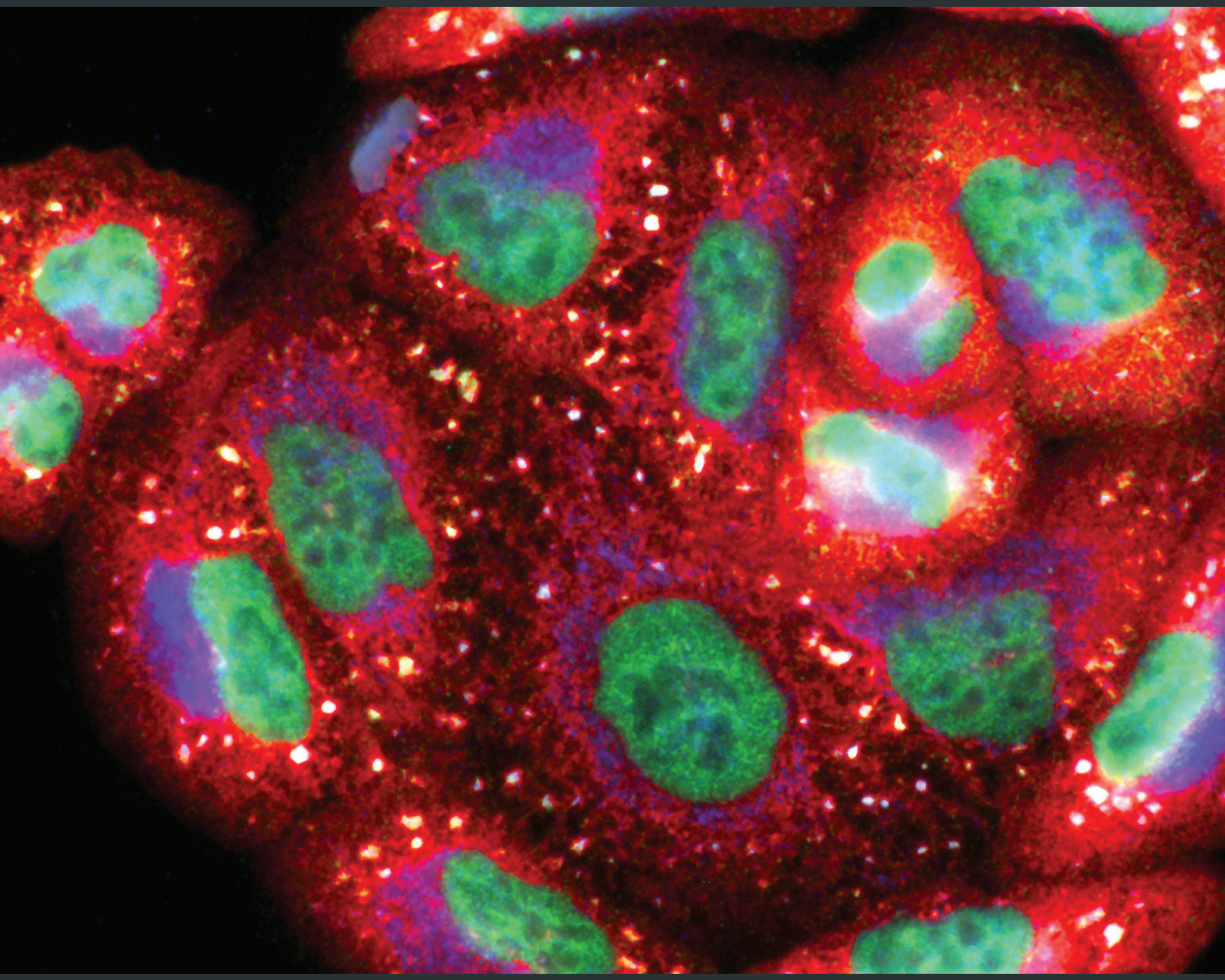
Oxidative Medicine and Cellular Longevity

Hydrogen Sulfide Signaling in Oxidative Stress and Aging Development

Guest Editors: Guangdong Yang, Steven S. An, Yong Ji, Weihua Zhang, and Yanxi Pei



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Editorial

Hydrogen Sulfide Signaling in Oxidative Stress and Aging Development

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Just in the last decade, the knowledge of the physiologic functions of hydrogen sulfide (H_2S) in medicine and biology has been tremendously accumulated [1–3]. H_2S has emerged to be one important member of the family of gasotransmitters, together with nitric oxide (NO) and carbon monoxide (CO) [1, 2]. The ubiquitous distribution of H_2S-producing enzymes and the potent chemical reactivities of H_2S in biological system make this molecule unique in regulating cellular and organ functions in both the mammalian and plants [1–3]. Pathophysiological abnormalities related to altered H_2S metabolism and function have been demonstrated in many cases [2, 3]. H_2S possesses a great therapeutic potential in age-associated diseases by modulation of oxidative stress [4, 5].

This special issue contains review paper and research articles that focus on the topics of H_2S signaling in oxidative stress and aging development, including discussions on the potency and efficiency of H_2S in dealing with various diseases. A number of contributions have addressed the protective role of H_2S in cardiovascular diseases and diabetes. In an original research article, H. Yu et al. demonstrate that H_2S decreases NADPH oxidase activity and reactive oxidative species (ROS) production, which lead to reduced mean arterial pressure and heart rate in spontaneously hypertensive rats. H_2S, as an antioxidant, may be a potential target for cardiovascular diseases. A research article by S. Jin and colleagues compares H_2S generation in ageing diabetic mouse hearts, and they find that H_2S levels are reduced in the diabetic heart due to the alterations in H_2S-producing enzymes, which might be related with the pathogenesis of diabetic cardiomyopathy. Y. Zong and colleagues explore the possible effects of endogenous H_2S on endothelial apoptosis under high-salt stimulation, and their data validate that supplementation of H_2S donor markedly inhibits vascular endothelial cell oxidative stress and mitochondria-related apoptosis induced by high salt. Q. Wang and colleagues report that H_2S antagonizes advanced glycation end-products induced-epithelial sodium channel activity by targeting the ROS/PI3K/PTEN pathway in A6 cells. The authors conclude that H_2S may provide protection against hypertension in diabetic patients. In a review paper by Y. Shen and colleagues, the underlying mechanisms for the cardioprotective effects of H_2S against myocardial infarction, arrhythmia, hypertrophy, heart failure, and so forth are discussed. Some mechanisms, including antioxidative action, preservation of mitochondrial function, reduction of apoptosis, anti-inflammatory responses, angiogenic actions, regulation of ion channel, and interaction with NO, are mostly responsible for the cardioprotective effect of H_2S.

Some papers in this special issue describe new insights into the therapeutic potential in fibrosis. In a review paper, S. Zhang and colleagues summarize studies that supplement with exogenous H_2S mitigates the severity of fibrosis in

various experimental animal models. The protective role of H_2S in the development of fibrosis is primarily attributed to its antioxidation, antiapoptosis, anti-inflammation, proangiogenesis, and inhibition of fibroblasts activities. K. Song and colleagues continue on with this topic that H_2S protects fibrosis diseases that relate to heart, liver, kidney, and other organs. In a research article, G. Meng and colleagues provide new evidence on the protective role of GYY4137, a slow-releasing H_2S donor, in myocardial fibrosis by inhibiting oxidative stress, blocking TGF-β1/Smad2 signaling pathway, and decreasing in expression of α-SMA. Further clinical studies are needed to translate this potential to clinical use.

D. Wu and colleagues highlight the recent findings regarding the role of H_2S in ischemia-reperfusion (I/R) injury. In their paper, the authors proposed that treatment with H_2S or its donors in proper dose range and time frame will exhibit more potent therapeutic effects against I/R injury in further preclinical research and clinical application. A review article by W. Zhang and her colleagues addresses the reciprocal interaction between H_2S and calcium ion channels and transporters through different mechanisms, all of which are essential for the maintenance of intracellular calcium homeostasis by H_2S. In an original research article, L. Zhang and colleagues explore the role of H_2S in human gastric neoplasias. Their data point that H_2S level is lower in non-cancerous gastric samples in comparison with human gastric carcinoma mucosa, and the authors further prove that H_2S induces apoptosis and inhibits cell migration and invasion of gastric cancer cells by regulating apoptosis related proteins. The therapeutic application of H_2S donors against gastric cancer development can be realized.

In a review article, B. Wu and colleagues discuss the latest research on the interaction of H_2S with oxygen sensing under hypoxia condition. Emerging evidence has elucidated an important protective role of H_2S in hypoxia-mediated damage in many mammalian systems. By regulating the functions of hypoxia-inducible factors and the activation of carotid bodies, H_2S acts as important oxygen/hypoxia sensor.

Not only has it acted as a signalling molecule in mammalian system, but also overwhelming evidence has demonstrated that H_2S plays important roles in diverse physiological processes in plants. J. Zhu and Y. Pei discuss in a review article the physiological implications of H_2S in plants. H_2S modulates various defence responses in plants, including growth and development, abiotic stress, heavy metal toxicity, drought and osmotic stress, hypoxia, senescence, and maturation by interacting with plant hormones, hydrogen peroxide, NO, CO, and other molecules. The same research group also provides evidence that H_2S alleviates cadmium-induced cell death in Chinese cabbage roots, and they further verify that, by upregulating antioxidant enzyme activities, H_2S removes excessive ROS and reduces cell oxidative damage induced by cadmium. In one original research article, Y. Zhang and colleagues demonstrate that H_2S acts as an antioxidant in delaying cell apoptosis and enhancing α-amylase secretion regardless of the presence of gibberellic acid in barley aleurone layers. In addition, D.-B. Zhu and colleagues investigate the effects of SO_2 pretreatment on H_2S and ROS accumulation in germinating wheat seeds, and their data suggest that SO_2 could increase endogenous H_2S accumulation and the antioxidant capability and decrease endogenous aluminum content in wheat grain to alleviate aluminum stress. SO_2 may be reduced to H_2S by sulfite reductase, thus contributing to H_2S production.

The articles presented in this special issue highlight the current advances in the research field of H_2S in medicine and biology. These articles not only enrich our understanding of how H_2S regulation of oxidative stress in various disorders occurs but also provide evidence on the therapeutic potential of H_2S against aging development and other disorders. We hope that readers will find these contributions interesting and informative.

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Guangdong Yang
Steven S. An
Yong Ji
Weihua Zhang
Yanxi Pei

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Review Article

Role of Hydrogen Sulfide in Ischemia-Reperfusion Injury

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Ischemia-reperfusion (I/R) injury is one of the major causes of high morbidity, disability, and mortality in the world. I/R injury remains a complicated and unresolved situation in clinical practice, especially in the field of solid organ transplantation. Hydrogen sulfide (H_2S) is the third gaseous signaling molecule and plays a broad range of physiological and pathophysiological roles in mammals. H_2S could protect against I/R injury in many organs and tissues, such as heart, liver, kidney, brain, intestine, stomach, hind-limb, lung, and retina. The goal of this review is to highlight recent findings regarding the role of H_2S in I/R injury. In this review, we present the production and metabolism of H_2S and further discuss the effect and mechanism of H_2S in I/R injury.

1. Introduction

Ischemia-reperfusion (I/R) is a well-recognized pathological condition that is characterized by an initial deprivation of blood supply to an area or organ followed by subsequent vascular restoration and concomitant reoxygenation of downstream tissue [1]. I/R can develop as a consequence of trauma, hypertension, shock, sepsis, organ transplantation, or bypass surgery leading to end-organ failure such as acute renal tubular necrosis, bowel infarct, and liver failure. I/R can also occur under various complications of vascular diseases such as stroke and myocardial infarction [1, 2]. Several pathophysiologic mechanisms have been proposed as mediators of the damage induced by I/R, such as activation of the complement system and leukocyte recruitment, endoplasmic reticulum stress, calcium overload, reduction of oxidative phosphorylation, increased free radical concentration, development of the no-reflow phenomenon, endothelial dysfunction, and activation of signaling pathways of apoptosis, necrosis, and/or autophagy [1, 3]. Many studies have shown that there are three time frames in the protection against I/R injury: before the index ischemic episode (ischemic preconditioning), during ischemia (ischemic conditioning), and at the onset of reperfusion (ischemic postconditioning) [4, 5]. Currently, several therapeutic gases have been shown to play a role in the treatment of I/R injury, including hydrogen, nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S) [6].

H_2S is a colorless, flammable, and water-soluble gas with the characteristic smell of rotten eggs. In the past several centuries, H_2S had been known only for its toxicity and environmental hazards [7, 8]. It elicits its toxic effects by reversibly inhibiting cytochrome c oxidase (CcO), preventing oxidative phosphorylation and lowering the production of adenosine triphosphate (ATP). Recently, there has been growing evidence that H_2S plays a broad range of physiological and pathophysiological functions [9, 10], including induction of angiogenesis [11], regulation of neuronal activity [9], vascular relaxation [12], glucose homeostatic regulation [13], and protection against I/R injury in heart, liver, kidney, lung, and brain [14–18]. The abnormal metabolism of H_2S could result in an array of pathological disturbances in the form of hypertension, diabetes, atherosclerosis, heart failure, sepsis, inflammation, erectile dysfunction, cataracts, asthma, and neurodegenerative diseases [10]. In addition, H_2S can also interact with other specific molecules, including NO [19], CcO [20], catalase [21], myoglobin [21, 22], hemoglobin [21, 22], Kelch-like ECH-associated protein 1 (Keap1) [23], cysteine residues on ATP-sensitive potassium (K_{ATP}) channels [24], epidermal growth factor receptor [25], and vascular

endothelial growth factor receptor 2 [25, 26]. Considering H_2S is involved in numerous biological processes, it is now widely accepted that H_2S functions as the third signaling gasotransmitter, along with NO and CO [9].

With the deepening of research on H_2S and I/R injury, the role that H_2S plays in attenuating I/R injury has begun to be elucidated. In this review, we highlight recent studies that provide new insight into the production and metabolism of H_2S and discuss the role and mechanism of H_2S on I/R injury.

2. Production and Metabolism of H_2S

2.1. Endogenous Production of H_2S. H_2S is endogenously generated in mammalian cells via both enzymatic and nonenzymatic pathways, although the nonenzymatic pathway is less important in H_2S production [27]. With regard to the enzymatic pathway, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) are two pyridoxal-5′-phosphate-(PLP-) dependent enzymes, which use either L-cysteine or L-cysteine together with homocysteine as their principal substrates to produce H_2S [9]. Unlike CBS and CSE, 3-mercaptopyruvate sulfurtransferase (3-MST) is a PLP-independent enzyme, which uses 3-mercaptopyruvate (3MP) as a substrate to produce H_2S. 3MP is a metabolite of L-cysteine and α-ketoglutarate by cysteine aminotransferase (CAT) [9]. CSE and CBS are cytosolic enzymes with tissue-specific distributions. CBS is predominantly expressed in the central nervous system and is also found in liver, kidney, ileum, uterus, placenta, and pancreatic islets. CSE is abundant in heart, liver, kidney, uterus, ileum, placenta, and vascular smooth muscle. CSE is the most relevant H_2S-producing enzyme in the cardiovascular system [9, 27]. CAT and 3-MST are localized both in cytosol and mitochondria, but the majority of these two enzymes are present in the mitochondria [9]. They have been found in the heart, kidney, liver, lung, thymus, testis, brain, and thoracic aorta and are apparently important for H_2S production in the brain and vasculature [9, 27, 28]. Furthermore, a recent study has demonstrated that D-cysteine (a negative control of L-cysteine) can be metabolized to achiral 3MP by D-amino acid oxidase and can be used as a substrate for 3-MST to produce H_2S in both kidney and brain [29]. During the enzymatic pathway, H_2S can be immediately released or stored in a form of bound or acid-labile sulfur in the cells [30].

Apart from enzymatic pathway, endogenous H_2S can also be produced through nonenzymatic processes that are less well understood [27, 30, 31]. Nonenzymatic production of H_2S occurs through glucose, inorganic, and organic polysulfides (present in garlic), glutathione, and elemental sulfur [30, 31]. H_2S can be generated from glucose either via glycolysis (>90%) or from phosphogluconate via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (<10%) [7, 27, 30]. Glucose could react with cysteine, methionine, or homocysteine to produce gaseous sulfur compounds such as H_2S and methanethiol [7, 8, 30]. H_2S is also produced through direct reduction of glutathione and elemental sulfur. Reduction of elemental sulfur to H_2S is mediated through reducing equivalents of the glucose oxidation pathways such as nicotinamide adenine dinucleotide and NADPH [7, 8]. Thiosulfate is an intermediate of sulfur metabolism from cysteine and H_2S formation from thiosulfate through a reductive reaction involving pyruvate, which acts as a hydrogen donor [7, 8, 32, 33]. In addition, garlic and garlic-derived organic polysulfides could induce H_2S production in a thiol-dependent manner, such as diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), and S-allyl cysteine (SAC) [30–34].

2.2. Exogenous Source of H_2S. H_2S gas has been considered as the authentic resource of exogenous H_2S [35]. Recent studies have shown that H_2S gas plays important roles in promoting angiogenesis [11], ameliorating type II diabetes [13], and protecting against myocardial I/R injury [36]. However, H_2S gas is not an ideal resource due to a possible toxic impact of H_2S excess and difficulty in obtaining precisely controlled concentration [35]. Currently, a number of H_2S-releasing compounds have already been successfully designed and developed. These compounds could be mainly divided into two types, including the "H_2S donors," which release H_2S as the only mechanism of action, and the "H_2S-releasing hybrid drugs," also known as "dirty drugs" in which H_2S release is an ancillary property which accompanies a principal mechanism of the hybrid drugs [35]. Inorganic sulfide salts, such as sodium hydrosulfide (NaHS), sodium sulfide (Na_2S), and calcium sulfide, have been widely used as H_2S donors [7, 8, 35]. As the maximum concentration of H_2S released from these salts can be reached within seconds, they have been called fast-releasing H_2S donors [35]. However, the effective residence time of these donors in tissues may be very short because H_2S is highly volatile in solutions [35]. Ideal H_2S donors for therapeutic purposes should generate H_2S with relatively slow-releasing rates and longer periods of treating time. Recently, many slow-releasing H_2S donors (Table 1) and H_2S-releasing hybrid drugs (Table 2) have been designed and synthesized to increase the treatment efficacy of H_2S.

2.3. Metabolism of H_2S. In order to maintain a proper physiological balance of its metabolism, H_2S can be broken down through several enzymatic and nonenzymatic processes [7, 10, 37]. The main pathway of H_2S catabolism occurs in mitochondria. Mitochondrial oxidative modification converts H_2S into thiosulfate through several enzymes including quinone oxidoreductase, S-dioxygenase, and S-transferase. Thiosulfate could be further converted into sulfite, which is catalyzed by thiosulfate : cyanide sulfurtransferase. Sulfite is then rapidly oxidized to sulfate by sulfite oxidase. Therefore, sulfate is a major end-product of H_2S metabolism under physiological conditions [7, 10, 37, 38]. The secondary mechanism of H_2S catabolism is the methylation to methanethiol and dimethylsulfide via thiol S-methyltransferase in the cytosol [10, 37, 38]. The third pathway of H_2S metabolism is the interaction of H_2S with methemoglobin that leads to sulfhemoglobin, which is considered as a possible biomarker of plasma H_2S [10, 37, 38]. These three pathways are considered the main processes of H_2S catabolism in mammals. Furthermore, recent studies have shown that H_2S could be converted into sulfite via minor oxidative routes in activated neutrophils [10, 37].

Table 1: The biological characteristics of slow-releasing H_2S donors.

Compounds	H_2S release mechanisms	Therapeutic effects	References
GYY4137	Hydrolysis	Vasodilation	[86]
		Anti-inflammation	[19]
		Anticancer	[87]
		Protection of mitochondrial function	[88]
		Regulation of oviductal embryo transport and myometrial contractility	[89, 90]
		Antithrombotic	[91]
ADT	Metabolized by carboxylesterases	Neuroprotection against oxidative stress	[92]
		Protection of blood-brain barrier integrity	[55]
ADT-OH	Metabolized by carboxylesterases	Neuroprotection against oxidative stress	[92]
		Vasorelaxation	[93]
		Antineuroinflammation	[94]
AP39	Metabolized by carboxylesterases	Protection against oxidative mitochondrial DNA damage	[95]
S-Aroylthiooximes	Hydrolysis	Unknown	[96]
S-Propargyl-cysteine	Hydrolysis	Angiogenesis promotion	[97]
		Anticancer	[98]
		Cardioprotection	[99]
		Anti-inflammation	[100]
SG-1002	Activation after oral administration	Cardioprotection	[101]
4-Hydroxythiobenzamide	Hydrolysis	Improvement of wound healing	[102]
Arylthioamides	Thiol activation	Unknown	[103]
N-(benzoylthio)benzamides	Hydrolysis	Unknown	[104]
S-Propyl cysteine	Hydrolysis	Cardioprotection	[99]
N-Acetylcysteine	Hydrolysis	Protection against oxidative stress	[105]
N-Acetylcysteine ethyl ester	Hydrolysis	Protection against oxidative stress	[105]
SAC*	Hydrolysis	Protection against oxidative stress	[99]
PhNCS	Thiol activation	Unknown	[106]
PhNCS-COOH	Thiol activation	Unknown	[106]
Lawesson's reagent	Hydrolysis	Anti-inflammation	[107]
		Protection against gastric damage	[108]
Dithioperoxyanhydrides	Thiol activation	Vasorelaxation	[35]
Thioglycine	Bicarbonate activation	Unknown	[109]
L-Thiovaline	Bicarbonate activation	Unknown	[109]
Thioamino acids	Bicarbonate activation	Vasorelaxation	[109]
Phosphorodithioates	Hydrolysis	Protection against oxidative stress	[35]
S-SH compounds	Thiol activation	Myocardial I/R protection	[110]
N-(acylthio)-benzamides	Thiol activation	Unknown	[104]
H_2S photo-donor **5**	Light activation	Unknown	[111]
gem-Dithiol compounds	Light activation	Unknown	[35]
Allyl isothiocyanate	Thiol activation	Unknown	[112]
Benzyl isothiocyanate	Thiol activation	Unknown	[112]
4-Hydroxybenzyl isothiocyanate	Thiol activation	Unknown	[112]
Erucin	Thiol activation	Unknown	[112]
Sinigrin	Hydrolysis	Unknown	[112]
Poly(ethylene glycol)-ADT	Metabolized by carboxylesterases	Unknown	[113]
S-memantine	Thiol activation	Protection against ischemic neuronal death	[114]
ACS1	Metabolized by carboxylesterases	Neuroprotection	[115]
		Anticancer	[116]

*This compound is also a derivative of garlic.

Table 2: The biological characteristics of H_2S-releasing hybrid drugs.

Compounds	Parent drugs	Therapeutic effects	References
ACS2	Valproic acid	Anticancer	[116]
		Antiangiogenesis	[117]
ACS6	Sildenafil	Proerectile	[118]
		Neuroprotection	[119]
		Protection against oxidative stress	[120]
ACS14	Aspirin	Protection against oxidative stress	[121]
		Prevent the progression of atherosclerosis	[122]
		Antiaggregatory	[123]
		Protection against I/R injury	[124]
		Modulation of thiol homeostasis	[125]
		Neuroprotection	[115]
ACS15*	Diclofenac	Anticancer	[126]
		Antiosteolysis	[127]
		Anti-inflammation	[128]
		Antiangiogenesis	[117]
ACS18	Sulindac	Anticancer	[126]
		Antiangiogenesis	[117]
ACS21	Salicylic acid	Protection against I/R injury	[124]
ACS32	Diclofenac	Antiosteolysis	[127]
ACS33	Valproic acid	Anticancer	[129]
		Inhibition of histone deacetylase activity	[129]
ACS67	Latanoprost	Regulation of insulin secretion	[114]
		Neuroprotection	[85]
ACS83	L-DOPA	Anti-inflammation	[130]
ACS84	L-DOPA	Anti-inflammation	[131]
		Neuroprotection	[132]
ACS85	L-DOPA	Anti-inflammation	[118]
ACS86	L-DOPA	Anti-inflammation	[118]
ATB-284	Unknown	Prevention against irritable bowel syndrome	[133]
ATB-337*	Diclofenac	Anti-inflammation	[134]
ATB-343	Indomethacin	Anti-inflammation	[135]
ATB-345	Naproxen	Anti-inflammation	[136]
ATB-346	Naproxen	Anti-inflammation	[136]
		Anticancer	[137]
ATB-429	Mesalamine	Anti-inflammation	[138]
		Abirritation	[139]
HS-aspirin (HS-ASA)	Aspirin	Anticancer	[140]
Compound **8e**	3-n-Butylphthalide	Antithrombosis	[141]
H2S-EXP 3174	Active metabolite of losartan	Vasorelaxation	[142]
NOSH-aspirin (NBS-1120)	Aspirin	Anticancer	[143]
		Anti-inflammation	[144]
NOSH-naproxen (AVT-219)	Naproxen	Anti-inflammation	[145]
NOSH-sulindac (AVT-18A)	Sulindac	Anti-inflammation	[145]
S-diclofenac*	Diclofenac	Anti-inflammation	[146]
		Protection against I/R injury	[146]
S-zofenopril	Zofenopril	Improvement of vascular function	[147]

*These compounds are remarkably similar to each other.

3. H_2S and I/R Injury

3.1. H_2S and Myocardial I/R Injury. Myocardial ischemia is a common clinical symptom characterized by low pH values, low oxygen, and high extracellular potassium concentration, which may cause arrhythmias, cardiac dysfunction, myocardial infarction, and sudden death [3, 5, 6]. The damaged myocardial structure and decreased heart function induced by ischemia can be repaired with subsequent reperfusion. The effectiveness of reperfusion depends on the duration and severity of prior ischemia [6, 39]. However, myocardial reperfusion could also activate a complex inflammatory response, which may finally lead to myocardial ischemia/reperfusion injury (MIRI), such as arrhythmias, myocardial stunning, microvascular dysfunction, and myocyte death [2, 40]. Therefore, it is necessary to develop effective cardioprotective strategies and agents against MIRI to improve myocardial function and to reduce the risk of cardiovascular events [4]. H_2S is now considered as an endogenous signaling molecule which plays an important role in the cardiovascular system [6, 15, 27]. In the heart, H_2S is produced in the fibroblasts, myocardium, and blood vessels from L-cysteine by CSE, CBS, and 3-MST and accumulates at relatively high local concentrations [6, 27, 30]. An accumulating body of evidence indicates that exogenous or endogenous H_2S could exert cardioprotection against MIRI in cardiac myocytes, isolated hearts, and intact animals. However, it is currently difficult to define the precise underlying mechanisms for this protection. A summary of what is known about the mechanisms by which H_2S and its donors-induced cardioprotection against MIRI is shown in Table 3.

3.2. H_2S and Hepatic I/R Injury. Liver I/R-induced injury represents a continuum of organic processes that could produce profound liver damage and ultimately lead to morbidity and mortality [41, 42]. Hepatic I/R injury has now been considered a worldwide health problem and usually occurs in liver transplantation, hemorrhagic shock and resuscitation, trauma, liver resection surgery, and aortic injury during abdominal surgery [41–43]. Hepatic I/R injury can be categorized into warm I/R and cold storage reperfusion injury, which share a common mechanism in the disease aetiology [41, 42]. Increasing number of experimental and clinical studies indicate that pathways/factors involved in the hepatic I/R injury include liver Kupffer cells and neutrophils, intracellular calcium overload, oxidative stress, anaerobic metabolism, mitochondria, adhesion molecules, chemokines, and proinflammatory cytokines [41, 42, 44, 45]. Despite significant advances in surgical techniques and perioperative cares, hepatic I/R injury remains one of the major complications in hepatic resection and transplantation [46]. Novel agents/drugs exhibiting antioxidative, anti-inflammatory, and cytoprotective activities may be possible candidates for protecting the liver from I/R injury [46]. Recent studies have shown that H_2S could significantly attenuate hepatic I/R injury in several ways, including inflammation, apoptosis, oxidation, and AKT activation (Table 4). The results suggest that H_2S has a protective effect against hepatic I/R injury, and targeting H_2S may present a promising approach against I/R-induced liver injury.

3.3. H_2S and Renal I/R Injury. Acute kidney injury (AKI) is a common and serious complication of critical illness and is associated with high morbidity, mortality, and resource utilization [25, 47, 48]. Renal I/R injury is one of the leading causes of AKI in many clinical settings [47, 48]. Renal I/R injury often arises from shock and various surgical procedures such as kidney transplantation and resection [47–49]. H_2S plays important physiological and pathological roles in the kidney [48]. For instance, it participates in the control of renal function and increases urinary sodium excretion via both tubular and vascular actions in the kidney [50]. CSE deficiency in mice could lead to reduced renal H_2S production and increase severity of damage and mortality after renal I/R injury, which indicates that H_2S may play a role in alleviating renal I/R injury [14]. More recently, there is growing evidence regarding the beneficial effects of H_2S on ameliorating renal I/R injury mainly via a variety of antioxidant, antiapoptotic, and anti-inflammatory effects (Table 5). These studies indicate that H_2S and its donors may be of benefit in conditions associated with renal I/R injury, such as renal transplantation.

3.4. H_2S and Cerebral I/R Injury. Ischemic cerebrovascular disease is one of the most common disorders that greatly threaten human health with high morbidity, disability, and mortality [51]. Cerebral I/R injury is mainly characterized by a deterioration of ischemic but potentially salvageable brain tissue of an ischemic injury after reperfusion [52, 53]. There are a number of risk factors involved in cerebral I/R injury, such as excitotoxicity, mitochondrial dysfunction, formation of free radicals, breakdown of the blood-brain barrier (BBB), edema, neuroinflammation, and apoptosis [52–54]. Emerging evidences indicate that H_2S functions not only as a neuromodulator, but also as a neuroprotectant in the central nervous system [18, 55–57]. In an *in vivo* model of cerebral I/R injury, treatment with low concentration of H_2S decreased the infarct size and improved the neurological function via antiapoptotic effect, implying that H_2S has a therapeutic role in cerebral ischemic stroke [18, 57]. DAS, an H_2S donor, could also protect the brain from I/R injury partly via its antiapoptotic effects [58]. ADT, another H_2S donor, decreased the infarct size and protected BBB integrity by suppressing local inflammation and nicotinamide adenine dinucleotide phosphate oxidase 4-derived ROS generation [55]. However, it is notable that the effects of H_2S on cerebral I/R injury are controversial [56]. Treatment with a higher dose of exogenous H_2S donor could deteriorate the effects of cerebral I/R injury [18, 59]. These opposite effects of H_2S on cerebral I/R injury may be partially associated with the concentration of H_2S in brain. This research offers a novel insight for future studies on the cytoprotective effects of a proper dose of H_2S on central nervous system degenerative diseases, such as Alzheimer's disease and Parkinson's disease.

3.5. H_2S and Intestinal I/R Injury. Intestinal I/R injury is considered to be a major and frequent problem in many clinical conditions, including intestinal mechanical obstruction, abdominal aortic aneurysm surgery, cardiopulmonary

Table 3: Effects of H_2S and its donors in myocardial I/R injury.

Experimental models	Effects	Proposed mechanisms	References
Myocardial I/R *in vivo* (rat)	NaHS (0.2 mg/kg, prior to R) protects against the effects of haemorrhage-induced I/R	Upregulation of the protein kinase B/endothelial nitric oxide synthase pathway	[148]
Regional myocardial I/R *in vivo* (rat)	NaHS (3 mg/kg, 15 min prior to I) shows cardioprotective effects	Combination of antiapoptotic and anti-inflammatory effects	[149]
Isolated perfused heart *ex vivo* (rat)	NaHS (100 μM, plus histidine buffer solution, prior to R) enhances cardiac performance	Prevention of apoptosis and preservation of the phosphorylative system	[150]
Isolated perfused heart *ex vivo* (rat)	NaHS (0.1–100 μM, at the onset of R) protects rat heart against I/R injury	Mitochondrial K_{ATP} channel opening	[151]
Primary cultured neonatal cardiomyocytes (rat)	NaHS (25–200 μM, 30 min prior to H) protects cardiomyocytes from oxidative stress	Inhibition of mitochondrial complex IV and enhancement of SOD activity	[152]
Isolated perfused heart *ex vivo* (rat)	NaHS (10 μM, at the onset of R) protects isolated rat hearts from I/R injury	Activation of the Janus kinase 2/signal transducer and activator of transcription 3 signaling pathway	[153]
Isolated perfused heart *ex vivo* (rat)	NaHS (40 μM, throughout the experiment) provides myocardial protection	Possibly activation of the expression of heat shock protein 72	[154]
Isolated perfused heart *ex vivo* (rat)	L-cysteine (0.1–10 mM, 10 min before I until 10 min after R) induces limitation of infarct size	Dependent on H_2S synthesis	[155]
Myocardial I/R *in vivo* (rat)	NaHS (14 μM/kg, 7 days before myocardial I/R) significantly reduces the myocardial infarct size	Antiapoptotic, antioxidative, and anti-inflammatory activities	[156]
Isolated perfused heart *ex vivo* (rat)	NaHS (100 μM, prior to I) significantly decreases the duration and severity of I/R-induced arrhythmias	Mitochondrial K_{ATP} channel opening	[157]
Isolated perfused heart *ex vivo* (rat)	NaHS (100 μM, prior to I) significantly decreases myocardial infarct size and improves heart contractile function	Activation of K_{ATP}/PKC/ERK1/2 and PI3K/Akt pathways	[158]
Isolated cardiac myocytes (rat)	NaHS (100 μM, prior to I) increases cell viability, percentage of rod-shaped cells, and myocyte contractility	K_{ATP}/PKC dependent induction of COX-2 expression and nitric oxide-induced COX-2 activation	[159]
Myocardial I/R *in vivo* (mice)	H_2S (100 ppm, prior to I) has protective properties in I/R injury	Reduction of myocardial ROS production and the inhibition of inflammation, necrosis, and fibrogenesis	[36]
Regional myocardial I/R *in vivo* (pig)	Na_2S (100 μg/kg bolus + 1 mg/kg/hr infusion, 10 min prior to R) improves myocardial function and reduces infarct size	Anti-inflammatory properties	[160]
Regional myocardial I/R *in vivo* (pig)	Na_2S (100 μg/kg bolus + 1 mg/kg/hr infusion, throughout the experiment) reduces myocardial infarct size	Antiapoptotic activities	[161]
Regional myocardial I/R *in vivo* (rat)	NaHS (0.1–10 μM, 10 min prior to I until 10 min into R) results in a concentration-dependent limitation of infarct size	Mitochondrial K_{ATP} channel opening	[162]
Myocardial I/R *in vivo* (rat)	NaHS (0.2 mg/kg, prior to R) protects against the effects of haemorrhage-induced I/R	Protection against oxidative stress	[163]
Primary cultured neonatal cardiomyocytes (rat)	NaHS (1–100 μM, 30 min prior to H) shows concentration-dependent inhibitory effects on cardiomyocyte apoptosis induced by H/R	Induction of phosphorylation of GSK-3 and inhibition of mitochondrial permeability transition pore opening	[164]
Myocardial I/R *in vivo* (mice)	Na_2S (0.1 mg/kg, 7 days prior to I) attenuates myocardial I/R injury	Activation of nuclear factor erythroid-2-related factor-2 signaling in an Erk-dependent manner	[165]

Table 3: Continued.

Experimental models	Effects	Proposed mechanisms	References
Myocardial I/R *in vivo* (rat)	NaHS (14 μM/kg, 7 days prior to I) inhibits apoptosis of cardiomyocytes induced by myocardial I/R	Enhancement of the phosphorylation of apoptosis repressor with caspase recruitment domain	[166]
Myocardial I/R *in vivo* (mice)	Na_2S (10–500 μg/kg, prior to R) limits infarct size and preserves left ventricular function	Inhibition of myocardial inflammation and preservation of both mitochondrial structure and function	[167]
Myocardial I/R *in vivo* (mice)	Na_2S (100 μg/kg, 1 h prior to I) reduces myocardial infarct size	miR-21-dependent attenuation of ischemic and inflammatory injury	[168]
Myocardial I/R *in vivo* (mice)	Na_2S (100 μg/kg, 24 h prior to I) reduces myocardial infarct size	Combination of antioxidant and antiapoptotic signaling	[169]
Isolated perfused heart *ex vivo* (rabbit)	Allitridum (60 μM, prior to I) reduces myocardial infarct size	Activation of PKC	[170]
Myocardial I/R *in vivo* (mice)	DATS (200 μg/kg, prior to R) significantly reduces infarct size and increases myocardial contractile function	Preservation of endogenous hydrogen sulfide and increase of nitric oxide bioavailability	[32]
Myocardial I/R *in vivo* (mice)	Na_2S (100 μg/kg, prior to R) protects against the structural and functional deterioration of the left ventricle	Protection against oxidative stress and mitochondrial dysfunction	[15]
Isolated perfused heart *ex vivo* (rat)	NaHS (50 μM, prior or post to I) protects against cardiac I/R injury	Phosphorylation of mammalian target of rapamycin C2	[171]
Myocardial I/R *in vivo* (rat)	NaHS (3 mg/kg, 15 min prior to I) significantly reduces myocardial infarct size	Mitochondrial K_{ATP} channel opening	[172]
Primary cultured neonatal cardiomyocytes (rat)	NaHS (30 μM, 30 min prior to H) attenuates cardiomyocyte apoptosis and enhances cell viability	Protection of cardiomyocytes against I/R-induced apoptosis by stimulating Bcl-2	[173]
Isolated perfused heart *ex vivo* (mice)	Na_2S (10 μM, 40 seconds after the start of R) markedly improves the recovery of myocardial function	Nitric oxide synthase 3-dependent signaling pathway	[174]
Myocardial I/R *in vivo* (rat)	NaHS (14 μM/kg/d, 6 d prior to I) markedly reduces heart infarct size and has great improvement in blood pressure	Upregulation of survivin	[175]
Myocardial I/R *in vivo* (pig)	NaHS (0.2 mg/kg, prior to R) markedly reduces myocardial infarct size and improves regional left ventricular function	Higher expression of phospho-GSK-3β and lower expression of apoptosis-inducing factor	[176]

H/R: hypoxia/reoxygenation; SOD: superoxide dismutase; PKC: protein kinase C; ERK1/2: extracellular signal regulated kinase 1/2; PI3K (PtdIns3K): phosphatidylinositol 3-kinase; Akt (PKB): protein kinase B; COX-2: cyclooxygenase-2; ROS: reactive oxygen species; GSK-3: glycogen synthase kinase-3.

bypass, strangulated hernias, liver and intestinal transplantation, mesenteric artery occlusion, shock, and severe trauma [60–64]. This injury can lead to the development of systemic inflammatory response syndrome and multiple organ dysfunction syndrome [62, 63]. Although many advanced treatments have been applied to clinical research, the mortality induced by intestinal I/R injury remains very high [61, 63]. Therefore, it is urgent to develop new therapeutic agents/drugs for the treatment of intestinal I/R injury. Recent studies have shown that H_2S has anti-ischemic activity in the intestinal I/R model. NaHS could significantly reduce the severity of intestinal I/R injury and dramatically increase the activities of SOD and glutathione peroxidase (GSH-Px) in both serum and intestinal tissue, which suggests that H_2S protects against intestinal I/R injury by increasing the levels of antioxidant enzymes [63]. In addition, administration of NaHS after the onset of ischemia can attenuate I/R-induced damage of intestinal tissues both *in vitro* and *in vivo* [65]. These observations provide new insight regarding the potential use of H_2S as a therapeutic agent to limit intestinal I/R injury.

3.6. H_2S and Gastric I/R Injury. Gastric I/R injury is an important and common clinical problem which could lead to mucosal injury [66]. A number of clinical conditions contribute to gastric I/R injury, including peptic ulcer bleeding, vascular rupture or surgery, ischemia gastrointestinal disease, and hemorrhagic shock [66]. However, there are few satisfactory clinical methods in the treatment of gastric I/R injury [67]. H_2S has been found to play an important role in protecting against gastric I/R injury. Endogenous H_2S had a protective effect against gastric I/R in rats by enhancing the antioxidant capacity through increasing the contents of GSH and SOD [68]. Another study has shown that NaHS and L-cysteine could protect the gastric mucosa against I/R damage mainly mediated by altering mRNA expression and

Table 4: Effects of H_2S and its donors in hepatic I/R injury.

Experimental models	Effects	Proposed mechanisms	References
Hepatic I/R *in vivo* (rat)	NaHS (28 μM/kg, prior to R) attenuates the injured hepatic function and the synthetic action of hepatocytes	Inhibition of lipid peroxidation and inflammation reactions	[177]
Hepatic I/R *in vivo* (mice)	NaHS (1.5 mg/kg, 1 h prior to I) protects against hepatic I/R injuries	Activation of the PtdIns3K-AKT1 pathway	[17]
Hepatic I/R *in vivo* (rat)	NaHS (14 μM/kg, 30 min prior to I) significantly attenuates the severity of liver injury and inhibits the production of lipid peroxidation	Antioxidant and antiapoptotic activities	[46]
Hepatic I/R *in vivo* (rat)	DAS (1.75 mM/kg, 12–15 h prior to I) protects the liver from warm I/R injury	Induction of heme oxygenase-1 and inhibition of cytochrome P450 2E1	[178]
Hepatic I/R *in vivo* (mice)	Na_2S (1 mg/kg, 5 min prior to R) protects the murine liver against I/R injury	Upregulation of intracellular antioxidant and antiapoptotic signaling pathways	[179]
Hepatic I/R *in vivo* (mice)	H_2S (100 ppm, 5 min prior to R) protects the liver against I/R injury	Reduction of necrosis, apoptosis, and inflammation	[180]
Hepatic I/R *in vivo* (mice)	NaHS (14 and 28 μM/kg, 30 min prior to I) attenuates hepatic I/R injury	Weaken the apoptosis through the inhibition of c-Jun N-terminal protein kinase 1 signaling pathway	[181]
Hepatic I/R *in vivo* (rat)	NaHS (12.5, 25 and −50 μM/kg, 5 min prior to I) reduces liver damage after perioperative I/R injury	Inhibition of mitochondrial permeability transition pore opening, reduction of cell apoptosis, and activation of Akt-GSK-3β signaling	[182]

Table 5: Effects of H_2S and its donors in renal I/R injury.

Experimental models	Effects	Proposed mechanisms	References
Renal I/R *in vivo* (mice)	NaHS (1 mg/kg, 15 min prior to I) rescues mice from the injury and mortality	Modulation of oxidative stress	[14]
Renal I/R *in vivo* (mice)	H_2S (100 ppm, before and after treatment) shows protective effects on survival, renal function, apoptosis, and inflammation	A hypometabolic state induced by H_2S	[183]
Renal I/R *in vivo* (pig)	Na_2S (100 μg/kg, 10 min prior to R) results in a marked reduction in kidney injury and preserves glomerular function	Anti-inflammatory effects	[184]
Isolated perfused kidney *ex vivo* (pig)	H_2S (0.5 mM, 10 min before and after R) ameliorates the renal dysfunction	Activation of K_{ATP} channels	[185]
Renal I/R *in vivo* (mice)	NaHS (100 μM/kg, 30 min prior to I) significantly attenuates I/R injury-induced renal dysfunction	The increase in expression of CSE	[186]
Renal I/R *in vivo* (rat)	NaHS (100 μM/kg, 15 min prior to I and 5 min prior to R) attenuates renal I/R injury	Antiapoptotic and anti-inflammatory effects	[187]
Warm renal I/R *in vivo* (rat)	NaHS (150 μM, at time of renal pedicle clamping and during R) improves long-term renal function and decreases long-term inflammation	Antiapoptotic and anti-inflammatory effects	[188]
Warm renal I/R *in vivo* (rat)	NaHS (150 μM, during I and R) increases renal capillary perfusion and improves acute tubular necrosis and apoptosis	Decrease of leukocyte migration and inflammatory responses	[189]
Renal I/R *in vivo* (pig)	Na_2S (2 mg/kg, 2 h prior to I) attenuates tissue injury and organ dysfunction	Antioxidant and anti-inflammatory effects	[190]
Renal I/R *in vivo* (rat)	NaHS (100 μg/kg, 20 min prior to I or 10 min prior to R) protects against renal I/R injury	Antioxidant and antiapoptotic effects	[191]

plasma release of proinflammatory cytokines [69]. Furthermore, NaHS and L-cysteine also showed gastroprotective effects against I/R injury by Keap1 s-sulfhydration, nuclear factor-kappa B dependent anti-inflammation, and mitogen-activated protein kinase dependent antiapoptosis pathway [66]. Thus, H_2S and its donors may have potential therapeutic value in acute gastric mucosal lesion, which is often caused by I/R.

3.7. H_2S and Hind-Limb I/R Injury. I/R injury can occur in skeletal muscle during elective surgery (i.e., free tissue transfer) and lower extremity arterial occlusion [70, 71]. Limb I/R injury may result in a series of postreperfusion syndromes, such as crush syndrome, compartment syndrome, and myonephropathic-metabolic syndrome [72]. Currently, clinical practice mainly focuses on reducing the duration of ischemia to minimize the ischemic injury in skeletal muscle [70, 71]. Therapeutic interventions that change the biochemical environment during the ischemic and/or reperfusion period may result in amelioration of subsequent cellular damage [71]. Treatment with NaHS for 20 minutes before the onset of hind-limb ischemia or reperfusion could result in significant protection against the cellular damage induced by I/R [71, 73]. However, administration of NaHS for 1 minute before reperfusion did not show any protection against limb I/R Injury [73]. Whether H_2S could protect against limb I/R injury in a dose- and time-dependent manner needs further investigation.

3.8. H_2S and Lung I/R Injury. Lung I/R injury occurs in various clinical conditions such as lung transplantation, cardiopulmonary bypass, trauma, cardiac bypass surgery, sleeve lobectomy, shock, pulmonary embolism, resuscitation from circulatory arrest, and reexpansion pulmonary edema [16, 74–77]. Lung I/R injury is characterized by increased pulmonary vascular resistance, worsened lung compliance, poor lung oxygenation, edema, and increased pulmonary endothelial permeability [16, 78]. Currently, there is no effective therapy available for the lung I/R injury. The precise mechanism of lung I/R injury needs to be further elucidated [16, 74]. A recent study has shown that preperfusion with H_2S could attenuate the lung I/R injury by reducing lung oxidative stress [16], which suggests that administration of H_2S or its donors might be a novel preventive and therapeutic strategy for lung I/R injury.

3.9. H_2S and Retinal I/R Injury. Retinal I/R injury is a common clinical condition and is associated with the loss of neurons, morphological degeneration of the retina, loss of retinal function, and ultimately vision loss [79, 80]. Emerging evidence suggests that retinal I/R injury plays an important role in the pathologic processes of several ocular diseases such as diabetic retinopathy, retinopathy of prematurity, acute glaucoma, and retinal vascular occlusion [81, 82]. Retinal I/R injury often results in visual impairment and blindness because of the lack of effective treatment [81, 83]. One recent study has indicated that rapid preconditioning with inhaled H_2S can mediate antiapoptotic effects and thus protect the rat retina against I/R injury [84]. ACS67, a H_2S-releasing derivative of latanoprost acid, possesses neuroprotective properties and could attenuate retinal ischemia *in vivo* and decrease the oxidative insult to RGC-5 cells (retinal ganglion cells) *in vitro* [85]. These results suggest that H_2S represents a novel and promising therapeutic agent to counteract neuronal injuries in the eye [84]. Further studies are needed to prove the neuroprotective propensity of H_2S in retinal I/R injury using a postconditioning approach.

4. Concluding Remarks

H_2S is now considered as the third signaling gasotransmitter which plays a broad range of physiological and pathophysiological functions, including vascular relaxation, induction of angiogenesis, regulation of neuronal activity, and glucose homeostatic regulation. H_2S can be endogenously generated via both enzymatic and nonenzymatic pathways and mainly metabolized through three pathways in mammals. However, whether H_2S could be generated and metabolized via another pathway should be further studied and confirmed. In addition, more efforts should be made to illuminate the expressions and functions of H_2S-generating enzymes in different organ and tissue. In order to increase the treatment efficacy of H_2S, a number of slow-releasing H_2S donors and H_2S-releasing hybrid drugs have been successfully designed, synthesized, and proved to be effective *in vitro*, *ex vivo*, and *in vivo*. Novel synthetic strategy should be developed to extend the exposure time of H_2S donor. Agents/drugs with antiapoptotic, antioxidative, anti-inflammatory, and antitumor effects could be conjugated with H_2S donor to enhance their therapeutic effects. Furthermore, new drug targeting carrier systems should be designed to effectively transport the H_2S donor to the targeted organ or tissue.

I/R is a pathological condition that is characterized by an initial deprivation of blood supply to an area or organ followed by the subsequent restoration of perfusion and concomitant reoxygenation. Novel mechanisms associated with I/R need to be further studied and illuminated in addition to the existing pathophysiologic mechanisms. Increasing number of studies have shown that H_2S could protect against I/R injury in many organs and tissues, such as heart, liver, kidney, brain, intestine, stomach, hind-limb, lung, and retina. Whether H_2S could exert protection against I/R injury in other organs and/or tissues need to be further demonstrated. In addition, the molecular targets of H_2S in I/R injury are also needed to be clarified. Ischemic preconditioning, conditioning, and postconditioning are three time frames in the protection against I/R injury. Proper time frame and optimal duration of treatment should be confirmed according to the physicochemical property of H_2S-releasing compounds. Considering different doses of H_2S-releasing compounds may exert different therapeutic effects, proper dose range should also be further explored to obtain a better therapeutic efficacy. Currently, researches into the molecular mechanisms of H_2S in I/R injury using animal experiments have made some progress. Clinical evidence-based research should also be useful in further exploring the little-understood field of the role of H_2S in I/R injury. In addition, longer-term studies are

required to determine whether H_2S treatment permanently improves organ function following I/R injury and whether this effect reduces long-term morbidity and mortality.

In conclusion, with the rapid developments of design and synthetic strategies, as well as better understanding of the precise mechanisms behind the role of H_2S in I/R injury, treatment with H_2S or its donors in proper dose range and time frame will exhibit more potent therapeutic effects against I/R injury in further preclinical research and clinical application.

Conflict of Interests

The authors declare no conflict of interests related to this work.

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Research Article

Cardiac H_2S Generation Is Reduced in Ageing Diabetic Mice

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Aims. To examine whether hydrogen sulfide (H_2S) generation changed in ageing diabetic mouse hearts. *Results*. Compared to mice that were fed tap water only, mice that were fed 30% fructose solution for 15 months exhibited typical characteristics of a severe diabetic phenotype with cardiac hypertrophy, fibrosis, and dysfunction. H_2S levels in plasma, heart tissues, and urine were significantly reduced in these mice as compared to those in controls. The expression of the H_2S-generating enzymes, cystathionine γ-lyase and 3-mercaptopyruvate sulfurtransferase, was significantly decreased in the hearts of fructose-fed mice, whereas cystathionine-β-synthase levels were significantly increased. *Conclusion*. Our results suggest that this ageing diabetic mouse model developed diabetic cardiomyopathy and that H_2S levels were reduced in the diabetic heart due to alterations in three H_2S-producing enzymes, which may be involved in the pathogenesis of diabetic cardiomyopathy.

1. Introduction

The consumption of soft drinks, which contain high concentrations of fructose, has markedly increased during the last three decades. This has paralleled the increased prevalence of obesity and insulin resistance that are associated with the development of type 2 diabetes and cardiovascular disease [1, 2]. Meta-analyses have also suggested that the consumption of fructose primarily from soft drinks is related to a risk for diabetes [3]. It has been predicted that by 2030, more than 366 million people, among which approximately 196 million people will be between the ages of 60 and 79 years, will be afflicted by type 2 diabetes [4].

The development of diabetic complications, such as cardiovascular disease, is a major cause of mortality among older diabetic patients. As first reported by Rubler et al. [5] in 1972, diabetic cardiomyopathy (DCM) is defined as structural and functional abnormalities in the myocardium in diabetic patients that occurs independently of coronary artery disease and/or hypertension. DCM is characterized by early-onset diastolic dysfunction and late-onset systolic dysfunction. It is a prolonged progression and finally results in heart failure. Multiple mechanisms for the development of DCM have been proposed, including excess oxidative stress, impaired calcium homeostasis, mitochondrial dysfunction, and activation of apoptotic signalling pathways [6–10]. Endothelial dysfunction markedly alters angiogenesis and induces micro- and macrovascular complications in the diabetic heart, which also further contributes to the aetiology of this disease [11]. However, the pathogenesis of DCM remains incompletely understood. Thus, more details are required to further delineate the basic mechanisms underlying DCM. Animal models in DCM research, such as those for drug treatment, high fat diets, and genetic mutations, are commonly used; however, none of the abovementioned models are without limitations. Thus, to better understand the pathogenesis of DCM, a long-term rodent model that possibly mimics human DCM would be advantageous.

Hydrogen sulfide (H_2S) was recognized as the third gasotransmitter to be identified after nitric oxide and carbon

monoxide and is synthesized endogenously from L-cysteine primarily via the action of two enzymes, cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS) [12–14]. CSE is primarily involved in maintaining cardiovascular function, whereas CBS has an important role in the central and peripheral nervous systems [15, 16]. Recent studies also discovered a third H_2S producing enzyme, 3-mercaptopyruvate sulfurtransferase (3-MST), which generates H_2S in the brain as well as in the vascular endothelium [17, 18]. H_2S has been shown to provide robust protection to various organs after ischemia-reperfusion injury [19–21], stroke [22], and inflammatory disorders [23–25]. In recent years, accumulating evidence derived from cell culture, animal models, and clinical studies suggests that lower H_2S levels may play a role in the pathogenesis of diabetes mellitus and its associated complications [24, 26, 27]. However, it is not known whether H_2S generation is changed in ageing diabetic mice with DCM.

Therefore, the aims of this study were to investigate any functional and structural changes in ageing diabetic mouse hearts that resulted from long-term (15 months) feeding of a high-fructose diet and to examine for any changes in the levels of endogenous H_2S and expression of the three H_2S-producing enzymes involved in the pathogenesis of DCM in these mice.

2. Materials and Methods

2.1. Animals and Treatments. Male C57BL/6J mice (8-week-old) from the Department of Laboratory Animal Science, Fudan University were housed at constant temperature (22 ± 2°C) and humidity (60%) with a 12 h dark-light cycle and unrestricted access to food and water. After acclimatization for 2 weeks, mice were randomly divided into two different groups: one group received tap water and the other group received water that contained 30% fructose for 15 months. All our animal experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH) of the United States and approved by the Ethics Committee of Experimental Research, Fudan University Shanghai Medical College.

2.2. Glucose Tolerance and Insulin Tolerance Tests. For a glucose tolerance test, glucose levels were measured using glucose strips (Onetouch; Johnson) in blood obtained from the tail vein immediately prior to and at 15, 30, 60, 90, and 120 min after an intraperitoneal (IP) injection of a 25% glucose solution (2 g/kg body weight) into mice that were fasted for 16 h. Insulin sensitivity was tested by IP injection of 0.5 units/kg body weight of recombinant human insulin (Humulin 70/30, Eli Lilly and Company) and plasma glucose measurements were in tail vein blood obtained at 0, 15, 30, 60, 90, and 120 min after this injection in mice that were fasted for 4 h. Areas under the curve (AUC) were determined using the trapezoidal rule.

2.3. The 24 h Water and Food Intakes and 24 h Urine Volumes. To record 24 h water and food intakes and collect 24 h urine samples, individual mouse was placed in a metabolic cage (Tecniplast, Italy). Starting 3 days before the collection period, mice were acclimatized to this new environment for 6 h each day.

2.4. Echocardiography. To test left ventricular function, mouse two-dimensional echocardiography was performed using a Vevo770 ultrasound device (VisualSonics Inc.), as previously described [28]. Mice were anaesthetized with isoflurane (1%), and M-mode images of the left ventricle were recorded. All measurements were averaged for five consecutive cardiac cycles. Left ventricular internal dimension systole (LVIDs), left ventricular internal dimension diastole (LVIDd), left ventricular end-systolic volume (LVESV), left ventricular end-diastolic volume (LVEDV), ejection fraction and fractional shortening (LVEF and LVFS) were measured to evaluate heart function.

2.5. Biochemical Analyses. At the end of the experimental period, mice were fasted for 12 h and then euthanized with 6% chloral hydrate. Blood samples were collected, and their glucose levels were monitored using blood glucose strips (Onetouch; Johnson). Then, plasma was prepared by centrifuging the blood samples at 3000 rpm for 15 min. The plasma levels of triglycerides (TG), total cholesterol (CHO), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), blood urea nitrogen (BUN), and creatinine (Cr) were determined by automatic biochemical analyzer (Cobas 6000, Roche). The BUN/Cr index was calculated.

2.6. Morphological and Histological Analyses. A heart was surgically removed to determine the heart to body weight ratio (HW/BW × 100%). For histological analysis, the ventricles were excised, fixed in 10% formalin for 48 h before dehydration using a graded ethanol series, embedded in paraffin, sectioned at 4 μm thickness and stained with hematoxylin and eosin (HE) and with Masson's trichrome.

2.7. Immunofluorescence Microscopy. Paraffin embedded myocardial tissues were subjected to immunofluorescence for the detection of CBS (Santa Cruz Biotechnology Company) and CD31 (Abcam Company) which were incubated with the antibodies at a dilution of 1 : 100, overnight at 4°C. After washing, the sections were incubated with Alexa Fluor 488 and Alexa Fluor 594 (Life Technologies) secondary antibody at 37°C for 1 h in the dark. Then, sections were incubated with 4′,6-diamidino-2-phenylindole (DAPI) at room temperature for 5 min to stain nuclei. Fluorescent signals were observed under a fluorescence microscope (Olympus).

2.8. Western Blot Analysis. Frozen left ventricle tissues were lysed with ice-cold RIPA buffer. Proteins were extracted and quantified using BCA reagent (Shen Neng Bo Cai Corp.). Protein samples were separated on 10% SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore-Upstate). The membranes were blocked with 5% non-fat milk at room temperature for 1 h and then incubated with antibodies directed against CSE, CBS, 3-MST, Collagen I,

Bax, Bcl-2 (Santa Cruz Biotechnology Company), and Collagen III (Abcam Company) at 4°C overnight. After washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Specific bands were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific-Pierce).

2.9. Cell Culture and Treatment. Primary neonatal rat cardiac ventricular myocytes (NRCMs) were collected as previously described with some modifications [29]. Briefly, the ventricles of new born Sprague-Dawley rats (1–3 days old) regardless of sex were minced and digested with 0.125% trypsin. Isolated cardiomyocytes were cultured in Dulbecco's modified Eagle's medium/F-12 (DMEM/F12, Life Technologies) supplemented with 10% (v/v) fetal bovine serum (FBS, Thermo Fisher Hyclone), penicillin/streptomycin (100 units), and 0.1 mmol/L 5-bromo-2′-deoxyuridine and maintained in an incubator (37°C with 5% CO_2). Cells were then cultured in medium containing either normal glucose (5.5 mmol/L, NG group) which served as a normal control or high glucose (33 mmol/L, HG group) for 72 h. Meanwhile, different concentrations (10, 50 and 100 μmol/L) of sodium hydrosulfide (NaHS, a donor of H_2S) were added in the medium of the HG group, and the control cells were treated with the vehicle. NaHS treatment was repeated every 6 h during the entire treatment period of 72 h. L-Glucose (27.5 mmol/L) was added to medium containing normal glucose (5.5 mmol/L) to make osmotic pressure equal to high glucose.

2.10. Cell Viability Assays. The viability of NRCMs which were cultured in 96-well plates was measured by using the Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies), according to the manufacturer's instructions. The absorbance of CCK-8 was obtained with a microplate reader at 450 nm. The values were normalized to the NG group.

2.11. Annexin V–FITC/Propidium Iodide Staining for Detecting NRVMs Apoptosis. Cellular apoptosis was determined using the Annexin V–FITC apoptosis detection kit (Dojindo Molecular Technologies), according to the manufacturer's instructions. NRCMs were stained with Annexin V–FITC and propidium iodide (PI) and then the percentage of cell apoptosis was then determined using flow cytometry with a BD FACSCalibur platform (BD Biosciences). The apoptotic ratio was calculated according to the percentage of Annexin V positive apoptotic cells of the total cells. Fluorescent signals were also observed under a laser confocal microscope (Zeiss).

2.12. Detection of ROS Levels. ROS levels in NRVMs were determined by dihydroethidium (DHE, Sigma-Aldrich) fluorescence using confocal microscopy. After treatments for 72 h, cells were washed with PBS and incubated with DHE (10 μmol/L) at 37°C for 30 min in the dark. Then, DHE was removed by washing. Fluorescent signals were observed (excitation, 488 nm; emission, 610 nm) under a laser confocal microscope (Zeiss). The values were normalized to the NG group.

Table 1: Physiological and biochemical results for control and ageing diabetic mice induced by a 30% fructose solution fed for 15 months.

	Control ($n = 10$)	30% Fructose ($n = 16$)
Body weights (g)	27.90 ± 0.72	34.19 ± 0.70**
Total cholesterol (mM)	1.54 ± 0.04	2.00 ± 0.06**
Triglycerides (mM)	0.46 ± 0.04	0.38 ± 0.02
HDL cholesterol (mM)	1.13 ± 0.06	1.30 ± 0.07
LDL cholesterol (mM)	0.26 ± 0.02	0.47 ± 0.04**
BUN (mM)	6.68 ± 0.49	6.07 ± 0.34
Cr (μM)	9.56 ± 0.5	7.54 ± 0.42**
BUN/Cr index	0.7 ± 0.04	0.82 ± 0.05

Results are means ± SEM. $^{**}P < 0.01$ versus control; HDL, high density lipoprotein; LDL: low-density lipoprotein; BUN: blood urea nitrogen; Cr: creatinine; BUN/Cr blood urea nitrogen/creatinine.

2.13. Measurement of H_2S Content. H_2S levels in plasma, urine, and cell culture medium were measured according to previously described methods [30], and H_2S levels in heart tissues and NRCMs were measured with some modifications. Briefly, heart tissues and NRCMs were homogenized in ice-cold Tris-HCl (100 mmol/L, pH 8.5) followed by centrifugation at 12,000 g for 20 min at 4°C. Thirty μL supernatant was used to detect H_2S and proteins in the supernatant were quantified using BCA reagent (Shen Neng Bo Cai Corp.). H_2S concentrations were determined using a curve generated with sodium sulfide (0–40 μmol/L) standards, and the H_2S concentrations in plasma, urine, and cell culture medium were expressed as μmol/L. H_2S concentrations in heart tissues and NRCMs were divided by the protein concentrations and were expressed as μmol/g of protein.

2.14. Statistical Analyses. Results were expressed as means ± SEM. Statistical analysis was performed using an SPSS software package, version 13.0 (SPSS, Inc., Chicago, IL, USA). The results for three or more groups were compared using one-way ANOVA followed by Student-Newman-Keuls test. Comparisons between two groups were made using Student's t-test. P of <0.05 was considered significant.

3. Results

3.1. Long-Term High-Fructose Feeding Induces Obesity and Type 2 Diabetes in Mice. Compared to mice that were fed water only, mice that were fed a 30% fructose solution for 15 months exhibited characteristics typical of a severe diabetic phenotype, including marked obesity, hyperglycaemia, and dyslipidemia, but there were no differences in BUN and BUN/Cr index (Table 1, Figure 1(a)). The mice that were fed with fructose solution also had increased 24 h urine volumes and water intake at the end of treatment as compared with those of the controls, although there were no differences in their 24 h food intakes (Table 2).

Regarding glucose tolerance and insulin tolerance tests, as expected, mice that were fed the fructose solution developed both impaired glucose tolerance and insulin resistance (Figures 1(b)–1(e)).

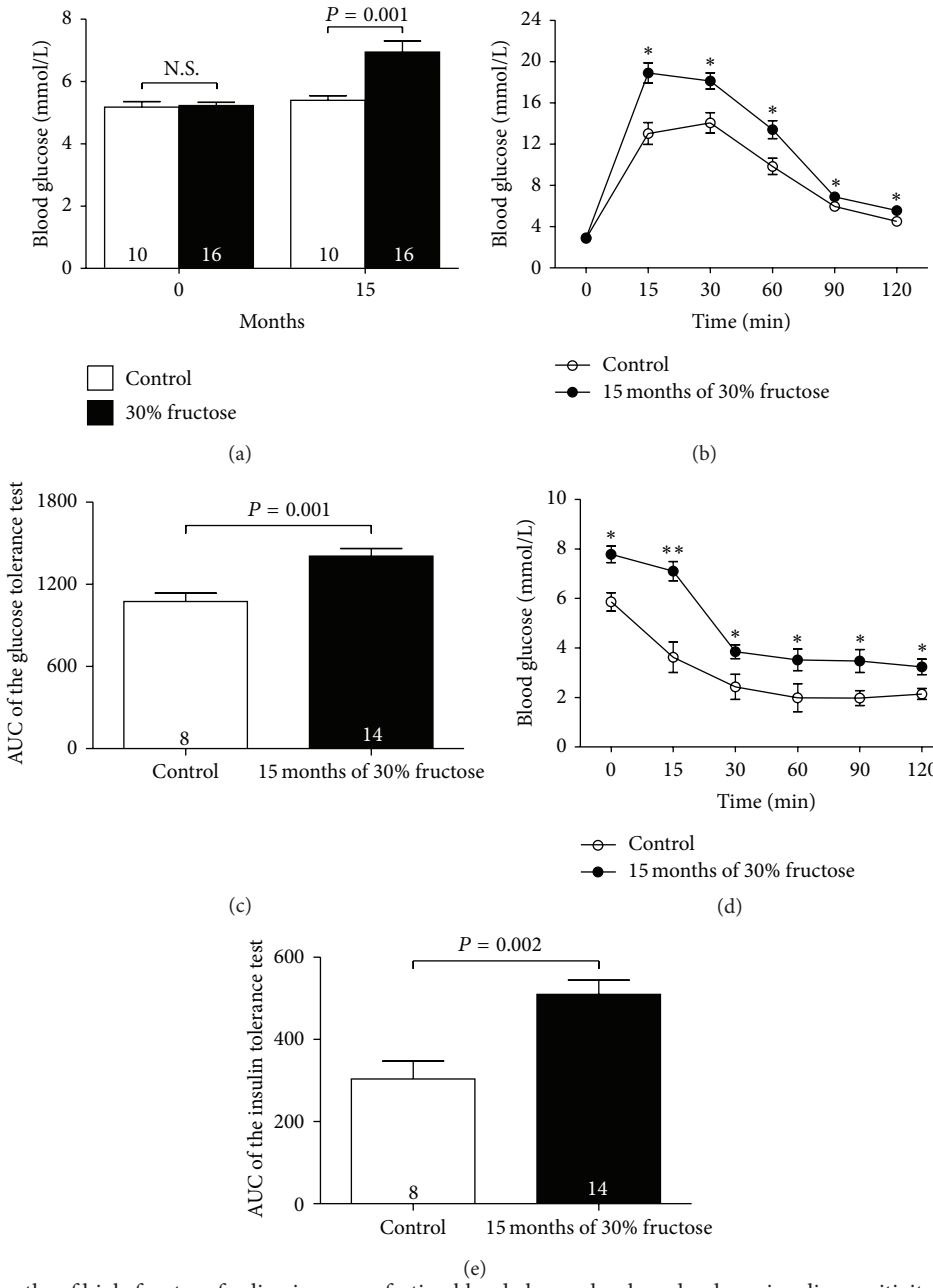


Figure 1: Fifteen months of high-fructose feeding increases fasting blood glucose levels and reduces insulin sensitivity and glucose tolerance in mice. (a) Fasting blood glucose levels of control and high-fructose-fed mice at the beginning and after 15 months of the experimental period. (b) Representative glucose tolerance test curves for control and high-fructose-fed mice after 15 months. (c) Area under the curve (AUC) of glucose tolerance test results was determined for each animal using the trapezoidal rule. (d) Representative insulin tolerance test curves for control and high-fructose-fed mice after 15 months. (e) Area under the curve (AUC) of insulin tolerance test results was determined for each animal using the trapezoidal rule. Results are means ± SEM. $^{*}P < 0.05$ versus control; $^{**}P < 0.01$ versus control.

Table 2: Twenty-four-hour metabolic characteristics of control and ageing diabetic mice induced by a 30% fructose solution fed for 15 months.

	Control ($n = 9$)	30% Fructose ($n = 16$)
24 h water intake (mL)	3.74 ± 0.31	6.04 ± 0.38**
24 h food intake (g)	0.62 ± 0.19	0.32 ± 0.15
24 h urine volume (mL)	1.02 ± 0.16	2.13 ± 0.25**

Results are means ± SEM. $^{**}P < 0.01$ versus control.

3.2. Ageing Diabetic Mice Exhibit Cardiac Dysfunction. To assess the effects of the long-term high-fructose diet on cardiac function, we used echocardiography to measure cardiac physiological parameters. The representative M-mode images were showed in Figure 2(a). Echocardiography examinations revealed that the left ventricular ejection fraction (LVEF) and fractional shortening (LVFS) were significantly reduced in these mice (Figures 2(b) and 2(c)), whereas the left ventricular internal dimension systole (LVIDs), left

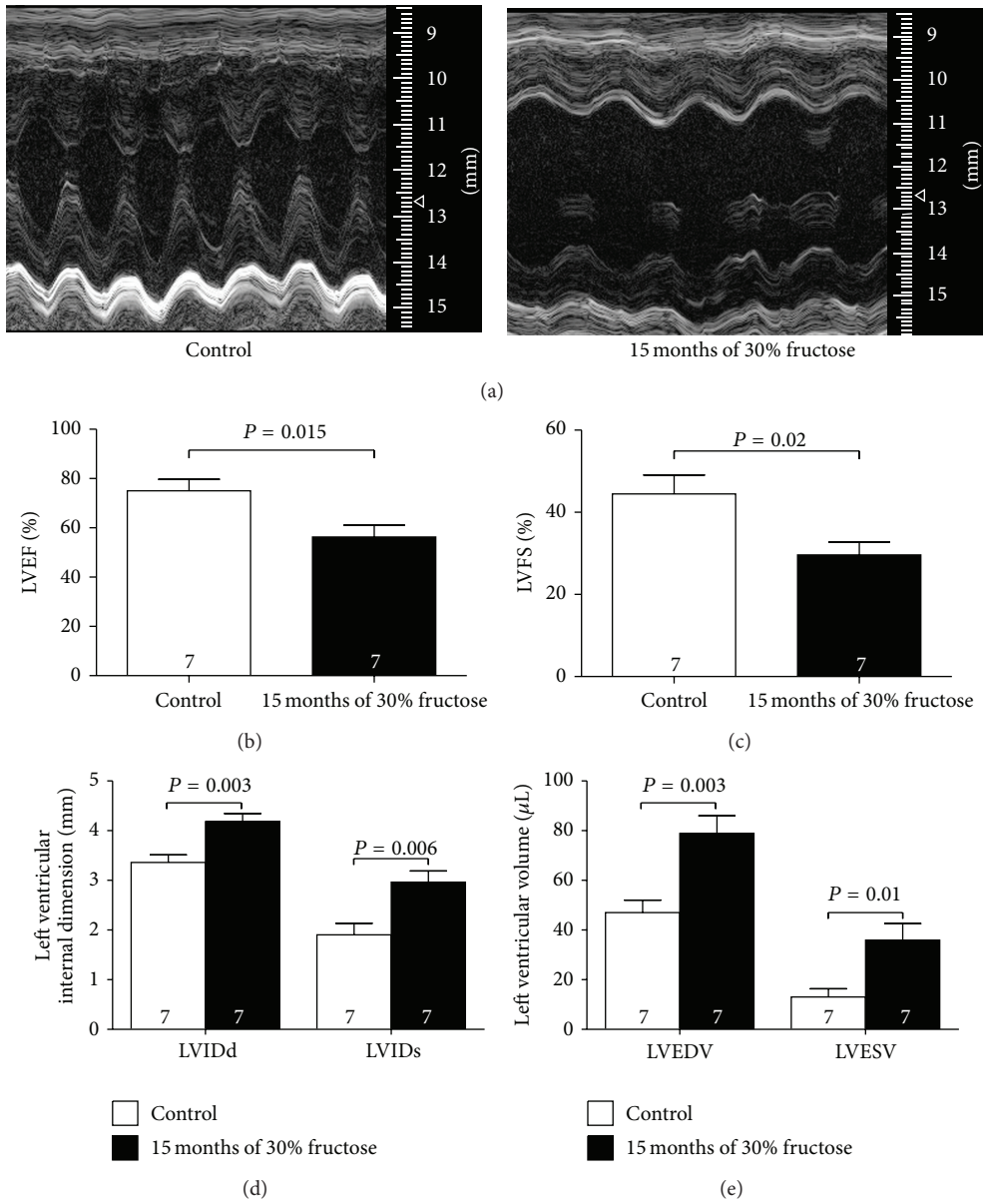


Figure 2: Fifteen months of high-fructose feeding induces cardiac dysfunction. (a) Representative M-mode images. (b–e) Echocardiographic parameter analysis. LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; LVIDs, left ventricular internal dimension systole; LVIDd, left ventricular internal dimension diastole; LVESV, left ventricular end-systolic volume; LVEDV left ventricular end-diastolic volume. Results are means ± SEM. A P of <0.05 was considered significant.

ventricular internal dimension diastole (LVIDd), left ventricular end-systolic volume (LVESV), and left ventricular end-diastolic volume (LVEDV) were increased in the high-fructose-induced diabetic mice (Figures 2(d) and 2(e)). These findings indicated that these mice had impaired cardiac function.

3.3. Ageing Diabetic Mice Exhibit Cardiac Remodelling and Apoptosis. After 15 months of high-fructose feeding, increased HW/BW ratio (Figure 3(a)) and increased cardiomyocyte cross-sectional areas (CSA) were found in the ageing diabetic mice (Figures 3(b) and 3(c)). Masson's trichrome staining showed markedly increased interstitial collagen volumes in these mice as compared with those of the controls (Figure 3(d)). There was also higher expression of Collagen I and Collagen III proteins in these ageing diabetic mice (Figure 3(e)). In addition, Bax/Bcl-2 ratio was also significantly increased in the myocardium of these mice (Figure 3(f)). All of these indicated that myocardial remodelling and apoptosis had occurred in the ageing diabetic mice.

3.4. Reduced H_2S Production in Ageing Diabetic Mice. In the long-term high-fructose-induced diabetic mice, H_2S levels in both plasma and urine were significantly lower than those

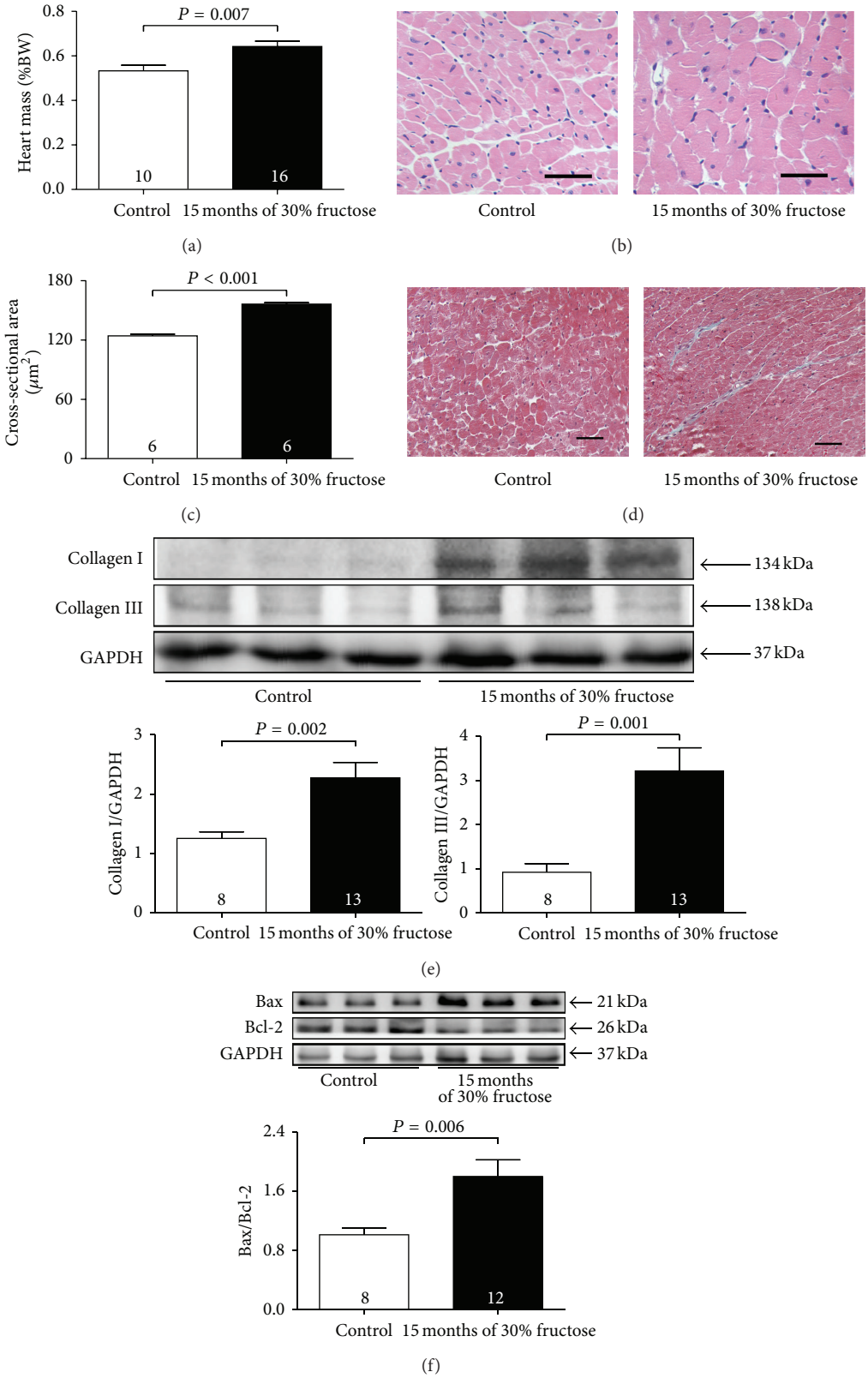


Figure 3: Fifteen months of high-fructose feeding induces cardiac remodelling and apoptosis. (a) Heart to body weight ratio (HW/BW × 100%). (b) Representative HE-stained left ventricular sections (scale bar = 250 μm). (c) Quantitative analysis of cross-sectional areas (CSA). (d) Representative Masson's trichrome-stained left ventricular sections (scale bar = 250 μm). (e) Representative Western blots and quantitative analysis for Collagen I and Collagen III protein expression in the myocardium. GAPDH was used as the internal control. (f) Representative Western blots and quantitative analysis for Bax and Bcl-2 protein expression in the myocardium. GAPDH was used as the internal control. Results are means ± SEM. A P of <0.05 was considered significant.

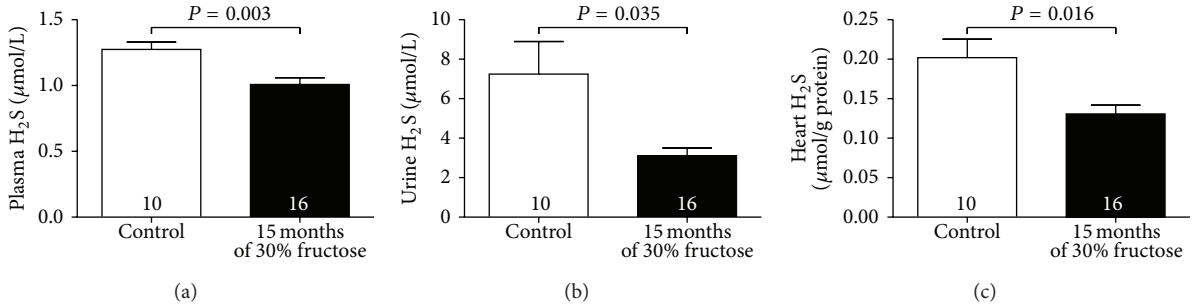


Figure 4: Fifteen months of high-fructose feeding results in reduced H_2S levels in plasma, urine, and heart tissues. (a) H_2S levels in plasma, (b) H_2S levels in urine, and (c) H_2S levels in heart tissues. Results are means ± SEM. A P of <0.05 was considered significant.

in control mice (Figures 4(a) and 4(b)). We also examined whether H_2S production was reduced in the diabetic heart. As shown in Figure 4(c), H_2S production was significantly reduced in the left ventricular tissues of these mice as compared with that in the controls.

3.5. Expression of H_2S-Producing Enzymes Is Altered in Ageing Diabetic Mice. Because the H_2S levels were low in the diabetic heart and H_2S production depends on CBS, CSE, and 3-MST enzymes, we determined the expression levels of these three enzymes in heart samples. Western blot analysis revealed bands of 61 kDa, 45 kDa, and 33 kDa, which corresponded to CBS, CSE, and 3-MST, respectively. CSE and 3-MST protein expression levels were significantly reduced in the high-fructose-induced diabetic mice as compared with those of controls, whereas CBS protein expression levels were significantly increased in the heart tissues of diabetic mice (Figure 5(a)). The double-staining immunofluorescence showed that CBS protein expression was significantly increased in cardiomyocytes and interstitial but not in coronary vessels (Figure 5(b)).

3.6. Reduction of Endogenous H_2S Involves in High Glucose-Induced Myocardial Injury. To confirm whether endogenous H_2S is involved in the diabetic myocardial injury, NRCMs were incubated in normal glucose (5.5 mmol/L) and high glucose (33 mmol/L) for 72 h to mimic the hyperglycemia in DCM *in vitro*. As shown in Figure 6, NRCMs which were exposed to high glucose resulted in a significantly decreased cell viability (Figure 6(a)), increase in apoptosis rate (Figures 6(b) and 6(c)), and overproduction of ROS (Figures 6(d) and 6(e)). Meanwhile, high glucose-induced myocardial injury was accompanied by a decrease of H_2S levels in NRCMs (Figure 6(f)).

3.7. Exogenous H_2S Attenuates High Glucose-Induced Myocardial Injury. To determine whether exogenous H_2S attenuated high glucose-induced myocardial injury, different concentrations (10, 50, and 100 μmol/L) of NaHS (a donor of H_2S) were added in the media of the HG group, which were repeated every 6 hours during the entire treatment period of 72 h. After adding NaHS in to the cell culture media, H_2S concentration peaked around 5–30 min and diminished afterwards eventually (Figure 7(a)). H_2S levels in NRCMs were also increased at the end of treatment (Figure 7(b)). Exogenous H_2S could suppress the high glucose-induced myocardial injury, leading to an increase in cell viability (Figure 7(c)) and a decrease in apoptotic rate (Figures 7(d) and 7(e)), preventing ROS generation (Figures 7(f) and 7(g)).

4. Discussion

In this study, we established an ageing diabetic mouse model by feeding mice water with 30% fructose for up to 15 months to investigate any effects of long-term high-fructose feeding on the mouse cardiovascular system. This resulted in two important findings: (1) long-term high-fructose consumption was associated with diabetic cardiomyopathy (DCM) and (2) H_2S levels were reduced in the ageing diabetic heart because of alterations in the three H_2S-producing enzymes.

Despite recent advances in care and management, diabetes and its associated complications continue to be a major global public health problem, which is gradually worsening, particularly in the developing nations. Although genetic predisposition is an important aetiology of this disease, environmental factors, such as diet and physical activity, are also involved. In particular, long-term consumption of overly nutritious diets that are enriched in fructose and fats can cause initiation of obesity and insulin resistance, which result in development of type 2 diabetes and its associated complications [31, 32].

Although increased coronary atherosclerosis is the major cause of death among diabetic patients, particularly elderly patients, there is an increased risk for the development of heart failure that is independent of coronary artery disease and hypertension. This adverse situation is referred to DCM, which is characterized by cardiac remodelling, fibrosis, progressive cardiac dysfunction and independent of coronary artery disease [33, 34]. However, therapeutic strategies to effectively prevent or reduce diabetic heart failure are still unavailable because of our incomplete understanding of the underlying mechanisms. Thus, an animal model that can mimic the extremely protracted pathogenesis of DCM is required.

