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Yi-Chun Zhu *Editor*

Advances in Hydrogen Sulfide Biology

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Preface

Hydrogen sulfide (H_2S) has originally been found to regulate the function of a few organs including the cardiovascular system and the brain. In the recent two decades, more organs/systems/biological processes have been found to be regulated by H_2S including the kidney, nutritional metabolism, gastrointestinal tract, microbiota, immune system, inflammation, autoimmune diseases, cell senescence, and cancer. The mechanisms underlying the H_2S actions on such a variety of biological processes remain unknown. More recent studies show that epigenetic mechanisms are also involved in mediating the H_2S effects. Moreover, the interaction between H_2S and other gasotransmitters implies not only the complexity of the H_2S mechanisms but also the importance of H_2S signaling as one of the basal components in physiology and diseases.

An ultimate question on the mechanisms underlying the comprehensive biological effects of H_2S is about the direct target molecules to trigger the signaling networks to mediate the H_2S effects. H_2S might function as a "ligand" and its target molecules serve as "receptors." The established theory to explain the interaction between a ligand and its receptor is the "key-lock" or "hand-in-glove" mechanisms which are based on conformational matching between the ligand and its receptors. However, H_2S is a gas molecule too small to possess a three-dimensional structure sufficient for conformational matching with its potential "receptors." In the chapter "A Common Molecular Switch for H_2S to Regulate Multiple Protein Targets," we proposed a mechanism based on an interaction between the frontier molecular orbitals of H_2S and its target proteins. In such a model, the interaction between the "ligand" and the "receptors" is not based on the concept of conformational matching, but on an interaction between the sulfur atom of H_2S and the oxidized sulfur atom of the cysteine residues in the target proteins. This is actually a nucleophilic attack that occurred between two sulfur atoms. Only the oxidized sulfur atom is recognized and subsequently attacked by H_2S, while oxidization of the sulfur atom of the sulfhydryl group of the cysteine residues results in the formation of a reversible disulfide bond between two cysteine residues which might regulate the conformation and function of the proteins targeted by H_2S. Since endogenous H_2S levels have been found to be changed in a series of disease status, the conformation and function of potential H_2S targets might be changed in parallel with the changes in H_2S levels. We name such a reversible disulfide bond as a "molecular switch" for H_2S regulation. It might be due to the very small size of the H_2S molecule which facilitates its

penetration into the large protein molecules to get access to potential disulfide bonds, H_2S is found much potent in opening such disulfide bonds than other endogenous reducing factors at concentrations of equivalent reducing potent. Such a "molecular switch" is first found in the intracellular kinase domain of vascular endothelial growth factor receptor 2 (VEGFR2) to regulate VEGFR2 function and angiogenesis. In fact, VEGFR2 belongs to the receptor tyrosine kinases (RTKs) family which includes important members such as the insulin receptor (IR), epidermal growth factor receptor (EGFR), and thrombopoietin (TPO) receptor. Interestingly, H_2S has been found to activate these RTK members to mediate a variety of physiological functions, i.e. facilitating glucose uptake by increasing IR sensitivity, inducing Na^+/K^+-ATPase endocytosis, and inhibition in renal tubular epithelial cells by targeting EGFR and promoting the generation of megakaryocytes/platelets by activating the TPO receptor. All these RTK members share a similar intracellular kinase domain. It gives rise to a hypothesis that H_2S might target some common motif contained in various protein molecules to activate multiple signaling pathways. The idea deserves in-depth validation in the future. This may not only find how H_2S recognizes and activates its target proteins but also uncovers a new mechanism of the interaction between a very small ligand and its "receptors" beyond the established model of ligand–receptor interaction.

In the chapter "H_2S and the Kidney," Balakuntalam Kasinath and Hak Joo Lee describe how H_2S regulates kidney function. H_2S is endogenously generated in the kidney. H_2S regulates basal renal functions including an increase in glomerular filtration rate (GFR) by increasing renal blood flow. H_2S also regulates tubular functions by acting on some transporter and channels. H_2S is also involved in renal diseases. The deficiency of H_2S generation plays a role in many types of renal disorders and administration of H_2S might provide protection. H_2S is found to be involved in kidney-related diseases such as renovascular hypertension, preeclampsia, aging kidney, and adrenal cell carcinoma.

In the chapter "The Role of H_2S in the Metabolism of Glucose and Lipids," Jinsong Bian and colleagues describe the role of H_2S in the metabolism of major nutrients. H_2S is involved in diabetes, obesity, and metabolic syndrome by playing a central role in the metabolism of glucose and lipids. Potential therapeutic approaches based on the pharmacological regulation of H_2S in the disorder of glycolipid metabolism are also discussed.

Jingxin Li and colleagues describe the role of H_2S in the guts. In the chapter "The Role of H_2S in the Gastrointestinal Tract and Microbiota," the role of H_2S in the physiology and diseases in the gastrointestinal system is reviewed and discussed. In addition to the conventional biosynthesis pathways in gastrointestinal tissues, a unique route to generate H_2S in the gut is by using sulfides produced in food catabolism as raw materials. H_2S regulates gastrointestinal motility, secretion, absorption, as well as the endocrine cells and stem cells. The role of H_2S in the interaction between gastrointestinal microorganisms and host is also reviewed.

One of the major recent advances in the field of H_2S biology is its role in the immune system. In the chapter "H_2S and the Immune System," Peter Rose and colleagues describe the pro- and anti-inflammatory effects of H_2S in

mammalian systems depending on the status of the subjects as well as the dosage used and the route of administration of this molecule. The signaling mechanisms underlying the regulation of H_2S in the immune cells are also described. The roles of H_2S in several immune-related diseases including arthritis, atherosclerosis, sepsis, and respiratory diseases have been reviewed.

H_2S seems to participate in various signaling networks in the immune system. This topic is discussed by Madhav Bhatia and colleagues in the chapter "H_2S and Its Interaction with Other Players in Inflammation." Interaction of H_2S with substances P, NO, and CO in the immune response is reviewed. The pathways regulating reactive oxidant species (ROS), adhesion molecules, and leukocyte infiltration are described in various inflammatory diseases.

In the chapter "An Update of the New Therapeutic Approach of Hydrogen Sulfide in Inflammation and Immune Response," Yizhun Zhu and colleagues provide a more focused review on autoimmune diseases regulated by H_2S which regulates various immune cells in different disease conditions. The therapeutic potential of the H_2S donors is discussed, in particular, as an anti-inflammatory drug to treat rheumatoid arthritis.

The role of H_2S in the cardiovascular system is indeed the most important facet of this gas molecule and is reviewed extensively in two previous books edited by Hideo Kimura and Philip Moore. In the following two chapters, some most recent advances and ideas in this direction are reviewed and discussed.

Firstly, the epigenetic mechanisms underlying the cardiovascular effects of H_2S are reviewed by Qian Ding and Yizhun Zhu in their chapter "The Cardiovascular Effects of H_2S: The Epigenetic Mechanisms" where the roles of DNA methylation, histone modification, and noncoding RNAs in the cardiovascular effects of H_2S are reviewed.

Secondly, the interaction between H_2S and other gas molecules in the cardiovascular system is reviewed by Junbao Du and colleagues in their chapter "Interaction among H_2S and Other Gasotransmitters in Mammalian Physiology and Pathophysiology". H_2S interacts with other gasotransmitters including NO, CO, and SO_2 to regulate the function of cardiovascular system and also to be involved in disease status.

Some important roles of H_2S, e.g. in cancer or cell senescence, have been reviewed in the above chapters regarding particular mammalian systems.

All the above chapters show that H_2S is a fundamental signaling molecule found so far to be involved in almost every mammalian system. However, future works are required to uncover the mechanisms underlying such extensive biological effects. If the "molecular switch" mechanism mentioned in Chapter 1 can be applied to the findings described in the rest of the chapters remains to be examined with futures works. Moreover, are there any uncovered mechanisms beyond the "molecular switch" mechanism underlying the interaction between H_2S and its direct target molecules? In addition, the development of H_2S donors with proper pharmacokinetics would be valuable for future therapeutic approaches.

Shanghai, China Yi-Chun Zhu

Contents

A Common Molecular Switch for H_2S to Regulate Multiple Protein Targets

Bei-Bei Tao and Yi-Chun Zhu

1 Introduction

Numerous studies have reported that hydrogen sulfide (H_2S) has numerous biological effects in different organs [1–11]. Especially, H_2S has many protective effects on the cardiovascular system. For instance, H_2S can lower high blood pressure [12] and ameliorate hypertension-induced myocardial remodeling in spontaneously hypertensive rats [13, 14]. H_2S also has protective effects in myocardial injury [15] and myocardial infarction [16].

In 2007, our research group found that H_2S has a significant pro-angiogenic effect in vitro and in vivo and the signaling molecules involved are PI3K/Akt [17]. After that, different research groups all reported the findings that both exogenous and endogenous H_2S can promote the process of angiogenesis [18–32].

Along with the numerous protective effects reported by research groups worldwide, there remains an important scientific problem. The problem is what is the "receptor" of H_2S or what is the target of it. As we all know, H_2S must have been acting upon certain proteins or other biological molecules in cells since it could not exert any effect acting upon nothing. Usually, a ligand can structurally match and then combine with its receptor, which leads to conformational change and activity increase of the receptor, ultimately resulting in a certain biological effect. However, as a gaseous molecule with a molecular weight of only 32 Dalton, H_2S is much smaller compared with its target protein. It probably could not interact with its "receptor" the same way as the ligand–receptor interaction. Therefore, there must have to be a different mechanism between H_2S and its "receptors," or target proteins.

As the research on H_2S goes on, several target proteins of H_2S and the molecular mechanisms have been discovered. In 2009, Asif K. Mustafa reported that H_2S can directly sulfhydrate the cysteine residue in GAPDH [33]. This is the first piece of evidence about the interaction between H_2S and its "receptor." After that, other papers reported several other target proteins of H_2S with the same modification (sulfhydration) on certain cysteine residues in proteins. All those findings are supporting the sulfhydration theory in H_2S–"receptor" interaction mode.

However, in our research, we failed to find any modification between H_2S donor NaHS and any of the 20 basic amino acids in a cell-free reaction system with mass spectrometry. Surprisingly, we found that H_2S can break up the disulfide bond in cystine and then transform cystine into cysteine with both mass spectrometry and Raman analysis. Meanwhile, we found that VEGFR2 is a

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"receptor" of H_2S in vascular endothelial cells, and there exists a newly found disulfide bond C1045-C1024 located in the intracellular kinase domain near the activation loop of VEGFR2. The disulfide bond C1045-C1024 vanished after the administration of H_2S donor NaHS with a significantly increased activity of this tyrosine kinase receptor [34]. In addition to VEGFR2, we found several other "receptors" of H_2S with a disulfide bond molecular switch in these target proteins, such as insulin receptor [35], EGFR [36], etc. Therefore, we proposed the disulfide bond molecular switch regulation as another theory in H_2S–"receptor" interaction mode.

Both the sulfhydration theory and the disulfide bond molecular switch regulation theory are supported by large amounts of experimental data. Therefore, the key problem is what indeed the more direct effect of H_2S, or whether they exist in the different micro-environment of target proteins, or whether both of them are related to each other. We will review the papers about these two theories in recent years and try to discuss the above problems, to understand more about the interaction mode between H_2S and its "receptors."

2 Sulfhydration

2.1 H_2S Increases the Sulfhydration Level of Proteins

Sulfhydration is an important post-translational modification of proteins. Sulfhydration modification on cysteine residues usually leads to an increase in protein activity or a more stable state. Therefore, sulfhydration can enhance the biological effects of those proteins. In addition, sulfhydration of cysteine residues can protect them from oxidation to maintain a normal function of the proteins. It is reported that sulfhydration is a more common post-translational modification than nitrosylation and about 25–50% of proteins in mouse liver lysates are sulfhydrated [37].

As shown in the papers, the process of sulfhydration is as follows. Persulfides are found to be formed in sulfurtransferases and cysteine desulfurases or be formed through the reaction with sulfenic acids, S-nitrosothiols, disulfides, polysulfides, etc. [38].

In 2009, Asif K. Mustafa reported that GAPDH from mouse liver lysate is a target protein of H_2S and H_2S can sulfhydrate (adding sulfur to the free thiol group) the Cys150 residue in GAPDH to form an -SSH state. Sulfhydration of GAPDH physiologically increased its catalytic activity by several fold and it can inhibit its interaction with Siah1 resulting in the protection of cell apoptosis. In this paper, Asif K. Mustafa used the modified biotin switch method and mass spectrometry to analyze the sulfhydration state [33].

After that, from 2009 till now, it is reported that sulfhydration of proteins by H_2S is playing an important role in the cardiovascular system [39]. Besides, sulfhydration also exists in neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, and traumatic brain injury [40].

There are many newly found H_2S receptors, such as GAPDH, Keap1, Sirt1, ATP5A1 (Table 1). These target proteins of H_2S all have one or two special cysteine residues which can be sulfhydrated by H_2S resulting in a significant change in protein activity. For instance, GAPDH can be sulfhydrated at Cys150, Keap1 can be sulfhydrated at Cys151, ATP5A1 can be sulfhydrated at Cys244 and Cys294... More target proteins with the modification sites are shown in Table 1.

2.2 The Methods for Analyzing Sulfhydration

2.2.1 Modified Biotin Switch Assay (Fig. 1a)

The biotin switch is previously used for nitrosylation assessment. And then, it was modified to detect S-sulfhydration. Therefore, this method has been named "modified biotin switch assay."

Table 1 Categories and functions of protein S-sulfhydration in the cardiovascular system

Protein	Cite	Effect	Method	References	Date
GAPDH	Cys150	Increase catalytic activity	Modified biotin switch assay Jaffrey and Snyder [41]	Mustafa et al. [33]	2009
Keap1	Cys151	Regulated Nrf2 activation to protect against cellular aging induced by oxidative stress	Modified biotin switch assay Nishida et al. [42]	Yang et al. [43]	2013
NF-κB	P65 subunit Cys38	Mediate the antiapoptotic effect of NF-kB	Modified biotin switch assay Mustafa et al. [33]	Sen et al. [44]	2012
Glutathione peroxidase 1		Clear free radicals and inhibit oxidative stress, reduced lipid peroxidation, and increased antioxidant defense	Modified biotin switch assay Mustafa et al. [33]	Cheung and Lau [45]	2018
Sirt1	Conserved zinc finger domains: CXXC domain (C371/374; C395/398)	Enhanced SIRT1 binding to zinc ion then promoted its deacetylation activity and increased SIRT1 stability, thus reducing atherosclerotic plaque formation	Modified biotin switch assay Cai et al. [46]	Du et al. [47]	2019
ATP5A1	Cys244 and Cys294	Maintain ATP synthase in a physiologically activated state, thereby supporting mitochondrial bioenergetics	Modified biotin switch assay Mustafa et al. [33]	Modis et al. [48]	2016
KLF5	Cys664	H_2S decreased KLF5 promoter activity; reduced KLF5 mRNA expression; attenuated specificity protein 1 binding activity with KLF5 promoter to prevent myocardial hypertrophy	"Tag-Switch" Method Zhang et al. [49]	Meng et al. [50]	2016
Sp1	Cys68 and Cys755	Maintaining vascular health and function	Modified biotin switch assay Paul and Snyder [51]	Saha et al. [52]	2016
MEK1	Cys341	Facilitates the translocation of phosphorylated ERK1/2 into the nucleus, where it activates PARP-1, mediates DNA damage repair, and cells are protected from senescence.	Modified biotin switch assay	Zhao et al. [53]	2014
P66Shc	Cys59	Enhance antioxidant effect	Modified biotin switch assay	Xie et al. [54]	2014
PTP1B	Cys215	Inhibiting PTP1B activity and thereby promoting PERK activity, alters the endoplasmic reticulum stress response	Cysteinyl labeling assay that utilizes a biotinylated iodoacetic acid probe Boivin et al. [55]	Krishnan et al. [56]	2011
PP2A		Inhibition of PP2A to activate the phosphorylation of AMPK	Modified biotin switch assay or the maleimide assay	Shimizu et al. [57]	2018
PPARγ	Cys139	Increases PPARγ activity, thereby promotes glucose uptake and lipid storage	Modified biotin switch assay Mustafa et al. [33]	Cai et al. [46]	2016
Kir6.1	Cys43	Causes vascular endothelial and smooth muscle cell hyperpolarization and	Modified biotin switch assay	Yang et al. [58]	2011

(continued)

Table 1 (continued)

Protein	Cite	Effect	Method	References	Date
		vasorelaxation by activating the ATP-sensitive			
MuRF1	Cys44	Prevent myocardial degradation in the cardiac tissues of db/db mice	Modified biotin switch assay Meng et al. [39]	Sun et al. [59]	2020
Hrd1	Cys115	H_2S regulates VAMP3 ubiquitylation via Hrd1 S-sulfhydration at Cys115 to prevent CD36 translocation in diabetes	Modified biotin switch assay	Yu et al. [60]	2020
Human antigen R	Cys13	S-sulfhydration of human antigen R prevents its homodimerization and activity, which attenuates the expression of target proteins such as CD62E and cathepsin S, to preserve endothelial cell function and delay atherogenesis	Modified biotin switch assay	Bibli et al. [61]	2019

An alkylating agent S-methyl methanethiosulfonate (MMTS) was used to block thiol in proteins. The persulfides group can be conjugated with N-[6-(biotinamido)hexyl]-30-(20-pyridyldithio) propionamide (biotin-HPDP). Then, the biotinylated protein representing the level of protein S-sulfhydration was immunoprecipitated by western blotting [33].

This method has been widely used in sulfhydration testing. Since the persulfide group and the thiol group have similar reactivity to MMTS, this method can provide false-positive results in analyzing sulfhydration modification [62, 63].

2.2.2 Maleimide Assay

Fluorescent maleimide reacts with both unmodified and modified sulfhydryl groups. DTT can reduce the modified cysteines, representing S-sulfhydration, which results in a decrease in fluorescent intensity. Then the fluorescent intensity can be detected by SDS-PAGE [64].

Unfortunately, the maleimide assay can also be used to determine nitrosylation and sulfenic

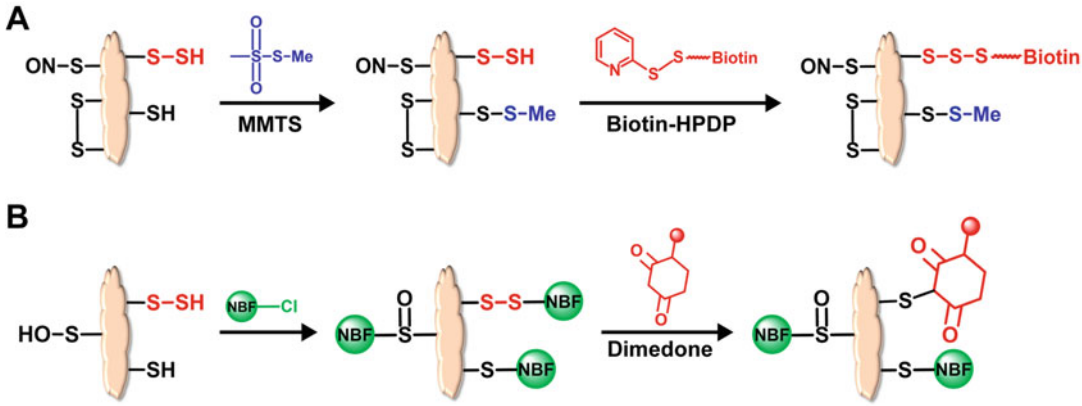

Fig. 1 Modified biotin switch method for S-sulfhydration (**a**) and dimedone switch method for S-sulfhydration (**b**)

acids, which decreases the credibility in detecting sulfhydration [65, 66].

2.2.3 Tag-Switch Method

Methylsulfonybenzothiazole (MSBT) is used to block thiols. Then, a reagent containing nucleophile and biotin will label only persulfides and there is no binding with the blocked thiol groups. Finally, the labeled persulfides representing S-sulfhydration can be conjugated with streptavidin and be further tested by western blotting [54, 67].

However, this method has no higher sensitivity which has posed a problem for S-sulfhydration measurement. Wedmann et al. reported an improved tag-switch method with new cyanoacetic acid derivatives such as fluorescent BODIPY moiety or the Cy3-dye for more sensitivity [68].

2.2.4 Mass Spectrometry Analysis

After thiols in protein samples have been blocked with MSBT, persulfide groups in samples can form biotin-labeled adducts by reacting with CN-biotin. Then, these biotin-labeled protein samples were digested into peptides by digestive enzymes, for instance, trypsin, for further mass spectrometry analysis [67]. The sulfhydrated proteins will be identified through their peptide fingerprints by comparing the MS results with the protein database. Besides, the location of the sulfhydrated cysteine can also be ascertained. However, it was very difficult to block the protein samples completely, and therefore, we cannot avoid false-positive results by using this method [67, 69].

Considering the difficulty in distinguishing unmodified and modified thiol groups, we can use mass spectrometry to find out sulfhydrated groups by using recombinant proteins. After digested into peptide fingerprints, recombinant protein peptides can be tested with LC-MS/MS. The sulfhydrated groups have an extra S group than the free thiols. Therefore, they can be located by comparing the results with the protein database under their unmodified state. Our group has been using LC-MS/MS and our software Create-Compare 2.0 for locating disulfide bonds, which can be used for locating sulfhydrated groups also [34].

2.2.5 Dimedone Switch Method (Fig. 1b)

4-chloro-7-nitrobenzofurazan (NBF-Cl) can react with both persulfide groups and free thiols, as well as sulfenic acids. It is used for the blocking and detection of thiols, amines, and sulfenic acids. After NBF-Cl reacting with persulfide groups, there shows a characteristic absorbance maximum at 412 nm. Then, the addition of dimedone results in a fast disappearance of the 412 nm peak. The two main products in the reaction mixture are the 4-thio-7-nitrobenzofurazan (NBF-SH) and dimedone labeled persulfide groups confirmed by electrospray-ionization time-off light (ESI-TOF) mass spectrometry (MS)/MS analysis. It is a versatile, chemoselective persulfide labeling approach to selectively detect S-sulfhydration [70].

2.3 Existing Problems in S-Sulfhydration Theory for Hydrogen Sulfide

2.3.1 Why the Sulfhydration of Free Thiols in Cysteine Residues by H_2S Has Not Been Found in a Cell-Free Reaction System?

To investigate the reaction between H_2S and free thiols in cysteine residues, we put H_2S donor NaHS and L-cysteine, both resolved in ddH_2S, together. After the reaction, the reaction mixture was tested by mass spectrometry. The result showed that the free thiols in cysteine residues have not been modified and no sulfhydration of thiols has been found [34].

Since large amounts of papers reported the S-sulfhydration by hydrogen sulfide, why this phenomenon has not been seen in such a cell-free reaction system?

2.3.2 Why the S-Sulfhydration of Free Thiols in Cysteine Residues Cannot Happen in All the Cysteine Residues in Protein?

Each protein usually has many cysteine residues. However, the S-sulfhydration of the free thiols in cysteine residues only exists in specific sites. Why one cysteine residue is different from another cysteine residue? And how does hydrogen sulfide decide to modify this cysteine residue other than the cysteine at another site?

3 Molecular Switch of Disulfide Bond

3.1 The Molecular Switch of a Disulfide Bond in Target Proteins of H_2S (Fig. 2)

3.1.1 Target Protein of H_2S: Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) are a subclass of tyrosine kinases, which are involved in the control of cell viability, growth, migration, differentiation, and metabolism [76]. There are 58 known RTKs [76]. All RTKs have similar protein structures, including an extracellular ligand-binding domain, a transmembrane domain, an intracellular domain containing a para membrane regulatory region, a tyrosine kinase domain (TKD), and a C-terminal tail [71]. Dysfunction of the RTK signals usually result in many human diseases, especially tumors.

RTK is usually activated by receptor-specific ligands. The ligand binds to the extracellular domain of RTK, and then the receptor is activated by dimerization and/or oligomerization. The conformational changes of RTKs lead to the trans-autophosphorylation of TKD, thus making TKD present an activated conformation. Self-phosphorylation of RTKs also recruits and activates a variety of downstream signaling molecules, including SRC homology-2 (SH2) or phosphotyrosine binding (PTB) domains, thus activating key cellular signaling pathways in cells [77, 78].

In 2013, we reported that VEGFR2 (vascular endothelial growth factor receptor 2) in vascular endothelial cells is a "receptor," or target protein of H_2S. Instead of finding the S-sulfhydration of free thiols in cysteine residues in recombinant VEGFR2 protein, we discovered that there has been a newly found disulfide bond between C1024 and C1045 in the intracellular kinase domain of VEGFR2 (Fig. 2). This disulfide bond C1045-C1024 in recombinant VEGFR2 protein can be broken up after treated with NaHS, with an increase in protein activity. Besides, the mutation of C1045, which inhibits the formation of such disulfide bond, significantly increased the kinase activity of recombinant VEGFR2 proteins and abrogated the further enhancement in kinase activity after H_2S treatment. In addition, migration of vascular endothelial cells HUVECs has been increased and cannot be further increased by H_2S treatment, after the cells have been transfected with a mutated VEGFR2 (789-end) or VEGFR2 C1045A (789-end) [34]. Figure 2 shows the crystal structure of the tyrosine kinase domain of VEGFR2 [71].

In addition to VEGFR2, other proteins with the molecular switch of disulfide bond for H_2S have been discovered. For instance, EGFR (Cys797/Cys798) [36], insulin receptor [36], TPO [79], etc.

In investigating the role of H_2S in renal sodium and water homeostasis, we found that H_2S decreased the activity of Na^+/K^+-ATPase and promoted its endocytosis in renal tubular epithelial cells. siRNA interference of epidermal growth factor receptor (EGFR) abrogated the effect. H_2S increased phosphorylation of EGFR in renal tubular epithelial cells as well as in kidneys of chronic salt-loaded rats. H_2S transiently bonded to and directly activated EGFR. The certain disulfide bond in EGFR intracellular kinase domain was broken up by H_2S. Mutations of EGFR Cys797 (human) or Cys798 (rat) significantly increased the activity of EGFR [36] (Fig. 2). Figure 2 shows the crystal structure of the tyrosine kinase domain of EGFR [36].

In investigating if H_2S can promote glucose uptake, we found that treatment with sodium

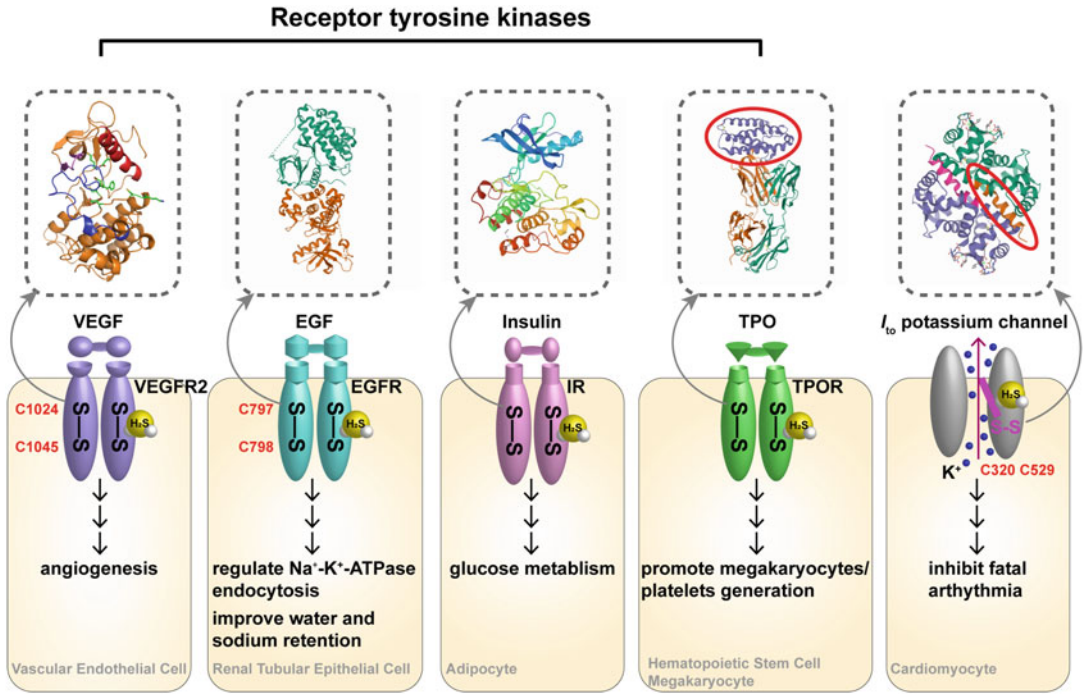

Fig. 2 The common molecular switch, disulfide bond, is located in the intracellular domain of target proteins for H_2S. The crystal structure of target domains (or part of the target domains) for H_2S is indicated in red circles. The origin of the structures shown for VEGFR2, EGFR, IR, TPOR, and the I_{to} potassium channel: the tyrosine kinase domain of VEGFR2Δ50 (M806-D1171, ΔT940–E989) [71]; the tyrosine kinase domain of EGFR (G672-P966) [72]; the tyrosine kinase domain of IR (S981-K1283) [73]; the D. Human thrombopoietin functional domain complexed to neutralizing antibody TN1 Fab (C7-C151) [74]; and the crystal structure of the complex between KChIP1 and Kv4.2 N1-30 (M217-P237) [75]

hydrosulfide (NaHS, an H_2S donor) significantly increased glucose uptake in both myotubes and adipocytes. These effects were blocked by RNA interference of the insulin receptor (IR). The phosphorylation of IR was increased by NaHS. Besides, glucose uptake was reduced in the cells after RNA interference of the H_2S-generating enzyme cystathionine γ-lyase (CSE). The recombinant protein of IR reacted with NaHS for 1 h in a cell-free system. The result showed that NaHS, at concentrations of 50 and 400 μM, significantly increased the activity of the enzyme [36]. Given that IR is also a receptor tyrosine kinase, there probably also a disulfide bond existing in the intracellular domain of IR as a target for H_2S. Figure 2 shows the crystal structure of the tyrosine kinase domain of IR [73].

In investigating the role of H_2S in hematopoiesis using a mice model of myelosuppression and cultured fetal liver cells, we found that H_2S significantly promoted megakaryocytes generation and increased platelet levels. These effects were abrogated in thrombopoietin (TPO) receptor knockout mice (c-mpl$^{-/-}$ mice). However, these effects of H_2S promoted megakaryocytes/platelets generation still exist in TPO knockout mice (TPO$^{-/-}$ mice). These results suggested that the target of H_2S was probably TPO receptors, not the ligand TPO. The TPO receptor, as a tyrosine kinase receptor, might also have a disulfide bond in its intracellular kinase domain acting as the target for H_2S. This hypothesis needs to be further investigated [79]. Figure 2 shows the crystal structure of the human thrombopoietin

functional domain complexed to neutralizing antibody TN1 Fab [74].

3.1.2 Target Protein of H_2S: Ion Channels

It has been reported that H_2S has regulatory roles in ion channels. H_2S inhibits L-type calcium channels in cardiomyocytes [80]. Exogenous H_2S protects myocardial ischemia-reperfusion injury by opening K_{ATP} [81]. H_2S inhibits Cav3.2 T-type Ca^{2+} channels, which play a central role in sensory nerve excitability [82].

In exploring the mechanisms underlying the potential regulation of H_2S on the ion channels, we found that H_2S showed a novel inhibitory effect on Ito potassium channels, and this effect was blocked by mutation at the Cys320 and/or Cys529 residues of the Kv4.2 subunit. H_2S broke the disulfide bond between a pair of oxidized cysteine residues; however, it did not modify single cysteine residues. H_2S extended action potential duration in epicardial myocytes and regularized fatal arrhythmia in a rat model of myocardial infarction. In conclusion, H_2S targets the Cys320/Cys529 motif in Kv4.2 to regulate the I_{to} potassium channels. However, H_2S did not change the function of other ion channels, including I_{K1} and I_{Na} [76]. Figure 2 shows the crystal structure of the complex between KChIP1 and Kv4.2 N1–30 [75].

3.1.3 Common Points of the Mechanism in Targeting "Receptors" by H_2S

There are several common points in targeting those "receptors" by H_2S.

Although the "receptors" of H_2S we previously found are all transmembrane proteins, for instance, receptor tyrosine kinase and ion channels, the target sites (the disulfide bond as a molecular switch) of H_2S are all in the intracellular domain of those "receptors." The maintenance of metabolic functions will be prohibited when ROS levels are high in cells. Besides, the maintenance of ROS in low levels inside cells is important to allow the redox-reduction changes to exit sensitively for the regulation [83]. We know that there are also disulfide bonds in the extracellular domain of transmembrane proteins. However, the stronger oxidative environment outside the cells might probably inhibit the reducing ability of H_2S.

Breaking up disulfide bonds acting as molecular switches is necessary for the normal biological function of tyrosine kinase receptors. Without the disulfide bond broken, the kinase activity of tyrosine kinase receptors in dimerization will still be in an inhibited state. In our previous studies, the siRNA-mediated knockdown of CSE attenuated VEGF-induced migration of vascular endothelial cells and also attenuated VEGF-induced phosphorylation of VEGFR2 at Tyr1175 site. The results illustrate endogenous H_2S is required for VEGF to promote the migration of vascular endothelial cells [34].

In addition to the activating role of H_2S on its tyrosine kinase receptors by breaking up the disulfide bonds as molecular switches, H_2S exerts an inhibitory effect upon Ito potassium channels [76]. Whether H_2S is playing an activating role or an inhibitory role on target proteins depends on the role of a disulfide bond in proteins.

3.1.4 The Comparison Between H_2S and Other Reducing Species in Cells

Although endogenous H_2S is not the only reducing molecule in mammalian cells, it is the smallest one. Therefore, it is much easier for H_2S to reach the disulfide bonds which are embedded underneath the surface of proteins.

In previous experiments, we compared H_2S with a range of biological thiols, including glutathione, Cys, cysteinyl glycine, and homocysteine at concentrations with equal reducing potency. Comparison of ion intensity ratios between the cleaved ion and the peptide containing the Cys1045-Cys1024 S–S bond showed that H_2S was the most potent reducing factor in breaking S–S bonds as compared with these biological thiols (Fig. 3) [34].

3.1.5 H_2S Donors (Fig. 4)

The decrease of endogenous H_2S production is usually related to different kinds of diseases [1, 84]. Therefore, the administration of H_2S donors, for example, GYY4137 [85], FW1256

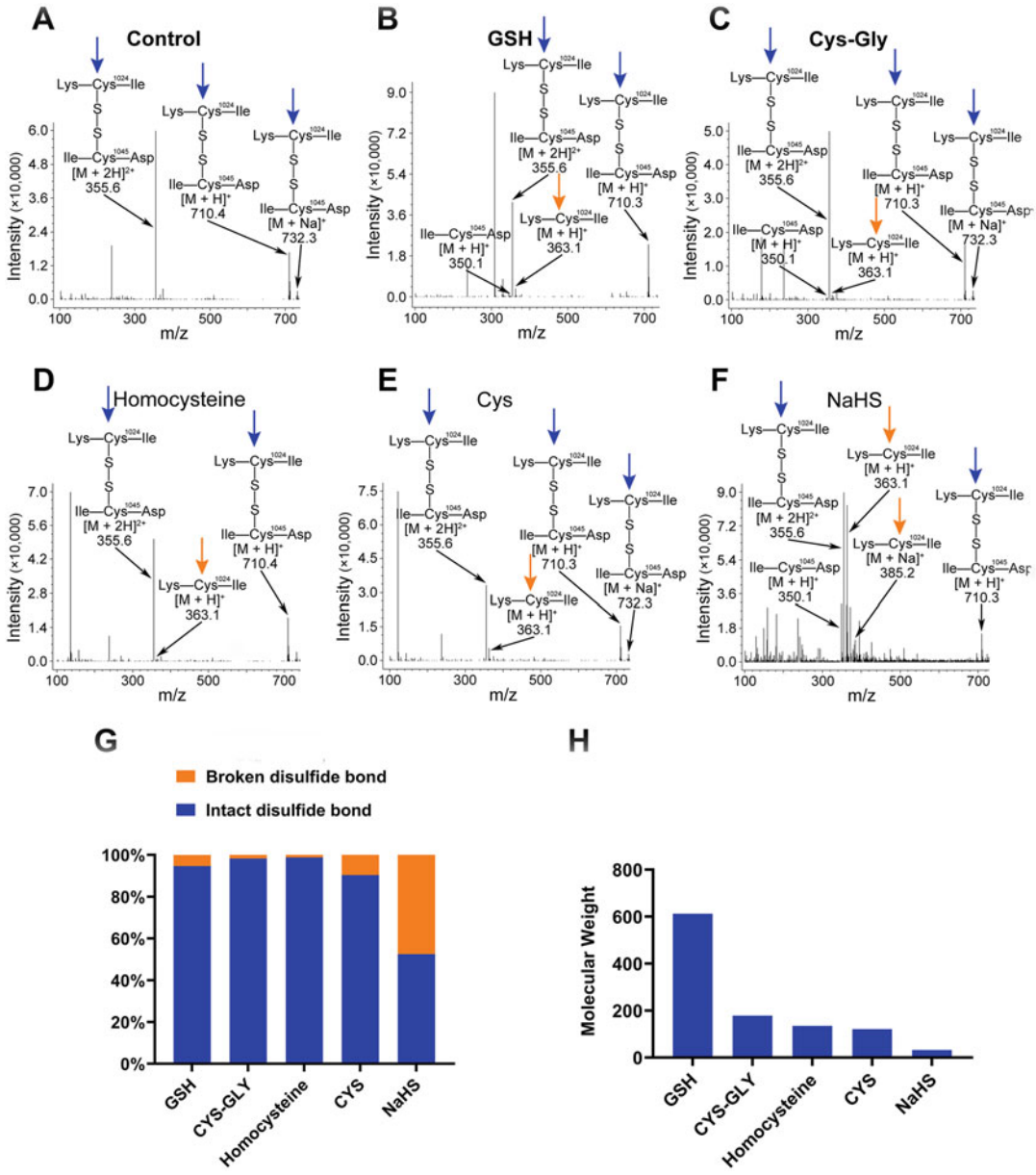

Fig. 3 H_2S was the most potent reducing factor in breaking S–S bonds as compared with other biological thiols (created from Fig. 4 C–H from ARS 2013 Aug 10;19(5):448–64)

[86], SG-1002 [87], etc., can be used to increase H_2S level for treatment.

S-propargyl-l-cysteine (SPRC) is a structural analogue of s-allylcystene (SAC). Unlike NaHS and other inorganic salts, SPRC releases H_2S through endogenous H_2S-producing enzyme CSE or CBS. Therefore, SPRC is called an endogenous H_2S donor. Studies have shown that SPRC can improve the proliferation of endothelial cells and has an anti-inflammatory effect on myocardial cells [88, 89]. SPRC has a cardioprotective effect in vivo by inhibiting

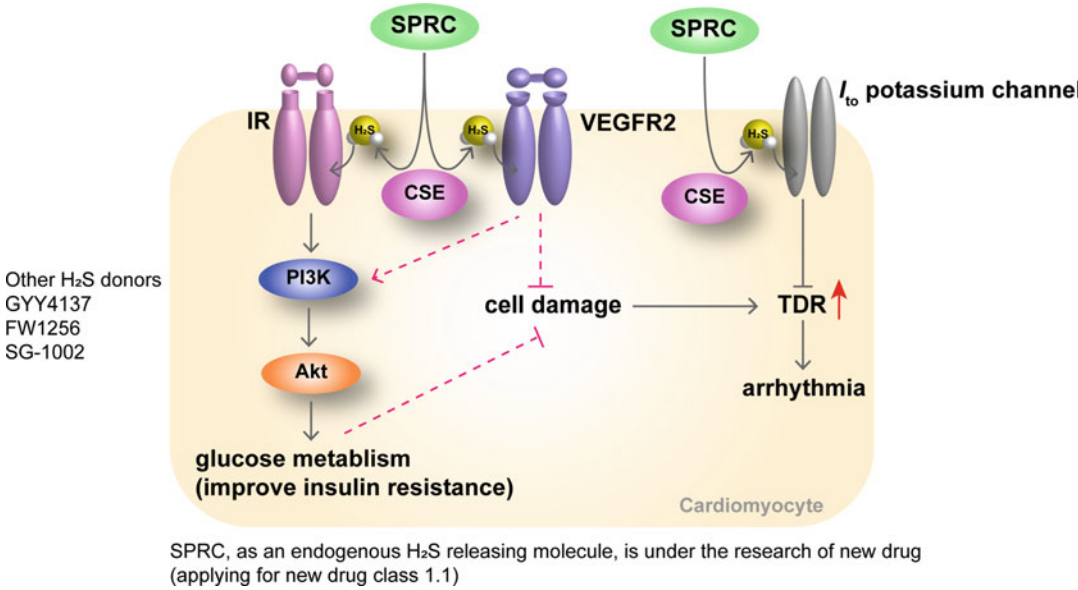

Fig. 4 Nonspecific and specific H_2S-releasing drugs. SPRC releases H_2S through endogenous H_2S-producing enzyme CSE or CBS

mitochondrial dysfunction and promoting S-mercapto of CaMKII [90]. Besides, SPRC also has anti-cancer and anti-hypoxic/ischemia effects and improves A_β-induced neuronal damage [91].

Considering that H_2S might be oxidized and lost its reducing ability, different endogenous H_2S-producing enzyme might have different target proteins. Therefore, using a substrate of a certain enzyme usually results in a more specific distribution of H_2S close to certain targets to avoid nonspecific adverse effects.

3.2 The Methods for Analyzing Disulfide Bonds in Proteins

3.2.1 Mass Spectrometry [34]

Amino acids or peptides are loaded to a SHIMADZU mass spectrometer (LCMS-IT-TOF), and the MS analysis was operated with a capillary temperature of 200 °C. The spray voltage was 2.4 kV. Data acquisition and analysis were performed using the SHIMADZU LCMS solution (Shimadzu Scientific Instruments).

3.2.2 Raman Spectroscopy [34]

HORIBA Jobin Yvon LabRam-1B Raman spectrometer (HORIBA Scientific) can be used for Raman spectroscopy. The excitation source was a helium-neon laser with a wavelength of 632.8 nm and an excitation power of 4.3 mW. The Raman S-S stretching band occurs in a vibrational spectrum of 488–504 cm^{-1} and the S–H stretching band occurs in the range of 2500–2600 cm^{-1}.

3.2.3 Fluorescence Labeling of Reactive Cys Thiols [36, 92]

Recombinant proteins are subjected to immunoprecipitation with antibody and then are incubated with 10μM NEM for 2 h at room temperature. After being washed with PBS (pH 7.4), 100 μM Cy3 maleimide is added and incubated for 30 min and then washed. After treated with 25 mM DTT for 1 h at room temperature, the immunoprecipitates were reacted with 100 μM

Cy5 maleimide for 30 min at room temperature and washed. The double-labeled immunoprecipitates are separated with SDS-PAGE. Fluorescent images and fluorescence levels are acquired and quantified.

3.3 Existing Problems in the Molecular Switch Theory for H_2S

As a reducing agent, H_2S can reduce the disulfide bond in a two-step reaction [34]. However, H_2S can easily be oxidized. Therefore, to maintain the reducing state of H_2S generated from endogenous H_2S-producing enzymes, the distance between the endogenous H_2S-producing enzyme and the target protein of H_2S should not be too far. Otherwise, H_2S might probably be in its oxidized form when it crosses a long distance and finally reaches its target. Besides, there might be different target proteins for each H_2S-producing enzyme, CTH, CBS, or MPST, since the endogenous H_2S is prone to react with nearby disulfide bonds in target proteins.

4 Which Theory Is the Truth, or Which Mechanism Is More Important?

4.1 The Specificity of S-Sulfhydration Measurement

Because of the similarity in chemical characteristics, the free thiols (unmodified thiols) and sulfhydration of thiols (modified thiols) cannot be easily discriminated against. Therefore, it might probably produce false-positive results. In 2019, a more specific method has been found and can be used to measure the S-sulfhydration of cysteine residues with more accuracy to find out more target proteins and the target cites of H_2S. Besides, this more accurate method can be used in combination with the RNA interference method. After RNA interference of CSE, CBS, or MPST, dependently, we can observe the S-sulfhydration of different target proteins by endogenous H_2S generated from different H_2S-producing enzymes.

4.2 Persulfides Might Be Intermediates in the Process of Breaking Up Disulfide Bonds by H_2S

In our previous studies, we found that it is an HS^-, not a gaseous form of H_2S in water solution, that can break up disulfide bonds. The breaking-up process contains two steps. One step is that one molecule of HS^- can break up the disulfide bond through a nucleophilic attack reaction, producing -SSH (persulfides) as an intermediate. The other step is that another molecule of HS^- can break up the S-S bond in -SSH, reducing the persulfides into free thiols and producing H_2S_2. H_2S_2 is not stable and it can quickly change into H_2S and S [34].

Reaction 1:

$$HS^- + C_3H_6NO_2 - S - S - C_3H_6NO_2 \rightarrow C_3H_6NO_2 - S^- + HS - S - C_3H_6NO_2$$

Reaction 2:

$$HS^- + HS - S - C_3H_6NO_2 \rightarrow C_3H_6NO_2 - S^- + HS - SH$$

In this process, the persulfide has transiently appeared in the reaction by the method of mass spectrometry.

4.3 It Is Probably H_2S_n, Not Reducing the Form of H_2S, Producing Persulfides by Adding a Sulfur Atom to Free Thiol Directly (Fig. 5)

Dr. Kimura points out in his review that H_2S cannot add extra sulfur to free thiol in proteins when it is in a reducing form. However, H_2S can be easily transformed into an oxidized form H_2S_n by reacting with ROS. Then, H_2S_n can oxidize free thiols into persulfides.

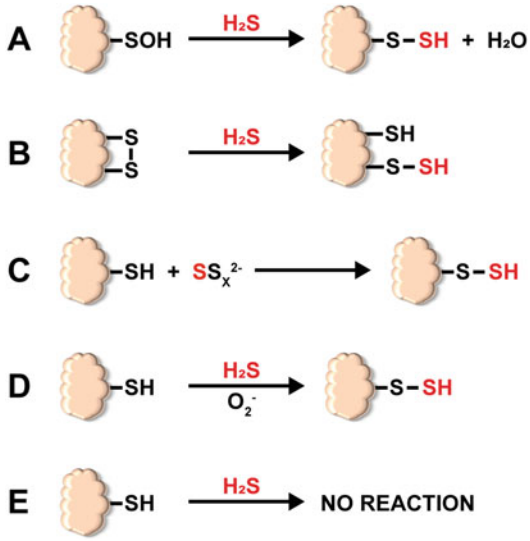

Fig. 5 Reaction mechanisms for S-sulfhydration formation. S-sulfhydration can be induced by H_2S on cysteine sulfenic acids (Cys-SOH) (**a**) or cysteine disulfides (-S-S) (**b**), or by polysulfides on cysteine thiols (Cys-SH) (**c**). H_2S induces S-sulfhydration on cysteine thiols in oxidation conditions (**d**–**e**)

It is also reported by other scientists that H_2S cannot directly undergo an oxidizing reaction since it is a reducing agent. This reaction is impossible due to the thermodynamic constraints. H_2S can only modify free thiols directly when it is in an oxidizing environment [39, 44–46]. Incubation of proteins, such as GAPDH, BSA, or immunoglobulins with H_2S, led to no detectable protein S-sulfhydration [49, 93] confirming the theory [38].

5 Future Directions

Finding out target proteins and target mechanism and precisely locating the target cites of H_2S are of great value in H_2S biology. Finding out which theory is more important, S-sulfhydration theory, or the disulfide molecular switch theory, is essential in understanding the role of H_2S of both exogenous and endogenous origin.

5.1 Using or Finding Out More Accurate and Specific Methods When Testing Persulfides

Exploring more specific methods for sulfhydration assessment with lower false-positive results can provide us more accurate knowing for persulfides produced by H_2S.

5.2 Observing the Structure of Target Proteins and the Modification of the Proteins Directly

By expressing and purification recombinant target proteins, we can observe directly the structure of these proteins and the specific modification or reaction in certain cites of proteins produced by H_2S, using the method of cryo-EM and protein crystallization and X-ray. The sulfhydration modification and the molecular switch disulfide bond can both be presented directly when the structure is observed in these methods. Therefore, the problem of these two theories will be finally resolved.

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Hydrogen Sulfide and the Kidney

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Abbreviations

3-MST	3-mercaptopyruvate sulfurtransferase
AAT	Aspartate amino transferase
ACC	Acetyl-CoA carboxylase
AE1	Anion exchanger-1
AKI	Acute kidney injury
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AQP2	Aquaporin-2
AT1	Angiotensin receptor type 1
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
Bnip3	BCL2/adenovirus E1B 19kDA-interacting protein
CBS	Cystathionine β-synthase
ccRCC	Clear cell renal cell carcinoma
cGMP	Cyclic guanosine monophosphate
CHF	Congestive heart failure
CKD	Chronic kidney disease
CO	Carbon monoxide
CSE	Cystathionine γ-lyase
DAO	D-amino acid oxidase
DKD	Diabetic kidney disease
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
ENaC	Epithelial Na channel
eNOS	Endothelial NO synthase
ER	Dietary restriction
ERK	Extracellular-signal-regulated kinase
ESKD	End stage kidney disease
FGF23	Fibroblast growth factor 23
FOXO3	Forkhead box O3
GFR	Glomerular filtration rate
GSK-3β	Glycogen synthase kinase-3β
H_2S	Hydrogen sulfide
HIF	Hypoxia inducible factor
HO-2	Hemoxygenase-2
HS	Hydrosulfide
IGF1	Insulin-like growth factor 1
IL	Interleukin
iNOS	Inducible NO synthase
IRI	Ischemic renal injury
IRβ	Insulin receptor β
JNK	c-Jun N-terminal kinase
KIM-1	Kidney injury molecule-1
MAP kinase	Mitogen-activated protein kinase
miR	microrna
MMP	Matrix metalloprotease
mTORC1	Mechanistic target of rapamycin complex 1
NAD	Nicotinamide adenine dinucleotide
NaHS	Sodium hydrosulfide
NFκB	Nuclear factor κ B
NMDA	N-methyl D-aspartate
NO	Nitric oxide
NOX	NADPH (nicotinamide adenine dinucleotide phosphate) oxidase

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Nrf-2	Nuclear factor erythroid-2 related factor-2
PARP-1	Poly(ADP ribose) polymerase-1
PI 3 kinase	Phosphoinositide 3 kinase
PKG-II	cGMP activated protein kinase G-II
PlGF	Placental growth factor
PTEN	Phosphatase and tensin homolog
RCC	Renal cell carcinoma
ROS	Reactive oxygen species
SASP	Senescence associated secretory phenotype
sFlt1	Soluble fms-like tyrosine kinase
sGC	Soluble guanylyl cyclase
Sirt	Sirtuin
TGFβ	Transforming growth factor β
TNFα	Tumor necrosis factor α
VEGF	Vascular endothelial growth factor

1 Introduction

The kidney is a versatile organ that performs a wide variety of functions. In addition to waste elimination via urine generation, the kidney regulates blood pressure, body content of sodium and other electrolytes, volume in intracellular, interstitial, and extracellular compartments, acid-base balance, hormone synthesis and degradation, erythropoiesis, and bone metabolism. In order to perform these extensive functions, the kidney requires large amounts of energy; accordingly, the proximal tubular epithelial cells which account for majority of kidney cells and perform most of the reabsorption function are rich in mitochondria; incidentally, mitochondria are an important site of hydrogen sulfide (H_2S) generation and action. Either acute or chronic impairment in kidney function can be associated with abnormalities in nearly all other organ systems. The kidney also demonstrates a remarkable ability to regenerate itself following acute injury. In view of the extensive effects of kidney on critical aspects of human physiology, it is important to know the biological controllers of kidney function. It is in this context that recent discoveries have highlighted a fundamental role for H_2S.

Evolution of life on the Earth has a close relationship with H_2S [1]. As primordial life appeared approximately 3.8 billion years ago, the Earth's environment was rich in H_2S and lacked oxygen. The emerging life forms probably used H_2S as a source of energy. With the increase in atmospheric oxygen by photosynthetic bacteria (the Great Oxidation Event), terrestrial sulfur was oxidized to sulfate which was reduced to H_2S in the presence of Fe^{++} and bacteria capable of reducing sulfate. Eukaryotes are thought to have emerged under these conditions [2]. It is likely that the organ systems in the evolving life forms, including the kidneys, were affected by H_2S, a legacy that is still seen at the present time. Additionally, both humans and select bacteria share common steps in metabolism of H_2S [2], supporting the view that H_2S has had an intimate role in organic evolution.

2 Synthesis of H_2S in the Kidney

This topic has been reviewed in detail recently [3]. Kidney synthesizes H_2S during metabolism of L-cysteine in the trans-sulfuration pathway that involves two enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) (Fig. 1). CSE is more abundant than CBS in the kidney [4]. These enzymes normally reside in the cytosol although they are capable of nuclear translocation [5]. CSE is expressed by glomerular podocytes, mesangial, and endothelial cells in addition to majority of tubular epithelial cells [6]. CBS has been identified in the tubular epithelial cells [7]. In addition, mitochondrial 3-mercaptopyruvate sulfurtransferase (3-MST) also contributes to H_2S synthesis in the kidney. Recent findings by Shibuya have shown that metabolism of D-cysteine in the peroxisome is another source of H_2S generation in the kidney [8]. In fact, this pathway is said to generate several fold more of the gas than the trans-sulfuration pathway [8]. With a pKa of 6.8, most of H_2S is present as HS^- in cells [9, 10]. H_2S degradation results in the formation of thiosulfate. A part of

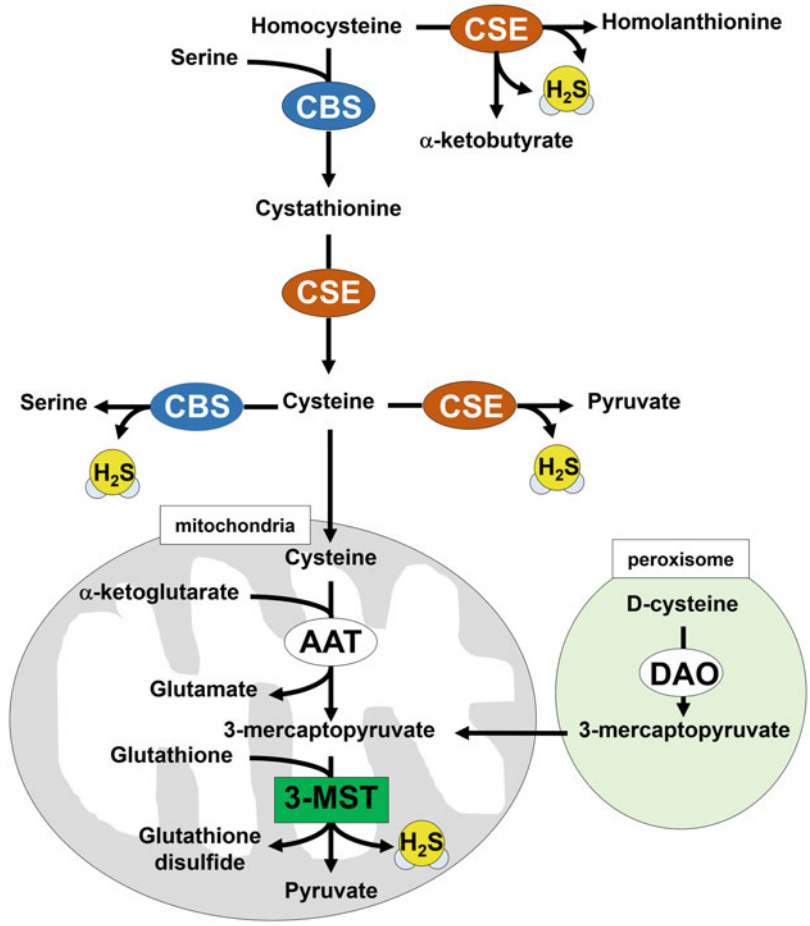

Fig. 1 A simplified schematic of H_2S synthesis in the kidney. H_2S synthesis is catalyzed by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) in the cytoplasm (trans-sulfuration pathway). Mitochondrial generation of H_2S by 3-mercaptopyruvate sulfurtransferase (3-MST) and peroxisomal generation of H_2S by D-amino acid oxidase (DAO) are also shown. AAT = Aspartate amino transferase

thiosulfate is further converted to sulfite which is oxidized to sulfate; thiosulfate is also used as a sulfur donor to synthesize H_2S. The content of H_2S in cells and extracellular environment has been variably reported due to differences in assays employed.

Urinary sulfate and thiosulfate levels have been linked to dietary intake of sulfur containing amino acids and systemic H_2S [11, 12]. Circulating thiosulfate undergoes unimpeded filtration at the glomerulus [13], and both reabsorption and secretion occur in the proximal tubule [14]. Sulfate is also filtered completely at the glomerulus following which most of it is reabsorbed at the proximal tubule by transporters Na-Sulfate 1 (Na-Si 1) and Sat-1 [15]. Caution should be exercised in interpreting urinary levels of these metabolites as indices of H_2S metabolism as kidney injury can alter function of the proximal tubules. Additionally, bacterial contribution to H_2S generation in the body can also contribute to urinary metabolites of H_2S.

Cellular Transport of H_2S It is unclear how H_2S moves in and out of cells. Diffusion has been suggested as a mechanism [16]. In erythrocytes, anion exchanger-1 (AE1), a Cl^--HCO_3^- exchanger, has been implicated in H_2S transport [17]. This scenario involves movement of H_2S from the intracellular to the extracellular phase by diffusion or via a channel. In the extracellular space H_2S dissociates into HS^- and H^+ resulting in lowering the pH (acidification). This is followed by entry of HS^- into cells in exchange for Cl^- facilitated by AE1. HS^- combines with H^+ in the intracellular fluid to become H_2S. It is

not known whether this erythrocyte model applies to the kidney. A shorter form of AE1 that differs from the erythrocyte AE1 at the amino terminal is present in the basolateral membrane of α-intercalated cells of the kidney collecting ducts [18]; it remains to be seen if it can transport H_2S. A channel mediated transport is also a possibility; a HS^- channel exists in *Clostridium difficile*, a bacterium [9]. Recently, methemoglobin has been proposed as a carrier of H_2S in the blood which can facilitate its function at locations away from its sites of generation [19].

2.1 H_2S in Renal Physiology

In addition to waste clearance function, the kidney is involved in several other functions including generation of hormones such as erythropoietin and 1:25 dihydroxy vitamin D3, drug metabolism, and glucose generation by gluconeogenesis. Recent studies have shown that H_2S has far reaching effects on several of these functions.

We will first briefly review the structural and functional organization of the kidney. The basic unit of the kidney is a nephron which consists of a capillary tuft, the glomerulus, and the tubule. The unique aspect of the kidney glomerulus is that it is situated between the afferent and the efferent arterioles thereby encountering higher hydrostatic pressures than capillaries elsewhere in the body [20]. The kidneys are highly vascular organs receiving nearly a quarter of the cardiac output through the renal arteries. The blood reaching the glomerulus undergoes filtration across the capillary wall. The unfiltered blood in glomerular capillary is conveyed out of the glomerulus through the efferent arteriole. Once out of the glomerular area, the efferent arteriole branches into capillaries that surround the tubules. This arrangement serves the important purpose of returning to systemic circulation the water, ions and solutes reclaimed by the tubules during postglomerular processing of the glomerular filtrate. This return occurs via renal venules which unite to form the major renal vein; the latter drains into the inferior vena cava and systemic circulation.

Glomerular filtration is the first step in urine formation [20]. The determinants of glomerular filtration are renal afferent arteriolar blood flow, difference in hydrostatic pressure across the glomerular capillary, difference in osmotic pressure across the glomerular capillary, and the surface area and permeability characteristics of the glomerular basement membrane. The glomerular basement membrane is a condensed form of extracellular matrix containing type IV collagen, laminin, proteoglycans, and many other molecules. Glomerular endothelial cells and podocytes, specialized epithelial cells, synthesize the glomerular basement membrane during development and postnatally. Nearly 20% of plasma in the glomerular capillary becomes the ultrafiltrate which can amount to nearly 160–170 liters per day. The ultrafiltrate has the same composition of the plasma except for proteins. Circulating cells in blood are mostly prevented from entering the ultrafiltrate. Charge and size barrier function of the glomerular capillary wall inhibits loss of albumin and other plasma proteins during filtration thereby conserving them. The glomerular filtration rate (GFR) is an important measure of waste clearance function of the kidney. Studies by Xia et al. have shown that renal arterial infusion of H_2S in the form of sodium hydrosulfide (NaHS) in doses that do not affect systemic blood pressure results in increase in GFR by augmenting renal blood flow [21]. Conversely, infusion of inhibitors of CSE and CBS, enzymes in the trans-sulfuration pathway, reduce renal blood flow indicating that the latter is under constitutive regulation by H_2S. Renin is an enzyme which catalyzes the conversion of angiotensinogen to angiotensin I. Angiotensin converting enzyme removes 2 amino acids from angiotensin I to generate angiotensin II, which causes marked vasoconstriction including of the afferent arteriole. H_2S can inhibit renin activity [22] to cause afferent arteriolar vasodilation and augment blood flow into the glomerular capillary leading to increase in GFR.

Downstream from the glomerular capillary, the filtrate enters the tubular part of the nephron

which is organized into distinct anatomical and functional segments: proximal tubule, descending and ascending loops of Henle, distal convoluted tubule, connecting duct, and cortical and medullary collecting ducts. The lining epithelia have different morphologic and transport characteristics depending on the tubular segment. These properties are not rigidly maintained. Recent investigations employing single cell RNA sequencing have shown that there are new cell types of epithelial cells that originate from a transitional cell, particularly in the collecting tubules [23]. The major function of the proximal tubule is reabsorption of most of water and electrolytes such as Na^+ from the glomerular filtrate, and return them to systemic circulation via the capillary arrangement described above. The major cation in the glomerular filtrate is Na^+. By means of coordinated actions of Na^+-H^+ exchanger 3 in the apical membrane and Na^+-K^+ ATPase in the basolateral membrane, most of the filtrate Na^+ is reabsorbed in the proximal tubule [24]. Additionally, Na^+ reabsorption is also coupled to glucose, sulfate, phosphate, and amino acids at this site [25]. The remaining Na^+ is reabsorbed in the downstream segments. In the loop of Henle, Na^+-K^+-$2Cl^-$ transporter aids in Na^+ reabsorption, whereas Na^+-Cl^- co-transporter assists in Na^+ recovery in the distal convoluted tubule. Epithelial Na channel (ENaC) is the main conduit for Na^+ reclamation in the collecting duct which occurs in exchange for K^+ under the control of aldosterone, a mineralocorticoid hormone synthesized by the adrenal cortex [26].

In studies reported by Xia, H_2S infusion increases Na^+ and K^+ excretion [21]. The former was attributed to inhibition of Na^+-K^+-$2Cl^-$ transporter in the ascending loop of Henle and Na^+-K^+ ATPase [21]. Additionally, H_2S can also inhibit Na^+-H^+ exchanger 3 as shown in studies on cells in culture [27]. H_2S may also inhibit Na^+ reabsorption by ENaC in the collecting duct. Aldosterone stimulation of Na^+ reabsorption by ENaC involves reactive oxygen species (ROS) [28]. In *Xenopus* A6 cells hydrogen peroxide augments opening of ENaC by stimulating phosphoinositide 3 kinase (PI 3-kinase); the kinase activity is augmented by inhibiting phosphatase and tensin homolog PTEN, its constitutive inhibitor [29]. H_2S abolished hydrogen peroxide inhibition of PTEN restoring its function leading to reduction in PI 3-kinase activity and ENaC opening. Thus, inhibition of ENaC could contribute to an increase in Na^+ excretion by H_2S. Na^+ -K^+ ATPase located in the basolateral membrane is important for transporting Na^+ out of tubular cell cytoplasm. H_2S inhibits its activity by promoting endocytosis of its subunits in in vitro studies employing renal tubular epithelial cells [30]. H_2S can activate the receptor for epidermal growth factor, a receptor tyrosine kinase, leading to activation of PI 3-kinase. NaHS administration reduced blood pressure and increased urinary Na^+ excretion in rats with desoxycorticosterone-induced hypertension; increase in EGF receptor-PI 3-kinase signaling pathway was also seen in the kidneys of NaHS treated hypertensive rats [27]. Cysteine 797/798 residue in the EGF receptor was found to be important in H_2S stimulation. It is unclear if this regulation involves persulfidation. It is interesting to note the contrast in H_2S regulation of PI 3-kinase, i.e., ENaC inhibition by H_2S requires suppression of PI 3-kinase activity [29], whereas inhibition of Na^+ -K^+ ATPase by H_2S involves stimulation of the kinase, indicating the importance of context in the recruitment of signaling pathways.

Water reclamation in distal nephron is dependent on aquaporin-2 (AQP2) water channel protein [31]. Lithium, a commonly employed drug in the management of bipolar disorders, inhibits AQP2 resulting in excessive urine formation, polyuria, which can be a bothersome side effect. Recent studies have shown that H_2S stimulates AQP2 expression and distribution to the cell membrane in the principal cells of the collecting duct; this results in increase in water reabsorption, the effect being mediated by the cAMP system; H_2S also inhibited lithium-induced diuresis [32] which has potential clinical application in alleviating polyuria. Lithium also causes CKD characterized mainly by tubulointerstitial fibrosis [33]. It is important to explore if H_2S can alleviate this complication which can lead to

discontinuation of what may have been an effective treatment.

The area in the kidney outside the glomerulus and the tubules is called the renal interstitium. It contains two zones, i.e., the area around the afferent arteriole that contains the renin synthesizing perivascular interstitial cells [34, 35], and the area around the tubules and peritubular blood vessels called the tubulointerstitium. The latter area contains immunologically active cells such as the dendritic cells, monocytes, macrophages, and the resident stellate fibroblast-like cells which synthesize erythropoietin [34]. Synthesis of erythropoietin, a maturation factor for red blood cells, is regulated by ambient oxygen tension in the tubulointerstitium.

Regulation of acid-base balance is an important function of the kidney. The role of H_2S in this function has not been well studied. A positive association between urinary sulfate excretion and net acid excretion has been reported in human kidney transplant subjects; however, there was no correlation with serum bicarbonate or systemic pH [12].

2.2 Gasotransmitters in Oxygen Sensing

Recent studies have revealed a role for H_2S in oxygen sensing by the carotid body, which participates in the regulation of ventilation, heart rate, and blood pressure [36, 37]. Interestingly, gasotransmitters interact in this context [38]. The carotid body monitors oxygen tension in systemic circulation, and its chemoreceptors induce changes in ventilation in response to changes in systemic oxygen tension.

In states of normoxia, glomus cells in the carotid body generate CO by the action of hemoxygenase 2, which results in increase in cGMP through the action of soluble guanylyl cyclase, leading to stimulation of protein kinase G (PKG). PKG, in turn, promotes phosphorylation of Ser-377 on CSE resulting in inhibition and reduction in the production of H_2S (Fig. 2). The consequence of reduced H_2S generation is decrease in neural output from the carotid body.

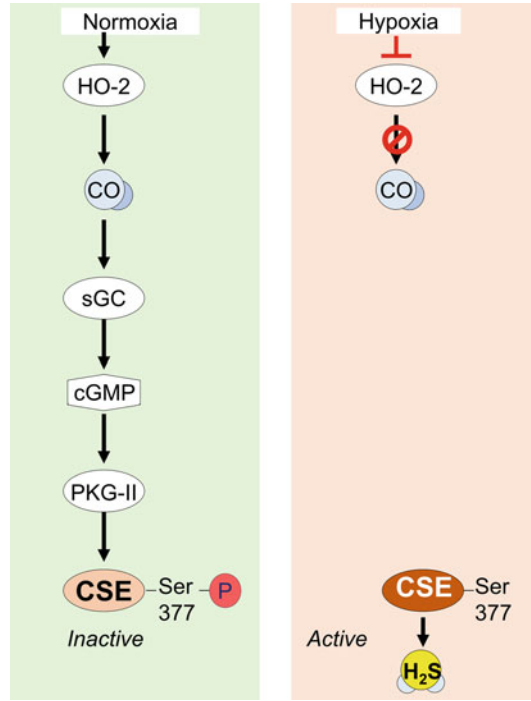

Fig. 2 H_2S and oxygen sensing. Under normoxic conditions, CO generated by hemoxygenase-2 (HO-2) in the carotid body stimulates soluble guanylyl cyclase (sGC) to increase cGMP expression which leads to increased activity of phosphokinase G (PKG) II. The latter enzyme phosphorylates CSE on Ser-377 resulting in inhibition of H_2S generation and neural output from the carotid body. When hypoxia occurs, CO generation by HO-2 is reduced leading to decreased cGMP and inhibition of PKG-II activity. This results in decreased phosphorylation of CSE, stimulation of H_2S generation, and increase in carotid body neural output leading to stimulation of respiration

During hypoxia such as that seen during obstructive apnea, HIF1α activity increases with generation of ROS via NOX2. Augmented ROS production inhibits hemoxygenase 2 leading to reduced generation of CO and decrease in PKG. This removes PKG-mediated inhibition of CSE and H_2S generation (Fig. 2). Increase in H_2S production leads to increase in neural activity, and stimulation of catecholamine production in the adrenal medulla with consequent hypertension. Inhibition of CSE by chemical inhibitors, or by genetic manipulation ($CSE^{+/-}$ mice), or use of CO donors rescue mice from

hypoxia-induced hypertension [39]. The medullary region of the kidney is hypoxic compared to the cortex. It is important to study whether H_2S participates in oxygen sensing in the kidney. Other vascular beds in the heart and gastrointestinal tract seem to employ H_2S as an oxygen sensor [40, 41].

3 H_2S in Acute Kidney Injury (AKI)

Sudden reduction in the existing kidney function can occur in three ways: (1) Due to diseases that affect the perfusion of the kidney (pre-renal, e.g., reduced blood pressure due to loss of extracellular fluid, congestive heart failure), (2) Due to intrinsic renal events affecting the parenchyma of the kidney (e.g., acute tubular necrosis, glomerulonephritis, tubulointerstitial nephritis), or (3) Due to obstruction to the flow of urine in the urinary tract (post-renal, e.g., prostatic enlargement). Serum creatinine is a common index of GFR, and an increase of about 0.3 mg/dl over the normal values is indicative of AKI. It is important to note that for AKI to occur as indicated by the rise in serum creatinine both the kidneys should be affected by pathology; injury to one kidney in a person with *normally* functioning two kidneys at baseline will not result in kidney injury because the uninvolved kidney compensates and maintains the kidney function. However, in a person with a single functioning kidney, or in a patient with previous chronic kidney disease, pathology affecting a single kidney is enough to cause AKI. Deficiency of H_2S generation has been found to play a role in many types of AKI.

3.1 AKI Due to Intrinsic Damage to Renal Parenchyma

Ischemic Renal Injury (IRI) Critical events in IRI include reduced blood flow to the renal parenchyma followed by reperfusion resulting in oxidative stress, inflammation, and apoptosis of tubular epithelial cells. Bos and associates reported that kidney CSE mRNA and protein, and CBS mRNA were decreased post-ischemia [6]. IRI resulted in more severe kidney injury and greater mortality in mice lacking CSE compared to wild type controls; administration of NaHS ameliorated kidney injury and improved mortality [6]. Other studies have confirmed the protective role of H_2S in IRI [42, 43].

IRI is particularly relevant in the setting of kidney transplantation. Removal of the kidney from the donor interrupts blood supply, and implantation in the recipient subjects the kidney to reperfusion. The kidney mRNA content of CSE correlated directly with the integrity of kidney function at 2 weeks in the post-transplant period suggesting ability to generate H_2S offers protection against IRI [6]. Inhalational administration of H_2S induces a hypometabolic state, maintains mitochondrial integrity, markedly mitigates apoptosis and influx of inflammatory cells, and promotes survival in mice [44]. Inclusion of NaHS in the preservative fluid helps to minimize kidney injury by in a rat model of kidney transplantation [45].

Epidemiologic studies have linked urinary metabolites of H_2S, sulfate, and thiosulfate with outcomes after kidney transplantation [12]. Urinary thiosulfate levels have been used as an index of systemic H_2S levels as they appear to correlate with the amount of inhaled or intravenously administered H_2S [46]. However, gut bacterial contribution to urinary sulfate metabolites may be of concern. Multiple regression analysis accounting for confounding factors was conducted in 707 renal transplant recipients. Data showed that urinary sulfate and thiosulfate levels directly correlated with the clearance function of the kidney, and inversely with hypertension and parameters of cardiovascular disease such as serum levels of h-c reactive protein, and pro-brain type natriuretic peptide [12]. Furthermore, mortality in transplant recipients correlated with reduced urinary sulfate and thiosulfate levels; there was a significant gradient between the lowest and highest tertiles of urinary sulfate and thiosulfate and mortality. Urinary sulfate could reflect an acid load which could contribute to metabolic acidosis in transplant patients.

Although there was a significant correlation between urinary sulfate and acid excretion by the kidney it did not interfere with the salutary association with preservation of glomerular filtration rate and decreased mortality.

Mitochondrial generation of H_2S from D-cysteine via the action of 3-MST can also protect against IRI. AP-39 is an agent that is directed to mitochondria by triphenyl phosphonium, and contains dithiolethione which releases H_2S [47]. AP-39 offered partial protection against IRI in rodents by ameliorating oxidative stress and inflammation [48].

In another model of IRI employing body cooling followed by rewarming the kidney expression of all 3 enzymes (CBS, CSE, and 3-MST) was decreased; administration of dopamine shielded against ischemic kidney injury by maintaining the expression of the three enzymes and stabilizing H_2S generation [49]. There is an instance in nature in which animals undergo physiological ischemia and reperfusion and yet resist IRI, i.e., hibernation. During hibernation the animals undergo torpor characterized by reduction in ventilation, cardiac output, and metabolism [50]. During arousal from this state blood flow is restored, and yet animals do not manifest IRI [50]. Interestingly, epithelial structural integrity is maintained during hibernation [51]. A torpor-like state can be induced in mammals by the administration of H_2S; metabolites of H_2S show differential regulation during periods of torpor and arousal [52]. Inhalational H_2S slows the metabolic rate, similar to hibernation, and protects the kidney following ischemia as reviewed above [44]. The ability of H_2S to lower body temperature by itself has been questioned recently [53]. If one understands the mechanism of kidney protection during hibernation and arousal, it could help develop strategies for preservation of kidney integrity during transplantation.

Restriction of intake of dietary calories also protects against IRI of the kidney and the liver by increasing H_2S generation; this could be partly due to restriction of dietary methionine [54]. Taken together, these studies show that constitutive H_2S generation is important for kidney defense against IRI. In diverse models of IRI of the kidney, H_2S deficiency occurs, and restoring it could be a therapeutic strategy.

Rhabdomyolysis Crush injuries are commonly encountered in the battlefield, and following earthquakes and vehicular accidents. Traumatic injury to the skeletal muscle (rhabdomyolysis) in these instances is commonly associated with acute tubular injury due to myoglobin and other substances released by the injured muscle. In this situation, kidney injury can be additionally brought on by hypotension and IRI. In a rat model of crush injury-induced AKI, administration of NaHS mitigated indices of kidney injury such as increase in kidney injury molecule-1 (KIM-1) expression, oxidative stress, and apoptosis, and reduced the elevation in serum creatinine indicating preservation of kidney clearance function [55].

Nephrotoxic Injury Acute tubular injury can also occur due to administration of nephrotoxic compounds such as antibiotics and chemotherapeutic agents.

