generally agreed that HNO has significant potential to function as an antioxidant and hence further investigation is necessary to explore its prospective therapeutic benefits.

6.1. Perspectives. In recent few years, a few research groups have demonstrated the formation of novel intermediate species during the reaction between H_2S and NO. In the initial work, the mixture of various NO donors and H_2S generated an intermediate formation with general properties similar to an S-nitrosothiol. Later, HSNO (thionitrous acid) was considered as the most likely S-nitrosothiol candidate [154]. Shortly after that discovery, a few of reports suggested HNO generation from the reaction between NO and H_2S donors [49, 75]. The endogenous production of HNO is also speculated, and lots of efforts have been put in developing reliable HNO detection methods in order to understand endogenous HNO generation. Several approaches including electrochemical analysis [155], high-performance liquid chromatography [156], and mass spectrometry [157] have been used to detect HNO in various biological samples. However, these methods either lacked sensitivity or specificity towards endogenously generated HNO. Hence, novel HNO

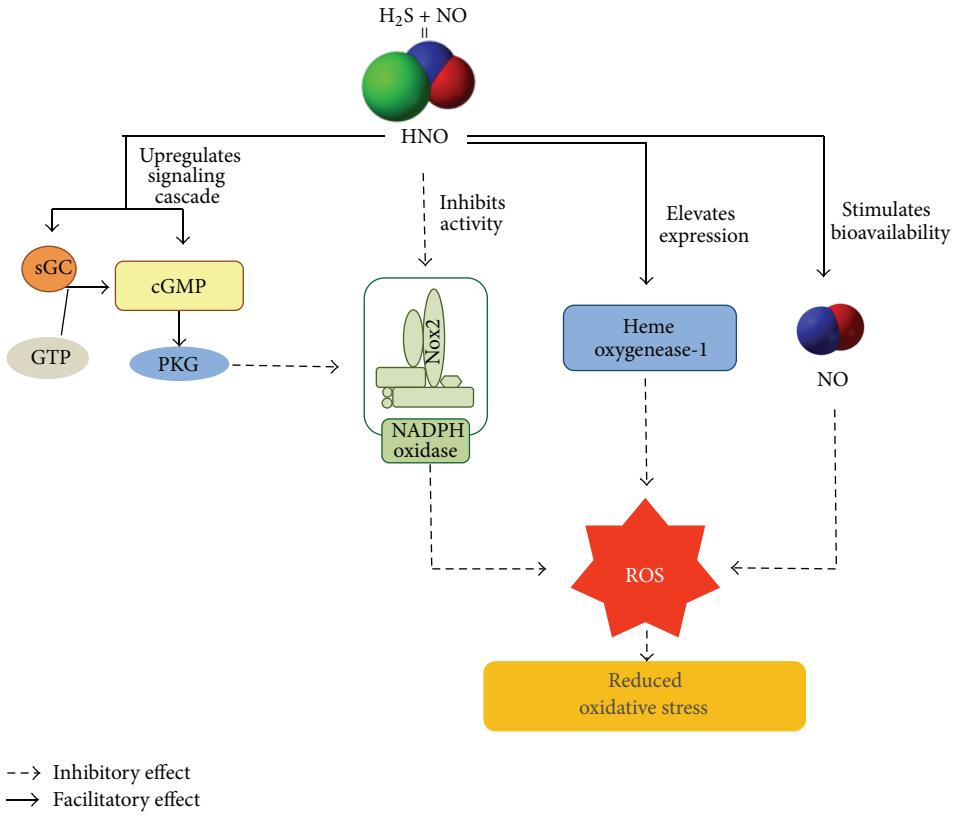

Figure 6: Role of H_2S-NO interaction in oxidative stress in CVS. The primary target of action of HNO is NADPH oxidase, which is the main culprit enzyme for endogenous synthesis of ROS in aging CVS. HNO can inhibit its activity in both sGC-cGMP dependent and independent ways. HNO is also known to strengthen anti-inflammatory response by elevating heme oxygenase-1 expression. HNO increases the diminished bioavailability of NO in oxidative stress, resulting in proper functioning of heart and blood vessels.

detection approaches including Cu^{2+}-medicated fluorescent probes and HNO-specific electrodes were adopted [155, 158]. Recently, a novel coumarin-based fluorescent probe, P-CM, was developed. It has been applied for selective quantitative detection of HNO in bovine serum samples [159]. Jing et al. successfully detected lysosomal HNO in cell system (RAW 264.7 macrophage cells) and *in vivo* using a newly synthesized a near-infrared fluorescent probe Lyso-JN [160].

It is speculated that HNO is produced endogenously in low concentration and perhaps insufficient to elicit any significant physiological effect [49]. The mechanisms proposed are still under investigation and include NO reduction [161], reaction of S-nitrosothiol with thiols [162], and reaction catalyzed by NOS. HNO is biochemically different from NO and H_2S and is known to show increased cardiac tissue contractility [163]. Furthermore, studies have implied that endogenous HNO production is elevated during pathological situations such as inflammation [7, 164]. Thus HNO and its derivatives are rapidly drawing attention as a potential pharmacological target in treatment of congestive cardiac failure [165] and acute heart failure [166].

However even with the favorable results, there are some concerns raised about the physiological relevance of these studies. For one thing, all these studies have utilized the supraphysiological concentrations of NO and H_2S. Furthermore, the use of exogenously added NO donors, instead of stimulating endogenous source of NO, are also debatable. For example, SNP does not release NO spontaneously and thus fails to mimic endogenous production and response of NO [167]. The known reactivity of SNP and H_2S further complicates the matter as it might generate products different from those generated in biological reaction between H_2S and NO [47, 49, 154].