Animal models in DCM research are quite common. However, the drawback of these models is that they only

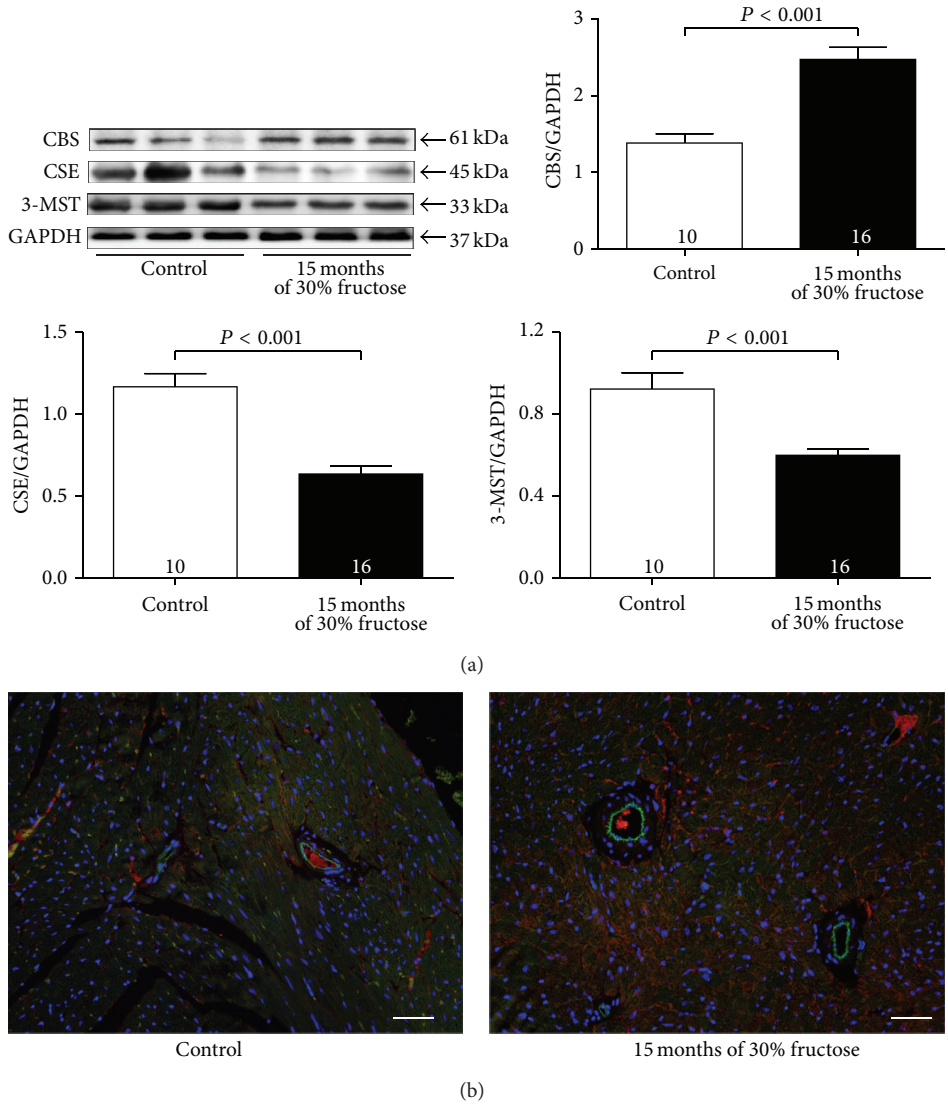


Figure 5: Fifteen months of high-fructose feeding alters CBS, CSE, and 3-MST protein expression. (a) Representative Western blots and quantitative analysis for CBS, CSE, and 3-MST expression in the myocardium. GAPDH was used as the internal control. (b) Representative double-staining immunofluorescence showing the distribution of CBS (red) in the cardiomyocytes and vessels (labelled by CD31, green) from control or ageing diabetic mice (scale bar = 250 μm). Results are means ± SEM. A P of <0.05 was considered significant.

mimic a short term for DCM but do not mimic it long term. Thus, to better understand the pathogenesis of DCM, a long-term rodent model mimicking as best as possible human DCM would be of great help. In this study, ageing diabetic mice were induced by feeding with a 30% fructose water solution for 15 months (at the end, mice were 17 months old), and these mice were overweight, hyperglycaemic, insulin resistant, and dyslipidemic by the end of the study.

This long-term fructose feeding also caused morphological changes in mouse heart tissue, increased interstitial collagen deposition and expression and increased heart/body weight ratios, indicative of cardiac hypertrophy and fibrosis. The increased Bax/Bcl-2 ratio also indicated cardiomyocyte apoptosis in these mice. M-mode echocardiography confirmed that LVEF and LVFS were significantly reduced along with an increased LV volume, which suggested hyperglycaemia-induced cardiac dilation and dysfunction. In contrast, mice that were fed tap water only for the same period remained healthy. This was consistent with the results of previous reports that showed the developmental stages of cardiomyopathy in db/db diabetic mice [35, 36].

A number of mechanisms have been proposed to contribute to the development of diabetic cardiomyopathy, including increased oxidative stress [37], altered calcium homeostasis [38], activation of apoptotic signals [39], and reduced angiogenesis [40]. H_2S is the third gasotransmitter to be identified after nitric oxide and carbon monoxide, and it is endogenously generated by three enzymes: CBS, CSE, and 3-MST. H_2S is involved in numerous pathophysiological and physiological functions due to its antiapoptotic [20], antioxidative [21] anti-inflammatory [41, 42], and proangiogenic activities [43, 44] in mammals, and reduced endogenous H_2S

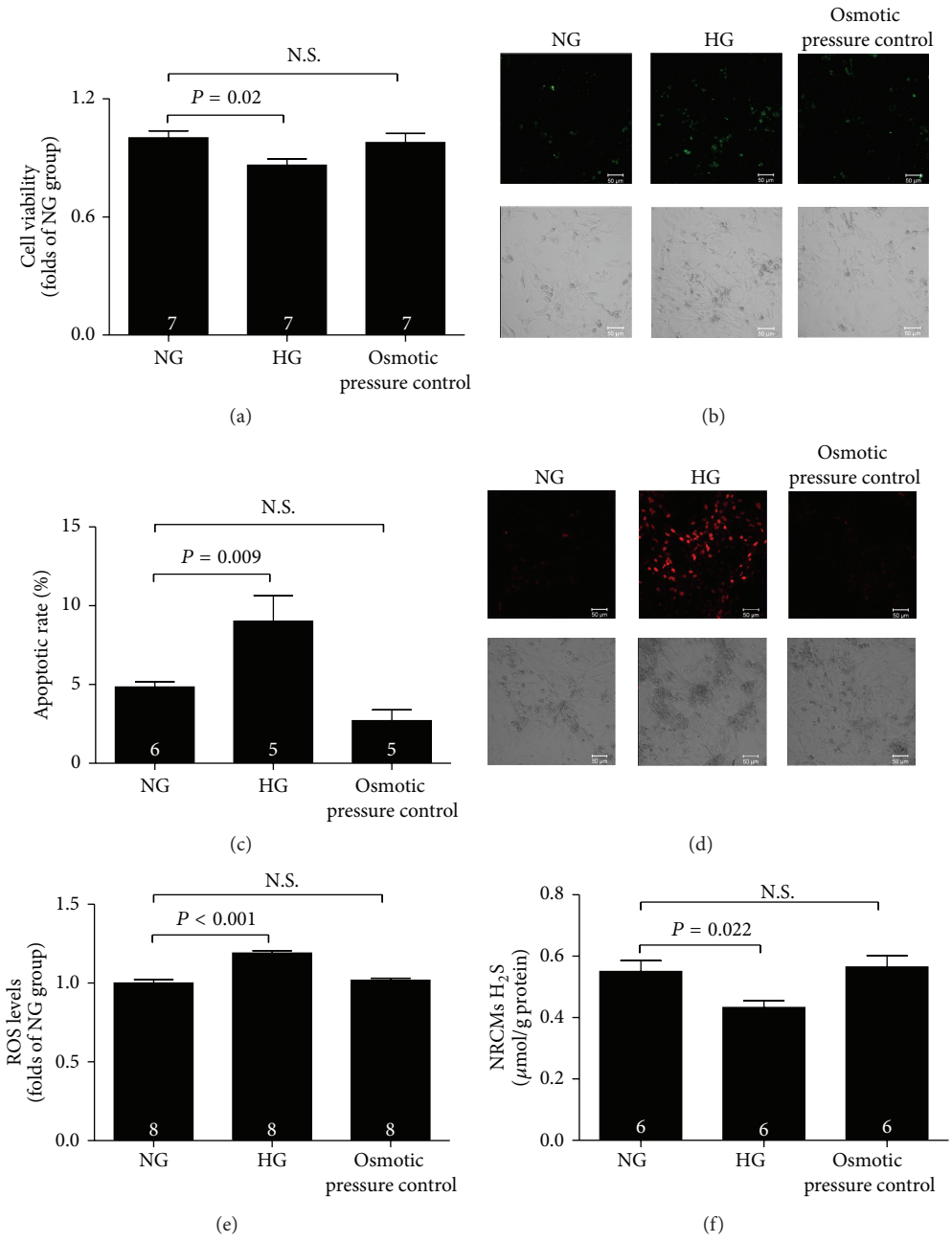


Figure 6: Reduction of endogenous H_2S involves in high glucose-induced myocardial injury. (a) Neonatal rat cardiac ventricular myocytes (NRCMs) viability measured by CCK-8 assay at the end of the treatment for 72 h. (b) Representative images of cardiomyocyte apoptosis detected by a laser confocal microscope at the end of the treatment for 72 h. (c) Quantitative analysis for cardiomyocyte apoptosis determined by flow cytometry. (d) Representative images of ROS levels in NRCMs detected by a laser confocal microscope at the end of the treatment for 72 h. (e) Quantitative analysis for ROS levels in NRCMs. (f) H_2S levels in NRCMs at the end of the treatment for 72 h. NG group, normal glucose (5.5 mmol/L); HG group, high glucose (33 mmol/L); Osmotic pressure control group, normal glucose (5.5 mmol/L) + L-glucose (27.5 mmol/L). Results are means ± SEM. A P of <0.05 was considered significant.

levels are related to various diseases. However, information on endogenous H_2S levels in the hearts of ageing diabetic mice with DCM is fairly limited. Therefore, it would be premature to conclude whether the change in H_2S generation in the ageing diabetic mouse heart is involved in DCM. Our results indicated that circulating, heart and urine H_2S levels in long-term high-fructose-fed diabetic mice were lower than those in control mice, which was similar to the results of previous studies of diabetic patients and animals [24, 45, 46]. To confirm whether endogenous H_2S is involved in the diabetic myocardial injury, NRCMs were exposed to high glucose (33 mmol/L) for 72 h to mimic the hyperglycemia in DCM *in vitro* and resulted in a significant decrease in cell viability, increase in apoptosis rate, and overproduction of

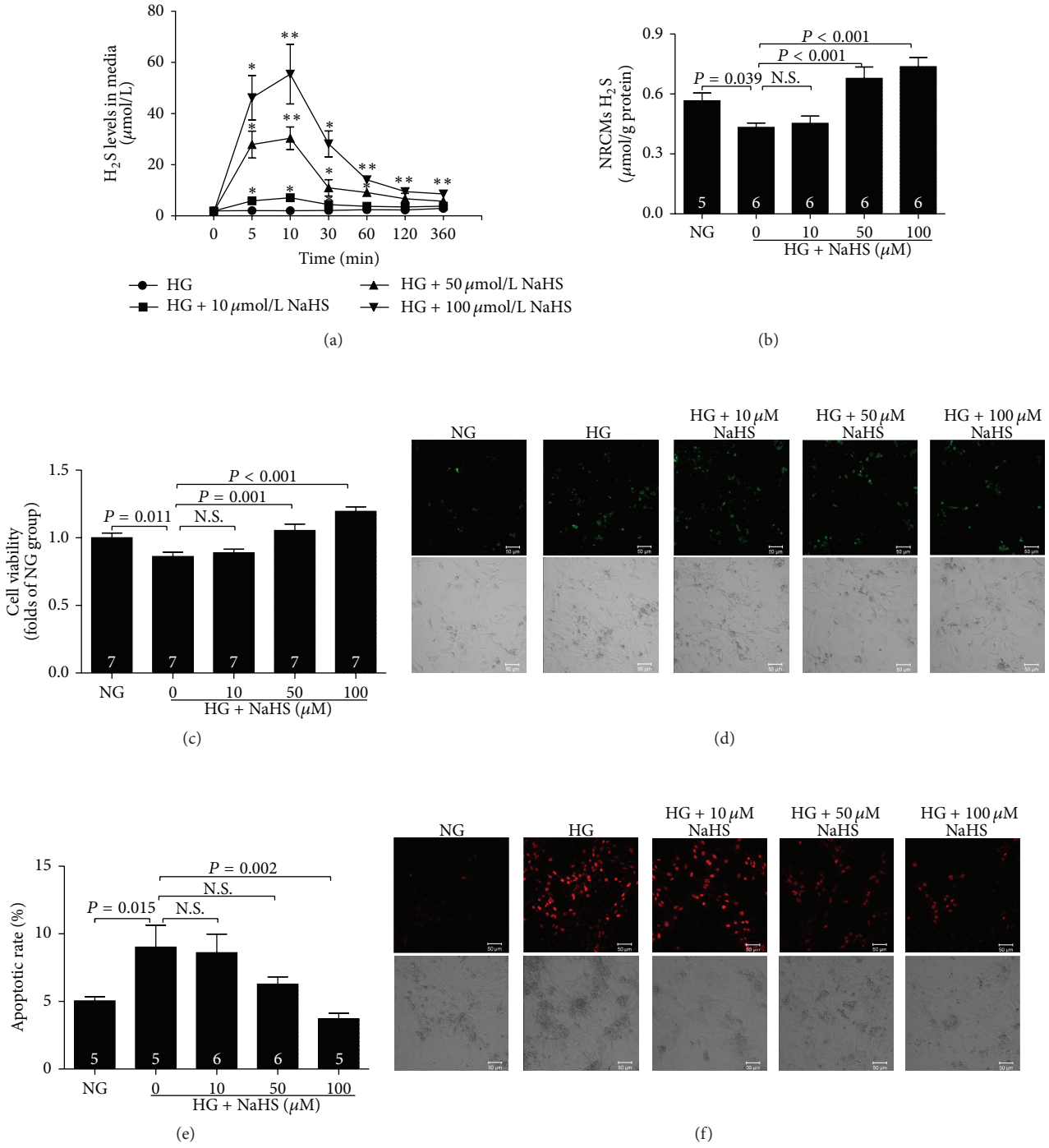
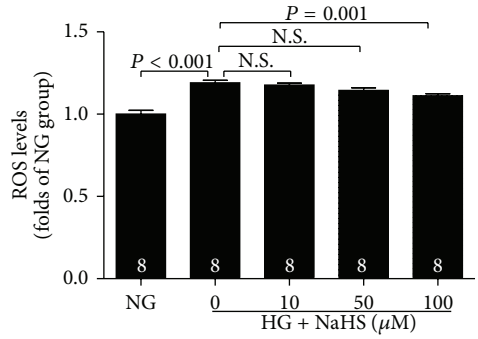


Figure 7: Continued.



(g)

Figure 7: Exogenous H_2S attenuates high glucose-induced myocardial injury. (a) Exogenous NaHS caused transient increase of H_2S level in cell culture media within 6 hours. $^*P < 0.05$ versus HG group; $^{**}P < 0.01$ versus HG group ($n = 4$ in each group). (b) H_2S levels in neonatal rat cardiac ventricular myocytes (NRCMs) at the end of the treatment for 72 h. (c) NRCMs viability measured by CCK-8 assay at the end of the treatment for 72 h. (d) Representative images of cardiomyocyte apoptosis detected by a laser confocal microscope at the end of the treatment for 72 h. (e) Quantitative analysis for cardiomyocyte apoptosis determined by flow cytometry. (f) Representative images of ROS levels in NRCMs detected by a laser confocal microscope at the end of the treatment for 72 h. (g) Quantitative analysis for ROS levels in NRCMs. NG group, normal glucose (5.5 mmol/L); HG group, high glucose (33 mmol/L). Results are means ± SEM. A P of <0.05 was considered significant.

ROS, accompanied by a decrease of H_2S levels. Exogenous H_2S could suppress the high glucose-induced myocardial injury by preventing ROS generation, inhibiting cardiomyocyte apoptosis and promoting cell viability.

CSE, a key enzyme involved in H_2S production in the cardiovascular system, was downregulated, which might have contributed to the reduced H_2S levels. These findings were consistent with those in previous studies. Zhang et al. reported that glucose induced SP1 phosphorylation via p38 MAPK activation, which resulted in decreased CSE promoter activity and the subsequent downregulation of the expression of CSE gene [47]. Notably, for the first time, we report that 3-MST protein expression was also reduced in long-term high-fructose-fed diabetic mice. CBS was reported to be mainly expressed in nervous system, but this enzyme has also been shown to exist in the cardiovascular system [48]. In our study, CBS existed in the cardiac tissue and was upregulated after the long-term fructose feeding. The double-staining immunofluorescence further showed that CBS protein expression was significantly increased in cardiomyocytes and interstitial but not in coronary vessels. Although CBS was also reported to be upregulated in some studies [42, 49], we considered that CBS upregulation might be due to a compensatory response for hyperhomocystinaemia (HHcy). High-fructose-fed diabetic mice typically also have HHcy [50]. In the transsulfuration pathway, homocysteine (Hcy) condenses with serine to form cystathionine, which is catalyzed by CBS. To metabolize this excess Hcy, CBS is upregulated; meanwhile, Hcy level can be lowered by inducing transgenic human CBS (Tg-hCBS) [51]. However, this increase in CBS is not sufficient to cause an increase in overall H_2S generation, because the expression of both CSE and 3-MST, the other two H_2S-producing enzymes in cardiovascular system, are downregulated, and the defined mechanisms underlying CBS upregulation remain to be further studied.

As discussed above, there are conflicting reports regarding the regulation of H_2S-producing enzymes in diabetes. Several *in vivo* studies have also reported different protein expression levels for CSE or CBS in various tissues. There were reductions in both CSE protein expression and CSE activity, which could have resulted in impaired H_2S levels both in the liver tissues of STZ-treated T1D rats and PBMCs isolated from T1D patients [52]. CBS and CSE were also lower in hyperglycaemic Akita mice [53]. Yamamoto et al. reported that CSE expression was markedly reduced in the diabetic kidney, whereas CBS expression was unaffected in the proximal tubules of diabetic kidneys in CaMTg mice [54]. However, Suzuki et al. did not find any notable changes in the expression of CSE or CBS in the brain, heart, kidney, lung, liver, or thoracic aorta of rats subjected to STZ-induced diabetes [55]. On the other hand, several studies suggested that the expression of CBS and/or CSE was increased in the pancreas, liver, and kidney of STZ-diabetic rats [56, 57]. Similar conflicting reports regarding the protein expression of H_2S producing enzymes based on *in vitro* studies can be found [58–60].

These conflicting findings on the expression of H_2S-producing enzymes may be due to the different responses of different organs and different cell types. This may also depend on the stage or severity of the disease. These questions as well as the actual molecular regulation of these enzymes need to be further investigated. Despite the controversy on the expression of the three H_2S-producing enzymes, it appears that endogenous H_2S plays an important role in the development of diabetes and its complications.

5. Conclusion

In conclusion, our results suggest that ageing diabetic mice induced by long-term high-fructose consumption developed

diabetic cardiomyopathy and that H_2S levels were reduced in the diabetic heart due to alterations in the expression of the three H_2S-producing enzymes, which might be involved in the pathogenesis of DCM.

Conflict of Interests

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

Acknowledgments

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Research Article

The Hydrogen Sulfide Donor NaHS Delays Programmed Cell Death in Barley Aleurone Layers by Acting as an Antioxidant

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H_2S is a signaling molecule in plants and animals. Here we investigated the effects of H_2S on programmed cell death (PCD) in barley (*Hordeum vulgare* L.) aleurone layers. The H_2S donor NaHS significantly delayed PCD in aleurone layers isolated from imbibed embryoless barley grain. NaHS at 0.25 mM effectively reduced the accumulation of superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and malondialdehyde (MDA), promoted the activity of superoxide dismutase (SOD), guaiacol peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX), and decreased those of lipoxygenase (LOX) in isolated aleurone layers. Quantitative-PCR showed that NaHS treatment of aleurone tissue led to enhanced transcript levels of the antioxidant genes *HvSOD1, HvAPX, HvCAT1*, and *HvCAT2* and repressed transcript levels of *HvLOX* (lipoxygenase gene) and of two cysteine protease genes *HvEPA* and *HvCP3-31*. NaHS treatment in gibberellic acid- (GA-) treated aleurone layers also delayed the PCD process, reduced the content of $\cdot O_2^-$, and increased POD activity while decreasing LOX activity. Furthermore, α-amylase secretion in barley aleurone layers was enhanced by NaHS treatment regardless of the presence or absence of GA. These data imply that H_2S acted as an antioxidant in delaying PCD and enhances α-amylase secretion regardless of the presence of GA in barley aleurone layers.

1. Introduction

Programmed cell death (PCD), a response of plants to biotic and abiotic stresses, can also occur during the normal course of development [1]. Cereal aleurone layers undergo gibberellic acid- (GA-) stimulated PCD process following germination and therefore provide a convenient model for studying PCD [2]. PCD in barley aleurone layers occurs only after cells become highly vacuolated and is accompanied with loss of plasma membrane integrity and increased cysteine protease activity [3]. However, the hallmarks of apoptosis in animal cells, including internucleosomal DNA ladders and formation of apoptotic bodies, are not observed in aleurone cells [4]. Reactive oxygen species (ROS) such as superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals are key players in the PCD process in both plants and animals [1, 4, 5]. For instance, intracellular H_2O_2 overproduction or exogenous H_2O_2 application results in a rapid death in GA-treated aleurone protoplasts [6]. Antioxidant enzymes such as catalase (CAT), guaiacol peroxidase (POD), ascorbate peroxidase (APX), and superoxide dismutase (SOD) are responsible for ROS scavenging, thereby keeping homeostatic levels of ROS. PCD in plants is also accompanied by increased protease activity [2, 7]. For instance, cysteine protease activation is instrumental in PCD of soybean cells, while ectopic expression of cystatin, a cysteine protease inhibitor gene, inhibits cysteine protease activity and blocks PCD [7]. What is more, the increase in cysteine protease and aspartic protease activities is also observed in GA-treated barley aleurone layers [2].

Hydrogen sulfide (H_2S), similar to nitric oxide (NO) and carbon monoxide (CO), is an important endogenous gaseous signaling molecule in animal cells [8]. Accumulating evidence shows that H_2S is involved in various processes in plants, such as response to pathogen attack, seed germination, root organogenesis, abiotic stress tolerance, guard

cell movement, and postharvest senescence of fruits and vegetables [9–17]. In particular, during abiotic stresses and postharvest storage, H_2S acts as an antioxidant to counteract excessive ROS to promote seed germination and alleviate postharvest senescence [10, 15, 16]. More recently, H_2S is found to delay PCD in GA-treated wheat aleurone layers by modulation of glutathione (GSH) and heme oxygenase 1 expression [18]. However, whether H_2S has a role in regulating PCD in barley aleurone layers treated with GA or not and whether ROS and ROS-scavenging enzymes participate in the role of H_2S are still unknown. In the present research, we find that the H_2S donor NaHS effectively delays PCD in barley aleurone layers regardless of the presence of GA, through the enhancement in antioxidant enzyme genes expression and antioxidant enzyme activity and decrease in protease gene expression.

2. Materials and Methods

2.1. Materials and Treatments. Grains of barley (*Hordeum vulgare* L.) were kindly supplied by Jiangsu Academy of Agricultural Sciences, Jiangsu Province, China. Grains were surface-sterilized as described by Chrispeels and Varner [19]. In brief, embryo end of the grain was removed, and fifteen half-grains were imbibed in distilled water at 25°C for 3 d on Petri dishes and further used for NaHS or gibberellic acid (GA) plus $CaCl_2$ treatment. H_2S donor NaHS and GA were purchased from Sigma.

2.2. Cell Viability Analysis in Barley Aleurone Layers. Barley half-grains pretreated with water for 3 d were incubated in different concentrations of NaHS (0, 0.005, 0.025, 0.05, 0.25, or 0.5 mM) or 0.25 mM NaHS + 5 μM GA (in 10 mM $CaCl_2$) at 25°C for 5 d prior to isolation of aleurone layers from half-grains. To determine the number of dead cells, three aleurone layers per treatment were stained with 0.4% trypan blue [20] for 10 min and observed with Nikon Eclipse 80 i fluorescence microscope (Nikon, Japan). The percentage of dead cells was determined by the calculation of blue or purple cells compared to the total number of cells in randomly selected fields from three different aleurone layers per treatment.

2.3. Detection of Reactive Oxygen Species Using Fluorescent Probe. A reactive oxygen species kit 2′,7′-dichlorodifluorescein diacetate (DCHF-DA) (Cayman Chemical, America) which is a fluorogenic probe in living cells was used to detect ROS content [21]. Three aleurone layers per treatment were rinsed with water three times and incubated with 5 μM DCHF-DA for 20 min at 37°C in the dark according to manufacturer's instructions. The fluorescence of dichlorofluorescein DCF (excitation at 488 nm, emission at 525 nm) was observed using a Nikon Eclipse 80 i fluorescence microscope (Nikon, Japan). Nonstained aleurone layers were used as negative control. The experiment was repeated three times and similar results were obtained.

2.4. Determination of the Contents of Superoxide Anion, Hydrogen Peroxide, and Malondialdehyde. $\cdot O_2^-$, H_2O_2, and MDA contents were measured according to the method in [22]. Embryoless half-grains were pretreated with sterile water for 3 d and incubated in sterile H_2O, 0.25 mM NaHS, and 0.25 mM NaHS + 5 μM GA. Three independent experiments with three replicates of 15 half-grains (0.45 ± 0.001 g) were sampled for each treatment every 24 h until the fifth day.

2.5. Assays of the Activity of Antioxidant Enzymes and Lipoxygenase. Activity of SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), APX (EC 1.11.1.11), and POD (EC 1.11.1.7) was determined according to García-Limones et al. [23] and that of LOX (EC 1.13.11.12) followed the description by Surrey [24]. Frozen grain samples (0.45 ± 0.001 g) were homogenized with 1 mL of 200 mM ice-cold phosphate buffer (pH 7.8) containing 1.0 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 12,000 g at 4°C for 20 min, and the supernatant was used for activity measurement.

For LOX, three independent replicates of 15 half-grains (0.45 ± 0.001 g) in three independent experiments per treatment were homogenized with 1 mL of 200 mM phosphate buffer (pH 6.0). The homogenate was centrifuged at 15,000 g at 4°C for 10 min, and the supernatant was used for the enzyme assay. The assay mixture in a total volume of 3 mL contained 200 mM borate buffer (pH 6.0), 0.25% linoleic acid, 0.25% tween-20, and 50 μL of enzyme extract. The reaction was carried out at 25°C for 5 min, and the activity of LOX was determined in the presence of linoleic acid by monitoring the changes in absorbance at 234 nm.

2.6. Quantitative PCR Analysis. Total RNA was isolated from five aleurone layers using the plant RNeasy kit (Forgene, China) according to the manufacturer's instructions. Total RNA (500 ng) from different treatments was used for first-strand cDNA synthesis in a 20 μL reaction volume containing 4 μL 5 × PrimeScript RT Master Mix (TaKaRa). Quantitative PCR was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Premix Ex Taq (TaKaRa Bio Inc, China) according to the manufacturer's instructions. cDNA was amplified by PCR using the following primers: *HvActin* (accession number: LOC548170) forward (5′-TCTCACGGACTCCCTTT-3′) and *HvActin* reverse (5′-CACTGAGCACGATGTTTC-3′); *HvCAT1* (accession number: HVU20777) forward (5′-AAGACCGTTTCCTCCAGC-3′) and reverse (5′ATTCAAGGCTACCGCACA-3′); *HvCAT2* (accession number: HVU20778) forward (5′-CGCCTTCAAGCCCAACCCA-3′) and reverse (5′-TTCTCCCTCTTTCCAACCAC-3′); *HvSOD1* (accession number: JQ364454) forward (5′-CGATAGCCAGATTCCTTTG-3′) and reverse (5′-TCCACCAGCATTTCCAGTA-3′); *HvAPX* (accession number: AJ006358) forward (5′-CTACTACTGCTGCTACTATGCG-3′) and reverse (5′-CACTGACAGCGTTCAAGGTAT-3′); *HvLOX* (accession number: AJ966349) forward (5′-CCGCTCTGACCCATTTCG-3′) and reverse (5′-TGCTCCTTGACCTCCACCTT-3′); *HvICY* (accession number: AJ536590) forward (5′-TCGTCGTGCCGTTTACTC-3′) and

reverse (5′-TTGGCCTTCTTGTTGTGC-3′); *HvEPA* (accession number: HVU94591) forward (5′-CCCGTGTCGGTGGCAATA-3′) and reverse (5′-GCATCCTGATGTAACCCTTCTC-3′); *HvCP3-31* (accession number: AB377533) forward (5′-ACAACCTCCGCTACATCG-3′) and reverse (5′-CCCTTCTTCCTCCAGTCG-3′). Relative gene expression was presented as values relative to control *HvActin* transcript level, after normalization to the control *HvActin* transcript levels.

2.7. Assays of Secreted α-Amylase Activity. Embryoless barley half-grains were incubated in distilled water for 3 d and then treated with various concentrations of NaHS in presence or absence of 20 μM GA and 10 mM $CaCl_2$.

Agar-starch medium (containing 4% agar and 0.1% starch) was used to detect α-amylase activity secreted by aleurone layers in NaHS treatment without GA and $CaCl_2$ for 24 h. Aleurone layers which were prepared as described above were placed on agar-starch medium for 16 h after which the agar-starch was stained with 0.6% I_2 and 6% KI solution to show digested starch zones. The experiment was repeated three times and similar results were obtained.

Twenty embryoless half-grains were imbibed in distilled water at 25°C for 3 d on Petri dishes and incubated in Erlenmeyer flasks which contained different concentrations of NaHS in 20 μM GA and 10 mM $CaCl_2$. Incubation medium was sampled after 24 h and heated at 70°C for 15 min to eliminate β-amylase activity. Amylase secreted to the medium was visualized in 10% native PAGE gels by the starch-iodine method according to [25]. To visualize α-amylase activity, the gel was incubated at 25°C for 30 min in 50 mM PBS (pH 7.0) containing 1% boiled soluble starch. After being washed three times with distilled water, the gel was stained with 0.6% I_2 and 6% KI solution. The experiment was repeated three times and similar results were obtained.

The DNS method for the determination of secreted α-amylase activity in medium was performed in 0.01 M sodium acetate buffer, pH 5.4. The reaction mixture containing 1% soluble starch was incubated at 25°C for 5 min without substrate. Then, the reaction was initiated by adding the substrate and was continued for an additional 10 min at 37°C. The reaction was terminated and hydrolysis was determined with 3,5-dinitrosalicylic acid reagent as modified by Noelting and Bernfeld [26].

2.8. Statistical Analysis. Statistical significance in all experiments was tested by one-way analysis of variance (ANOVA), and the results are expressed as the mean values ± standard deviation (SD) of three independent experiments with three replicates for each. Fisher's least significant differences (LSD) were calculated following a significant ($P < 0.01$ or $P < 0.05$) t-test.

3. Results

3.1. Programmed Cell Death in Barley Aleurone Layers Is Delayed by the H_2S Donor NaHS. To test the effect of H_2S on the PCD process, water-pretreated barley half-grains were incubated in different concentrations of NaHS for 5 days. Aleurone layers are isolated from half-grains and stained with trypan blue to visualize dead cells. NaHS treatments ranging from 0.005 to 0.5 mM significantly decrease cell death compared with water controls (Figures 1(a) and 1(b)). Only 9% of cells die in aleurone layers treated with 0.25 mM NaHS while approximately 67% of cells of aleurone layers incubated in water undergo PCD. As shown in Figure 1, NaHS at 0.25 mM is most effective in delaying PCD in barley aleurone layers, and this concentration is used in subsequent experiments.

A time course of cell death in aleurone layers treated with 0.25 mM NaHS is shown in Figures 1(c) and 1(d). After 7 days incubation in water, about 90% aleurone cells are dead in contrast to only 45% cell death in NaHS-treated layers. Together, these findings show that barley aleurone cells undergo PCD naturally in the absence of GA and that the H_2S donor NaHS effectively delays the PCD process.

3.2. NaHS Treatment Reduces the Accumulation of Reactive Oxygen Species in Non-GA-Treated Barley Aleurone Layers. Because ROS are tightly associated with the promotion of PCD in barley aleurone cells [6], we examine the contents of $\cdot O_2^-$, H_2O_2, and MDA in non-GA-treated barley aleurone layers in the presence and absence of NaHS. As shown in Figure 2(a), $\cdot O_2^-$ content in control barley aleurone layers accumulates rapidly during the 5 days of incubation. However, $\cdot O_2^-$ content in NaHS-treated layers accumulates slowly until day 3 and keeps stable on day 5.

The assay of H_2O_2 shows that layers incubated in NaHS produce less H_2O_2 than those incubated in water (Figure 2(b)). H_2O_2 content increases rapidly in control aleurone layers during the whole incubating time, whereas a slower increase in H_2O_2 content was observed in NaHS treatment on the first two days followed by a plateau.

MDA is determined as an index of lipid peroxidation. As shown in Figure 2(c), MDA content increases rapidly in water controls until day 4 followed by a decrease. In contrast, NaHS treatment significantly lowers the level of MDA (Figure 2(c)).

We use the ROS-sensitive fluorescent probe DCHF-DA to visualize the production of ROS in barley aleurone layers (Figure 2(d)). Fluorescence from layers which are incubated in 0.05 and 0.25 mM NaHS is much less intense than water controls. More weak fluorescence is detected in tissue incubated in 0.25 mM NaHS.

3.3. Effects of NaHS on Antioxidant Enzymes and Lipoxygenase in Non-GA-Treated Barley Aleurone Layers. We examine the activity of the ROS metabolizing enzymes SOD, POD, CAT, APX, and LOX in barley aleurone layers that are incubated in 0.25 mM NaHS and water (Figure 3). The activity of SOD increases to maximum on day 3 and then declines in NaHS-treated layers. In contrast, SOD activity in water controls fluctuates slightly up to day 3 followed by a significant decrease (Figure 3(a)).

Figure 3(b) shows changes in POD activity in NaHS-treated and water control layers. NaHS significantly increases POD activity on day 1 and remains high until day 4.

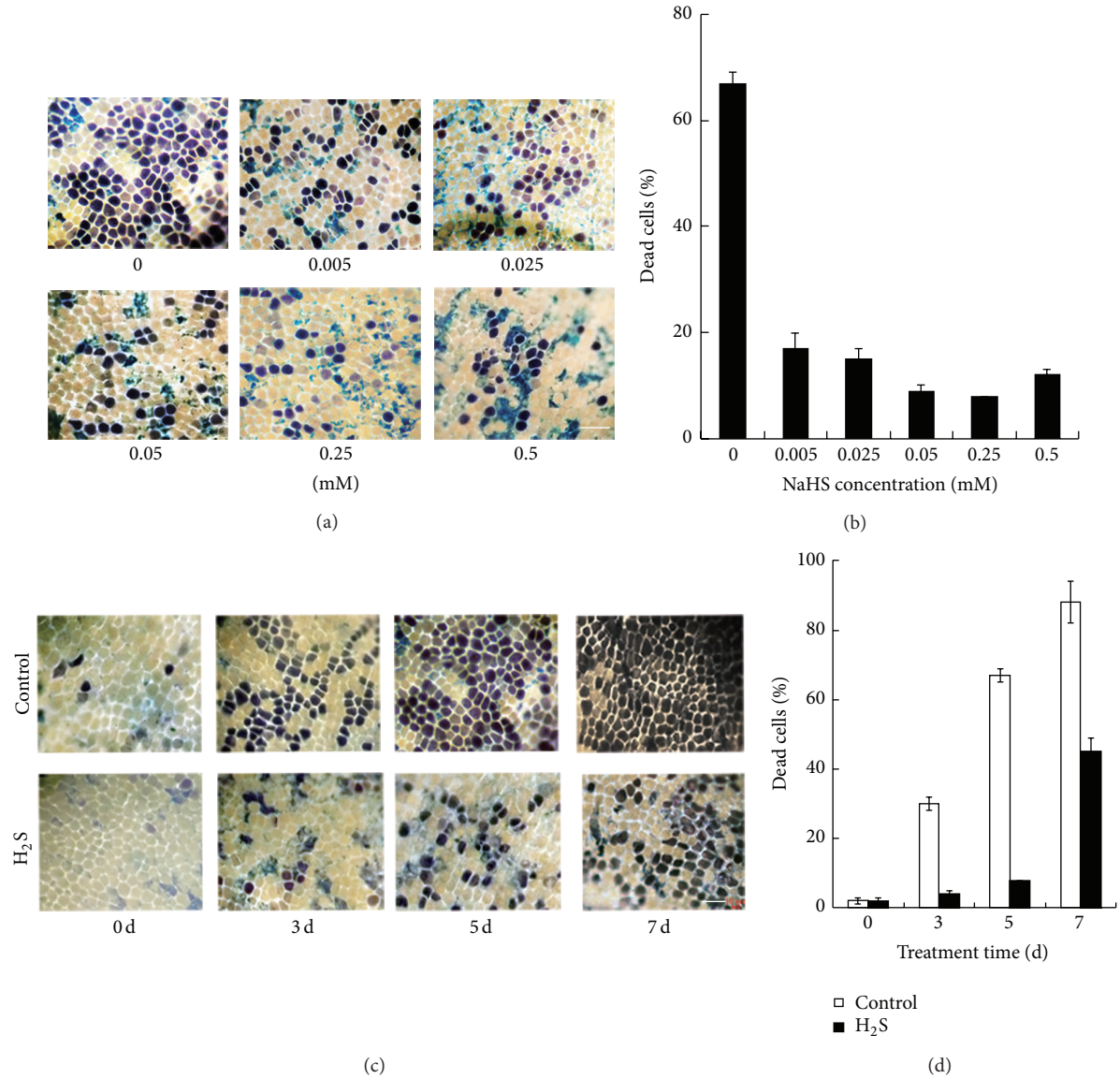

Figure 1: Effect of H_2S donor NaHS on cell viability in barley aleurone layers. ((a), (b)) Aleurone layers are incubated in different concentrations of NaHS (0, 0.005, 0.025, 0.05, 0.25, and 0.5 mM) for 5 d at 25°C. After staining with trypan blue, the images are obtained by light microscopy with blue or purple indicating dead cells. ((c), (d)) Time course of PCD in barley aleurone layers treated with NaHS (H_2S) or water (Control). Aleurone layers are incubated in 0.25 mM NaHS or water for 0, 1, 3, 5, and 7 d at 25°C and are stained with trypan blue. Digital images of barley aleurone layers ((a), (c)) and percentages of dead cells ((b), (d)) are shown. Bar, 50 μm. Data are means ± SD of three different aleurone layers per treatment.

In comparison, POD activity in water controls increases gradually and peaks on day 3 followed by a sharp decline. NaHS treatment maintains significantly higher levels of POD activity compared with water control during the whole treatment time. APX activity increases during the first 3 days of incubation and peaks on day 3 followed by a decrease in both NaHS-treated and water controls. However, APX activity in NaHS treatment is always significantly higher than that of control (Figure 3(c)).

Changes in CAT activity are shown in Figure 3(d). In both NaHS and water controls, CAT activity increases gradually up to day 3 and then decreases sharply. However, CAT activity from NaHS-treated layers is always significantly higher than those in control layers.

Figure 3(e) shows the changes in LOX activity in barley aleurone layers. LOX activity in water control increases dramatically and peaks on day 3 followed by a decrease. In contrast, NaHS treatment significantly decreases LOX activity, being about 50% of that of water control on day 3.

3.4. Transcript Analysis of HvSOD1, HvCAT1, HvCAT2, HvLOX, Cysteine Protease (HvCP3-31 and HvEPA), and Cystatin (HvICY) in Non-GA-Treated Barley Aleurone Layers. We examine the expression of *HvSOD1*, *HvCAT1*, *HvCAT2*,

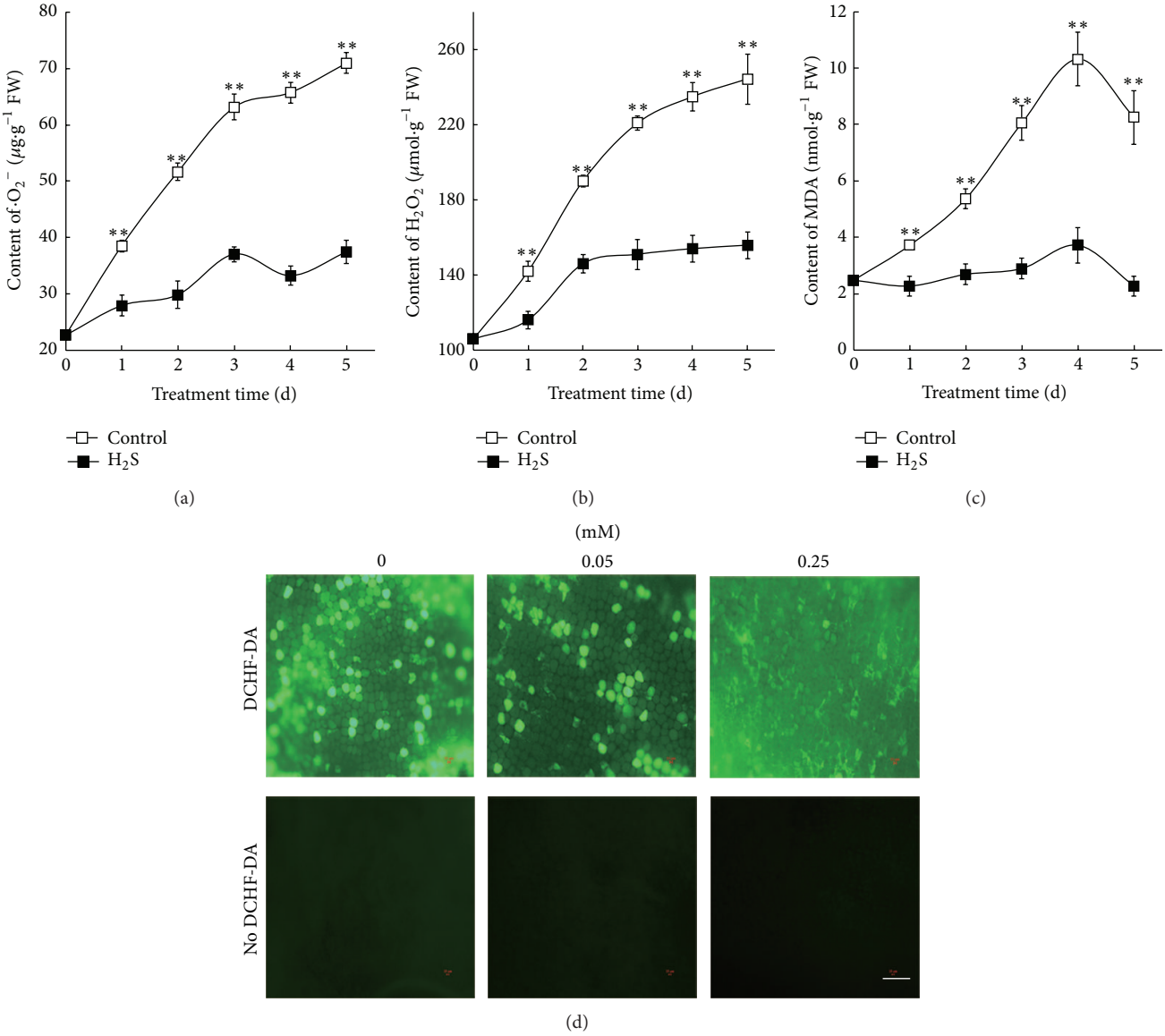

Figure 2: Effects of NaHS on the contents of $\cdot O_2^-$ (a), H_2O_2 (b), and MDA (c) in barley aleurone layers. Aleurone layers treated with 0, 0.05, and 0.25 mM NaHS for 1 d are incubated DCHF-DA and are observed by fluorescence microscopy (d). Bar, 100 μm. Data are expressed as means ± SD of three independent experiments with three replicates of 15 grains per treatment. The symbols $*$ and $**$ mean significant difference at $P < 0.05$ and $P < 0.01$ between NaHS (H_2S) and water (control) treatment, respectively.

HvLOX, the cysteine proteases *HvCP3-31* and *HvEPA,* and cystatin (*HvICY*) in NaHS-treated barley aleurone layers and water controls (Figure 4). Compared with water controls, NaHS induces higher expression of *HvSOD1*, *HvAPX*, *HvCAT1,* and *HvCAT2* on days 1 and 5. *HvLOX* expression increases in water control layers on days 1 and 5 compared with day 0, while NaHS treatment sustains lower transcript of *HvLOX* than water control, especially on day 5. PCD in barley aleurone layers is accompanied with increased cysteine protease activity [3]. Accordingly, we determine the expression of the cysteine proteinases *HvEPA* and *HvCP3-31* and the cystatin *HvICY* in NaHS treatment and water control. The expression of *HvEPA* and *HvCP3-31* increases in water controls on days 1 and 5, whereas their expression is much lower in NaHS-treated tissue. The expression of *HvICY* was enhanced in NaHS-treated layers, whereas less transcript of *HvICY* is observed in water controls.

3.5. NaHS Delays PCD in GA-Treated Barley Aleurone Layers. PCD in barley aleurone layers is tightly regulated by GA and abscisic acid (ABA). We therefore assess whether H_2S can ameliorate PCD in GA-treated barley aleurone layers. As shown in Figure 5(a), the accumulation of dead cells increases rapidly from 24 to 96 h in GA-treated barley aleurone layers, whereas 0.25 mM NaHS treatment significantly delays the rate of PCD. After incubation for 96 h, about 90% cells in

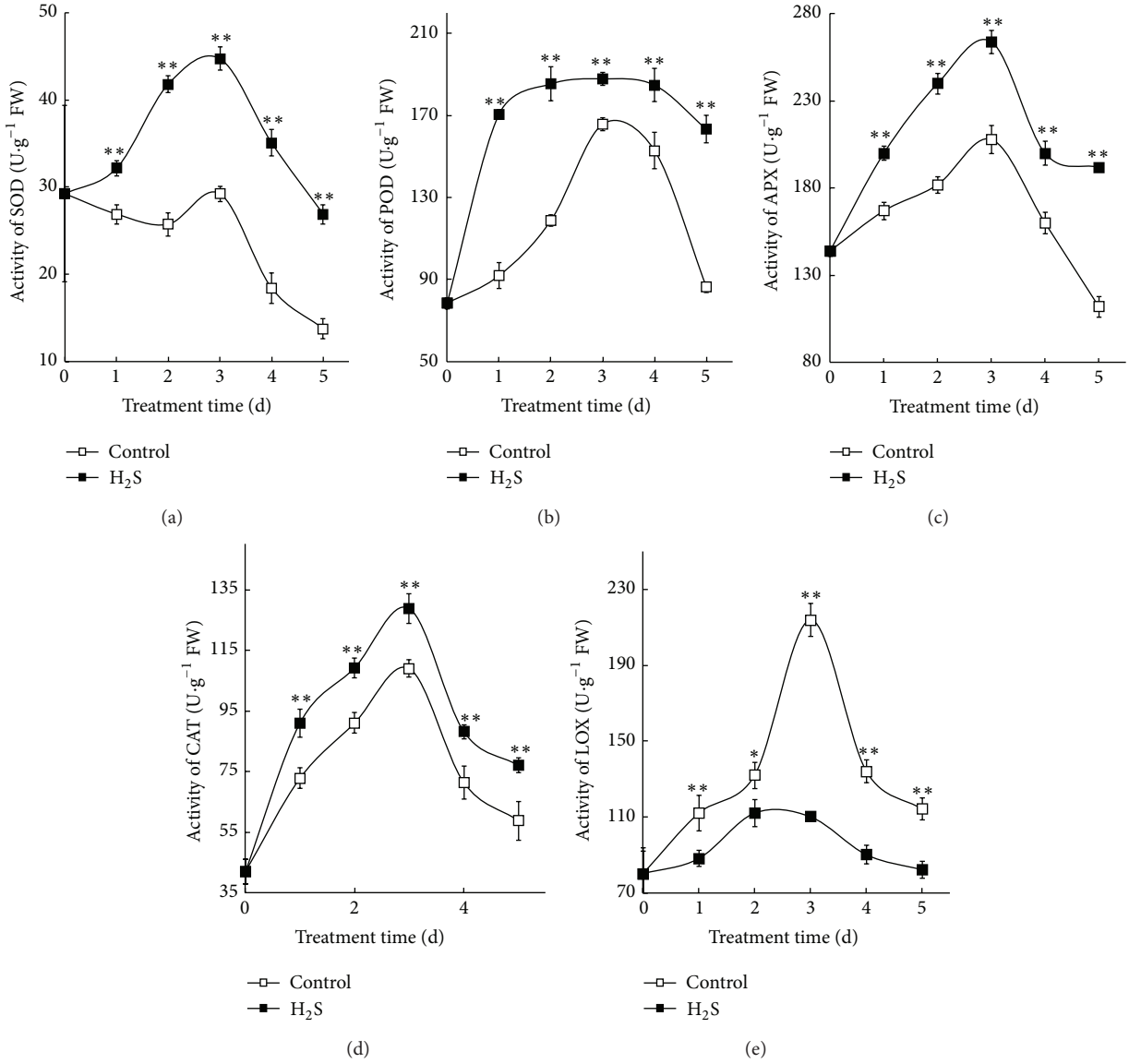

Figure 3: Effects of NaHS on the activity of SOD (a), POD (b), APX (c), CAT (d), and LOX (e) in barley aleurone layers. Data are expressed as means ± SD of three independent experiments with three replicates of 15 grains per treatment. The symbols $*$ and $**$ mean significant difference at $P < 0.05$ and $P < 0.01$ between control and T, respectively.

GA-treated layers are dead, while half of cells are still alive in NaHS-treated layers (Figure 5(b)). In water control, much less cells undergo PCD compared with the counterpart of GA and GA plus NaHS.

Determination of $\cdot O_2^-$ content shows that NaHS treatment maintains lower levels of $\cdot O_2^-$ in GA-treated barley aleurone layers (Figure 5(c)). After a rapid increase during the first 2 days of incubation, the content of $\cdot O_2^-$ in GA-treated layers decreases till day 4. In contrast, $\cdot O_2^-$ content in NaHS plus GA treatment increases more slowly until day 3. A comparable but lower $\cdot O_2^-$ content was observed in water control compared with NaHS plus GA treatment.

Figure 5(d) shows the effect of H_2S on POD activity in GA-treated barley aleurone layers. In both NaHS treatment and GA control, POD activity in GA-treated barley aleurone layers increases gradually up to day 3 and day 2, respectively, and decreases thereafter. However, the activity of POD in NaHS-treated aleurone layers is always significantly higher than those in water controls and GA treatment alone.

Figure 5(e) shows that NaHS treatment maintains lower levels of LOX activity compared with water control during the first 2 days of GA treatment. LOX activity in GA treatment increases dramatically on day 1 followed by a gradual decrease, while, in NaHS-treated tissue, the activity increases more slowly till day 3 followed by a decline. After day 3, LOX activity in NaHS plus GA is higher than that in GA treatment.

3.6. H_2S Donor Promotes α-Amylase Secretion in Barley Aleurone Layers Regardless of GA. Secretion of α-amylase is

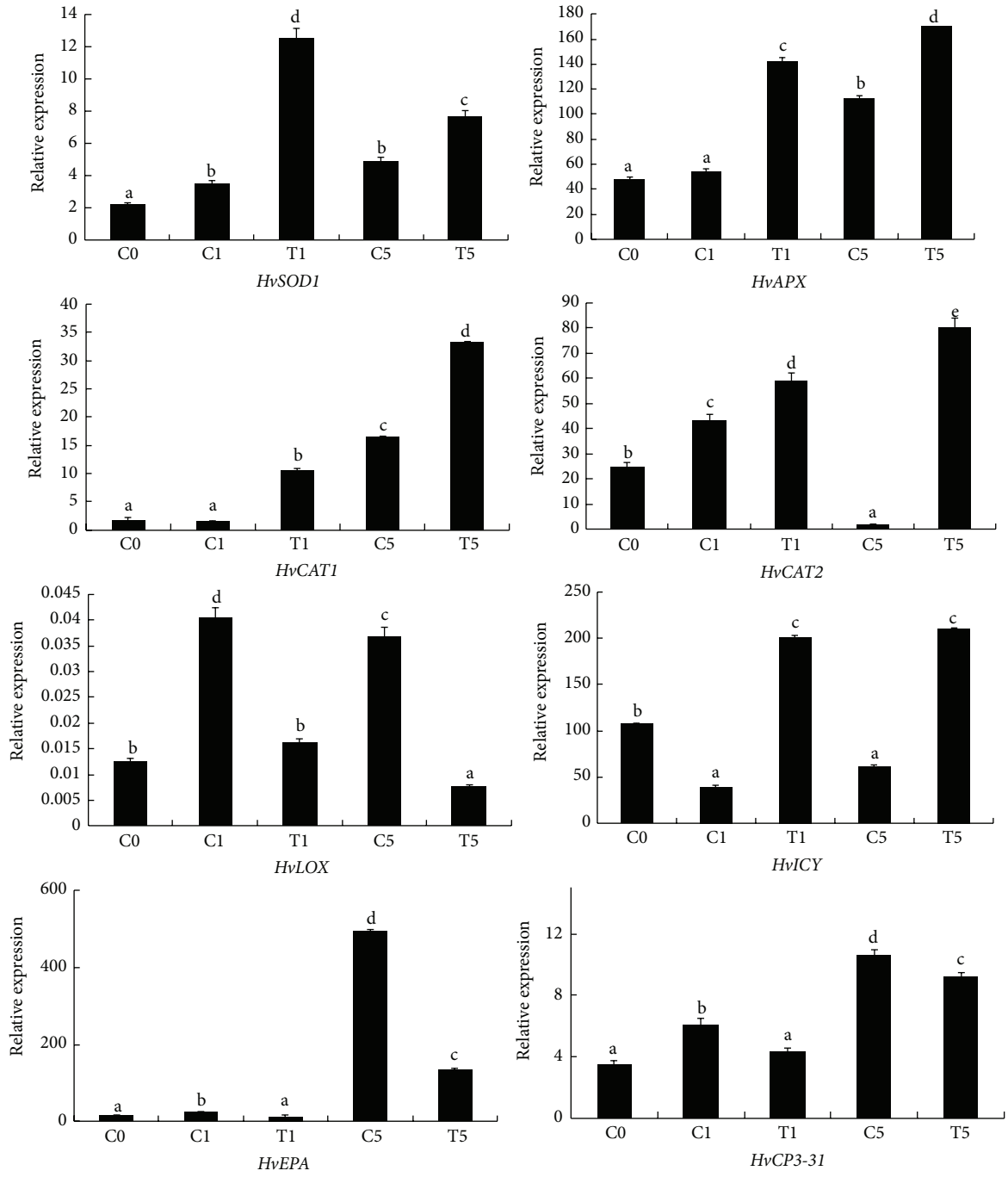

Figure 4: Effects of H_2S on the expression of *HvSOD1*, *HvCAT1*, *HvCAT2*, *HvLOX*, cystatin (*HvICY*), and the cysteine proteases *HvCP3-31* and *HvEPA* in barley aleurone layers. Aleurone layers are incubated in NaHS (T) or water (C) and total RNA is obtained at 0, 1, and 5 d. Means and SD values are calculated from three independent experiments. Within each identified gene, bars with different letters are significantly different in comparison with the corresponding control at $P < 0.01$ according to Fisher's least significant differences (LSD).

a characteristic response of aleurone cells to GA. We therefore test whether the ameliorating effect of H_2S on PCD affects the release of α-amylase. As shown in Figure 6(a), NaHS promotes α-amylase release in water-treated aleurone layers. In the presence of GA, NaHS treatment also enhances α-amylase release from barley aleurone layers (Figure 6(b)) with 0.5 mM NaHS exhibiting optimal effect. Figure 6(c) shows the time changes in α-amylase secretion in water control, GA-treated aleurone layers, and GA plus 0.25 mM NaHS treatment. The accumulation of α-amylase in incubation medium in GA-treated layers increases and peaks on day 3 followed by a plateau but addition of 0.25 mM NaHS brings about a more rapid increase till day 4 followed by a decrease on day 5 (Figure 6(c)). The activity of α-amylase released following

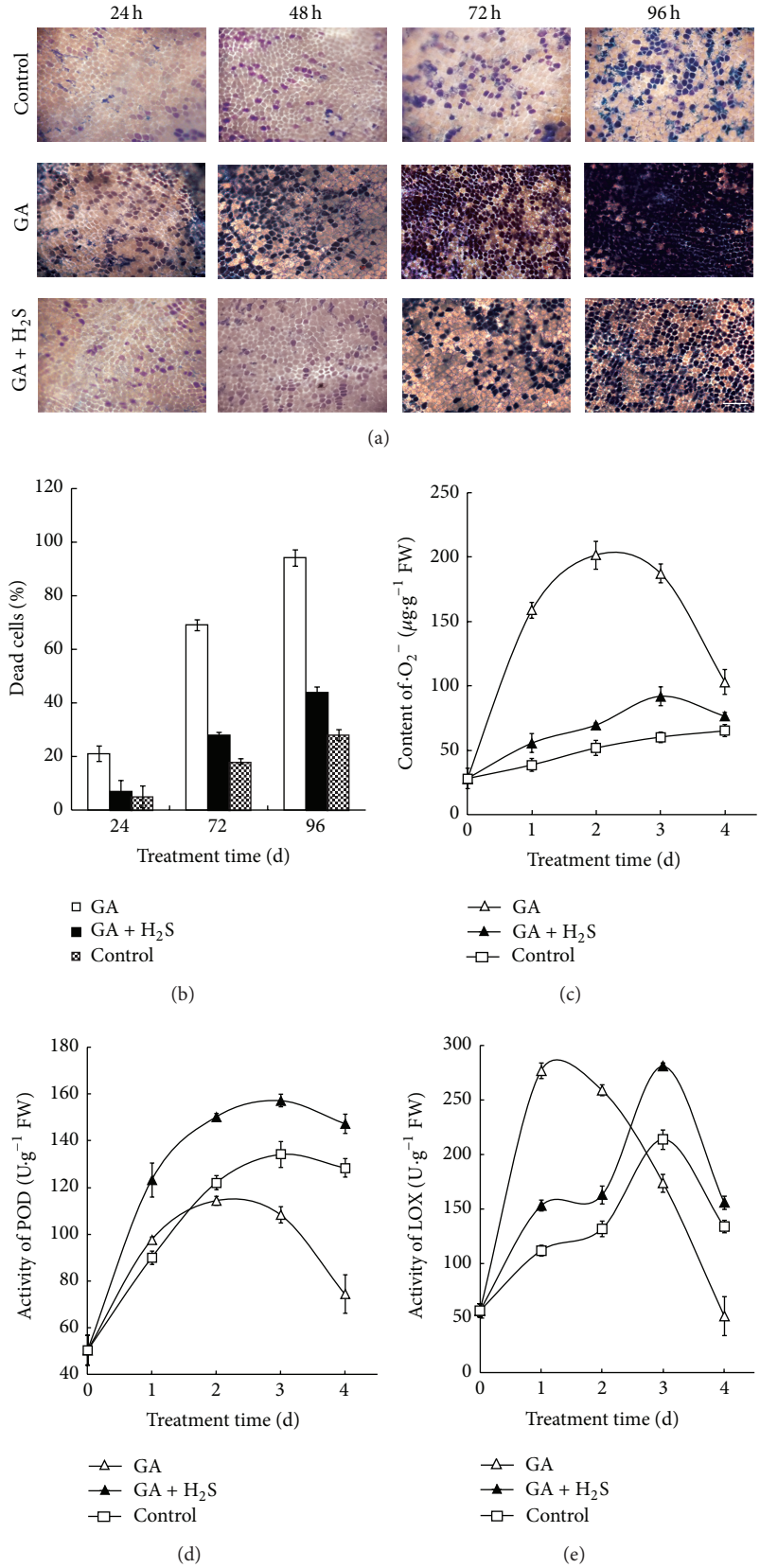

Figure 5: NaHS delays PCD in GA-treated barley aleurone layers. Aleurone layers are incubated in water, GA, or GA + NaHS (GA + H_2S) and, after being stained with trypan blue, images are obtained by light microscopy (a) and the percentage of dead cells is shown in (b). Content of $\cdot O_2^-$ (c), activity of POD (d), and LOX (e) are measured on 0, 1, 2, 3, and 4 d. Bar, 100 μm. Data in (c) and (d) are expressed as means ± SD of three independent experiments with three replicates of 15 grains per treatment.

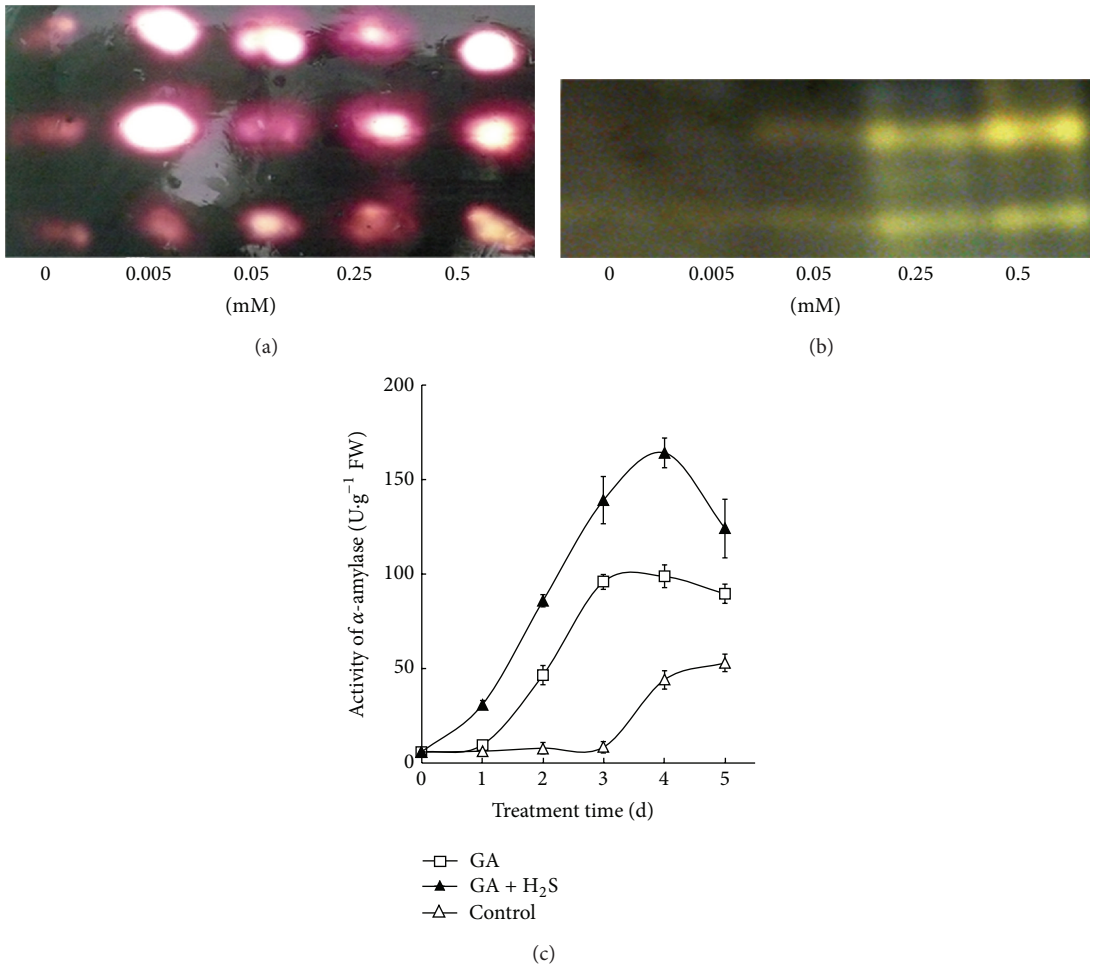

Figure 6: NaHS increases the activity of α-amylase in incubation medium of barley aleurone layers in the absence (a) and presence ((b), (c)) of GA. Barley aleurone layers isolated from treated embryoless half grains are incubated in 0, 0.005, 0.05, 0.25, or 0.5 mM NaHS for 24 h ((a), (b)). After incubation, aleurone layers are placed on agar-starch medium (containing 4% agar and 0.1% starch) for 16 h. Agar plates are stained with I_2-KI solution to detect the activity of α-amylase (a). (b) shows native PAGE analysis of α-amylase activity in incubation medium surrounding the aleurone layers. (c) indicates secreted α-amylase activity in incubation medium surrounding barley aleurone layers treated with water (control), GA, or GA + NaHS (GA + H_2S) at different times of incubation. Data in (c) are expressed as means ± SD of three independent experiments with three replicates of 20 embryoless half grains per treatment.

GA + NaHS treatment is significantly higher than that of layers incubated only in GA during the whole treatment time. As expected, much lower α-amylase activity was only observed in water control after 3 days of incubation. Together, this result indicates that H_2S delays PCD in barley aleurone layers and meanwhile promotes α-amylase release regardless of the presence of GA.

4. Discussion

H_2S participates in multiple processes in plants [27]. In this paper, we show that H_2S delays PCD in barley aleurone layers regardless of the presence or absence of GA. In the absence of GA, PCD in barley aleurone layers is evident on day 3 of incubation, while H_2S delays PCD at an optimal concentration of 0.25 mM (Figure 1). Consistent with the reports that GA accelerates PCD process in barley aleurone layers, GA treatment triggers cell death in about 90% aleurone cells at 72 h (Figure 5(a)). GA-induced cell death is slowed by the addition of NaHS and this H_2S donor also prolongs the phase of α-amylase production in GA-treated layers. The promoting effect of NaHS on α-amylase synthesis and its prolongation of cell survival indicate that 2.5 mM NaHS does not affect aleurone cell function.

ROS, such as $\cdot O_2^-$ and H_2O_2, are inducers of PCD in plant and animal cells [1]. It is reported that the peroxidation of membrane lipids and damage to the plasma membrane can occur when the rate of ROS production overcomes the cells' ability for scavenging ROS [27]. Overproduction of ROS and oxidative damage are universal events in PCD in plant cells [28]. In this paper, we show that the content of $\cdot O_2^-$ increases in parallel with cell death in GA-treated layers (Figure 5(c)). In non-GA-treated layers, the burst of $\cdot O_2^-$ and H_2O_2 and

the accumulation of MDA are also accompanied by PCD (Figure 2). These results suggest that ROS play a key role both in GA-treated [29] and in non-GA-treated aleurone layers (Figures 2(a), 2(b), and 5(c)).

Aleurone cells contain a suite of ROS-metabolizing enzymes. GA-induced PCD in layers is accompanied by a decline in activity of ROS metabolizing enzymes which leads to increased susceptibility of aleurone cells to ROS [29]. A novel aspect of our work is that NaHS treatment effectively reduces the accumulation of ROS in barley aleurone layers regardless the presence of GA (Figures 2 and 5), thereby delaying PCD process in these cells. We propose that the H_2S donor reduces ROS accumulation in layers by increasing the activity of ROS-scavenging enzymes. The data in present study show that H_2S treatment maintains significantly higher POD activity in GA-treated layers (Figure 5(d)) and higher SOD, POD, CAT, and APX activity in non-GA-treated layers (Figure 3). The increased activity of ROS-scavenging enzymes in NaHS-treated likely promotes the cell's ability to metabolize ROS. In addition, LOX activities which are responsible for lipid peroxidation are downregulated in NaHS-treated aleurone layers at early stage of treatment (Figures 3(e) and 5(e)). Meanwhile, quantitative-PCR analysis shows that expression of *HvSOD1*, *HvAPX*, *HvCAT1,* and *HvCAT2* genes in non-GA-treated layers is maintained at higher levels in NaHS treatment compared with water controls (Figure 4). Consistent with lower LOX activity in NaHS-treated aleurone layers, the accumulation of LOX transcripts is also reduced (Figure 4). In summary, H_2S slows down ROS-induced PCD in barley aleurone layers probably by enhancing the activity and expression of ROS-scavenging enzymes and reducing the peroxidation of membrane lipids.

Consistently, Xie et al. [18] found that H_2S delayed GA-triggered PCD in wheat aleurone layers by increasing GSH content and heme oxygenase-1 gene expression. Here we provide evidence that H_2S can alleviate both natural PCD and GA-triggered PCD through the modulation of antioxidant enzyme activities and their expression. Compared with the slow natural PCD process, the present study also confirms the pivotal role of GA in triggering PCD (Figure 5).

The role of ROS in GA and ABA signaling in barley aleurone cells is recently clarified [30], in which they found that the production of H_2O_2, a type of ROS, was induced by GA in aleurone cells but suppressed by ABA. Furthermore, exogenous H_2O_2 appeared to promote the induction of α-amylases by GA by promoting the expression of *GAMyb* and α-amylase genes, whereas antioxidants suppressed the induction of α-amylase. Unexpectedly, we found that H_2S reduces ROS accumulation and delays PCD process in barley aleurone layers in the presence or absence of GA and meanwhile promotes the secretion of α-amylase, suggesting that the antioxidant H_2S works through an unknown way to regulate α-amylase secretion and antioxidants do not always suppress the induction of α-amylase. Besides, the activation of α-amylase by H_2S in the absence of GA implies that α-amylase can be secreted independent of GA signaling pathway. Therefore, the present findings advance our knowledge on the relations between PCD process and α-amylase secretion and the independence of α-amylase secretion and GA pathway.

The activation of cysteine proteases was instrumental in the PCD of soybean cells, while cystatin, an endogenous cysteine protease inhibitor gene, inhibited cysteine protease activity and blocked PCD in these cells [7]. In this paper, we show that H_2S downregulates the transcriptions of two barley cysteine proteinases, *HvEPA* and *HvCP3-31,* in non-GA-treated barley aleurone layers, thereby delaying cell component degradation and PCD process.

5. Conclusion

In summary, we report the role of H_2S in delaying PCD in barley aleurone layers regardless of the presence or absence of GA without repressing α-amylase induction, suggesting that the function of H_2S may be universal in regulating plant PCD. PCD in plant cells is regulated by many internal and external factors, such as the hormones (GA and ABA), Ca^{2+}, ROS, and NO [6]. It will be interesting to know whether H_2S is involved in other signals and how α-amylase is induced by H_2S in the presence or absence of GA in cereal aleurone cells.

Conflict of Interests

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

Authors' Contribution

Ying-Xin Zhang and Kang-Di Hu contributed equally to this work.

Acknowledgments

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Research Article

Downregulation of Endogenous Hydrogen Sulfide Pathway Is Involved in Mitochondrion-Related Endothelial Cell Apoptosis Induced by High Salt

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Background. The study aimed to investigate whether endogenous H_2S pathway was involved in high-salt-stimulated mitochondria-related vascular endothelial cell (VEC) apoptosis. *Methods*. Cultured human umbilical vein endothelial cells (HUVECs) were used in the study. H_2S content in the supernatant was detected. Western blot was used to detect expression of cystathionine gamma-lyase (CSE), cleaved-caspase-3, and mitochondrial and cytosolic cytochrome c (cytc). Fluorescent probes were used to quantitatively detect superoxide anion generation and measure the *in situ* superoxide anion generation in HUVEC. Mitochondrial membrane pore opening, mitochondrial membrane potential, and caspase-9 activities were measured. The cell apoptosis was detected by cell death ELISA and TdT-mediated dUTP nick end labeling (TUNEL) methods. *Results*. High-salt treatment downregulated the endogenous VEC H_2S/CSE pathway, in association with increased generation of oxygen free radicals, decreased mitochondrial membrane potential, enhanced the opening of mitochondrial membrane permeability transition pore and leakage of mitochondrial cytc, activated cytoplasmic caspase-9 and caspase-3 and subsequently induced VEC apoptosis. However, supplementation of H_2S donor markedly inhibited VEC oxidative stress and mitochondria-related VEC apoptosis induced by high salt. *Conclusion*. H_2S/CSE pathway is an important endogenous defensive system in endothelial cells antagonizing high-salt insult. The protective mechanisms for VEC damage might involve inhibiting oxidative stress and protecting mitochondrial injury.

1. Introduction

High-salt diet as an important risk factor of hypertension could interrupt body homeostasis, resulting in cardiovascular diseases and even life-threatening events. High-salt insult can promote the proliferation of vascular smooth muscle cells induced by angiotensin II, significantly increase blood pressure in salt-sensitive rats, and accelerate hypertensive process and the development of cardiovascular disease. Moreover, it can change small artery structures in normal-diet rats.

Numerous studies have been conducted to investigate the mechanisms for hypertension. Previous studies indicated that high-salt insult promoted hypertension in association with activating angiotensin-aldosterone system [1], damaging renal natriuresis function [2], enhancing sympathetic activity [3], increasing the extracellular capacity, and eventually leading to sodium and water retention [4]. In addition, high salt could destroy the transfer passage of paraventricular nucleus Gαi-protein gating signals [5]. However, the mechanisms for high-salt-induced hypertension remain unclear. Vascular endothelial cell serves as a junction of blood flow and vessel wall. Maintenance of vascular endothelium integrity is essential for smooth blood flow, which plays an important role in maintaining vascular homeostasis [6]. Endothelial dysfunction is the initial cause of the onset and development of a variety of cardiovascular diseases [7]. Importantly, vascular

endothelium dysfunction plays a key role in the pathogenesis of salt-sensitive hypertension, while high-salt stimulation could damage vascular endothelial function [8]. High salt led to endothelial cell cortex sclerosis via compromising epithelial sodium channels [9] and contributing to intracellular edema and mitochondrion swelling [10] while the mechanisms for intracellular structural changes and endothelial dysfunction are unidentified. In normotensive rats, high-salt diet increased production of reactive oxygen species in striated muscle and superior mesenteric arteries, activating the oxidative stress system [11, 12]. Therefore, we speculated that high-salt-induced oxidative damage might be involved in the development of cell damage induced by high salt. H_2S is known as a gas with rotten egg stink and toxicity. Recent studies suggest that endogenous H_2S exerts biological effects and acts as a novel gaseous signal molecule [13–19]. Several studies indicated that H_2S/CSE pathway was downregulated in the development of hypertension, while H_2S supplementation could significantly lower blood pressure and reverse aortic remodeling [20, 21]. It was also implied that H_2S could protect human umbilical vein endothelial cells (HUVECs) via antagonizing oxidative stress-related pathways [22]. Hence, we proposed a hypothesis that downregulation of endogenous H_2S/CSE might be involved in mitochondrion-related endothelial cell injury induced by high-salt exposure.

Therefore, the present study was undertaken to explore the possible effects of endogenous H_2S on endothelial apoptosis under high-salt stimulation and its mechanisms.

2. Materials and Methods

2.1. Cell Culture. HUVECs were purchased from Lifeline Cell Technology, USA. The 3–7 generations of HUVEC were used in this experiment. HUVECs were cultured in the medium which was supplemented with 0.2% FBS, 0.1% rh VEGF, 0.1% rh IGF-1, 0.1% rh FGF-b, 0.1% ascorbic acid, 0.1% rh EGF, 0.1% heparin, 0.1% Hydrocort, and 5% L-glutamine (Lifeline Cell Technology, USA) in an incubator containing 5% CO_2 at constant temperature of 37°C. The cells were cultured in exogenous growth factors-free medium and synchronized for 12 h before each experiment. The concentration of sodium in the synchronization medium was 137 ± 1.0 mmol/L. Sodium chloride (Sinopharm, Shanghai, China) was added to culture medium at different concentrations including 150 mmol/L, 200 mmol/L, and 250 mmol/L.

2.2. Measurement of Endogenous H_2S Content in HUVEC. Endogenous H_2S in cells was measured using a fluorescent probe (provided by Professor Xinjing Tang, Peking University Health Science Centre, China) as described previously [23]. The culture supernatant was collected to test H_2S levels in HUVEC. Then, the slides were washed with PBS (0.01 mol/L) for three times and fixed in prewarmed 4% paraformaldehyde at room temperature for 20 min. After washing with PBS three times, the slides were stained in the working liquid of fluorescent probe for 30 min at 37°C. The slides were mounted by antifade solution (Applygen, Beijing, China) after washing with PBS. Then, the slides were detected as blue fluorescent by laser confocal scanning microscope. The concentration of H_2S in culture supernatant was detected by the free radical analyzer TBR4100 (World Precision Instruments, USA). First, the ISO-H_2S-100 sensor was polarized with PBS buffer solution (pH 7.2, 0.05 mol/L) to achieve a stable baseline current (usually between 100 and 2000 pA). Then, the sensor was calibrated by the standards, in which the concentration of H_2S was 0.5 μmol/L, 1 μmol/L, 2 μmol/L, 4 μmol/L, 8 μmol/L, and 16 μmol/L, respectively. The current output jumped rapidly after each sample and then plateaus. As soon as it reached a plateau, the next sample was injected. The reduction difference between the peak and baseline was recorded (pA). Then, the calibration curve was constructed by plotting the reduction difference (pA) against the concentration (μmol/L) of H_2S. The sensor tip was immersed into each cultural supernatant sample in about 10–15 mm deep, and the concentration of H_2S was calculated via the equation.

2.3. Western Blotting Analysis. Expressions of CSE, cytochrome c (cytc), and caspase-3 as well as cleaved-caspase-3 in HUVEC were detected by western blot. The total protein of HUVEC was extracted using ice-cold cell lysis buffer containing 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L PMSF, 1 mmol/L EDTA, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS. The cells were washed with ice-cold PBS for three times, soaked in ice-cold lysis buffer for 30 min, and scraped off. Cell lysate was subsequently centrifuged at 12000 g for 10 min at 4°C, and supernatant was reserved. Protein concentration was detected using BCA method. Equal amount of protein sample (60 μg) was loaded, and SDS-PAGE electrophoresis of 10% or 12% separation gel was conducted. The separated proteins were transferred onto a nitrocellulose membrane for 2 h at 200 mA after electrophoresis. The membranes were blocked with 5% dried skimmed milk for 1 h. Then, the primary antibodies CSE (Sigma, USA) (dilution 1 : 200), cytc (Santa Cruz, USA) (dilution 1 : 200), caspase-3 (Cell Signaling Technology, USA) (dilution 1 : 1000), and cleaved-caspase-3 (Cell Signaling Technology, USA) (dilution 1 : 200) were added, respectively, and incubated at 4°C overnight. The second antibody was incubated for 1 h at room temperature after washing. LumiGLO chemiluminescence reagent was used and exposed to films. Quantification of blots was analyzed using an AlphaImager (San Leandro, CA, USA).

2.4. Preparation of Mitochondrial Protein. Separation of mitochondrion and cytoplasm was conducted using Mitochondria Isolation Kit (Applygen, Beijing, China). The HUVECs were mixed thoroughly with Mito solution after centrifugation at 800 g for 5 min, at 4°C. The supernatant was collected in a fresh tube on ice after centrifuging at 800 g for 5 min, at 4°C twice. Supernatant containing cytoplasm was then collected after centrifuging at 10000 g for 20 min, at 4°C, while precipitate containing mitochondria was processed for further centrifugation. The precipitate was washed with Mito solution and centrifugated at 12000 g for 10 min, at 4°C. The sedimentation was dissolved in Mito solution.

2.5. Detection of Superoxide Anion in HUVEC by Dihydroethidium (DHE) and CellROX Green Reagent. DHE (Beyotime, Shanghai, China) was used to detect superoxide anion in HUVEC. The medium covering the cells was changed after washing three times with PBS. Then, the cells were incubated with DHE probes for 30 min at 37°C, avoiding light exposure. Subsequently, the cells were mounted in new cultural medium for observation under a fluorescence microscope. Moreover, CellROX Green Reagent (Thermo Fisher, MA, USA) was used to quantify the generation of superoxide anion in HUVEC. HUVECs were plated in a 96-well plate. CellROX Green Reagent at a final concentration of 5 μmol/L was added in the medium and incubated with cells at 37°C for 60 min. The cells were then washed with PBS and analyzed on Fluoroskan Ascent Fluorometer (Thermo Fisher, MA, USA). Fluorescence intensity was analyzed at the excitation/emission wavelengths of 488/520 nm.

2.6. Detection of Mitochondrial Superoxide Anion in HUVEC by MitoSOX Reagent. MitoSOX Red Mitochondrial Superoxide Indicator (Thermo Fisher, MA, USA) was used to detect the generation of mitochondrial superoxide anion in HUVEC and MitoTracker Green FM (Thermo Fisher, MA, USA) was used to label mitochondria. The HUVEC on slides were covered with MitoSOX (5 μmol/L) and MitoTracker probes (100 nmol/L) after washing with PBS. The slides were incubated for 20 min at 37°C, avoiding light. Then, the HUVECs were fixed in prewarmed 4% paraformaldehyde at room temperature for 15 min after washing with warm PBS for three times. The slides were mounted by antifade solution (Applygen, Beijing, China) after washing with PBS. Cells on the slides were detected by a laser scanning confocal microscope. Red fluorescence indicated mitochondrial superoxide anion and green fluorescence indicated labeled mitochondria.

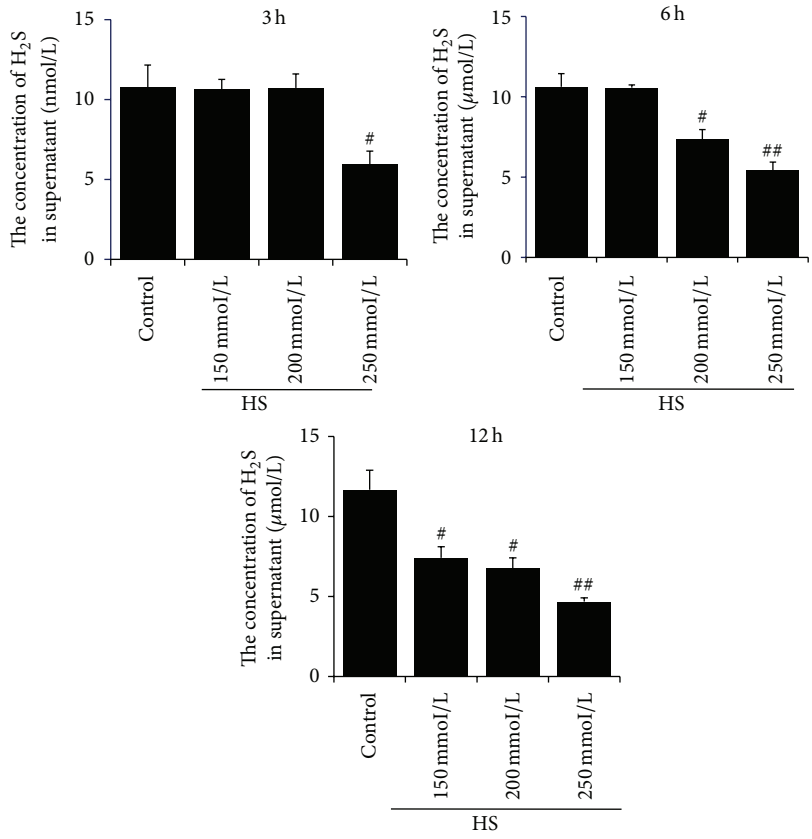
2.7. Detection of Mitochondrial Permeability Transition Pore (MPTP) Opening in HUVEC. Cell MPTP Assay Kit (Genmed, Shanghai, China) was used to test the MPTP opening in HUVEC. Experiment rationale was that calcein-AM could gather in mitochondrion presenting green fluorescent staining, whereas, being released into the cytoplasm via the opening of MPTP, fluorescent quenching could occur. Culture medium was discarded and slides were rinsed gently with cleaning solution. Slides were subsequently incubated with staining working solution for 30 min at 37°C, protecting from light. 4% paraformaldehyde was used to fix the cells for 15 min after rinsing for three times with cleaning solution. Finally, the slides were mounted with antifade solution (Applygen, Beijing, China) after washing with PBS three times and were immediately examined using a laser confocal microscope.

2.8. Measurement of Mitochondrial Membrane Potential in HUVEC. Mitochondrial membrane potential in HUVEC was measured by Mitochondrial Membrane Potential Assay Kit with JC-1 (Beyotime, Shanghai, China). When mitochondrial membrane potential is high in living cells, JC-1 accumulates in matrix in the form J-aggregates presenting red fluorescence; while mitochondrial membrane potential is low in apoptotic cells, JC-1 cannot aggregate, and the JC-1 monomer presents green fluorescence. Decreased mitochondrial membrane potential suggests apoptosis. Briefly, JC-1 working solution and cell medium were mixed at the ratio of 1 : 1. Then, the slides seeded with HUVEC were incubated with the mixture for 20 min at 37°C in the dark. The cells were fixed in prewarmed 4% paraformaldehyde at room temperature for 20 min after washing with ice-cold JC-1 buffer solution twice. After washing three times with PBS, the antifade solution (Applygen, Beijing, China) was used to mount the slides. Analysis was conducted immediately using a laser confocal scanning microscope.