Gentamicin Kidney Injury Gentamicin and related aminoglycoside antibiotics are commonly employed to treat serious infections. Aminoglycosides are concentrated by the proximal tubule, and in toxic doses they lead to tubular necrosis. Administration of NaHS, and, H_2S generated from garlic derived diallyl sulfides alleviate gentamicin-induced kidney injury ([56–58]). A recent study has proposed an interaction between gasotransmitters, H_2S, CO, and NO in gentamicin nephrotoxicity. Gentamicin administration resulted in tubular injury, rise in serum creatinine, and proteinuria which were associated increase in inflammatory markers, decrease in anti-inflammatory defense, and increase in inducible NOS and renal tissue NO content, whereas eNOS expression was decreased [59]. NaHS administration inhibited renal tubular injury, improved renal function, decreased inflammation by shoring up anti-

inflammatory defense, and inhibited iNOS induction resulting in decrease in renal tissue NO content while augmenting eNOS expression. These salutary effects of NaHS were reduced by zinc protoporphyrin, an inhibitor of hemoxygenase-1; the latter catalyzes release of CO. These data suggest that gentamicin administration is associated with increase in NO generation via iNOS, and the protective effects of H_2S are partly mediated by CO [59].

***Cis*-Platinum Kidney Injury** Platinum based compounds are extensively used in the treatment of cancers. Among them, *cis*-platinum is well known to cause kidney injury by inducing oxidative stress and inflammation. Nearly 30% of patients treated with *cis*-platinum develop AKI which limits its use [60]. *Cis*-platinum is transported into the kidney proximal tubular epithelial cells by organic cation transporter-2 and copper transport protein-1 located on the basolateral membrane [60]. Initial studies employing rats reported that *cis*-platinum-induced kidney injury was associated with increase in the expression of CSE and H_2S generation leading to inflammation and apoptosis; it was ameliorated by CSE inhibitor propargylglycine [61]. It is important to note that PAG may have other properties such as inhibition of aminotransferases [62]. However, same conclusion was drawn in another study employing GYY4137, a long acting H_2S donor [63]; concerns about the dose and solvent employed in preparation of the agent have been raised [64]. Other studies have shown an opposite role for H_2S in this disease. Administration of NaHS ameliorated *cis*-platinum kidney injury by inhibiting oxidative stress, inflammation, and apoptosis [65]. *Cis*-platinum decreases the expression of renal cortical CSE leading to reduction in H_2S generation in rats [64]. Different H_2S donors were compared for their efficacy to alleviate *cis*-platinum kidney injury. In vitro studies employing porcine renal proximal tubular epithelial cells (LLC-PK1) showed that whereas the short acting NaHS and long acting GYY4137 were able to inhibit kidney injury, AP-39, a mitochondrially targeted donor of H_2S, was not. The mechanism of H_2S protection involved inhibiting oxidative stress due to ROS generated by NADPH oxidase (NOX), and protecting against apoptosis by decreasing MAP kinase activity, particularly Erk and JNK [64]. Inhibition of NOX appeared to involve persulfidation of p47phox, an important component of the oxidase. Administration of NaHS and GYY4137 effectively inhibited clinical indices of *cis*-platinum induced AKI in mice in association with suppression of tubular epithelial cell apoptosis [64]. Polysulfides (H_2Sn) are agents that have higher number of sulfane sulfur atoms than H_2S. They can manifest stronger properties than H_2S. H_2S_3 may be derived from H_2S, or, interestingly, their production may be catalyzed by 3-MST and rhodanase [66]. Similar to H_2S, sodium tetrasulfide inhibited *cis*-platinum-induced apoptosis and the associated activation of MAP kinases and NOX and ROS generation [67]. Sodium tetrasulfide increased the translocation of nuclear factor erythroid-2 related factor-2 (Nrf-2) to the nucleus which was dependent on Akt activation. Nrf-2 is a well-known inducer of anti-oxidant genes [68]. Intraperitoneal administration of sodium tetrasulfide to rats ameliorated *cis*-platinum-induced apoptosis of tubular epithelial cells, and resolved increase in serum creatinine, corresponding to in vitro data in LLC-PK1 cells [67]. Inflammation induction by *cis*-platinum involves NFκB-induced TNFα synthesis which was inhibited by NaHS [69, 70]. Garlic derived diallyl sulfides have been reported to alleviate *cis*-platinum kidney injury by inhibiting oxidative stress, NFκB regulated inflammation, and apoptosis [71, 72].

Recent studies have shown an additional mechanism of kidney injury due to *cis*-platinum. NAD-dependent deacetylase sirtuin-3 (Sirt-3) is a mitochondrial deacetylase which removes acetyl groups from superoxide dismutase-2, ATP synthase β [73], and optic atrophy-1 protein [74], leading to increase in the mitochondrial anti-oxidant capacity and function. Sirt-3 is decreased in the kidney in *cis*-platinum induced kidney damage, and augmenting its expression by

transplantation of mesenchymal stem cells protects kidney function [75]. Since H_2S at small concentrations can lead to mitochondrial DNA repair, suppression of ROS production, and improvement in ATP production [76], whether H_2S can protect against mitochondrial damage by *cis*-platinum has been explored by focusing on Sirt-3. Administration of NaHS to *cis*-platinum treated mice resulted in preservation of kidney function, reduction in tubular cell apoptosis, mitochondrial fragmentation and ROS production, and increasing ATP content [77]. Using both an in vivo model and in vitro cell culture of renal proximal tubular epithelial cells, the authors demonstrated that NaHS reversed *cis*-platinum-induced reduction in Sirt-3 expression. *Cis*-platinum augmented the acetylation of superoxide dismutase-2, optic atrophy-1, ATP synthase β, and increased ROS generation; these were reversed by H_2S by improving the deacetylation function of Sirt-3. Furthermore, NaHS increased the sulfhydration of cysteine residues in the zinc finger motif of Sirt-3, which was found to be required for its protective action against *cis*-platinum-induced toxicity.

In summary, multiple pathways are recruited by *cis*-platinum to cause kidney injury. Most studies favor a protective role for H_2S in *cis*-platinum kidney toxicity. Development of agents that release H_2S which can be acceptable to humans may permit increase in the use of *cis*-platinum in cancer management. Role of H_2S in cis-platinum kidney injury has been reviewed in depth recently ([65, 78]).

3.2 AKI Due to Post-Renal Causes

Obstructive Kidney Injury Obstruction to the flow of urine out of the kidney due to abnormalities in ureter, bladder, prostate, or urethra results in AKI due to back pressure on *both* kidneys. Song et al. showed that kidney obstruction leads to reduction in CBS content and decreased H_2S generation [79]. This was linked to inflammation, generation of TGFβ, and transformation of interstitial fibroblasts to myofibroblasts leading to renal interstitial fibrosis. These abnormalities were prevented by the administration of NaHS. In other investigations, obstruction was reported to reduce CSE expression; CSE knockout mice displayed more severe injury [80]. H_2S deficiency in the obstructed kidney has been reported to promote TGFβ-induced epithelial to mesenchymal transition leading to fibrosis [81]. This transition appears to involve the β-catenin and Erk pathways that are inhibited by H_2S [82]. The role of H_2S and other gasotransmitters in obstructive kidney injury has been expertly reviewed [83].

3.3 Role of H_2S in AKI: A Counterpoint

In contrast to the ameliorative effect of H_2S in the aforementioned models of AKI, an injurious role for the gasotransmitter has been proposed in AKI encountered in burns and failure of multiple organs. Compared to wild type mice, CSE knockout mice were protected from thermal injury-induced kidney abnormalities [84]. Endotoxemia-induced AKI is reported to be decreased in $CBS^{+/-}$ mice indicating mediation by CBS-generated H_2S [85]. A mediator role for H_2S in AKI due to IRI has also been suggested [86]. Clearly, more work is needed to clarify the role of H_2S in AKI.

4 Chronic Kidney Injury (CKD)

Progressive kidney injury results in replacement of functioning renal parenchyma with fibrosis, the structural hallmark of CKD. Diverse mechanisms are involved in this process. CKD can progress to end stage kidney disease (ESKD) requiring replacement of kidney function by dialysis or transplantation. CKD and ESKD result in shortened life span and significant morbidity. The currently available treatment modalities may slow the progression of CKD but are unable to stem the disease process altogether with recovery of normal kidney function; this indicates that we

have not fully understood the underlying mechanisms. Thus, there is a need for new approaches to CKD.

Replacement of functioning parenchyma by fibrosis leads to glomerulosclerosis and tubulointerstitial fibrosis. More recently, the classic glomerulo-centric approach is making room for a tubulo-centric approach. Tubulointerstitial fibrosis is characterized by myofibroblast proliferation, matrix deposition, tubular atrophy, and capillary rarefaction [87]. Myofibroblasts may originate from the capillary pericytes, stem cells [87], or through epithelial to mesenchymal transition, and the topic is hotly debated. Proximal tubular epithelial cells play a central role in tubulointerstitial fibrosis; they undergo direct injury, reprogram their metabolism, and secrete molecules that promote matrix synthesis, e.g., angiotensin II, TGFβ [88, 89]. In CKD chronic changes occur in the vessel wall due either to the disease causing CKD, or to accompanying hypertension and atherosclerosis; this results in ischemic injury to the nephron. As such, CKD can be viewed as a composite of glomerular, tubular, and vascular injury.

There are several reasons for exploring the role of H_2S in CKD: (1) Low levels of H_2S, CBS, and CSE have been reported in circulating leukocytes in hemodialysis patients [90]. Since dialysis patients are at high risk for infections whether correcting H_2S deficiency has an effect on leukocyte defense against infections should be studied. Levels of serum lanthionine and homolanthionine products in the H_2S biosynthetic pathway are increased in dialysis patients [10]. In studies on endothelial cells in vitro lanthionine inhibited H_2S production which coincides with decreased CBS expression; lanthionine also reduced glutathionylation of CBS thereby reducing its activity [91]. In another study, plasma H_2S levels were lower and homocysteine levels higher in non-dialysis CKD patients, and associated with low mRNA levels of CSE and CBS but higher 3-MST expression in circulating mononuclear cells [92]. (2) The most common cause of death in ESKD is atherosclerotic vascular disease. Kuang et al. found a negative correlation between plasma H_2S levels and left ventricular ejection fraction [92]. Recent investigations have shown an important protective role for CSE in maintaining endothelial integrity and delaying atherosclerosis [93] supporting further exploration of the gasotransmitter in CKD-associated cardiovascular disease. (3) Urinary sulfate excretion was inversely associated with graft failure in a large cohort of kidney transplant patients suggesting that reduced ability of H_2S generation may contribute to graft failure [94]. In support of the protective role of H_2S in the transplant setting, CSE mRNA content in the transplanted kidney at the time of implantation directly correlates with kidney functional integrity in the post-transplant period [6].

Animal models have been employed to study the role of H_2S in CKD. Studies in rodents that have undergone removal of 5/6 of renal parenchyma have shown reduced content of CSE, CBS, and 3-MST in association with ROS generated by increased NOX 4 activity indicating that reduced ability to generate H_2S allows oxidative stress [95]. Dietary methionine restriction leads to increased H_2S generation in the kidney [54]. Administration of low methionine diet to mice with 5/6 nephrectomy resulted in protection as manifested by reduced impairment in GFR and albuminuria in association with decreased indices of inflammation and fibrosis [96]; these observations imply that the protection was at least partly due to H_2S generation.

Rodents with hyperhomocysteinemia have been extensively studied as another model of CKD. Homocysteine accumulation occurs when either the expression and/or activity of CBS is impaired, and leads to proteinuria (Fig. 1) [97, 98]. In a feedback manner, hyperhomocysteinemia inhibits the expression of CBS, CSE, and 3-MST [99] leading to H_2S deficiency, the effect on CSE being attributable to hypermethylation of its promoter [100]. The phenotypes of kidney injury in this model include hypertension, vascular injury attributable to matrix metalloprotease (MMP)-9, proteinuria, oxidative and nitrosative stress, inflammation, and mitophagy [101–103]. Administration of

NaHS to CBS heterozygous knockout mice resolves these kidney abnormalities [104, 105] demonstrating that H_2S deficiency underlies renal dysfunction. Vascular pathology including reduced renal cortical blood flow with consequent kidney fibrosis, hypertension, and impaired GFR has been linked to reduced eNOS in $CBS^{+/-}$ mice with hyperhomocysteinemia. eNOS undergoes homocysteinylation impairing its function. These abnormalities were mitigated by treatment with NaHS [106].

Administration of sodium thiosulfate reduced vascular injury in another model of CKD in rats by mobilizing calcium in the deposits in the vessel wall, heart, and kidney, and promoting its urinary excretion suggesting a beneficial role for H_2S; however, the bone strength was impaired indicating that careful studies on unwanted effects of sodium thiosulfate and H_2S are needed [107].

As stated above, angiotensin II and TGFβ contribute to tubulointerstitial fibrosis in CKD; epithelial-mesenchymal transition (EMT) has been suggested as an important underlying mechanism. An in vitro study showed that EMT induced by angiotensin II was mediated by TGFβ in rat renal tubular epithelial cells; H_2S abolished EMT by preventing dimerization of the TGFβ resulting in an inactive monomer [108]. One may recall here the TGFβ antagonizing actions of H_2S in obstructive kidney injury [79]. EMT is an area of intense controversy. Lineage tracing of cells involved in renal tubulointerstitial fibrosis showed pericyte as the origin of myofibroblasts; the authors questioned whether EMT of proximal tubular epithelial cells significantly contributes to the tubulointerstitial pool of myofibroblasts, i.e., proximal tubular epithelial cells do not assume a mesenchymal phenotype, and migrate to the interstitium as myofibroblasts [109]. More recently, Translation Ribosome Affinity Purification approach was employed to identify genes in proximal tubular epithelial cells involved in fibrosis in an obstruction model; the data showed that less than 3% of proximal tubular epithelial cells showed evidence of EMT in situ [89].

4.1 Anemia in CKD

Anemia is one of the serious complications of CKD. Lack of erythropoietin is a major mechanism for anemia although additional mechanisms exist. The renal interstitial stellate fibroblast-like cells synthesize erythropoietin mainly in the cortico-medullary junction [34, 35]. The partial pressure of oxygen in renal cortex is 50 mm Hg and decreases to about 30 mm Hg in the medulla in the rat [110] providing a hypoxic environment for erythropoietin synthesis. In response to anemia and hypoxia the kidney generates more erythropoietin. There is evidence that H_2S regulates erythropoietin synthesis. Cell culture studies have shown that induction of erythropoietin synthesis by hypoxia involves HIF1α and HIF2α; the latter may exert a greater influence [111, 112]. H_2S generated by CBS is likely involved in increase in HIFs because blockers of CBS abolished hypoxia-induced erythropoietin production. Further confirmation of role of H_2S in erythropoietin metabolism has come from studies in patients on dialysis and mice lacking CSE. Hypoxia-induced increase in erythropoietin and hemoglobin was blunted in CSE knockout mice that could be mitigated by exogenous H_2S suggesting a role for H_2S and CSE [113].

Important insights into the complex role of H_2S in erythropoiesis have emerged from recent studies. CBS homozygous knockout mice die within 4 weeks and display anemia due to suppressed erythropoiesis associated with iron overload in several tissues; these abnormalities could be partially ameliorated by restoration of CBS via an adenoviral vector [114, 115]. Anemia in CBS knockout mice is macrocytic, and is associated with increase in blood levels of iron, hepcidin, and IL-6, an inflammatory cytokine. Studies on bone marrow showed decreased population of erythroid precursors, and reduced expression of enzymes involved in heme synthesis such as δ-aminolevulinate synthase-2 and ferrochelatase. Bone marrow content of transferrin receptor 1, involved in iron import into the cell, and the iron release protein ferroportin-1 was decreased indicating defective cellular uptake of

iron and its release. The liver content of erythropoietin, its receptor, and its upstream regulator HIF2α was decreased in CBS knockout mice; however, whether erythropoietin synthesis of in the kidney was affected is not clear.

In contrast to the role of CBS in erythropoiesis described above, CSE has been suggested to inhibit erythropoiesis. CSE knockout mice manifest polycythemia or increased numbers of red blood cells in circulation in association with elevated heme content in plasma and liver [116]. Interestingly, there was no evidence of hypoxia on arterial blood gas analysis. The investigators attributed these changes to increase in the hepatic expression of coproporphyrinogen oxidase, a mitochondrial enzyme that converts coproporphyrinogen III to protoporphyrinogen IX in the heme synthesis pathway. Using a reporter assay, they found that liver cell coproporphyrinogen oxidase gene promoter activity was increased when CSE activity was inhibited indicating that it was under H_2S regulation. Mitochondrial respiration was found to be increased in isolated mitochondria from the liver of CSE knockout mice.

Taken together these data suggest that H_2S could play an important regulatory role in erythropoiesis. Clearly, further investigation is needed to parse out the exact role of H_2S, CSE, and CBS in erythropoiesis. Additionally, it is important to explore if H_2S participates in oxygen sensing in the kidney.

CKD affects nearly every organ system with significant morbidity. CKD-associated neuropathy and bone disease are major and common complications that can cause significant morbidity. Cardiovascular complications including ischemic heart disease and congestive heart failure are the leading cause of death in CKD. Investigation is needed to elucidate the role of H_2S in these complications.

4.2 Kidney Injury in Chronic Congestive Heart Failure (CHF)

Decreased cardiac output, the hallmark of CHF, leads to reduced systemic perfusion and hypoxia including in the kidney. This results in maladaptive responses including stimulation of renin–angiotensin–aldosterone axis and the sympathetic nervous system. Inhibition of these responses forms an important strategy in the treatment of CHF. Acute or chronic CHF can result in kidney injury (types 1, 2 cardiorenal syndrome, respectively) [117]. Administration of a H_2S donor, JK-1, improved cardiac function in a mouse model of pressure overload CHF created by transverse aortic constriction, an example of type 2 cardiorenal syndrome [118]. The study was conducted over 18 weeks with the administration of JK-1 being delayed for 3 or 10 weeks after aortic constriction to mimic the clinical situation where patients seek medical attention after the onset of symptoms. H_2S administration delayed for 3 weeks still resulted in attenuation of renin-angiotensin axis, catecholamines, cardiac fibrosis resulting in improved cardiac structure and function. In addition, kidney fibrosis and impairment in its function were also improved by the H_2S donor [118]. This exciting report should stimulate more work on H_2S aimed at understanding and mitigating cardiac and kidney injury in other models of type 2 cardiorenal syndrome and in acute models (type 1 cardiorenal syndrome). Additionally, AKI and CKD can also secondarily affect heart function (types 3, 4 cardiorenal syndrome); these models should also be explored for the contribution of H_2S.

4.3 Obesity and Kidney Injury

Obesity is an ever-growing problem around the world. At the present time nearly 40% of the population in the USA is obese, and projections are that this will increase to nearly 50% by 2030 [119]. Kidney injury in obesity, without contribution from hyperglycemia, manifests as enlargement of the glomerulus, glomerulosclerosis, tubulointerstitial fibrosis, and a variable degree of proteinuria [120]. Obesity increases the risk of type 2 diabetes and obese subjects are at a higher risk of CKD and proteinuria [121]. Reduced renal expression of CBS but not CSE was observed in the kidneys of mice with

high fat diet-induced obesity [122]. High fat diet-induced obesity in mice was associated with matrix increase in the kidney, and indices of inflammatory molecules; these were reversed by intraperitoneal administration of NaHS [123]. Investigation of H_2S in obesity-induced kidney injury is still in its infancy. More detailed investigations are needed.

4.4 Diabetic Kidney Disease (DKD)

DKD is the single most common cause of CKD progressing to ESKD in the West. Both type 1 diabetes due to insulin lack and type 2 diabetes due to resistance to insulin actions cause clinically significant kidney injury in nearly a third of patients. The clinical course is characterized by an initial rise in GFR followed by progressively increasing proteinuria including albuminuria, hypertension, and eventually progressive loss of GFR. During this process, hypertrophy of the kidney is seen early followed by increase in the accumulation of matrix proteins leading to glomerulosclerosis and of the tubulointerstitial fibrosis. The role of H_2S in DKD has been reviewed extensively [124–127].

H_2S has a complex role in diabetes. Streptozotocin is commonly used to induce type 1 diabetes in rodents; H_2S is implicated in streptozotocin-induced injury to insulin synthesizing β cells in the pancreatic islets of Langerhans [128]. On the other hand, there is good clinical and experimental evidence that DKD is associated with reduced synthesis of H_2S. Reduced circulatory H_2S levels have been reported in type 2 diabetes [129], and in patients with ESKD due to DKD [130]. In rodents with type 1 and 2 diabetes, kidney H_2S generation is reduced due to decreased expression of CBS and CSE, which could be due to MMP-9 [7, 131–134]. That H_2S deficiency contributes to the well-known phenotypes of DKD such as oxidative stress, fibrosis, and albuminuria is supported by their amelioration by administering H_2S releasing agents such as NaHS, GYY4137, and S-propargyl-cysteine [133–136]. H_2S protects against DKD in two ways, i.e., it can reduce blood glucose levels, and it affects pathogenetic mechanisms.

One mechanism by which H_2S reduces hyperglycemia is by stimulating insulin receptor mediated glucose uptake in peripheral tissues as has been shown in rats with type 2 diabetes [137].

TGFβ mediates fibrogenic response of the kidney in diabetes [138, 139]. Increase in renal TGFβ expression in DKD is inhibited by NaHS [133, 134]. As mentioned above, tubulointerstitial fibrosis is a major aspect of progressive CKD. In addition to the direct effects of high glucose on tubular epithelial cells and renal fibroblasts in promoting tubule-interstitial fibrosis, hyperglycemia can induce chronic ischemia due to peritubular capillary injury and rarefaction. This can be mitigated by H_2S administration [140, 141]. Pathologic remodeling of renal blood vessels has been linked to *N*-methyl D-aspartate (NMDA) receptor-1 being activated by MMP-9 [132]. In the setting of DKD, opening of the NMDA receptor-1 has been linked to calcium influx into the mitochondria leading to opening of the mitochondrial permeability transition pore and ROS generation; these events are inhibited by GYY4137, an H_2S donor [142].

Some understanding of the cellular actions of H_2S in the context of DKD has been gained. Increased protein synthesis is required for kidney hypertrophy, and matrix accumulation contributing to renal fibrosis. Signaling pathways, particularly, the (PI 3kinase)-Akt-mechanistic target of rapamycin complex 1 (mTORC1) pathway, are important regulators of mRNA translation, a rate limiting step in protein synthesis [143]. Increase in the activity of this signaling axis has been documented in early stages of DKD in rodents [144–149]. Inhibition of mTOR by rapamycin administration ameliorates kidney hypertrophy, and onset of laminin accumulation in mice with type 2 diabetes [149, 150]. In addition to Akt, mTOR activity and mRNA translation are under the control of AMP-activated protein kinase (AMPK) through the TSC1-TSC2-Rheb axis. Normally, AMPK keeps mTORC1 and protein synthesis in check in kidney cells. AMPK is regulated by the ratio of ATP to AMP in cells. Hyperglycemia increases

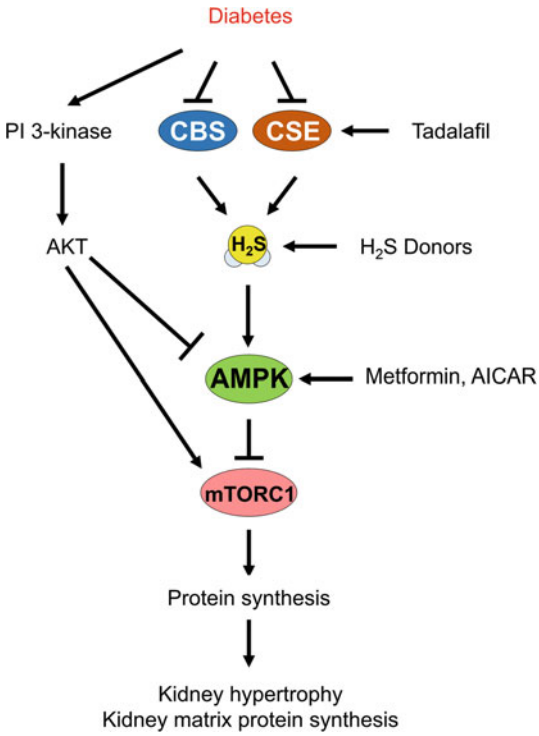

Fig. 3 Kidney H_2S regulation in Diabetes. Reduced expression of CBS and CSE leads to decreased H_2S generation. AMPK activity is reduced, whereas PI 3-kinase-Akt-mTORC1 axis is stimulated leading to increase in protein synthesis required for kidney hypertrophy and matrix protein synthesis. Administration of tadalafil, H_2S donors, and AMPK stimulators restore AMPK activity, resulting in inhibition of mTORC1, and reduction in kidney hypertrophy and matrix protein synthesis

ATP/AMP ratio and inhibits AMPK [151]. Reduction in AMPK activity removes the break on mTORC1 allowing protein synthesis to continue (Fig. 3). Akt can also inhibit AMPK by serine phosphorylation [152, 153] providing an additional mechanism for AMPK inhibition in the kidney in diabetes. Administration of AMPK stimulating agents such as metformin and AICAR at doses that do not alter hyperglycemia inhibits kidney hypertrophy [146, 154]. Interestingly, metformin increases kidney H_2S content in a dose-dependent manner in mice [155]. Whether metformin regulates expression and/or activity of CSE, CBS, or 3-MST in the kidney should be explored. It will be interesting to study if metformin stimulation of AMPK is H_2S-dependent. The salutary role of AMPK in ameliorating diabetes- and obesity-induced kidney injury has been confirmed by others [154, 156–158]. Given the desirable property of AMPK in amelioration of DKD, we examined if H_2S has a regulatory effect on AMPK. High glucose-induced protein synthesis and hypertrophy in kidney epithelial cells were blocked by NaHS [7]. This was dependent on induction of AMPK activation by its upstream regulator, calcium calmodulin kinase kinase-β and possibly, liver kinase B-1 (LKB-1). Activated AMPK inhibited mTORC1, and reactions involved in both the initiation and elongation phases of mRNA translation [7]. These data provide a signaling basis for the beneficial effect of H_2S in DKD.

Since NaHS is intolerable by humans due to its smell, we searched for an alternative. Because NO and H_2S share many properties such as vasodilation and increased blood flow, we examined if tadalafil, a phosphodiesterase 5 inhibitor, and inducer of NO could be an alternative. In vitro studies in renal glomerular epithelial cells that express podocyte markers showed that tadalafil also inhibited high glucose-induced hypertrophy, matrix protein synthesis by down regulating mTORC1 activity, and key steps in the initiation and elongation phases of mRNA translation, similar to NaHS [159]. The inhibitory effects of tadalafil on high glucose-induced mTORC1 and protein synthesis required AMPK stimulation. Tadalafil also increased H_2S generation by increased expression of CSE by regulating its mRNA translation rather than transcription. The salutary effects of tadalafil on high glucose-induced injury required activation of guanylyl cyclase and NO generation via inducible NOS (iNOS). Since iNOS is generally associated with tissue injury, its role should be clarified in animal models of DKD. In summary, the sequential steps are as follows: tadalafil promotes NO generation, stimulates soluble guanylyl cyclase leading to induction of CSE expression and H_2S generation; in turn, H_2S stimulates AMPK to inhibit mTORC1 and synthesis of proteins including matrix proteins. Thus, under tadalafil, signaling

pathways of two gasotransmitters, NO and H_2S, are integrated. Our findings are in line with a randomized double blinded multi-center clinical trial. PF-00489791, a phosphodiesterase 5 inhibitor similar to tadalafil, reduced proteinuria in patients with DKD [160].

Multiple other pathogenic pathways are interrupted in DKD by H_2S administration, and are briefly reviewed below.

The renin–angiotensin axis is of central importance in DKD. Angiotensin II binds to angiotensin receptor type 1 (AT1) to initiate hemodynamic and cell biologic effects that contribute to glomerular hypertension, renal fibrosis, and progressive kidney failure [127]. Inhibition of this axis is widely employed in the treatment for DKD and non-diabetic CKD. Diabetes in rats was associated with increase in renal parenchymal angiotensin II and AT1 expression in the kidney; these changes were inhibited by NaHS [134]. H_2S also reduces renin generation [22]. Intrinsic cells of the nephron are capable of expressing components of this system and initiate local injury in diabetes. High glucose promotes mesangial cell synthesis of angiotensinogen, angiotensin converting enzyme, and AT1 in vitro along with that of TGFβ and collagen; these effects are dependent on ROS generated by NOX and they are inhibited by H_2S [136].

Renal vascular bed is another locus of H_2S action in DKD. Employing Akita mice with type 1 diabetes, MMP-9 knockout mice, and [MMP-9 +/− Akita] mice, Kundu et al. have linked ROS generation to induction of MMP-9 leading to H_2S reduction in DKD. This leads to induction of NMDA receptor and increase in Connexins 40 and 43 resulting in renovascular remodeling [132]. Diabetes-induced changes in renal vasculature result in ischemia, which can contribute to DKD. Careful measurements of renal blood flow confirmed that correcting H_2S deficiency reduced renal blood vessel resistance and improved blood flow; this was associated with amelioration of renal dysfunction including fibrosis [161]. Improvement in renal capillary blood flow by bolus administration of NaHS has been shown in OVE26 mice with type 1 diabetes [141]. Sen's group has employed barium sulfate angiography to demonstrate reduced vessel area in the kidney in Akita mice which was restored to normal following administration of GYY4137, an H_2S donor [131].

High glucose increases the generation of ROS and induces oxidative stress in DKD. This was inhibited by NaHS in type 1 diabetic rats by recruiting Nrf-2, a transcription factor that augments expression of anti-oxidant molecules [134]. Poly (ADP ribose) polymerase-1 (PARP-1), a regulator of DNA strand repair, is increased in DKD; PARP-1 knockout mice are protected from diabetic kidney injury [162]. GYY4137 administration ameliorated the increase in ROS generation in the kidney and reduced kidney PARP-1 expression in Akita mice [131]. NADPH oxidase 4 is a major source of ROS production in the kidney and contributes to renal hypertrophy, accumulation of matrix proteins, and proteinuria [163–166]. We studied the interaction between NOX4 and H_2S in an in vitro model of high glucose-induced kidney injury employing proximal tubular epithelial cells [167]. High glucose increased the expression and activity of NOX 4 that was abolished by NaHS in an AMPK-dependent manner. Interestingly, the salutary effects of NaHS were NO-dependent demonstrating coordination between two gasotransmitters. As reviewed above, the signaling systems of NO and H_2S are also integrated in oxygen sensing operations of the carotid body, and in tadalafil effects on high glucose-induced kidney cell injury [159] (Figs. 2 and 3). Finally, it should be pointed out that the source of ROS in the kidney in DKD is a hotly debated topic. Dugan and associates have shown that mitochondria are not the source of ROS in DKD in diabetic rodents [154]. It is possible that ROS may originate from extra-mitochondrial sources in kidney cells.

Inflammatory injury is another pathway of kidney injury in DKD. Infiltration of monocytes into renal parenchyma is well documented in DKD [168]. Inflammatory injury mediated by activation of NFκB in diabetic rodents was

suppressed by NaHS in rats with type 1 diabetes [134].

John and associates have provided further insights into regulation of matrix metabolism in DKD by H_2S [131]. As mentioned above, MMP-9 has been implicated in the reduced expression of CSE in DKD. In Akita mice with type 1 diabetes, increase in the kidney mRNA, and protein expression of MMPs-9 and 13, and types I and IV collagen was associated with reduced expression of MMP-14; these changes were reversed by GYY4137, which also reduced blood glucose level modestly [131]. Synthesis of collagen in DKD is under the regulation of HIF1 [169]. DKD in Akita mice was characterized by mRNA and protein elevation of HIF1α, and regulatory molecules involved in the synthesis and folding of collagens; these changes were also restored to normal by the administration of GYY4137 [131]. Interestingly, the kidney expression of microRNA (miR)-194, a major miR expressed in the kidney, was decreased in Akita mice and was restored by the administration of GYY4137 [131]. Using glomerular endothelial cells in culture, the authors went on to show that high glucose-induced changes in ROS generation, changes in MMPs, collagens, and regulators such as HIF1α could all be reversed by an miR-194 mimic, suggesting a fundamental role for this miR in DKD. Taken together, these data imply that H_2S exerts inhibitory control on transcription of several important genes that have roles in ROS generation, and matrix changes; GYY4137-induced reduction in blood glucose could assist in these changes. Additionally, H_2S regulates the expression of miR-194, a major miR in the kidney. Whether there are binding sites for miR-194 in the 3' UTRs of the genes mentioned above or whether mIR-194 exerts its effects indirectly need clarification. Also, the mechanism by which H_2S affects the production of miR-194 also needs more study.

Clinical reports are appearing that lend support for a role of H_2S deficiency in DKD. We have already referred to the beneficial effect of phosphodiesterase-5 inhibitor in a clinical trial [160]. In another study, urine samples from patients enrolled in two large DKD trials (Sun-MACRO and PREVEND) were analyzed for their sulfate content, which may reflect systemic H_2S metabolism. Urinary sulfate levels inversely correlated with albuminuria and progression of DKD, implying that lower conversion of H_2S into sulfate was associated with faster progression of the disease [170]. On multivariate analysis, urinary sulfate was found to be an independent protection factor supporting the notion that higher H_2S production capacity protects against DKD. As pointed by the authors, a cautionary note is required; some of the urinary sulfate can originate in the bacteria in the gastrointestinal tract [171]. The same group of investigators have reported that urinary sulfate and not thiosulfate levels inversely correlated with all-cause mortality in the general population [172].

In summary, further exploration of H_2S role in DKD is likely to yield more insights into mechanisms of injury, and provide an opportunity to test clinical intervention. Issues in DKD that could be addressed in future could include the following. H_2S is a well-known cause vasodilation and an increase in blood flow [173]. As discussed above infusion of H_2S into renal artery increases blood flow into glomerulus and GFR [21]. Since GFR is increased in early stages of diabetes, it will be interesting to study whether H_2S mediates increased glomerular perfusion. Studies in vitro have shown that tadalafil amelioration of high glucose-induced kidney cell injury requires integration between NO and H_2S [159]. Additionally, H_2S seems to interact with CO, the third gasotransmitter. H_2S augmented hemoxygenase-1 expression in human mesangial cells and podocytes in culture implying generation of CO [174]. Thus, another area for future exploration could be the interaction between H_2S, CO, and NO in DKD.

5 Hypertension

Blood pressure is a product of cardiac output and peripheral resistance of the arterial tree. Hypertension is one of the most common disorders affecting nearly a billion people around the

world [175, 176]. When the cause of hypertension is not known, as it is the case in most cases, it is called primary hypertension. The remaining subjects have secondary hypertension attributable to a disease, e.g., renovascular hypertension, disorders of hormones such as aldosterone and catecholamines, renal disease, and vascular anomalies including coarctation of the aorta. Elevated blood pressure has far reaching effects on both morbidity and mortality including atherosclerosis, coronary artery disease, heart failure, CKD, and stroke [177]. There is overwhelming evidence that control of BP mitigates many of these consequences [178].

Currently available medications to treat hypertension are fraught with the problems of intolerable side effects leading to non-compliance, and/or, inefficient blood pressure control. This has prompted a search for more novel approaches. All three gasotransmitters have extensive vascular effects including lowering of blood pressure. Thus, they are prime candidates for exploration in the development of new anti-hypertensive agents. They have additional local cell biologic effects on the target tissues such as the heart, brain, blood vessels, and the kidney that makes them even more attractive as potential therapeutic candidates. The author wishes to point out that the literature of H_2S in hypertension is vast, and only a very brief account will be given here due to space considerations. The topic of H_2S in hypertension has been deftly reviewed recently [179–181].

The involvement of H_2S in hypertension was brought to focus by studies in CSE knockout mice that had a mixed genetic background (C57BL6 + 129Sve) [173]. The investigators concluded that H_2S deficiency was the cause of hypertension by excluding other mechanisms of hypertension, and ameliorating hypertension by the administration of NaHS [173]. It should be noted that other investigators have not observed hypertension in CSE knockout mice. In one study, investigators found that while there was no systolic hypertension at baseline by telemetrically monitored blood pressure measurement the mean and diastolic blood pressures were *lower* in the $CSE^{-/-}$ mice; these could be attributed to NO as infusion of L-NAME restored the blood pressures to control levels [182]. In this study, blood pressure was measured in conscious mice and the genetic background of mice (C57BL6) was different from the previously cited study [173]. Lack of hypertension was also reported in another study; the difference could be due to genetic background (C57BL6) and the method of blood pressure measurement [183].

There is ample evidence for a role of H_2S in many commonly employed animal models of hypertension. Circulating H_2S levels are low in spontaneously hypertensive rats, and are associated with oxidative stress; these abnormalities are rectified by NaHS [184]. In salt sensitive Dahl rats, decreased H_2S production may be due to reduced CBS expression both of which could be rescued by NaHS administration resulting in restoration of blood pressure [185]. Reduced vasculature content of CBS and CSE has been reported in dexamethasone model of hypertension [186].

Renovascular hypertension is a common cause of secondary hypertension. The experimental model for studying this form of hypertension involves clipping one of the two normal kidney arteries. Interruption in blood supply augments renin generation by the perivascular interstitial cells around the afferent arteriole leading to increase in the synthesis of angiotensin II, which increases blood pressure by vasoconstriction and increasing renal Na^+ reabsorption in the kidney. H_2S ameliorates hypertension in this model by diverse mechanisms. It decreases renin expression by inhibiting cAMP [22]. H_2S interrupts AT1 activation by angiotensin II and oxidative stress [187]. Similar amelioration of hypertension, oxidative stress, and endothelial dysfunction by H_2S also occurs in a model of hypertension caused by direct infusion of angiotensin II [188]. Similar salutary effects of H_2S were seen in the angiotensin II model following infusion of NaHS and sodium thiosulfate, a metabolite of H_2S although only NaHS was able to reduce intrarenal hypertension [189]. H_2S induced effects on hypertension have been attributed to the activation of K^+-ATP channels [189–191].

Excessive Na^+ retention by the kidney is an important pathogenic event in hypertension. As discussed in the Renal Physiology section, H_2S inhibits renal Na^+ reabsorption at various sites along the nephron. H_2S reduces the activity of Na^+-H^+ exchanger 3 which is another major regulator of Na^+ reabsorption in the proximal tubule [27]. H_2S may inhibit Na^+-K^+-$2Cl^-$ co-transporter, and, reduce the activity of Na^+-K^+ ATPase by promoting endocytosis in renal epithelial cells [21, 30]. Aldosterone-induced ENaC opening in the distal nephron is also inhibited by H_2S by stimulating PTEN and suppressing PI 3-kinase activity [29]. It will be important to explore if H_2S has a role in regulation of other contributing mechanisms in hypertension involving neurologic and endocrine systems.

An emerging theme in studies on hypertension is the dialogue between NO and H_2S. NO deficiency in the angiotensin II model of hypertension was corrected by H_2S [188]. Hypotension due to H_2S administration in normotensive rats is corrected by decreasing NO generation and vice versa [192]. This crosstalk may be model specific, e.g., these gasotransmitters may not communicate in the spontaneously hypertensive rats [192]. In contrast to the aforementioned studies, H_2S has been reported to scavenge and decrease NO in the peripheral arteries [182].

5.1 Preeclampsia

Around 2–5% of pregnant women develop hypertension and proteinuria after the 20th week of gestation in the absence of CKD and previous hypertension; they are said to have preeclampsia [193, 194]. Fetal growth can be affected due to decrease in placental perfusion from remodeling of the spiral arteries. Preeclampsia is characterized by excessive production of soluble fms-like tyrosine kinase (sFlt1), a splice variant of vascular endothelial growth factor receptor-1 (VEGFR1), and decrease in placental growth factor (PlGF). While PlGF is a vasodilator, sFlt1 is a vasoconstrictor. In preeclampsia the ratio of sFlt1 to PlGF is increased [195]. Placentae obtained from women with preeclampsia demonstrate increased expression of mitochondrial ROS, reactive nitrogen species, and sFlt1 [196]. sFlt1 overproduction results in decrease in VEGF availability, and contributes to endothelial dysfunction and impaired vascular relaxation [194]. Circulating H_2S levels and single nucleotide polymorphisms in CBS and CSE have been reported in preeclampsia patients [197, 198]. In the rat model of preeclampsia generated by an adenovirus-induced sFlt1 overproduction, administration of NaHS effectively reduced blood pressure and ameliorated proteinuria [199]. In mice CSE inhibition has been shown to increase sFlt1 production which is inhibited by NaHS [200]. This indicates that H_2S deficiency is upstream of placental sFlt1 overproduction in preeclampsia.

miRs appear to have an important regulatory role in preeclampsia. miR-20a, −20b, and -200c may be involved in reducing placental VEGF synthesis, and this can be corrected by NaHS [201]. The mechanism by which NaHS inhibits sFlt1 generation may involve miR-133b [202]. In view of mitochondrial dysfunction in preeclampsia, AP39 a mitochondrial H_2S donor has been tested in cell culture employing human primary cytotrophoblasts isolated from placenta [196]. In response to hypoxia, mimicking in vivo placental status, cytotrophoblasts showed increased expression of sFlt1 and HIF1α, and mitochondrial ROS generation; all these changes were prevented by AP39 which also increased H_2S content in the cells [196]. Thus, H_2S deficiency appears to be a proximal event in the pathogenesis of preeclampsia. It will be important to investigate if placental deficiency of H_2S contributes to remodeling of spiral arteries leading to placental ischemia. The life time risk for hypertension and CKD appears to be higher in women with preeclampsia; intrauterine growth retardation in fetus may also result in eventual susceptibility to cardiovascular disease [203]. Thus, urgent work is needed to test the therapeutic potential of H_2S donors in preeclampsia to prevent both short-term and long-term pathology in both the mother and the fetus.

6 Renal Cell Carcinoma

Cancers of the genitourinary tract such as prostate carcinoma, urothelial malignancies affecting the bladder, pelvicalyceal compartment, ureter, and renal cell carcinoma (RCC) pose diagnostic and therapeutic challenges. They can remain undetected until the malignancy has spread beyond their anatomical confines. RCC remains asymptomatic until late in the disease, and in nearly a third of the patients it is metastatic at diagnosis [204]. Clear cell RCC (ccRCC) is the most common variety, and is commonly characterized by inactivation of Von Hippel Lindau protein resulting in persistence of HIF1α and HIF2α.

The role of H_2S in malignancy is controversial, and is probably, tumor-specific [205]. Invasiveness of ccRCC has been correlated with increased expression of CBS [206]. In contrast, H_2S has been suggested to induce apoptosis in RCC cells. In RCC the expression of CSE is reduced and linked to resistance to apoptosis suggesting provision of H_2S from internal or external sources may promote tumor cell death [207]. It is possible that H_2S plays a nuanced role in ccRCC. Whether HIFs regulate CBS expression in ccRCC should be of interest. The topic of H_2S role in cancer including genitourinary malignancies has been reviewed in depth recently [205].

7 Aging Kidney

The goal of gerontology is not only to understand mechanisms underlying aging-related changes and prolong life span but also to improve the quality of life, i.e., health span. Achieving health span requires structural and functional integrity of organ systems including the kidney [208]. Aging is associated with nephrosclerosis, a composite term comprising glomerulosclerosis, tubular atrophy, interstitial fibrosis, and arteriolosclerosis affecting the blood vessels. Functioning nephrons are injured during aging, and replaced by scar tissue. Vascular changes result in ischemia, and contribute to loss of functioning parenchyma. GFR loss occurs commonly in healthy aging adults although it is not universal [209, 210]. Older adults are at higher risk for AKI and CKD. In mice nephrosclerosis is associated with albuminuria and reduced GFR seen as elevation of serum cystatin C [211]. In the marmoset, a non-human primate, nephrosclerosis is associated with proteinuria but stable serum creatinine [212].

Several studies have established links between aging and H_2S metabolism. Dietary restriction (DR) is a well-established method of prolonging life span and improves functions of organ systems including that of the kidney. DR in mice resulted in preservation of GFR, and reduction in albuminuria, renal fibrosis, mitochondrial DNA damage, and oxidative stress [213]. Authors showed that aging in ad libitum fed mice inhibited kidney parenchymal Sirt-1 and stimulated PI 3-kinase-Akt pathway leading to acetylation and phosphorylation of FOXO3, inhibiting its nuclear translocation; this resulted in reduced BCL2/adenovirus E1B 19kDA-interacting protein (Bnip3), decreased autophagy, and promoted mitochondrial damage and oxidative stress. These abnormalities were prevented by DR which restored the activity of Sirt-1, inhibited PI 3-kinase-Akt axis, deacetylation, and dephosphorylation of FOXO3 promoting its nuclear translocation; this led to increased Bnip3, resulting in increased autophagy, and maintenance of mitochondrial integrity [213]. Studies employing parabiosis involving shared blood circulation between young and aged mice showed that kidney injury was ameliorated by augmenting autophagy, and inhibiting apoptosis and inflammation [214]. In these studies, the role of H_2S in autophagy impairment in kidney aging was not explored.

Hine et al have elegantly shown that the salutary effects of DR in improving resistance to ischemic injury to the kidney, and in extending life span in models across the evolutionary spectrum are dependent on H_2S generated via the trans-sulfuration pathway [54]. The aspect of DR that results in increased H_2S generation relates to dietary methionine, an essential amino acid. Dietary methionine administration reduces

H_2S generation, whereas its restriction promotes it via the trans-sulfuration pathway [54]. A recent study in mice showed that the longevity extension by DR is lost when it is supplemented with essential amino acids which includes methionine [215]. Aging associated nephrosclerosis, monocyte infiltration, impaired waste clearance function, and oxidative stress were resolved by DR which was also shown to be dependent on essential amino acid restriction. DR promoted CSE expression in the kidney and generation of H_2S [215]. The renal parenchymal methionine content increased in ad libitum fed mice, and in mice on DR supplemented with essential amino acids whereas it was decreased in mice on DR alone; this established a connection between dietary methionine content, methionine metabolism in the kidney, and H_2S generation, i.e., H_2S generation is decreased with increase in renal parenchymal methionine. Mortality and kidney injury were more severe in mice given DR supplemented with essential amino acids; since there was no increase in cancer more severe kidney injury could have contributed to higher mortality. Further direct demonstration of the role of dietary methionine consisted of removing methionine from the dietary supplement of essential amino acids being added to DR; this thoughtful strategy showed that life span extension and ameliorative effect of DR on kidney injury which was lost when essential amino acids were added back could be regained by only excluding methionine [215]. Dietary methionine restriction has been shown to be effective in mitigating aging-related structural and functional changes in the Fischer 344 rats [216]. The difficulty in applying DR to humans to improve health span and life span is the difficulty in compliance over time. Additionally, whether restriction of methionine, an essential amino acid, results in pathologic effects is of concern.

Vascular aging is associated with reduced activity of NAD-Sirt1 axis; H_2S administration in the form of NaHS leads to activation of this axis and restoration of vascular health [217]. One may recollect here that Sirt-1 is also reduced in aged kidney [213].

Other mechanisms underlying kidney impairment in aging involve oxidative stress, hemodynamic changes, renin-angiotensin system activation, and growth factors [218]; however, we do not fully understand the process. In DKD and other causes of CKD activation of Akt-mTORC1 signaling pathway and renal fibrosis are prominent features in which H_2S plays an important role. The main aspect of kidney changes in aging is also fibrosis, but the role of H_2S in the process has not been thoroughly investigated. Hou et al. reported decreased H_2S generation due to reduced expression of CSE and CBS in the aged kidney [219]. This was accompanied by reduced nuclear localization of Nrf-2 leading to decreased expression of anti-oxidant factors such as superoxide dismutase-2 and hemoxygenase-1. NaHS administration decreased fibrosis and albuminuria, promoted nuclear translocation of Nrf-2 resulting in restoration of superoxide dismutase-2 and hemoxygenase-1. Reduced expression of CSE leading to decrease in H_2S and NO content in the kidney was also found in the galactose model of accelerated aging which was mitigated by NaHS [220].

We have investigated the role of H_2S in kidney aging from the perspective of signaling reactions that control protein synthesis. Increased matrix protein synthesis contributes to renal fibrosis. In aged mice the renal cortical content of CBS and CSE was decreased along with reduced generation of H_2S (Fig. 4) [221]. This occurred in association with increase in the expression of matrix proteins such as laminin and collagen type 1α. We found stimulation of insulin receptor-Akt-mTORC1 signaling pathway which controls protein synthesis (Fig. 4). It is important to mention here that reduced autophagy in the aged kidney is associated with stimulation of Akt-mTORC1 axis [213]. These changes resemble signaling dysregulation in the kidney injury seen in DKD [143, 144, 149, 222]. We have previously reported that AMPK inhibits mTORC1 in the kidney in normal mice, and that its activity is inhibited in DKD, which facilitates mTORC1 activation [146]. AMPK activity was decreased in the aging kidney (Fig. 4) [221]. As is widely

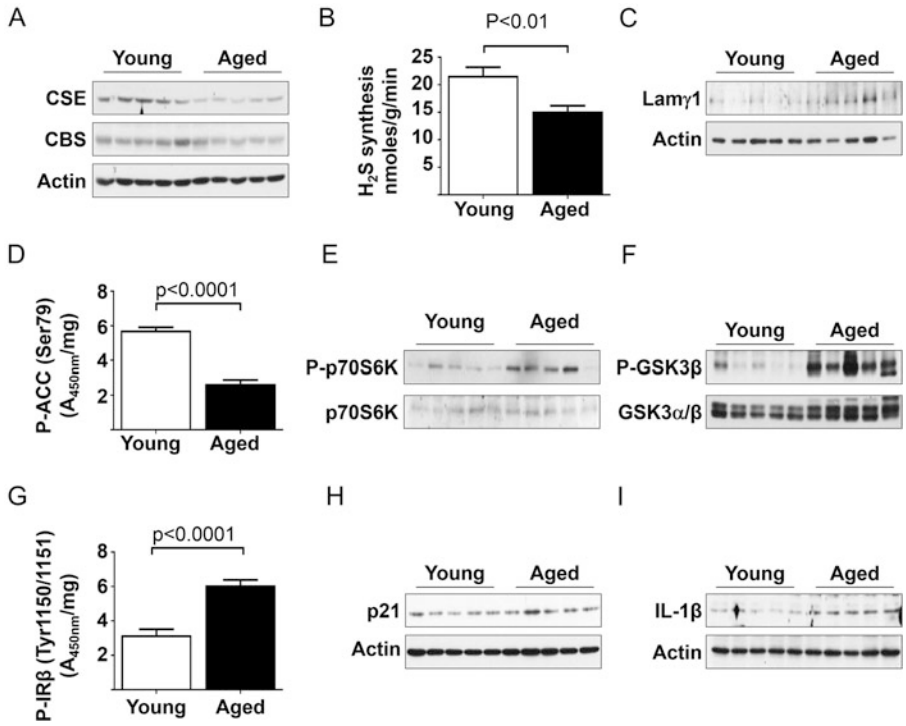

Fig. 4 H_2S deficiency in kidney in aging mice. Aging leads to (**a**) reduced expression of CSE and CBS, and (**b**) decreased H_2S generation. This is associated with (**c**) increased expression of matrix protein, laminin, (**d**) reduced AMPK activity as shown by reduced acetyl-CoA carboxylase (ACC) phosphorylation on ELISA, (**e**) stimulation of mTORC1 as demonstrated by increased phosphorylation of p70 S6 kinase (p70S6K), (**f**) increased Akt activity as indicated by increased glycogen synthase kinase (GSK) 3β phosphorylation, and (**g**) activation of insulin receptor (IRβ) as shown by greater tyrosine phosphorylation on ELISA. (**h**, **i**) increased expression of p21 and IL-1β was seen, consistent with senescence associated secretory phenotype (SASP). Data are from Lee HJ, et al., GeroScience 40: 163, 2018. Used with permission of the American Aging Association

recognized now, during aging a subset of cells undergo growth arrest called cell senescence; these senescent cells secrete many proteins including pro-inflammatory cytokines, which is termed senescence associated secretory phenotype (SASP) [223]. The aged kidney demonstrated SASP seen as increased expression of p21, and interleukins 1 and 6 (Fig. 4) [221].

We tested whether correction of H_2S deficiency can rectify kidney injury in aging. 18- to 19-month old mice were randomized to receive orally administered NaHS in water vs. water alone for 5 months. NaHS administration resulted in reduction in glomerulosclerosis; aspects of SASP were inhibited (Fig. 5). Signaling pathway analysis showed that NaHS activated AMPK and inhibited the insulin receptor-Akt-mTORC1 axis providing a mechanistic basis for reduced synthesis of fibrogenic matrix proteins (Fig. 5) [221]. It is likely that inhibition of mTORC1 leads to increase in autophagy which has been shown to ameliorate aging-related kidney injury [213]. Functional assays of the kidney showed that NaHS administration improved GFR, measured by the serum level of cystatin C, and reduced urinary albumin losses (Fig. 5). These data point to the therapeutic potential of H_2S in slowing the aging associated kidney impairment and increasing health span. Additionally, SASP can be a target of intervention to improve kidney function in aging. As it can be related to mTORC1 activation, inhibitors of that kinase such as rapamycin may be effective [224]. Removal of senescent cells improves

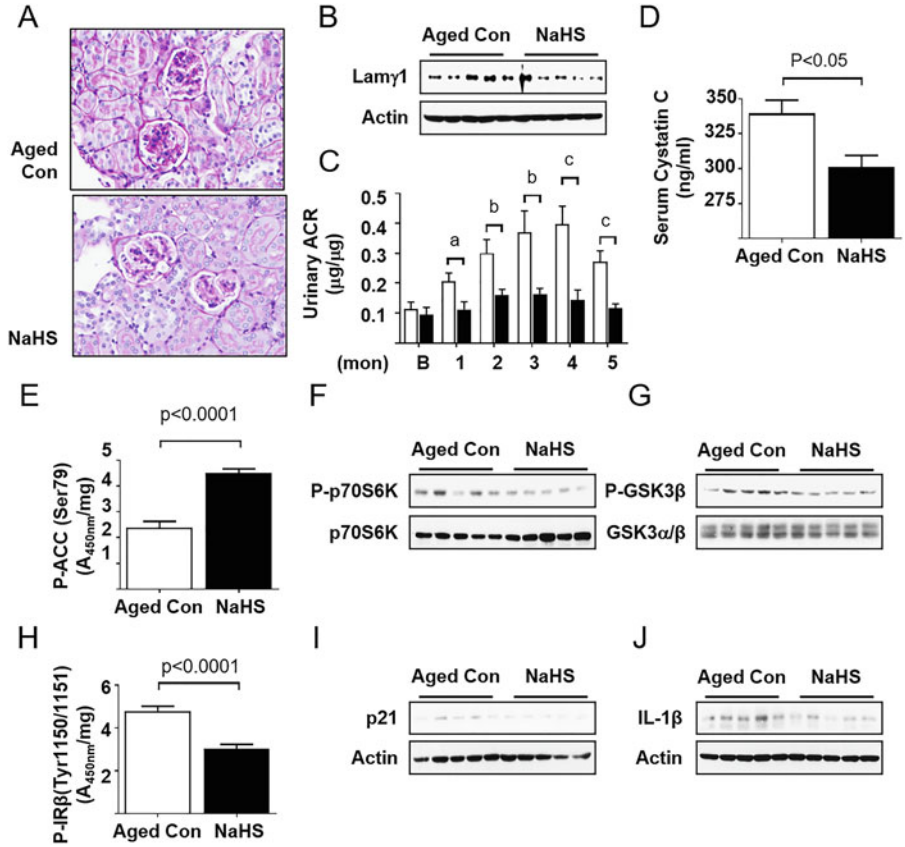

Fig. 5 H_2S administration ameliorates aging associated kidney injury deficiency in mice.18–19-month old mice were randomized to receive water alone vs. NaHS in water for 5 months. NaHS administration was associated with the following: (**a**) reduced glomerulosclerosis, and (**b**) decreased laminin content. This was associated with (**c**) decrease in age related increase in albuminuria, ($a = p < 0.05$, $b = p < 0.01$, $c = p < 0.001$ by two-way ANOVA), and (**d**) reduced cystatin C, indicating prevention of functional deterioration. NaHS administration resulted in the following: (**e**) stimulation of AMPK activity as shown by increased ACC phosphorylation, (**f**) inhibition of mTORC1 activity as demonstrated by decreased phosphorylation of p70S6K, (**g**) inhibition of Akt activity as indicated by decreased GSK3β phosphorylation, and (**h**) inhibition of IR as shown by diminished tyrosine phosphorylation on ELISA. (**i**, **j**) NaHS reduced the expression of p21 and IL-1β suggesting amelioration of SASP. Data are from Lee HJ, et al., GeroScience 40: 163, 2018. Used with permission of the American Aging Association

kidney function [225, 226]. Currently, senolytic therapy is an area of active investigation in geroscience. Signaling events in aged kidney are schematically shown in Fig. 6.

Marmosets are non-human primates. As they are evolutionarily closer to the humans than rodents, small and easy to handle, and have a brief lifespan over which to test interventions, they can form excellent models to study mechanisms of human aging [227]. We explored kidney aging in marmosets [212]. Nephrosclerosis and proteinuria were associated with reduced expression of CBS and H_2S generation in the kidney cortex of aged male marmosets. These changes were accompanied by reduced AMPK activity and stimulation of insulin-like growth factor (IGF1) receptor-Akt-mTORC1 pathway. Pro-fibrogenic TGFβ-Smad3 pathway is activated in the kidney of aging mice [211]; activation of this pathway was also seen in the renal cortex of

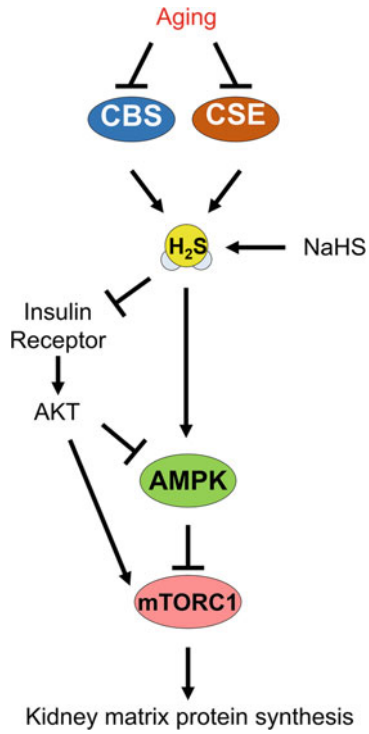

Fig. 6 Kidney H_2S regulation in Aging. Kidney expression of CBS and CSE is reduced resulting in decreased H_2S generation. AMPK activity is reduced, whereas insulin receptor-Akt-mTORC1 axis is stimulated leading to increased matrix protein synthesis. NaHS, an H_2S donor, stimulates AMPK, and inhibits insulin receptor-Akt axis resulting in inhibition of mTORC1 and synthesis of matrix proteins

aged marmosets [212]. Aging kidney in female marmosets showed similar structural and functional injury although there were differences in signaling reactions [212]. These data show conservation of key signaling reactions in kidney aging between mice and marmosets. Marmosets can be studied to understand human kidney aging, and test interventions to ameliorate kidney injury.

αKlotho is an aging-related hormone. Mice with αKlotho deficiency develop aging phenotypes, whereas transgenic mice age slower [228]. Klotho is expressed both in a circulating form and as a transmembrane protein. It is synthesized by the distal tubular epithelial cells in the kidney [229]. αKlotho deficiency occurs in the aged kidney, and soluble αKlotho levels inversely correlate with loss of GFR [212, 230]. Aging-related reduction in GFR has been linked to *αKLOTHO* SNPs 1818TT and 370SS in humans [231]. αKlotho has been shown to inhibit signaling by the insulin receptor and IGF1 receptor [228] suggesting that its deficiency may contribute to activation of these receptors in the kidneys of aged mice and marmosets, respectively [212, 221]. αKlotho inhibits angiotensin II-induced kidney injury [232]. Renin- angiotensin II axis has been proposed as a pathway that contributes to kidney injury in aging [218]. Since H_2S inhibits renin generation in the kidney [22] and activity of angiotensin converting enzyme [233] it is possible that αKlotho is involved in its effects [234].

αKlotho is an obligate cofactor for fibroblast growth factor 23 (FGF23) receptor; the FGF23 receptor + klotho complex augments phosphate excretion by inhibiting Na^+-dependent phosphate transporter in the kidney [235, 236]. FGF23 also inhibits 25 hydroxy-vitamin D 1α hydroxylase enzyme critical for the synthesis of 1:25 dihydroxy vitamin D [235]; this can have far reaching effects on bone metabolism in aging and CKD. These observations indicate that interaction between H_2S and αKlotho in the aged kidney needs more exploration.

In summary, H_2S deficiency is a proximal event in the pathways that lead to aging associated kidney injury. It appears to be an evolutionarily conserved mechanism. Thus, H_2S can serve as a target for intervention to protect kidney integrity in aging.

8 Conclusion and Future Directions

Even though exploration of H_2S in kidney physiology and pathology is in its infancy, it is already apparent that it is a fundamental regulator. Similar to most biological agents the role of H_2S as a mediator vs. ameliorator of kidney injury depends on the context. We need investigation of its role in glomerulonephritis, inherited kidney diseases such as polycystic kidney disease, and in target tissue injury in CKD such as the bones and the

neurologic system. More work is needed in the area of malignancies of the genitourinary tract. Most of the data to date have emerged from animal models, and there is an urgent need for exploration in the clinical setting although some progress is being made [170]. In developing interventional strategies to potentiate H_2S, dietary restriction of calories, dietary methionine restriction, and natural sources of H_2S such as diallyl sulfide are candidates. Compliance with calorie restriction is likely going to be a hurdle, and nutritional deficiency from lack of methionine will need to be explored. Development of H_2S delivering therapeutic agents has included structural modification of nonsteroidal anti-inflammatory agents [237]. Because nonsteroidal agents can aggravate kidney injury, particularly in the setting of AKI and CKD, toxicity of such agents could be an issue. H_2S suppressing agents are also in development for application in clinical situations in which H_2S inhibition may be of benefit [238].

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The Role of H_2S in the Metabolism of Glucose and Lipids

Hai-Jian Sun, Zhi-Yuan Wu, Xiao-Wei Nie, and Jin-Song Bian

1 Introduction

Glucose and lipids are the major sources of energy for human body [1]. The normal metabolism of glucose and lipids is strictly modulated in healthy individuals [2]. One of the most abundant lipids, cholesterol, is a key component of cell membranes in mammals [2]. The blood glucose is precisely regulated in the physiological range of 70–110 mg/dL under healthy conditions, which is orchestrated by a complicated network of numerous hormones from several metabolic organs/tissues including the livers, pancreas, brain, intestine tissue, and adipose as well as skeletal muscle (Fig. 1) [3–5]. Defects in glucose and lipid metabolism may result in severe diseases including metabolic syndrome, cardiovascular diseases, diabetes, and obesity [2]. Notably, the prevalence of obesity and diabetes and their complications as well as the healthcare cost remain major socioeconomic problems [6, 7]. Thereafter, it is urgent to elucidate the underlying mechanisms of glucose/lipid dysfunction for the evolution of novel therapeutic approaches for glycolipid metabolism disorder-related diseases.

Hydrogen sulfide (H_2S) has long been regarded as a toxic environment pollutant with a rotten egg smelling [8, 9]. However, mounting evidence indicates the physiological significance of H_2S because of its role in a wide range of physiological and pathological activities in mammal systems [10], especially in the cardiovascular system [11, 12], central nervous system [13–15], and other systems [5, 16–18]. As such, H_2S has gained recognition as the third gaseous signaling molecule along with nitric oxide (NO) and carbon monoxide (CO) [19]. It has been reviewed that endogenous H_2S is produced by both enzymatic and non-enzymatic pathways in mammal tissues. The former pathway requires the actions of three enzymes: cystathione β-synthase (CBS), cystathione γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST) [20–22]. Emerging evidence suggests that H_2S also actively regulates the metabolism of glucose and lipids, and its dysregulation is implicated in glycometabolic disorders [23]. Specifically, H_2S promotes glucose generation via activation of

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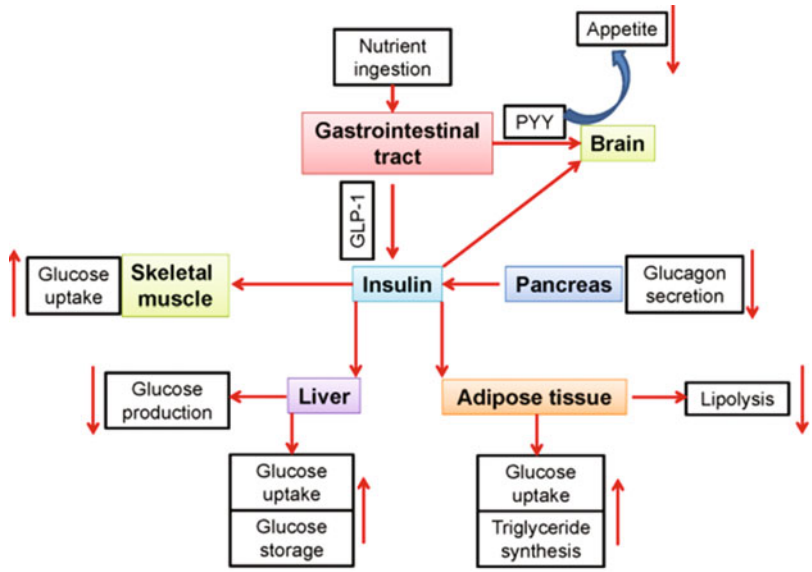

Fig. 1 The precise regulatory mechanisms for glucose homeostasis network. Glucose homeostasis is strictly regulated by neuropeptides, glucose substrates, and various hormones including insulin, the released insulin acts on the brain, intestine, adipose tissue, pancreas, liver, and skeletal muscle to control blood glucose. GLP-1, glucagon-like peptide-1; PYY, peptide YY

gluconeogenesis and glycogenolysis in the livers [24], but prevents lipolysis in adipocytes [25]. The glucose uptake into adipocytes and skeletal muscle is stimulated by H_2S [26, 27], but the glucose uptake into hepatocytes is inhibited by H_2S [24]. Moreover, H_2S is found to sensitize insulin signaling in the liver tissues, adipose tissues, and skeletal muscle [28, 29], yet blocks insulin secretion from pancreatic β cells [30]. These observations indicate a sophisticated role of H_2S in glucose/lipid metabolism in health and diseases. In this chapter, we will outline the published studies regarding the roles of H_2S in glucose/lipid metabolism, and continue to describe the molecular mechanisms and physiological/pathological significance of H_2S on organs and tissues that are required for homeostatic maintenance of circulating glucose/lipid levels. Future directions highlighting the H_2S-mediated monitoring of glucose/lipid metabolism under physiological and pathophysiological conditions are also discussed.

2 H_2S and Glucose Metabolism

Glucose is an indispensable substrate for mammalian cells, affecting various physiological functions, such as protein and nucleic acid synthesis, cell growth, and fatty acid synthesis [31]. The metabolism homeostasis of glucose is crucial for maintaining normal body function, whereas disturbances in glucose metabolism might lead to abnormal blood glucose levels or energy supply barrier [32]. Needless to say, the most glucose metabolism disorder is diabetes. Diabetes and its complications represent major socioeconomic issues worldwide [33]. Therefore, it is of utmost importance to understand the precise mechanisms that regulate glucose homeostasis and their dysregulation in the pathophysiology of diabetes. In 1990, the first experiment was performed to examine the roles of H_2S in glucose homeostasis [34], the authors showed that inhalation of low dose of H_2S elevated the circulating blood glucose in postpartum rats [34]. By contrast, animal studies have shown that supplementation with L-cysteine, a precursor for H_2S biosynthesis, or a diet containing L-cysteine improves insulin resistance by ameliorating glucose metabolism [35–39]. However, the underlying mechanisms of H_2S or L-cysteine in glucose modulation are not elucidated in these studies. As research continues to deepen, the mechanistic studies regarding the roles of H_2S in glucose homeostasis have come to light through the

examination of insulin synthesis and secretion. Moreover, the actions of H_2S on glucose metabolism-target tissues, including the livers, adipose tissue, and skeletal tissue, are also gradually clarified [5, 27, 40]. It is believed that the investigations of the involvement of H_2S in glucose metabolism are of great interests in the prevention and treatment of diabetes. Next, the roles of H_2S in metabolic hormone secretion and the downstream signaling pathways in target organs/tissues will be described.

3 H_2S and Insulin Secretion

There is no doubt that the pancreas plays a foremost role in glucose metabolism through the release of pancreatic hormones, such as insulin and glucagon [41]. Insulin is a well-known metabolic hormone that decreases blood glucose, thus the therapeutic strategies aiming at improving insulin secretion and sensitivity are a major research focus for diabetes [42]. Both CSE and CBS are abundantly expressed in pancreatic islets; however, the CSE mRNA levels are higher in pancreatic islets than those of CBS [43, 44]. The production of H_2S is significantly downregulated in CSE knockout (KO) mice, and a CSE inhibitor PPG (DL-propargylglycine) obviously reduces H_2S generation in normal pancreatic islets from mice [45]. It is documented that the expressions of CSE and CBS in the pancreas are remarkably augmented in streptozotocin (STZ)-induced diabetic rats, whereas the decreased enzymes are rescued by exogenous insulin treatment [46]. Intriguingly, the production of H_2S is inhibited in high glucose-treated INS-1E cells and rat pancreatic islets, this effect may be mediated by SP1 and p38 mitogen-activated protein kinase (MAPK) phosphorylation [47]. These observations suggest a delicate interplay between H_2S and insulin release. Actually, pancreatic H_2S levels are greater in Zucker diabetic rats, and inhibition of pancreatic H_2S production with PPG increases serum insulin level and lowers hyperglycemia [43]. It is likely that insulin release is reduced in diabetic rats because of high pancreatic production of H_2S. Downregulation of the higher H_2S system in pancreatic tissues seemingly benefits diabetic prevention and treatment.

Accumulating evidence shows that H_2S may be an endogenous inhibitor of insulin secretion from pancreatic β cells [45, 48]. Generally, H_2S could affect the functions of pancreatic β cells via preventing the release of insulin [30, 44], and influencing the cellular apoptosis induced by various stimuli [49, 50]. Mechanistically, the suppressive actions of H_2S on insulin secretion may be mediated by opening K_{ATP} channels in insulin secreting cells [51], inhibiting L-type voltage-dependent Ca^{2+} channels [30], and inducing pancreatic β cell apoptosis [52]. In terms of pancreatic β cell apoptosis, another group has demonstrated that H_2S prevents high glucose-induced pancreatic β cell apoptosis through increased total glutathione levels [53]. The discrepancies about the effects of H_2S on pancreatic β cell apoptosis may be caused by different treatments used in those experiments. It should be noted that the effects of exogenous and endogenous H_2S on pancreatic β cell survival may be significantly different. As such, a comparison of endogenous H_2S levels with exogenous H_2S levels may be difficult because of the uncertain physiological range of H_2S in pancreatic β cells.

Given the inhibitory effects of H_2S on insulin release, it is natural to consider that H_2S may be detrimental in the context of diabetes. As a matter of fact, endogenous H_2S does not always serve as a foe. For instance, increased H_2S production with L-cysteine could attenuate oxidative stress-induced apoptosis in pancreatic β cells [53]. The persistently elevated inflammatory response is a driving force for islet cell damage and diabetic complications, thus anti-inflammation in pancreatic β cells may be a very useful approach to treat diabetes [54]. Intriguingly, H_2S donor sodium hydrosulfide (NaHS) could attenuate inflammatory factors-evoked injury in primary cultured pancreatic β cells or MIN6 cells [55]. Moreover, knockout of CSE aggravates oxidation-related insults in islets from mice fed a high-fat diet (HFD), affecting neither insulin secretion nor blood glucose levels in HFD-induced diabetic

mice [50]. It is likely that CSE-produced H_2S protects pancreatic β cell from glucotoxicity, and thus it may prevent the onset/development of type 2 diabetes induced by HFD [50].

Based on the above observations, the biphasic effects of H_2S on insulin secretion may be associated with different stages during diabetic development (Fig. 2). It may be hypothesized that hyperglycemia-induced upregulations of CSE/H_2S may be an adaptive response at the early stage, and the augmented H_2S production may confer anti-oxidative and anti-inflammatory activities, thereby protecting pancreatic β cells against hyperglycemia. With the progression of diabetes, the further increased H_2S generation will then prevent insulin secretion through reducing ATP production, and opening KATP channels in insulin secreting cells [51], as well as inhibiting L-type voltage-dependent Ca^{2+} channels [30]. Finally, if the hyperglycemia state exits for a long period, the endogenous H_2S may exceed its threshold, thus inducing endoplasmic

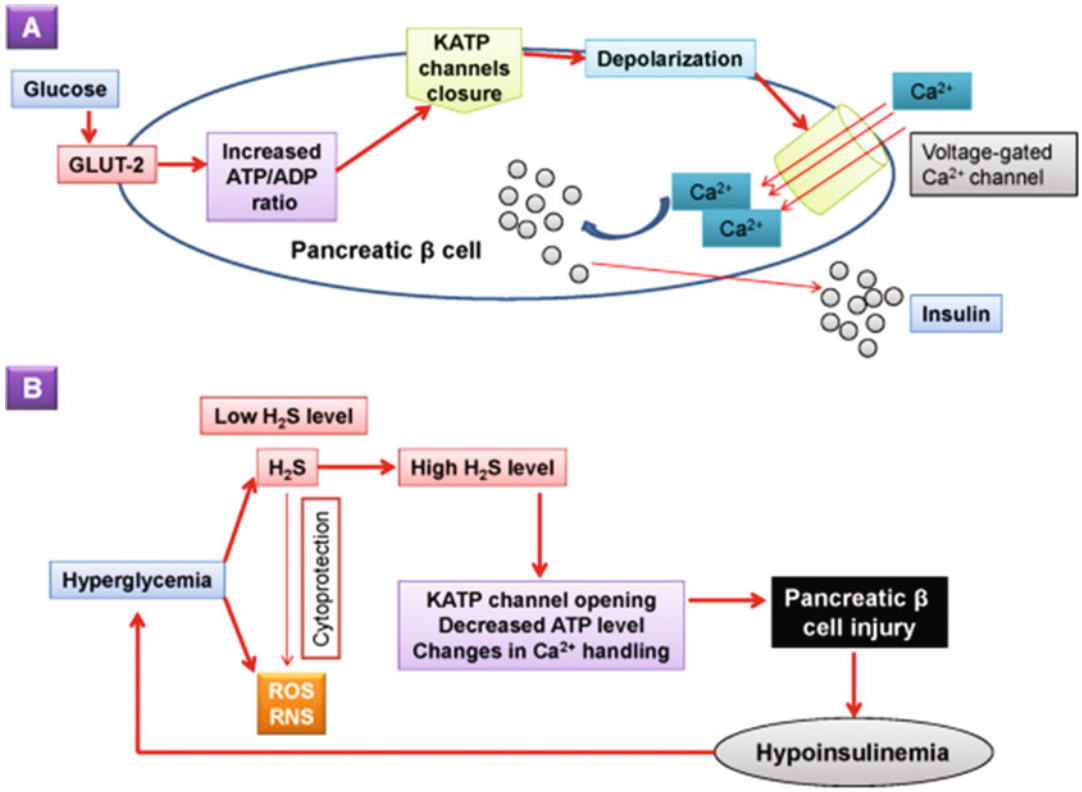

Fig. 2 A proposed sketch showing H_2S-mediated effects on insulin secretion from pancreatic β cells. (**a**) Under normal conditions, glucose enters the pancreatic β cells via GLUT-2 and then is subject to glucose metabolism, accompanied by the increased ATP/ADP ratio. This might induce the closure of KATP channels and depolarization of β cells. After that, the voltage-gated Ca^{2+} channel is open and allowing Ca^{2+} to enter the cells. The increased intracellular Ca^{2+} concentration stimulates insulin exocytosis and releases from β cells. (**b**) In the early stage of diabetes, hyperglycemia may upregulated CSE-derived H_2S levels (low H_2S levels), thus serving as a protective mechanism by neutralizing oxidative/nitrosative stress and autoimmune attack. When H_2S levels continue to increase, the intra-islet H_2S production leads to decreased insulin generation through stimulating KATP channels, inactivating voltage-gated Ca^{2+} channel, and inhibiting glucose metabolism. Eventually, the persistently increased glucose may lead to β cell toxicity, resulting in lower circulating insulin levels and higher hyperglycemia. ADP, adenosine-diphosphate; ATP, adenosine-triphosphate; GLUT, glucose transporter; KATP, ATP-sensitive potassium channel; RNS, reactive nitrogen species; ROS, reactive oxygen species

reticulum stress-induced apoptosis in pancreatic β cells [52]. Overall, therapeutic strategies for diabetes can be achieved by administration of H_2S at the early stage of diabetes or blocking endogenous H_2S generation from pancreatic tissues at its late stage.

