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Review Article

The Drug Developments of Hydrogen Sulfide on Cardiovascular Disease

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The recognition of hydrogen sulfide (H_2S) has been evolved from a toxic gas to a physiological mediator, exhibiting properties similar to NO and CO. On the one hand, H_2S 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, H_2S 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 H_2S 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 H_2S 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 (H_2S) 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, H_2S, 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 H_2S has been changed from that of a noxious gas to a gasotransmitter with vast potential in pharmacotherapy. At the end of the 1980s, endogenous H_2S 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 H_2S were unveiled. Recently, H_2S, 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

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

	Nitric oxide	Carbon monoxide	Hydrogen sulfide
Formula	NO	CO	H_2S
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 K_{Ca} channels and nitrosylation	Increase cGMP, activate K_{Ca} channels	Increase cGMP and cAMP, activate K_{ATP} channels and sulfhydration
Application on human	Pulmonary hypertension, lung transplantation, and ARDS	Not available	Not available

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 H_2S are summarized in Table 1.

This review is prepared for researchers, who are interested in H_2S 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. H_2S on "inflammation," "redox status," and "cardiovascular disease" summarizes the currently novel findings of the effects of H_2S and underlying mechanisms.

2. Physical and Biological Characteristics

H_2S, 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, H_2S is a weak acid which hydrolyzes to hydrogen ion and hydrosulfide and sulfide ions as following: $H_2S \leftrightarrow H^+ + HS^- \leftrightarrow 2H^+ + S^{2-}$. The pKa at 37°C is 6.76. When H_2S is dissolved in physiological solution (pH 7.4, 37°C), it yields approximately 18.5% H_2S and 81.5% hydrosulfide anion (HS^-), as predicted by the Henderson-Hasselbalch equation [4]. H_2S could be oxidized to sulfur oxide, sulfate, persulfide, and sulfite. H_2S 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.

The toxic effect of H_2S 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. H_2S 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, H_2S affects many organ systems including the lung, brain, and kidney.

H_2S 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 H_2S by chemical transformation and enzymatic reactions [5]. Structures of natural food-releasing H_2S on digestion are shown in Figure 1. Consuming mushrooms, garlic, and onions, which contain chemicals and enzymes responsible for the transformation of the sulfur compounds, is responsible for H_2S production in the human gut [6]. Human body produces small amounts of H_2S and uses it as a signaling molecule. In different species and organs, the concentration of H_2S varies in different levels. In Wistar rats, the normal blood level of H_2S is 10 μM [7]; while in Sprague-Dawley rats, the plasma level of H_2S increases to 46 μM [8]; in human, 10–100 μM H_2S in blood was reported [9]. The tissue level of H_2S is known to

