Review Article
The Drug Developments of Hydrogen Sulfide on Cardiovascular Disease

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Received 30 March 2018; Accepted 27 May 2018; Published 29 July 2018

Academic Editor: Mohamed M. Abdel-Daim

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The recognition of hydrogen sulfide (H2S) has been evolved from a toxic gas to a physiological mediator, exhibiting properties similar to NO and CO. On the one hand, H2S is produced from L-cysteine by enzymes of cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS), 3-mercaptopyruvate sulfurtransferase (3MST) in combination with aspartate aminotransferase (AAT) (also called as cysteine aminotransferase, CAT); on the other hand, H2S is produced from D-cysteine by enzymes of D-amino acid oxidase (DAO). Besides sulfide salt, several sulfide-releasing compounds have been synthesized, including organosulfur compounds, Lawesson’s reagent and analogs, and plant-derived natural products. Based on garlic extractions, we synthesized S-propargyl-L-cysteine (SPRC) and its analogs to contribute our endeavors on drug development of sulfide-containing compounds. A multitude of evidences has presented H2S is widely involved in the roles of physiological and pathological process, including hypertension, atherosclerosis, angiogenesis, and myocardial infarcts. This review summarizes current sulfide compounds, available H2S measurements, and potential molecular mechanisms involved in cardioprotections to help researchers develop further applications and therapeutically drugs.

1. Introduction

In an evolutionary perspective, the synthesis and catabolism of hydrogen sulfide (H2S) by living organisms antedates the evolution of vertebrate. Bacteria and archaea produce and utilize the stinking gas as one of the essential sources for their survival and proliferation. For many decades, H2S, the colorless gas with a strong odor of rotten gas, is recognized as a toxic gas and an environmental pollutant. The mechanism of its toxicity is a potent inhibition of mitochondrial cytochrome c oxidase, which is the important enzyme that is closely related with chemical energy in the form of adenosine triphosphate (ATP). Sulfide, together with cyanide, azide, and carbon monoxide (CO), all can inhibit cytochrome c oxidase which leads to chemical asphyxiation of cells.

In the last two decades, the perception of H2S has been changed from that of a noxious gas to a gasotransmitter with vast potential in pharmacotherapy. At the end of the 1980s, endogenous H2S is found in the brain [1]. Then, its enzymatic mechanism, physiological concentrations, and specific cellular targets were described in the year 1996 [2]. Subsequently, the physiological and pharmacological characters of H2S were unveiled. Recently, H2S, followed with NO and CO, is identified as the third gasotransmitter by Wang [3]. The three gases share some common features. They are all colorless and poisonous gases. With the exception of gas
pressure in atmosphere, they can dissolve in water at different solubility. All these small signaling molecules possess significant physiological importance, like anti-inflammation and antiapoptosis. The similarities and differences of the features of NO, CO, and H2S are summarized in Table 1.

This review is prepared for researchers, who are interested in H2S and sulfide-containing compounds, on drug development of cardiovascular disease. Therefore, some key issues were discussed, like "donors and inhibitors" to support choosing the sulfide-releasing chemicals and specific inhibitors. Readers could depend on the precision of currently measuring methods to decide the analyzing techniques. The toxic effect of H2S on living organisms has been recognized for nearly 300 years, and until recently, it was believed to be a poisonous environmental pollutant with minimal physiological significance. H2S is more toxic than hydrogen cyanide and exposed to as little as 300 ppm in the air for just 30 min is fatal to human. The level of odor detection of sulfide by the human nose is at a concentration of 0.02–0.1 ppm, 400-fold lower than the toxic level. As a broad-spectrum toxicant, H2S affects many organ systems including the lung, brain, and kidney.

H2S is often produced through the anaerobic bacterial breakdown of organic substrates in the absence of oxygen, such as in swamps and sewers (anaerobic digestion). It also results from inorganic reactions in volcanic gases, natural gas, and some well waters. Digestion of algae, mushrooms, garlic, and onions is believed to release H2S by chemical transformation and enzymatic reactions [5]. Structures of natural food-releasing H2S on digestion are shown in Figure 1. Consuming mushrooms, garlic, and onions with the characteristic foul odor of rotten eggs, is known for decades as a toxic gas and an environmental hazard. It is soluble in water (1 g in 242 ml at 20°C). In water or plasma, H2S is a weak acid which hydrolyzes to hydrogen ion and hydrosulfide and sulfide ions as following: H2S ↔ H+ + HS− ↔ 2H+ + S2−. The pKa at 37°C is 6.76. When H2S is dissolved in physiological solution (pH 7.4, 37°C), it yields approximately 18.5% H2S and 81.5% hydrosulfide anion (HS−), as predicted by the Henderson-Hasselbalch equation [4]. H2S could be oxidized to sulfur oxide, sulfate, persulfide, and sulfite. H2S is permeable to plasma membranes as its solubility in lipophilic solvents is fivefold greater than in water. In other words, it is able to freely penetrate cells of all types.

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Table 1: Comparison of nitric oxide, carbon monoxide, and hydrogen sulfide.

|                              | Nitric oxide | Carbon monoxide | Hydrogen sulfide |
|------------------------------|--------------|-----------------|-----------------|
| **Formula**                  | NO           | CO              | H2S             |
| **Color and odor**           | Colorless; a mild, sweet odor | Colorless; odorless | Colorless; smell like rotten egg |
| **Free radical**             | Yes          | No              | No              |
| **Flammable**                | No           | No              | Yes             |
| **Toxicity**                 | Yes          | Yes             | Yes             |
| **Inhibition of mitochondrial cytochrome c oxidase** | Yes | Yes | Yes |
| **Resources**                | L-arginine or nitrite | Protohaem IX | L/D-cysteine |
| **Intermediate products**    | L-NG hydroxyarginine, citrulline | Biliverdin IX-α | Cystathionine, L-cysteine, α-ketobutyrate, and pyruvate |
| **Enzymes**                  | eNOS, iNOS, and nNOS | HO-1, HO-2, and HO-3 | CBS, CSE, 3MST/AAT, and DAO |
| **Vascular effect**          | Vasodilation, angiogenesis | Vasodilation, angiogenesis | Vasodilation, angiogenesis |
| **Inhibition inflammation**  | Yes          | Yes             | Yes             |
| **Antiapoptosis**            | Yes          | Yes             | Yes             |
| **Haem effect**              | Yes          | Yes             | Yes             |
| **Molecular targets**        | Soluble guanylate cyclase (sGC) | Soluble guanylate cyclase (sGC) | K_ATP (ATP-gated potassium) channel |
| **Targeting outcome**        | Increase cGMP, activate KCa channels and nitrosylation | Increase cGMP, activate KCa channels | Increase cGMP and cAMP, activate K_ATP channels and sulfhydration |
| **Application on human**     | Pulmonary hypertension, lung transplantation, and ARDS | Not available | Not available |

2. Physical and Biological Characteristics

H2S, a colorless and flammable gas with the characteristic foul odor of rotten eggs, is known for decades as a toxic gas and an environmental hazard. It is soluble in water (1 g in 242 ml at 20°C). In water or plasma, H2S is a weak acid which hydrolyzes to hydrogen ion and hydrosulfide and sulfide ions as following: H2S ↔ H+ + HS− ↔ 2H+ + S2−. The pKa at 37°C is 6.76. When H2S is dissolved in physiological solution (pH 7.4, 37°C), it yields approximately 18.5% H2S and 81.5% hydrosulfide anion (HS−), as predicted by the Henderson-Hasselbalch equation [4]. H2S could be oxidized to sulfur oxide, sulfate, persulfide, and sulfite. H2S is permeable to plasma membranes as its solubility in lipophilic parts.
be higher than its circulating level. The concentration of endogenous H$_2$S has been reported up to 50–160 μM in the brains of rat, human, and bovine [1, 10, 11]. Significant amounts of H$_2$S are generated from vascular tissues, and this production varies among different types of vascular tissues. For instance, the homogenates of thoracic aorta yielded more H$_2$S than that of portal vein of rats [8]. Furne et al. reported that in situ tissue H$_2$S level through analyzing the gas space over rapidly homogenized mouse brain and liver was only 15 nM [12].