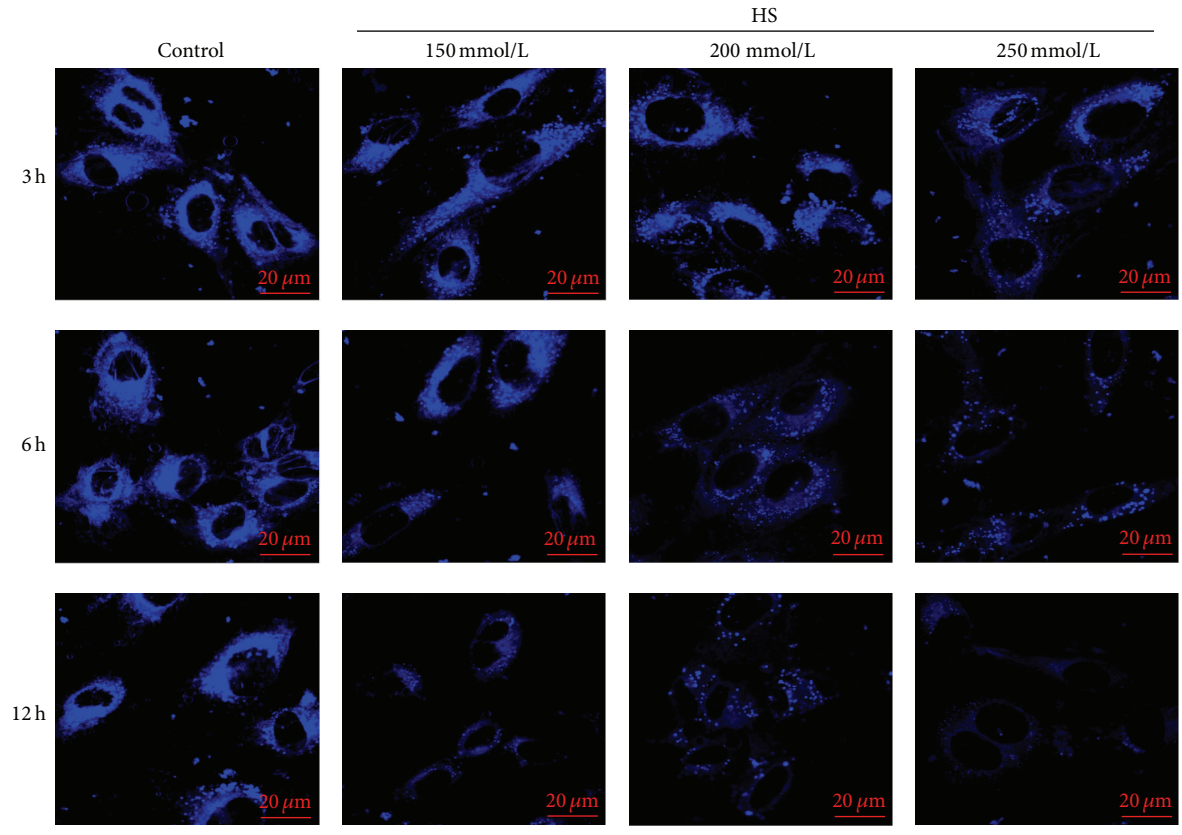
2.9. In Situ and Quantitative Measurement of Caspase-9 Activity Assay in HUVEC. Change of *in situ* caspase-9 activity in HUVEC was detected by living cells caspase-9 activity fluorescence staining kit (Genmed, Shanghai, China). Briefly, after washing twice with PBS buffer, the cells on glass slide were incubated with fluorescence probe FITC-LEHD-FMK, a FITC labeling inhibitor of caspase-9, in room temperature for 30 min. 4% paraformaldehyde was used to fix the cells in room temperature for 30 min after washing three times with PBS buffer. Then the antifade solution was used to mount the slides. Observations were made immediately under a laser confocal scanning microscope.

Change of caspase-9 activity in HUVEC was quantified using cell caspase-9 colorimetric activity kit (Genmed, Shanghai, China). The assay was based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) cleavage by caspase-9 from the labeled substrate Ac-LEHD-pNA. The free pNA was quantified using a microtiter plate reader at 405 nm. Briefly, after washing twice with PBS buffer, the cells were incubated with lysis buffer on ice for 30 min. The cells were subsequently scraped off carefully and centrifuged for 5 min at 16000 g in a microcentrifuge. The supernatant was collected in a fresh tube on ice. The BCA method was used for protein concentration determination. Then 50 μg sample and substrate buffer were put into 96-well plate in order and incubated for 90 min at 37°C. Finally, samples were read in a microtiter plate reader (Bio-Rad, Hercules, CA, USA) at 405 nm.

2.10. In Situ and Quantitative Detection of Apoptosis in HUVEC by Using TdT-Mediated dUTP Nick End Labeling (TUNEL) Assay and ELISA Assay. In situ apoptosis of HUVEC was detected with *in situ* Cell Death Detection Kit, Fluorescein (Roche, Mannheim, Germany) in accordance with the instructions of the manufacturer. Briefly, the cells on slides were washed for three times with PBS. Then, the slides were covered with TUNEL reaction mixture and incubated for 60 min at 37°C in dark. The cells were fixed in prewarmed 4% paraformaldehyde at room temperature for 15 min after washing with PBS. Then, the antifade solution was used to mount the slides after washing three times with PBS, and the slides were immediately transferred for analysis under a laser confocal scanning microscope. Moreover, the quantitative detection of DNA fragments in HUVEC was

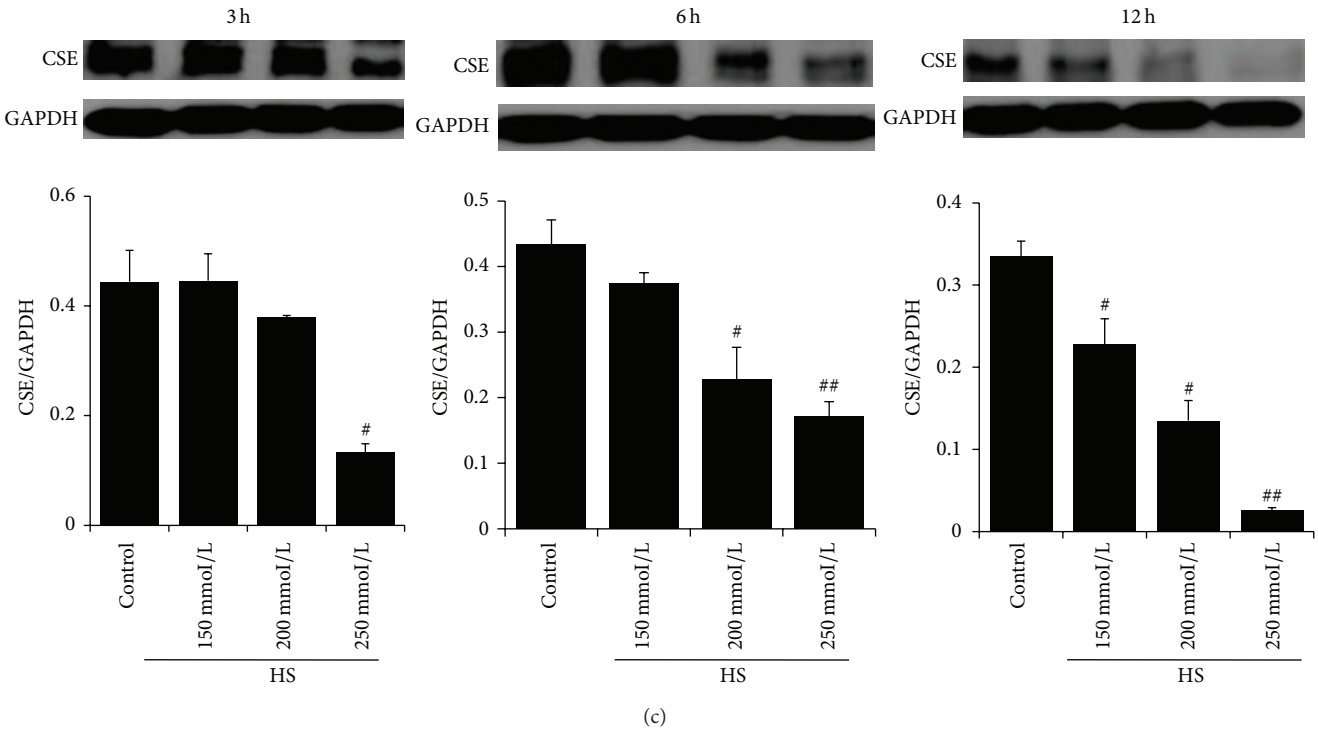


(a)



(b)

Figure 1: Continued.



(c)

Figure 1: Changes in endogenous H_2S pathway in human umbilical vein endothelial cells (HUVECs). (a) The concentration of endogenous H_2S in supernatant detected by the free radical analyzer TBR4100; (b) the production of endogenous H_2S in HUVEC determined by H_2S fluorescent probe; and (c) CSE protein expression in HUVEC analyzed by western blotting. Control: the concentration of sodium was 137 mmol/L. HS: high salt; the concentration of sodium was 150 mmol/L, 200 mmol/L, and 250 mmol/L, respectively. The scale in (b) represented 20 μm. $^{\#\#}P < 0.01$; $^{\#}P < 0.05$ versus control group.

measured by Cell Death Detection ELISA^{PLUS} Kit (Roche, Mannheim, Germany) according to the suggested protocol of the manufacturer. The assay is based on a quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies directed against DNA and histones, respectively. This allows the specific determination of mono- and oligonucleosomes in the cytoplasmatic fraction of cell lysates. Briefly, the cell lysate was added into a streptavidin-coated microplate. A mixture of anti-histone-biotin and anti-DNA-POD was added and incubated. During the incubation period, the antibody-nucleosome complexes were bound to the microplate by the streptavidin. After a washing step, the unbound components were removed. The amount of nucleosomes by the POD retained in the immunocomplex was quantitatively determined by a microtiter plate reader (Bio-Rad, CA, USA) at 405 nm.

2.11. Statistical Analysis. Data are described as mean ± SE. SPSS 13 (SPSS Software, Inc., Chicago, IL, USA) was used for data analysis. Independent-samples t-test or one-way ANOVA followed by either LSD test was conducted and $P < 0.05$ was considered statistically significant.

3. Results

3.1. The Endogenous H_2S Pathway Was Downregulated in High-Salt- (HS-) Stimulated HUVEC. Compared with control group, H_2S synthesis in HUVEC was significantly reduced with prolonged sodium chloride (NaCl) incubation. After the treatment with 250 mmol/L NaCl medium for 3 h, H_2S content in the culture supernatant of HUVEC was significantly decreased (Figure 1(a)), and the fluorescence of H_2S probes weakened (Figure 1(b)). H_2S content in the culture supernatant was significantly decreased after incubation with 200 mmol/L and 250 mmol/L NaCl medium for 6 h, respectively, with parallel decrease of the H_2S probe fluorescence in HUVECs (Figure 1(b)). Moreover, 12 h incubation with 150 mmol/L, 200 mmol/L, and 250 mmol/L NaCl medium demonstrated a significantly decreased H_2S content in HUVEC culture supernatant (Figure 1(a)) as well as reduced H_2S probe fluorescence (Figure 1(b)). The results of CSE protein expression in HUVEC were consistent with the variation trend of H_2S content (Figure 1(c)).

3.2. H_2S Inhibited Superoxide Anion Generation in High-Salt-Induced HUVEC. Compared with control group, the superoxide anion generation in HUVEC of HS group represented by both DHE staining image and fluorescence quantification with CellROX Green Reagent on fluorometer was significantly increased (Figures 2(a) and 2(b)). However, when pretreated with NaHS, the superoxide anion generation in high-salt-induced HUVEC was decreased markedly (Figures 2(a) and 2(b)). Furthermore, the change of superoxide anion generation in mitochondria of HUVEC detected by MitoSOX probe was in accordance with the change of superoxide anion generation in whole HUVEC (Figure 2(c)).

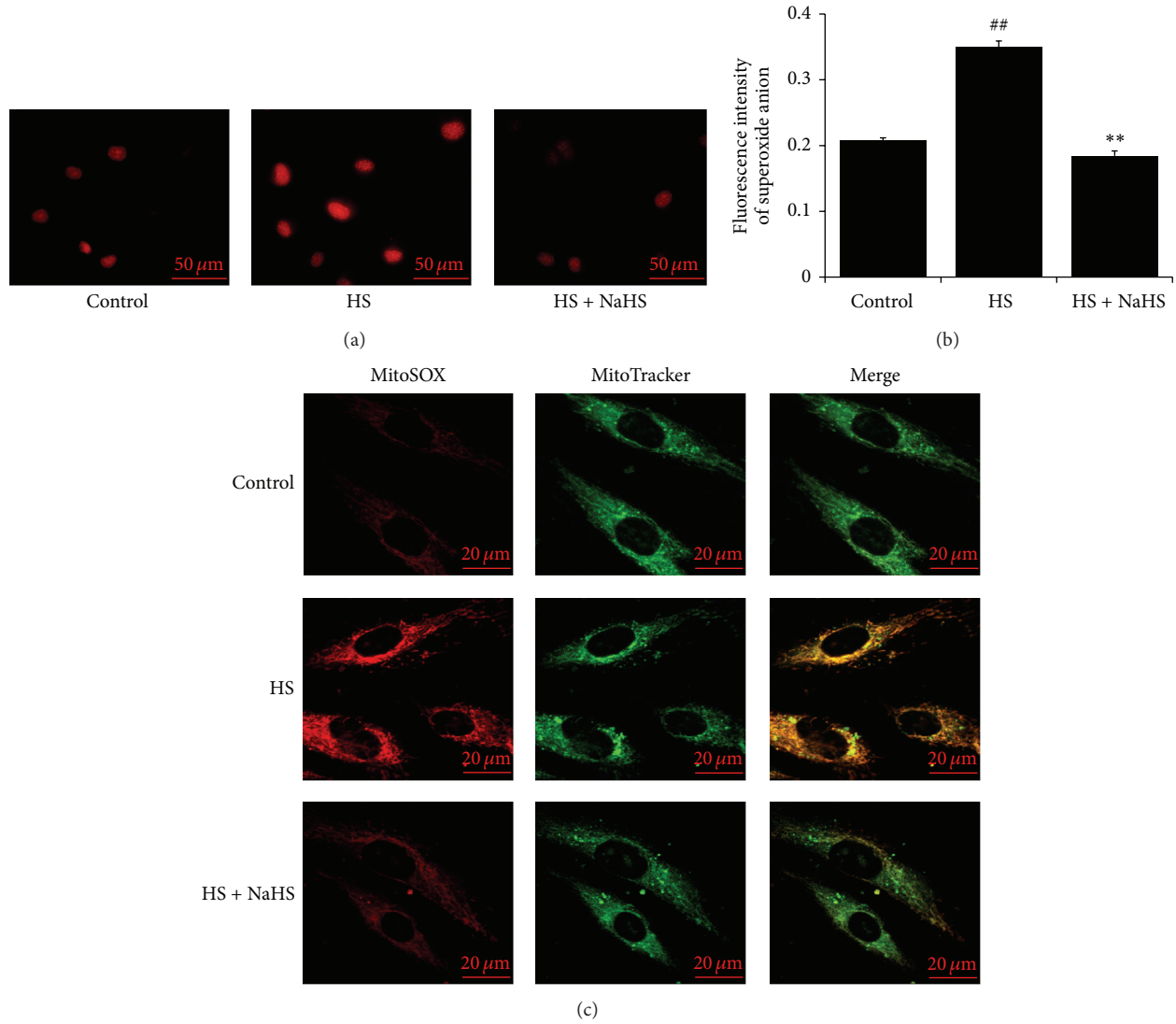


Figure 2: Changes in superoxide anion generation in human umbilical vein endothelial cells (HUVECs). (a) Fluorescent micrographs of superoxide anion generation in HUVEC detected by DHE probes. Red fluorescence indicates levels of superoxide anion generation in HUVEC; (b) quantification analysis of fluorescent intensity of superoxide anion generation in HUVEC detected by CellROX Green Reagent on Fluoroskan Ascent Fluorometer; (c) superoxide anion generation in HUVEC mitochondria detected by MitoSOX probes. Control: the cell treated with 137 mmol/L sodium. HS: high salt, the cell treated with 200 mmol/L sodium for 6 h. HS + NaHS: the cell pretreated with 200 μmol/L NaHS for 30 min following 200 mmol/L sodium for 6 h. $^{\#\#}P < 0.01$ versus control group; $^{**}P < 0.01$ versus HS group.

3.3. H_2S Inhibited Mitochondrial Dysfunction in High-Salt-Induced HUVEC. In HS group, the mitochondrial membrane potential was significantly reduced (Figure 3(a)) and mitochondrial permeability transition pore significantly opened (Figure 3(b)) relative to that of the control group, whereas H_2S donor increased mitochondrial membrane potential (Figure 3(a)) and closed mitochondria permeability transition pore in HS-treated HUVEC (Figure 3(b)).

3.4. H_2S Antagonized High-Salt-Induced Release of Cytc from Mitochondria into Cytoplasm in HUVEC. Western blot results showed no significant difference of the total cytc among different groups. Interestingly, compared with control group, high-salt treatment significantly downregulated cytc protein expression in mitochondria of HUVEC ($P < 0.05$) and markedly increased cytoplasmic cytc ($P < 0.01$) (Figure 4), whereas, in the presence of NaHS, the cytoplasmic cytc protein expression was profoundly downregulated ($P < 0.01$) and mitochondria cytc was upregulated ($P < 0.01$) (Figure 4) in HS + NaHS-treated HUVEC as compared with that of HS-treated HUVEC.

3.5. H_2S Inhibited Caspase-9 Activation in High-Salt-Treated HUVEC. Compared with the control group, incubation with high salt for 6 h markedly enhanced the green fluorescence indicating caspase-9 activities in HUVEC and semiquantitative analysis suggested profoundly increased caspase-9

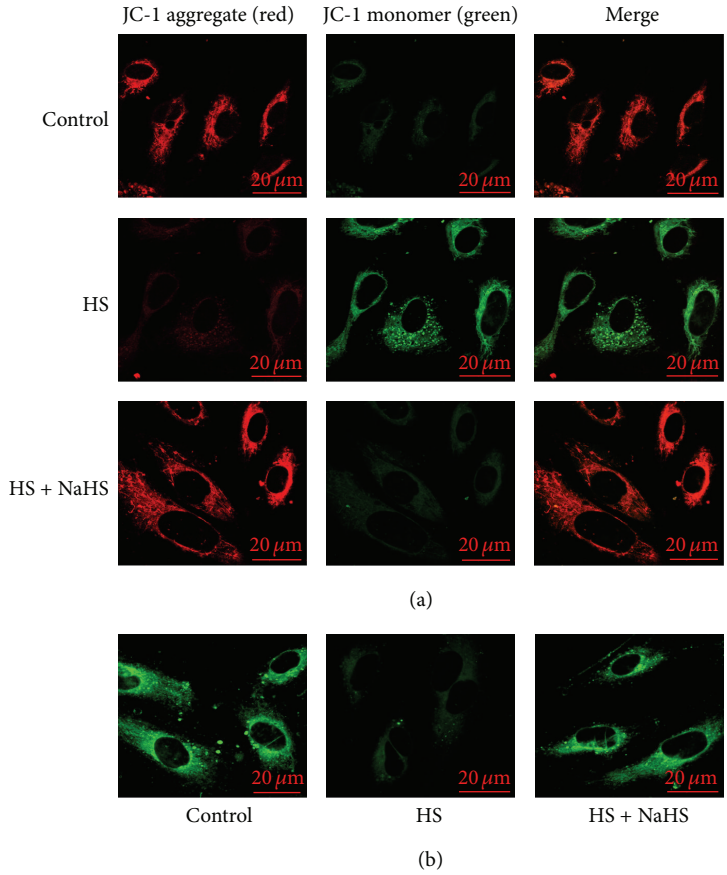


Figure 3: Changes in mitochondrial membrane potential and mitochondrial permeability transition pore (MPTP) opening in human umbilical vein endothelial cells (HUVECs). (a) Change of mitochondrial membrane potential detected by JC-1 fluorescent probe and examined by laser confocal microscope, with red fluorescence presenting JC-1 aggregate and green JC-1 monomer. (b) Changes of MPTP opening in HUVEC detected by calcein-AM as a fluorescence indicator by laser confocal microscopy. The green fluorescence quenching represented MPTP opening. Control: the cell treated with 137 mmol/L sodium. HS: high salt, the cell treated with 200 mmol/L sodium for 6 h. HS + NaHS: the cell pretreated with 200 μmol/L NaHS for 30 min following 200 mmol/L sodium for 6 h.

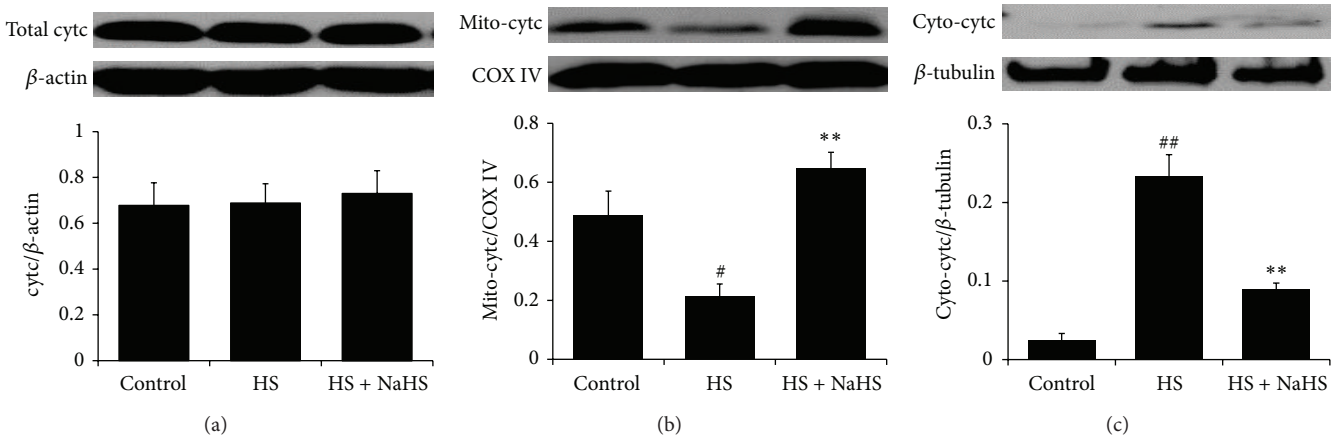


Figure 4: Cytochrome c (cytc) protein expression and distribution in human umbilical vein endothelial cells (HUVECs). Control: the cell treated with 137 mmol/L sodium. HS: high salt, the cell treated with 200 mmol/L sodium for 6 h. HS + NaHS: the cell pretreated with 200 μmol/L NaHS for 30 min following 200 mmol/L sodium for 6 h. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ versus control group; $^{**}P < 0.01$ versus HS group.

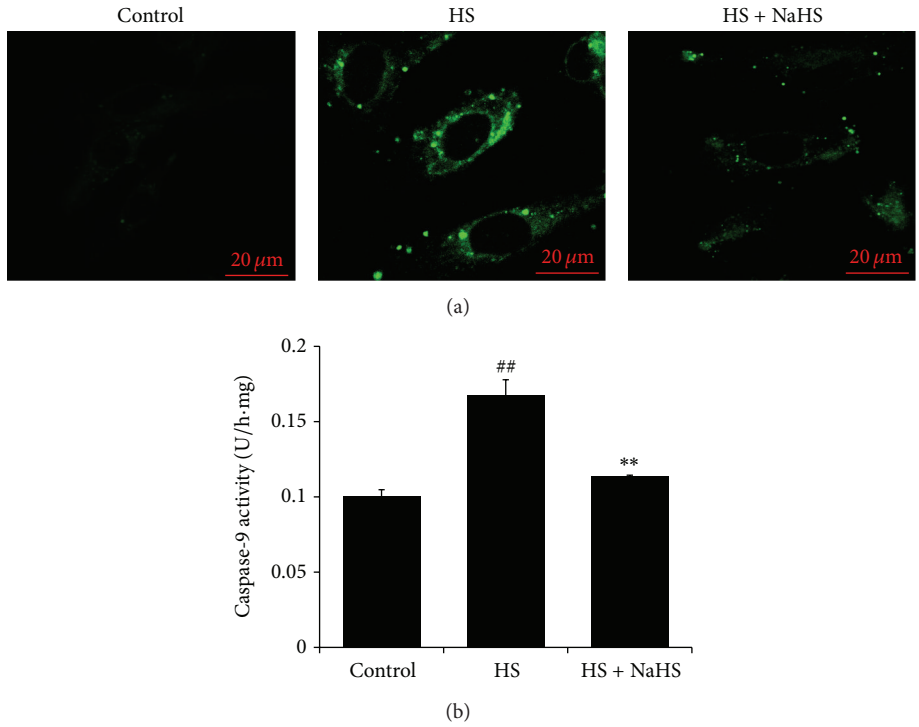


FIGURE 5: Caspase-9 activities in human umbilical vein endothelial cells (HUVECs). (a) Caspase-9 activities in HUVEC detected by living cells caspase-9 fluorescence staining kit. (b) Quantitative analysis of caspase-9 activities in HUVEC. Control: the cell treated with 137 mmol/L sodium. HS: high salt, the cell treated with 200 mmol/L sodium for 6 h. HS + NaHS: the cell pretreated with 200 μmol/L NaHS for 30 min following 200 mmol/L sodium for 6 h. $^{\#\#}P < 0.01$ versus control group; $^{**}P < 0.01$ versus HS group.

activity (Figure 5), while H_2S administration reversed the high-salt-induced caspase-9 activities (Figure 5).

3.6. H_2S Reversed HS-Induced HUVEC Apoptosis. After incubation with high salt (200 mmol/L) for 6 h, the ratio of cleaved-caspase-3/caspase-3 in HUVECs was markedly increased ($P < 0.01$) (Figure 6(a)), the amount of nucleosomes in the HUVEC lysate representing DNA fragmentation was increased ($P < 0.01$) (Figure 6(b)), and the green fluorescence intensity in the nuclei of TUNEL-positive HUVECs was augmented (Figure 6(c)). Interestingly, H_2S donor inhibited caspase-3 activation, and DNA fragmentation in HUVECs was treated with high salt (P all <0.01) (Figures 6(a) and 6(b)) and weakened the green fluorescence intensity in the nuclei of TUNEL-positive HUVECs (Figure 6(c)).

4. Discussion

High-salt diet is an important risk factor associated with hypertension. Recently, several studies have focused on investigating the direct impact of high salt on blood vessels. Liu et al. [24] found that high salt could promote the proliferation of vascular smooth muscle cells induced by angiotensin II. A recent study by Dmitrieva and Burg [25] proved that high salt might increase the secretion of von Willebrand factor (vWF) in vascular endothelial cells, resulting in hypercoagulability and thrombosis. Vascular endothelial cells serve as the primary barrier maintaining vascular function and structural stability. In the present study, we found that increased concentration of extracellular NaCl promoted caspase-3 activities and apoptosis in endothelial cells as demonstrated by TUNEL assay, confirming that high salt could stimulate endothelial cell apoptosis. Then, subsequently, the possible mechanism involved was examined.

The cardiovascular protective effects of endogenous H_2S have been widely reported. Yang et al. [20] discovered that H_2S generated by vascular endothelial cells played an important regulatory role in maintaining vascular structure and function and CSE deficiency was involved in the abolished endothelium-dependent vasodilation and hypertention in mutant mice. Shen et al. [22] reported H_2S encouraged the proliferation and migration of endothelial cells and played a protective role in vascular endothelial cells. In our present study, H_2S content in both vascular endothelia and culture supernatant showed a time-dependent reduction after high-salt treatment in addition to downregulated expression of CSE, a key enzyme for H_2S synthesis. Nevertheless, supplementing H_2S donor markedly inhibited endothelial cell apoptosis induced by high salt, suggesting that high-salt-induced downregulation of endogenous H_2S pathway resulted in inadequate protective effects of H_2S on vascular endothelial cells, which might be one of the mechanisms associated with high-salt-mediated vascular endothelial damage. Then, the possible protective mechanism by which endogenous H_2S

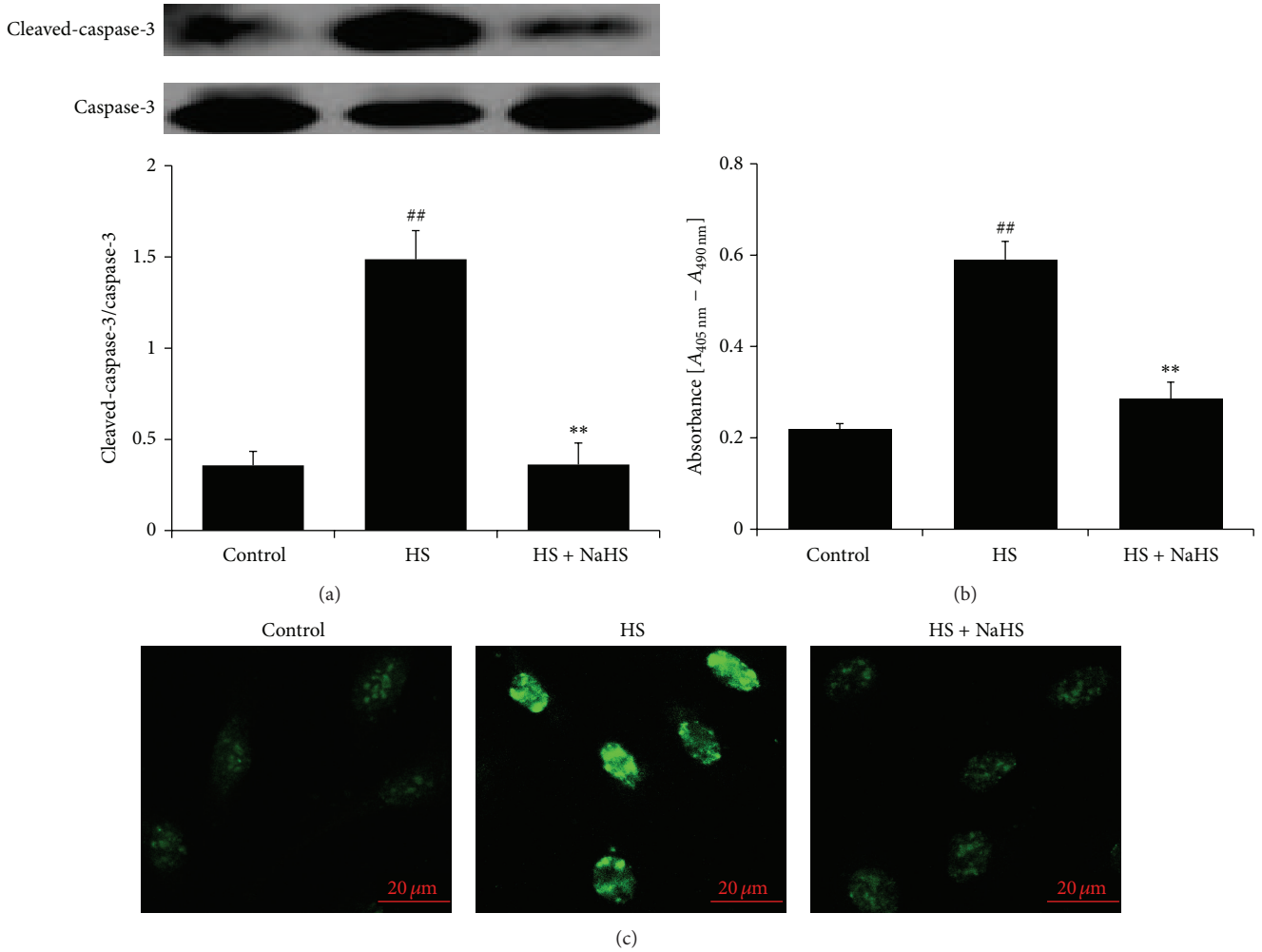


Figure 6: Apoptosis and caspase-3 activities in human umbilical vein endothelial cells (HUVECs). (a) Caspase-3 activities in HUVEC detected by western blot; (b) DNA fragmentation in HUVEC detected by Cell Death Detection ELISA. (c) HUVEC apoptosis detected by *in situ* Cell Death Detection Kit, Fluorescein. Control: the cell treated with 137 mmol/L sodium. HS: high salt, the cell treated with 200 mmol/L sodium for 6 h. HS + NaHS: the cell pretreated with 200 μmol/L NaHS for 30 min following 200 mmol/L sodium for 6 h. $^{\#\#}P < 0.01$ versus control group; $^{**}P < 0.01$ versus HS group.

protected against high-salt damage in vascular endothelia was investigated.

Animal experiments revealed that high-salt diet increased production of microvascular reactive oxygen species in rat striated muscles as well as promoted superoxide anion generation in the superior mesenteric arteries, contributing to oxidative stress *in vivo* [11, 12]. Other studies established antioxidant effects of H_2S in multiple systems of the body including the central nervous system, cardiovascular system, and respiratory system [19, 22, 26]. In the present study, DHE fluorescent probe demonstrated that high-salt stimulation significantly augmented superoxide anion generation in HUVEC. Considering that mitochondrion was one of major sources producing oxygen free radicals, in particular, increased production and decreased removal of oxygen free radicals caused by abnormal mitochondrial structures and dysfunctional mitochondria remain an important mechanism of cellular oxidative stress injury. Therefore, further study was designed to examine oxygen free radical generation in mitochondria using mitochondrial oxygen radical-specific fluorescent probe MitoSOX. The results showed that high salt promoted oxygen free radical generation in mitochondria, which was abolished in the presence of H_2S. Therefore, we speculate that mitochondria play a crucial role in high-salt-induced vascular endothelial injury and likely mediate protective effects of endogenous H_2S on vascular endothelia.

Mitochondria-related apoptosis is one of the important mechanisms for apoptosis [27]. Previous studies showed that excessive generation of mitochondrial ROS might cause lipid peroxidation of mitochondrial membranes, thereby destroying mitochondrial membrane potential and promoting mitochondrial MPTP opening; meanwhile, the combination of cytc and endometrial center phospholipid molecules was broken, releasing the "free" cytc. Increased mitochondrial permeability encouraged the leakage of cytc and other proapoptotic molecules into the cytoplasm, activating caspase-9

and caspase-3, initiating apoptosis cascade, and eventually culminating to cell death [28]. Our results showed that, under high-salt treatment, mitochondrial membrane potential in vascular endothelia was significantly reduced along with increased MPTP opening and release of mitochondrial cytc to cytoplasm, activating cytoplasmic caspase-9. In contrast, H_2S donor restored mitochondrial membrane potential in vascular endothelia, inhibited MPTP opening, and blocked the leakage of mitochondrial cytc, establishing that H_2S antagonized the mitochondria-mediated apoptosis induced by high salt.

Taken together, this study demonstrated for the first time that high salt damaged vascular endothelial cells through downregulating H_2S/CSE pathway, which might subsequently result in augmented endothelial cell oxidative stress, increasing mitochondria-mediated endothelial cell apoptosis.

The limitation of this study was the relatively high dose of sodium used in the experiment. Although recent study showed that HUVECs adapted sodium well at 380 mosmol/kg (or 190 mmol/L sodium), maintaining a normal appearance of the cells and a logarithmic growth for two weeks [25], and the concentration of 500 mosmol/kg (or 250 mmol/L sodium) was used in the *in vitro* experiment [29], there was a limited elevation of plasma sodium concentration (about 2–6 mmol/L) in salt-sensitive hypertensive patients and animal models with high-salt diet [30, 31]. Moreover, extreme hypernatraemia (196 mmol/L) only occurred in a small part of patients [32]. Furthermore, sodium concentration at 150 mmoL and above is very likely to affect the membrane potential of various cells and tertiary protein structure of multiple enzymes and accordingly the enzyme activity, which might interrupt the specificity of the protection of endogenous H_2S/CSE pathway on the HUVECs. Therefore, the effect of a reasonable high-salt insult which was in accordance with clinical change in plasma sodium in the patients and animal model with high-salt diet on the HUVEC needs further investigation.

Conflict of Interests

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

Authors' Contribution

Yanfang Zong and Yaqian Huang contributed equally to this work.

Acknowledgments

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Review Article

Hydrogen Sulfide: A Therapeutic Candidate for Fibrotic Disease?

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Fibrotic diseases including chronic kidney disease, liver cirrhosis, idiopathic pulmonary fibrosis, and chronic disease account for 45% mortality in the developed countries and pose a great threat to the global health. Many great targets and molecules have been reported to be involved in the initiation and/or progression of fibrosis, among which inflammation and oxidative stress are well-recognized modulation targets. Hydrogen sulfide (H_2S) is the third gasotransmitter with potent properties in inhibiting inflammation and oxidative stress in various organs. Recent evidence suggests that plasma H_2S level is decreased in various animal models of fibrotic diseases and supplement of exogenous H_2S is able to ameliorate fibrosis in the kidney, lung, liver, and heart. This leads us to propose that modulation of H_2S production may represent a promising therapeutic venue for the treatment of a variety of fibrotic diseases. Here, we summarize and discuss the current data on the role and underlying mechanisms of H_2S in fibrosis diseases related to heart, liver, kidney, and other organs.

1. Introduction

Fibrotic disease refers to a group of clinical entities including chronic kidney disease, liver cirrhosis, idiopathic pulmonary fibrosis, and chronic heart failure, featured by chronic inflammatory diseases [1, 2]. As an important pathological feature, fibrosis is also present in many autoimmune diseases such as scleroderma, Crohn's disease, and systemic lupus erythematosus and affects the long-term survival of the graft as well as tumor metastasis patients [3]. In essence, fibrosis is a dysregulated wound healing process. It involves multiple cellular events such as the recruitment of inflammatory cells, the release of profibrotic cytokines, and the activation of collagen-producing cells including fibroblast, epithelial cells, and bone marrow stromal cells [4]. If highly progressive, fibrosis will eventually lead to the formation of permanent scars, irreversible organ dysfunction, and even death. In developed countries, fibrotic diseases account for nearly 45% morbidity and mortality [5]. Unfortunately, there are few effective therapies in most organ fibrosis, and the validated antifibrotic agents are even fewer. To date, the inhibitors of renin-angiotensin-aldosterone system (RAAS) are the primary medications for renal fibrosis and myocardial remodeling, but the application of these drugs is limited when serum creatinine rises above 3.5 mg/dL [6, 7]. Numerous novel therapeutic targets for fibrosis have been proposed, but monotarget therapy or simple combined treatment seems ineffective [8]. Since fibrosis is a disorder associated with multiple molecules and processes, small molecules interacting with several molecular targets of the fibrosis cascade would be promising for the treatment of fibrotic diseases.

Hydrogen sulfide (H_2S) is the third gaseous transmitter secondary to nitric oxide (NO) and carbon monoxide [9]. For many decades, it was recognized as a poisonous gas because of its ability to inhibit cytochrome c oxidase in a similar manner to hydrogen cyanide. Recently, a lot of independent work demonstrates that H_2S can be endogenously produced by cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE), and 3-mercaptopyruvate sulphurtransferase (3-MST) in mammal organs and exerts important physiological and pathological effects. For instance, H_2S is able to regulate vascular tone, inflammation, oxidative stress, cardiac contractility, nociception, insulin secretion, and resistance, and so forth. These have been intensively and extensively

discussed and reviewed elsewhere [10]. Recently, it is interesting to find that plasma H_2S level is decreased in many animal models of fibrotic diseases and the supplement of exogenous H_2S partially reverses fibrosis in the fibrotic organs [11–13]. In this review, we summarize the recent evidence of the antifibrotic effect of H_2S in various types of organs and discuss its underlying antifibrotic mechanisms, with the emphasis on its anti-inflammatory and antioxidative effects on fibrogenesis. Finally, we discuss the current obstacles for the application of H_2S in the treatment of organ fibrosis.

2. Protective Effect of H_2S against Fibrotic Diseases

2.1. H_2S and Chronic Kidney Disease. Tubulointerstitial fibrosis is the final common pathway of chronic kidney disease (CKD) regardless of the initial injuries [14]. Pathologically, renal fibrosis is featured by the deposition of collagen I, collagen III, and collagen IV, the accumulation of myofibroblasts and extracellular matrix proteins, and the infiltration of inflammatory cells. Three H_2S-producing enzymes, CBS, CSE, and 3-MST, have been found in various parts of the kidney and produce H_2S in a synergistic way [15]. Recently, we and other researchers consistently demonstrated that CBS is the predominant H_2S-generating enzyme located in proximal renal tubules, while CSE is sporadically expressed in glomeruli, renal arterioles, and interstitium [13, 16].

H_2S plays physiological roles in maintaining normal function of kidney. For instance, H_2S acts as an oxygen sensor monitoring the oxygen contents of the renal medulla and regulating the regional blood flow in renal cortex [17]. It also increases the glomerular filtration rate and exhibits diuretic property by excreting sodium and kalium ion into the urine [15].

Accumulating evidence suggests that H_2S may inhibit fibrosis in CKD. The plasma H_2S level has been found reduced in 5/6 nephrectomy rats and uremia patients [18, 19]. Moreover, in heterozygous $cbs^{+/-}$ mice with unilateral nephrectomy, a CKD model featured by proteinuria, the expressions of collagen and matrix metalloproteinase-2 and metalloproteinase-9 were markedly enhanced [20]. We recently found that, at a relatively lower dosage, H_2S donor NaHS alleviated renal fibrosis in rats with unilateral urethral obstruction (UUO) [13]. Since UUO model is featured with progressive tubulointerstitial fibrosis without confounding causative factors such as proteinuria, hypertension, and uremia toxin, our study indicates a direct link between H_2S and renal fibrosis. Moreover, CBS-derived H_2S may be relevant in maintaining homeostasis in the kidney under normal conditions and may be suppressed under pathological conditions, because the amount of CBS protein level in proximal tubules was considerably and immediately reduced after UUO injury, while CSE expression in the renal interstitium was compensatorily enhanced and CSE inhibitor DL-propargylglycine (PAG) aggravated renal fibrosis [13]. This finding is consistent with a previous study, in which CBS reduction was earlier than the increase of CSE (6 h versus 24 h) after renal ischemic reperfusion [21]. Therefore, current data suggest that targeting CBS rather than CSE may be more promising in modulating endogenous H_2S generation for the treatment of kidney fibrosis, particularly in the early period of renal fibrosis.

Another noteworthy effect of H_2S on renal fibrosis is its inhibition on RAAS. The kidney contains all the components of RAAS. Angiotensin II is a potent profibrotic factor, which can stimulate collagen synthesis through the TGF-β1 [22] dependent and independent signaling pathway [23]. H_2S can not only lower the serum angiotensin II level in renovascular hypertension animal model by downregulating the cellular cAMP production [24], but also counteract the hypertension, proteinuria, and renal damage induced by angiotensin II [25]. Recent data suggest that renin is also implicated in the progression of renal fibrosis and direct rennin inhibition with Aliskiren attenuating inflammation and fibrosis induced by UUO [26]. It has been found that H_2S is able to inhibit the activity of angiotensin-converting enzyme (ACE) in human endothelial cells although the ACE mRNA expression is not altered [27]. Moreover, endogenous H_2S can suppress the release of renin in As4.1 and renin-rich renal cells [28]. Taken together, all the data indicate that H_2S may represent a novel venue for drug development to treat renal fibrosis, particularly when serum creatinine is beyond 3.5 mg/dL and the application of RAAS inhibitors is limited.

2.2. H_2S and Hepatic Fibrosis. The liver is an important organ to maintain the plasma H_2S homeostasis by regulating its production and elimination. Hepatic H_2S production is mainly determined by CBS and CSE, both of which are rich in the liver. On the other hand, H_2S is mainly eliminated in the liver via oxidation [29]. Similar to the kidney, CBS is more important in maintaining the normal liver function compared with CSE and 3-MST during physiological conditions [30].

The primary functions of H_2S in the liver are associated with the regulations of lipid and glucose metabolism. H_2S is critical for maintaining the normal lipid profile. The serum triglyceride and fatty acid levels were increased in *cbs* deficient mice [31], while high fat diet to *cbs* deficient mice enhanced serum total cholesterol and low density lipoprotein (LDL) cholesterol levels but decreased high density lipoprotein (HDL) cholesterol [32]. In addition, NaHS inhibited the insulin-stimulated absorption of glucose and decreased the glycogen content in HepG2 cells, indicating its beneficial effect on insulin resistance and diabetes [33].

Recent data also demonstrated that H_2S may be implicated in hepatic fibrosis. Liver cirrhosis is associated with reduced serum H_2S level. Tan et al. reported that NaHS (10 μmol/kg, i.p.) significantly ameliorated liver fibrosis and portal hypertension induced by tetrachloride, while PAG (30 mg/kg, i.p.) resulted in converse effects [12]. Fan et al. also found that NaHS (500 μM) inhibited hepatic stellate (HSC-T6) cell proliferation caused by ferric nitrilotriacetate [34]. However, the antifibrotic action of H_2S on liver fibrosis may be of limited clinical relevance, because the primary causes of hepatic fibrosis are viral hepatitis and nonalcoholic fatty liver disease, not chemical intoxication. Moreover, inconsistent evidence stems from *cbs* genetic mutant mice. A study demonstrated that 3–8-week-old *cbs* deficient mice

exhibited no signs of liver fibrosis [35], while another study revealed that the liver fibrosis was evident in 8–32-week *cbs* deficientmice [36]. Surely, it can be argued that the difference may be related to the mice age. As a result, more studies are needed to validate the antifibrotic effect of H_2S in different animal models of hepatitis in the future.

2.3. H_2S and Pulmonary Fibrosis. H_2S can also be produced by CBS and CSE in the lung [37]. The expression pattern of the three H_2S-producing enzymes varies among different species and cell types. In bovine pulmonary tissues, CSE is predominantly expressed in vascular smooth muscle cells (SMCs), while CBS is mainly located in endothelial cells [38]. In murid animals, CSE is found in blood vessel as well as airway SMCs in rat lung tissues, but CBS and CSE are colocalized in arterial and airway SMCs, endothelial cells, and SMCs [39]. Both CBS and CSE have been detected in human airway SMCs and lung fibroblast MRC5 cells [40].

H_2S affects various respiratory system diseases. Serum H_2S levels are significantly lower in chronic obstructive pulmonary disease patients than in control subjects [41]. H_2S inhibits chronic inflammation, airway, and vascular remodeling and thus exhibits therapeutic effects on asthma and pulmonary hypertension [42, 43]. More importantly, the antifibrotic effect of H_2S on pulmonary fibrosis has been recently reported. The serum H_2S levels were decreased in the rats treated with bleomycin on day 7, compared with controls, while CSE mRNA expression was increased on days 7 and 28 [11]. Intraperitoneal injections with NaHS at 1.4 μmol/kg and 7 μmol/kg twice a day significantly reduced the contents of hydroxyproline and malondialdehyde (MDA) in the lung, although the significance was not obtained between these two dosages of NaHS [11]. Consistently, an *in vitro* study showed that 100 μmol/L H_2S inhibited the proliferation, migration, and transdifferentiation of human lung fibroblast stimulated by fetal bovine serum [44].

2.4. H_2S and Cardiac Fibrosis. Adverse cardiac remolding is an important event that may eventually lead to chronic heart failure. Partial manifestation of cardiac remolding is the formation of interstitial fibrosis, featured by the activation of cardiac fibroblast associated with excessive formation of extracellular matrix within the myocardium. It is well known that the local RAAS is activated in chronic heart failure and plays an important role in cardiac remolding. Locally released angiotensin II is able to stimulate the proliferation of cardiac fibroblasts and increase the collagen production by activating the AT1 receptor [45]. Recent data suggests that aldosterone is also able to induce cardiac fibrosis by exhibiting proinflammatory effects and directly promoting the cardiac fibroblasts proliferation and collagen synthesis [46]. Although the production of cardiac renin is debatable, mast cells constitute a major source of renin after myocardial infarction, contributing to the formation of local angiotensin II [47].

H_2S generation in the cardiovascular system is largely ascribed to CSE although CBS mRNA is detected in the tumoral tissue of the heart [48]. Similar to other organs, H_2S is able to block cardiac fibrosis in various heart diseases. The first study on the association between cardiac fibrosis and H_2S was presented by Shi et al., who demonstrated that exogenous administration of NaHS (10–90 μmol/kg/day, i.p.) for three consecutive months markedly ameliorated the left ventricular remodeling and cardiac fibrosis in spontaneously hypertensive rat [49]. Similar results were obtained in streptozotocin-induced diabetic rats in which NaHS (14 μmol/kg/day, i.p.) administration for 7 weeks attenuated the expressions of matrix metalloproteinase-2 (MMP-2), procollagen-1, and TGF-β1 in the left ventricle [50]. Moreover, administration with the same dose of NaHS (14 μmol/kg/day, i.p.) for 14 days ameliorated collagen deposition in the left ventricle induced by abdominal aortic coarctation [51].

Recent evidence demonstrated that H_2S exhibited antifibrotic effects in the heart by inhibiting the local RAAS. Exogenous H_2S attenuated angiotensin II-induced hypertension, cardiac fibrosis, and oxidative stress in rats [52]. *In vitro* study also confirmed the antiangiotensin II effect of H_2S. For instance, NaHS (50–100 μmol/L) inhibited the cardiac fibroblasts proliferation induced by 10% fetal bovine serum and angiotensin II [53]. Liu et al. reported that NaHS ameliorated isoproterenol-induced heart failure through the suppression of renin degranulation from cardiac mast cells [54]. As the renin level can be reduced by H_2S, it is reasonable to hypothesize that H_2S is able to decrease the aldosterone level in the heart. Future studies are needed to validate this hypothesis.

The effects of H_2S on various cells associated with fibrosis are summarized in Figure 1.

3. Cellular and Molecular Mechanisms of the Antifibrotic Effects of H_2S

3.1. H_2S Inhibits Myofibroblast Activation. Myofibroblast activation and proliferation are two key cellular events of fibrosis [4]. Once activated by injury or chronic inflammation, the fibroblast deposits extracellular matrix protein such as fibronectin within the cell and secrets fibrogenic cytokines such as TGF-β1, which in turn contributes to fibroblast differentiation. Although the origin of myofibroblasts is still debatable, a recent study using fate map technology revealed multiple origins of renal myofibroblasts, with 50% from resident fibroblasts, 35% from bone marrow, 10% arising from the endothelial-to-mesenchymal transition, and 5% from the epithelial-to-mesenchymal transition [55].

A multitude of *in vitro* studies demonstrated that H_2S reduced the proliferation and differentiation of fibroblasts in various types of organs. In the kidney, NaHS at 100 μmol/L suppressed the fetal bovine serum stimulated fibroblast proliferation by reducing DNA synthesis and the expressions of proliferating cell nuclear antigen and c-Myc within the cells. NaHS also blocked TGF-β1-induced fibroblasts differentiation through inhibiting the phosphorylation of Smad3 and mitogen-activated protein kinases [13]. Similar results were obtained in pancreatic stellate cells. NaHS (12.5–500 μmol/L) decreased the cell number in a dose-dependent manner and halted the cell cycle progression at the G0/G1 check-point [56]. NaHS also inhibited the pancreatic stellate cells migration stimulated by 10% fetal bovine serum [56].

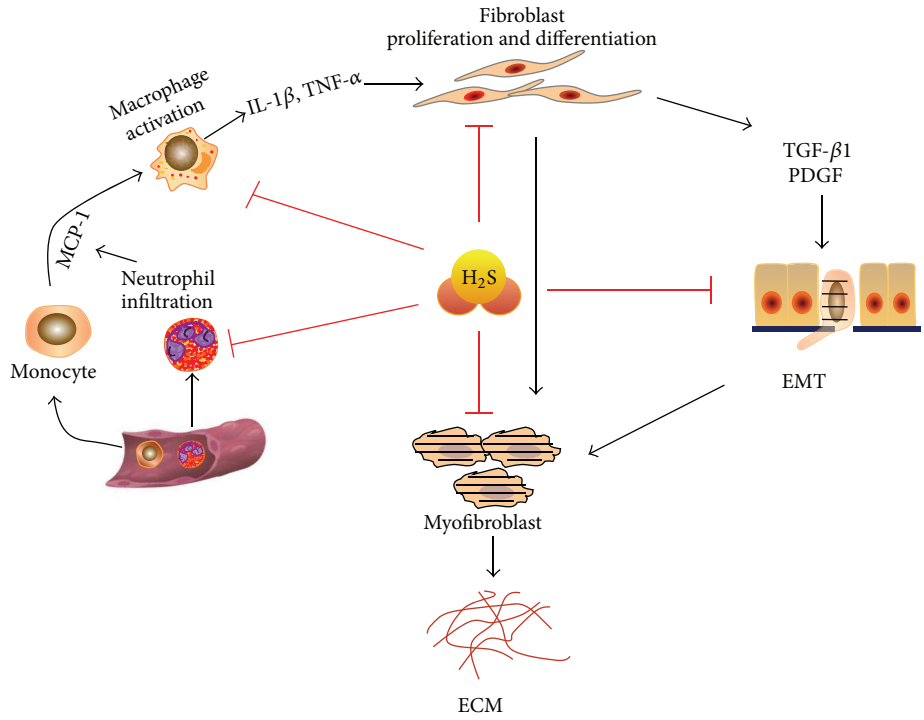

Figure 1: The effects of H_2S on various cells associated with fibrosis. H_2S inhibits neutrophil infiltration and macrophage activation during the inflammation response. H_2S inhibits fibroblast proliferation and differentiation to myofibroblasts. In epithelial cells, H_2S inhibits the epithelial-mesenchymal transition (EMT) process induced by various insults and cytokines. ECM: extracellular matrix.

Lastly, in human alveolar epithelial cells (A549 cells), H_2S (100 μmol/L) reversed the TGF-β1-induced epithelial-mesenchymal transition by suppressing the expression of E-cadherin and increasing the vimentin levels within the cells [57].

3.2. H_2S Exhibits Antifibrotic Effect by Inhibiting Inflammation. Inflammation plays a crucial role in the development and progression of fibrosis. The damaged epithelial cells and platelets may produce various chemotactic factors which further recruit neutrophils and macrophages to the damaged tissues [58]. Infiltration of these myeloid cells functions as a two-edged sword on fibrosis. On one hand, these cells are important for wound healing as they eliminate fibrin clots and cellular debris in the sites of injury. On the other hand, they secrete a variety of inflammatory mediators that may damage the surrounding tissues. If not properly eliminated, neutrophils and macrophages are capable of augmenting the inflammatory responses and eventually result in the formation of permanent scars.

It has been well recognized that macrophage serves as the most important immune cells associated with fibrosis. UUO animal model revealed a large quantity of macrophage infiltration in the renal interstitium in the early stage (3 days) after injury [59]. Macrophage ablation considerably reduced the production of various growth factors including TGF-β1 in various clinical settings [60]. Recent studies show that macrophage phenotype transition is implicated in modulating inflammation and fibrosis [61]. Classically activated macrophage (M1) exhibits proinflammatory and death-promoting effects [61]. Activated M1 cells also express peroxisome proliferator-activated receptor-α (PPAR-α) and PPAR-γ whose inhibitors have been proved to inhibit fibrosis progression [62]. By contrast, alternatively activated macrophage (M2) that is induced by IL-4 exhibits anti-inflammatory effects and promotes cell survival and proliferation [61]. Activated M2 cells also express enzyme arginase-1 (Arg1), through which M2 cells compete with T_{help} cells and myofibroblasts for L-arginine, and affect the production of L-proline and collagen [63].

A large body of evidence confirms the anti-inflammation property of H_2S in various types of cells and organs, except at the super physiological range of concentrations. We verified that lower doses of NaHS (0.1–1 μmol/kg/day, i.p.) reduced the infiltration of CD68 positive cells and decreased the expression of inflammatory cytokines including IL-1β, TNF-α, and monocyte chemoattractant protein-1 in the renal cortex at seven days after UUO injury. In contrast, CSE inhibitor PAG and higher dose of NaHS (10 μmol/kg/day, i.p.) increased the number of macrophage in the renal interstitium [13]. In line with this, another study showed that NaHS (10 μmol/kg/day, i.p.) markedly reduced the serum levels of TNF-α, IL-1β, IL-6, and soluble intercellular adhesion molecule (ICAM)-1 in carbon tetrachloride (CCl4) induced cirrhosis rats [12]. Apart from supplementation with NaHS, the modulations of endogenous H_2S level with pharmacologic and genetic approaches *in vitro* also support the anti-inflammatory action of H_2S in macrophage and other related cells. For example, we found that raw264.7 macrophage expressed CSE, and CSE upregulation inhibited the macrophage activation and

TNF-α generation caused by oxidized low density lipoprotein through the suppression of nuclear transcription factor-κB (NF-κB) as well as the mitogen-activated protein kinase including the ERK1/2 and JNK [64]. In addition, Du et al. also reported that CBS overexpression or NaHS supplement could promote rotenone-treated microglia (central nervous system resident macrophage) transition from M1 toward M2 phenotype [65]. Thus, all the data suggest that H_2S and its synthesis enzyme may be involved in regulating macrophage activation and its phenotype transition.

Apart from macrophage, neutrophils also participate in inflammation of fibrotic diseases. Evidence shows that neutrophils are present in most forms of glomerulonephritis (GN) such as acute poststreptococcal GN, immunoglobulin A nephropathy, and lupus nephritis [66]. Except a few forms of GN, most GN will eventually progress into renal fibrosis. Once recruited to the injury sites, neutrophils are activated via phagocytosis of immune complex or bacteria, generate reactive oxygen species (ROS), and degranulate multiple cytotoxic proteins including cationic serine proteases. Emerging evidence demonstrates that lower dose of NaHS is capable of reducing neutrophils infiltration and the damage in pancreas and decreasing the serum levels of P-selectin, E-selectin, ICAM-1, and VCAM-1 [67]. In support of this, plasma H_2S levels are negatively correlated with the number of neutrophil and eosinophil in inflamed lung and joint disease [68]. Taken together, the evidence highly suggests that H_2S may also exhibit its antifibrotic effect by suppressing neutrophils infiltration.

3.3. H_2S Inhibits Fibrosis via Regulating Redox Homeostasis. ROS play crucial roles in the activation and differentiation of myofibroblasts [69]. NADPH oxidase (NOX) is a primary source of ROS by catalyzing the electron transmission from NADPH to oxygen. Such an effect differentiates NOX from other oxidases such as xanthine oxidase, which produces ROS as a by-product of their primary function [70]. There are seven types of NOX, among which NOX1, NOX2, and NOX4 are expressed in both rodent and human kidneys. Numerous studies show that NOX is associated with fibrotic diseases. NOX expression in fibroblasts can be induced by a variety of cytokines including TGF-β1, angiotensin II, and PDGF [71]. It has been reported that NOX expression was higher in lung fibroblasts isolated from idiopathic pulmonary patients than controls, and the colocalization of NOX and α-SMA was observed in samples from autoimmune hepatitis [72]. *In vivo* studies also confirm the association of NOX and fibrosis. The NOX inhibitor or knockdown with siRNA ameliorated the fibrosis progression in renal, pulmonary, and liver fibrosis [15, 73, 74].

In addition to ROS, reactive nitrogen species (RNS) are also implicated in fibroblast-to-myofibroblast differentiation. In mammal cells, NO is generated by NO synthases (NOS) with L-arginine and oxygen as the substrate and NADPH as the cofactor. NO stimulates guanylyl cyclase (GC) and catalyzes guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), which subsequently regulates the cGMP-dependent kinases (PKG) and enhances the intracellular calcium level [75]. NO signaling is critical for maintaining fibroblast phenotype. It has been found that TGF-β1 significantly decreased the NOS expression/activity and NO production in the dermal fibroblast while the NOS inhibitor N_w-nitro-L-arginine methylester (L-NAME) enhanced the collagen contents within the cells. By contrast, the NO donor, sodium nitroprusside (SNP) inhibited the prostatic fibroblast differentiation into myofibroblast and L-arginine suppressed the TGF-β1-induced collagen production in dermal fibroblasts [76]. Consistently, the *in vivo* study also showed that NOS inhibition aggravated fibrosis in the kidney, heart, and penile [77–79].

Emerging data demonstrate that H_2S acts as the scavenger of oxidant species. Notably, mammal cells have a high capacity of H_2S oxidation in the mitochondria. The oxidation produces various products including persulfide, sulfite, thiosulfate ($S_2O_3^{2-}$), and sulfate (SO_4^{2-}), most of which are excreted by the kidney in the form of sulfate [80]. *In vitro* studies reveal that H_2S donor (NaHS or Na_2S) suppressed the protein oxidation caused by HOCl, $ONOO^-$, and $^{\bullet}NO$. Moreover, NaHS suppressed lipid oxidation and NOX activity. For example, NaHS reduced the MDA content in bleomycin-induced pulmonary fibrosis model, as well as the MDA formation in the lung tissue incubated with free radical-generating system *in vitro* [11]. In fact, H_2S has been demonstrated to produce antioxidative effects in addition to acting as a direct ROS/RNS scavenger. For instance, Jung et al. reported that NaHS administration increased the expression/activity of catalase, copper-zinc superoxide dismutase, and manganese superoxide dismutase and also elevated the glutathione level in UUO animal model [81].

3.4. Energy-Sensing Molecules and H_2S in Treating Fibrosis. Adipose tissue has been viewed as a passive fat storage depot; however, recent data suggest that adipose tissue is the largest endocrine organ of the body [82]. It secretes numerous cytokines that are involved in various physiological and pathological processes such as energy and cell metabolism, inflammation, oxidative stress, and fibrosis as well. Among the cytokines, adiponectin and its downstream signaling such as 5′-AMP-activated protein kinase (AMPK) have been highlighted in fibrotic diseases [82]. Classically, AMPK is activated when the cellular ATP is depleted under low caloric circumstances. Recently, mounting evidence shows that AMPK not only acts as an energy sensor, but also regulates redox signals. It is thus implicated in various pathological processes, such as neurodegeneration, cancer, and fibrosis. For example, acting through the AMPK pathway, adiponectin administration normalized the albuminuria and improved the podocyte function in adiponectin knockout mice [83]. Furthermore, AMPK activation with AICAR (5-aminoimidazole-4-carboxamide ribonucleotide) considerably reduced the mesangial matrix production and the urinary TGF-β1 levels [84]. AMPK activation also regulated the lipid accumulation and alleviated the kidney fibrosis induced by high fat diet [85, 86].

The metabolic inhibition property of H_2S has been recently reported both *in vivo* and *in vitro*. It has been found that inhaled H_2S induced a hibernation-like state in mice whose production of carbon dioxide and oxygen

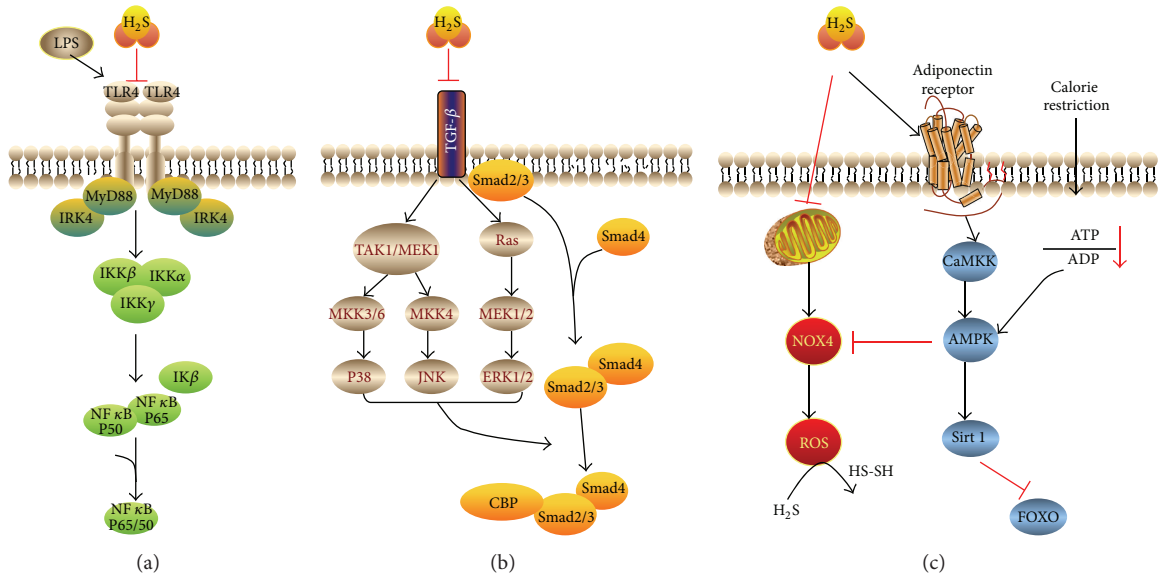

FIGURE 2: The schematic summarization for the signaling mechanisms of the antifibrotic effects of H_2S. (a) H_2S inhibits LPS-induced inflammation through the Toll-like receptor-NF κB signal transduction. (b) H_2S ablates the activation of TGF-β1 and MAPK kinases pathway. (c) H_2S regulates redox production by inhibiting the expression/activity of NOX4 in addition to direct reaction with some reactive oxygen species (ROS). Lastly, H_2S is able to modulate cellular metabolism and homeostasis by activating AMPK and sirtuin 1.

consumption were decreased approximately 90%. The core body temperature of the animal also reduced to the ambient temperature and their heart rates were relatively lower than the controls [86]. Exposure to various concentrations of NaHS reduced mitochondria membrane potential in renal proximal tubular cells (NRK52e) [87]. The mechanisms of the hypometabolism-inducing effect of H_2S are not well classified. One hypothesis is that H_2S inhibits cytochrome c oxidase and ATP generation and thus activates AMPK. We recently demonstrated that H_2S activated AMPK in a calmodulin-dependent protein kinase kinase β (CaMKKβ) manner, which is critical for the anti-inflammatory actions of H_2S in the brain [88]. Similarly, a recent study reported that NaHS stimulated and restored AMPK phosphorylation which was reduced by high glucose in renal glomerular epithelial cells [89]. Other molecules may also be involved in the hypometabolism-inducing effect of H_2S. For instance, sirtuin family members are important energy-related molecules as well as the downstream signal molecules of AMPK [90]. Sirtuins are mainly activated by calorie restriction and the deficiency of sirtuin perpetuates renal fibrosis and aging process [91, 92]. Recent data suggests that H_2S increases the activity of sirtuin 1 induced by aging in human umbilical vein endothelial cell [93]. Therefore, H_2S may produce antifibrotic effects by acting on the energy-sensing molecules.

The antifibrotic mechanisms of hydrogen sulfide are presented in Figure 2.

4. Limitations and Perspectives

Although the antifibrotic property of H_2S has been demonstrated in various animal models, current evidence mainly comes from the studies with the administration of exogenous H_2S donor NaHS and Na_2S in treating animal models of fibrotic diseases. The effect of endogenous H_2S with genetically modified animals on organ fibrosis is still limited. The use of heterozygous *cbs*$^{+/-}$ mice with unilateral nephrectomy reveals the role of endogenous H_2S in renal fibrosis, but its function in liver fibrosis is not confirmed [20, 36]. As the expression of CBS or CSE was reported to decrease in fibrotic diseases, the use of animals with overexpression of H_2S-producing enzymes is needed in studying the effect of H_2S on organ fibrosis. A transgenic mouse with CSE overexpression in the heart has been established and may be helpful in addressing the therapeutic effect of H_2S on cardiac fibrosis in the future [94]. Recently, the intermediate-conductance Ca^{2+}-activated K^+ channel ($K_{Ca}3.1$) has been proposed as the "switch" molecule of fibrotic disease because it regulates the proliferation, migration, and differentiation of renal and pulmonary fibrosis-producing cells [95–97]. Mouse with genetic mutation $K_{Ca}3.1$ also confirms the antifibrotic effect of this ion channel in renal fibrosis [98]. As H_2S is able to inhibit the big conductance of Ca^{2+}-activated K^+ channel (BK_{Ca}) [99], it is interesting to further explore whether or not the antifibrotic effect of H_2S correlates with the inhibition of $K_{Ca}3.1$ ion channel.

Another limitation of the current studies is the difference in applied dose range of NaHS in combating fibrosis because the toxicity of H_2S is always a concern. Lower doses of NaHS (1–10 μmol/kg/day, i.p.) seem to be more effective in inhibiting renal, pulmonary, and liver fibrosis [11–13], but higher doses of NaHS (30 μmol/kg/day, i.p.) are required for reversing the cardiac fibrosis in spontaneously hypertensive rats [49]. As the plasma H_2S level in mammal tissues is still debatable, varying from 0.15 to 300 μmol/L, due to the different measurements [100, 101], it is difficult to answer

which dose of NaHS is mimicking the physiological relevance of H_2S in animal tissues. Therefore, the development of fast, selective, and efficient detection method for sulfide monitoring is required for the booming research field of H_2S biology.

Last but not least, the development of proper H_2S-releasing agents is required to treat fibrotic diseases in a controlled way. GYY4137 and SG1002 are two orally administrated H_2S-releasing compounds that have been proven to be beneficial in various diseases such as diabetes, hepatocellular carcinoma, and chronic heart failure [102–104]; however, their effects on fibrotic diseases have not been determined. More researches are therefore needed to explore the effectiveness of these and other similar agents on various fibrotic diseases.

Conflict of Interests

There is no potential conflict of interests to be disclosed.

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Research Article

Sulfur Dioxide Enhances Endogenous Hydrogen Sulfide Accumulation and Alleviates Oxidative Stress Induced by Aluminum Stress in Germinating Wheat Seeds

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Aluminum ions are especially toxic to plants in acidic soils. Here we present evidences that SO_2 protects germinating wheat grains against aluminum stress. SO_2 donor ($NaHSO_3$/Na_2SO_3) pretreatment at 1.2 mM reduced the accumulation of superoxide anion, hydrogen peroxide, and malondialdehyde, enhanced the activities of guaiacol peroxidase, catalase, and ascorbate peroxidase, and decreased the activity of lipoxygenase in germinating wheat grains exposed to Al stress. We also observed higher accumulation of hydrogen sulfide (H_2S) in SO_2-pretreated grain, suggesting the tight relation between sulfite and sulfide. Wheat grains geminated in water for 36 h were pretreated with or without 1 mM SO_2 donor for 12 h prior to exposure to Al stress for 48 h and the ameliorating effects of SO_2 on wheat radicles were studied. SO_2 donor pretreatment reduced the content of reactive oxygen species, protected membrane integrity, and reduced Al accumulation in wheat radicles. Gene expression analysis showed that SO_2 donor pretreatment decreased the expression of Al-responsive genes TaWali1, TaWali2, TaWali3, TaWali5, TaWali6, and TaALMT1 in radicles exposed to Al stress. These results suggested that SO_2 could increase endogenous H_2S accumulation and the antioxidant capability and decrease endogenous Al content in wheat grains to alleviate Al stress.

1. Introduction

Aluminum ions (Al^{3+}) together with silicon and iron are the three most abundant mineral elements in soil. Whereas silicon and iron are required for plant growth, Al is toxic. Many different mechanisms have been advanced to explain Al toxicity in plants [1, 2]. One of the primary causes of Al toxicity is oxidative stress due to accumulation of reactive oxygen species (ROS), such as the superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), bringing about lipid peroxidation in plant cells [3–5]. Plants have developed several strategies to counteract oxidative stress caused by Al, such as activation of antioxidants, and exudation of organic acids as a mechanism for Al exclusion [6]. Recently, a range of signaling molecules, such as inositol 1,4,5-triphosphate (IP_3), salicylic acid, hydrogen peroxide (H_2O_2) and nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S), were found to participate in plant's resistance to Al-induced oxidative stress [4, 7–10].

Sulfur dioxide (SO_2) is a colorless, nonflammable gas with a penetrating odor. Low concentrations of SO_2 have been found to play a physiological role *in vivo* in animal models, participating in various biological processes [11]. The physiological processes regulated by SO_2 in animals include cardiac function [11], inhibition of L-calcium channels in cardiomyocytes [12], and improvement in pulmonary vascular structural remodeling [13]. In plants, the toxic effects of SO_2 on growth and development have been extensively studied [14, 15]. Exposure to high concentrations of SO_2 can cause visible foliar damage, a decline in photosynthesis, an inhibition of plant growth, and structural disorganization and cell death [16–19]. On the other hand, many reports show

that low levels of atmospheric SO_2 might be beneficial to plants [20]. SO_2 can be metabolized and used as a sulfur source for plant growth, especially when the sulfur supply in soil is insufficient for normal growth [20]. Recently, low concentrations of SO_2 were found to induce transcriptome reprogramming associated with oxidative signaling and biotic defence responses in plants, suggesting a physiological role of SO_2 in plant [21].

In plants, sulfate is taken up from soil by high-affinity transporters. Sulfate is largely transported to shoots where it can be activated by ATP via ATP sulfurylase in the leaves. The product is reduced by 5′-adenylylsulfate (APS) reductase to sulfite which can be reduced to H_2S by sulfite reductase [22]. SO_2 can also be produced endogenously from sulfur-containing amino acids [23]. The endogenous production of SO_2 also suggests that it has a physiological role in plants.

In order to establish the role of SO_2 in alleviating Al stress, we investigated the effects of SO_2 pretreatment on H_2S and ROS accumulation and the antioxidant system in whole wheat grains and in wheat radicles. We also analyzed endogenous H_2S and Al content as a means of understanding the mechanism of the role of SO_2. We speculated that SO_2 might act as an antioxidant molecule to alleviate Al toxicity during wheat grain germination.

2. Materials and Methods

2.1. Materials and Treatments. Wheat (*Triticum aestivum* L.) grains were supplied by the Anhui Aidi Agricultural Technology Co., Ltd., Anhui Province, China. Sodium bisulfite ($NaHSO_3$) and anhydrous sodium sulfite (Na_2SO_3) were used as sulfur dioxide (SO_2) donors according to Laisk et al. [24]. Wheat grains were sterilized by 0.1% $HgCl_2$ for 3 min and washed extensively with H_2O and then dried with filter papers. Wheat grains of similar size were selected and allocated randomly in Petri dish (9 cm diameter × 1.2 cm depth, 50 grains per dish). Wheat grains were germinated in H_2O or aqueous solutions of $AlCl_3$ at 5, 10, 15, 20, 25, 30, 60, and 90 mM for 48 h at 25°C and the length of coleoptiles and radicles and radicle number were recorded. To test the protective role of SO_2 on germination and seedling growth of wheat grains under Al stress, grains were pretreated with H_2O or 0.4, 0.8, 1.2, 1.6, or 2.0 mM SO_2 donor for 12 h and subsequently subjected to a semi-inhibitory $AlCl_3$ concentration (15 mM). $AlCl_3$ solutions were renewed every 12 h and geminating grains were sampled every 12 h for further analysis.

2.2. Determination of MDA, $O_2^{\bullet-}$, and H_2O_2. The contents of MDA, $O_2^{\bullet-}$, and H_2O_2 were determined by the method of Zhang et al. [25].

2.3. Assays of LOX, CAT, APX, and POD Activities. Activity of lipoxygenase (LOX, EC 1.13.11.12) was determined following the description by Surrey [26] and those of catalase (CAT, ECl.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), and guaiacol peroxidase (POD, EC 1.11.1.7) were assayed according to Hu et al. [27]. Wheat grains were homogenized in ice-cold 50 mM phosphate buffer (pH 7.8) containing 1.0 mM EDTA. The homogenate was centrifuged at 15,000 g at 4°C for 10 min. The supernatant was used for activity determination.

2.4. Assays of Reducing Sugars and Soluble Protein. Wheat grains (0.5 ± 0.05 g) were ground in 5 mL of phosphate buffer (pH 7.0, 200 mM), the homogenate was centrifuged at 10,000 g for 30 min, and the supernatant was used for detection of reducing sugars and soluble protein content. Reducing sugar content was measured according to Miller [28].

For detection of soluble protein, 0.1 mL supernatant was mixed with 0.9 mL H_2O and 5 mL Coomassie brilliant blue for 5 min and the absorbance recorded at 595 nm using the method described by Bradford [29].

2.5. Preparation of Wheat Radicles. Wheat grains were geminated in H_2O for 36 h in the dark at 25°C and the average of radicle length was approximately 1.0 cm. The geminated wheat grains were pretreated with or without 1 mM SO_2 donor for 12 h and then exposed to 0 or 400 μM $AlCl_3$ for 48 h.

2.6. Detection of Plasma Membrane Integrity, Al Accumulation, and ROS Production in Radicles. Plasma membrane integrity of wheat radicles was detected following the method of Yamamoto et al. [30]. Radicles were stained with Evans blue solution (0.025% [w/v] Evans blue in 100 μM $CaCl_2$, pH 5.6) for 10 min, then washed three times with 100 μM $CaCl_2$ solutions, and photographed. Staining intensity of Evans blue is positively correlated with more damaged plasma membrane.

Al content in radicles was visualized by staining tissues with hematoxylin. Hematoxylin stain was prepared as described by Polle et al. [31]. Wheat radicles were washed with H_2O for 30 min and then stained with solution of 0.2% hematoxylin and 0.02% $NaIO_3$ for 30 min at room temperature. Radicles were then immersed in H_2O for 30 min to remove excess stain and photographed. Staining intensity of hematoxylin is positively correlated with Al uptake.

ROS distribution in radicle tips was detected by 2′,7′-dichlorofluorescin diacetate (DCFH-DA) following the method of LeBel et al. [32]. Radicle tips were incubated in a solution containing 100 μM $CaCl_2$ and 10 μM DCFH-DA for 20 min and then washed three times with H_2O. The fluorescence was detected with a Nikon 80i microscope (excitation at 488 nm and emission at 525 nm). For each treatment, ten individual roots from ten seedlings were examined and similar results were obtained.

2.7. Real-Time Quantitative RT-PCR Analysis in Wheat Radicles. Radicle tips were prepared for RNA extraction according to Li et al. [33]. Total RNA was isolated by grinding with liquid nitrogen according to the manufacturer's instructions (CWBIO, Beijing, China). cDNA was generated from total RNA with a reverse transcription kit (Prime Script RT Master Mix, Takara, Kyoto, Japan). Quantitative PCR was performed using a StepOnePlus Real-Time PCR

Table 1: Inhibitory effect of Al stress on the germination of wheat grains. Wheat grains were exposed to 0, 5, 10, 15, 20, 25, 30, 60, or 90 mM $AlCl_3$ for 48 h.

Al^{3+} concentration (mM)	Germination percentage (%)	Radicle length (cm)	Coleoptile length (cm)	Radicle number (50 grains)
0	64 ± 1.2^{a}	3.1 ± 0.8^{a}	1.5 ± 0.3^{ab}	178 ± 7.8^{a}
5	66 ± 1.1^{a}	2.7 ± 0.5^{ab}	1.6 ± 0.2^{a}	168 ± 8.9^{a}
10	51 ± 2.3^{b}	1.9 ± 0.3^{b}	1.3 ± 0.3^{ab}	162 ± 7.6^{a}
15	35 ± 3.8^{c}	1.1 ± 0.2^{c}	1.1 ± 0.2^{bc}	142 ± 6.3^{b}
20	28 ± 4.2^{cd}	0.7 ± 0.3^{cd}	0.8 ± 0.2^{cd}	80 ± 5.6^{c}
25	21 ± 5.1^{de}	0.4 ± 0.3^{d}	0.6 ± 0.2^{de}	68 ± 6.1^{c}
30	15 ± 4.7^{ef}	0.2 ± 0.2^{d}	0.3 ± 0.3^{e}	45 ± 5.3^{d}
60	8 ± 5.2^{f}	0.1 ± 0.1^{d}	0.3 ± 0.2^{e}	22 ± 3.5^{e}
90	7 ± 6.3^{f}	0 ± 0^{e}	0.2 ± 0.2^{e}	0 ± 0^{f}

Values are the means ± SD ($n = 6$). Values are the means ± SD ($n = 6$). Different letters mean significance of difference between different treatments ($P < 0.05$).

System (Applied Biosystems, Foster City, CA, USA) with SYBR Premix Ex Taq (TaKaRa Bio Inc., China) according to the manufacturer's instructions. cDNA was amplified by PCR using the following primers: Taβ-actin forward (5′-CTATCCTTCGTTTGGACCTT-3′) and reserve (5′-AGCGAGCTTCTCCTTTATGT-3′); TaWali1 forward (5′-CTGATGGAGTCGAGCAAGG-3′) and reserve (5′-CCGAAGTAGCGATTTAGGAGT-3′); TaWali2 forward (5′-AGCCTACTGCTCCGCCTTGT-3′) and reserve (5′-CGTTTCGTCGGCATCTCC-3′); TaWali3 forward (5′-GACGAGCCCTAAGAAGACG-3′) and reserve (5′-CACGGAGCAATGACAACAG-3′); TaWali5 forward (5′-TGGACCCTGCAAGAAGTAC-3′) and reserve (5′-GCTGAACAACAAGCAACACC-3′); TaWali6 forward (5′-TACGGAATAGACAGGACAAGG-3′) and reserve (5′-CAGCATTTCGGGAACTCG-3′); TaALMT1 forward (5′-TGCCACGCTGAGTAAAGG-3′) and reserve (5′-CGCTGACGCTACGAAGAA-3′). Relative gene expression was presented as values relative to the corresponding gene expression in control, after normalization to the control Taβ-actin transcript levels.

2.8. Statistical Analysis. Statistical significance was tested by one-way ANOVA, and the results are expressed as the mean values ± SD (standard deviation) of three independent experiments. Each experiment was repeated three times.

3. Results

3.1. Inhibitory Effect of Al on Wheat Grain Germination. The effect of Al stress on wheat seedling growth and development was examined following incubation of grain in $AlCl_3$ with concentrations ranging from 5 mM to 90 mM (Table 1). At concentrations of 5 mM or below, germination percentage, coleoptile length, and radicle number are almost unaffected, but radicle length was reduced by 13%, suggesting that the radicle is the primary target of Al toxicity. At 15 mM Al, germination percentage was almost halved compared with that of control and this concentration was selected for further experiments. At 90 mM Al, radicle growth was completely inhibited, but very stunted coleoptile growth was still observed.

3.2. SO_2 Donor Ameliorates Al Stress in Germinating Wheat Grain. To establish whether the SO_2 donor Na_2SO_3/$NaHSO_3$ had a toxic effect on wheat grain germination, grains were germinated in different SO_2 donor concentrations ranging from 0.4 to 2.0 mM for 36 h (see Table S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/612363). Table S1 shows there was no significant change in germination percentage, coleoptile length, radicle length, or radicle number between water control and SO_2 donor treatment, establishing that the concentrations of SO_2 donor used in this work exhibited no visible toxic effects. To test the ability of SO_2 donor to alleviate Al stress, wheat grains were pretreated with SO_2 donor concentrations ranging from 0.4 to 2.0 mM for 12 h prior to incubation with 15 mM Al (Table 2 and Figure 1). At all SO_2 donor concentrations used, SO_2 pretreatment was effective in alleviating the toxic effects of Al in a dose dependent manner. The optimal SO_2 donor concentration for alleviating Al stress was 1.2 mM, a concentration where the germination percentage was increased by 51%, radicle and coleoptile length by 28% and 26%, respectively, compared with those exposed to Al. This result clearly shows that SO_2 alleviates Al-induced inhibition of wheat grain germination and seedling growth.

3.3. Effect of SO_2 Donor on the Contents of Reducing Sugars and Soluble Protein in Al-Stressed Wheat Grain. Figure 2(a) shows the changes in reducing sugars in germinating wheat grains preincubated in SO_2 donor or H_2O for 12 h followed by incubation in Al for 48 h. Within 12 h pretreatment in H_2O and 24 h of Al treatment, the content of reducing sugar decreased gradually, whereas reducing sugar in the SO_2 donor pretreatment remained stable and slightly increased at 24 h. Thereafter reducing sugar content increased steadily in both treatments followed by a slight decrease at 48 h. The content of reducing sugars in SO_2 donor pretreated grain was always significantly higher than the counterpart of only Al treatment.

The content of soluble protein increased gradually and peaked on 24 h of Al stress followed by a slight decrease (Figure 2(b)). Though the mean values of soluble protein in

Table 2: Effects of SO_2 donor pretreatment on wheat grain germination under 15 mM Al^{3+} stress. Wheat grains were pretreated with 0, 0.4, 0.8, 1.2, 1.6, and 2.0 mM SO_2 for 12 h and subsequently subjected to 15 mM $AlCl_3$ for further 48 h, and then germination was investigated.