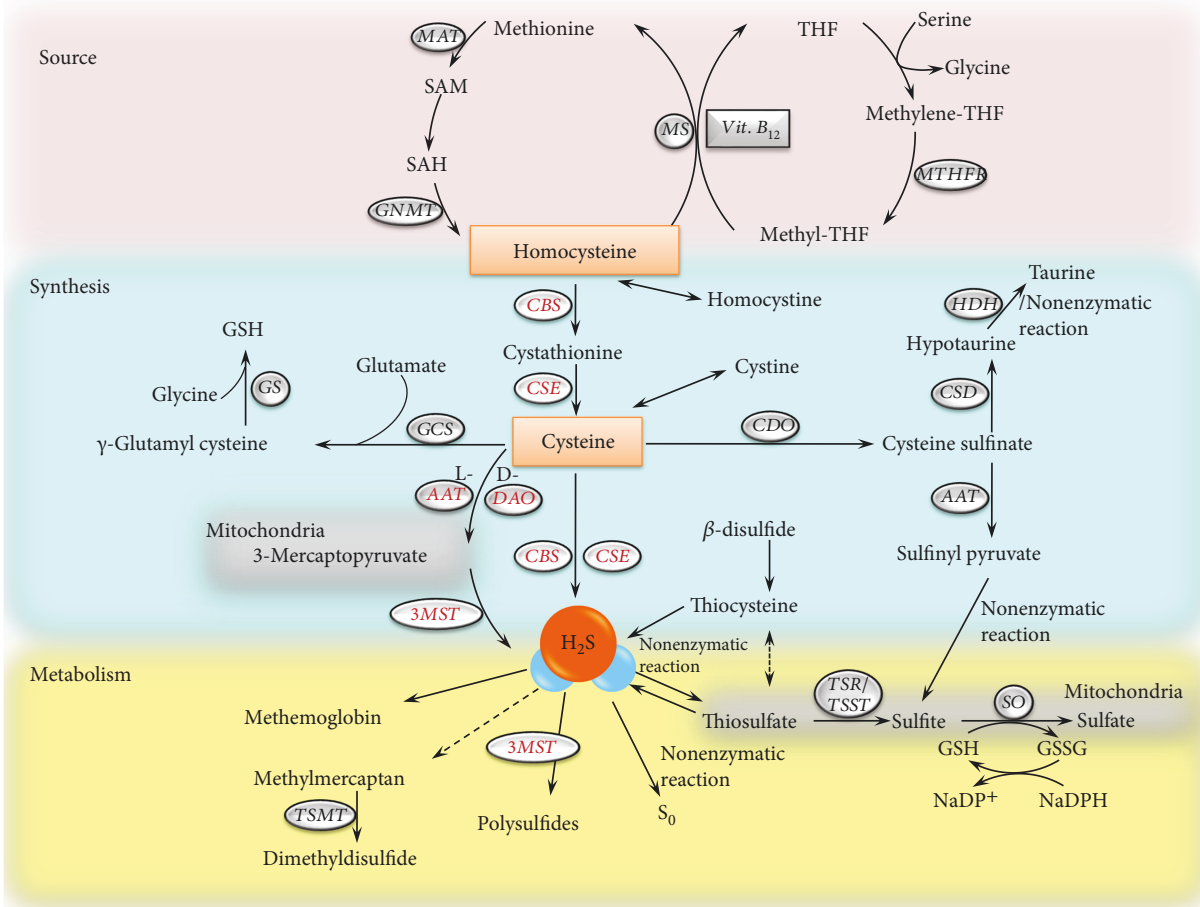


Figure 1: Synthesis and catabolism of H_2S. AAT: aspartate aminotransferase; CDO: cysteine dioxygenase; CSE: cystathionine γ-lyase; HDH: hypotaurine dehydrogenase; GCS: γ-glutamyl cysteine synthase; GS: glutathione synthase; MAT: methionine adenosyltransferase; MS: methionine synthase; S0: elemental sulfur; SAM: S-adenosylmethionine; THF: tetrahydrofolate; TSST: thiosulfate sulfurtransferase; CBS: cystathionine β-synthase; CSD: sulfinate decarboxylase; DAO: D-amino acid oxidase; H_2S: hydrogen sulfide; GNMT: glycine N-methyltransferase; GSH: glutathione; 3MST: 3-mercaptopyruvate sulfide transferase; MTHFR: methylenetetrahydrofolate reductase; SAH: S-adenosylhomocysteine; SO: sulfite oxidase; TSR: thiosulfate reductase; TSMT: thiol S-methyltransferase.

be higher than its circulating level. The concentration of endogenous H_2S has been reported up to 50–160 μM in the brains of rat, human, and bovine [1, 10, 11]. Significant amounts of H_2S 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_2S than that of portal vein of rats [8]. Furne et al. reported that in situ tissue H_2S level through analyzing the gas space over rapidly homogenized mouse brain and liver was only 15 nM [12].

3. Synthesis and Catabolism of H_2S

H_2S is endogenously formed by both enzymatic and nonenzymatic pathways [3]. The enzymatic procedure of synthesizing H_2S, 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_2S 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 cysteine to H_2S, 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_2S [18]. The two synthesis pathways of producing H_2S 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_2S-

Table 2: Characteristics of H_2S-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 Ca^{2+}/calmodulin
Inhibitors	D,L-propargylglycine, β-cyano-L-alanine	Hydroxylamine, aminooxyacetate
Functional roles	H_2S production in the liver and smooth muscle	H_2S production in the brain and nervous system

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 H_2S by origin of exogenous cysteine in different rat blood vessels was tail artery > aorta > mesenteric artery [21].

A third enzymatic reaction contributing to H_2S production has recently been identified in brain and vascular endothelium, that is, 3-mercaptopyruvate 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-mercaptopyruvate (3MP) by AAT; then, the intermediate product is converted to H_2S 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 H_2S 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 H_2S, whereas vascular SMCs likely absorb 3-mercaptopyruvate or other sources to generate H_2S which exerts as a vasodilator.

The fourth enzymatic pathway was recently reported by Shibuya et al. [26] that produces H_2S from D-cysteine by D-amino acid oxidase (DAO). Different from using L-cysteine to produce H_2S by CBS, CSE, and 3MST/AAT, which are pyridoxal 5′-phosphate- (PLP-) dependent enzymes, D-cysteine pathway generates H_2S 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 H_2S 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 H_2S from D-cysteine in concentration-dependent manner, but that from L-cysteine was not influenced by I2CA [26]. This new enzymatic H_2S-producing pathway is integrated into the part of "synthesis" in Figure 1.