3. Synthesis and Catabolism of H$_2$S

H$_2$S is endogenously formed by both enzymatic and nonenzymatic pathways [3]. The enzymatic procedure of synthesizing H$_2$S, in mammalian tissues, is involved in two pyridoxal 5′-phosphate-dependent enzymes: cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS) [13–15]. As shown in Figure 1, H$_2$S is catalyzed from the desulfhydration of L-cysteine, a sulfur containing amino acid derived from alimentary sources, produced by the transsulfuration pathway of L-methionine to homocysteine or liberated from other endogenous proteins [16, 17]. As the intermediate, CBS catalyzes homocysteine together with serine to yield cystathionine, which is converted to cysteine, α-ketobutyrate, and NH$_4^+$ by CSE. The two pyridoxal 5′-phosphate-dependent enzymes both or either catalyze the conversion of cystathionine to H$_2$S, pyruvate, and NH$_4^+$. CSE also could catalyze a β-disulfide elimination reaction that results in the production of thiocysteine, pyruvate, and NH$_4^+$. Thiocysteine is associated with cysteine or other thiols to form H$_2$S [18]. The two synthesis pathways of producing H$_2$S are illustrated in Figure 1.

The two enzymes are widespread in mammalian tissues and cells and also in many invertebrates and bacteria [19]. The activity of CSE is chiefly concentrated in the liver, heart, vessels, kidney, brain, small intestine, stomach, uterus, placenta, and pancreatic islets; whereas, the amount of CBS is mainly located in the brain, liver, kidney and ileum, uterus, placenta, and pancreatic islets [20]. The locations of H$_2$S-
producing enzymes are seen in Table 2. In several species, the liver is the common organ containing the two enzymes in abundance. According to the research of Zhao et al., the intensity rank of biosynthesis of H2S by origin of exogenous cysteine in different rat blood vessels was tail artery > aorta > mesenteric artery [21].

A third enzymatic reaction contributing to H2S production has recently been identified in brain and vascular endothelium, that is, 3-mercaptoppyruvate sulfurtransferase (3MST) in combination with aspartate aminotransferase (AAT) (also called cysteine aminotransferase, CAT) [22, 23], seen in Figure 1. In mitochondria, L-cysteine and α-ketoglutarate as substrates can be converted to 3-mercaptoppyruvate (3MP) by AAT; then, the intermediate product is converted to H2S by 3MST [23]. In the brain, 3MST is found in neurons [24] and astrocytes [25], while CBS in astrocytes [24]. It could speculate that the two enzymes of catalyzing H2S play different roles in the nervous system. In vascular tissues, 3MST could be detected in both endothelial cells and vascular smooth muscle cells (SMCs), while AAT just occurs in endothelial cells. From another perspective, only vascular endothelial cells in vessel could utilize the two enzymes to produce H2S, whereas vascular SMCs likely absorb 3-mercaptoppyruvate or other sources to generate H2S which exerts as a vasodilator.

The fourth enzymatic pathway was recently reported by Shibuya et al. [26] that produces H2S from D-cysteine by D-amino acid oxidase (DAO). Different from using L-cysteine to produce H2S by CBS, CSE, and 3MST/AAT, which are pyridoxal 5′-phosphate- (PLP-) dependent enzymes, D-cysteine pathway generates H2S by PLP-independent enzyme [27]. Similar to 3MST on mitochondria, DAO localizes to peroxisomes in mitochondrial fractions [28]. D-cysteine is metabolized by DAO in peroxisomes to achiral 3MP, which is also generated from L-cysteine by AAT [27, 29]. 3MP then is metabolized to final H2S through 3MST, due to the vesicular trafficking between mitochondria and peroxisomes [30]. The key enzyme in new D-cysteine pathway, DAO was verified by DAO-selective antagonist I2CA, which suppressed the production of 3MP and H2S from D-cysteine in concentration-dependent manner, but that from L-cysteine was not influenced by I2CA [26]. This new enzymatic H2S-producing pathway is integrated into the part of "synthesis" in Figure 1.

The nonenzymatic route of yielding H2S is the conversion of elemental sulfur and transformation of oxidation of glucose. The nonenzymatic route is presented in vivo, involving phosphogluconate (<10%), glycolysis (>90%), and glutathione (<5%) [3].

In the pathway of H2S production, there are several important amino acids: homocysteine and D-cysteine. Besides the generation of H2S pathway, homocysteine is related to folate cycle and methionine cycle [31], the latter of which is participated in methionine, SAM and SAH, as previously stated. As the bridge of the two cycles, homocysteine could be remethylated to methionine by interacting with methylenetetrahydrofolate (methyl-THF) and vitamin B12 as cofactor under the synthesis of methionine synthase (MS). Methyl-THF is transformed from methylenetetrahydrofolate (methylene-THF) by methylenetetrahydrofolate reductase (MTHFR). Tetrahydrofolate (THF) is generated by remethylation and converted to methylene-THF, thus integrated the folate cycle. In another cycle, methionine is transformed to S-adenosylmethionine (SAM) by methionine adenosyltransferase (MAT) and then is converted to S-adenosylhomocysteine (SAH), which is subsequently hydrolyzed to homocysteine by glycine N-methyltransferase (GNMT). The cycles of homocysteine can assist researchers to link the studies of upstream and downstream of H2S, as illustrated in Figure 1. The second interesting amino acid is D-cysteine, because mammalian enzymes generally metabolize L-amino acids, except a little few like D-aspartate and D-serine [29]. Previously, D-cysteine is widely used as a negative control for L-cysteine until discovered as a highly effective H2S-producing source by Hideo group [26]. As the key enzymes in D-cysteine pathway, DAO is localized in the cerebellum and kidney, together with 3MST [26]. After birth, the level of DAO increased then reached maximal at 8 weeks in mice, while the level of 3MST was quite high at birth but slightly reduced at 8 weeks in mice [27]. Taken together, the level of H2S through D-cysteine pathway rose after birth and rocketed to maximal at 6 weeks [27]. The level of H2S generated from L-cysteine was much lower than that from D-cysteine and remains in a certain amount over time. Additionally, the generation of H2S from D-cysteine is 80 times more efficient than that from L-cysteine in the kidney [26]. Moreover, the generation of H2S from D-cysteine in the kidney is 7 times higher than that in the cerebellum, which is the region producing highest level of H2S from D-cysteine than other parts in the brain [26]. Since H2S has presented significant therapeutic potentials on anti-inflammation, antioxidation, antiapoptosis, antimitochondrial dysfunction, and energy

| Table 2: Characteristics of H2S-producing enzymes. |
|-----------------------------------------------|
| **Cystathionine γ-lyase (CSE)** | **Cystathionine β-synthase (CBS)** |
| **Localization** | Liver, heart, vessels, kidney, brain, adipose, small intestine, stomach, uterus, placenta, and pancreatic islets | Brain, liver, kidney and ileum, uterus, placenta, and pancreatic islets |
| **Activators** | Pyridoxal 5′-phosphate | Pyridoxal 5′-phosphate, S-adenosyl-L-methionine, and Ca2+/calmodulin |
| **Inhibitors** | D,L-propargylglycine, β-cyano-L-alanine | Hydroxylamine, aminoxyacetate |
| **Functional roles** | H2S production in the liver and smooth muscle | H2S production in the brain and nervous system |
reservoir, the new D-cysteine pathway in the kidney and cerebellum may provide researchers new ideas of finding therapeutic approaches on brain and kidney diseases, such as kidney transplantation.

Cysteine metabolism is engaged in three major routes. Apart from the conversion of H$_2$S, one path is oxidation of -SH group by cysteine dioxygenase (CDO) to cysteine sulfinate, which is decarboxylated to hypotaurine by cysteine sulfinate decarboxylase (CSD) and then further transformed to taurine by a nonenzymatic reaction or by hypotaurine dehydrogenase (HDH) or which is converted to sulfanyl pyruvate, subsequently to sulfite and further sulfate. Another path from cysteine is synthesis GSH by glutathione synthase (GS) from γ-glutamyl cysteine, which is originated from cysteine and glutamate catalyzed by γ-glutamyl cysteine synthase (GCS). Besides H$_2$S, cysteine metabolism is integrated in Figure 1 for helping researchers to find out the potential associations.