Although additional investigations are warranted to establish the exact mechanisms of action of H_2S and NO, the importance and necessity of these two functional molecules in regulation of mammalian cardiovascular system are beyond debate. Our knowledge on these two gasotransmitters is expanding continuously and it is now evident that interaction between their molecular pathways is increasingly investigated as the future direction for the research in the area of gasotransmitters.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Vasorelaxant Effect of a New Hydrogen Sulfide-Nitric Oxide Conjugated Donor in Isolated Rat Aortic Rings through cGMP Pathway

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Endothelium-dependent vasorelaxant injury leads to a lot of cardiovascular diseases. Both hydrogen sulfide (H_2S) and nitric oxide (NO) are gasotransmitters, which play a critical role in regulating vascular tone. However, the interaction between H_2S and NO in vasorelaxation is still unclear. ZYZ-803 was a novel H_2S and NO conjugated donor developed by H_2S-releasing moiety (*S*-propyl-L-cysteine (SPRC)) and NO-releasing moiety (furoxan). ZYZ-803 could time- and dose-dependently relax the sustained contraction induced by PE in rat aortic rings, with potencies of 1.5- to 100-fold greater than that of furoxan and SPRC. Inhibition of the generations of H_2S and NO with respective inhibitors abolished the vasorelaxant effect of ZYZ-803. ZYZ-803 increased cGMP level and the activity of vasodilator stimulated phosphoprotein (VASP) in aortic rings, and those effects could be suppressed by the inhibitory generation of H_2S and NO. Both the inhibitor of protein kinase G (KT5823) and the inhibitor of K_{ATP} channel (glibenclamide) suppressed the vasorelaxant effect of ZYZ-803. Our results demonstrated that H_2S and NO generation from ZYZ-803 cooperatively regulated vascular tone through cGMP pathway, which indicated that ZYZ-803 had therapeutic potential in cardiovascular diseases.

1. Introduction

Due to the important role in cellular signal transduction, hydrogen sulfide (H_2S) and nitric oxide (NO) are identified as gaseous transmitters. In vascular tissue, NO is synthesized from L-arginine by nitric oxide synthase (NOS) and it is considered to be the endothelium-derived relaxing factor. Evidence showed that in hypertensive patients the NO generation in endothelium cells was damaged [1]. Moreover, NO could prevent platelet activation and promote vascular smooth muscle cells proliferation [2]. H_2S has been considered to be toxic gas for decades. But increasing study indicated that H_2S could be protective against apoptosis and oxidative stress in cardiomyocytes [3]. H_2S is synthesized from L-cysteine by cystathionine-γ-lyase (CSE) in vascular tissue [4]. Early study showed that H_2S was a vasoactive factor and it could relax rat aorta through K_{ATP} channels in vascular smooth muscle cells [5]. H_2S also had endothelium-independent vasorelaxation via vascular smooth muscle cells voltage-gated Ca^{2+} channels [6].

However, the interaction between H_2S and NO is still controversial. Some researchers found that H_2S could directly inhibit eNOS activity to suppress NO synthesis in rat and mouse aortic rings [7]. Study also showed that the deletion of H_2S caused the increase of iNOS mRNA and protein expression levels in mice [8]. However, NO could enhance H_2S generation, and H_2S also could increase iNOS expression level under interleukin-1β (IL-1β) stimulation in vascular tissue [5, 9]. Coletta et al. reported that H_2S and NO are mutually dependent to regulate endothelium-dependent vasorelaxation [10]. On the other hand, Whiteman et al. [11] and Ali et al. [12] found that the vasorelaxant effect of NO was inhibited by H_2S.

Hypertension is caused by persistently high arteriotony, and it is a risk for the development of coronary heart disease, cerebrovascular diseases, heart failure, and so on [13].

Both H_2S and NO can induce vascular relaxation to protect against hypertension; however the interaction effect and mechanism between them are still controversial. In this study, we synthesise a new slow-releasing H_2S-NO donor, termed as ZYZ-803, to investigate the cross talk between H_2S and NO in vasorelaxation effect.

2. Methods and Materials

2.1. Chemical Synthesis. Both 2-amino-3-prop-ynylsulfanyl-propionic acid (10 mmol) and Boc anhydride (10 mmol) were dissolved, subsequently stirred in tetrahydrofuran, eventually saturated with $NaHCO_3$ (60 mL) for 5 h. The solution was then diluted with EtOAc (15 mL) and H_2O (15 mL). The pH was also adjusted to 2–2.5 and then separate organic extracts were washed with brine and dried over Na_2SO_4. The crude material was purified by silica gel chromatography to give 2-(N-Boc-amino)-3-prop-2-ynylsulfanyl-propionic acid as oleaginous solid. Furoxan oxide was made as described previously [14]. Briefly, saturated aqueous sodium nitrite (217 mmol) was added into a solution of cinnamyl alcohol (75 mmol) in glacial acetic acid (15 mL) and then stirred for 1 h. The solution was diluted with H_2O (15 mL) and extracted with diethyl ether (20 mL) for four times. The separate organic extracts were washed with brine and dried over $MgSO_4$. The crude material was purified by silica gel chromatography to give furoxan as oleaginous solid. 2-(N-Boc-amino)-3-prop-2-ynylsulfanyl-propionic acid (0.6 mmol), furoxan oxide (0.6 mmol), N,N′-diisopropylcarbodiimide (0.6 mmol), and DPTS (0.6 mmol) were dissolved and stirred in DCM (50 mL) at room temperature for 5 h. The reaction mixture was diluted with DCM and washed three times with H_2O (15 mL). The organic layer was separated and dried over Na_2SO_4 (60 mL) and then solvent was removed by rotary evaporation. The crude product was eluted by 5% MeOH/PE from silica gel. The final product was oleaginous solid and verified by ^1H nuclear magnetic resonance spectroscopy.

2.2. Chemicals and Antibodies. The following antibodies were used: anti-vasodilator stimulated phosphoprotein (VASP) antibody, anti-p-VASP antibody, anti-eNOS antibody, and anti-p-eNOS antibody were purchased from Cell Signaling Technology, and anti-CSE and anti-β-actin-antibody were purchased from Santa Cruz.

All drugs were purchased from Sigma unless otherwise stated.

2.3. Animals. Male Sprague-Dawley (SD) rats (6-7 weeks old) were maintained on standard conditions and were free to receive food and water. Animals were handled according to the Guide for the Care and Use of Laboratory Animals, published by the US National. Experimental procedures were managed according to the local ethical committee of Fudan University.