SO_2 donor concentration (mM)	0.0	0.4	0.8	1.2	1.6	2.0
Germination percentage (%)	37 ± 3.3^a	42 ± 2.7^a	44 ± 3.5^a	56 ± 3.8^a	48 ± 2.7^a	47 ± 3.1^a
Length of radicle (cm)	1.42 ± 0.6^a	1.72 ± 0.4^a	1.80 ± 0.7^a	1.82 ± 0.7^a	1.78 ± 0.8^a	1.62 ± 0.4^a
Length of coleoptile (cm)	4.64 ± 0.4^a	4.70 ± 0.6^a	5.20 ± 0.5^a	5.83 ± 0.8^a	5.40 ± 0.8	5.23 ± 0.6^a
Radicle number (50 grains)	127 ± 7.3^a	135 ± 8.1^a	139 ± 8.1^a	148 ± 7.9^a	130 ± 6.7^a	119 ± 7.1^a

Values are the means ± SD ($n = 6$). Different letters mean significance of difference between different treatments ($P < 0.05$).

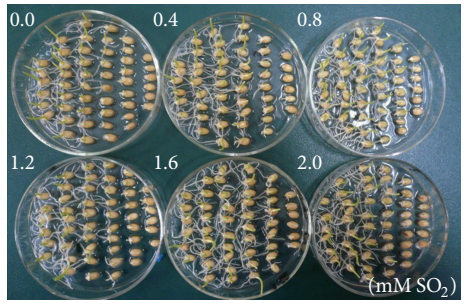

Figure 1: Effects of SO_2 pretreatment on wheat grain germination under 15 mM Al stress. Wheat grains were pretreated with 0, 0.4, 0.8, 1.2, 1.6, and 2.0 mM SO_2 for 12 h, subsequently subjected to 15 mM Al for further 48 h, and then photographed.

SO_2 donor pretreatment were higher than those pretreated in H_2O, they are not significantly different.

3.4. Effect of SO_2 Donor Pretreatment on Contents of Endogenous H_2S, $O_2^{\bullet-}$, H_2O_2, and MDA. H_2S, which can be produced from sulfite, is involved in plant growth regulation including various abiotic stresses [8, 22]. To investigate whether exogenous SO_2 application can induce endogenous H_2S production, we measured the concentration of H_2S in Al-stressed wheat grain. Generally, H_2S accumulated during wheat grain germination following pretreatment with water or SO_2, but SO_2 donor pretreatment significantly enhanced H_2S concentration at 12 h of pretreatment and 12 h, 36 h of Al stress (Figure 3(a)).

To study the protective role of SO_2 in the Al-stressed wheat grain, reactive oxygen species $O_2^{\bullet-}$, H_2O_2, and malondialdehyde (MDA) were determined with time. As shown in Figure 3(b), a rapid accumulation of $O_2^{\bullet-}$ was observed when H_2O-pretreated grains were exposed to Al. During the first 12 h of Al exposure, the increase in $O_2^{\bullet-}$ content was very rapid, but this was followed by a slow decrease. In contrast, the content of $O_2^{\bullet-}$ in SO_2 pretreatment increased slowly till 36 h of Al stress followed by a decrease. SO_2 pretreatment maintained significantly lower level of $O_2^{\bullet-}$ in Al-stressed wheat grains compared with grains incubated in H_2O and exposed to Al.

H_2O_2 in both treatments increased gradually during pretreatment time and 36 h of Al stress and decreased at 48 h (Figure 3(c)). However, H_2O_2 content in SO_2 pretreatment was significantly lower than that in water pretreatment when exposed to Al stress.

During the 12 h pretreatment time, no significant difference was observed in MDA content in wheat grains whether pretreated with SO_2 donor or H_2O (Figure 3(d)). After exposure to Al, the content of MDA in water pretreated grains increased rapidly till 48 h of Al stress. An increase of MDA content was also observed in SO_2 pretreatment at 12 h of Al stress, but thereafter MDA content remained stable until 36 h. SO_2 pretreatment dramatically reduced the amount of MDA from 24 h to 48 h of Al stress in comparison with grains pretreated in water.

3.5. Effects of SO_2 Donor Pretreatment on POD, CAT, APX, and LOX Activities. Activities of POD, CAT, APX, and LOX were determined with time in SO_2 donor and H_2O-pretreated grains exposed to Al (Figure 4). Figure 4(a) shows the time course of POD activity following pretreatment in SO_2 donor or H_2O for 12 h when POD activity showed almost a twofold increase. During Al stress, POD activity exhibited a gradual increase in both treatments, but SO_2 pretreatment maintained significantly higher level of POD activity during Al stress.

The activity of CAT increased almost twofold during 12 h pretreatment with H_2O or SO_2 donor (Figure 4(b)). After exposure to Al, CAT activity in water pretreatment decreased gradually till 48 h of Al stress, suggesting that CAT activity is very sensitive to Al stress. In contrast, CAT activity in SO_2 pretreatment increased steadily and decreased only slightly at 48 h of Al stress.

As shown in Figure 4(c), SO_2 pretreatment enhanced APX activity in Al-stressed wheat grain. A rapid increase in APX activity occurred during the pretreatment time in H_2O and SO_2. Within the first 12 h of Al stress, APX activity in H_2O-pretreated grains decreased sharply, whereas SO_2 donor pretreatment enhanced APX activity slightly. Thereafter APX activity increased steadily in water pretreated grain, whereas its activity in SO_2 donor pretreatment fluctuated slightly. The APX activity in SO_2 donor pretreated grains was always significantly higher than the counterpart of water pretreatment.

An increase in LOX activity was observed during the first 24 h of Al stress in SO_2 and H_2O-pretreated grains (Figure 4(d)). However, the increase of LOX activity in water pretreatment was more rapid than after SO_2 pretreatment. Thereafter LOX activity in water pretreatment showed a sharp decrease at 36 h of Al stress, while its activity in SO_2 pretreatment decreased at 48 h. At 12 and 24 h of Al stress, SO_2 pretreatment maintained significantly lower level of

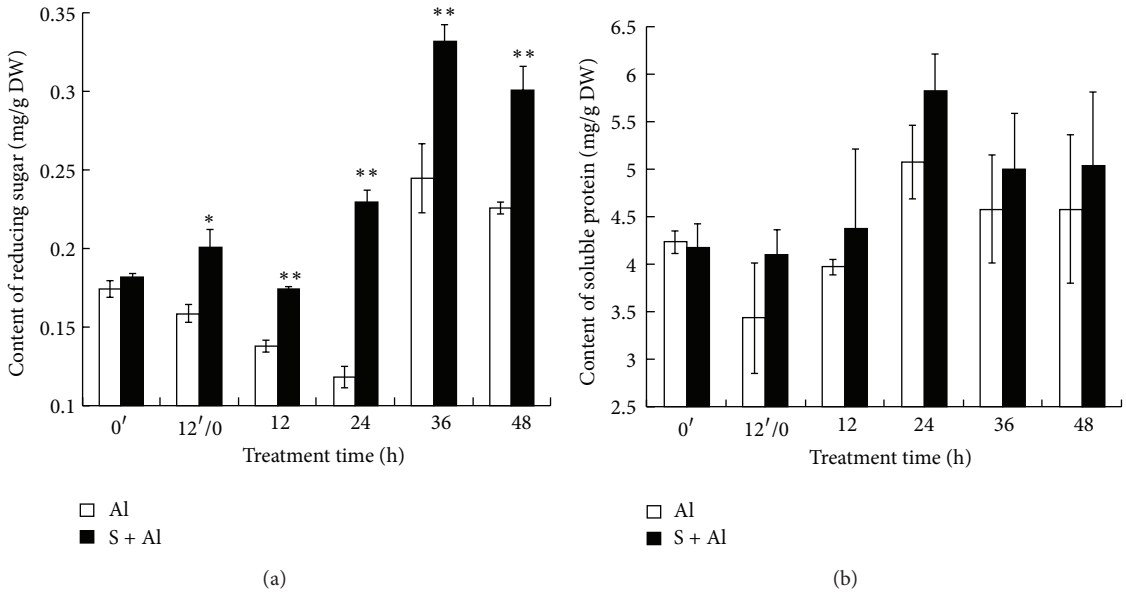

FIGURE 2: Effect of SO_2 pretreatment on the contents of reducing sugar and soluble protein in Al-treated grain as shown in (a) and (b), respectively. Wheat grains were pretreated with water (Al) or 1.2 mM SO_2 donor (S + Al) for 12 h (shown from 0′ to 12′/0 h of pretreatment time) and then exposed to 15 mM Al for further 48 h (shown as 12′/0, 12, 24, 36, and 48 h). The symbols $*$ and $**$ in this figure and following ones stand for significant difference between Al-treated grains with and without SO_2 pretreatment at $P < 0.05$ and $P < 0.01$, respectively.

LOX, while at 36 h LOX activity in SO_2 pretreatment was higher than that of water pretreatment.

3.6. Effects of SO_2 Donor Pretreatment on Localization of Al, Lipid Peroxidation, and ROS Production. To detect ROS production in the radicle tips, we used DCFH-DA fluorescence to indicate ROS accumulation. As shown in Figure 5(a), Al treatment induced higher level of ROS in radicle as intense DCFH-DA fluorescence, while SO_2 donor pretreatment for 12 h followed by Al stress significantly reduced fluorescence. Figure 5(b) shows DCFH-DA fluorescence in maturation zone in radicles. Similarly, intense fluorescence in SO_2 donor pretreatment followed by Al stress was much weaker than that in water pretreated plus Al-stressed radicles, suggesting that SO_2 donor was effective in alleviating oxidative stress in radicles. SO_2 donor treatment alone showed comparable fluorescence intensity as observed in water control.

The radicles were stained with Evans blue to show membrane integrity. The radicles treated with Al alone were stained extensively with Evans blue, while Al-stressed radicles pretreated with SO_2 donor for 12 h were less stained (Figure 5(c)), suggesting SO_2 donor serves to protect cell membrane from Al-induced damage. SO_2 donor treatment alone showed similar Evans blue staining to water control, suggesting no visible damaging effect of SO_2 on radicles.

The hematoxylin staining was used to detect Al accumulation in radicles. As shown in Figure 5(d), the radicles of water control and SO_2 treatment incubated with hematoxylin showed no dark staining but wheat radicles treated with Al alone were stained intensively. In contrast, radicles pretreated with SO_2 donor for 12 h and then exposed to Al for 48 h showed much weaker staining compared with Al stress, especially in the elongation zone.

3.7. Effect of SO_2 Donor Pretreatment on the Relative Expressions of Aluminum Stress Related Genes. We determined the changes in gene expression of aluminum stress related genes in wheat radicles. Radicles were pretreated with or without 1 mM SO_2 donors for 12 h and then exposed to Al for 48 h. As shown in Figure 6, Al stress induced higher expression of TaWali1, TaWali2, TaWali3, TaWali5, and TaWali6 (wheat aluminum induced) in radicles, while pretreatment with SO_2 donor for 12 h followed by Al stress alleviated such expression increase. Besides, the gene expression of TaALMT1 (Al-activated malate transporter) was also attenuated by SO_2 pretreatment.

4. Discussion

In solution, SO_2 is dissociated from its sulfite derivatives ($NaHSO_3/Na_2SO_3$ 1 : 3 M/M) [34]. Thus $NaHSO_3/Na_2SO_3$ (1 : 3 M/M) was chosen as an SO_2 donor in our study. Similar to the observation that H_2S could promote wheat grain germination and alleviate oxidative damage against Al stress [8], our results show that SO_2 donor pretreatment alleviates Al stress in germinating wheat seedlings. Wheat grains pretreated for 12 h with the SO_2 donor show an increase in germination percentage, coleoptile length, radicle length, and radicle numbers of wheat. The increase in the contents of reducing sugars and soluble protein suggests that nutrients in wheat grains pretreated with SO_2 donor are rapidly mobilized to provide energy to grain germination. SO_2 donor maintained lower level of H_2O_2, $O_2^{\bullet-}$, and MDA probably

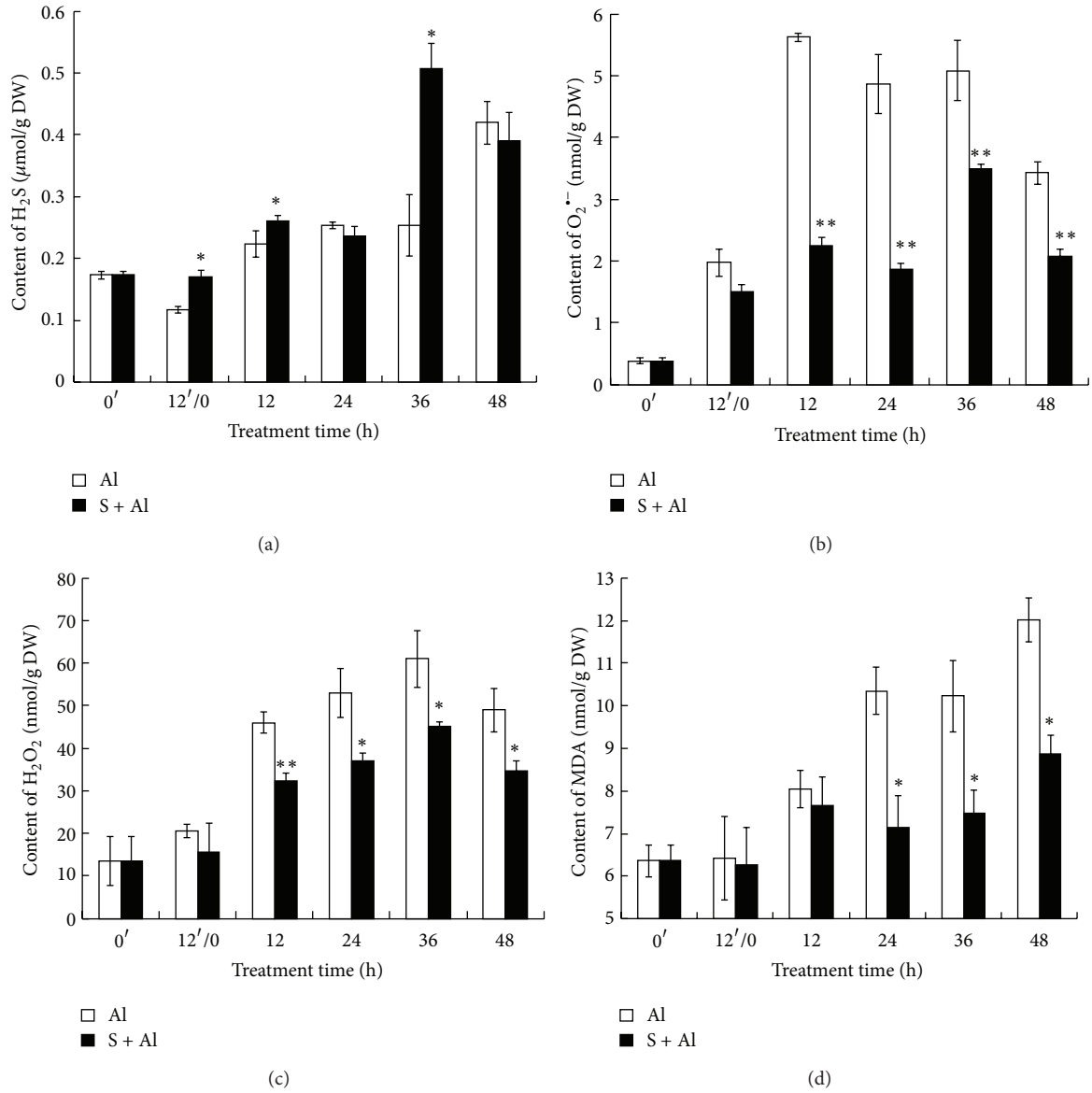

Figure 3: Effects of SO_2 pretreatment on the accumulation of endogenous H_2S (a), superoxide anion ($O_2^{\bullet-}$) (b), hydrogen peroxide (H_2O_2) (c), and malondialdehyde (MDA) (d) in germinating wheat grains under Al stress. The numbers (0′, 12′/0, 12, 24, 36, and 48) or letters (CK or SO_2) presented are the same as mentioned in Figure 2. Al: Al stress without SO_2 pretreatment; S + Al: Al stress with SO_2 pretreatment.

by activation of the antioxidant system. These results suggest that SO_2 acts as an antioxidant and may function in a way that is similar to what the effects of H_2S, CO, and NO do in plants exposed to heavy metal stress [10, 35].

Sulfite can be reduced by sulfite reductase to H_2S, which is incorporated into O-acetylserine via O-acetyl(thiol)lyase to form cysteine [22]. In RNA interfered mutant of sulfite reductase (SiR), sulfide synthesis in younger leaves was decreased by the impaired SiR activity [36]. In the present study, exogenous SO_2 application can induce endogenous H_2S production in Al-stressed wheat grains (Figure 3(a)), suggesting the interplay between sulfite and the formation of H_2S.

Consistent with previous observations [7], our results show that Al stress caused overproduction of ROS in wheat. To mitigate and repair oxidative damage, plants have evolved an efficient antioxidant system that includes enzymes such as SOD, CAT, and APX that function to scavenge ROS [37]. SOD catalyzes the dismutation of the superoxide radical $O_2^{\bullet-}$ and H^+ into H_2O_2. CAT, APX, and POD are responsible for the elimination of H_2O_2 generated by SOD. Al stress brings about a dramatic increase in H_2O_2 and $O_2^{\bullet-}$. The elevated levels of H_2O_2 and $O_2^{\bullet-}$ suggest that antioxidant enzymes in Al-stressed wheat do not efficiently scavenge the overproduction of ROS, and this can result in lipid peroxidation or plasma membrane inhibiting grain germination and seedling growth [8]. Our data show that pretreatment of wheat with SO_2 donor activates antioxidant enzymes including POD, CAT, and APX.

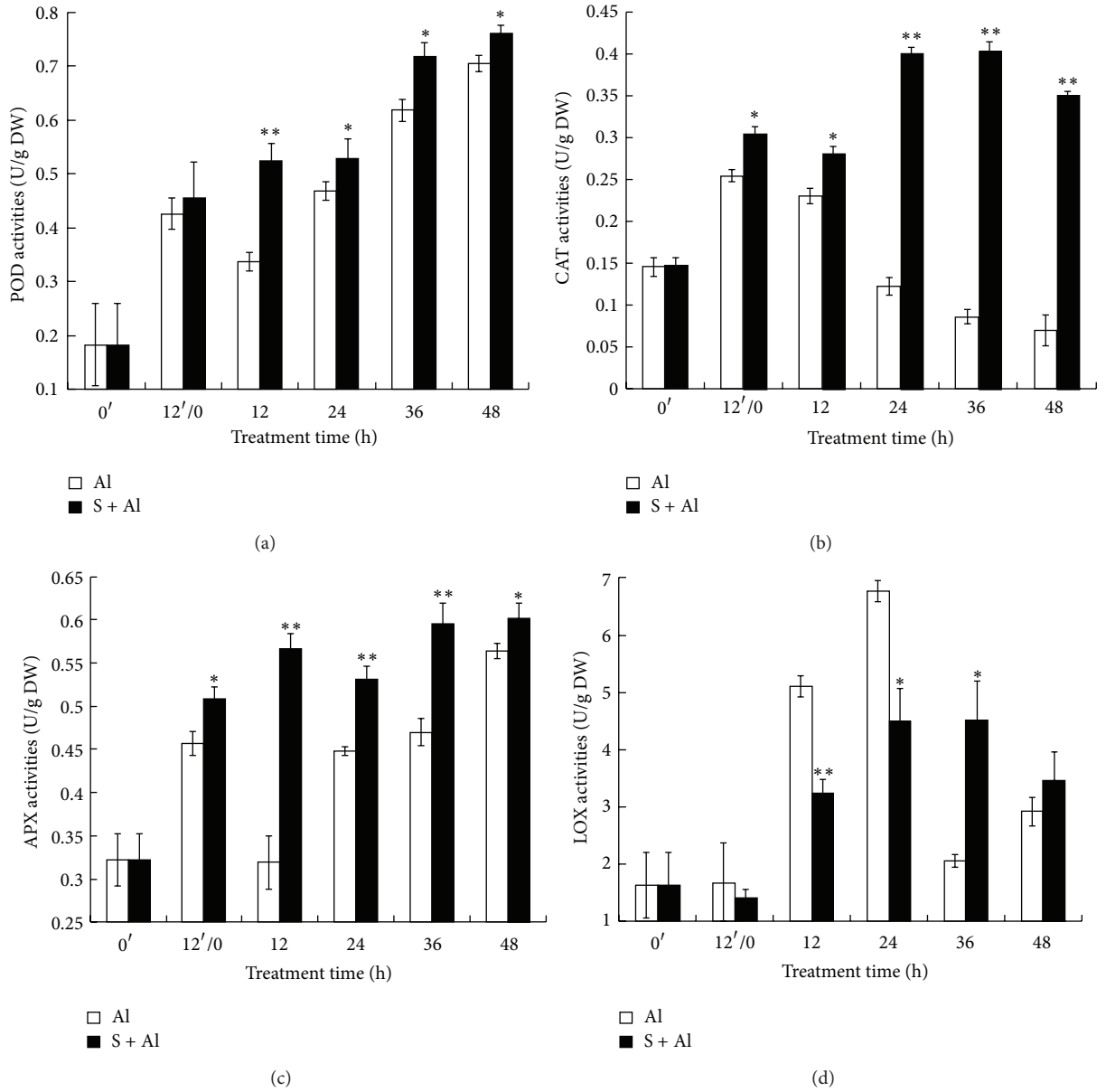

Figure 4: Effect of SO_2 donor pretreatment on the activities of POD (a), CAT (b), APX (c), and LOX (d) in germinating wheat grains under 15 mM Al stress. Grains were treated and the number or letters presented are the same as mentioned in Figure 2. Al: Al stress without SO_2 pretreatment; S + Al: Al stress with SO_2 pretreatment.

LOX, which catalyzes oxygenation of polyunsaturated fatty acids into lipid hydroperoxides, is considered an indicator of oxidative stress during responses to various environmental stresses [9]. Pretreatment with SO_2 donor lowers LOX activity in Al-stressed wheat radicles compared to seedlings pretreated with H_2O and exposed to Al. The lowering of LOX by SO_2 pretreatment also helps to explain the lower MDA content of Al-stressed grain. Taken together, these data suggest that SO_2 donor reduced oxidative stress by modulation of the antioxidant system.

Our data indicate that the radicle is the primary target for Al toxicity. DCFH-DA fluorescence assay shows that Al incubation induces higher accumulation of ROS in radicle tips and maturation zone. SO_2 donor pretreatment effectively reduces ROS content in subsequent Al stress, suggesting the role of SO_2 in alleviating oxidative stress. Correspondently, Al stress causes membrane injury to radicles, while SO_2 donor effectively alleviates such injury. To understand whether SO_2 donor helps to reduce Al accumulation in radicles, hematoxylin staining was used to indicate Al and the results show that SO_2 donor obviously reduces Al content in radicles, implying a potential role of SO_2 donor treatment as a strategy to reduce Al uptake.

In response to Al stress, many gene expressions are activated, for instance, TaWali (wheat aluminum induced), aluminum-activated malate transporter (TaALMT1) [38–41].

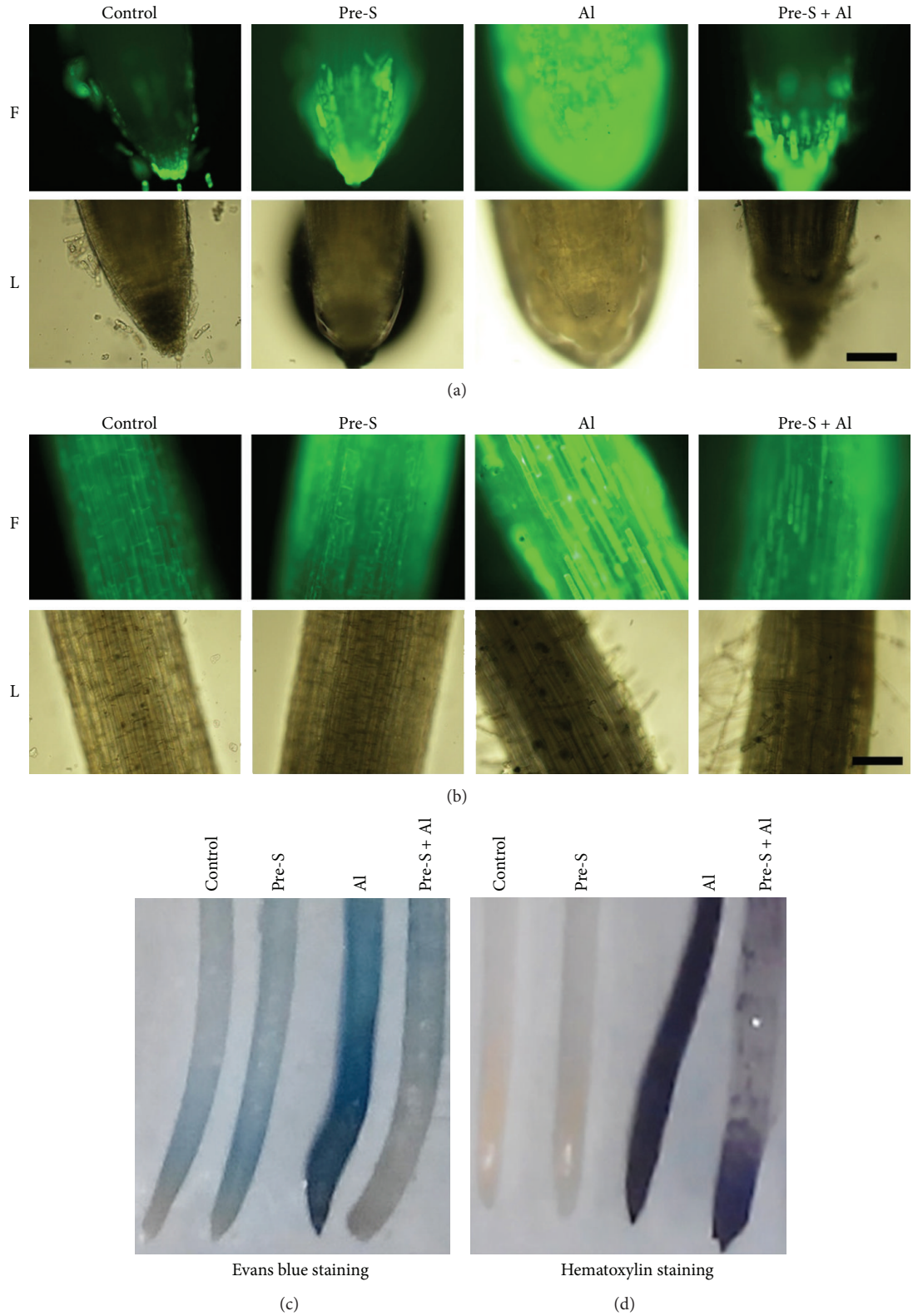

Figure 5: ROS staining ((a) on radicle tips; (b) on maturation zone; bar: 200 μm), Evans blue staining (c), and hematoxylin staining (d) in wheat radicles. Initially, wheat grains were geminated in water for 36 h. Then four treatment groups were done as follows, control, 60 h in H_2O; Pre-S, pretreatment with 1 mM SO_2 donor for 12 h, and then exposed to H_2O for 48 h; Al, 12 h in H_2O prior to exposure to 400 μM $AlCl_3$ for 48 h; Pre-S + Al, 12 h in 1 mM SO_2 donor pretreatment followed by 400 μM $AlCl_3$ for 48 h.

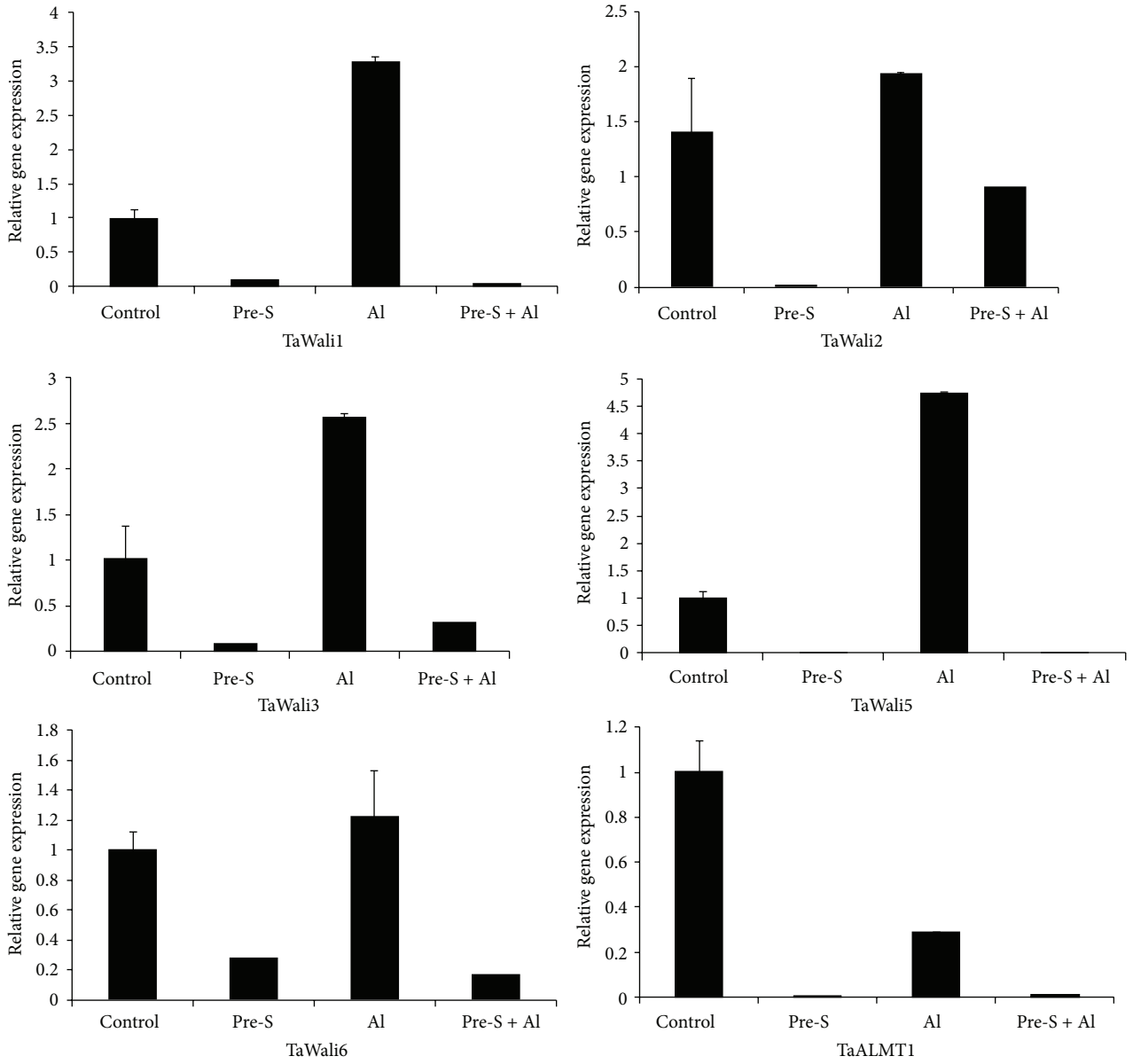

Figure 6: Effect of SO_2 donor pretreatment on relative gene expression of TaWali1, TaWali2, TaWali3, TaWali5, TaWali6, and TaALMT1 in wheat radicals exposed to Al stress. Initially, wheat grains were geminated in water for 36 h. Then four treatment groups were done as follows, control, 60 h in H_2O; Pre-S, pretreatment with 1 mM SO_2 donor for 12 h, and then exposed to H_2O for 48 h; Al, 12 h in H_2O prior to exposure to 400 μM $AlCl_3$ for 48 h; Pre-S + Al, 12 h in 1 mM SO_2 donor pretreatment followed by 400 μM $AlCl_3$ for 48 h.

Relative gene expression analysis shows that Al treatment induces higher expression of TaWali, while these gene expression levels are reduced by SO_2 donor pretreatment, suggesting the response to Al stress is attenuated in SO_2 donor pretreatment.

5. Conclusion

In the present study, SO_2 acts as an antioxidant signal to reduce ROS damage in wheat grains and radicles caused by Al stress. Besides, SO_2 also decreases Al uptake. The induced higher level of H_2S suggests an intricate interplay of SO_2 and H_2S in plants. Exogenous application of SO_2 may be reduced to H_2S by sulfite reductase, thus contributing to H_2S production. H_2S in itself acts as an antioxidant signaling molecule in plants' response to abiotic stress. Thus the nature of SO_2/sulfite functions in alleviating Al stress still needs further research.

Conflict of Interests

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

Authors' Contribution

Dong-Bo Zhu, Kang-Di Hu, and Xi-Kai Guo contributed equally to this work.

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Review Article

Interaction of Hydrogen Sulfide with Oxygen Sensing under Hypoxia

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Based on the discovery of endogenous H_2S production, many in depth studies show this gasotransmitter with a variety of physiological and pathological functions. Three enzymes, cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (MST), are involved in enzymatic production of H_2S. Emerging evidence has elucidated an important protective role of H_2S in hypoxic conditions in many mammalian systems. However, the mechanisms by which H_2S senses and responses to hypoxia are largely elusive. Hypoxia-inducible factors (HIFs) function as key regulators of oxygen sensing, activating target genes expression under hypoxia. Recent studies have shown that exogenous H_2S regulates HIF action in different patterns. The activation of carotid bodies is a sensitive and prompt response to hypoxia, rapidly enhancing general O_2 supply. H_2S has been identified as an excitatory mediator of hypoxic sensing in the carotid bodies. This paper presents a brief review of the roles of these two pathways which contribute to hypoxic sensing of H_2S.

1. Introduction

H_2S had been known mostly due to its unpleasant smell and fierce toxicity before its physiological importance was studied [1]. Production of H_2S in mammalian cells had been considered almost as a metabolic waste, until the physiological roles of this small molecular were elucidated in the central nervous system and the cardiovascular system nearly two decades ago [2, 3]. H_2S is now identified as a well-known gasotransmitter which plays a critical role in both physiological regulation and pathophysiological processes of different mammalian tissues [4, 5]. Endogenous H_2S levels have been reported in different mammalian systems, ranging from 50 to 160 μM [6, 7]. The enzymatic production of endogenous H_2S were identified, mostly composed of cystathionine β-synthase (CBS) [8–10], cystathionine γ-lyase (CSE) [11–13], and 3-mercaptopyruvate sulfurtransferase (MST) [14–16].

H_2S and oxygen (O_2) are mutually exclusive on the Earth. During the Permian period, the depletion of oxygen and accumulation of H_2S in the oceans and the air caused great extinctions of more than 90 percent of life species [1, 17]. Physicochemical reactions in living cells require enough molecular oxygen O_2 for essential metabolic processes. O_2 serves as the terminal electron acceptor in the system of oxidative phosphorylation which produces high-energy phosphate bond in ATP [18, 19]. Insufficient oxygen supply to tissues, organs, or cells constitutes hypoxia. Hypoxia is evoked by several kinds of causes which all lead to oxygen-partial-pressure (PO_2) in arterial blood less than 40 mm Hg [19, 20]. Several lines of evidence point to an important protective role of H_2S in hypoxic conditions. However, the mechanisms by which H_2S senses and responses to hypoxia are largely elusive. A master regulator of hypoxia in mammalian cells is hypoxia-inducible factor-1 (HIF-1), which activates the transcription of >100 target genes under hypoxic contexts [18, 21]. HIF-1 is involved in H_2S-mediated angiogenesis under hypoxia [22, 23]. With the remarkable sensitivity and the prompt speed to response to hypoxia, the carotid bodies play a unique role in O_2 sensing [24, 25]. It has been shown that H_2S is involved in the regulation of

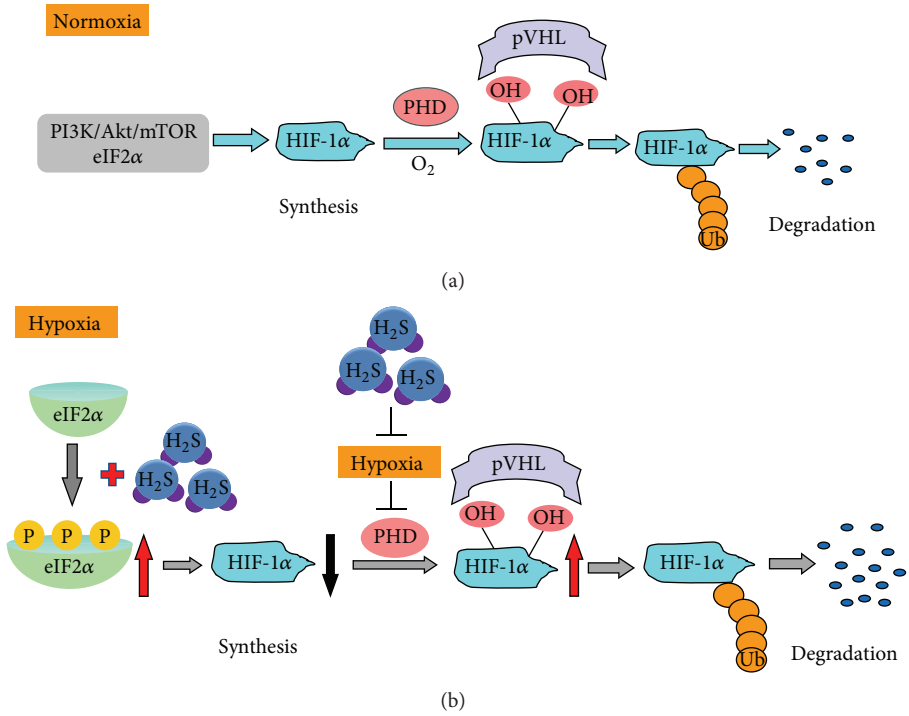

Figure 1: Pathways regulating HIF-1α synthesis/degradation under normoxia and schematic illustration of H_2S effects on HIF-1α accumulation under hypoxia. (a) HIF-1α protein translation under normoxia is mainly dependent on activation of the PtdIns3K-Akt mammalian target of rapamycin (mTOR). HIF-1α is hydroxylated by the prolyl hydroxylase (PHD) under normoxia. Hydroxylated HIF-1α is then bound by the von Hippel Lindau protein (pVHL). This complex in turn recruits a ubiquitin ligase that targets HIF-1α for its proteasomal degradation. (b) Under hypoxia, H_2S induces phosphorylation of translation initiation factor 2α (eIF2α). Phosphorylated eIF2α inhibits HIF-1α translation. In addition, H_2S decreases cellular oxygen (O_2) consumption under hypoxia and reverses hypoxia-induced inhibition of PHD activity. Thus, H_2S enhances degradation of HIF-1α. Abbreviations: Ub, ubiquitin.

sensory activity in the carotid bodies [26, 27]. In genetically distinct rat models, variations in endogenous H_2S levels have been reported to be closely correlated with variations of carotid body O_2 sensing in both hypoxia-induced pulmonary edema and essential hypertension [28]. In this paper, we review physiological responses of H_2S to hypoxia and focus particularly on the regulating effects of H_2S on HIF-1 action and the O_2 sensing role of H_2S in the carotid bodies.

2. H_2S and HIF-1 under Hypoxia

2.1. Hypoxia and Hypoxia-Inducible Factor-1. HIF-1 was first identified as the protein responsible for the hypoxia-evoked transcription of erythropoietin [29, 30]. HIF-1 is a heterodimer consisting of the inducibly regulated HIF-1α subunits and the constitutively expressed HIF-1β subunits [31]. Since the expression of HIF-1β is abundant in cells, HIF-1α determines the protein level and the transcriptional activity of HIF-1 [21]. The HIF-1 heterodimer combines with the hypoxia-responsive element in target genes, thus enhancing target genes transcription [21].

Under normoxic conditions, although continuously transcribed and translated, the protein levels of HIF-1α are hard to be detected in normal cells due to HIF-1α protein fast degradation. Tow specific proline residues within the oxygen-dependent degradation (ODD) domain in HIF-1α are hydroxylated by the prolyl hydroxylase (PHD) under normoxia. Hydroxylated HIF-1α is then bound by the von Hippel Lindau protein (VHL) [32]. This complex in turn recruits a ubiquitin ligase that targets HIF-1α for its proteasomal degradation [33] (Figure 1). PHD activity is inhibited under hypoxic conditions, thus resulting in HIF-1α stabilization. Other pathways and regulators are also involved in HIF-1α degradation [21].

Compared with the accumulated studies on HIF-1α degradation, translational control of stress-survival protein HIF-1α under hypoxia is far more beyond our understanding. HIF-1α protein translation under normoxia is dependent on activation of the PtdIns3 K-Akt mammalian target of rapamycin (mTOR) and the mitogen-activated protein kinases (MAPK) pathways [20, 21]. Under hypoxia-induced stress, phosphorylation of the translation initiation factor 2α (eIF2α) and inhibition of mTORC1 activity have been suggested to account for HIF-1α protein expression inhibition [21] (Figure 1). Certain antitumour compounds which inhibit HIF-1α translation contribute to eIF2α phosphorylation [34, 35]. There is evidence that other factors are involved in HIF-1α translation regulation, that is, studies on the 5′UTR of HIF-1α which contains an internal ribosome-entry-site [21].

2.2. Effects of H_2S on HIF-1 Accumulation. Nitric oxide (NO), the first identified gasotransmitter, has been demonstrated

to affect HIF-1 action [36, 37]. An early report showed that NO donors inhibited HIF-1 action under both hypoxia and hypoxia-mimetic conditions [36]. In later studies, by overexpressing the human iNOS to increase endogenous NO production, NO was shown to enhance HIF-1 protein accumulation and its activity [37]. The second gasotransmitter carbon monoxide (CO) has also been shown to regulate HIF-1 protein levels and activity in certain contexts [38]. A series of recent studies showed that H_2S, like NO and CO, plays an important role in the regulation of HIF-1 function under hypoxia. *Caenorhabditis elegans* (*C. elegans*) is an attractive model for hypoxia relative research [6]. This animal model was used to study the influence of H_2S on HIF-1. In *C. elegans*, the EGg laying defective- (EGL-) 9 is responsible for HIF-1 hydroxylation [39]. HIF-1 was closely correlated with increased tolerance of H_2S exposure in *C. elegans* [39]. Animals with the HIF-1 null mutation did not survive from exposure to only 15 ppm H_2S, while wild-type worms survived with high viability even exposure to 50 ppm H_2S [39]. Mutations in either EGL-9 or VHL-1 induced an overabundance of HIF-1, which caused animals to tolerate 150 ppm H_2S [39]. It is strongly indicated that HIF-1 is required when animals response to H_2S. H_2S treatment, as well as hypoxia, elevated the levels of HIF-1 protein and its activity [39]. However, H_2S and hypoxia caused different expression patterns of a HIF-1 target gene [39].

The effects of H_2S on HIF-1 in higher organisms have been investigated. In vascular smooth muscle cells, the interaction between H_2S and HIF-1 was tested under $CoCl_2$-induced hypoxia-mimetic conditions [40]. In a concentration of 300 μM, NaHS induced upregulation of HIF-1α mRNA and proteins [40]. HIF-1α transcription activity in the same context was also increased [40]. The authors suggested that H_2S upregulates HIF-1α gene transcription. However, HIF-1α synthesis during hypoxia is largely regulated at the level of translation rather than transcription [21]. It is worthy of mentioning that although $CoCl_2$ treatment could induce HIF-1α accumulation to mimetic hypoxic stress, the model itself is not essential hypoxia and may cause other cellular changes [1].

Some later studies provided further understanding of the influence of H_2S on HIF-1 in mammalian cells. It is reported that 1 mM NaHS (H_2S donor) decreased hypoxia-induced HIF-1α protein accumulation and HIF-1 target gene expression in mammalian cell lines [22]. However, H_2S had no similar effect on HIF-1 in hypoxia-mimetic conditions [22]. In contrast, another study showed that NaHS at relatively low concentrations (10–100 μM) treatment decreased HIF-1α protein levels in HEK293T, Hep3B, and EA.hy926 cells under both hypoxia (1% O_2) and hypoxia-mimetic conditions [23]. The controversial observations will be considered in subsequent sections.

2.3. Mechanisms of H_2S Effects on HIF-1 Accumulation. In the *C. elegans* study, H_2S-induced HIF-1 accumulation was dependent on EGL-9, indicating that H_2S may prevent HIF-1 degradation [39]. In the same study, H_2S-evoked HIF-1 increase was independent of VHL-1, which implies that *C. elegans* may have a special HIF-1 degradation pathway. However, hypoxia-mediated HIF-1 accumulation in mammals is largely dependent on VHL-1. It has been shown that VHL-independent degradation pathways play important roles in controlling HIF-1 levels [21]. CYSL-1 is homologous to CBS which is a key enzyme for H_2S production in mammalian cells [41]. CYSL-1 identified from genetic screens negatively regulated EGL-9 and thus enhanced HIF-1 stability [41]. It was proposed that H_2S promoted the interaction of EGL-9 and CYSL-1 to cause HIF-1 accumulation in *C. elegans* under hypoxia [41].

Kai and colleagues showed that H_2S did not affect HIF-1 levels in EB8 cells under hypoxia, compared with control cells [22]. The fact that EB8 cells have no mtDNA suggested that mitochondria was involved in H_2S-inhibited HIF-1 activation [22]. The authors found that 1 mM NaHS inhibited mitochondrial oxygen consumption and thus increased the oxygen level in hypoxic cells. H_2S did not inhibit the stabilization of HIF-1α protein under hypoxia-mimetic conditions [22]. The authors concluded that H_2S promoted HIF-1α degradation under hypoxia, due to NaHS-induced mitochondrial oxygen consumption inhibition (Figure 1). However, they did not provide the direct evidence that the speed of HIF-1α degradation was altered in the presence of H_2S. On the other hand, it is worthy of mentioning that most of the key observations in their study were obtained with NaHS at 1 mM. This high concentration of H_2S is clearly not within the physiological range of endogenous H_2S and may have toxic effects on cells for long-time treatments [1, 42].

NaHS at 10–100 μM has been used in different studies to reflect physiologically relevant concentrations of H_2S in vivo [43, 44]. In a study using NaHS at 10–100 μM, NaHS significantly lowered HIF-1α protein levels under both hypoxia (1% O_2) and hypoxia-mimetic conditions (DFX or $CoCl_2$) [23]. Although inducing HIF-1α accumulation, both DFX and $CoCl_2$ have no effect on oxygen partial pressure in the culture medium [23]. It is suggested that oxygen partial pressure change or the alteration of mitochondrial respiration may not be involved in H_2S-induced HIF-1α downregulation under hypoxia. Both HIF-1α degradation and HIF-1α ubiquitination were not changed by NaHS treatment [23]. In contrast, cycloheximide (CHX), a translation inhibitor, blocked the effect of NaHS on HIF-1α protein levels [23], suggesting that H_2S mediates HIF-1α translation suppression. The authors further demonstrated that the key mechanism for H_2S-induced HIF-1α downregulation was H_2S-evoked repression of HIF-1α protein translation, rather than an effect on the ubiquitin proteasomal degradation pathway (Figure 1). Eukaryotic translation initiation factor 2α (eIF2α) is a critical regulatory molecule for eukaryotic initiation of translation [21]. eIF2α is responsible for the transformation of GDP to GTP, an essential step for translation start. Phosphorylation of eIF2α at Ser51 prevents the reformation of the eIF-2 ternary complex and thus inhibits protein translation [21]. Certain antitumour compounds which essentially decrease HIF-1α translation contribute to eIF2α phosphorylation [34, 35]. In eIF2α knockdown cells exposed to hypoxia, H_2S-induced reduction of HIF-1α was partially reversed [23]. Therefore, HIF-1α translational suppression is associated with

H_2S-induced eIF2α phosphorylation in hypoxic contexts (Figure 1).

In summary of this section, it is likely that two key factors, the extent of hypoxia together with the concentration of H_2S in the same condition, determine the final result of the action of H_2S on HIF-1. H_2S-induced upregulation of HIF-1 was observed in a *C. elegans* model. *C. elegans* naturally lives where O_2 level is lower than that in the air [6, 7]. The animals have no circulatory system. O_2 delivery in this species is dependent on diffusion. *C. elegans* are able to survive from O_2 depletion, due to an anoxia-induced suspended animation state [6, 7]. In contrast, models in which H_2S-triggered downregulation of HIF-1 come from high organisms which are highly oxygen-demanded. The differences in both species and oxygen demand may explain the opposite regulational patterns in the action of H_2S on HIF-1. On the other hand, the effect of H_2S on HIF-1 regulation may be dose-dependent. Differences in NaHS concentrations may be correlated with various observations in abovementioned studies. Thus, further studies on the role of endogenous H_2S may provide further understanding of the interaction between H_2S and HIF-1.

Emerging evidence indicates that VHL-independent HIF-1α degradation has an important role in controlling HIF-1α levels. Although this kind of pathways seems to be less dependent on oxygen levels in cells, that is, receptor of activated protein kinase C- (RACK1) mediated HIF-1α degradation [21], and further research is still required to solve whether these pathways are involved in the effect of H_2S on HIF-1 in hypoxic contexts. On the other hand, translational regulation of HIF-1α under hypoxia is largely elusive. Therefore, whether H_2S targets other factors which potentially regulate HIF-1α translation is another challenge. Moreover, it is not yet clear how H_2S-mediated HIF-1α regulation contributes to the protective role of H_2S under hypoxia.

3. H_2S and Hypoxic Sensing in the Carotid Body

3.1. O_2 Sensing in the Carotid Body. With the remarkable sensitivity and the fast speed to hypoxic response, the carotid body plays a unique role in O_2 sensing [19]. Carotid bodies are small sensory organs located at the bifurcation of the common carotid artery [14, 19]. Changes in O_2 levels of arterial blood rapidly active the carotid bodies, which in turn transduce sensory information to brainstem neurons [14]. The final response in the central nervous system regulates vital functions including breathing, heart rate, and blood pressure to increase ventilation and systemic delivery of oxygen [15]. Carotid bodies are primarily composed of two cell types: glomus cells (also called type I cells) and sustentacular cells (also called type II cells) [15]. Accumulated evidence suggests that type I cells are the primary site of hypoxic sensing in carotid bodies. NO and CO inhibited carotid body activity [15]. Neuronal nitric oxide synthase- (nNOS-) generated NO has been proposed as an important mediator of efferent inhibition of the carotid body [25, 45]. Heme oxygenase-2 (HO-2), a key enzyme for CO production, is expressed in glomus cells of many mammalian carotid bodies [24, 46]. Exogenous application of CO at low concentrations and HO inhibitors inhibited and stimulated the carotid body activity, respectively [16, 24].

3.2. Effects of CSE/H_2S on Carotid Body Activity. Emerging evidence demonstrated that H_2S is involved in sensing and response to hypoxia in many tissues that possess the ability to sense hypoxia. H_2S stimulated catecholamine secretion from chromaffin cells in trout [30]. H_2S regulated the O_2-sensing signal in trout chemoreceptors [47]. It was proposed that the balance between endogenous H_2S production and its oxidation by available O_2 contributed to H_2S-mediated O_2 sensing [47]. Recent studies showed that H_2S, like NO and CO, is another gas regulator for hypoxic sensing in carotid bodies. Both CSE [26] and CBS proteins [27] are expressed in glomus cells. Rat carotid body expresses mRNAs for both CBS and CSE [48]. Hypoxia increased H_2S generation in both mouse and rat carotid bodies [26]. Hypoxia-induced H_2S production and secretion of catecholamine were significantly decreased in CSE knockout mice or in wide-type mice treated with CSE inhibitors DL-propargylglycine (PPG) [26]. CSE knockout mice exhibited severely impaired function of carotid body sensing to hypoxia. The similar observation was shown in rats treated with PPG [26]. Exogenous application of H_2S donor, NaHS, increased the sensory excitation of the carotid bodies from mice and rats [26, 27]. The patterns of NaHS action on carotid bodies are similar to that of hypoxia. H_2S produced by CSE may function as an excitatory mediator for the sensory excitation by hypoxia [26] (Figure 2).

3.3. Potential Effects of CBS/H_2S on O_2 Sensing in Carotid Body. It is worthy of mentioning that, in CSE knockout mice, the basal levels of H_2S in the carotid bodies were half reduced compared to those of control wild-type animals [26]. It is likely that CBS may also be responsible for H_2S production in the carotid bodies. Aminooxyacetic acid (AOA) and hydroxylamine (HA), two inhibitors of CBS, suppressed carotid body and ventilatory responses to hypoxia [27]. This study suggested that CBS-catalyzed H_2S also plays a role in excitatory carotid bodies sensing under hypoxia (Figure 2). Compared to CSE inhibitor PPG, AOA and HA are nonspecific inhibitors [1]. HA inhibits many heme-containing enzymes [49, 50], while AOA generally targets aminotransferase [51, 52]. Given the pharmacologic nonspecific inhibition, further studies need to elucidate the effects of endogenous CBS on O_2 sensing in carotid bodies. In addition, the relative importance of H_2S/CSE and H_2S/CBS on carotid bodies hypoxic sensing needs to be established.

The role of H_2S/CBS in O_2 sensing in tissues, other than carotid bodies, has been revealed in recent studies. CBS is a nuclear encoding heme protein [8]. The levels of CBS proteins in liver mitochondria are regulated in a Lon protease-mediated O_2-dependent pathway [8]. Under normoxic conditions, a low level of CBS proteins was shown in mitochondria [8]. Hypoxia inhibited Lon protease activity and thus increased the accumulation of CBS in mitochondria

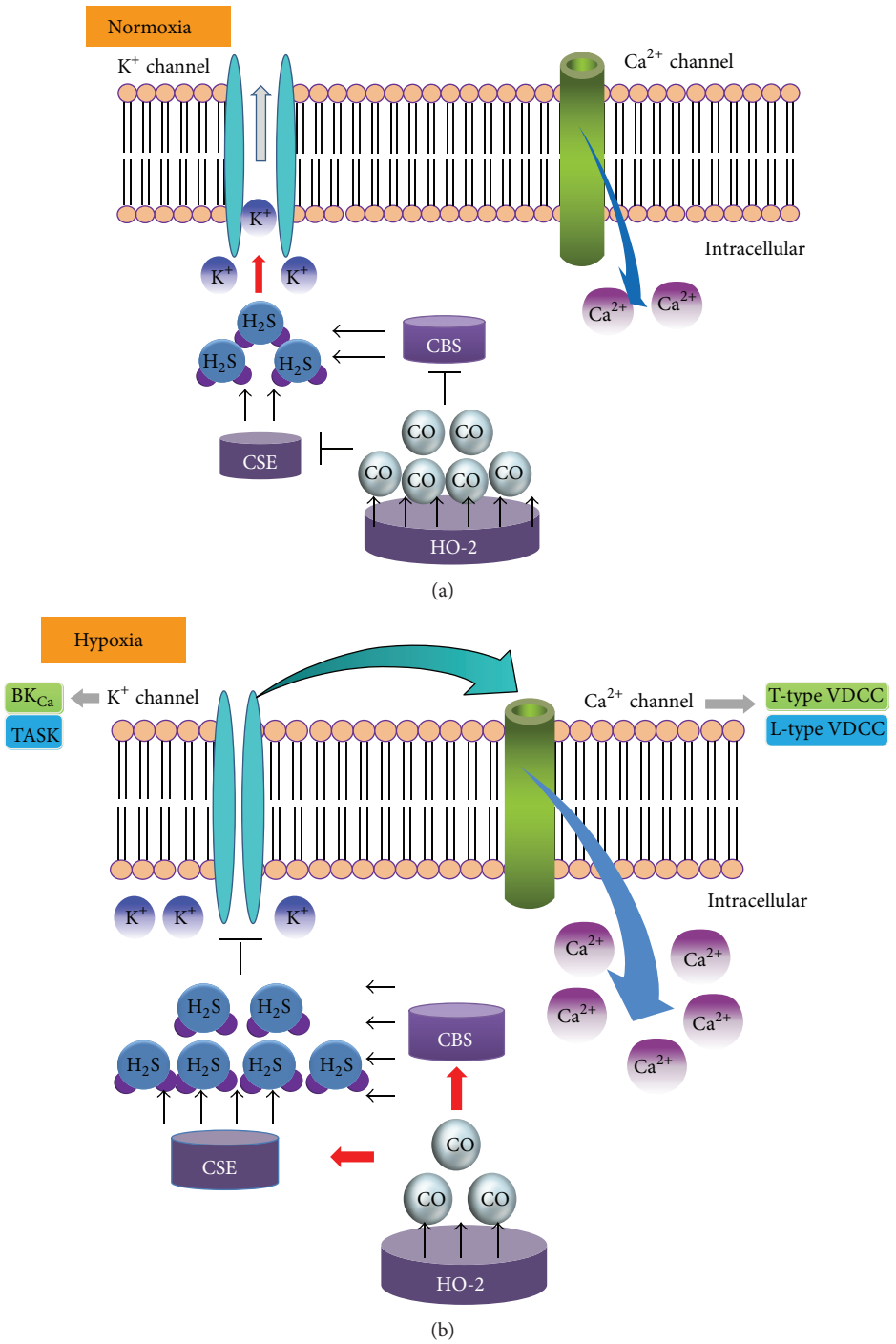

Figure 2: Potential interaction of heme oxygenase-2- (HO-2-) generated CO with cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS) in glomus cells of the carotid body. (a) Under normoxia, HO-2-generated CO is relatively high, resulting in inhibition of both CSE and CBS activity. H_2S production is relatively low, contributing to low sensory activity in glomus cells. (b) Under hypoxia, CO generation from HO-2 is reduced, resulting in removal of CO inhibition on CSE and CBS activity. H_2S levels are increased due to enhanced CSE and CBS activities. Increased H_2S may activate Ca^{2+} channels due to membrane depolarization via the inhibition of K^+ channels, which in turn excites sensory responses in glomus cells.

[8]. Reoxygenation recovered Lon protease activity and thus accelerated the degradation of CBS proteins [8]. This study indicates that CBS accumulated in mitochondria is oxygen-sensitive. CBS serves as a CO-sensitive modulator of H_2S in liver [53]. Studies using recombinant CBS indicated that CO bound to the prosthetic heme in CBS and thus specifically inhibited CBS activity [53]. In livers of heterozygous CBS knockout mice, overproducing CO had no effect on H_2S generation. Another further study showed that CBS-catalyzed H_2S functioned as a vasodilator in the cerebral circulation [54]. CO produced by HO-2 was O_2-dependent in the brain. Endogenous CO negatively regulated CBS activity under normoxia, while, under hypoxia, CBS activity was increased due to the decrease of CO production [54]. However, it is not clear whether the interaction between CO and CBS affects the sensory action of carotid body.

3.4. Mechanisms of Regulating Carotid Body Activity by H_2S. Recent studies showed that hypoxia induces the opening of voltage-dependent Ca^{2+} channels (VDCC) through inhibition of K^+ channels in carotid bodies [14, 15]. VDCC activation increases intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and thus enhances the release of neurotransmitters under hypoxia [15, 19] (Figure 2). NaHS inhibited big-conductance Ca^{2+}-sensitive K^+ (BK_{Ca}) channels [27, 48], background K^+ current (TASK) [55], and elevated $[Ca^{2+}]_i$ in glomus cells [55]. NaHS-induced elevation of $[Ca^{2+}]_i$ was abolished in the absence of extracellular Ca^{2+} [55, 56] or in case that the depolarization was prevented by voltage clamping at the resting membrane potential [55]. In cultured rat glomus cells, NaHS-induced elevation of $[Ca^{2+}]_i$ was blocked by nifedipine, a L-type VDCC blocker [56]. Similar finding was observed in both astrocytes [57] and rat neurons [58]. These findings support the view that L-type VDCC may be involved in H_2S-induced elevation of $[Ca^{2+}]_i$ in carotid bodies [56] (Figure 2). A series of studies demonstrated that H_2S was also involved in the activation of T-type VDCC [59–61]. More recently, it is suggested that $Ca_V3.2$ T-type VDCC contributed to H_2S mediated carotid body response to hypoxia [62]. $Ca_V3.2$ was the major T-Type VDCC isoform expressed in the carotid body [62] (Figure 2). In brief, all abovementioned observations indicate that H_2S may function as a physiological excitatory mediator of the carotid body sensory response to hypoxia (Figure 2). Moreover ion channels are closely correlated with this regulation (Figure 2).

In addition, exogenous application of H_2S inhibited mitochondrial function in rat glomus cells [55]. The author proposed that the inhibitory effect of H_2S on TASK was due to inhibition of oxidative phosphorylation. Given that mitochondrial reactive oxygen species is speculated to mediate carotid body action [63, 64], whether mitochondria is directly involved in H_2S-mediated excitatory sensing in carotid bodies needs to be demonstrated. In addition, it is reported that neurotransmitters were involved in H_2S-induced sensory excitation of carotid bodies. Acetylcholine (ACh) and adenosine triphosphate (ATP) are two excitatory neurotransmitters in the carotid bodies of both cats and rats [27, 65, 66]. Inhibition of purinergic receptors using pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid, or application of hexamethonium, a blocker of nicotinic cholinergic receptors, prevented NaHS-evoked sensory excitation of the mouse carotid body [27]. This study indicates that the release of ATP/ACh from glomus cells is correlated with H_2S-induced sensory excitation. However, Na_2S (another H_2S donors) significantly reduced the release of both ACh and ATP in the cat carotid body [65]. The effect of H_2S on neurotransmitters release needs to be further established. Whether neurotransmitters release is associated with H_2S-regulated carotid body activity is not clear.

3.5. Hypoxia-Induced H_2S Increase: Evidence for Interaction of CSE with HO-2. In CSE knockout mice and rats treated with PPG, hypoxia failed to increase H_2S generation in both mouse and rat carotid bodies [26]. These observations suggested that hypoxia-induced H_2S production may be due to increased CSE activity. Hemeoxygenase-2 (HO-2) converts heme to CO [67]. HO-2 is constitutively expressed in type I cells of mammals [16]. It has been demonstrated that CO is an inhibitory mediator for carotid bodies hypoxia sensing [16] (Figure 2). Under normoxia, pharmacological inhibition of HO-2 greatly increased H_2S generation [26]. In contrast, a CO donor inhibited the hypoxia-evoked H_2S production in the carotid body [26]. These couple of findings suggested that CO may be a negative regulator for H_2S production in the carotid body (Figure 2). In CSE knockout mice, the HO-2 inhibitor had no effect on H_2S generation. Under hypoxia, reduced CO generation may improve H_2S generation and the sensory action of the carotid body [26]. Taken together, it was proposed that H_2S generation is negatively controlled by CO-inhibited CSE activity [26] (Figure 2). Given that CSE does not contain a heme group, this enzyme is unlikely to be bound by CO [1, 14]. To date, the mechanisms by which CO inhibits CSE activity are still unknown.

3.6. H_2S-Mediated Sensory Activity of Carotid Bodies in Diseases. More recently, inherent variations in CO-mediated H_2S have been shown to contribute to reflex variation of carotid body O_2 sensing in three genetically distinct rat strains, including Sprague-Dawley (SD) rats, Brown-Norway (BN) rats, and spontaneous hypertensive (SH) rats [28]. In comparison with SD rats, BN rats display a profoundly reduced ventilatory response to hypoxia and also develop pulmonary edema when challenged with hypoxia [28, 68]. SH rats, compared with SD rats, exhibit an exaggerated response to hypoxia [69] and this alteration is vital for the development of essential hypertension [70]. Variability in kinetic properties of HO-2 was shown in liver microsomes from SD, BN, and SH rats, consistent with changes in CO levels from these three rat strains [28]. The authors proposed that variability of HO-2 activity of the liver was similar to that of the carotid bodies, based on the observation that hypoxic responses of the liver microsomes paralleled those of carotid bodies in the respective strain [28]. Impaired hypoxic sensitivity in BN rats was associated with augmented basal levels of CO and reduced H_2S [28]. In contrast, the exaggerated hypoxic response in SH rats was correlated with

lower CO and higher basal H_2S [28]. Different substrate affinities for HO-2 resulted in various basal levels of CO and H_2S generation [28]. Taken together, this series of findings further demonstrates that CO-H_2S-mediated O_2 sensing is a fundamental mechanism in the carotid body chemosensory reflex [28].

4. Summary and Perspective

An abundant and continuous supply of O_2 is essential for survival of all mammalian cells. Reduced O_2 availability or hypoxia evokes many important physiological responses and changes, for the sake of increasing oxygen delivery. The activation of carotid bodies is a sensitive and prompt response to hypoxia, rapidly enhancing general O_2 supply. The family of HIFs, master regulators of cellular O_2, induces an elegant series of delayed changes through activating target genes expression under hypoxia.

Emerging evidence shows that H_2S plays a crucial role in O_2 sensing, through regulating carotid body activity and HIF-1 action. The regulation of HIF-1 accumulation via exogenous H_2S has been shown, but the mechanisms for this effect are largely elusive and controversial. The patterns for H_2S-regulated HIF-1 may be dose-dependent. Further studies need to identify the role of endogenous H_2S on HIF-1 action. Compared with many studies on the role of H_2S on HIF-1α, few have been done to elucidate the interaction between H_2S and HIF-2α. Glomus cells had higher expression of HIF-2α than that of HIF-1α [71]. Heterozygous HIF-1α gene-knockout mice had no carotid bodies responses to hypoxia [72]. Carotid bodies from mice with heterozygous knockout of HIF-2α gene exhibited exaggerated responses to hypoxia [73]. It was proposed that a balance between HIF-1α and HIF-2α contributed to changes of redox homeostasis in the carotid body [15]. Based on this hypothesis, would H_2S affect HIF-1α and/or HIF-2α in the carotid body? Endogenously generated H_2S has been shown as a stimulator of carotid body activity under hypoxia. Although CO has been shown to negatively regulate CSE-catalyzed H_2S production, the accurate molecular mechanisms by which CO affects CSE activity are still unknown. Moreover, the effect of CBS/H_2S on carotid body action is elusive.

One of the most recently identified mechanisms for H_2S effect is *S*-sulfhydration [74–76]. The potential targets of *S*-sulfhydration in H_2S-mediated O_2 sensing remain to be determined. In addition, a novel study showed that H_2S activated protein kinase G (PKG) Iα oxidation by inducing disulfide formation [77]. The key mediator in that event is polysulfides. H_2S rapidly converts to polysulfides when H_2S contacts O_2 or hydrogen peroxide [1, 77]. It is not yet clear whether polysulfides are involved in H_2S-mediated carotid body response to hypoxia or H_2S-regulated HIF functions.

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 Alleviates Cadmium-Induced Cell Death through Restraining ROS Accumulation in Roots of *Brassica rapa* L. ssp. *pekinensis*

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Hydrogen sulfide (H_2S) is a cell signal molecule produced endogenously and involved in regulation of tolerance to biotic and abiotic stress in plants. In this work, we used molecular biology, physiology, and histochemical methods to investigate the effects of H_2S on cadmium- (Cd-) induced cell death in Chinese cabbage roots. Cd stress stimulated a rapid increase of endogenous H_2S in roots. Additionally, root length was closely related to the cell death rate. Pretreatment with sodium hydrosulfide (NaHS), a H_2S donor, alleviated the growth inhibition caused by Cd in roots—this effect was more pronounced at 5 μM NaHS. Cd-induced cell death in roots was significantly reduced by 5 μM NaHS treatment. Under Cd stress, activities of the antioxidant enzymes were significantly enhanced in roots. NaHS + Cd treatment made their activities increase further compared with Cd exposure alone. Enhanced antioxidant enzyme activity led to a decline in reactive oxygen species accumulation and lipid peroxidation. In contrast, these effects were reversed by hydroxylamine, a H_2S inhibitor. These results suggested that H_2S alleviated the cell death caused by Cd via upregulation of antioxidant enzyme activities to remove excessive reactive oxygen species and reduce cell oxidative damage.

1. Introduction

Recently, hydrogen sulfide (H_2S) has become appreciated as an endogenous signaling molecule, after nitric oxide and carbon monoxide [1]. In the 1980s, H_2S release in plants was discovered [2]. Some genes encoding these enzymes, which are responsible for endogenous H_2S generation, were recently cloned in higher plants. Two cysteine desulfhydrases with the ability to decompose cysteine to pyruvate, ammonia, and H_2S have been identified: L-cysteine desulfhydrase (LCD) with L-cysteine as substrate and D-cysteine desulfhydrase (DCD) with D-cysteine as substrate [3, 4]. Since then, Álvarez et al. reported a novel L-cysteine desulfhydrase, named DES1, which is an *O*-acetylserine(thiol)lyase homolog [5]. Some enzymes with similar function are being discovered, but detailed information remains limited.

As a signal molecule, H_2S plays a vital role in regulating the growth and development of plants; moreover the important effects of H_2S in plants response to some stresses have been intensely discussed in recent years. Increasing amounts of evidence illustrate the physiological functions of H_2S in the growth and development of plants, such as enhancing photosynthesis, regulating seed germination, stomatal movement, root formation, and flower senescence [6–10]. In addition, H_2S, as a pivotal role in plant response to environmental stimuli, such as improving drought resistance, coping with heat stress, enhancing freezing tolerance, and involvement in plants response to heavy metal, osmotic, and salt stresses, has also been reported [11–15]. The protective roles of H_2S alleviating stresses have focused on promoting antioxidant capacity to decrease reactive oxygen species (ROS) accumulation or interacting with other signaling molecules. However,

it is just the beginning of studying H_2S-mediated stress responses, and the potential molecular mechanisms remain ambiguous.

Cadmium (Cd) is a major environmental pollutant and can be easily transported from the roots to other parts of plant. Cd also displays deleterious effects on seed germination, growing development, and photosynthesis [16, 17]. Treatment with high Cd concentrations can trigger programmed cell death (PCD) or necrosis in tobacco and *Arabidopsis* cell cultures [18, 19]. These negative effects of Cd were found to be mediated by ROS accumulation.

ROS including hydrogen peroxide (H_2O_2), superoxide radical ($O_2^{\bullet-}$), hydroxyl radical, and single oxygen are generated unavoidably in processes of glycolysis and photosynthesis, which are important to energy production and storage strategies for aerobic microbes and plants. In plants, ROS are continuously produced predominantly in chloroplasts, mitochondria, and peroxisomes [20]. Under normal growth conditions, ROS are produced and maintained at a low level in vivo by a complex antioxidant system. Low molecular-weight antioxidants (ascorbic acid, glutathione reduced, carotenoids, and tocopherols) and antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase (POD), and catalase (CAT) in plant can scavenge ROS [21]. Under stress conditions, ROS formation exceeds the capacity of antioxidant system scavenging and results in oxidative stress. The cellular Cd intoxication mechanism is known to disturb redox homeostasis by indirectly stimulating ROS production in the train of oxidative damage through oxidizing lipids, proteins, DNA, and carbohydrates. Malondialdehyde (MDA), the product of lipid peroxidation, can cause membrane components cross-linking and polymerization [22]. In this way, the structure of cell membranes may be destroyed.

Our research concerns Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) which has a close genetic relationship with *A. thaliana* and a clear genetic background. Because of having greater biomass than *A. thaliana*, the root growth relating index of Chinese cabbage can be detected more easily. The aim of this work is to provide more evidence for the potential mechanisms of H_2S mitigation of developmental inhibition caused by Cd stress. We investigated changes on root elongation, ROS content, lipid peroxidation level, electrolyte leakage percentage (ELP), cell death, and DNA damage. The results showed that H_2S could decrease cell death rate to alleviate Cd-induced growth inhibition through regulating the ROS balance in Chinese cabbage roots.

2. Materials and Methods

2.1. Plant Material and H_2S Treatment. A commercial variety of Chinese cabbage (Jinyu75) was used. Plants were grown in nutrient soil : vermiculite (1 : 1, v/v) in growth chambers at 23°C with a photoperiod of 16/8 h (light/dark). Light ($160\ \mu Em^{-2}\ s^{-1}$) was supplied by cool white fluorescent tube. At 3 days after emergence, seedlings were irrigated by cadmium chloride ($CdCl_2$) solutions of different concentrations (0, 5, 10, and 20 mM). Gene expressions were determined after Cd exposure for 24 h. Root length, H_2S production, and physiological indexes were determined after 48 h of Cd exposure. Pretreatment with sodium hydrosulfide (NaHS), a H_2S donor, was performed for 24 h before Cd treatment. Pretreatment of 1 mM hydroxylamine, an inhibitor of H_2S generation, was performed for 4 h before Cd treatment.

2.2. Detection of Cell Death. Cell death was measured according to the method of Turner and Novacky [23] with some modifications. Roots (2 cm long) were incubated in 0.25% Evans blue solution for 15 min and washed with water for 10 min. The trapped Evans blue was released from the roots by homogenizing with 1.0 mL of 80% alcohol. The homogenate was incubated for 15 min in a water-bath at 50°C and centrifuged at 10,000 ×g for 10 min. The absorbance of supernatant was measured at 600 nm and calculated on the basis of fresh weight.

2.3. Detection of DNA Fragmentation. The fragmented DNA was extracted with a DNA purification kit (Beyotime, C0008) according to the manufacturer's protocols. The eluent containing DNA was subjected to electrophoresis on a 1.5% agarose gel. DNA bands were observed and analyzed electrophoretically by an ultraviolet gel documentation system (Bio-Rad, USA).

2.4. Determination of H_2S Production Rate. Endogenous H_2S was determined by the method of Sekiya et al. [2] with some modifications. Roots were homogenized in 1 mL of 50 mM phosphate buffer solution (PBS, pH 7.0) containing 0.1 M EDTA and 0.2 M ascorbic acid. The homogenate was mixed with 0.5 mL of 1 M Tris-HCl in an Erlenmeyer flask to release H_2S, and H_2S was absorbed in a test tube containing 1% zinc acetate located in the bottom of the Erlenmeyer flask. After 15 min of reaction, 0.15 mL of 3.5 mM H_2SO_4 solution, containing 5 mM dimethyl-*p*-phenylenediamine, was added to the test tube and then 0.15 mL of 50 mM $FeCl_3$ was added. After 15 min, absorption at 667 nm was measured.

2.5. Analysis of Transcript Levels. The total RNA of roots was extracted using RNAiso plus (Takara, D325A). The cDNA was synthesized using a Reverse Transcription System Kit (Takara, D6110A). Real-time PCR was performed by a real-time PCR (Bio-Rad, California, USA) detection system. The list of primers was supplied in Table S1 (see Supplementary Material available online at http://dx.doi.org/10.1155/2015/804603). All reactions were repeated independently at least three times. Statistical analysis was performed using iQ5 software (Bio-Rad, California, USA).

2.6. Measurement of Intracellular H_2O_2, $O_2^{\bullet-}$, and ROS Levels. H_2O_2 and $O_2^{\bullet-}$ were measured according to a previously described method [24] with some modifications. For cytochemical visualization of H_2O_2, intact roots were immersed in 1% solution of 3,3-diaminobenzidine, incubated at room temperature for 2 h, illuminated until appearance of brown precipitate, and then decolorized with 95% alcohol. For cytochemical visualization of $O_2^{\bullet-}$, intact roots were immersed

in a 0.1% solution of nitroblue tetrazolium in 50 mM PBS (pH 7.4), at room temperature, and illuminated until the blue formazan precipitate appeared and then decolorized with 95% alcohol. ROS was determined by fluorimetric assay. Root tissues were incubated with 25 mM 2′,7′-dichlorofluorescein diacetate at 37°C for 30 min. Results were observed by fluorescence microscopy (488 nm) and fluorescence intensity was analyzed with LSM 5 software (ZEISS, Germany).

2.7. Measurement of MDA and Electrolyte Leakage Percentage (ELP). MDA content was assayed as described by Heath and Packer [25] with some modifications. Root tissues were homogenized with 5% trichloroacetic acid (TCA). After centrifuging at 10,000 ×g for 5 min, supernatant was mixed with 5% TCA containing 0.68% thiobarbituric acid. The mixture was heated at 98°C for 30 min and centrifuged at 7500 ×g for 5 min. The absorbance of the supernatant was measured at 532,600 and 450 nm.

ELP was measured using an electrical conductivity meter according to Lutts et al. [26]. Roots were washed with distilled water and 0.2 g of samples were placed in tubes containing 10 mL of distilled water and then incubated at 25°C for 1 h. Then the electrical conductivity of the bathing solution (EC1) was read. Samples were then placed in a water-bath at 95°C for 30 min and the second reading (EC2) was determined after cooling to 25°C. ELP was calculated as ELP = (EC1/EC2) × 100%.

2.8. Enzyme Extraction and Activity Measurements. Activities were analyzed by the methods described by Li et al. [27] with some modifications. Roots were homogenized in 1.5 mL of 50 mM PBS buffer containing 1 mM EDTA and 1% polyvinylpyrrolidone. The homogenate was centrifuged at 10,000 ×g for 10 min at 4°C and the supernatant was used to examine the activity of antioxidant enzymes.

Total SOD activity was measured colorimetrically at 560 nm, based on the ability of $O_2^{\bullet-}$ generated by the riboflavin system under illumination to reduce nitroblue tetrazolium. CAT activity was determined at 290 nm for 3 min, using 1 mL reaction mixture containing 50 mM PBS buffer, 2% H_2O_2, and 50 μL of supernatant. One unit of CAT activity was defined as a decrease of absorbance of 0.01 min^{-1}. A mixture containing 50 mM PBS buffer (pH 7.0), 3% guaiacol, 2% H_2O_2, and 50 μL of enzyme extract was used to measure POD activity at 470 nm for 3 min. POD activity was expressed as increase of absorbance of 0.01 per min as one enzyme unit. APX activity was determined as the decrease in A_{290} for 3 min in 1 mL of reaction mixture containing 50 mM PBS buffer (pH 7.0), 15 mM ascorbate, 30 mM H_2O_2, and 50 μL of enzyme extract. One unit of APX activity was defined as a decrease of absorbance of 0.01 per min.

2.9. Statistical Analysis. All experiments were performed in triplicate. Data were presented as mean ± SE. One-way analysis of variance was used for multiple comparisons using SPSS 17.0 software (IBM SPSS, Chicago, USA).

3. Results

3.1. Cell Death due to Cd Stress Arrests Root Growth. To explore the negative effects of Cd exposure on Chinese cabbage roots, 3-day-old seedlings were treated with $CdCl_2$ at increasing concentrations (0, 5, 10, and 20 mM) for 48 h. The root length was significantly ($P < 0.05$) inhibited by 5 mM $CdCl_2$ treatment compared to controls (Figure 1(a)). Furthermore, growth inhibition of roots after Cd exposure decreased in a dose-dependent manner ($R^2 = 0.9878$, $P < 0.05$). With 10 and 20 mM $CdCl_2$ treatment, the growth inhibitions were 71.56% and 86.52%, respectively. Moreover, the black spots and necrosis of the roots tips can be observed. Thus, 5 mM $CdCl_2$ was chosen for further experiments.

Because Evans blue can stain the cell walls of dead cells, it was used to indicate dead cells. Roots treated with $CdCl_2$ showed a deeper level of dye compared with controls, and the quantity of Evans blue extracted from roots sharply increased (Figure 1(b)). The content of Evans blue was 4.6 times higher for 20 mM $CdCl_2$ treatment than controls. Furthermore, the length of roots was inversely related to the death rate of root cells ($y = -16.681x + 130.24$, $R^2 = 0.9968$, $P < 0.05$). These results demonstrated that Cd inhibited root growth due to causing the death of root cells.

3.2. H_2S Is Involved in Response to Cd Stress. To investigate the relationship between Cd stress and endogenous H_2S in Chinese cabbage, the expression of genes *BraLCD*, *BraDCD1*, and *BraDES1* (Figure 2(a)) and the production rate of H_2S were examined (Figure 2(b)). The endogenous H_2S emission was stimulated by Cd stress. Firstly, after a range of concentrations (0, 5, 10, and 20 mM) of Cd treatment for 24 h, the relative expressions of *BraDCD1* and *BraDES1* were upregulated. However *BraLCD* expression level was not increased. The expression level of *BraDES1* differed significantly under the 5 mM treatment, and expression level under the 20 mM Cd treatment was 4.7 times of the control. Secondly, as Cd concentration increased, the H_2S production also gradually increased.

H_2S was applied to verify the role of H_2S in enhancing plant tolerance to Cd stress. Seedlings were pretreated with different concentrations (5, 20, 50, 80, and 100 μM) of NaHS and then exposed to Cd. Inhibition of root growth was markedly ($P < 0.05$) alleviated by 5 μM NaHS, and the other concentrations of exogenous NaHS pretreatment had no significant effect and even showed some negative effects (Figure 2(c)). Thus, 5 μM NaHS was used in further experiments.

3.3. H_2S Influenced the Cd-Induced Cell Death and DNA Damage. There were fewer dead cells in controls and the NaHS treatment alone, but Cd exposure notably increased cell mortality (Figure 3(a)). When seedlings were pretreated with 5 μM NaHS for 24 h before 5 mM Cd treatment, the content of Evans blue was reduced by 31.9% ($P < 0.05$) compared with Cd treatment alone. The content of Evans blue differed significantly between Cd and HA + Cd treatments.

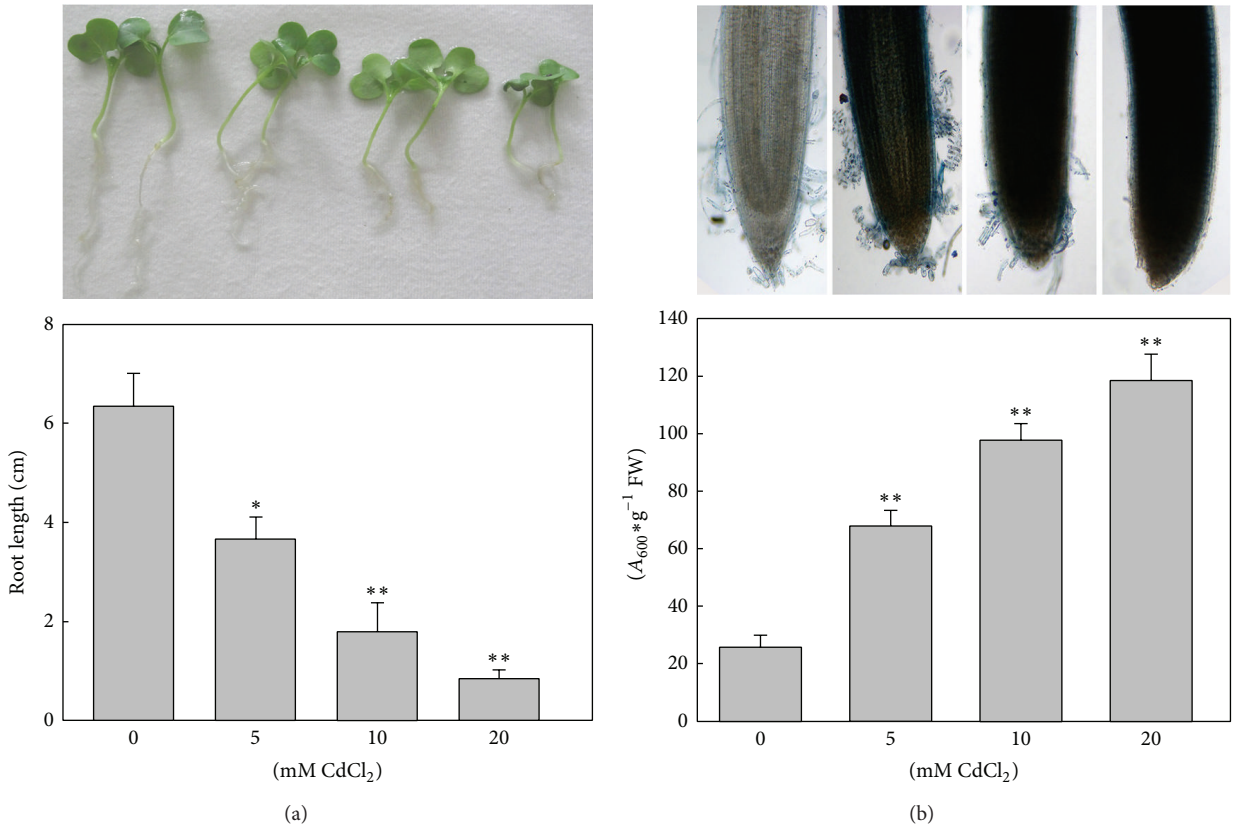

Figure 1: Influence of Cd stress on root development and cell death in Chinese cabbage roots. (a) Root growth under Cd stress. (b) Roots stained with Evans blue observed under a light microscope and quantitative analysis of cell death caused by Cd exposure. Three-day-old seedlings were exposed to different concentrations of Cd (0, 5, 10, and 20 mM) for 24 h. Data are mean ± SE of three independent repeats. Using one-way ANOVA and compared with controls, significance is shown by $^{*}P < 0.05$, $^{**}P < 0.01$.