The concentration of H$_2$S is not only determined by the rate of formation but also by degradation of H$_2$S. Dissolved gaseous H$_2$S is in a pH-dependent equilibrium, with hydro sulfide anions (HS⁻) and sulfide anions (S²⁻), which can be catabolized to any sulfur-containing molecule. Sulfide, via nonenzymatic route, is catabolized to thiosulfate, which could be catalyzed to sulfite by thiourea reductase (TSR) in the livers, brains, or kidneys, or by thiourea sulfurtransferase (TSST) in the livers, sequentially oxidized to sulfate via sulfite oxidase (SO) by a glutathione- (GSH-) dependent reaction. The last product is excreted in urine [32]. H$_2$S could be broken down by rhodamine, methylated to CH$_3$SH, sequestered by methemoglobin, interacted with superoxide or NO, and scavenged by metallo- or disulfide-containing molecules such as oxidized glutathione [18, 19]. The major routes of degradation of H$_2$S through nonenzymatic oxidation of sulfide also yield elemental sulfur, polysulfides, dithionate, and polythionates. Among them, polysulfides could be produced through the enzymatic way via 3MST [33–35] and the chemical interaction of H$_2$S with NO [36]. The whole schematic version of source, synthesis, and metabolism of H$_2$S is depicted in Figure 1.

4. Donors and Inhibitors of H$_2$S

4.1. The Donors of H$_2$S

4.1.1. Sulfide-Containing Salts. Sodium hydrogen sulfide (NaHS) and disodium sulfide (Na$_2$S) are the common H$_2$S-releasing chemicals in research of hydrogen sulfide. These sodium salts purchased from pharmaceutical companies are usually aquo compounds, like NaHS·12H$_2$O, Na$_2$S·9H$_2$O, or anhydrous forms. The products of sodium hydrogen sulfide and disodium sulfide should be white. The pills with yellow color predicate the anhydrous forms have been converted to hygroscopic blocks and should not be purchased. White sulfide products are likely to have greater purity, but may contain sodium salts of thiosulfate or higher oxidation state sulfur oxyanions [37]. Contamination by trace metal ions may also be important, as these catalyze oxidation processes. The sulfides should therefore be reserved in a vacuum desiccator to minimize oxidation.

The solution of NaHS, at physical pH and room temperature, hydrolyzes to sodium ion, hydrosulfide as following: NaHS ↔ Na$^+$ + HS$^-$ Solutions of HS$^-$ are sensitive to oxygen, converting mainly to polysulfides, indicated by the appearance of yellow color. Hence, solutions of fresh prepared NaHS should be clear and put to use immediately. The purity of sulfides could be measured by determining the sulfide content either by titration with bromate, as described in standard analytical chemistry texts, or by UV spectroscopy in the case of sodium hydrogen sulfide, at pH 9, which has an absorption maximum at 230 nm with a molar absorptivity of 7200 l/mol/cm [38].

Considering the unstable chemical properties of NaHS and Na$_2$S, some researchers introduce another donor of H$_2$S, calcium sulfide (CaS), which is more steady [39]. CaS can be found as one of the effective components in a traditional herb, named “hepar sulfuris calcareum,” usually applied to homeopathic remedy. Oral administration of CaS will be decomposed to more H$_2$S in stomach acid environment. This review postulates CaS may carry out hypotension, arguing from its catabolism, relationship of calcium supplementation and blood pressure, dosage design, and traditional application of homeopathic remedy on infection.

4.1.2. H$_2$S-Releasing Molecules. Thioacetamide is an organosulfur compound with the formula C$_3$H$_7$NS. This white crystals solid is soluble in water and serves as a source of sulfide ions in the synthesis of organic and inorganic compounds [40]. For lab safety, thioacetamide is carcinogen class 2B and has hepatotoxicity. Thioacetamide was widely used in classical qualitative inorganic analysis as an in situ source for sulfide ions.

Some research laboratories developed H$_2$S releasers. Lawesson’s reagent is a chemical compound used in organic synthesis as a thiation agent and is also a H$_2$S releaser. Lawesson’s reagent is first synthesized in 1956 during a systematic study of the reactions of arenes with P$_4$S$_{10}$ [41]. After much time, it is first made popular by Sven-Olov Lawesson for introducing a thiation procedure as an example of a general synthetic method for the conversion of carbonyl to thio carbonyl groups [41]. 2,4-Bis (4-methoxyphenyl)-1,3,2,4-dithia diphenphosphate 2,4-disulfide, Lawesson’s reagent, has a four-membered ring of alternating sulfur and phosphorus atoms. Normally in higher temperatures, the central phosphorus/sulfur four-membered ring can open to form two reactive diithiophosphine ylides (R-PS2), which decompose to release H$_2$S. As its strong and unpleasant smell, it is best to prepare Lawesson’s reagent within a fume hood and treat all glassware used with a decontamination solution before taking the glassware outside the fume hood.

Based on Lawesson’s compound, a series of compounds are synthesized. Professor Moore’s lab reports that morpholin-4-ium-4-methoxyphenyl (morpholino) phosphinodithioate (GY4137) releases H$_2$S slowly both in vitro and in vivo. It has been proved that GYY4137 has vasodilator and antihypertensive activities and a useful H$_2$S-releasing chemical in the study of biological effects of H$_2$S [42]. In a later experiment, administration of GYY4137 to lipopolysaccharide- (LPS-) induced rats displays its anti-
Na₂S, which is pure, pH neutral, and stable. IK-1001 has been from the company Ikaria, is an injectable form of IK-1001, from the company Ikaria, is an injectable form of (http://www.antibe-therapeutics.com). The compound, IK-

bowel disease, joint pain, and irritable bowel syndrome tures of natural food-releasing H₂S on digestion are shown in Figures 2 and 3. Structures of H₂S-releasing molecules are shown in Figure 2.

Considering pharmacological effects and adverse effects of H₂S donors which are made up of well-established parent compounds and H₂S-releasing moieties. CTG Pharma developed ACS series H₂S-releasing compounds to meet their interests on the aspects of hypertension, metabolic syndrome, thrombosis, and arthritis (http://www.ctgpharma .com). Antibe Therapeutics synthesizes several ATB series H₂S-releasing derivatives for the treatments of inflammatory bowel disease, joint pain, and irritable bowel syndrome (http://www.antibe-therapeutics.com). The compound, IK-1001, from the company Ikaria, is an injectable form of Na₂S, which is pure, pH neutral, and stable. IK-1001 has been used several basic studies and processed into clinical trials. One is a phase I safety trial for assessing pharmacokinetics of intravenous IK-1001 (ClinicalTrials.gov ID: NCT00879645). Another is a phase II efficacy trial which administers IK-1001 in patients undergoing surgery for a coronary artery bypass graft (ClinicalTrials.gov ID: NC100858936). The effects of some H₂S-releasing compounds are shown in Table 3.

4.1.3. Natural Products Containing Sulfur. Digestion of algae, mushrooms, garlic, and onions is believed to form H₂S by chemical transformation and enzymatic reactions [5]. Structures of natural food-releasing H₂S on digestion are shown in Figures 2 and 3. Nearly all the allium families are sulfur-rich containing. Several publication reports enumerated functional activities of garlic. It exhibits hypolipidemic, antimicrobial, antiplatelet, and procirculatory effects [44–46]. It also demonstrates immune enhancement and provides anti-cancer, antiinflammatory, and anti proliferative that are interesting in chemopreventive interventions. Additionally, aged garlic extract possesses hepatoprotective, neuroprotective, and antioxidative activities [47]. The major sulfur-containing compounds in intact garlic are γ-glutamyl-S-allyl-L-cysteine and S-allyl-L-cysteine sulfoxides (alliin). Both are abundant as sulfur compounds, and allin is the primary odorless, sulfur-containing amino acid, a precursor of allicin, methiin, (+)-S-(trans-1-propenyl)-L-cysteine sulfoxi-

desulfurase [59]. The CBS locus is mapped to chromosome 21 (21q22.3) [60]. Several specific blockers for CSE and CBS are currently available. D,L-Propargylglycine (PAG) and b-cyano-L-alanine selectively inhibit CSE [8]. L-Cysteine metabolites, including ammonia, H₂S, and pyruvate, cannot inhibit CSE activity [61]. CBS is inhibited by hydroxylamine (HA) and aminoxyacetate (AOAA) albeit

![Figure 2: Structures of H₂S-releasing molecules.](image)
these chemicals are not selective inhibitors of CBS [2]. The relationships between H$_2$S-producing enzymes and their inhibitors are summarized in Table 2.