2.4. Isolated Rat Aortic Ring Assay. SD rats (8–10 weeks of age) were anesthetized by isoflurane inhalation and dissected to obtain thoracic aortas, which were subsequently cleaned from connective tissue. Rings (2-3 mm in length) were cut and placed in organ bath (10 mL) filled with oxygenated (95% O_2 to 5% CO_2) Krebs-Henseleit solution at 37°C, mounted to isometric force transducers, and connected to a data acquisition system. The aortic rings were allowed to equilibrate at a basal tension of 2 g for 1 h. Subsequently rings were contracted with phenylephrine (PE; 1 μmol/L) until a plateau was reached. In order to identify the integrity of the endothelium, an acetylcholine (Ach) was operated on PE-contracted rings. Rings that relaxed less than 80% were abandoned, unless the removal of endothelium was specified. 60 min later, a series of experiments 1–5 were performed. As least five aortic rings were carried out for each experiment, which were from different rats.

2.4.1. Series 1. This series of experiments were performed to determine the effects of cumulative concentrations (1–100 μmol/L) of *S*-propyl-L-cysteine (SPRC), furoxan, SPRC + furoxan, and ZYZ-803 on the sustained contractile response to PE (1 μmol/L) in endothelium-contained and endothelium-uncontained aortic rings.

2.4.2. Series 2. This series of experiments were performed to determine the time course (1–60 min) of 100 μmol/L SPRC, furoxan, SPRC + furoxan, and ZYZ-803 on the sustained contractile response to PE (1 μmol/L) in endothelium-intact aortic rings.

2.4.3. Series 3. In this series of experiments, the effects of cumulative concentrations (1–100 μmol/L) of ZYZ-803 on the sustained contractile response to PE (1 μmol/L) were detected in endothelium-intact aortic rings. In order to investigate the role of endogenous H_2S and NO generations in PE-contracted aortic rings, the endothelium-intact aortic rings were incubated with PAG (1 mmol/L) and L-NAME (50 μmol/L) for 60 min, respectively.

2.4.4. Series 4. This series of experiments were performed to investigate whether K_{ATP} channels played a key role in the vasorelaxant effect of cumulative concentrations (1–100 μmol/L) ZYZ-803 on PE-induced (1 μmol/L) contraction. The endothelium-intact aortic rings were incubated with glibenclamide (10 μmol/L) for 60 min.

2.4.5. Series 5. In this series, the vasorelaxant effect of cumulative concentrations (1–100 μmol/L) ZYZ-803 on PE-induced (1 μmol/L) contraction was studied under the inhibition of the activity of VASP. The endothelium-intact aortic rings were incubated with KT5823 (1 μmol/L) for 60 min.

2.5. Detection of H_2S and NO Level. Aortic rings were incubated with 100 μmol/L SPRC, furoxan, or ZYZ-803 for 1 h in organ bath filled with oxygenated (95% O_2 to 5% CO_2) Krebs-Henseleit solution at 37°C. Rings were homogenized and subsequently centrifuged (12000 r/min, 15 min) and eventually the supernatant was collected. The H_2S level in aortic rings was measured as descripted previously [15]. 75 μL homogenized aortic ring matrix was mixed with 250 μL zinc

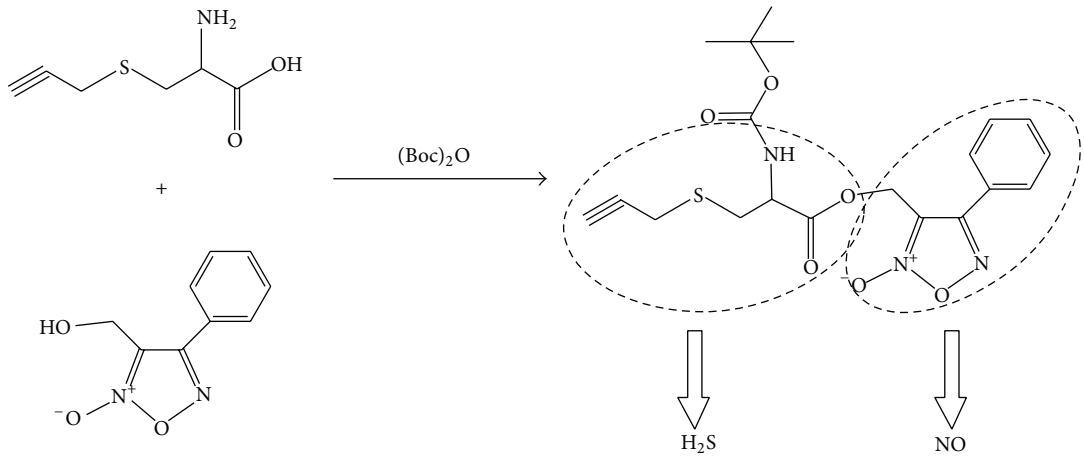

Figure 1: Chemical synthesis of ZYZ-803. Boc: *t*-butyloxycarbonyl.

acetate (1%, w/v), 425 μL distilled water, 133 μL *N*-dimethyl-*p*-phenylenediamine sulfate (20 mmol/L, 7.2 mmol/L HCl), and 133 μL $FeCl_3$ (30 mmol/L, 1.2 mmol/L HCl) for 10 min incubation at room temperature. 250 μL trichloroacetic acid (10%) was added and then centrifuged at 14,000 r/min, 5 min. The absorbance of samples at 670 nm was detected by the microplate reader (Infinite 1000, Tecan Systems Inc.). The NO level in aortic rings was measured with Griess Assay Kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol.

2.6. Western Blot Analysis. Aortic rings were homogenized with homogenization buffer (Beyotime Institute of Biotechnology). And western blot analysis was used to determine the protein expression level as previously described [3]. The images were captured by Image System (Bio-Rad) with an enhanced chemiluminescence detection kit (Millipore). Protein concentration was determined by BCA assay kit (Pierce).

2.7. Detection of Cyclic GMP (cGMP) Level. The cGMP level in aortic rings was measured with cGMP ELISA kit (R&D Systems) according to the manufacturer's protocol.

2.8. Statistical Analysis. All data show mean ± SEM, statistical analysis was performed using one-way ANOVA, and post hoc pairwise comparisons were performed using Prism graph. A p value < 0.05 was considered significant difference.