4 H_2S-Mediated Glucose Metabolism in the Livers

The livers are important glucose-producing organs, and it is also a necessary location for the control of blood glucose homeostasis through regulating glycogen synthesis (glycogenolysis) and de novo glucose synthesis (gluconeogenesis) [56]. Hepatic malfunction of gluconeogenesis and glycogenolysis may induce aberrant glucose metabolism and even diabetic development [57]. The livers are also chief H_2S-producing organs as the H_2S-generating enzymes CBS, CSE, and 3-MST are extensively expressed in the livers, and they are responsible for liver H_2S generation to distinct extents [58].

Importantly, the expression levels of CSE are upregulated in the livers of diabetic rats and HepG2 cells with insulin resistance [24, 46, 59]. It is reported that gene deficiency of CSE exhibits a significantly decreased gluconeogenic system in the livers [60]. As an important gluconeogenic regulator, peroxisome proliferator-activated receptor-c coactivator-1α (PGC-1α) is a critical transcription factor that upregulates the gluconeogenic rate-limiting enzymes, such as glucose 6-phosphatase (G6Pase), fructose 1,6-bisphosphatase (FBPase), and phosphoenolpyruvate carboxykinase (PEPCK) [61]. Therefore, H_2S-stimulated glucose production in hepatocytes requires the increased protein expressions and activity of hepatic PGC-1α G6Pase, FBPase, and PEPCK (Fig. 3) [24, 60]. Furthermore, the enhanced glucose production by H_2S in the livers is partially ascribed to activation of the cyclic adenosine

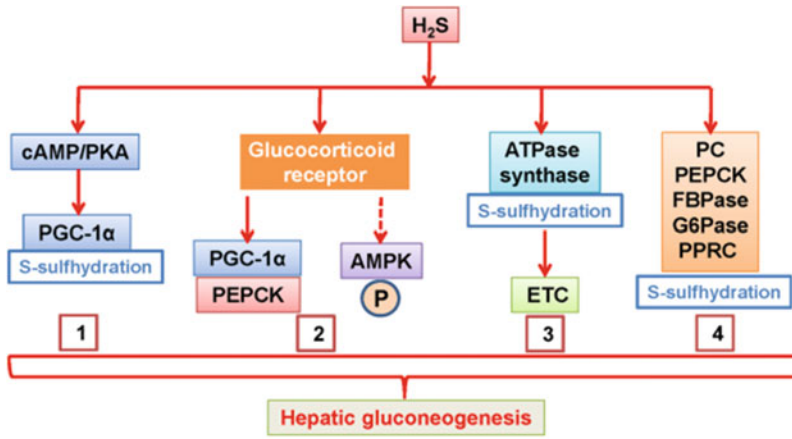

Fig. 3 Role of H_2S in hepatic gluconeogenesis. The stimulatory effects of H_2S on hepatic gluconeogenesis are dependent on the following several mechanisms: (1) activating the cAMP/PKA signaling pathway to upregulate PGC-1α protein expression as well as induction of PGC-1α S-sulfhydration; (2) promoting the PGC-1a and PEPCK protein expression and inactivation of AMPK through glucocorticoid receptor pathway; (3) upregulating the activity of ETC via complex II and ATPase synthase S-sulfhydration; (4) upregulations of both protein levels and activities (by S-sulfhydration) of PC, PEPCK, FBPase, G6Pase, and PPRC. *AMPK* AMP-activated protein kinase, *cAMP* cyclic adenosine monophosphate, *ETC* electron transport chain, *FBPase* fructose 1,6-bisphosphatase, *G6Pase* glucose 6-phosphatase, *PC* pyruvate carboxylase, *PEPCK* phosphoenolpyruvate carboxykinase, *PGC-1α* peroxisome proliferator-activated receptor-coactivator-1α, *PKA* protein kinase A, *PPRC* peroxisome proliferator-activated receptor-c coactivator-related protein

monophosphate (cAMP)/protein kinase A (PKA) and glucocorticoid signaling pathways [60]. Exogenous H_2S donor NaHS also stimulates gluconeogenesis and glycogen breakdown through PEPCK in the livers [60, 62]. Additionally, H_2S can inhibit glucose uptake and glycogen storage in HepG2 hepatocytes via reduced glucokinase activity [24].

The ability of H_2S to inhibit glucose uptake and utilization in HepG2 cells and primary hepatocytes is also attributed to AMP-activated protein kinase (AMPK) de-phosphorylation and inactivation [24]. In fact, activation of AMPK stimulates uptake and utilization of glucose in hepatocytes as well as reducing hepatic glycogenolysis and gluconeogenesis [63]. As a consequence, the lower AMPK activity is likely responsible for the higher hepatic glycogen content observed in the livers from CSE knockout mice under the fed state. Very recently, inactivation of the AMPK signaling pathway by CSE deficiency promotes hepatic gluconeogenesis, thus contributing to obesity-related insulin resistance [29]. These phenomena could be reversed by supplementation with NaHS. Nevertheless, NaHS treatment ameliorates HFD-induced obesity and metabolism disorders in wild-type mice, suggesting that H_2S is a critical player in obesity-associated insulin resistance. These findings reveal a double-edged sword effects of the CSE/H_2S system in obesity associated with insulin resistance. Targeting the CSE/H_2S system may serve as a valuable therapeutic strategy for insulin resistance in obesity.

5 H_2S-Mediated Glucose Metabolism in the Adipose Tissue

Adipose is one of the most plenteous tissues in the human body [64]. Within the adipose system, adipocytes, a class of extremely specialized cells, are capable to take up a large amount of triglycerides in response to excessive energy and to mobilize them during the period of energy shortages [65, 66]. Apart from lipid regulation, the adipose tissue is also considered to be an endocrine organ to regulate glucose homeostasis [67]. The adipose tissue turns the extra glucose into fat when blood glucose increases, and in turn, the stored fat within the adipose tissue could be utilized for energy when blood glucose decreases [67]. It is well accepted that adiposity is implicated in insulin resistance, whereby lipid moieties from white adipose tissue interfere with insulin signaling [5]. It has been found that the circulating H_2S levels are downregulated in obese population [68]. However, it is still unclear whether the changed circulating H_2S levels affected glucose metabolism in the adipose tissue. As a result, the underlying mechanisms by which dysregulated circulating H_2S level is a driving force in adipose metabolic disorder are not investigated.

It is established that the CSE/H_2S system is observed in both rat adipocytes and preadipocytes, and H_2S may be involved in insulin resistance in adipocytes [69]. The authors have shown that H_2S (10–1000 μM) diminishes insulin-induced glucose uptake into adipocytes, whereas inhibition of CSE enhances this effect [69]. Additionally, H_2S also participates in TNF-α-induced insulin resistance in adipocytes [70]. These studies suggest the involvement of H_2S in the pathophysiology of adipose insulin resistance. However, this notion is challenged by the contradictory actions of H_2S on glucose metabolism in the adipose tissues. In other words, H_2S may, in fact, play a beneficial role in glucose regulation in the adipose tissue. For example, H_2S is essential for maintenance of glucose metabolism in the adipose tissues as H_2S promotes glucose uptake and utilization in 3T3L1 adipocytes through GLUT4 translocation to cell surface and its activation [71, 72]. In an in vivo study, H_2S donor is established to increase glucose uptake in both myotubes and adipocytes, thus decreasing blood glucose, and improving insulin sensitivity in Goto-Kakizaki (GK) diabetic rats [27]. Upon exposure to high glucose, the CSE expressions at both protein and mRNA levels are significantly downregulated, paralleled with increased monocyte chemotactic protein 1 (MCP-1) production in 3 T3-L1 adipocytes [73]. These effects are counteracted by overexpression of CSE or a

source of exogenous H_2S, NaHS [73]. Sulfhydration of peroxisome proliferator-activated receptor γ (PPARγ) in the adipose tissues is required for H_2S to improve insulin resistance in HFD-fed mice [26]. These findings suggest that activation of PPARγ or inhibition of inflammation by H_2S may improve insulin resistance in adipocytes. Similar to PPARγ activation, the cellular phosphatidylinositol 3,4,5-trisphosphate (PIP3), a positive regulator of phospholated Akt, is activated by H_2S, this may be responsible for H_2S-mediated glucose metabolism and utilization in 3 T3-L1 adipocytes [71]. Likewise, supplementation of H_2S triggers the phosphorylation of insulin receptor (IR), PI3K, and Akt, which synergistically promotes glucose intake in 3 T3-L1 adipocyte [27]. From this collection of evidence, H_2S plays a regulatory role in glucose metabolism and insulin signaling in adipocytes. It is clear that the effects of H_2S on glucose metabolism in the adipose tissue are sophisticated and even contradictory. The reason for this discrepancy might be attributed to the different dose and treatment time period of H_2S, experimental models, and the distinct stage of diabetic development. Certainly, H_2S-based strategies that aim at increasing glucose uptake and utilization in the adipose tissue may be a cornerstone of diabetic research.

6 H_2S-Mediated Glucose Metabolism in the Skeletal Muscle

Skeletal muscle is also an insulin-sensitive organ, which is responsible for roughly 85% of glucose uptake in the whole body by itself or via a crosstalk with the livers and adipose tissue [74, 75]. To be sure, a significant amount of CBS and CSE proteins are detected in human skeletal muscle [76]. All three H_2S-producing enzymes, including CBS, CSE, and 3-MST, are present in rat skeletal muscle [27, 77, 78]. Surprisingly, all known H_2S-generating enzymes are undetectable in mouse skeletal muscle [76, 79].

As of yet, the roles of H_2S in the skeletal muscle-mediated glucose metabolism is still limited. Human studies have found that acute H_2S inhalation induces the shift from aerobic to anaerobic metabolism, coupled with accumulation of lactate in the circulation [80]. This metabolic shift may be relied on the downregulated citrate synthase enzyme level in the skeletal muscle during exercise [80]. It is undefined whether any changes occur in blood glucose level after H_2S inhalation in this study. Xue and colleagues have illustrated that knockdown of CSE decreases glucose uptake in rat L6 myotubes [27]. The same group has also shown that exogenous H_2S promotes the absorption of glucose in L6 myotubes in the presence of insulin [27]. It appears that H_2S sensitizes the skeletal muscle to insulin signaling, resulting in increased glucose uptake in the skeletal muscle tissue from type 2 diabetes. Although the H_2S-generating enzymes are absent in the mouse skeletal muscles, it is reasonable that the mouse skeletal muscles may be biologically influenced by circulating H_2S level. As H_2S acts as an insulin sensitizer in the rat skeletal muscles [27], it should be the same in the murine skeletal muscle. Unfortunately, there is still a lack of evidence that H_2S plays a functional role in mouse skeletal muscle glucose metabolism under both physiological and pathophysiological conditions. As the current understanding of H_2S biology/pharmacology is rapidly evolving, it is highly possible that the roles and precise mechanisms of H_2S in the skeletal muscle glucose metabolism will be progressively identified.

7 H_2S-Mediated Glucose Metabolism in the Gastrointestinal Tract

In recent years, evidence has emerged to recognize H_2S as a mediator of several functions of the gastrointestinal tract [81]. It is well known that the required nutrients are first processed by the hormones from the gastrointestinal tract, and these hormones play a crucial role in the modulation of glucose or lipid homeostasis [82]. As an incretin hormone from intestinal L cells upon food intake, glucagon-like peptide-1 (GLP-1) is

established to enhance glucose-provoked insulin secretion from pancreatic β cells [83]. Moreover, GLP-1 may be a stimulator for pancreatic β cell proliferation and proinsulin gene expressions [84]. Peptide YY (PYY), an energy-regulating hormone secreted from gastrointestinal tract is a potential appetite-suppressing hormone, which is co-localized with GLP-1 in intestinal L cells [85]. Studies have shown that H_2S inhibits the production of GLP-1 and PYY from a murine enteroendocrine cell line [86]. The authors have found that both endogenous and exogenous H_2S inhibit oleic acid-induced GLP-1 and PYY release, and this effect is completely abolished by PPG, suggesting a key role of CSE in GLP-1 and PYY generation from the gastrointestinal tract. The aforementioned studies indicate that the endogenous generation of H_2S in the gut wall likely plays a central role in the modulation of gastrointestinal tract functions and subsequent glucose metabolism under both physiological and pathophysiological circumstances [33].

8 H_2S and Lipid Metabolism

Similar to diabetes, obesity is also a major global health burden with high prevalence worldwide. Over-nutrition leads to expanding the adipose tissue loading, together with ectopic deposition of lipid in the muscle, livers, and insulin-producing pancreatic islets [87, 88]. The excessive lipid deposition might trigger a lipotoxic stress that elicits inflammation response and insulin resistance in the adipose tissue, livers, and muscle, followed by a disruption of glucose/lipid homeostasis [89]. Specifically, exceeding lipid synthesis and transport in the livers is a critical step for driving lipogenesis and lipoprotein profiles that ultimately lead to atherosclerosis [90]. In the following sections, we will outline the involvement of H_2S in pathologies of lipid metabolism disturbance in the context of obesity, metabolic syndrome, or diabetes, especially lipid regulation by H_2S in the livers and adipose tissue.

9 H_2S-Mediated Lipid Metabolism in the Adipose Tissue

In the process of obesity, of central interest is the formation of adipocytes, which is also known as adipogenesis [91]. Impaired adipogenesis in the adipose tissue is thought to be a primary player in obesity and its associated metabolic complications [92]. As a consequence, a comprehensive understanding of the molecular mechanisms of adipogenesis may be helpful to develop more efficient strategies for the management of obesity and metabolic disorders [93]. The CSE/H_2S system is a potent adipogenesis regulator as it promotes the differentiation of 3 T3-L1 preadipocytes into mature 3 T3-L1 adipocytes [73], a finding later confirmed by other research groups [26, 94]. During the course of 3 T3-L1 adipocyte differentiation, the expression levels of CBS, CSE, and 3-MST are raised in a time-dependent manner [94]. Exogenous H_2S treatment upregulates the levels of genes associated with adipogenesis-related genes, such as fatty acids binding protein 4 (FABP4/aP2), peroxisome proliferator-activated receptor γ (PPARγ), and CCAAT/enhancer-binding protein a (CEBPa) in 3 T3-L1-derived adipocytes [94]. These observations are consistent with the findings that the lower body weight and adipose tissue accumulation are present in both CSE knockout mice and CBS knockout mice [95, 96].

By contrast, diallyl trisulfide (DATS), a potential stimulator for H_2S release in biological systems, is reported to suppress adipogenesis in 3 T3-L1 cells [97, 98]. Blockade of extracellular signal regulated kinases (ERK) by its specific inhibitor PD98059 abolishes the suppressive effects of DATS on the process of adipogenesis [98]. These findings suggest that DATS prevents adipogenesis in an ERK-dependent fashion. Despite the opposite results, whether the inhibitory effects of polysulfides on adipogenesis are directly mediated by released H_2S remains questionable. Anyway, it is obvious that H_2S plays a

critical role in adipogenesis, thus impacting lipid metabolism homeostasis. Notably, the roles of H_2S in the development of obesity-related adipogenesis is not yet clear and more research on the H_2S system in the adipose tissue needs to be conducted in the future.

Lipolysis is defined as the breakdown of fats and other lipids by hydrolysis to release fatty acids and glycerol into the circulation when energetically desirable [99]. The appropriate regulation of lipolysis is widely accepted as an alternative therapeutic avenue in the management of obesity and metabolic diseases [100]. Unraveling the underlying mechanisms of lipolysis may open up novel therapeutic targets against metabolic disorders and obesity. The first experiment to test the involvement of H_2S in lipolysis was carried out by Geng et al. [25]. Geng and colleagues have found that inhibition of the endogenous H_2S system stimulates lipolysis in a protein kinase A/perilipin/hormone-sensitive lipase-dependent pathway [25]. Accordingly, inhibition of lipolysis by H_2S may blunt the increased fat mass in obese mice [25]. This viewpoint is further confirmed by a later finding that treatment with GYY4137 (a slow-releasing H_2S donor) or NaHS remarkably enhances the generation of lipid droplets and also mitigates adrenergic receptor-stimulated lipolysis in 3 T3-L1 cells [94]. Recently, it is found that perilipin-1 sulfhydration is a major direct target for H_2S-mediated inhibition of lipolysis since gene deletion of perilipin-1 eliminates the inhibitory effects of H_2S on lipolysis [101]. Totally, H_2S may function as a lipolysis suppressor, and inhibition of lipolysis by H_2S may promote and sensitize insulin response in adipocytes. These speculations still await further in-depth research.

However, the functional roles of H_2S in the manipulation of adipose lipolysis are still controversial. Using the microdialysis method, H_2S donor sodium sulfide (Na_2S) provokes dose-dependent stimulation of glycerol formation in fat cells, even though its effect is less than that of isoproterenol [64]. The effects of Na_2S on lipolysis may be associated with cAMP release, since Na_2S-mediated effects on lipolysis are prevented by a PKA inhibitor, KT5720 [64]. Furthermore, it has been shown that H_2S release is remarkably higher in rats fed by a HFD [64]. These preliminary results suggested that the cAMP/PKA pathway mediated H_2S-induced adipose tissue lipolysis. It is likely that upregulation of the CSE/H_2S system in the adipose tissue might lead to increased lipolysis in obese animals. Based on the above results, H_2S might grant a dual role in adipose lipolysis, and more studies are indispensable to test the exact effects of H_2S on adipose lipolysis in both health and diseases. However, it is well recognized that deficiency of H_2S may result in adipose inflammatory response in obesity and metabolic syndrome [28, 102–104]. Hence, the link between the H_2S deficiency-mediated adipose inflammation and lipid metabolism in the adipose tissue deserves further studies.

10 H_2S-Mediated Lipid Metabolism in the Livers

The livers play a critical role in the metabolism of nutrient and lipid [105]. Dysregulated lipid metabolism may be a possible mechanism for liver lipotoxicity [106]. In health, the lipid metabolism in the livers is orchestrated by hormones, transcription factors, nuclear receptors, and other intracellular signaling pathways [107]. The overproduction of lipids in the livers disrupts the hepatic function and leads to many hepatic diseases, such as non-alcoholic fatty liver disease (NAFLD), liver cirrhosis, and liver cancer [108]. A better understanding of the molecular mechanisms of hepatic lipid metabolism will facilitate the development of novel therapeutic targets for hepatic metabolic disorders [109]. NAFLD is a broad spectrum hepatic lipid metabolism disorder without an alcohol abuse history [110–112]. The prevalence of NAFLD is sharply increasing and its pathological mechanisms are complicated and incompletely understood.

Moreover, the livers are important organs for the production and clearance of H_2S [113]. Studies have shown that CBS, CSE, and 3-MST are

abundantly expressed in the liver tissues, and they are responsible for H_2S generation to distinct extents in the livers [58]. Hepatic H_2S system participates in insulin sensitivity, glycolipid metabolism, mitochondrial biogenesis, and bioenergetics [33, 58, 62, 114]. The circulating H_2S levels are relevant to the severity of NAFLD patients [115, 116]. Deficiency of CBS elevates endogenous homocysteine levels [117], and hyperhomocysteinemia, in turn, could promote fatty liver through impaired lipid metabolism and oxidative injury [118–120]. As expected, it has been revealed that CBS-deficient mice spontaneously develop hepatic steatosis in rodents [121]. Under HFD condition, hepatic lipid accumulation is exacerbated in mice with CSE deficiency, as evidenced by higher total cholesterol and aggravated liver dysfunction [29, 122]. Notably, this effect could be rescued by exogenous H_2S treatment, confirming that hepatic lipid metabolism disorder is caused by deficiency of CSE-derived H_2S [29, 122]. In line with CSE knockout mice, 3-MST-deficient mice exhibit normal liver functions upon a normal diet, but with increased anxiety-like behaviors [123]. However, hepatic 3-MST protein expression is obviously augmented in mice and patients with NAFLD, and partial knockdown of 3-MST significantly ameliorates hepatic steatosis in HFD-fed mice [124]. The mechanisms involving in the detrimental role of 3-MST in NAFLD may be mediated by inhibiting the CSE/H_2S pathway [124]. These observations suggest that 3-MST may be a crucial stimulator for accelerating the development of NAFLD. In the progression of NAFLD, the contradictory result of 3-MST with CSE and CBS may be derived from the unique catalytic activities of 3-MST. It is demonstrated that reaction of 3-MST with 3-mercaptopyruvate produces persulfide-containing intermediates, leading to liver damage [125]. In summary, the above results provide ample evidence that the H_2S system is a critical regulator for lipid metabolism in the livers, and recovery of the H_2S system may be an important strategy for the treatment of fatty liver disease.

Studies have shown that the expression levels of CBS and CSE are upregulated in the livers from HFD mice [126]. In agreement with this, supplementation with tyrosol upregulates hepatic CSE and CBS expression as well as H_2S formation in HFD mice [127], this observation is related with the prevention of HFD-elicited hepatic lipid peroxidation [127]. NaHS is revealed to decrease the deposition of cholesterol and triglyceride via downregulated fatty acid synthase and upregulated carnitine palmitoyltransferase-1 in the livers from obese mice [128]. A substrate for endogenous H_2S production, S-Propargyl-cysteine (SPRC) is able to reduce lipid accumulation in the livers of mice with NAFLD [129]. Exogenous H_2S is found to mitigate the fatty liver through ameliorating lipid metabolism and antioxidant properties [128]. Treatment with NaHS markedly reduces hypertriglyceridemia and ameliorates NAFLD in HFD mice by amplifying the AMP-activated protein kinase AMPK/mammalian target of rapamycin (mTOR) pathway [130]. A recent study further demonstrates that H_2S donor GYY4137 effectively mitigates liver lipid deposition in STZ- and HFD-induced mice, and the therapeutic benefit of H_2S is dependent on Keap1 S-sulfhydration-mediated Nrf2 activation. [131]. These published papers suggest that H_2S might be a therapeutic candidate against NAFLD through restoring the lipid metabolism homeostasis.

It is worthwhile to note that H_2S treatment enhances adipocyte numbers but improves hepatic insulin resistance in mice fed by a HFD [26, 132]. Actually, H_2S is is an inducer of adipogenesis in adipose tissue, while it serves as an inhibitor of lipid production and storage in the liver. These studies suggested that H_2S may confer a biphasic effect in the fat tissues and liver tissues. Moreover, the direct regulatory roles and potential mechanisms of H_2S in hepatic lipid accumulation, storage, and depletion are still in its infancy. In this regard, future research is necessary to elucidate the effects of H_2S on the molecular mechanisms related with lipogenesis,

lipolysis, and lipoprotein secretion during the development of NAFLD.

11 Conclusions and Further Directions

In this chapter, we aimed to highlight the importance of H_2S in glucose/lipid metabolism under physiological and pathophysiological conditions. We apologize to many scientists who contributed greatly to the field of H_2S-mediated glucose/lipid metabolism if their works are not appropriately mentioned. The roles of H_2S in obesity, metabolic syndrome, and diabetes will be an expanding research area. As mentioned above, exogenous administration of H_2S restrains glucose-induced insulin release from pancreatic β cells, yet increases glucose production in the livers. It appears that H_2S sensitizes adipocytes and skeletal muscle, yet desensitizes hepatocytes to insulin signaling pathway. These results suggest that H_2S may grant differential signaling transduction systems in different tissues with regard to the insulin signaling pathway. The contradicting reports about the roles of H_2S in diabetes and insulin resistance may be owned to the lack of standardized methodology and variation in models and study designs. H_2S supplementation can be beneficial or deleterious dependent on the early or late stage of insulin resistance. In other words, H_2S-derived therapeutics for diabetes should aim at decreasing H_2S level in the pancreas at the late stage as H_2S inhibits insulin release from pancreatic β cells and overproduction of H_2S may induce pancreatic β cell apoptosis. However, most research focus on the protective effects of H_2S in diabetic complications, such as diabetic nephropathy, cardiomyopathy, encephalopathy, and endothelial dysfunction-related diseases. To sum up, our current knowledge of H_2S-mediated effects on glucose homeostasis in the livers, adipose tissue, and skeletal muscle is still deficient. More human and animal studies are required to comprehensively understand the physiological and pathophysiological roles of H_2S in glucose metabolism. A full understanding of the impacts of H_2S on glucose metabolic parameters would be considerably informative.

In the context of lipid metabolism, the involvement of H_2S in both adipose tissues and liver tissues seems to be a general phenomenon. However, H_2S-mediated specific ways in which lipids accumulate/decomposition in both adipose tissues and liver tissues under various experimental conditions are largely unknown. Thus, evaluating these characteristics and molecular mechanisms of lipid metabolism would undoubtedly advance our knowledge of the importance of H_2S in systematical lipid metabolism. H_2S production from the adipose tissues and liver tissues is critically involved in lipid metabolism. Despite that a significant advance has been achieved in this field over the last several decades, more original work is still necessary. In particular, several effects of H_2S on insulin sensitivity and lipolysis in the adipose tissues are controversial. The underlying mechanisms whereby H_2S regulates lipid metabolism are fully uncharacterized. Because of these discrepancies, the use of H_2S donors for the treatment of metabolic pathologies must be treated with caution. Most importantly, the H_2S system that links the interactions between lipid and glucose metabolism in health and disease may be of utmost importance for therapy of metabolic diseases.

Taken together, a complicated relationship between H_2S and glucose/lipid metabolism in the body's homeostasis is becoming to be unraveled. Despite that the potential mechanisms that underlie these processes are not fully clarified. Their significant importance to body health indicates clinical implications for future preclinical or clinical transformation of H_2S. There is no doubt that H_2S will be a prominent node in a complex interaction network for regulating glucose/lipid metabolism under both physiological and pathophysiological conditions.

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The Role of H_2S in the Gastrointestinal Tract and Microbiota

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Abbreviation

3-MP	3-Mercaptopyruvate
3-MST	3-Mercaptopyruvate sulfurtransferase
AC	Adenylate cyclase
AOAA	Amino-oxyacetic acid
Apr	Adenosine-5′-phosphosulfate reductase
APS	Adenosine-phosphosulphate
BCA	β-cyano-L-alanine
CAT	Cysteine aminotransferase
CBS	Cystathionine β-synthase
CD	Crohn's disease
CRC	Colorectal cancer
CRD	Colorectal distension
CSE	Cystathionine γ-lyase
CT	Cholera toxin
CVH	Chronic visceral hyperalgesia
DADS	Diallyl disulfide
DAO	D-amino acid oxidase
DRG	Dorsal root ganglia
Dsr	Dissimilatory sulfite reductase
DSS	Dextran sodium sulfate
FD	Functional dyspepsia
FGID	Functional gastrointestinal diseases
H_2S	Hydrogen sulfide
HAEC	Congenital megacolon-associated enterocolitis
HSCR	Congenital hirschsprung disease
IBS	Irritable bowel syndrome
ICCs	Interstitial cells of Cajal
IJP	Inhibitory junction potential
ISCs	Intestinal stem cells
LDHA	Lactate dehydrogenase A
L-NAME	N(G)-Nitro-L-Arginine Methyl Ester
MDSCs	Marrow-derived suppressor cells
MLCP	Myosin light chain phosphatase
MP	Myenteric plexus
NANC	Noncholinergic and nonadrenergic
NCI	Neonatal colitis
NMD	Neonatal maternal deprivation
NOS	Nitric oxide synthase
NSAID	Non-steroidal anti-inflammatory drugs
PAG	dl-propargylglycine
SMP	Submuscular plexus
SQR	Sulfide quinone oxidoreductase
SRB	Sulfate-reducing bacteria
TEA	Tetraethylamine
TLR	Toll-like receptor
TRPA1	Transient receptor potential ankyrin 1 channel
TRPV1	Transient receptor potential vanilloid 1
UC	Ulcerative colitis

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1 Introduction

Hydrogen sulfide (H_2S) is a gas with a pungent odor. Twenty years ago, it was discovered as a physiological mediator. Later, H_2S-producing

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enzymes, including cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), 3-mercaptopyruvate sulfurtransferase (3-MST), etc., have been found in the brain, angiocarpy, kidney, gastrointestinal tract, and many other tissues and organs of mammals. These enzymes are involved in the endogenous production of H_2S in the body. But among these tissues and organs, the gastrointestinal tract is special because there are a variety of commensal microorganisms in the gastrointestinal tract of humans and other animals, and some of them can also produce H_2S by using sulfides produced in human food catabolism as raw materials. We call them microbial-derived H_2S (Fig. 1). In recent years, targeting the source and mechanism of H_2S in the gastrointestinal tract, a lot of research and progress has been made on the physiological regulation of H_2S on the intestine and the interaction between H_2S, intestinal microorganisms, and their hosts. Closely related to clinical practice, some of these studies have provided credible evidence and new theoretical foundations for more comprehensively and deeply revealing the occurrence, development process, and mechanism of diseases.

2 H_2S Production and Mechanisms in the Gastrointestinal Tract

2.1 H_2S Produced by Gastrointestinal Tissues

As is already known, the endogenous H_2S is produced from L-cysteine by two pyridoxal 5-′-phosphate (PLP)-dependent enzymes, cystathionine β-synthase (CBS), and cystathionine γ-lyase (CSE) and PLP-independent 3-mercaptopyruvate sulfurtransferase (3-MST) [1, 2]. Shibuya, N et al. reported a pathway involved in the production of H_2S by 3-MST and D-amino acid oxidase (DAO) in the mammalian cerebellum and kidney cells [3]. It is currently known that H_2S can be synthesized in many tissues of several mammals including the gastrointestinal tract. It has a wide range of effects on different target organs and tissues. So far, protein expressions of CBS and CSE have been observed in human gastric [4] and colon [5] tissues, and in the entire gastrointestinal tract (stomach, duodenum, jejunum, ileum, colon) of rats [5]. In addition, mRNA and protein expressions of 3-MST have been observed in rat stomach [6], and DAO expression in rat jejunum [7]. Protein expressions of CBS and CSE have been observed in the stomach [4], jejunum [8], ileum [9], and colon [10] of mice. Additionally, D-cysteine and DAO-dependent H_2S production pathway has been observed in the stomach of mice [11]; mRNAs of CBS, CSE, and 3-MST have also been found in chicken intestinal tissues [12] (shown in Table 1).

It was found in our previous studies that the protein expressions of CBS and CSE were present in the gastroscopy biopsies of normal healthy volunteers and patients with functional dyspepsia (FD), and the CBS content of FD patients was significantly lower than that of normal controls [4]. It is suggested that there may be a correlation between the abnormality of the H_2S production pathway and the pathogenesis of FD. Whether the abnormal H_2S synthesis is involved in the pathogenesis of FD or FD causes the disorder of H_2S synthesis requires further research. This evidence suggests that the H_2S-producing enzymes CBS, CSE, 3-MST, and DAO are structurally expressed in the gastrointestinal tract, but their expression is highly species-specific and tissue-specific.

In mammals and some other animals, the methionine contained in their food is partly converted into homocysteine through the methionine cycle. The latter is an important intermediate product in methionine metabolism. CBS and CSE then catalyze homocysteine to cystathionine and release H_2S through two different pathways. The first pathway is to link homocysteine with serine [13], and the second with cysteine [14]. Cystathionine is catalyzed by CSE in an α, γ-elimination reaction to produce cysteine, which, together with α-ketobutyrate, produces 3-mercaptopyruvate (3-MP) under the action of cysteine aminotransferase (CAT). Then the sulfur atom is transferred to the receptor under the action of 3-MST before H_2S is released [15]. In the

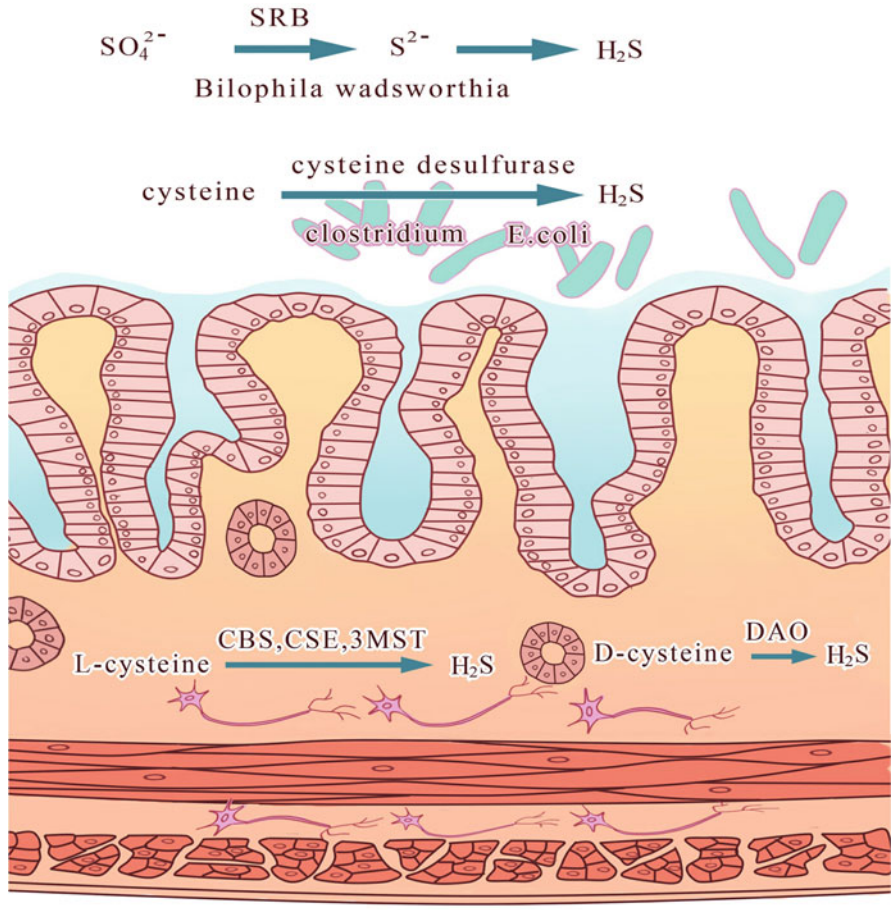

Fig. 1 H_2S production in the gastrointestinal tract. There are two main sources of hydrogen sulfide in the gastrointestinal tract: H_2S-producing enzymes in the gastrointestinal tract, including cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), 3-mercaptopyruvate sulfurtransferase (3-MST), D-amino acid oxidase (DAO) synthesize H_2S with L-cysteine or D-cysteine as substrate; another part of gastrointestinal H_2S is derived from the fermentation and metabolic processes of intestinal sulfate-reducing bacteria (SRB) on the inorganic sulfate and sulfite in unabsorbed food residues

whole process, the roles of CBS and CSE are diverse. They catalyze various reactions starting from cystine and cysteine with the release of H_2S [16, 17], including catalyzing homocysteine to form homomethionine by γ-replacement, catalyzing homocysteine to form α-Ketobutyrate by α, γ-elimination, catalyzing cysteine to form serine or lanthionine by β-replacement, catalyzing cysteine and cystine to form pyruvate by α, β-elimination. H_2S entering the mitochondrial matrix is further metabolized into sulfate and thiosulfate through sulfide quinone oxidoreductase (SQR), persulfide dioxygenase (ETHE1), sulfite oxidase, and rhodanese [18].

It has been reported that CBS and CSE are mainly distributed in the cytoplasm, and some studies have shown that CBS post-translationally modified by the small ubiquitin-like modifier-1 can be shuttled into the nucleus where it associates with the nuclear scaffold [19]. 3-MST is distributed in the cytoplasm and mitochondria. Since the concentration of cysteine in mitochondria is significantly higher than that in the cytoplasm, the catalytic process of 3-MST

Table 1 Expression of CBS, CSE, 3-MST, DAO in gastrointestinal tract of different species

Species and intestinal tract	CBS	CSE	3-MST	DAO	References
Human stomach	Yes	Yes	Unknown	Unknown	[4]
Human duodenum	Unknown	Unknown	Unknown	Unknown	
Human jejunum	Unknown	Unknown	Unknown	Unknown	
Human ileum	Unknown	Unknown	Unknown	Unknown	
Human colon	Yes	Yes	Unknown	Unknown	[5]
Rat stomach	Yes	Yes	Yes	Unknown	[5, 6]
Rat duodenum	Yes	Yes	Unknown	Unknown	[5]
Rat jejunum	Yes	Yes	Unknown	Yes	[5, 7]
Rat ileum	Yes	Yes	Unknown	Unknown	[5]
Rat colon	Yes	Yes	Unknown	Unknown	[5]
Mouse stomach	Yes	Yes	Unknown	Yes	[4, 11]
Mouse duodenum	Unknown	Unknown	Unknown	Unknown	
Mouse jejunum	Yes	Yes	Unknown	Unknown	[8]
Mouse ileum	Yes	Yes	Unknown	Unknown	[9]
Mouse colon	Yes	Yes	Unknown	Unknown	[10]
Chicken small intestine	Yes	Yes	Yes	Unknown	[12]

may mostly occur in mitochondria [20]. The oxidative metabolism of sulfide occurs in the mitochondria of mammals, and the oxidation pathway is highly sensitive to H_2S concentration [21]. DAO that catalyzes D-cysteine to produce 3-MP is present in the peroxisome [22], and 3-MST is mainly located in the mitochondria [15]. Mitochondria and peroxisomes exchange multiple metabolites through a special form of vesicle trafficking [23], which makes it possible for the two enzymes, DAO and 3-MST, to produce H_2S through the interaction of these two organelles (Fig. 2).

2.2 Gastrointestinal Microorganisms-Derived H_2S

Another part of gastrointestinal H_2S is derived from the fermentation and metabolic processes of intestinal sulfate-reducing bacteria (SRB) on the inorganic sulfate and sulfite in unabsorbed food residues.

The digestion methods currently known in mammals include physical digestion, chemical digestion, and microbial digestion. One of the main functions of the human intestinal microbiota is to help obtain nutrients and energy from our diet. This is one of the important foundations for how we can live in harmony with them. Food residues unabsorbed by the proximal intestine are transported to the distal intestine, where they undergo primary fermentation to produce short-chain fatty acids (such as acetate, propionate, and butyrate) and gases (such as H_2 and CO_2). These fermentation products not only are the source of carbon, nitrogen, and energy for the microorganisms but also affect the host. In addition to maintaining the redox balance as much as possible during the fermentation process, it is also necessary to maximize the energy produced [24]. In the human intestine, H_2-consuming microorganisms include methanogens, acetogens, and SRB, which produce methane, acetate, and H_2S, respectively [25].

SRB, a type of anaerobic microorganisms including bacteria and archaea, uses sulfate as the electron acceptor of the respiratory chain terminal to oxidize organic matter and hydrogen to obtain energy. They are widely distributed in the digestive tract of humans and animals. Most of the SRBs currently found belong to the Deltaproteobacteria, and the main species of intestinal SRBs belong to the Desulfovibrionaceae family, Deltaproteobacteria class. Up to now, isolated from intestinal samples and identified by PCR technology, they are comprised of Desulfovibrio desulfuricans and Desulfovibrio intestinalis,

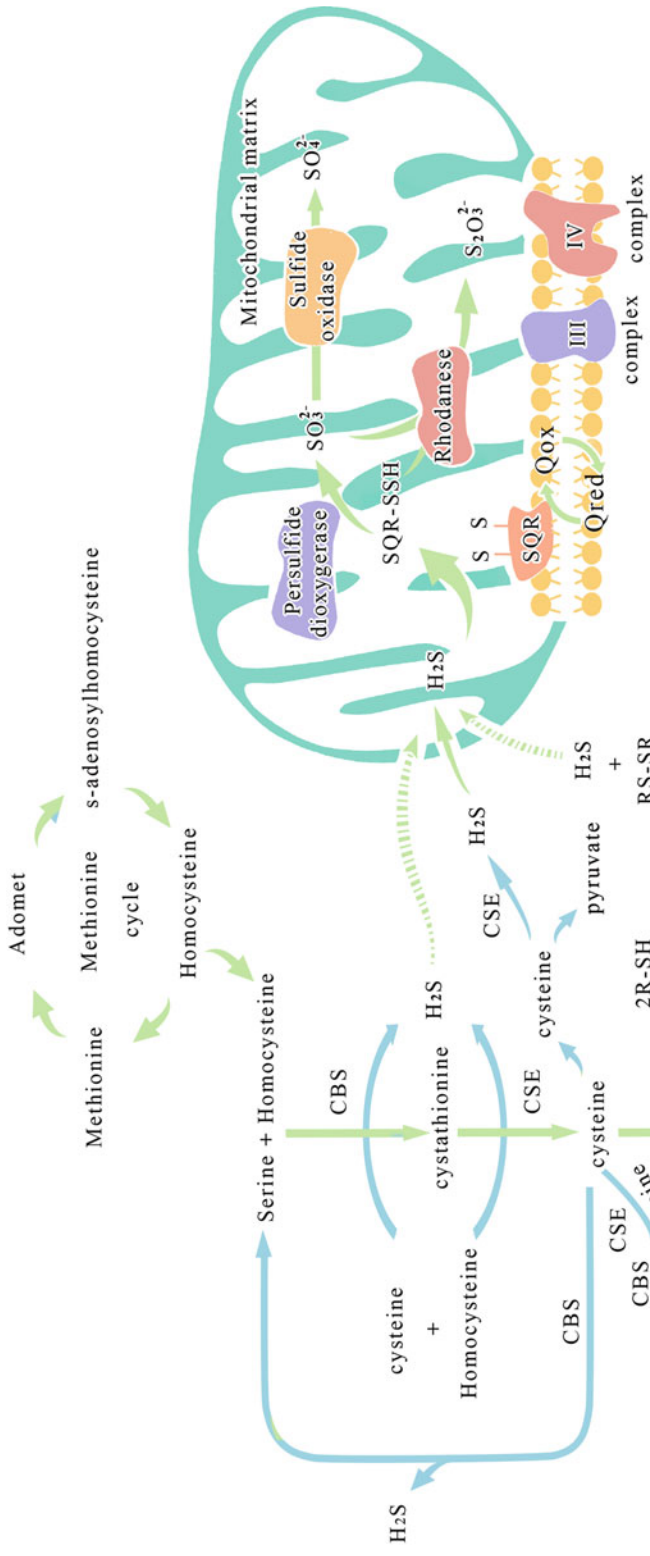

Fig. 2 Mechanism of hydrogen sulfide formation catalyzed by enzymes. Methionine contained in food is partly converted into homocysteine through the methionine cycle. CBS and CSE then catalyze homocysteine to cystathionine and release H_2S through two different pathways. The first pathway is to link homocysteine with serine, and the second with cysteine. In the whole process, CBS and CSE catalyze various reactions starting from cystine and cysteine with the release of H_2S. H_2S entering the mitochondrial matrix is further metabolized into sulfate and thiosulfate through sulfide quinone oxidoreductase (SQR), persulfide dioxygenase (ETHE1), sulfite oxidase, and rhodanese

Desulfovibrio fairfieldensis, Desulfomonas pigra, Desulfovibrio Vulgaris. Among them, Desulfovibrio desulfuricans, Desulfovibrio fairfieldensis, Desulfomonas pigra are isolated from the human intestine and distributed in the colon and rectum of healthy people and patients with colitis [26–29].

SRB, also called dissimilatory sulfate-reducing prokaryotes, basically features the reduction of sulfate to sulfur ions employing dissimilatory reduction, that is, using sulfate as the terminal electron acceptor of the respiratory chain. Although the mechanism of sulfate reduction has not been fully elucidated, what has been observed is that it follows at least three steps: sulfate first binds ATP to form adenosine-phosphosulphate (APS), and then forms sulfite under the catalysis of adenosine-5-′-phosphosulfate reductase (Apr), and lastly forms sulfur ion through a series of electron transfer processes under the catalysis of dissimilatory sulfite reductase (Dsr) [30].

SRB has a wide range of electron donors, among which short-chain fatty acids are commonly seen. For example, lactic acid, butyric acid, malic acid, propionic acid, fumaric acid, etc. Amino acids, ethanol, and hydrogen can also be used as respiratory electron donors for SRB [31]. Many Desulfovibrio can use hydrogen as the only energy source, indicating that the oxidation of hydrogen with sulfite as the terminal electron acceptor is an energy-saving mechanism [32].

In addition to SRB, other bacterial species in the colon cavity can convert cysteine to H_2S [33]. Many bacterial groups, including Clostridium, Escherichia, Salmonella, Klebsiella, Streptococcus, and Enterobacter convert cysteine to H_2S, pyruvate, and ammonia by activating cysteine desulfurase [34].

Recent studies have shown that Bilophila Wadsworth, the anaerobic opportunistic pathogen in the human intestinal microorganism, can convert taurine (2-aminoethanesulfoniate), a microbial substrate rich in the intestine, into H_2S. A key enzyme in this pathway is isethionate sulfite-lyase, a member of the glycyl radical enzyme family. This enzyme catalyzes a new, radical-based C-S bond-rupture reaction to convert isethionate (2-hydroxyethanesulfonate) to sulfite and acetaldehyde [35].

3 Physiological Effects of H_2S on the Gastrointestinal Tract

3.1 H_2S Regulation Over Gastrointestinal Motility

3.1.1 H_2S Regulatory Targets for Gastrointestinal Motility

Regarding the role of H_2S in regulating gastrointestinal motility, in some reports, it can promote contraction of the gastrointestinal tract [36, 37], but in more reports, it promotes relaxation instead. The targets of H_2S may include gastrointestinal smooth muscle, interstitial cells of Cajal (ICCs), and enteric inhibitory neurons.

H_2S of physiological concentrations can inhibit the spontaneous contraction of gastric and jejunal smooth muscle in rats, reduce the frequency and amplitude of smooth muscle contraction [38]. ACh-mediated contraction of the guinea-pig ileum circular muscle can be concentration-dependently relaxed by sodium hydrosulfide (NaHS), a donor of H_2S [2]. NaHS can also exert an inhibitory effect on smooth muscles (human, rat, and mouse colons) and jejunum (mouse) [39]. NaHS reduces the contraction of the circular muscle strips of the mouse gastric fundus and distal colon caused by $PGF_{2\alpha}$ in a concentration-dependent manner; potassium channel blockers such as glibenclamide, apamin, and scorpion venom cannot affect this effect of NaHS [40]. In one of our studies, it was found that NaSH or L-cysteine could relax muscle strips in the gastric body or fundus of mice. In the in vivo experiments, the mouse gastric fundus pretreated with L-cysteine (1 mM) intraperitoneal injection showed a significant increase in compliance with dilation stimuli, and the CBS inhibitor aminooxy acetic acid (AOAA) rather than CSE inhibitor dl-propargylglycine (PAG) can reverse this effect. At the same time, we found that the expression of CBS proteins in gastric fundus tissues showed a change of first increase and

then decrease with the extension of feeding time (0–30 min) after fasting, suggesting that H_2S as a gas transmitter participates in the regulation of gastric diastolic function [4].

In the gastrointestinal tract, smooth muscle function can be indirectly regulated by ICCs, which can generate electrical signals that are transmitted to smooth muscle cells through gap junctions. There is a discrepancy in whether CBS and CSE are expressed on ICCs. There are reports on the presence of CSE immunohistochemical staining in ICCs of guinea-pig colon [41], and also no presence of mRNAs of CBS or CSE in ICCs of mouse colon. However, NaHS can indeed affect ICCs, no matter whether ICCs can release H_2S or not [39]. NaHS (0.1–0.3 mM) can depolarize ICCs and non-selectively activate cation channels, thereby activating pacemaker cells and slightly increasing spontaneous calcium waves; while NaHS (1 mM) can activate K_{ATP} channels, and increase mitochondrial calcium uptake, thereby inhibiting ICCs. None of the above effects can be blocked by adenylate cyclase (AC) inhibitor ODQ or nitric oxide synthase (NOS) inhibitor N(G)-Nitro-L-Arginine Methyl Ester (L-NAME) [42]. Electrophysiological experiments and intracellular calcium analysis show that high-concentration NaHS (0.5-1 mM) can inhibit the pacing current in ICCs isolated and cultured from the small intestine of mice [42]. However, in the presence of low-concentration NO, low-concentration NaHS will enhance the inhibitory effect of NO on the pacemaker system, indicating that there may be interactions between these two gas transmitters [43]. H_2S also has different effects on intestinal motility patterns. The video recordings revealed two rat colon motor patterns: low-frequency high-amplitude propulsive ripples and high-frequency low-amplitude non-propulsive ripples. Propulsive ripples drain the contents to the distal end of the colon, while non-propulsive ripples may participate in mixed and segmented motor patterns to promote intestinal absorption. These two motor patterns may be related to pacemaker cells in the submuscular plexus (ICC-SMP) and the myenteric plexus (ICC-MP) of the colon wall [44]. The administration of 1 mM NaHS on the serosa side does not alter the amplitude, duration, or frequency of slow-wave activity produced by ICC-SMP in full thickness rat colon specimens, but can inhibit the rhythmic propulsive motor complexes produced by ICC-MP in the colon [45].

In rats, cyclic depolarizations and slow waves generate myogenic contractions of low frequency and high frequency, respectively. ICCs located near the submuscular plexus (SMP) generate slow waves. Inhibitory junction potential (IJP) consists of a fast purinergic inhibitory junction potential (IJPf) followed by a slow nitrergic inhibitory junction potential (IJPs) component leading to relaxation. According to the study of electric field stimulation of different programs, it is found that for tissues devoid of ICC-SMP, single pulse protocols increase purinergic and nitrergic IJP amplitude in a voltage-dependent manner. In tissues with intact ICC-SMP, nitrergic and purinergic responses are reduced and slow waves maintain their intrinsic frequency and increase their amplitude under nerve-mediated hyperpolarization, indicating that inhibitory neurons modulate colonic spontaneous motility and the principles determining post-junctional responses are the frequency of firing that determines the neurotransmitter/receptor involved, the transwell gradient and the origin and nature of each myogenic activity [46]. Motor neurons in the intestinal wall can spontaneously release the inhibitory neurotransmitter NO in vitro, so when intestinal plexus blocker tetrodotoxin (TTX) is added to the incubation solution, the frequency, and amplitude of smooth muscle contraction increase because TTX blocks the release of inhibitory transmitters [47]. Incubated with PAG and AOAA, human colonic smooth muscle samples can undergo depolarization. In the presence of TTX, NaHS can decrease the spontaneous contraction of smooth muscle in a concentration-dependent manner and block nerve-mediated increased frequency and amplitude of cholinergic and

tachykinin contractions reversibly [48]. These results suggest that H_2S can regulate intestinal mobility by promoting the function of enteric inhibitory neurons.

3.1.2 Regulatory Mechanism of H_2S on Gastrointestinal Motility

Regulation of H_2S over gastrointestinal motility involves a variety of intracellular mechanisms, including concentration changes in cyclic guanosine monophosphate (cGMP), cyclic adenosine monophosphate (cAMP), and cell membrane hyperpolarization caused by direct or indirect activation of K^+ channels [49].

NaHS (10–100 μM) reverses the vasodilation caused by isoproterenol and salbutamol in a concentration-dependent manner, and significantly reduces the accumulation of cAMP in vascular smooth muscle cells induced by forskolin. However, in the presence of NOS inhibitor L-NAME (100 μM), or the aortic ring endothelial exfoliation, NaHS-induced vasoconstriction weaken, Glibenclamide (10 μM) cannot reduce the blood vessel contraction caused by NaHS, proving that the vasoconstriction effect of H_2S involves the AC/cAMP pathway [50], and also suggesting that there is an interaction between H_2S and NO in the regulation of vascular smooth muscle cell contraction.

When aortic tissues are exposed to CSE inhibitor PAG or β-cyano-L-alanine (BCA), the cGMP content decrease, and when CSE expression is silenced by siRNA transfection, intracellular cGMP content also decrease, confirming that endogenous H_2S promotes cGMP accumulation, and the non-selective phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine greatly reduces this effect of H_2S. These findings provide evidence for H_2S as an inhibitor of phosphodiesterase (PDE) activity [51]. In colonic smooth muscle from rabbit, mouse, and human, endogenous and exogenous H_2S inhibits PDE5 activity and increases the level of phosphorylation of cGMP and inositol triphosphate (IP3) receptor. In turn, the cGMP/PKG pathway can increase CSE-derived H_2S and the level of NO-induced cGMP, suggesting a new model of interaction between NO and H_2S in colonic smooth muscle cells [52].

The action of H_2S on different ion channels in the cell membrane is also one of its important mechanisms for regulating gastrointestinal motility. NaHS has a dual effect on the spontaneous contraction of guinea-pig gastric antrum muscle strips: the excitatory effect mediated by inhibiting the K_V channel, and the inhibitory effect mediated by activating the K_{ATP} channel [53]. H_2S dose-dependently inhibits contraction of rat jejunal circular muscles, in part through the K_{ATP} channel and myosin light chain phosphatase (MLCP) rather than the exogenous or enteric nervous system, visceral afferent nerve, NO, K_{Ca} channel [54]. In our previous research, it was found that H_2S can induce biphasic effects on rat duodenal intestinal motility: the transient excitatory effect might be mediated by activation of transient receptor potential vanilloid 1 (TRPV1) channels in sensory nerve terminals with the consequent release of substance P. The long-lasting inhibitory effect might be mediated by the activation of K_{ATP} channels in the smooth muscle cells [55]. H_2S regulates the contractile activity of the colonic smooth muscle, potentially by modifying the sulfhydryl groups of L-type calcium channels [56]. In addition, the ionic mechanism of H_2S also involves K_{ATP} and small conductance calcium-activated potassium channels ($K_{Ca}2.2$/$K_{Ca}2.3$; SKCa) [39, 45], Nav1.5 voltage-dependent Na^+ channels [57], TRPV1, TRPA1 cation channels [41, 55, 58], and large conducting calcium-activated potassium channels BKCa [59].

However, inconsistent with the negative regulatory effect of H_2S on gastrointestinal motility reported by most studies, there are also reports claiming that exogenous H_2S sulfide Kv4.3 can depolarize cell membranes of gastric antrum smooth muscle, thereby increasing its basic tension [36]. Low-concentration H_2S depolarizes the cell membrane by inhibiting the Kv current, resulting in an increase in L-type Ca^{2+} current, intracellular calcium ions, and the tension of the gastric fundus smooth muscle cells in mice [37].

Smooth muscle contraction is calmodulin-dependent because of the activation of myosin

light chain kinase and inhibition of MLCP. NaHS can relax the contraction of mouse gastric fundus muscle strips caused by $PGF_{2\alpha}$ in a concentration-dependent manner, and the MLCP inhibitor calyculin A reduces NaHS-induced relaxation, suggesting that H_2S at least in part causes gastric fundus relaxation in mice by activating MLCP [40]. Exogenous (NaHS) and endogenous (L-cysteine) H_2S reduce the contraction induced by carbachol in isolated rabbit gastric smooth muscle cells. The reason is that the dephosphorylation of the myosin light chain caused by the activation of MLCP and the inhibition of Rho kinase and PKC activity [60]. H_2S induces the sulfur hydration reaction of RhoA, which is related to the inhibition of RhoA activity. It is speculated that the sulfidation of RhoA by H_2S results in the inhibition of RhoA and Rho kinase activity and muscle relaxation [60, 61].

The sulfhydrylation of proteins that regulate different functions and the production of polysulfides may to some extent reveals the reason for the diverse mechanisms of action of H_2S [62, 63]. Not only that, noncholinergic and nonadrenergic (NANC) inhibitory neurotransmitters released by the enteric nerve include H_2S, NO, ATP, vasoactive intestinal peptide (VIP), CO, and so on. In several physiological digestive reflexes that cause smooth muscle relaxation in the gastrointestinal tract, it is common that multiple NANC transmitters work in concert [49].

3.2 Regulation of H_2S Over Gastrointestinal Sensation

The sensation produced by the afferent impulse of the visceral receptor is called visceral sensation. The suitable stimulus of the visceral receptor is the natural one in the body. The afferent impulse of the visceral receptor, which causes a variety of reflex activities, plays an important role in the regulation of visceral function. Visceral afferent impulses are relatively vague, diffuse, difficult to accurately locate, and generally does not produce consciousness. The number of sensory nerve fibers in the viscera, mixed in sympathetic and parasympathetic nerves, is less than that on the general surface. Afferent impulses enter the spinal cord along the nerves or the brain stem along the cranial nerves, causing corresponding reflex activities. Visceral pain is present in almost all parts of the body. These parts, though sensitive to tension and pressure, show no reaction to incised wounds.

Given the above characteristics of visceral sensation, many studies on H_2S have focused on its role in visceral nociceptive sensation. According to different animal models and ways of administration, H_2S has been reported to exert either pronociceptive or antinociceptive effects in the gastrointestinal tract [64]. It produces analgesic effects in rodent visceral pain models by activating K_{ATP} channels and causes transactivation and internalization of the mu-opioid receptor (MOR) in neuron-like cells through a mechanism that requires phosphorylation of protein kinase B (AKT), suggesting that the analgesic effect of H_2S involves opioid receptors [65]. Our recent research has revealed that H_2S downregulates colonic afferent nerve impulses in mice through a neuronal nitric oxide synthase (nNOS)-dependent mechanism. NaHS can significantly reduce the autonomous discharge of the colonic afferent nerves. The non-specific K^+ channel inhibitor tetraethylamine (TEA) can reduce the relaxing effect of NaHS on intestinal smooth muscle, but it does not block its inhibition of afferent neural activity. The inhibitory effect of NaHS on the intestinal afferent nerve can be eliminated by NOS inhibitor L-NAME (1 mM), specific nNOS inhibitor NPLA (1μM), or N-type Ca^{2+} channel blocker ω-conotoxin GVAS (1μM). Compared with wild-type mice, CBS +/− mice are more sensitive to colorectal distension (CRD) [66].

Many of the currently known studies are about the role and mechanism of H_2S in promoting visceral nociception. In the mouse colon, the role of luminal H_2S in promoting visceral nociception may be related to activation or sensitization of T-type Ca^{2+} channels in primary afferent neurons [67]. In an irritable bowel syndrome (IBS)-like rat model with chronic visceral hyperalgesia (CVH), CBS expression was

significantly increased in colon dorsal root ganglia (DRGs), and CBS inhibitors markedly attenuated the abdominal withdrawal reflex scores in response to colorectal distension in rats with CVH [68]. The rapid phosphorylation of extracellular signal-regulated kinase (ERK) in the spinal dorsal horn is often used as a marker for spinal neurons after somatic and colonic nociception [69]. By giving mibefradil, a strict T-type Ca^{2+} channel blocker, the colonic pain, and hyperalgesia in mice caused by H_2S can be eliminated. $ZnCl_2$ can inhibit Cav3.2, but not Cav3.1 and Cav3.3. When two Zn^{2+} chelators of TPEN and diisoglyceric acid are administered intracolonally, the pain-inducing effect induced by NaHS can be mimicked, suggesting that H_2S and other Zn^{2+} chelators can clear Zn^{2+} on the cavity surface and cause colonic pain by activating T-type Ca^{2+} Channel, which is likely to be Cav3.2 subtype [70]. In the rat colon, luminal H_2S can activate the upregulated Cav3.2 channel in early colitis and cause excitement of sensory nerves, resulting in protective effects on colonic mucosal cells [71]. Studies have also reported that NaHS/H_2S-induced mechanical hyperalgesia in mice requires both Cav3.2 channels and activation of transient receptor potential ankyrin 1 channel (TRPA1) [72]. Neuropathic pain induced by anticancer drug paclitaxel may also be related to the activation of T-type Ca^{2+} channels and increased CSE activity, rather than upregulation of protein levels [73]. In the CVH model caused by neonatal maternal deprivation (NMD), the expression of CBS in colon-associated DRGs was significantly increased, and its change was quite consistent with the process of enhanced visceral movement caused by CRD. The CBS inhibitor AOAA produces antinociceptive effects in a dose-dependent manner. The P65 content in the nucleus of NMD rats is increased. Intrathecal injection of the P65 inhibitor PDTC can significantly reduce the expression of CBS and the nociceptive response produced by CRD [74]. The sensitization of Na^+ channels on colon-associated DRG neurons in NMD and neonatal colitis (NCI) rat models may also be mediated by CBS/H_2S [75–77]. In addition, the activation of adrenergic signals and Toll-like receptor 4 (TLR4) and NF-kappaB are also involved in the role of H_2S in visceral hypersensitivity [78, 79]. Recent studies have shown that NaHS can increase the spontaneous excitatory postsynaptic currents of spinal glia neurons through a presynaptic mechanism, thereby regulating the transmission of pain signals [80].

3.3 Effects of H_2S on Gastrointestinal Secretion and Absorption

There are currently some disputes over the role of H_2S in regulating gastrointestinal secretion. Some scholars have suggested that gastrointestinal epithelial cells may defend and eliminate the potential harm of bacterial-derived H_2S by utilizing their electrolytes and fluid transport mechanisms [81]. Krueger et al. studied the effect of NaHS on the secretion of guinea pigs and human colon with Using Chamber and found that TRPV1 blocker: capsazepine, AMG9801, SB705498, BCTC; LY294002 (PI3K inhibitor); SKF96365 (store operated calcium channel blocker); 2-APB (inositol triphosphate blocker) and atropine can significantly reduce NaHS-induced secretion. Neurokinin-1, -3 receptor blockers can inhibit the effect of NaHS in the guinea-pig colon, while neurokinin-1, -2 receptor blockers can restrain NaHS response in the human colon. NaHS and capsaicin show cross-desensitization. NaHS induces capsazepine and LY294002 sensitive afferent discharge. These results suggest that exogenous H_2S evokes mucosal secretion by targeting TRPV1 expressing afferent nerves which activate cholinergic secretomotor neurons in a species dependent manner via neurokinin-1,-2,or-3 receptors [82]. It has also been reported that NaHS mucosal perfusion increases gastric HCO_3^- secretion, which is mediated by capsaicin-sensitive afferent neurons. This process relies on NO and prostaglandins, not K_{ATP} channels [83]. Interestingly, intraperitoneal injection of NaHS can significantly reduce gastric juice pH; K_{ATP} channel blocker glibenclamide and AC inhibitor SQ22536 can eliminate its effects: indicating that NaHS can act on the K_{ATP} channel and activate the AC/cAMP

pathway to promote gastric acid secretion [84]. Endogenous and exogenous H_2S can reduce intestinal fluid secretion and Cl-loss caused by cholera toxin (CT). The CSE inhibitor PAG reverses the effect of L-cysteine but cannot reverse that of AC inhibitor SQ22536, cAMP inhibitor bupivacaine, PKA inhibitor KT-5720, and AMPK inhibitor AICAR, suggesting that the anti-CT-induced diarrhea mechanism of H_2S in mice may not include the AC/cAMP/PKA pathway and AMPK [85].

It has been reported that H_2S can activate K^+ channels to exert its physiological role. A previous study of ours tried to find out whether H_2S acts on the K^+ channels on the basal surface of jejunal epithelial cells to hyperpolarize the cell membrane and then regulates the Na^+ coupled nutrient transport on the mucosal side [8]. In order to test our hypothesis, we performed Western Blot and immunohistochemistry experiments and detected the expression of CBS and CSE protein levels in rat jejunum with serosa and muscularis removed. In the immunohistochemical specimens of rat jejunum, CBS and CSE localization showed certain specificity: CBS was localized in the jejunal villus tip and crypt cells, while CSE mainly in crypt cells (Fig. 3a, b) [8]. L-cysteine (10 mM) can significantly increase the amount of H_2S production in rat jejunal mucosal homogenate, and this effect can be dose-dependently attenuated by the CBS inhibitor AOAA rather than the CSE inhibitor PAG (Fig. 3c–f) [8].

Through Using Chamber, we recorded the basal short-circuit current (Isc) of the rat jejunal mucosa and compared the short-circuit current after L-alanine and glucose were added with the basic ones. It was found that L-cysteine can significantly increase Isc (the change of which represents the combined transepithelial transport of Na^+ and L-alanine). The effect of L-cysteine is largely suppressed by the CBS inhibitor AOAA, rather than the CSE inhibitor PAG, which is similar to what we previously observed in the H_2S generation experiments (Fig. 4a–f) [8]. The non-selective K^+ channel blocker glibenclamide can block the effect of L-cysteine (Fig. 4g) [8]. AOAA, PAG, L-serine does not affect L-alanine transport (Fig. 3h–k) [8]. L-cysteine also enhances glucose transport by jejunal epithelium, and the combined application of AOAA and PAG can significantly inhibit its effect (Fig. 5) [8]. In a rat oral glucose tolerance test (OGTT), intraperitoneal injection of L-cysteine can significantly increase blood glucose in the tail and hepatic portal veins of mice after oral glucose administration for 30 min. AOAA can significantly inhibit this effect of L-cysteine and reduce blood glucose at 30 min after oral glucose administration (Fig. 6) [8]. Our results show that L-cysteine may activate basal K^+ channels through CBS-derived H_2S pathways to promote jejunal nutrient absorption. Based on these findings, we believe that the therapeutic modification of H_2S signals may be an attractive approach for the treatment of future nutrition-related diseases.

3.4 Regulation of H_2S on Gastrointestinal Endocrine Cells

In the past ten years, gastrointestinal microbes have become potential regulators of the body metabolism, partly due to the endocrine cells and hormones that are of considerable species and quantity in the gastrointestinal tract. Some of these hormones are distributed in both the gastrointestinal tract and the central nervous system. The intestinal microbiota and its fermentation products can intervene in the enteroendocrine system during colonization. For example, short-chain fatty acids significantly increase glucagon-like peptide 1 (GLP-1) in human and mouse postprandial plasma [86, 87], and indole stimulates GLP-1 secretion in acute treatment in vitro [88].

As mentioned earlier, H_2S as a bacterial metabolite is produced by SRB and some other bacteria. Whether H_2S can regulate the metabolism of the body through intestinal hormones is currently a question of great concern for researchers, because this may provide a very attractive treatment option for the increasing people suffering from metabolic diseases in the world.

By supplementing the diet with prebiotic chondroitin sulfate for 4 weeks, it was found

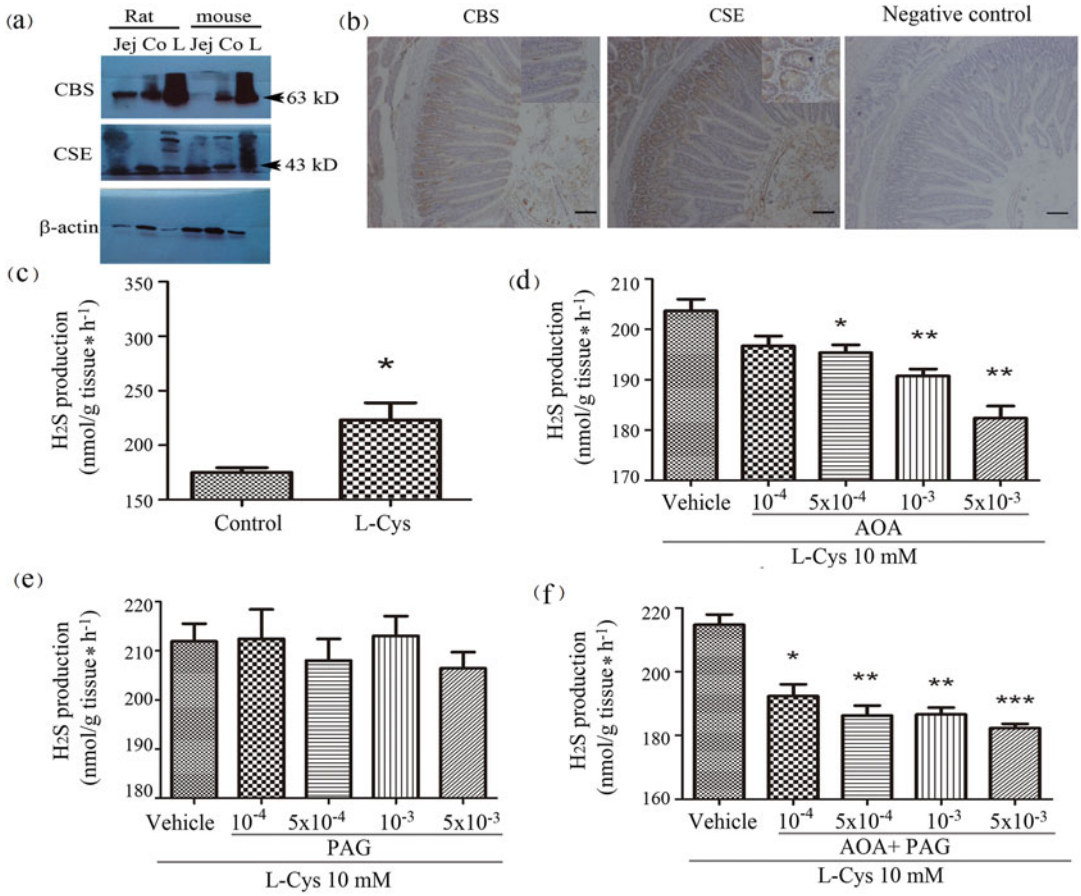

Fig. 3 Existence of an H_2S-generating pathway in intestinal epithelium. (**a**) Representative results of Western blot analysis for cystathionine-b-synthase (CBS) and cystathionine-c-lyase (CSE) in the intestinal epithelium. Jej, jejunum; Co, colon; L, liver. (**b**) Immunochemistry results for CBS and CSE in the rat jejunum, showing that CBS was detected in absorptive cells near the tips of the villi and in crypt cells of the jejunum. Immunoreactivity for CSE was primarily localized to crypt cells of the jejunum. The results shown are from a single experiment and are representative of three different specimens. Scale,100μm. Inset: 200×. (**c**–**f**) Under basal (control) condition, H_2S was generated by the homogenate of rat jejuna mucosa. Incubation of the homogenate with 10 mmol/L L-cysteine (L-Cys) significantly increased H_2S production compared with basal values. Aminooxyacetic acid (AOA), but not D,L-propargylglycine (PAG), significantly inhibited the L-Cys-induced increase in H_2S production. Data represent the mean SEM ($n = 6$ or 8 rats). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with control (From Fig. 1 in Clin Exp Pharmacol Physiol. 2016;43 (5):562–568)

that mice treated with chondroitin sulfate had elevated Desulfovibrio higher levels in the feces and increased colonic and fecal H_2S concentration, enhanced GLP-1 and insulin secretion, improved oral glucose tolerance, and reduced food consumption. H_2S donors (NaHS and GYY4137) directly stimulate GLP-1 secretion in murine L-cells (GLUTag) through the p38 mitogen-activated protein kinase (p38-MAPK) pathway [89]. It was also found that GYY4137 can partially inhibit the secretion of ghrelin in rat primary gastric culture cells through the AKT pathway. CSE immunofluorescence was found throughout the stomach primary culture

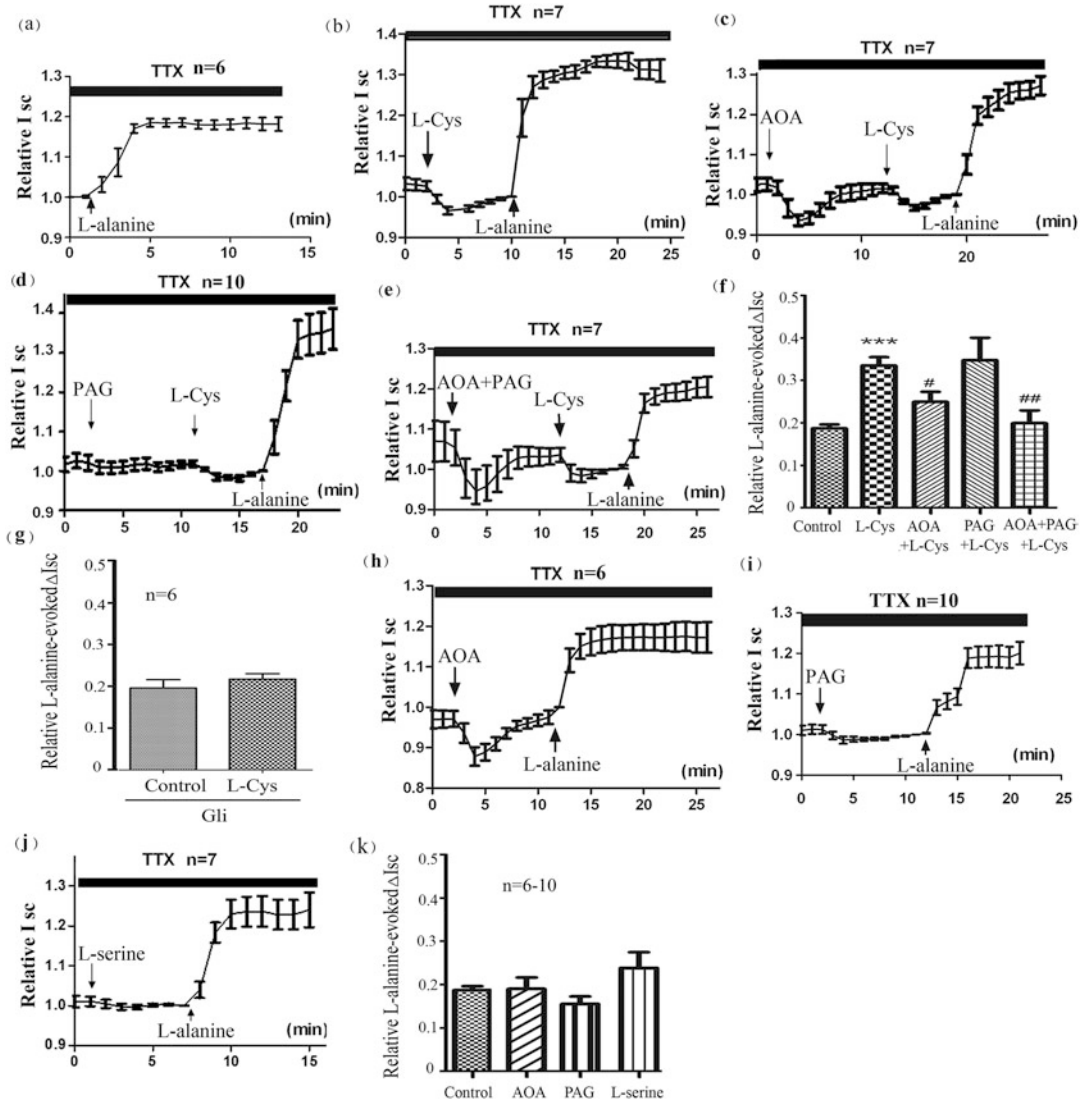

Fig. 4 L-cysteine (L-Cys), from which H_2S can be generated, enhances L-alanine transport in the rat jejunum. (**a**) L-alanine (2×10^{-2} mol/L) induced an increase in the short-circuit current (I_{sc}). The change in I_{sc} represents sodium ion transport coupled with L-alanine transport across the epithelium. (**b**, **f**) Serosal administration of L-Cys (10^{-3} mol/L) enhanced the L-alanine-induced increase in I_{sc}. (**c**–**f**) The effects of L-Cys were largely attenuated by amino-oxyacetic acid (AOA), but not D, L-propargylglycine (PAG). (**g**) Glibenclamide blocked the effects of L-Cys on Na^+–L-alanine cotransport. (**h**, **i**, **k**) Neither AOA nor PAG had any effect on the response to L-alanine. (**j**, **k**) The amino acid L-serine (10^{-3} mol/L) did not exert any L-Cys-like effects on L-alanine-induced increase in $I_{sc.}$ Data are the mean standard error of the mean (SEM). $^{***}P < 0.001$ compared with control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with L-Cys (From Fig. 2 in Clin Exp Pharmacol Physiol. 2016;43 (5):562–568)

preparation. Expression was not restricted to ghrelin producing cells; however, all ghrelin producing cells were positive for CSE. Inhibition of CSE stimulated ghrelin secretion in primary gastric culture. In mice, GYY4137 treatment prolonged the postprandial drop of circulating ghrelin and caused reduced food consumption up to 4 h after treatment. These results suggest

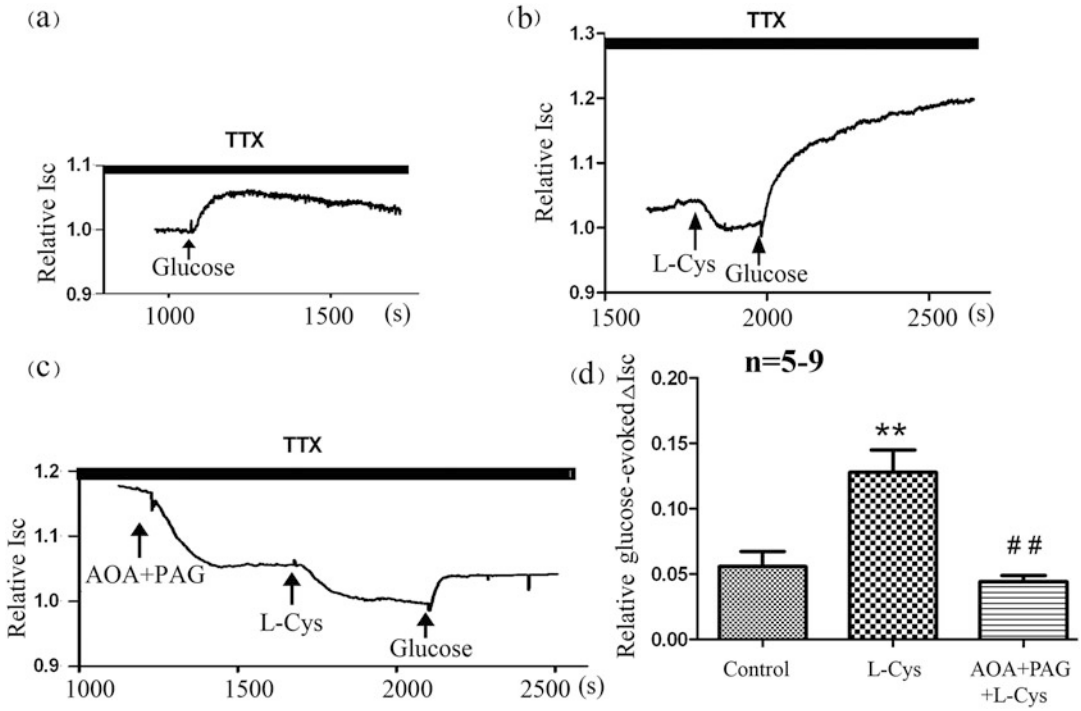

Fig. 5 L-Cysteine (L-Cys), from which H_2S can be generated, augments glucose transport in the rat jejunum. (**a**) Glucose (2×10^{-2} mol/L) increased the short-circuit current (I_{sc}). (**b**) The effects of glucose on I_{sc} were enhanced by L-Cys ($n = 9$), but the effects of L-Cys were attenuated by the administration of (**c**) amino-oxyacetic acid (AOA) and (**d**) D, L-propargylglycine (PAG; $n = 5$). Data are the mean standard error of the mean (SEM). $^{**}P < 0.01$ compared with control; $^{\#\#}P < 0.01$ compared with L-Cys (From Fig. 3 in Clin Exp Pharmacol Physiol. 2016;43 (5):562–568)

that the stomach possesses the ability to generate H_2S, and that ghrelin producing cells may be regulated by this gas in both an autocrine and paracrine manner [90].