The nonenzymatic route of yielding H_2S 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 H_2S production, there are several important amino acids: homocysteine and D-cysteine. Besides the generation of H_2S 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 B_{12} 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 H_2S, 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 H_2S-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 H_2S through D-cysteine pathway rose after birth and rocketed to maximal at 6 weeks [27]. The level of H_2S generated from L-cysteine was much lower than that from D-cysteine and remains in a certain amount over time. Additionally, the generation of H_2S from D-cysteine is 80 times more efficient than that from L-cysteine in the kidney [26]. Moreover, the generation of H_2S from D-cysteine in the kidney is 7 times higher than that in the cerebellum, which is the region producing highest level of H_2S from D-cysteine than other parts in the brain [26]. Since H_2S has presented significant therapeutic potentials on anti-inflammation, antioxidation, antiapoptosis, antimitochondrial dysfunction, and energy

reservation, 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_2S, 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 sulfinyl 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_2S, cysteine metabolism is integrated in Figure 1 for helping researchers to find out the potential associations.

The concentration of H_2S is not only determined by the rate of formation but also by degradation of H_2S. Dissolved gaseous H_2S is in a pH-dependent equilibrium, with hydrosulfide anions (HS^-) and sulfide anions (S^{2-}), which can be catabolized to any sulfur-containing molecule. Sulfide, via nonenzymatic route, is catabolized to thiosulfate, which could be catalyzed to sulfite by thiosulfate reductase (TSR) in the livers, brains, or kidneys, or by thiosulfate 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_2S could be broken down by rhodanese, methylated to CH_3SH, sequestrated 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_2S 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_2S with NO [36]. The whole schematic version of source, synthesis, and metabolism of H_2S is depicted in Figure 1.

4. Donors and Inhibitors of H_2S

4.1. The Donors of H_2S

4.1.1. Sulfide-Containing Salts. Sodium hydrogen sulfide (NaHS) and disodium sulfide (Na_2S) are the common H_2S-releasing chemicals in research of hydrogen sulfide. These sodium salts purchased from pharmaceutical companies are usually aquo compounds, like $NaHS{\cdot}12H_2O$, $Na_2S{\cdot}9H_2O$, 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.

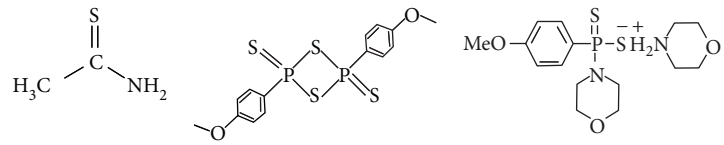
The solution of NaHS, at physical pH and room temperature, hydrolyzes to sodium ion, hydrosulfide as following: $NaHS \leftrightarrow Na^+ + HS^-$. Solutions of HS^- are sensitive to oxygen, converting mainly to polysulfides, indicated by the appearance of yellow color. Hence, solutions of fresh prepared 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_2S, some researchers introduce another donor of H_2S, 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_2S 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_2S-Releasing Molecules. Thioacetamide is an organosulfur compound with the formula C_2H_5NS. This white crystalline 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_2S releasers. Lawesson's reagent is a chemical compound used in organic synthesis as a thiation agent and is also a H_2S releaser. Lawesson's reagent is first synthesized in 1956 during a systematic study of the reactions of arenes with P_4S_{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 thiocarbonyl groups [41]. 2,4-Bis (4-methoxyphenyl)-1,3,2,4-dithiadiphosphetane 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 dithiophosphine ylides (R-PS2), which decompose to release H_2S. 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 (GYY4137) releases H_2S slowly both in vitro and in vivo. It has been proved that GYY4137 has vasodilator and antihypertensive activities and a useful H_2S-releasing chemical in the study of biological effects of H_2S [42]. In a later experiment, administration of GYY4137 to lipopolysaccharide- (LPS-) induced rats displays its anti-



(a) (b) (c)

Figure 2: Structures of H_2S-releasing molecules.

inflammatory effect by increasing plasma anti-inflammatory cytokine IL-10 and reducing plasma proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and nitrite/nitrate, C-reactive protein, and L-selectin [43]. Structures of H_2S-releasing molecules are shown in Figure 2.

Considering pharmacological effects and adverse effects of H_2S, some pharmaceutical factories join in working on H_2S donors which are made up of well-established parent compounds and H_2S-releasing moieties. CTG Pharma developed ACS series H_2S-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 H_2S-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 Na_2S, 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: NCT00858936). The effects of some H_2S-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 H_2S by chemical transformation and enzymatic reactions [5]. Structures of natural food-releasing H_2S 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 anticancer, antimutagenic, and antiproliferative 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-cysteines and S-allyl-L-cysteine sulfoxides (alliin). Both are abundant as sulfur compounds, and alliin is the primary odorless, sulfur-containing amino acid, a precursor of allicin, methiin, (+)-S-(trans-1-propenyl)-L-cysteine sulfoxide, and cycloalliin [48].