3. Results

3.1. The Vasorelaxant Effect of ZYZ-803 on the Sustained Contraction Induced by PE. SPRC, synthesized by our group, has been proved that it could increase H_2S concentration through promoting CSE expression [16]. Furoxan is a NO donor, and it can release NO in the presence of thiols [14]. In this study, we made SPRC react with *t*-butyloxycarbonyl (Boc) anhydride and then coupled it with furoxan by ester bond to develop a new H_2S-NO conjugated donor, termed as ZYZ-803 (Figure 1). In endothelium-uncontained aortic rings, ZYZ-803 could not significantly relax the PE-induced contraction (Figure 2(a)). However, exposure of endothelium-contained aortic rings to ZYZ-803, at 1–100 μmol/L, caused a significant and concentration-dependent relaxation (Figure 2(b)). It indicated that the vasorelaxant effect of ZYZ-803 was endothelium-dependent. And at a concentration of 100 μmol/L, ZYZ-803 caused maximal relaxation that was about 85%. After treatment with SPRC or furoxan, the vasorelaxant response curve was shifted to the ZYZ-803 dose-dependent curve right (Figures 2(c)-2(d)). And, at the same vasorelaxant effect, ZYZ-803 was at least 100-fold or 1.5-fold more potent than SPRC or furoxan, respectively. Treatment with the mixture of SPRC and furoxan also caused less vasorelaxant effect than that of treatment with ZYZ-803 in PE-contracted endothelium-contained aortic rings (Figure 2(e)).

3.2. ZYZ-803 Stimulated Vasorelaxation Slowly. To investigate the vasodilator rate, we measured the time course of vasorelaxant effects of testing compounds. At 100 μmol/L, SPRC did not cause significant relaxation of PE-contracted endothelium-contained aortic rings (Figure 3(a)). At the same concentration, furoxan could induce the relaxation effect of endothelium-contained aortic rings significantly under sustained contraction induced by PE (Figure 3(b)). However, this vasorelaxant effect was so rapid and unstable. Treatment with 100 μmol/L ZYZ-803 caused remarkable and time-dependent vasorelaxant effect in PE-induced contracted aortic rings. In the meanwhile, the vasorelaxant response to ZYZ-803 was much slower and sustained long residual action than that to furoxan (Figure 3(c)). Interestingly, the mixture of SPRC and furoxan only induced rapid vasorelaxant effect, which was unlike the effect of ZYZ-803 (Figure 3(d)).

3.3. Vasorelaxant Effect of ZYZ-803 on PE-Induced Contractions after Inhibition of CSE and/or eNOS. All of SPRC, SPRC + furoxan, and ZYZ-803 could induce the generation of H_2S in aortic rings. In the meantime, the H_2S level in ZYZ-803 treatment was the highest one among these three treatments (Figure 4(a)). A similar result was observed in the level of NO

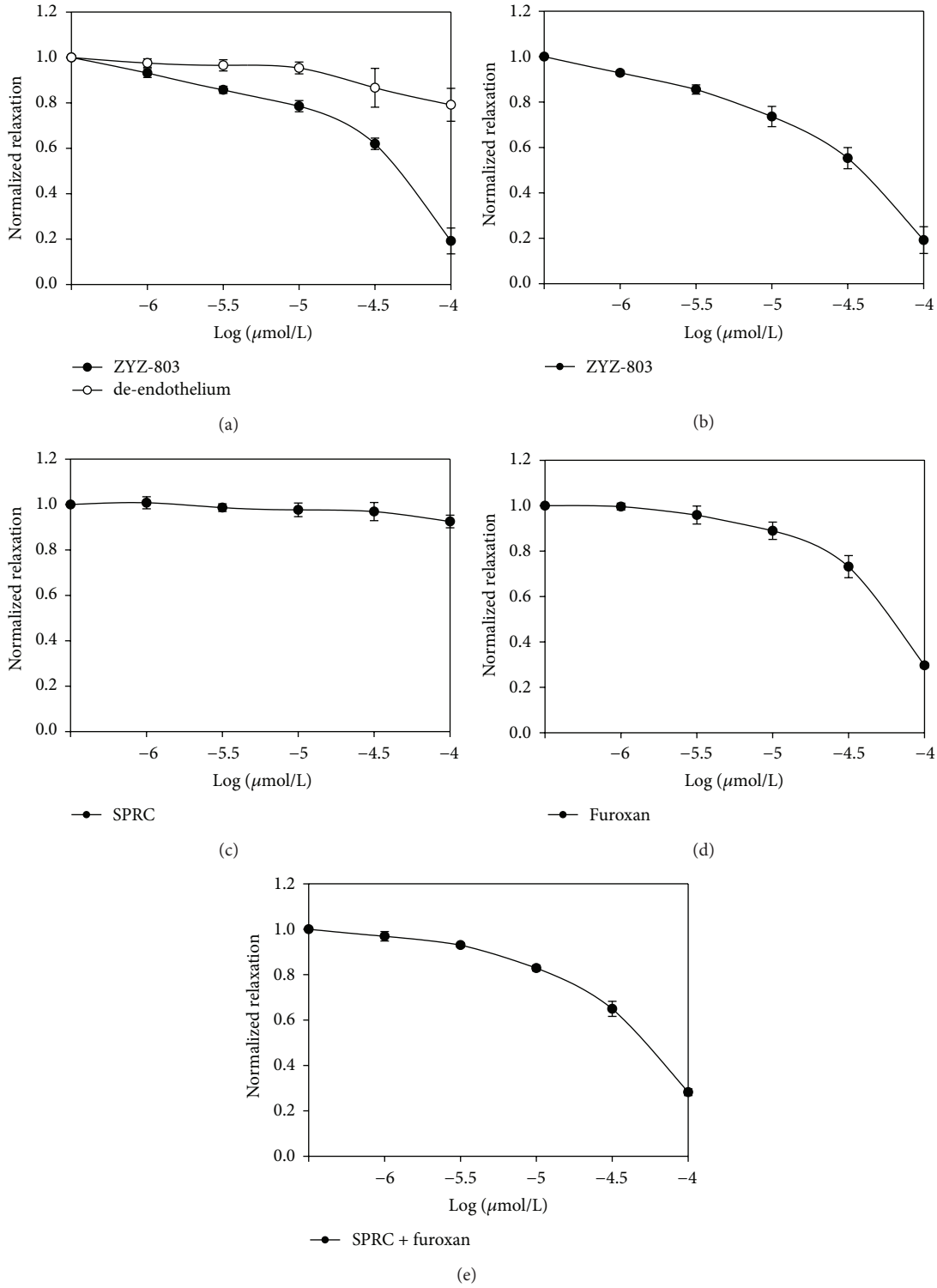

Figure 2: ZYZ-803 could dose-dependently relax the sustained contraction induced by PE in aortic rings. (a) The vasorelaxant effect of ZYZ-803 on the sustained contraction induced by PE was endothelium-dependent. The vasorelaxant effect of ZYZ-803 (b), the vasorelaxant effect of SPRC (c), the vasorelaxant effect of furoxan (d), and the vasorelaxant effect of the mixture of SPRC and furoxan (e) on the sustained contraction induced by PE in endothelium-contained aortic rings. Data represent mean ± SEM, $n \geq 5$ for each group.