To confirm the above results concerning cell death, DNA-ladder assays were performed based on DNA or chromatin fragmentation after cell death. Genomic DNA degradation was not apparent in controls (Figure 3(b)). Strong nuclear-DNA random cleaving appeared with Cd treatment and weakened with NaHS + Cd treatment but it was more serious in the HA + Cd treatment.

A further phenotype test was carried out using exogenous H_2S or HA to treat seedlings (Figure 3(c)). As expected, the NaHS pretreatment greatly improved root growth under Cd stress. The HA + Cd treatment greatly inhibited the elongation of roots.

3.4. H_2S Lowered the Cd-Induced Accumulation of ROS. In order to determine whether H_2S could regulate ROS content in roots to alleviate Cd-induced cell death, we colored H_2O_2 and $O_2^{\bullet-}$ in the roots cells and measured ROS with a fluorescence probe. Notable accumulations of H_2O_2 and $O_2^{\bullet-}$ took place in roots treated with 5 mM $CdCl_2$ (Figures 4(a) and 4(b)). Both brown and blue precipitates were diminished in seedlings pretreated with NaHS for 24 h before 5 mM Cd treatment, compared with Cd treatment. Furthermore, the fluorescence intensity of ROS in roots treated with Cd was significantly increased by 62.8% ($P < 0.05$) compared with control. Simultaneously, NaHS + Cd treatment reduced ROS levels in roots by 36.9% (Figure 4(c)). The HA + Cd treatment even further increased the fluorescence intensity (by 34.5%) derived from Cd stress. Exogenous H_2S significantly decreased the ROS accumulation caused by Cd stress. In contrast, pretreatment with an inhibitor of endogenous H_2S increased the ROS content in roots.

3.5. H_2S Reduced the Cd-Induced Accumulation of MDA and ELP. Exposure to 5 mM $CdCl_2$ caused significant MDA overproduction, and NaHS + Cd significantly reduced MDA content (Figure 5(a)). Generation of MDA decreased by 35.4% in Chinese cabbage treated with NaHS + Cd compared with Cd treatment. In the presence of HA, MDA content following Cd treatment was about twice that for Cd alone.

There was a significant increase (42.5%) of ELP in roots for 48 h of Cd treatment compared with controls (Figure 5(b)). Roots with NaHS + Cd treatment showed a 27.7% decrease in ELP compared to Cd-exposed roots; and the HA + Cd treatment showed a slight increase compared with Cd treatment. As expected, 5 μM NaHS pretreatment lowered the MDA content and the Cd-induced ELP. This

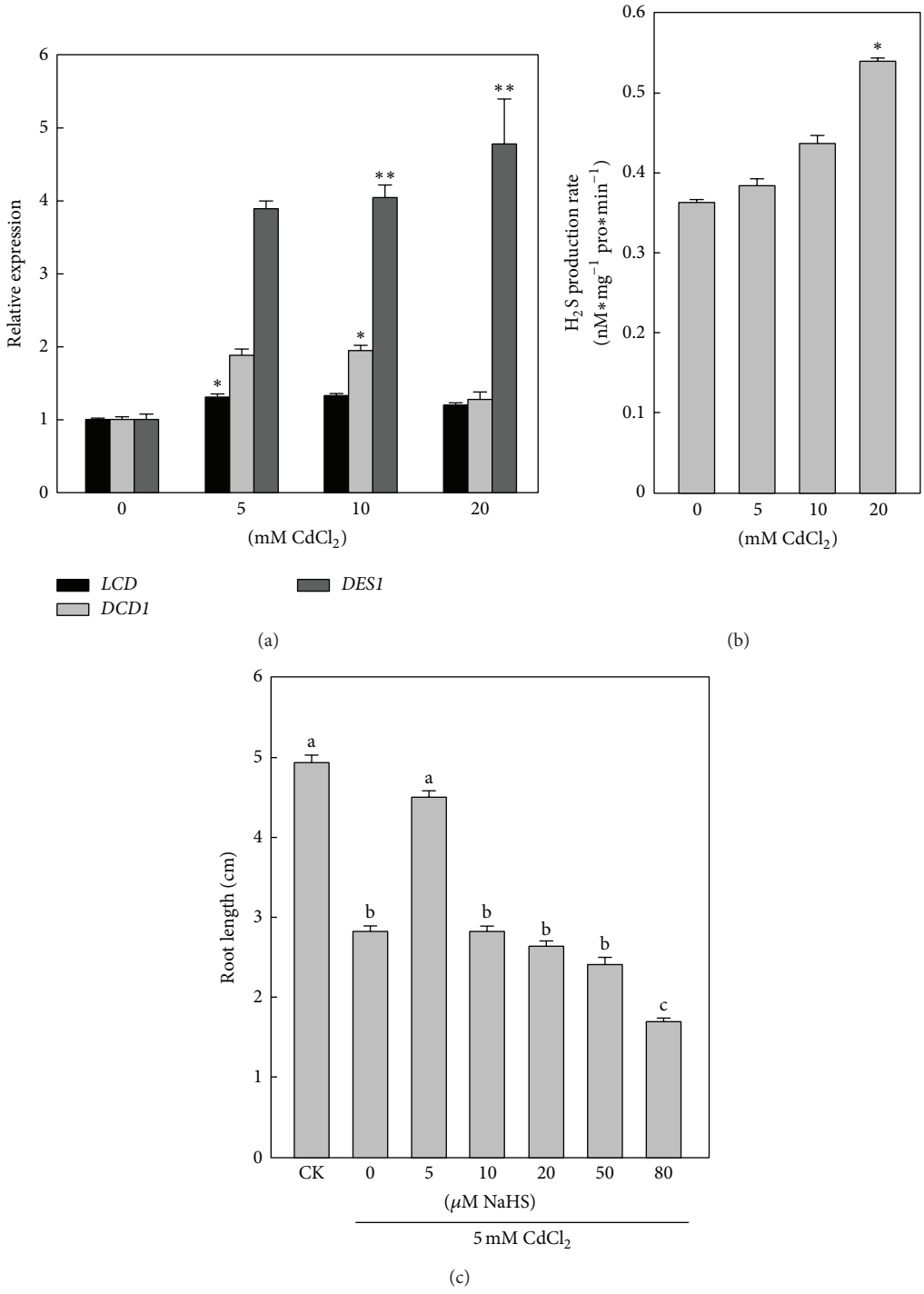

Figure 2: Emission of H_2S and its positive effect on root growth inhibition caused by Cd stress in Chinese cabbage. (a) The expressions of H_2S synthase-encoding genes *LCD*, *DCD1*, and *DES1* under Cd stress. (b) The production rate of H_2S under increasing concentrations of Cd. (c) Effects of different concentrations of NaHS on root development under 5 mM Cd treatment. Three-day-old seedlings were exposed to different concentrations of Cd (0, 5, 10, and 20 mM) for 48 h. Data are mean ± SE of three independent repeats. Using one-way ANOVA and compared with controls, significance is shown by $^{*}P < 0.05$, $^{**}P < 0.01$; LSD was used for multiple comparisons; different letters indicate significant differences ($P < 0.05$).

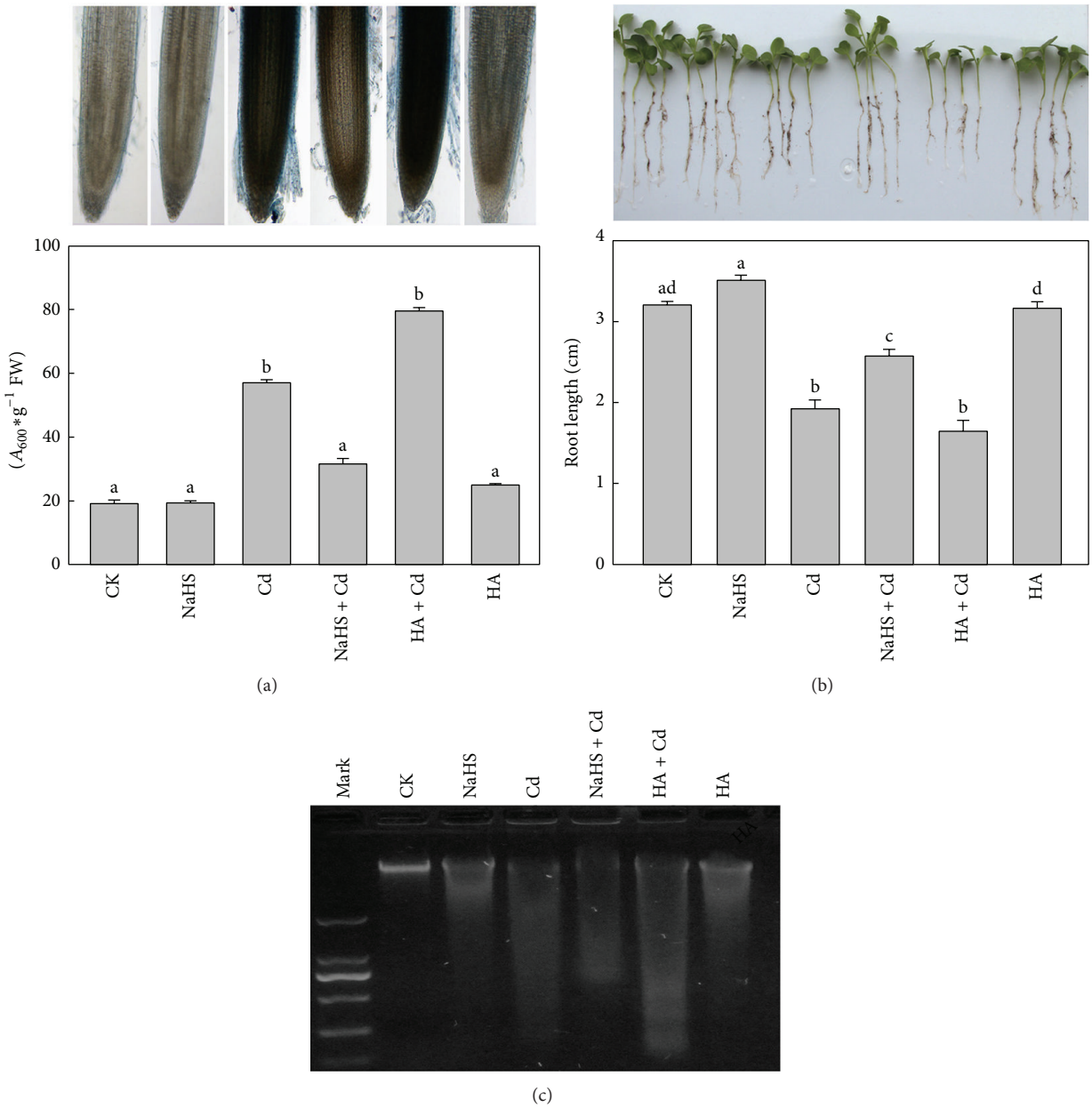

FIGURE 3: Effect of H_2S on Cd-induced root cell death in Chinese cabbage. (a) Roots stained with Evans blue observed under a light microscope and quantitative analysis of cell death caused by Cd stress. (b) DNA damage analysis. (c) Root growth phenotypes. CK: control; NaHS: fumigated with 5 μM NaHS for 24 h; Cd: 5 mM Cd treatment for 48 h; NaHS + Cd: seedlings fumigated with 5 μM NaHS for 24 h and then treated with 5 mM Cd for 48 h; HA + Cd: seedlings treated with 1 mM HA for 4 h and then treated with 5 mM Cd for 48 h; HA: seedlings treated with 1 mM HA for 4 h. Data are mean ± SE of three independent repeats. LSD was used for multiple comparisons; different letters indicate significant differences ($P < 0.05$).

result showed that suppression of cell membrane lipid peroxidation could guarantee structural integrity of the cells and improve plant tolerance to Cd stress.

3.6. H_2S Regulated the Activity of Enzymes Scavenging ROS. The activities of SOD, CAT, POD, and APX were determined in an exploration of the effect of H_2S on inhibition of ROS accumulation through enhancing activity of ROS-scavenging enzymes. There was a significant increase in activities of antioxidant enzymes (SOD, CAT, POD, and APX) in Chinese cabbage roots exposed to Cd. Activities of CAT in roots with NaHS + Cd treatment increased by 89% ($P < 0.01$) compared to Cd alone. SOD activity significantly increased 65% ($P < 0.05$) in NaHS + Cd treatment compared to Cd alone. Both SOD and CAT activities decreased with HA + Cd treatment. However, there was only slight increase being observed in APX and POD activities with NaHS + Cd treatment. Seedlings with NaHS treatment alone showed no significant

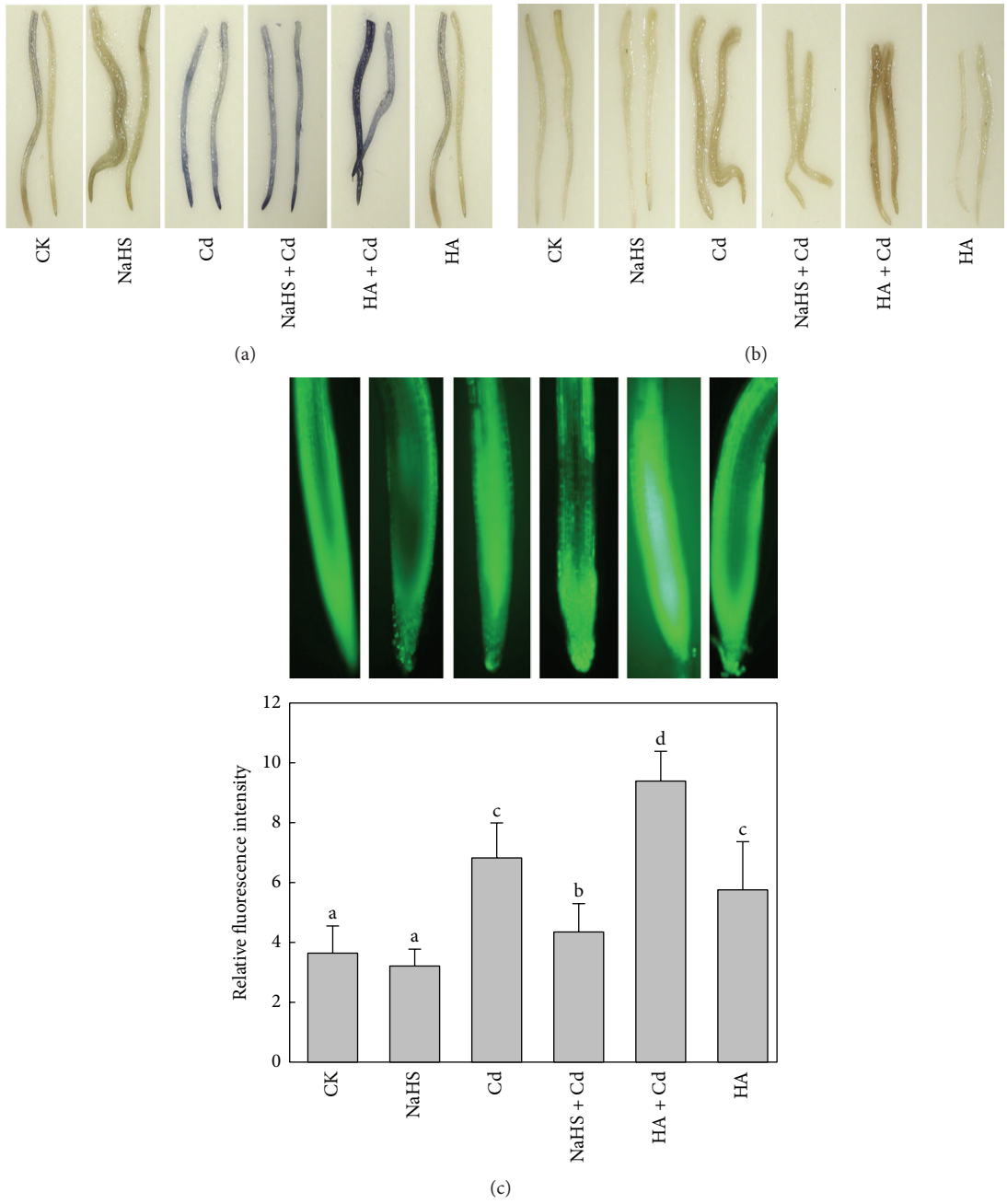

FIGURE 4: Effect of H_2S on Cd-induced ROS accumulation in Chinese cabbage roots. (a) Histochemical detection of H_2O_2. (b) Histochemical detection of $O_2^{\bullet-}$. (c) Fluorescence probe staining of ROS observed under fluorescence microscopy at 488 nm and relative fluorescence intensity of ROS. CK: control; NaHS: fumigated with 5 μM NaHS for 24 h; Cd: 5 mM Cd stressed for 48 h; NaHS + Cd: seedlings fumigated with 5 μM NaHS for 24 h and then treated with 5 mM Cd for 48 h; HA + Cd: seedlings treated with 1 mM HA for 4 h and then treated with 5 mM Cd for 48 h; HA: seedlings treated with 1 mM HA for 4 h. Data are mean ± SE of three independent repeats. LSD was used for multiple comparisons; different letters indicate significant differences ($P < 0.05$).

effects on activity of the four antioxidant enzymes. Analysis of the four antioxidant enzymes revealed that the activities of CAT and SOD were more sensitive to regulating by H_2S. The massive increase in activity of antioxidant enzymes could explain the significant decreases in ROS production.

4. Discussion

Numerous studies have focused on H_2S as a regulator or a signal molecule in plants and its participation in response to diverse stresses. This study aimed to explore the mechanism

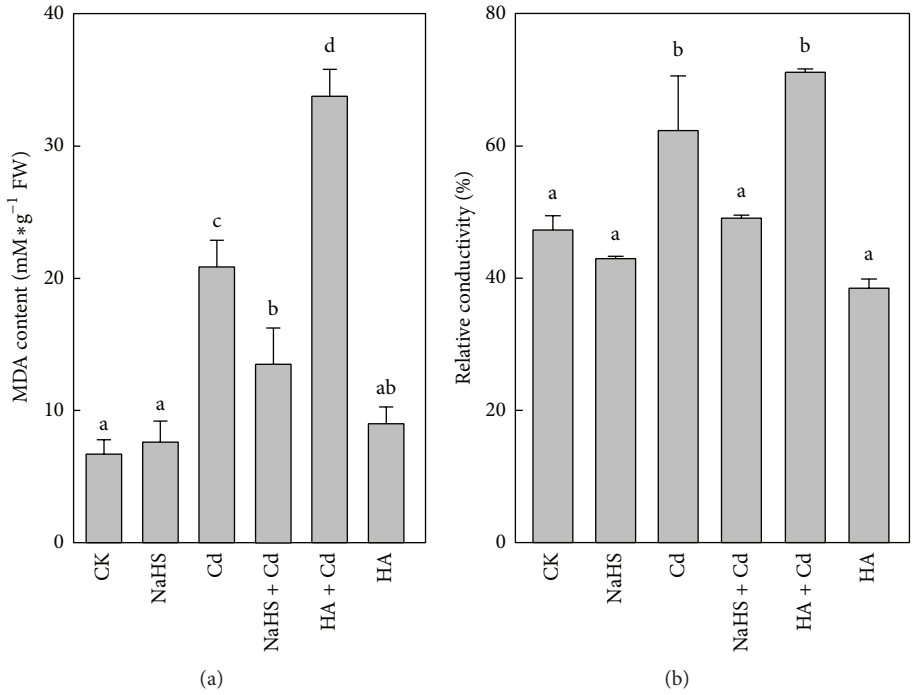

FIGURE 5: Effect of H_2S on Cd-caused oxidation damage in Chinese cabbage roots. (a) Content of MDA. (b) Electrolyte leakage percentage. CK: control; NaHS: fumigated with 5 μM NaHS for 24 h; Cd: 5 mM Cd stressed for 48 h; NaHS + Cd: seedlings fumigated with 5 μM NaHS for 24 h and then treated with 5 mM Cd for 48 h; HA + Cd: seedlings treated with 1 mM HA for 4 h and then treated with 5 mM Cd for 48 h; HA: seedlings treated with 1 mM HA for 4 h. Data are mean ± SE of three independent repeats. LSD was used for multiple comparisons; different letters indicate significant differences ($P < 0.05$).

of the positive effects of H_2S on the plant growth inhibition caused by Cd. Cd stress has been reported to cause much physiological, biochemical, and structural damage in plants [16] with growth inhibition being the most directly perceived. We found that Cd-reduced growth inhibition was positively correlated with cell death in roots of Chinese cabbage (Figure 1). Cd treatment of tobacco cell cultures and onion roots eventually triggers either necrosis or PCD [18]. It is clear that Cd stress can induce cell death, but the mechanisms have remained unclear until now. However, the cells death mediated by ROS is notable [28]. In dead cells the orderly degradation of genomic DNA, which can be detected by DNA-ladder formation, is an important biochemical marker of PCD. In contrast, the random cleaving of nuclear DNA is a characteristic of cell necrosis [29]. Our results showed that genomic DNA was randomly degraded with 5 mM $CdCl_2$ treatment, indicating induced acute cell necrosis in Chinese cabbage roots (Figure 3(b)).

In this experiment, we confirmed that Cd stress could result in the activation of cystein-desulfhydrase encoding genes transcription and H_2S production (Figures 2(a) and 2(b)). Jin et al. [11] reported that drought stress triggered the expression of *AtLCD* and increase of the endogenous H_2S production. In our present study, under Cd treatment, expression of *BraDES1* was mainly activated in roots of Chinese cabbage (Figures 2(a) and 2(b)). Both LCD and DES1 could specifically metabolize L-cysteine to form H_2S, and pyridoxal phosphate was required as a cofactor. We speculate that H_2S is generated through different pathways and helps plants responding to various stresses.

In Figure 2(c), seedlings were treated by different concentrations of NaHS and then Cd treatment. The data indicated that the elongation inhibition of Cd was significantly alleviated under 5 μM NaHS pretreatment only. The pretreatment of 100 μM NaHS had a toxicological effect on roots, and the difference was not observed in the other concentration of NaHS pretreatments. The results had consistency with the report that H_2S has a narrowness of the transition zone between physiological and toxicological levels. As such, H_2S can quickly cause the opposite effect when H_2S concentration further increased [30].

Lipid peroxidation products are reportedly enhanced in shoot and root tissues of plants treated with Cd [31]. In this way, cell membranes may have their lipid composition modified and so their structure can be destroyed [32]. As mentioned above, H_2S maybe protects the membrane integrity through depressing lipid peroxidation. Results provided evidence that H_2S plays an important role in the response of Chinese cabbage to Cd stress. Seedlings pretreated with 5 μM NaHS not only attenuated growth inhibition and cell death effectively but also decreased ROS accumulation significantly in roots upon 5 mM Cd treatment. Oxidative damage is represented by lipid peroxidation and ELP. Cd-induced ROS production was drastically

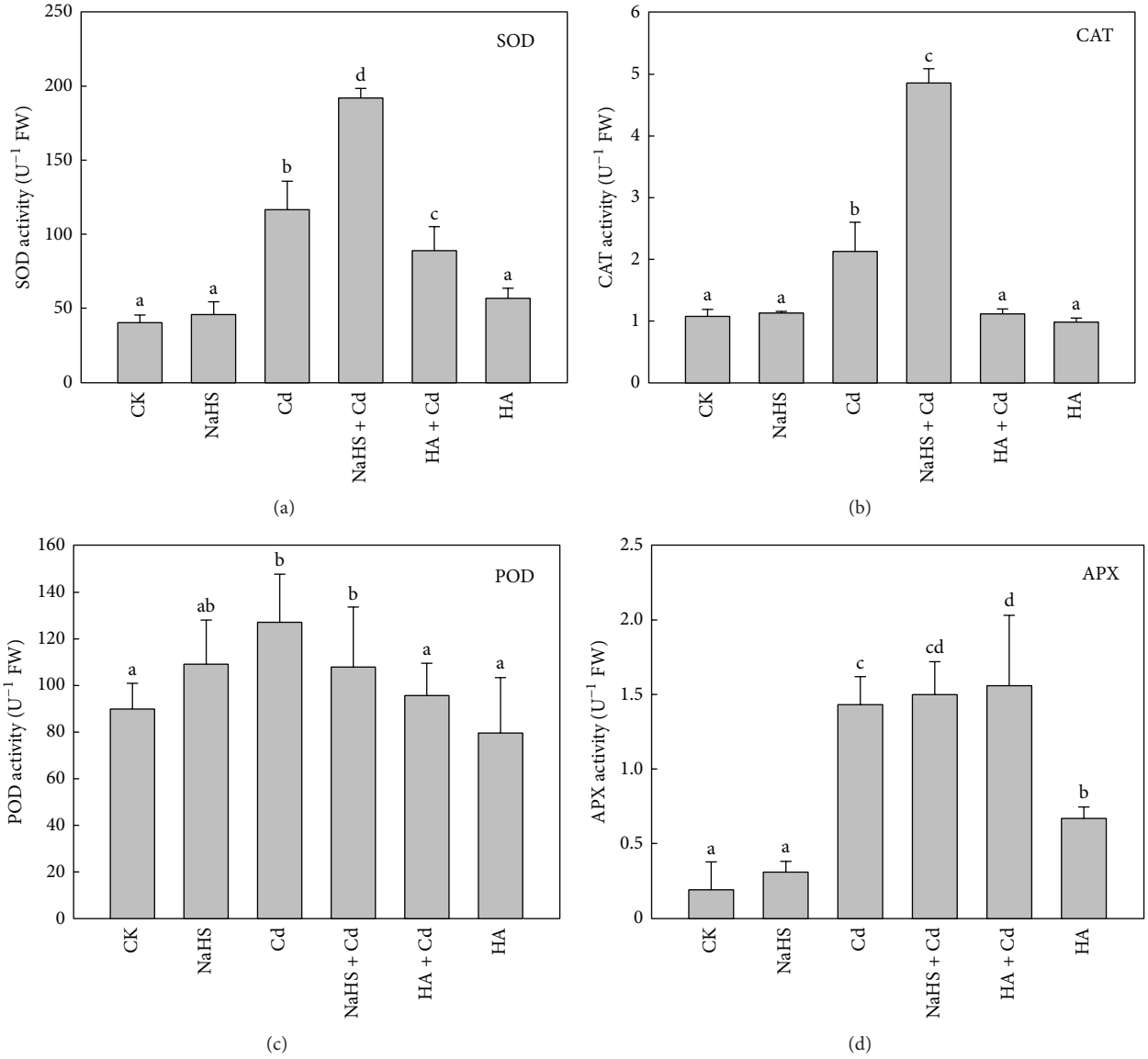

FIGURE 6: Effect of H_2S on antioxidant enzyme activity in Chinese cabbage roots. Activities of (a) SOD, (b) CAT, (c) POD, and (d) APX. CK: control; NaHS: fumigated with 5 μM NaHS for 24 h; Cd: 5 mM Cd stressed for 48 h; NaHS + Cd: seedlings fumigated with 5 μM NaHS for 24 h and then treated with 5 mM Cd for 48 h; HA + Cd: seedlings treated with 1 mM HA for 4 h and then treated with 5 mM Cd for 48 h; HA: seedlings treated with 1 mM HA for 4 h. Data are mean ± SE of three independent repeats. LSD was used for multiple comparisons; different letters indicate significant differences ($P < 0.05$).

decreased by NaHS treatment (Figure 4), consistent with significantly reducing MDA content and ELP in Cd-treated roots (Figure 5).

It was well known that, under Cd stress, ROS levels can rise to excessive levels, with oxidative damage and cell death as a consequence. Thus, continuous control of ROS and their metabolism is imperative under stress conditions. Plants have several antioxidant enzymes to scavenge ROS: SOD catalyzes superoxide radicals to H_2O_2 by very rapid dismutation; CAT and several kinds of peroxidases such as POD and APX then scavenge the H_2O_2 [33]. Chen reported that H_2S significantly inhibited H_2O_2 and $O_2^{\bullet-}$ production in leaves and roots of barely [13]. Our results demonstrated that H_2S could significantly reduce the ROS accumulation caused by Cd stress (Figure 4). Current research has shown that, as a signal molecule, H_2S regulates the metabolism and balance of ROS through upregulating the capacity of the antioxidant system to remove excess ROS. In this research, SOD, CAT, POD, and APX activities were determined to confirm the positive role of H_2S in eliminating ROS. Our results showed that Cd stress could improve the activities of SOD, CAT, POD, and APX in roots. Meanwhile, NaHS + Cd treatment led to a significant increase in SOD and CAT activities. In contrast, POD and APX activities showed no difference between Cd and NaHS + Cd treatments. We concluded that H_2S was involved in scavenging ROS accumulation via mainly enhancing SOD

and CAT activities and partially stimulating POD and APX activities (Figures 6(a)–6(c)).

H_2S-mediated tolerance to Cd stress in Chinese cabbage was related to the modulation of ROS homeostasis. However, H_2S as a signal molecule and its embedded mechanisms of activating these antioxidant enzymes are little understood. In 2009, Mustafa et al. [34] performed the first study of protein S-sulfhydration in mammalian cells. Using a mass spectrometry assay they found that a large number of proteins were S-sulfhydrated under physiological conditions including glyceraldehyde-3-phosphate dehydrogenase, with its activity enhanced by protein S-sulfhydryl modulation. Catalase, related to elimination of H_2O_2, was also S-sulfhydrated. Since then, 176 proteins were identified in leaves of *A. thaliana* and some of them have also been found in mammalian systems [35]. Therefore, further study is needed to verify that sulfhydration of antioxidant enzymes is the signaling mechanism of H_2S regulating these enzymes activities.

In conclusion, H_2S had significant beneficial effects on Cd-exposed Chinese cabbage plants. It effectively blocked elevation of ROS, leading to activation of enzymes (SOD, CAT, POD, and APX) for ROS removal. H_2S prevented plant from cell death caused by oxidant stress and so alleviated Cd-reduced growth inhibition.

Conflict of Interests

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

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Review Article

Hydrogen Sulfide as a Potential Therapeutic Target in Fibrosis

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Hydrogen sulfide (H_2S), produced endogenously by the activation of two major H_2S-generating enzymes (cystathionine β-synthase and cystathionine γ-lyase), plays important regulatory roles in different physiologic and pathologic conditions. The abnormal metabolism of H_2S is associated with fibrosis pathogenesis, causing damage in structure and function of different organs. A number of *in vivo* and *in vitro* studies have shown that both endogenous H_2S level and the expressions of H_2S-generating enzymes in plasma and tissues are significantly downregulated during fibrosis. Supplement with exogenous H_2S mitigates the severity of fibrosis in various experimental animal models. The protective role of H_2S in the development of fibrosis is primarily attributed to its antioxidation, antiapoptosis, anti-inflammation, proangiogenesis, and inhibition of fibroblasts activities. Future studies might focus on the potential to intervene fibrosis by targeting the pathway of endogenous H_2S-producing enzymes and H_2S itself.

1. Introduction

Hydrogen sulfide (H_2S), known for decades as a noxious and toxic gas, has been recognized recently as a third gasotransmitter, together with its two counterparts nitric oxide (NO) and carbon monoxide (CO) [1]. H_2S plays important regulatory roles in different physiologic and pathologic conditions, including hypertension, angiogenesis, neurodegenerative diseases, inflammation, and metabolic syndrome, to name a few [2]. H_2S is endogenously generated in various tissues through the transsulfuration pathway by two pyridoxal-5′-phosphate-dependent enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), with L-cysteine and homocysteine (Hcy) as the substrates. Besides, 3-mercaptopyruvate sulfurtransferase (MST) may produce H_2S through the cooperation with cysteine aminotransferase [1, 2].

Fibrosis is a complicated process, in which the tissue repair after injury is too strong or out of control, and thus results in excessive formation of fibrous connective tissue. This process could be triggered by various factors such as inflammation, immunity, toxicant, ischemia, or hemodynamic changes [3]. Fibrosis can occur in multiple tissues or organs including lung, heart, liver, and kidney. The main pathological changes of fibrosis are the increased fibrous connective tissue and decreased parenchymal cells, which can cause organ structural damage, functional decline, and even failure, seriously threatening to human health [4].

Previously, H_2S was considered as a toxic environmental pollutant, which can cause early lung damage, chronic lung inflammation, and pulmonary fibrosis [5, 6]. However, the physical role of H_2S in the development of fibrosis has attracted significant attention recently. Numerous studies have demonstrated that endogenous and exogenous H_2S play a critical role in the development of fibrosis in lung [7–10], liver [11–13], kidney [14, 15], and heart [16–18]. This review focuses on the protective roles of H_2S in fibrosis pathogenesis.

2. Altered Endogenous H_2S/Its Producing Enzymes in Fibrosis Pathogenesis

The H_2S-producing enzymes/endogenous H_2S pathways are involved in the development of fibrosis. The deficiency in endogenous CBS/H_2S or CSE/H_2S system is responsible for fibrosis [8, 19]. Downregulation of CBS and CSE expression/activity and decreased plasma H_2S levels were observed in patients with hepatocirrhosis [20–23]. And animal models of various organ fibrosis demonstrated the significant decrease of the endogenous H_2S level in plasma and tissues and the H_2S-producing enzymes, whereas the administration exogenous H_2S could inhibit the fibrosis development [9, 13, 15, 18, 19, 24, 25].

2.1. H_2S and Pulmonary Fibrosis. Pulmonary fibrosis, a chronic and progressive interstitial lung disease, is triggered by various factors like organic and inorganic particles, chemicals, radiation, and infections. Its common steps are fibroblastic foci formation, exaggerated extracellular matrix (ECM) deposition, and eventually leading to the destruction of the lung parenchymal architecture [26–28]. The H_2S-producing enzymes (CSE, CBS, and/or MST) are expressed in human and animal lungs [29], and the physiologic plasma concentration of H_2S in healthy animals and humans ranges from 10 to 300 μmol/L [30, 31]. The alteration of H_2S-producing enzymes and endogenous H_2S levels are associated with the development of pulmonary fibrosis. In a rat model of bleomycin- (BLM-) induced pulmonary fibrosis, plasma H_2S content and lung tissue CSE activity (H_2S production rate) in experimental groups were downregulated by 44% and 27%, respectively, on day 7, while the CSE mRNA level in the lungs treated with BLM was 34% and 143% higher than that in controls on day 7 and day 28, respectively. These results may be due to the compensatory mechanism for the decreased H_2S in the body [9]. The lung hydroxyproline content, as a marker of collagen deposition, was increased by 43% in BLM-treated group on day 7 and 100% on day 28, along with histologic changes of inflammatory cells infiltration, fibroblast proliferation, and collagen deposition, whereas intraperitoneal injection of sodium hydrosulfide (NaHS, 1.4 and 7 μmol/kg body weight, resp.) twice a day decreased the hydroxyproline content and remarkably attenuated the severity of lung fibrosis [9]. These results were further supported by Cao et al. [10] and Li et al. [32]. Consistent with the BLM-induced pulmonary fibrosis, reduced endogenous H_2S levels in plasma and lung tissue were observed in another rat model of passive smoking-induced pulmonary fibrosis, accompanied by the upregulation of type I collagen expression and the occurrence of typical histopathological changes of pulmonary fibrosis, whereas NaHS administration at 8 μmol/kg once daily remarkably upregulated H_2S level and inhibited the passive smoking-induced pulmonary fibrosis [28].

According to the above mentioned studies, exogenous H_2S (NaHS) is considered to have protective effect against pulmonary fibrosis at a relatively low dose from 1.4 μmol/kg body weight twice daily to 28 μmol/kg once daily [9, 10, 28, 32]. However, high concentrations of H_2S (50~500 ppm) can cause bronchiolitis obliterans (BO) and pulmonary edema and eventually lead to chronic inflammation and pulmonary fibrosis [2, 6, 33]. Except the protective role of H_2S proved by extensive studies including animal models and *in vitro* experiments [7–10, 28, 32, 34], the altered expression of endogenous H_2S-producing enzymes and the levels of endogenous H_2S in patients with pulmonary fibrosis are unknown yet.

2.2. H_2S and Hepatic Fibrosis. Similar to pulmonary fibrosis, hepatic fibrosis is a dynamic process in response to a variety of stimuli such as ethanol, viral infection, and toxins, leading to the destruction of the architecture of liver parenchyma, followed by excessive ECM deposition, fibrous tissues formation, and cirrhosis (the final pathological stage of hepatic fibrosis) [35, 36]. Three endogenous H_2S-producing enzymes (CSE, CBS, and MST) are all present in the liver [37]. CSE is expressed in the cytosol of hepatocytes, hepatic stellate cells (HSCs), hepatic artery, portal vein, and the terminal branches of the hepatic afferent vessels, while CBS is mainly expressed in the cytosol of hepatocytes, hepatic artery, and portal vein [2, 38]. MST is predominantly localized in mitochondria and cytosolic fractions of pericentral hepatocytes [39]. CSE and CBS are the primary contributors to H_2S production in the liver [40]. Under physiological conditions, CSE accounts for 97% of the H_2S output in liver [40]. The metabolic levels of H_2S and its producing enzymes were observed to change in human hepatic fibrosis and cirrhosis, as well as in animal and cellular models of hepatic fibrosis [11, 13, 20, 23, 38, 41]. In patients with cirrhosis-induced portal hypertension, plasma H_2S levels were significantly lower than healthy controls and correlated inversely with the disease severity by Child-Pugh score (42.6 ± 4.7 μmol/L, 33.5 ± 7.7 μmol/L, and 22.2 ± 7.9 μmol/L in group Child-Pugh score of A, B, and C, resp., but 43.5 ± 6.2 μmol/L in control group) [20]. This decrease stemmed from a reduction of CBS and CSE expression/activity [21–23, 42]. Whether schistosomiasis cirrhosis-induced portal hypertension (SPH) model of rabbit or bile duct ligation- (BDL-) or carbon tetrachloride- (CCl_4-) induced cirrhosis model of rat, both revealed reduced H_2S level in serum and liver tissues by 20% to 80% [13, 20, 38, 41] and decreased CSE protein expression by approximately 30% to 80% [13, 20, 38, 43]. Meanwhile, plasma H_2S concentrations showed a clear descending trend during the progression of cirrhosis [13, 20]. These results suggest that endogenous H_2S generated by H_2S-producing enzymes might involve the pathogenesis of human and animal hepatic fibrosis.

To further prove the protective role of endogenous H_2S, the effects of exogenous H_2S supplementation in the progress of hepatic fibrosis were investigated in animal models [11–13, 38, 44]. NaHS administration significantly elevated the serum levels of H_2S, decreased the portal pressure, attenuated hyaluronic acid level (HA, a serum fibrosis index), downregulated hepatic hydroxyproline content, reduced the number of collagenous fibers, and eventually alleviated the pathologic features of hepatic fibrosis induced by schistosomiasis, bile duct ligation, or carbon tetrachloride in animal models

[11–13, 20, 38, 44]. For example, in cirrhotic rats induced by CCl_4, intraperitoneal injection with NaHS (10 μmol/kg body weight, every two days for 12 weeks) significantly elevated the serum levels of H_2S by an average of about 1.33-fold, reduced the mean level of serum HA by 38.6%, decreased both the number of collagenous fibers and the hydroxyproline content in livers by 40%, downregulated the alpha-smooth muscle actin (α-SMA, a marker of fibrosis) by 50%, and reduced the portal pressure by about 30% [13]. Similarly, NaHS inhibited CCl_4-induced liver fibrosis in rats at a dosage of 56 μmol/kg body weight once daily. *In vitro*, HSCs isolated from BDL-induced cirrhosis rats had a 40% downregulated CSE expression and an 80% drop of H_2S production [38]. Besides, Fan et al. [11, 12] investigated the effects of exogenous H_2S on HSCs activation by ferric nitrilotriacetate (Fe-NTA, 500 μg/L) and found that incubation with various concentrations of NaHS (0, 100, 200, or 500 μmol/L) resulted in a dose-dependent inhibition in HSC proliferation and induction of G1 phase cell cycle arrest and a downregulated expression of collagen I protein.

These researches above indicate that H_2S plays a protective role against hepatic fibrosis at a low level of 10~56 μmol/kg/day [13, 38, 44], while high concentration of H_2S may cause hepatotoxicity [37, 45]. Exposure to a deadly concentration of H_2S (500~1000 ppm) caused abnormal liver function (elevated ALT and AST) in human, as reported previously [46]. Animal studies also demonstrated different degrees of hepatic damage such as enlarged paled livers and severe hyperemia after H_2S exposure at high concentrations (63 to 500 ppm) [33].

2.3. H_2S and Renal/Kidney Fibrosis. Renal fibrosis, including glomerular sclerosis and tubulointerstitial fibrosis, is the hallmark of progressive renal disease of virtually any etiology such as glomerular hyperfiltration, hyperperfusion and hyperpressure, and ischemia/reperfusion injury. It is characterized by renal parenchymal cells injury and death, interstitial inflammatory cells infiltration, fibroblasts proliferation and myofibroblast transformation, excessive ECM deposition, and fibrogenesis [47, 48]. Similar to liver and lung, renal tissues express all the three endogenous H_2S-producing enzymes [39]. CBS is predominantly expressed in renal proximal tubules, while CSE is mainly located in renal glomeruli, proximal tubules, interstitium, and interlobular arteries [14]. Besides, MST is also localized in proximal tubular epithelium in the kidney [39]. CBS and CSE are abundant in renal tissues and produce H_2S in kidney in a combined manner [49]. Under normal physiological conditions, the expression of CSE protein in kidney is 20-fold higher than that of CBS, appearing to be the main H_2S-forming enzyme in the kidney [2, 40].

The alterations of endogenous H_2S metabolism and its producing enzymes in renal fibrosis are well studied in *in vivo* models [14, 15, 19, 50, 51]. Unilateral ureteral obstruction- (UUO-) induced model is a commonly used experimental model for renal interstitial fibrosis, which can be easily manipulated with respect to timing, severity, and duration through surgical intervention [47]. After unilateral ureteral obstruction, CBS and CSE expressions in ureteral obstructive mice kidneys were gradually downregulated in a time-dependent pattern, consistent with decreased plasma and tissue H_2S levels and aggravated interstitial fibrosis in the kidney after UUO [15, 19]. For instance,the protein expression was downregulated by 30.3%, 62.1%, and 70.5% on days 7, 14, and 21 for CBS, respectively, and decreased by 27.2%, 58.2%, and 74.1% for CSE, accompanied by the time-dependent decrease in plasma H_2S level by 10.56%, 11.76%, and 22.01% after UUO [19]. Accordingly, the area of tubulointerstitial fibrosis was increased by 13.11-fold on day 7, by 31.35-fold on day 14, and by 55.33-fold on day 21, respectively [19]. Song et al. [14] found that the CBS expression was nearly completely ablated by obstructive injury on day 14, but CSE was increased when compared to the contralateral kidney. Meanwhile, H_2S production in the obstructed kidney or in plasma was dramatically reduced, along with a significant accumulation of collagen fibrils and an enhanced renal expression of α-SMA and fibronectin on day 14 after operation [14]. Notably, the increase in CSE expression in obstructive kidney could be explained by a "compensatory mechanism" as mentioned by the author, attempting to maintain the H_2S level [14]. These results suggest that the expression of H_2S-producing enzymes and H_2S levels in obstructive kidney and plasma reflect the severity of renal interstitial fibrosis in the UUO-induced model. Thus, the endogenous plasma H_2S might be an ideal biomarker for renal fibrosis.

UUO-induced animal model revealed that exogenous H_2S could impact the development of renal fibrosis [14, 15, 19, 52]. For instance, Zhao et al. [19] demonstrated that intraperitoneal injection with NaHS (1.4 or 7.0 μmol/kg, twice daily immediately after operation) significantly increased plasma and tissue H_2S concentration in the obstructive kidney and decreased the area of renal tubulointerstitial fibrosis. Jiang et al. [52] also found that NaHS treatment (89 μmol/kg, i.p. injection once daily, 3 days before surgery and thereafter continuously for 9 days) reduced the development of interstitial fibrosis and the fibrous area in the obstructed kidneys. In addition, NaHS administration also alleviated the pathological changes of renal fibrosis in other models of renal injury triggered by gentamicin-induced nephrotoxicity [53] or streptozotocin-induced diabetic nephropathy [50, 51]. However, the metabolic changes of H_2S and its producing enzymes as well as its therapeutic potentials in patients with renal fibrosis need to be further studied.

2.4. H_2S and Cardiac Fibrosis. Cardiac fibrosis is a pathological process initiated by some harmful stimuli such as myocardial injury, mechanical stretch, and inflammatory stimuli and followed by the proliferation and migration of cardiac fibroblasts and myofibroblasts transdifferentiation. This process then causes excessive ECM deposition and abnormalities in cardiac structure and function including hypertrophy, failure, and arrhythmias [54, 55]. In mammals, CSE is abundant in heart, vascular smooth muscle, and vascular endothelial cells and is the most relevant H_2S-producing enzyme in the cardiovascular system [56, 57]. CBS

mRNA has been found in endocardium cells and atrial and ventricular myocardial cells [58], yet its protein expression is actually rare in mammalian cardiovascular system [2]. MST is predominantly localized in cardiomyocytes in the heart and in the vascular endothelium, but its contribution to H_2S production is significantly lower [39, 59].

Growing evidence has suggested that endogenous H_2S and its producing enzymes involve the development of cardiac fibrosis. Enzymes expressions including CSE and CBS and endogenous H_2S levels were reduced in different animal models of cardiac fibrosis [17, 25, 60–62]. Simultaneously, decreases in endogenous H_2S and its producing enzymes were negatively correlated with the severity of cardiac fibrosis [17, 25, 60–62]. For instance, endogenous H_2S concentration in plasma decreased by about 15% in a rat model of cardiac fibrosis induced by pressure overload, and the hydroxyproline content in the cardiac tissue increased by about 65%, with an extensive deposition of collagen in the left ventricle (LV) tissue [25]. In volume overload-induced mouse model of cardiac fibrosis, endogenous H_2S production and CSE protein expression in the heart were reduced by 60~80% and 35%, respectively, while the expressions of matrix metalloproteinase- (MMP-) 2/9 and tissue inhibitors of metalloproteinase- (TIMP-) 1/3 were robustly increased, along with a 1.24-fold rise of total hydroxyproline and an increase in collagen deposition [17, 60]. The similar alterations of endogenous H_2S-producing enzymes and H_2S were also seen in mouse model of cardiac fibrosis mediated by myocardial infarction induced by ligation of left anterior descending coronary artery. For example, the expressions of CSE and CBS proteins in hearts were decreased by about 80% and 60%, respectively, after myocardial infarction [62], and the plasma H_2S concentration was significantly decreased to $53.3 \pm 2.7\,\mu$mol/L compared to the controls ($65.1 \pm 1.5\,\mu$mol/L), while the percentage of fibrosis size to total area of left ventricle in rats of myocardial infarction was more than twofold higher than that in controls ($25.7 \pm 1.2\%$ versus $12.5 \pm 0.5\%$; $P < 0.01$) [61].

The protective effect of exogenous H_2S in cardiac fibrosis has been demonstrated in various animal models [17, 18, 25, 60–66]. In a model of spontaneously hypertensive rats (SHR), chronic treatments with NaHS (i.p. 10, 30, and 90 μmol/kg/day, for 3 months) were all effective in reducing indexes of perivascular and interstitial fibrosis in the heart including perivascular collagen area-to-luminal area ratio (PVCA/LA) and collagen volume fraction (CVF) [65]. In pressure overload-induced heart failure model of mouse by transthoracic or abdominal aortic banding, administration of NaHS (po, 30 μmol/L, or i.p. 14 μmol/kg/day) significantly upregulated the levels of endogenous H_2S in plasma and reduced the deposition of collagen in perivascular and intracardiac parenchymal tissue [25, 64]. Similarly, NaHS (30 μmol/L) or sodium thiosulfate ($Na_2S_2O_3$, 3 mg/mL) ameliorated the decreased CSE expression, normalized the reduced H_2S production, mitigated the expressions of MMP-2/9 and TIMP-1/3, and then attenuated the collagen deposition in LV tissues in a volume overload-induced heart failure model of mouse [17, 60]. The role of exogenous H_2S in alleviating cardiac fibrosis is also confirmed in the mouse model of myocardial infarction induced by ligation of left anterior descending coronary artery [18, 61, 62, 66]. In the NaHS-treated group of rats after myocardial infarction, H_2S concentration in plasma, CSE protein content, and mRNA expression in LV myocardium were all significantly increased, compared with vehicle-treated group ($69.5 \pm 4.6\,\mu$mol/L versus $53.3 \pm 2.7\,\mu$mol/L for plasma H_2S level, 0.66 ± 0.04 versus 0.51 ± 0.03 for CSE protein, and 0.94 ± 0.03 versus 0.72 ± 0.03 for CSE mRNA, resp.) [61]. Simultaneously, NaHS supplementation prevented the increase of MMP-2 and MMP-9 expression in the border zone of infracted tissues on day 14 [18] and decreased ratio of the fibrosis size to total area of LV at the end of the 6th week, compared with vehicle-injected controls ($12.5 \pm 0.5\%$ versus $25.7 \pm 1.2\%$) [61].

3. Protective Mechanisms of H_2S in the Development of Fibrosis

3.1. H_2S and Oxidative Stress. Oxidative stress, resulting from an increased production of free radicals including reactive oxygen and nitrogen species (ROS and RNS) and an overwhelmed antioxidant defense system, plays a prominent role in the progression of fibrosis [17, 67–70]. ROS mainly consists of superoxide anion, hydroxyl radical ($HO^{\bullet}$), and hydrogen peroxide, and RNS mainly consists of nitric oxide, nitrogen dioxide, and peroxynitrite. There are two kinds of antioxidant system in our body: one is enzymatic antioxidant system including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase 1 (GPx1), heme oxygenase 1 (HO-1), and NAD(P)H: quinone oxidoreductase 1 (NQO1). The other is nonenzymatic antioxidant system, including vitamin C, vitamin E, glutathione (GSH), thioredoxin-1 (Trx-1), melatonin, α-lipoic acid, carotenoids, and trace elements (copper, zinc, and selenium). Multiple studies revealed that ROS/RNS could activate profibrotic mediators (e.g., protease activated receptor-1/2 (PAR-1/2), a disintegrin and metalloproteinase-12 (ADAM-12), MMP-2/9, and TIMP-1/3) and suppress antifibrotic factors such as TIMP-4 and β1-integrin, leading to parenchymal cells apoptosis, fibroblasts activation, and collagen deposition [17, 68, 69, 71]. By directly scavenging oxygen free radicals [15, 72, 73], inhibiting lipid peroxidation [9, 15, 52], modulating the balance of MMPs/TIMPs and ADAM-12/β1-integrin axis [17, 59, 63, 64], and activating antioxidant system [15, 52, 63, 65, 73, 74], H_2S reduces the intracellular redox environment and alleviates oxidative stress-induced damage. Given the important role of oxidative stress in the pathogenesis of fibrosis, it is reasonable to suspect that endogenous H_2S/H_2S-producing enzymes pathway inhibits the development of fibrosis by its antioxidative action.

In animal models of lung fibrosis, bleomycin stimulated inflammatory cells to generate excess ROS and then promoted oxidative stress such as lipid peroxidation (LPO), resulting in activation of profibrotic mediators like PAR-2 and MMPs 2/9, apoptosis of epithelial cell, and accumulation of collagen, thus leading to pulmonary fibrosis [71, 75, 76]. NaHS administration attenuated lung malondialdehyde (MDA, a marker of tissue fibrosis) and hydroxyproline formation in a rat model of bleomycin-induced pulmonary

fibrosis [9]. In another rat model of pulmonary fibrosis induced by chronic cigarette smoke exposure, H_2S could significantly decrease cigarette smoking-induced oxidative stress-related indexes like MDA and ROS in serum and lung tissue and enhance the activities of serum SOD and GPx, which is associated with the activation of nuclear factor E2-related factor (Nrf2) and the upregulation of HO-1 and Trx-1 proteins in the lung tissue [28]. In CCl_4-induced animal model of cirrhosis and Fe-NTA-induced *in vitro* model of hepatic fibrosis, the overproduction of ROS triggers lipid peroxidation, along with deficient antioxidants such as GSH, causes the imbalance between oxidative stress and antioxidant defense, and finally promotes HSCs proliferation and collagen synthesis in liver [11–13, 44, 68, 77]. Exogenous H_2S inhibits the Fe-NTA-induced elevation of intracellular ROS level and inhibition of proliferation of HSC cells, attenuates the CCl_4-induced elevation of hepatic MDA level, and decreases in hepatic GSH level, resulting in the reduction of collagen deposition in liver tissue, which are related to the inhibition of phosphorylated p38 MAPK (mitogen-activated protein kinase) and activation of phospho-Akt signaling pathway [11–13]. Similarly, exogenous H_2S supplement reversed these pathophysiological changes of fibrosis in kidney in animal models through inhibition of oxidative stress and recovery of antioxidant defense system, which may be attributed to the activation of Nrf2 and the upregulation of its downstream targets including HO-1 and NQO1 proteins in the renal tissue [15, 51–53].

In cardiac fibrosis induced by chronic volume/pressure overload or persistent hyperglycemia, H_2S ameliorates oxidative stress, decreases levels of MMP-2/9, TIMP-1/3, and ADAM-12, and increases the levels of TIMP-4 and β1-integrin [17, 59, 60, 63–65]. In addition, H_2S itself and the increased H_2S-derived thiols (principally GSH) are involved in the maintenance of enzymatic activation of antioxidant enzymes (e.g., CAT and SOD) in the myocardium [63, 65, 66]. NADPH oxidase 2 and NADPH oxidase 4 (Nox2 and Nox4), as a major source of ROS, play a critical role in profibrotic responses in cardiac fibroblasts and ischemic myocardium; moreover, Nox-generated ROS can mediate the conversion of fibroblasts into myofibroblasts via an extracellular signal-regulated kinase 1/2- (ERK1/2-) dependent signaling pathway; exogenous H_2S significantly inhibits cardiac Nox2/4 expression, ROS generation, and ERK1/2 phosphorylation [18, 78].

In short, H_2S can prevent the development of fibrosis by regulating the balance between oxidation and antioxidation and the detailed mechanisms underlying its antioxidant and antifibrotic effects involve the inhibition of phosphorylated p38 MAPK and Nox4-ROS-ERK1/2 signaling pathway and the activation of phospho-Akt and Nrf2-induced antioxidant signaling pathway [9, 11–13, 15, 17, 18, 28, 51–53, 74, 78].

3.2. H_2S and Inflammation. Inflammation has been reported to be the initial stage in the development of fibrosis [79, 80], which causes parenchymal cells apoptosis, fibroblasts proliferation, and ECM deposition, ultimately leading to irreversible fibrotic injury [81, 82]. Treatment with H_2S significantly decreases the infiltration of inflammatory cells, downregulates proinflammatory cytokines like inducible nitric oxide synthase (iNOS), tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-4, IL-6, and IL-8, and then inhibits the progression of fibrosis [10, 13–15, 18, 28, 51, 52, 78].

Inflammatory responses are associated with pulmonary fibrosis [79]. In the process of pulmonary fibrosis induced by bleomycin, different inflammatory cells including neutrophils and eosinophils appeared in the alveoli and interstitium at the early stage, followed by foci of collagen deposition, while large area of fibrosis instead of inflammatory infiltration was observed at the late stage [10]. Similarly, significant fibrosis and inflammatory cell infiltrations also emerged in the lung of smoking rats [28]. Nuclear factor-kappa B (NF-κB) regulates the generation of a lot of proinflammatory cytokines including high-sensitivity C-reactive protein (hs-CRP), TNF-α, IL-1, IL-6, and IL-8 and promotes the proliferation of fibroblast and the formation of fibrosis [10, 28, 75, 83]. MAPK, which consists of three major members, ERK1/2, c-Jun N-terminal kinase (JNK), and p38, also plays an important role in regulating inflammation and fibrosis [84]. In addition, Th1/Th2 balance may play a vital role in the processes of inflammation and fibrosis, as Th2 cytokines such as IL-4 mediate inflammatory response and then enhance the fibrotic process by augmenting fibroblasts proliferation and collagen production, while Th1 cytokines like interferon-(IFN-) γ have inhibitory effects on fibroblast proliferation and collagen synthesis [85]. Studies have demonstrated that H_2S can suppress the expression of NF-κB p65 and inflammatory markers like hs-CRP, TNF-α, IL-1β, and IL-6, inhibit the phosphorylation of MAPKs, and regulate Th1/Th2 balance by elevating the ratio of IFN-γ/IL-4, thus alleviating inflammation and delaying the progression of pulmonary fibrosis [10, 28].

Chronic inflammation and the associated regenerative wound healing responses are strongly associated with the development of hepatic fibrosis and cirrhosis [86]. CCl_4-induced cirrhotic rats showed significantly high levels of serum proinflammatory cytokines including TNF-α, IL-1β and IL-6, and soluble ICAM-1 (intercellular cell adhesion molecule-1), while simultaneous administration of NaHS resulted in a significant decrease of these cytokines, along with alleviated collagenous fibers in the liver [13]. Interstitial inflammation plays an important role in the priming and progression of renal fibrosis as it can induce apoptosis in tubular cells and promote extracellular matrix production, fibroblast proliferation, and epithelial to mesenchymal transition [80, 81]. Exogenous H_2S was found to mitigate the renal interstitial inflammatory response through the inhibitions of NF-κB and MAPKs signal pathways and then result in fibrogenesis suppression in kidney [14, 15, 51]. Similarly, supplementation with exogenous H_2S significantly decreased mRNA and protein levels of inflammatory biomarkers (iNOS, TNF-α, ICAM-1, and VCAM-1 (vascular cell adhesion molecule-1)) in the border zone of infracted myocardium tissues and also reduced granulocyte influx into necrotic areas to some extent [18, 78]. Furthermore, studies demonstrated that the heat shock protein "HO-1" had an inhibitory effect on the inflammatory and fibrotic responses in injured myocardium and

H_2S therapy markedly increased HO-1 protein expression in both the ischemic heart and angiotensin II- (Ang II-) stimulated cardiac fibroblasts, accompanied by ameliorative cardiac fibrosis and inflammation in ischemic myocardium [18, 87].

Taken together, H_2S may exert its anti-inflammatory and antifibrotic effect through inhibiting the activation of NF-κB and MAPKs (p38, JNK, and ERK), as well as upregulating the ratio of Th1/Th2 and the expression of HO-1 protein [10, 14, 15, 17, 28, 51, 87].

3.3. H_2S and Fibroblasts. Numerous studies have shown that fibroblasts proliferation, migration, and differentiation to myofibroblasts, along with excessive ECM production, are key events of fibrosis [18, 47, 88–91]. H_2S could mediate these inhibitory effects by suppressing the activation of proliferation-related genes, protein kinases, signalling pathways, and ion channels [7, 8, 11, 14, 16, 18, 44].

The migration, proliferation, and myofibroblasts transdifferentiation of lung fibroblasts like MRC5 cells, as well as epithelial-mesenchymal transition (EMT) of alveolar epithelial cells like A549 cells, are closely associated with the pathogenesis of pulmonary fibrosis [7, 8]. Studies have reported that incubation with H_2S significantly inhibited the proliferation, migration, and myofibroblasts differentiation of MRC5 cells and the EMT of A549 cells through suppressing ERK phosphorylation and (transforming growth factor beta-1) TGF-β1-Smad2/3 signaling pathways [7, 8, 92]. In the process of liver fibrosis, HSCs activation is characterized by a transformation from quiescent vitamin A-rich cells to myofibroblasts with enhanced proliferation, fibrogenesis and ECM synthesis, and contractility [89]. NaHS treatment could alleviate hepatic fibrosis by reducing the contractility of HSCs and attenuating ECM deposition through the downregulation of calcium influx and collagen I protein expression in activated HSCs [11]. In addition, TGF-β1 is a profibrogenic agent in liver injury and hepatic fibrosis [93, 94]. Shen et al. [44] elucidated that exogenous H_2S could downregulate TGF-β1 expression, prevent HSC activation and proliferation, reduce ECM synthesis, and consequently have antifibrosis effect on liver. For renal fibroblasts, NaHS decreased the cell number and the DNA synthesis of normal rat kidney fibroblasts (NRK-49F), which was associated with the decreased expressions of proliferation-related genes including proliferating cell nuclear antigen (PCNA) and c-Myc [14]. NaHS treatment blocked the transdifferentiation of quiescent renal fibroblasts and tubular epithelial cells into myofibroblasts by inhibiting the TGF-β1/Smad3 and MAPKs (ERK, p38, and JNK) signaling pathways in the UUO-induced kidney fibrosis models [14, 15, 95]. Similarly, exogenous H_2S was found to suppress Ang II-mediated cardiac fibroblast activation and profibrotic activity by repressing Nox4-ROS-ERK1/2 signaling pathway [18]. In addition, NaHS effectively reduced proliferation and myofibroblast transformation of atrial fibroblasts via inhibition of TGF-β1 function and the activities of BK_{Ca}, I_{to}, and IK_{ir} channels [16].

The inhibitory effects of H_2S on these fibroblasts activation and the underlying mechanisms are summarized as follows: (1) H_2S downregulates the expressions of proliferation-related genes including PCNA and c-Myc, which are associated with its antiproliferative effect [14]; (2) H_2S reduces the phosphorylation of MAPKs (p38, JNK, and ERK), increases the phosphorylation of Akt, and thus further suppresses fibroblasts proliferation and migration [7, 8, 12, 51]; (3) H_2S inhibits myofibroblast transformation of fibroblasts with blockade of TGF-β1-Smad signaling pathway [14, 16, 44, 50, 51, 96]; (4) H_2S suppresses fibroblasts activation by repressing Nox4-ROS-ERK1/2 signaling pathway [18]; (5) H_2S negatively modulates the activity of calcium and potassium channels through downregulating Ca^{2+} influx, large conductance Ca^{2+}-activated K^+ current (BK_{Ca}), transient outward K^+ current (I_{to}), and inwardly rectifying K^+ current (IK_{ir}) in fibroblasts and then reducing fibroblast proliferation and myofibroblast transdifferentiation [11, 16, 97, 98].

3.4. H_2S and Apoptosis. H_2S has both a proapoptotic effect on fibroblasts and an antiapoptotic effect on parenchymal cells, which depends on the regulation of cell cycle, apoptosis-related factors, and apoptotic signaling pathways, thus interfering with the progress of fibrosis [11, 17, 34, 52, 61, 64, 66]. During the normal wound healing, the number of fibroblasts is reduced through apoptosis [96]. However, it has been reported that fibroblast is resistant to Fas-mediated apoptosis in the process of pulmonary fibrosis [99]. Baskar et al. [34] found that H_2S propelled MRC5 fibroblast cells towards apoptotic death by inducing DNA damage and cell cycle arrest at G1 phase and activating various apoptosis-related factors, such as p53, p21, ku70, ku8, Bax, and cytochrome c.

In the pathogenesis and development of liver fibrosis, cell cycle arrest and apoptosis of activated HSCs play an important role [11, 12, 100]. Fan et al. [11, 12] found that NaHS induced a significant increase in the percentage of cells in the G1 phase, with a corresponding decrease in the percentage of cells in the S phase, indicating that NaHS inhibited HSC proliferation by inducing G1 phase arrest. Meanwhile, they discovered that Fe-NTA treatment increased the apoptotic rate of HSC-T6 cells, but NaHS administration led to two contradictory results. In the early stage of hepatic fibrosis, oxidative stress contributed to the activation and transformation of quiescent HSCs and simultaneously promoted the proapoptotic activity of HSCs. And during this period, NaHS might inhibit apoptosis through antioxidant effect, with decreased phospho-p38 and increased phospho-Akt proteins [12, 35]. On the contrary, NaHS treatment resulted in a significantly higher apoptotic rate in HSC-T6 cells treated with Fe-NTA in the later stage, which might be due to the increased oxidative stress [11, 12]. The above two phenomena are consistent with a previous study, which has shown that proapoptotic or antiapoptotic activity depends on the stage of fibrosis [100].

H_2S propels fibroblasts towards apoptosis by inducing DNA damage and cell cycle arrest at G0/G1 phase, as well as stimulating apoptosis-related factors including p53, p21, ku70, ku8, Bax, and cytochrome c [11, 34]. On the other hand, H_2S reduces parenchymal cells apoptosis by inhibiting TNF-α-mediated proapoptotic signaling pathway, regulating the MMP/TIMP axis, and elevating the ratio of

antiapoptotic factors [insulin like growth factor-I (IGF-I), Bcl-2] to proapoptotic factors [Fas ligand (Fas-L), Bax, caspases, and cytochrome c], thus eventually leading to resistance to fibrosis [14, 17, 52, 61, 64, 66].

3.5. H_2S and Angiogenesis. As sensitive to anoxia and ischemia, the heart is the most liable to undergo dysfunction or failure after myocardial ischemia or infarction, accompanied by structural abnormalities including cardiomyocyte loss and myocardial fibrotic remodeling in peri-infarct and infarct area [18, 101]. Angiogenesis, referring to the spontaneous blood vessel formation and/or the growth of new blood vessels from preexisting vessels, is a complex biological process characterized by ECM remodeling as well as endothelial cell growth, migration, and assembly into capillary structures [2, 74]. As angiogenesis promotes the delivery of both oxygen and energy substrates for tissue repair after injury, it is vital for various physiological or pathological events, like normal growth and development, wound healing, or repair after myocardial infarction [2, 102]. Interestingly, previous studies have revealed that H_2S protects against fibrosis by a proangiogenic effect [62, 74, 103]. In different mouse models of cardiac fibrosis induced by myocardial infarction or pressure overload-mediated heart failure, exogenous H_2S supplement upregulated the expression of vascular endothelial growth factor (VEGF, a proangiogenic factor) and its receptors including tyrosine kinase receptor (flk-1) and fms-like tyrosine kinase (flt-1) as well as the phosphorylation of endothelial NO synthase (eNOS) and the bioavailability of NO but downregulated the expression of antiangiogenic factors such as endostatin, angiostatin, and parstatin, accompanied by augmented vascular density and reduced intermuscular and perivascular fibrosis in heart tissues [62, 74]. The proangiogenic effect of H_2S is associated with the proliferation and migration of endothelial cell through the activation of several cellular signaling pathways including the PI-3 K/Akt, the MAPKs (e.g., ERK1/2 and p38), ATP-sensitive potassium (K_{ATP}) channels, and VEGF-eNOS-NO pathway [74, 103]. Although exogenous H_2S inhibits the cardiac fibrosis partially by proangiogenic effect, no much evidence is from lung, liver, and kidney fibrosis.

These protective mechanisms of hydrogen sulfide in the development of fibrosis have been also depicted in Figure 1.

4. Concluding Remarks

In this review, we have demonstrated that endogenous or exogenous H_2S at a physiological or relatively low concentration plays a protective role in the development of fibrosis in lung, liver, kidney, and heart, with its antioxidant, antiapoptotic, anti-inflammatory, proangiogenic properties and its inhibitory effect on fibroblasts activation. These results imply that the endogenous H_2S-producing enzymes and H_2S pathway might be a potential therapeutic target for fibrosis. However, we still have a long way to go before a complete understanding of the physical functions of H_2S in fibrosis pathogenesis. The following issues serve only as examples.

(i) The degree in endogenous H_2S-producing enzymes and H_2S was related to the severity of fibrosis. Although it will be interesting to investigate further whether endogenous H_2S could be a promising biomarker for fibrosis, it appears to be premature to correlate the measured blood/tissue levels of H_2S with the severity of fibrosis or the related biological outcomes after H_2S treatment, as long as the real or exact blood and tissue H_2S concentrations are still unknown and/or undetectable due to the lack of appropriate techniques.

(ii) As fibrosis can occur in many tissues and organs with a similar pathogenesis, it is reasonable to speculate that endogenous H_2S-producing enzymes and H_2S would play an important role in all kinds of fibrosis in the body like pancreatic fibrosis and skin fibrosis.

(iii) Supplementation with exogenous H_2S such as NaHS not only directly increases H_2S levels in the body, but also keeps, restores, or even promotes the expression of endogenous H_2S-producing enzymes like CSE and CBS to produce endogenous H_2S [15, 18, 19, 50, 51, 60–62, 66]. This positive feedback mechanism is largely unknown and merits further investigation.

(iv) Although H_2S mitigates the fibrosis development through its antioxidant, antiapoptotic, and anti-inflammatory properties as mentioned above, the exact molecular mechanism is still unknown. Mustafa et al. [104] initially found that H_2S physiologically modified some proteins' activation by S-sulfhydration. Similar to methylation or acetylation, S-sulfhydration is now recognized as another mode of posttranslational modification. Recent studies found H_2S can induce Keap1 s-sulfhydration, promote Nrf2 dissociation from Keap1, enhance Nrf2 nuclear translocation, and eventually stimulate mRNA expression of Nrf2-targeted downstream genes such as glutamate-cysteine ligase and GSH reductase to protect against oxidative stress-induced cellular senescence and ischemia/reperfusion injury [105, 106]. Given the important role of Nrf2 in oxidative stress and the development of fibrosis [28], it is reasonable to suspect that H_2S might modify the Keap1/Nrf2 pathway by S-sulfhydration to protect against fibrosis development.

(v) Recent research showed organ fibrosis is related to the decrease of autophagy [107, 108]. Impairment of autophagy by TGF-β1 or IL-17A promoted fibrogenesis in pulmonary fibrosis [107, 108], while autophagy activation via IL-17A blockage decreased the production of collagen, attenuated fibrosis, and increased survival in the murine model of bleomycin-induced fibrosis [108]. Autophagy plays a complex regulatory pathway in liver fibrosis, with profibrogenic effects relying on the activation of hepatic stellate cells, but with antifibrogenic properties via indirect hepatoprotective and anti-inflammatory properties, as also seen in kidney fibrosis [109–111]. These studies

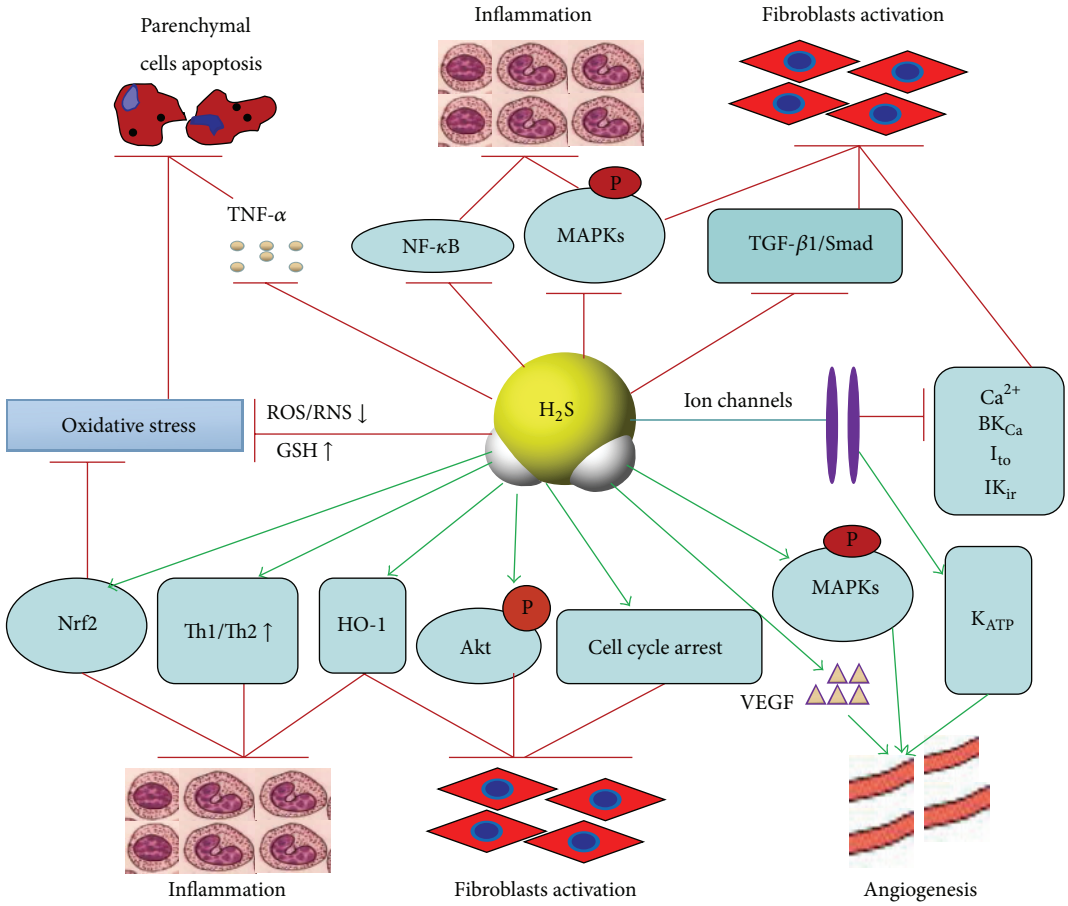

Figure 1: The main mechanisms of H_2S-mediated protection against fibrosis development. H_2S plays a complex role in the development of fibrosis. Besides as a reductant to directly scavenge oxygen free radicals, H_2S exerts its inhibitory effect on fibrosis by anti-inflammation, selectively anti- or proapoptosis, proangiogenic effect, and suppression of fibroblasts activation. Many signaling pathways such as NF-κB, Akt, MAPKs, TGF-β1/Smad, and HO-1 and the activity of calcium and potassium channels are involved in the process of antifibrosis of H_2S. The excitatory effects are denoted by the lines with arrow ends, and the inhibitory effects are indicated by the lines with bar ends. ROS/RNS: reactive oxygen and nitrogen species; GSH: glutathione; TNF-α: tumor necrosis factor-α; NF-κB: nuclear factor-kappa B; Akt: protein kinase B; MAPKs: mitogen-activated protein kinases; TGF-β1: transforming growth factor beta-1; Nrf2: nuclear factor E2-related factor; HO-1: heme oxygenase 1; VEGF: vascular endothelial growth factor; K_{ATP}: ATP-sensitive potassium channels.

imply that autophagy might involve the pathogenesis of fibrosis. Interestingly, H_2S can play its biologic roles via autophagy regulation. Exogenous H_2S can induce/enhance autophagy to inhibit the proliferation of colon epithelial cells or reduce hyperglycemia-induced matrix remodeling by glomerular endothelial cells via signaling pathways of AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) [112, 113]. In other conditions, exogenous H_2S can also suppress excessive activation of autophagy to protect against cigarette smoking-induced left ventricular systolic dysfunction [114] and alleviate traumatic brain injury or ischemia-reperfusion injury [115, 116]. Given the important role of autophagy in the pathogenesis of fibrosis and the regulatory function of H_2S for autophagy and fibrosis, it is reasonable and interesting to hypothesize that exogenous or endogenous H_2S might inhibit the development of fibrosis via targeting with autophagy or autophagy-associated signaling pathways.

(vi) The protective role of endogenous or exogenous H_2S in fibrosis pathogenesis is convincing from various animal models, but much remains unknown of its role in the pathogenesis of human fibrosis. Further clinical studies are needed to translate this potential to clinical use.

Conflict of Interests

The authors have declared no conflict of interests regarding the publication of this paper.

Authors' Contribution

Shufang Zhang and Chuli Pan contributed to the acquisition and analysis of the data and the initial draft writing of

this paper. Feifei Zhou, Zhi Yuan, and Wei Cui contributed to the collection and interpretation of data. Huiying Wang contributed to the collection of the revised paper. Gensheng Zhang contributed to the concept of the review, the revision of this paper, and the final approval of the version to be published. Shufang Zhang and Chuli Pan contributed equally to this work.

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Review Article

Physiological Implications of Hydrogen Sulfide in Plants: Pleasant Exploration behind Its Unpleasant Odour

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Recently, overwhelming evidence has proven that hydrogen sulfide (H_2S), which was identified as a gasotransmitter in animals, plays important roles in diverse physiological processes in plants as well. With the discovery and systematic classification of the enzymes producing H_2S *in vivo*, a better understanding of the mechanisms by which H_2S influences plant responses to various stimuli was reached. There are many functions of H_2S, including the modulation of defense responses and plant growth and development, as well as the regulation of senescence and maturation. Additionally, mounting evidence indicates that H_2S signaling interacts with plant hormones, hydrogen peroxide, nitric oxide, carbon monoxide, and other molecules in signaling pathways.

1. Introduction

Hydrogen sulfide (H_2S) is a colorless, flammable gas with the characteristic odor of rotten eggs. It was widely considered to be just a toxic gas for nearly 300 years mostly due to its unpleasant smell. The breakthrough in the effort to link endogenous H_2S levels and functional changes came when the possible role of H_2S as an endogenous neuromodulator in the brain was reported [1]. The focus on enzymes generating H_2S was another breakthrough in 2001 [2]. The initial work concluded that H_2S was a physiological vasodilator and regulator of blood pressure, which stimulated research on H_2S physiology [3]. In plants, H_2S has been revealed as a crucial player in the regulation of normal plant physiological processes, including seed germination, root morphogenesis, photosynthesis, and flower senescence [4–8]. It was also shown to be an important messenger in plant defense signaling against various abiotic stresses at physiological concentrations [9–13]. In this review, we discuss recent progress that increases our understanding of H_2S synthesis and signaling functions in plants.

2. H_2S Synthesis

In mammalian cells, H_2S is physiologically generated by pyridoxal-5′-phosphate-dependent enzymes, including cystathionine beta-synthase, cystathionine gamma-lyase, and 3-mercaptopyruvate sulfurtransferase (3-MST), during cysteine (Cys) metabolism [3, 14]. H_2S is generated in plants via both enzymatic and nonenzymatic pathways, although the latter only accounts for a small portion of H_2S production. Figure 1, with the enzymes highlighted, demonstrates the production of H_2S in *Arabidopsis thaliana*.

Several candidate Cys-degrading enzymes have been reported to exist in different plant species (shown in Table 1). In the model plant *A. thaliana*, the enzymes that produce H_2S can be roughly divided into two categories. One class of these enzymes is Cys desulfhydrases (CDes), which degrade Cys into H_2S, ammonia, and pyruvate in a stoichiometric ratio of 1 : 1 : 1 and require pyridoxal 5′-phosphate as a cofactor [15]. L-Cys desulfhydrase is one of the enzymes that decompose L-Cys and was first discovered in the sulfur metabolism of tobacco cultured cells [16]. D-Cys desulfhydrase 1 specifically uses D-Cys as its substrate, and D-Cys desulfhydrase 2

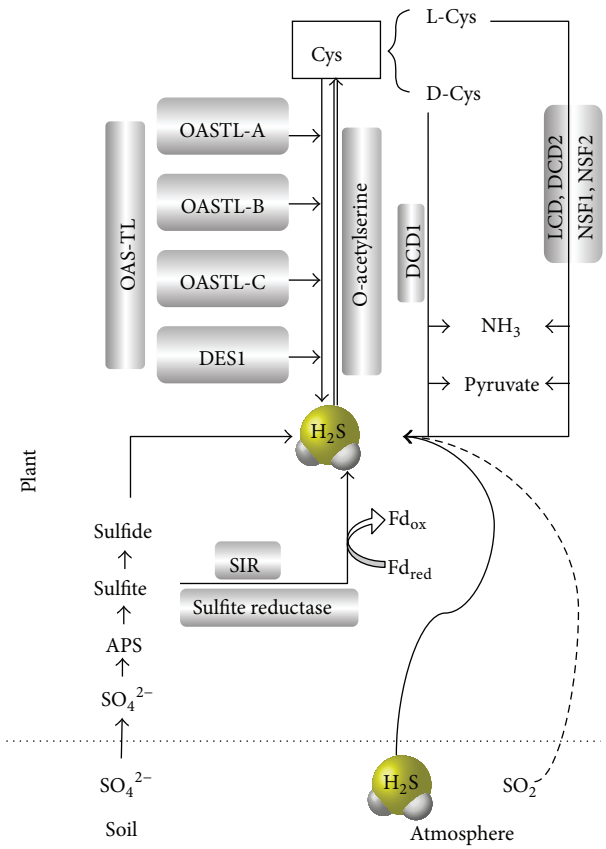

Figure 1: An overview of H_2S generation in higher plants (adapted from Papenbrock et al., 2007). APS: adenosine 5′-phosphosulfate; Fd_{red}, Fd_{ox}: reduced and oxidized ferredoxin; SIR: sulfite reductase.

degrades L/D-Cys simultaneously [17, 18]. The production of H_2S by CDes has been confirmed in various areas of biology [9, 11, 14, 15, 19, 20]. CDes are Cys desulfhydrases with singular functions in desulfuration. Their mRNA levels were significantly higher in the stems and cauline leaves than in the roots, rosette leaves, and flowers of *A. thaliana* [9].

Another class of the enzymes is O-acetyl-L-serine (thiol) lyase (OAS-TL), which is responsible for the incorporation of inorganic S into Cys, and free H_2S appears to be released only in a minor reaction [21]. During an incubation period, the enzyme formed about 25 times more Cys than H_2S, in a molar ratio, per mg protein [22]. Nine OAS-TL genes have been identified in *A. thaliana*, which are located in the cytosol, mitochondria, or plastid [23]. Recently, DES1 was reported as a frequent novel L-Cys desulfhydrase, which, based on sequence feature alignments, belongs to the OAS-TL family [24–28]. The *Km* value for L-Cys in the DES1 reaction is 13-fold lower than that for OAS in the OAS-TL reaction, indicating a much higher affinity of DES1 for L-Cys as a substrate [2]. The biochemical characterization of the T-DNA insertion mutant *des1* reveals that the total intracellular Cys concentration increased by approximately 25% [28]. However, as a member of the OAS-TL family, its function in synthesizing H_2S has not been clearly studied. *In vitro*, the reaction of OAS-TL is a net H_2S-consuming reaction [22].

Table 1: Enzymes and coding genes related to H_2S generation in *Arabidopsis*.

Substrate	Enzyme	Cellular localization	Locus	Reference
L-Cys	AtLCD	Cytoplasm	At3g62130	[16]
	AtNFS1/Nifs1	Mitochondria	At5g65720	[27]
	AtNFS2/Nifs2	Plastid	At1g08490	[28]
	DES1	Cytoplasm	At5g28030	[24]
	OASTL-A1	Cytoplasm	At4g14880	[25]
	OASTL-A2	Cytoplasm	At3g22460	[30]
	OASTL-B	Plastid	At2g43750	[26]
	OASTL-C	Mitochondria	At3g59760	[27]
D-Cys	AtDCD1	Mitochondria	At1g48420	[17]
L/D-Cys	AtDCD2	Mitochondria	At3g26115	[18]
Unknown	PLP-dependent transferase superfamily	Chloroplast	At5g26600	[15]
Unknown	NAC domain containing protein 1	Unknown	At1g01010	[15]

At: *Arabidopsis thaliana*, Cys: cysteine, DCD: D-Cys desulfhydrase, DES: desulfhydrase, LCD: L-Cys desulfhydrase, NAC: N-acetyl-L-cysteine, NFS: nitrogenase Fe-S cluster, OASTL: O-acetyl-L-serine(thiol)lyase, and PLP: pyridoxal 5′-phosphate.

Thus, the statement that DES1 is the only enzyme involving in the degradation of Cys is open to question [24, 28, 29].

In addition, Nifs/NFS, with L-Cys desulfhydrase-like activity, is also potentially involved in H_2S production [31, 32]. Two genes, At5g26600 and At1g01010, in *A. thaliana* have been identified that encode proteins with CDes structural features [15], and 3-MST is also related to H_2S production in plants [33].

3. Physiological Functions of H_2S in Plants

H_2S has been reported to play important roles in diverse physiological processes in plants. Research on the endogenous H_2S of higher plants can be traced back to 1978, when H_2S was observed to be released from leaves of cucumber, corn, and soybean [34]. Leaves of older plants contain higher H_2S concentrations than younger plants [35]. A recent study showed that the mRNA levels of *CDes* were gradually elevated in a developmental stage-dependent manner [9]. The importance of H_2S in the regulation of plant growth, development, and senescence has emerged.

The improvement in seed germination rates due to exogenous H_2S treatments was confirmed. H_2S or HS^-, rather than other sulfur-containing components derived from the exogenous H_2S donor, NaHS, contributed to the promotion of seed germination [4]. NaHS preferentially affects the activity of endosperm β-amylase and maintains lower levels of malondialdehyde and hydrogen peroxide (H_2O_2) in germinating seeds [7]. In addition, the application of NaHS to seedling cuttings of sweet potato promoted the number and length of adventitious roots [5]. At the same time, H_2S

modulates the expression of genes involved in photosynthesis and thiol redox modification to regulate its photosynthesis [36]. It is hypothesized that an increase in the stomatal density also contributes to this process [37]. The osmotic-induced decrease in the chlorophyll concentration could be alleviated by spraying the NaHS solution [6]. H_2S was also found to delay flower opening and senescence in cut flowers and branches [8]. These effects occur in a dose-dependent manner. In the cytosol, H_2S negatively regulates autophagy and modulates the transcriptional profile of *A. thaliana* using *des1* [38]. H_2S strongly affects plant metabolism at most stages of life and causes statistically significant increases in biomass, including higher fruit yields [39].