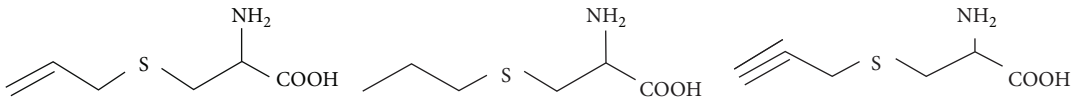
S-allylcysteine (SAC), a major transformed product from γ-glutamyl-S-allyl-L-cysteine, is a sulfur amino acid detected in the blood that is verified as both biologically active and bioavailable [49], as seen in Figure 3. SAC has been enumerated in several research investigations mediating protective effects in neural system and cardiovascular system by the inhibition of cell damage in the neuron, heart, and endothelium. In neural system, it is reported that SAC may attenuate Aβ-induced apoptosis [50] and destabilize Alzheimer's Aβ fibrils in vitro [51]. SAC prohibits cerebral amyloid, cerebral inflammation, and tau phosphorylation in Alzheimer's transgenic mouse model harboring Swedish double mutation [52]. In stroke-prone spontaneously hypertensive rats, intaking SAC diminishes incidence of stroke, impairs behavioral syndromes, and abates mortality induced by stroke [53]. SAC inhibits free radical production, lipid peroxidation, and neuronal damage in rat brain ischemia [54]. In cardiovascular system, SAC can help the acute myocardial infarction rats survived by significantly lowering mortality and reducing infarct size [55].

S-propyl-L-cysteine (SPC) and S-propargyl-L-cysteine (SPRC) are structural analogues of SAC, differing only in the propargyl and allyl moiety, respectively, while containing the same cysteine structure as shown in Figure 3. Wang et al., from our lab, reported that SPRC exhibited stronger cardioprotective effects than SAC in reducing mortality, increasing cell viability, reducing heart infarct size, lowering LDH and CK levels and activities, and having antioxidant properties [56]. These data suggest that the propargyl group of SPRC further increases the affinity and/or activity of SPRC towards the enzyme CSE as compared to SAC, where SPRC treatment is shown to have an increased CSE expression and activity to produce H_2S for coping with ischemic damage. This observation suggests that the cardioprotective effects involving the CSE/H_2S pathway were more effective using SPRC compared to SAC. Recently, our lab reported that SPRC showed neuroprotective effects of cognitive impairment and inhibition of neuronal ultrastructure damage in Aβ-induced rats, affords a beneficial action on anti-inflammatory pathways [57]. SPRC has been demonstrated the anticancer effect on gastric cancer at high doses 50 mg/kg/d and 100 mg/kg/d [58]. The effects of SAC and SPRC are shown in Table 3.

4.2. The Inhibitors and Regulators of H_2S. The production of H_2S from cysteine by tissue/cell homogenate is decreased by the presence of inhibitors of H_2S-producing enzymes, which are mainly attributed to CSE and CBS. CSE is also named as cysteine desulfhydrase [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, H_2S, and pyruvate, cannot inhibit CSE activity [61]. CBS is inhibited by hydroxylamine (HA) and aminooxyacetate (AOAA) albeit

Table 3: H_2S-releasing compounds used in basic scientific researches.

Compounds	Constituents	Effects on research fields
SAC	S-allylcysteine	Protection on cardiovascular and neural systems
SPRC	S-propargyl-cysteine	Anticancer, anti-inflammation, and antihypoxic/ischemia and impairs cognition and Aβ-induced neuronal damage
GYY4137	Morpholin-4-ium-4-methoxyphenyl (morpholino) phosphinodithioate	Antagonizes endotoxic shock though anti-inflammatory effects
ACS-6	A H_2S-donating sildenafil	Inhibits superoxide formation and $gp91^{phox}$ expression in porcine PAECs
ACS-14	A H_2S-releasing aspirin	Regulates redox imbalance, such as GSH formation, HO-1 promoter activity, and isoprostane suppression
ACS-15	A H_2S-releasing derivative of diclofenac	Arthritis
ACS-67	A H_2S-releasing derivative of latanoprost acid	Glaucoma; retinal ischemia
ATB-284	A H_2S-releasing derivative of trimebutine	Irritable bowel syndrome
ATB-337	A H_2S-releasing derivative of diclofenac	Gastrointestinal damage induced by NSAIDs
ATB-346	A H_2S-releasing derivative of naproxen	Acute and chronic joint pain
ATB-429	A H_2S-releasing derivative of mesalamine	Inflammatory bowel disease and antinociceptive and anti-inflammatory effects
IK 1001	Calcium-cross-linked alginate polymer	Suspended animation, multiple hypoxic/ischemic conditions, cardiac remodeling, and congestive heart failure



S-allyl-L-cysteine (SPAC) S-propyl-L-cysteine (SPC) S-propargyl-L-cysteine (SPRC)

Figure 3: The chemical structures of SAC, SPC, and SPRC.

these chemicals are not selective inhibitors of CBS [2]. The relationships between H_2S-producing enzymes and their inhibitors are summarized in Table 2.