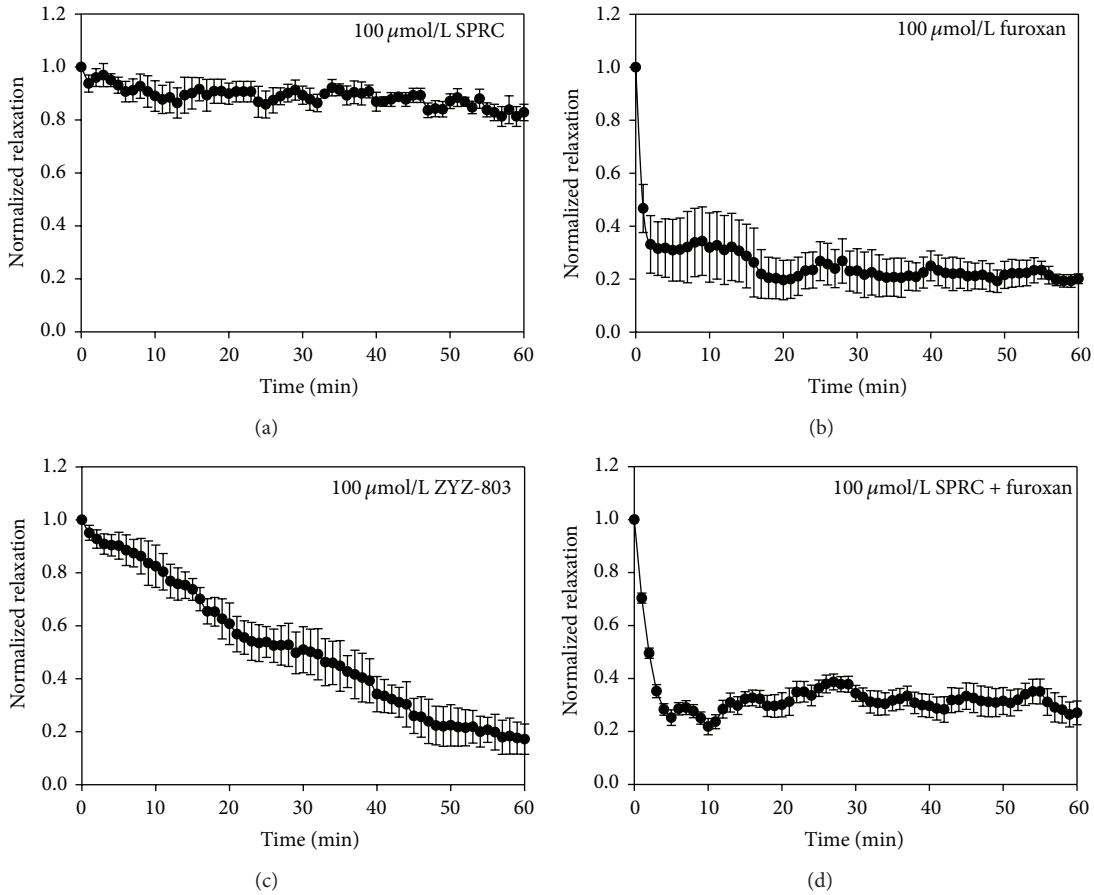

Figure 3: The time course of vasorelaxant effects of testing compounds in aortic rings on the sustained contraction induced by PE. (a) The vasorelaxation time-effect curve of 100 μmol/L SPRC. (b) The vasorelaxation time-effect curve of 100 μmol/L furoxan. (c) The vasorelaxation time-effect curve of 100 μmol/L ZYZ-803. (d) The vasorelaxation time-effect curve of 100 μmol/L furoxan plus 100 μmol/L SPRC. Data represent mean ± SEM, $n \geq 5$ for each group.

in aortic rings. ZYZ-803 caused more generation of NO than SPRC + furoxan or furoxan alone (Figure 4(b)). As shown in Figure 4(c), ZYZ-803 could increase CSE expression and eNOS activity dose-dependently. Considering that H_2S and NO had good vasorelaxant effects, the further experiment was to identify the interaction between H_2S and NO. As shown in Figure 4(d), both CSE inhibitor PAG and eNOS inhibitor L-NAME, as well as the mixture of PAG and L-NAME, could suppress the vasorelaxant of ZYZ-803. And the inhibitory vasorelaxation of PAG + L-NAME was more severe. It indicated that both H_2S and NO played a key role, and these two gases were mutually promoted in the regulation of vascular tone.

3.4. Vasorelaxant Effect of ZYZ-803 on PE-Induced Contractions after Inhibition of K_{ATP} Channel. Early studies indicated that H_2S and NO could regulate vascular tone through opening K_{ATP} channel [5, 17, 18]. In order to assess the role of K_{ATP} channel in the vasorelaxant effect of ZYZ-803, we did the test in PE-contracted endothelium-contained aortic rings that were pretreated with glibenclamide, a K_{ATP} channel inhibitor. As shown in Figure 5, 1–100 μmol/L ZYZ-803 relaxed the contraction evoked by PE in a dose-dependent manner. However, the vasorelaxant response to ZYZ-803 was significantly abolished in the presence of glibenclamide. This result demonstrated that ZYZ-803 was a K_{ATP} channel opener to relax vascular to.