The currently known regulations of H$_2$S-producing enzymes are glutamate and its receptors, S-adenosylmethionine (SAM), hormones, and other gasotransmitters—NO and CO. In the brain, electrical stimulation and excitatory neurotransmitter, glutamate, rapidly increase CBS activity in Ca$^{2+}$/calmodulin-dependent manner [62]. Both α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) glutamate receptors and N-methyl-D-aspartate (NMDA) are involved in this effect. SAM is an intermediate product of methionine metabolism and a major donor of methyl groups. This allosteric regulator can activate CBS by approximately twofold [2]. Sex hormones seem to regulate brain H$_2$S, since CBS activity and H$_2$S level are higher in male than in female mice and castration of male mice decreases H$_2$S formation [16]. Sodium nitroprusside, a nitric oxide donor, increases the activity of brain CBS in vitro; however, this effect is NO-independent and results from chemical modification of the enzyme’s cysteine groups [63]. In contrast, NO itself may bind to and inactivate the CBS. Interestingly, CO is a much more potent CBS inhibitor than NO and it is suggested that CBS may be one of the molecular targets for CO in the brain [64, 65]. In homogenates of the rat aorta, NO donors acutely increase CSE-dependent H$_2$S generation in a cGMP-dependent manner [21]. Moreover, prolonged incubation of cultured vascular smooth muscle cells in the presence of NO donors increases CSE mRNA and protein levels [8]. The physiological significance of NO in the regulation of H$_2$S production is also supported by the observation that circulating H$_2$S level as well as CSE gene expression and enzymatic activity in the cardiovascular system are reduced in rats chronically treated with NOS inhibitor. Thus, NO is probably a physiological regulator of H$_2$S production in the cardiovascular system. Recently, the inhibitors of 3MST were selected by high-throughput screening (HTS) of a large chemical library (174,118 compounds) with the H$_2$S-selective fluorescent probe, HSip-1, which discovered compound 3 presented very high selectivity for 3MST over other H$_2$S/sulfane sulfur-producing enzymes and rhodanese [66]. This study provides these compounds as useful chemical tools for investigating the physiological roles of 3MST.

5. H$_2$S Measurements

5.1. Spectrophotometric Method. The principle of spectrophotometric method of H$_2$S depends on the formation of methylene blue. H$_2$S is chemiadsorbed by zinc acetate and transformed into stable zinc sulfide. The sulfide is recovered by extraction with water. In contact with an oxidizing agent such as ferric chloride in a strongly acid solution, it reacts with the N,N-dimethyl-p-phenylenediammonium
The methylene blue method has been designed to a different protocol. A common method is adding NNDPD and ferric chloride to the plasma or homogenized tissue and then developing color and colorimetric estimating immediately. Owing to the volatile character of H₂S, researchers modify the protocol, like using a filter paper to augment the contact surface and prolong the contact time [67, 68]. Based on published papers and previous experience, our lab revised the assay for H₂S by placing a sample in an airtight vessel with a central tube. The central tube contains a filter paper wick saturated with zinc acetate. The purpose of the filter paper wick is for trapping H₂S to zinc sulfide. The reactions are initiated by mixing of strong acid with the sample, which sulfide is driven out and adsorbed onto the wick. The driving time is usually 30–120 minutes which is modified based on lab condition and optimization in the sorts of samples. Reactions are stopped by injecting 0.5% trichloroacetic acid (TCA). After gas evolution and wick absorption, the sulfide in the central tube reacts with NNDPD in present of Fe²⁺ ion. All in all, making reasonably accurate measurements of such an evanescent and reactive gas in biological tissues is difficult. Indeed, the chemical nature is usually 30–120 minutes which is modified based on lab condition and optimization in the sorts of samples. This colorimetric method is not only widely used on the determination of H₂S on serum in animal experiment but also widely used on the activity of CSE/CBS enzyme on tissues or cells. The concentrations of H₂S are reflected on the different shades of color of methylene blue and calculated by the plotting H₂S standard curve.

Two points need to be made. Firstly, most researchers’ assay H₂S using the spectrophotometric assay involves acidifying zinc acetate-treated (to “trap” free H₂S) biological samples in the presence of a dye and observing a color change. This assay actually measures total sulfide and not the gas H₂S. Secondly, H₂S is either broken down rapidly in the body by enzymes, sequestered by binding to hemoglobin, or can react chemically with a number of species abundant in tissues, including superoxide radical [69], hydrogen peroxide [67], peroxynitrite [70], and/or hypochlorite [71]. All in all, making reasonably accurate measurements of such an evanescent and reactive gas in biological tissues is difficult. Indeed, the chemical nature of gases such as H₂S, NO, and CO might render it nonsensical even to try and measure them in body fluids or tissues.

5.2. Sulfide Ion-Selective Electrode. A sulfide ion-selective electrode (SISE) is immersed in an aqueous solution containing the ions to be measured, together with a separate, external reference electrode. The electrochemical circuit is completed by connecting the electrodes to a sensitive millivoltmeter using special low-noise cables and connectors. A potential difference is developed across the SISE membrane when the sulfide ions diffuse through from the high concentration side to the lower concentration side.

At equilibrium, the membrane potential is mainly dependent on the concentration of the target ion outside the membrane and is described by the Nernst equation. Briefly, the measured voltage is proportional to the logarithm of the concentration, and the sensitivity of the electrode is expressed as the electrode slope in millivolts per decade of concentration. Thus, the electrodes can be calibrated by measuring the voltage in sulfide standard solution. Testing samples can then be determined by measuring the voltage and plotting the result on the calibration graph. The use of sulfide ion-selective electrode suffers from precipitation of metal sulfide, for example, sliver sulfide (Ag₂S) from the filling solution on the electrodes.

Reproducibility is limited by factors such as temperature fluctuations, drift, and noise. The electrode can be used at temperatures from 0 to 100°C and only used intermittently at temperatures above 80°C. Interfering ions, like mercury, must be absent from all sulfide sample. In aqueous solution, H₂S is dissolved into HS⁻ and S²⁻. In acid solution, sulfide is chiefly in the form of H₂S, while in the intermediate pH range (up to approximately pH 12), almost all the sulfide is in the form HS⁻. Only in very basic does the sulfide exist primarily as free ion (S²⁻). The SISE from Thermo Scientific supplies sulfide antioxidant buffer could maintain a fixed level of H₂S.

Nevertheless, the alkaline condition of antioxidant buffer is regarded as an influencing factor to SISE measurements in plasma. Initially, mixing samples to antioxidant buffer is reported to generate protein desulfuration and artificially increased sulfide values [72]. It is also observed that placing 5% bovine serum albumin into antioxidant buffer leads to a surging reading of total sulfide measured by SISE in the first 20 minutes and following slow accumulation in 3 hours [73].

5.3. Fluorescent Probe Assays. Currently, there are more and more labs that choose to use fluorescent probes to assay the
concentrations of real-time H$_2$S, sensitively, selectivity, and biologically compatible. There are 3 types of fluorescent probes for H$_2$S detections: reduction-based, nucleophilic-based, and metal sulfide-based.

Reaction-based fluorescent probes for H$_2$S detection are designed based on the reducing ability of H$_2$S [74]. The firstly developed fluorescent probes by Lippe and colleagues were probes SF1 and SF2 based on the H$_2$S-mediated reduction from an aryl azide to an aryl amine [75]. After adding NaHS for 1 hour, probes SF1 and SF2 detected 7- and 9-fold fluorescent increase, respectively. Probes SF4–7 were improved by the same lab with enhanced sensitivity and cellular retention [76]. The group of Peng and colleagues simultaneously reported another fluorescent probe DNS-Az through the reduction of a sulfonyl azide to a sulfonamide with faster kinetics than aryl azide reduction but less adaption [77].