3.5 H_2S Regulation Over Gastrointestinal Stem Cells

As H_2S is an ancient gas involved in the origin of life on the earth, it is naturally speculated whether there is a special relationship between it and stem cells as the origin of cells, such as whether H_2S participates in regulating the proliferation and differentiation of stem cells or affects other functions of stem cells.

The current research on the effects of H_2S on stem cells mainly involves mesenchymal stem cells (MSCs) distributed in different tissues. MSCs as progenitor cells can self-renew and also exhibit multilineage differentiation. They can maintain tissue homeostasis by orchestrating several cell types (such as osteoblasts, chondrocytes, muscle cells, and adipocytes).

The results show that endogenous H_2S regulates MSCs through several possible mechanisms. It controls the proliferation and differentiation of neural stem cells by activating ERK1/2 and enhances the proliferation and survival of human-induced pluripotent stem cell-derived MSCs by activating the PI3K/Akt pathway [91].

MSC can produce H_2S, and endogenous H_2S plays a vital role in maintaining MSC function to ensure bone homeostasis [92]. In CBS knockout mice, osteoblast differentiation and proliferation

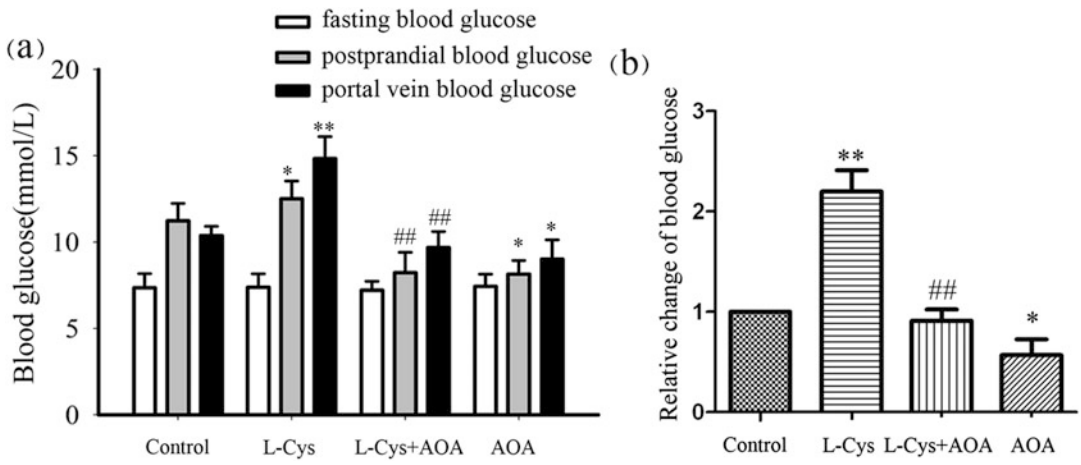

Fig. 6 L-Cysteine(L-Cys) elevates postprandial blood glucose levels in vivo. (**a**) Int4raperitoneal injection of L-Cys (100 mg/kg) significantly increased blood glucose concentration 30 min after oral administration of glucose. This effect was completely inhibited by simultaneous injection of amino-oxyacetic acid (AOA; $n = 5$). AOA alone also decreased blood glucose concentrations 30 min after glucose administration. (**b**) The relative change of blood glucose is calculated with the ratio of the change of portal vein blood glucose after co-administration of glucose and drugs/basal change of portal vein blood glucose after oral administration of glucose. Data are the mean standard error of the mean ($n = 6$). $^{*}P < 0.05$; $^{**}P < 0.01$ compared with control; $^{\#\#}P < 0.01$ compared with L-Cys (From Fig. 4 in Clin Exp Pharmacol Physiol. 2016;43 (5):562–568)

are impaired [93, 94]. Some of the involving mechanisms are abnormal intracellular Ca^{2+} influx caused by decreased sulfhydrylation of the Ca^{2+} transient receptor potential channels (TRPV3, TRPV6, and TRPM4), and impaired Wnt/β-catenin signaling [93]. NaHS, when not reaching the toxic threshold, can reduce human osteoclast differentiation in a dose-dependent manner. The inhibition of human osteoclast differentiation is related to the downregulation of RANKL-dependent intracellular ROS in human osteoclasts [95].

Under ischemic conditions, H_2S has protective effects on MSCs and mature adipocytes, which are mediated by increased expression of anti-apoptotic genes such as Bcl2 [96]. MSCs have the potential to promote heart repair after acute myocardial infarction. However, MSC treatment is limited by stem cell apoptosis after transplantation. It has been shown that hypoxia and serum deficiency can significantly induce MSC apoptosis. CSE overexpression, which enhances the endogenous H_2S level, protects MSCs from apoptosis via attenuation of the mitochondrial injury pathway, inhibition of endoplasmic reticulum stress, and activation of the PI3K/Akt signaling pathway [97]. These findings suggest that the regulation of the CSE/H_2S system may be a therapeutic approach to promote the viability of transplanted MSCs.

Studies on the direct effects of H_2S on gastrointestinal stem cells are currently rare, but we can learn a few facts from several recent studies. In mammalian intestines, stem/progenitor cells are located in intestinal crypts, and many enteric microbial metabolites may be involved in regulating stem/progenitor cell activity. Butyric acid is an important bacterial metabolite that effectively inhibits intestinal stem/progenitor cell proliferation at physiological concentrations. Under normal homeostasis, mucus and colonic epithelium formed barrier is likely to prevent butyric acid from reaching the stem/progenitor cells in the crypt. But when the intestine is acutely injured or damaged by inflammatory bowel disease, the stem/progenitor cells in the body may be exposed to butyrate, leading to inhibited proliferation and delayed wound repair. The mechanism

of action of butyric acid depends on the transcription factor Foxo3. These results indicate that mammalian crypt structure protects stem/progenitors cell proliferation to some extent by consuming butyrate formed a metabolic barrier with differentiated colonic cells [98].

Like butyric acid, H_2S is also one of the metabolites of intestinal microorganisms, and it can pass freely through the cell membrane. Further research needs to be done to learn whether it can reach colonic crypts more easily than butyric acid. Our previous immunohistochemistry confirms that, compared with CBS that is mainly located in the villi, CSE is more likely to be located in the crypt (Fig. 2b). This may provoke us to consider whether both enzyme-derived and microbial-derived H_2S, having the opportunity to contact stem/progenitor cells in the intestinal tract, can further regulate them anyway.

Adjacent to Paneth cells, intestinal stem cells (ISCs) are located at the bottom of the intestinal crypts. They reshape the intestinal epithelium based on dietary signals. A recent study indicates that short-term fasting enhances the organoid-forming ability of ISC alone or when co-cultured with Paneth cells. Gene set enrichment analysis was used to detect nuclear receptor peroxisome proliferator-activated receptor (PPAR) family targets, which contain genes related to fatty acid oxidation (FAO). Researchers have confirmed mRNA and protein expression of selective PPAR targets Pdk4, Cpt1a, and Hmgcs2 in ISCs and crypts, and mRNA levels of Pdk4, Cpt1a, and Hmgcs2 in fasted mice were found to be much higher than those in mice fed ad libitum. Untreated intestinal organoids from fasting mice have a greater ability to self-renew, and the use of the irreversible Cpt1a inhibitor etomoxir can eliminate the promoting effect of fasting on organoid production. There is no difference in the number of primary organoids in each crypt is between wild-type mice and Cpt1a knockout mice fed ad libitum, but there are significant differences between fasted wild mice and Cpt1a knockout mice, indicating that fasting increased stem cell activity in an FAO-dependent manner [99]. However, the effect of short-term fasting on intestinal H_2S and whether H_2S is involved in increased ISC activity caused by fasting remains unknown.

In addition to the above mentioned, H_2S also participates in the regulation of other physiological and pathophysiological functions of the gastrointestinal tract, such as immunity and inflammation. However, because of the source, the form of action, and the complexity of the mechanism of H_2S, it is not appropriate to discuss H_2S alone in separation from intestinal microorganisms and the host gastrointestinal mucosal barrier. So in the next part, we will focus on the interaction between H_2S, intestinal microorganisms, and the host, as well as the correlation between the disrupted dynamic balance between the three and some clinical gastrointestinal diseases.

4 Interaction Among H_2S, Gastrointestinal Microorganisms, and the Host

4.1 Effect of H_2S on Gastrointestinal Microorganisms

At present, we know that the human microbiome is mainly composed of bacteria, fungi, and viruses. They participate in the processing of nutrients and the metabolism of the human body and building the body's protective barrier in cooperation with the immune system. There are more than 160 types of symbiotic bacteria in the human gastrointestinal tract [100]. In nature, bacteria can form biofilms to reduce exposure to drugs and immunity. The gastrointestinal microbes of healthy individuals are mainly derived from four kinds of bacteria: Bacteroides, Trichoderma, Proteus and Actinomycetes [101]. As mentioned earlier, the SRB colonized in the intestinal tract obtains energy by oxidation of organic compounds and reduces sulfate to H_2S.

Intestinal bacteria or host cells produced endogenous or exogenous H_2S, by affecting the intestinal flora in a variety of ways, reduces gastrointestinal inflammation and damage and promotes repair. H_2S can positively affect many aspects of bacterial-epithelial interactions. There

is evidence that H_2S donors may favorably regulate the intestinal flora and promote biofilm formation and integrity, especially in the improvement treatment of diseases such as ulcerative colitis (UC) and Crohn's disease (CD) [102].

To understand the changes in bacterial biofilms in experimental colitis and the effects of endogenous and exogenous H_2S on these changes, researchers used fluorescence in situ hybridization to examine the flora. The results show that in normal mice and rats, the intestinal microbiota is sandwiched between the sterile mucus layer and the feces in a linear structure. In mice and rats with colitis, the bacterial biofilm is severely damaged and the microbiota is chaotic. The biofilm fragments of these pathogenic microorganisms closely adhere to the host tissue and enter the lamina propria, and the depletion of mucus particles in the epithelial cells is also obvious. In CSE knockout mice, the mucus layer is thin, showing mild colitis, and bacteria are in close contact with epithelium. In rats treated with CSE inhibitors, depletion of epithelial mucus particles is more pronounced than that in the control group. After being treated with garlic-derived H_2S donor diallyl disulfide (DADS) for 1 week, the inflammation of the colon subsided, the recovery of biofilm tissue accelerated, and bacteria displacement reduced in rats with colitis. This suggests that CSE-derived H_2S contributes significantly to the formation and stability of colonic microbiota biofilms and helps to enhance epithelial integrity and mucus barrier function [103].

Studies have shown that exogenous H_2S can inhibit the growth of pathogenic bacteria in the cecum of rats with back burns, promote the growth of bifidobacteria and lactic acid bacteria, and help maintain the integrity of the intestinal biological barrier in burned rats [104].

Higher doses of DADS (30 mM/kg) can reduce gastric damage caused by non-steroidal anti-inflammatory drugs (NSAID) [105]. While causing ileal ulcers in rats, NSAID leads to an increase of Escherichia coli, Klebsiella, Proteus, Bacillus, and other Gram-negative bacteria in the intestine by 37.3% [106], and promotes intestinal damage through tumor necrosis factor (TNF-α) and TLR4-mediated pathways [107]. But when given ATB-346, a derivative of naproxen (a traditional NSAID), that can release H_2S, the displacement of intestinal microorganisms reduces [108].

4.2 Effects of H_2S on the Gastrointestinal Tract of the Host

Evidence from experimental models indicates that concentration changes in bacterial metabolites in the large intestine cavity, such as H_2S and butyric acid, may exert an important beneficial or harmful effect on colonocyte metabolism and physiology in aspects of mitochondrial energy metabolism, reactive oxygen production, gene expression, DNA integrity, proliferation, and vitality [109].

There is no clear and consistent evidence on the effect of H_2S on epithelial cell integrity [110]. No damage to the gastrointestinal tract tissue is found after H_2S donors are given to dogs or rodents for 2 weeks. No gastrointestinal epithelial damage and inflammation is observed visually or histologically, even when high doses of DADS (60 mM/kg) are given twice daily for 5 consecutive days [108]. However, the administration of H_2S can maintain the normal structure of mucus and microbiota and the integrity of the mucus barrier [103]. GYY4137 can attenuate the increased permeability of colonic monolayer cells induced by lipopolysaccharide or TNF-a/IFN-g in vitro. Besides, for in vivo models of endotoxemia, intraperitoneal injection of GYY4137 has protective effects on intestinal barrier dysfunction [111]. On the other hand, inhibition of endogenous H_2S production will increase the sensitivity of the mucosa to injury, delay the healing, increase the expression of proinflammatory cytokines, reduce the synthesis of COX-2 and related prostaglandins, and increase the number of mucosal granulocytes [103, 112–114]. H_2S has protective effects on post-cardiac arrest cardiopulmonary resuscitation (CA/CPR)-induced intestinal mucosal damage in

rabbits, and its mechanism may be related to up-regulated tight junction protein ZO-1, alleviated oxidative stress, reduced inflammation, and inhibited apoptosis and autophagy [115]. H_2S has a protective effect on TNF-α/IFN-γ induced Caco-2 monolayer intestinal epithelial barrier impairment, which may be achieved through inhibition of NF-kB P65-mediated myosin light chain phosphorylation signaling pathway [116].

The epithelium and the mucus layer together form the intestinal barrier, serving as the first line of defense against multiple toxic compounds from the intestinal lumen [117]. Mucus is composed of a highly glycosylated polymer network of mucin, which is connected to the intestinal epithelium by disulfide bonds produced by goblet cells. The pore size of this hydrated gel-like structure in the inner mucus layer does not allow bacteria to penetrate. Some studies have reported an increase in SRB content in the intestinal mucosa flora and feces of patients with inflammatory bowel disease (IBD) [118, 119], or compared with controls, specific types of SRB increase [27]. Compared with healthy controls, patients with ulcerative colitis (UC) have increased intestinal SRB-produced sulfide [120]. A recent study shows that sulfide can effectively reduce disulfide bonds, and promote mucin degeneration and entry of microorganisms to the mucus layer [121]. These results suggest that intestinal SRB-produced sulfide will drive mucin degeneration, thereby promoting microorganisms to enter intestinal epithelium. Enhanced microbial pathways lead to bacterial penetration and translocation, which triggers an immune response and helps the initiation and/or development of IBD [122].

The different effects of H_2S on the intestinal epithelium may be partly attributed to the supply and demand relationship between it and the host cell mitochondria. In the presence of sulfide and succinate or L-α-glycerophosphate, colonocyte mitochondria obtain maximum respiratory rates, and this oxidation is accompanied by mitochondrial energization. In contrast, other cell types not naturally exposed to a high concentration of sulfide showed much lower oxidation rates. Mitochondria showed a very high affinity for sulfide that permits its use as an energetic substrate at low micromolar concentrations, hence, below the toxic level. However, if the supply of sulfide exceeds the oxidation rate, poisoning renders mitochondria inefficient [123]. NaHS (20 mM–40 mM) can transiently increase the oxygen consumption of colonic cells through the SQR pathway, but at concentrations higher than 65 mM, NaHS can reduce oxygen consumption by inhibiting mitochondrial cytochrome oxidase C. In rat models fed with high protein, H_2S production in rat colon increases, and correspondingly, the expression of the SQR gene also increases [124]. This indicates that within a certain range, an increase in H_2S in the intestine can promote the function of the H_2S as a detoxifying enzyme system in colonocyte.

4.3 Effects of Microorganisms on the Gastrointestinal Tract of the Host

Decreasing the proportion of protective bacteria like butyrate-producing and other short-chain fatty acid bacteria, and increasing the abundance of pathogenic bacteria, will increase the body's exposure to antigens, thereby inducing the body's immune response, which might be one of the main pathogenesis of gastrointestinal inflammatory or immune diseases [125].

By characterizing the microbial community at the colonic mucosa-lumen interface of newly-onset children with Crohn's disease (CD), it has been found that there is no change in the microbial diversity, but the relative abundance of Enterobacteriaceae, Veillonella, Clostridium, Neisseria, and Haemophilus increases. One-quarter of these bacteria can produce H_2S by fermenting sulfur-containing amino acids, and the relative abundance of these H_2S producers is positively correlated with the severity of inflammation. Characterization of the host proteome shows that mitochondrial proteins involved in H_2S oxidation, such as Eth1, thiosulfate sulfurtransferase (TST), and mitochondrial respiratory complexes III-IV, are the major proteins downregulated in CD. The significant

downregulation of these proteins has been linked to the depletion of butyrate producers and a large number of pathogenic bacteria, many of which are known to be effective H_2S producers. These results indicate that mitochondria are regulated, as well as disturbed, by H_2S_producing microbiota in patients with CD [126].

Chronic inflammatory diseases are generally thought to arise from interactions between susceptible host genes and environmental factors. Genetic defects in TLR1 promote acute enteric infection of Yersinia enterocolitica, and changes in the cellular immune response are also present in this model. By promoting the recruitment of neutrophils, Yersinia's metabolism of the respiratory electron receptor tetrathionate is increased. These events lead to permanent changes in the anti-immune family, microbiome composition, and chronic inflammation, which persist long after the Yersinia clearance. Therefore, acute infection of intestinal pathogenic microorganisms can lead to long-term changes in the immunity and microbiota of genetically susceptible individuals, leading to chronic inflammatory diseases [127].

The causal relationship between SRB and chronic inflammation is still elusive, and more prospective experiments may be needed for proof. Lactobacillus treatment can reduce SRB in the human intestine [128]. A putative prebiotic, Glycomacropeptide reduces Desulfovibrio in the cecum and feces of mice, which is related to the anti-inflammatory effects of spleen cells and reduced plasma concentrations of the proinflammatory cytokines TNF-α, IL-1β, IFN-γ, and IL-12 [129]. Vitro studies have shown that active SRB can interact with human intestinal epithelial cells and form biofilms on them, leading to apoptosis of epithelial cells [130]. When a combination of D. indonesiensis and SRB with anaerobic bacteria in patients with colitis is intragastrically given to sterile mice with colitis, SRB contributes to the mucosal immune response in the steady state and facilitates recruitment of lamina propria cells and phagocytic cells, and T lymphocytes in the mesentery. On the other hand, the increase in intestinal SRB after gavage aggravates chemical colitis, which may be achieved by inducing Th17 cytokines, IL-6, and IL-17A [131]. SRB has been observed to make direct contact with the epithelial barrier in the colon tissue of patients with UC, indicating that disorder of mucosal epithelial barrier contributes to SRB invasion of the mucosal epithelium under inflammatory conditions [130].

4.4 Effects of Host Gastrointestinal Epithelium and Diet on H_2S and Intestinal Microorganisms

H_2S is an important metabolic fuel for the gastrointestinal epithelium. Colon epithelial cells produce ATP during the process of oxidizing H_2S. The epithelium is both a physical barrier that prevents potentially harmful substances in the intestine from entering the basal side and a metabolic barrier that oxidizes H_2S produced by microorganisms. Defects in epithelial barrier function may cause H_2S in the intestinal cavity to enter the lamina propria and affect the normal function of the gastrointestinal tract [109, 123, 132].

Bacterial metabolites can affect the pH value of the colonic lumen. In turn, changes in the luminal pH may influent the absorption of intestinal lumen produced compounds by colonic cells [109]. PH value of mucosa side depends on the acid-base concentration of complex formed by intestinal secretions and bacterial metabolites, among which hydrogen and bicarbonate secreted by the colonic mucosa are important determinants [133]. Short-chain fatty acids and other microorganisms-derived acidic metabolites such as branched-chain fatty acids, organic acids, and H_2S are also involved. Ammonia intervenes in the lumen pH as a weak base. At low pH, the H_2S/HS^- ratio in the large intestine will increase, it will be easy for H_2S to penetrate the biofilm, and a lower pH will increase the H_2S concentration entering the basal side from the intestine, to amplify its inhibitory effect on colonocyte mitochondria at high concentrations [132].

It has been increasingly recognized that the composition and metabolic activity of the intestinal microbiota can be regulated by dietary

proteins, which in turn affects health. Regulation between the microbiome and dietary proteins can be bidirectional. In response to changes in dietary protein components, significant changes occur in microbial metabolites, including short-chain fatty acids, sulfides, and methane, which are toxins associated with the development of colon cancer and inflammatory bowel disease. Providing a high-protein diet may increase the risk of diseases associated with protein-fermenting pathogens and bacteria. These changes in the microbiota can affect the intestinal barrier and the immune system by regulating gene expression in related signaling pathways and the secretion of metabolites [134].

4.5 Gastrointestinal Diseases Related to H_2S and H_2S-Producing Gastrointestinal Microorganism

4.5.1 Inflammatory Bowel Disease (IBD)

With the changes in people's life and diet habits in the past few decades, the incidence of immune-related gastrointestinal diseases such as IBD has increased significantly, attracting the attention of clinical medical workers and researchers. Although its pathogenesis is unclear, many factors that are involved in its pathogenesis and development, including the effect of H_2S and H_2S-producing microorganisms in the gastrointestinal tract on IBD, have been extensively studied.

Although the role of H_2S in IBD is still controversial, to elucidate the complex role of H_2S in the colon and understand the exact nature of its interaction with the colon still helps make the pharmacological regulation of H_2S production and metabolism potential targets for future treatment of multiple colon diseases [135].

A large number of studies have shown that the destruction of the mucus barrier plays an important role in the worsening of IBD, especially the change of the mucus barrier in ulcerative colitis that has been supported by public data and widely accepted. The application of fluorescence in situ hybridization reveals the importance of the mucus barrier in maintaining the symbiotic relationship between the host and the bacteria. Studies have shown that the use of H_2S, short-chain fatty acids, prebiotics and probiotics, neutrophil elastase inhibitors, and other drugs can regulate the function of the mucus barrier and provide potential treatment options for this disease [136].

In the three tested models, diet-induced homocysteinemia (Hhcy) significantly aggregates colitis. None of the three colitis models show an increase in colonic H_2S synthesis after colitis induction. Injecting H_2S into Hhcy rats can significantly reduce the severity of colitis. Compared with wild-type mice, interleukin-10 (IL-10) -deficient mice have reduced H_2S synthesis in the colon, and 40% increased serum homocysteine. By injection of IL-10 into IL-10-deficient mice, colonic H_2S synthesis can be recovered, and serum homocysteine levels are significantly reduced. These results indicate that the exacerbation of Hhcy colitis is partly due to impaired colonic H_2S synthesis. In addition, IL-10 has a new role in promoting H_2S production and homocysteine metabolism [137].

Diallyl sulfide (DAS) and DADS, two garlic-derived sulfur compounds, reduce inflammation and damage induced by dinitrobenzene sulfonic acid in the body. In IFN-γ stimulated intestinal epithelial cells, DADS reduced IP-10 and IL-6 levels, while DAS inhibited NO production and STAT-1 expression, confirming the protective effect of DAS and DADS on experimental IBD [138].

In vitro model, GYY4137 reverses the significantly increased permeability of Caco-2 cells and downregulates the expression of tight junction proteins (ZO-1, couldin4, and occludin) caused by dextran sodium sulfate (DSS). In the in vivo model, CBS silencing caused by siRNA transfection further proves that the endogenous H_2S system is involved in DSS-induced inflammatory responses and mediates barrier functions. GYY4137 reduces intestinal permeability and up-regulates tight junctions, reduces inflammatory responses, and improves the function of the intestinal barrier [139].

Exogenous H_2S donors such as diallyl trisulfide (DATS) have been used as anti-inflammatory

mediators. This exogenous H_2S spreads through the plasma membrane to achieve its biological functions, including inhibiting the excessive production of proinflammatory cytokines, inhibiting cell adhesion macrophages from acting on vascular endothelium, and repairing colonic inflammatory tissue [140].

In DSS-induced colitis mice, there is no significant change in colonic CSE (related to H_2S production) activity and protein levels, and repeated use of CSE inhibitors did not or only slightly reduce the symptoms of colitis. In contrast, colonic rhodanase (related to H_2S detoxification) activity, protein, and mRNA levels are significantly reduced. Consistent with the onset of colitis, it is followed by an increase in rhodanase activity in red blood cells. These data suggest that IBD may be related to the impaired detoxification of H_2S resulted from downregulated or inhibited colonic rhodanase, and the delayed enhancement of rhodanase activity in red blood cells may be a compensatory event for IBD [141].

Congenital megacolon-associated enterocolitis (HAEC) is the most common cause and cause of death for congenital Hirschsprung disease (HSCR). Compared with the control group, the expression of CBS and CSE significantly reduces in ganglia and ganglion-free intestines of the HSCR group ($p < 0.003$). Confocal microscopy shows the expression of CBS and CSE in smooth muscle, ICCs, platelet-derived growth factor-alpha receptor-positive cells, intestinal neurons, colonic epithelium in the HSCR group is significantly lower than that in the control group. This indicates that decreased expression of CBS and CSE in HSCR patients may affect mucosal integrity and colonic contractility, thereby making HSCR patients more prone to HAEC [142].

On the other side of the shield, there are also a lot of researches supporting the negative impact of H_2S in IBD. Studies on the volatile organic compounds in the breath of IBD patients show that compared with healthy controls, the concentrations of H_2S, acetic acid, propionic acid, and butyric acid in the breath of IBD patients are higher and relative concentrations of pentane and other volatile organic compounds are weakly correlated with simple clinical activity indicators. It is speculated that H_2S and these carboxylic acids may be exhaled biomarkers for intestinal bacteria overgrowth [143].

Patients with IBD have an increased risk of developing colorectal cancer (CRC). Tumor immune evasion is a major problem for colorectal cancer. Preclinical and clinical evidence indicates that marrow-derived suppressor cells (MDSCs) promote tumor growth and development by suppressing T cells and regulating the innate immune response. Marrow mesenchymal stem cells consist of two different subtypes of immature myeloid cells: CD11b + Ly6G + Ly6C (low) with granulocyte phenotype (G-MDSCs) and CD11b + Ly6G (-Ly6C) with monocyte phenotype (M-MDSCs). To better clarify the role of the H_2S pathway in innate immune-mediated IBD, the researchers use a model of innate immune-mediated IBD that is induced by hepatic bacilli (Hh) infection. G-MDSCs and M-MDSCs in the colon and spleen of Hh-infected mice show a significant time-dependent increase. Long-term oral administration of H_2S donor DATS can reduce colon inflammation by limiting the recruitment of colonic G-MDSCs of Hh-infected mice. It is concluded that the L-cysteine/H_2S pathway may be a new participant in the immunosuppressive mechanism and is responsible for promoting the development of colitis-related cancer [144].

Changes in microbial populations and metabolism during intestinal inflammation are key events related to intestinal homeostasis and disrupted immune tolerance. There is a serious imbalance between the number of intestinal bacteria and their relationship with the host in patients with IBD, which is considered to be part of the complex mechanism that triggers and develops intestinal inflammation. A large number of SRB has been found in the intestine of IBD patients. SRB not only directly interacts with intestinal epithelial cells during intestinal inflammation, but also may promote intestinal damage by generating high levels of H_2S [145].

A mixed culture of SRB communities obtained from healthy mice and colitis mice shows differences in the distribution of sulfate-reducing microorganism communities. The amount of H_2S produced in the mixed culture of ulcerative colitis

mice was 1.39 times that in healthy mice. Meta-analysis is used to observe the occurrence of SRB and its metabolic process in healthy people and IBD patients, suggesting the important role of Desulfovibrio in small bowel diseases [146].

Researchers have suggested using the term "the microflora core of gut metabolism" to describe key microbial flora, which more accurately reflects the functional importance of metabolically active microbial flora. The microflora core includes functional groups of microorganisms with similar metabolic functions: butyric acid-producing bacteria, propionic acid-producing bacteria, acetic acid-producing bacteria (glycogen), hydrogenated microorganisms (reducing acetone, sulfate-reducing bacteria, methanogenic bacteria), bacteria that produce and use lactic acid, bacteria involved in bile acid metabolism, bacteria that metabolize proteins and amino acids, vitamin-producing microorganisms, oxalic acid-degrading bacteria, and so on. Disorders of microbial metabolism may be the root cause of many human diseases. The main clinical variants of metabolic disorders are due to disrupted microbial synthesis of short-chain fatty acids (mainly butyric and propionic acid) and increased production of H_2S, ammonia, and secondary bile acids (especially deoxycholate) by bacteria. This metabolic disorder eventually leads to IBD or CRC and contributes to the onset of the chronic liver, kidney, and cardiovascular diseases [147].

4.5.2 Colorectal Cancer (CRC)

Many studies have demonstrated the promoting role of H_2S in cancer biology. CBS, CSE, 3-MST are highly expressed in a variety of cancers. The effect of inhibiting CBS has shown anti-tumor activity, especially in colon, ovarian, and breast cancers, while the results of inhibiting CSE or 3-MST have not been found in cancer cells. Interestingly, high-dose or prolonged H_2S treatment in vivo induces apoptosis of cancer cells while retaining non-cancer fibroblasts. Therefore, a bell-shaped model is proposed to explain the role of H_2S in cancer development. To be specific, endogenous H_2S or relatively low levels of exogenous H_2S may exhibit cancer-promoting effects, while high-dose or prolonged exposure to H_2S may cause the death of cancer cells. An in-depth understanding of the role and mechanism of H_2S in cancer can provide new strategies for cancer treatment [148].

CBS is upregulated in biopsies of the precancerous adenomatous polyp. Increased expression of CBS in adenomatoid colonic epithelial cell line induces the characteristics of metabolism and gene expression profiles of CRC cells, with more than 350 differentially expressed genes. These genes overlap significantly with glycolysis, hypoxia, and colon cancer cell phenotype-related gene sets, including genes regulated by NF-kappaB, KRAS, p53, and Wnt signaling, downregulated genes after E-cadherin knockout, and genes related to the increased outer matrix, cell adhesion, and epithelial-mesenchymal transition. Differentially expressed metabolites involve intermediates of the glycolytic pathway, the nucleotide sugars, and the pentose phosphate pathway. Compared with wild mice, CBS heterozygous mice (CBS (+/−)) have a reduced number of colonic crypt abnormalities caused by mutagens, proving that the activation of the CBS/H_2S axis promotes the occurrence of colon cancer [149].

CBS promotes the growth of colon and ovarian cancer in preclinical models. Conversely, its tumor-suppressive effect has also been reported in other cancer types, suggesting that the role of CBS in tumor growth and development is related to the environment [150].

In many cancer lesions, 3-MST is upregulated compared to that in surrounding normal tissue. In vitro studies have revealed that 3-MST upregulation is particularly prominent in cancer cells that recover from oxidative damage and/or develop into a multi-drug resistant phenotype, and 3-MST drug inhibitors and the use of 3-MST silencing methods can inhibit cancer cell proliferation [151].

GYY4137 of low concentration enhances mitochondrial function and glycolysis in HCT116 cells. siRNA-mediated transient silencing of lactate dehydrogenase A (LDHA) attenuates the stimulation of GYY4137 to mitochondrial respiration but fails to inhibit

glycolysis. H_2S induces sulfur hydration of Cys163 in recombinant LDHA and stimulates LDHA activity. Studies have confirmed that LDHA is involved in the stimulation of H_2S on mitochondrial respiration of colon cancer cells [152].

The inhibition of H_2S synthesis can significantly increase the sensitivity of colon cancer cells to 5-FU. miRNA sequencing reveals that miR-215-5p is one of the miRNAs with the most significant change in expression level after CBS knockout. Epidermal growth factor (EREG) and thymidine synthetase (TYMS) are expected to be potential targets for miR-215-5p. Reducing H_2S synthesis can significantly reduce the expression of EREG and TYMS. These results suggest that inhibition of H_2S synthesis can reverse the acquired resistance of colon cancer cells to 5-FU, which may be mediated by EREG and TYMS [153].

Using in situ responses of cuprous oxide (Cu_2O) and high concentrations of endogenous H_2S in colon cancer sites, researchers have developed a new drug for the diagnosis and treatment of colon cancer and confirmed its advantages in photoacoustic imaging and photothermal therapy in the in vitro and in vivo experiments [154].

The S-arylthio oxime functionalized amphiphilic block copolymer micelles prepared by the researchers can slowly release H_2S. Compared with Na_2S and GYY4137, this new type of H_2S-releasing micelles can significantly reduce the survival rate of HCT116 colon cancer cells, indicating that H_2S release kinetics may determine the selective toxicity of H_2S to tumor cells [155].

Colonocytes are adapted to exposure to higher concentrations of H_2S because they have a potent mitochondrial H_2S oxidation pathway. SQR is present in the apex of the human colonic crypt, but in CRC epithelium, this gradient localization from the apex to the crypt disappears. Mitochondrial H_2S oxidation changes the bioenergetics of the cell, leading to the reduction of NAD^+/NADH redox pairs. The resulting lack of electron receptors leads to a lack of uridine and aspartate and enhances glutamine-dependent reductive carboxylation. The metabolic characteristics of the H_2S-induced stress response are partially mapped to redox-sensitive nodes in central carbon metabolism, suggesting that CRC tissues and cell lines counteract the anti-tumor growth effect of H_2S through driving mitochondrial metabolic reprogramming [156].

Collecting multi-component data and building genomic-scale microbial metabolism models enable researchers to examine microbial communities, community functions, and interactions in a new way, for example, to evaluate H_2S production in CRC based on fecal, mucosal, and tissue samples collected from the same individual inside and outside the tumor site, and then use 16 s rRNA microbial community and abundance data to screen and validate metabolic models. Using the open-source platform MICOM, the metabolic flux of H_2S is tracked through a defined microbial community, which can represent the sample community inside or outside the tumor [157].

Sulfur metabolizing microorganisms are related to the occurrence of CRC. The researchers investigate its relationship with the risk of developing CRC using data from a large prospective study of men. By collecting follow-up data of 51,529 male health professionals since 1986, the relationship between sulfur metabolizing bacteria in stool and the risk of CRC is analyzed in 26 years. The results indicate that factors related to high-sulfur microbial diet scores include increased consumption of processed meat and low-calorie beverages and reduced consumption of vegetables and beans. Increased sulfur microbial diet scores are associated with a risk of the distal colon and rectal cancer [158].

African Americans (AAs) have a higher incidence of CRC than non-Hispanic whites (NHWs). It is hypothesized that sulfur-induced bacterial abundance in the colonic mucosa may be an environmental risk factor of CRC that distinguishes AA and NHW, so the researchers compared the sulfite bacterial abundance in the uninvolved colon mucosa of AAs and NHWs patients with CRC and the normal controls and investigated correlations between bacterial indicators, race, disease status, and dietary intake. Regardless of disease status, AAs harbor more

sulfite bacteria than NHWs. Compared with the control group, the F. falciparum-specific dsrA is more abundant in the AAs group. Linear discriminant analysis of the 16 s rRNA gene sequence reveals that 5 sulfite gene genuses were more abundant in AAs cases. The intake of fat and protein and the daily meat consumption in AAs is significantly higher than that in NHWs, and a variety of dietary components are associated with higher sulfite bacteria abundance. These results suggest that sulfur pathogens are a potential environmental risk factor for CRC in AAs [159].

The link between CRC and intestinal flora has been established, but the specific microbial species and their role in canceration are still an active area for research. The researchers collected specimens of colorectal tumors and normal adjacent tissues and mucosal from 83 patients with colon cancer undergoing partial or total colectomy. The status of mismatch repair (MMR) in each tumor sample is determined and divided into defective MMR (dMMR) or proficient MMR (pMMR) tumor subtypes. Using genomics and metabolomics research methods, it is found that H_2S-producing amino acid metabolites are increased in dMMR-CRC. The combination of tumor biology and microbial ecology highlights the unique microbial, metabolic, and ecological characteristics of dMMR and pMMR-CRC [160].

4.5.3 Functional Gastrointestinal Disease (FGID)

Traditionally, functional gastrointestinal diseases (FGID), such as irritable bowel syndrome (IBS) and functional dyspepsia (FD), refer to diseases defined by specific symptoms but lacking evidence of changes in tissue structure or biochemical indicators that cause these symptoms. But this definition is now being overturned by more and more evidence. The low-grade inflammation of the gastrointestinal tract, the activation of the immune system, and the disorder of gastrointestinal flora have been confirmed to be involved in the occurrence of FGID.

There is evidence that acute gastrointestinal infections often occur before IBS [161, 162]. Results of questionnaires and colon biopsies based on gastrointestinal symptoms show that the prevalence of IBS is 3.6 times greater in patients with colon spirochetosis than that in patients not infected [163].

With appropriate testing, some patients with FD or IBS will be found to be suffering from low-grade inflammation [164]. In addition to gastrointestinal mucosal inflammation, increased cytokines appear in these patients, suggesting activation of the immune system [165–167]. The reason for the changes in the immune system may partially depend on the defect of the intestinal mucosal barrier [168–170], and we have already mentioned the damage to the barrier by H_2S and H_2S-producing intestinal microorganisms [121, 122].

The density of bacteria colonized in the gastrointestinal mucosa plays a key role in the intensity of meal-related symptoms in FD patients and is closely related to impaired quality of life [171]. In the in vitro starch fermentation experiments, the gut microbiota of IBS patients produces more sulfide than healthy controls, but less butyrate [172].

Visceral pain is regarded as one of the most common symptoms of FGID, but since many mechanisms are involved in the production and maintenance of this pain, satisfactory results are often not obtained in the treatment of FGID. Earlier we mentioned the role of H_2S in visceral nociceptive sensation, which has a dual effect of promoting pain and analgesia. Recently, it has been suggested that intestinal microorganisms may be new hope for treating visceral pain [173]. Studies using germ-free mice have shown that the presence of intestinal commensal bacteria is necessary for the development of pain sensitivity, and the absence of microorganisms causes the inactivated response to stimuli in germ-free mice [174]. Transplanting feces from patients with IBS into the intestines of mice can reproduce their symptoms to a certain extent [175]. Therefore, microorganisms may become new targets for the treatment of FGID. Some people have proposed the use of probiotics and prebiotics to improve visceral pain and reduce visceral hypersensitivity [176]. However, the type and number of microorganisms, the role of metabolites of

microorganisms in FGID, and the mechanism of action need to be further explored.

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Hydrogen Sulfide and the Immune System

Peter Rose, Yi-Zhun Zhu, and Philip K. Moore

1 Introduction

Hydrogen sulfide (H_2S) is now accepted as the third gaseous mediator identified in mammals alongside NO and CO. This simple molecule is synthesized by a range of cells and tissues whereby it functions as a signalling molecule [1, 2]. At the molecular level H_2S reportedly alters the activities of transcription factors important in inflammatory signalling like nuclear factor kappa beta (NF-κB), and nuclear factor erythroid 2–related factor 2 (Nrf-2). More widely, this same molecule can activate several kinases such as p38 mitogen-activated protein kinase (p38 MAPK), c-JunNH_2-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), phosphoinositide 3-kinase – protein kinase B (PI-3 K-Akt), AMP-activated protein kinase and protein kinase C (PKC). Furthermore, other proteins including p53, proliferator-activated receptor γ, NAD-dependent deacetylase sirtuin-1 (SIRT1), SIRT3, and mechanistic target of rapamycin (mTOR) may also play important roles in the inflammatory properties of H_2S (reviewed in, [3–6]). Collectively, this body of work points to a spectrum of complex molecular networks that influence the response of cells to H_2S, many of which have distinct roles in cytoprotection ([7], vascular function [8–10], neurological systems [11–13], tissue repair and healing [14, 15], apoptosis and the cell cycle [16], in aging [17, 18], mitochondrial function and energy metabolism and biogenesis [19, 20], and in inflammation [21, 22]. In these networks, H_2S functions at multiple levels, acting as an antioxidant and interactions with other signalling molecules like nitric oxide to form biologically active nitrosothiols and related species [23, 24]. The interplay between H_2S and these pathways contributes to the effects of H_2S in the immune system.

In tissues and cells the biosynthesis and catabolism of H_2S are governed by the interplay between the biosynthetic enzymes cystathionine β synthase (CBS, EC 4.2.1.22), cystathionine-γ-lyase (CSE, EC 4.4.1.1), and 3-mercaptopyruvate sulfurtransferase (3-MST, EC 2.8.1.2), with enzymes involved in H_2S detoxification, namely Ethylmalonic encephalopathy protein 1 (Ethe1, EC: 1.13.11.18) and mitochondrial sulfide quinone oxidoreductase (SQR1, EC 1.8.5.4), (Fig. 1a) also important. Much has been reported on these two

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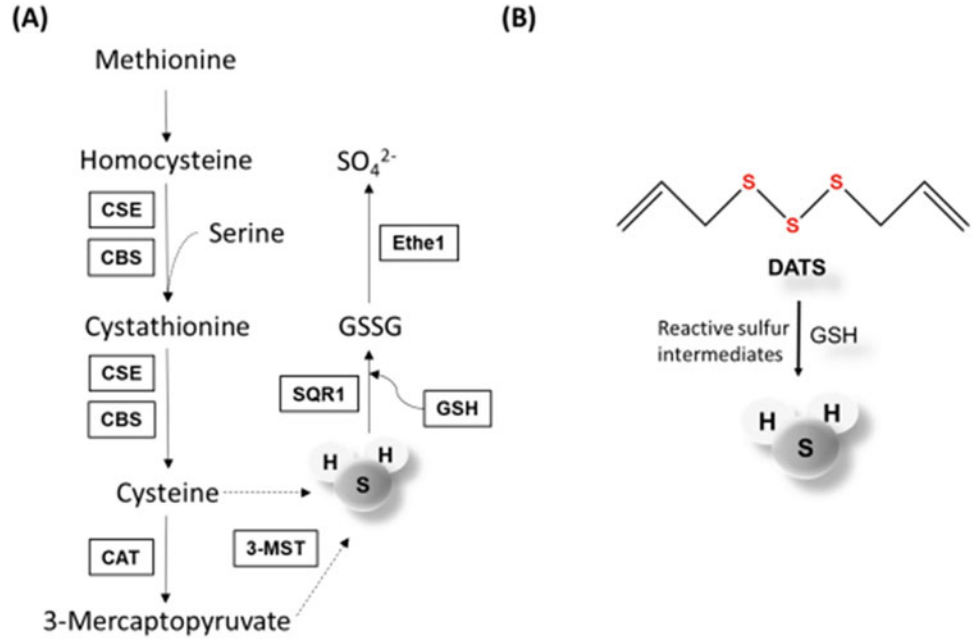

Fig. 1 Hydrogen sulfide biosynthesis in mammalian cells is governed by the expression and activities of three key metabolic enzymes namely cystathionine gamma lyase (CSE), cystathionine beta synthase (CBS), and 3-mercaptopyruvate sulfotransferase (3-MST), respectively. These enzymes are responsible for the majority of H_2S produced in cells and tissues and their expression and activities respond to inflammatory stimulation. The H_2S produced can either function as a signalling molecule or alternatively be detoxified by the enzymes sulfide quinone oxidoreductase (SQR1) or ethylmalonic encephalopathy protein 1 (Ethe1), (A). (B) Additional sources of H_2S are potentially derived from the metabolism of exogeneous sulfur compounds like diallyl trisulfide in reactions with cellular thiol molecules like glutathione

opposing groups of enzyme, and how they influence tissue H_2S levels covered in greater detail in [25, 26]. In general, cellular pools of H_2S are governed by the rates of catabolism of cysteine by the enzymes CBS, and CSE, and by the activity of 3-MST towards the substrate, 3-mercaptopyruvate. CBS and CSE are the major H_2S generating enzymatic systems in mammalian tissues; with CBS prominently expressed in the brain, nervous system, liver, and kidney. In contrast, CSE is largely expressed in the liver and in vascular and non-vascular tissues. The enzyme 3-MST produces H_2S in a coupled reaction with the enzyme cysteine aminotransferase. In addition, it is worth noting that pools of H_2S may also be generated following the ingestion of dietary sulfur compounds including the allium derived compound diallyl trisulfide. However, these routes of H_2S production are beyond the scope of the current work (Fig. 1b; see reviews by [27, 28] for further details).

The detoxification of H_2S in cells and tissues can occur via chemical as well as enzymatic routes involving sulfide quinone oxidoreductase (SQR; E.C. 1.8.5.4) and tethylmalonic encephalopathy 1 (Ethe1; E.C. 1.13.11.18) systems are [29, 30]. Interestingly, loss of function of Ethe1 promotes H_2S mediated toxicity in humans, and is a recognized metabolic disorder. In addition to enzymatic routes of elimination, chemical processes are also important in mediating the loss for this molecule. These processes occurring following the oxidation of H_2S in chemical reactions driven by O_2, myoglobin and/or transition metals to generate thiosulphate and other oxidation products. Importantly, dysregulation in endogenous H_2S production, coordinated by either one or the other of these enzymatic pathways, will

influence the severity, progression, and rates of recovery from episodes of disease where H_2S is important. Interestingly, many of the pathophysiological conditions known to be affected by H_2S, often show signs of dysregulation in the immune response and in inflammatory signalling networks, viz. diabetes, cancer(s), and arthritis. Critically, loss in the capacity of tissues to produce H_2S via reduction in the activities and expression of CSE, CBS, 3-MST often correlates with these observed changes, and points to a role of this molecule in these conditions. This has led many researchers to question what function H_2S plays in inflammation, and has led to the testing of several H_2S donor compounds in multiple inflammatory models. While much of this research is still ongoing, this work will hopefully help to unravel the biological significance of H_2S in the immune system.

2 H_2S and Inflammation

In mammalian cells and tissues the inflammatory process is tightly coordinated in tissue specific responses to infection and stress. Inflammatory networks in turn initiate cellular cascades involving leukocytes and lymphocytes that once activated initiate inflammatory networks. These processes involving, (1) the mobilization of inflammatory cells like macrophages to the site of infection and, (2) the biosynthesis and release of inflammatory molecules like prostaglandins (PG), various cytokines (CK), leukotrienes (LK), and other molecules including the gaseous mediators, NO, CO, and H_2S [31]. The latter molecules are important, since they are involved in coordinating cell signalling responses needed to activate gene expression, and the synthesis of proteins that play important roles in the inflammatory response. Combined, these networks exist to allow for the removal of invading pathogen, viz. bacteria, viruses, foreign bodies, and fungi. In recent times, it has become clear that dysregulation in inflammatory signalling networks occurs during aging and in many common diseases including some cancer(s), arthritis, diabetes, and other conditions that fall under the umbrella of "metabolic syndrome" [32, 33]. In many of these pathophysiological conditions the over production of pro-inflammatory mediators leads to altered cellular proliferation and damage to tissue. Consequently, researchers have begun to define the role of gaseous mediators in inflammatory processes and whether loss of production of these molecules is important in altered immune responses. With regard to H_2S, the advent of newer research tools, viz. H_2S donor molecules, novel inhibitors of H_2S biosynthesis enzymes, and gene knockout technologies, has allowed researchers to gain a better picture of how gaseous signalling molecules function in the immune system [34]. Indeed, in our laboratory and that of others, it has been shown that H_2S is an important mediator of inflammation and evidence from this research will be covered in the current chapter.

The part played by H_2S in the immune system is complicated, and researchers are only now gaining a picture of the role of this molecule in various inflammatory conditions. Importantly, H_2S can have both pro-and anti-inflammatory effects in mammalian cells and tissues. One of the earliest reports of the part played by H_2S in inflammation was by Li and colleagues [35]. In a model of endotoxin-induced inflammation, *E. coli* lipopolysaccharide (LPS) was used to mimic septic shock in mice. Findings from this research revealed that the administration of LPS increased plasma H_2S concentrations and the rates of synthesis of this molecule in liver and kidney tissues. Concomitantly, the increased production of H_2S correlated with the accumulation of myeloperoxidase (MPO; a marker of tissue neutrophil infiltration and inflammation) in the lung, liver, and kidney tissues of animals. This research was the first to provide evidence that H_2S can be pro-inflammatory in mammalian systems. Using sodium hydrosulfide (NaHS), a fast H_2S releasing donor molecule to manipulate tissue H_2S levels, the released H_2S caused a marked increase in lung inflammation, lung, and liver MPO activity, and raising of plasma tumor necrosis factor alpha (TNF-α) concentrations. Critically, these pro-inflammatory markers were reduced using the H_2S biosynthetic enzyme inhibitor, DL-propargylglycine (PAG). Findings

from this work showing that the manipulation of H_2S homeostatic systems influences the inflammatory response in various animal models. Further research, using a murine model of cecal ligation and puncture-induced sepsis, also confirm the pro-inflammatory nature of H_2S. In this instance, treatment with NaHS induces the production of pro-inflammatory mediators, driven largely by the activation of NF-κB signalling pathway [36]. In addition, in human fibroblast the addition of NaHS increases the production of TNF alpha, and IL-8, and drives an increase in gene expression and protein levels of cyclooxygenase II (COX-2) and IL-8. In the chicken, H_2S is pro-inflammatory by causing the production of NO, TNF-α, and IL-1β, increasing hydrogen peroxide (H_2O_2), the lipid peroxidation product malondialdehyde (MDA), and elevated inducible nitric oxide synthase (iNOS) activity in the spleen of animals. In contrast, the levels or activities of the cellular antioxidant glutathione (GSH), catalase, and superoxide dismutase were found to be diminished. Collectively, these events leading to necroptosis via the induction of mitogen-activated protein kinase (MAPK) and the activation of NF-κB [37]. Atmospheric H_2S gas is also reported to activate the LR4/Myd88/NF-κB signalling pathway and to initiate the formation of the NLRP3 Inflammasome in broiler thymus tissues [38]. In this instance, H_2S increased IL-1β, IL-4, and IL-10 levels, and downregulated IL-12 and IFN-γ production; this finding going someway in explaining the immunotoxic effects of H_2S. H_2S treatment also initiates the phosphorylation of Src family kinases (SFKs) to promote inflammation in mouse pancreatic acinar cells [39]. In addition, in a murine polymicrobial sepsis model, H_2S treatment stimulated the induction of COX-2 and the production of prostaglandin E metabolite (PGEM) to promote inflammation [40]. Wang et al. have shown that CSE expression is important in driving the rates of H_2S mediated persulfidation (widely known as sulfhydration), on cysteine-38 of the NF-κB p65 subunit and the nuclear translocation of NF-kB in bone-metastatic PC3 cells. This resulting in an increase in IL-1β expression and the promotion of cell invasion [41]. Clearly, further studies are needed to define the regulatory effects of H_2S on NF-κB signalling systems under differing inflammatory stimuli and disease models. These findings indicate that the administered of non-physiological levels of H_2S can be pro-inflammatory in mammals.

In other studies evidence points to H_2S having anti-inflammatory effects in isolated cells and in various animal tissues. Indeed, in human osteoarthritis chondrocytes, exposure of isolated cells to low concentrations of H_2S has an anti-inflammatory effect, suppressing IL-8, COX-2, inducible nitric oxide (iNOS), and matrix metalloproteinase-13 (MMP13) protein expression. This finding correlates with significant reductions in the synthesis of pro-inflammatory mediators like prostaglandin E2 (PGE2) and NO [42]. Similarly, in C28/12 chondrocyte cells, exposure of cells to low concentrations of NaHS inhibits IL-1β, interleukin 6 (IL-6), and IL-8 expression and reduces leucocyte adhesion [43]. This finding supporting an anti-inflammatory role for H_2S. The non-steroid anti-inflammatory drug (NSAID), S-diclofenac, a H_2S-releasing derivative of diclofenac, appears to be anti-inflammatory in animal models [44, 45]. These anti-inflammatory properties are more pronounced with the H_2S releasing derivative than that of the parental molecule, diclofenac. Other H_2S releasing NSAIDs like ADT-OH, a derivative of anethole dithiolethione (ADT), is a slow release hydrogen sulfide (H_2S) donor and also exhibits anti-inflammatory activity [44]. As such, lots of evidence now points to a likely role of H_2S in the immune system, and that this molecule exhibits both pro- and anti-inflammatory effects that are dependent on the time, duration of exposure and relative tissue concentrations.

2.1 H_2S and Inflammatory Signalling; Impacts on NF-kB Systems

The inflammatory response is mediated by the activation of cell signalling systems that regulates

the expression of genes and proteins involved in the production of both pro- and anti-inflammatory molecules. These mediators have the capacity to drive inflammation both in surrounding tissues and at distal sites, and are key players in orchestrating the recruitment of inflammatory cells found in the general circulation such as leukocytes. In mammalian systems, one important component of the inflammatory signalling systems is that of the transcription factor Nuclear factor Kappa beta (NF-kB). NF-kB is representative of one of the most widely characterized cell signalling networks and plays a pivotal role in driving inflammatory cascades in response to tissues injury and stress. NF-kB is also important in other cellular processes including cellular development, maturation, survival, and proliferation [46, 47]. The molecular mechanisms responsible for NF-κB action are now widely known and have led to the characterization of a canonical and non-conical pathway. The conical system is composed of Rel and p50 proteins and the protein complex is localized to the cytoplasm of cells as an inactive form, and bound to the inhibitory protein Iκβα. Following an inflammatory stimulus like for example the exposure of cells to lipopolysaccharide, IκB kinase (IKK) is activated leading that phosphorylates the Iκβα protein. This leads to the ubiquitination of Iκβα and drives its degradation by the proteasome (the classic canonical pathway). Consequently, once IκB has been degradation the active NF-κB complex can translocate to the nucleus, and binds to its respective promoter sequence and driving gene transcription [48, 49]. When NF-kB is activated it causes the biosynthesis of pro-inflammatory cytokines such as members of the interleukin family, interleukin (IL)-1β, IL-6, various prostaglandins PGE and PGE2, and other widely recognized inflammatory mediators like tumor necrosis factor alpha (TNF-α). Interestingly, molecules like TNF alpha can then activate other kinases and in turn drive other signalling events such as programmed cell death cascades [50]. In the non-canonical NF-κB pathway, the p100 subunit of the transcription factor is phosphorylated by IKKα kinase following exposure to various ligands, and once activated NF-kB translocates to the nucleus, binds to DNA, and drives the expression of inflammatory genes. Importantly, dysregulation in NF-kB signalling systems have been observed across a spectrum of separate inflammatory conditions like inflammatory bowel disease, arthritis, and asthma as well as several cancers including lymphoma, liver cancer, lung cancer, and breast cancer.

Regarding a potential role of H_2S and its impact on NF-kB signalling, Oh et al. showed that non-cytotoxic concentrations of H_2S are able to inhibit NO production and reduced the expression of inducible NO synthase (iNOS), in lipopolysaccharide stimulated RAW264.7 macrophages [51]. Reductions in the production and expression of these inflammatory markers correlated with the prevention of NF-κB activation in cells pre-treated with H_2S. Similarly, in the rat, H_2S inhibits NF-κB activation following LPS injected [44]. Further research in this area has confirmed the inhibitory action of H_2S on the NF-kB signalling system, and its ability to temper the production of pro-inflammatory molecules like IL-1β, IL-6, TNF-α, PGE-2; by virtue of reducing the expression of proteins such as COX-2 and iNOS in various cell types and animal tissues [52–54]. Consequently, it is now widely reported that H_2S treatment suppress NF-kB activity in mouse and rat models of heart ischemic reperfusion injury, neuroinflammation [55, 56], and oxidized low-density lipoprotein-(ox-LDL) induced macrophage inflammation. Interestingly, recent work points to the fact that H_2S drives persulfidation of the p65 unit of NF-kB that consequently causes the retention of NF-κB in the cytosol of cells, reduces NF-kB DNA binding, and inhibits the production of inflammatory molecules [57]. Persulfidation of the p65 unit of NF-kB also appears to be important in regulating the expression of anti-apoptotic proteins that are important in conferring cytoprotective [58]. In view of the ability of H_2S to mitigate inflammatory signalling, researchers have taken inspiration from this earlier work and focused their efforts on the development of novel H_2S releasing molecules. As mentioned, H_2S-releasing derivatives of diclofenac (c.f. diclofenac alone) have been developed and shown to exhibit

enhanced anti-inflammatory activity [44, 59]. Other H_2S releasing drug entities, including H_2S-releasing mesalamine reduces colitis-associated leukocyte infiltration and expression of several pro-inflammatory cytokines [60], others enhance ulcer healing [59], and inhibit aspirin-induced leucocyte adherence in mesenteric venules, and reduces leukocyte infiltration in an air pouch model and decreasing carrageenan-induced hind paw edema [61]. The anti-inflammatory properties of a selection of H_2S releasing donor molecules are summarized in Table 1. Based on the available research many H_2S donor molecules are anti-inflammatory, and have the capacity to inhibit the NF-kB signalling pathway, this property seems to be an important mechanism of action for these compounds. By way of illustrating this fact, the allyl cysteine analogue, S-propargyl-cysteine (SPRC), has been used to preserves tissue levels of H_2S in the rat hippocampus in animals subjected to LPS treatment. Furthermore, SPRC afforded beneficial actions by inhibiting TNF-α production and TNF-α receptor 1 (TNFR1) and amyloid-β(Aβ) generation. These effects correlated with the inhibition of LPS driven IκBα degradation and the activation of NF-κB p65 [55]. Similarly, Pan et al. showed that SPRC exerted its inhibitory effect on TNF-α-stimulated inflammation in endothelial cells through scavenging ROS, inhibiting JNK1/2/NF-κB pathways, and attenuating the expression of adhesion molecules [70]. The main impacts of H_2S on NF-kB signalling system are summarized in Fig. 2.

2.2 Role of H_2S in Inflammatory Conditions

In the last two decades many research groups from around the world have collectively explored the functional role of H_2S and its impact on inflammation in numerous cell and animal models. A large body of this work has focused on conditions in which inflammation is a significant contributor to the development and severity of the ailment and includes studies focused on tissue injury, joint disease, viz. arthritis, sepsis, and the inflammatory role of H_2S in the respiratory tract and cardiovascular system. Findings from this body of work are expansive, and some of these studies will be described below.

2.3 H_2S and Tissue Injury

Burns can cause significant tissue injury and based on the severity of the damage are often associated with high morbidity and mortality rates. In recent times researchers have begun to unpick the function of H_2S and its association with the severity of tissue injury (reviewed in [71]). Findings from several burn injury models indicate that H_2S is a critical component of the inflammatory response associated with tissue damage. Indeed, in mice, H_2S is protective in burn and smoke-induced lung injury and decreases mortality and increases median survival rates in animals. H_2S inhibits IL-1B levels and increases the concentration of the anti-inflammatory cytokine IL-10 in the lung. Histological evaluation of lung tissues showed that H_2S diminished protein oxidation and improved the condition of the lung [72]. In other research, burn injuries decrease plasma H_2S levels and increased levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-8 and decreasing IL-10 levels. However, the replenishment of tissue H_2S using NaHS reduced the inflammatory response induced by burn damage [73]. More recently, Ahmad and Szabo reported on a complex association between the benefits of inhibiting H_2S biosynthesis (using aminooxyacetic acid; AOAA) and the replenishment of tissue H_2S levels using the mitochondrially targeted H_2S donor [10-oxo-10-[4-(3-thioxo-3H-1,2-dithiol-5-yl) phenoxy]decyl]triphenyl-phosphonium (AP39). In this example, burn injury caused an increase in the markers, alkaline phosphatase (ALP) and alanine aminotransferase (ALT), amylase and creatinine. In contrast, treatment with either AOAA or AP39 reduced the levels of ALP, ALT, amylase, and creatinine, and suppressed inflammatory mediator production and multi-organ injury [74]. Furthermore, in CSE−/− deficient mice, burn injury stimulates the accumulation of myeloperoxidase and lipid peroxidation in heart,

Table 1 Example studies testing the effects of H_2S releasing donor molecules in models of inflammation

Compound	Model	Effect	References
Sodium hydrosulfide	Endotoxin-induced inflammation in the mouse	Pro-inflammatory effect: Increased lung and liver MPO activity, and raised plasma TNF alpha concentration	Li et al. [35]
GYY4137	Endotoxin-induced inflammation in the rat	Inhibition of LPS-induced rise in plasma TNF-α, IL-1beta, IL-6, nitrite/nitrate, C-reactive protein, and L-selectin post-administration (2 h). In RAW 264.7 macrophages reduces LPS-mediated NF-kB activation, iNOS and COX2 expression, and the production of PGE(2) and nitrate/nitrite	Li et al. [62]
H_2S-releasing naproxen derivative, ATB-346	Ligature induced periodontitis in rats	Inhibition of bone defect and histological markers of damage like flatness of the gingival epithelium, chronic inflammatory cell infiltration and loss of connective tissue in the gingival papillae. Naproxen and ATB-346 inhibited the increase of gingival IL-1β and IL-6 secondary to periodontitis, but IL-10 was unaffected	Herrera et al. [63]
pH-controlled H_2S donor (JK-1)	Mouse model of aspirin elicited gastric lesions	JK-1 promoted reductions in aspirin-induced gastric lesion area and the tissue levels and content of Il-6, TNF-α, and COX-2 protein expression. Inhibition of oxidative stress markers for lipid peroxidation and restored glutathione content in animal tissues.	Yang et al. [64]
FW1256	LPS-treated mice	Concentration dependently decreased TNFα, IL-6, PGE2 and NO generation in LPS-stimulated RAW264.7 macrophages and BMDMs. FW1256 also significantly reduced IL-1β, COX-2 and iNOS mRNA and protein in LPS-stimulated RAW264.7 macrophages. In vivo, FW1256 administration also reduced IL-1β, TNFα, nitrate/nitrite and PGE2 levels in LPS-treated mice	Huang et al. [65]
S-diclofenac	Carrageenan-evoked hindpaw oedema in the rat	Reduced hindpaw swelling and carrageenan-evoked rise in hindpaw myeloperoxidase activity reflecting tissue neutrophil infiltration and levels in hindpaw PGE(2), nitrite/nitrate content.	Sidhapuriwala et al. [45]
ADT-OH	LPS-induced in vivo neuroinflammation model	ADT-OH promoted M2 polarization of BV2 cells and in vivo suppressed M1 and promoted M2 gene expression	Zhou et al. [66]
Thiol-activated gem-dithiol-based H_2S donors (TAGDDs)	Respiratory syncytial virus infection mouse model.	Reduction in neutrophils recruited to the airways. Decreased the production of the pro-inflammatory cytokines IL-1α, IL-1β, IL-6, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte-colony stimulating factor (G-CSF) in RSV-infected mice	Bazhanov et al. [67]
AP-39	Murine model of combined hemorrhagic shock and blunt chest trauma	High dose AP39 attenuated systemic inflammation and reduced the expression of iNOS and IκBα expression in lung tissue	Welper et al. [68]
SPRC	Acute LPS model and chronic turpentine model in mice	SPRC reduced serum hepcidin, improves transferrin saturation, and maintained erythrocyte membrane integrity. Reduces hepatic JAK2/STAT3 activation and decreased serum IL-6 content and hepatic Il-6 mRNA	Wang et al. [69]

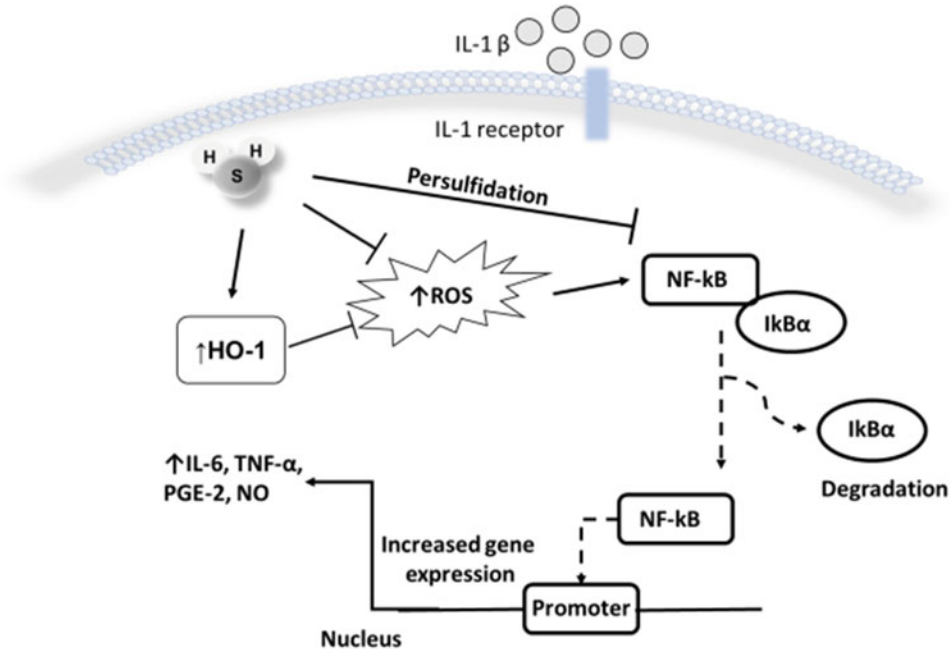

Fig. 2 Hydrogen sulfide can reduce inflammatory signalling systems by driving cellular rates of persulfidation of the transcription factor NF-kB, by acting as an antioxidant to scavenge reactive oxygen species or by stimulating heme oxygenase 1 expression. Reduced NF-kB signalling tempers the rates of production of pro-inflammatory molecules including TNF-β and PGE-2

lung, liver, and kidney tissues of wildtype mice but the levels are significantly reduced in CSE knockout animals. In parallel the levels of inflammatory mediators including TNF-α, IL-1β, IL-4, IL-6, IL-10, and IL-12 are significantly lower in the plasma of CSE−/− animals [75]. In other knockout animals, viz. 3-MST, similar patterns of protection are also reported [76]. Collectively, these studies pointing to the fact that the inhibition of H_2S biosynthetic enzymes appears protective in some burn models. Clearly, further work is needed to determine the molecular mechanisms of action for these protective effects. In other research studies burn injury significantly increases plasma H_2S levels in the liver, and lung tissues of mice, and that this correlates with increased CSE mRNA expression in tissues. In this instance, increased H_2S/CSE pathway expression was associated with systemic inflammation, and increased MPO activity and organ damage. Endogenous H_2S synthesis therefore appeared to contribute to inflammatory damage following burn injury [77]. Interestingly, roles for neuropeptide substance P as a critical mediator of burn-induced acute injury have been postulated [78].

2.4 H_2S and Joint Disease

Dysregulation in H_2S biosynthesis occurs in the osteoarthritic (OA) joint, in rheumatoid arthritis (RA), and in gout [52, 79–81]. The manipulation of H_2S levels and the testing of several H_2S releasing donor molecules have long been reported to be beneficial when used in animal models of arthritic diseases [82–84]. The molecular mechanisms of action ascribed to the therapeutic action of these molecules are linked to the known anti-inflammatory properties of H_2S [42, 52, 85–87], the inhibitory effects of this

molecule on cartilage destruction via reduced expression of matrix metalloproteinases at sites of damage [84, 88] and to reduce gonarthrosis [89]. Other important mechanisms of action include the induction of cytoprotective systems in the joint [82, 90], and reductions in the production of pro-inflammatory mediators [91]. Not surprisingly, in the last two decades, researchers have tried to characterize the molecular mechanisms attributed to the functional roles of H_2S in joint diseases. Short-term treatment with NaHS to fibroblast-like synoviocytes (FLSs) isolated from rheumatoid arthritis patients downregulates IL-1beta expression and inhibits p44/42 MAPK (ERK1/2) activation in cells; long-term exposure rates have apposing effects and appear to activate p38 MAPK and ERK1/2 and to upregulation of IL-6 expression [87]. Other cellular studies show H_2S treatment reduces NO, PGE-2, IL-6, and MMP13 released from isolated human chondrocytes cells stimulated with IL-1β. Moreover, CSE expression and its activity is induced by treatment of cells with IL-1β, TNF-α, IL-6 or LPS [92]. Research using the prototypic H_2S donor drug GYY4137 (a slow-releasing H_2S molecule), reduces lipopolysaccharide (LPS) evoked release of inflammatory mediators in isolated human synoviocytes (HFLS) and articular chondrocytes (HAC). Moreover, in vivo GYY4137 reduced acute joint inflammation in a complete Freund's adjuvant (CFA) model of acute joint inflammation in the mouse. In this instance, GYY4137 reduces synovial fluid myeloperoxidase (MPO) and N-acetyl-β-D-glucosaminidase (NAG) activity. In turn, GYY4137 decreases TNF-α, IL-1β, IL-6, and IL-8 production [93]. Similarly, the H_2S releasing naproxen derivative ATB-346 inhibits carrageenan-induced synovitis in the rat [94]. In other mechanistic work, Wu et al. recently demonstrated a link between H_2S production and jumonji domain containing protein 3 (JMJD3). In synovial fibroblasts (SFs) from RA patients and in the joints of arthritic mice, both CSE and JMJD3 are upregulated. Overexpression CSE was reported to inhibit JMJD3 expression and was accompanied by reduced inflammation in IL-1β-treated SFs [95, 96]. Similarly, in C57bl/6 mice, the H_2S donor, sodium sulfide (Na_2S) reduces leukocyte trafficking in kaolin/carrageenan induce joint inflammation but appears to offer no observable effect on joint pain [97]. Lastly, roles for other H_2S generating enzymes have come to light. Roles for the H_2S producing enzyme 3-MST in cartilage calcification and OA development have been reported. 3-MST expression in human cartilage is negatively correlated with calcification and the severity of OA. Moreover, in 3-MST−/− knockout mice joint calcification and OA severity is reported to increase as compared to wildtype animals. The inhibition of 3-MST appears to enhance mineralization and IL-6 secretion in chondrocytes [83].