3.5. The Vasorelaxant Effect of ZYZ-803 through cGMP Pathway. cGMP was considered to be one of the second messengers that regulate vascular tone under physiological conditions. The cellular level of cGMP is the balance of synthesis and degradation. cGMP is synthesized by soluble guanylyl cyclase (sGC) [19]. Previous study had shown that NaHS could time- and dose-dependently increase cGMP level in rat aortic smooth muscle cells [20], and NO could also increase cGMP level in mice aortic rings [21]. Considering the critical role of cGMP in vasorelaxation, we studied whether there was any effect of ZYZ-803 on cGMP concentration. As shown in Figure 6(a), the level of cGMP was elevated by ZYZ-803 treatment, whereas this effect was attenuated by PAG and/or L-NAME treatment. cGMP can activate its downstream signaling molecule protein kinase G (PKG). We found that the vasorelaxant effect of ZYZ-803 was inhibited when PKG inhibitor KT5823 was used in PE-induced contraction aortic rings (Figure 6(b)). VASP serine-239 is

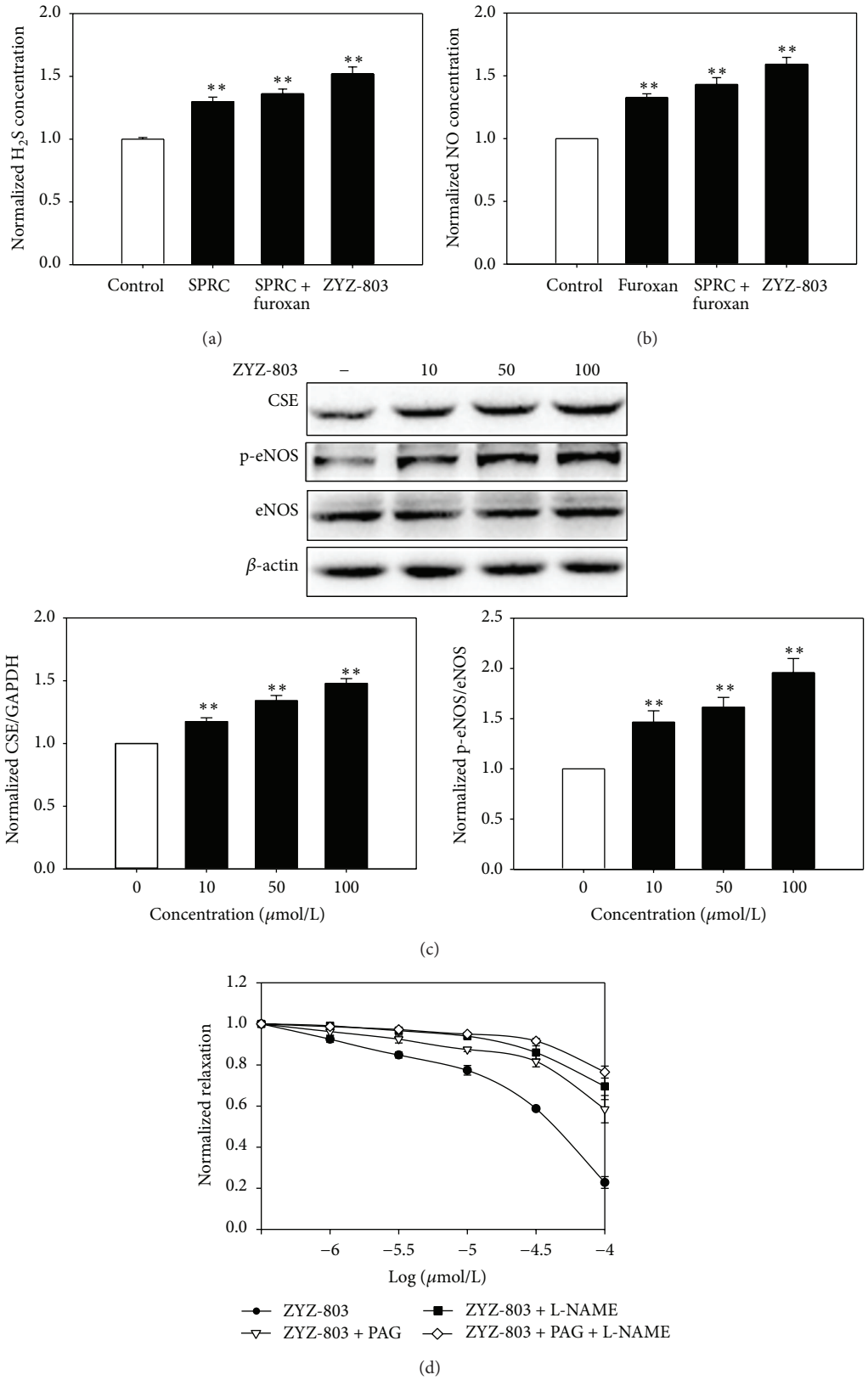

Figure 4: Vasorelaxant effect of ZYZ-803 on PE-induced contractions was suppressed upon inhibition of endogenous H_2S or NO generation. (a) The concentration of H_2S in aortic rings after SPRC, SPRC + furoxan, and ZYZ-803 treatments. (b) The concentration of NO in aortic rings after furoxan, SPRC + furoxan, and ZYZ-803 treatments. (c) The expressions of CSE, eNOS, and p-eNOS after ZYZ-803 (10, 50, and 100 μmol/L) treatments. (d) The vasorelaxant effect of ZYZ-803 after L-NAME, PAG, and L-NAME + PAG treatments on PE-induced contractions in aortic rings. Data represent mean ± SEM, $n \geq 5$ for each group. $^{**}p < 0.01$ compared with control group.