Later, various fluorophores were developed for H$_2$S measurement with different colors and targeting specific organelles. Fluorescent probes SHS-M1 and SHS-M2 were reported by Bae et al. to detect mitochondrial moiety by incorporating triphenylphosphonium group [78]. SulpHensor by Yang et al. was designed to detect lysosome moiety due to the morpholine group [79]. AzMC was reported by Thorson et al. to screen CBS based on coumarin [80]. Other functional groups that can be reduced by H$_2$S were utilized in the design of fluorescent probes, like nitro group. Montoya and Pluth reported fluorescent probe HSN-1, which incorporates a nitro group into the 1,8-naphthalimide scaffold, but with greater thiol cross-reactivity than azide probes [81]. This weakness was attenuated by Wang et al. that increased electron-rich aromatic system on the nitro-based probe [82]. The concept of H$_2$S-mediated reduction was extended to other fluorophore scaffolds by several laboratories [83–85].

Nucleophilic-based fluorescent probe for H$_2$S detection is designed based on the strong nucleophilic HS$^-$ hydrolyzed from H$_2$S at physiological pH (pH = 7.4) [86]. Qian et al. used this concept to develop fluorescent probes, SFP-1 and SFP-2, which allowed fluorescence switching via HS$^-$ addition to aldehyde and underwent an intramolecular Michael addition to unsaturated acrylate ester to form a thioacetal, producing stable tetrahydrothiophene with strong fluorescence [87]. Qian et al. designed the probes with an aldehyde group ortho to an $\alpha,\beta$-unsaturated acrylate methyl ester on an aryl ring, which trapped H$_2$S and modulated a fluorescence response through decreased photoinduced electron transfer (PET) quenching of the product [87]. Disulfide bond cleaved by H$_2$S was utilized by Liu et al. and Peng et al. to develop WSP1–5, which persulfide group, like 2-thiopyridine, intramolecular nucleophilic attacked on the ester moiety to release great fluorophore [88, 89]. 50–500 $\mu$M H$_2$S in bovine plasma and 250 $\mu$M H$_2$S in cells could be detected by this probe. Reversible nucleophilic addition was exploited by Chen et al., as CouMC, to track real-time H$_2$S fluxes due to fast and potentially reversible fluorescence [90].

Metal sulfide-based fluorescent probe for H$_2$S detection is based on the phenomenon that heavy metal ions such as Fe$^{3+}$ and Cu$^{2+}$ quench the fluorescence of a nearby fluorophore [91]. Zinc sulfide complex was utilized to design a selective fluorescent probe of H$_2$S by Galardon et al. by releasing a coumarin dye [92]. Choi chose copper sulfide precipitation to design the fluorescent sulfide sensor [93]. Later, Sasakura et al. developed it to HSip-1, which possessed a cyclen macrocycle with fluorescein and binds Cu$^{2+}$ to release unbound cyclen-AF, displaying greater fluorescence [94]. The measuring range of this probe for sulfide could be 10–100 $\mu$M. Hou et al. improved the copper-containing probe to a lower detection limit of 1.7 $\mu$M [95]. Another strength of metal precipitation-based probes is that they respond to turn on within seconds, allowing the real-time H$_2$S detection [96]. Researchers may choose one of these fluorescent probes depended on their facilities, reagents, targeted organelles, and sensitivity ranges.

5.4. Other Analyzing Methods. Carbon nanotube (CNT) was introduced by Wu et al. for measuring low-concentration and nanoquantity H$_2$S [97, 98]. One of the benefits of unfunctionalized CNT in analyzing H$_2$S is due to the special bond with H$_2$S, but other proteins kept in serum. H$_2$S concentrations are reflected by the intensity of the fluorescence of the unfunctionalized CNT, due to the two values in a linear relationship. The lowest H$_2$S concentration that can be tested is 20 $\mu$M and smallest quantity of H$_2$S is 0.5 $\mu$g. The series of experiments are trying to establish a new sensor to measure micro- or nanoquantity H$_2$S, comprising unfunctionalized CNT as a transducer and LSM fluorescence as a signal acquisition modality.

Polarography is a voltammetric measurement which makes use of the dropping mercury electrode or the static mercury drop electrode. The value of diffusion current depends on the speed of electroactive material (samples) diffusing to dropping mercury electrode. This principle contributes to the measurement of the concentration of analytes. Polarography is well known for the application of quantitative measurements of O$_2$ (polarographic oxygen sensor, POS) and NO (polarographic nitric oxide sensor, PNOS). By recent years of the appreciation of the third gas transmitter, H$_2$S, several analytical methods are utilized, including polarography. A novel polarographic hydrogen sulfide sensor (PHSS) has been developed for the study of H$_2$S-producing rates and consumption in mammalian tissues, with resolution of 10 nM [99]. The polarographic sulfide sensor is also applied to the investigation of kinetics of sulfide metabolism in organisms living in sulfide-rich environment [100]. PHSS permits direct and simultaneous measurement of H$_2$S gas in biological fluids without sample preparation. PHSS has provided an alternative method for sulfide measurement.

Gas chromatography is a recent method described by Levitt et al. as a unique chemiluminescence-based technique to measure free and acid-labile H$_2$S in multiple tissues from mouse [101]. The tissues were first submerged in 50 mM glycine-NaOH buffer (pH 9.3) and homogenized. The homogenates were then transferred to syringes, which were sealed and flushed with N$_2$. The homogenate in alkaline extraction turns to acidification to pH 5.8 by adding sodium hydrogen phosphate solution (pH 5.5). After vigorous mixture, the gas space was removed to gas chromatography to analyze free H$_2$S concentration. Next, adding 50% trichloroacetic acid to the syringe, the gas was collected to test the
acid-labile H$_2$S concentration. The flow rate of N$_2$ was 25 ml/min. The concentration of H$_2$S was calculated by the plotting H$_2$S standard curve.

High-performance liquid chromatography (HPLC) is used to separate the sulfide mixture. Togawa et al. reported that using monobromobimane (MBB) with dithiothreitol (DTT) reacted with bound sulfide to counter invade agents will turn to target host tissues, making deeply more serious damage. H$_2$S regulating inflammation and injury was initially contradictory, but in recent years, more studies supported that H$_2$S inhibited the process of inflammation, except at high concentration [105]. This mediator possibly exerts its anti-inflammatory effects through reduction of leukocyte-endothelial cell adhesion [106], action on ATP-sensitive K$^+$ channels [107], scavenging of toxic free radicals [108], elevation of cyclic AMP and/or cyclic GMP [70, 71], and inhibition of nuclear factor-$\kappa$B (NF-$\kappa$B) and proinflammatory cytokines (e.g., COX-2 [109], iNOS [110], and interleukin-(IL-)1$\beta$, IL-6 [111]).

Various diseases could be found inflammatory response, like atherosclerosis, ischemia-reperfusion, and colitis. Contributing to anti-inflammatory molecular mechanisms of this novel gasotransmitter, it is not surprising that H$_2$S may participate in the process of resolution of a variety of inflammatory diseases. In atherosclerosis, H$_2$S exerts its potent inhibitor of leukocyte adherence to vascular endothelium [112]. Meanwhile, the generation of reactive oxygen species (ROS), activation of NF-$\kappa$B, increased expressions of cell adhesion cytokines, and induction of apoptosis, which were all regarded as the key promoters of pathology, were all found suppressed by H$_2$S [112, 113]. These mechanisms of action described for H$_2$S may explain that H$_2$S can diminish the plagues in arteries and attenuate the atherosclerotic injury, suggesting the character of anti-inflammation of H$_2$S is a benefit for the vascular protection.