H_2S also plays pivotal roles in plant responses or adaptation under biotic and abiotic stress conditions. Early studies concerning H_2S emissions in plants were associated with plant responses to pathogens as part of sulfur-induced resistance [40]. In 2008, H_2S was found to be an important cellular signal for the first time, highlighting the protective effect of H_2S against copper stress [4]. Thereafter, a stream of publications on various positive effects of H_2S and H_2S signaling in plants emerged. Soon, H_2S was shown to alleviate the effects of aluminum, cadmium, chromium and boron toxicity, drought and osmotic stress, heat stress, hypoxia, and other stresses [9, 11–13, 20, 41–43]. Most of these reports discussed, as analogies with animal systems, how H_2S signaling is important for plant protection against stress.

Stomatal movement is very important in plant responses to environmental stimuli, and a key target of H_2S signaling in plants is the specialized guard cell. Recent studies have reported that H_2S is responsible for drought stress relief by inducing stomatal closure in *A. thaliana* [9, 20]. These observations are consistent with a previous report in both *Vicia faba* and *Impatiens walleriana* [30]. Similarly, H_2S was confirmed to be a novel downstream indicator of nitric oxide (NO) during ethylene-induced stomatal closure [44]. However, the effect of H_2S on stomatal movement has been a controversial topic. Another research group reported that exogenous H_2S induced stomatal opening by reducing the accumulation of NO in guard cells of *A. thaliana* and a crop plant, *Capsicum annuum* [45, 46]. The reasons for these different observations are not clear and require further study. The difference may simply be due to the different experimental materials and methods. The purpose of stomatal closure is to reduce the moisture loss under drought stress, and the induction of stomatal opening is to enhance photosynthesis and reduce the photorespiration.

4. Cross-talk of H_2S with Other Signals

Plants perceive and respond to H_2S, but studies on the mechanisms of H_2S functioning in plant responses to stress are very limited. An overview of our current understanding of plant H_2S signaling is shown in Figure 2. H_2S is particularly active and may interact with and modify numerous other signals. Thus, there may be multiple routes of H_2S perception and signaling to be unraveled.

Several lines of evidence point to an interrelationship between H_2S and plant hormones in plant defenses. Abscisic acid (ABA) is produced in large amounts in plants under various abiotic stresses. Under drought stress, the expression of *CDes* was significantly upregulated, and the production rate of H_2S from these plants also increased [9]. Subsequently, the relationship between H_2S and ABA was reported based on a deficiency of H_2S in the *lcd* mutant that had a weakened ABA induction of stomatal closure, which indicated that the induction of stomatal closure by ABA was partially dependent on H_2S. As H_2S was also involved in the expression regulation of ion-channel genes, H_2S may be a critical component of ABA-induced stomatal closure via ion channels. At the same time, H_2S influenced the expression of ABA receptors, and the influence of H_2S may have begun upstream of the ABA signaling pathway. Therefore, the above results showed that H_2S interacted with ABA in the stomatal regulation responsible for drought stress in *A. thaliana* [20]. Indole acetic acid (IAA) showed a rapid increase in different plants treated by exogenous H_2S [5], and ethylene (Eth) could induce H_2S generation [44]. In addition, gibberellic acid (GA) and jasmonic acid (JA) were also involved in the H_2S signal transduction process. H_2S can alleviate the GA-induced programmed cell death in wheat aleurone cells [47], and H_2S may function downstream of H_2O_2 in JA-induced stomatal closure in *V. faba* [48].

H_2O_2 is another signaling molecule in plants, especially in guard cells. Abiotic stress induces synthesis of both H_2S and H_2O_2; yet it is unclear how these two molecules work in concert in the physiological process. H_2S may represent a novel downstream component of the H_2O_2 signaling cascade during JA-induced stomatal movement in *V. faba* [48]. Pretreatment of H_2O_2 could improve the germination percentage of *Jatropha curcas* seeds, and this improvement was mediated by H_2S [49]. These results suggest that H_2O_2 is upstream of H_2S. However, there is plenty of evidence to the contrary. H_2S inhibited the cadmium influx through the plasma membrane calcium channels, which were activated by H_2O_2 [50]. H_2S can participate in enhancing plant resistance to abiotic stress via the improvement of antioxidant systems, such as heavy metal stress, osmotic stress, heat stress, and hypoxia stress [4–7, 10, 42, 43, 49].

Recent evidence suggests that H_2S also plays a role in the NO and carbon monoxide (CO) signaling pathway. In bermudagrass, sodium nitroprusside (SNP, a NO donor) and NaHS combined treatments showed that NO signaling could be blocked by H_2S inhibitors and scavengers, indicating that NO-activated H_2S was essential for the cadmium stress response [51]. Additional evidence showed that both NaHS and GYY4137 reduced the NO accumulation to a large extent in *A. thaliana* epidermal cells [45]. In sweet potato seedlings, a rapid increase in endogenous H_2S and NO was sequentially observed in shoot tips treated with NaHS. A similar phenomenon in H_2S donor-dependent root organogenesis was observed in both excised willow shoots and soybean seedlings. These results indicated that the process of H_2S-induced adventitious root formation was likely mediated by IAA and NO and that H_2S acts upstream in IAA and NO signaling transduction pathways [5]. Similarly, heme oxygenase 1 functions as a downstream component in H_2S-induced adventitious root formation by the modulation

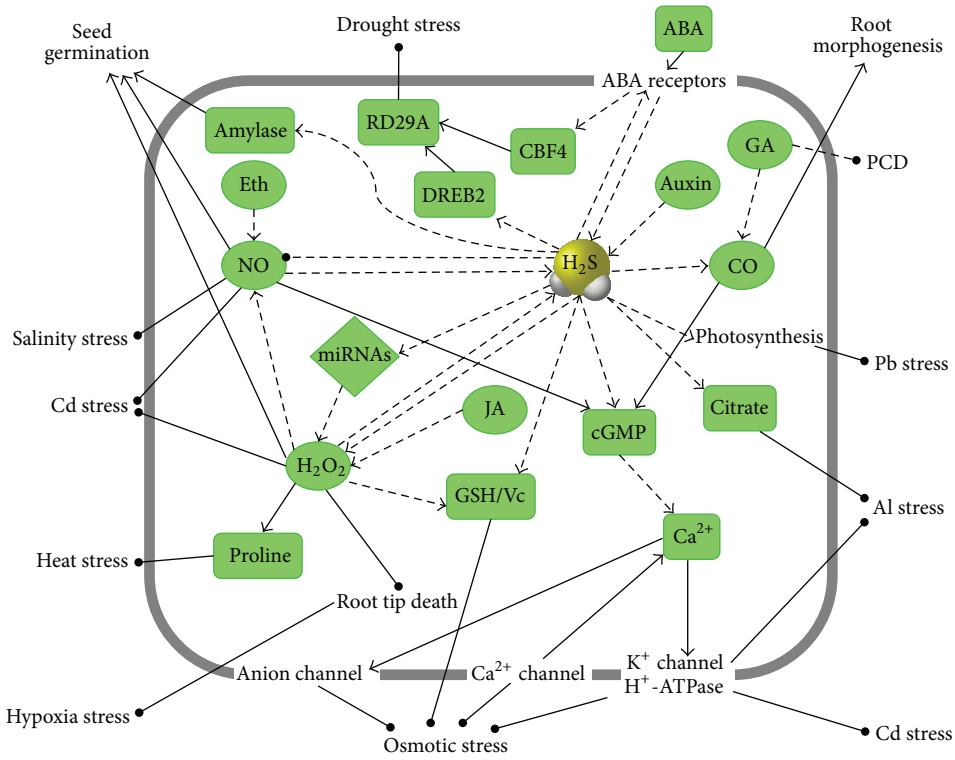

FIGURE 2: Generalized model of H_2S signaling in response to abiotic stress in plants. Solid line arrows depict stimulatory effect; dashed cents arrows represent the putative interaction; gray bold rectangle indicates cell membrane. ABA: abscisic acid; CBF: C-repeat binding factor; CO: carbon monoxide; DREB: dehydration responsive element; Eth: ethylene; GA: gibberellic acid; cGMP: cyclic guanosine monophosphate; GSH: glutathione; H_2O_2: hydrogen peroxide; H_2S: hydrogen sulfide; JA: jasmonic acid; NO: nitric oxide; PCD: programmed cell death; RD: responsive to desiccation.

of expression of related genes, which suggested that CO was involved in H2S-induced cucumber adventitious root formation [52].

Additionally, growing evidence suggests that H_2S signaling interacts with calcium (Ca) signaling pathways. Ca^{2+} confers structure and rigidity to the cell wall and regulates plant processes through calmodulin. Li et al. (2013) showed that NaHS pretreatment could improve the entry of extracellular Ca^{2+} into tobacco suspension cultured cells mediated by intracellular calmodulin to increase the heat tolerance [41]. At the level of transcription, the expression of Ca^{2+} channel coding genes decreased, whereas Ca^{2+}-ATPase and Ca^{2+}-H^+ cation antiporters were elevated in the *lcd* mutant. This was in accordance with stronger Ca^{2+} fluorescence in the wild type than in the *lcd* mutant [20]. These results suggest that Ca signaling plays an important role in the mechanism of H_2S.

Numerous studies showed that, during the enhancement of plant resistance, many substances changed simultaneously. H_2S plays an ameliorative role in protecting plants by increasing the proline content against aluminum toxicity and heat stress [10, 12, 41]. Aluminum-induced citrate secretion was also significantly enhanced by NaHS pretreatment [10]. During the NaHS preincubation period the grain β-amylase activity increased, improving seed germination [7].

5. Conclusions and Perspectives

The mechanisms by which H_2S is generated still remain unresolved, and elucidating how it is made by different plant cells under different conditions is clearly a research priority. H_2S is a key factor in the tolerance of cells to the oxidative stress induced by a range of abiotic conditions, including heavy metal toxicity, drought and osmotic stress, hot stress, hypoxia and other stresses. This probably involves the activation of antioxidant defenses, the induction of stomatal closure, and the enhanced expression of genes encoding resistance-associated enzymes. In these processes, plant hormones, H_2O_2, NO, CO, and Ca signaling participate in H_2S signal transduction, resulting in a complex signaling network.

There are numerous unanswered questions and important areas for further research, concentrated in the following areas. (1) Owing to the promiscuous chemical properties of H_2S, it is problematic to achieve adequate specificity and selectivity for its measurement. At present, the physiological H_2S level was measured by various techniques such as the methylene blue method, monobromobimane, gas chromatography, ion selective electrodes, and fluorescent probes [53]. The diverse detection methods resulted in magnitude differences in measured biological sulfide levels, which will certainly attract increasing attention. (2) The

mechanism of H_2S functions performed at the protein level. Until now, a great number of studies focused on protein S-sulfhydration, which is impossible to determine directly by chemical analyses. But in mammals, there have been many results indicating that this process might occur by the transition of intermediate links, such as positional changes and interactions with associated proteins. Moreover, if H_2S can thiolate proteins, it may have the same effect on DNA. (3) Even though H_2S is a short-lived molecule, it is an extremely active one. The mechanisms by which either H_2S or other molecules participating in H_2S signaling function are also important. Thus, elucidation of the H_2S complex signaling network is clearly a research priority.

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

Superoxide Mediates Depressive Effects Induced by Hydrogen Sulfide in Rostral Ventrolateral Medulla of Spontaneously Hypertensive Rats

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Hydrogen sulfide (H_2S) plays a crucial role in the regulation of blood pressure and oxidative stress. In the present study, we tested the hypothesis that H_2S exerts its cardiovascular effects by reducing oxidative stress via inhibition of NADPH oxidase activity in the rostral ventrolateral medulla (RVLM). We examined cell distributions of cystathionine-β-synthase (CBS) and effects of H_2S on reactive oxygen species (ROS) and mean arterial blood pressure (MAP) in spontaneously hypertensive rats (SHRs). We found that CBS was expressed in neurons of the RVLM, and the expression was lower in SHRs than in Wistar-Kyoto rats. Microinjection of NaHS (H_2S donor), S-adenosyl-l-methionine (SAM, a CBS agonist), or Apocynin (NADPH oxidase inhibitor) into the RVLM reduced the ROS level, NADPH oxidase activity, and MAP, whereas microinjection of hydroxylamine hydrochloride (HA, a CBS inhibitor) increased MAP. Furthermore, intracerebroventricular infusion of NaHS inhibited phosphorylation of $p47^{phox}$, a key step of NADPH oxidase activation. Since decreasing ROS level in the RVLM reduces MAP and heart rate and increasing H_2S reduces ROS production, we conclude that H_2S exerts an antihypertensive effect via suppressing ROS production. H_2S, as an antioxidant, may be a potential target for cardiovascular diseases.

1. Introduction

H_2S is an important gasotransmitter as are nitric oxide, carbon monoxide, and ammonium [1–4]. Endogenous H_2S is produced by three enzymes, cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase in conjunction with cysteine aminotransferase. In the brain, the production of H_2S is mainly catalyzed by CBS [5–7].

H_2S participates in the regulation of numerous physiological functions [8]. In the central nervous system (CNS), H_2S exerts important multifaceted neuromodulatory effects. Evidence highlights a crucial role of H_2S in the development of hypertension. For example, Yang et al. found that genetic deletion of CSE in mice resulted in hypertension [9]. Systemic administration of H_2S donors and precursors decreased mean arterial pressure (MAP) in various models of hypertension [10–13]. Nevertheless, mediation of H_2S in the cardiovascular center has been controversial [14–16].

The rostral ventrolateral medulla (RVLM), where sympathetic premotor neurons are located, is connected with other cardiovascular nuclei that regulate sympathetic nerve activity [17, 18]. Reactive oxygen species (ROS) in the RVLM plays a pivotal role in the pathogenesis of hypertension and heart failure [19–22]. Overproduction of $O_2^{\bullet-}$ and H_2O_2 contributes to hypertension by increasing sympathetic outflow to blood vessels [23–25]. Thus, upregulation of endogenous antioxidants is potentially an effective therapeutic strategy for cardiovascular diseases. A recent study indicates that neurons were protected by the antioxidant effect of H_2S [26].

However, its role in central cardiovascular mechanisms remains unclear. The present study was undertaken to assess the hypothesis that H_2S exerts antihypertensive effects by decreasing ROS production by inhibiting NADPH oxidase activity in the RVLM.

2. Materials and Methods

2.1. Animals and Agents. Male spontaneously hypertensive rats (SHRs), weighing 280–310 g, were supplied by the Experimental Animal Center of Department of Physiology and Pathophysiology, Shanghai Medical College, Fudan University. They were housed socially (3–5 per cage with food and water ad libitum) and kept on a 12-hour light/12-hour dark cycle. Studies were approved by the Ethics Committee of Experimental Research, Shanghai Medical College, Fudan University. NaHS, lucigenin, S-adenosyl-l-methionine (SAM), and hydroxylamine hydrochloride (HA) were purchased from Sigma. Apocynin (APO) was purchased from Calbiochem, and the antibodies (anti-CBS, anti-MAP-2, anti-GFAP, and $p47^{phox}$ antibodies) were purchased from Jackson and Abcom. BCA kits were purchased from Beyotime.

2.2. Immunofluorescence Staining and Laser Confocal Microscopy. Rats were anaesthetized with chloral hydrate (300 mg/kg ip) and then transcardially perfused with 150 mL saline followed by 250 mL 4% paraformaldehyde in 0.1 M sodium phosphate buffer (0.1 M PB; pH 7.4). Brains were rapidly dissected and postfixed in the same fixative solution at 4°C for 6 h and then transferred sequentially into 20 and 30% sucrose in 0.1 M PB for cryoprotection. Transverse serial medullary sections (30 μm thick) were cut with a microtome (Reichert-Jung) 1.5–1.7 mm rostral to the obex according to Paxinos and Watson's atlas. Sections were immersed in 4% paraformaldehyde for 10 min followed by 6 × 5 min washing in 0.01 M phosphate-buffered saline (0.01 M PBS; pH 7.4). Free floating sections were incubated in 2% BSA and 0.2% Triton X-100 in 0.01 M PBS for 30 min at 37°C to eliminate nonspecific staining, and they were then exposed to antibodies for 1 h at 37°C, plus an additional 24 h at 4°C for the first primary antibody. The sections were then washed three times in TBST and incubated with fluorescent secondary antibodies for 60 min for confocal microscopy (Zeiss LSM510, Jena, Germany).

2.3. Microinjection into the RVLM. Rats were anesthetized with a mixture of urethane (700 mg/kg) and α-chloralose (35 mg/kg) and intubated to facilitate ventilation. The left femoral artery was cannulated to monitor blood pressure and heart rate. Body temperature was maintained between 37°C and 37.5°C during the experiment with a temperature-controlled table. Then, rats were mounted in a stereotaxic frame and a micropipette tip (outer diameter 10–30 μm) was inserted into the RVLM [27] for microinjection (1.8 to 2.1 mm lateral to the midline, 2.6 to 3.3 mm caudal to interaural line, and 0.3 to 0.9 mm from the ventral surface). Injection sites were confirmed histologically.

2.4. Intracerebroventricular Infusion. A lateral ventricular cannula was implanted after the general surgical procedures. Anesthetized rats were placed in a stereotaxic frame and a small hole was made in the skull (1.2–1.4 mm lateral to midline and 0.8–1.0 mm posterior to bregma). A 10 mm stainless steel guide cannula (22 gauges) was lowered 4 mm below the surface of the skull and fixed with cranioplastic cement. A stainless steel injector was introduced through the guide cannula to 0.5 mm beyond its tip.

2.5. Measurement of Superoxide Production. The lucigenin-enhanced chemiluminescence assay was used to determine superoxide production as previously described [28]. After infusion of various agents, the ventrolateral medulla was removed and homogenized in a 0.02 mol/L phosphate-buffered saline (PBS), pH 7.4, containing 0.01 mM EDTA. The homogenate was centrifuged at 1000 g for 10 min at 4°C to remove nuclei and cell debris. Supernatant was obtained immediately for $O_2^{\bullet-}$ measurement. Background chemiluminescence was used for assessing $O_2^{\bullet-}$. An aliquot of supernatant (100 μL) was then added to buffer (2 mL) containing lucigenin (5 μmol/L) and measured for chemiluminescence. $O_2^{\bullet-}$ production was calculated and expressed as mean light unit per mg protein.

2.6. Measurement of NADPH Oxidase Activity. NADPH oxidase activity in the ventrolateral medulla was determined by a luminescence assay. The preparation was identical to that for $O_2^{\bullet-}$. The luminescent assay was performed in PBS buffer containing 0.01 M/L EGTA and 5 μM/L lucigenin as the electron acceptor and 100 mM/L NADPH as the substrate. After dark adaptation, background counts were recorded and a tissue homogenate (1 μL protein sample) was added. The chemiluminescence value was recorded at 1 min intervals for 30 min. $O_2^{\bullet-}$ production was measured after addition of NADPH to the incubation medium with and without a flavoprotein inhibitor of NADPH oxidase, Apocynin.

2.7. Western Blot Analysis. After having been anesthetized, rat medullas were rapidly removed and frozen immediately in liquid nitrogen until being homogenized in cell lysis buffer, followed by centrifugation at 12000 g for 15 min at 4°C. The supernatant was obtained for protein concentration. Then, protein samples were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking at room temperature in 5% BSA for 1 h, the membrane was incubated with various primary antibodies at 4°C overnight and then washed three times in TBST buffer and incubated with 1 : 5000 dilutions of anti-mouse IgG. Visualization was made with an enhanced chemiluminescent kit. Band densities on Western blot were quantified with β-actin as internal control.

2.8. Statistical Analysis. Data were analyzed with statistical software SigmaSTat (SPSS 17.0) and expressed as the mean ± SEM. One-way ANOVA with repeated measures was used as appropriate to assess group means followed by the Bonferroni post hoc tests. Probability values of $P < 0.05$ were considered significant.

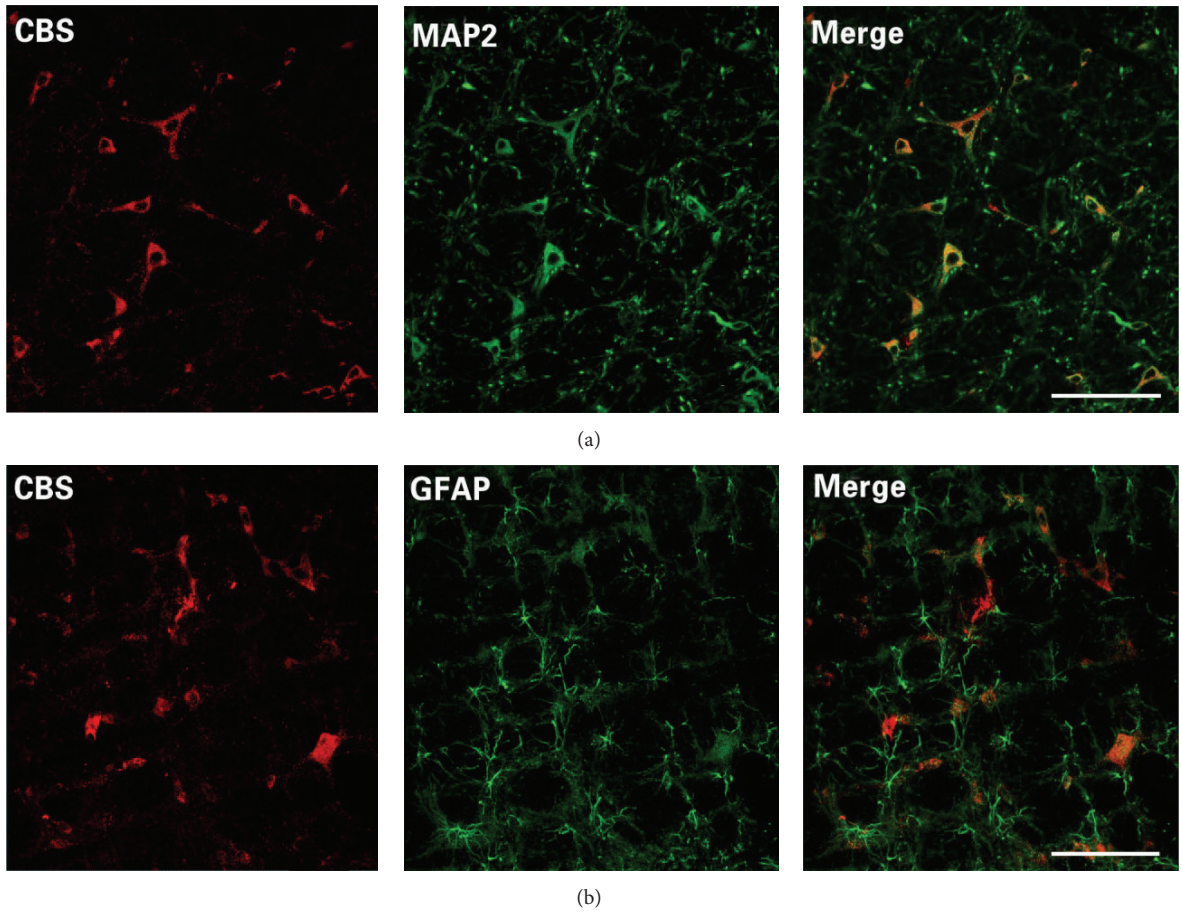

Figure 1: CBS expression in RVLM neurons. Confocal images showed that CBS immunoreactivity is colocalized with a neuronal marker (MAP2: upper panels) but not a glia marker (GFAP: lower panels).

3. Results

We studied H_2S induced antihypertensive effects in SHRs by examining molecular mechanisms involved in the RVLM from 4 different aspects.

3.1. Expression of CBS. Cellular distribution of CBS was identified by immunofluorescent stain coupled with laser confocal microscopy. CBS immunoreactivity was found in neuronal cells, but not in glia cells (Figure 1). CBS expressions in the RVLM were confirmed by Western blot assay, which were the same in SHRs and WKY rats at 8 weeks of age; however, expression was lower in SHRs at 17 weeks of age (Figure 2).

3.2. Effects of H_2S on MAP and HR. Microinjection of NaHS (400 pmol/0.1 μL) into the RVLM significantly decreased mean arterial blood pressure (MAP) and heart rate (HR) (Figure 3). Typically, MAP returned to baseline within 10–20 min. Similarly, microinjection of S-adenosyl-l-methionine (SAM, a CBS agonist, 10 pmol/0.1 μL) or Apocynin (APO, a NADPH oxidase inhibitor, 10 nmo/0.1 μL) decreased MAP. On the other hand, microinjection of hydroxylamine hydrochloride (HA, a CBS inhibitor, 9 nmol/0.1 μL) increased MAP (Figure 4). These results support a link between H_2S and ROS and provide novel evidence for regulation of hemodynamics by exogenous and endogenous H_2S in the RVLM.

3.3. Effect of H_2S on $O_2^{\bullet-}$ Production and NADPH Oxidase Activity. Microinjection of NaHS (400 pmol), SAM (10 pmol/0.1 μL), APO (10 nmol/0.1 μL), or Tempol (a SOD mimetic, 50 nmol/0.1 μL) decreased the level of superoxide anion ($O_2^{\bullet-}$) in the RVLM (Figure 5(a)). NADPH oxidase is a major enzyme for superoxide production in the brain. To determine whether the decrease of ROS results from inhibition of this enzyme, we assessed the activity of NADPH oxidase and found that microinjection of NaHS, SAM, and APO decreased NADPH oxidase activity significantly (Figure 5(b)).

3.4. Effect of H_2S on Phosphorylation of NADPH Oxidase. Phosphorylation of $p47^{phox}$ subunit is an important step for activation of NADPH oxidase. Thus, we examined the effect of intracerebroventricular infusion of NaHS on phosphorylation of $p47^{phox}$ serine residues. We found that NaHS significantly decreased serine phosphorylation of $p47^{phox}$ in

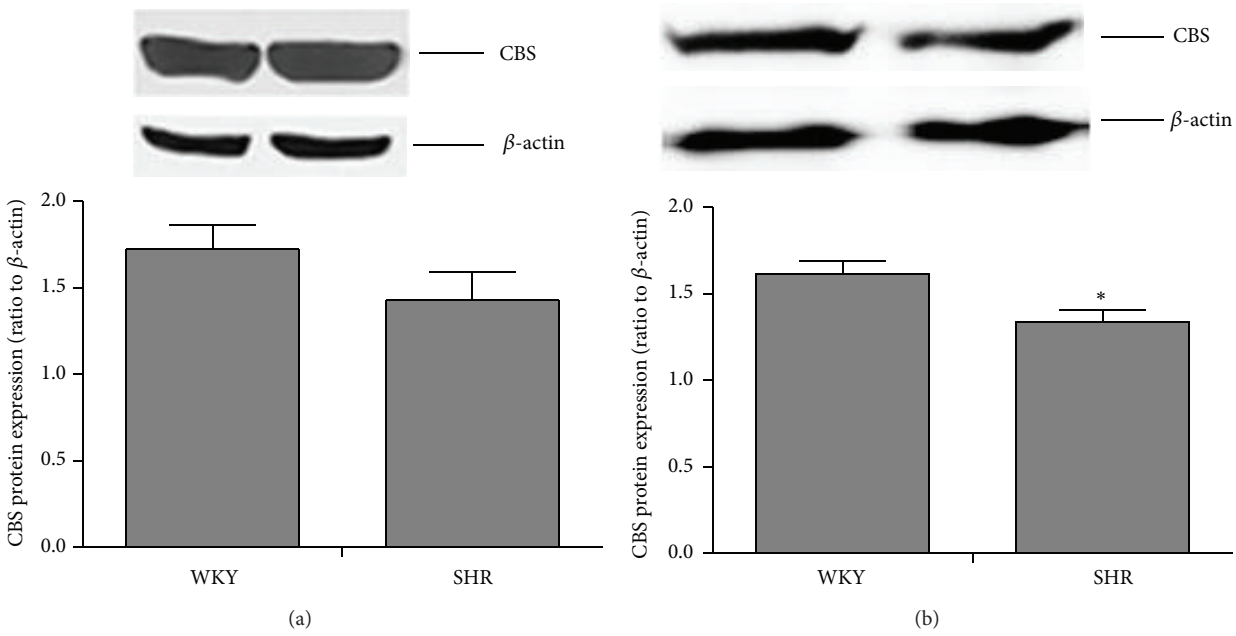

Figure 2: CBS is expressed less in the RVLM of hypertensive rats. CBS protein expression in WKY rats ($n = 5$) and SHR ($n = 6$) at 8 weeks (a) and 17 weeks (b). $^{*}P < 0.05$, SHR versus WKY. Please note that the difference in CBS expression occurred only at 17 weeks of age, when hypertension developed.

the RVLM (Figure 6), supporting that NaHS reduces production of superoxide via suppression of serine phosphorylation of $p47^{phox}$.

4. Discussion

Our results provide the first evidence demonstrating that NADPH oxidase derived superoxide mediates the antihypertensive effects of H_2S in the RVLM. Our statement is supported by the following 4 findings: (1) CBS was expressed in RVLM neurons, which provides an anatomical basis for the regulation; (2) increasing exogenous or endogenous H_2S in the RVLM decreased NADPH oxidase activity, superoxide anion, and MAP; (3) decreasing ROS produced the same depressive effects; (4) infusion of NaHS inhibited phosphorylation of $p47^{phox}$, a key step of NADPH oxidase activation.

H_2S can be produced endogenously in various parts of the body in the heart, kidney, liver, and CNS. CBS is significantly expressed in the CNS, especially in the hippocampus and cerebellum, as well as the cerebral cortex and brain stem [29]. CBS has been identified in astrocytes, microglia, and neurons [30–32]. However, its cellular distribution in the RVLM is unknown. Our data revealed that CBS proteins were expressed mainly in RVLM neurons, but not glial cells (Figure 1). Furthermore, the level of CBS proteins in the RVLM was lower in SHRs than in WKY rats (Figure 2), which is consistent with a recent report of intracerebroventricular infusion with NaHS [33]. It is interesting to note that the difference in CBS expression did not occur until hypertension developed.

Accumulating evidence highlights the crucial role of H_2S homeostasis in hypertension. A transient hypotensive effect was first reported in anesthetized rats with administration of H_2S donors [4]. The CSE-L-cysteine pathway was downregulated and H_2S was effective in reducing MAP and vascular remodeling in SHRs [12]. However, direct evidence for blood pressure control was reported in CES gene deficient mice [9]. Administration of H_2S donors and precursors decreases MAP in various hypertensive models (chronic inhibition of nitric oxide synthase, two-kidney-one-clip, and SHRs) [9–13]. The antihypertensive effect of H_2S has also been studied by infusion of NaHS into the RVLM cardiovascular center [15, 31]. The RVLM receives neuronal input from the paraventricular nucleus, solitary tracts nuclei, and so forth and then sends the signal to the spinal cord to regulate MAP and HR [17, 18]. Microinjection of NaHS (200, 400, and 800 pmol) into the RVLM decreases MAP, HR, and renal sympathetic nerve activity in a dose-dependent manner in SD rats [31]. Consistent with this study, our current results show that microinjection of NaHS (400 pmol) into the RVLM significantly decreased MAP and HR. Furthermore, we demonstrated that increased endogenous H_2S by microinjection of SAM (a CBS agonist) or decreased ROS by infusion of Apocynin produced the same depressive effects, while microinjection of HA (a CBS inhibitor) increased MAP, supporting that H_2S is a negative regulator for blood pressure in the RVLM.

Overproduction of ROS is critical for the pathogenesis of cardiovascular diseases, including hypertension and heart failure [21, 34, 35]. The baseline ROS, including $O_2^{\bullet-}$ and H_2O_2, in the RVLM is elevated in hypertensive animals [36, 37]. Elevated ROS in the brain increased MAP and sympathoexcitation, probably because of an upregulation of AT_1 receptor and NADPH oxidase [38, 39]. It has been

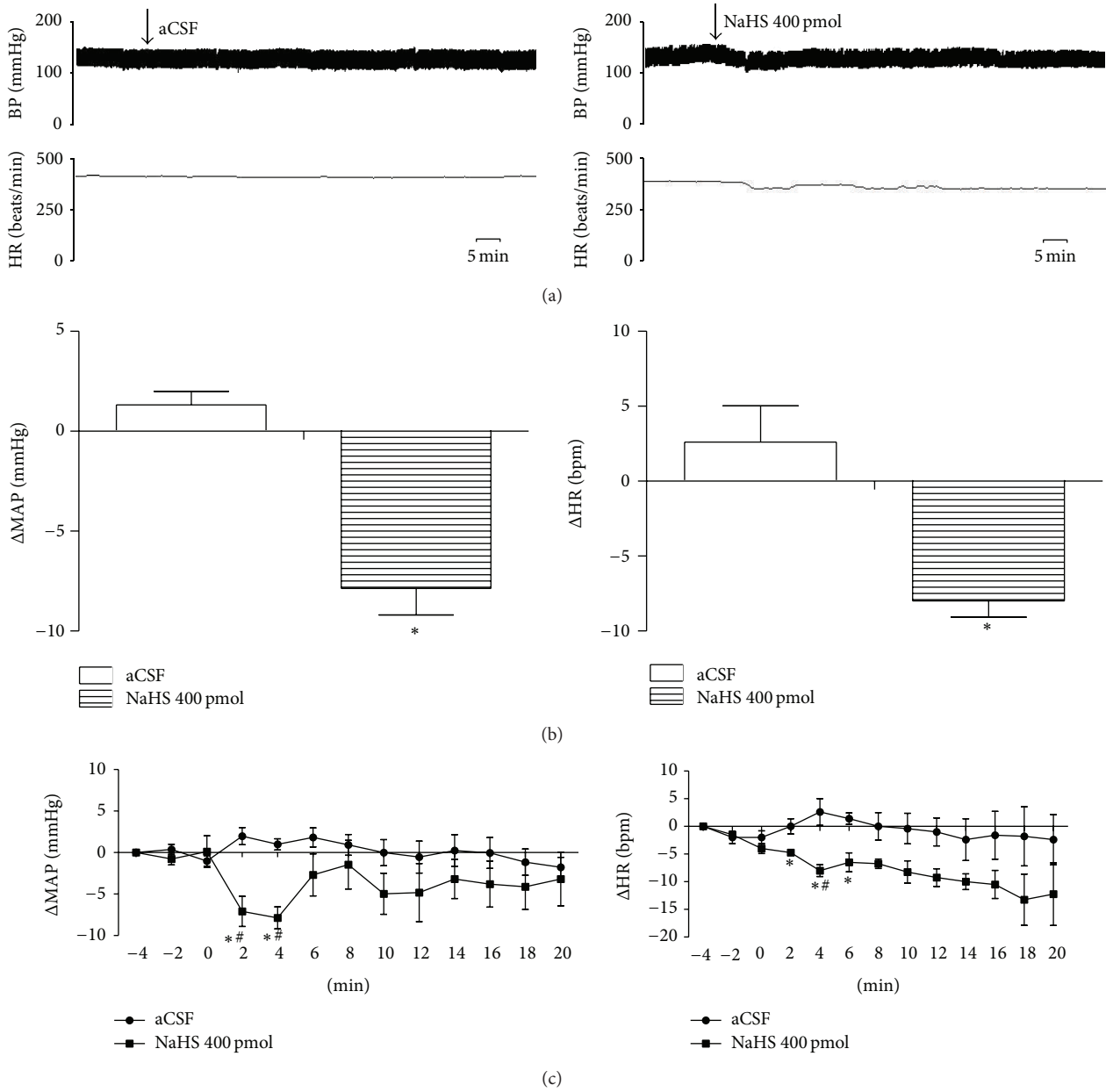

Figure 3: Microinjection of NaHS (400 pmol) into the RVLM decreased MAP and HR in SHRs. (a) Typical MAP and HR traces in response to the microinjection. (b) Maximal changes detected during the response. (c) Time courses of MAP and HR in response to microinjections of aCSF, artificial cerebral spinal fluid ($n = 5$), or NaHS ($n = 4$). $^{*}P < 0.05$ versus aCSF control group.

reported that NO exerts antihypertensive effects by inhibiting NADPH oxidase and thus reduces $O_2^{\bullet-}$ production [40–42]. Since H_2S also exerts an antihypertensive effect, we speculate that H_2S operates with the same mechanism. Indeed, exogenous (microinjection of NaHS) and endogenous (microinjection of SAM) H_2S decreased NADPH oxidase activity and $O_2^{\bullet-}$ production. Our hypothesis is further supported by the decreased $O_2^{\bullet-}$ with the addition of Apocynin (a NADPH oxidase inhibitor) or Tempol (a cell membrane-permeable SOD mimetic). It is worth noting that increasing H_2S by microinjection of NaHS or SAM decreased MAP and HR, while decreasing ROS by microinjection of Apocynin decreased MAP only. We speculate that H_2S may exert additional influence on HR through another mechanism. Further studies are needed to verify this plausibility.

ROS can be produced by xanthine oxidase, cytochrome P450, mitochondrial respiratory chain enzyme, or NADPH oxidase, which is the major enzyme for superoxide production in the brain. Its activation is initiated by serine phosphorylation of its cytosolic regulatory $p47^{phox}$ subunit [43, 44]. We found that NaHS infusion significantly decreased phosphorylated $p47^{phox}$ levels in the RVLM, which would decrease enzyme activity of NADPH oxidase and superoxide production. Furthermore, microinjection of Apocynin

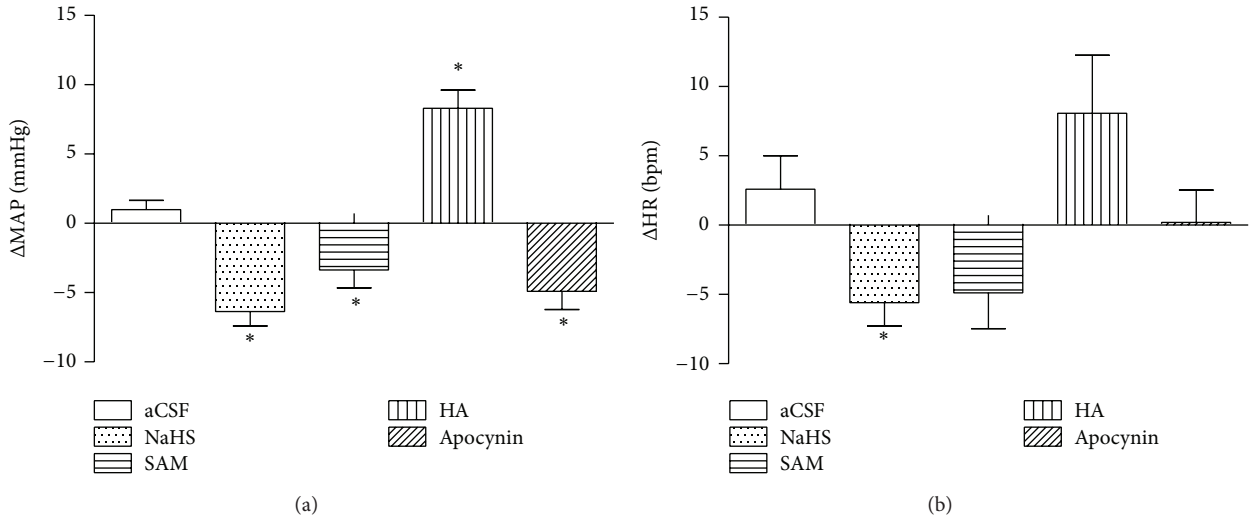

Figure 4: Maximal responses in MAP (a) and HR (b) to microinjections of different agents into the RVLM in SHRs. aCSF (control), $n = 5$; NaHS (H_2S donor), $n = 7$; SAM (a CBS agonist), $n = 5$; HA (a CBS inhibitor), $n = 5$; and APO (NADPH oxidase inhibitor), $n = 5$. $^*P < 0.05$ versus aCSF group.

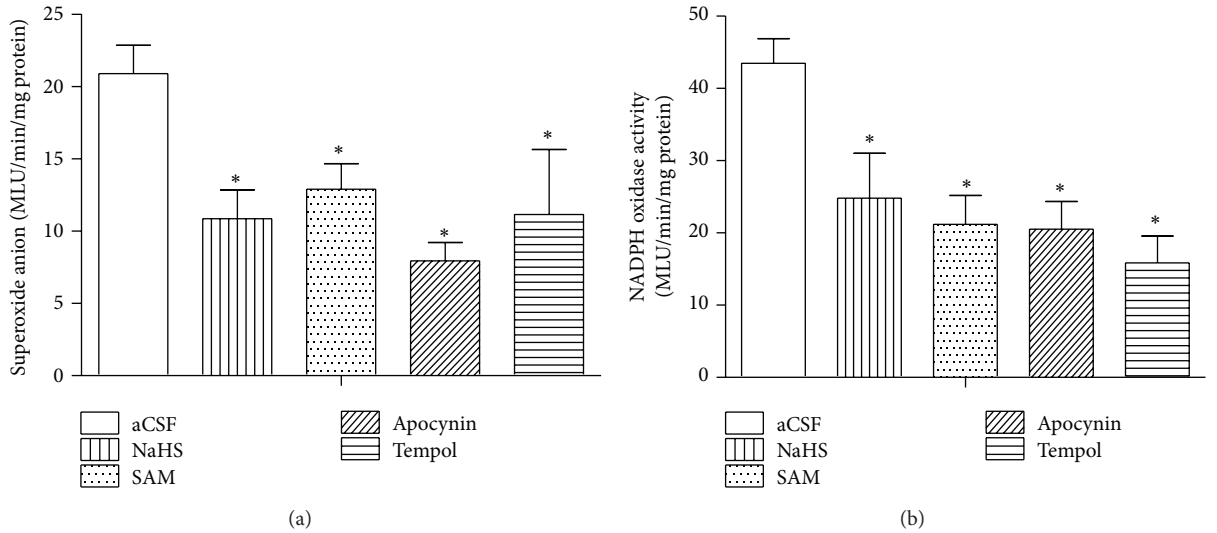

Figure 5: Infusion of various depressive agents suppressed NADPH oxidase activity and superoxide production in the RVLM of SHRs. Tissue levels of superoxide anion (a) and NADPH oxidase activity (b) after infusion of aCSF (artificial cerebral spinal fluid, $n = 9$), NaHS (H_2S donor, $n = 5$), Apocynin (NADPH oxidase inhibitor, $n = 5$), SAM (a CBS agonist, $n = 4$), or Tempol (SOD mimetic, $n = 4$). $^*P < 0.05$ versus aCSF group.

decreased blood pressure. Muzaffar et al. observed that H_2S downregulated NADPH oxidase and inhibited $O_2^{\bullet-}$ formation in pulmonary arterial endothelial cells, and this effect could be canceled by inhibitors of PKA, but not by inhibitors of PKG, indicating that the effect of H_2S on NADPH oxidase may be mediated by the adenylyl cyclase-cAMP-PKA pathway [45]. Taken together, our results suggest that NADPH oxidase-derived superoxide mediates H_2S induced central depressive effects. Since NADPH oxidase is composed of membrane-bound ($gp91^{phox}$ and $p22^{phox}$) and cytoplasmic ($p47^{phox}$, $p40^{phox}$, and $p67^{phox}$) subunits and small molecules (GTPase Rac1 and/or Rac2), the role of each component of the enzyme in the mediation requires further exploration.

In summary, present studies demonstrated that the H_2S metabolic system was present in the RVLM, and central administration of H_2S into the RVLM decreased phosphorylation of NADPH oxidase, NADPH oxidase activity, and $O_2^{\bullet-}$ production and reduced MAP and HR in SHRs, whereas decreasing H_2S by microinjection of a CBS antagonist increased MAP. Yet our data support that H_2S in the RVLM may decrease MAP mediated through NADPH oxidase, which is largely based on correlation, and a direct

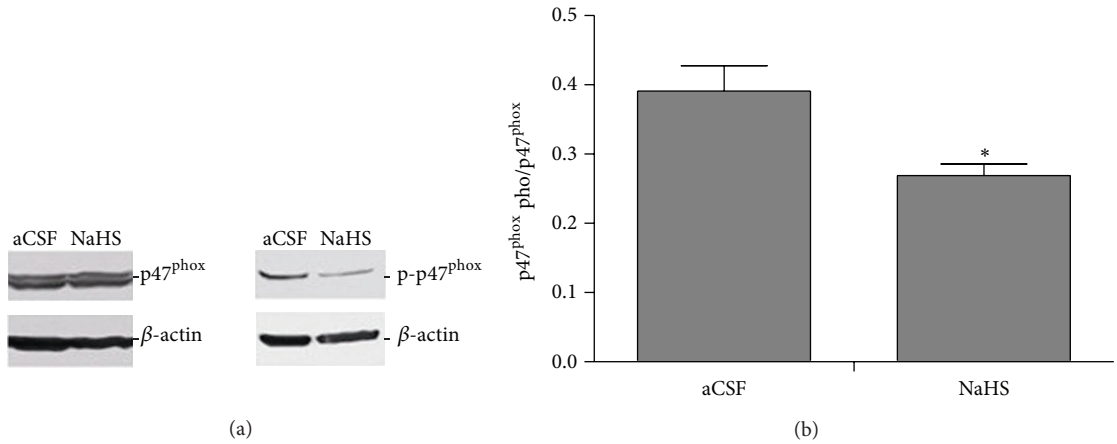

FIGURE 6: Exogenous H_2S suppressed $p47^{phox}$ phosphorylation of NADPH oxidase in the RVLM. Western blots show that $p47^{phox}$ phosphorylated/p-47 protein levels after intracerebroventricular infusion of aCSF or NaHS. Representative gel: (a) representative densitometric analysis and (b) group data ($n = 5$); $^*P < 0.05$ versus aCSF group.

mediation is not conclusive. Further studies are still needed. Nevertheless, since overproduction of superoxide in the CNS is involved in the etiology of hypertension, we expect that the H_2S-NADPH oxidase-superoxide system may be an effective therapeutic target in preventing hypertension.

Conflict of Interests

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

Acknowledgments

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Review Article

The Cardioprotective Effects of Hydrogen Sulfide in Heart Diseases: From Molecular Mechanisms to Therapeutic Potential

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Hydrogen sulfide (H_2S) is now recognized as a third gaseous mediator along with nitric oxide (NO) and carbon monoxide (CO), though it was originally considered as a malodorous and toxic gas. H_2S is produced endogenously from cysteine by three enzymes in mammalian tissues. An increasing body of evidence suggests the involvement of H_2S in different physiological and pathological processes. Recent studies have shown that H_2S has the potential to protect the heart against myocardial infarction, arrhythmia, hypertrophy, fibrosis, ischemia-reperfusion injury, and heart failure. Some mechanisms, such as antioxidative action, preservation of mitochondrial function, reduction of apoptosis, anti-inflammatory responses, angiogenic actions, regulation of ion channel, and interaction with NO, could be responsible for the cardioprotective effect of H_2S. Although several mechanisms have been identified, there is a need for further research to identify the specific molecular mechanism of cardioprotection in different cardiac diseases. Therefore, insight into the molecular mechanisms underlying H_2S action in the heart may promote the understanding of pathophysiology of cardiac diseases and lead to new therapeutic targets based on modulation of H_2S production.

1. Introduction

Hydrogen sulfide (H_2S) has been thought of to be just a toxic gas with a strong odor of rotten eggs for hundreds of years. However, with the advancement of scientific technology over the years, researchers have discovered that H_2S takes part in a series of physiological and pathological processes in mammals. A pioneering study reported by Abe and Kimura [1] in 1996 determined that H_2S facilitated the induction of hippocampal long-term potentiation by enhancing the activity of N-methyl-D-aspartate (NMDA) receptors. From then on, scientific interest has grown in the investigation of the function of H_2S as a gasotransmitter.

Now H_2S has been regarded as a novel gaseous signaling molecule, similarly to nitric oxide (NO) and carbon monoxide (CO) [2, 3]. H_2S is endogenously produced by several enzymes, including cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST) along with cysteine aminotransferase (CAT) [4–7]. The distributions of these enzymes' expressions are tissue specific. CBS is the critical enzyme for H_2S production in the nervous system and CSE is the major H_2S-producing enzyme in the cardiovascular system [8]. A number of studies have demonstrated that H_2S may be involved in a multitude of pathophysiologic processes, such as oxidative stress, inflammation, apoptosis, and angiogenesis [3]. In recent years, growing evidence has showed that H_2S is a critical regulator of heart functions and plays a protective role in the pathogenesis and development of heart diseases.

In this review, we summarize the biosynthesis and physiological functions of H_2S and explore its emerging pathogenic significance in several heart diseases including myocardial ischemia/reperfusion (I/R) injury, myocardial infarction, arrhythmias, cardiac hypertrophy, cardiac fibrosis,

and heart failure. Furthermore, we also discuss the molecular mechanisms involved in the cardioprotective effects of H_2S and how these might be used therapeutically to overcome some of the heart diseases.

2. Biosynthesis and Metabolism of H_2S

H_2S is a small molecule which can pass through cell membranes freely. The basal level of its production in mammalian tissues is determined by the activity of three key enzymes: CBS, CSE, and 3-MST together with CAT (Figure 1). Recent studies have provided a broader picture of enzyme distribution; for example, CBS is expressed in brain, liver, kidney, ileum, uterus, placenta, and pancreatic islets, and it is the predominant producer of H_2S in the central nervous system [9–11]. CSE is the main H_2S-generating enzyme in the cardiovascular system and is also found in the liver, kidney, ileum, thoracic aorta, portal vein, uterus, and placenta and is weakly detected in the brain [9, 10, 12, 13]. 3-MST, along with CAT, is a third H_2S-producing enzyme in neurons, vascular endothelium, and the retina [14–17]. Both CBS and CSE are pyridoxal-5-phosphate- (PLP-) dependent enzymes and located in cytosol; they use L-cysteine as their principal substrate to produce H_2S [18]. Unlike CBS and CSE, 3-MST and CAT have been found in both mitochondria and cytosol, although approximately two-thirds of 3-MST exists in the mitochondria [19]. 3-MST produces H_2S from 3-mercaptopyruvate (3MP), which is produced by CAT from L-cysteine and α-ketoglutarate [17]. In addition to the above pathway, Kimura group discovered a novel pathway for the production of hydrogen sulfide from D-cysteine in mammalian cells [20]. D-Cysteine is metabolized by d-amino acid oxidase (DAO) to 3MP, which is a substrate for 3-MST to produce H_2S. This pathway is functional only in the kidney and the brain, particularly in the cerebellum.

H_2S can undergo several catabolic pathways in order to maintain a proper physiological balance of its metabolism under physiological conditions. Firstly, once deprotonated, HS^- is rapidly oxidized in the mitochondria to form thiosulfate (nonenzymatic conversion), followed by further conversion into sulfite and finally into sulfate, the major end product of H_2S metabolism [21]. Secondly, H_2S can also be methylated by thiol S-methyltransferase to form dimethylsulfide and methanethiol. Lastly, H_2S can react with methemoglobin to form sulfhemoglobin [22]. Metabolic labeling studies with $Na_2{}^{35}S$ have indicated tissue specific differences in sulfide catabolism rates and in product distribution [23]. Rat liver converts sulfide primarily to sulfate, kidney to a mixture of thiosulfate and sulfate, and lung predominantly to thiosulfate. These biosynthetic and degradative pathways for H_2S will likely prompt more interest into the translational cardioprotective potential of this gasotransmitter in the future.

3. Disturbance of Endogenous H_2S Generation in Heart Diseases

The discovery of CSE in the rat heart as well as identification of H_2S as an important modulator is a breakthrough in the investigation of the role of H_2S in heart function. Increasing evidence has demonstrated that disturbed H_2S production is relevant to heart disease. In clinical patients, Jiang et al. [24] found plasma H_2S levels were significantly lowered in coronary heart disease (CHD) patients compared with that in angiographically normal control subjects. Moreover, in CHD patients, plasma H_2S levels in unstable angina patients and acute myocardial infarction patients were significantly lower than that in stable angina patients. In addition, Polhemus et al. [25] found that heart failure (HF) patients had marked reductions in circulating H_2S levels compared to age matched controls. In experimental animal model, studies also show that the endogenous production of H_2S is significantly reduced in many heart diseases, including myocardial ischemia, myocardial infarction- (MI-) induced or arteriovenous fistula-induced HF, and spontaneous, pulmonary, or hyperhomocysteinemia-induced hypertension [26]. These findings imply that cardiac disease may impair the endogenous synthesis of H_2S, which may further exacerbate the disease state. Meanwhile, these findings are clear evidence which support the involvement of endogenous H_2S in maintaining basal physiological functions of the heart.

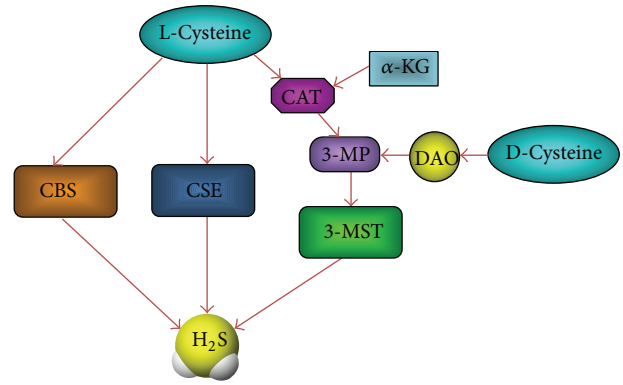

Figure 1: Biosynthesis pathways of endogenous H_2S. Cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) use L-cysteine as a substrate to produce H_2S. However, 3-mercaptopyruvate sulfurtransferase (3-MST) uses 3-mercaptopyruvate (3-MP) as a substrate to form H_2S. 3-MP is produced by cysteine aminotransferase (CAT) from L-cysteine in the presence of α-keto glutarate (α-KG); on the other hand, it is also produced by D-amino acid oxidase (DAO) from D-cysteine.

4. Role of H_2S in Heart Diseases

Recently, H_2S has been widely recognized as a cardioprotective agent for majority of cardiac disorders. Growing evidence has revealed that H_2S improves cardiac function and cardiac complications in different pathogenic conditions, such as myocardial I/R injury, myocardial infarction, cardiac arrhythmia, cardiac hypertrophy, myocardial fibrosis, and heart failure (Figure 2).

4.1. Myocardial I/R Injury. I/R injury is one critical cause of tissue destruction and often leads to heart failure. Although reperfusion relieves ischemia, it also results in a complex

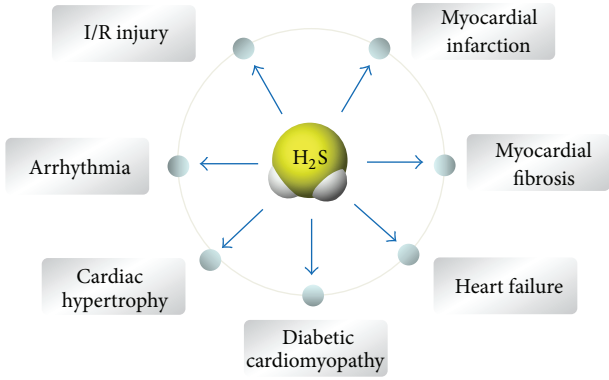

FIGURE 2: Cardioprotective effects of H_2S in different heart disease. H_2S protects the heart against myocardial ischemia/reperfusion injury, myocardial infarction, arrhythmia, myocardial fibrosis, cardiac hypertrophy, heart failure, and diabetic cardiomyopathy.

reaction that leads to cell injury caused by inflammation and oxidative damage [27]. A growing body of evidence indicates that H_2S is involved in myocardial I/R injury. H_2S postconditioning effectively protects isolated rat hearts against I/R injury via activation of the JAK2/STAT3 signaling pathway, an important component of the survivor activating factor enhancement (SAFE) pathway [28]. In another study, sulfur dioxide (SO_2) preconditioning can significantly reduce I/R-induced myocardial injury *in vivo*, which is associated with increased myocardial antioxidative capacity and upregulated H_2S/CSE pathway [29]. H_2S infusion but not bolus administration markedly reduced myocardial infarct size and improved regional left ventricular function in a porcine I/R model by suppressing cardiomyocyte apoptosis and autophagy [30]. Furthermore, NaHS pretreatment protects isolated rat hearts against I/R injury by inhibition of mitochondria permeability transition pore (MPTP) opening [31]. Our group also found pharmacologic inhibition of CSE resulted in an increase in infarct size in a rat I/R model; conversely, H_2S replacement displayed myocardial protection [32]. Additionally, cardiac specific CSE overexpressed in transgene mice significantly reduced infarct size and improved cardiac function compared to the wild-type group after 45 minutes of ischemia and 72 hours of reperfusion [33]. These findings reveal that both exogenous donors and endogenously elevated H_2S serve to protect heart against I/R injury and may serve as an important therapeutic target.

4.2. Myocardial Infarction. Myocardial infarction (MI) is the leading cause of death worldwide. It occurs when a coronary artery is occluded, leading to insufficient oxygen supply to the myocardium and resulting in death of cardiomyocytes and nonmyocyte cells [34, 35]. More and more evidence indicates that H_2S has direct benefits for myocardial infarction. Our group demonstrated for the first time that decreased H_2S levels in the plasma were associated with an increased infarct size and mortality. NaHS significantly decreased the infarct size of the left ventricle and mortality after acute MI in rats [36]. We also found S-propargyl-cysteine (SPRC), a novel modulator of endogenous hydrogen sulfide, could protect against MI by reducing the deleterious effects of oxidative stress through increased CSE activity and plasma H_2S concentration [37]. Moreover, we found that increased CSE and H_2S levels *in vivo* by miR-30 family inhibitor can reduce infarct size, decrease apoptotic cell number in the peri-infarct region, and improve cardiac function in response to MI [38]. Qipshidze et al. [39] also found that administration of H_2S remarkably ameliorated infarct size and preserved left ventricular function during development of MI in mice. This cardioprotective effect was associated with the improvement of angiogenesis due to inhibition of antiangiogenic proteins and stimulation of angiogenic factors such as vascular endothelial growth factor (VEGF). In another study, Xie et al. [40] found that H_2S preconditioning effectively promoted mesenchymal stem cells (MSCs) survival under ischemic injury and helped cardiac repair after myocardial infarction in rats.

4.3. Cardiac Arrhythmias. Cardiac arrhythmias are an important problem in coronary I/R therapy and constitute a major risk for sudden death after coronary artery occlusion [41]. The primary causes for I/R-induced arrhythmias are considered to be the endogenous metabolites, such as reactive oxygen species (ROS), calcium, thrombin, and platelet activating factor, produced and accumulated in the myocardium during reperfusion.

Zhang et al. [42] found that reperfusion with NaHS after ischemia attenuated arrhythmias in the isolated Langendorff-perfused heart and improved cardiac function during I/R. These effects could be blocked by the ATP-sensitive potassium (K_{ATP}) channel blocker glibenclamide, indicating that the cardioprotective effect of H_2S against arrhythmias during reperfusion at least partially depends on the opening of K_{ATP} channel. Bian et al. [43] also found that blockade of endogenous H_2S synthesis increased both the duration of I/R-induced arrhythmias and the severity of the arrhythmias. However, preconditioning with 100 μM NaHS attenuated arrhythmias in the isolated heart, increased cell viability, and improved cell function in cardiac myocytes during I/R, and these effects may be mediated by protein kinase C (PKC) and sarcolemmal K_{ATP} channels. Connexin 43 (Cx43) is the principal connexin in the mammalian ventricle and has been proven to have a close association with arrhythmia [44]. Huang et al. [45] found that H_2S ameliorated the expression of Cx43 in cardiac tissue, which indicated that endogenous H_2S may play an important role in regulating heart function and arrhythmia. Furthermore, Yong et al. [46] found that lowered H_2S production during ischemia may cause overstimulation of the β-adrenergic function which was closely linked with the incidence of ventricular arrhythmias. Exogenous application of H_2S negatively modulated β-adrenergic function by inhibiting adenylyl cyclase activity and finally protected heart against cardiac arrhythmias.

Based on these findings, H_2S replacement therapy may be a significant cardioprotective and antiarrhythmic intervention for those patients with chronic ischemic heart disease whose plasma H_2S level is reduced.

4.4. Myocardial Fibrosis. Cardiac fibrosis is characterized by net accumulation of extracellular matrix proteins in the

cardiac interstitium and contributes to both systolic and diastolic dysfunction in many processes of cardiac disorders [47]. Although the fibroblast activation and proliferation are important for maintaining cardiac integrity and function early after cardiac injury, the development of fibrous scar tissue in the infarct zone often leads to chronic complications and functional insufficiencies [48].

Mishra et al. [49] found cardiac fibrosis and apoptosis in chronic heart failure (CHF) were reversed by administration of H_2S, which was associated with a decrease in oxidative and proteolytic stresses. In addition, Huang et al. [45] revealed that H_2S markedly prevented the development of cardiac fibrosis and decreased the collagen content in the cardiac tissue by inhibiting the activity of intracardiac Ang-II. It is well known that multiple potassium channels are expressed in cardiac ventricular fibroblasts [50], whereby their modulations may have major significance in cardiac fibrosis. Sheng et al. [51] found that H_2S potentially modulate cardiac fibrosis by inhibiting large conductance Ca^{2+}-activated K^+ current (BK_{Ca}), transient outward K^+ current (Ito), and Ba^{2+}-sensitive inward rectifier K^+ current (IK_{ir}), independent of K_{ATP} channels, leading to decreased proliferation and suppression of transforming growth factor-β1- (TGF-β1-) induced myofibroblast transformation of atrial fibroblasts. Our previous finding has demonstrated that H_2S therapy significantly attenuated ischemia-induced cardiac fibrosis in chronic heart failure rats [52]. We also found that treatment with H_2S substantially inhibited AngII-stimulated cardiac fibroblasts, as evidenced by the reduction in α-SMA and type I collagen expression as well as effective suppression of the fibrotic marker CTGF. In addition, we proved that the pharmacologic supplementation of exogenous H_2S attenuated fibrotic and inflammatory responses induced by MI. The beneficial effects of H_2S, at least in part, were associated with a decrease of Nox4-ROS-ERK1/2 signaling axis and an increase in heme oxygenase-1 (HO-1) expression [53].

4.5. Cardiac Hypertrophy. Cardiac hypertrophy, usually considered as an effective compensation mechanism, can maintain or even increase cardiac output. However, in the long term, persistent hypertrophy will ultimately result in cardiac dilatation, decreased ejection fraction, and subsequent heart failure [54]. Pathological hypertrophy usually occurs in response to chronically increased pressure overload or volume overload, or following MI.

A large number of experiments confirm that H_2S play a positive role in protecting heart against cardiac hypertrophy. Lu et al. [55] demonstrated that H_2S could improve cardiac function and reduce myocardial apoptosis in the isoproterenol- (ISO-) induced hypertrophy rat model by reducing Nox4 expression and ROS production in the mitochondria. Treatment of mice with sodium sulfide (Na_2S) leads to less cardiac hypertrophy and left ventricular dilatation as well as improved left ventricular function after the induction of heart failure in a thioredoxin 1- (Trx1-) dependent manner [56]. In addition, pharmacologic H_2S therapy during heart failure serves to mitigate pathological left ventricular remodeling and reduce myocardial hypertrophy, oxidative stress, and apoptosis [49]. In an endothelin-induced cardiac hypertrophy rat model, Yang et al. [57] found that H_2S treatment could decrease left ventricular mass index, volume fraction of myocardial interstitial collagen, and myocardial collagen content and improve cardiac hypertrophy. In another hypertrophy model induced by abdominal aorta coarctation, Huang et al. [58] revealed that exogenous administration of H_2S significantly suppressed the development of cardiac hypertrophy and also greatly downregulated the Ang-II levels in cardiac tissue, suggesting that H_2S plays a pivotal role in the development of pressure overload-induced cardiac hypertrophy. Interestingly, Padiya et al. [59] showed that administration of freshly prepared homogenate of garlic, which have been shown to generate H_2S after interaction within cellular proteins, can activate myocardial nuclear-factor-E2-related factor-2 (Nrf2) through PI3K/AKT pathway and attenuate cardiac hypertrophy and oxidative stress through augmentation of antioxidant defense system in fructose-fed insulin resistance rats.

5. Heart Failure

Heart failure (HF) is a heterogeneous syndrome that can result from a number of common disease stimuli, including long-standing hypertension, myocardial infarction, or ischemia associated with coronary artery disease. The pathogenesis of HF has not been fully elucidated and the current treatments for HF are woefully inadequate. H_2S therapy has recently been shown to ameliorate ischemic-induced heart failure in a murine model. Cardiac-restricted overexpression of CSE in mice resulted in increased endogenous H_2S production and a profound protection against ischemia-induced heart failure and decreased mortality [60]. In contrast, knockout of CSE in murine models of heart failure showed worsened myocardial function and greater infarct size [61].

In a hypertension-induced heart failure model, it has been demonstrated clearly that H_2S decelerated progression to adverse remodeling of the left ventricle and induced angiogenesis in the myocardium [62]. Polhemus et al. [63] also found H_2S therapy attenuated left ventricular remodeling and dysfunction in the setting of heart failure by creating a proangiogenic environment for the growth of new vessels. In another model of pressure overload-induced heart failure, mice administered Na_2S exhibited enhanced proangiogenesis factors, such as matrix metalloproteinase- (MMP-) 2, and suppressed antiangiogenesis factors, including MMP-9 [64]. H_2S also play a protective role in volume overload-induced CHF by upregulating protein and mRNA expression of HO-1 [65].

Local cardiac renin-angiotensin system (RAS) is required for the development of heart failure and left ventricular remodeling. Liu and coworkers [66] have demonstrated that treatment with NaHS could protect against isoproterenol-induced heart failure by suppression of local renin levels through inhibition of both mast cell infiltration and renin degranulation in rats, suggesting a novel mechanism for H_2S-mediated cardioprotection against heart failure. Our group found NaHS markedly inhibited cardiac apoptosis and improved mitochondrial derangements, both of which led to cardioprotection in a rat model of heart failure [52].

In addition, we also showed that NaHS decreased the leakage of cytochrome c protein from the mitochondria to the cytoplasm, improved mitochondrial derangements, and increased CSE mRNA and protein levels in heart failure rats [52]. SPRC, reported also as ZYZ-802, could reduce infarct size and improve cardiac function in a rat model of MI-induced heart failure via antiapoptosis and antioxidant effects as well as angiogenesis promotion [67, 68]. All these illustrate that the CSE/H_2S pathway plays a critical role in the preservation of cardiac function in heart failure.

5.1. Diabetic Cardiomyopathy. Diabetic cardiomyopathy (DCM) is a distinct primary disease process which occurs independently of coronary artery disease and hypertension, resulting in structural and functional abnormalities of the myocardium leading to HF [69]. Increasing evidence has proved that H_2S plays a positive role in regulating diabetic myocardial injury.

A current study [70] showed that both plasma H_2S levels and plasma H_2S synthesis activity were significantly reduced in the streptozotocin- (STZ-) induced diabetic rats. In addition, H_2S was also decreased in the plasma of type 2 diabetic patients compared with age matched healthy controls [71]. These findings suggest the involvement of H_2S in diabetic pathological processes. Xu et al. [72] found exogenous H_2S exerted a protective effect against high glucose- (HG-) induced injury by inhibiting the activation of the p38 MAPK and ERK1/2 pathways and preventing oxidative stress in H_9C_2 cells. Wei et al. [73] also reported that a novel H_2S-releasing molecule GYY4137 probably protected H_9C_2 cells against HG-induced cytotoxicity by activation of the AMPK/mTOR signal pathway. Moreover, H_2S may reduce HG-induced oxidative stress by activating Nrf2/ARE pathway and may exert antiapoptotic effects in diabetic myocardium by inhibiting JNK and p38 MAPK pathways and activating PI3K/Akt signaling [74]. Interestingly, Padiya et al.'s study [59] showed that administration of raw garlic homogenate in insulin resistance fructose fed rat activated myocardial Nrf2 by increasing H_2S level and activating PI3K/AKT pathway and attenuated cardiac hypertrophy and oxidative stress through augmentation of antioxidant defense system. In another study, using a STZ-induced diabetes model in rats, Zhou et al. [74] demonstrated an important therapeutic potential of the H_2S pathway in DCM. They found that daily administration of NaHS had anti-inflammatory, antioxidative, and antiapoptotic effects and rescued the decline in heart function in the STZ + NaHS group. Furthermore, Peake et al. [75] found that exogenous administration of Na_2S attenuated myocardial I/R injury in db/db mice, suggesting the potential therapeutic effects of H_2S in treating a heart attack in the setting of type 2 diabetes.

6. Molecular Mechanisms of H_2S-Induced Cardioprotection

Similar to NO and CO, the effects of H_2S on the heart are mediated via a diverse array of cellular and molecular signals. The mechanisms by which H_2S protects against cardiac diseases are through antioxidative action, preservation of mitochondrial function, reduction of cardiomyocyte apoptosis, anti-inflammatory responses, angiogenic action, regulation of ion channel, and increasing the production of NO (Figure 3).

6.1. Antioxidative Action. Oxidative stress is a process due to an imbalance between prooxidant and antioxidant systems. Oxidative stress-induced cellular injury is often caused by excessive formation of ROS, such as superoxide anion (O^{2-}), hydroxyl radical (OH^{-}), peroxynitrite ($ONOO^{-}$), and hydrogen peroxide (H_2O_2). The occurrence of the majority heart diseases is associated with ROS generation, including myocardial I/R injury, cardiac hypertrophy, myocardial fibrosis, and arrhythmias. H_2S has been reported as a strong antioxidant and widely proposed to protect the cardiac system through its antioxidant role. The robust antioxidant actions of H_2S are associated with direct scavenging of ROS and/or increased expressions and functions of antioxidant enzymes.

Sun et al. [76] found that H_2S inhibited mitochondrial complex IV activity and increased the activities of Mn-SOD and CuZn-SOD and decreased the levels of ROS in cardiomyocytes during I/R. H_2S decreased lipid peroxidation by scavenging hydrogen peroxide and superoxide in a model of isoproterenol-induced myocardial injury [77]. The activation of Nrf2 dependent pathway mediated by H_2S results in upregulated gene expression of specific factors, such as HO-1, gluthatione reductase, glutathione S-transferase, thioredoxin, and catalase, which play role in endogenous antioxidant defense. Furthermore, H_2S has an inhibitory effect on phosphodiesterase-5 (PDE-5), which results in decreased NADPH oxidase formation, and the level of antioxidant enzymes increases [78]. Besides these mechanisms, H_2S also acts as a direct scavenger to neutralize cytotoxic reactive species like peroxynitrite [79] and directly destroys organic hydroperoxides of pathobiological importance, like fatty acid hydroperoxides (LOOHs) [80]. Collectively, these findings suggest that H_2S is capable of preventing the generation of ROS, scavenging ROS, and strengthening the endogenous antioxidant system.

6.2. Preservation of Mitochondrial Function. Mitochondrial function is compromised under hypoxic conditions or in the presence of increased ROS [81]. Growing evidence has shown that H_2S has the ability to protect mitochondria and ultimately improve respiration and promote biogenesis. Elrod and colleagues [33] found a dose dependent reduction of oxygen consumption in isolated murine cardiac mitochondria after hypoxia, and the administration of H_2S was shown to improve the recovery of posthypoxic respiration rate significantly. Moreover, electron microscopy showed a notable reduction in mitochondrial swelling and increased matrix density in mice after treatment with H_2S, further suggesting a prominent role of H_2S in the preservation of mitochondrial function in the cytoprotection. In addition, H_2S can affect mitochondria of cardiac cells by inhibition of cytochrome c oxidase in a potent and reversible way, which leads to preservation of mitochondrial structure and function [52]. H_2S may protect mitochondrial function by

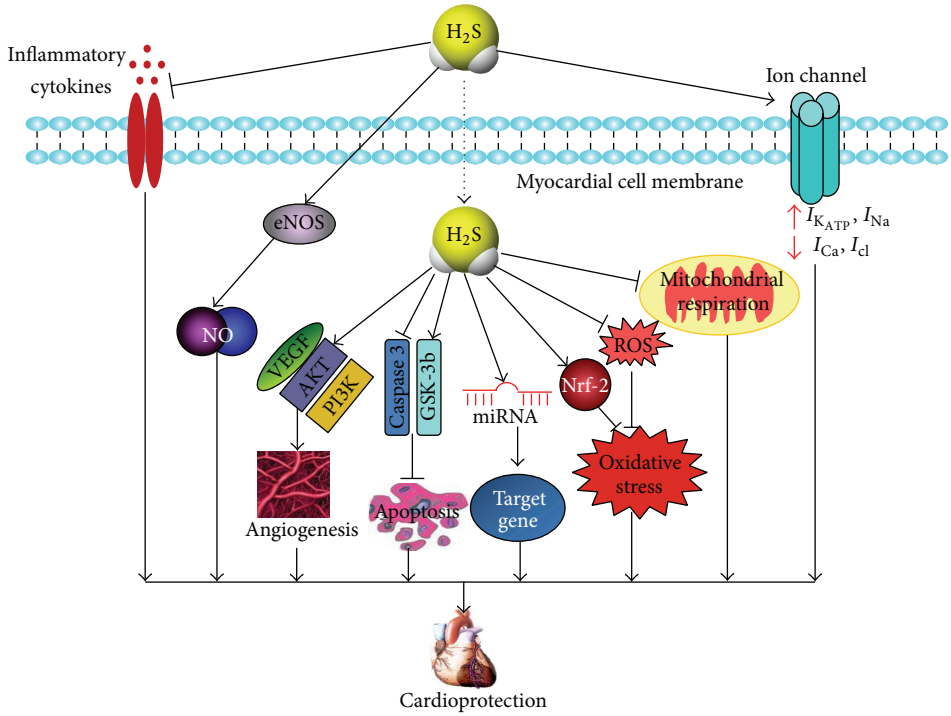

Figure 3: Different signaling pathways activated by H_2S showing the cardioprotective effects. H_2S can protect heart against diseases via different mechanisms: H_2S prevents inflammatory response mediated by inflammatory cytokines. H_2S stimulates angiogenesis by increasing the expression of VEGF and activating phosphatidylinositol 3-kinase (PI3K) and Akt. H_2S activates endothelial nitric oxide synthase (eNOS) and augments NO bioavailability. H_2S significantly protects against cardiomyocyte apoptosis by suppressing the activation of caspase-3 and upregulating the expression of glycogen synthase kinase-3 (GSK-3β). H_2S plays its role by regulating the expression of miRNA. H_2S also protects mitochondrial function via inhibition of mitochondrial respiration. H_2S exerts antioxidative action by activating nuclear-factor-E2-related factor-2 (Nrf2) dependent pathway and scavenging of ROS. H_2S opens K_{ATP} channels, increases Na^+ channels (Nav) current, and inhibits L-type Ca^{2+} channels and chloride channels, to produce cardioprotective effects.

inhibiting respiration, thus limiting the generation of ROS and diminishing the degree of mitochondrial uncoupling, leading to decreased infarct size and preserved function [33]. Furthermore, H_2S preserved mitochondrial function after reperfusion as noted by increased complex I and II efficiency, leading to downregulated mitochondrial respiration and subsequent cardioprotective effects during myocardial I/R injury [82]. Downregulation of MPTP can reduce mitochondrial membrane potential depolarization and consequently inhibit the activation of proapoptotic protein [83]. It is reported that H_2S can affect mitochondrial targets via upregulation of the reperfusion injury salvage kinase pathway, which is able to inhibit the opening of mitochondria permeability transition pores (MPTP) [84].

6.3. Antiapoptosis. There is increasing proof that H_2S has antiapoptotic actions. Most data indicate the antiapoptotic effects of H_2S are mainly due to the preservation of mitochondrial function, and many of the cytoprotective actions of H_2S during ischemic states may be a result of potent actions on mitochondria [85]. It is reported that H_2S significantly protected against high glucose-induced cardiomyocyte apoptosis by altering Bax and Bcl-2 gene expression [86]. Moreover, It is found that NaHS treatment suppressed the activation of caspase-3 and reduced apoptotic cell numbers in both mice [33] and swine [87], suggesting that H_2S was capable of inhibiting the progression of apoptosis after I/R injury.

Survivin is an antiapoptotic gene implicated in the initiation of mitochondrial-dependent apoptosis. In an *in vivo* I/R rat model, our group found administration of NaHS for 6 days before surgery significantly upregulated survivin mRNA and protein expressions by 3.4-fold and 1.7-fold, respectively [32], suggesting another way of action for H_2S-induced cardioprotection.

The activity of glycogen synthase kinase-3 (GSK-3β), which has been proposed as a viable target in the ischemic heart injury, is associated with both apoptosis and cell survival. Osipov et al. [30] found that H_2S infusion increased the expression of the phosphorylated form of GSK-3β significantly. Similarly, Yao et al. [88] also demonstrated that NaHS upregulated the phosphorylation of GSK-3β (Ser9) expression and subsequently resulted in inhibiting the opening of MPTP, preventing apoptosis and protecting the heart against ischemic damage.

6.4. Anti-Inflammation. Inflammation is involved in the main pathological processes of ischemic heart disease. For example, cytokines mediate the development of ischemic injury in the heart and depress myocardial function [89]. IL-6 and IL-8 are released on myocardial I/R damage and then

increase neutrophil adhesion and inflammatory responses [90]. TNF-α plays multiple roles in the pathogenesis of myocardial I/R injury by inducing endothelium adhesion molecules, allowing for neutrophil infiltration, increasing the production of ROS, amplifying the inflammatory response, and having direct myocardial depressant and apoptotic actions [91].

Studies have shown that H_2S may play dual roles in inflammatory process. Whiteman and Winyard [92] reviewed 14 studies showing an anti-inflammatory effect of H_2S and 15 studies showing a proinflammatory effect of H_2S. However, the anti-inflammatory effect of H_2S plays a dominant role in heart disease. In myocardial I/R experiments, Elrod et al. [33] have demonstrated that, at the time of heart reperfusion, H_2S decreased the number of leukocytes within the ischemic zone as well as neutrophils within the myocardial tissue. The evaluation of inflammatory cytokines revealed myocardial levels of IL-1β to be markedly reduced after administration of H_2S. Additionally, H_2S was found to potently reduce *in vivo* leukocyte-endothelial cell interactions. Using the ischemic porcine heart, Sodha et al. [93] found that NaHS treatment decreased the level of TNF-a, IL-6, and IL-8 as well as the activity of myeloperoxidase. Therefore, H_2S restrained the extent of inflammation and limited the extent of MI by preventing leukocyte transmigration and cytokine release. In another study, the H_2S donor, Na_2S and NaHS were both able to inhibit leukocyte adherence and the resultant inflammatory pathology via activation of K_{ATP} channels [94].

In the lipopolysaccharide-induced inflammatory response of rat embryonic ventricular myocardial cells (H_9C_2 cells), our group also found [95] that SPRC prevented nuclear factor-κB (NF-κB) activation and suppressed LPS-induced extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation and intracellular reactive oxygen species (ROS) production. In addition, SPRC induced phosphorylation of Akt, attenuated LPS-induced mRNA and protein expression of tumor necrosis factor-α (TNF-α), and inhibited mRNA expression of intercellular adhesion molecule-1 (ICAM-1) and inducible nitric oxide synthase (iNOS). Therefore, SPRC produced an anti-inflammatory effect in LPS-stimulated H_9C_2 cells through the CSE/H_2S pathway by impairing IκBα/NF-κB signaling and by activating PI3K/Akt signaling pathway. These studies provide strong evidence of the function of H_2S as anti-inflammatory agent.

6.5. Angiogenesis. The cardioprotective role of H_2S could also be due to its angiogenic action on the ischemic area in the heart. Angiogenesis plays a pivotal role in the early stage of wound healing. In *in vitro* studies, incubation with low micromolar concentrations of H_2S increased endothelial cell number, cell migration, and capillary morphogenesis on matrigel [96]. Chicken chorioallantoic membranes, an *in vivo* model of angiogenesis, displayed increased branching and lengthening of blood vessels in response to 48 h treatment with H_2S [97]. Aortic rings isolated from CSE knockout mice exhibited markedly reduced microvessel formation. Additionally, in a wound healing model, topically applied H_2S accelerated wound closure and healing [97].

Angiogenesis is very important in chronic ischemia as poorly vascularized tissue will result in loss of function. Therefore, increasing myocardial vascularity and perfusion in concert with cardiac myocyte growth are critical to prevent the progression of heart failure. In a hypertension-induced heart failure model, administration of H_2S induced angiogenesis in the myocardium and decelerated the progression of left ventricle remodeling [63]. In a similar heart failure model, NaHS treatment improved cardiac function and mitigated transition from compensatory hypertrophy to heart failure, which was associated with a significant increase in capillary density [98]. In another MI model, H_2S supplementation showed improvement of heart function and mitigation of cardiac remodeling by increasing angiogenic vessels and blood flow in MI mice [39].

Multiple signaling mechanisms are involved in the angiogenic action of H_2S, including activation of K_{ATP} channels [99]. By using the K_{ATP} channel inhibitor glibenclamide, Papapetropoulos et al. [97] found that K_{ATP} channel was involved in H_2S-stimulated angiogenesis. Additionally, H_2S can stimulate angiogenesis through phosphatidylinositol 3-kinase (PI3K) and Akt activation [96]. H_2S can also activate hypoxia inducible factor-1a (HIF-1a) and thus increase expression of VEGF [100]. VEGF is a key growth factor in physiological angiogenesis and induces angiogenesis in myocardial ischemia and MI. H_2S is reported to promote angiogenesis in a MI model by increasing the expression of VEGF and its specific receptors such as the tyrosine kinase receptor-flk-1 and the fms-like tyrosine kinase-flt-1 [39]. It is also reported that H_2S can regulate the matrix metalloproteinase/tissue inhibitor of metalloproteinase (MMP/TIMP) axis to promote VEGF synthesis and angiogenesis [98]. Furthermore, Zhu group identified VEGFR2 as a receptor for H_2S for inducing angiogenesis in vascular endothelial cells and found that an intrinsic inhibitory Cys1045–Cys1024 disulfide bond acted as a molecular switch for H_2S to regulate the structure and function of VEGFR2. VEGFR2 was directly activated by H_2S suggesting that VEGFR2 acted as a direct target molecule for H_2S in vascular endothelial cells [101].

6.6. Regulation of Ion Channel. The effects of H_2S on heart electrophysiology have been reported. There are two different types of Ca^{2+} channels (L-type and T-type) in the myocardial membrane. L-type Ca^{2+} channels are absolutely essential for maintaining the electrophysiological basis for the plateau phase of action potentials and for excitation-contraction (EC) coupling [102]. Whole patch clamp experiments in rat cardiomyocytes revealed that NaHS negatively modulates L-type Ca^{2+} channels composed by the CaV1.2 subunits in rat cardiomyocytes [103–105]. T-type Ca^{2+} channels can be reexpressed in atrial and ventricular myocytes in a variety of pathological conditions such as cardiac hypertrophy and heart failure and participate in abnormal electrical activity and EC coupling [106]. A recent report has showed that NaHS (10 μM–1 mM) selectively inhibits Cav3.2 T-type Ca^{2+} channels which are heterologously expressed in HEK293 cells [107].

K_{ATP} channels are located on the surface of cell membranes and mitochondria and are widely distributed in the myocardium. The opening of K_{ATP} channels is an important endogenous cardioprotective mechanism involved in cardiac ischemia preconditioning. The K_{ATP} channel opening generates outward currents and causes hyperpolarization, which reduces calcium influx via L-type Ca^{2+} channels and prevents Ca^{2+} overload. Tang and coworkers [108] found evidence that NaHS (100 μM) opened the K_{ATP} channels in vascular smooth muscle cells. Furthermore, H_2S may also indirectly activate the K_{ATP} channels by inducing intracellular acidosis [109]. By activation of the K_{ATP} channels, H_2S shortens action potential duration (APD) and produces cardioprotective effects [110, 111], though H_2S has no significant effect on the amplitude of action potential and resting potential [104].

Study has demonstrated that voltage-dependent Na^+ channels (Nav) can be regulated by H_2S. In Native Nav from jejunum smooth muscle and recombinant Nav (Nav1.5) heterologously expressed in HEK293, Strege et al. [112] found NaHS increased peak sodium currents and also right-shifted the voltage dependence of Na^+ current inactivation and activation. This effect could extend beyond the jejunum, since Nav1.5 is also expressed in other tissues. In the heart, Nav1.5 gives rise to the upstroke of the cardiac action potential; thus, it is possible that H_2S may have the same effect on the Nav expressed in the heart.