2.5 H_2S and Sepsis

Sepsis is caused by a severe systemic infection leading to a systemic inflammatory response. Severe sepsis and septic shock are one of the leading causes of mortality among intensive care units and postoperative care patients [98, 99]. In recent times, the importance of this condition has fuelled several discoveries relating to the role of H_2S in sepsis, however, much still remains to be discovered. To date, researchers have explored the influence of H_2S in conditions linked to sepsis and some scientists suggest that this molecule could be a useful biomarker linked to the severity of this condition [100–102]. Current evidence from studies in animal models indicates a complex role of H_2S in this condition. For instance, in male Wistar rats subjected to acute endotoxemia (treated with *Escherichia coli* lipopolysaccharide (LPS), circulatory failure (hypotension and tachycardia) and an increase in ALT, AST (markers for hepatic injury), lipase (indicator of pancreatic injury), and creatine kinase (indicator of neuromuscular injury) are reported. These observations correlate with an increase in myeloperoxidase (MPO) activity, and in the expression and activities of H_2S synthesizing enzymes CSE and CBS. Importantly, inhibition of H_2S biosynthesis reduces hepatocellular, pancreatic, and neuromuscular injury but not circulatory failure

[103]. H_2S also acts as an important mediator of inflammation in models of cecal ligation and puncture (CLP) induced sepsis. In this model the expression of CSE mRNA is elevated in the liver of animals and plasma H_2S levels are increased. This indicating that the H_2S biosynthetic pathway is responsive to CLP. Interestingly, treatment with PAG significantly reduced sepsis-associated systemic inflammation, as evidenced by increased mortality, and decreased MPO activity and histological changes in lung and liver. Further assessment of the anti-inflammatory effects of PAG showed that this inhibitor could significantly reduce the mRNA and protein levels of IL-1β, IL-6, TNF-α, monocyte chemotactic protein-1 (MCP-1), and macrophage inflammatory protein-2 (MIP-2) in lung and liver tissues. These changes were coupled with a committed decreased in the nuclear translocation and activation of NF-κB. In contrast, replenishment of tissue H_2S levels using the fast donor molecule NaHS provokes an increase in sepsis-associated systemic inflammation by virtue of increased NF-κB activation. Again, these experimental observations are indicative of a pro-inflammatory role for H_2S in sepsis and associated organ injury in this model [36]. Additional confirmation of these early studies have now been reported by several additional researchers. Indeed, the inhibition of CSE activity using siRNA techniques in monocytes/macrophages attenuates inflammation in cecal ligation and puncture-induced sepsis in the mouse [104]. In other research, H_2S has been shown to upregulate substance P in models of polymicrobial sepsis-associated lung injury [36], and neurokinin-1 receptor (NK-1R) levels [105], and to drive tissue damage in acute lung injury in mice [106], and to promote liver damage in endotoxemic rats [107]. In addition, H_2S restores hepatic l-arginine levels and asymmetric dimethylarginine (ADMA) handling in endotoxemia [108], and is important in the regulation of hypothermic state induced by endotoxic shock [109]. A link between H_2S as an important regulator of cholecystokinin-octapeptide (CCK-8) alleviated acute lung injury in animals has also been proposed [110]. Common to sepsis is the increased production of free radical species and associated oxidative damage. Interestingly, H_2S reduces the levels of oxidative and nitrosative stress along with inflammation in some sepsis models. Elevations in NO, a key contributor to mitochondrial oxidative stress during endotoxemia can be inhibited using the H_2S donor, GYY4137 [111]. These studies painting a complex picture relating to H_2S and its role in sepsis related conditions. Other anti-inflammatory effects attributed to H_2S include improved regional blood flow in models of (CLP) induced septic shock. In this instance, delayed treatment with NaHS, and reduced inflammatory signalling caused reductions in plasma levels of IL-1β, IL-5, IL-6, TNF-α, and levels of high-mobility group protein 1 (HMGB1) [112]. In the heart, H_2S has a protective role towards sepsis-induced damage in a mechanism involving the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) signalling pathway [113]. Exogenous H_2S reduces serum myocardial enzyme levels, decreased the levels of the inflammatory factors tumor necrosis factor (TNF)-α and interleukin (IL)-6, and increased the level of anti-inflammatory IL-10. Moreover, induction of PI3K/Akt signalling corresponded with increased phosphorylation status of total PI3K and Akt levels. H_2S treatment was also reported to reduce the levels of pro-apoptotic proteins, Bcl-2-associated X protein (Bax), and caspase levels [113]. The slow release H_2S donor, GYY4137 attenuates LPS or TNF-α/IFN-γ induced Caco-2 monolayer permeability by decreasing the expression of tight junction proteins, and reduces TNF-α/IFN-γ levels in plasma. Moreover, GYY4137 is cytoprotective in colon epithelial cells in mice with endotoxemia [114]. Similarly, GYY4137 reduces LPS-mediated pulmonary injury and neutrophil infiltration, and inhibits LPS evoked production of pro-inflammatory cytokines, in a mechanism involving heme oxygenase-1 (HO-1), [115]. The induction of these protective systems are important in the protection against damage induced during sepsis. Additional studies using pig models have shown that sepsis reduces CSE expression and that this property has been

proposed to contribute to sepsis-associated cardiac dysfunction [116]. However, similar studies using GYY4137 report no effect in a pig model of resuscitated septic shock in which cardiac and kidney function or systemic inflammatory response are assessed [117]. In other models of sepsis, namely urinary derived sepsis and associated systemic inflammatory response syndrome (SIRS), H_2S appears to be anti-inflammatory. Indeed, Chen et al. found that in a rabbit model of sepsis the levels of TNF-α, IL-10, and NF-κB are increased while in NaHS treated animals TNF-α and NF-κB levels are reduced and IL-10 levels elevated [118]. Furthermore, the relationship between NF-κB and H_2S in sepsis-induced myocardial injury has also gained some interest. In a recent study by Li et al. H_2S was reported to downregulate the NF-κB subunit p65 and TNF-α levels while increasing IL-10 levels in the rat model [119]. Importantly, the anti-inflammatory properties of H_2S correlated with significant reduction in the mortality rates in H_2S treated animals and reduction in pathological scores of cardiac tissue damage in animals. Similarly, in CBS knockout heterozygous (CBS+/−) mice mitochondrial mediated apoptosis in the adrenal gland and adrenal insufficiency is increased. However, GYY4137 treatment restored adrenal function and eliminated mitochondria mediated apoptosis via persulfidation of the target protein ATP Synthase F1 Subunit Alpha (ATP5A1) in male mice [120]. In other knockout systems, the loss of CSE amplifies the inflammatory response in a cascade involving the NADPH oxidase 4 (Nox4) ROS signalling system in septic mice and macrophages [120]. Similarly, in rabbits the inhibition of endogenous H_2S production using PAG exacerbates the inflammatory response during urine-derived sepsis-induced kidney injury [102].

Few investigations have assessed H_2S levels in patients suffering from sepsis. However, early research by Goslar et al. indicated that the plasma levels of sulfide are elevated during shock [121], and increased plasma sulfide concentrations are inversely correlated with blood pressure and cardiac function. Indeed, ICU patients with higher total plasma sulfide levels have higher mortality compared to patients with lower levels [101]. Furthermore, Chen et al. recently compared sepsis-associated acute kidney injury (SA-AKI), in AKI patients and healthy controls [122]. In AKI patients, levels of creatinine (Cre), urea nitrogen (BUN), tumor necrosis factor-α TNF-α and IL-1β, and myeloperoxidase (MPO) activity, as well as concentrations of MDA and H_2O_2, were significantly increased. In contrast, plasma level of H_2S was reduced in the same group of patients. Using an AKI mouse model (receiving an intraperitoneal LPS injection), Chen et al. found that diminished levels of H_2S correlated with a decline of 3-MST activity and expression. Moreover, replenishment of H_2S preserved kidney function in animals, and reduced markers of inflammation, oxidative stress and the expression of TLR4, NLRP3, and caspase-1 [122].

2.6 H_2S and the Respiratory Tract; Overview of H_2S, Chronic Obstructive Pulmonary Disease (COPD) and Asthma

H_2S is recognized as playing important roles in the respiratory tract (RT), and is involved in the regulation of airway tone, inflammation, fibrosis, pulmonary circulation, as well as in tempering oxidative stress, and in controlling rates of cellular proliferation and apoptosis. Altered levels of H_2S are found in several respiratory diseases such as chronic obstructive pulmonary disease (COPD), asthma, and some viral infections [123, 124]. Changes in the levels of H_2S in sputum from patients with cystic fibrosis (CF) are inversely correlate with the amount of sputum produced [125]. Moreover, in allergic rhinitis (AR) higher levels of H_2S, CSE, and CBS mRNA and protein expression are found in patients suffering this condition [126]. However, the significance of these findings requires further exploration.

The pathophysiological links between the development of chronic obstructive pulmonary disease (COPD) and H_2S have yet to be fully elucidated. COPD is a debilitating disease associated with inflammation and structural

remodelling in the lung. Researchers have shown that serum H_2S concentration increase in patients with stable COPD and are decreased in patients with acute exacerbation of COPD (AECOPD) [127]. In addition, in AECOPD patients with low serum H_2S levels, increased neutrophil proportion and lower lymphocyte proportion in sputum are found. This finding points to a potential association with H_2S and inflammation in the lungs of COPD patients [128]. Additional studies have shown that exhaled levels of H_2S in patients suffering from COPD correlates with that of nitric oxide (NO). At present, the biological significance of this interplay between these two molecules has yet to be addressed, however, it is feasible that measures of these two molecules could be useful in the development of non-invasive biomarker of airway inflammation in pulmonary diseases [129, 130]. In peripheral lung tissue CSE mRNA is increased but CBS mRNA decreased in COPD patients [131]. Similarly, H_2S can be detected in the exhaled breath of COPD patients suffering from lower respiratory tract infections [132]. A key question from these human studies is whether dysregulation in H_2S has a functional role in these respiratory conditions. Studies using animal models suggest this may be the case since treatment of C57/BL6 mice with NaHS prevents ozone induce lung injury by partially reducing Nod-like receptor pyrin domain containing 3 (NLRP3)-caspase-1 activation, and p38 mitogen-activated protein kinase phosphorylation and decreased Akt phosphorylation [133]. Furthermore, H_2S can inhibit cigarette smoke-induced endoplasmic reticulum stress (ERS) and apoptosis in bronchial epithelial cells [134], and reduces the expression of ERS markers including glucose-regulated protein-78 (GRP78), and C/EBP homologous protein (CHOP), and caspase-12 in pulmonary artery endothelial cells (PAECs) [135]. Other research indicates that H_2S inhibits fetal calf serum stimulated phosphorylation of ERK-1/2 and p38 mitogen-activated protein kinases (MAPKs) in airway smooth muscle cells (ASM) isolated from non-smoker and smoker but not that of COPD patients. Importantly, H_2S could repressed ASM proliferation and cytokine release in cells [136]. In the work of Guan et al. NaHS could prevent cigarette smoke (CS) induced airway remodelling, epithelial-mesenchymal transition (EMT), and collagen deposition in mouse lungs [17]. Mechanistically, H_2S reduced cigarette smoke extract (CSE) induced collagen deposition and oxidative stress in human bronchial epithelial 16HBE cells. This observation corresponded with the upregulation of SIRT1 expression, and the inhibition of the TGF-β1/Smad3 signalling pathway in vivo and in vitro [137]. The same group has also reported that NaHS increases the expressions of tight junction proteins zonula occludens-1 (ZO-1), occludin, and claudin-1, and has the capacity to reduce apoptosis and the secretion of pro-inflammatory cytokines including TNF-α, IL-6, and IL-1β in CS-exposed mouse lungs and in A549 cells via suppression of the PHD2/HIF-1α/MAPK signalling pathway [138]. Findings from this work indicating that H_2S inhibits CS-induced inflammation, injury, and apoptosis in alveolar epithelial cells by attenuating mitochondrial dysfunction, in a mechanism involving sirtuin 1 [17].

In other respiratory conditions such as asthma, dysregulation in the levels of H_2S are also reported, and are linked to altered function in lung tissues [123]. For example, in ovalbumin (OVA) treated rats, a standard approach to induce eosinophilic asthma through Th2 mediated inflammatory response, H_2S levels are diminished in pulmonary tissues. Moreover, serum H_2S levels positively correlate with the levels of H_2S in lung tissues and peak expiratory flow. In addition, using the same animal model, H_2S is reported to negatively correlates with the proportion of eosinophils and neutrophils in bronchoalveolar lavage fluid (BALF), and the infiltration of inflammatory cells in airway, along with goblet cell and airway smooth muscle hyperplasia [139]. This has led some researchers to suggest H_2S is a potential biomarker of asthma [140]. In mice, the lack of CSE expression increases airway hyperresponsiveness (AHR) and lung inflammation [141]. While other studies report that the lower abundance of CSE expression and H_2S production enhances type-2-immunoreaction and renders a higher incidence

of allergic asthma at a young age in mice [142]. Mechanistically, NaHS seems to be protective in epithelium cells by virtue of its ability to reduce airway inflammatory cell infiltration, by inhibiting the production of IL-4, IL-5, and IL-25, and to lessen the activation of caspase 3 and FasL following OVA challenge in mice [143].

2.7 H_2S and the Cardiovascular System

The manipulation H_2S levels in the cardiovascular system either via altered biosynthetic enzyme expression or delivery of H_2S via donor molecules markedly attenuates myocardial injury and improves cardiac function. Indeed, a whole spectrum of evidence shows H_2S has beneficial effects in numerous cardiovascular disorders, including atherosclerosis, ischemic, and heart diseases, with some of these effects likely due to the immunomodulatory properties of this molecule. As described in the current chapter, H_2S is important in the regulation of immune cell functions, i.e. T-cell activation and proliferation, monocyte and polymorphonuclear cell apoptosis, leukocyte adhesion and infiltration, and inflammatory cytokine release by immune cells. Research shows that H_2S and associated donors are anti-inflammatory in models of cardiovascular diseases [10, 144]. In conditions such as atherosclerosis, a major cause of death in developed and developing countries, dysregulation in inflammation is a key driver of this disease. Typically, over many years, vascular inflammation, vascular smooth muscle cell proliferation and migration, elevated rates of oxidative stress, thrombus formation, monocyte infiltration and differentiation, and lesion-resident macrophage are converted into foam cells. Collectively, these processes drive the severity and rates of atherosclerosis and damage in the cardiovascular system. Endogenous and exogenous sources of H_2S reduced ROS levels and temper the production of cytokines, and is seen as cytoprotective [7, 145, 146]. Indeed, in genetic mouse models of atherosclerosis, viz. ApoE−/− CSE−/− mice, H_2S inhibits the progression of atherosclerosis [147, 148]. Part of these protective effects stem from the preservation of endothelial function that prevents increased ROS production, and the expression of various proteins like the adhesion molecules, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion protein 1 (VCAM-1), [149]. These effects are a consequence of diminished endothelial inflammation via reduced NF-kB signalling. Other research shows H_2S impacts on inflammatory cell function that is known to be important in the cardiovascular system, viz. macrophages. Indeed, several studies show H_2S can inhibit foam cell formation, and macrophage lipid metabolism [150–154], and the recruitment of immune cells to the cardiovascular system [155]. At the molecular level, H_2S suppresses the expression of the C-C chemokine receptor type 2 (CCR2)-monocyte chemotactic protein-1 (CCL2), CX3CR1-CX3CL1, and CCR5-CCL5 [156], and reduces macrophage infiltration and promotes plaque stability. H_2S also alters the function of other immune cells including T-helper (Th) cells; cells that play a critical role in mediating adaptive immunity. Naive CD4 + T cells may differentiate into different lineages of Th cells, including Th1, Th2, Th17, and Treg cells; Treg cells can be recruited to atherosclerotic plaques and are found to limit lesion progression in experimental models by downregulating inflammatory responses [157]. In the work of Yang et al. reduced H_2S levels impaired CD4 + Foxp3+ Treg cell differentiation and function. Treatment with H_2S rescued Treg-cell-deficient phenotypes in CBS−/− mice and partially rescue autoimmunity in CBS−/− mice [158]. Taken together, H_2S donor molecules may offer a novel therapeutic approach for the treatment of chronic immune-inflammatory responses in atherosclerosis.

Functional roles of H_2S in heart failure are also recognized. Heart failure is an inability of the heart to adequately meet the metabolic needs of the body. Heart failure results from conditions like atherosclerosis, and other comorbidities such as diabetes and obesity, with each condition having an inflammatory component that precedes tissue injury. Acute MI is largely determined by the extent of myocardial tissue loss, however, it is

now known that inflammation also plays a role in cardiac remodelling following MI. Kondo et al. reported that circulating H_2S levels are reduced in experimental models of heart failure. Importantly, CSE deficient mice exhibit greater cardiac dilatation and dysfunction compared to wildtype mice after transverse aortic constriction [159]. Often accompanying these changes is the gradual increases in inflammation including leukocyte activation and release of inflammatory mediators, with the progression of ventricular dysfunction. Indeed, several inflammatory biomarkers including C-reactive protein, and various inflammatory cytokines like TNF-α and IL-6 are increased in heart failure, and leukocytosis is associated with disease progression [160]. To date, the molecular mechanisms of H_2S action in heart failure are attributed to this molecule causing a decrease in Nox4/ROS/ERK1/2 signalling and increased HO-1 expression [161], reduced recruitment of CD11b + Gr-1+ cells in infarct myocardium and peripheral blood, and to attenuate cardiac dilation in chronic ischemia-mediated infarcted myocardium in mice [162]. Consequently, H_2S is widely reported to be able to upregulate a spectrum of endogenous antioxidants molecules and proteins like glutathione peroxidase (GPx1) and HO-1, and improves cardiac function after heart failure via stimulating angiogenesis [22, 163–166].

Another area of research gaining attention is the part played by H_2S in myocardial infarction (MI). MI is the leading cause of death worldwide and is caused by reductions in the oxygen-carrying capacity of blood, combined with nutrient starvation, cardiomyocytes become insensitive to oxygen, leading to MI. Reducing rates of inflammation and the recruitment of inflammatory cells to sites of damage has been suggested as a promising therapeutic approach for treating MI and reperfusion injury [167, 168]. MI and reperfusion injury trigger a complex inflammatory response in the injured myocardium, leading to leukocyte infiltration and the release of cytokines including IL-6, IL-8, and TNF-α. In mammals, decreased levels of H_2S in plasma are linked with increased infarct size and mortality. Moreover, the administration of NaHS and other H_2S donor molecules, viz. SPCR and GYY4137, can decrease infarct size of the left ventricle and improve MI-associated mortality in animals [169, 170]. Based on the available studies, the cardioprotective mechanism of H_2S appear to be associated with the ability of this molecule to promote vasodilation, to act as an antioxidation, reduce levels of apoptosis in damaged cells, and to reduce the production of pro-inflammatory molecules [70]. Indeed, exogenous H_2S administration or overexpression of the H_2S-producing enzyme CSE reduces leukocytes and neutrophil infiltration within the ischemic zone and reduces myocardial inflammatory cytokine production. H_2S treatment can also improve post-MI cardiac remodelling and dysfunction in wildtype and CSE deficient mice, by reducing infarct size and mortality, and stimulating M2 polarization of macrophages at the early stage of MI [164]. Researchers have also shown that H_2S suppresses myocardial inflammatory responses by inhibition of NLRP3 inflammasome activation a mechanism dependent on microRNA-21 [171]. Furthermore, some H_2S donors like S-propargyl-cysteine (SPRC, a novel endogenous H_2S modulator) can prevent LPS-induced TNF-α, ICAM-1, and iNOS expression in cardiomyocytes via altering CSE expression and H_2S production. Increased H_2S levels inhibits the NF-κB signalling pathway, and the induction of PI3K/Akt signalling [149]. Consequently, many studies have now confirmed a beneficial role of H_2S in the myocardium and this is partly attributed to the anti-inflammatory properties of this molecule [113, 172–175].

3 Alternate Mechanisms of Action Linked to the Anti-Inflammatory Effects of H_2S

In recent years, there has been considerable interest aimed at developing therapeutics that take advantage of the anti-inflammatory properties of H_2S and has led to the identification of several novel cellular targets. These will be addressed below.

3.1 Alternate Signalling Pathways and Epigenetic Mechanisms

H_2S has the capacity to mitigating the inflammatory response in mammalian cells and tissues via several additional signalling systems. In recent years, H_2S has been reported to induce Sirtuin signalling in various mammalian cell lines [12, 176–181] and in various animal models [155, 182–185]. Xin et al. recently showed that H_2S reduces hepcidin expression in Huh7 cells and in mouse tissues via reduction in IL-6 secretion and following deacetylation of sirtuin-1 (SIRT1); SIRT1 a protein that mediates signal transducer and activator of transcription 3 (STAT3), [186]. Several H_2S donor molecules and/or the manipulation of the expression levels of H_2S biosynthetic enzymes can alter STAT3 activity, and has been shown to be important in ischemic post-conditioning [187], cancer cell proliferation [188], anemia of inflammation [69]. Regarding inflammatory signalling, NaHS inhibits exogenous ATP-stimulated inflammatory responses and Aβ1–42 production in both BV-2 and primary cultured microglial cells causing a reduction of pro-inflammatory cytokines, ROS and the activation of nuclear factor-κB (NF-κB) pathway via suppression of the STAT3 system [189]. Other H_2S donor molecules including SPRC reduced serum hepcidin, improved transferrin saturation, and maintained erythrocyte membrane integrity likely due to reduced hepatic JAK2/STAT3 activation [69]. Furthermore, in CSE−/− mice, H_2S deficiency increases indoleamine 2, 3-dioxygenase 1 (IDO1) expression and activity and stimulates the NF-κB and the STAT3 pathways. Additionally, H_2S donors including GYY4137 and NaHS effectively restricted tumor development in H22 HCC-bearing mice via downregulating IDO1 expression and inducing T-effector cells [190]. Other researchers indicate that exogenous H_2S minimized liver injury and decreased the expression of STAT3 in liver tissue of Methotrexate (MTX) challenged rats, an anticancer and immunosuppressive agent with occasional hepatotoxic effects [191].

Epigenetic mechanisms linked to H_2S treatment have also received consideration in recent times, and appear to be important in explaining some of the known inflammatory effects of H_2S. Importantly, H_2S acts as an inhibitor of histone H3 acetylation, leading to the suppression of chromatin opening, and decreased gene transcription of the pro-inflammatory cytokines IL-6 and TNF-α. In addition, H_2S can inhibit angiotensin II induced downregulation of microRNA precursor 129 (miR-129), CBS, CSE, and IL-10 while increasing IL-17A and DNA (cytosine-5)-methyltransferase 3A (DNMT3a) in the hypertensive kidney [192]. In CBS deficient mice the activity of histone deacetylase 3 (HDAC3) is reduced, and treatment with NaHS can activate HDAC3, and downregulated inflammatory signalling, via runt-related transcription factor 2 (RUNX2) persulfidation. Moreover, in the study by Lin et al. H_2S attenuated oxidative stress induced mitochondrial reactive oxygen species (mtROS) production and NLRP3 inflammasome activation via persulfidation of c-Jun at position cys269 in macrophage cells [177]. Persulfidation of c-Jun enhanced its transcriptional activity.

3.2 H_2S and the Inflammasome

While researchers have reported much on the influence of H_2S on NF-kB signalling systems it is only now that we are beginning to understand how this molecule impacts on other components of the inflammatory process. One area that has gained interest in the last decade is how H_2S influences the formation of protein complexes called inflammasomes. To date, a number of inflammasomes have been characterized, viz. NLRP1, NLPR3, NLRC4, and AIM2 inflammasomes [193]. Of interest here, is the NLRP3 inflammasome since this protein complex has received the most focus in the field of H_2S research. This protein complex is formed by the NLRP3 sensor molecule, the adaptor protein ASC, and the effector caspase-1. NLRP3 is activated by a spectrum of stimuli including pathogen-associated molecular patterns

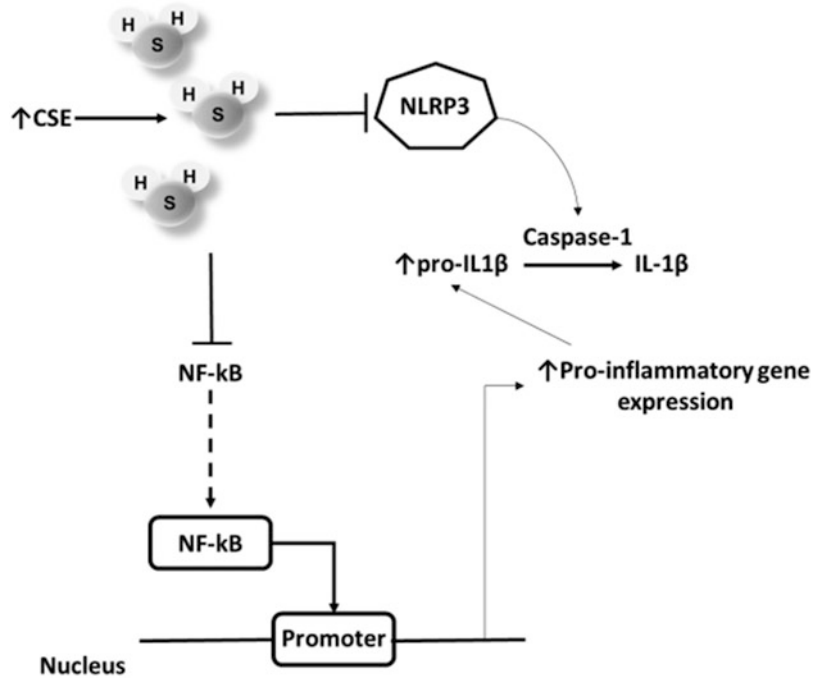

Fig. 3 The inhibitory effect of H_2S on inflammasome activation. Inhibition of NLRP3 drives reduction in the production of the pro-inflammatory molecule IL-1β

(PAMPs) and damage-associated molecular patterns (DAMPs) that induce signalling events that cause the upregulation of NLRP3 and pro-IL-1β transcription/translation [194]. In turn, NLRP3 assembly activates caspase-1 and converts any pro-IL-1β formed into its active form IL-1β [195]. Dysregulation in inflammasome activation is associated with autoimmune and autoinflammatory diseases. The part played by H_2S in inflammasome formation and activation is only now coming to light (Fig. 3). Huang et al. reported that some of the known cardioprotective effects of H_2S via the suppression of cardiomyocyte inflammation and apoptosis via the inhibition of the TLR4/NF-κB pathway, and downstream activation of the NLRP3 inflammasome in high glucose mediated cytotoxicity in h9c2 cardiac cells [196]. Similarly, in collagenase-induced intracerebral hemorrhage in the rat, H_2S production is decreased in the brain and correlates with reductions in CBS expression. Administration of S-adenosyl-L-methionine (SAM), a CBS-specific agonist, or NaHS, reportedly restores brain and plasma H_2S levels and reduces brain edema, microglial accumulation, and neurological deficits. Part of the mechanism for these beneficial effects was the inhibition of the P2X7R/NLRP3 inflammasome cascade [197]. Some H_2S donor molecules are also reported to influence the activation and formation of inflammasomes. For example, in isolated murine and human macrophages, sodium thiosulfate and GYY4137 can inhibit monosodium urate crystal induced IL-1β secretion, XO/caspase-1 activities, mitochondrial reactive oxygen species (ROS) production, and ASC oligomerization [198]. Consequently, H_2S has now been reported to prevent inflammasome activation in mammalian cells induced by free fatty acids in Raw 264.7 cells [199], and oxidative stress induced inflammasome formation and activity [177]. In addition, H_2S reduces lipopolysaccharide (LPS) + Adenosine Triphosphate (ATP)-induced inflammation by inhibiting nucleotide-binding oligomerization domain-like receptor 3 (NLRP3) inflammasome activation and promoting autophagy in L02 cells [200], and tempers NLRP3 in dextran sulfate sodium (DSS)-induced colitis [201]. Other researchers have reported that the inhibition of inflammasome

formation and inflammation by H_2S in spontaneous hypertensive rats preserves endothelial function [202], that H_2S reduces high glucose induced retinal and adipocyte mediated inflammation [41, 203], renal injury and fibrosis [204], diabetes-accelerated atherosclerosis [205], and liver injury [206, 207]. Collectively, in most of these studies, H_2S appears to attenuate cellular damage, apoptosis, and reduces the expression of NLRP3, ASC, pro-caspase-1, caspase-1, IL-1β, IL-18, and caspase-3, respectively.

3.3 Antioxidant Properties of H_2S

Free radicals are ubiquitous to life and serve a number of important roles in biological systems [208]. Dysregulation in the production of these molecules occurs in a number of pathophysiological conditions whereby the oxidation of proteins, carbohydrates, and nucleotide bases present in DNA and RNA is believed to contribute to disease severity. Free radical production originates from organelles such as mitochondria, and via the activities of enzymes like xanthine oxidase (EC 1.17.3.2), and NADPH oxidase (EC 1.6.3.1). It is common to find elevated levels of reactive oxygen species (ROS), comprising of superoxide (O_2. -) and hydroxyl ($OH^{\cdot}$) radicals and the non-radicals hydrogen peroxide (H_2O_2), during episodes of cellular stress. In addition, reactive nitrogen and chlorine species like peroxynitrite (ONOO-) and hypochlorous acid (HOCl) are also produced. In a healthy individual basal free radical production is important in normal cellular processes particularly in immune defences. However, elevated rates of production leads to oxidative stress; a term indicative of increased rates of tissue damage mediated by free radical species. Increased free radical production occurs in arthritis, sepsis, cardiovascular disease, COPD, and many other common disorders and in the aging process [209]. In these conditions, ROS can damage lipids, proteins, cellular membranes, and DNA promoting cellular damage, or the activation of signalling cascades linked to cell proliferation, apoptosis, and inflammation or cells of the immune system [210, 211]. Normally, these damaging effects can be mitigated by the induction of cellular antioxidant defences comprising of enzymes like catalase (EC 1.11.1.21), superoxide dismutase (EC 1.15.1.1), and various repair enzymes. Allied to this induction is the increased synthesis of small molecular weight antioxidants such as glutathione (GSH), and the activation of Nrf2, a key transcription factor involved in the regulation of cytoprotective enzymes. Interestingly, H_2S acts as a one-electron chemical reductant (a nucleophile) that can scavenge free radicals via single electron or hydrogen atom transfer processes. Indeed, at physiological pH (pH 7.4), and a temperature of 37 °C, approximately 80% of the H_2S molecules dissociate forming HS^- (hydrosulfide anion). HS^- anions act as one-electron chemical reductants with the capacity to quench free radical species via hydrogen atom transfer or by single electron transfer [212]. The reactivity of the HS^- anion with oxygen is enhanced by the presence of divalent metal ions and can facilitate the release of NO in reactions with S-nitrosothiols [213, 214]. Other noted reactions of the HS^- anions can occur in the presence of hypochlorous acid and hydrogen peroxide to yield hydrogen disulfide (H_2S_2); this molecule in turn can be a source of H_2S in reactions with thiols [215]. It is now widely accepted that H_2S can scavenge reactive oxygen, nitrogen, and chlorine species, viz. superoxide [216], hydrogen peroxide [217, 218], peroxynitrite [219], and hypochlorous acid [220] (reviewed by [221]). In addition, H_2S seems to be important in the radical generating and scavenging properties of the tetracycline antibiotics Doxycycline [222]. H_2S treatment is widely reported to reduce the damaging effects of free radical in cells by preventing free radical generation and by inducing cytoprotective systems. Indeed, H_2S can inhibit ROS generating enzymes like NADPH Oxidase [161, 188, 223]. Moreover, at the cellular level, Sun et al. showed that pre-treating rat neonatal cardiomyocytes with NaHS reduced the levels of ROS during hypoxia/reoxygenation (H/R). The mechanisms for these protective effects seem to correlate with the ability of H_2S to inhibit mitochondrial

complex IV activity and to increase the activities of superoxide dismutases (SODs), including Mn-SOD and CuZn-SOD [224]. Interestingly, other researchers have shown that H_2S can be oxidized by SOD in the presence of oxygen to produce polysulfides (mainly H_2S_2, and to a lesser extent H_2S_3 and H_2S_5), [225]. Importantly, some polysulfide compounds act as potential sources of H_2S and possess antioxidant like properties themselves. Using electron paramagnetic resonance (EPR) spin trapping techniques, Misak and colleagues found that the polysulfide (Na_2S_4) was a potent scavenger of the O_2- and cPTIO radicals as compared to H_2S [226]. Additional studies of other antioxidant enzymes indicate that the enzyme catalase is also important in the metabolism of H_2S. In this instance, catalase can function as either a sulfide oxidase or sulfide reductase; roles that are important in the metabolism of a range of sulfur species and that are dependent on relative oxygen levels [227]. In addition, evidence now points to a role of cellular antioxidant systems as important sites in the production and metabolism of H_2S and polysulfide species in cells [228]. Canonical inhibitors of ROS antioxidant pathways alter reactive sulfur species in HEK293 cells independent of ROS levels. Decreasing intracellular glutathione (GSH) with l-buthionine-sulfoximine (BSO) or using diethyl maleate (DEM) reduced H_2S production but does not impact on polysulfide levels (H_2S_n) in cells. Treatment of cells with auranofin, a glutathione reductase inhibitor, decreases H_2S and H_2S_n but after 2 days H_2S_n levels recover. The inhibition of peroxiredoxins with conoidin A decreased H_2S and increased H_2Sn. The inhibition of glutathione peroxidase using tiopronin increases H_2S levels in cells. Clearly additional research is needed to understand the impact of these systems on tissue H_2S levels but this new body of work points to a complex system of biochemical processes that can influence sulfur metabolism. Critically, in the last few years polysulfides have been recognized as important molecules possessing a range of bioactive properties in mammalian cells. It is likely that these molecules are of great importance in biological systems and various physiological processes, however, more work is needed in this area (reviewed by [5, 229]).

3.4 H_2S and Nrf-2

In addition to the synthesis of intracellular antioxidants during episodes of stress, mammalian cells also rely on enzymatic systems that protect cellular constituents from the damaging effects of exogeneous and endogenous molecules such as ROS. These antioxidant enzymes detoxify a whole spectrum of molecules to less harmful forms. In relation to the immune system, ROS production can occur via respiration, and various enzymatic systems including NADPH oxidases enzymes (NOX), [230]. NOX enzyme is widely expressed in cells and tissues of mammals including many immune cell types and play important roles in immune defence and cell signalling systems such as in the cardiovascular system [231], the central nervous system [232], and in rheumatic diseases [233]. To prevent ROS mediated cellular damage, a counter response is initiated that involves increased expression and activities of cytoprotective enzymes. The expression of these proteins is mediated by the transcription factor, Nrf-2, [234]. Nrf-2 is a basic leucine-zipper protein, and a master regulator involved in combating oxidative stress and cellular damage induced endogenous and exogenous molecules. Gene expression occurs via the binding of Nrf-2 to the antioxidant responsive element (ARE) located in the promote regions of a spectrum of enzymes needed to combat the damaging effects of toxic molecules. During episodes of stress, Nrf-2 is liberated from its cytoplasmic anchor, Kelch-like ECH-associated protein (Keap 1), releasing Nrf-2, and allowing for its translocation to the nucleus of cells. Here, Nrf-2 can drive transcription of cytoprotective genes that provides protection against the damaging effects of a whole spectrum of separate compounds. Several researchers have shown that H_2S can induces the Nrf-2 signalling pathway in various disease models including hemorrhagic shock [235], lung injury [236], hyperoxia-induced acute lung injury [237], ischemia-

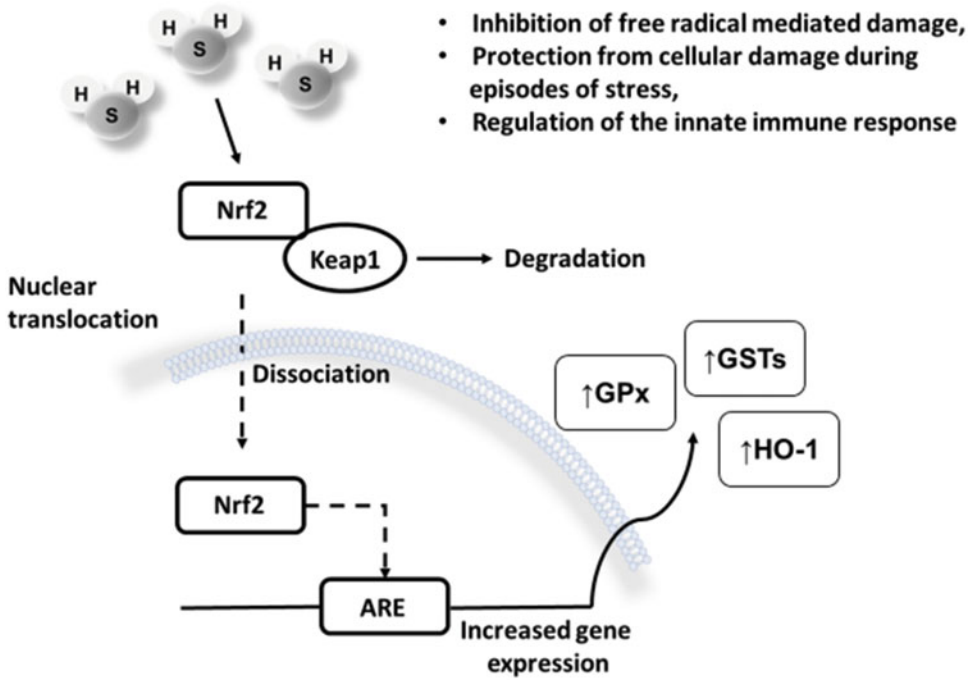

Fig. 4 During episodes of inflammation, reactive oxygen species and other reactive molecules are generated, and these can damage cellular components like proteins, lipids, and DNA. To mitigate the damaging effects of these molecules the transcription factor Nrf2 upregulates the expression of a spectrum of enzymes critical for detoxification and cellular protection. Hydrogen sulfide is a recognized inducer of this cytoprotective system in mammalian cells

reperfusion injury [238], galactosamine/lipopolysaccharide induced acute liver failure [239], human osteoclast differentiation and function [240], and diabetic nephropathy [66]. Additional research point to the protective role of H_2S in models of diabetic cardiomyopathy [241], left ventricular remodelling in smoking rats [242], and critical Limb Ischemia [243], that is mediated by Nrf-2 signalling. The ability of H_2S to drive Nrf2 networks appear to be critically important in describing some of the known protective and antioxidant effects of this molecule (Fig. 4). Indeed, in more recent times, research has shown that H_2S induced Nrf-2 signalling is important in the immune system and likely contributes to the anti-inflammatory effects ascribed to this molecule. In macrophages H_2S mediates the inhibition of NF-κB and the secretion of the pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), but induces the antioxidant Nrf-2/HO-1 pathway in cells [244]. The novel H_2S modulator, S-propargyl-cysteine (also known as ZYZ-802), can induces HO-1 expression and Nrf-2 activation in a model of rheumatoid arthritis [90]. H_2S has been found to induce Nrf-2 signalling in models of mycoplasma mediated inflammation [245], in dextran sulfate sodium (DSS)-induced colitis [201], to ameliorate endothelial dysfunction with hypertension and NLRP3 inflammasome formation [202], induce Nrf-2 signalling in *Mycoplasma pneumoniae* infected human mononuclear THP-1 cells [246], and in a model of paraquat-Induced acute liver injury [206]. It is now clear that H_2S induces the Nrf-2 system in a range of disease models and this has led some investigators to speculate that a common mechanism of action may be responsible. Interestingly, H_2S plays an important role in protein persulfidation a recently characterized post-

translational modification that occurs to reactive cysteine residues. Persulfidation of protein induced by H_2S is important in the regulation of several signalling pathways [4, 5]. To date, several studies have shown that persulfidation appears to be important in H_2S mediated Nrf-2 signalling. Indeed, persulfidation of Keap1 at cysteine-151, cysteine-226, and cysteine-613 residues drives dissociation of the Nrf-2/Keap1 complex leading to increased expression of antioxidant genes and protection against cellular senescence [247]. This post-translational modification appears to be important in preventing ischemia-reperfusion induced oxidative stress and inflammatory responses in cells. Treatment with NaHS increases in GSH levels, and decreases lipid peroxidation products, reactive oxygen species generation and the levels of NO, IL-6 and TNF-α [248]. Persulfidation has been reported in additional studies and shown to be important in reducing oxidative stress, inflammation, and cellular damage. To date, H_2S mediated persulfidation of Nrf-2 has been observed in models of high-salt diet-induced renal oxidative stress and kidney injury [65], diabetes-accelerated atherosclerosis [249], sulfur mustard induced lung injury [250], oxidative stress induced hepatocyte damage [251], and paraquat poisoning [206].

4 Conclusions

Over the last two decades, H_2S has now gained interest from the academic community and is recognized as an important gasotransmitter that has roles in various physiological and biochemical processes including the immune system. It is now widely reported that dysregulation in the production of this molecule is found in pathophysiological conditions spanning most organ systems, viz. heart, kidney, and brain. Moreover, loss in the capacity to maintain tissue H_2S occurs in several diseases and is generally linked to altered immune function. While new targets sensitive to changes in H_2S will likely be identified in the near future, our understanding of this molecule in the immune system has progressed steadily over the last few years. Importantly, this has led to the development of a range of H_2S donor molecules that can be used to manipulate immune signalling systems in various diseases. Consequently, these molecules will provide new tools to explore the function of H_2S in the immune system and drive new breakthroughs in the area.

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Hydrogen Sulfide and its Interaction with Other Players in Inflammation

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1 Introduction

Inflammation is a protective response of vascularized tissue to invading pathogens or endogenous signals, resulting in tissue destruction or injury. In broader context, inflammation is a host response to damaged cells or tissue injury to clear injurious stimuli and begin a healing process. Redness (rubor), swelling (tumor), pain (dolor), heat (calor) that result in loss of function (functio laesa) are the clinical ("cardinal") signs of inflammation [1, 2]. There is often a spontaneous and rapid host response to an injurious factor which is caused by elevated blood flow into damaged tissues, mainly from arteriolar (vascular) dilatation and opening of capillary beds. There is also an increase in the migration of leukocytes with increased adhesion of leukocytes to the vascular endothelium cells via adhesion molecules. Once at the site of injury/infection, leukocytes ingest and attempt to destroy the pathogens, leading to the release of toxic residues and possibly tissue injury. These activities result in a series of biochemical mediators such as vasoactive amines and arachidonic acid [3]. In addition, ROS are activated in inflammation, including the release of proteases to fight against invading pathogens. However, the activity of ROS and proteases has a deleterious effect on the surrounding tissue. A complex arrangement of chemokines and cytokines is released by both vascular endothelium and inflammatory cells, resulting in the regulation of lymphocyte function via activating (e.g., IL-4, IFNβ, IL-2, and IFN α) or inhibiting (e.g., TGF-β and IL-10) the immune response. Additionally, there is activation of inflammatory cells or stimulation of hematopoiesis and leukocyte growth and differentiation by cytokines [3]. Physiological gaseous transmitters such as nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S) have been proposed to be involved in these inflammatory processes [4].

Over many years, hydrogen sulfide (H_2S) has only been viewed as a poisonous gas, despite its existence in the atmosphere at low concentrations. According to Occupational Safety and Health Administration, its exposure limit is 20 parts per million (ppm) [5]. If exposure to H_2S is greater than 700 ppm, it can result in sudden death [5]. Numerous deaths and accidents caused by the exposure of high concentrations of H_2S have been reported. Various reports have identified cardiovascular, respiratory, metabolic, neurological outcomes as a result of high H_2S exposure. Crude petroleum, putrefying water, sewage plants, and hot springs are found to be the common sources of H_2S. Despite its lethal activity for centuries, H_2S can be synthesized endogenously to regulate both physiological and pathophysiological states in living organisms

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[6]. H_2S is considered a relatively newer nominee to the family of biological gasotransmitters which previously only consisted of NO and CO. Other than inflammation, it is also involved in oxidative stress, gut motility, ulcer healing, vascular tone, cryoprotection, neuromodulator, memory loss hormone secretion, apoptosis, and pancreatic β-cells (insulin secretion) [7, 8].

This review summarizes studies illustrating the importance of H_2S in inflammatory diseases (Fig. 1), the characteristics of H_2S, its activities with other inflammatory mediators, and interrelationship with other gasotransmitters. The evidence collected in recent years has clearly shown H_2S as an inflammatory mediator. However, according to some investigators, the specific activity of H_2S in inflammation is still ambiguous. We will also be illustrating brief insights about the ambiguous nature of H_2S according to different studies.

2 H_2S in Inflammatory Diseases

2.1 Sepsis

Sepsis is a life threatening and pathophysiological condition accompanied by systemic inflammatory response syndrome (SIRS) which is caused by a dysregulated host response to infection. Sepsis when followed by a minimum of one organ damage is identified as severe sepsis. Septic shock is the progressive condition of patients with sepsis which results in the underlying circulatory, metabolic and cellular abnormalities that are associated with higher mortality risk than sepsis alone [9–12]. As per the recommendations of the Society of Critical Care Medicine and the European Society of Intensive Care Medicine, the definitions of sepsis and septic shock have been updated [11]. Based on their recommendation, organ malfunction/dysfunction during sepsis can be categorized by the Sequential [Sepsis-related] Organ Failure Assessment (SOFA) Score [13]. Despite advances in health care, sepsis remains a major health problem worldwide, with reports on incidences rising. This is likely to reflect the aging population, increased rate of comorbidities that elevate susceptibility to infection, and growing numbers of immunocompromised patients like those suffering from malignancies, organ transplant, or HIV infection. Based on the survey data in the USA, sepsis incidence in 2003 was reported to be 359 per 100,000 population, which transforms into annual number of 535 cases per 100,000 population in 2009 (49% increase), with valuation costing above $20 billion and 5.2% of the overall United States hospital expense in 2011 [13, 261]. Even though the correct statistics of the incidence is still unknown, conservative estimates reflect that sepsis is a major factor for mortality and critical illness worldwide and in-hospital mortality rate continues to be high at 25–30% [13]. Sepsis and its eventful progressive condition are the systemic response to infection caused by various inflammatory mediators, such as chemokines and cytokines.

H_2S has been proposed to promote inflammation in animal models of sepsis, such as that induced by cecal ligation puncture model (CLP) or lipopolysaccharide (LPS) in mice or rats. LPS-induced endotoxemia was employed in early studies to investigate the role of H_2S in sepsis. The significant elevation of H_2S plasma concentration, cystathionine γ–lyase (CSE) activity, and CSE mRNA levels in kidney and liver tissue involved in elevated inflammatory response and multiple organ damage demonstrated excess production of endogenous H_2S [254]. CSE is the predominant enzyme responsible for H_2S-synthesis in the peripheral organs and vascular system [216]. The detailed biosynthesis of H_2S will be discussed in the biosynthesis section. Administration of sodium hydrogen sulfide (NaHS) aggravated endotoxemia, leading to multiple organ damage [254]. However, pretreatment with the CSE inhibitor DL-propargylglycine (PAG) resulted in inhibition of these symptoms. These results highlight that H_2S has pro-inflammatory activity in LPS-induced endotoxemia. Conversely, other studies have

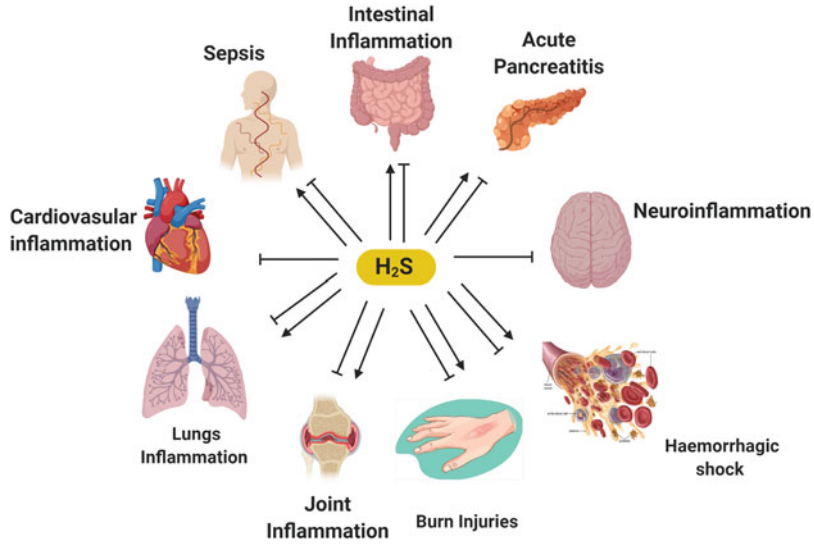

Fig. 1 Schematic diagram of H_2S involvement in various inflammatory diseases

reported anti-inflammatory roles of H_2S. Studies using slow H_2S-releasing donors GYY4137 (morpholin-4-ium-4-methoxyphenyl (morpholino)phosphinodithioate), Lawesson's reagent, and S-diclofenac reportedly reduced leukocyte infiltration, cytokine and eicosanoid production, and NF-κB activation in an LPS-induced endotoxemia model [255, 259, 260]. To avoid the limitations of LPS-induced endotoxemia (it does not reflect the cytokine profile or hemodynamic alterations of human sepsis), another comprehensive mice model which portrays the clinical condition of sepsis has been used to study the inflammatory role of H_2S [14]. Various studies have used CLP-induced sepsis to demonstrate significant elevation in the expression of CSE and H_2S levels which are involved in leukocyte infiltration and organ injury. Liver H_2S synthesis and plasma levels were significantly elevated after 8 h in CLP-induced sepsis compared to sham operation. This model also showed significant increase in liver CSE mRNA expression. When used prophylactically and therapeutically, PAG significantly decreased sepsis-induced systemic inflammation, as reflected by reduced MPO activity and histological changes in liver and lungs, reducing mortality in CLP-induced sepsis. However, the administration of NaHS, an H_2S donor, resulted in the exacerbation of sepsis-associated systemic inflammation. Attenuation of H_2S formation and administration of NaHS reveals that H_2S plays an important role in pro-inflammation to regulate the severity of sepsis and associated organ injury [14]. The mechanism of the involvement of H_2S in sepsis has been highlighted in several studies . For instance [15], PAG used as both a prophylactic and therapeutic agent showed significantly reduced mRNA and protein expression levels of IL-1β, IL-6, TNF-α, MCP-1, and MIP-2 in liver and lungs, with declined nuclear translocation and activation of NF-κB. Furthermore, PAG caused significant reduction of lung permeability along with plasma alanine aminotransferase activity. However, administration of NaHS resulted in increased NF-κB activation and significant exacerbation of sepsis-associated systemic inflammation. In addition, BAY11-7082 (an inhibitor of NF-κB) also attenuated H_2S-induced inflammation in lungs. These studies reflect the role of H_2S in the increased production of pro-inflammatory mediators and aggravation of systemic inflammation in sepsis through a pathway involving NF-κB activation.

Another study [16] demonstrated H_2S-mediated inflammatory response through the regulation of extracellular signal related kinases (ERK) pathway in CLP-induced sepsis.

The silencing of CSE in macrophages via siRNA in CLP-induced sepsis revealed the activity of H_2S and macrophages in sepsis. Administration of siRNA inhibited inflammation in both liver and lungs in CLP-induced sepsis mice, as supported by the significant decrease in MPO activity, and expression of cytokine and chemokine levels. These findings demonstrated pro-inflammatory activity of H_2S synthesized via CSE in macrophages in sepsis and highlighted CSE gene silencing via siRNA as a novel therapeutic approach for sepsis. Furthermore [17], we have used CSE knockout mice to study CSE/H_2S activity in sepsis-induced liver, lung injury, and inflammation. In this study, wild type CLP-induced mice demonstrated an increase in CSE expression, H_2S synthesis, MPO activity, and cytokines/chemokines levels, through activation of ERK1/2-NF-κB p65. However, in the CSE knockout mice, these were reversed, demonstrating the inhibition of inflammation. In a more recent study [18], we demonstrated the presence of LSEC damage in CLP-induced sepsis, as supported by the elevation in defenestration and gap formation. Several studies have highlighted LSEC defenestration formations as the key characteristic or even an early phenome non in the development of liver infection or inflammation. LSECs have been demonstrated to influence the pathogenesis of inflammatory disease by inducing recruitment of leukocytes through chemokines and adhesion molecules [262]. CSE knockout mice demonstrated reduced defenestration and gaps formation even in sepsis, suggesting that CSE/H_2S signaling plays a role in the regulation of the LSEC [18].

In contrast, studies have reported a protective effect of H_2S in CLP-induced sepsis. For example, the administration of H_2S donors (NaHS and Lawesson's reagent) has been shown to improve neutrophil migration and survival rate via a mechanism involving the activation of K_{ATP} channels in CLP-induced sepsis [19]. Furthermore, the administration of NaHS increased survival rates in mice by attenuation of the C/EBP homologous 10 (CHOP) in CLP-induced sepsis [20]. Depending on the model used, H_2S can play both pro-inflammatory and anti-inflammatory roles. In the CLP-induced sepsis mice model, gene deletion and pharmacological inhibition of H_2S has shown the pro-inflammatory effects of H_2S [48]. In contrast, models which have used the slow-release H_2S donors show anti-inflammatory effects of H_2S. H_2S has a bell-shaped biphasic dose response curve in which low and high concentrations show opposing effects. Low concentrations/slow H_2S releasing compounds are associated with anti-inflammatory properties, whereas high concentrations show pro-inflammatory properties.

2.2 Acute Pancreatitis

Acute pancreatitis is an inflammatory disease with acute inflammation at a local site in the pancreas and is in some cases followed by systemic inflammatory response syndrome (SIRS) which leads to multiple organ failure. It is often secondary to excess alcohol consumption, biliary disease, or viral infections, with increasing incidence. In severe cases, the mortality rate can be up to 30–50% [21, 22]. There is auto-digestion of the pancreas, failure of microcirculation, and increase of free oxygen radicals secreted from the injured cells. The pathophysiology of acute pancreatitis is characterized by acinar cell injury and release of pro-inflammatory chemokines, cytokines, substance P, and gaseous transmitters [23].

H_2S plays an important role in the pathophysiology of acute pancreatitis as evident with in vitro and preclinical studies [23–26]. Pancreatic acinar cells highly express both CBS and CSE [25] and pancreatic homogenates demonstrate H_2S synthesizing activity [27]. Endogenous H_2S has been shown to mediate the inflammatory response [27]. Mouse pancreas expresses CSE mRNA and plasma levels of H_2S increase upon induction of acute pancreatitis in mice [27]. When mice were treated with DL-propargylglycine (PAG), an irreversible inhibitor of CSE, the H_2S synthesizing

activity of mouse pancreatic homogenates was attenuated [27]. Furthermore, prophylactic or therapeutic treatment of mice with PAG reduced disease severity [26]. This was evident from decreased acinar cell injury, myeloperoxidase (MPO) activity, and histological evidence [27].

Severe acute pancreatitis is associated with lung injury with increase in acinar cell injury and MPO activity [27]. This lung injury is mediated by accumulation of neutrophils. PAG treatment also protected mice against lung injury in severe acute pancreatitis [26]. CBS also plays an important role in acute pancreatitis pathogenesis and lung injury. Mice treated with caerulein produced H_2S and NH_3 in the pancreas and lungs [28]. Treatment of mice with aminooxyacetate, a reversible CBS inhibitor, reduced the synthesis of H_2S and NH_3 in the pancreas [28]. Thus, results from both pancreatic acinar cells and mice show that acute pancreatitis is associated with increased H_2S synthesis and inhibition of H_2S synthesis provides protection against the disease severity. The increase in H_2S synthesis is followed by release of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6, among others. Macrophage response in primary pancreatic acinar cells showed that several macrophage proteins, monocyte chemoattractant protein (MCP1), macrophage inflammatory protein MIP-1a and MIP-2 were increased upon exposure to caerulein [26]. Furthermore, PAG treatment reduced the CSE mRNA expression along with MCP-1, MIP-1a, and MIP2.

H_2S acts through the activation of the transcriptional factor NF-κB and downstream pathways to induce a cascade of inflammatory genes [29]. In this study, H_2S activated the NF-κB pathway and increased phosphorylation of Src-family kinases in caerulein-treated pancreatic acinar cells [29]. Severe acute pancreatitis is associated with pancreatic acinar cell death which can be mediated through either apoptosis or necrosis [30]. Necrosis has been shown to positively correlate with the severity of acute pancreatitis [31]. Acute necrotizing pancreatitis is the most severe form with coagulation necrosis of the glandular cells and fat tissue. High mortality rate up to 20% has been seen in patients with pancreatic necrosis [32]. A necrosis–apoptosis imbalance has been proposed in acute pancreatitis and shifting this imbalance in favor of apoptosis could ameliorate the disease severity.

Necrosis is "accidental" cell death which is accompanied by a strong inflammatory response. Necrosis leads to the release of cellular contents which induces inflammation through the activation and infiltration of neutrophils. In a study of severe acute pancreatitis mice model treated with either exogenous H_2S (NaHS) or PAG, administration of exogenous H_2S worsened necrosis [32]. H_2S regulates the expression of apoptotic proteins to reduce apoptosis. H_2S increased the expression of Bcl-2 which inhibits apoptosis and reduced the expression of Bax protein which stimulates apoptosis. PAG, however, reduced the expression of Bcl-2 and increased the expression of Bax to promote apoptosis. Thus, inhibition of H_2S synthesis with PAG reduced necrosis and increased apoptosis, reducing the disease severity.

Another mechanism for the action of H_2S in the inflammatory response is through the autophagy pathway by activation of AMPK and inhibition of mTOR [33]. Impaired autophagy is a pathological response in acute pancreatitis [34]. There is accumulation of autophagic vacuoles and pre-mature activation of trypsinogen [35]. Autophagy in acini proceeds with the initiation of autophagosome and fusion of autophagosome with lysosome [36, 37]. The impairment in autophagy is worsened with the exogenous H_2S donor NaHS [33]. Mice with gene deletion of Atg5, involved in autophagic vesicle formation, is resistant to caerulein-induced acute pancreatitis [35]. This shows that the autophagy pathway is involved in the pathophysiology of acute pancreatitis and exogenous H_2S worsens it. This suggests that the toxic effects of H_2S are mediated through the autophagy pathway [38].

H_2S is involved in regulation of intracellular energy metabolism [39]. Levels of HIF-1α increase after 8-12 hours post-acute pancreatitis induction in mice [40]. HIF-1α provides relief against the metabolic stress and promotes apoptosis over necrosis by reducing the translocation of

NF-κB to nuclei. It prevents the increase in concentration of calcium and decreases the mitochondrial membrane potential which suppresses the glycolysis pathway. Most of the studies reveal the pro-inflammatory role of H_2S in acute pancreatitis; however, some studies suggest that H_2S also has anti-inflammatory effects in acute pancreatitis [41, 42]. These studies show that H_2S inhibits the NF-κB expression and decreases the pro-inflammatory cytokines, chemokines, and adhesion molecules production [43, 44]. It inhibits the synthesis of TNF-α in hemorrhagic shock-induced rats [45]. It also shows anti-apoptotic activity [46, 47].

2.3 Lung Inflammation

H_2S reportedly plays varying roles in different lung inflammatory diseases, including asthma and chronic obstructive pulmonary disease (COPD). The effect of the gasotransmitter differs depending on several factors: whether exposure is acute or long-term, and if endogenous or exogenous H_2S is being studied.

COPD is an inflammatory disease that is characterized by (partially reversible) progressive obstructed airflow and inflamed airways [49]. Although cigarette smoking is the main risk factor associated with COPD, other causes have also been identified [50]. While investigating the role of long-term (3–64 years) ambient exposure to H_2S on lung function, asthma, and COPD in Rotorua, a city located on a geothermal field in the Taupo Geothermal Zone of New Zealand, Bates et al. (2015) discovered no evidence of lung function decrement or increased asthma or COPD risk [51]. The results of the cross-sectional study differ from previous ecological studies which elucidated that there may be health risks associated with H_2S exposure in the city which is surrounded and filled with geysers, boiling water, and boiling mud pools. Previously, patients with stable COPD showed higher H_2S serum levels than healthy controls and those with acute exacerbation of COPD [50]. In contrast, CSE expression is decreased in the lung tissue of smokers (without COPD) and patients with COPD compared to healthy patients. Although protein expression is lower, CSE mRNA expression is higher in patients who have COPD and patients who are smokers compared to healthy counterparts, even though there is no significant difference in H_2S levels in lung tissue in each group [52].

Cigarette smoke induces endoplasmic reticulum stress and endoplasmic reticulum stress-induced apoptosis [53], but exogenous H_2S inhibits this in bronchial epithelial cells and consequently prevents lung tissue damage in rat lung [54, 55]. While investigating the role of H_2S on the anti-inflammatory activity of theophylline, a non-selective phosphodiesterase inhibitor that is used to treat COPD, Chen et al. (2006) found no significant change in H_2S levels between patients treated with theophylline and those that were not, although theophylline-treated patients showed improved symptoms and reduced sputum neutrophils. Further studies are needed to elucidate the effect of H_2S on treatments for COPD and other lung diseases.

2.4 Joint Inflammation

Arthritis, the inflammation of one or more joints, is typically characterized by swelling due to fluid accumulation in the tissue surrounding the affected joints. There are different types of inflammatory arthritis; rheumatoid arthritis (RA), psoriatic arthritis (PsA), systemic lupus erythematosus (SLE, lupus), gout, and ankylosing spondylitis (AS). The role of H_2S has been studied in the different types of joint inflammation. Patients with RA and gout, two common forms of inflammatory arthritis, had significantly elevated levels of H_2S in the synovial fluid, compared to plasma H_2S levels, although plasma H_2S levels in patients with RA and gout were not significantly different to plasma levels in healthy counterparts [56]. This indicates an elevation in H_2S levels at the inflammatory site, the joint, but not systemically. This may be important therapeutically, as H_2S could be targeted specifically to mediate local inflammation. Furthermore, no correlation was found between H_2S plasma

levels and disease activity in patients with RA and gout. However, a correlation was identified between synovial fluid H_2S levels and disease activity in RA patients [56].

In an older study [57], carrageenan was administered via intra-plantar (i.p) injection to rats to trigger inflammatory hind-paw edema formation. This caused an increase in the H_2S synthesizing enzyme activity, but pretreatment with DL-propargylglycine (PAG), a CSE irreversible inhibitor, significantly reduced inflammation in a dose-dependent manner. Although this study was able to show that H_2S is involved in intra-plantar carrageenan-induced hind-paw edema, the underlying molecular mechanism has not yet been established.

Other studies looking at the role of H_2S in inflamed joints have indicated conflicting results, with some implicating H_2S as an anti-inflammatory molecule [58, 59] and another confirming its pro-inflammatory activity [60].

2.5 Cardiovascular Inflammation

Cardiovascular (or heart) diseases are the leading cause of mortality and morbidity worldwide. They are typically multifaceted and are caused by varying disorders of the heart and its circulation, which results in immense health and economic burden. H_2S has pathogenic significance in several cardiovascular diseases including myocardial ischemia/reperfusion injury and atherosclerosis. Inflammation was shown to be a prominent hallmark of these cardiovascular diseases [64, 75].

Myocardial ischemia/reperfusion injury is a major cause of tissue destruction that often results in heart failure [61]. Ischemia, insufficient blood flow to an organ or area of the body, particularly heart muscles, is prevalent in heart diseases like myocardial infarction [62]. Re-establishing coronary blood flow using reperfusion techniques is critical to save viable myocardium [63]. While reperfusion relieves ischemia, it involves complex inflammatory processes and oxidative damage that can injure ischemic myocardial tissue and cause cardiomyocyte death, hence the term "reperfusion injury" [64]. H_2S has been demonstrated to play a role in myocardial ischemia/reperfusion injury.

Pre-conditioning (administration of H_2S before the onset of ischemia) [65–67] and post-conditioning (administration of H_2S during reperfusion) [68, 69] have been investigated, and H_2S has been shown to play a role in reducing infarct size in myocardial ischemia/reperfusion injury [70, 71]. Administration of H_2S before reperfusion in a rat hemorrhagic shock model was found to protect against the effects of hemorrhage induced ischemia/reperfusion by reducing inflammatory cytokines and the expression of iNOS as well as by upregulating the Akt/eNOS pathway [72]. This same effect was not observed when H_2S was given post-perfusion, indicating that the timing of administration of exogenous H_2S is important [72]. Furthermore, regulating endogenous H_2S by cardiac-specific overexpression of CSE reduced the extent of injury [73].

Atherosclerosis is an inflammatory condition that is crucial in the underlying pathology of cardiovascular diseases such as coronary artery disease and ischemic gangrene, as well as heart failure and stroke [74, 75]. The presence of plaques and lesions is linked to the morbidity and mortality related to the condition [76]. These lesions are termed complicated when they are infiltrated with red blood cells which lyse and release hemoglobin [75]. Hemoglobin can react with plaque lipids to form covalently cross-linked, oxidized hemoglobin species that exert varying pro-oxidant and pro-inflammatory effects from sensitizing vascular endothelial cells to lipid peroxidation [77–79]. Sulfide prevents oxidative formation of hemoglobin cross-links and demonstrates cytoprotective effects by mediating the reduction of ferrylHb (an oxidized hemoglobin species) [80]. Chemically, H_2S is a reductant that is capable of reducing lipid hydroperoxide in complicated lesions with hemorrhage [81]. Elevated CSE expression is proposed to have a atheroprotective effect; the subsequent H_2S produced inhibits the creation of pro-oxidant and pro-inflammatory lipid mediators, cross-linked

hemoglobin species, and downstream endothelial response [80].

The expression of chemokines (CCL2, CX3CL1, and CCL5) and their receptors (CCR2, CX3CR1, and CCR5) have been shown to be crucial in the development of atherosclerotic lesions [82, 83]. H_2S can protect against the formation of atherosclerotic lesions in mice and prevent CX3CR1 expression on stimulated macrophages via peroxisome proliferators-activated receptor-γ pathway as well as downregulate CX3CL1 expression by suppressing NF-κB activation [84].

2.6 Hemorrhagic Shock

Hemorrhagic shock describes the process of the body shutting down due to excessive blood loss. Reduced tissue perfusion results in insufficient delivery of oxygen and other essential nutrients that are essential for cellular function. Hemorrhagic shock is considered the second leading cause of death in trauma patients. Van de Louw and Haouzi (2012) [85] used a urethane anesthetized rat model (a lethal model) to determine whether H_2S increases during hemorrhagic shock associated with O_2 deficit and found that blood H_2S did not increase in untreated hemorrhagic shock. However, when treated with hydroxocobalamin (cobalt), there was no improvement in survival observed, even though cobalt was able to increase the ability of blood and kidneys to oxidize exogenous H_2S in vitro. The authors suggest that this could imply that blood levels of H_2S cannot be used as a marker for hemorrhagic shock.

Alternatively, a trauma-hemorrhagic shock and resuscitation model revealed that intraperitoneal administration of exogenous H_2S (NaHS) preserved organs by maintaining intracellular balance and improved survival rate. A significant decrease was also seen in oxidative stress in the rats treated with NaHS, suggesting H_2S may have oxidative effects in hemorrhagic shock [86].

Finally, inhibitors of CSE preventing the synthesis of H_2S partially restored mean arterial blood pressure and heart rate after hemorrhagic shock in anesthetized rats, indicating that endogenous H_2S plays a role in hypotension observed in hemorrhagic shock [87]. As this effect was only seen 60 minutes after blood withdrawal, the effect of H_2S may not be immediate, but H_2S may be involved in the later stages of hemorrhagic shock.

2.7 Intestinal Inflammation

Contrasting evidence is available regarding the role of H_2S in intestinal inflammation. It shows either pro-inflammatory or anti-inflammatory effects based on the model used, endogenous vs exogenous source of H_2S, slow-release vs fast H_2S donors for the bioavailability, and the route of administration [88].

H_2S is synthesized in the colonic tissue mainly by CBS and CSE enzymes. CSE is mainly expressed in the stomach, whereas CBS is expressed in colon [89]. H_2S causes vasodilation of ileum smooth muscles to increase colonic secretion [90, 91] and has been shown to protect rats from ischemia-reperfusion injury [92]. High levels of H_2S have been found in inflammatory bowel disease and colorectal cancer [93]. NaHS, a fast-release H_2S donor, showed concentration-dependent cell proliferation up to 200 μM, whereas higher levels of 1000μM showed opposite effects and inhibited cellular proliferation in HCT116 and SW480 cells [94]. This effect of H_2S is ascribed to its bell-shaped biphasic dose response curve. Whether H_2S will produce a stimulatory or inhibitory effect depends on the rate of H_2S synthesis, slow vs fast-release donors used, and concentration of the donor relative to the endogenous H_2S production [93]. It has been suggested that H_2S donors with different rates of H_2S production can have a both quantitative and qualitative variance in effects.

Ulcerative colitis is mucosal inflammation of the colon with extensive damage to the epithelium layer. Neutrophils and eosinophils accumulate at the site of inflammation with crypt abscesses. H_2S has been suggested to play a role in etiology of ulcerative colitis. Increased fecal H_2S levels have been seen in patients and

therapeutic reduction of sulfide levels provided relief from ulcerative colitis [95, 96]. Sulfate-reducing bacteria (SRB) in the colon uses short-chain fatty acids, alcohols, and hydrogen as substrates to reduce sulfur-containing amino acids. These are gram-negative obligate anaerobes that colonize the gastrointestinal tract. Some studies show that SRB can be genotoxic to gut epithelium [97], whereas other studies report the cytoprotective effects of SRB [98, 99]. SRB is responsible for the endogenous H_2S production in colonic tissues.

Intestinal inflammation is characterized by disruption of the intestinal homeostasis and immune tolerance [100]. Mice models of colitis and human studies show chronic intestinal inflammation [101]. When the intestinal epithelial gets damaged, the immune cells are exposed to high levels of microbes and their metabolites. Increased populations of SRB have been reported in the intestines of patients with ulcerative colitis and inflammatory bowel disease [95, 96, 100]. SRB interacts with the intestinal epithelial cells and synthesizes high levels of H_2S to damage the epithelium [100]. SRB form a biofilm on the mucosal epithelium and the high levels of H_2S cause mucolysis and mucus layer degradation, followed by apoptosis and inflammation.

2.8 Burn Injuries

Burn injuries are associated with a heightened inflammatory response. Activation and infiltration of neutrophils and polymorphonuclear cell trafficking lead to the tissue injury associated with burns [102]. Depending upon the percentage of burns, studies have shown either anti-inflammatory or pro-inflammatory effects of H_2S. In early stages of burn injury, H_2S shows pro-inflammatory effects, whereas later stages of burn injury are associated with anti-inflammatory and wound-healing effects [103]. Both CBS and CSE gene deletion show a beneficial effect in mice models with burn injury. In both mice and patients with burn injury, circulating plasma levels of H_2S were increased.

Severe burns, with more than 25% of full body burn injury, are associated with SIRS, sepsis, and multi-organ failure [103]. Severe burns are associated with increase in liver H_2S synthesizing activity, CSE mRNA expression in liver and lungs, and increased MPO activity showing lung injury [104]. PAG treatment reduced the plasma H_2S levels and CSE mRNA expression in the liver and lungs. Administration of exogenous NaHS at induction of burn injury worsens the severity of burn injury. NaHS activates NF-κB expression in the liver and lungs which heightens the inflammatory response. This suggests that H_2S plays an important role in the pathogenesis of burn injury.

H_2S can also activate the MAPK pathway in the cultured skin macrophages of burned rats [105]. The mRNA and protein levels of ERK1/2, p38, and JNK1/2 were increased. Application of a topical MAPK inhibitor reduced the inflammation in burn injury [106]. An optimum effect was achieved with immediate application with reduction in plasma levels of IL-6 and MIP-2 and dermal expression of IL-6, MIP-2, and IL-1β.

2.9 Neuroinflammation

Several studies have reported that H_2S can protect against neuroinflammation. Physiological levels of H_2S induce hippocampal long-term potentiation through NMDA-receptor mediated increase in K_{ATP} channel currents [107–109]. It also regulates the synaptic activity of neurons and glia by increase of intracellular Ca^{2+} ion concentration [110]. H_2S has been shown to be associated with cognitive processes such as learning and memory in humans and mice models. A decrease in the expression of CBS in the hippocampus of rats resulted in learning and memory defects [111]. It has been suggested that H_2S deficits could be associated with memory impairment in Schizophrenia patients [112]. Furthermore, decreased CBS activity and reduced H_2S synthesis in Alzheimer's disease patients may be responsible for the cognitive decline. Reduced plasma levels of H_2S were observed in patients with cerebrovascular disease, vascular

dementia, and Alzheimer's disease [113]. Administration of NaHS could ameliorate the learning and memory deficits in rats by decreasing apoptosis in hippocampal CA1 region [114].

Studies using cultured microglia and astrocytes show that H_2S has an endogenous anti-inflammatory role [115, 116]. The NLRP3 inflammasome is activated in response to intracerebral hemorrhage in rats which can downregulate endogenous H_2S generation in brain [117]. Treatment with H_2S donors or CBS-specific antagonist attenuated this response by suppressing the P2X7 receptor. Methamphetamine-induced inflammation in rats resulted in oxidative stress through free radical generation and apoptotic cascades and an increase in pro-inflammatory cytokines such as TNF-α, IL-1b, and IL-6 [118]. In this model, hippocampal neurons were protected against neurotoxicity by H_2S through inhibition of apoptosis and neuroinflammation. Regulation of several signaling pathways such as the MAPK pathway, PI3k/Akt signaling, and NF-κB pathway has been proposed to mediate the mechanism of action of H_2S in neuroinflammation [119].

3 H_2S in Vasodilation

Although vasodilation in inflammation has been under-studied, it is considered to have an important part in the progression of acute inflammation, as local blood flow influences the amount of exudate produced [120, 121]. Several mediators, such as histamine, and various eicosanoids, have been shown to regulate blood flow. However, the mechanisms involved are not well understood [121]. Critical drop in blood pressure also has a role in the failure and dysfunction of vital organs, leading to systemic inflammatory response such as sepsis and endotoxemia. In addition, reports have indicated that gasotransmitters, mainly H_2S, have vasodilating properties that can be biochemically associated with the alteration of several ion channels. H_2S directly targets ATP-sensitive potassium, resulting in a H_2S-dependent activation [122] (Fig. 2). This role of H_2S in K_{ATP} channels has been shown in the nervous, cardiovascular, respiratory, and gastrointestinal systems [123–128]. The stimulation of K_{ATP} channels by H_2S can result in cysteine alteration of several channels' subunits like the sulfonylurea receptor 1, sulfonylurea receptor 2B, and the pore-forming protein, $K_{ir}6.1$. Furthermore, it results in the elevation of channel amplitude and current eventually causing overall channel activation and hyperpolarization of membrane. In terms of $K_{ir}6.1$ cysteine was clearly demonstrated to form a persulfide under H_2S influence which mediates its activity [129]. The use of biotin switch technique has suggested the persulfide formation of SUR2B [130]; however, the specific residue by H_2S has not been recognized till now [131]. This method involves the utilization of methanethiosulfonate (MMTS) to inhibit thiols before synthesis of persulfides. However MMTS also inhibits hydropersulfides and may cause an issue with the outcome of this experimental setting [132]. The cysteine position at Cys6 and Cys26 of SUR1 is affected by H_2S [133]. It is probable that these cysteines are converted to persulfides, but until now it has not been experimentally proven. Apart from K_{ATP} channels, small and intermediate -conductance K_{Ca} channels (IK_{Ca}, SK_{Ca}) are stimulated by H_2S [134] and in terms of IK_{Ca} a formation of persulfide has been exhibited. Even though the direct effect of H_2S on SK_{Ca} channels has not been assured so far, the expression of SK_{ca} 2.3 was increased by H_2S and inhibited by the deletion of the CSE gene. This highlights the strong influence of H_2S in channel activity, showing H_2S is highly influential in the endothelium derived hyperpolarizing factor (EDHF) [129].

Further regulation of channel activity of H_2S has been demonstrated for Cav1.2 L-type and CAV3.2 T-type voltage dependent calcium channels [135–138] even though the activity seemed to be cell or tissue type specific as shown by opposing results. Recent studies also directed channel activity alterations in dependence of H_2S regarding BK_{Ca} channels [94, 139, 140], TRPA1 [141] and TRPV1 [142], voltage-gated sodium ($Na_V1.5$) channels, and chloride channels. In addition, H_2S has also been seen to mediate vasorelaxation through the

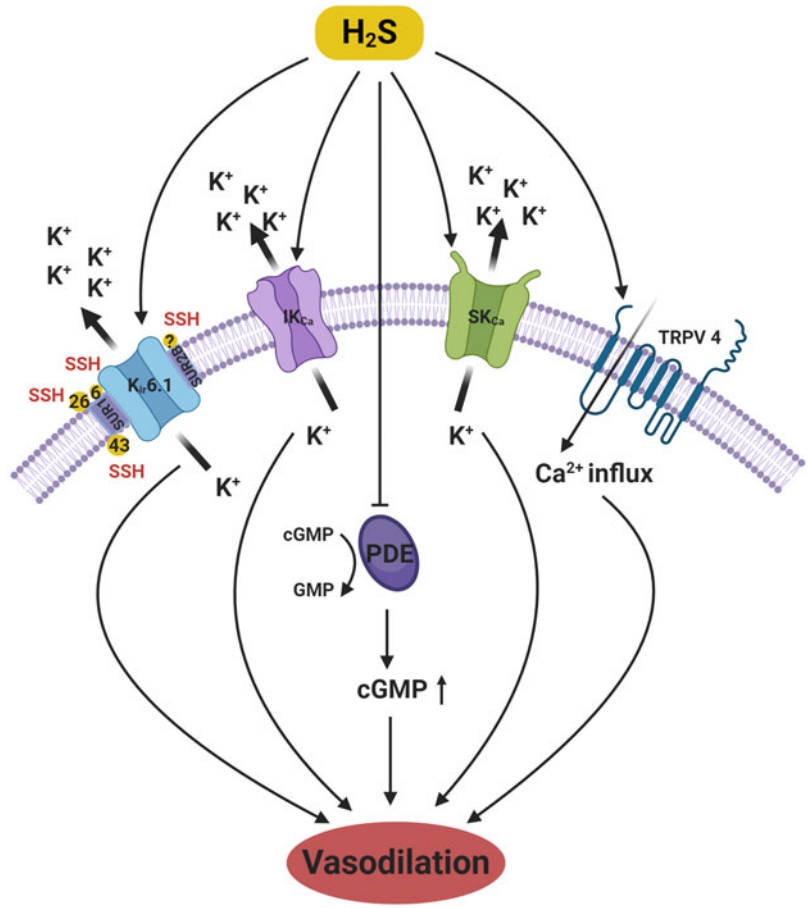

Fig. 2 H_2S vasodilatory effects. H_2S-induced persulfide (SSH) formation on cysteine residues (yellow circles, SH) at ATP-sensitive potassium channel subunits such as SUR2B, SUR1, and Kir6.1 results in channel activation, as well as stimulates endothelial SK_{Ca}/IK_{Ca} resulting in vasodilation. H_2S mediated vasorelaxation through the activation of TRPV 4 induced Ca2+ influx within endothelial cells. H_2S non-selectively blocks phosphodiesterases (PDE) resulting in increased cGMP expression and vasodilation

activation of TRPV 4 induced Ca^{2+} influx and eBK channel activation within EC [143]. Even though H_2S has been shown to act in the persulfide formation, detailed investigations have not been done; therefore, more studies should be performed to answer this question. Furthermore, H_2S non-selectively attenuates phosphodiesterases as demonstrated by Bucci et al. and others [144, 145] resulting in elevated cGMP levels and vasodilation (Fig. 2).