The currently known regulations of H_2S-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_2S, since CBS activity and H_2S level are higher in male than in female mice and castration of male mice decreases H_2S 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_2S 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_2S production is also supported by the observation that circulating H_2S 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_2S 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_2S-selective fluorescent probe, HSip-1, which discovered compound 3 presented very high selectivity for 3MST over other H_2S/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_2S Measurements

5.1. Spectrophotometric Method. The principle of spectrophotometric method of H_2S depends on the formation of methylene blue. H_2S 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

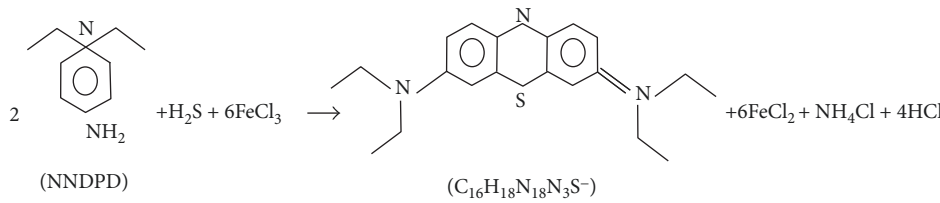


Figure 4: The equation of spectrophotometric method of H_2S.

(NNDPD) ion to yield methylene blue ($C_{16}H_{18}N_3SCl$). The equation is shown in Figure 4:

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 H_2S, 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 H_2S 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 H_2S 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 Fe^{2+} ion. The absorbance of the resulting solution at 670 nm was measured with a microplate reader. This method was improved by Ishigami et al. through the release of H_2S from acid-labile sulfur using acids as an artifact, which leads H_2S absorbed immediately and stored as bound sulfur [24].

This colorimetric method is not only widely used on the determination of H_2S on serum in animal experiment but also widely used on the activity of CSE/CBS enzyme on tissues or cells. The concentrations of H_2S are reflected on the different shades of color of methylene blue and calculated by the plotting H_2S standard curve.

Two points need to be made. Firstly, most researchers' assay H_2S using the spectrophotometric assay involves acidifying zinc acetate-treated (to "trap" free H_2S) biological samples in the presence of a dye and observing a color change. This assay actually measures total sulfide and not the gas H_2S. Secondly, H_2S 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 H_2S, 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 (Ag_2S) 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, H_2S is dissolved into HS^- and S^{2-}. In acid solution, sulfide is chiefly in the form of H_2S, 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 (S^{2-}). The SISE from Thermo Scientific supplies sulfide antioxidant buffer could maintain a fixed level of H_2S.

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_2S, sensitively, selectively, and biologically compatible. There are 3 types of fluorescent probes for H_2S detections: reduction-based, nucleophilic-based, and metal sulfide-based.

Reaction-based fluorescent probes for H_2S detection are designed based on the reducing ability of H_2S [74]. The firstly developed fluorescent probes by Lippert and colleagues were probes SF1 and SF2 based on the H_2S-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_2S 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_2S were utilized in the design of fluorescent probes, like nitro group. Montoya and Pluth reported the 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_2S-mediated reduction was extended to other fluorophore scaffolds by several laboratories [83–85].

Nucleophilic-based fluorescent probe for H_2S detection is designed based on the strong nucleophilic HS^- hydrolyzed from H_2S 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 intermolecular 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 α,β-unsaturated acrylate methyl ester on an aryl ring, which trapped H_2S and modulated a fluorescence response through decreased photoinduced electron transfer (PET) quenching of the product [87]. Disulfide bond cleaved by H_2S 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 μM H_2S in bovine plasma and 250 μM H_2S in cells could be detected by this probe. Reversible nucleophilic addition was exploited by Chen et al., as CouMC, to track real-time H_2S fluxes due to fast and potentially reversible fluorescence [90].

Metal sulfide-based fluorescent probe for H_2S 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_2S 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 μM. Hou et al. improved the copper-containing probe to a lower detection limit of 1.7 μM [95]. Another strength of metal precipitation-based probes is that they respond to turn on within seconds, allowing the real-time H_2S 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_2S [97, 98]. One of the benefits of unfuctionalized CNT in analyzing H_2S is due to the special bond with H_2S, but other proteins kept in serum. H_2S concentrations are reflected by the intensity of the fluorescence of the unfuctionalized CNT, due to the two values in a linear relationship. The lowest H_2S concentration that can be tested is 20 μM and smallest quantity of H_2S is 0.5 μg. The series of experiments are trying to establish a new sensor to measure micro- or nanoquantity H_2S, comprising unfuctionalized 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 gasotransmitter, H_2S, several analytical methods are utilized, including polarography. A novel polarographic hydrogen sulfide sensor (PHSS) has been developed for the study of H_2S-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_2S 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_2S 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_2S concentration. Next, adding 50% trichloroacetic acid to the syringe, the gas was collected to test the