Growing studies show that chloride channels play an important role in normal physiological function in myocardial cells, but abnormal changes can be found in pathological conditions such as myocardial ischemia and arrhythmias. Malekova et al. [113] investigated the effect of H_2S on single-channel currents of chloride channels using the patch clamp technique and found that NaHS inhibited the chloride channels by decreasing the channel open probability in a concentration dependent manner. The inhibitory effect of H_2S on the chloride channels may be involved in the biological actions of H_2S in the heart.

6.7. Interaction with NO. H_2S protects cardiac muscles from I/R injury by increasing the production of NO [114]. H_2S is known to interact with the other biological mediators and signal transduction components to produce its effects in the cardiovascular system. H_2S can activate endothelial nitric oxide synthase (eNOS) through phosphorylation at the S1177 active site and augment NO bioavailability [61], highlighting that there is an interaction between NO and H_2S of physiological significance. There is evidence that NO and peroxynitrite react with H_2S to form a novel nitrosothiol, which has been proposed to regulate the physiological effects of both NO and H_2S [115]. Moreover, mice treated with the H_2S donor, diallyl trisulfide (DATS), showed marked increases in plasma nitrite, nitrate, and nitrosylated protein (RXNO) levels 30 minutes after injection [116].

In CSE knockout mice, the levels of H_2S and bound sulfane sulfur in tissues and blood as well as the levels of NO metabolites were decreased significantly. However, administration of H_2S rescued the heart form I/R injury by activating eNOS and increasing NO availability. In addition to these observations in CSE knockout mice, the administration of H_2S failed to protect the cardiac muscle from I/R injury in eNOS defective mutant mice [114]. Similar results were also obtained by Kondo et al. [61] in a mouse model of pressure overload-induced heart failure, which suggests that H_2S protects the heart by upregulating eNOS phosphorylation accompanied by increasing NO production. Interestingly, plasma H_2S levels, CSE gene enzymatic activity, and expression in the cardiovascular system were reduced in rats after treated with a NOS inhibitor chronically, indicating the physiological significance of NO in the regulation of H_2S production in the cardiovascular system [117].

6.8. Regulation of miRNA Expression. MicroRNAs (miRNAs) are evolutionarily conserved molecules that modulate the expression of their target genes by mRNA degradation or translational repression, and they may participate in various physiological and pathological processes of heart diseases [118]. An increasing body of evidence shows that H_2S exerts its role by regulating the expression of miRNA. Shen et al. [119] found H_2S was involved in regulating the expression of drought associated miRNAs such as miR-167, miR-393, miR-396, and miR-398 and their target genes, and therefore improved the tolerance of Arabidopsis to drought. A recent study [120] demonstrated that H_2S played a role in the protection of hepatic I/R injury in the young rats by down-regulating the expression of miR-34a, which resulted in the promotion of Nrf-2 signaling pathway. More importantly, Liu et al. [121] found H_2S inhibited cardiomyocyte hypertrophy by upregulating miR-133a. In addition, H_2S donor, Na_2S, would attenuate myocardial injury through upregulation of protective miR-21 and suppression of the inflammasome, a macromolecular structure that amplifies inflammation and mediates further injury [122]. These data suggest a new mechanism for the role of H_2S and indicate that miRNA could be a new target of H_2S in cardiac disorders.

7. H_2S-Based Therapeutic Potential for Heart Diseases

More and more H_2S donors with varying chemical and pharmacological properties have been reported as potential therapeutics. Among them, Na_2S and NaHS were the first H_2S-releasing agents studied in the cardiac system [33, 123]. As inorganic salts, Na_2S and NaHS have the advantage of rapidly increasing H_2S concentration within seconds, but they also rapidly decline within tissue and could exert adverse side effects because of rapid increases in H_2S at high concentrations [124]. This somewhat limits their therapeutic potential. Thus, it is important to develop novel H_2S-releasing drugs used to treat heart diseases.

Synthetic H_2S-releasing compounds have been developed. GYY4137, a water-soluble compound capable of releasing H_2S slowly, has been reported to protect against high glucose-induced cytotoxicity by activation of the AMPK/mTOR signal pathway in H_9C_2 cells [73]. SG-1002 [61] and penicillamine based donors [125] are examples of synthesized H_2S donors whose release is more precisely controlled.

H_2S therapy with SG-1002 resulted in cardioprotection in the setting of pressure overload-induced heart failure via upregulation of the VEGF-Akt-eNOS-NO-cyclic guanosine monophosphate (cGMP) pathway with preserved mitochondrial function, attenuated oxidative stress, and increased myocardial vascular density. Penicillamine based donors showed potent protective effects in an *in vivo* murine model of myocardial I/R injury.

In recent years, some natural plant-derived compounds, such as garlic, have been found to produce H_2S. Naturally occurring H_2S donors such as DATS, a polysulfide derived from garlic, is known to protect against myocardial I/R injury in mice through preservation of endogenous H_2S [126]. It also has been shown to protect against hyperglycemia-induced ROS-mediated apoptosis by upregulating the PI3 K/Akt/Nrf2 pathway, which further activates Nrf2-regulated antioxidant enzymes in cardiomyocytes exposed to high glucose [127]. Additionally, organic sulfide donors derived from garlic, such as diallyl disulfide (DADS), attenuate the deleterious effects of oxidized LDL on NO production [128] and protect the ischemic myocardium. SAC (S-allylcysteine), another derivative of garlic, significantly lowers mortality and reduces infarct size following MI [129]. SPRC, a structural analogue of SAC which was synthesized by our group, was found to protect against myocardial ischemic injury both in *in vivo* and *in vitro* studies through the increase in CSE activity and plasma H_2S concentration [130]. SAC and SPRC are both cardioprotective in MI by modulating the endogenous levels of H_2S, reducing the deleterious effects of oxidative stress and preserving the activities of antioxidant-defensive enzymes like SOD [37]. As novel H_2S releasing agents or H_2S donors develop, these novel agents should ultimately address the clinically relevant issues such as sustained release or half-life, route of administration, tissue specificity, and low toxicity.

8. Conclusion and Perspectives

Following in the footsteps of NO and CO, H_2S is rapidly emerging as a critical cardiovascular signaling molecule. We have summarized the current knowledge on the function of H_2S in heart disease and discussed the possible molecular mechanisms involved in its cardioprotective effect. Although the complete actions of this gas remain under investigation and the underlying mechanisms should be further elucidated, the therapeutic options relating to heart disease are extremely promising. We also reviewed the current H_2S donors which have been verified to have the therapeutic potential for heart disorders. Most of the current H_2S donors have the drawback of rapid degradation and difficult to control. Furthermore, whether the therapeutic effects of these donors in animal studies can be transferable to clinical studies needs to be determined. However, we believe a long-acting donor with controlled H_2S release will be developed. In short, a better understanding of the function of the H_2S in heart disease as well as development of novel H_2S-based therapeutic agents may be helpful to reduce the risks of heart disease in the future.

Conflict of Interests

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

Acknowledgments

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Research Article

Hydrogen Sulfide Donor GYY4137 Protects against Myocardial Fibrosis

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Hydrogen sulfide (H_2S) is a gasotransmitter which regulates multiple cardiovascular functions. However, the precise roles of H_2S in modulating myocardial fibrosis *in vivo* and cardiac fibroblast proliferation *in vitro* remain unclear. We investigated the effect of GYY4137, a slow-releasing H_2S donor, on myocardial fibrosis. Spontaneously hypertensive rats (SHR) were administrated with GYY4137 by intraperitoneal injection daily for 4 weeks. GYY4137 decreased systolic blood pressure and inhibited myocardial fibrosis in SHR as evidenced by improved cardiac collagen volume fraction (CVF) in the left ventricle (LV), ratio of perivascular collagen area (PVCA) to lumen area (LA) in perivascular regions, reduced hydroxyproline concentration, collagen I and III mRNA expression, and cross-linked collagen. GYY4137 also inhibited angiotensin II- (Ang II-) induced neonatal rat cardiac fibroblast proliferation, reduced the number of fibroblasts in S phase, decreased collagen I and III mRNA expression and protein synthesis, attenuated oxidative stress, and suppressed α-smooth muscle actin (α-SMA), transforming growth factor-β1 (TGF-β1) expression as well as Smad2 phosphorylation. These results indicate that GYY4137 improves myocardial fibrosis perhaps by a mechanism involving inhibition of oxidative stress, blockade of the TGF-β1/Smad2 signaling pathway, and decrease in α-SMA expression in cardiac fibroblasts.

1. Introduction

About two-thirds of cells within the heart consist of non-myocardial cells, including fibroblasts, smooth muscle cells, and endothelial cells, of which more than 90% are fibroblasts. Cardiac fibrosis, a common pathological process which occurs in the context of many different heart diseases, may increase myocardial stiffness, hamper ventricular diastolic and systolic function, and eventually trigger heart failure [1]. Excessive accumulation of collagen fibers in the heart is one of the main manifestations of myocardial fibrosis [2]. Methods for retarding cardiac fibrosis are rare but

include treatment with some antihypertensive drugs [3, 4], inhibitors of matrix metalloproteinases [5], microRNA intervention [6], and stem cell transplantations [7]; however, none of these treatment provides satisfactory outcomes in the clinic.

Hydrogen sulfide (H_2S), the third gasotransmitter discovered after nitric oxide and carbon monoxide, plays a key role in modulating cardiovascular function [8–10]. Much evidence now confirms a role for H_2S in a wide range of physiological and pathological processes in the cardiovascular system, including blood pressure lowering, vasorelaxation, cardioprotection, and inhibition of atherosclerosis [11–15]. The effect of H_2S on fibrosis has been studied previously by several groups. Using whole cell patch clamping, NaHS has been found to inhibit human atrial fibroblast proliferation induced by transforming growth factor-β1 (TGF-β1) or 20% fetal bovine serum (FBS). Preconditioning of fibroblasts with NaHS decreases basal expression of Kv4.3 (encode I(to)), but not KCa1.1 (encode BK(Ca)) and Kir2.1 (encode IK(ir)) [16]. Furthermore, H_2S attenuates TGF-β1-stimulated Kv4.3 and α-smooth muscle actin (α-SMA) expression, in parallel with its ability to inhibit TGF-β-induced myofibroblast transformation [16]. Huang et al. reported that H_2S suppressed cardiac fibrosis induced by pressure overload, possibly by inhibiting the activity of intracardiac angiotensin II (Ang II) and by modifying the expression of cx43 [17]. NaHS also inhibits Ang II-induced expression of α-SMA, connective tissue growth factor (CTGF), and type I collagen and additionally upregulates the expression of heme oxygenase-1 (HO-1), in cardiac fibroblasts [18].

Nevertheless, the pathogenesis of myocardial fibrosis is complex and the precise role of H_2S in modulating myocardial fibrosis *in vivo* and cardiac fibroblast proliferation *in vitro* remains unclear. One reason for such lack of clarity to date may be the reliance on NaHS as H_2S donor in previous studies. NaHS has been very widely used to evaluate the biology of H_2S and has provided useful information about the pharmacological effects of this gas. However, NaHS releases copious amounts of H_2S over a very short time frame (seconds) and as such is unlikely to mimic the time course of H_2S release *in vivo* [19]. With this in mind, we have therefore now evaluated the biological effects in this system of GYY4137 (morpholin-4-ium-methoxyphenyl-morpholino-phosphinodithioate), which releases low concentrations of H_2S slowly (hours) in aqueous solution at physiological pH and temperature [20]. Previous work with GYY4137 has revealed its ability to reduce systolic blood pressure (SBP) in spontaneously hypertensive rats (SHR) [20], highlighting a potential beneficial therapeutic effect on myocardial fibrosis, which is a common and important complication of hypertension.

The aims of the present study were therefore to examine whether GYY4137 is able to attenuate myocardial fibrosis in SHR *in vivo* and Ang II-induced cardiac fibroblast proliferation *in vitro* and to elucidate the mechanism of such effects. The present data raise the novel possibility that treatment with slow-releasing H_2S donors can provide a novel therapeutic approach to reduce the development of myocardial fibrosis.

2. Materials and Methods

2.1. Treatment of Animals. Male SHR and Wistar-Kyoto (WKY) rats at 12 weeks of age were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Animals were housed at constant temperature (20–22°C) and humidity (45–55%), with a 12 h light-dark cycle and fed a standard rat chow (SLAC) and water *ad libitum*. Following a 3-day acclimatization period, normotensive WKY rats served as controls (WKY group, $n = 10$) and age-matched SHRs were randomly divided into 4 groups ($n = 10$ per group) assigned different dosages of GYY4137: 0 (SHR group), 10 (GYY10 group), 25 (GYY25 group), or 50 (GYY50 group) mg/kg/d. GYY4137 was given by intraperitoneal injection once daily over a 4-week period. WKY and SHR control groups received the same volume of physiological saline instead of drug once daily over the same time period. During treatment, SBP was measured by the tail-cuff method with a Visitech BP-2000 blood pressure analysis system (Visitech Systems, Apex, NC, USA) under minimal restraint.

Animal experiments were performed in accordance with the NIH Guidelines for Care and Use of Laboratory Animals. This study was approved by the Committee on Animal Care of Nanjing Medical University.

2.2. Echocardiography. After treatment, cardiac systolic function was evaluated in pentobarbital-anesthetized rats using an echocardiography system (Visual Sonics Vevo 2100, VisualSonics, CA) equipped with a 12 MHz linear-array transducer. Two-dimensional (2D) images were obtained in the parasternal long-axis and short-axis views as well as apical two- and four-chamber views. Left ventricular (LV) ejection fraction (EF) and fractional shortening (FS) were derived by goal-directed, diagnostically driven software.

2.3. Histology. After 4 weeks of treatment as above, rats were anesthetized with pentobarbital and killed and the heart was excised immediately. The upper mid-level LV slices were dehydrated and embedded in paraffin. Two sequential 5-μm-thick sections were obtained from each heart. The sections were stained with the collagen-specific picrosirius red (Sigma, St. Louis, MO). A total of 15 myocardial images from each slide were captured by light microscopy (Leica, Germany) and analyzed using the Image Pro Plus program (Media Cybernetics, Bethesda, MD, USA). For each image, the collagen volume fraction (CVF) in LV was determined as the ratio of collagen surface area to myocardial surface area. Quantitative evaluation of perivascular fibrosis was also performed, by imaging a field surrounding an intramyocardial artery and determining the ratio of perivascular collagen area (PVCA) to lumen area (LA).

2.4. Myocardial Collagen Measurement. Myocardial hydroxyproline concentration was determined as previously described [21]. Myocardial collagen was extracted and digested with cyanogen bromide (CNBr) according to the procedure described previously [22]. The remaining portion of the CNBr-digested collagen sample was subjected to

acid hydrolysis and hydroxyproline was determined as a measure of non-cross-linked (soluble) collagen. The amount of cross-linked (insoluble) collagen in the myocardium was determined based on total myocardial collagen amount. The ratio between cross-linked and non-cross-linked collagen was taken as an index of the degree of collagen cross-linking [3].

2.5. Culture and Treatment of Cardiac Fibroblasts. Sprague-Dawley rats, 1–3 days old, were anesthetized with ether prior to euthanasia. Hearts were removed immediately and ventricles were separated from the atria, trisected, and digested completely with 0.25% trypsin (Beyotime, Haimen, China) at 37°C for 7–10 cycles. All supernatants from each cycle, except the first, were pooled. Dulbecco's modified Eagle's medium (DMEM, Wisent Inc., Canada) with 10% FBS (Wisent Inc., Canada) equal in volume to the supernatants was added to terminate digestion, and the mixture was centrifuged for 4.5 minutes at 2000 g. The cell pellet was resuspended in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Dispersed cells were incubated for 1.5 h in a 5% CO_2 incubator. Nonmyocytes attached to the bottom of the dishes were subsequently incubated with DMEM supplemented with 10% FBS for an additional 2–4 days. Confluent cardiac fibroblasts (CFs) were treated with trypsin and subcultured. Subconfluent (>70% confluency) CFs grown in culture dishes from the second to third passages were used in all experiments. The medium was changed to DMEM supplemented with 0.5% FBS for 24 h. Confluent cells were preincubated with different concentrations of GYY4137 (12.5 μM, 25 μM, and 50 μM) for 4 h, followed by Ang II (Sigma-Aldrich, St. Louis, MO; 10^{-7} M) stimulation for an additional 24 h. Culture medium without GYY4137 was used as a vehicle control.

2.6. Cell Count. Numbers of CFs were determined by cell counting Kit-8 (CCK-8, Beyotime, Shanghai, China), according to the manufacturer's directions.

2.7. Cell Cycle Analysis. After treatment as above mentioned, the CFs were harvested by trypsinization, washed in phosphate buffered saline (PBS), and resuspended in cold 70% ethanol. Finally, propidium iodide (20 μg/mL) staining solution was added to the samples and cell cycles were analyzed on a flow cytometry (BD FACSCalibur, Ann Arbor, MI). Results were acquired from 10,000 cells.

2.8. Measurement of Hydroxyproline in Cell Culture Medium. After treatment, cell culture medium was collected for measuring hydroxyproline content according to the manufacturer's instructions (Beyotime, Shanghai, China).

2.9. Quantitative Real-Time Polymerase Chain Reaction. Quantitativereal-time PCR analysis was used to measure mRNA expression with 18S ribosomal RNA as a control. Total RNA was extracted from myocardium or CFs using Trizol reagent (Takara, Otsu, Shiga, Japan). RNA (500 ng) was added as a template to reverse-transcriptase reactions carried out using the PrimeScript RT Master Mix Kit (Takara, Otsu, Shiga, Japan). PCRs were carried out with the resulting cDNAs using the SYBR Green Premix (Takara, Otsu, Shiga, Japan) with ABI 7500 Real-Time PCR System (ABI, Carlsbad, CA). Experimental Ct values were normalized to 18S and relative mRNA expression was calculated versus a reference sample. Each sample was run and analyzed in triplicate. Primers used for amplification were collagen I: 5′-AGGGTCATCGTGGCTTCTCT-3′ and 5′-CAGGCTCTTGAGGGTAGTGT-3′; collagen III: 5′-AGCGGAGAATACTGGGTTGA-3′ and 5′-GATGTAATGTTCTGGGAGGC-3′; TGF-β1: 5′-GCCCTGGACACCAACTATTGC-3′ and 5′-GGAGCGCACGATCATGTTGG-3′; and 18S: 5′-AGTCCCTGCCCTTTGTACACA-3′ and 5′-GATCCGAGGGCCTCACTAAAC-3′.

2.10. Western Blotting Analysis. Protein samples were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA). After blocking at room temperature in 5% w/v nonfat milk with TBST buffer (Tris-HCl 10 mM, NaCl 120 mM, and Tween-20 0.1%; pH 7.4) for 2 h, membranes were incubated overnight with the appropriate primary anti-collagen I, anti-collagen III, anti-TGF β1 (1 : 1000, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Smad2, anti-p-Smad2 (1 : 500, Bioworld Technology, St. Louis Park, MN), or anti-GAPDH (1 : 6000, Sigma-Aldrich, St. Louis, MO) antibodies, at 4°C, followed by horseradish peroxidase- (HRP-) conjugated secondary antibody at room temperature for 2 h. Proteins were visualized by enhanced chemiluminescence substrate (ECL, Pierce, Rockford, IL).

2.11. Immunofluorescence Staining. After treatment, the cells were blocked with 10% bovine serum albumin (Solarbio, Beijing, China) and incubated with primary antibody against α-SMA (1 : 1000, Santa Cruz Biotechnology, CA), collagen I, collagen III, or negative IgG control for 16 h at 4°C. Immunoreactivity was visualized using Alexa Fluor 488 or Alexa Fluor 555 conjugated IgG (Beyotime, Haimen, China, 1 : 1000). Cells were counterstained with DAPI (5 μg/mL, Beyotime, Haimen, China) and then evaluated under a fluorescence microscope (Nikon, Tokyo, Japan).

2.12. Measurement of Reactive Oxygen Species. Detection of intracellular reactive oxygen species (ROS) was accomplished by the use of a 2′,7′-dichlorofluorescein-diacetate (DCFH-DA, 10 μM) liposoluble probe according to the manufacturer's instructions (Beyotime, Shanghai, China). This probe is hydrolyzed to 2′,7′-dichlorodihydrofluorescein (DCFH), which is available for oxidation by ROS to produce fluorescent 2′,7′-dichlorofluorescein (DCF). The fluorescence, with its intensity in proportion to the amount of ROS, was measured at 488 nm (excitation) and 528 nm (emission) by fluorescence microscopy (Nikon TE2000, Tokyo, Japan) and flow cytometry (BD FACSCalibur, Ann Arbor, MI).

2.13. Statistical Analysis. All data are expressed as mean ± standard error of mean (SEM) and analyzed using one-way

ANOVA with Tukey's posttest analysis for comparison of intra- as well as intergroup variance. Statistical significance was taken when $P < 0.05$.

3. Results

3.1. GYY4137 Decreases Systolic Blood Pressure in SHR. SHR at 12 weeks of age treated with GYY4137 for 4 weeks at 25 or 50 mg/kg/d, but not at 10 mg/kg/d, exhibited a significant reduction in SBP as measured by the tail-cuff method (Figure 1(a)). However, there was no significant difference of EF or FS between groups (Figures 1(b)-1(c)), suggesting that GYY4137 does not affect cardiac systolic function in SHR of this age and that the lowering of SBP by GYY4137 is likely not explained by changes in cardiac function.

3.2. GYY4137 Attenuates Myocardial Fibrosis in SHR. Picrosirius red stains myocardial cells yellow and collagen fiber red under light microscopy. A few of perivascular collagen fibers were seen in the myocardium of WKY rat, while a number of perivascular collagens accumulated along the myocardial interstitial matrix of SHRs. CVF and PVCA/LA ratio, two important indexes to evaluate the degree of myocardial interstitial and perivascular fibrosis, respectively, were quantitated. SHR exhibited increased CVF and PVCA/LA as compared to WKY rats ($P < 0.01$). GYY4137 (25 mg/kg/d and 50 mg/kg/d) treatment for 4 weeks caused a reduction in collagen-specific staining as well as CVF and PVCA/LA ratio ($P < 0.01$, Figure 2). Collectively these data show that GYY4137 efficiently attenuates myocardial fibrosis in SHR.

3.3. GYY4137 Improves Collagen Property in SHR. Total hydroxyproline content and expression of collagen I and III mRNA were higher in LV myocardium of SHR ($P < 0.01$), suggesting accumulation of collagen in SHR as compared to WKY rats. After GYY4137 administration at doses of 25 mg/kg/d or 50 mg/kg/d, both hydroxyproline content and collagen I and III mRNA expression in myocardium decreased ($P < 0.05$ and $P < 0.01$, Figures 3(a)–3(c)). SHR at 16 weeks of age exhibited an increase in both non-cross-linked (soluble) collagen and cross-linked (insoluble) collagen in myocardium ($P < 0.01$), and GYY 4137 at 25 mg/kg/d or 50 mg/kg/d evoked a decrease in cross-linked collagen without an increase in non-cross-linked collagen, and indeed, at the 50 mg/kg/d dosage, GYY4137 elicited a decline in the ratio of cross-linked to non-cross-linked collagen ($P < 0.01$, Figures 3(d)-3(e)).

3.4. GYY4137 Inhibits Ang II-Induced Cardiac Fibroblasts Proliferation. To determine whether GYY4137 inhibits CF proliferation, neonatal rat CFs were preincubated with different concentrations of GYY4137 for 4 h and then exposed to Ang II (10^{-7} M) for an additional 24 h. CF number was evaluated by cell count analysis (represented as an OD value) and content of hydroxyproline. Ang II increased CF number and hydroxyproline concentration in the medium, whilst preincubation with GYY4137 for 4 h inhibited CF proliferation ($P < 0.05$, Figures 4(a)-4(b)). Flow cytometry evaluation suggested that GYY4137 reduced the number of fibroblasts in the S phase of the cell cycle following Ang II-stimulation ($P < 0.05$, Figure 4(c)).

3.5. GYY4137 Reduces Ang II-Induced Collagen Synthesis in Cardiac Fibroblasts. Increase in collagen types I and type III is the predominant phenotype in cardiac fibrosis [23]. We therefore examined whether GYY4137 suppresses collagen synthesis after Ang II stimulation. Compared with medium-treated vehicle control, Ang II increased expression of collagen I and III at both the mRNA and protein levels, whose effect was attenuated by GYY4137 pretreatment ($P < 0.05$, Figures 5(a)–5(d)). Immunofluorescence staining was also carried out to further confirm that GYY4137 indeed inhibits collagen I and collagen III synthesis in Ang II-stimulated CFs (Figure 5(e)).

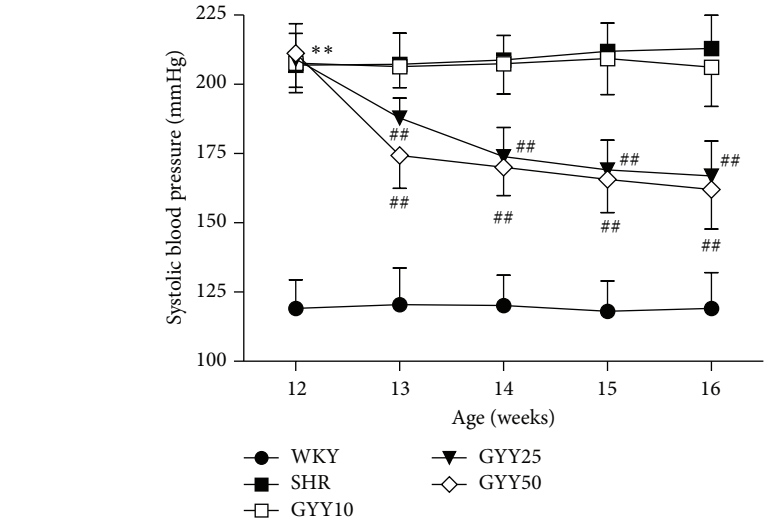
3.6. GYY4137 Blocks Ang II-Induced α-SMA and TGF-β1/Smad2 Expression in Cardiac Fibroblasts. Expression of α-SMA, one of the most robust markers of myofibroblast differentiation [24], was also measured by immunofluorescence. Expression of α-SMA was enhanced after Ang II stimulation, and this effect was attenuated by GYY4137 (50 μM) pretreatment (Figure 6).

TGF-β/Smad signal pathway facilitates the progression of myocardial fibrosis [25]. As expected, exposure of CFs to Ang II enhanced expression of TGF-β1 and phosphorylation of Smad2. Moreover, treatment with GYY4137 decreased TGF-β1 expression as well as Smad2 phosphorylation ($P < 0.01$, Figure 7).

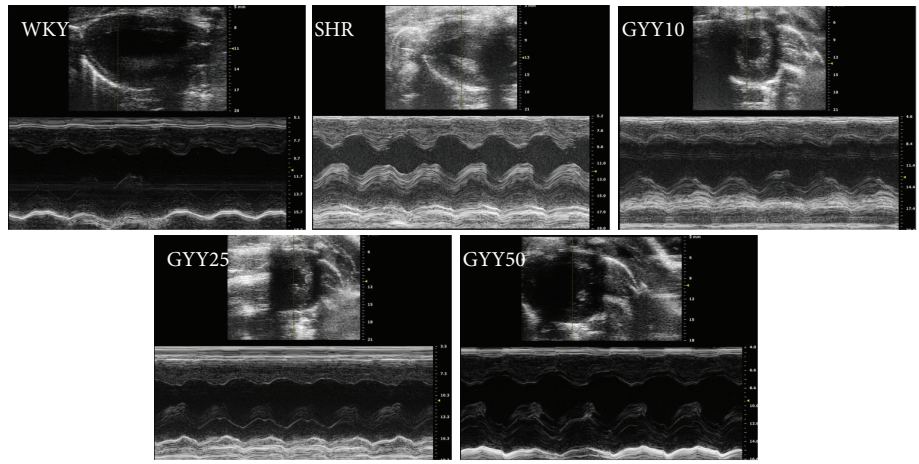
3.7. GYY4137 Suppresses Ang II-Induced Oxidative Stress in Cardiac Fibroblasts. Compared with vehicle-treated control, Ang II induced severe oxidative stress in CFs as evidenced by increased intensity of DCFH fluorescence both on fluorescence microscopy and flow cytometry. These parameters were effectively restored by pretreatment with GYY4137 ($P < 0.05$, Figure 8).

4. Discussion

It has previously been reported that H_2S protects against fibrosis in different systems. In a rat model of bleomycin-induced pulmonary fibrosis, the H_2S donor diallyl sulfide attenuated excessive collagen production and extracellular matrix (ECM) protein expression [26]. In a rat model of unilateral ureteral obstruction, NaHS inhibited renal fibrosis by attenuating the production of collagen and of ECM, as well as the expression of α-SMA [27]. In mouse liver fibrosis induced by carbon tetrachloride, H_2S blocked cell cycle activation and proliferation of stellate cells, thereby lessening liver fibrosis and reducing the accumulation of ECM [28]. Each of these examples suggests that H_2S has antifibrotic effect in different tissues and organs. Moreover, Shi et al. found that chronic (3 months) NaHS treatment decreased interstitial fibrosis in SHR [29]. On the other hand, it is well established that NaHS promotes apoptotic cell death of cultured fibroblasts and smooth muscle cells and additionally releases copious amounts of H_2S over a short time frame



(a)



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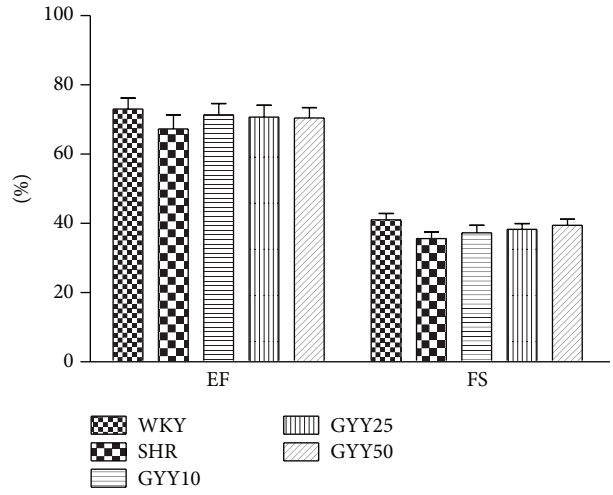

(c)

Figure 1: Effect of GYY4137 on systolic blood pressure and cardiac systolic function in SHR. Male SHR and WKY rats at 12 weeks of age were given GYY4137 by intraperitoneal injection at doses of 10 mg/kg/day (GYY10), 25 mg/kg/day (GYY25), or 50 mg/kg/day (GYY50) for 4 weeks. (a) Systolic blood pressure (SBP) was measured every week by the tail-cuff method. (b) Representative 2D M-mode echocardiograms in rat hearts after GYY4137 treatment for 4 weeks. (c) Quantitative analysis of left ventricular ejection fraction (EF) and fractional shortening (FS) with echocardiography. Plots represent mean ± SEM; $n = 8$–10. Statistical significance: $^{**}P < 0.01$ compared with WKY; $^{\#\#}P < 0.01$ compared with SHR.

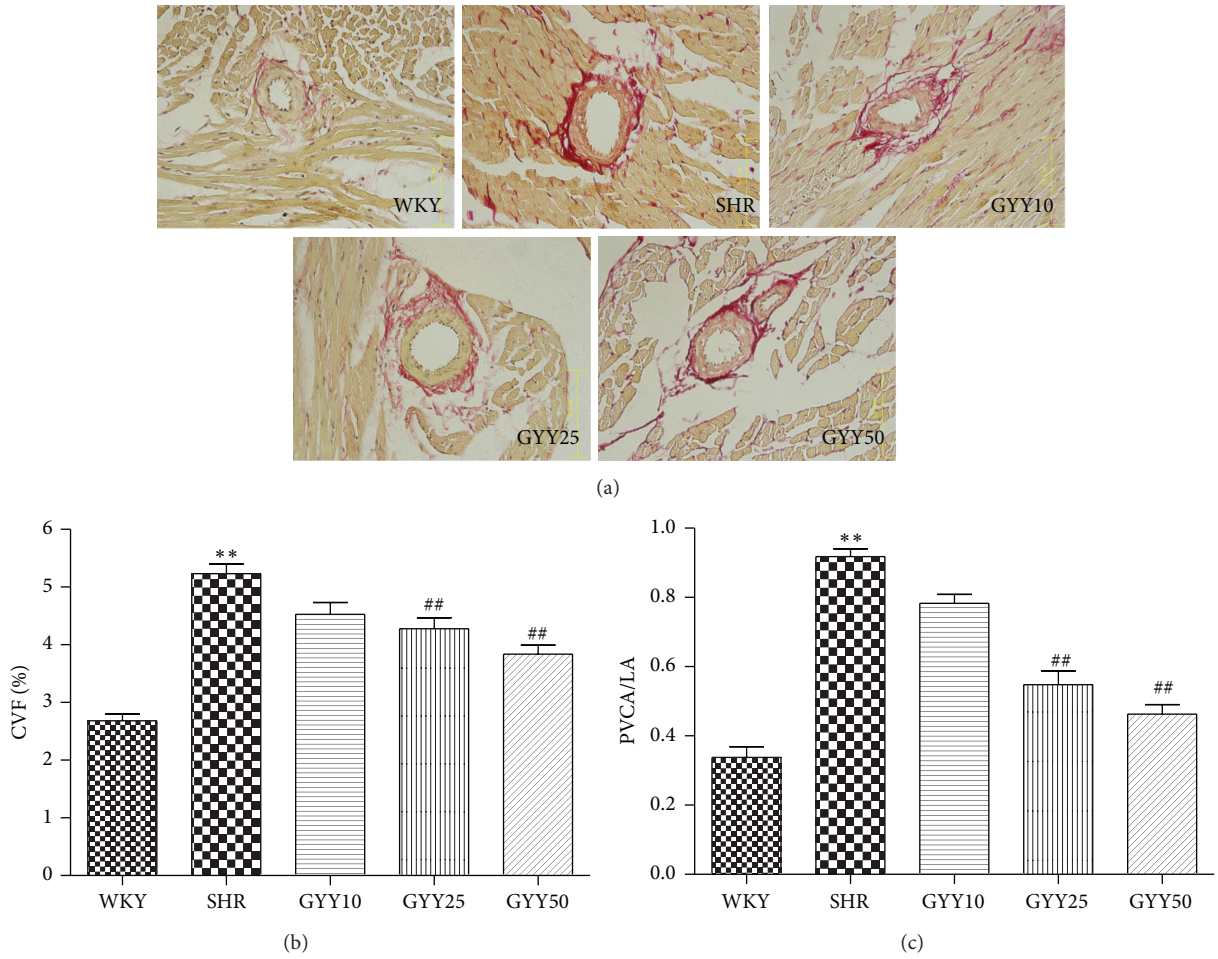

Figure 2: Effect of GYY4137 on myocardial fibrosis in SHR. Male SHR and WKY rats at 12 weeks of age were given GYY4137 by intraperitoneal injection at doses of 10 mg/kg/day (GYY10), 25 mg/kg/day (GYY25), or 50 mg/kg/day (GYY50) for 4 weeks. (a) Histological examination with picrosirius red staining of myocardium. (b) Collagen volume fraction (CVF) in left ventricle (LV) interstitial regions. (c) The ratio of perivascular collagen area (PVCA) to lumen area (LA) in perivascular regions. Plots represent mean ± SEM; $n = 10$. Statistical significance: $^{**}P < 0.01$ compared with WKY; $^{\#\#}P < 0.01$ compared with SHR.

(seconds), which does not effectively mimic physiological concentrations of H_2S *in vivo* and might be harmful [30]. However, previous studies have not evaluated specifically for possible toxicity of NaHS, and therefore whether the observed antifibrotic effect was simply a manifestation of H_2S toxicity was unclear. Since NaHS is not an ideal H_2S donor, several other H_2S related compounds, including GYY4137 (a slow-releasing H_2S donor), have been synthesized in order to better evaluate the physiological role(s) of H_2S. When incubated in aqueous buffer (pH 7.4, 37°C), release of H_2S from GYY4137 was slow. H_2S increased for 15 minutes and then keeps a stabilized concentration up to 75 minutes. After administration (intravenous or intraperitoneal) of GYY4137 to anesthetized rats, plasma H_2S concentration was increased at 30 minutes and remained elevated over the 180-minute time course of the experiment [20]. Moreover, GYY4137 did not cause detectable cytotoxicity or alter the cell cycle profile or p53 expression of cultured rat vascular smooth muscle cells; additionally, it did not trigger signaling pathways leading to cell death; and chronic treatment of conscious animals with GYY4137 at 133 μmol/kg reduced SBP in SHR [20]. In the present work, daily GYY4137 administration for 4 weeks effectively reduced the degree of myocardial fibrosis in 16-week SHR as evidenced by collagen-specific staining. Not only total myocardial collagen amount but also perivascular fibrosis was also inhibited by GYY4137. These data demonstrate that GYY4137 has a powerful antifibrotic effect in the myocardium.

Excess collagen accumulation increases myocardial stiffness, impairs ventricular diastolic and systolic function, results in cardiac electrophysiological disorders, and eventually leads to heart failure; however, collagen deposition is also vital in maintaining myocardial structure and systolic heart function [31]. Therefore, it is important to study the details of alterations in collagen amount to comprehensively evaluate possible effects on myocardial fibrosis. Increase in myocardial cross-linked collagen, not simply total collagen, contributes to enhancement of stiffness, and the accumulation of cross-linked collagen decreases cardiac compliance in SHR. Cross-linked collagen is relatively resistant to degradation

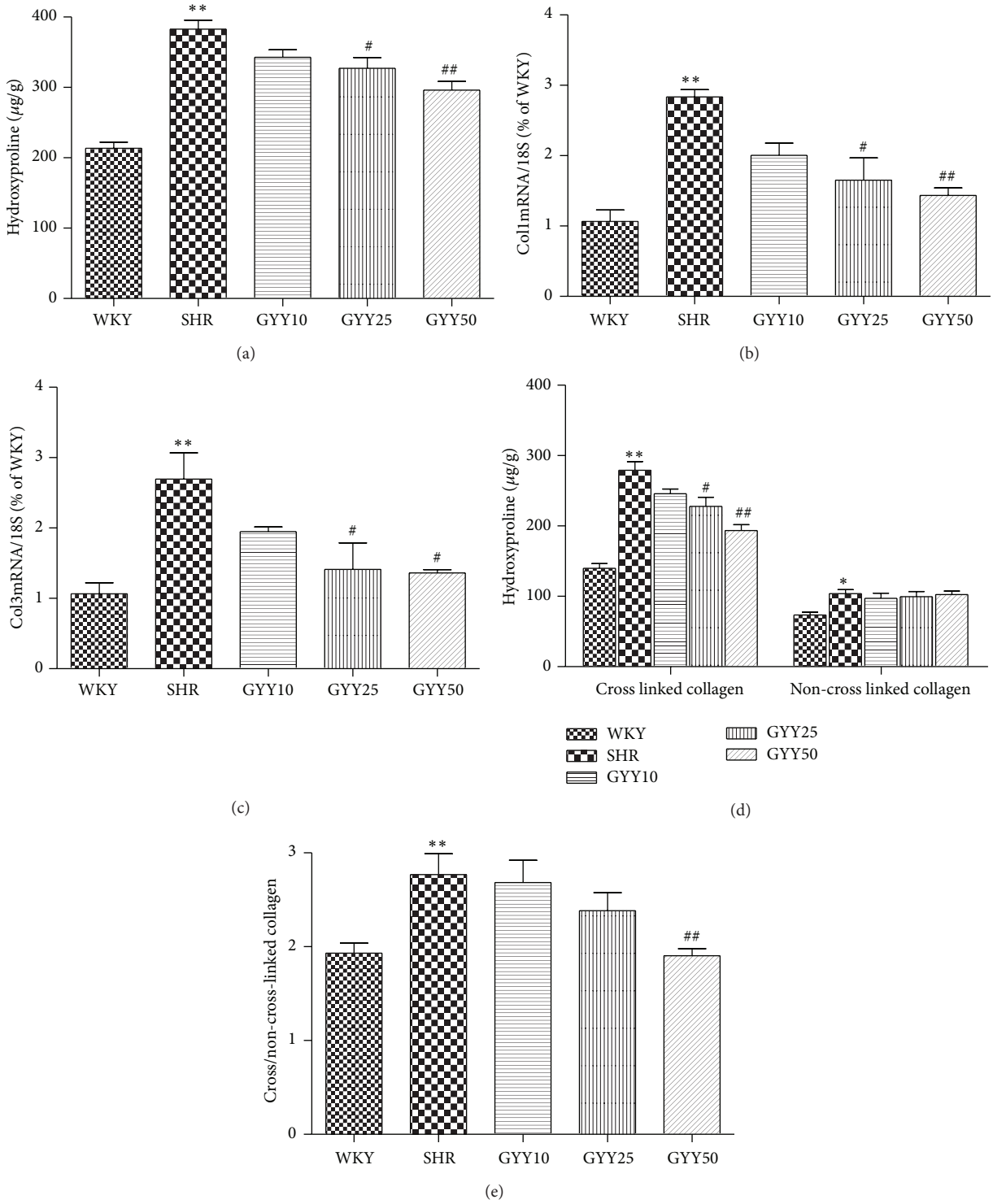

Figure 3: Effect of GYY4137 on collagen property in SHR. (a) Hydroxyproline concentrations in myocardium. (b-c) Quantification of collagen I and collagen III mRNA expression in myocardium was carried out with real-time RCR. (d) The amount of cross-linked and non-cross-linked collagen in the myocardium was evaluated as hydroxyproline content. (e) The ratio between cross-linked and non-cross-linked collagen was used as an index of the degree of collagen cross-linking. Plots represent mean ± SEM; $n = 10$. Statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$ compared with WKY; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with SHR.

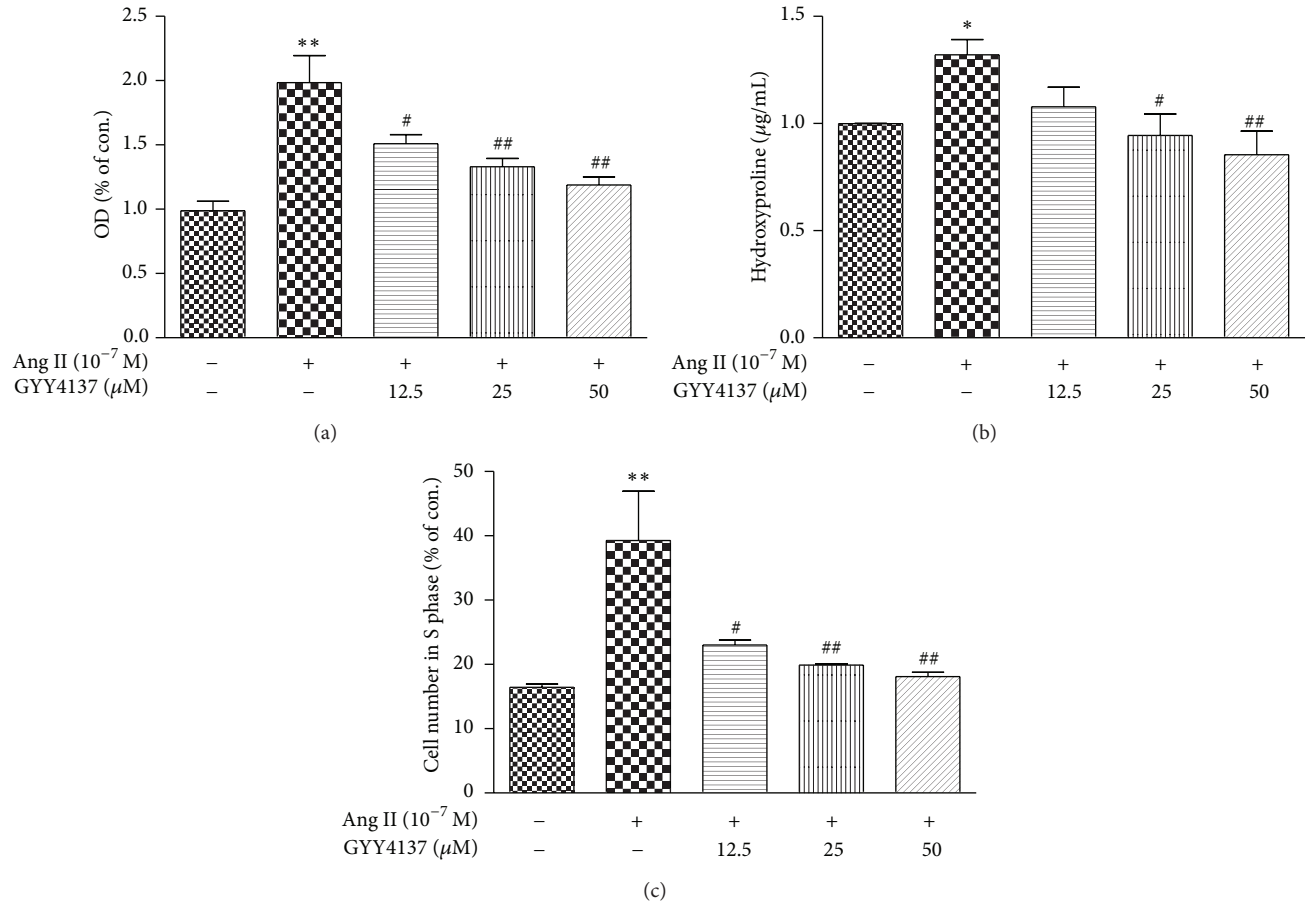

Figure 4: Effect of GYY4137 on Ang II-induced cardiac fibroblasts proliferation. Neonatal rat cardiac fibroblasts were pretreated with different concentrations of GYY4137 (12.5 μM, 25 μM, and 50 μM) for 4 h followed by Ang II (10^{-7} M) stimulation for an additional 24 h. (a) The number of cells was represented as an OD value using a cell count assay. (b) Content of hydroxyproline in cell culture medium was determined. (c) Cell cycle was analyzed by flow cytometry. Plots represent mean ± SEM from 3–5 independent experiments. Cells treated with culture medium served as vehicle control (con.). Statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$, compared with con. group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with Ang II stimulation alone.

by proteases, which accelerates matrix accumulation [32]. In our study, increased accumulation of both cross-linked and non-cross-linked collagen was seen in myocardium of SHR; however, only cross-linked but not non-cross-linked collagen was affected by GYY4137; this might be expected to give rise to favourable functional effects in terms of reduced myocardial stiffness. This is the first study, to our knowledge, to demonstrate an inhibitory effect of H_2S on cross-linked collagen content.

Fibroblasts are the main source of ECM during the process of myocardial fibrosis [33]. Ang II, one of the most important humoral factors which accelerate cardiac fibrosis, was used to stimulate CF proliferation. Fibrillar collagens, types I and III, are major structural proteins of the myocardial collagen matrix. Collagen type I, constituting 85% of total collagen, is a rigid protein (the tensile strength of collagen is 50–100 MPa and approaches that of steel) and is usually present in the form of thick fibers. The concentration of collagen type I determines the tissue stiffness of the myocardium. Collagen type III is more distensible and forms a fine reticular network. Myocardial fibrosis exhibits excess collagen and disarranged architecture in myocardium [34]. Our study found that Ang II increased numbers of fibroblasts as well as secretion of collagen, which were suppressed by GYY4137 administration. Moreover, levels of collagen types I and III were both attenuated by GYY4137.

It has been reported that NaHS increases the percentage of hepatic stellate cells (HSCs, the major cell type in hepatic fibrosis) in the G1 phase, whilst decreasing the percentage of cells in the S phase correspondingly [28]. NaHS also retards breast cancer cells transitioning from G1 to G0 phase and inhibits breast cancer cell division [35]. A 48 h treatment of diallyl sulfide induced G0/G1 cell cycle arrest in HeLa human cervical cancer cells [36]. These studies showed the potential ability of H_2S to inhibit cell proliferation by regulating the cell cycle and hence DNA synthesis. In the present work, we found that cells in S phase increased after Ang II stimulation, which was rescued by GYY4137, indicating that GYY4137 might be able to inhibit cardiac fibroblast proliferation by reducing the number of cells in S phase.

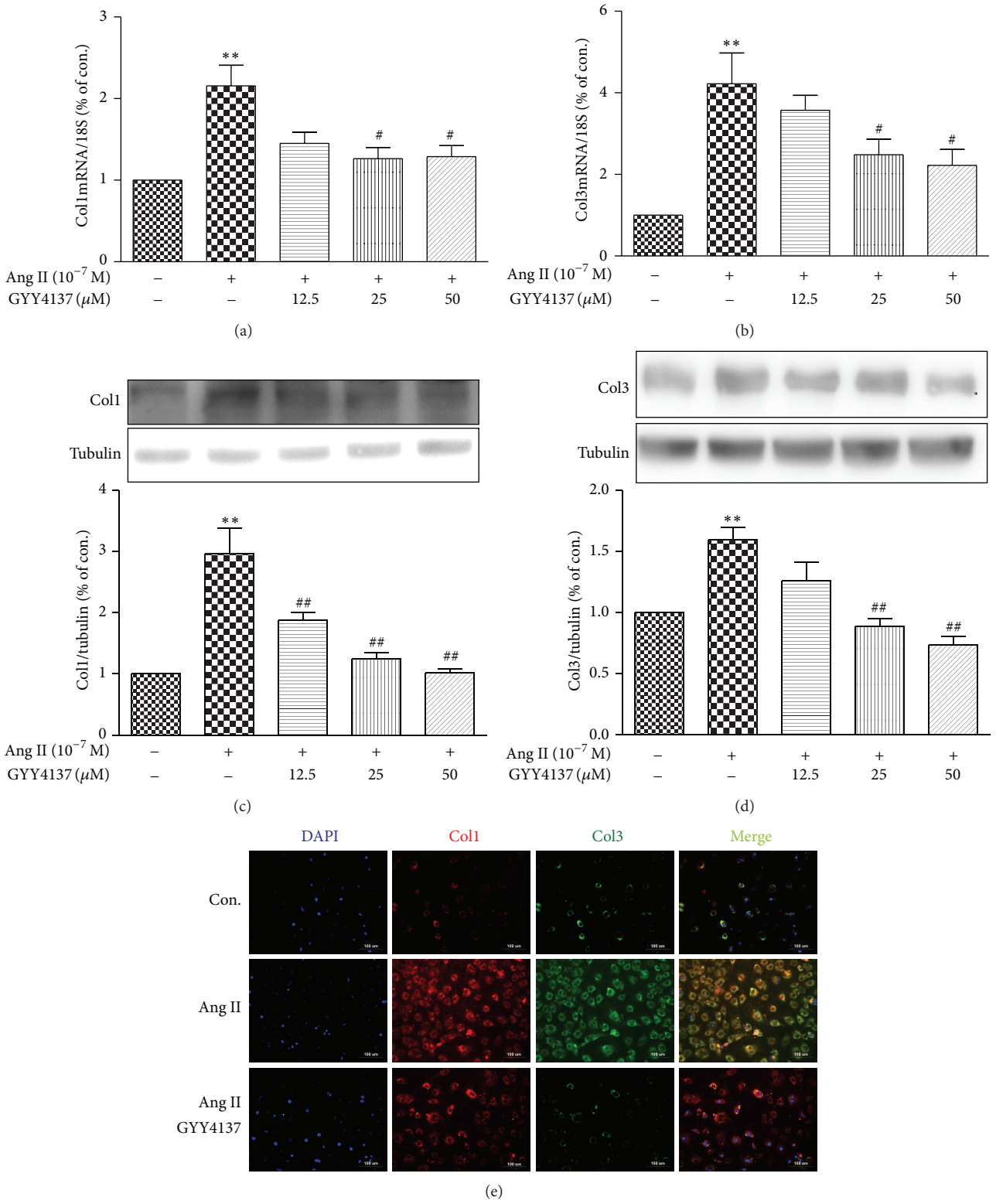

FIGURE 5: Effect of GYY4137 on Ang II-induced collagen synthesis in cardiac fibroblasts. Neonatal rat cardiac fibroblasts were pretreated with different concentrations of GYY4137 for 4 h followed by Ang II (10^{-7} M) stimulation for an additional 24 h. (a-b) Quantification of collagen I and collagen III mRNA expression in myocardium was carried out with real-time RCR. (c-d) Cell lysates were tested for collagen I and collagen III protein expression by western blotting. Tubulin was probed as a loading control. (e) Cellular collagen I and III were visualized using Alexa Fluor 555 or Alexa Fluor 488 conjugated IgG, respectively, by immunofluorescence staining. The nuclei were counterstained with DAPI (scale bar: 100 μm). Plots represent mean ± SEM from 4–9 independent experiments. Cells treated with culture medium served as vehicle control (con). Statistical significance: $^{**}P < 0.01$, compared with con. group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with Ang II stimulation alone.

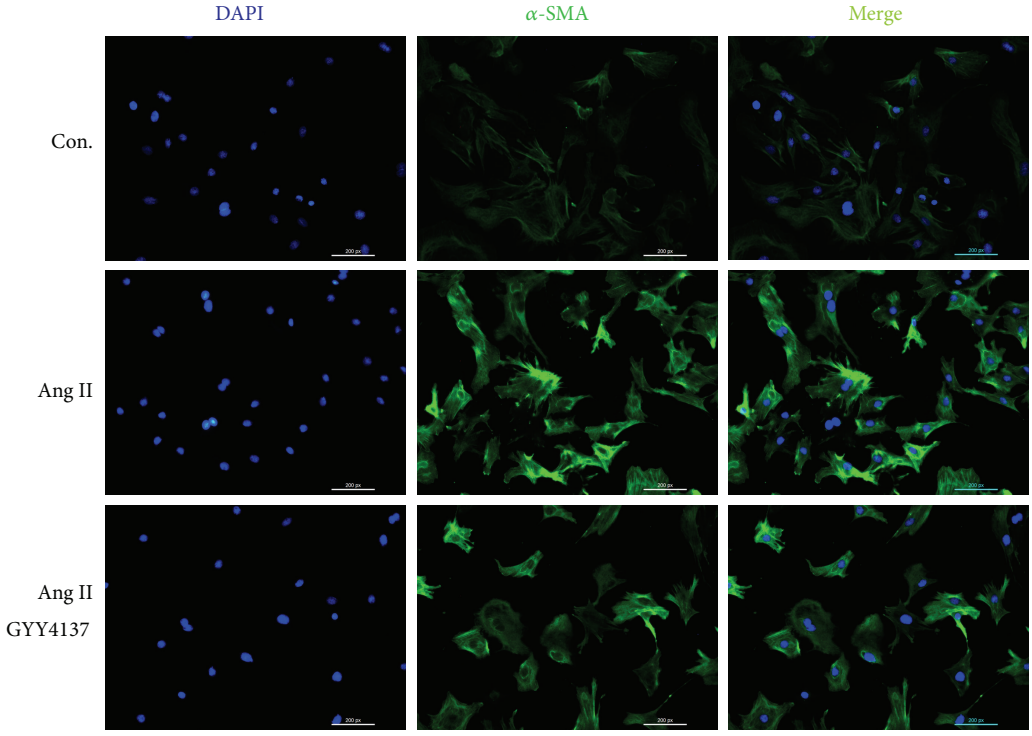

Figure 6: Effect of GYY4137 on α-SMA expression in cardiac fibroblasts. Neonatal rat cardiac fibroblasts were pretreated with different concentrations of GYY4137 for 4 h followed by Ang II (10^{-7} M) stimulation for an additional 24 h. Cellular α-SMA was visualized using Alexa Fluor 488 conjugated IgG by immunofluorescence staining. The nuclei were counterstained with DAPI (scale bar: 100 μm).

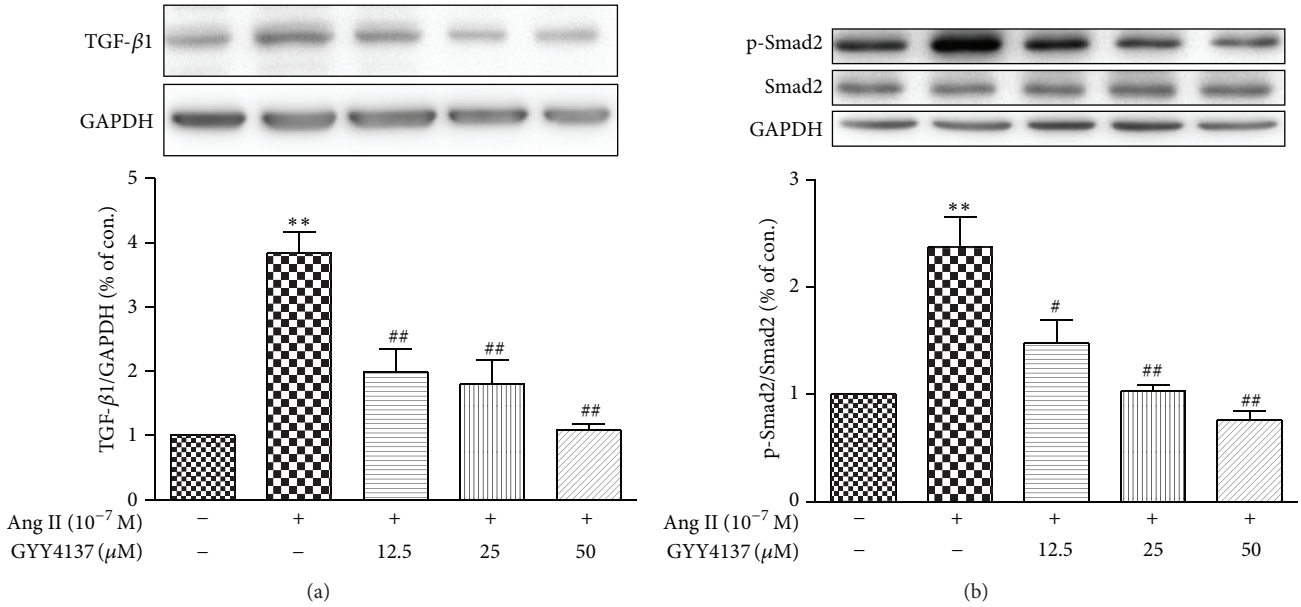

Figure 7: Effect of GYY4137 on TGF-β1/Smad2 expression in cardiac fibroblasts. Neonatal rat cardiac fibroblasts were pretreated with different concentrations of GYY4137 for 4 h followed by Ang II (10^{-7} M) stimulation for an additional 24 h. Cell lysates were tested for TGF-β1 (a) and Smad2 protein (b) expression by western blotting. GAPDH was probed as a loading control. Plots represent mean ± SEM from 4 independent experiments. Cells treated with culture medium served as vehicle control (con.). Statistical significance: $^{**}P < 0.01$, compared with con. group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with Ang II stimulation alone.

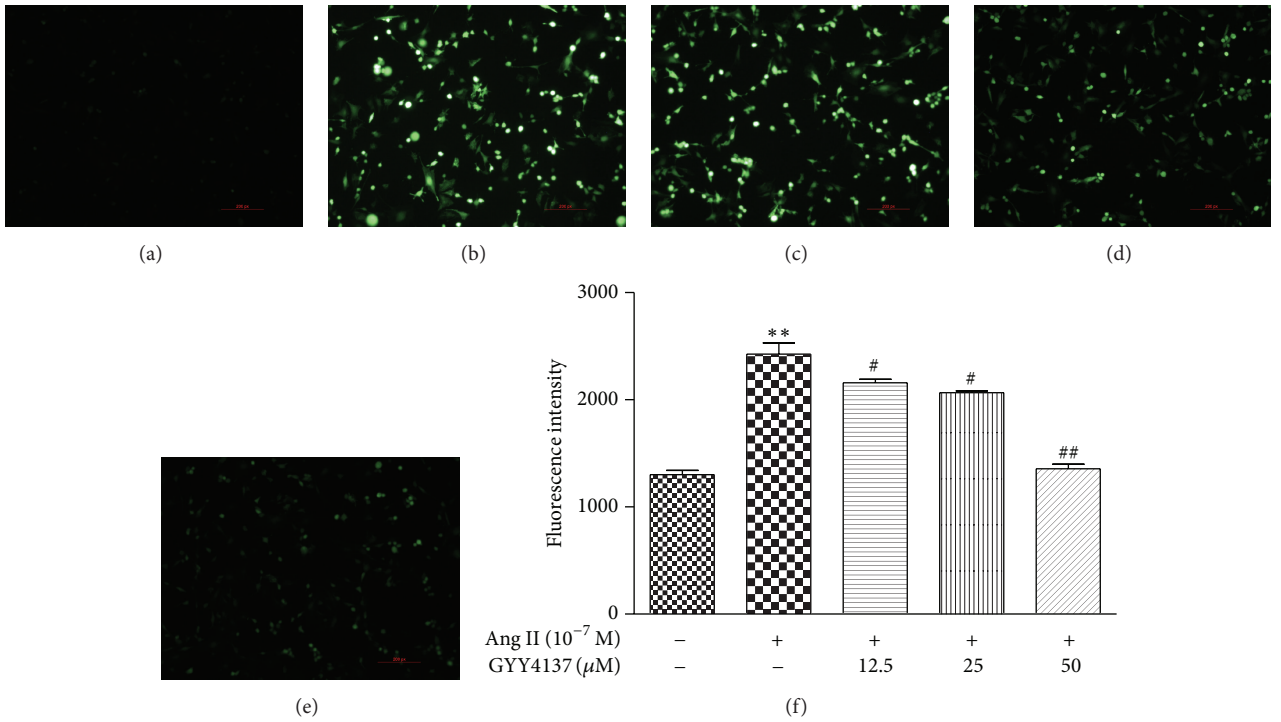

Figure 8: Effect of GYY4137 on Ang II-induced oxidative stress in cardiac fibroblasts. Neonatal rat cardiac fibroblasts were pretreated with different concentrations of GYY4137 for 4 h followed by Ang II (10^{-7} M) stimulation for an additional 24 h. (a–e) Levels of ROS in cardiomyocytes were measured by DCFH-DA (10 μM) fluorescence staining (scale bar: 100 μm). (a) Cells treated with culture medium served as vehicle control (con.); (b) Ang II stimulation alone; (c-d) cells pretreated GYY4137 (12.5 μM, 25 μM, and 50 μM), respectively, for 4 h followed by Ang II (10^{-7} M) stimulation for an additional 24 h. (f) Fluorescence was estimated directly by flow cytometry. Plots represent mean ± SEM from 4 independent experiments. Cells treated with culture medium served as vehicle control (con.). Statistical significance: $^{**}P < 0.01$, compared with con. group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with Ang II stimulation alone.

Myofibroblasts have a particular ultrastructure between smooth muscle cells and fibroblasts, manifesting characteristics of both. Compared with ordinary fibroblasts, myofibroblasts, as the main source of ECM deposition, have greater ability to proliferate and secrete collagen [37]. α-SMA is one of the most robust markers of myofibroblast differentiation [38]. Previous studies have confirmed that H_2S can inhibit the expression of α-SMA in obstructive nephropathy-induced renal fibrosis and carbon tetrachloride-induced liver fibrosis [27, 39]. Moreover, H_2S attenuates TGF-β1-stimulated human atrial fibroblast proliferation via moderating their differentiation towards myofibroblasts [16]. Notably, in our study, α-SMA was reduced by GYY4137, suggesting that inhibition of fibroblast conversion into myofibroblasts by H_2S may act as a vital mechanism to antagonize Ang II-induced profibrotic effects in CFs.

The molecular mechanisms regulating myocardial fibrosis are complex and as yet incompletely defined. Increasing evidence suggests that TGF-β1 signaling pathways play an important role in collagen synthesis [40]. It has been shown that H_2S regulates the TGF-β1 pathway in multiple cell lines and tissues. For example, supplementation with H_2S attenuates high-glucose-induced renal mesangial cells proliferation rate and production of TGF-β1 [41]. NaHS treatment also attenuates TGF-β1 levels in unilateral ureteral obstruction-induced kidney fibrosis in mice [42]. In the liver fibrosis model induced by carbon tetrachloride, exogenous H_2S inhibits the expression of TGF-β1 and improves the liver fibrosis [28]. Moreover, NaHS treatment for 9 weeks prevented myocardial collagen remodeling in SHR by a mechanism involving inhibition of collagen synthesis via TGF-β1/Smad2/3 signaling pathway [43]. Our data show that GYY4137 inhibits the expression of TGF-β1 and Smad2 phosphorylation in Ang II-stimulated CFs. This action might therefore be responsible, at least in part, for the attenuation of cardiac fibroblast proliferation.

H_2S may exert cardioprotection via its antioxidative effects. Thus, H_2S protects against from myocardial ischemia/reperfusion injury by reducing ROS production and accumulation in the myocardium [44]. H_2S also suppresses high glucose-induced cardiomyocyte apoptosis by attenuating ROS generation [45, 46]. Redox-sensitive signaling pathways play an important role in myocardial fibrosis and tempol (a powerful antioxidant) attenuates fibrosis in a ROS-inhibition-dependent manner in the renal fibrosis model induced by unilateral ureteral ligation [47]. Another study demonstrated that H_2S was protective against gentamicin-induced nephrotoxicity in rats due to its antioxidant effect [48]. H_2S also ameliorated cardiac fibrosis by decreasing oxidative stress in chronic heart failure [49]. However, the detailed mechanism by which H_2S exerts its antioxidative effects is unclear. H_2S can elicit vasoprotection by both

scavenging O_2^- and reducing vascular NADPH oxidase-derived O_2^- production, in an acute oxidative stress model with xanthine oxidase or with the O_2^- generator pyrogallol [50]. H_2S has also been found to inhibit H_2O_2-mediated mitochondrial dysfunction in human endothelial cells by preserving the activities and protein expression levels of the antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase, and glutathione-S-transferase [51]. H_2S also exerts neuroprotective effect by enhancing uncoupling protein 2-mediated antioxidation and subsequently suppressing ROS-triggered endoplasmic reticular stress [52]. H_2S increases the nuclear localization of NF-E2 related factor 2 (Nrf2), a transcription factor that regulates the gene expression of a number of antioxidants (including heme oxygenase-1 and thioredoxin 1) during myocardial ischemia/reperfusion injury and renal interstitial fibrosis in diabetic rats [53, 54]. In accordance with these studies, our data demonstrate that the protective effect of GYY4137 against cardiac fibrosis depends, at least partly, on its antioxidant ability. However, similar to other H_2S donors, the antioxidative effect of GYY4137 might be related to both direct scavenging activity and indirect regulation of antioxidant enzymes expression.

There are some limitations to our study. As stated above, the detailed antioxidative mechanism of GYY4137 in the context of myocardial fibrosis and Ang II-stimulated cardiac fibroblast proliferation is unclear and needs to be elucidated further. In addition, whether GYY4137 regulates ECM degradation, another important process in myocardial fibrosis, is not known. How GYY4137 decreases the expression of TGF-β1 also needs to be better defined.

In conclusion, we provide evidence that GYY4137 decreases myocardial fibrosis, which may be related to inhibition of oxidative stress, blockage of TGF-β1/Smad2 signaling pathway, and decrease in expression of α-SMA in cardiac fibroblasts. The present data raise the possibility that H_2S may be of value in the treatment of cardiac fibrosis and related cardiovascular diseases which are underpinned by oxidative stress.

Conflict of Interests

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

Authors' Contribution

Guoliang Meng, Jinbiao Zhu, Yujiao Xiao, Zhengrong Huang, and Yuqing Zhang contributed equally to this study.

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Review Article

Interaction of H_2S with Calcium Permeable Channels and Transporters

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A growing amount of evidence has suggested that hydrogen sulfide (H_2S), as a gasotransmitter, is involved in intensive physiological and pathological processes. More and more research groups have found that H_2S mediates diverse cellular biological functions related to regulating intracellular calcium concentration. These groups have demonstrated the reciprocal interaction between H_2S and calcium ion channels and transporters, such as L-type calcium channels (LTCC), T-type calcium channels (TTCC), sodium/calcium exchangers (NCX), transient receptor potential (TRP) channels, β-adrenergic receptors, and N-methyl-D-aspartate receptors (NMDAR) in different cells. However, the understanding of the molecular targets and mechanisms is incomplete. Recently, some research groups demonstrated that H_2S modulates the activity of calcium ion channels through protein S-sulfhydration and polysulfide reactions. In this review, we elucidate that H_2S controls intracellular calcium homeostasis and the underlying mechanisms.

1. Introduction

Hydrogen sulfide (H_2S) was thought for hundreds of years to be a toxic gas that smelled like rotten eggs, but the gas is now believed to be a molecule involved in intensive physiological and pathological processes [1], such as protecting the heart against acute myocardial infarction [2, 3] and ischemia/hypoxia injury, regulating blood pressure [4], mediating smooth-muscle relaxation [5], and inhibiting insulin release and renin activity [6, 7]. H_2S, as an endogenous gasotransmitter, can be mainly generated by pyridoxal-5′-phosphate- (PLP-) dependent cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), which interconvents the sulfuration from intracellular L-methionine and L-cysteine to produce H_2S [8]. In addition, 3-mercaptopyruvate sulfurtransferase (3-MST) and cysteine aminotransferase (CAT) produce H_2S from cysteine through the combined actions of both enzymes [9].

An increasing amount of evidence has demonstrated that H_2S regulates cellular biological signaling through modulating calcium ion channels and their related transporters [10, 11], such as L-type calcium channels (LTCC), T-type calcium channels (TTCC), sodium/calcium exchangers (NCX), transient receptor potential (TRP), β-adrenergic receptors, and NMDA receptors. This review presents the current research on H_2S to better understand its regulation of calcium channels, with a special emphasis on mechanisms.

2. The Regulatory Mechanism of H_2S Interacting with Calcium ion Channels

2.1. Voltage-Dependent Calcium Channels (VDCC). Ca^{2+} serves as an important second messenger in both excitable and nonexcitable cells. Voltage-dependent calcium channels (VDCC), store-operated calcium channels (SOCs), and

G-protein coupled receptors (GPCRs) are responsible for calcium influx from extracellular fluids. Alterations in intracellular calcium levels trigger physiological responses, including cardiac muscle contraction, vascular dilatation, hormone secretion, and neurotransmitter release [12–16].

The family of VDCCs includes L-, T-, N-, and P/Q-subtypes, which differ in their cellular and subcellular distributions and functional properties [17, 18]. For example, T-type calcium channels (TTCCs) are involved in regulating cellular excitability [19], N and P/Q type channels mediate fast evoked neurotransmitter release [14], and L-type calcium channels (LTCCs) mediate excitation-contraction coupling in the heart and muscles, insulin secretion, and calcium-dependent gene transcription [20].

LTCCs are integral in excitation/contraction coupling and are one of the main channels for extracellular Ca^{2+} influx in myocardial cells. In 2002, Zhao and Wang first reported that H_2S could directly inhibit calcium influx from LTCCs in smooth-muscle cells [21]. Moreover, in 2009, Sun et al. further demonstrated that H_2S, as a novel inhibitor of LTCC, has negative inotropic effects in rat cardiomyocytes [22]. In a recent study, Avanzato et al. investigated the role of H_2S in regulating VDCCs and the related functional effects on the cardiomyoblast cell line H9c2. They found that H_2S inhibits LTCCs and TTCCs in H9c2. Pretreatment with NaHS (a donor of H_2S) prevented cell death via H_2O_2 through inhibiting LTCCs. Their results were the first to demonstrate that H_2S protects rat cardiomyoblasts against oxidative stress through inhibition of LTCCs [23]. In addition, Tang et al. suggested that exogenous and endogenous H_2S inhibited pancreatic insulin secretion by inhibiting LTCCs activity. They confirmed that NaHS reversibly decreased LTCC current density in a concentration-dependent manner in CSE WT pancreatic beta cells. Furthermore, they observed that DL-propargylglycine (an inhibitor of CSE) increased the basal LTCC activity in beta cells from CSE WT mice, but not in pancreatic beta cells from CSE-KO mice. Pancreatic beta cells from CSE-KO mice displayed a higher LTCCs density than those from WT mice. These results suggested that a novel mechanism for regulating insulin secretion was related to the CSE/H_2S system, which controlled LTCC activity [24]. Recently, some data showed that exogenous and endogenous H_2S can modify cystein residues of different proteins through S-sulfhydration. The –SH from sulfhydryl donor is transformed to free cysteine sulfhydryl and forms covalent persulfide (–SSH) [25, 26]. In 2012, Zhang and his coworkers showed that NaHS inhibited the peak amplitude of the L-type calcium current in a concentration-dependent manner and could be partly inhibited by the oxidant sulfhydryl modifier diamide (DM). They explained that dithiothreitol (DTT), a reductant that transforms disulfide bridges into sulfhydryl groups in cysteine-containing proteins, could significantly reverse NaHS-induced inhibition of calcium current from LTCCs. Their results suggested that H_2S inhibited L-type calcium currents depending on the sulfhydryl group in rat cardiomyocytes [27] (Figure 1).

TTCCs are classified into three T-type channel subtypes, Cav3.1, Cav3.2, and Cav3.3. There have been reports about the T-type channels being activated by H_2S in neurons [28–30]. In the pain pathways, Cav3.2 in the peripheral terminals of nociceptors and dorsal horn spinal neurons appears to promote peripheral nociception and central nociceptive sensitization [28]. H_2S may function as a neuromodulator in sensory transmission. There is evidence that chemotherapy-induced neuropathic pain is blocked by ethosuximide, which is known to block TTCCs. Systemic administration of DL-propargylglycine and β-cyanoalanine, irreversible and reversible inhibitors of CSE, respectively, also abolished neuropathic pain. Okubo et al. demonstrated that $Ca_v3.2$ and CSE at the protein level are upregulated, which induced a significant increase in H_2S level. H_2S facilitated pain sensation by targeting $Ca_v3.2$ TTCCs. The $H_2S/Ca_v3.2$ pathway appears to play a role in the maintenance of surgically evoked neuropathic pain [31]. Intraplantar administration of NaHS causes mechanical hyperalgesia in rats, an effect reversed by mibefradil (a T-type Ca^{2+} channel blocker), and also enhances membrane currents through the TTCC in NG 108-15 cells and mouse dorsal root ganglion neurons [29, 30]. Their data suggested that spinal and peripheral NaHS/H_2S facilitates the expression of Cav3.2 TTCCs in the primary afferent and/or spinal nociceptive neurons, leading to sensitization of nociceptive processing and hyperalgesia [31]. Sekiguchi et al. demonstrated that endogenous and exogenous hydrogen sulfide facilitate T-type calcium channel currents in Cav3.2-expressing HEK293 cells [32]. In contrast, Elies et al. reported an inhibitory effect with high doses of NaHS on Cav3.2-overexpressing HEK cells [33]. Their data were the first preliminary evidence that H_2S negatively modulates endogenously expressed TTCCs in a myoblast cell line. In spite of the opposite opinion in the effects of NaHS on TTCCs in different research groups, H_2S regulating the activity of TTCC has been confirmed widely. However, most of the evidence suggests that H_2S elevates the activities of TTCCs and increases the amplitudes of T-type Ca^{2+} currents in different cell lines.

2.2. β-Adrenergic Receptors. Cardiac excitation-contraction coupling is under the direct control of the adrenergic nervous system. In the heart, the β-adrenergic receptor (AR), a G-protein coupled receptor, activates the associated adenylyl cyclase (AC)-cAMP-protein kinase A (PKA) pathway [34]. β-Adrenoceptor-coupled stimulatory G proteins lead to an increased intracellular cAMP level and stimulate protein kinase A (PKA) to mediate phosphorylation of LTCCs and finally increase contractile function [35–37]. Some reports have observed that H_2S content in the heart was significantly reduced in a cardiac ischemia [38] and overstimulation of the β-adrenergic system by isoproterenol (ISO, β-adrenoceptor agonist) models [39]. Yong and his coworkers revealed that H_2S may negatively modulate β-adrenoceptor function via inhibiting adenylyl cyclase activity [40]. They found that ISO (10^{-9}–10^{-6} M), in a concentration-dependent manner, increased the twitch amplitude of ventricular myocytes, which was attenuated by NaHS (10^{-5}–10^{-3} M) in a dose-dependent manner. The amplitudes and maximal velocities (±dL/dt) for myocyte twitch

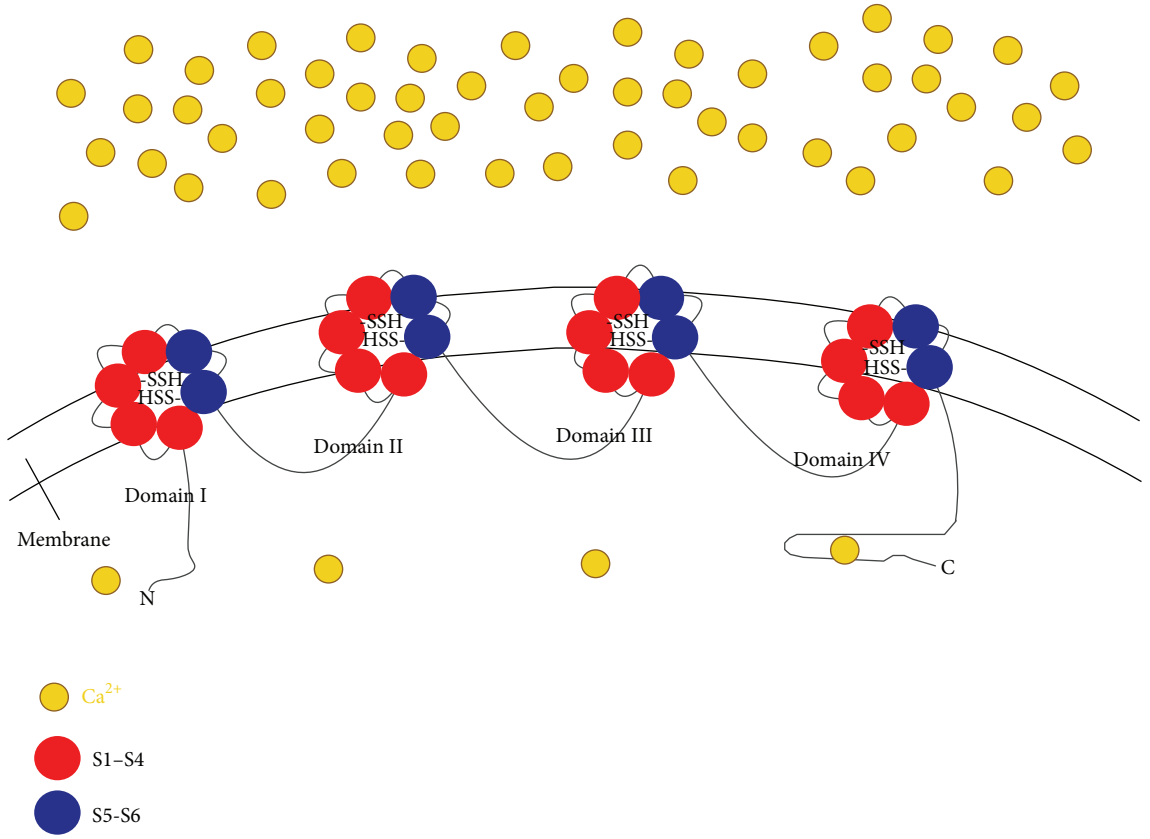

FIGURE 1: Hydrogen sulfide regulating L-type calcium channels by S-sulfhydration. LTCC consists of a pore-forming α subunit which contains four homologous domains (I–IV), each with six transmembrane segments (S1–S6). The S1–S4 segments are the voltage sensor, and the S5-S6 segments form the channel pore and selectivity filter. The cartoon demonstrated that H_2S modifies the –SH from sulfhydryl donor which is transformed to free cysteine sulfhydryl and forms covalent persulfide (–SSH).

and EI-$[Ca^{2+}]_i$ transient amplitudes were enhanced by ISO, forskolin (an adenylyl cyclase activator), 8-bromoadenosine-3′,5′-cyclic monophosphate (an activator of protein kinase A), and Bay K-8644 (a selective LTCC agonist). Administration of NaHS (100 μM) significantly attenuated the effects of only ISO and forskolin. Moreover, NaHS reversed the ISO-induced cAMP increase and forskolin-stimulated adenylyl cyclase activity. Thus, they postulated that H_2S may negatively regulate β-AR function through inhibition of the cAMP/PKA pathway. In addition, some studies found that the plasma concentration of H_2S in patients with coronary heart disease [41] and in the setting of ISO overstimulation significantly decreased endogenous H_2S production, which implies that a reduced H_2S level caused by ischemia and β-adrenoceptor overstimulation may result in impairment of the negative modulation of H_2S on the β-adrenoceptor system and hence calcium overload.

2.3. Sodium Calcium Exchanger (NCX). The sodium calcium exchanger (NCX) is one of the key players in the regulation of intracellular calcium homeostasis. In a physiological condition, NCX, a nonselective cation channel, may induce the influx of 3 Na^+ into cells in exchange for the efflux of 1 Ca^{2+} [42]. However, in pathological conditions, such as ischemia/reperfusion, hypoxia, and heart failure, NCX function could be reversed, with one Ca^{2+} moving inward and three molecules of Na^+ going out of the cell [43]. H_2S may stimulate Ca^{2+} influx into endothelial cells (ECs) by recruiting the reverse-mode for the NCX [44–46]. To confirm the role of NCX in NaHS-dependent Ca^{2+} signaling, KB-R 7943 (20 μM), a selective inhibitor of the reverse-mode, was used in the experiment. Moccia and his coworkers' data showed that NaHS failed to elicit a $[Ca^{2+}]_i$ elevation in ECs pretreated with KB-R 7943. In addition, the amplitude of the Ca^{2+} response was significantly lower in ECs activated by the H_2S donor in the presence of KB-R 7943. Taken together, these findings hinted at NCX as a key mediator of NaHS-elicited Ca^{2+} inflow in rat aortic ECs. To further determine the effect of sulfide signaling on the NCX, several studies investigated NCX expression and function in HeLa cells. They observed increased levels of NCX1 mRNA, protein, and activity after 24 h of GYY4137 (morpholin-4-ium-4-methoxyphenyl(morpholino) phosphinodithioate, a slow releasing H_2S donor) treatment. This increase was accompanied by elevated cAMP due to GYY4137 treatment, which was completely abolished when NCX1 was silenced. An increased cAMP level would point to upregulation of the β-adrenergic receptors. Thus, Cheng et al. investigated

the relationship of β-adrenergic receptors with the NCX1 in the presence and/or absence of H_2S and determined the physiological importance of this potential communication using GYY4137 [47]. Indeed, GYY4137 increased expression of the β1 and β3 (but not β2) adrenergic receptors, suggesting that sulfide signaling played a role in regulating the NCX1 and β1 and β3 adrenergic receptors and their colocalization.

2.4. Transient Receptor Potential (TRP) Channels. A growing body evidence has shown that H_2S and neuronal excitation induce calcium ion influx in astrocytes, and the interaction between neurons and astrocytes regulates synaptic activity [48–50]. TRP channels were found to mediate the responses to H_2S in the urinary bladder and sensory neurons [51]. Although the effects of H_2S on transient receptor potential (TRP) channels are not completely clear, Kimura et al. demonstrated that polysulfides of H_2S-derived signaling molecules stimulated TRP channels in the brain [52]. They suggested that H_2S induced Ca^{2+} influx in astrocytes through generating polysulfides of TRP. They administered sodium polysulfides, Na_2S_3, in their experiments, which induced Ca^{2+} influx in a concentration-dependent manner. They also confirmed that this astrocyte response to H_2S was suppressed by the TRP channel blockers La^{3+} and Gd^{3+}. To further reveal the mechanism for Na_2S_3-induced TRP channel opening, the TRPA1 channel inhibitors HC-030031 and AP-18 and TRPA1 siRNA were used. Their data showed that, in the presence of the inhibitors or TRPA1 siRNA, Na_2S_3 could not induce Ca^{2+} influx through the TRPA1 channel. Liu et al. showed that H_2S maintained mesenchymal stem cell function via regulation of Ca^{2+} channel sulfhydration [53]. They found that NaHS-treated bone marrow mesenchymal stem cells (BMMSCs) induced Ca^{2+} influx with a limited contribution from intracellular Ca^{2+} storage. They also found that DTT, by reducing the disulfide bonds in proteins and increasing the number of residual sulfhydryl proteins, elevated NaHS-induced Ca^{2+} influx in BMMSCs. Diamide, by reducing the number of sulfhydryls and 2-sulfonatoe-methanethiosulfonate (MTSES), a nonpermeable reagent able to reduce free sulfhydryls only on the outer cytomembrane, could reduce NaHS-induced Ca^{2+} influx in BMMSCs. These results revealed that free sulfhydryls affect NaHS-induced Ca^{2+} influx. The above results suggested that polysulfides, as H_2S-derived bioactive molecules, stimulate TRP channels, providing a new molecular mechanism for sulfide-induced signaling.