Ischemia-reperfusion (I/R) is identified as an acute endogenous inflammatory response that characterizes release of toxic free radicals, leucocyte-endothelial cell adhesion, and platelet-leucocyte aggregation [114]. In porcine myocardial I/R model, therapeutic sulfide improved myocardial function and diminished infarct size though decreased levels of inflammatory cytokines (IL-6, IL-8, and TNF-α), reduced left ventricular pressure, and improved coronary microvascular reactivity [115]. A similar tissue protection of H$_2$S was also found in hepatic I/R injury by inhibition of inflammation (lipid peroxidation, IL-10, ICAM-1, and TNF-α) and apoptosis (caspase-3, Fas, and Fas ligand) [116]. Another study suggested that the cardioprotective effects of H$_2$S may be mediated by opening the mitochondrial K$_{ATP}$ channel and second window of protection caused by endotoxin [117].

Colitis is a one form of gastrointestinal inflammation and ulceration. Administration of H$_2$S-generating agents or precursor for H$_2$S synthesis, L-cysteine, has been shown to significantly accelerate ulcer healing [118, 119]. This ability of H$_2$S to enhance gastrointestinal resistance attracts investigators to exploit novel treatments of gastrointestinal injury and inflammation, like H$_2$S-releasing derivative of NSAIDs to reduce the adverse drug reaction of NASIDs, retarding gastrointestinal ulcer healing [120]. Evidence of H$_2$S in resolution of colitis in rats or mice studies showed that administration of H$_2$S donor significantly inhibited the severity of colitis with marked reduction of granulocyte infiltration into colonic tissue. In inflamed colon, H$_2$S production was highly increased via CSE, CBS, or other enzymatic
pathways [121, 122]. Once H$_2$S synthesis was inhibited, the colitis tended to worsen the inflammation with thickening of the smooth muscle, perforation of bowel wall, and even death [110].

7. H$_2$S in Redox Status

7.1. H$_2$S Direct Effects on Toxic Free Radicals. In a weak acid, H$_2$S dissociates in equilibrium with hydrosulfide anion (HS$^-$) and sulfide anion (S$^{2-}$). Under physiological conditions, the amounts of H$_2$S and HS$^-$ are early equal within the cell, whereas extracellular fluid and plasma exist approximately the ratio of 20% H$_2$S, 80% HS$^-$, and 0% S$^{2-}$. HS$^-$ is a potent one-electron reductant that eliminates free radicals by donating single electron. Hydrogen disulfide (H$_2$S$_2$), a kind of hydrogen polysulfide (H$_2$S$_n$), is the production of oxidation of HS$^-$ by two-electron oxidants, like hypochlorous acid [123] and hydrogen peroxide [124]. Additionally, the chemical interaction between H$_2$S and NO also produced H$_2$Sn by activating transient receptor potential ankyrin 1 (TRPA1) channels [36]. H$_2$S$_2$, a highly reactive oxidizing chemical, generates H$_2$S by reacting with thiol [125] or disproportionation [123, 126]. H$_2$S$_2$ and H$_2$S$_3$ were reported to generate redox regulators CysSSH and GSSH via 3MST in the brain of wild-type mice but not in those of 3MST-KO mice [34, 35, 127, 128].

H$_2$S is considered as an endogenous reducing agent which is produced in response to oxidative stress [129, 130]. Evidence showed that H$_2$S is a highly reactive molecule and may easily react with other compounds, especially with reactive oxygen and nitrogen species. H$_2$S reacts with at least four different ROS: superoxide radical anion [69], hydrogen peroxide [67], peroxynitrite [70], and hypochlorite [71]. All these compounds are highly reactive, and their reactions with H$_2$S result in the protection of proteins and lipids against RNS/RNS-mediated damage [70, 71] and myocardial injury induced by homocysteine in rats [131].

7.2. H$_2$S Protects Mitochondria against Oxidative Stress. Mitochondrial injury is an important source of reactive oxygen species (ROS), which is involved in a range of pathologies, such as ischemia-reperfusion, atherosclerosis, and toxin exposure [132]. Under oxidative stress conditions, mitochondria will show unstable mitochondrial membrane potential ($\Delta \varphi_{m}$), redox transitions, and negative changes in the mitochondrial permeability transition (MPT) pore and the inner membrane anion channel (IMAC) [133]. Our lab found that H$_2$S can reduce the H$_2$O$_2$-induced injury in HUVTECs via increasing ATP production, saving mitochondrial ultrastructure, stabilizing mitochondrial membrane intact, decreasing ROS and MDA, and rising antioxidants. The same situation was also unveiled in H$_2$O$_2$-stimulated isolated rabbit aorta that H$_2$S ameliorated mitochondrial dysfunction through improving O$_2$ consumption and ATP production, protecting mitochondrial respiration chain complexes activities and matrix enzymes, decreasing mitochondrial membrane permeability, and inhibiting mitochondrial ROS levels. These effects of H$_2$S indicated that the antioxidative ability of H$_2$S is through increasing antioxidants and prohibiting ROS levels and also preserving mitochondrial function to reduce the production of toxic free radicals.

8. H$_2$S in Cardiovascular System

8.1. Hypertension. Before identified as the third gasotransmitter, H$_2$S has been speculated to regulate an array of physiological processes in regulating cardiovascular functions, distinctive from its toxicological effect. A great number of studies have been carried on investigation of the modulating of blood pressure by exogenous and endogenous H$_2$S. Early at the end of the last century, it is first reported that H$_2$S relaxes the contracted smooth muscles (SM) induced by 1 $\mu$M norepinephrine in rat thoracic aorta and portal vein [134]. The relaxations in these tested aortas and veins present a NaH$_3$ dose-dependent manner, but the potency of relaxation by exogenous H$_2$S in the thoracic aorta is less than the portal vein, even by 10$^{-7}$M NaH$_3$, which are around 25% and 90%, respectively. The data also showed that the relaxation effects of H$_2$S and NO can be enhanced by each other. 30 $\mu$M NaH$_3$ can augment the loosening effect of NO by up to 13-fold. Thus, endogenous cysteine and glutathione do not have synergistic effect with NO. Subsequently, the vasorelaxant effect of H$_2$S was found in vivo of SD rats, ex vivo of aortic rings, and in vitro at rat aortic smooth muscle cells [15], which was a literature that first demonstrated the underlying mechanism of vasorelaxation, a consequence of opening K$_{ATP}^+$ channels. Interestingly, it has been found that H$_2$S induces endothelium-dependent vasorelaxation with many common mechanistic traits of hyperpolarizing factor [135]. CSE knockout mice lacked the methacholine-induced endothelium-dependent vasorelaxation in mesenteric arteries and showed higher resting membrane potential of SMCs, while hyperpolarization of SMCs induced by methacholine was observed in endothelium-intact mesenteric arteries at wild-type mice [136]. Administration of exogenous H$_2$S hyperpolarized both SMCs and vascular endothelial cells in wild-type and CSE knockout mice [136]. Removal of functional endothelium attenuated vasorelaxation of rat aorta [137] and rat mesenteric artery [138]. It appears that vasorelaxation of H$_2$S is induced on both SMCs and endothelial cells, instead of previous research discussions mainly focusing on SMCs.

A multitude of H$_2$S-induced vasodilation studies have investigated the activation of K$_{ATP}^+$ channels. One possible mechanism involved in the activation of K$_{ATP}^+$ channels by H$_2$S was opening K$_{ATP}^+$ channels and increasing K$^+$ currents resulted in hyperpolarizing membrane of smooth muscle cells [139]. The explanation of the opening of K$_{ATP}^+$ channels by H$_2$S was that cysteines on K$_{ATP}^+$ channels of SMCs were S-sulfhydrated, leading to hyperpolarization [140]. Cys443 of the inwardly rectifier (Kir) potassium channels subunit Kir 6.1 was sulfhydrated by NaH$_3$, eliciting the binding to phospholipid phosphatidylinositol (4,5)-bisphosphate (PIP2) together with decreased association of ATP [140]. Additionally, the vasodilatation effect of H$_2$S was inhibited significantly by either using a calcium-free bath solution or with the normal bath solution, but in the presence of nifedipine, a voltage-gated Ca$^{2+}$ channel inhibitor, on aortic rings [8],
indicates that the vascular effects of H₂S are also likely mediated by the attenuation of intracellular inward Ca²⁺ currents. Not only H₂S hyperpolarizes ion channels on blood vessels to possess the relaxant effects but also endothelium generates H₂S by increasing catalytic activity of CSE through calcium-calmodulin, indicating that the H₂S formation may be involved in vascular activation to reduce blood pressure [141]. Moreover, H₂S exerts cardioprotective effect by relieving vascular structural remodeling observed during hypertension, including suppression of VSMC proliferation via the activation of cardiac extracellular signal-regulated kinase (ERK) and/or Akt pathway [137] and attenuation of collagen accumulation through reduction of collagen type I level, [3H] thymidine and [3H] proline incorporation, and [3H] hydroxyproline secretion in the SHR [142] and through nitrogen-activated protein kinase (MAPK) pathway [143]. As endothelium-derived relaxing factors (EDRF), H₂S and NO have “cross-talk” on the calcium mobilization [144], activation of eNOS [145–148], PI3K/Akt signaling [145], soluble guanylate cyclase (sGC) [145, 149], and cGMP [150, 151]. However, whether NO is directly involved in the anti-hypertensive effects of H₂S has to be further investigated by a NO deficiency model induced to hypertension and treated by sulfide-rich compounds.