4 H_2S and Reactive Oxidant Species (ROS)

ROS are generally characterized as partially reduced metabolites of oxygen which have powerful oxidizing capabilities. These are harmful to cells at higher concentrations but mediate signaling pathways at lower concentrations. ROS oxidize proteins and lipids which alter cellular composition and damage DNA. At physiological concentrations, ROS act as signaling molecules which regulate cell growth, differentiation, apoptosis, and senescence. Chronic ROS production is associated with the progression of inflammatory disease [258].

H_2S acts as an antioxidant in neuroblastoma SH-SY5Y cells by blocking peroxynitrite-induced tyrosine nitration and cytotoxicity along with hypochlorous acid protein oxidation [146, 147]. In addition, H_2S reduces homocysteine resulting in lipid peroxidation [148] in plasma and kidneys and myocardial ischemia reperfusion

injury [149, 150]. This demonstrates the protective effects against ROS and reactive nitrogen species (RNS)-mediated damage of proteins and membranes. It is an unusual mechanism for H_2S to directly scavenge for oxidants. Although H_2S can react directly with ROS with the reaction kinetic [151], its endogenous concentration is very low, enabling H_2S to function as an antioxidant [152–154]. However, H_2S has a cytoprotective effect at the cellular level by increasing glutathione (GSH) production through activation of cystine/cysteine transporters and GSH redistribution to mitochondria [155–158] (Fig. 3). H_2S also elevates the expression of nuclear transcription factor Nrf2, resulting in increased expression of GSH-producing genes, GSH synthesis and distribution [159, 160] (Fig. 3a). Furthermore, H_2S inhibits the catabolism of GSH to normalize intracellular GSH [6]. As GSH influences many inflammatory diseases such as chronic kidney disease [161], sepsis [158], and chronic obstructive pulmonary disease [162] it suggests that the anti-inflammatory effects of H_2S are mediated through regulation of cellular GSH.

Apart from the antioxidant effects of H_2S, its role as a pro-oxidant has also been reported. H_2S can induce ROS production and result in cell death [162, 163]. Cytochrome P450 inhibitors such as cimetidine and benzylimidazole have been shown to reduce the production of H_2S-induced ROS (Fig. 3b). In another study, the antioxidant activity of GSH was depleted by NaHS in isolated hepatocytes (Fig. 3b) which could be prevented with antioxidant enzymes and ferric chelators [164]. H_2S also inhibits catalase activity by heme binding [165]. The exposure of the H_2S to non-transformed intestinal epithelial cells (IEC-18) increased the NADPH/NADP ratio, decreased intracellular redox environment, and suppressed mitochondrial respiratory activity [166]. The increased oxidative stress by H_2S caused genomic DNA damage in HT29-Cl.16E cells and Chinese hamster ovaries [167–169] (Fig. 3b).

5 The Role of Adhesion Molecules and Leukocyte Infiltration

Inflammation is predominantly influenced by adhesion molecules like selectin, integrin, and immunoglobulins which are important in mediating the infiltration of leukocytes from the bloodstream to the inflammatory site. Following endothelial cell activation, the expression of these cell adhesion molecules is upregulated by various chemokines and cytokines. This activation promotes the interaction of endothelial cells with leukocytes. Selectins are involved in the initial interaction of activated endothelial cells and leukocytes, while integrin and IG superfamily CAM are involved in the firm adhesion of the cells and subsequent steps [170]. Several studies have highlighted the role of H_2S as the amplification precursor for these processes. In caerulein-treated pancreatic acinar cells, H_2S elevated the expression of intercellular adhesion molecules ICAM-1 and neutrophil adhesion via nuclear factor (NF-κB) and SRC-family kinase pathway. In this study, H_2S increased the expression of ICAM-1 in caerulein-stimulated pancreatic acini via SFK family phosphorylation. Attenuation of SFKs inhibited H_2S-induced ICAM-1 expression, via the inhibition of NF-κB activation. This effect of SFK attenuation on NF-κB activation happens simultaneously with IκBα degradation [29]. Furthermore, H_2S-treated acinar cells showed increased neutrophil attachment, and inhibition of SFK reduced the neutrophil attachment [29]. In sepsis model, endogenous H_2S elevated leukocyte rolling and attachment in mesenteric venules with increase in mRNA and protein expression of adhesion molecules (ICAM-1, E-selectin, and P-selectin) in the liver and lungs and PAG treatment attenuated these activities. Similarly, exogenous H_2S increased leukocyte rolling and adherence, including the tissue level expression of adhesion molecules [16]. Administration of NaHS in normal mice led to lung inflammation, increasing the expression of adhesion molecules and neutrophil uptake in the lungs. These changes were inhibited by

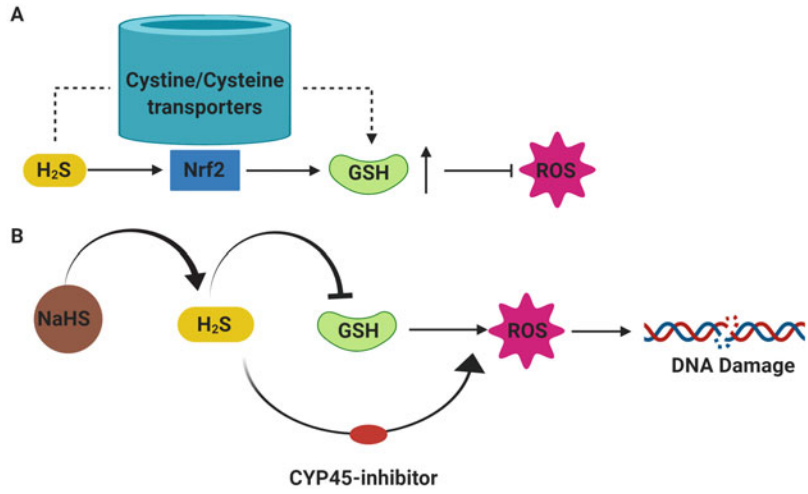

Fig. 3 H_2S role in reactive oxygen species. A) H_2S protects cells by increasing the role of cysteine/cysteine transporters resulting in increased GSH synthesis. In addition, H_2S elevates the expression of nuclear transcription factor Nrf2, resulting in increased expression of glutathione producing genes and elevated glutathione synthesis. B) H_2S donor NaHS reduces the antioxidant activity of glutathione. Furthermore, H_2S enhances oxidative stress causing DNA damage. In addition, CYP45-inhibitor (red sphere) inhibits H_2S induced ROS formation

pretreatment with BAY 11-7082, a NF-κB inhibitor. Furthermore, chemokine CXCR2 was upregulated in neutrophils in mice treated with H_2S with increased MIP-2-induced translocation of neutrophils. Therefore, H_2S regulates the infiltration of leukocyte by modulating the expression level of adhesion molecules during the inflammatory response [16] (Fig. 4).

On the contrary, H_2S inhibited leukocyte–endothelial adhesion in rat models when treated with H_2S donors and non-selective inhibitors of CBS and CSE [171]. H_2S downregulated the ICAM-1 expression in endothelial cells exposed to high glucose induced vascular damage. Wang et al. showed H_2S mediated attenuation of ICAM-1 expression in both in vivo and in vitro models, exerting its antiatherogenic effect. In another study, S-propargyl-cysteine (hydrogen sulfide donor) inhibited the activity of TNF-α in endothelial cells inhibiting JNK12/NF-κB activation and adhesion molecules expression. Similarly, H_2S attenuated expression of ICAM-1 and VCAM-1 (Fig. 4) via the upregulation of heme oxygenase 1 and inhibition of NF-κB pathway [172, 173]. In atherosclerosis mouse model, H_2S demonstrated protection of vascular tissues from atherogenic damage by reducing vessel intimal proliferation and attenuating adhesion molecule expression. Another pivotal anti-inflammatory protein that is involved in the reduction of leukocyte adherence is annexin A1 (AnxA1). It is synthesized and released via neutrophils to induce the termination of the inflammatory response. A study showed a possible relationship between H_2S and AnxA1 wherein Anxa1 wild type mice administered with NaHS showed decreased leukocyte adhesion and migration in inflamed microcirculation, which was not detected in AnaA1 KO mice [174].

6 H_2S and Neurogenic Inflammation

Substance P is a large protein that is synthesized in the ribosome and is widely distributed in the central and peripheral nervous system of vertebrates [175]. In mammals, the preprotachykinin-A (PPT-A) gene encodes substance P. Substance P exerts its biological activity

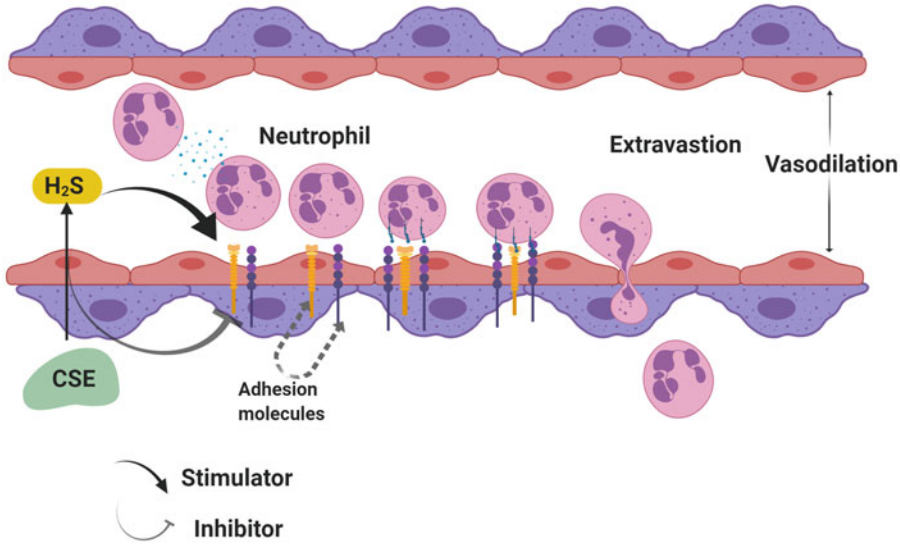

Fig.4 Hydrogen sulfide role in the activity of adhesion molecules and leucocyte recruitment. H_2S induces both increased and reduced expression of adhesion molecules, which are responsible for migration of leukocytes through the sub-endothelial space

via neurokinin G protein-coupled receptors, namely neurokinin 1 receptor (NK-1R), neurokinin 2 receptor (NK-2R), and neurokinin 3 receptor (NK-3R) [176]. Transient receptor potential vanilloid 1 (TRPV1) is a non-selective cation channel that participates in pain and neurogenic inflammation. Its activation results in the release of neuropeptides, such as substance P from both peripheral and central neuron terminals. Substance P regulates behavioral activity, neural degeneration and survival, respiratory and cardiovascular functions, as well as triggering the emetic reflex [175]. In the central nervous system, substance P acts as a neurotransmitter of pain information and regulates autonomic reflexes. The pathophysiological role in inflammation was demonstrated by abnormal levels of substance P, substance P-nerve fibers, and NK-1R in the inflammatory diseases. This is supported by the positive effect of NK-1R−/− and NK-1R antagonists in inflammatory animal models [177]. Although there is numerous evidence that substance P has a neuropeptide origin, animal studies have shown its production via inflammatory cells, namely lymphocytes, eosinophils, macrophages, and dendritic cells [178–181]. Substance P facilitates the release of oxygen radicals, cytokines, arachidonic acid derivatives, and histamine which cause tissue damage. This is followed by leukocyte infiltration, thereby elevating the inflammatory response [182]. Substance P also evokes local vasodilation, which affects vascular permeability, resulting in the translocation and aggregation of leukocytes to tissue [183]. Substance P elicits an influx of eosinophils and neutrophils in the human dermis which upregulates both P-selectin and E-selectin [184].

H_2S has been demonstrated to influence substance P in various inflammatory diseases. The interrelationship of H_2S and substance P was examined in a study where pre- or post-treatment of PAG significantly reduced PPT-A gene expression, subsequently decreasing substance P synthesis in the lungs. However, exposure to NaHS led to further elevation of substance P in sepsis. PPT-A gene silencing and pretreatment of NK-1R antagonist L703606 inhibited H_2S from exacerbating lung inflammation. Furthermore, septic mice with PPT-A gene deletion or that

were pretreated with L703606 did not show further elevation in lung permeability after exposure to NaHS [185]. Therefore, this study highlighted that H_2S upregulates the production of substance P, which aggravates lung injury and lung inflammation through NK-1R pathway activation.

In another study, H_2S was involved in systemic inflammation and consequently caused multiple organ damage, a feature of sepsis, through TRPV1-induced neurogenic inflammation [186]. Administration of TRPV1 antagonist, capsazepine, 30 minutes before CLP procedure significantly inhibited systemic inflammation and multiple organ damage caused by sepsis, as demonstrated by decreased liver and lung MPO activation and histological data supporting reduced pulmonary and hepatic injury. In addition, capsazepine reduced sepsis-mediated mortality. Subcutaneous injection of NaHS enhanced aggravation of CLP-induced mortality but capsazepine reversed these detrimental effects. In the presence of PAG, capsazepine had no significant effect on hepatic CSE activity and plasma H_2S level when compared with PAG-induced inhibition of systemic inflammation and multiple organ damage in sepsis. This suggested that capsazepine is not involved in endogenous H_2S production, implying that H_2S is an upstream pathway for the TRPV1 activation and can have a major role in controlling the production and release of neuropeptides in sepsis (Fig. 5). This result was the first report of H_2S-mediated systemic inflammation and multiple organ damage via TRPV1-induced neurogenic inflammation during sepsis [186]. Similarly, in another study [187], SP was generated by H_2S-mediated neurogenic inflammation during sepsis via TRPV1 activation. The capsazepine-treated group showed significant inhibition of pulmonary and circulating levels of substance P in a CLP sepsis mouse model. Capsazepine also attenuated NaHS-induced substance P activation, but there was no significant effect of PAG on substance P levels in both lung and plasma. In addition, capsazepine significantly inhibited the levels of H_2S-mediated inflammatory chemokines, cytokines, and adhesion molecules and protected against liver and lung injury in sepsis. However, in the absence of H_2S, capsazepine had no significant effect on the PAG-induced inhibition of sepsis-related inflammatory response. In addition, capsazepine strongly attenuated phosphorylation of IκBα and ERK1/2 and inhibition of NF-κB signaling, even in the presence of NaHS. Conversely, capsazepine did not influence PAG-induced attenuation in the sepsis model. As a whole, this study directed that H_2S activates the TRPV1 channel, causing an increase in substance P production and inducing ERK-NF-κB signaling in neurogenic inflammation (Fig. 5). H_2S has been shown to stimulate prostaglandin E metabolite and cyclooxygenase-2 in sepsis-induced acute lung injury through TRPV-1 channel activation. In this study, NaHS treatment in the CLP mouse model resulted in the significant overproduction of COX-2 and PGEM in the lungs [187]. The administration of NaHS as an exogenous H_2S donor, as well as the attenuation of endogenous H_2S production with PAG demonstrated the significance of H_2S in the overproduction of COX-2 and PGEM in sepsis. Capsazepine treatment showed the effect of H_2S in COX-2 and PGEM upregulation in sepsis via TRPV1 channel [188]. Inhibition of TRPV1 with capsazepine resulted in the inhibition of H_2S-induced COX-2 and PGEM production. However, no significant change was observed with PAG-induced inhibition in both inflammatory agents in septic lungs, indicating that H_2S regulates the sensory neurogenic agents during sepsis in a complementary manner with TRPV1. Overall, this demonstrates that H_2S increases the expression of COX-2 and PGEM which regulate the neurogenic inflammatory response through activation of TRPV1, resulting in lung inflammation and injury in CLP-induced polymicrobial sepsis [188].

As in sepsis, substance P plays a key role in inflammation in acute pancreatitis [189–199]. Substance P induced chemokine production in pancreatic acini cells by stimulating the inflammatory response [200–202]. Substance P also enhanced chemokine production in neutrophils and macrophages, key inflammatory mediators [203–206].

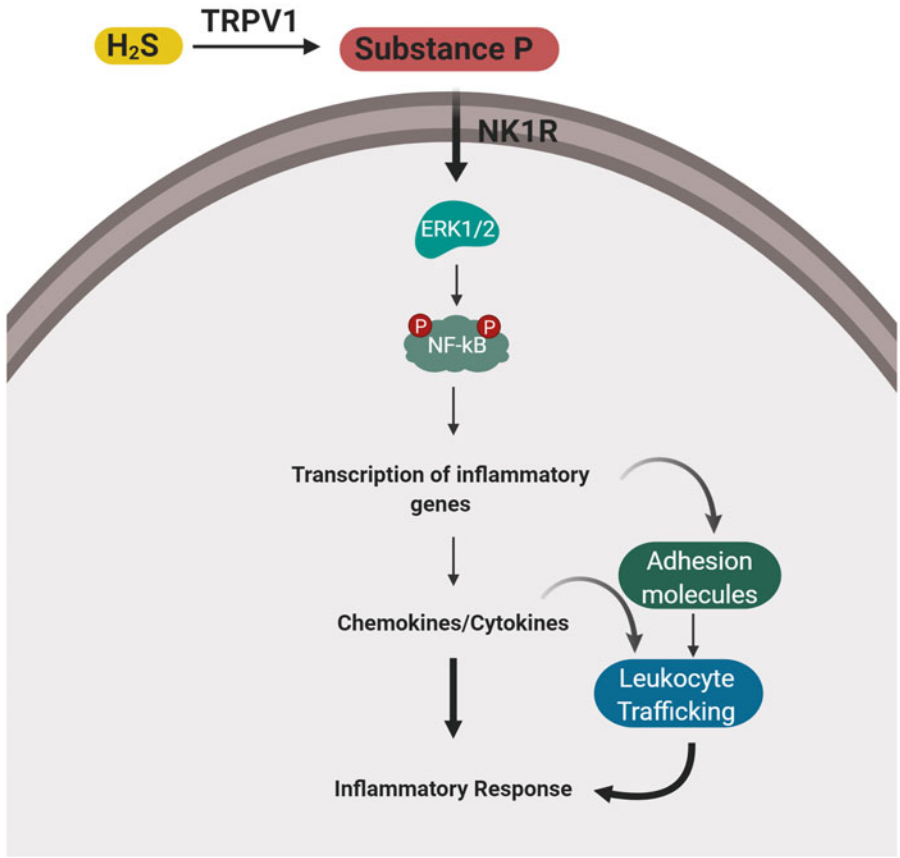

Fig.5 H_2S interrelationship with substance P. Substance P is activated by the H_2S upregulation via TRPV1 channel. Substance P induced via NK-1R plays a vital role in inflammation. Substance P/NK-1R induces increase in chemokines/cytokines and adhesion molecules expression depends on the activation of ERK1/2 mediated NF-κB signaling

Both prophylactic and therapeutic use of PAG in caerulein-mediated acute pancreatitis showed significant attenuation in substance P levels in plasma, lung, and pancreas [207]. A significant decrease was observed in the PPT-A mRNA level and NK-1R mRNA level in both the lung and pancreas, which has been demonstrated to protect against acute pancreatitis and associated lung injury. The elevated production of SP and increased expression of NK-1R in both the pancreas and lungs aggravated inflammation and tissue damage in both organs as supported by myeloperoxidase activity, hyperamylasemia, and histological analysis of the tissue injury. Although no evidence of increased pulmonary H_2S synthesizing activity was observed in the caerulein group in an acute pancreatitis model, increased pancreatic H_2S synthesizing activity resulted in increased plasma H_2S which can increase both PPT-A and NK-1R mRNA expression and eventually increase substance P production, in the lung [207]. These results demonstrated the influence of H_2S in the inflammatory response regulated via SP/NK-1R pathway during acute pancreatitis. Similarly, in another study, the inhibition of endogenous H_2S synthesis with PAG significantly attenuated caerulein-mediated elevation of substance P production and expression of PPT-A and NK-1R expression in isolated pancreatic acini cells

[25]. To show the direct influence of H_2S-mediated inflammation in acinar cells, NaHS treatment significantly upregulated substance P synthesis, PPT-A expression, and NK-1R expression. Furthermore, knockdown of the PPT-A gene and inhibition of H_2S production have resulted in decreased expression of TLR4 in acute pancreatitis, which may imply the interaction between SP/H_2S is via TLR4 and NF-κB signaling pathways. Recently, H_2S and SP levels were studied in patients with sepsis [208]. This study demonstrated that both circulating levels of H_2S and substance P were significantly increased in septic patients. The increase in H_2S subsequently regulated SP levels, demonstrating that H_2S is involved upstream of SP in the pathological process of inflammatory diseases in animal models [208] (Fig. 5).

The interrelation of CSE/H_2S with SP and NK-1R in modulating LSEC fenestrations in CLP-induced sepsis has been investigated. CSE knockout (KO) mice reversed liver sieve defenestration and gap formation in LSECs by decreasing SP/NK-1R signaling in CLP-induced sepsis [209]. CLP-induced sepsis elevated liver CSE and H_2S production and plasma H_2S levels, which increased SP levels in the liver, lungs, and plasma, and also increased NK-1R levels in both liver and lungs. In contrast, CSE KO mice resulted in decreased sepsis-induced SP production and NK-1R levels in the liver, lung, and plasma, demonstrating H_2S synthesized via CSE regulates the SP/NK-1R signaling pathway in sepsis. In addition, PPT-A KO mice prevented defenestration and gap formation in LSECs induced by sepsis, as suggested by the preservation of the fenestration and small number of gaps [209].

7 H_2S, NO, and CO.

7.1 Biosynthesis

Recent studies suggest that H_2S, NO, and CO work synergistically and are equally important. There is significant evidence that the gasotransmitters interact in ways that affect each other's biosynthesis, activity, and biological response [210]. The biosynthesis and interrelationship of the three gases in inflammation will be further discussed.

Endogenous H_2S is synthesized via enzymatic pathways and non-enzymatic (labile) sources. Cystathionine-γ-lyase (CSE), cystathionine-β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3-MST) are the three mammalian enzymes known to generate hydrogen sulfide from sulfur amino acids, namely homocysteine and L-cysteine [4]. The tissue distribution and expression levels of these enzymes varies between locations, particularly where the enzymes are jointly expressed [211]. For example, while it is known that the kidney and liver express all three at high levels, there is 60-fold expression of CSE compared to 20-fold expression of CBS [212]. While both CSE and CBS are cytosolic and widely expressed in cells and tissues, CSE functions primarily in peripheral organs and the vascular system, while CBS acts predominantly in the central nervous system [213]. The activity of these enzymes is reliant on the presence of the coenzyme pyridoxal-5-′-phosphate (PLP) (the active form of vitamin B6) [214]. CSE majorly catalyzes H_2S synthesis via the α,β-elimination of cysteine [215]. Alternatively, CBS condenses cysteine and homocysteine to generate H_2S. Once synthesized, H_2S functions in a negative feedback mechanism to reduce the activity of CBS and CSE [216].

H_2S is also reportedly generated from D-cysteine by D-amino acid oxidase (DAO) and 3-MST in the cerebellum and kidney [217]. More commonly, 3-MST is involved in the catalysis of 3-mecaptopyruvate to pyruvate, forming a 3-MST persulfide. Thioredoxin and dihydrolipoic acid reduce the persulfide to produce H_2S [218]. Although the activity of 3-MST has been reported in certain tissues, CSE and CBS are regarded as the major contributing enzymes in the production of endogenous H_2S [212].

Endogenous H_2S is also produced via non-enzymatic pathways, albeit at lower and less significant concentrations. Acid-labile sulfur and bound sulfane-sulfur are stored in cells and

released as H_2S under acidic conditions or in the presence of reducing agents, respectively [219].

NO is an inorganic molecule that is synthesized from L-arginine by a family of enzymes called nitric oxide synthase (NOS) [220] (Fig. 6). Three major NOS isoforms have evolved: neuronal (nNOS), inducible (iNOS), and endothelial (eNOS) [221]. nNOS is primarily expressed in neurons to regulate cardiac catecholamines, while iNOS is produced in macrophages during early immune defense response [222]. eNOS is found in platelets and it is involved in diastolic relaxation and reduction of oxygen consumption in cardiac muscles via pancreas-synthesized NO [223].

CO is generated in the human body by the metabolism of haem to produce CO, iron, and biliverdin via heme oxygenase (HO) [4] (Fig. 6). Similar to NOS, HO has three isoforms: inducible HO-1, HO-2, and HO-3 (the latter two being constitutive forms) [224].

8 Interrelationship between H_2S with NO and CO in Inflammation

Like H_2S, other gasotransmitters are also likely to be involved in inflammation. iNOS and HO-1 pathways involved in the biosynthesis of NO and CO have been markedly demonstrated to be involved in the inflammatory progression and production of large quantities of NO and CO, respectively [225]. If these gases are exposed simultaneously during inflammation, the three could interplay with one another in various ways, influencing each other's biosynthesis and biological reactivity. The NO/iNOS signaling pathway has been demonstrated to increase HO-1 expression in different kinds of cells [225, 226]. This increased expression of HO-1 via NO is independent of cyclic guanosine monophosphate (cGMP) synthesis, as exogenous cGMP does not exert any effect, and engages in the regulations of transcription factors, mainly nuclear factor-erythroid 2 related factor-2 (Nrf2) [226]. CO has been demonstrated to have influence in the NO/iNOS system. CO is capable of binding to the hemoprotein of iNOS and deactivating iNOS, thereby inhibiting NO synthesis [227] (Fig. 6). Also, the CO/HO-1 system may be responsible for attenuating NO synthesis via inhibition of iNOS expression [228], highlighting the possibility of interaction between CO/HO-1 and NO/iNOS pathway in inflammatory regulation.

Ashino and colleagues [229] conducted a time-dependent experiment to show the expression level of HO-1 and iNOS in LPS-treated macrophages. They observed a 6 hour delay between iNOS and HO-1 expression, as the expression of HO-1 reached its maximum level 6 hrs after LPS treatment, while iNOS reached its maximum level after 12 hrs. When treated with LPS, iNOS knockdown macrophages did not show HO-1 expression, demonstrating the involvement of NO/iNOS in the expression of HO-1. Furthermore, this study also stimulated Nrf2 knockdown macrophages with LPS, to investigate its effects in iNOS and HO-1 expression. LPS significantly increased iNOS expression, but did not affect HO-1 expression, indicating CO/HO-1 attenuates iNOS expression. This evidence indicates that there may be an interaction between NO and CO.

There are also studies suggesting the interrelationship of H_2S and NO, including CO. A study performed by Anuar et al. [230] demonstrated that NO decreases the production of H_2S in LPS-treated rats. Using nitroflurbiprofen as a NO donor, the effects of NO release were compared with the parent drug flurbiprofen. The results showed the attenuation of LPS-mediated elevation of kidney and liver H_2S synthesis and CSE mRNA, which was induced via inhibition of NF-κB. However, no effects were observed by the treatment of flurbiprofen in animals.

Oh et al. (2006) reported that H_2S was able to attenuate NO synthesis and iNOS expression through HO-1 expression in LPS-treated RAW264.7 macrophages. Additionally, H_2S mediated HO-1 expression via ERK activation, which resulted in the decrease of iNOS expression that was further reduced by L-cysteine treatment. Additionally, the pre-incubation of H_2S with LPS-treated macrophages diminished

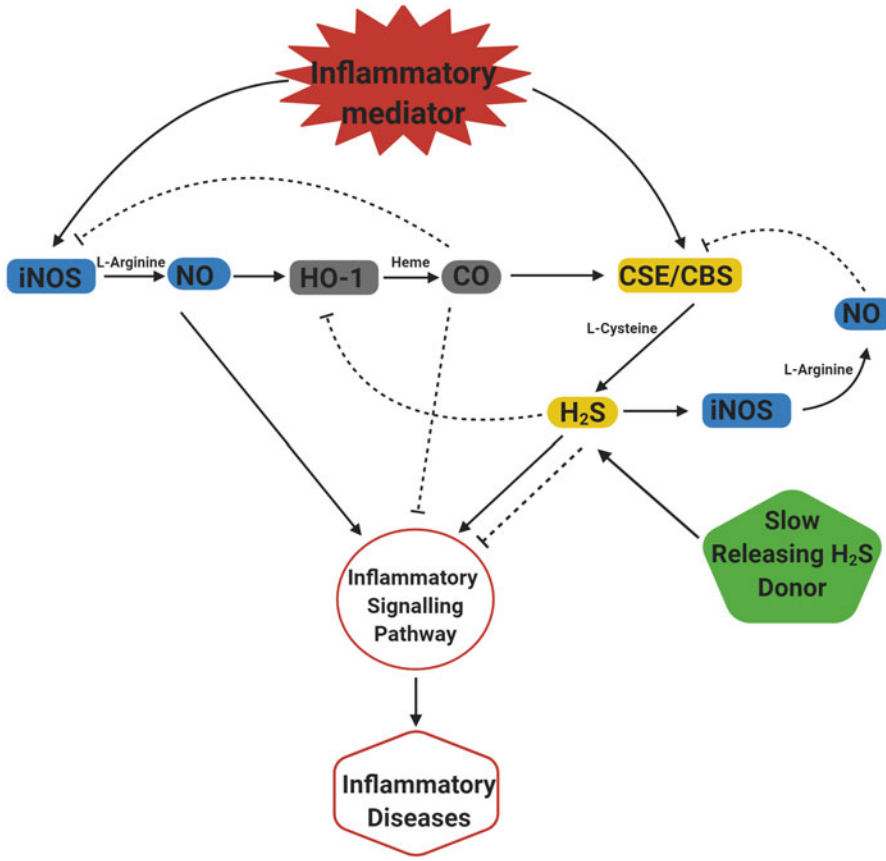

Fig. 6 Possible interrelation of H_2S/CSE/CBS, NO/iNOS, and CO/HO-1 pathways in inflammation. Inflammatory mediators (e.g., LPS) regulate iNOS expression, leading to elevated NO synthesis which excites inflammatory pathways to eradicate pathogens. However, significantly increased production of NO can be harmful to the host. HO-1 expression level is mediated in response to high levels of NO, leading to elevated CO synthesis that blocks both NO/iNOS and inflammatory signaling pathways. Pathogenic mediators also regulate CSE/CBS expression, consequently causing elevated H_2S synthesis that might excite inflammatory signaling pathways. H_2S synthesized immensely from CSE/CBS enzymes could block NO/iNOS pathway, likely through excitation of CO/HO-1 pathway in macrophages. Other studies have reported anti-inflammatory roles of slow H_2S-releasing donors

NF-κB activation. Interestingly the same activity was demonstrated by CO in the same treatment condition [231]. This showed that all three gasotransmitters are interlinked in inflammation. Overall, the activity of H_2S was responsible for diminishing NF-κB that is involved in the activation of HO-1/CO pathway, highlighting the probability of the H_2S/CSE system interaction with HO-1/CO system [228] (Fig. 6).

After investigating the role of different H_2S donors in LPS-treated macrophages, Whitman et al. (2010) reported similar results with Oh et al. The main focus was on the activity of GYY4137, a slow-releasing H_2S donor, on LPS-induced synthesis/expression of inflammatory mediators. They demonstrated the dose-dependent inhibition of GYY4137 on the release of pro-inflammatory mediators IL-6, IL-β, and TNF-α as well as the inhibition of NO synthesis, likely through NF-κB inactivation. However, the authors also mentioned NaHS as a pro-inflammatory mediator with a biphasic

nature; at higher concentrations, NaHS elevated the production of IL-1β, IL-6, NO, PGE2, and TNF-α [232]. Overall, this study illustrated the complexity of H_2S in the inflammatory process, demonstrating that the inflammatory process is not only dependent on H_2S concentration, but also on the rate of H_2S production.

Furthermore, an intriguing study by Hua et al. (2013) [233] demonstrated that the administration of NaSH inhibited inflammatory cell infiltration in injured tissue, alleviated cardiac edema, and limited myocardial lesions, supporting the evidence for inflammation reduction in a mouse model of myocarditis. In contrast, PAG administration reversed this, verifying H_2S as an anti-inflammatory molecule. Administration of NaSH reduced the expression of iNOS mRNA, which was highly expressed in the disease model, while HO-1 mRNA was elevated. Therefore, the authors concluded that H_2S maintains its cardioprotective activity via inhibiting overproduction of NO in inflammation and by increasing the levels of HO-1 [233]. The author also mentioned the overexpression of iNOS in the myocarditis model and the attenuation of H_2S synthesis resulted in aggravated inflammation. As described previously, inducible nitric oxide synthase (iNOS) and heme oxygenase-1 (HO-1) are the main enzymes involved in the synthesis of NO and CO, respectively; therefore, this shows that H_2S interplays with NO and CO in inflammation (Fig. 6).

Recently, a similar study supporting Oh et al. was performed in the lungs of endotoxemic mice [234]. GYY4137 restored the histopathological changes caused by LPS in the lungs. Administration of NG-nitro-L-arginine methyl ester (L-NAME), NOS inhibitor, increased antioxidant biomarkers (total antioxidant capacity(T-AOC), catalase (CAT), and superoxide dismutase (SOD)), but reduced expression of 3-nitrotyrosine (3-NT), a marker of peroxynitrite (ONOO-) action in the endotoxemic lung. L-NAME treatment also inhibited inflammation by reducing pulmonary expression of IL-6, IL-8, and myeloperoxidase (MPO) and increasing expression of anti-inflammatory cytokine IL-10. GYY4137 administration demonstrated the reverse activity of endotoxin-induced oxidative/nitrative stress, as supported by reduced levels of malondialdehyde (MDA), hydrogen peroxide (H_2O_2) and 3-NT and increased levels of antioxidant biomarkers, resulting in inhibition of endotoxin-induced acute lung inflammation. Besides this, GYY4137 also attenuated the expression of iNOS and synthesis of NO in the endotoxemic lung [234].

The effect of pretreated H_2S (0.06-1.5 mM), with or without IL-1β, on cultured human (osteoarthritis) OA chondrocytes was analyzed in an in vitro model of cartilage inflammation. In this study, H_2S treatment attenuated IL-1B-induced inflammation, as evidenced by the reduced secretion of H_2S which suppressed the secretion of NO, PGE2, and MMP-13 in OA chondrocytes [235]. Furthermore, the genetic expression of iNOS, COX-2, and MMP-13 was analyzed by reverse transcription quantitative polymerase chain reaction (RT-qPCR), while the analysis of the expression of signaling molecules associated with MAPKs and NF-κB was performed by western blot. These revealed that H_2S significantly reduced the gene expression of iNOS, MMP-13, and COX-2 and also inhibited the activation of NF-κB pathway, demonstrating the reversal effect of IL-1β on cultured human OA chondrocytes. This study demonstrates H_2S downregulates the NO/iNOS pathway while simultaneously attenuating the inflammatory response induced by IL-1β on cultured human OA chondrocytes [235].

Additionally, the effects of another slow-releasing H_2S donor, FW1256, have been studied in LPS-induced macrophage cells as well as in LPS-treated mouse model [236]. This H_2S donor showed a concentration-dependent reduction of TNF, IL-6, PGE2, and NO synthesis in LPS-induced macrophage cell lines. In addition, FW1256 markedly decreased IL-1, COX-2, and iNOS mRNA and protein expression in LPS-induced RAW macrophages. In a LPS-induced sepsis mice model, FW1256 administration resulted in the inhibition of IL-1, nitrate/nitrite, TNF, and PGE2 expression [236] (Fig. 6).

H_2S and CO have been demonstrated to act as important endogenous mediators of

gasoprotection in acute gastric mucosal damage. Acetylsalicylic acid is mainly recognized as anti-inflammatory drugs. However, there is the occurrence of the adverse effect of acute gastric mucosal damage caused by its dose–effect response. These adverse effects of acetylsalicylic acid result in significantly decreased gastric blood flow (GBF), H_2S synthesis, CO level, mRNA and protein levels of CSE, 3-MST, and HO-2 but elevates mRNA and protein expression for CBS, HO-1, Nrf-2, HIT-1 α, IL-1 β, iNOS, COX2 in gastric mucosa, and COHb level in blood [237]. Pretreatment with CORM-2 (CO donor) or NaHS, without PAG, reduced ASA-damage and elevated GBF. CORM-2 increased CO concentration, mRNA or protein expression of HO-1, and Nrf-2 and reduced the levels of CBS, COX-2, HIF-1 α, IL-1 β, iNOS, and H_2S synthesis in gastric mucosa and COHb level in the blood. NaHS treatment resulted in increased mRNA and protein expression of CSE, COX-1 and reduced mRNA level of IL-1 β and COHb concentration in blood. Furthermore, both of these molecules acted via sGC/cGMP pathway and showed anti-inflammatory activity by reducing the expression levels of gastric mucosal pro-inflammatory markers and preventing COHb blood level increase. CO ameliorated hypoxia and also regulated Nrf expression, but H_2S did not show these activities. Interestingly, this study demonstrated the involvement of H_2S in gasoprotection mediated via the activation of CO. However, the beneficial role of CO in the gastric mucosa appears to be independent of H_2S in the ASA-injured stomach [237].

A study reported an increase in the acute lung injury during endotoxic shock when treated with H_2S, which results in reduced eNOS activity, but increased iNOS activity, consequently leading to an increase in the synthesis of NO. In addition, H_2S also activated the HO-1/CO system in lung tissue during endotoxic shock [238] (Fig. 6).

9 Is H_2S Biphasic in Nature?

As described above, H_2S has the potential to function directly and indirectly as a pro- and anti-inflammatory mediator. Literature has demonstrated the biphasic nature of H_2S in multiple models of different inflammatory diseases in animals. Studies related to endogenous and exogenous H_2S have demonstrated conflicting results of pro- [14, 23, 32, 104, 239, 240] and anti- [19, 171, 241–244] inflammatory effects in various inflammatory diseases. H_2S has also been found to have a dichotomous role (activatory and inhibitory) in relation to adhesion molecules, leukocyte infiltration [16, 29, 170–174], and ROS [146, 147, 152–154, 162–164] activity in inflammation. These conflicting outcomes may be due to the different animal models used, which leads to altered pathogenesis and subsequent induction of the inflammatory response. Therefore, the activity of H_2S in different animal models may have led to different outcomes in the pathogenesis of various diseases.

However, similar models have also been reported to have different outcomes as described earlier, which may be due to inconsistencies in the dosage, source of drug, route and time of administration. These conflicting differences make it challenging to directly compare different studies, suggesting extra attention is required while interpreting the role of H_2S in inflammation via the use of exogenous H_2S donors. During the production of free radicals, higher concentrations of H_2S have resulted in the deletion of glutathione, release of intercellular iron and pro-apoptotic activity, resulting in cell toxicity [245–247]. In fact, higher concentrations of sulfide and H_2S synthesized by the inflammatory cells as non-specific protection against LPS have been demonstrated to injure host tissue [248, 249]. Although lower concentrations of H_2S may demonstrate anti-inflammatory [228], cytoprotective [73, 250], and antioxidant effects and physiological concentration may have some beneficial effects [251], apoptotic effects have also been observed [252, 253]. The role of H_2S in neurogenic-inflammation and vasodilation in inflammatory conditions imply that H_2S can function as a pro-inflammation mediator.

Besides sulfide salts such as NaHS and Na_2S which are renowned for large, quick bolus release of H_2S, slow-releasing donors also exist

that facilitate controlled, low concentration release of H_2S, including GYY4137 [234], FW1256 [236], and s-diclofenac [41]. The controlled, slow-releasing H_2S donors have been highlighted to show anti-inflammatory activity in various inflammatory disease models where NaHS was previously reported to be a pro-inflammatory mediator, as demonstrated in pancreatitis [41], LPS-induced endotoxemia [254, 255], and LPS-stimulated macrophages [232]. Also, in other animals models such as carrageenan-induced hind-paw edema [171], acute knee joint inflammation [256], and myocardial ischemia-reperfusion injury [257] slow-releasing H_2S donors have demonstrated an anti-inflammatory effect. Furthermore, slow-releasing H_2S donors limit the release and anti-inflammatory effects of other gasotransmitters such as NO and CO [231, 233]. It is interesting that till date, there are no reports of slow-releasing H_2S donors as pro-inflammatory mediators. This clearly states inflammatory activity is not only dependent on H_2S concentration, but also on the rate of H_2S production/release. This has opened the new horizon for scientists to study the anti-inflammatory potential of these donors.

10 Conclusion

Studies on the role of H_2S in inflammatory diseases and its interaction with the other players in the inflammatory pathway reveal a key role of H_2S in inflammation. Many studies show H_2S as a pro-inflammatory mediator, and some studies suggest its anti-inflammatory effects. A number of contradicting issues still remain, such as dosage, source of drug, route and time of administration. Work with appropriate animal models of disease has led to the translational studies on the role of H_2S in the clinic. Definite studies of the molecular mechanism of H_2S-mediated signaling in biological systems can lead to the development of new therapeutic strategies or solutions for the treatment of inflammatory diseases.

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Hydrogen Sulfide: a Novel Immunoinflammatory Regulator in Rheumatoid Arthritis

M. Li, Jian-Chun Mao, and Yi-Zhun Zhu

1 Introduction

H_2S is now regarded as a third gaseous signaling transmitter, next to nitric oxide (NO) and carbon monoxide (CO). H_2S is characterized by a smell of rotten egg which can be endogenously produced in several mammalian tissues, such as the brain, cardiovascular system, liver, lung, and kidney [1]. Recent theories have raised the question that life may originate from the heat generated by the process of hydrothermal circulation which provides energy mainly from H_2S [2].

Bernardino Ramazzini, an Italian physician, is probably the first one to describe the toxicity of H_2S in 1713, which is the earliest study on H_2S [3]. After that, the toxic effects of H_2S on mammals have been known over the centuries. Until 1996, Abe and Kimura suggested the role of endogenous H_2S as a neuromodulator, starting a new era of H_2S research in biology and medicine [4].

Over the past two decades, the concept of H_2S has evolved from a toxic gas to a tiny signaling molecule with potential clinical relevance [5, 6]. H_2S is a small lipophilic molecule that can penetrate cell membranes without the requirement for any specific transporter [7]. It diffuses rapidly into the cytoplasm, exerting multiple biological effects on various targets [8]. The biological effects of H_2S are multiple and sometimes opposite, depending on its concentration. In 2002, Zhao W. and Wang R. firstly found that H_2S showed a biological effect in the vascular system, which was able to induce the relaxation of vascular smooth muscle [9]. H_2S has widely been demonstrated to have cardio-protective [10–14] and pro-angiogenic effects [15, 16] in vivo and in vitro, as well as inhibitory effect on platelet aggregation [17] and macrophage migration [18], cytoprotective effect [19], anti-oxidant effect [20], and anti-apoptotic activity [21]. However, the frequently reported role of H_2S is anti-inflammation [22–28]. The potential effects of H_2S were also discussed in several review articles [29–32]. In summary, we depicted the roles of H_2S in Fig. 1. This review addresses the relevance of H_2S in the research area of RA.

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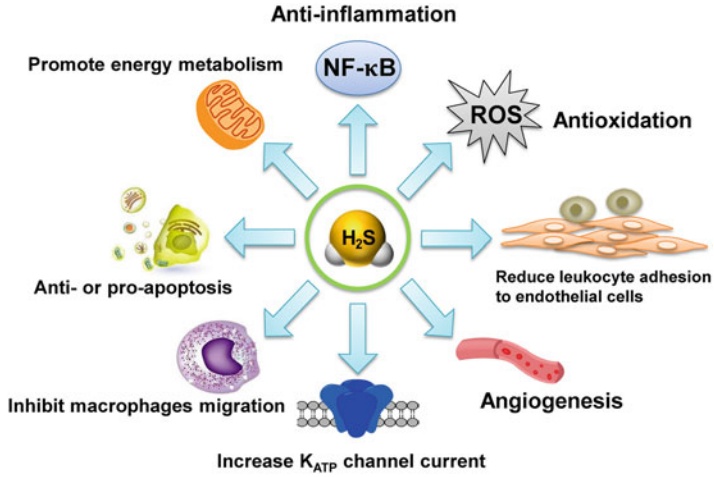

Fig. 1 General description of pharmacological effects of H_2S. The anti-inflammatory effect of H_2S is due to its ability to inhibit NF-κB signaling pathway. It improves angiogenesis through KATP channel/MAPK pathway activation. On the other hand, it avoids the adhesion of leukocytes to endothelial cells via intercellular cell adhesion molecule-1 (ICAM-1) and further inhibits the production of inflammatory cytokines. Moreover, H_2S have pro- or anti-apoptotic effects depending on the cell type and its concentration. Interestingly, it is also able to show an antioxidant property as a reductant to activate Keap1/Nrf2 pathway. In myocardial infarction (MI), H_2S suppressed macrophages migration and promotes the mitochondrial biogenesis in macrophages, resulting in the conversion of M1 to M2 macrophages

2 Hydrogen Sulfide

2.1 Physicochemical Properties of H_2S

H_2S is a colorless and flammable gas. The fate of H_2S after generation is unique compared with NO and CO because it dissociates to HS^- and S^{2-} in solution. Due to the volatile nature of H_2S, it can be easily predicted that the equilibrium will constantly shift, resulting in the formation of H_2S and quick releasing from the solution ($H_2S \leftrightarrow H^+ + HS^-$, $HS^- \leftrightarrow H^+ + S^{2-}$). Some reports showed that half of H_2S could escape from the medium in 5 min and even shorter time [33–35]. This may partially contribute to the dramatic variations on the reported concentrations of H_2S in tissues, plasma, and certain experiments [36–38]. Otherwise, there are limits of measurement techniques and the quantification of biologic H_2S levels is under debate [39–41].

Free sulfide is a weak acid that dissociates in the body fluids with pH 7.4 to yield ~20% of H_2S and ~ 80% of HS^- and negligible amounts of S^{2-} as its pKa1 = 6.8 and pKa2 > 12 at 37 °C [42]. Although the physiological pH is about ~7, different organelles range from pH 4.7 to 8, which means the ratio of $S^{2-}/HS^-/H_2S$ is variable [43]. The cell membranes allow rapid diffusion of H_2S through the lipid bilayer [7]. However, HS^- is not permeable and requires specific transporter to enter the cell [44, 45]. Therefore, the existing form of H_2S is pH-dependent and affects its membrane permeability.

H_2S is often considered as a reductant since the sulfur atom is at −2 oxidation state, which is the same with GSH and cysteine. However, the respective reduction potential of S^0/H_2S, GSSG/GSH, and cystine/cysteine is −0.23, −0.24, and − 0.34 V [46]. Additionally, the physiological level of H_2S is much lower than that of GSH or cysteine. For example, in plasma samples, free H_2S level is in the pmol/mg protein range, but GSH and cysteine concentrations are in the nmol/mg protein range [39, 47]. Hence, free H_2S is less likely to be an effective reductant under normal physiological conditions.

However, H_2S undergoes complex oxidation processes and generates thiosulfate, elemental sulfur, sulfenic acids, persulfides, polysulfides, sulfite, and sulfate. These oxidative products of H_2S can be involved in protein post-translational modification (PTM) and low molecular thiol interaction. Low molecular thiols or cysteine residues of proteins can be modified via S-sulfhydration, a special PTM of protein cysteine residues, in which the -SH from a sulfhydryl donor is transferred to cysteine sulfhydryls to form a covalent persulfide (-SSH) in the target proteins [48].

2.2 Endogenous Synthesis of H_2S

H_2S is produced in mammalian organisms by enzymatic and non-enzymatic pathways. In enzymatic pathways, H_2S is generated in the tissues by three cysteine metabolizing enzymes: cystathionine-β-synthase (CBS, EC 4.2.1.22), cystathionine-γ-lyase (CSE or CTH, EC 4.4.1.1) and 3-mercaptopyruvate sulfurtransferase (3-MST or MPST, EC 2.8.1.2) [30, 49, 50]. As shown in Fig. 2A, CBS represents the main H_2S-generating enzyme in the brain, whereas CSE dominates in the cardiovascular system [51, 52]. The activity of 3-MST seems to be highest in the adrenal cortex [53]. The expression of the enzyme was also reported in the brain and erythrocytes [54, 55]. As research progressed, all enzymes can be found in the lungs, liver, kidneys, and gastrointestinal tract [56–58]. CBS and CSE are exclusively cytosolic enzymes, while 3-MST is found mainly in the mitochondria of cells [59].

CBS and CSE are two pyridoxal 5′-phosphate-(PLP) dependent enzymes which are a part of the

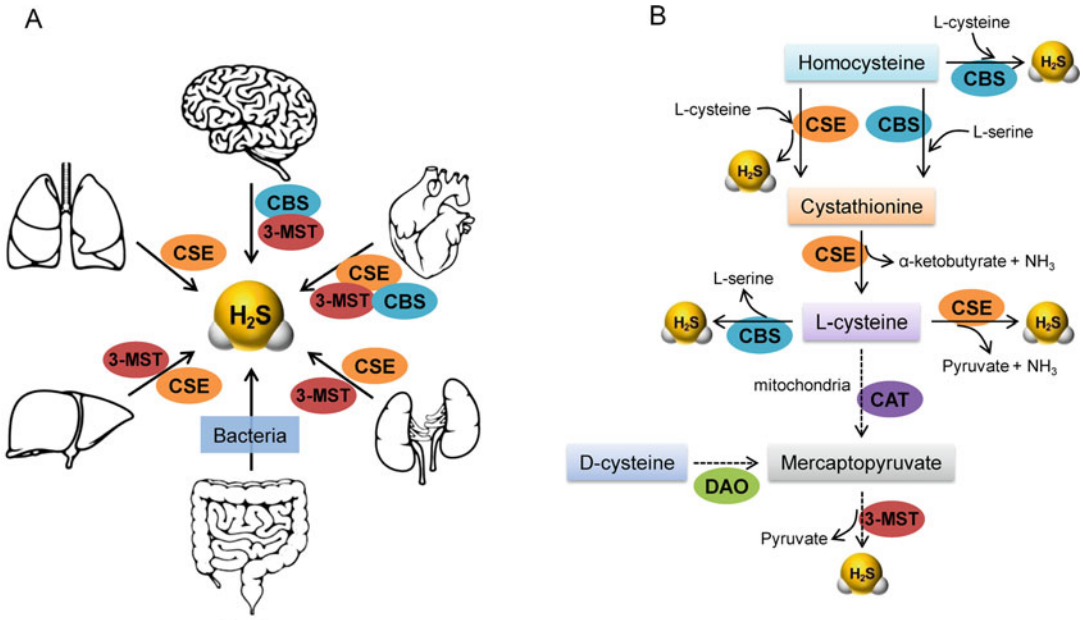

Fig. 2 The histological distribution of H_2S producing enzymes/bacteria (**a**) and endogenous metabolic pathways (**b**). In fact, CBS, CSE and 3-MST exist in various organs and cell types. However, CBS in the brain, CSE in the cardiovascular system and 3-MST in the kidney are mostly reported and studied. CBS and CSE produce H_2S from homocysteine and Lcysteine. In addition, CSE catalyzes the breakdown of cystathionine to L-cysteine which supplies more H S sources. In mitochondria, CAT utilizes L-cysteine to produce mercaptopyruvate which is the substrate of 3-MST, and DAO resolves D-cysteine to produce mercaptopyruvate. Therefore, 3-MST transfers the thiol from mercaptopyruvate and release H_2S

reverse transsulfuration pathway. As depicted in Fig. 2B, in the presence of L-cysteine, CBS generates H_2S via a β-replacement reaction along with the production of L-serine. When L-homocysteine is available, the rate of H_2S generation can be accelerated by 23-fold compared with that from L-cysteine alone [60]. Additionally, CBS catalyzes the condensation of homocysteine and L-serine which results in the formation of L-cystathionine and H_2O [61]. This reaction is a critical step for the biosynthesis of L-cysteine which can be further used as an H_2S producing substrate. CSE catalyzes the α,-γ-elimination of cystathionine to cysteine, α-ketobutyrate, and NH_3. H_2S is produced by CSE either by the breakdown of cysteine to pyruvate, H_2S, and NH_3 or by the use of homocysteine and cysteine to produce cystathionine and H_2S [62, 63]. However, 3-MST does not play a role in the transsulfuration pathway. Cysteine aminotransferase (CAT) utilizes L-cysteine to produce mercaptopyruvate, the substrate of 3-MST. Also, D-amino acid oxidase (DAO) resolves D-cysteine to produce mercaptopyruvate [64–66]. 3-MST transfers the thiol from mercaptopyruvate to itself. In the presence of ambient reductants, 3-MST-bound persulfide is removed to release H_2S [67, 68]. In essence, 3-MST acts as a sulfur carrier, rather than an H_2S producer, as the sequential reactions catalyzed by CAT and 3-MST generate sulfane sulfur [69].

In the non-enzymatic pathway, sulfate-reducing bacteria (SRB) produce H_2S resides in the gastrointestinal (GI) tract (Fig. 2A). Although the bacterial source of H_2S has been known for centuries, it was considered as only a by-product of sulfur metabolism. As reviewed by Shatalin et al. analysis of bacterial genomes had revealed that most bacteria had orthologs of mammalian CBS, CSE, or 3-MST [70]. SRB use sulfate as an electron acceptor in the process of dissimilatory sulfate reduction and finally produce H_2S [71]. In a study of healthy adults in the USA, SRB colonized approximately 50% of their gut, and *Desulfovibrio piger* was the primary H_2S producer [72].

3 H_2S and Rheumatoid Arthritis

RA is a common autoimmune disease that is associated with progressive disability, systemic complications, and early death [73]. In RA, T cells, B cells, and macrophages infiltrate into the synovium and sometimes organize into discrete lymphoid aggregates with germinal centers (Fig. 3) [74]. As reported in increasing papers, H_2S shows a protective effect in RA via targeting innate immune cells (macrophages) [34] and adaptive effector (FLS) [75].

3.1 Pathogenesis of RA

3.1.1 Immune-Related Cellular Disorders

At the cellular level, the synovium in RA contains abundant myeloid cells and plasmacytoid dendritic cells which express cytokines (IL-12, 15, 18, and 23), human leukocyte antigen (HLA) class II molecules, and co-stimulatory molecules that are necessary for antigen presentation and T-cell activation [76, 77]. Although RA is usually considered as a disease mediated by type 1 helper T (Th1) cells [78], attention has increasingly focused on the role of Th17 cells which produce IL-17A, 17F, 21, 22, and TNF-α [79, 80]. Transforming growth factor β (TGF-β), IL-1β, 6, 21, and 23, derived from macrophages and dendritic cells, provide a milieu that supports the differentiation of Th17 cells and suppresses the differentiation of Treg cells, thus shifting T-cell homeostasis toward inflammation. IL-17A, which synergizes with TNF-α to promote the activation of fibroblasts and chondrocytes, is currently being targeted in clinical trials [81]. In a CIA model, administration of peritoneal cells (PCs) reduced Th17 cells, induced Treg cells, and ameliorated the severity of CIA [82].

Humoral adaptive immunity also takes a part in RA driven by T helper cells [83]. B cells infiltrate into the synovia and mainly aggregate with T cells, which is closely related to the expression of factors such as a proliferation-

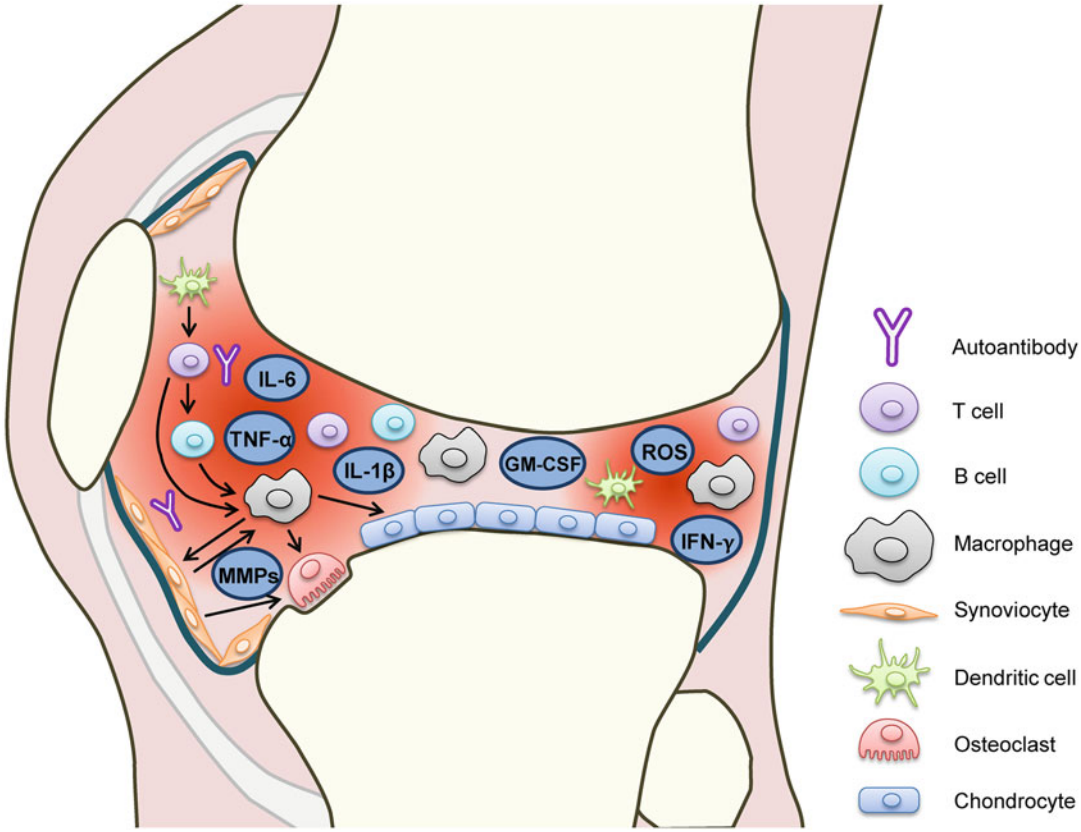

Fig. 3 Immune-related pathogenesis of RA in the knee joint. A step-wise progression can begin with the activation of innate immunity by stimulating dendritic cells in the presence of some environmental or gene factors. Then, dendritic cells recruit and activate T lymphocytes, which stimulate B cells, macrophages, synoviocytes, chondrocytes and osteoclasts in succession accompanied with the secretions of pro-inflammatory and bone destructive cytokines (i.e. IL-1β, IL-6, TNF-α, and MMPs). Therefore, in the synovial membrane and adjacent bone marrow, adaptive and innate immune pathways integrate to promote tissue remodeling and damage, driving the chronic phase in RA

inducing ligand (APRIL), B-lymphocyte stimulator (BLyS), and CC and CXC chemokines (e.g., CC chemokine ligand 21 and CXC chemokine ligand 14) [84, 85]. B lymphocytes play a pathologic role in the development and propagation of the disease, as B lymphocytes are recruited to the synovial fluid, where they contribute to local inflammation through the secretion of pro-inflammatory mediators (cytokines, chemokines, micro-RNAs) and present antigens to T cells [86]. Besides, activated Fc Receptor-like 4 (FcRL4) positive B cells are a component of the local autoimmune response, and express receptor activator of nuclear factor kappa-B ligand (RANKL) to contribute to joint destruction [87]. A pathogenic role for a cluster of differentiation (CD) 20^{+} B cells was confirmed by the efficacy of rituximab in RA patients, indicating that the treatment anti-CD20 B cells might be of importance in the therapy of RA [88].

Additionally, increased numbers of dendritic cells (DCs) are present in the synovium and synovial fluid of inflamed joints in RA patients compared with OA patients [89]. In RA, they trigger differentiation and activation of the auto-reactive T cells as well as innate immune effector

functions via presenting self-peptides [90]. Many application methods to achieve antigen-specific therapy were reviewed recently [91]. In the context of inflammation, DCs play a role as antigen-presenting cells (APCs) and a producer of pro-inflammatory cytokines (i.e., IL-23 and IL-12), which induce the differentiation of Th17 cells and Th1 subsets, relevant in joint inflammation [92].

Furthermore, macrophages act as an active role in the pathogenesis of RA because of the high expression of pro-inflammatory cytokines and matrix metalloproteinases (MMPs), and they are APCs for T cells and B cells, representing a source of osteoclast precursors in an inflammatory context [93]. In particular, the number of macrophages increases dramatically and they are central to the pathophysiology of RA. They constitute the main source of pro-inflammatory cytokines and chemokines to activate a wide range of immune and non-immune cells and secrete diverse tissue degrading enzymes that drive chronic inflammation, tissue destruction, and pain responses in RA [94]. Macrophages act through the release of cytokines (e.g., TNF-α, IL-1, 6, 10, 12, 23), reactive oxygen intermediates, nitrogen intermediates, production of prostanoids, and matrix-degrading enzymes, phagocytosis, and antigen presentation [95]. Therefore, macrophages are central effectors of RA and can be used to predict the possible efficacy of anti-rheumatic treatment [96]. Macrophages can be divided into two extreme subsets, classical activation (M1), and alternatively activation (M2) [97]. The symptoms and signs of RA are exacerbated with the increase of M1 which is the main part of macrophages, while M2 secrete anti-inflammatory cytokine (e.g., IL-10) to improve RA [98]. In a recent study, macrophage depletion with clodronate-containing liposomes (Clo-lip) could ameliorate the incidence and development of RA [99]. Macrophages from sirtuin 1 (SIRT1) transgenic (Tg)-mice exhibited enhanced polarization of M2 phenotype macrophages and reduced polarization of M1 phenotype macrophages. In line with these observations, SIRT1-Tg mice showed less histological signs of arthritis [100]. Eventually, macrophages induce FLS hypertrophy by TNF-α and IL-1β, which perpetuates the inflammation, generating a chronic loop [74]. In RA, FLS is hyperproliferative in the presence of inflammatory macrophages and produces cytokines and proteases, apart from acquiring an aggressive, tumor-like phenotype due to transcriptional mechanisms of imprinting and epigenetic changes, which lead to cartilage destruction and drive joint inflammation [101]. RA-FLS also secretes many pro-angiogenic factors like fibroblast growth factor, vascular endothelial growth factor (VEGF), hypoxia-inducible factors (HIFs), and IL-18, which promote new blood vessel formation, pannus growth, and inflammation [102]. Therefore, FLS is another key target in RA therapy.

3.1.2 Key Signaling Pathways

At the molecular level, some pathogenic signaling pathways involved in RA are concluded in Fig. 4. Nuclear factor κB (NF-κB) is abundantly activated in the synovium of RA patients and regulates multiple genes that contribute to inflammatory and immune responses, such as IL-6, IL-8, TNF-α, iNOS, and COX-2 [103, 104]. Animal models of inflammatory arthritis support the concept that NF-κB plays a very active role in the development and progression of arthritis [105, 106]. NF-κB activation prior to the onset of clinical manifestations of arthritis has been found in both murine CIA and rat AIA models [107, 108]. Targeting NF-κB is an effective therapeutic strategy in many animal models of arthritis [109–113].

Highly expressed receptor activator of nuclear factor-κB (RANK) and its ligand RANKL are indispensable regulators of bone erosion in RA [114]. RANKL is highly expressed in the synovial tissue of RA patients with active disease [115]. After binding with RANK, RANKL triggers the recruitment of an adaptor molecule TNF receptor-associated factor 6 (TRAF6), resulting in the activation of NF-κB, Protein kinase B (PKB/AKT), mitogen-activated protein kinase (MAPKs) (i.e., extracellular regulated kinase (ERK), c-Jun N-terminal kinase (JNK),

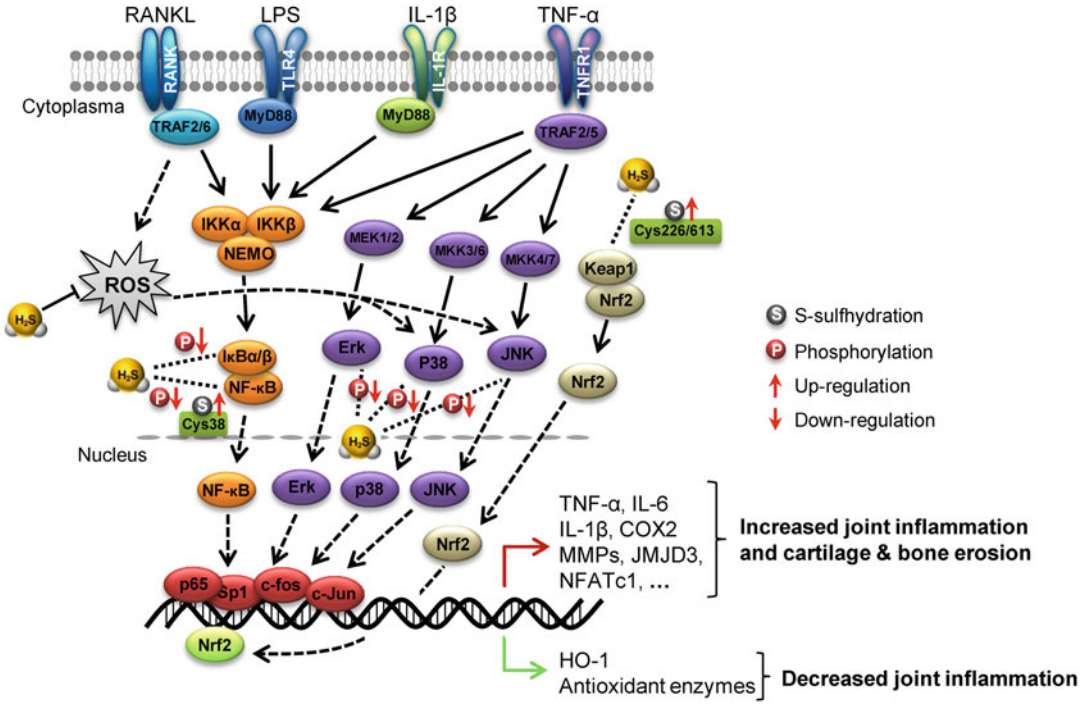

Fig. 4 Pathogenic signaling pathways and H_2S-related targets in RA. After binding with RANK, RANKL recruits TRAF-6 and activates NF-κB pathway, as well as NADPH oxidase to produce ROS. Consequently, ROS stimulates p38 and JNK, which can also be activated by TNF-α. Pro-inflammatory (i.e. IL-6 and TNF-α) and bone destructive (i.e. MMPs and collagenases) cytokines are highly expressed after the activation of NF-κB and MAPK pathways. However, Keap1/Nrf2 pathway plays a protective role in RA as it promotes the production of HO-1 and other antioxidant enzymes to ameliorate the oxidative stress in the cavity. Firstly, as a reductant, H_2S can scavenge ROS directly. On the other hand, H_2S can also S-sulfhydrates the Cys226/613 of Keap1 to release Nrf2. Secondly, H_2S suppresses the phosphorylation of IκBα and NF-κB, and increases the S-sulfhydration of NF-κB (p65) at Cys38, resulting in the inhibition of NF-κB pathway. Thirdly, H_2S decreases the phosphorylation of Erk/p38/JNK to inhibit MAPK pathway

and p38), and nuclear factor of activated T cells c1 (NFATc1) [116]. Hence, the RANKL/RANK signaling pathway is a complementary therapeutic target in RA. Denosumab (RANKL-specific human monoclonal antibody) is currently used for treating osteoporosis, osteosarcoma, multiple myeloma, and bone metastasis [117].

The MAPKs are also key pro-RA regulators that stimulate the production of cytokines and MMPs in RA [118, 119]. All three kinase families (ERK, JNK, and p38) are highly expressed in rheumatoid synovial tissue [120] and closely related to NF-κB [121]. All are constitutively expressed in cultured synoviocytes and will be rapidly phosphorylated in the presence of pro-inflammatory cytokines. The MAPKs have attracted considerable attention as potential therapeutic targets in RA. Preclinical studies showed that p38 inhibitors were effective in murine CIA model and rat AIA model [122–124]. A selective JNK inhibitor, IQ-1S, inhibited MMP 1 and MMP3 gene expressions induced by IL-1β in human FLS and significantly attenuated the development of CIA [125]. Upstream kinases that activate the MAPKs, for example, mitogen-activated protein kinase kinase 3 (MKK3), MKK4, MKK6, and MKK7, are also activated in RA synovium and can form signaling

complexes that integrate external environmental stresses to generate an appropriate cellular response [126, 127].

In RA, excessive reactive oxygen/nitrogen species (ROS/RNS) arise from the activation of NADPH oxidase (NOX) 2 and iNOS which may modulate inflammation-related cell signaling pathways [128]. Kelch-like ECH-associated protein 1 (Keap1) is bound to the inactivated form of nuclear factor erythroid 2-related factor 2 (Nrf2), suppressing antioxidant enzymes. Dissociation from Keap1 stabilizes Nrf2, while binding to Keap1 promotes the degradation of Nrf2 [129]. In the nucleus, Nrf2 binds to the promoter regions of antioxidant genes via the antioxidant response element (ARE) [130]. Anti-oxidative proteins, such as hemeoxygenase-1 (HO-1), are then transcribed by ARE [131]. In RA, Nrf2 deficiency promotes increased cytokine production and ROS following infection by bacteria or lipopolysaccharide (LPS) [132]. In Nrf2 knockout mice, cartilage damage was more severe and there was more oxidative damage during antibody-induced arthritis [133]. This suggests that these mice have an impaired ability to neutralize ROS resulting in increased susceptibility to oxidative stress.

3.2 Anti-RA Effect of H_2S

In RA, there is no doubt that immune responses cause inflammation and inflammatory cytokines stimulate immune cells as well as FLS, chondrocytes, and osteoclasts [73]. Here, we introduce the anti-inflammatory potential of H_2S in RA therapy via different targets (Fig. 4).

The effect of H_2S on joint inflammation was investigated in the past due to its anti-inflammatory effect. The beneficial effect of H_2S seems to be dose-dependent as different studies demonstrated opposite results [134–137]. Between the inflammatory cells involved in RA, macrophages play a key role [98, 138, 139], which can polarize into different phenotypes and mediate the immune/inflammatory reactions. In murine macrophages stimulated with LPS, low concentration of H_2S inhibited the activation and synthesis of several pro-inflammatory mediators such as TNF-α, NF-κB, IL-6, and IL-1β [140]. However, at higher concentrations, H_2S stimulated the production of pro-inflammatory molecules by macrophages and human FLS [30, 75]. Kloesch et al. demonstrated the dual effect of H_2S on RA FLS in two different experiments, showing that short-term exposure to H_2S induced IL-6 expression, while long-term exposure had the opposite effect [141, 142].

In the last decade, the molecular mechanisms of H_2S on anti-inflammation were widely investigated in macrophages and FLS. Inhibitor of κB (IκB)/NF-κB is an important signaling pathway in inflammation and the key target of H_2S. Oh et al. demonstrated for the first time that H_2S inhibited NF-κB signaling in RAW264.7 cells stimulated with LPS [143]. Subsequently, in human FLS isolated from RA patients, DATS significantly attenuated the production of key inflammatory cytokines due to its inhibitory effect on the NF-κB pathway [144]. H_2S-dependent persulfide formation at Cys38 of p65 retained NF-κB in the cytosol and inhibited its DNA binding activity, resulting in reduced inflammation [145]. Additionally, other studies confirmed that H_2S inhibited the NF-κB dependent expression of pro-inflammatory cytokines (e.g., IL-1β, IL-6, TNF-α, COX-2, and iNOS) in macrophages, cardiac cell line, chondrocytes, and myoblast cell line [140, 146–148].

Although targeting NF-κB is closely related to the anti-RA effect of H_2S, other mechanisms are also involved. NaHS partially blocked IL-1-β-induced IL-6 and IL-8 expression by regulating p38 and ERK1/2 MAPK in chondrocyte cell line C-28/I2 [149]. Similarly, Kloesch et al. showed that in FLS isolated from RA patients, administration of NaHS transiently decreased IL-1-β-induced synthesis of IL-6, which was independent of NF-κB activation but dependent on p44/42 (ERK1/2) MAPK deactivation [141]. SPRC, also named as ZYZ-802, is known as an endogenous H_2S modulator which activates the Nrf2 signaling pathway by S-sulfhydrating Keap1 to play a role in antioxidant stress in the

AIA rat model [150]. In another research, the author reported the specific S-sulfhydration locus that H_2S targeted Keap1 at cys226 and cys613, leading to its dissociation from Nrf2, consequently enhancing Nrf2 nuclear translocation and further activation of related genes [151]. Furthermore, NaHS up-regulated Nrf2 protein expression, its nuclear translocation, and the transcription of the two key downstream antioxidant genes peroxiredoxin-1 and NAD(P)H dehydrogenase quinone 1 in $CD11b^+$ human monocytes (osteoclasts precursors), inhibiting human osteoclast differentiation [152]. Similarly, in the dextran sulfate sodium (DSS)-induced mouse colitis, NaHS inhibited the activation of NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome and activated Nrf2 [153]. It suggested that H_2S shows a potential therapeutical role in erosive diseases of bone by activating a sustained antioxidant response. In the CIA mouse model, over-expression of CSE elevated H_2S production and inhibited Jumonji domain-containing protein 3 (JMJD3, a histone H3K27 demethylase) expression via the transcription factor Sp-1, which was accompanied by reduced inflammation in IL-1β-treated synovial fibroblasts [154]. Moreover, NaHS treatment activated another epigenetic enzyme histone deacetylase 3 (HDAC3), down-regulated inflammatory signaling, and maintained runt-related transcription factor 2 (RUNX2) sulfhydration, eventually ameliorating osteoclastogenesis [155].

On the other hand, H_2S donors may be used as biochemical factors able to induce cartilage and bone repair in the context of arthritis as the chronic inflammatory process leads to the irreversible disruption of the cartilage. Mesenchymal stem cells (MSCs) can express chondrogenic potential and improve the repair of cartilage [156–158]. Recently, it was reported that the pre-treatment of MSCs with NaHS protected MSCs upon hypoxia-ischemic injury via Akt and ERK1/2 pathways [159]. Furthermore, NaHS promoted the success of MSC therapy in rats with isoprenaline-induced heart failure [160]. A sponge H_2S-releasing silk fibroin loaded with GYY4137 was able to support MSCs adhesion, proliferation, and viability, and induce a significant increase in differentiation to mature osteoblasts [161].