2.5. N-Methyl-D-aspartate Receptors (NMDARs). N-Methyl-D-aspartate receptors (NMDARs) form glutamate-gated ion channels that are widely expressed in the central nervous system and are highly permeable to calcium ions, which are essential for regulating synaptogenesis, use-dependent synaptic remodeling, and long-term plastic changes in synaptic strength [54]. H_2S, as a neuromodulator, elevates the activity of N-methyl-D-aspartate (NMDA) receptors to facilitate the induction of hippocampal long-term potentiation (LTP), a synaptic model of memory formation [48, 55]. Nagai et al. demonstrated that H_2S enhances the neuronal response to glutamate and induces Ca^{2+} waves in astrocytes [49]. Glial cells communicate with surrounding cells by increasing the intracellular concentration of Ca^{2+} and propagating the signal as Ca^{2+} waves that occur in glia, and neurons show Ca^{2+} oscillations and intracellular Ca^{2+} waves. Because astrocytes elicit intracellular Ca^{2+} waves by electrical stimulation and application of NMDA in mixed cultures of neurons and astrocytes, astrocytes have been suggested to respond directly to a neurotransmitter released from neurons excited by NMDA or electrical stimulation [56–59]. La^{2+} and Gd^{3+}block Ca^{2+} waves and inhibit Ca^{2+} channels; La^{2+} and Gd^{3+}may inhibit the exocytosis of glutamate or some factor from neurons when neurons are stimulated by NMDA. However, La^{2+} and Gd^{3+}block H_2S-initiated waves in pure astrocyte culture, showing that Ca^{2+} is most likely involved in the propagation step. H_2S released in response to neuronal excitation may activate Ca^{2+} channels to induce Ca^{2+} waves in astrocytes. H_2S may therefore mediate signals between neurons and glia. H_2S is released from neurons or glia by neuronal excitation and increases the intracellular concentration of Ca^{2+} by activating Ca^{2+} channels in astrocytes and to a lesser extent causes release from intracellular Ca^{2+} stores. An elevated intracellular Ca^{2+} triggers the induction of Ca^{2+} waves that propagate to the neighboring astrocytes [60–63]. H_2S enhances the activity of NMDA receptors by reducing the cysteine disulfide bond in the hinge region of the ligand-binding domain of NMDA receptors, and polysulfides further enhance this activity by adding bound sulfane sulfur to the receptors. Polysulfides activate the TRPA1 channels in astrocytes to induce Ca^{2+} influx, which facilitates the release of the gliotransmitter D-serine to enhance the activity of NMDA receptors. By these integrated mechanisms, H_2S along with polysulfides may facilitate the induction of LTP [64].

3. Conclusions and Perspective

An increasing amount of evidence has clearly demonstrated that H_2S is associated with relevant biological processes, such as cardiac systolic function, sensory transduction, antiapoptotic function, and neuroprotection [65]. These functions are closely related to H_2S regulating various calcium ion channels and transporters [66]. Many studies cited in this review investigated the fact that polysulfides of calcium ion channels, which are modified by H_2S, have been found to elevate the activity of TRP, TTCC, and NMDARs and to inhibit LTCC through the mechanism of sulfhydration. Furthermore, H_2S could upregulate the activities of the NCX1 and β1 and β3 adrenergic receptors and their colocalization. Altered effects of H_2S on calcium ion channels under different pathophysiological conditions are being investigated. Extensive research on the mechanisms of H_2S modulation of calcium signaling will provide new insights into the physiological function of H_2S.

Conflict of Interests

No conflict of interests, financial or otherwise, was declared by any of the authors.

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Research Article

Hydrogen Sulfide Prevents Advanced Glycation End-Products Induced Activation of the Epithelial Sodium Channel

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Advanced glycation end-products (AGEs) are complex and heterogeneous compounds implicated in diabetes. Sodium reabsorption through the epithelial sodium channel (ENaC) at the distal nephron plays an important role in diabetic hypertension. Here, we report that H_2S antagonizes AGEs-induced ENaC activation in A6 cells. ENaC open probability (P_O) in A6 cells was significantly increased by exogenous AGEs and that this AGEs-induced ENaC activity was abolished by NaHS (a donor of H_2S) and TEMPOL. Incubating A6 cells with the catalase inhibitor 3-aminotriazole (3-AT) mimicked the effects of AGEs on ENaC activity, but did not induce any additive effect. We found that the expression levels of catalase were significantly reduced by AGEs and both AGEs and 3-AT facilitated ROS uptake in A6 cells, which were significantly inhibited by NaHS. The specific PTEN and PI3K inhibitors, $BPV_{(pic)}$ and LY294002, influence ENaC activity in AGEs-pretreated A6 cells. Moreover, after removal of AGEs from AGEs-pretreated A6 cells for 72 hours, ENaC P_O remained at a high level, suggesting that an AGEs-related "metabolic memory" may be involved in sodium homeostasis. Our data, for the first time, show that H_2S prevents AGEs-induced ENaC activation by targeting the ROS/PI3K/PTEN pathway.

1. Introduction

Advanced glycation end-products (AGEs) are modified proteins or lipids that become nonenzymatically glycated and oxidized after contacting aldose sugars and polypeptides. High levels of glucose react with proteins to form adduct AGEs in diabetes mellitus [1]. Increasing evidence suggests that AGEs play an important role in the development of diabetic nephropathy. Typically, proteins after being directly cross-linked to AGEs can change cellular structure and function or may interact with a combination of different cell surface receptors [1, 2]. The long-term progression of diabetic complications in kidney could be a metabolic memory phenomenon. In other words, even after hyperglycemia is efficiently controlled previous exposure of the target cells to high glucose (HG) may still cause the persistence of its deleterious effects. Hypertension is a major complication in diabetes and is the cause of the increasing morbidity and mortality in diabetic patients. Hypertension alone accounts for nearly 85% of cardiovascular disease (CVD) risk factors. Since diabetic patients tend to have higher blood pressure than nondiabetic patients [3], after becoming hypertensive they should have even higher risk for CVD. Therefore, control of the development of hypertension in diabetic patients is very critical for preventing CVD. Recent studies have demonstrated that AGEs are upregulated in hypertensive diabetic subjects, particularly in distal nephron cells [4]. AGE accumulation mediates proliferation, migration, metabolic memory, and inflammatory gene expression in the distal nephron, which is thought to accelerate hypertension development in diabetes [5]. However, the detailed mechanisms underlying hypertension in diabetic patients are not fully understood.

The epithelial sodium channel (ENaC) mediates Na^+ absorption across epithelial cells in the kidney collecting duct, lung, distal colon, and sweat duct. Na^+ transport is critical for Na^+ homeostasis and thus plays a vital role in maintaining salt balance and systemic blood pressure. ENaC excess activation causes hypertension, as seen in Liddle's syndrome [6]. In type 1 and type 2 diabetic animal models, the expression levels of ENaC were increased in cortical collecting duct cells. Cultured with AGEs, ENaC was increased at both mRNA and protein levels in mouse CCD cells [7]. Therefore, it is very possible that AGEs may be involved in the development of hypertension in diabetes, at least, in part, by stimulating ENaC function.

Hydrogen sulfide (H_2S) is an important intercellular gaseous messenger molecule that regulates multiple physiological and pathological processes. Accumulating evidence has shown that H_2S protects against a number of organ injuries. One of the primary mechanisms of H_2S protection is antioxidation, as it either enhances reduced glutathione (GSH, a major cellular antioxidant) [8] or directly scavenges superoxide [9], H_2O_2 [10], and peroxynitrite [11] to suppress oxidative stress. Our previous studies suggest that H_2S could protect H_2O_2-induced ENaC activity in A6 cells [12]. Therefore, we hypothesized that AGEs might elevate ENaC activity and that H_2S might protect against this elevation. The present study shows that H_2S prevents AGEs-induced ENaC activation by targeting the ROS/PI3K/PTEN pathway.

2. Materials and Methods

2.1. Cell Culture. A6 cells are an established renal cell line derived from the *Xenopus laevis* distal nephron segment, which is an appropriate cell model for studying ENaC [12]. A6 cells were purchased from American Type Culture Collection (Rockville, MD, USA) and grown in the medium consisting of 3 parts of DMEM/F-12 (1 : 1) medium (Gibco, USA), 1 part of H_2O, 15 mM $NaHCO_3$ (total Na^+ = 101 mM), 2 mM L-glutamine, 10% fetal bovine serum (Invitrogen, USA), 25 units/mL penicillin, and 25 units/mL streptomycin, as previously described [13]. A6 cells were cultured in plastic flasks in the presence of 1 μM aldosterone at 26°C and 4% CO_2. After the cells reached 70% confluence, they were subcultured on polyester membranes of *Transwell* inserts (Corning Costar Co, USA) for confocal microscopy or *Snapwell* inserts (Corning Costar Co, USA) for cell-attached patch-clamp analysis. To allow for polarization, cells were cultured for at least 2 to 3 weeks before performing experiments.

2.2. Patch-Clamp Recording. ENaC single-channel currents were recorded using a cell-attached patch-clamp configuration with an Axopatch-200B amplifier (Axon Instruments, USA) as described previously [14, 15]. A6 cells were thoroughly washed with NaCl solution containing (in mM) 100 NaCl, 3.4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES, adjusted to pH 7.4 with NaOH. This NaCl solution was used as bath solution for recordings and used to fill the electrodes. The reagents were added to the bath solution from either basolateral side or apical side. Borosilicate glass electrodes had tip resistance of 7–10 MΩ when filled with NaCl solution. Experiments were conducted at room temperature (22–25°C). The data were acquired by application of 0 mV pipette potential and were sampled at 5 kHz and low-pass filtered at 1 kHz with Clampex 10.2 software (Molecular Devices, Sunnyvale, CA, USA). Prior to analysis, the single-channel traces were further filtered at 30 Hz. The total number of functional channels in the patch was determined by observing the number of peaks detected on the current amplitude histograms during at least 10 min recording period. The open probability (P_O) of ENaC before and after chemical application was calculated with Clampfit 10.2 (Molecular Devices, Sunnyvale, CA, USA). Control ENaC activity was recorded for 2 min after forming the cell-attached mode and ENaC activity stabilized. A single patch was typically recorded for at least 30 min before any experimental manipulation.

2.3. Confocal Laser Scanning Microscopy Analysis. Studies were performed using confocal microscopy (Olympus Fluoview 1000, Japan) as previously described [12, 13, 16]. A6 cells were washed twice with NaCl solution prior to the performance of any experiments. Immediately following experimental manipulation, the polyester membrane support was quickly excised and mounted on a glass slide with a drop of NaCl solution to keep the cells alive. A6 cells grown on Transwell inserts were loaded with 2.5 μM 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA), a membrane-permeable ROS-sensitive fluorescent probe (Invitrogen, USA) that becomes fluorescent when oxidized. Prior to exogenous AGEs or 3-AT application, A6 cells were treated with an iron chelator, 50 μM 2,2′-dipyridyl for 3 min [17]. Labeled cells were washed twice in modified DPBS before confocal microscopy analysis. ROS levels were measured by fluorescence intensity.

2.4. Western Blotting. A6 cells were cultured as described above. Cell lysates (100 μg) were loaded and electrophoresed on 10% SDS-polyacrylamide gels for 60–90 min. Gels were blotted onto polyvinylidene fluoride (PVDF) membranes for 1.5 h at 200 mA. After 1 h of blocking in 5% nonfat dry milk in phosphate-buffered saline (PBS), PVDF membranes were incubated with a rabbit polyclonal primary antibody (1 : 2,000) against catalase (Abcam, ab16731) overnight at 4°C and then incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, USA, 1 : 5000) for 1 h at room temperature after four vigorous washes. Finally, blots were visualized by chemiluminescence using the ECL Plus Western blotting detection system (Bio-Rad, USA).

2.5. Chemicals and Reagents. Unless otherwise noted, all chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO, USA). AGEs were purchased from Cell Biolabs (San Diego, USA). All solutions were premade and stored in a −20°C freezer or freshly made before use.

2.6. Data Analysis. Data are presented as mean ± S.E. Statistical analysis was performed with SigmaPlot and SigmaStat

software (Jandel Scientific, CA, USA). Student's t-test was used to compare pre- and posttreatment activities. Analysis of variance (ANOVA) was used to make multiple comparisons among various treatment groups. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. H_2S Reverses AGEs-Induced ENaC Activity in A6 Cells. To investigate whether AGEs enhance ENaC activity, we performed cell-attached patch-clamp experiments. Because in diabetic patients AGEs are delivered to renal epithelial cells from the blood, we applied AGEs to the basolateral side of A6 cell monolayer to mimic the *in vivo* AGEs delivery. We compared ENaC P_O in cell-attached patches from four experimental groups: control (basolateral incubation of A6 cells with 200 μg/mL BSA for 24 h), AGEs (cells treated with basolateral 200 μg/mL AGEs for 24 h), NaHS (cells treated with 0.1 mM NaHS for 30 min; in addition, NaHS at 0.05, 0.1, or 0.3 mM does not affect cell viability [12]), and AGEs + NaHS (cells treated with basolateral 200 μg/mL AGEs for 24 h and then incubation with 0.1 mM NaHS 30 min). After treatment with AGEs, ENaC P_O was significantly increased, from 0.22±0.03 (control; $n = 10$) to 0.50±0.03 (AGEs; $n = 10$; $P < 0.01$ compared to control). Consistent with our previous studies [12], NaHS did not affect ENaC P_O compared with control (0.23 ± 0.03; $n = 10$; $P > 0.05$). The AGEs-induced increase in ENaC P_O was reversed by NaHS (0.25 ± 0.01; $n = 10$; $P < 0.01$ compared to AGEs and $P > 0.05$ compared to control) (Figure 1). These results suggest that AGEs strongly stimulate ENaC activity in A6 cells and that H_2S exerts a sufficient protective effect on AGE-induced ENaC activity.

3.2. Inhibition of Catalase Activity Mimics the Effect of AGEs on ENaC. Because AGEs potently inhibit catalase and ROS regulates ENaC [12, 13, 18], we reasoned that AGEs might stimulate ENaC by increasing ROS levels via inhibition of catalase. Therefore, we used a catalase inhibitor, 3-AT [19], to treat A6 cells. As shown in Figure 2(a), ENaC activity was significantly upregulated by application of 20 mM 3-AT to the basolateral bath; ENaC P_O was increased from 0.22 ± 0.03 (control; $n = 10$) to 0.57 ± 0.07 ($n = 9$; $P < 0.01$ compared to control) (Figure 2(b)). We have to note that 20 mM 3-AT led to an increase in osmolarity from 268 ± 2 mOsmol/kg ($n = 3$) to 302 ± 3 mOsmol/kg ($n = 3$). However, increasing in osmolarity up to 400 mOsmol/kg (adjusted by sucrose) did not affect ENaC P_O in A6 cells (data not shown), which suggests that the effect of 3-AT on ENaC is not due to changes in osmolarity. The combined application of AGEs to the basolateral bath and 3-AT to the apical bath did not further upregulate ENaC ($P_O = 0.63±0.03$; $n = 9$; $P > 0.05$ compared to 3-AT alone) (Figures 2(a) and 2(b)). These results suggest that AGEs and 3-AT may activate ENaC through the same pathway associated with catalase activity and accumulation of ROS.

3.3. H_2S Attenuates Both AGEs- and 3-AT-Induced Oxidative Stress in A6 Cells. AGEs are known to inhibit catalase expression [18]. The inhibition of oxidative stress accounts for the cardioprotective effects of H_2S during ischemia/reperfusion (I/R) [20, 21]. However, it is unknown whether H_2S can reduce AGE-induced or 3-AT-induced oxidative stress in A6 cells. Therefore, we examined intracellular ROS levels with an ROS-sensitive fluorescent probe, DCF (refer to Section 2), in the presence of AGEs, AGEs + NaHS, 3-AT, or 3-AT + NaHS. Our results show that pretreatment of A6 cells with AGEs or 3-AT induced significant increase in intracellular fluorescence intensity. These results suggest that exogenous AGEs or 3-AT significantly elevated intracellular ROS in A6 cells. Furthermore, application of 0.1 mM NaHS for 30 min led to a significant decrease in intracellular ROS levels, as suggested by reduced fluorescence intensity upon incubation with NaHS (Figures 3(a)–3(d); $n = 7$). Our Western blotting data show that AGEs caused a significant decrease in catalase expression in A6 cells (Figure 3(e); $n = 6$). These results together suggest that AGEs increase intracellular ROS via inhibition of catalase and H_2S significantly attenuates AGEs and 3-AT induced ROS accumulation in A6 cells.

3.4. TEMPOL Abolishes Both AGEs- and 3-AT-Induced Activation of ENaC. 2,2,6,6-Tetramethyl-1-piperidinyloxy (TEMPOL) is a well-known scavenger used to remove ROS from the cells. To confirm the role of ROS in stimulating ENaC activity, we added 250 μM TEMPOL to the basolateral bath. Our data show that even under control conditions ENaC P_O was significantly decreased by addition of TEMPOL, from 0.30 ± 0.03 (before) to 0.15 ± 0.02 (15 min after TEMPOL) in A6 cells ($n = 6$; $P < 0.01$) (Figure 4(a)). ENaC P_O was also decreased by TEMPOL, from 0.52±0.05 ($n = 7$) to 0.33±0.05 in AGEs treated cells ($n = 7$; $P < 0.01$) (Figure 4(b)) or from 0.51 ± 0.02 ($n = 7$) to 0.27 ± 0.02 in 3-AT treated cells ($n = 7$; $P < 0.01$) (Figure 4(c)). These data suggest that AGEs stimulate ENaC in A6 cells via a pathway closely associated with altered intracellular ROS.

3.5. AGEs Elevate ENaC P_O through the PTEN and PI3K Pathways. It is known that the tumor suppressor phosphatase and tension homolog (PTEN) reduces the cellular concentration of $PI(3,4,5)P_3$ and acts as a negative regulator of PI3K signaling pathways [22]. Our previous data show that increased intracellular ROS regulates ENaC via increased apical $PI(3,4,5)P_3$ by affecting both PTEN and PI3K [12, 13]. Therefore, we tested whether AGEs-induced activation of ENaC is mediated by PTEN or PI3K. Consistent with our previous findings [12], ENaC P_O was increased by ~70% (from 0.30 ± 0.03 to 0.51 ± 0.03; $n = 6$; $P < 0.05$) in A6 cells treated with a specific PTEN inhibitor, $BPV_{(pic)}$ (Figure 5(a)). In AGEs-pretreated cells, ENaC P_O was also significantly elevated by $BPV_{(pic)}$, from 0.50±0.02 to 0.67±0.04 ($n = 6$; $P < 0.05$), but with a less extent (~34%) (Figure 5(b)). LY294002, a PI3K inhibitor, significantly decreased ENaC P_O in untreated control A6 cells (from 0.29±0.02 to 0.23±0.04, reduced ~21%; $n = 6$; $P < 0.01$) (Figure 5(c)). However, in AGEs-pretreated cells, LY294002 reduced ENaC P_O by ~46% (0.46±0.03 versus 0.25±0.04; $n = 6$; $P < 0.01$) (Figure 5(d)). These data together suggest that the inhibition of TPEN and PI3K is involved in AGEs-induced activation of ENaC in A6 cells.

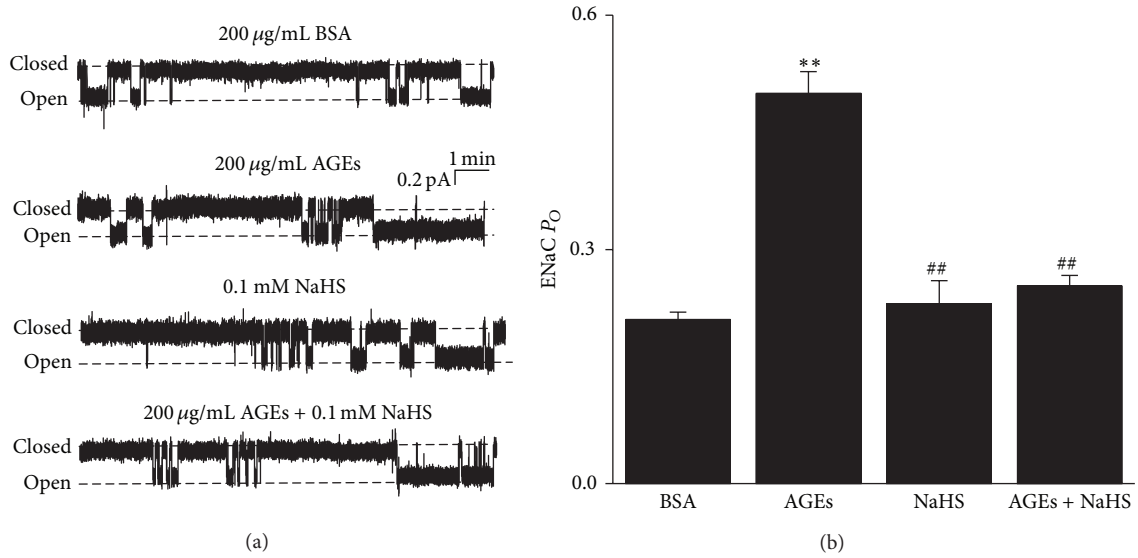

Figure 1: AGEs-induced activation of ENaC is reversed by 0.1 mM NaHS in A6 cells. (a) The representative ENaC single-channel current recorded from A6 cells, respectively, treated with basolateral 200 μg/mL BSA (control; top trace), basolateral 200 μg/mL AGEs, apical 0.1 mM NaHS, and basolateral 200 μg/mL AGEs + apical 0.1 mM NaHS (bottom trace) for 24 h. (b) Summary plot shows that AGEs treatment significantly increased ENaC P_O, which was reversed by H_2S treatment ($n = 10$ for each individual experimental set; ** indicates $P < 0.01$ compared to control; ## indicates $P < 0.01$ compared to AGEs treated cells).

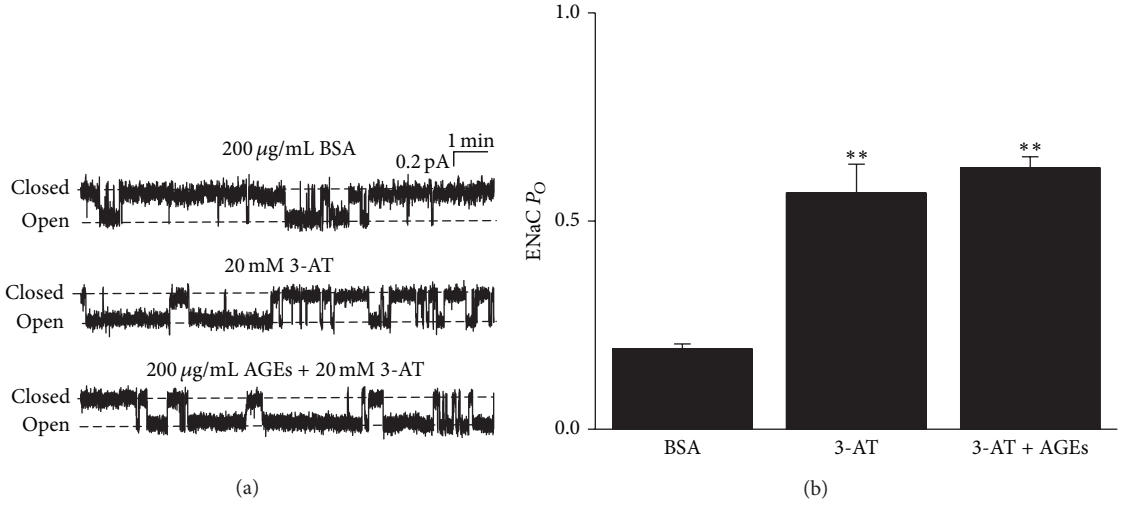

Figure 2: 3-Aminotriazole (3-AT) mimics the effect of AGEs on ENaC P_O. (a) The representative single-channel currents of ENaC recorded under control conditions (basolateral 200 μg/mL BSA for 24 h; top), after apical 20 mM 3-AT treatment for 30 min (middle), or after 24 h AGE treatment followed by treatment with apical 20 mM 3-AT for 30 min (bottom). (b) Summary plots show that ENaC P_O was significantly, respectively, increased after 20 mM 3-AT treatment ($n = 10$ for control and $n = 9$ for 3-AT group; ** indicates $P < 0.01$ compared to control). Addition of 3-AT to AGEs did not further increase ENaC P_O compared to 3-AT alone ($n = 9$ for AGEs + 3-AT group; $P > 0.05$).

3.6. Metabolic Memory Effects of AGEs on ENaC Activity. Since AGEs potently upregulate ENaC activity, we hypothesized that AGEs may exert a sustained stimulatory effect on ENaC after withdrawing AGEs, which is called "metabolic memory." To test this hypothesis, we firstly treated the A6 cells with the medium containing BSA for 24 h and then removed BSA from the medium to continuously culture the cells for 72 h. We also cultured the cells with a medium containing AGEs for 24 h and then removed AGEs from the medium (AGEs-free) followed by continuously culturing these cells for another 72 h. As seen in Figure 6, removal of BSA did not affect ENaC P_O (Figures 6(a) and 6(c)). Consistent with previous results, AGEs significantly increased ENaC P_O (0.50 ± 0.03; $n = 7$); interestingly, ENaC P_O remained at the similar high levels in the cells 72 h after removal of AGEs ($n = 10$; $P > 0.1$) (Figures 6(b) and 6(c)). These results suggest that AGEs regulate ENaC with metabolic memory.

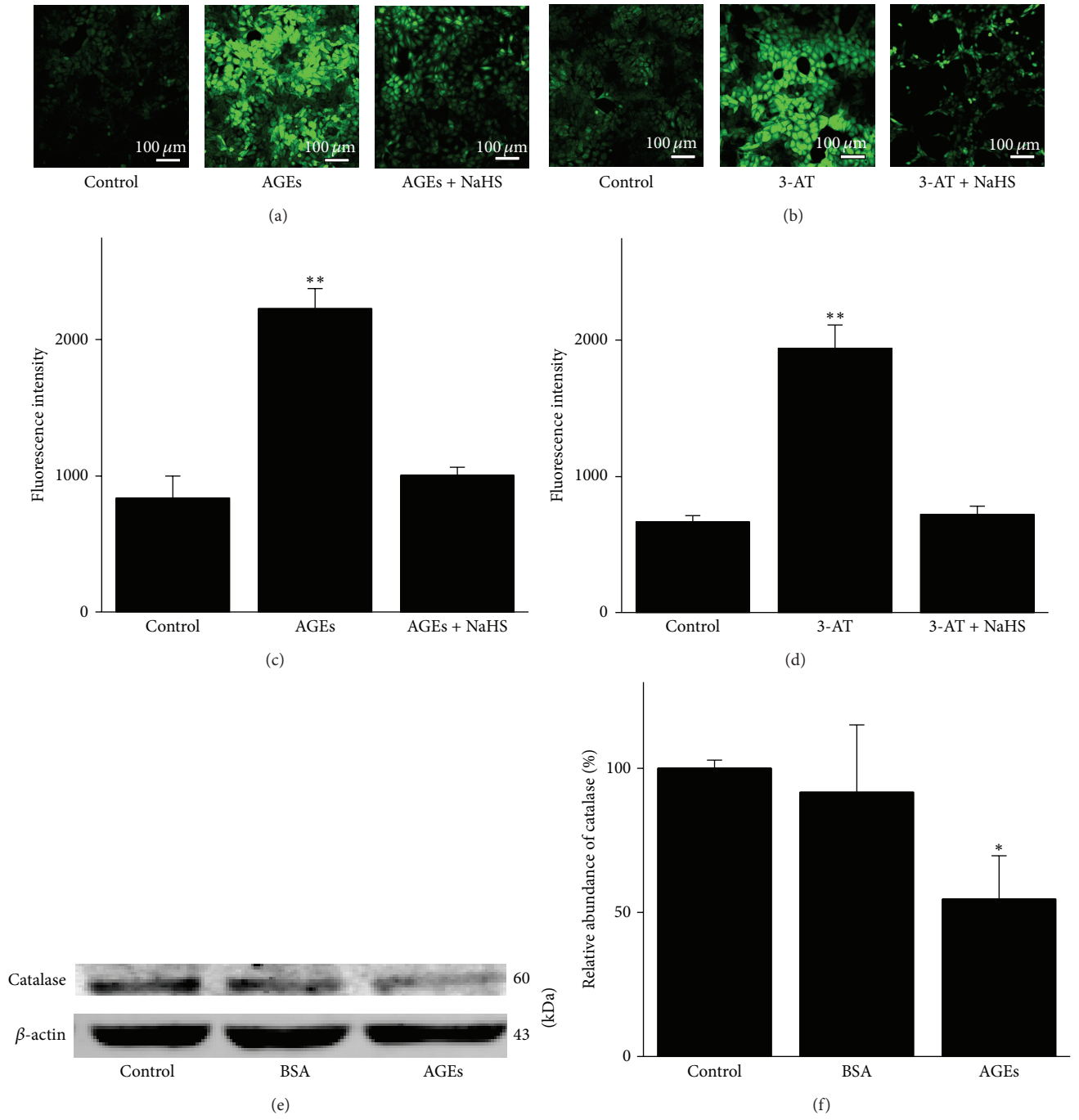

Figure 3: H_2S ameliorates AGE- or 3-AT-elicited oxidative stress and AGEs reduce catalase expression in A6 cells. (a) The left image shows that there was a residual level of intracellular ROS under control condition; the middle image shows a significant increase in intracellular ROS upon application of basolateral 200 μg/mL AGEs; the right image shows that the AGE-induced increase in intracellular ROS was abolished by 0.1 mM NaHS treatment. (b) The left image shows that there was a residual level of intracellular ROS under control conditions; the middle image shows a significant increase in intracellular ROS upon application of apical 20 mM 3-AT; the right image shows that the 3-AT-induced increase in intracellular ROS was also abolished by 0.1 mM NaHS treatment. (c) and (d) Summarized bar graphs show the mean fluorescence intensities under indicated experimental conditions ($n = 7$ for each experimental condition; $**$ indicates $P < 0.01$ compared to control). (e) and (f) Western blot demonstrating that expression levels of catalase were suppressed by AGEs ($n = 6$, $*$ represents $P < 0.05$ compared to control).

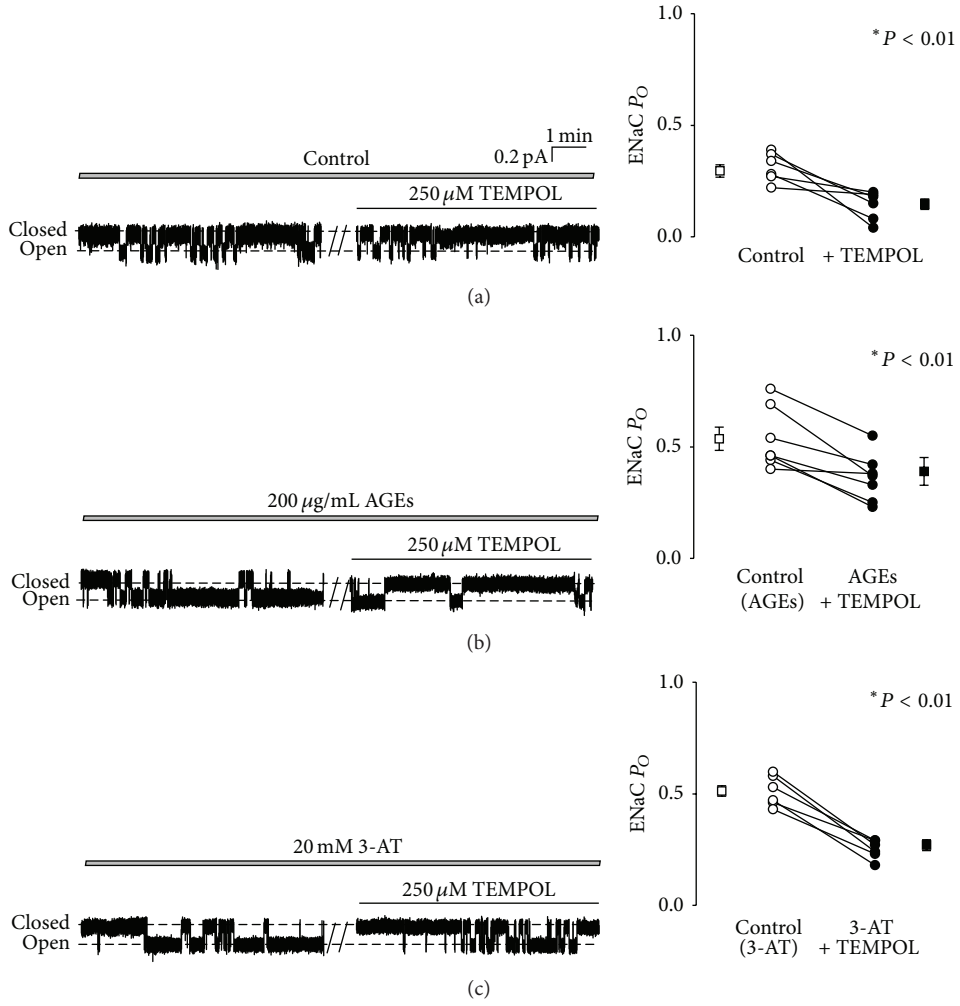

Figure 4: TEMPOL abolishes the effects of AGEs and 3-AT on ENaC activity. (a) ROS extraction by 250 μM TEMPOL significantly decreased ENaC P_O ($n = 6$ paired experiments; $*$ represents $P < 0.01$). (b) and (c) TEMPOL significantly reduced ENaC activity in cells pretreated with 200 μg/mL AGEs (b) or in the cells pretreated with 20 mM 3-AT (c) ($n = 7$ paired experiments; $*$ represents $P < 0.01$).

4. Discussion

Our major findings in this study are as follows: (1) AGEs stimulate ENaC by elevating intracellular ROS via inhibition of catalase; (2) NaHS reverses the effects of AGEs on ENaC activity by reducing AGEs-induced accumulation of intracellular ROS; (3) AGEs stimulate ENaC with "metabolic memory"; and (4) AGEs strongly activate ENaC through ROS/PTEN/PI3K singling pathways.

AGEs are produced by long-term challenge with high glucose and polypeptides. AGEs are the important pathogenic factors in diabetic nephropathy; however, almost all previous studies have focused on the mechanisms how AGEs take their effects on glomerular and vascular cells [23]. The effects of AGEs on fine sodium absorption in renal tubule cells, particularly in collecting ducts, have rarely been explored. It has previously been reported that circulating AGEs correspond to approximately 50 mg/mL of AGEs in diabetic patients [24]. Because the amount of AGEs decreases in the urine of diabetic patients, therefore we used *Snapwell* insert to culture A6 cells in order to mimic the biological environment. We then applied AGEs to the basolateral membrane, where renal collecting tubules should be exposed to high concentration of AGEs *in vivo*. Because AGEs may be concentrated in renal tissues *in vivo* and the corresponding levels of AGEs *in vitro* have not been conclusively determined, we examined the effects of a variety concentration of AGEs (up to 500 mg/mL) on cellular viability. Our data show that incubation of A6 cells with AGEs (up to 500 μg/mL) for 24 h did not affect cell viability (data not shown). Therefore, the effects of AGEs on ENaC should not be due to nonspecific effects on cell viability because in all the experiments A6 cells were treated with only 200 μg/mL AGEs. Instead, our results show that AGEs stimulate ENaC in A6 cells through catalase inhibition and subsequently an increase in intracellular ROS levels. We propose that catalase is a major player, because 3-AT, a catalase inhibitor, mimics the effects of AGEs on ENaC activity and intracellular ROS levels, albeit 3-AT at a concentration of 20 mM may lead to saturated increase in ENaC activity; this might result in a possibility that there

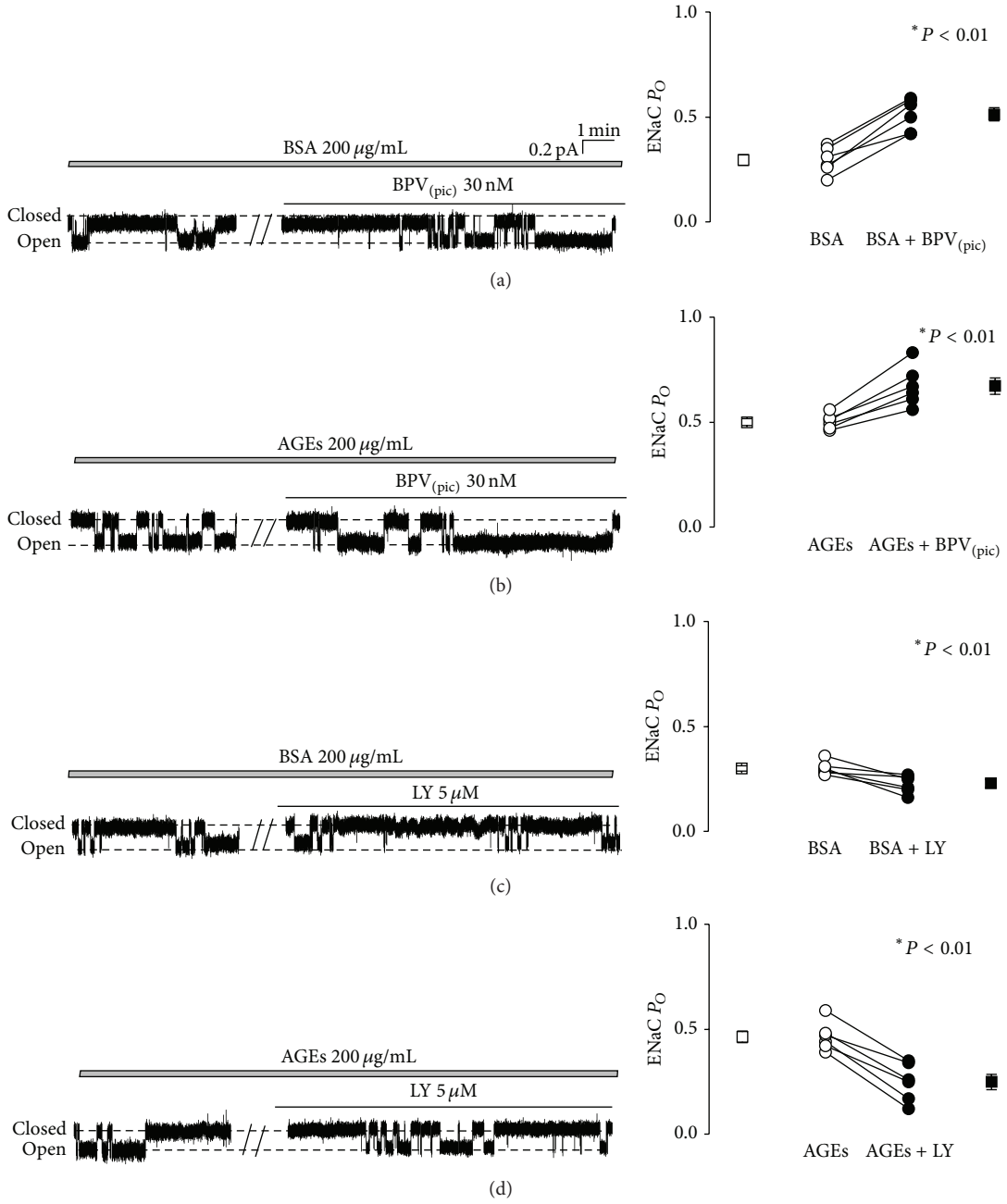

Figure 5: AGEs activate ENaC via PI3K and PTEN signaling pathways. (a) and (b) ENaC activity in A6 cells treated either with basolateral 200 μg/mL BSA or with basolateral 200 μg/mL AGEs, before and after addition of 30 nM $BPV_{(pic)}$ to the apical bath. (c) and (d) ENaC activity in A6 cells treated as in (a) and (b), before and after addition of 5 μM LY294002 to the apical bath; the data show that a PI3K inhibitor, LY294002, significantly inhibits ENaC activities under control condition and in the presence of AGEs. Four breaks between the traces indicate 20 min omitted recording periods. Summarized P_O of ENaC before and after application of each reagent were shown on the right. $n = 6$ paired experiments. $*$ indicates $P < 0.01$.

is a catalase-independent effect of AGEs on ENaC activity. However, our data show that AGEs suppress the expression levels of catalase and that in AGEs treated cells 3-AT was no longer able to further activate ENaC. We also propose that ROS plays a critical role in downstream catalase, because, in the presence of ROS scavenger, TEMPOL, AGEs- and 3-AT-induced activation of ENaC were almost completely abolished. Our studies clearly suggest that ROS mediates the AGEs-induced activation of ENaC in A6 cells. Our results are also consistent with previous studies, where the amiloride-sensitive short-circuit currents across A6 cell monolayer were significantly reduced by extraction of intracellular ROS with TEMPOL [25].

It is well documented that oxidative stress is a primary cause of diabetes-induced kidney injury, which may be involved in diabetic hypertension [1, 2]. Recently, we have

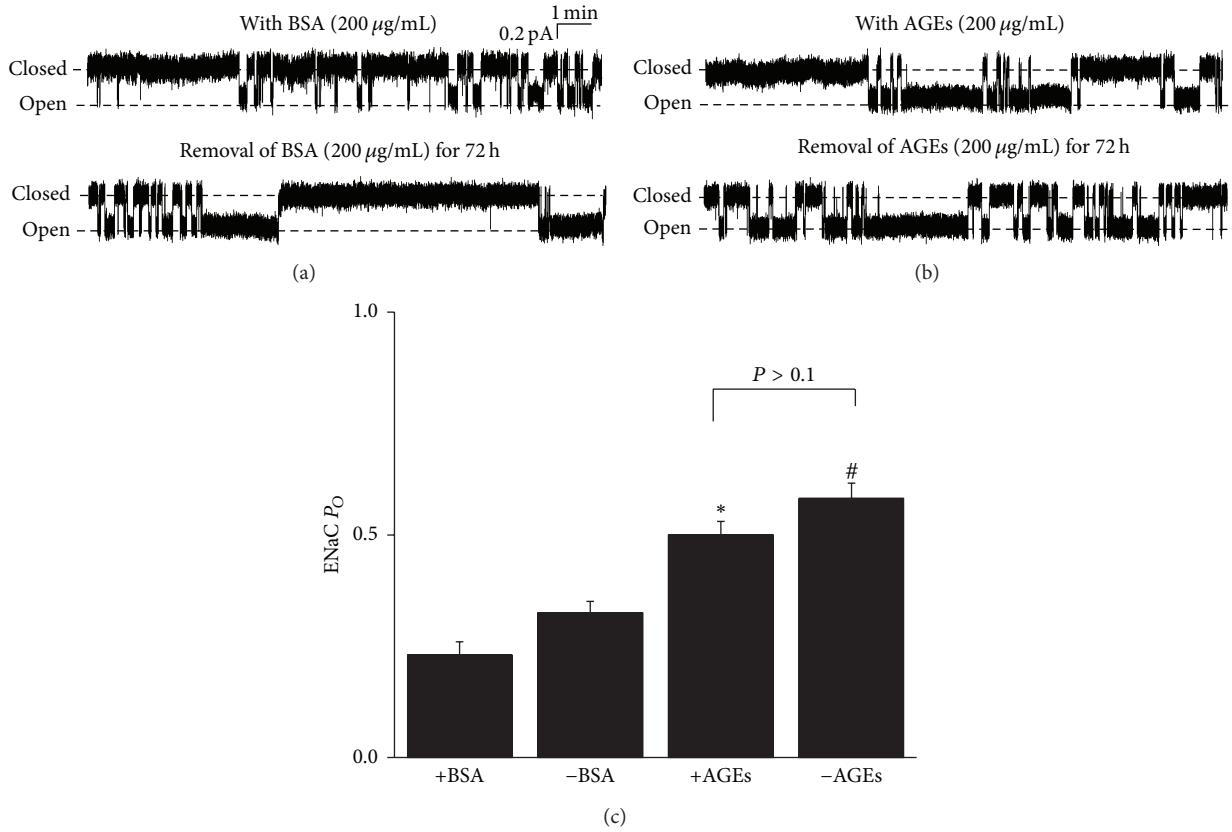

Figure 6: AGEs-induced aberrant activation of ENaC in A6 cells exerts "metabolic memory." (a) Representative ENaC single-channel currents recorded either from an A6 cell in the presence of basolateral 200 μg/mL BSA or from an A6 cell after removal of 200 μg/mL BSA for 72 h. (b) Representative ENaC single-channel currents recorded either from an A6 cell in the presence of basolateral 200 μg/mL AGEs from an A6 cell after removal of 200 μg/mL AGEs for 72 h. (c) Summarized bar graph shows that basolateral AGEs significantly increased ENaC P_O and the ENaC P_O remained at the same levels after removal of AGEs for 72 h (n = 7–10; $*$ and #, resp., indicate $P < 0.01$).

shown that H_2O_2-induced increase in ENaC activity can be reversed by NaHS (a H_2S donor) in A6 cells [12]. H_2S is an endogenous gaseous mediator that exerts various physiological and pathophysiological effects *in vivo*, including antioxidative stress and anti-inflammatory response in heart, liver, kidney, and other organs [26–28]. It was reported that NaHS provided cytoprotection in human neuroblastoma cells exposed to D-galactose and that H_2S may have potential antiaging effects through a reduction of ROS and AGEs formation [29]. As an endogenous signaling molecule, H_2S can be as high as 0.1 mM in human blood [30] or about 1.6 nmol/mg in intact rat kidneys [31]. Therefore, a final concentration of 0.1 mM NaHS which was used in this study should represent the physiological concentrations, as we reported previously [12]. Our results show that AGEs-induced activation of ENaC and accumulation of intracellular ROS were completely reversed by 0.1 mM NaHS. These results suggest that H_2S exerts a protective effect against elevation of intracellular ROS and ENaC activity induced by AGEs.

Our previous studies suggest that an increase in intercellular ROS leads to elevation of $PI(3,4,5)P_3$ near the apical compartment of A6 cells [12, 13], which is known to stimulate ENaC [32, 33]. Since the levels of $PI(3,4,5)P_3$ near the apical compartment of A6 cells are governed by both PTEN and PI3K [12, 13], we examined both PTEN and PI3K inhibitors on ENaC activity in the absence or in the presence of AGEs. Consistent with previous findings [12, 13], it appears that both PTEN and PI3K are involved in the activation of ENaC by AGEs. Interestingly, it has been reported that there are reduced H_2S levels in diabetic rats and that H_2S can increase cellular $PI(3,4,5)P_3$ levels and can enhance glucose utilization in adipocytes by activating PI3K and inhibiting PTEN [34, 35]. Moreover, we have shown that in distal nephron epithelial cells increased intracellular ROS elevates $PI(3,4,5)P_3$ levels near the apical membrane compartment via PTEN/PI3K to stimulate ENaC [12, 13]. However, it would be difficult to determine whether PTEN and PI3K equally contribute to effects of AGEs on ENaC activity.

Finally, this study also provides an evidence for AGEs to stimulate ENaC via a metabolic memory phenomenon which occurs in the long-term progression of diabetic complications in kidney. This phenomenon describes a surprising persistence of the deleterious effects of high glucose even after hyperglycemia has been tightly controlled. This cellular memory phenomenon was revealed by large-scale multicenter clinical trials such as the Diabetes Control and Complications Trial (DCCT) and the Follow-Up Observational Epidemiology of Diabetes [5]. Interestingly, we found that

the effect of AGEs on ENaC activity in A6 cells lasted, at least, for 72 h after removal of AGEs, suggesting that there is a metabolic memory phenomenon in Na^+ homeostasis. This metabolic memory may play an important role in sustaining activation of ENaC, which accounts for the development of hypertension in diabetic patients.

5. Conclusion

AGEs significantly stimulate ENaC activity in A6 cells via inhibition of catalase and the effects of AGEs can be reversed by NaHS. Inhibition of catalase activity accounts for both oxidative stress induced by AGEs and elevation of $PI(3,4,5)P_3$ near the apical membrane compartment via PTEN/PI3K signaling pathways, thereby regulating ENaC activity. Finally, the effect of AGEs on ENaC activity exerts a metabolic memory phenomenon in A6 cells.

Conflict of Interests

The authors have no conflict of interests to declare.

Authors' Contribution

Qiushi Wang and Binlin Song contributed equally to this work.

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Research Article

An Anticancer Role of Hydrogen Sulfide in Human Gastric Cancer Cells

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Hydrogen sulfide (H_2S) can be synthesized in mammalian cells by cystathionine γ-lyase (CSE) and/or cystathionine β-synthase (CBS). Both CSE and CBS are expressed in rat gastric tissues but their role in human gastric neoplasia has been unclear. The aims of the present study were to detect CSE and CBS proteins in human gastric cancer and determine the effect of exogenous NaHS on the proliferation of gastric cancer cells. We found that both CSE and CBS proteins were expressed in human gastric cancer cells and upregulated in human gastric carcinoma mucosa compared with those in noncancerous gastric samples. NaHS induced apoptosis of gastric cancer cells by regulating apoptosis related proteins. Also, NaHS inhibited cancer cell migration and invasion. An antigastric cancer role of H_2S is thus indicated.

1. Introduction

Hydrogen sulfide (H_2S) has long been considered as a toxic gas with smell of rotten eggs. H_2S can be generated endogenously in the mammalian tissues [1, 2]. H_2S can be produced in mammalian cells from sulfur-containing L-cysteine with the catalyzation by pyridoxal-5′-phosphate-dependent enzymes-cystathionine γ-lyase (CSE) and/or cystathionine β-synthase (CBS) [1, 3]. H_2S has been recognized as one of the four gasotransmitters together with NO, CO, and ammonium [2], and it plays an important regulatory role in numerous physiological or pathophysiological processes in our body. CSE is dominantly expressed in the cardiovascular system and H_2S functions as a vasodilator and cardiac protector [1, 4–8]. In contrast, CBS is mainly expressed in nervous system [1, 2]. In recent years it has been found that gastric tissues express both CSE and CBS [9]. The anti-inflammatory protective effect of H_2S on the integrity of gastric mucosa has been investigated [10], but the role of H_2S in gastric neoplasia and the underlying mechanisms are unknown. To this end, we studied expression of CSE and CBS in human gastric cancer samples and explored the effects of exogenous H_2S donor NaHS on the phenotypic functions of gastric cancer SGC7901 cells.

2. Materials and Methods

2.1. Gastric Tissue Samples. Ten human gastric carcinoma samples were obtained from patients during gastrectomy surgery (9 males and 1 female aged from 50 to 72). Cancer samples were taken from the center of tumor and noncancerous samples were obtained 5–7 cm away from tumors. All samples were verified by pathology tests. All work was conducted in accordance with the Declaration of Helsinki and Ethical approval was given by the Ethics Committee of Harbin Medical University (number 2008-8).

2.2. Cell Culture and Reagents. SGC 7901 cells were purchased from the Cell Bank of the Institute of Life Science, Chinese Academy of Sciences, Shanghai, China. Cells were cultured in Dulbecco's modified Eagle medium (DMEM, HyClone) supplemented with 10% fetal bovine serum (FBS) (HyClone). NaHS, DL-propargylglycine (PPG), and hydroxylamine (HA) were from Sigma (St. Louis, MO, USA). Anti-CSE and anti-CBS monoclonal antibodies were from Abnova (Taiwan). The antibodies for Bax, cytochrome C, caspase 3, cyclin D1, p21, p27, and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-MMP-2 and anti-MMP-9 antibodies were from Thermo Scientific (Waltham, USA).

2.3. Cell Viability Assay. Cell viability was determined by measuring the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye absorbance of viable cells. Briefly, cells were seeded in 96-well microtiter plates with 4,000 cells per well. After growing for 24 hours, cells were exposed to the indicated concentrations of NaHS, PPG, and HA for another 24 hours. Cells of each well then were incubated with 20 μL MTT for 4 hours at 37°C. Formazan crystal formed in viable cells was solubilized by adding 150 μL DMSO. Absorbance at 490 nm was measured on ELx800 microtiter plate reader (BioTek).

2.4. Fluorescence Microscopy. For apoptosis assay, 5×10^4 cells per well were plated on 24-well plates and cultured for 24 hours before the indicated concentrations of NaHS, PPG, and HA were added and cultured for 24 hours. Cells were washed and exposed to 10 mg/L Hoechst 33258 at room temperature in the dark for 10 min and 10 mg/L propidium iodide (PI) for 10 min. The cells were observed under a fluorescence microscope (Olympus IX71).

2.5. RT-PCR. SGC7901 cells were washed with ice cold phosphate-buffered saline (PBS) and scraped using a rubber policeman and collected into an Eppendorf tube. One mL of Trizol (Invitrogen) was added and total RNA was extracted according to manufacturer's instructions. RNA was treated with RNase-free DNase (Promega) and extracted again. 1 μg of RNA was reverse-transcribed into the first strand cDNA, and PCR was run under the condition 1 cycle of 94°C for 2 min, 32 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 min and then 1 cycle of 72°C for 4 min. Primers designed for the PCR were as follows: CSE (forward primer: 5′-GTTTCTTGCAAAACTCTCTTGG-3′ and reverse primer: 5′-TTCTTGAGGAAAATCTCAGCAT-3′); CBS (forward primer: 5′-TCTCACATCCTAGACCAG-TACC-3′ and reverse primer: 5′-CTTGTCCACCACCGT-CCTGTCC-3′). RT minus reactions also were included.

2.6. Western Blotting. Human gastric tissues were snap-frozen in liquid nitrogen after gastrectomy and stored under −80°C. Before being lysed, 100 mg of tissue samples was ground in liquid nitrogen in a mortar and powders were poured with liquid nitrogen into an Eppendorf tube, and 100 μL of lysis buffer was added and put on ice for half an hour. For SGC7901 cells, cells from a 25 cm^2 flask were washed twice in ice cold PBS and lysed in 100 μL RIPA lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 2 mM sodium orthovanadate, and 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride) on ice. Cell lysates were then centrifuged at 14,000 g for 20 min at 4°C. The supernatant was recovered and protein concentration was detected by Bradford method. Proteins of 20–40 μg were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane (Pall Corp.). The membrane then was immunoblotted with the indicated primary antibodies and detected by using horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence (Pierce). Densitometric analysis was performed by Quantity One Analysis Software (Bio-Rad).

2.7. Cell Migration Assay. In this assay, 2×10^5 cells/mL were seeded in 12-well plates and cultured to confluence. A 200 μL pipette tip was used to make a straight scratch. For the control group, DMEM containing 0.1% serum was used, whereas, for H_2S group, NaHS was added in addition to DMEM and 0.1% serum. After 12 h, migration distance of cells was calculated.

2.8. Cell Invasion Assay. Invasion assay was carried out in 12-well plate with hanging PET inserts (pore size, 8 μm) (Millipore). The PET membranes were coated with Matrigel (Sigma). In the upper compartment, 400 μL of cells (1.5×10^5/mL) were seeded with DMEM containing 0.1% serum. To the lower compartment, 1.5 mL of DMEM containing 10% fetal bovine serum was added as a chemoattractant. After 20 h of incubation, cells on the upper side of the inserts were removed. Then cells that transmigrated through the Matrigel and membrane were fixed and stained with 0.1% crystal violet. Cell numbers were counted in ten randomly selected fields under a light microscope with 200 time magnification.

2.9. Statistical Analysis. All data were expressed as mean ± SEM. ANOVA was used to compare values of test and control samples. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Upregulation of CSE and CBS Protein Expression in Gastric Carcinoma. To investigate the role of H_2S in gastric tumorigenesis, we analyzed the expression of CSE and CBS in 10 gastric primary tumors with the adjacent nontumor gastric tissues. We found that the expression of both CSE and CBS proteins was significantly higher in gastric carcinomas than in adjacent noncancerous gastric tissues ($n = 10$, $P < 0.05$) (Figure 1). We then detected CSE and CBS expression in human gastric cancer cells, SGC 7901 cell line. As shown in Figure 1(b), RT-PCR displayed 282 bp of expected CSE PCR product and 317 bp CBS product. Western blotting revealed major bands for CSE and CBS proteins. Both CSE and CBS were expressed at transcription and protein levels.

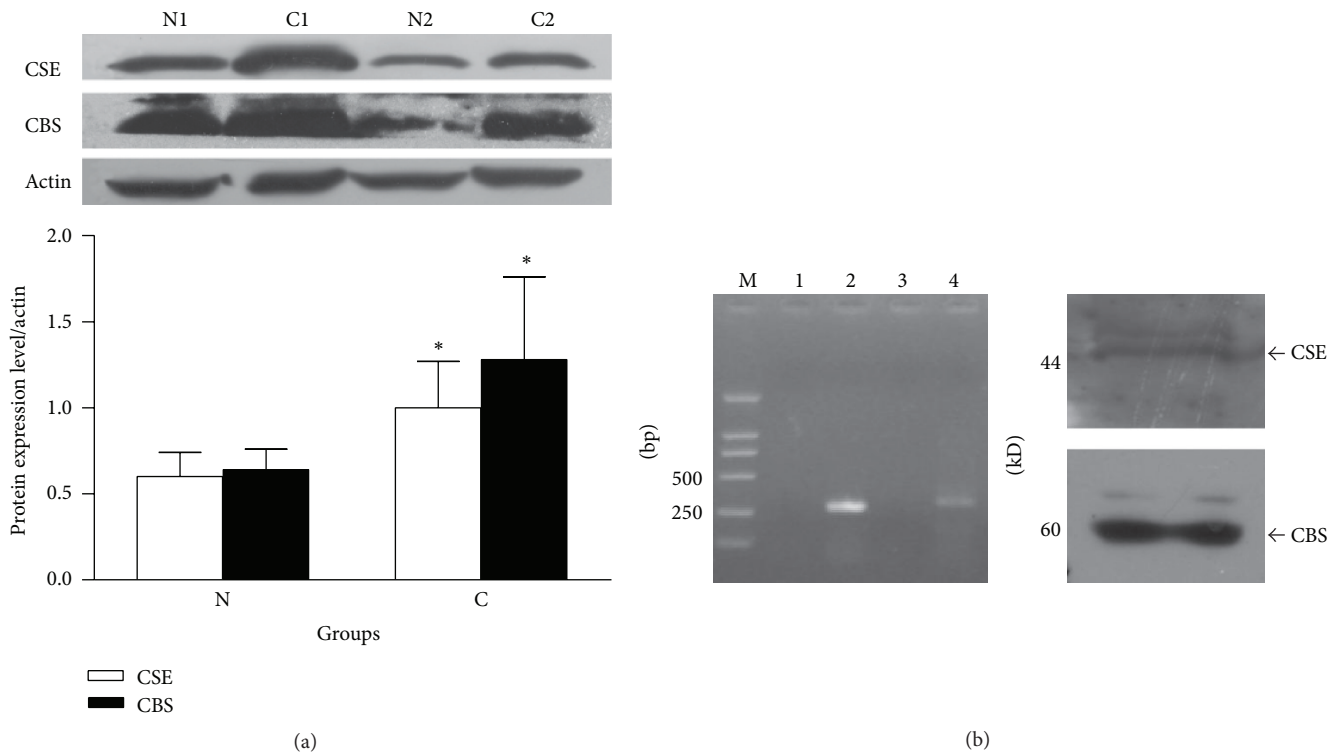

Figure 1: Upregulation of CSE and CBS expression in gastric carcinoma. (a) Tissue lysates from the gastric carcinoma and adjacent noncancerous tissue were immunoblotted with anti-CSE or anti-CBS antibodies. The 2 representative pairs of samples were shown. C, gastric carcinoma; N, adjacent noncancerous tissue. The right panel indicates the quantitative representation. $n = 10$, $^{*}P < 0.05$ versus corresponding N group. (b) The expression of CSE and CBS in gastric cancer SGC 7901 cells. The left panel shows CSE and CBS mRNA expression in SGC7901 cells. Expected CSE and CBS RT-PCR products are 282 bp and 317 bp, respectively. M: DL2,000 DNA marker; 1: no reverse transcription control for CSE; 2: CSE RT-PCR; 3: no reverse transcription control for CBS; 4: CBS RT-PCR. The right panel shows CSE or CBS protein expression in SGC7901 cells. Protein sizes were 44 kDa and 60 kDa, respectively.

3.2. H_2S Reduced Cell Viability of SGC 7901 Gastric Cancer Cells. To assess the effect of H_2S on cell viability of cloned gastric cancer cells, we exposed these cells to the indicated concentrations of NaHS. When cells seeded at low density to the plates, NaHS treatment significantly increased cell death, compared with the control, in a concentration-dependent manner at concentrations from 0.2 to 0.8 mM (Figure 2). Cell viability was enhanced by PPG alone, but not by HA alone (Figure 2).

3.3. H_2S Induces Apoptosis of SGC 7901 Gastric Cancer Cells. To investigate whether H_2S is involved in apoptosis, we performed apoptosis test using Hoechst-Propidium Iodide staining of cells with different treatments. As shown in Figure 3, NaHS treatment enhanced apoptotic rate of cells. PPG increased mitotic rate. The levels of apoptosis-related proteins, Bax, Cyt C, and caspase 3 were increased after NaHS treatment (Figure 4). We next sought to reveal the role of NaHS on the expression of cell cycle proteins. Cyclin D1 was upregulated during 0.5 h, 2 h, and 8 h, but downregulated at 12 h of NaHS treatment. On the other hand, cell cycle inhibitors $p21^{waf1/cip1}$ and $p27^{kip1}$ were downregulated by NaHS in a time-dependent manner (Figure 5).

3.4. NaHS Inhibited Gastric Cancer Cell Migration and Invasion. We further examined the effect of NaHS on SGC7901 cell migration. As shown in Figure 6, 0.8 mM NaHS significantly reduced cell migration in a scratch assay. NaHS-induced delay of coverage of the scratched area by cell migration is unlikely due to the reduced cell proliferation because the assay was carried out in presence of 0.1% serum to essentially stop cell proliferation. To evaluate the contribution of H_2S on cell invasion, we added NaHS to the upper inserts of Boyden Chambers. As shown in Figure 7, 0.8 mM NaHS inhibited cancer cell invasion. To further determine the mechanisms of involvement in cell invasion, we tested MMP-2 and MMP-9 expression during NaHS treatment. As shown in Figure 8, 0.8 mM NaHS significantly attenuated MMP-2 expression, but there was no significant effect of NaHS observed on MMP-9 level.

4. Discussion

The effects of H_2S on the cardiovascular system [7, 11–14] and the liver [3] have been intensively investigated in recent years. The involvement of H_2S in the regulation of physiological gastric functions has also been explored [9, 10]. But its role in gastric malignancy has been unknown. Because H_2S

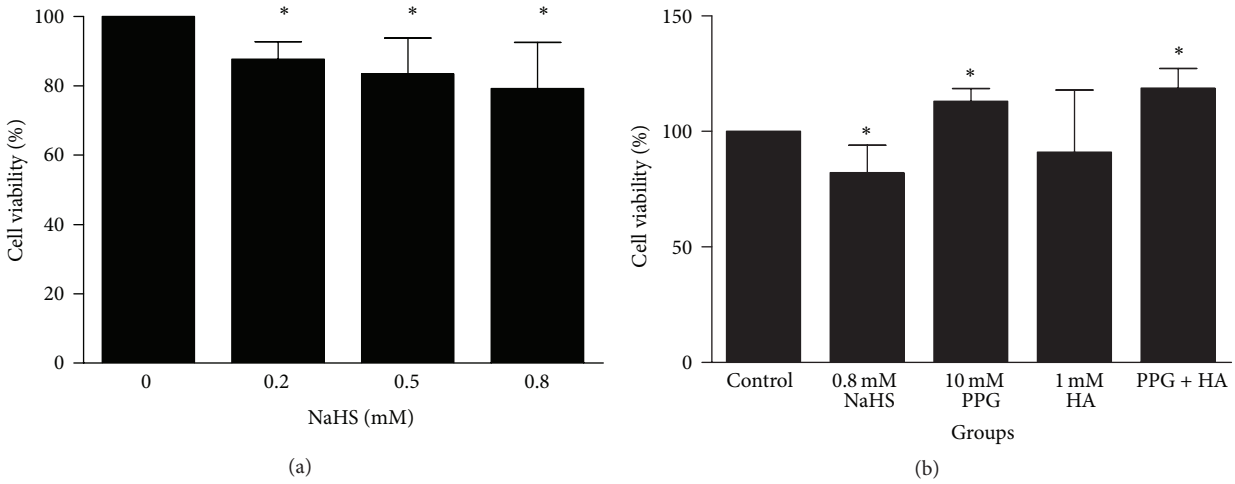

FIGURE 2: The effect of NaHS on SGC7901 cell viability. (a) NaHS significantly reduces cell viability at the concentrations of 0.2, 0.5, and 0.8 mM. The cells were treated with NaHS for 24 h. (b) Effects of PPG and HA on cell viability. Data were obtained from three independent experiments. $^*P < 0.05$ versus control. $n = 3$.

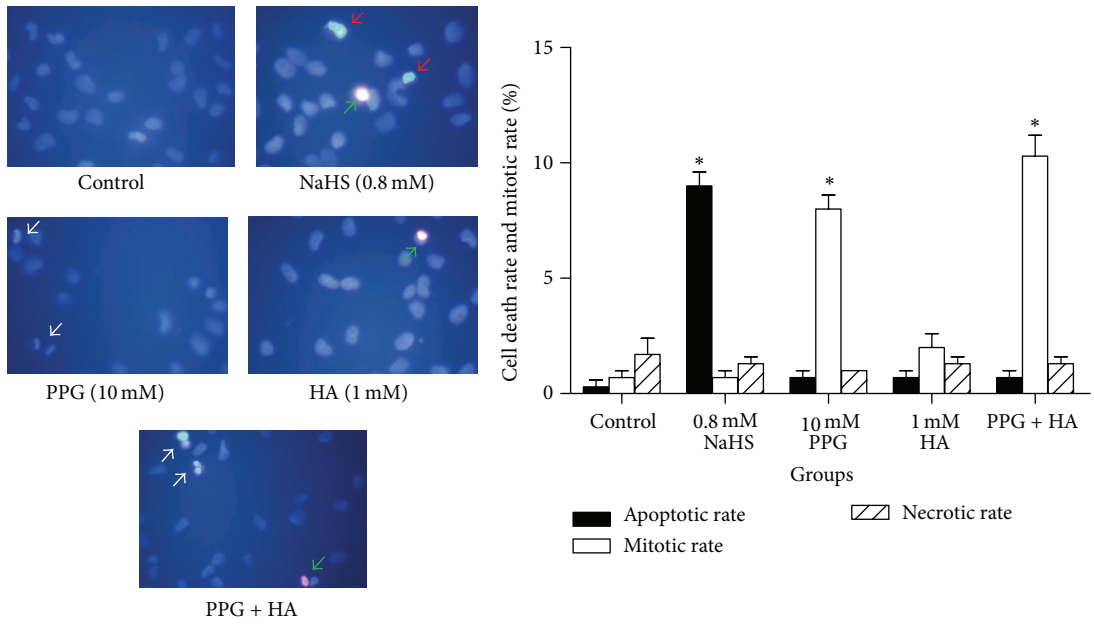

FIGURE 3: NaHS induced apoptosis of gastric cancer. Apoptosis of gastric cancer cells was determined by Hoechst and propidium iodide staining. Red arrow indicates apoptotic cell nuclei; white arrow is used to indicate mitotic nuclei and green arrow to necrotic nuclei. $^*P < 0.05$ versus control. $n = 3$.

regulates cell growth, proliferation [15], and apoptosis [15–17], we speculated that this gasotransmitter may be involved in gastric hyperplasia.

To test our hypothesis, we firstly collected human gastric cancer tissue samples and performed western blotting to determine the expression levels of H_2S-producing enzymes CSE and CBS. Our result showed that CSE and CBS were both expressed in noncancerous gastric tissues. Interestingly, both CSE and CBS were upregulated in gastric carcinoma mucosa. This indicates that the production of endogenous H_2S is elevated in cancerous tissues as a compensatory mechanism against the cancer development. Alternatively, this upregulation may promote tumor growth as a pathogenic mechanism. Our pharmacological intervention study with NaHS, an exogenous H_2S donor, on cultured SGC7901 cells helps to exclude the tumor-promoting role of H_2S since NaHS treatment inhibits cancer cell proliferation, migration, and invasion. NaHS has a fast releasing rate in aqueous solution and produces one-third of free H_2S at neutral pH. NaHS decreased cancer cell viability at concentrations as low as 200 μM (Figure 2). This concentration of NaHS will give 60–70 μM free H_2S. Although this concentration is still

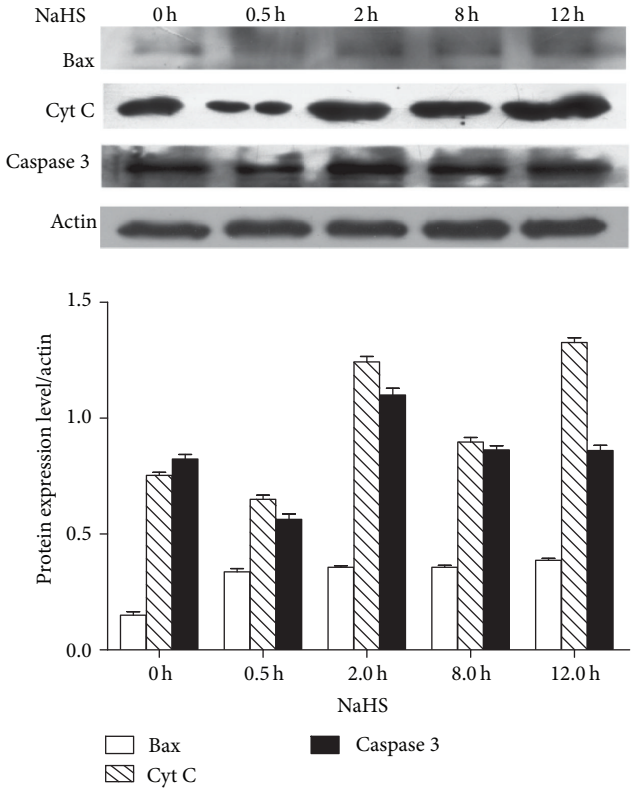

FIGURE 4: NaHS increased the levels of Bax, caspase 3, and Cyt C in SGC7901cells, detected by western blotting.

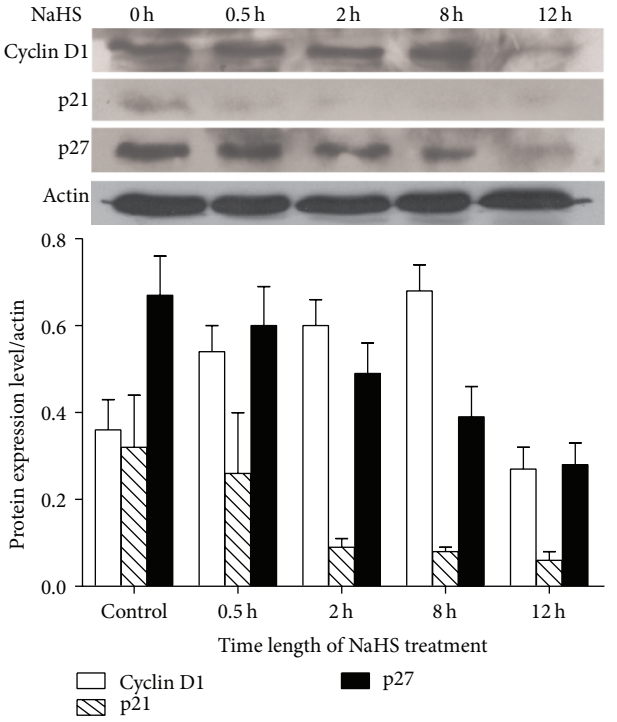

FIGURE 5: The effect of NaHS on the expression of cell cycle proteins, cyclin D1, p21, and p27 by western blotting. Cyclin D1 was upregulated, but p21 and p27 were downregulated by 0.8 mM NaHS incubation.

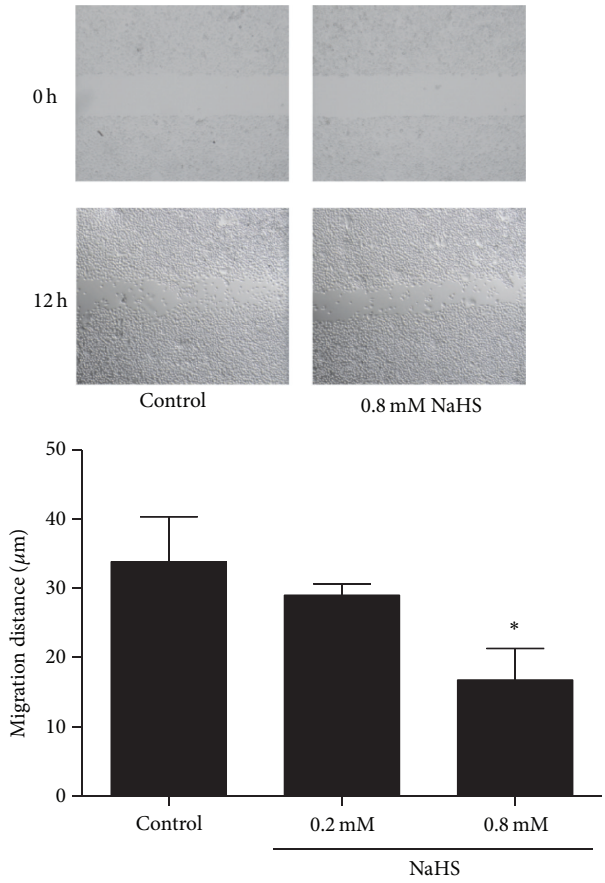

FIGURE 6: NaHS reduces cancer cell migration. Gastric cancer cells, SGC7901, were cultured in the absence or presence of NaHS. The effects of NaHS on cell migration were determined by a scratch assay. $^{*}P < 0.05$ versus control. $n = 3$.

higher than the estimated physiological range of H_2S in the circulation at low micromolar to high nanomolar levels, it is certainly perceivable as a therapeutic concentration. Moreover, the concentration of H_2S on the epithelial surface of the stomach mucosa can reach 0.5 mM, because H_2S generated by CSE and CBS in gastric mucosa can be retained by the gastric adherent mucus gel layer [18].

We also determined the role of endogenous H_2S in cancer cell viability by testing the effects of CSE inhibitor PPG and CBS inhibitor HA on the viability of SGC7901 cells. PPG alone, but not HA alone, increased cell viability (Figure 2). Furthermore, PPG increased mitotic rate of SGC7901 cells, but HA alone failed to do so (Figure 3). These results indicate that CSE, rather than CBS, plays a major role in gastric production of H_2S [9] and gastric cancer development. Future determination of endogenous H_2S levels in normal gastric tissues and gastric cancer tissues will strengthen this notion. Our previous studies have shown that H_2S was endogenously produced in a colon cancer cell line (WiDr) and colon tissues through the activities of both CSE and CBS. Butyrate and NaHS decreased cell viability in a dose-dependent manner. However, silence of CBS, not CSE, decreased butyrate-stimulated H_2S production and reversed butyrate-inhibited

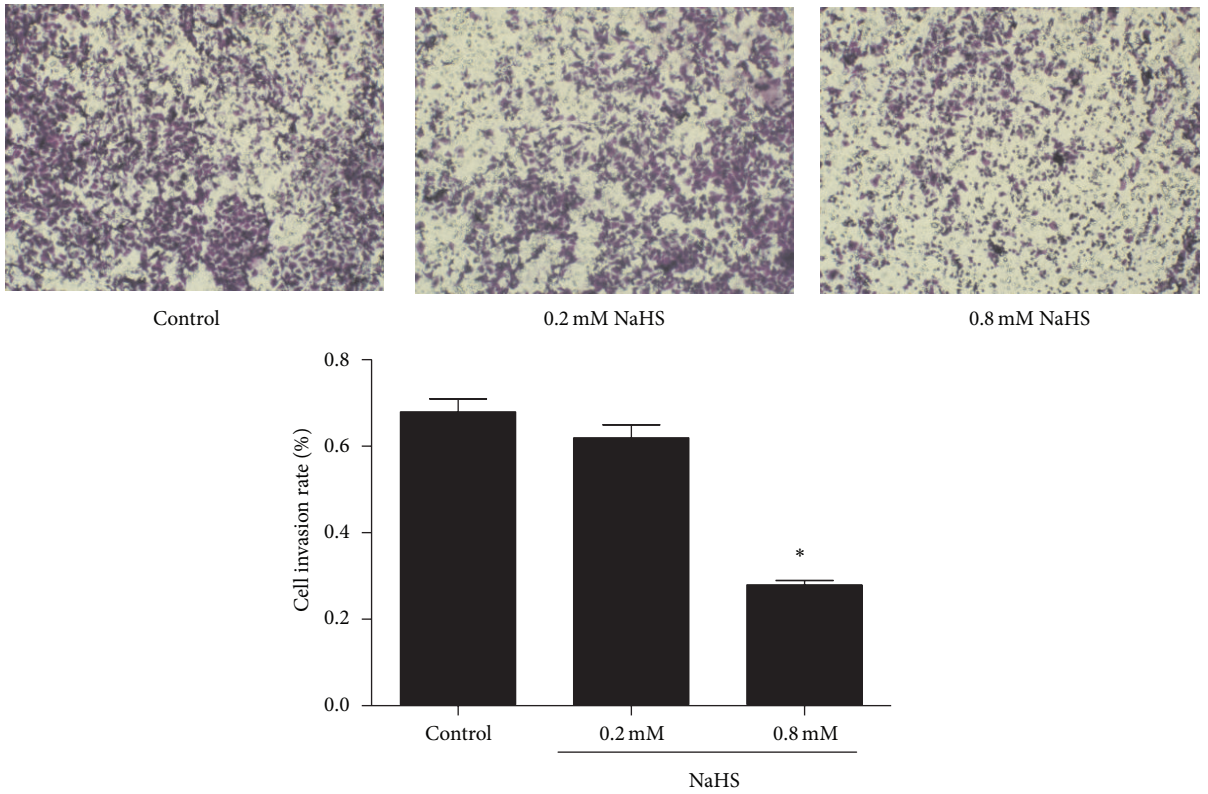

Figure 7: NaHS inhibits cancer cell invasion. Cancer cell invasion was performed in Boyden Chambers with hanging inserts. Cells transmigrated through the matrix gel were calculated based on the cells seeded on the upper chambers. NaHS was added to the upper chambers and cell invasion ratio was determined. $^{*}P < 0.05$ versus control. $n = 3$.

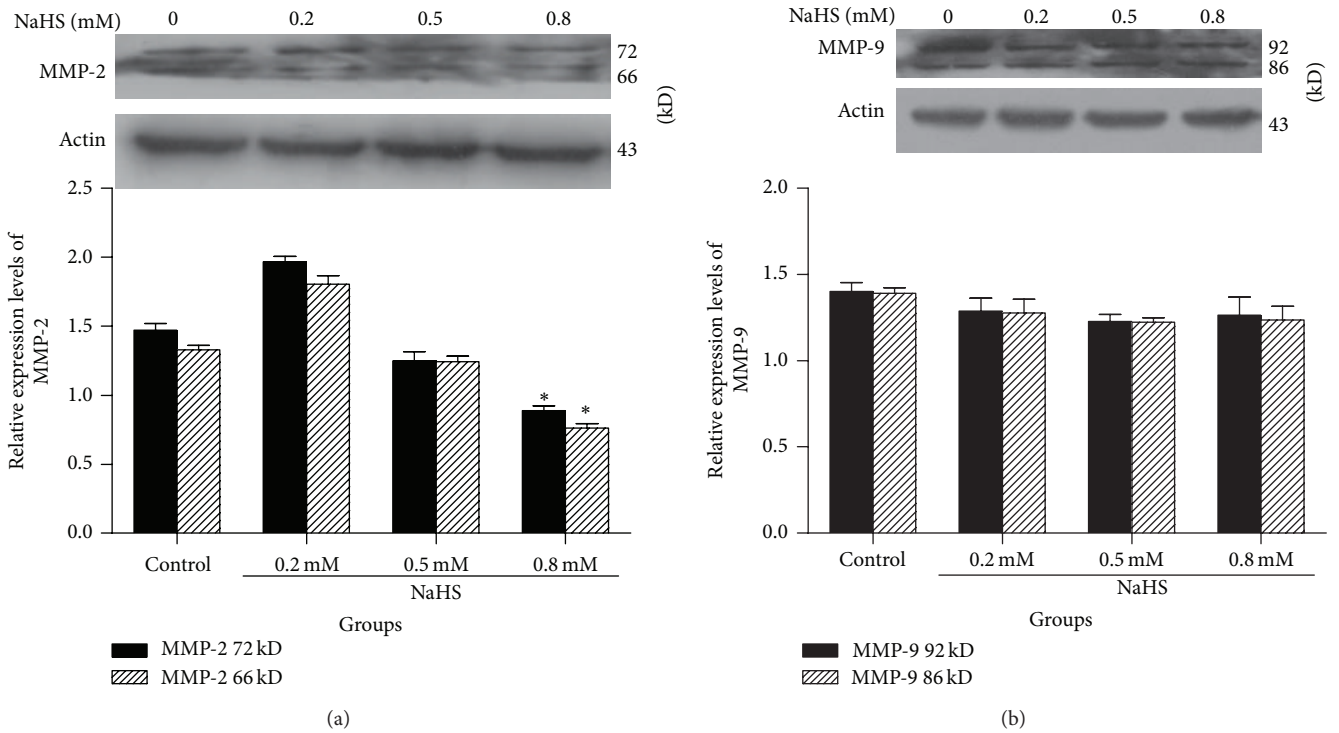

Figure 8: The protein expression levels of MMP-2 and MMP-9 during NaHS treatment. Cell lysate of SGC7901 treated with NaHS was immunoblotted with MMP-2 or MMP-2 antibody and protein expression level of both proteins was determined. (a) MMP-2 expression and (b) MMP-9 expression were determined. $^{*}P < 0.05$ versus control. $n = 3$.

cell viability [19]. It appears that CSE and CBS play different roles in endogenous H_2S production along gastrointestinal tract.

The proapoptotic effect of NaHS on SGC7901 cells is demonstrated in this study. This effect may be mediated by NaHS-induced upregulation of Bax, Cyt C, and caspase 3. The activation of intrinsic pathway during apoptosis is triggered by Bax translocation to mitochondria, followed by cytochrome C release from mitochondria and activation of caspase 3. We also assessed the changes in cell cycle control protein levels, including oncogene cyclin D1 and tumor suppressor genes $p21^{waf1/cip1}$ and $p27^{kip1}$ expression, with NaHS treatment. The expression of cyclin D1 protein was increased with 8 h of incubation with NaHS, but decreased at 12 h of NaHS incubation. This expression pattern of cyclin D1 was accompanied by the decreased expression of $p21^{waf1/cip1}$ and $p27^{kip1}$ proteins. Cell cycle progression is controlled by cyclins and cyclin-dependent kinase. Cyclin D1 is a major oncogene overexpressed in many types of cancers. Elevated expression of cyclin D1 shortens G1 phase of the cell cycle to facilitate cell cycle progression through G1 checkpoint. Both $p21^{waf1/cip1}$ and $p27^{kip1}$ proteins are cyclin-dependent kinase inhibitors (CDKI). Decreased expression of $p21^{waf1/cip1}$ and $p27^{kip1}$ proteins of gastric carcinoma cells by NaHS treatment suggests that more cancer cells may proceed through the G1 checkpoint to S and G2 phases.

To determine the effect of H_2S on cancer cell migration and invasion, we carried out the "scratch" assay on cultured confluent SGC7901 cells. Our finding showed that NaHS inhibited cancer cell migration and invasion. For cell invasion to occur, extracellular matrix must be degraded. MMPs are potent proteinases for cancer cells to degrade the matric gel. MMP-2 and MMP-9 are two major matrix metalloproteinases [20]. Our immunoblotting tests suggested that the inhibitory effects of H_2S on cell invasion might be through the downregulation of MMP-2, not MMP-9. H_2S inhibits cell migration (the initial step for cell invasion) and MMP-2 expression (critical step for cell invasion) and, therefore, blocks gastric cancer cell invasion.

In conclusion, endogenous hydrogen sulfide may play an anticancer role in gastric malignance development by regulating multiple steps. Our in vitro cell culture study shows the potential of a H_2S donor in restricting the growth and migration of gastric cancer cells. These observations should be extended to whole animal in vivo studies, such as using gastric cancer-implanted or gastric cancer-metastasis animal models, before the therapeutic application of H_2S donors against gastric cancer development can be realized.

Disclosure

Li Zhang and Qi Qi are the co-first authors.

Conflict of Interests

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

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