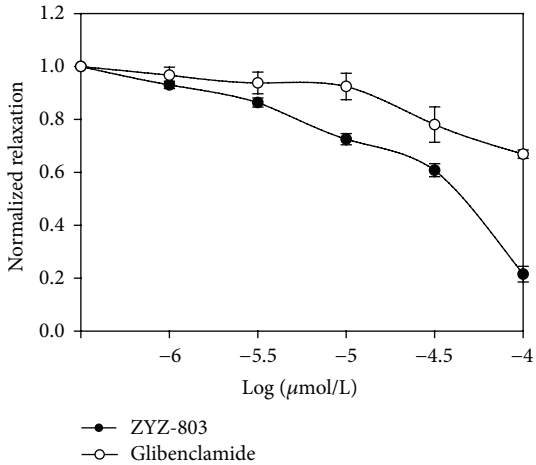

Figure 5: Vasorelaxant effect of ZYZ-803 on PE-induced contractions was suppressed after inhibition of K_{ATP} channel. The vasorelaxant effect of ZYZ-803 was abolished on PE-induced contractions in aortic rings after glibenclamide treatment. Data represent mean ± SEM, $n \geq 5$ for each group.

the major phosphorylation site of PKG, and it was used as the marker of PKG activity. In aortic rings, treatment with ZYZ-803 dose-dependently increased the phosphorylation level of VASP at serine-239 site, and PAG and/or L-NAME could inhibit VASP activity (Figure 6(c)). The inhibitory effects of PAG + L-NAME on cGMP level and VASP activity were more severe than that of PAG or L-NAME alone. These results indicated the cooperation of H_2S and NO on cGMP/VASP pathway in vascular tissue.

4. Discussion

Endothelium-dependent vasorelaxation injury is known to lead many cardiovascular diseases, such as hypertension, atherosclerosis, and heart failure [19]. NO generation from eNOS is considered to be endothelium-derived relaxing factor [21]. Like NO, H_2S is an endogenous gaseous transmitter and it also can regulate vascular tone under physiological and pathological conditions [22]. Growing evidence has shown that the interactions between H_2S and NO play an important role in vasorelaxation; however the precise nature of the interaction is still unclear. Furoxan is a NO donor that is used for NO bioactivity widely in preclinical studies [23]. SPRC is an effective endogenous H_2S donor, which could increase CSE expression and activity to protect against ischemia reperfusion injury [16]. In the current study, we conjugated furoxan with SPRC to develop a novel releasing H_2S and NO donor, termed ZYZ-803. We used ZYZ-803 to study the interaction between H_2S and NO and investigated the therapeutic potential of ZYZ-803 for vasorelaxation.

Endothelial cells exist between blood vessels and blood, which is a single layer of cells. It can secrete a variety of bioactivator substances to regulate vascular functions, like vascular tone, blood pressure, blood coagulation, and migration of leukocytes. NO is secreted in endothelial cells to relax blood vessel through cGMP pathway. And NO donors have vasorelaxant effect to decrease blood pressure. It has been reported that the concentration of H_2S in hypertension rats is lower than that in normal rats [24]. H_2S caused a dose-dependent relaxation from preconstricted aortic rings through K_{ATP} channel [5]. Growing evidence has shown the interactions between H_2S and NO play an important role in vasorelaxation. Hosoki et al. found that H_2S and NO regulated vasorelaxation in the synergistic effect [25]. Nevertheless, Zhao and Wang reported that the vasorelaxant activity of NO was inhibited by H_2S [22]. NO reacted together with H_2S to produce a new molecule that exhibited little vasorelaxant activity [12]. However, Eberhardt et al. showed that nitroxyl was generated by the chemical reaction of H_2S and NO, which could stimulate vasorelaxation [26]. These results suggested that the complex cross talk between H_2S and NO is of great importance. In this paper, we found that, at the same concentration, ZYZ-803 had more competitive vasorelaxant effect compared with SPRC and furoxan alone. This result indicated that H_2S and NO could mutually potentiate relaxant effect of one another in aortic rings.

In this study we found that SPRC, furoxan, and ZYZ-803 showed different time-effective curve in vasorelaxation. 100 μmol/L SPRC did not show a significant relaxation in aortic rings. At the same concentration, the vasorelaxant effect of furoxan was effective and rapid. However, a rapid relaxation of blood vessels leads to a range of side effects, such as vascular damage, low blood pressure, and rapid heart rate. 100 μmol/L ZYZ-803 relaxed aortic rings stably, enduringly, and more effectively than furoxan. Several studies suggested that the effective concentration of H_2S to relax vessels was high, usually in 300 μmol/L–1 mmol/L [27–29]. SPRC was linked to furoxan by an ester bond. With the ester bond dissociated slowly, SPRC and furoxan could stimulate the generations of gasotransmitters. Therefore, different from the mixture of SPRC and furoxan, ZYZ-803 could generate H_2S and NO over a long time period and performed more significant than SPRC + furoxan in vasorelaxant effect. Indeed, ZYZ-803 may be a good strategic treatment for vascular chronic diseases. *In vivo*, the generation of gasotransmitters is a slow progression and their concentrations are low under physiological condition. Initial studies demonstrated that some donors released a large amount of H_2S or NO during a short time, which caused a variety of side effects and increased the toxicity of these donors [30, 31]. Furthermore, in previous studies, the doses of gasotransmitter donors were high in diseases treatments due to the effumability of gases. ZYZ-803 released H_2S and NO slowly and lasted for a long time, which were close to the physiological environment. Compared with treatment of SPRC and furoxan alone, treatment of ZYZ-803 had fewer side effects with lower concentration. So ZYZ-803 will be a useful tool for biological researches.

In vascular tissue, H_2S is generated from smooth muscle cells, endothelial cells, and perivascular adipose cells by CSE. And NO is generated by eNOS in endothelial cells [19]. Furoxan could increase the level of H_2S induced by SPRC, and SPRC could increase the level of NO induced by furoxan. Moreover, the H_2S and NO levels of treatment with ZYZ-803 were higher than those of SPRC and/or furoxan.