3.3 H_2S-Donors as Potential Anti-RA Drugs

Numerous studies that investigate the biological roles of H_2S in vivo or in vitro use fast-dissolving salts, such as NaHS, Na_2S, or Lawesson's reagent (LR), to generate H_2S in a few seconds or minutes. This rapid burst of H_2S is not likely a good mimic for in vivo physiologic H_2S release, which occurs in smaller quantities [162] and much more slowly [163]. Here we introduce several promising exogenous H_2S donors in the anti-inflammation area including (RA).

3.3.1 ATB-346

ATB-346 (Fig. 5A), developed by Antibe Therapeutics, is an H_2S-releasing derivative of naproxen that progressed most into clinical development (with phase 2 trials completed). The first scientific literature of ATB-346 was published in 2010 [164]. Naproxen is considered the most cardiovascular-safe drug among these nonsteroidal anti-inflammatory drugs (NSAIDs) [165]. It exhibited comparable or even superior anti-inflammatory activity to equimolar doses of naproxen in adjuvant-induced arthritis (AIA) rat model, but with substantially reduced gastrointestinal toxicity [164]. In zymosan-induced arthritis (ZIA) rats, ATB-346 was more effective than naproxen in terms of various nociceptive responses and multiple inflammatory cellulars and biochemical parameters [166]. However, in the rat model of carrageenan arthritis, ATB-346 and naproxen were found to be comparably effective [167]. A phase 2a clinical trial was performed in which a low dose of ATB-346 (250 mg once daily) was evaluated for its pain-relieving properties in patients with osteoarthritis (OA). In this trial, a once-daily dose of ATB-346 produced a 4.3 units' reduction of the Western Ontario-McMaster University Arthritis Index (WOMAC) pain score on day 4, with a further decrease to 7.6 units on day 10, at a very high level of

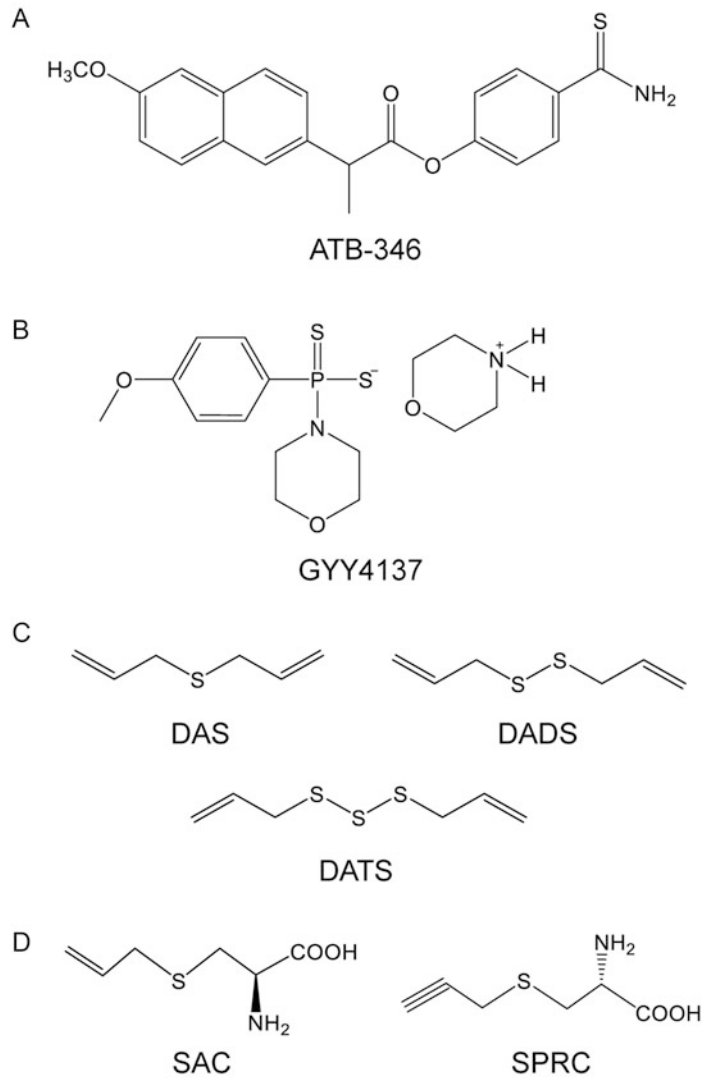

Fig. 5 Chemical structures of ATB-346 (**a**), GYY4137 (**b**), DAS/DADS/DATS (**c**), and SAC/SPRC (**d**)

statistical significance in comparison to baseline pain ($P < 0.001$).

3.3.2 GYY4137

In 2008, GYY4137 (morpholin-4-ium-4-methoxyphenyl (morpholino) phosphinodithioate) (Fig. 5B) was reported as a new type of H_2S-releasing compound [168]. GYY4137 can slowly produce H_2S both in vitro and in vivo. In an endotoxic shock rat model, GYY4137 was administered to conscious rats 1 or 2 h prior to LPS stimulation, and suppressed the rise of pro-inflammatory cytokines (e.g., tumor necrosis factor α (TNF-α), interleukin (IL)-1β and IL-6) and lung myeloperoxidase (MPO) activity in plasma, while plasma concentration of the anti-inflammatory cytokine IL-10 was increased [169]. GYY4137 also significantly inhibited LPS-induced release of pro-inflammatory mediators such as IL-1β, IL-6, TNF-α, NO, and PGE2 in RAW264.7 macrophages, as well as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in human fibroblast-like synoviocyte (FLS) and articular chondrocytes [170, 171]. In a mouse complete Freund's adjuvant (CFA) model of acute joint

inflammation, GYY4137 decreased TNF-α, IL-1β, IL-6, and IL-8 concentrations to protect the knee joints [170].

3.3.3 Diallyl Sulfide (DAS), Diallyl Disulfide (DADS), and Diallyl Trisulfide (DATS)

Multiple lines of evidence support that DAS, DADS, and DATS (Fig. 5C), both oil-soluble sulfur ingredients in garlic which supply H_2S in cell and animal models, exert anti-inflammatory and antioxidant effects. DAS prevents IL-1β and monosodium urate (MSU) crystal-induced COX-2 up-regulation in synovial cells and chondrocytes and ameliorates crystal-induced synovitis potentially through a mechanism involving nuclear factor-kappa B (NF-κB) [172]. In the CFA-induced arthritic rat model, the treatment with DADS provoked significant reductions in paw volume, edema formation, arthritic score, and organ indices, together with significant improvement in body weight and decreased the expression of pro-inflammatory cytokines [173]. Moreover, Liang et al. confirmed that DATS could decrease cell viability and introduce apoptosis in RA-FLS [144]. DATS significantly attenuated the production of key inflammatory cytokines induced by RA-FLS cells following treatment with TNF-α due to its inhibitory effect on the NF-κB and Wnt signaling pathways. Additionally, DATS regulated the immune function by restoring the balance between type 17 helper T (Th17) cells and regulatory T (Treg) cells in the collagen-induced arthritis (CIA) mouse model, which are two important cell types in the pathogenesis of RA.

3.3.4 S-Propargyl-Cysteine (SPRC)

SPRC (also named as ZYZ-802, Fig. 5D) is a structural analog of S-allylcysteine (SAC) (Fig. 5D), which is a garlic-derived organosulfur-containing amino acid. SPRC, originally innovated by Zhu's lab, has been studied extensively in preclinical studies as a H_2S donor reviewed by Wen and Zhu [174] as well as the recent update by Rose et al. [175]. In in vivo studies, SPRC, at a dose of 50 mg/kg, induced only a slight, although statistically significant, the elevation of circulating H_2S levels [176, 177]. The molecular and biochemical pathways associated with the effects of SPRC are multiple. They include the up-regulation of CSE, stimulation of Akt phosphorylation, inhibition of NF-κB activation, VEGF receptor activation followed by STAT3 activation, elevation of intracellular glutathione levels, reduction of cellular ROS levels, and improvement of cellular calcium handling [11, 177–181]. In the AIA rat model, SPRC suppressed joint swelling and down-regulated the production of multiple inflammatory mediators in the knee joints [150]. It showed an anti-arthritis effect via up-regulating the Nrf2/ARE signaling pathway. A recent study also reported in vivo efficacy with a controlled release form of the compound termed CR-SPRC (produced by solid dispersion technique with Eudragit RS30D as a carrier) in acute and chronic myocardial ischemia models [11, 13]. Considering this, SPRC may be developed into an H_2S slow-releasing compound for cardiovascular and RA study.

These compounds are novel and useful for investigating the impact of H_2S on the inflammatory joint disease, possibly offering new therapeutic approaches. Finally, we should mention that the pharmacological addition of H_2S-forming reagents is not the only way to supply H_2S exogenously. Beneficial anti-inflammatory and antioxidant effects on patients with chronic lung disease, chronic rhinosinusitis, and rheumatologic diseases were exhibited when H_2S was administered in the form of sulfurous mineral water [182–186].

4 Conclusions and Perspectives

Currently, the interest in the therapeutic role of H_2S in inflammatory diseases especially in RA is growing. Meanwhile, the evidences of the anti-inflammatory property of H_2S increased rapidly. H_2S shows inhibitory effect on the production of inflammatory cytokines in innate immune cells, as well as fibroblasts and chondrocytes. Moreover, H_2S plays a role as a radical scavenger in hypoxic condition which is usually associated

with RA. Interestingly, pre-treatment with H_2S protects MSCs and enhances its capacity of cartilage repair. Therefore, as an immuno-inflammatory regulator in RA, investigations of H_2S may produce useful information for clinical research and even anti-RA drug development.

Additionally, challenges in H_2S application definitely exist in current studies. First of all, we should concern about H_2S-related toxicity caused by high dose and quick release. Thus, slow-releasing H_2S pro-drug is a better way to solve the problem. Secondly, in order to exert a specific effect in specified tissue, H_2S should be directed by designed dosage form or conjugated with other clinical drugs as it can freely penetrate cell membrane, which may cause side effects via targeting other organs or tissues. Thirdly, there is a growing need to accurately monitor the concentration of H_2S in vivo and balance the efficacy and toxicity well. Further work is clearly required to substantiate the anti-RA effect of H_2S as it possibly offers a new therapeutic option for RA treatment.

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The Cardiovascular Effects of Hydrogen Sulfide: The Epigenetic Mechanisms

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1 Introduction

Hydrogen sulfide (H_2S) is a kind of colorless poisonous gas with the odor of rotten eggs, it was once considered as a metabolic waste in organisms, and it has now been proved to be an important biologically active gas signal molecule in the body. It has been discovered that H_2S can participate in the occurrence and development of certain cardiovascular diseases (CVD) through various mechanisms. Epigenetics is a kind of heritable gene expression changes without alteration of DNA sequence, the main mechanism of epigenetics includes DNA methylation, histone modification, and non-coding RNA mechanisms, these mechanisms work together to define genes specific expression [1]. Epigenetic links environment to genes, environmental factors act on genes through epigenetic modification, regulate specific genes expression, produce long-term phenotype changes that eventually lead to the occurrence of diseases, and affect the phenotype of diseases [2, 3]. CVD is a complex disease, mostly caused by genetic and environmental factors. The regulation between H_2S and epigenetics may have an important influence on the occurrence and development of CVD. This chapter mainly summarizes the H_2S cardiovascular effects and epigenetic regulation of H_2S on CVD.

2 Endogenous Synthesis of H_2S and Exogenous H_2S Donor

2.1 Endogenous Synthesis of H_2S

In the nineteenth century, H_2S was shown to be enzymatically synthesized by mammalian cells. The enzymes that synthesize H_2S are cystathionine-γ-lyase (CSE), cystathionine β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3-MST). Among them, H_2S synthesized by CSE and CBS catalyzes L-cysteine, and these two enzymes are widely expressed in various organs and have certain organ specificity. CBS is the main synthase of H_2S in the nervous system [4]. CSE is mainly expressed in the cardiovascular system. The previous study prepared CSE gene-deficient mice by the genetic method and measured that the content of H_2S in the aorta and arterioles decreased compared with the wild-type mice, indicating that CSE is the main

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enzyme of H_2S in the cardiovascular system [5]. 3-MST is mainly expressed in brain tissues and vascular endothelium [6]. Like nitric oxide (NO), endogenous H_2S freely penetrates the cell membrane in a simple diffusion manner without the need of specific membrane receptors, which have extensive physiological effects on the cardiovascular and central nervous systems [4], H_2S is considered to be the third endogenous gaseous signal molecule after NO and CO.

2.2 Exogenous H_2S Donor

Researchers have tried using multiple substances as exogenous donors of H_2S (commonly used H_2S donors shown in Table 1). Among the commonly used donors, NaHS is an exogenous donor dissociates with water and releases H_2S quickly, which has been used extensively for studying the function of H_2S [7]. Diallyl disulfide (DADS) and diallyl trisulfide (DATS) are sulfur-containing organic compounds found in several allium species like garlic, onion, leek, etc. It can be used as a donor of H_2S and has anti-oxidation, anti-inflammatory, and promoting vasodilation effects [8, 9]. Donors that slowly release H_2S include GYY4137 [10], DTT [11], AP39 [12], we synthesized s-propargyl-l-cysteine (SPRC, also known as ZYZ-802) based on garlic extract and its analogs, thus contributing to the development of sulfide compounds [13]. We also synthesized an H_2S-NO donor (named ZYZ-803), a slow-releasing H_2S-NO hybrid molecule [14], which may have more activities like regulated vascular tone through the cGMP pathway [15]. They may become H_2S in vitro supplements. Because H_2S has a short half-life period, it is different from the single high-concentration method used in the study, such as NaHS and Na_2S, and continuous low-concentration H_2S release may have more therapeutic significance, and the key scientific problem of H_2S donor drug research is how to improve the selectivity of H_2S donor molecules, release an appropriate concentration of H_2S in a certain part of the target, and limit its adverse reactions while playing a therapeutic role. In recent years, the nanomedicine-based H_2S delivery platform has made full use of the selective release of H_2S by small molecules and has made some progress [16, 17].

3 Cardiovascular Effects of H_2S

H_2S has been shown to have a wide range of physiological effects in the cardiovascular system (Shown in Fig. 1), such as modulating blood pressure, angiogenesis, inflammation, and smooth muscle cell growth or apoptosis, anti-oxidation, and protection of myocardium.

3.1 Anti-Inflammation

Series of studies indicate that H_2S plays an important and active role in regulating immune inflammation in the process of cardioprotection, treatment of myocardial equivalent overexpression of CSE or exogenous H_2S can significantly improve the myocardial damage and neutrophil infiltration or lymphocyte adhesion to vascular endothelial cells, down-regulate the release of IL-6, IL-8, TNF-α [18, 19]. Our laboratory research also demonstrates that H_2S exhibits pro-inflammatory activity in endotoxin-induced inflammation and suggests a new approach to the development of novel drugs for this condition, we further confirmed the results that the H_2S donor SPRC significantly induced CSE expression and regulated H_2S levels, thereby inhibiting LPS-induced ROS production in cardiomyocytes and NF-kB activation-mediated inflammatory factor TNF-α expression, which play a role in inhibiting cardiomyocyte inflammation, our data also reveal the inhibition of inflammatory hepcidin by SPRC and propose SPRC as a potential remedy against Anemia of inflammation (AI) [20–23].

3.2 Anti-Oxidant Stress

Oxidative stress is an intracellular metabolic process that occurs in oxygen imbalance, leading to superoxide anions generation. Many pieces of

Table 1 Commonly used H_2S donors

H_2S donors	Type	Release speed	References
NaHS	Classic donor	Quick	Caliendo et al. [7]
Na_2S	Classic donor	Quick	Caliendo et al. [7]
DADS	Sulfur-containing organic compound	Slow	Liu et al. [8]
DATS	Sulfur-containing organic compound	Slow	Benavides et al. [9]
GYY4137	Water-soluble hydrogen sulfide-releasing molecule	Slow	Li et al. [10]
DTT	Water-soluble hydrogen sulfide-releasing molecule	Slow	Powell et al. [11]
AP39	Mitochondria-targeted hydrogen sulfide donor	Slow	Szczesny et al. [12]
SPRC(ZYZ-802)	Synthesized molecule based on garlic extract	Slow	Wen et al. [13]
ZYZ-803	H_2S-NO hybrid molecule	Slow	Hu et al. [14]

evidence suggest that reactive oxygen species (ROS) are related to the occurrence of cardiovascular diseases, such as myocardial ischemia and reperfusion (MI/R) injury, and cardiac muscle hypertrophy. The presence of ROS causes lipid peroxidation, protein oxidation, and DNA damage, thus changing normal cell functions [24]. Our research provides new evidence that H_2S donor SPRC reduces the deleterious effects of oxidative stress by regulating endogenous H_2S levels and protecting the activity of antioxidant defense enzymes such as SOD, thereby having a cardioprotection effect on myocardial infarction (MI) [25]. H_2S can protect HUVECs from mitochondrial dysfunction and oxidative stress induced by H_2O_2 [26]. Other studies have found that NaHS not only inhibits angiotensin II (AngII) -induced rat cardiac myocyte hypertrophy and mitochondrial function impairment, up-regulates the expression of FoxO3a and SOD2, but also inhibits myocardial hypertrophy and oxidative stress induced by aortic constriction, and improves the mitochondrial function, this effect depends on SIRT3. These data show that H_2S

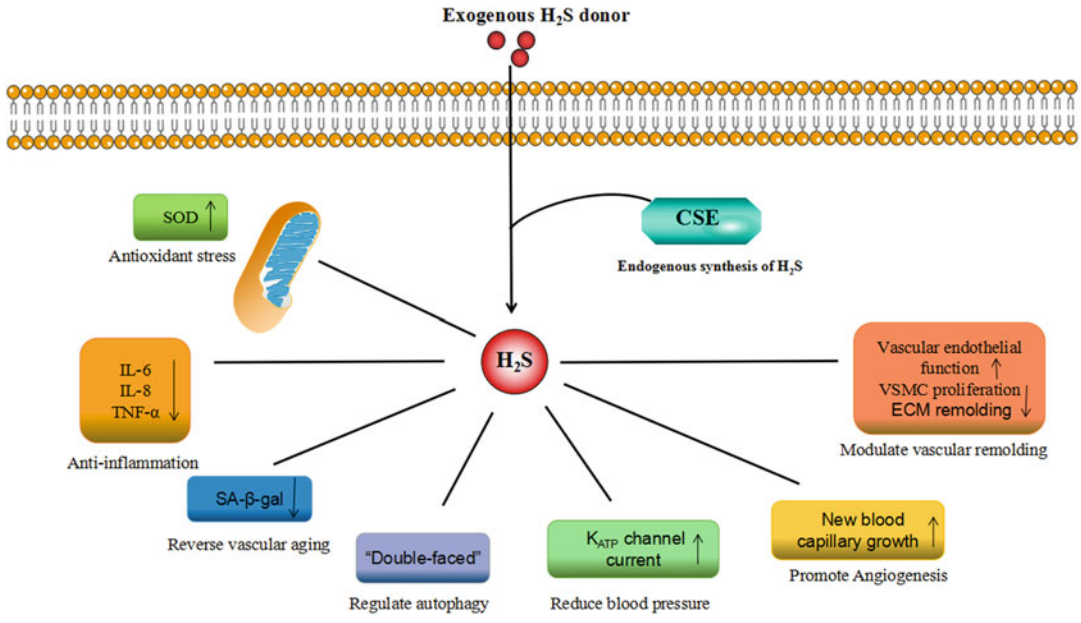

Fig. 1 *H_2S physiological effects in the cardiovascular system. H_2S* hydrogen sulfide, *CSE* cystathionine-γ-lyase, *SOD* superoxide dismutase, *IL6* interleukin-6, *TNF*-α tumor necrosis factor-α, *SA-β-gal* senescence-associated β-galactosidase, *VSMC* vascular smooth muscle cells, *ECM* extracellular matrix

inhibits oxidative stress during myocardial hypertrophy [27]. In aortic constriction-induced stress-overloaded heart failure mice, cardiac-specific CSE transgenes can enhance NO/cGMP signaling and mitochondrial homeostasis, reduce oxidative stress responses, and better preserve cardiac structure and function [28]. It has been reported that NaHS reduced the NADPH oxidase activity in the aorta of diabetic mice and restored its endothelial function [29]. In SD rats with β-adrenergic agonist isoproterenol-induced cardiomyopathy, NaHS inhibits oxidative stress and improves cardiac function by reducing NADPH oxidase activity and ROS production [30]. In rats with oxidative stress and myocardial hypertrophy induced by high salt, NaHS inhibits the oxidative stress response of myocardial tissues and reduces myocardial hypertrophy [31]. The above experiments show that reducing oxidative stress and mitochondrial damage plays a key role in H_2S protecting endothelial function and cardiac effects.

H_2S antioxidant function is partly due to the direct removal of ROS or inhibits ROS generation. As reported by Geng et al. [32], isoprenaline induces myocardium damaged, H_2S by eliminating O^- and H_2O_2 to reduce lipid peroxidation. In hypoxic/reoxygenated rat cardiomyocytes, H_2S reduces ROS in cardiomyocytes by inhibiting mitochondrial complex IV activity, enhancing superoxide dismutases (SOD) activity, and reducing ROS levels to protect cardiomyocytes [33].

Mitochondria are the main source of intracellular ROS. H_2S can reduce mitochondrial ROS production [34].

3.3 Regulates Vascular Aging

H_2S research has made great progress in the last 20 years, but there are few reports of H_2S role in aging. Vascular aging refers to vascular lumen dilation, vascular stiffness, and thickening, and one of the important changes in the body during vascular aging is the decreased number and function of vascular endothelial cells. The body and tissues rely heavily on the microvascular network to maintain oxygen supply, exchange nutrients, and excrete metabolic waste. Vascular senescence is the leading cause of aging and aging-related diseases, and H_2S may reverse vascular aging. It has been reported that H_2S can extend the lifespan and heat resistance of C.elegans, suggesting that the H_2S gas molecule may be an endogenous source of the aging regulator [35, 36]. Previous research found that use H_2O_2 to induce HUVEC senescence significantly reduces senescence-associated β-galactosidase (SA-β-gal) activity [37, 38].

Endothelial cell apoptosis and increased oxidative stress are the main reasons for aging to reduce angiogenesis. HUVEC treated with NaHS alone can increase SIRT1 protein expression, intracellular nicotinamide adenine dinucleotide (NAD^+) level, endothelial cell migration ability, and budding length, and significantly reduce aortic endothelial cells and HUVEC apoptosis induced by H_2O_2. It also cooperates with nicotinamide mononucleotide (NMN) to enhance the inhibitory effect on endothelial cell apoptosis. Treating 32-month-old mice with NaHS and NMN (NAD donor) for 4 weeks can significantly increase the density of their quadriceps capillaries and improve blood flow and tolerance to exercise [39].

3.4 Regulate Autophagy

Autophagy has been interestingly defined as cell "eat itself". When cells encountering various stresses from inside and outside, such as nutritional deficiencies, pathogen infection, hormone therapy, protein misfolding, and damage to organelles, autophagy becomes a remedy mechanism, in this process, cytoplasmic components and dysfunctional cell organs are surrounded by a double membrane, forming autophagosomes and transferred to lysosomal degradation and recycling to maintain homeostasis [40, 41]. Autophagy dysfunction can induce a variety of human diseases, and H_2S plays "double-faced" in the occurrence of autophagy.

On the one hand, it showed that cardiomyocytes autophagy happened during

hypoxia-reoxygenation injury, H_2S could activate mTOR, inhibited autophagy, and alleviated hypoxia-reoxygenation injury of cardiomyocytes [42]. On the other hand, researchers observed on the aged isolated rat heart and aging cardiomyocyte model, exogenous H_2S significantly restored the protective effect of ischemic postconditioning on the myocardium, such as reducing myocardial injury, infarct size, apoptosis, improving myocardial function, and increasing myocardium cell viability and autophagy by up-regulating AMPK pathway [43].

3.5 Modulate Vascular Remolding

Vascular remodeling is an important pathological basis for heart damage, mainly manifested as smooth muscle cell proliferation, hypertrophy and migration, endothelial cell dysfunction, and extracellular matrix collagen increase.

3.5.1 Regulation of Vascular Smooth Muscle Cell Proliferation and Apoptosis

Vascular smooth muscle is involved in the process of vasoconstriction. In pathological conditions vascular smooth muscle cells (VSMCs) can migrate into the intima promoting the development of atherosclerosis. Numerous studies show that H_2S can inhibit VSMC proliferation.

It has been reported that smooth muscle cell migration and growth ability from $CSE^{-/-}$mice were enhanced compared with wild-type mice, and the application of NaHS could significantly inhibit the smooth muscle cell migration and growth ability, reverse carotid ligation induction in $CSE^{-/-}$mice neointimal formation [44]. In high glucose-treated rat thoracic aortic smooth muscle cells, CSE activity and endogenous H_2S levels were reduced; NaHS administration significantly reversed VSMC proliferation induced by high glucose, animal experiments have found that calcium-sensitive receptors up-regulate endogenous H_2S production and prevent intima thickening in diabetic rats [45]. In high glucose-induced human pulmonary aortic smooth muscle cells, NaHS can keep mitochondria still, prevent its fission, and inhibit smooth muscle cell proliferation and migration [46]. In addition to inhibiting VSMC proliferation, exogenous H_2S and CSE overexpression can induce apoptosis in human aortic smooth muscle cells, and its mechanism is related to activation of ERK and p38 MAPK, up-regulation of p21 expression, and down-regulation of Cyclin D1 expression [47].

3.5.2 Improve Vascular Endothelial Function

H_2S plays a very important role in the maintenance of vascular endothelial function. The vasodilatation effect of H_2S on blood vessels depends on intact vascular endothelium. On the one hand, the hyperpolarization of the cell membrane is caused by direct excitation of K_{ATP} channels to cause vasodilation. On the other hand, the endothelial cells increase the conductance of potassium ions to achieve vasodilation. The protective mechanism of H_2S on the function of vascular endothelial cells may function as an endogenous opener of K_{ATP} channels [48], our group study showed that H_2S can directly activate vascular endothelial growth factor receptor 2 (VEGFR2) and specific knockout of VEGFR2 can inhibit H_2S-induced human vascular endothelial cells (HUVECs) migration [49], also the hybrid molecule ZYZ-803 can slowly release H_2S and NO in vivo to regulate endothelial homeostasis [50].

H_2S also inhibits apoptosis and the expression of endothelial inflammatory molecules, and promotes the proliferation of vascular endothelial cells [51].

3.5.3 Suppresses Extracellular Matrix Collagen Accumulation

The excessive accumulation of collagen is also the main reason for vascular remodeling.

Endogenous H_2S may be inhibited by regulating matrix metalloproteinases (MMPs)/Tissue inhibitors of metalloproteinases (TIMPs) expression, increasing the degradation of the collagen component of the blood vessel wall, reducing the collagen content and the deposition of extracellular matrix collagen, thereby slowing

down vascular remodeling [52]. Our study provides strong evidence that exogenous H_2S prevents cardiac remodeling by inhibiting the accumulation of extracellular matrix and increasing blood vessel density [53]. Another study reported that exogenous H_2S supplementation can reduce the formation of hydroxyproline and the content of type I colloid in the aorta of spontaneously hypertensive rats [54].

3.6 Regulate Blood Pressure

H_2S can temporarily reduce arterial blood pressure in SD rats and presented dose-dependent phenylephrine pre-construction in SD rats' aorta, which was dependent on the open channel of adenosine triphosphate-sensitive potassium channel (K_{ATP}). In in vitro vascular smooth muscle cells, H_2S directly increases the K_{ATP} channel current. In addition to the aorta, H_2S can also relax small resistance vessels such as the mesenteric artery [55]. Yang et al. [5] found that blood pressure in $CSE^{-/-}$ mice was significantly reduced, indicating that H_2S was a physiological vasodilator and blood pressure regulator. H_2S-induce vasodilatation through the non-endothelium-dependent manner. However, a small part of it is through the endothelium-dependent mechanism, the non-endothelial dependent mechanism includes activation of vascular smooth muscle K^+ channels, lowering intracellular pH, and generation suppresses [56–58]. Activated K^+ channels especially K_{ATP} channels are one of the main mechanisms of H_2S-induced vasodilation effect [55]. Acidification is another mechanism H_2S causes vasodilation. H_2S activates Cl^- /HCO_3^- exchanger, thereby reducing the intracellular pH. Lee et al. [58] reported that exogenous hydrogen sulfide sodium hydrosulfide (NaHS) reduces vascular smooth muscle intracellular pH in a dose-dependent manner, inhibition of Cl^-/HCO_3^- exchangers weakened NaHS-induced intracellular acidification and attenuated NaHS-induced aortic ring diastolic effect in SD rats. Wang et al. [59] reported that intracellular acidification can activate K_{ATP} subtype Kir6.1/SUR2B channel currents in vascular smooth muscle cells, and inhibition of K_{ATP} channel activity can reverse high-carbon acid acidosis-induced mesentery arterial relaxation. It is suggested that H_2S, K_{ATP} channels, and intracellular acidification have loops in regulating angiectasis. The endothelium-dependent mechanism is related to NO [56]. In rat aortic tissue, removing endothelial cells and blocking nitric oxide synthase can attenuate the vasodilation effect induced by H_2S [60], indicating that the vasodilation effect of H_2S partly depends on the endothelial cells and NO.

3.7 Regulate Angiogenesis

Angiogenesis is the formation of a new blood capillary growth process from the original vascular system. This process begins with the degradation of the local matrix, followed by endothelial cell proliferation, migration, and capillary germination [61].

A large amount of literature reports that H_2S can promote angiogenesis. We demonstrate the therapeutic potential of SPRC in ischemic disease through angiogenesis promotion via a novel STAT3-mediated mechanism [62]. Novel H_2S-NO donor ZYZ-803 promotes angiogenesis through the crosstalk between CaMKII and STAT3 [63]. In a mouse hindlimb ischemia model, H_2S promotes neovascularization [64]. Longchamp et al. [65] studied femoral artery ligated $CSE^{-/-}$mice and found that blood capillary density of ischemic tissue was low, and H_2S production was reduced; while high expression of CSE increased H_2S yield and vascular density in ischemic tissue. This effect does not depend on any other angiogenic stimuli, suggesting that CSE/H_2S can induce angiogenesis in vivo. In vitro experiments found that whether it is human umbilical vein endothelial cells (HUVEC) treated with CSE inhibitor DL-propargylglycine (PPG) or $CSE^{-/-}$endothelial cells, the bud length is reduced, indicating that angiogenesis in vitro also depends on CSE/H_2S. The angiogenesis effect of H_2S is partly through the inhibition of mitochondrial electron transport and oxidative phosphorylation, increasing glucose uptake and

glycolytic ATP production. In wild-type mice injected with vascular endothelial growth factor (VEGF), the capillary density of ischemic tissue increased significantly; $CSE^{-/-}$mice injected with VEGF did not have this effect. This indicates that CSE is involved in VEGF-induced angiogenesis.

4 H_2S and Cardiovascular Disease

In addition to regulating many physiological processes in the body, H_2S has important pathophysiological significance in the occurrence and development of diseases in the cardiovascular system (Fig. 2). Here is an introduction to the relationship between H_2S and cardiovascular system diseases.

4.1 Cardiovascular Diseases Protection

In addition to its vascular protection, H_2S also protects the heart from damage during myocardial infarction (MI), ischemia-reperfusion, heart failure, and myocardial fibrosis. Our precious works demonstrated that the endogenous H_2S was cardioprotective in the rat model of MI [66] and the H_2S may have the anti-infarct effect of MI by modulating migration of macrophage, which was achieved by activating the Src-FAK/Pyk2-Rac pathway [67], meanwhile, our study provides novel evidence that SAC (an organosulfur-containing compound extract from garlic) is protective in myocardial infarction via an H_2S-related pathway [68].

Our newly synthesized compound SPRC exerts cardioprotective effects [69], other SPRC related compounds of our group also showed cardioprotective activities, a novel leonurine-SPRC conjugate showed that it has a strong cardioprotective effect on hypoxia-induced neonatal rat ventricular myocyte injury. Part of its mechanism is related to increased production of hydrogen sulfide, anti-oxidative stress, and anti-apoptosis effects [70]. Furthermore, a controlled release formulation of SPRC (CR-SPRC) showed protective effects against myocardial infarction (MI) through the CSE/H_2S pathway and showed better cardioprotective effects than SPRC via extending the release of endogenous H_2S [71]. ZYZ-803 reduces endoplasmic reticulum (ER) stress-related necrosis after acute myocardial infarction (AMI) by down-regulating the RIP3-CaMKII signaling pathway [72], also our research suggests that H_2S may become a therapeutic agent for MI by promoting M2 macrophage polarization [73]. The new formulation of liposome ZYZ-802 improved the pharmacokinetics and optimized the concentration of H_2S in tissues and plasma. Liposome ZYZ-802 shows enhanced cardioprotection effect in vivo, it may inhibit TGF-β1/Smad signaling pathway by inhibiting myocardial fibrosis [74], inhibit NADPH Oxidase 4-related Signaling by NaHS attenuates myocardial fibrotic response [75]. In the neonatal rat cardiomyocytes model of hypoxia and reoxygenation, the administration of H_2S can increase the viability of cardiomyocytes and reduce the release of lactate dehydrogenase (LDH) [42]. H_2S can protect the myocardium from hypoxia-reoxygenation damage. Exogenous H_2S pretreatment or H_2S treatment after myocardial infarction can protect myocardial cells, reduce infarct size, and improve left ventricular function. The myocardial protective effect of H_2S is related to its protection of mitochondrial structure, opening of K_{ATP} channels, inhibition of JNK, and reduction of ROS levels [61, 76]. Liu et al. [77] reported that NaHS preconditioning reduces the apoptosis of H9c2 myocardial cells induced by doxorubicin through the PI3K/Akt/FoxO3a pathway and exerts myocardial protective effects.

4.2 H_2S and Hypertension

Numerous studies show reduced endogenous H_2S production or enzyme deficiency lack promotes the occurrence of hypertension. Chronic NaHS treatment can reduce the thickening of the myocardium, coronary intima, ROS productions, and interstitial fibrosis in spontaneously hypertensive

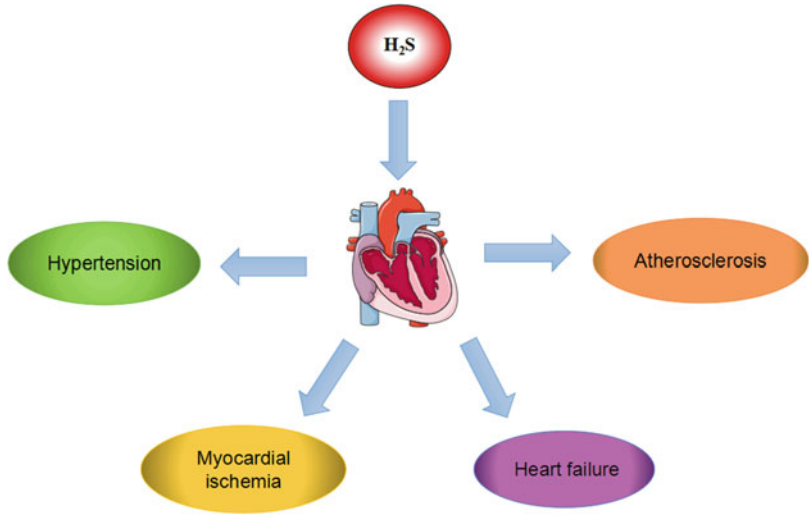

Fig. 2 *H_2S and cardiovascular diseases.* H_2S has pathophysiological significance in the occurrence and development of diseases in the cardiovascular system including hypertension, atherosclerosis, myocardial ischemia, and heart failure

rats [78]. Yang et al. [5] reported that $CSE^{-/-}$ mice showed significantly reduced endothelial-dependent vasodilation and hypertension, and their H_2S levels in serum, heart, aorta, and other tissues were significantly reduced. In salt-sensitive rats, high salt induces decreased endogenous H_2S levels and hypertension, while H_2S donors can antagonize salt-sensitive hypertension and reverse aortic structural remodeling [79]. In spontaneously hypertensive rats, plasma H_2S levels are reduced and mean arterial blood pressure is significantly increased compared to wild type. The use of NaHS can reduce blood pressure and oxidative stress and improve renal hemodynamics [80]. Similar results were seen in children with essential hypertension. Compared with healthy children with normal blood pressure, plasma H_2S levels in children with essential hypertension were significantly reduced, and systolic blood pressure was negatively correlated with plasma H_2S/Hcy (hydrogen sulfide/homocysteine) ratios [81]. The above results indicate that lower plasma H_2S levels can promote the occurrence of hypertension.

4.3 H_2S and Atherosclerosis

Atherosclerosis is a disease that occurs in the walls of arteries chronic inflammatory lesions. A large number of studies report that H_2S has important pathophysiological significance in the pathogenesis of atherosclerosis.

Our group demonstrates that H_2S has a protective effect on atherosclerosis formation at least in part by stabilizing atherosclerotic plaques in apolipoprotein E knockout mice [82]. In the $ApoE^{-/-}$ mouse atherosclerosis model, plasma H_2S levels were significantly reduced; the CSE inhibitor PPG further reduces plasma H_2S levels, increases aortic and plasma intercellular adhesion molecule-1 (ICAM-1) level, increases the area of aortic lesions; supplying NaHS increases plasma H_2S concentration, reduces aortic and plasma ICAM-1 levels, and reduces the area of aortic lesions [83]. However, the area of aortic lesions in $ApoE^{-/-}$ mice is smaller than that in $ApoE^{-/-}$/ $CSE^{-/-}$mice [84]. This shows that CSE deficiency promotes the development of atherosclerosis, and up-regulation of H_2S levels inhibits the formation of atherosclerosis, CSE/H_2S is an important intervention target for the prevention and treatment of atherosclerosis.

In a streptozotocin (STZ) induced $LDLR^{-/-}$ diabetic mouse model, the H_2S donor GYY4137 significantly reduced aortic atherosclerotic plaque area and aortic ROS levels. However, GYY4137 cannot reduce the area of atherosclerotic plaque in the aorta of $LDLR^{-/-}$/$Nrf2^{-/-}$double knockout mice, nor can it inhibit the formation of

enterocoelia macrophage-derived foam cells and oxidative stress in Nrf2$^{-/-}$ mice shows that H_2S inhibits diabetic atherosclerosis formation by Nrf2 [85].

In short, the mechanism of CSE/H_2S antagonizing atherosclerosis includes weakening endothelial dysfunction, anti-oxidative stress injury, regulating cell apoptosis, inhibiting the production of inflammatory factors, reducing the accumulation of macrophages at diseased sites, inhibiting foam cell formation [86], increasing the number of regulatory T cells, [87], etc.

4.4 H_2S and Myocardial Ischemia and Ischemia-Reperfusion Injury

Numerous studies have shown that exogenous H_2S can antagonize myocardial ischemia-reperfusion injury. Our study showed the cardioprotective effect of H_2S in ischemic-reperfusion experimental rats [88]. In CSE$^{-/-}$mice, the blood reperfusion injury is worse than that of the control group. The mechanism is associated with endothelial nitric oxide synthase (eNOS) loss activity, reduced NO levels, increased oxidative stress; exogenous H_2S restores eNOS activity and NO level in myocardium CSE$^{-/-}$ mice, it can reduce oxidative stress and myocardial injury, indicating that lack of CSE aggravates a myocardial ischemia-reperfusion injury, while exogenous H_2S can antagonize myocardial ischemia-reperfusion injury [89].

H_2S pretreatment can significantly antagonize myocardial ischemic injury, reduce the area of myocardial infarction, reduce troponin-I and oxidative stress levels. Further research found that H_2S can increase Nrf2 nuclear translocation and up-regulate PKC, and STAT-3 phosphorylation in the early pretreatment period, increase the expression of HO-1, thioredoxin 1, and heat shock protein 90 (Hsp90) in the post-treatment period, and reduce the activity of pro-apoptotic factors [90].

The treatment of H_2S during reperfusion can also significantly antagonize myocardial ischemia-reperfusion injury, reduce the area of myocardial infarction, and protect left ventricular function. Its mechanism is related to inhibiting myocarditis and protecting mitochondrial structure and function. Giving cardiac muscle-specific high expression of CSE antagonizes myocardial ischemia-reperfusion injury and reduces the area of myocardial infarction. These results indicate that myocardial ischemia-reperfusion injury can be alleviated by either exogenous H_2S administration or up-regulation of endogenous H_2S [76, 91].

It has also been reported that NaHS reduces myocardial cell Caspase-9 activity, up-regulates Bcl-2 expression, reduces p38 MAPK (p38 mitogen-activated protein kinase), and JNK (Jun N-terminal kinase) phosphorylation and NF-κB p65 (nuclear factor-kappaBp65) subunits nuclear translocation, and antagonizes myocardial ischemia-reperfusion injury [92]. Administration of H_2S donor ADT can increase AMPK activity and reduce the area of myocardial infarction after myocardial ischemia and reperfusion in rats [93]. Cardiac H_2S production and CSE expression were reduced in myocardial ischemia-reperfusion injury rats, and myocardial cell apoptosis and myocardial infarction were obvious; post-ischemic treatment increased H_2S level and CSE expression, and reduced rats cardiac ischemia-reperfusion injury; NaHS can inhibit oxidative stress, up-regulate the PI3K-Akt-GSK-3β pathway, and enhance the protective effect of ischemic post-processing on elderly hearts [94].

Diallyl trisulfide (DATS) is a polysulfide component in garlic oil that releases H_2S. After injecting DATS to ischemia-reperfusion mice, the area of myocardial infarction was reduced by increasing the H_2S level, the level of cardiac troponin I was reduced, the myocardial contractile function was improved, and myocardial ischemia-reperfusion injury was reduced. At the cellular level, DATS reduces mitochondrial respiration in a dose-dependent manner, improves mitochondrial coupling after reperfusion, and simultaneously activates eNOS, increasing NO bioavailability [95]. The above results provide an experimental basis for the application of H_2S

donors to clinically antagonize myocardial ischemia-reperfusion injury.

4.5 H_2S and Heart Failure

Our group previous study showed that H_2S attenuates cardiac dysfunction in a rat model of heart failure [96], and we further use SPRC or NaHS modulate blood H_2S levels, attenuated the development of animal heart failure, SPRC mediated S-sulfhydration of CaMKII was found to inhibit CaMKII activity and maintain cardiovascular homeostasis [97]. The novel ZYZ-803 (H_2S-NO hybrid molecule) augments synergistic effects against heart failure [98]. Our study provides the evidence for SPRC's therapeutic properties in reducing doxorubicin cardiotoxicity, which can be attributed to gp130-mediated activation of STAT3 signaling, which provide a new molecular basis and treatment strategy for H_2S donors to treat heart failure. In aortic coarctation-induced heart failure models, myocardial and circulating H_2S levels were significantly reduced; compared to wild-type mice, $CSE^{-/-}$ mice showed more severe heart enlargement and dysfunction after aortic coarctation. However, myocardial-specific CSE transgenic mice can maintain heart structure and function after aortic coarctation. These results indicate that H_2S levels decrease during heart failure while increasing CSE and increasing H_2S levels can protect heart function in heart failure animals [28].

It has been reported that heart structure and function of myocardial failure mice with overexpressed CSE were significantly improved compared with the control group; daily treatment of H_2S donor Na_2S during reperfusion or within 7 days after myocardial ischemia can also reducing oxidative stress and mitochondrial dysfunction, increasing AKT phosphorylation and Nrf2 nuclear translocation, inhibiting left ventricular structure and function deterioration. These data show that exogenous H_2S has a certain effect on ischemic heart failure [99].

Stress overload heart failure induced by aortic constriction model, using H_2S donor DATS can improve left ventricular remodeling and protect left ventricular function. DATS can increase myocardial VEGF expression, decrease the expression of angiostatin, and increase myocardial blood vessel density. It shows that H_2S can promote angiogenesis to improve left ventricular remodeling and dysfunction during heart failure [100].

5 Epigenetic Mechanism of the Cardiovascular Effects of H_2S

Epigenetics is a heritable change of gene expression without alteration of DNA sequence, the main mechanism of epigenetics includes DNA methylation, histone modification, and non-coding RNA mechanisms, epigenetic alternations are reversible process, enzymes that involved in the epigenetic regulations are named "writers, readers, and erasers" targeting those enzymes will affect epigenetic modification [101], and these modifications work together to define genes specific expression. The epigenetic mechanism plays an important role in the extensive cardiovascular effects of H_2S. We summarize the epigenetic related regulation of H_2S on CVD (shown in Table 2).

5.1 DNA Methylation

DNA methylation (showed in Fig. 3) refers to the methyl group of S-adenosylmethionine (SAM) transferred to the DNA sequence under the catalysis of DNA methyltransferase (DNMT) and methylation can be erased by ten-eleven translocation (TET) enzymes [102, 103]. Generally speaking, DNA methylation of mammals mainly on the cytosine of CpG (Cytosine-phosphoric acid-guanine) island produces 5mc, CpG island is a CpG-rich region, most of them are promoter regions [104]. DNA methylation level has an important effect on gene expression, usually, active genes of the promoter region are demethylated, while the silencing gene promoter region is hypermethylated. The CpG island's

Table 2 Epigenetic related regulation of H_2S on CVD

Targets	Major findings	Cells/Animals	Results	Effects	References
CSE	Hypermethylation of promoter regions	Macrophages	Reducing the CSE transcription and H_2S production	Contribute to the development of atherosclerosis or inflammation	Du et al. [106], Li et al. [107]
TFAM	Demethylation by H_2S	Smooth muscle cells and aorta tissues from mice	Maintain the normal replication mtDNA	Critical for cellular energy metabolism and mitochondrial biogenesis	Li and Yang [108]
SIRT1	Up-regulate by H_2S	Human umbilical vein endothelial cells	Improve endothelial cells function	Anti-aging and anti-atherosclerotic	Suo et al. [37], Zhang et al. [38]
	ZYZ-803-induce SIRT1 expression	Rats	Regulated angiogenesis through an SIRT1/VEGF/cGMP pathway	Regulate endothelial homeostasis	Hu et al. [50]
	NaSH increase the expression of SIRT1	H9c2 cardiomyocytes	Protects against cardiomyocytes apoptosis under oxidative stress	Cardiomyocytes protection	Wu et al. [110]
	NaHS improved expression of Sirt1/PGC-1α	Rat hearts	Protects isolated rat hearts from ischemia/reperfusion injury	Cardioprotection	Hu et al. [111]
	Endogenous CSE/H_2S directly sulfhydrated SIRT1	Mice	Enhanced SIRT1 binding to zinc ion promoted its deacetylation activity	Reducing atherosclerotic plaque formation	Du et al. [112]
HDAC6	Regulate CSE and producing H_2S	Mice	Improve endothelial function	Attenuates hypertension and atherosclerosis	Chi et al. [113], Leucker et al. [114]
JMJD3	Regulate CSE / H_2S system	Mice	Regulate inflammatory response	Prevent uncontrolled inflammation	Liu et al. [115], Wu et al. [116]
miR-30	Inhibiting miR-30 can up-regulate CSE expression	Rats	Increase H_2S production	Antagonize myocardial ischemic injury	Shen et al. [118]
miR-133a	Up-regulate by NaHS/Na_2S	Rat cardiomyocytes	Inhibit cardiomyocyte hypertrophy	Cardioprotection	Liu et al. [119], Kesherwani et al. [120]
	H_2S overexpress miR-133a	Cardiomyocyte	Reversed I/R-induced ER stress and cardiomyocyte apoptosis		Ren et al. [121]
miR-186	miR-186 was identified to bind to the 3'UTR of CSE	Human THP-1 macrophages	Inhibits CSE protein and mRNA expression	Increases macrophage lipid accumulation and secretion of pro-inflammatory cytokines	Yao et al. [122]

(continued)

Table 2 (continued)

Targets	Major findings	Cells/Animals	Results	Effects	References
miR-216a	Down-regulate the CSE and ABCA1 expression	Human THP-1 macrophages	Reducing cholesterol efflux	Modulate cholesterol levels	Gong et al. [123]
miR-21	Inhibits protein expression of CSE and SP-1	Aortic smooth muscle cells	Inhibit H_2S production	Stimulation of smooth muscle cell proliferation	Yang et al. [124]
	Na_2S inducing miR-21 expression	Mice	Inhibits myocardial cell apoptosis, necrosis myocardial inflammation	Reduces infarct size after M/IR injury	Toldo et al. [125]
miR-34a	Down-regulation of miR-34a expression can increase the expression of Nrf-2	Rats	Enhance the effect of H_2S	Attenuates I/R injury of aged rats	Huang et al. [126]
miR-1	H_2S pretreatment down-regulates miR-1	Rats	Reduces myocardial cell apoptosis	Improves cardiac muscle cell viability	Kang et al. [127]
miR-22	MiR-22/Sp-1 links estrogens with the up-regulation of CSE	Primary cultured neonatal female cardiomyocytes	Increase of anti-oxidative defense	Estrogenic cardioprotection	Wang et al. [129]
miR-640	Down-regulate by H_2S	ECs	Increases the level of HIF1A through the VEGFR2-mTOR pathway	Promotes angiogenesis	Zhou et al. [130]
miR-126-3p	Up-regulate by H_2S	HUVECs	Rescues high glucose-induced HUVECs migration dysfunction	Improve vascular function	Xue et al. [131]

CSE cystathionine-γ-lyase, *TFAM* mitochondrial transcription factor A, *mtDNA* Mitochondrial DNA; *SIRT1* sirtuin1, *VEGF* vascular endothelial growth factor, *cGMP* cyclic guanosine 5′-monophosphate, *PGC-1* peroxisome proliferator-activated receptorγcoactivator-1, *HDAC6* histone deacetylases 6, *JMJD3* jumonji domain containing 3; *I/R* ischemia-reperfusion, *ER* endoplasmic reticulum, ABCA1, ATP- binding cassette transporter A1, *MI/R* myocardial ischemia and reperfusion, *Nrf2* nuclear factor erythroid 2-related factor- 2, *SP-1* specificity protein-1, *ECs* endothelial cells; *HIF1A* hypoxia-inducible factor 1-α, *VEGFR2* vascular endothelial growth factor receptor 2, *HUVECs* human umbilical vein endothelial cells

abnormal hypermethylation will lead to gene silencing by recruiting chromatin-modifying enzymes or preventing transcription factors from binding to promoters and leading to disease [105]. Study shows that DNA hypermethylation of CSE promoters CpG rich regions may contribute to the development of atherosclerosis or inflammation by reducing the CSE transcription and H_2S production in macrophages [106, 107]. H_2S keeps mitochondrial DNA replication by demethylation of mitochondrial transcription factor A (TFAM), which maintains the normal replication of mitochondrial DNA (mtDNA), it is critical for cellular energy metabolism and mitochondrial biogenesis [108].

5.2 Histone Modification

Histone is the protein that assists DNA packaging to form nucleosomes, including the C-terminal domain and an unfolded N-terminal tail. Histone modification (showed in Fig. 4) changes the structure of chromatin. There are several histone modifications on N-terminal tails: methylation, acetylation, phosphorylation, ubiquitination, ubiquitin-like, deamidation, ADP ribosylation,

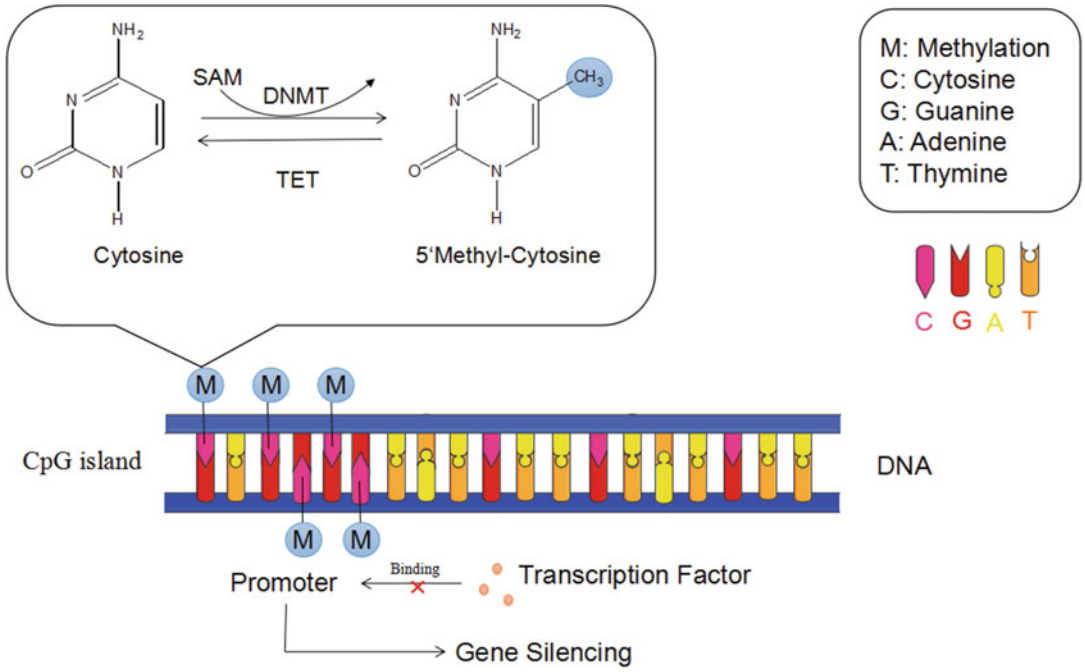

Fig. 3 *DNA methylation*. DNA methylation is the methyl group of *SAM* S-adenosylmethionine transferred to the DNA sequence under the catalysis of *DNMT* DNA methyltransferase and such methylation can be erased by *TET* ten-eleven translocation enzymes. DNA methylation mainly on the cytosine of CpG (Cytosine-phosphoric acid-guanine) island, produce 5mc; most of them are promoter regions. The CpG island's abnormal hypermethylation will lead to gene silencing by preventing transcription factors from binding to promoters

and proline isomerization, catalyzed by different enzymes, methylation and acetylation are the most widely studied modification methods [109]. Different histone modifications can activate or inhibit transcription, regulate gene expression, and guide cell differentiation.

SIRT1 is a histone deacetylase, which acetylates functional proteins, and has anti-aging and anti-atherosclerotic effects. Previous research found that H_2S can up-regulate SIRT1 activity of vascular endothelial cells and inhibit H_2O_2-induced endothelial cell senescence and improve endothelial cells function, and SIRT1 inhibitors impair H_2S inhibitory effect on aging which shows that H_2S prevents vascular endothelial aging by up-regulating SIRT1 activity [37, 38]. ZYZ-803, the slow-releasing H_2S-NO hybrid molecule of our group cooperatively regulated angiogenesis through a SIRT1/VEGF/cGMP pathway [50]. We demonstrate that H_2S also protects H9c2 cardiomyocytes against apoptosis under oxidative stress via SIRT1 Pathway [110]. Exogenous H_2S protects isolated rat hearts from ischemia/reperfusion (IR) injury via activating Sirt1/PGC-1α signaling pathway [111]. H_2S donor (NaHS or GY 4137) can reduce atherosclerotic plaque area in $ApoE^{-/-}$mice, reduce macrophage infiltration and aortic inflammation, and increase aorta and liver expression of SIRT1 mRNA; Endogenous H_2S can directly modify SIRT1 by S-sulfhydrylation, enhance its binding to zinc ions, and increase the deacetylation activity and stability of SIRT1, thereby reducing atherosclerotic plaque formation [112]. Furthermore, endothelium-specific SIRT1 gene knockdown reversed NaHS promotion of angiogenesis and improved tolerance to exercise. In 6-month-old endothelial-specific SIRT1 knockout mice, the density and number of lower limb muscle capillaries were lower than age and

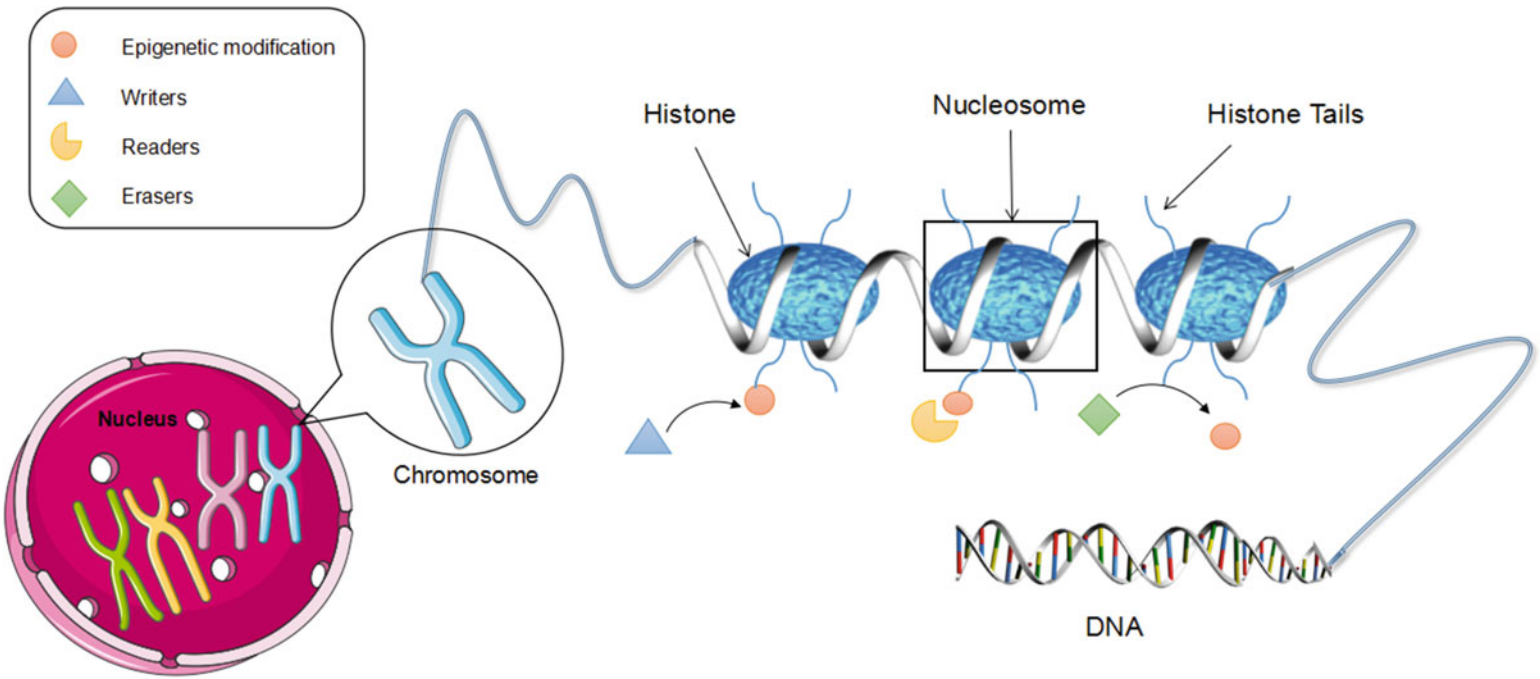

Fig. 4 *Histone modification*. Histone is the protein that assists DNA packaging to form nucleosomes and further assembled into chromosomes. Histone modification on N-terminal tails including methylation, acetylation, phosphorylation, ubiquitination, ubiquitin-like, deamidation, ADP ribosylation, and proline isomerization, catalyzed by different enzymes called "writers readers and erasers." Writers refer to the enzymes that add epigenetic modifications, readers refer to enzymes that bind to epigenetic modifications and alter gene activity and protein production, erasers refer to enzymes that remove epigenetic modifications

sex-matched wild-type mice. This further suggests that the H_2S reversal of vascular aging depends on SIRT1 [39].

One study suggests that the up-regulation of cystathionine-γ-lyase (CSE) and producing H_2S through histone deacetylases6 (HDAC6) inhibition attenuate hypertension, also improve endothelial function, and prevent the development of atherosclerosis [113, 114]. Studies suggest that H_2S could suppress histone acetylation leading to a decrease in the various pro-inflammatory cytokines gene transcription. Therefore, this mechanism may contribute to prove the previously anti-inflammatory effects of endogenous and exogenous H_2S [114].

Our group's previous study shows that inflammation can enhance the biosynthesis of the CSE/H_2S system, and then reduce the inflammatory response triggered by lipopolysaccharide (LPS) via regulating the expression of JMJD3. Therefore, the CSE/H_2S system represents a modification mechanism based on epigenetics to prevent uncontrolled inflammation [115], furthermore, inhibition of JMJD3 expression by the transcription factor Sp-1 may be the cause of negatively regulated inflammatory response by CSE [116].

5.3 Effects Mediated Via miRNAs

MicroRNA (miRNA) is an RNA small non-coding molecule that is involved in post-transcriptional regulation of RNA silencing and gene expression (showed in Fig. 5). Generally speaking, miRNA genes are transcribed in the nucleus to form the primary miRNA (pri-miRNA) and then cleaved by Drosha to form precursors-miRNAs (pre-miRNAs). The pre-miRNAs are exported to the cytoplasm by Exportin 5 and followed cleaved by Dicer to form mature miRNA duplex further unwind and one miRNA strand is added into the RNA-inducing silencing complex (RISC). A combination of a miRNA with its target mRNA leads to either transcription repression or mRNA decay [117]. miRNAs are involved in many physiological and pathophysiological effects and play an important role in the development of cardiovascular disease or related diseases such as high blood pressure, myocardial infarction, heart failure, and atherosclerosis. Recent studies show that H_2S can regulate miRNA expression during the above diseases.

Our group found that inhibiting miR-30 can up-regulate CSE expression and H_2S production in myocardial ischemia-reperfusion (I/R) rats and antagonize myocardial ischemic injury [118]. miR-133a is down-regulated in patients with diabetic heart failure. In neonatal rat cardiomyocytes, the administration of NaHS can up-regulate miR-133a and inhibit phenylephrine-induced cardiomyocyte hypertrophy [119]. Na_2S administration can increase miR-133a levels and inhibit hypertrophy of cardiac muscle cells induced by hyperhomocysteinemia [120]. miR-133a overexpression protects against I/R-induced endoplasmic reticulum stress and cardiomyocyte apoptosis and enhances cell movement [121]. Studies have shown that not only H_2S can regulate miRNA expression, but also regulate CSE expression under pathological conditions. In the human THP-1 macrophage model, miR-186 directly inhibits CSE protein and mRNA expression and increases macrophage lipid accumulation [122]; while miR-216a can down-regulate the CSE and ATP-binding cassette transporter A1 (ABCA1) expression, reducing cholesterol efflux from THP-1 macrophage-derived foam cells [123]. Overexpression of miR-21 in aortic smooth muscle cells inhibits protein expression of CSE and SP-1, inhibits H_2S production, stimulates smooth muscle cell proliferation, down-regulates smooth muscle cell differentiation-related genes, and regulates CSE/H_2S of smooth muscle cell proliferation and differentiation by targeting SP-1 [124]. In the myocardial ischemia and inflammation mouse model, Na_2S inhibits myocardial cell apoptosis and necrosis by inducing miR-21 expression, inhibits myocardial inflammation, and reduces infarct size after myocardial ischemia-reperfusion injury [125]. Down-regulation of miR-34a expression can increase the expression of Nrf-2 and its downstream targets, and enhance the effect of H_2S on liver I/R injury of aged rats

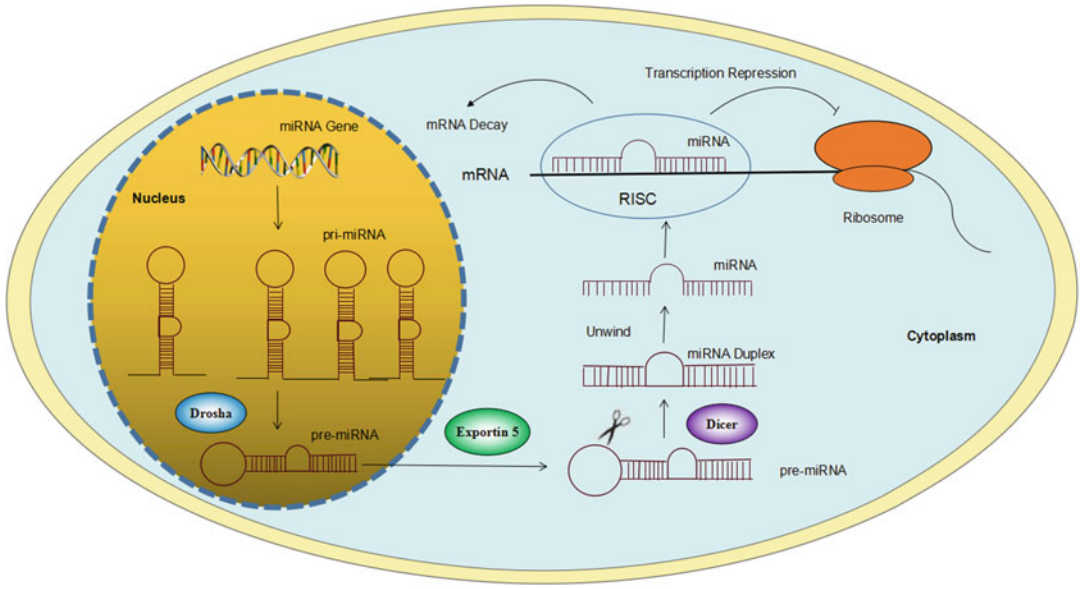

Fig. 5 *miRNA mechanism*. MicroRNA (miRNA) is an RNA small non-coding molecule, miRNA genes are first transcribed in the nucleus to form the *pri-miRNA* primary miRNA and then cleaved by Drosha to form *pre-miRNAs* precursors-miRNAs, which export to the cytoplasm by Exportin 5 and followed cleaved by Dicer to form mature miRNA duplex then unwind and one miRNA strand is added into the *RISC* RNA-inducing silencing complex. Combination of a miRNA with its target mRNA leads to either transcription repression or mRNA decay

[126]. Kang et al. [127] found that hypoxia and reoxygenation increased cardiac muscle cell death on neonatal rats, miR-1 expression was up-regulated, and Bcl-2 expression was decreased. H_2 S pretreatment down-regulates miR-1 expression and up-regulates Bcl-2 expression, reduces myocardial cell apoptosis and LDH release, and improves cardiac muscle cell viability. H_2S can also reduce myocardial cell apoptosis induced by ischemia-reperfusion injury in SD rats. miR-1 attenuates the protective effect of H_2S on cardiomyocytes by down-regulating Bcl-2 expression. These results indicate that inhibition of miR-1 expression may be an important mechanism for H_2S to exert myocardial protection effects. In rat ovariectomy models, myocardial miR-22 levels increased, and estrogen E2 treatment returned miR-22 expression to normal. At the same time, estrogens stimulate Sp-1 through the estrogen receptor-α mediated down-regulation of miR-22 that leading to the up-regulation of CSE, which in turn results in antioxidative defense. These studies show that miRNAs can regulate CSE expression and H_2S production [128, 129]. miR-640 plays a key role in mediating the angiogenic effect of H_2S; H_2S increases the level of hypoxia-inducible factor 1-α (HIF1A) through the VEGFR2-mTOR pathway by down-regulating the expression of miR-640 [130]. H_2S rescued high glucose-induced migration dysfunction of HUVECs by up-regulating miR-126-3p [131].

5.4 H_2S and the Transcription Factors

Nuclear factor erythroid 2-related factor- 2 (Nrf2) is an important antioxidant stress transcription factor that regulates the expression of many antioxidant and cytoprotective genes. ROS is an important risk factor for cardiovascular disease, which can cause endothelial cell apoptosis, activate NF-κB, increase the expression of adhesion molecules and cytokines, enhance monocyte

adhesion, make mitochondrial dysfunction [4, 132].

H_2S can activate Nrf2 signaling to inhibit oxidative stress, thereby inhibiting diabetes-induced atherosclerosis [85]. Exogenous H_2S inhibits oxidative stress induced endothelial cell autophagy through the Nrf2-ROS-AMPK signaling pathway [133]. H_2S pretreatment can activate myocardial ischemia mice Nrf2 signaling and up-regulate the antioxidant protein HO-1 and thioredoxin 1, and reduce myocardial ischemic injury [90]. NaHS can attenuate the combination of insulin-like growth factor 1 (IGF-1) and IGF-1R, and inhibit the proliferation of VSMCs induced by IGF-1 [134]. The transcription factor specificity protein-1 (SP-1) can bind to the promoter region of CSE and promote the phenotypic change of smooth muscle cells [135]. Its antioxidant function also depends on the Nrf2 signaling pathway [90]. Nrf2 is the nuclear basic leucine transcription factor NF-E2 (factor-erythroid2) family members, genes that regulate a variety of enzymes expressions, such as hemeoxygenase-1 (HO-1), thioredoxin (Trx), thioredoxin reductase (TrxR), glutathione reductase (GR), glutathione peroxidase (GPx), and catalase [136]. It is suggested that exogenous H_2S induced translocation of cardiomyocyte Nrf2 to the nucleus during myocardial infarction and up-regulated the expression of Trx 1 and HO-1 [90].

6 Conclusions

H_2S has a wide range of biological effects that arise in physiological processes, the gas signal molecule also has an important influence on the occurrence and development of cardiovascular diseases. H_2S and related enzymes are important and promising intervention targets for the prevention and treatment of these cardiovascular diseases. Epigenetics plays an important regulatory role in H_2S cardiovascular effects. Strengthening the basic and translational medicine research on H_2S is expected to provide a new understanding of the pathogenesis of cardiovascular disease and bring new strategies for its prevention and treatment, which has important theoretical and application value.

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Interaction among Hydrogen Sulfide and Other Gasotransmitters in Mammalian Physiology and Pathophysiology

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1 Introduction

Endogenous hydrogen sulfide (H_2S) plays various physiologically beneficial roles in different mammals. Together with nitric oxide (NO), carbon monoxide (CO), and sulfur dioxide (SO_2), it is a member of the "gasotransmitter" family. For centuries, they have been regarded as toxic and potentially lethal gases, and they are now considered to be important intracellular protective regulators with multiple physiological functions.

Although the gas NO was identified in the late eighteenth century, its biological effects were not discovered until 1980 [1]. NO was found to be produced endogenously and functions as a vasodilating molecule in 1987 [2]. NO is generated from guanidine nitrogen of L-arginine under the catalysis of NO synthase (NOS) which has three subtypes, namely, endothelial (eNOS), inducible (iNOS), and neuronal (nNOS) [3]. By the mid-1990s, CO was found to regulate vascular tone and hippocampal function of nervous system [4, 5]. Heme catabolism mediated by heme oxygenase (HO) is the main source of endogenous CO [6]. In the late 1990s, the third gas, H_2S, was discovered to be produced by the metabolism of sulfur-containing amino acids in the body [7]. The presence of H_2S can be detected in the brain, and it is involved in the regulation of learning and memory, playing a central regulatory role similar to neurotransmitters [8]. The gradual discovery of cystathionine γ-synthase (CBS) and cystathionine γ-lyase (CSE) as key enzymes for the production of H_2S further revealed their signal transduction pathways and extensive physiological functions [9–17]. Similar to H_2S, SO_2, which has long been considered a toxic gas and air pollutant, can also be endogenously generated by transamination of aspartate aminotransferase (AAT) through the metabolic pathway of sulfur-containing amino acids in mammals [18]. Since 2008, more studies suggest that SO_2 may act as a biologically active molecule to regulate the body's physiological activities [19–22].

As members of "gasotransmitter" family, NO, CO, H_2S, and SO_2 exhibit common characteristics as follows: (1) all of them are small gaseous molecules; (2) they can freely penetrate the cell membrane and play a biological effect through the independent way of membrane receptor; (3) they can be endogenously generated through a controllable enzymatic reaction; (4) they have specific regulatory roles in physiological state; (5) the biological effects of these molecules can be mediated by the intracellular second messenger or they can directly exert the

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biological effects without the mediation of intracellular second messengers, but through the clear cellular or molecular targets. Although the individual biological effects and signaling pathways of H_2S and other gasotransmitters have been extensively studied, the potential interactions among H_2S and other gasotransmitters have not been fully elucidated. In 2009, researchers found the possible interplay between H_2S and NO for the first time [23]. From that moment, more and more studies have suggested that H_2S and other gasotransmitters interact in their biosynthesis and various biological effects [24]. The great treatment potential of the gasotransmitters is further investigated through multiple preclinical and clinical researches. Here, we will review the biosynthesis and metabolism of gasotransmitters in mammals, as well as the known complicated interactions among H_2S and other gasotransmitters (NO, CO, and SO_2) and their effects on various aspects of cardiovascular physiology and pathophysiology.

2 Production and Metabolism of H_2S

H_2S is a colorless gas with the odor of rotten eggs. It is widely found in nature (volcanic eruption and hot spring), food (preserved eggs), industrial production (oil, rubber processing, etc.), and automobile exhaust. High concentration and rapid exposure can lead to lightning like death. H_2S can also be from the metabolism of bacteria in gastrointestinal tract and oral cavity. It is the main component of abnormal body odor such as halitosis. In the late 1990s, it was found that endogenous H_2S could be produced in the metabolism of sulfur-containing amino acids in mammals [7]. Later, it was found that H_2S was involved in the regulation of physiological functions of nervous system, circulatory system, digestive system, and other systems [9–17]. So far, the research on the biological effect of endogenous H_2S has become a hot issue in the field of life science and medicine.

Endogenous H_2S is produced in the metabolism of sulfur-containing amino acids in mammals. The key enzymes for its production include cystathionine-β-synthetase (CBS), cystathionine-γ-lyase (CSE), and 3-mercaptosulfurtransferase (MPST), in which CBS and CSE take L-cysteine as the substrate, pyridoxal phosphate as the coenzyme to generate H_2S, and MPST takes β-mercaptopyruvate as the substrate to generate H_2S. CBS mainly catalyzes the condensation of homocysteine and cysteine to form cystathionine, and releases H_2S at the same time; CSE catalyzes the decomposition of cystine to generate thiocysteine, which spontaneously degrades to generate cysteine and H_2S. The expression of three enzymes is also tissue-specific [25]. Previously, it was thought that CBS was mainly distributed in nervous system, MPST was mainly distributed in brain and red blood cells, and endogenous H_2S in cardiovascular tissue was mainly formed by CSE catalysis. However, recent studies have shown that the expression of CSE and MPST can also be detected in cardiovascular tissue, and the location of CSE in vascular tissue was also expanded from the former vascular smooth muscle cell to the vascular endothelial cell and vascular smooth muscle cell. Further intracellular localization studies showed that CSE was expressed in the endoplasmic reticulum and cytoplasm, and CBS was expressed in the endoplasmic reticulum. In addition to the above-mentioned enzymatic reaction to produce H_2S, it can also be produced through the reduction of sulfur elements mediated by non-enzymatic reaction, through the corresponding reduction products produced in the glucose oxidation process [26].

H_2S has good liposolubility and water solubility and its liposolubility is about 5 times of water solubility. Thus, it is easy to pass through cell membrane freely. H_2S dissolved in water can be partially hydrated into HS^- and S^{2-}, that is, $H_2S \rightarrow H^+ + HS^- \rightarrow 2H^+ + S^{2-}$ [27]. There are two forms of H_2S in mammals, i.e. physical dissolved H_2S gas (about 1/3) and chemical form HS^- (about 2/3). Sodium hydrosulfide is the most commonly used and widely recognized tool drug for H_2S research. It can be dissociated into Na^+ and HS^- in vivo. The latter combines with H^+ in vivo to generate H_2S, forming a dynamic

balance in vivo, which is beneficial to maintain the stability of H_2S content and pH value of internal environment in vivo. As for the metabolism of endogenous H_2S, most of them are oxidized in mitochondria to form thiosulfate and sulfate, and a few of them are converted into methanethiol and methanethiole by methylation metabolism in cytoplasmic solution [28]. H_2S in plasma can be removed by methemoglobin. The metabolites can be excreted through kidney, intestine, and lung within 24 h.