8.2. Atherosclerosis. Atherosclerosis is a chronic and slowly progressive cardiovascular disease that affects arterial blood vessels by thickening and hardening as consequences of the high plasma cholesterol concentrations, especially cholesterol in low-density lipoprotein [152]. Cholesterol deposition, lipid oxidation, cell adhesion, vascular inflammation, foam cell accumulation, smooth muscle cell migration, and plaque calcification are involved in different stages of the pathological process [153]. The cumulative plaques sequentially narrow the arterial lumen and restrict blood supply. Severe atherosclerotic lesions are the high risk factors of ischemic diseases such as stroke and heart attack [154].

Recent years, H₂S draws attentions from researchers by its cardiovascular protective effects, while there are not many studies on its effects on the progress of atherosclerosis. Fortunately, increasing evidence has indicated that H₂S plays a potentially significant role in a number of biological processes and potential cardiovascular protections, which suggest that H₂S may contribute to the inhibition of pathogenesis of atherosclerosis. First, H₂S shows inhibitory effects on the development of atherogenesis, such as oxidative stress, modified oxidation of LDL, cell adhesion, and calcification. In vascular smooth muscle cells (SMCs), low levels of NaHS (30 or 50 μM), a donor of H₂S, decrease reactive oxygen species, including H₂O₂, ONOO⁻, and O₂⁻ [155]. At the same time, NaHS also enhances the functions of antioxidant enzymes. In addition, H₂S inhibits atherogenic modification of LDL-induced HOCl in vitro (such as oxidized LDL, shortened as oxLDL). As a potent atherogenic agent, oxLDL particle is an important product of atherogenic oxidation that stimulates endothelial cells to express various adhesion molecules for consequent inflammatory reactions and formation of foam cells. Therefore, inhibition of oxLDL by potential treatments of H₂S implies that H₂S may interfere atherosclerotic progress [156]. Furthermore, H₂S attenuates atherosclerotic lesions by reducing cell adhesion molecules, such as ICAM-1, involving the NF-κB pathway in vivo and in vitro [112]. Adhesion molecules are the significant causes to promote bindings between monocytes and T lymphocytes to endothelial cells, which will lead to sequential inflammation and advanced process. Reduced expressions of adhesion molecules prohibit monocytes migration and later inflammation, which may also benefit in ameliorate atherosclerotic lesions. Lastly, calcification, presented in the advanced process of atherosclerosis, is a potent factor of plaque stability. There was a study that found the link between H₂S and plaque calcification [157]. In calcified arteries, H₂S level, CSE activity, and CSE mRNA were downregulated, while after administration of H₂S, a dose response was shown in the decreased vascular calcium content, Ca²⁺ accumulation, alkaline phosphatase (ALP) activity, and aortic osteopontin (OPN) mRNA. These changes speculated the effect on atherogenesis of H₂S might be induced by suppressing vessel calcification.

Second, H₂S possesses vascular protective capacities from inhibition of proliferation of vascular cells, such as intima and SMCs, and angiogenesis. It has been demonstrated that H₂S suppresses neointima hyperplasia on rat carotid after balloon injury [158]. In another balloon-injured artery experiment, NaHS (30 μmol/kg bodyweight) enhances methacholine-induced vasorelaxation and significantly ameliorates neointimal lesion formation. Additionally, evidences are also pointing to the fact that H₂S relieves apoptosis and proliferation of SMCs [159], SMCs migrate from the medial layer into the subendothelial space where they may proliferate, ingest modified lipoproteins, secrete extracellular matrix proteins, and contribute to lesion development. The suppression of proliferation of SMCs by H₂S can restrict atherosclerotic damages. Moreover, H₂S prevents the process of angiotensin II, and H₂S can restrain atherosclerotic damages. Moreover, H₂S prevents the process of angiotensin II [143, 160, 161]. Angiogenesis, ossification or calcification of a vessel, is an advanced change in the pathology of atherosclerosis. Its development leads to the narrowing of the caliber of an artery, stimulates thrombosis, or even worse generates the abrupture of unstable plaques. Vascular calcifications induced by vitamin D₃ and nicotine in rats are ameliorated by exogenous H₂S. The responses after administration of H₂S show the decreased calcium concentration in vessels, reduced expressions of angiogenesis, and accompanied acidic phosphatase and osteopontin.

Third, H₂S alleviates the vascular damage induced by an established risk factor, for instance, homocysteine. Homocysteine is an amino acid, biosynthesized from methionine and converted into cysteine and sulfur. Augmented levels of homocysteine in plasma, termed hyperhomocysteinemia, are considered as a high risk factor of atherogenesis. Early plaque development in apolipoprotein E-deficient mice, a knockout genetic model of atherosclerosis by 8 weeks high-cholesterol diet intake, could be enhanced by dietary supplementation with methionine or homocysteine [162]. A research shows that low concentrations of NaHS (30 or 50 μM), a H₂S donor, potentiates cell viability of rat aortic
SMCs by abating cytotoxicity and reactive oxygen species stimulated by hyperhomocysteinemia [163].

Although atherosclerosis is a chronic, systemic disease with multifactors involved in its initiation and progression, previous studies have shown that the specific characteristics and functions of H$_2$S may contribute to the inhibition of atherogenesis. The multiaspect recognitions of cardiovascular protective effects of H$_2$S provide a new avenue of antagonism towards this complicated cardiovascular disease.

8.3. Myocardial Injury. Plenty of work have documented that the CSE/H$_2$S pathway participates in the regulation of cardioprotective effects [155]. Administration of exogenous H$_2$S reduces "infarct-like" myocardial necrosis induced by isoproterenol in the rat [67, 164, 165]. This protection is accompanied with the reduced concentrations of H$_2$S in myocardium and plasma, decreased CSE protein activity, and upregulated CSE gene expression in myocardium [67]. NaHS attenuates the myocardial ischemic injury by evidences of reduced mortality and shrunk infarct size in vivo of rat and recovered SMC viability induced by hypoxia [67]. Further study discovers that 14 μmol/kg/d NaHS improves ECG and blood pressure and diminishes infarct size, as well as the greater survivin expression [165].

Oxidative stress injury is an important mechanism of myocardial injury. Direct or indirect antioxidantive effects will lead to cardioprotection from myocardial ischemia. The data in above literature reveal that NaHS may antagonize MDA production in vitro of myocytes by oxygen free radicals or directly react with hydrogen peroxide and superoxide anions [166]. Another experiment also proves that H$_2$S provided profound protection against ischemic injury by significant decreases in infarct size, circulating troponin I levels, and oxidative stress [67]. The protections by Na$_3$S in early and late preconditioning are all though stimulating the increased antioxidants, which could be itemized to the elevated Nrf2 in early stage and increased expressions of heme oxygenase-1 and thioredoxin 1 in late preconditioning. The antioxidiant effect of H$_2$S is also embodied in the preservation of mitochondrial functions and ultrastructure by Na$_3$S after myocardial ischemia-reperfusion (MI-R) injury [167]. These observations have been recently confirmed by cysteine analogues, SAC, SPC, and SPRC [168, 169]. The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase status are preserved by the cysteine analogues. The mitochondrial ultrastructure of cysteine analogues treatments appeared more normal than MI vehicle group. These evidences demonstrate the CSE/H$_2$S pathway is involved in reducing the deleterious effects of oxidative stress.