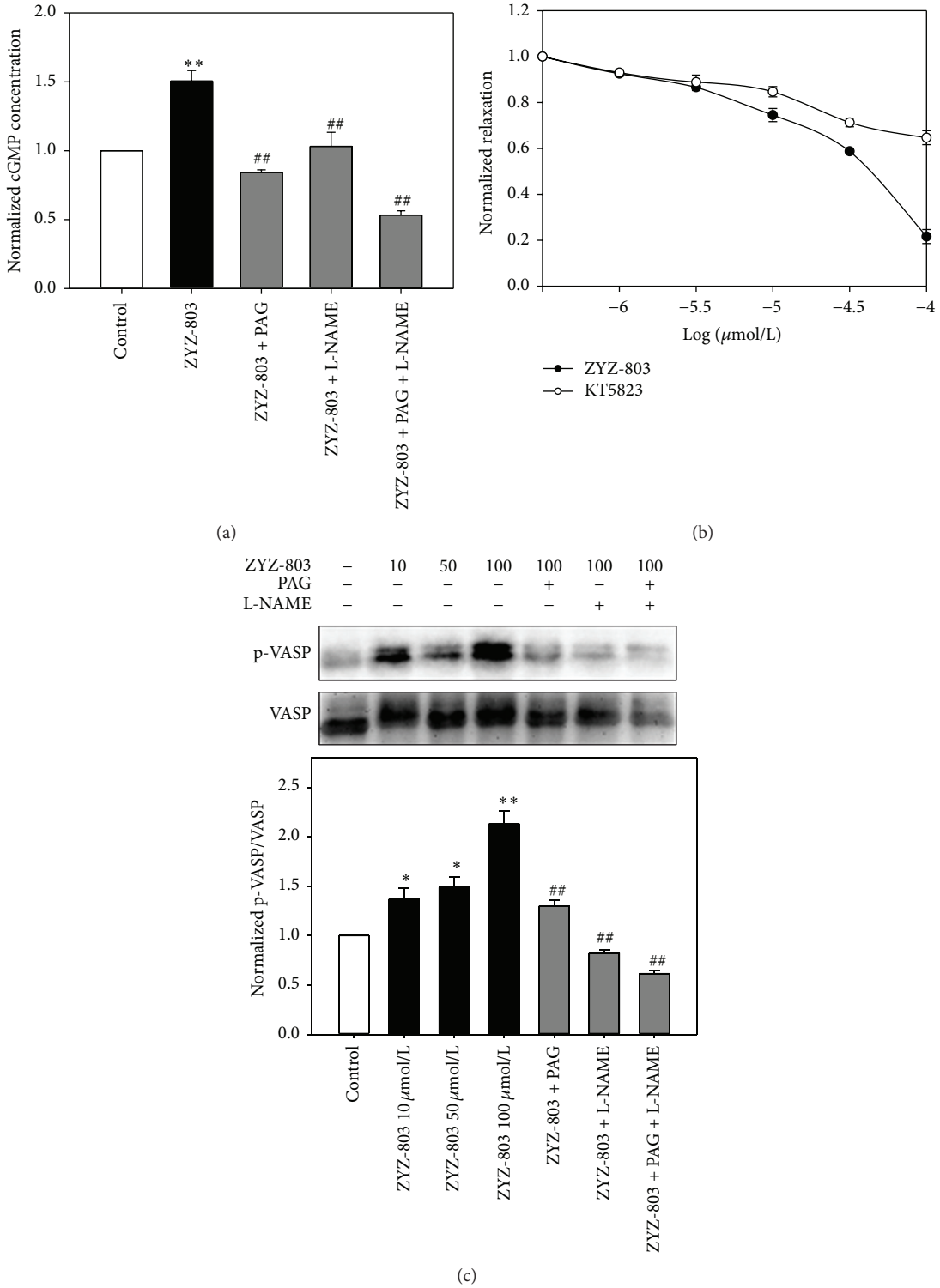

Figure 6: ZYZ-803 relaxed aortic rings on PE-induced contractions through cGMP pathway. (a) The ZYZ-803-induced cGMP increase was abolished after L-NAME, PAG, and L-NAME + PAG treatments. (b) The vasorelaxant effect of ZYZ-803 on PE-induced contractions was suppressed after KT5823 treatment in aortic rings. (c) The expression level of VASP and p-VASP in aortic rings. Data represent mean ± SEM, $n \geq 5$ for each group. $^{**}p < 0.01$ compared with control group. $^{\#\#}p < 0.01$ compared with ZYZ-803 group.

We speculated that the reason was that ZYZ-803 could keep concentrations of H_2S and NO in the effective dose range for a long time, which could stimulate CSE and eNOS to generate more H_2S and NO, respectively. ZYZ-803-induced vasorelaxation was abolished by L-NAME and/or PAG, which indicated that ZYZ-803 produced H_2S and NO through the stimulation of CSE and eNOS, which had synergetic effect on vasorelaxation.

It has been shown that both NO and H_2S are K_{ATP} channel openers in vascular tissue [5, 19]. In this study, we found that pretreatment with glibenclamide, the inhibitor of K_{ATP} channel, suppressed ZYZ-803-induced vasorelaxation. This result demonstrated that ZYZ-803 is a novel K_{ATP} channel opener in rat aortic rings. cGMP/PKG pathway plays a great role in the endothelium-dependent vasorelaxation. The second messenger cGMP can activate PKG and then induced K_{ATP} channel open. The ratio of p-VASP/VASP is a sensitive monitor to detect the cGMP/PKG pathway signaling [32]. Studies have shown that NO can activate sGC and increases intracellular cGMP level in vascular tissue [19]. Like NO, treatment with H_2S time- and concentration-dependently increases cGMP level in rat aortic smooth muscle cells [33]. In this study, ZYZ-803 could improve cGMP level and phosphorylation level of VASP in aortic rings. And these effects could be abolished by treatment with L-NAME and/or PAG, which indicated that H_2S and NO were mutually dependent on the activation of cGMP pathway.

5. Conclusions

In summary, we synthesized a novel H_2S-NO conjugated donor, ZYZ-803, which exhibited more potential in vasorelaxation compared with the parent drugs in aortic rings. ZYZ-803 opened K_{ATP} channel through cGMP pathway and exerted an effective, stable, and durable vasorelaxation. H_2S and NO could improve the generation of one another and created synergism in the regulation of vascular tone. ZYZ-803 could be very useful to study the interaction between H_2S and NO better in different physiological and pathological conditions, and it would open a new prospect in researches of gasotransmitters. In addition, ZYZ-803 exhibits powerful therapeutic potential in vasorelaxation, which sheds new light on further gaseous drugs design for the treatment of cardiovascular diseases.

Conflict of Interests

None of the authors has any conflict of interests to disclose.

Authors' Contribution

The contribution of each author was as follows: Yi Zhun Zhu, Dan Wu, and Qingxun Hu designed this study. Dan Wu, Qingxun Hu, and Fenfen Ma performed the experiments. Yi Zhun Zhu, Dan Wu, and Qingxun Hu analyzed the data and wrote the paper. All authors read and approved the final paper. Dan Wu and Qingxun Hu contributed equally to this work.

Acknowledgments

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