3 Production and Metabolism of NO

In 1998, Furchgott, Ignarro, and Murad won the Nobel Prize in medicine and physiology for their outstanding research contributions in the field of endogenous NO research in the cardiovascular field. NO was recognized as the first gasotransmitter in the body, creating a new field of gasotransmitter research. In the past 40 years, the experimental and clinical research on NO has been deepened gradually. NO is widely involved in the physiological and pathological regulation of various systems including cardiovascular, respiratory, neurological, and immune systems in the body [29–32].

NO is a kind of special small free radical molecule, which is soluble in water and fat and diffuses freely in and out of cells through membrane. Once it is produced, it disperses rapidly at the formation site and plays a role. NO has an unpaired free electron with extremely unstable chemical properties and high activity. Its half-life is only a few seconds. In the physiological solution rich in oxygen, NO can be rapidly oxidized to form nitrite and nitrate [33]. After entering the blood, it quickly combines with hemoglobin to form nitrosohemoglobin, which loses activity and prevents its function in the body. NO is stored and released in the form of S-nitrosothiol in vivo. The main metabolite in human body is nitrate, which is excreted by kidney.

Endogenous NO is produced from L-Arginine (L-Arg) by the action of nitric oxide synthase (NOS) and its cofactors [3]. Briefly, under the catalysis of NOS, L-Arg and oxygen molecules receive electron provided by the cofactor reduced nicotinamide adenine dinucleotide phosphate (NADPH) to produce L-guanidine and NO. In addition to L-Arg, small molecule peptides containing arginine are also substances for NO synthesis. At present, it is known that endogenous NO not only comes from vascular endothelial cells and inflammatory cells, but also has a complete L-Arg/NOS/NO pathway in vascular smooth muscle cells (VSMCs). Laser confocal results reveal colocation expression of three NOS, arginase, and soluble guanylyl cyclase (sGC) in VSMCs. Moreover, L-Arg uptake by VSMCs is with the help of the cationic amino acid transporter (CAT) in the membrane. The discovery of NO from VSMCs further promotes the vascular regulatory significance of endogenous NO.

In the process of NO synthesis, NOS is an important rate limiting enzyme. The changes of NOS gene expression and activity can affect NO production. At present, there are three types of NOS subtypes: (1) NOS I (~160 kD), which belongs to the constructive ROS (cNOS), mainly exists in the central and peripheral neurons. Therefore, it is also known as the neuronal NOS (nNOS). (2) NOS II (~130 kD) is induced in a variety of inflammatory cells stimulated by a variety of inflammatory factors, which promotes the rapid production of NO. Therefore, it is also called inducible NOS (iNOS). (3) NOS III (~133 kD), also a structural NOS, mainly exists in endothelial cells. Therefore, it is also known as endothelial NOS (eNOS).

The activities of eNOS and nNOS are regulated by Ca^{2+}/calmodulin, while the regulation of iNOS activity is not dependent on intracellular Ca^{2+} concentration. L-Arg isomorphism can compete with L-Arg to bind to NOS catalytic sites and inhibit endogenous NO production. However, recently, it has been found that long-term application of L-NAME at low dose can promote NO production through feedback. Another important regulatory mechanism of endogenous NO production in the physiological state is that NO inhibits NOS activity through a

feedback regulation. This inhibitory effect is very fast, which makes the reaction reach equilibrium before the enzyme catalyzes the third NO molecule synthesis. The inhibitory effect of NO on its synthetase may play an important role in maintaining the stability of NO physiological concentration. In addition, the intracellular transport of L-arginine is also one of the important links in the control of endogenous NO production.

4 Production and Metabolism of CO

CO is a colorless and tasteless gaseous molecule, which is produced in the incomplete combustion process of carbon containing compounds. It competently combines with oxygen to form carboxyhemoglobin, which reduces the oxygen carrying capacity of hemoglobin and causes hypoxia in the body tissues. CO is often called "silent killer." As early as 1952, Sjostrand found that CO could be produced during the degradation of hemoglobin in vivo [34]. However, it was not until the 1990s that people began to pay attention to the biological effects of endogenous CO. Many studies have shown that almost all organs, tissues, and cells in mammals can synthesize and release endogenous CO, which plays an important regulatory role in various systems in vivo, especially in cardiovascular system [35–40].

Heme oxygenase (HO)-mediated heme catabolism is the main source of endogenous CO [6]. HO uses NADPH as a cofactor to cut heme ring from α-methylene bridge to generate biliverdin, ferrous ion, and CO (heme + NADPH + H^+ + $2O_2$→biliverdin + Fe^{2+} + CO + $NADP^+$ + H_2O). The reaction takes place in cell microsomes. The rate and amount of CO produced by this pathway in human body are 0.4 ml/h and 16.5 μmol/L, respectively. Heme is the substrate of CO synthesis. 80–90% of heme comes from aging red blood cells and hemoglobin produced by ineffective hematopoiesis, while 10–20% comes from other heme proteins such as myoglobin, guanosine cyclase, cyclooxygenase, peroxidase, catalyst, and microsomal cytochrome. And other rare sources include membrane lipid peroxidation. CO is formed in the cell. After the biological effect is exerted, it diffuses into the blood, transports through hemoglobin, and is discharged from the lung. The other part combines with hemoglobin in the plasma to form carboxyhemoglobin. In the normal human body, the concentration of carboxyhemoglobin is 0.4–0.7%. Therefore, the endogenous CO production can be evaluated by the determination of CO removal rate and blood carboxyhemoglobin level.

The changes of endogenous CO synthesis and release are mainly regulated by HO. HO is an oxidase with multiple functions. So far, it has been proved that there are three isoenzymes in human and mammalian HO: (1) HO-1 is an inducible enzyme with a molecular weight of 32 kD, which can be induced by a variety of stimulating factors, such as hypoxia, hyperoxia, bacterial endotoxin, fever, shear stress, inflammatory factors, and some cytokines. (2) HO-2 is a structural type with a molecular weight of 36 kD. It is the main form of HO in physiological state. It is related to the function of CO as a neurotransmitter. HO-2 is usually not induced by various stimulants, but its activity is regulated by phosphorylation. (3) HO-3, with a molecular weight of 30 kD, has a weak catalytic effect on heme oxidation. It may be used as an oxygen sensor to regulate heme dependent gene expression. No HO-3 expression was found in vascular tissue. HO-1 and HO-2 mRNA can be detected in arteriovenous tissues, especially in vascular endothelial cells, smooth muscle cells, and adventitia. In basic state, HO-2 expresses more than HO-1. HO can be inhibited by synthetic and natural heme analogs, such as various metalloporphyrins. HO inducers include hemin, stannic chloride, arsenate, biological hormone, inflammatory cytokines, etc.

5 Production and Metabolism of SO_2

Similar to NO, CO, and H_2S, SO_2 is also one of the well-known air pollutants and industrial waste gas. In the past, its toxicology research has been

very thorough and extensive. However, biochemical research on amino acid metabolism has shown that the metabolic pathway of sulfur-containing amino acids starting with methionine in the body can generate SO_2 endogenously through enzymatic reaction. In recent years, our group has found that endogenous SO_2 production pathway can be detected in cardiovascular system, and endogenous SO_2 plays an important role in cardiovascular physiology and pathophysiology regulation, suggesting that endogenous SO_2 is expected to become a new cardiovascular gasotransmitter after NO, CO, and H_2S [41].

The formation of endogenous SO_2 in vivo is as follows: cysteine is oxidized by cysteine oxidase (CDO) to generate cysteinesulfinate, which is converted to β-sulfinylpyruvate by aspartate aminotransferase (AAT), and further spontaneously decomposes into SO_2 and pyruvate [18]. In addition, the oxidation of H_2S is also one of the ways to generate endogenous SO_2. The metabolic pathway of endogenous SO_2 in vivo is that it is metabolized to sulfite in the body and further oxidized to sulfate by sulfite oxidase, which is secreted into urine and excreted in vitro.

It was found that endogenous SO_2 production could be detected in the rat plasma, myocardium, and vascular tissues [19]. The serum SO_2 content is 15.54 ± 1.68 μmol/L. The content of SO_2 in each tissue is as follows (μmol/g protein): aorta (5.55 ± 0.35) > pulmonary artery (3.27 ± 0.21) > mesenteric artery (2.67 ± 0.17) and caudal artery (2.50 ± 0.20) > renal artery (2.23 ± 0.19) > myocardium (1.74 ± 0.16).

AAT that is the key enzyme of endogenous SO_2 production, also known as glutamic oxaloacetic transaminase (GOT), a pyridoxal phosphate-dependent transaminase, catalyzes the transfer of the amino group of aspartic acid to α-ketoglutarate to form oxaloacetic acid and glutamic acid and their reverse reactions. The structure of cysteinesulfinate is similar to that of aspartic acid. It can be used as a similar substance of aspartic acid to generate β-sulfinylpyruvate by AAT catalytic transamination, and then generate SO_2. AAT can be divided into two subtypes: AAT1 in cytoplasm, AAT2 in mitochondria. The activity, mRNA, and protein expression of AAT, the endogenous SO_2 producing enzyme, were also detected in the plasma, myocardium, and vascular tissues of rats. The activity of AAT in the plasma was 87 ± 18 U/L. AAT activity in each tissue was as follows (U/g protein): myocardium (4469 ± 278) > renal artery (188 ± 30) > tail artery (143 ± 36) > and mesenteric artery (112 ± 15) > pulmonary artery (96 ± 12) and aorta (88 ± 11). The sequence of AAT1 and AAT2 mRNA levels from high to low is myocardial tissue, renal artery, pulmonary artery, mesenteric artery, tail artery and aorta, which are consistent with AAT activity. AAT activity and AAT1 and AAT2 mRNA expression can be detected in myocardium and vascular tissues. AAT1 and AAT2 mRNA are mainly located in cardiomyocytes, vascular endothelial cells, and vascular smooth muscle cells near endothelium [42].

6 Interaction of H_2S with NO

6.1 Chemical Interaction between H_2S and NO to Form Hybrid Molecules

The addition of H_2S donor (NaHS) to different NO donors not only suppresses NO release, but also changes the effect of NO in the cell or tissue [43], indicating that crosstalk between H_2S and NO exists. There are two main forms of physically dissolved H_2S, namely, H_2S/HS^-, which have strong reducibility and can reduce NO, its oxidation products (such as nitrate and nitrite) or S-nitrosothiols (RSNOs, refers to thiols modified by NO, NO^+, or NO^-) to form different intermediates [27, 44, 45]. The action mode and biological effects of these intermediates may be the same as or different from their parental molecules through triggering identical or different signal transduction [46]. The mixture of NaHS and SNP could release nitrite in a time-dependent manner, suggesting a new substance named nitrosothiol generation (Fig. 1), which was further confirmed in liver tissues of rats administrated with lipopolysaccharide (LPS) and exogenous or endogenous H_2S [43]. They also

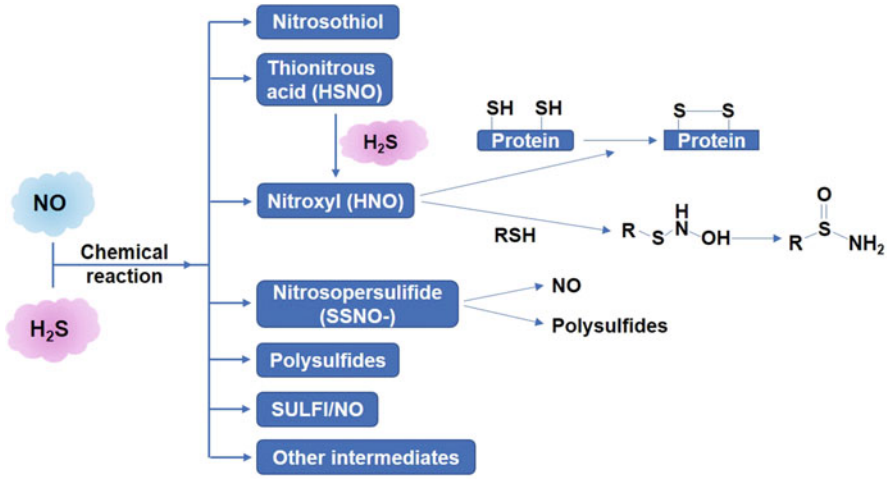

Fig. 1 Chemical reaction of H_2S with NO could generate several intermediates. And that HSNO reacts with H_2S could generate HNO, which acts on sulfhydryl group in target protein to produce RSNHOH or $RS(O)NH_2$ or induce disulfide bond formation between two free sulfhydryl groups nearby, thus changing conformations and functions of target protein. Besides, $SSNO^-$ can be decomposed into NO and polysulfides at pH 7.4

found that the nitrosothiol could not elevate cGMP level in a macrophage cell line RAW264.7 unless treatment with Cu^{2+} to release NO. But at that time, the characteristics of this nitrosothiol was not elucidated. Later, Filipovic et al. found that both the reaction of Na_2S with S-nitrosoglutathione (GSNO, a NO donor, belonging to RSNOs) or acidified nitrite and the reaction of NO with HS· produced thionitrous acid (HSNO) and suspected polysulfides (Fig. 1). NO^+, NO, and NO^- are generated by HSNO metabolism in the cell, and each product plays different physiological roles [45].

This group also reported that HSNO is short-lived, because it is easily reduced by H_2S to form other products including H_2S_2 and nitroxyl (HNO), the latter can also be produced from the reaction of NO donor SNP with H_2S [47]. Since HNO is weakly acidic ($pKa \approx 11.4$), it is the main existing form other than NO^- under physiological condition [48]. HNO is highly reactive to metalloproteins and reactive oxygen and nitrogen species, thus regulating the metabolism of metal ions (including Fe, Cu, and Mn), and the oxidation of many biomolecules [46]. In addition, HNO acts on sulfhydryl groups in protein to produce N-hydroxysulfenamide (RSNHOH) or sulfinamides [$RS(O)NH_2$] [49] or induce disulfide bond formation between two sulfhydryl groups nearby (Fig. 1), thus changing the conformations and functions of many important proteins containing redox-sensitive cysteines. It has been reported that HNO plays various physiological and pathophysiological effects, such as positive inotropy and cardiovascular protection in cell and animal models. The development of donors and detection methods for HNO has attracted the attention of more and more scientists. HNO donors include Angeli's salt ($Na_2N_2O_3$), Piloty's acid ($PhSO_2NHOH$), acyl nitroso and acyloxy nitroso compounds, metal nitrosyl complexes, and so on, with the first two being the most commonly used. HNO detection methods include traditional analytical methods with low sensitivity or selectivity and new methods with higher selectivity and specificity, such as various fluorescent probes based on copper, phosphine, or TEMPOL, membrane islet mass spectrometry, and electrochemical HNO detection [50, 51].

HSNO is unstable, because it is prone to isomerization through hydrogen transfer to form four different isomers. Cortese-Krott et al. reported that the chemical interplay of H_2S donor Na_2S with NO donor (DEA/NO or

SNAP) produces nitrosopersulfide ($SSNO^-$), polysulfides, and SULFI/NO at physiological pH [44] (Fig. 1). $SSNO^-$ is stable and will not be decomposed by thiols and cyanides. But it can be decomposed into NO and sulfane sulfur at pH 7.4, activating Keap1/Nrf2 signal and sCG/cGMP pathway, relaxing vascular tissue and VSMCs as well as downregulating blood pressure [44, 52]. Polysulfides form quickly when H_2S is exposed to NO. It is easily degraded by reducing agents including cysteine, GSH, and DTT. Polysulfides are found to cause vasodilation through activating PKG1α, downregulate blood pressure, promote arterial compliance, and regulate synaptic activity by activating TRPA1 channels [53]. SULFI/NO promotes sulfite generation to remove NO. It is decomposed to generate N_2O. SULFI/NO has a mild effect on blood pressure, but manifests significant positive inotropic action [44].

The chemical interaction between H_2S and NO and the subsequent generation of intermediates and products which might be new signal molecules are becoming a new research field. More and more studies are conducted to clarify the exact production mechanisms and biological importance of these hybrid molecules.

6.2 Regulation of NOS by H_2S

Besides direct chemical crosstalk, H_2S and NO influence each other's generation (Fig. 2). The first view is that H_2S enhances eNOS activity and NO generation. NaHS at concentration of 50–100 μM induced eNOS phosphorylation and thereby promoted NO production in HUVECs, but did not influence eNOS protein level. CSE insufficiency suppressed, but CSE overexpression upregulated NO production [54]. Another study conducted in bovine arterial endothelial cells showed that Na_2S at concentration of 150 μM induced NO generation [55]. H_2S upregulates eNOS activity to promote endogenous NO generation through many ways including phosphorylation of eNOS, sulfhydration to suppress its S-nitrosylated level, and upregulation of its dimeric active form. First, H_2S phosphorylates serine 1177 at eNOS via p38 MAPK-PI3K/Akt pathway activation [54]. Secondly, H_2S induces inositol triphosphate-mediated Ca^{2+} mobilization inside cells, upregulates the activity of K_{ATP} channels and revers mode of sodium-calcium exchanger, thereby elevating $[Ca^{2+}]_i$ level, leading to an increase in phosphorylated eNOS (serine 1177) and a decrease in S-nitrosylated eNOS level [56, 57]. Thirdly, H_2S sulfhydrates eNOS at cysteine-443 and reduces systemic oxidative stress to increase the stability of its dimeric active form [58] (Fig. 3). Furthermore, H_2S sulfhydrates proline-rich tyrosine kinase 2 to suppress its activity, resulting in a decline in phosphorylated eNOS at tyrosine 656 and an increase in eNOS activity [59] (Fig. 3).

While, the second view is that H_2S inhibits eNOS/NO pathway in rat aortas. Geng et al. found that H_2S treatment for 2–6 h suppressed NO production and eNOS activity in rat aortic tissues [60]. H_2S treatment for 2 h inhibited phosphorylated levels of Akt and eNOS (serine 1177), but did not affect eNOS protein expression. However, H_2S treatment for 4–6 h downregulated both the mRNA and protein expression of eNOS in HUVECs. Neither iNOS activity nor protein expression of iNOS and nNOS was affected by H_2S treatment for 2–6 h in aortic tissues and HUVECs. The inhibitory effect of H_2S on vascular eNOS/NO pathway was mediated at least partly by the opening of K_{ATP} channel [60]. In addition, Liu and Bian reported that pretreatment of NaHS for 10 min downregulated NO generation in rat aortic rings through activating HCO_3^- anion exchanger [61]. Kubo et al. also observed that H_2S incubation for 1 h directly inhibited the activity of purified bovine eNOS protein [62]. However, H_2S treatment for 10 min or 30 min did not influence eNOS activity in porcine aortic endothelial cells, cell lysates, or purified human eNOS protein [63]. These authors found that H_2S could decrease receptor agonist-stimulated eNOS activity and NO production through inhibiting Ca^{2+} mobilization and capacitative Ca^{2+} entry in porcine aortic endothelial cells, human

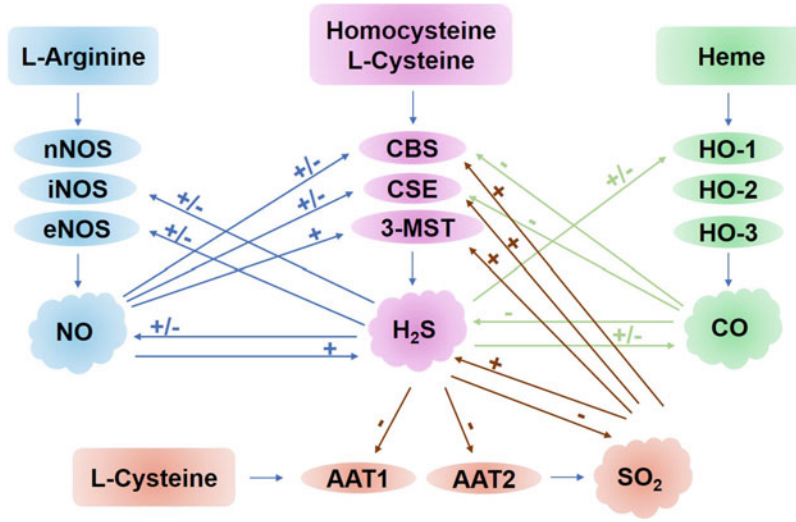

Fig. 2 Overview of endogenous NO, CO, H_2S, and SO_2 generation and the complex relationships between H_2S and the other three gasotransmitters. + represents activation; − represents inhibition

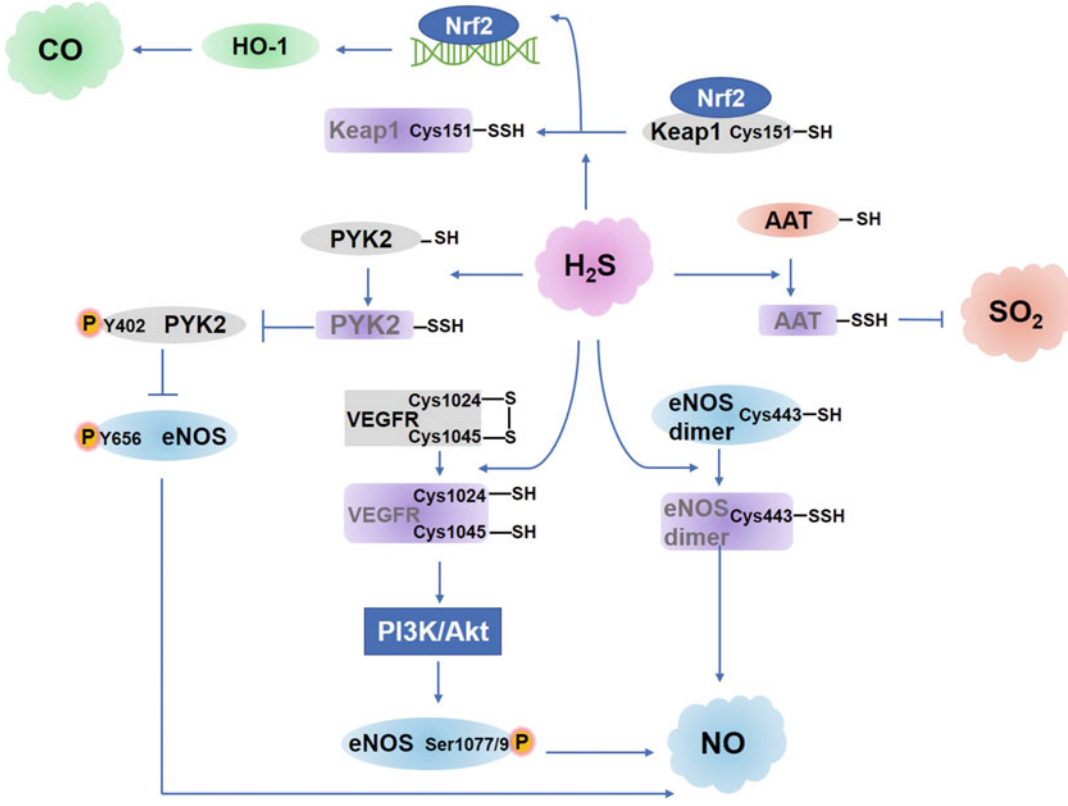

Fig. 3 H_2S regulates the generation of NO, CO, and SO_2 via sulfhydration. ↓ represents facilitate, ⊥ represents suppress

microvascular endothelial cells, and in smooth muscle cells from rat aorta and trachea.

Another finding showed that H_2S did not change NO generation in the basal state, but it promoted interleukin (IL)-1β-stimulated iNOS expression and NO generation in rat VSMCs via activating ERK1/2-mediated NF-κB pathway [64]. Na_2S facilitated NO production in ischemic tissues from the mice subjected with hind-limb ischemia both through increasing iNOS and nNOS expression and promoting nitrite reduction to NO in a xanthine oxidase (XO)-dependent fashion [65]. But in 25 mM of high glucose-stimulated rat VSMCs, the administration of H_2S donor NaHS or synthetic H_2S-releasing aspirin ACS14 for 24 h diminished the upregulated iNOS expression [66]. And in lipopolysaccharide-treated RAW264.7 macrophages, H_2S was also found to significantly downregulate iNOS expression and NO generation via promoting HO-1 expression to block NF-κB activation [67]. We found that H_2S inhibited NF-κB activation by sulfhydrating p65 protein at cysteine 38 in RAW265.7 macrophages [68]. Administration of NaHS for 8 weeks reduced iNOS activity and expression as well as NO concentration in the myocardial tissues of streptozotocin-induced diabetic rats [69]. H_2S also reduced NO generation in LPS-stimulated microglial cells through downregulating p38-MAPK signaling [70]. Therefore, the influence of H_2S on the activities and mRNA or protein expressions of these NOS isoforms is different. H_2S is reported to elevate or reduce the activities of eNOS or iNOS, or do not change iNOS and nNOS activities. The differences partly result from the different duration of H_2S treatment. For example, the stimulation of eNOS activity by H_2S is short-lived.

6.3 Regulation of H_2S Synthetases by NO

The regulation of H_2S-generating enzymes by NO is also complicated. The first view is that NO upregulates the activity and/or expression of H_2S-generating enzymes. Treatment with NO donor promoted H_2S production in normal rat vascular tissues and upregulated CSE mRNA expression in rat VSMCs [71, 72]. Administration of NOS inhibitor L-NAME to rats significantly downregulated plasma H_2S concentration, H_2S production, and CSE activity and mRNA expression in rat thoracic aortic tissues and superior mesenteric artery tissues [73]. In a high pulmonary blood flow-induced pulmonary hypertensive rat model, L-arginine treatment elevated plasma H_2S concentration, H_2S production rate, and CSE mRNA expression in lung tissues [74]. The increased CSE mRNA was mainly located in pulmonary artery SMCs. Administration of diabetic rats with nitrite could promote serum total sulfide concentration and the mRNA expressions of CSE, CBS, and MPST in soleus muscle as well as the CBS mRNA expression in adipose tissue and liver [75]. Further study showed that NO promoted H_2S generation through upregulating cGMP pathway. There is also a hypothesis that the active cysteine of CSE is likely to be modified by S-nitrosylation to elevate its activity [76]. The second view is that NO does not affect H_2S synthases. Chen et al. found that NO had no influence on the expression of H_2S synthases and H_2S content in endothelial cells [77]. The third view is that NO suppresses the activity of purified CSE protein but has no influence on the activity of CBS protein [78]. The fourth view is that NO inhibits CBS activity through binding to ferrous heme in CBS with high affinity ($K_d \leq 0.23$ μM) to form a penta-coordinate Fe (II)-NO complex [79]. Although CBS activity is inhibited, NO actually increases the generation of H_2S in this experimental environment. The reason may be related to tissue-specific modulation of H_2S generation or NO-induced non-enzymatic release of H_2S moieties from cellular macromolecules.

6.4 Competition of H_2S and NO in Protein Post-Translational Modification

H_2S-mediated sulfhydration and NO-mediated S-nitrosylation are two types of protein

post-translational modification, both of which can act on cysteine residues to regulate the conformation and function of their target proteins. The effects of these two modifications may be different or the same. For instance, H_2S-induced sulfhydration of cysteine-150 of GAPDH promotes its activity and facilitates it to combine with Siah, an E3 ligase, and then ubiquitinates PSD95 to cause its degradation in dendrites, eventually resulting in synapse loss and memory impairment [80]. While NO-induced GAPDH S-nitrosylation at cysteine-150 inhibits its activity and promotes its translocation into the nucleus, subsequently inducing the activation of p300/CBP and downstream p53 signal axis, which eventually leads to cell apoptosis [81]. In addition to GAPDH, NF-κB p65 can also undergo sulfhydration and S-nitrosylation. Either sulfhydration of p65 at cysteine-38 induced by H_2S or S-nitrosylation at this site induced by NO inhibits its DNA binding activity [68, 82]. Protein tyrosine phosphatases (PTPs) participate in many signaling pathways. Cysteine-215 of PTP1B could be S-nitrosylated by NO or sulfhydrated by H_2S to inhibit its catalytic activity, both of which are reversible [83]. S-nitrosylation of PTP1B blocks its irreversible inactivation caused by ROS and promotes endothelial insulin response [84, 85]. Phosphatase PTEN downregulates the content of phosphatidylinositol 3,4,5-triphosphate and the activity of PI3K/Akt pathway in cells. Low concentration of NO S-nitrosylates PTEN at cysteine-83 to inhibit its activity, thereby activating the downstream of PI3K/Akt signaling [83]. Endogenous H_2S sulfhydrates PTEN at cysteine-71 and cysteine-124 to prevent the S-nitrosylation and inactivation of PTEN caused by NO [86]. Future studies on the conformational changes of PTEN may explain why the two modifications at different cysteine residues inhibit each other. H_2S sulfhydrates eNOS at cysteine-443 to increase the stability of its dimeric form, which is the active form of eNOS catalyzing the production of NO [58] (Fig. 3). NO also S-nitrosylates eNOS at cysteine-443. NO has no effect on eNOS sulfhydration, while H_2S suppresses its S-nitrosylated level [56]. There are differences in the local concentration of H_2S and NO, and also, there are differences in the sensitivity of certain cysteinyl residues to the two gasotransmitters, which leads to a balanced and competitive relationship between sulfhydration or S-nitrosylation of the same cysteine sulfhydryl group to make the protein function normally.

6.5 Effect of H_2S–NO Interaction on Angiogenesis

Angiogenesis, as the name suggests, refers to new vessel growth from existing vasculature, which involves endothelial cell (EC) migration and proliferation and provides oxygen and nutrients for ischemic tissue. Increasing evidence show the crucial regulatory roles of NO and H_2S in angiogenesis [87, 88]. It is reported that both H_2S and NO stimulate angiogenesis. This effect of NO is mediated by the increased expression of VEGF, FGF, and MMP [89]. The activation of Akt signaling, K_{ATP} channels, and MAPK pathway participate in the facilitation of angiogenesis by H_2S [90]. In addition, VEGFR2 is a direct target that mediates the pro-angiogenesis of H_2S. H_2S specifically breaks the cysteine-1024-S-S-cysteine1045 disulfide bond in the intracellular kinase core of VEGFR2, which transforms this kinase core into active conformation, and then directly activates VEGFR2, leading to Akt phosphorylation and promoting angiogenesis [91].

There is an interaction between H_2S and NO in the mechanism for promoting angiogenesis (Fig. 4). H_2S inhibits PDE5A to reduce cGMP degradation, whereas NO induces sGC activation to promote the generation of cGMP in cells [92]. H_2S and NO eventually elevate cGMP levels and activate PKG/VASP, subsequently activating p38 and ERK signaling and promoting angiogenesis. Sirtuin-1 (SIRT1) is a crucial regulator of endothelial cell angiogenesis. The donor ZYZ-803, which releases H_2S and NO at the same time, promotes the expression of SIRT1, thereby increasing downstream VEGF and cGMP levels, and promoting angiogenesis [93]. H_2S activates Akt to promote angiogenesis, and Akt activation promotes eNOS

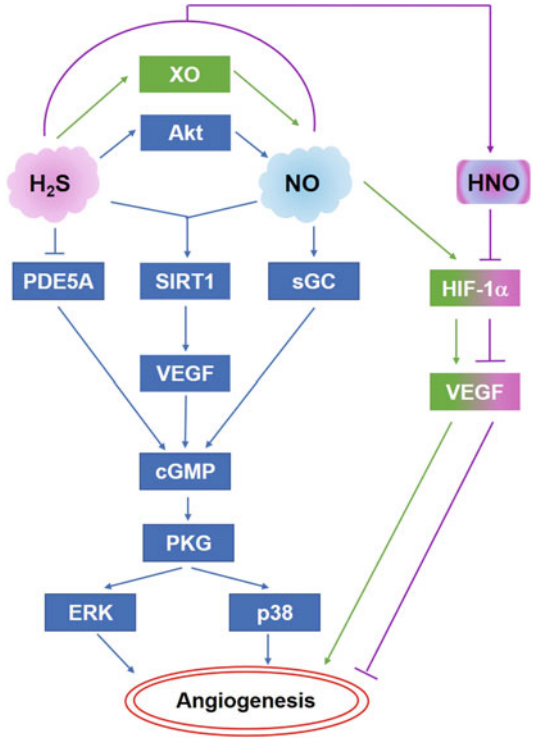

Fig. 4 Effect of H_2S–NO crosstalk on angiogenesis. ↓ represents facilitate, ⊥ represents suppress

phosphorylation at serine-1177 and increases the production of NO [94]. Altaany et al. found that H_2S activated p38-MAPK/Akt signaling to upregulate phosphorylated eNOS level, thereby promoting endothelial NO generation, which contributes to H_2S-stimulated endothelial cell proliferation and angiogenesis [54]. Na_2S facilitates NO production in ischemic muscle tissues from diabetic mice subjected with hind-limb ischemia both through increasing NOS expression and promoting nitrite reduction to NO in a XO-dependent fashion, thereby upregulating HIF-1α activity and expression as well as VEGF expression, which are helpful for H_2S to promote angiogenesis, increase hind-limb blood flow, and induce vascular remodeling in chronic ischemic tissues [65]. Similarly, elevated endogenous H_2S/CSE pathway was observed in gastrocnemius muscle tissues and plasma from permanent hind-limb ischemic mice subjected with ligation of femoral artery. Endogenous H_2S/CSE stimulates arteriogenesis and angiogenesis through increasing NO bioavailability to upregulate the concentration of downstream molecules including IL-6, VEGF, and bFGF and promote mononuclear cell recruitment in ischemic tissues [95]. H_2S has no effect on angiogenesis and wound healing in eNOS knockout mice. On the contrary, eliminating H_2S production by CSE gene deficiency abolishes NO-induced angiogenesis [96]. It suggests that the angiogenic effects of H_2S and NO need each other. H_2S reacts with NO to form HNO. IPA/NO, the donor of HNO, inhibits EC proliferation and re-endothelialization to suppress neointimal hyperplasia induced by carotid artery balloon injury [97]. In addition, HNO downregulates circulating VEGF level and HIF-1α protein content in tumor cells, reduces blood vessel density in mouse tumors, and inhibits angiogenesis [98].

6.6 Effect of H_2S–NO Interaction on Vascular Tension

NO is an important member of endothelial-derived relaxing factors [2]. It has a strong vasodilatory effect. The mechanisms include cGMP pathway activation, calcium-dependent potassium channel opening, protein S-nitrosylation, etc. [99]. H_2S can both relax and contract blood vessels, depending on its concentration. The concentration of NaHS greater than 100 μM (such as 200–1600 μM) causes VSMC relaxation [100]. Endogenous H_2S-elicited vasodilation contributes to maintain basal vascular tension and modulate physiological blood pressure [101]. And H_2S has a stronger relaxing effect on the aorta than on the pulmonary artery [102]. The mechanisms for mediating H_2S vasodilation include elevated K_{ATP} channel subunit expression [102, 103], K_{ATP} channels opening [71], extracellular calcium entry [104], increased calcium spark activity [105], activation of Cl^-/HCO_3^- exchanger [106], inhibition of mitochondria [107], reduction of cellular ATP levels [108], and the function of H_2S as a crucial adipocyte-derived relaxation factor [109]. The NaHS concentration below 100 μM reverses the

endothelium/NO-mediated relaxation [100]. The literature reported that 10 ~ 100 μM NaHS elicited vasoconstriction [100, 110]. It attributes to the reduced endothelial NOS and VSMC cAMP content, enhanced Na^+, K^+, $2Cl^-$ cotransport activity, and elevated calcium influx and ROS generation [88, 110].

The crosstalk between H_2S and NO in vascular tension modulation is complicated (Fig. 5). One view is that H_2S and NO cooperate to dilate blood vessels. H_2S boosted NO-caused aortic smooth muscle dilatory effect, and NO enhanced H_2S-induced thoracic aortic ring and portal vein ring relaxation [7]. In addition, H_2S and NO also have synergistic effect on the pulmonary artery relaxation [111]. The first mechanism is that H_2S and NO increase each other's production. H_2S augments eNOS activity to facilitate endogenous NO production. Using NOS inhibitor L-NAME or removing endothelium weakens H_2S-caused vasodilation [71], indicating that NO mediates the vasodilation effect of H_2S. NO upregulates H_2S production via inducing CSE/CBS expression or activation, which also contributes to the synergistic effect of NO and H_2S in relaxing blood vessels. The second mechanism is that both H_2S and NO increase cGMP content and then activate PKG/VASP. CSE deficiency reduced NO-stimulated increase in cGMP level, VASP activity, and vasodilation [96], while endogenous H_2S enhanced NO action through suppressing PDE activity to promote vasodilation [92], indicating that both H_2S and NO might target cGMP to relax blood vessels cooperatively. ZYZ-803 which simultaneously releases H_2S and NO exerts vasodilatory effect via cGMP-PKG signaling [93]. The third mechanism is that the reaction products of the interaction between H_2S and NO exert a stronger vasodilator effect. Simultaneous treatment of the pre-contracted isolated rat thoracic aortic rings or mesenteric arterial rings with GSNO and Na_2S has a more rapid and stronger vasodilation than using one of them alone. The synergistic response is attributed to the generated intermediates from H_2S/NO interplay, like polysulfides, $SSNO^-$, and HNO [112]. NO and polysulfides can be produced again from the degraded $SSNO^-$, which is the reason that the activation of sGC signaling caused by $SSNO^-$ may still exist. HSSNO which is generated by H_2S/NO reaction is speculated to exert powerful vasodilatory effect [113]. Additionally, H_2S reacts with NO to produce HNO, which is a novel endothelial-derived relaxation and hyperpolarization factor and can be generated endogenously in blood vessels [114]. The vasodilation of HNO is mediated by various mechanisms, including the activation of sGC and neuroendocrine TRPA1-CGRP pathway [115, 116]. HNO causes disulfide bond formation between cysteine-621 and cysteine-633 and between cysteine-651 and cysteine-665 of TRPA1, thereby activating TRPA1 channel, increasing intracellular calcium, releasing CGRP and eventually eliciting local and systemic vasorelaxation [116]. Unlike NO, vasodilation induced by HNO is resistant to tolerance in human arteries and veins [115]. Therefore, these studies indicate that H_2S and NO cooperatively exert vasodilation effect. The dynamic balance of H_2S and NO is essential for maintenance of vascular tension.

There is also a view that low concentration of H_2S inactivates NO to contract blood vessels (Fig. 5). It is found that SNP had no effect on vasodilation caused by H_2S in rat aortas, whereas 60 μM H_2S suppressed vasodilation of SNP [104]. Another study also confirmed that mixing H_2S with NO produced weaker vasodilation effect than NO alone [100], suggesting that H_2S might quench NO. Of note, NaHS contracts aortic rings with intact endodermis, but has no contractile function for those without endodermis, indicating that endothelial cells are indirectly involved in the vasoconstriction of H_2S. Moreover, H_2S (10 ~ 100 μM) concentration-dependently attenuated vasodilation caused by SNP, SNAP, or Ach, which exert vasodilatory effect via NO. And H_2S-induced vasocontraction was blocked by inhibition of endogenous NO generation in endothelial cells. Kuo et al. reported that H_2S contracted coronary artery when NO is present, whereas H_2S relaxed it when NO is absent [117]. H_2S is found to inhibit eNOS activity and NO production in rat aortic tissues [60]. Na_2S attenuates vasorelaxation caused by shear stress and facilitates

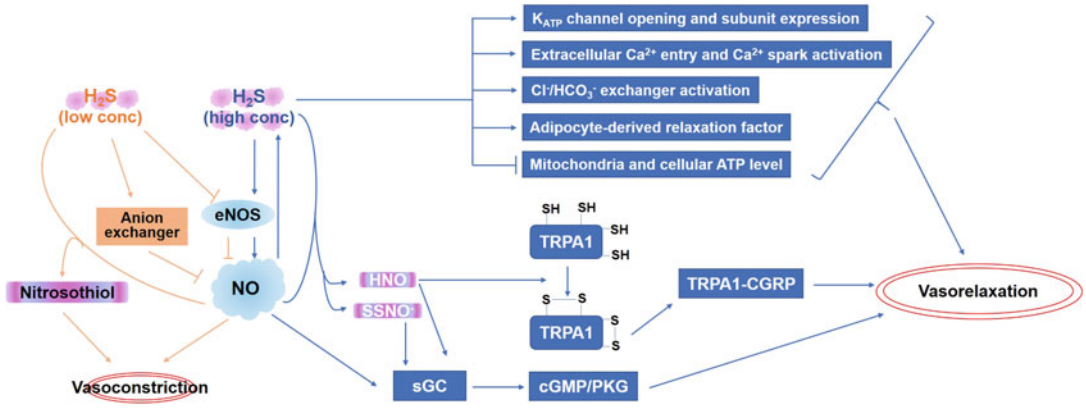

Fig. 5 Effect of H_2S–NO crosstalk on vascular tone regulation. ↓ represents facilitate, ⊥ represents suppress. Conc, concentration

vasoconstriction through inhibiting eNOS activity and NO production in mouse coronary arteries [118]. These results suggest that H_2S exerts vasoconstriction effect through suppressing endothelial NO bioavailability directly. In addition, researchers proposed that H_2S interacted with NO to produce a new molecule, namely, nitrosothiol, which will not stimulate cGMP production unless $CuCl_2$ is used to release NO [43]. Treating rat aortas with copper ions decomposes nitrosothiol into nitrite and nitrate, which can cancel vasoconstriction of H_2S, but does not affect vasodilation of H_2S, thus further confirming the existence of nitrosothiol in this organ incubation system [100]. H_2S inactivates or sequesters NO in this new molecular form, which contributes to its vasoconstriction. Bian's group revealed that H_2S activated anion exchanger, which made the bicarbonate ions enter the cells and made superoxide anions excrete from the cells, thereby inactivating NO and contracting blood vessels powerfully [61]. Then outside the cell, peroxynitrite ($ONOO^-$) is produced quickly from the reaction of superoxide anions and NO and is further eliminated by H_2S. And the decline of intracellular superoxide anion content could lead to the decrease of NO uptake by VSMCs [119]. Therefore, the results suggest that H_2S inactivates or sequesters NO to exert contraction effect on blood vessels.

The above conclusions suggest that in diseases related to reduced bioavailability of NO, such as ischemic heart disease, supplementation of exogenous H_2S can compensably relax the coronary arteries of patients, and benefit patients, but in individuals with normal NO bioavailability, H_2S may have the opposite effect through modulating NO [117]. Therefore, individualized use of H_2S may be needed in future clinical medication.

6.7 Effect of H_2S–NO Interaction on Heart Contractility

The regulation of NO on the basic contraction of cardiomyocytes is bidirectional. In the case of low levels (for example, 0.05 μM), it has positive inotropic effect through activating AC/cAMP/PKA signal and thereby augmenting $[Ca^{2+}]_i$ [120, 121]. In addition, NO produced by nNOS catalysis promotes cardiac contractility through S-nitrosylating sarcoplasmic ryanodine receptors [122]. High levels of NO ($\geq$ 10 μM) induces negative inotropic action [120]. The underlying mechanisms involve that the facilitation of cGMP signaling reduces the calcium sensitivity of myofilaments, and subsequently promotes myocardial relaxant effect [123]. And eNOS

participates in the suppressive effect of cGMP hydrolase (PDE5A) inhibitor on β-adrenergic induction of myocardial contraction [124]. And H_2S also attenuates myocardial contractility. The first mechanism is that H_2S decreases free sulfhydryl group of L-type Ca^{2+} channel to inactivate this channel and suppress its current [125, 126]. The second mechanism is that H_2S inactivates AC to suppress cAMP/PKA signaling [127]. The third is that H_2S induces the activation of K_{ATP} channel and mitochondrial membrane K_{ATP} channel [128, 129]. The fourth mechanism is that H_2S mitigates the anteroposterior load of heart through relaxing the arteries and veins and then reducing the venous reflux [128].

H_2S weakens the negative inotropic effect of NO, which may be due to the product HNO produced by the interaction of H_2S and NO can enhance myocardial contractility. NaHS at concentration of 50 μM did not markedly affect myocyte contraction, whereas mixing it with L-arginine, SNP, or DEA/NO could attenuate the negative inotropic action of these three NO-releasing agents [130]. HNO donor mimics but thiols abolish this positive inotropic action of a blend of NO and H_2S [130, 131]. And the production of HNO from the reaction of H_2S and NO was further confirmed [132]. These results indicate that HNO is responsible for the effect of H_2S–NO crosstalk on the heart contraction. Mechanistically, HNO-facilitated cardiac contractility is not related to cAMP/PKA and cGMP/PKG signaling [130], but is blocked by the treatment with NAC, indicating that a redox mechanism is involved [133]. HNO induces formation of heterodimers in the form of intermolecular disulfide bonds between cysteine-190 in tropomyosin and cysteine-257 in actin as well as between MLC1 and MHC, and then facilitates myofilament response to calcium ions, thereby enhancing myocardial contractility [134]. In addition, HNO promotes the transformation of phospholamban monomer into oligomer via forming disulfide bond to attenuate the suppressive effect of phospholamban on SERCA2a conformational flexibility and activity, thereby facilitating calcium ions uptake in sarcoplasmic reticulum, leading to cardiac inotropic and lusitropic action of HNO [135]. HNO could directly upregulate sarcoplasmic reticulum (SR) calcium pump activity and thiol-sensitive RyR2 function to promote calcium ions uptake and release from SR in myocytes [136]. These results suggest that myocardial contraction of HNO is closely related to redox. Moreover, a previous study showed that CGRP activation was responsible for the effect of HNO on enhancing myocardial contractility, because antagonizing CGRP receptor abolished the above-mentioned action of HNO [133]. The enhancement of cardiac contractility by CGRP had nothing to do with loading, but was only caused by the activation of cardiac sympathetic nerve, which was later found to negate the above-mentioned view [137]. Anyway, the positive inotropic effect of HNO is beneficial to the failing heart, making it a promising potential drug target for clinical treatment of congestive heart failure [138].

6.8 Effect of H_2S–NO Interaction on Oxidative Stress

Disturbance of the balance between generation and removal leads to excessive ROS which is the root cause of oxidative stress. Oxidative stress can cause inflammation, cell apoptosis, and endoplasmic reticulum stress, leading to cell damage, and participating in various diseases such as hypertension, heart disease, obesity, diabetes, senescence, and cancer.

H_2S resists oxidative stress and plays endothelial protective role through directly eliminating superoxide anions and decreasing the generation of superoxide anions originated from vascular NADPH oxidase [139]. And NO could S-nitrosylate p47phox to inhibit superoxide generation in microvascular ECs [140]. The high pKa of HNO and low dissociation energy of H–NO indicate that HNO easily provides hydrogen atoms, which may contribute to the extinction of active free radical intermediates [141]. HNO may prevent membrane from oxidative stress injury through its antioxidant effect, thereby maintaining the integrity of lipid membrane

[141]. HNO has a reducing property due to hydrogen atom supply. Its oxidation will result in NO release, and the latter has a strong antioxidant capacity [142]. The antagonistic effect of HNO on oxidative stress was observed in yeast, blood vessel, and hypertrophic myocardium [141, 143, 144]. Mechanistically, HNO was reported to activate sGC/cGMP pathway, subsequently downregulating Nox2 activity and expression as well as superoxide production in cardiomyocytes. This mechanism is responsible for antihypertrophic effect of HNO [144]. However, treating cerebral arteries with HNO antagonized angiotensin II-induced oxidative stress and vasoconstriction through rapidly and directly inactivating Nox2 unrelated to sGC/cGMP pathway. In view of previous reports that HNO acted on the cysteine of a variety of proteins to cause changes in protein conformation or activity, this group also speculated that HNO inactivated Nox2 via post-translational modifying its cysteine [143]. In addition, treating cardiac cells with HNO could also enhance HO-1 expression to increase nuclear Nrf2 level, both of which belong to antioxidant protein [145].

In an oxidative stress environment, NO reacts quickly with superoxide to form peroxynitrite, which aggravates oxidative stress and uncouples eNOS, thereby promoting superoxide production, decreasing NO release, and limiting bioavailability and actions of NO [146], while HNO is not sensitive to the reaction of superoxide, which makes it easier to retain its function under oxidative stress. Therefore, in the case of oxidative stress, damage to the NO system becomes an important pathogenesis of many diseases, and the retention of HNO function suggests that HNO has a good application prospect in diseases related to NO resistance.

6.9 Effect of H_2S–NO Interaction on Cardioprotection

NO is an important endogenous cardioprotective molecule. Either eNOS inhibition or nNOS deficiency aggravates cardiac injury caused by ischemia-reperfusion (I/R) or infarction, while NO donor supplementation attenuates this cardiac injury [147–149]. Mechanistically, NO activates sGC to upregulate cGMP generation and downstream PKG signal activity [150]. In addition, NO induces mitochondrial K_{ATP} channel opening but inhibits calcium overload [150–153]. H_2S is also an important endogenous cardioprotective molecule, which inhibits myocardial I/R injury, myocardial infarction, and prevents ventricular premature beats and fatal arrhythmias [154, 155]. The mechanisms include the opening of myocardial K_{ATP} channels [156], inhibition of L-type calcium channels, blockade of the disulfide bridge between cysteine-320/cysteine-529 residues of the Kv4.2 subunit and inhibition of I_{to} potassium channels in epicardial myocytes [155], activation of anti-apoptotic signals and PKC pathway [157, 158], and improvement of mitochondrial function [14, 159] (Fig. 6).

H_2S exerts a cardioprotective effect by upregulating the eNOS/NO pathway (Fig. 6). H_2S elevated serum and myocardial NO content. Both in the isoprenaline-induced myocardial injury model and in the rat ventricular myocyte injury model induced by severe metabolic inhibition, the application of L-NAME abolished the myocardial protection of H_2S, indicating the importance of NOS/NO pathway in the myocardial protective effect of H_2S [160, 161]. CSE-knockout mice showed elevated myocardial oxidative stress, decreased phosphorylation of eNOS at serine-1177, reduced eNOS cofactor BH4 level, declined NO bioavailability, and inhibited cGMP content, which further aggravated myocardial and liver I/R injury [162]. While exogenous H_2S supplementation restored eNOS/NO pathway activity and rescued myocardial and liver I/R injury aggravated by CSE deficiency. In eNOS gene knockout or phosphorylated site mutation mice, H_2S could not attenuate myocardial I/R injury [162], further suggesting that the myocardial protective effect of H_2S is mediated by eNOS/NO pathway activation. In addition, H_2S alleviated L-NAME-induced hypertensive heart damage by activating the K_{ATP}-mediated Akt/eNOS/NO pathway [163]. H_2S post-treatment activated Akt, PKC, and eNOS signals to prevent myocardial I/R

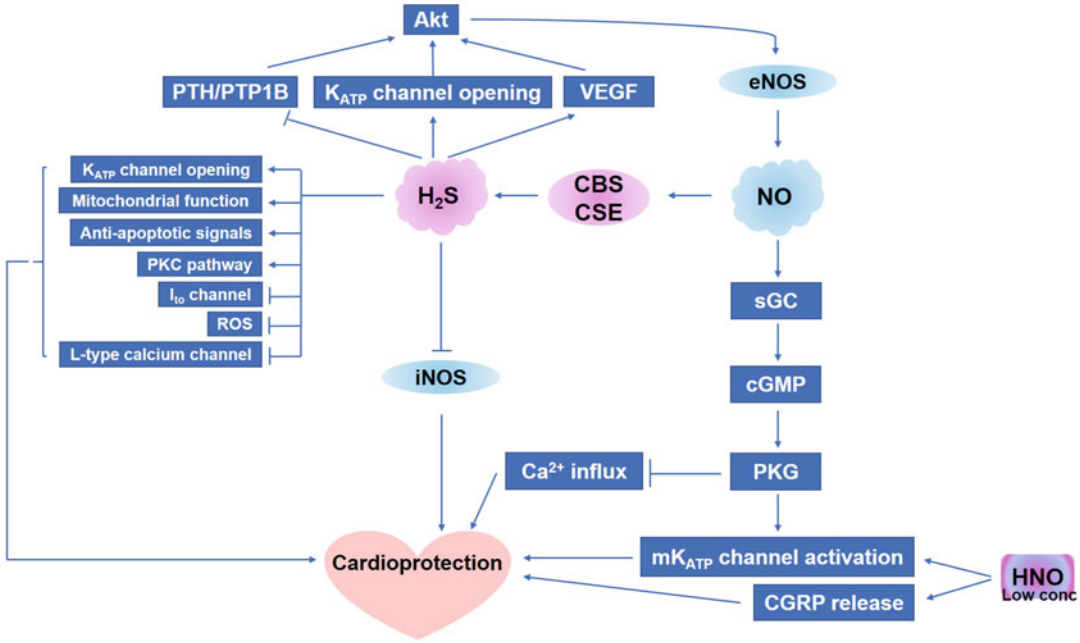

Fig. 6 Effect of H_2S–NO crosstalk on cardioprotection. ↓ represents facilitate, ⊥ represents suppress. Conc, concentration; mK_{ATP}, mitochondrial K_{ATP}; PTH, phosphatase and tensin homolog; PTP1B, protein tyrosine phosphatase 1B; I_{to}, transient outward potassium current

injury [164]. H_2S donor SG-1002 also exerted cardioprotection in pressure overload-stimulated heart failure through mitochondrial function preservation, ROS inhibition, and angiogenesis. The activation of VEGF/Akt/eNOS/NO/cGMP signaling mediated this protective effect of H_2S [165]. Na_2S increased the survival rate of mice subjected to sudden cardiac arrest due to an increase in phosphorylated eNOS level and NO content [166]. Thus, H_2S upregulates eNOS/NO pathway to exert cardioprotective effect. Conversely, NO also increases H_2S generation catalyzed by CBS and CSE [72].

Unlike eNOS, iNOS overexpression to catalyze production of large amounts of NO induces cytotoxicity and aggravates cardiac damage [167]. H_2S exerts a myocardial protective effect by inhibiting iNOS. In a mouse model of myocarditis caused by CVB3, the cardioprotection of H_2S was mediated by a decline in iNOS expression and downstream HO-1 signaling [168]. The expression of myocardial iNOS in STZ diabetic rats is positively correlated with the degree of myocardial damage [69]. H_2S prevents diabetic myocardial damage by reducing the activity and expression of iNOS [69].

In the myocardial protective effect, the interaction between H_2S and NO not only involves the regulation of each other's generating ability, but also involves the role of the newly produced molecule, HNO (Fig. 6). Pretreatment with HNO attenuated I/R-induced myocardial injury, as demonstrated by a decrease in infarct size, LDH level, and incidence of ventricular fibrillation but an increase in cardiac inotropy [169, 170]. This effect of HNO is similar to that of ischemic preconditioning, but it is more obvious than that of equimolar NO [169]. Mechanistically, HNO causes activation of mitochondrial K_{ATP} channel (mK_{ATP}), release of CGRP, and direct reaction with thiols [169, 171]. While, treatment with HNO at high concentration leads to postischemic myocardial damage, which is associated with the stimulation of neutrophil accumulation [172].

6.10 Effect of H_2S–NO Interaction on Hypertension

H_2S donor has biphasic response to the blood pressure of anesthetized rats. The pressor response was produced at a low dose of NaHS, and depressor response occurred at a high dose [100]. The pressor effect of H_2S is associated with the inhibition of eNOS activity [62] and/or extinguishment of NO [43]. Application of L-NAME could prevent the pressor response of H_2S, indicating that H_2S reacts with NO to generate a nitrosothiol-like compound and consumes NO, leading to the loss of NO-mediated vasodilation and an increase in blood pressure. H_2S prevents hypertension development and facilitates vasodilation in SHR model through increasing K_{ATP} subunits (SUR2B and Kir6.1) expression in VSMCs mediated by the activation of FXOX1 and FOXO3a [103, 173]. In this rat model, NaHS augments carotid sinus baroreceptor sensitivity through the upregulation of TRPV1 protein level and sulfhydration-mediated activation of this channel [174]. H_2S also inhibits vascular inflammation through downregulating NF-κB pathway in SHR rats [175]. The inhibitory effect of H_2S on VSMC proliferation via downregulation of MAPK pathway was also involved in the depressor effect of H_2S [176].

Some studies show that H_2S upregulates eNOS phosphorylation and NO bioavailability, thereby decreasing blood pressure [177]. Under physiological and pathophysiological conditions, H_2S coordinates the S-sulfhydration, S-nitrosylation, and phosphorylation of eNOS to fine-tune endothelial function. In endothelial cells, H_2S upregulates NO generation through calcium-mediated eNOS phosphorylation [178], Akt-dependent eNOS activation [96], or stabilizing eNOS activity [179]. Both L-NAME-induced hypertensive rats [72] and carotid arterial eNOS knockout mice [180] exhibited a low level of vascular CSE and H_2S. The supplementation of H_2S increased CSE or administration of its substrate L-cysteine suppressed hypertension formation [73, 180, 181], indicating that the decreased of H_2S/CSE pathway is involved in the pathogenesis of L-NAME-induced hypertension. However, intervention of CO/HO-1 did not improve the development of hypertension in the two models [182]. In angiotensin II-induced hypertensive mice, injection with NaHS elevated NO bioavailability, improved endothelial dysfunction, reduced oxidative stress and eventually decreased blood pressure [183]. Plasma H_2S content and aortic CSE activity and expression were decreased in the SHR, while the treatment of NaHS attenuated hypertension in the SHR through upregulating renal H_2S generation and NO bioavailability but suppressing renal RAS [101, 184, 185].

On the contrary, H_2S also affects NO generation by inhibiting nNOS and iNOS activities in a NO-dependent manner [186]. H_2S sustained-release donor, GYY4137, caused vasodilation in vitro and reduced blood pressure in vivo. It downregulated proinflammatory cytokines (TNF-α, IL-1β, IL-6) secretion and reduced COX-2 and iNOS expression in RAW264.7 macrophages treated with LPS [187].

HNO has been reported to reduce blood pressure in SHR model [188]. Since HNO exists in the body in a protonated form, it is not easily eliminated. Because of this, the aorta of angiotensin II-induced hypertensive mice still retains the diastolic response to HNO [189], suggesting that HNO may have a prospective effect in the treatment of hypertension.

6.11 Effect of H_2S–NO Interaction on Pulmonary Hypertension

H_2S alleviates pulmonary vascular remodeling and protects against pulmonary hypertension (PH) in the presence of hypoxia, monocrotaline (MCT), or high pulmonary blood flow [190–192]. Mechanistically, it relaxes pulmonary artery [111], inhibits pulmonary artery SMC proliferation [193] and promotes apoptosis [194], resists oxidative stress [195], suppresses pulmonary artery EC inflammation [196], and attenuates vascular collagen deposition [197].

Treatment with L-NAME to downregulate NO level could aggravate hypoxic PH and promote

plasma H_2S content and CSE activity in lung tissues of hypoxic rats. And the treatment with PPG to downregulate H_2S level also aggravated hypoxic PH and augmented plasma NO content and eNOS expression in lung tissues [198]. These results suggest that H_2S and NO inhibit each other in the development of hypoxic PH. In high pulmonary blood flow-caused PH model, treatment with L-arginine upregulated plasma and pulmonary H_2S content as well as CSE mRNA expression in pulmonary artery SMC to alleviate PH [74]. And H_2S downregulated but PPG upregulated pulmonary NO/NOS pathway [191, 199].

6.12 Effect of H_2S–NO Interaction on Diabetes

Impaired NO or H_2S pathway is involved in the onset of diabetes. Application of nitrite to increase NO level could alleviate carbohydrate metabolic abnormalities in high fat diet-fed STZ rats, which is an obese type 2 diabetic model. While application of H_2S donor at low dose (0.28 mg/kg) had no influence. Simultaneous treatment with H_2S donor and nitrite could enhance the effect of nitrite in improving metabolic abnormalities, as demonstrated by the improved carbohydrate metabolism, low serum glucose and insulin concentration, fine glucose tolerance and liver function, high GLUT4 level and strong antioxidant capacity in these obese diabetic rats [75]. The mechanisms responsible for this effect of H_2S are to enhance the eNOS activity [58] and biologically activate nitrite. The latter is supported by the fact that H_2S promotes NO release from nitrite via activation of xanthine oxidoreductase [65] and facilitates a new NO-releasing molecule, sulfinyl nitrite generation [200]. Moreover, H_2S makes sGC exist in the form of NO activation, and reduces cGMP degradation through suppressing PDE5 [201], so that cGMP is upregulated to promote insulin secretion.

In addition, H_2S protected against diabetic nephropathy by the inhibition of oxidative stress and inflammation in rat kidney tissues, while application of L-NAME attenuated this benefic role of H_2S [202], indicating that NO might be involved in H_2S action on diabetic nephropathy. Moreover, NOX4, which mainly produces ROS, was augmented in diabetic kidney tissue. NOX4 inhibition could attenuate diabetic kidney injury [203]. H_2S inhibited NOX4 expression in kidney proximal tubular epithelial cell stimulated with high glucose by activating AMPK signaling, which was reversed by L-NAME [204], indicating that NO might participate in H_2S action. H_2S upregulated the expression of iNOS but not eNOS. Inhibition of AMPK abolished the facilitation of H_2S on iNOS expression [204]. These findings suggest that H_2S induces iNOS expression via the activation of AMPK signaling, thereby inhibiting the increase of NOX4 induced by high glucose.

6.13 Effect of H_2S–NO Interaction on Gastrointestinal Tract, Immune System, and Nervous System

Both H_2S and NO could resist gastric mucosal injury and maintain mucosal barrier integrity. H_2S was reported to induce the generation of NO and PGE_2 via the activation of capsaicin-sensitive afferent neurons, thereby facilitating bicarbonate release and exerting a protective effect on the gastric mucosa [205, 206]. It indicates that H_2S has an important impact on the peripheral nervous system in the gastrointestinal tract. Considering that NSAIDs have serious gastrointestinal side effects, researchers have prepared NSAID releasing NO (NO-NSAID), NSAID releasing H_2S (HS-NSAID), and NSAID releasing both NO and H_2S in a dose-dependent manner in vitro and in vivo (NOSH-NSAID) [207, 208]. Subsequently, aspirin was used as a scaffold to develop NOSH-aspirin. In carrageenan-stimulated rat paw edema model, NOSH-aspirin had the same anti-inflammatory effects as aspirin through reducing paw volume and PEG_2 concentration in paw exudates. Treating rats with ASA upregulated plasma TNF-α, while NOSH-aspirin treatment reduced

it [208]. NOSH-aspirin had dose-dependent inhibitory effect on writhing response stimulated by acetic acid and inflammatory hyperalgesia stimulated by carrageenan, Freund's adjuvant or PGE_2, and the degree of inhibition was higher than that of aspirin did at the same dose [209]. Mechanistically, NOSH-aspirin downregulated IL-1β level and activated K_{ATP} channels to block the action of hyperalgesic mediator.

Moreover, H_2S suppressed LPS-induced NO production in microglial cells through downregulating p38-MAPK pathway [210]. It is assumed that H_2S may be a potential therapeutic target in the treatment of cerebral ischemia and neuroinflammatory diseases. Some studies showed that H_2S had a neuroprotective role in animal models of Parkinson's disease [211, 212].

7 Interaction of H_2S with CO

7.1 Regulation of H_2S Synthetases by CO

Due to the chemically inert state of CO, there are few reports on CO-mediated production of intermediates from H_2S and NO. CO inactivates CBS by combining with Fe^{2+}-CBS [213], leading to the decrease of H_2S generation. Due to the changes of methionine and S-adenosylmethionine levels, CO-mediated CBS inhibition may induce methylation or demethylation of different protein targets in different durations, which are related to re-methylation cycling [214]. The binding of CO to ferrous CBS was weaker than that of NO. HO-2/CO pathway inhibited CBS activity via the heme group of CO binding to histidine sites in CBS, thus regulating H_2S content, generating 6-coordinated CO-Fe(II)-histidine complex, turning CBS into CO sensing molecule [215].

O_2 tension is different between different tissues. CO, H_2S, and NO participate in complicated interactions with O_2 that modulates red blood cell levels and vascular tension, both of which play key roles in O_2 transport. The generation of CO and NO depends to some extent on the O_2 level in the cell [216], because molecular O_2 is necessary for the enzymatic activities of HO-2 and nNOS. Therefore, both CO produced by HO-2 and NO produced by nNOS are inhibited under hypoxic conditions that also modulate the steady-state expression of NOS at the mRNA and protein levels [217]. Under hypoxic conditions, the CO produced by HO-2 is downregulated, resulting in a decrease in the inhibitory effect of CO on CBS and an increase in H_2S contents, which subsequently promotes the carotid body sensory excitement [218]. However, under normoxic conditions, the O_2-dependent CO produced by HO-2 suppresses CSE activity, resulting in a decrease in H_2S contents and sensory activity in the carotid body [218]. Different from CBS, the CSE in the carotid body does not contain heme. Previous studies showed that inhibition of HO in VSMCs with ZnPP could upregulate CSE protein level and H_2S concentration [219], indicating that CO downregulates H_2S/CSE pathway in VSMC under physiological condition.

7.2 Regulation of HO by H_2S

H_2S also inhibits HO/CO pathway under physiological condition. Treatment of VSMC with PPG could enhance HbCO concentration and HO-1 protein level, but the treatment with NaHS inhibited them [219]. While H_2S augments the HO/CO pathway under pathophysiological condition (Fig. 3). NaHS augments both mRNA and protein expressions of HO-1 in hypoxic rat pulmonary arteries as well as plasma CO concentration, while CSE inhibitor PPG downregulates HO/CO pathway [220].

7.3 Interaction of H_2S with Transcription Factors Containing Heme

Studies have shown that gasotransmitters can regulate gene transcription through cross-talking with transcription factors containing heme as a prosthetic group. For instance, CO can activate neuronal PAS domain protein 2 (NPAS2), which

is an obligate dimer chaperone of BMAL1 and participates in modulating the circadian rhythm [221]. These data indicate that there is a correlation between heme biosynthesis and its degradation. H_2S can prevent the stimulation of Brg1 expression, which is the central catalytic subunit of the ATP-dependent chromatin remodeling complex SWI-SNF, although its related mechanism is still unclear [222]. The inhibitory effect of H_2S on the proliferation of VSMCs has been shown to be closely associated with the chromatin remodeling caused by Brg1 [223].

7.4 Effect of H_2S–CO Interaction on Pulmonary Hypertension

CO prevents hypoxic PH and vascular structural remodeling associated with increased Fas-mediated pulmonary VSMC apoptosis and reduced VSMC proliferation [224]. H_2S also protects against hypoxic PH and alleviates pulmonary vascular remodeling [190].

H_2S facilitated plasma CO concentration and HO-1 expression in hypoxic rat pulmonary artery, while the inhibition of H_2S production could downregulate HO/CO pathway [220]. Similarly, in high pulmonary flow-caused PH model, H_2S promoted but PPG reduced pulmonary CO generation and HO-1 expression [191, 199]. These results indicate that H_2S and CO play a synergistic effect in protecting against PH.

7.5 Effect of H_2S–CO Interaction on Nervous System

Liu et al. assumed that electrical acupuncture treatment prevents hypoxic injury via elevating CO level mediated by H_2S/CBS-CO/HO-1 and hypoxia inducible factor-1α (HIF-1α) system [225]. The results showed that electrical acupuncture treatment reduced CBS expression and upregulated the expression of HO-1 and HIF-1α in cortical cells of perinatal rats.

Moreover, in a rat model of recurrent febrile seizures, both H_2S and CO alone could reduce hippocampal damage. Administration of NaHS augmented plasma CO content as well as mRNA and protein expression of HO-1 in hippocampal neurons, while the inhibition of H_2S-generating enzyme CBS decreased them [226]. Administration of hemin to promote CO generation could facilitate plasma H_2S content as well as mRNA and protein expression of CBS in hippocampal neurons, while the inhibition of HO-1 inhibited them [226]. These results suggest that H_2S and CO play a synergistic role in recurrent febrile seizures development.

8 Interaction among H_2S, NO, and CO

H_2S suppresses NO production, iNOS gene expression, and NF-κB activation in LPS-induced macrophages through a mechanism involving the action of HO-1 and CO [67]. H_2S stimulated HO-1 expression and activation by activating ERK1/2 in RAW264.7 macrophages. H_2S suppressed iNOS protein expression and NO production in LPS-treated RAW264.7 macrophages, while application of CSE inhibitor, BCA, blocked the H_2S-inhibited NO production in LPS-treated macrophages. Inhibition of HO-1 by siRNA or inhibitor SnPP could block the H_2S-inhibited iNOS expression and NO production, while overexpression of HO-1 inhibited LPS-stimulated iNOS expression and NO generation. These findings indicated that the inhibitory effect of H_2S on iNOS/NO pathway is mediated by HO-1 expression. NO and H_2S could interact with each other. NO increases H_2S production, and H_2S suppresses NO production. H_2S suppresses NO production through inhibiting iNOS expression via upregulating HO-1/CO pathway in LPS-induced RAW264.7 macrophages. Also, H_2S inhibits LPS-induced NF-κB activity through HO–CO pathway [131].

9 Interaction of H_2S with SO_2

9.1 Regulation of AAT by H_2S

Endogenous H_2S inhibits endogenous SO_2 production. CSE knockout in endothelial cells downregulates H_2S level but upregulates SO_2 content, which are rescued by supplementation of H_2S donor. However, CSE knockdown does not affect protein expression of endogenous SO_2 producing enzyme AAT, but significantly enhances AAT activity, while supplementation of H_2S donor inhibits it. H_2S donor at concentration of 100 and 200 μM directly inhibits purified AAT protein activity. Mechanistically, H_2S inhibits AAT activity through sulfhydrating cysteine residues of AAT protein [227] (Fig. 3).

9.2 Effect of H_2S–SO_2 Interaction on Inflammation

Endogenous SO_2, as a compensatory defense system of decreased endogenous H_2S pathway, antagonizes the inflammatory response of endothelial cells. Treatment of endothelial cells with AAT activity inhibitor β-hydroxamate (HDX) to block the SO_2 production could aggravate endothelial cell inflammation, as demonstrated by the degradation of IκBα protein and the elevated levels of inflammatory cytokines including ICAM-1, TNF-α, and IL-6 in human umbilical vein endothelial cells (HUVECs). H_2S content is decreased but SO_2 level is increased in the lung tissues of monocrotaline (MCT)-induced pulmonary hypertensive rats. Administration of H_2S donor restores the inhibitory effect of MCT on H_2S generation and downregulates the elevated endogenous SO_2/AAT pathway through the sulfhydration of AAT protein. Application of HDX to inhibit the elevated SO_2 level aggravates the pulmonary vascular inflammatory response caused by the inhibited endogenous H_2S generation in MCT rats. These findings suggest that H_2S suppressed endogenous SO_2 production through decreasing AAT activity mediated by sulfenylating AAT protein. Endogenous SO_2 production was upregulated when endogenous H_2S/CSE pathway was inhibited. And endogenous SO_2, as a back-up defense system after the damage of endogenous H_2S system, plays an important anti-inflammatory role in ECs [227].

9.3 Effect of H_2S–SO_2 Interaction on Pulmonary Hypertension

SO_2 facilitates H_2S production in lung tissues to alleviate hypoxic PH development. Compared to control group, plasma H_2S content and lung tissue H_2S production were decreased in rat model of PH and pulmonary vascular remodeling under the condition of high pulmonary flow [228]. CSE mRNA expression in pulmonary arteries and lung tissue of rats with PH was also lower than that in control group [228]. These results indicate that endogenous H_2S/CSE pathway is downregulated in PH and pulmonary vascular remodeling caused by high pulmonary blood flow. Further study showed that in this PH model, supplementation of SO_2 donor attenuated PH and reduced the muscularization of pulmonary arteries; the production of H_2S, the protein expression of CSE, and the mRNA expression of CSE, 3-MST, and CBS in rat lung tissue were elevated [42]. SO_2 content, aspartate aminotransferase (AAT) activity, and the protein and mRNA expression of AAT2 in lung tissues of PH rats were also significantly decreased [42].

While, H_2S inhibited endogenous SO_2 pathway through sulfhydrating AAT to inhibit its activity. Endogenous SO_2 production was upregulated when endogenous H_2S/CSE pathway was inhibited in the model of MCT-induced PH. The increased endogenous SO_2 as a back-up defense system exerted an anti-inflammatory effect and delayed the progression of MCT-induced PH and pulmonary vascular structural remodeling [227]. Therefore, the interaction between these two gasotransmitters plays an important role in the modulation of pulmonary artery pressure and vascular remodeling.

10 Conclusions and Perspectives

In this article, we reviewed the production and metabolism of H_2S, NO, CO, and SO_2, and summarized the crosstalk among H_2S and the other three gasotransmitters and their effects on the cardiovascular, nervous, gastrointestinal, and immune system. As a member of the gasotransmitter family, H_2S has several similar biological reactivity and functions with NO, CO, and SO_2. H_2S and the other three gasotransmitters interplay with each other's synthases, thereby influencing their production. In addition, the chemical crosstalk of H_2S and NO generates new reaction products. They act as endothelial-derived relaxing factors to regulate blood vessel tension. They also promote angiogenesis and prevent heart damage. The interaction between H_2S and NO also plays an important role in regulating myocardial contractility, oxidative stress, hypertension, and diabetes. Additionally, CO inhibits the activity of H_2S synthases CBS and CSE, while H_2S increases the HO/CO pathway. H_2S suppresses the activity of AAT and then the level of SO_2, and endogenous SO_2, as a back-up compensatory system when the endogenous H_2S pathway is damaged, exerts a protective effect against endothelial cell inflammation and against pulmonary hypertension.

Many studies indicated that H_2S pathway might be used to treat a variety of diseases. However, due to the lack of selectivity of CBS and CSE inhibitors, caution should be exercised in some studies using currently available CBS and CSE inhibitors. In addition, due to the lack of inhibitors, the functional research of 3-MST is also hindered. The development of more selective synthase inhibitors will greatly improve the research in this field, which will provide solid evidence for the physiological role of these synthases in the modulation of smooth muscle tension, just as NOS inhibitors have done on NO.

Due to the interaction among H_2S and other gasotransmitters (such as NO), it may be very valuable to use one or two combinations of transgene models for enzyme silencing in future research. Data on the crosstalk among H_2S and the other three gasotransmitters (NO, CO, and SO_2) have just emerged. It will be interesting to uncover the effects of incorporating CSE-knockout background into other transgenic systems such as that of iNOS [229], eNOS [230], HO-1/2 [231, 232], or AAT1/2 knockout mouse models. For instance, how does the loss of each gas change the formation and content of circulating nitrosothiols? What are the consequences of this systemically? Can bioactive persulfides compensate for the loss of nitrosothiols? The current evidence shows that gases can affect mitochondrial function, energy metabolism, and tissue homeostasis, but the functional consequences of the combined defects in H_2S and NO, CO or SO_2 generation are not clear. Do these interactions, or lack of them, support metabolic disorders like diabetes or obesity? The development of these models will also be particularly helpful in the screening of hybrid donors of H_2S/NO, H_2S/CO, or H_2S/SO_2 [93, 208, 209].

HNO is generated endogenously through the reduction of NO, the reaction of S-nitrosothiol with thiol and NOS-catalyzed reactions [130, 233, 234]. It exerts positive inotropic effect in both normal and failing hearts [235], suggesting that it may be a potentially promising target for the treatment of congestive heart failure and acute heart failure [236, 237]. But all these studies have used super-physiological concentrations of NO and H_2S or used exogenous NO donors such as SNP instead of endogenous NO. This is somewhat controversial. In fact, the concentration of endogenous NO and H_2S is very low, and SNP cannot release NO spontaneously, and therefore, it cannot simulate the reaction of endogenous NO [238]. Moreover, the products produced by the direct reaction between these exogenous donors may be different from the products produced by the biological reaction of endogenous H_2S and NO [43, 130, 239]. Therefore, the physiological relevance of HNO and other reaction products needs further research to confirm. The mechanisms responsible for the interaction among H_2S and the other three gasotransmitters (NO, CO, or SO_2), and the interaction among their molecular pathways are also urgently needed for further study.

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