Furthermore, recent discoveries indicate the observed protection of H$_2$S is related to regulate leukocyte adhesion and leukocyte-mediated inflammation, increase anti-inflammatory cytokines, and reduce several proinflammatory cytokines [169]. The anti-inflammatory effect of H$_2$S is reflected in amplification of heat shock protein (HSP) 70, HSP 90, and cyclooxygenase-2 [115] and reduction of MPO activity [167], nuclear factor-κB (NF-κB), and interleukin (IL)-6, IL-8, and tumor necrosis factor-alpha (TNF-α) [167]. The cardioprotection of H$_2$S is associated with inhibition of cardiomyocyte apoptosis after myocardial injury. H$_2$S amplifies antiapoptosis proteins (Bcl-2, Bcl-xl) and inactivates proapoptogen (Bad) [115]. It is also suggested that H$_2$S ameliorates cardiomyocyte apoptosis after MI-R injury in vitro and in vivo, significant abatement of caspase-3 activity, and declining of the number of TUNEL positive nuclei, respectively [167].

Finally, multiple studies have elucidated a protective effect of K$_{ATP}$ channel activators in myocardial MI-R injury [168]. By virtue of the relaxant effect of H$_2$S as an opener of K$_{ATP}$ channels, it is easy to hypothesize that H$_2$S protects myocardial cells against ischemic injury. In the isolated Langendorff-perfused rat hearts, administrations of NaHS result in a dose-dependent limitation of infarct size induced by left coronary artery ligation and reperfusion, while this protective effect is abolished by K$_{ATP}$ channel blockers [170]. There is a report that H$_2$S preconditioning presents cardioprotective effects against ischemia though signaling pathways of K$_{ATP}$/PKC/ERK1/2 and P38/Akt [171]. Researchers may investigate additional molecular mechanisms to explain this ischemic injury in hearts not limited on stereotyped mechanisms, such as oxidative stress or potassium channels.

8.4. H$_2$S in Angiogenesis. The term "angiogenesis" is referred to the physiological process of blood vessel growth or vessel sprouting [172]. Blood vessel growth can benefit for delivering nutrients and waste and supplying immune surveillance [172]. Insufficient vessel growth has been linked to stroke, myocardial infarction, ulcerative disorders, hair loss, pre eclampsia, and neurodegeneration [173]. Embryonic development, menstrual cycle, hypoxia, inflammation, and tumor will stimulate angiogenic signals, such as vascular endothelial growth factor (VEGF), angiopoietin-2 (ANG-2), and fibroblast growth factors (FGFs) to sprout new endothelial cells and pericytes or vascular smooth muscle cells [173, 174].

H$_2$S has been displayed as an important regulator of angiogenesis through promoting endothelial proliferation, migration, and formations of tub-like structure and networks. Administration of H$_2$S increased proliferation and migration in bEnd3 microvascular endothelial cells and recovered microvessel sprouting in rat aortic rings of silencing CSE [145]. We discovered that SPRC, as a H$_2$S donor, enhanced HUVEC cell proliferation, adhesion, migration, and tube formation as well as the same effects in the rat aortic ring and Matrigel plug models [175]. In vivo studies of mouse hindlimb ischemia and rat myocardial ischemia provided additional evidence that SPRC ameliorated ischemic insults through augmenting angiogenesis [175]. Considering H$_2$S and NO share angiogenic effects, we synthesized H$_2$S-NO hybrid molecule, named ZYZ-803, to slowly release H$_2$S and NO [176]. As expected, ZYZ-803 presented significantly greater potency of angiogenesis than H$_2$S and NO alone [176]. Besides CSE-mediated effects, some studies showed that RNAi-mediated silencing CBS leads to a 40–50% decrease in HUVEC proliferation and 30% decrease in tube length on Matrigel [177]. Using AOAA, the CBS inhibitor developed a dose-dependent decrease of HUVEC proliferation...
rate, indicating that CBS is also involved in mitogenic effects of H\textsubscript{2}S [177]. Supplying 3MP, the 3MST substrate, facilitated wound healing and reserved mitochondrial functions which were associated with greater proliferation rates, proven by silencing 3MST to inhibit ECs growth and migration rates [178]. Taken together, H\textsubscript{2}S may be a potential proangiogenic agent, which is independent of the three synthesizing enzymes.

To determine how H\textsubscript{2}S regulates endothelial functions, most studies focused on the VEGF (also called as vascular permeability factor, VPF) signaling, which is the arguably crucial pathway in angiogenic responses both under healthy and pathophysiological circumstances [173, 179]. Silencing CSE and CSE inhibitor PAG reduced vessel length and branching stimulated by VEGF [145, 180]. Meanwhile, incubation of VEGF in HUVECs resulted in higher H\textsubscript{2}S synthesis and level [180]. Additionally, H\textsubscript{2}S presented as an endogenous stimulator of angiogenesis by increasing the activation of Akt, ERK, and p38, which are the downstreams of VEGF signaling [180]. Administration of glibenclamide, the K\textsubscript{ATP} channel blocker, reduced H\textsubscript{2}S-induced endothelial cells motility and prohibited H\textsubscript{2}S-triggered activation of p38, indicating K\textsubscript{ATP} channel was one of the H\textsubscript{2}S targets and may locate at upstream of p38 in this motility process [180]. We first developed SPRC as the H\textsubscript{2}S donor which activated and interacted with signal transducer and activator of transcription 3 (STAT3) to induce angiogenesis in vitro and in vivo [175]. We also discovered that ZYZ-803, releasing H\textsubscript{2}S and NO, regulates angiogenesis through SIRT1/VEGF/cGMP pathway [176]. However, as the STAT3 links to Akt signaling, ERK/p38, and K\textsubscript{ATP} channel still needs further investigations.

9. Conclusion and Perspectives

Over the last few decades, there are significant progress achieved in delineating the therapeutic potentials and molecular mechanisms underlying the actions of H\textsubscript{2}S on cardiovascular diseases [181], seen in Figure 6. The evidences elaborated above indicate that H\textsubscript{2}S derived from CSE, CBS, 3MST/AAT, or DAO reduces blood pressure, inhibits atherosclerotic progress, alleviates infarct myocardial injuries, and stimulates the angiogenic properties on endothelium. Therefore, several chemicals have been developed to test the therapeutical potentials for further drug development in human. In spite of compelling evidences in the literature for the role of exogenous and endogenous and H\textsubscript{2}S in vessel and myocardial protection, several questions regarding to precise mechanisms and regulations of H\textsubscript{2}S in the context of cardiovascular diseases need to be better understand. In quiescent, growing, and maturing vessels, does the generation of H\textsubscript{2}S generated by different cell types have any interaction and which one plays the major role? Is the H\textsubscript{2}S-mediated inflammation different in high blood pressure, angiogenesis, ischemic injury, and atherosclerosis? What is the exact manner of cross-talk between the three gas neurotransmitters, that is, NO, CO, and H\textsubscript{2}S? Interestingly, some studies showed obvious discrepancy by suggesting vasoconstrictor effects of H\textsubscript{2}S, instead of vasodilation actions. Further studies will be required to determine whether this discrepancy is due to dose of H\textsubscript{2}S, vascular response, oxygen tension, or experimental models. Finally, the posttranslational level of H\textsubscript{2}S-producing enzymes should be defined in the context of regulations and activities. After these tremendous growths of preclinical studies, we expect the sulfide-containing compounds will apply to clinics someday with considerable efficacy and safety.

Disclosure

This publication is an extension and based on the Dr. Ya-Dan Wen’s thesis (http://scholarbank.nus.edu.sg/bitstream/10635/777161/Wen%20Yadan_HT090143H_PhD%20thesis-v2.pdf).

Conflicts of Interest

There is no conflict of interest declared by the authors.
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