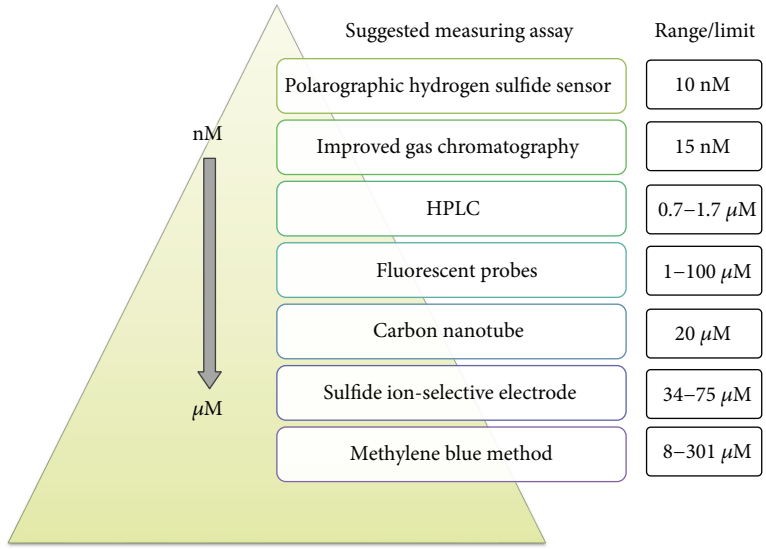


Figure 5: The ranges or limits of H_2S measurements.

acid-labile H_2S concentration. The flow rate of N_2 was 25 ml/min. The concentration of H_2S was calculated by the plotting H_2S 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 produce sulfide dibimane, which is separated from MBB by HPLC and detected by its fluorescent probes [102]. Recently, MBB assay without DTT was used to measure available H_2S in rat blood [103] and mouse plasma [104]. The ranges or limits of H_2S measurements are in Figure 5.

6. H_2S in Inflammation

Inflammation is an immune response to an injury or harmful stimuli, in order to self-protect the body from avoiding pathogen assaults and initiating healing process. However, the adaptive immune system fails to counter invading agents will turn to target host tissues, making deeply more serious damage. H_2S regulating inflammation and injury was initially contradictory, but in recent years, more studies supported that H_2S 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-κB (NF-κB) and proinflammatory cytokines (e.g., COX-2 [109], iNOS [110], and interleukin-(IL-) 1β, 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_2S may participate in the process of resolution of a variety of inflammatory diseases. In atherosclerosis, H_2S exerts its potent inhibitor of leukocyte adherence to vascular endothelium [112]. Meanwhile, the generation of reactive oxygen species (ROS), activation of NF-κB, 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_2S [112, 113]. These mechanisms of action described for H_2S may explain that H_2S can diminish the plaques in arteries and attenuate the atherosclerotic injury, suggesting the character of anti-inflammation of H_2S 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_2S 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_2S 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_2S-generating agents or precursor for H_2S synthesis, L-cysteine, has been shown to significantly accelerate ulcer healing [118, 119]. This ability of H_2S to enhance gastrointestinal resistance attracts investigators to exploit novel treatments of gastrointestinal injury and inflammation, like H_2S-releasing derivative of NSAIDs to reduce the adverse drug reaction of NASIDs, retarding gastrointestinal ulcer healing [120]. Evidence of H_2S in resolution of colitis in rats or mice studies showed that administration of H_2S donor significantly inhibited the severity of colitis with marked reduction of granulocyte infiltration into colonic tissue. In inflamed colon, H_2S production was highly increased via CSE, CBS, or other enzymatic

pathways [121, 122]. Once H_2S 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_2S in Redox Status

7.1. H_2S Direct Effects on Toxic Free Radicals. In a weak acid, H_2S dissociates in equilibrium with hydrosulfide anion (HS^-) and sulfide anion (S^{2-}). Under physiological conditions, the amounts of H_2S and HS^- are early equal within the cell, whereas extracellular fluid and plasma exist approximately the ratio of 20% H_2S, 80% HS^-, and 0% S^{2-}. HS^- is a potent one-electron reductant that eliminates free radicals by donating single electron. Hydrogen disulfide (H_2S_2), a kind of hydrogen polysulfide (H_2Sn), 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_2S and NO also produced H_2Sn by activating transient receptor potential ankyrin 1 (TRPA1) channels [36]. H_2S_2, a highly reactive oxidizing chemical, generates H_2S by reacting with thiol [125] or disproportionation [123, 126]. H_2S_2 and H_2S_3 were reported to generate redox regulators Cys-SSH and GSSH via 3MST in the brain of wild-type mice but not in those of 3MST-KO mice [34, 35, 127, 128].

H_2S is considered as an endogenous reducing agent which is produced in response to oxidative stress [129, 130]. Evidence showed that H_2S is a highly reactive molecule and may easily react with other compounds, especially with reactive oxygen and nitrogen species. H_2S 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_2S 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_2S 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 ($\Delta\Psi m$), 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_2S can reduce the H_2O_2-induced injury in HUVECs 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_2O_2-stimulated isolated rabbit aorta that H_2S 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_2S indicated that the antioxidative ability of H_2S is through increasing antioxidants and prohibiting ROS levels and also preserving mitochondrial function to reduce the production of toxic free radicals.

8. H_2S in Cardiovascular System

8.1. Hypertension. Before identified as the third gasotransmitter, H_2S 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_2S. Early at the end of the last century, it is first reported that H_2S relaxes the contracted smooth muscles (SM) induced by 1 μM norepinephrine in rat thoracic aorta and portal vein [134]. The relaxations in these tested aortas and veins present a NaHS dose-dependent manner, but the potency of relaxation by exogenous H_2S in the thoracic aorta is less than the portal vein, even by 10^{-3} M NaHS, which are around 25% and 90%, respectively. The data also showed that the relaxation effects of H_2S and NO can be enhanced by each other. 30 μM NaHS 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_2S 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_2S 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_2S 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_2S is induced on both SMCs and endothelial cells, instead of previous research discussions mainly focusing on SMCs.

A multitude of H_2S-induced vasodilation studies have investigated the activation of K_{ATP}^+ channels. One possible mechanism involved in the activation of K_{ATP}^+ channels by H_2S 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_2S was that cysteines on K_{ATP}^+ channels of SMCs were S-sulfhydrated, leading to hyperpolarization [140]. Cys43 of the inwardly rectifier (Kir) potassium channels subunit Kir 6.1 was sulfhydrated by NaHS, eliciting the binding to phospholipid phosphatidylinositol (4,5)-bisphosphate (PIP2) together with decreased association of ATP [140]. Additionally, the vasodilation effect of H_2S 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 H_2S are also likely mediated by the attenuation of intracellular inward Ca^{2+} currents. Not only H_2S hyperpolarizes ion channels on blood vessels to possess the relaxant effects but also endothelium generates H_2S by increasing catalytic activity of CSE through calcium-calmodulin, indicating that the H_2S formation may be involved in vascular activation to reduce blood pressure [141]. Moreover, H_2S 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 SHRs [142] and through mitrogen-activated protein kinase (MAPK) pathway [143]. As endothelium-derived relaxing factors (EDRF), H_2S 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 antihypertensive effects of H_2S 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 oxidization, 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 consequentially 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, H_2S 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 H_2S plays a potentially significant role in a number of biological processes and potential cardiovascular protections, which suggest that H_2S may contribute to the inhibition of pathogenesis of atherosclerosis. First, H_2S shows inhibitory effects on the development of atherogenesis, such as oxidative stress, modificated oxidation of LDL, cell adhesion, and calcification. In vascular smooth muscle cells (SMCs), low levels of NaHS (30 or 50 μM), a donor of H_2S, decrease toxic reactive oxygen species, including $H_2O_2^-$, $ONOO^-$, and O_2^- [155]. At the same time, NaHS also enhances the functions of antioxidative enzymes. In addition, H_2S 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 H_2S implies that H_2S may interfere atherosclerotic progress [156]. Furthermore, H_2S 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 H_2S and plaque calcification [157]. In calcified arteries, H_2S level, CSE activity, and CSE mRNA were downregulated, while after administration of H_2S, a dose response was shown in the decreased vascular calcium content, Ca^{2+} accumulation, alkaline phosphatase (ALP) activity, and aortic osteopontin (OPN) mRNA. These changes speculated the effect on atherogenesis of H_2S might be induced by suppressing vessel calcification.

Second, H_2S possesses vascular protective capacities from inhibition of proliferation of vascular cells, such as intima and SMCs, and angiosteosis. It has been demonstrated that H_2S 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 H_2S 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 H_2S can restrict atherosclerotic damages. Moreover, H_2S prevents the process of angiosteosis [143, 160, 161]. Angiosteosis, 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 abruption of unstable plaques. Vascular calcifications induced by vitamin D_3 and nicotine in rats are ameliorated by exogenous H_2S. The responses after administration of H_2S show the decreased calcium concentration in vessels, reduced expressions of angiosteosis, and accompanied acidic phosphatese and osteopontin.

Third, H_2S 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 H_2S 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_2S may contribute to the inhibition of atherogenesis. The multiaspect recognitions of cardiovascular protective effects of H_2S provide a new avenue of antagonism towards this complicated cardiovascular disease.

8.3. Myocardial Injury. Plenty of work have documented that the CSE/H_2S pathway participates in the regulation of cardioprotective effects [155]. Administration of exogenous H_2S reduces "infarct-like" myocardial necrosis induced by isoproterenol in the rat [67, 164, 165]. This protection is accompanied with the reduced concentrations of H_2S 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 antioxidative 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_2S provided profound protection against ischemic injury by significant decreases in infarct size, circulating troponin I levels, and oxidative stress [67]. The protections by Na_2S 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 antioxidant effect of H_2S is also embodied in the preservation of mitochondrial functions and ultrastructure by Na_2S 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 redox status are preserved by cysteine analogues. The mitochondrial ultrastructure of cysteine analogues treatments appeared more normal than MI vehicle group. These evidences demonstrate the CSE/H_2S pathway is involved in reducing the deleterious effects of oxidative stress.

Furthermore, recent discoveries indicate the observed protection of H_2S 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_2S 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_2S is associated with inhibition of cardiomyocyte apoptosis after myocardial injury. H_2S amplifies antiapoptosis proteins (Bcl-2, Bcl-xL) and inactivates proapoptogen (Bad) [115]. It is also suggested that H_2S 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_2S as an opener of K_{ATP} channels, it is easy to hypothesize that H_2S 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_2S preconditioning presents cardioprotective effects against ischemia though signaling pathways of K_{ATP}/PKC/ERK1/2 and PI3K/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_2S 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, preeclampsia, 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_2S 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_2S 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_2S 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_2S and NO share angiogenic effects, we synthesized H_2S-NO hybrid molecule, named ZYZ-803, to slowly release H_2S and NO [176]. As expected, ZYZ-803 presented significantly greater potency of angiogenesis than H_2S 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

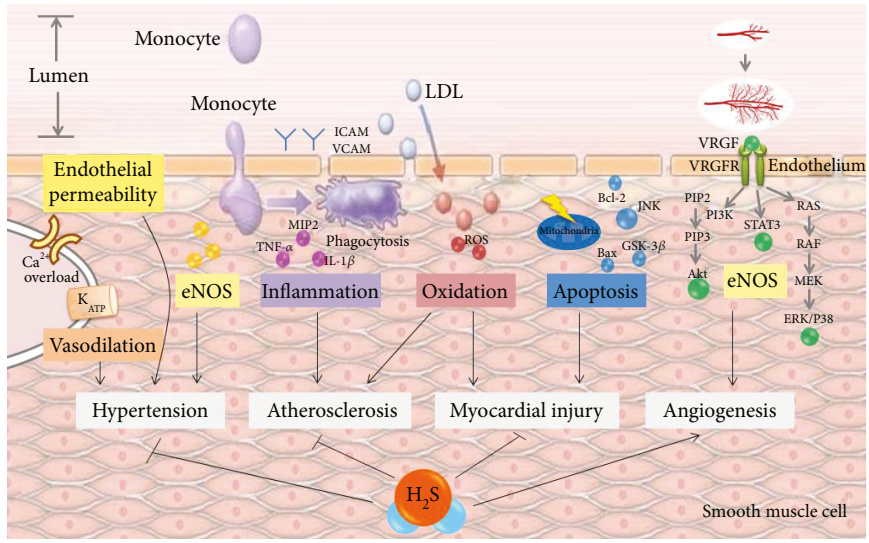


Figure 6: Schematic illustration of molecular mechanisms underlying H_2S-induced cardioprotection.

rate, indicating that CBS is also involved in mitogenic effects of H_2S [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_2S may be a potential proangiogenic agent, which is independent of the three synthesizing enzymes.

To determine how H_2S 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_2S synthesis and level [180]. Additionally, H_2S 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_{ATP} channel blocker, reduced H_2S-induced endothelial cells motility and prohibited H_2S-triggered activation of p38, indicating K_{ATP} channel was one of the H_2S targets and may locate at upstream of p38 in this motility process [180]. We first developed SPRC as the H_2S 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_2S and NO, regulates angiogenesis through SIRT1/VEGF/cGMP pathway [176]. However, how the STAT3 links to Akt signaling, ERK/p38, and K_{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_2S on cardiovascular diseases [181], seen in Figure 6. The evidences elaborated above indicate that H_2S 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_2S in vessel and myocardial protection, several questions regarding to precise mechanisms and regulations of H_2S in the context of cardiovascular diseases need to be better understand. In quiescent, growing, and maturing vessels, does the generation of H_2S generated by different cell types have any interaction and which one plays the major role? Is the H_2S-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_2S? Interestingly, some studies showed obvious discrepancy by suggesting vasoconstrictor effects of H_2S, instead of vasodilation actions. Further studies will be required to determine whether this discrepancy is due to dose of H_2S, vascular response, oxygen tension, or experimental models. Finally, the posttranslational level of H_2S-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/10635/77716/1/Wen%20Yadan_HT090143H_PhD%20thesis-v2.pdf).

Conflicts of Interest

There is no conflict of interest declared by the authors.

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