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

Ya-Qian Huang, Hong-Fang Jin, Heng Zhang, Chao-Shu Tang, and Jun-Bao Du

## 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

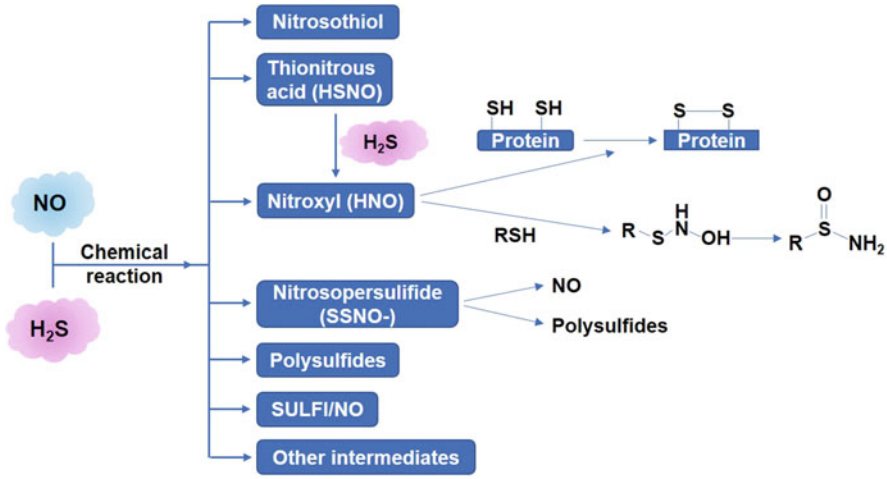


**Fig. 1** Chemical reaction of $H_2S$ with NO could generate several intermediates. And that HSNO reacts with $H_2S$ could generate HNO, which acts on sulfhydryl group in target protein to produce RSNHOH or $RS(O)NH_2$ or induce disulfide bond formation between two free sulfhydryl groups nearby, thus 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

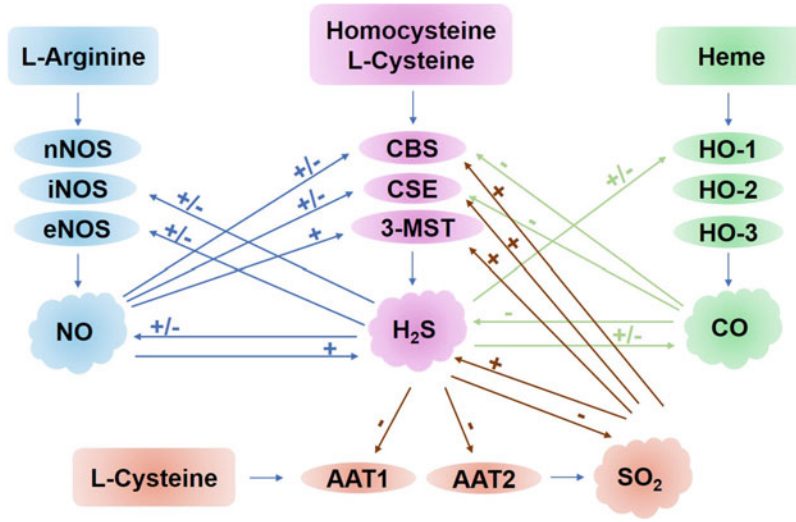


**Fig. 2** Overview of endogenous NO, CO, $H_2S$, and $SO_2$ generation and the complex relationships between $H_2S$ and the other three gasotransmitters. + represents activation; − represents inhibition

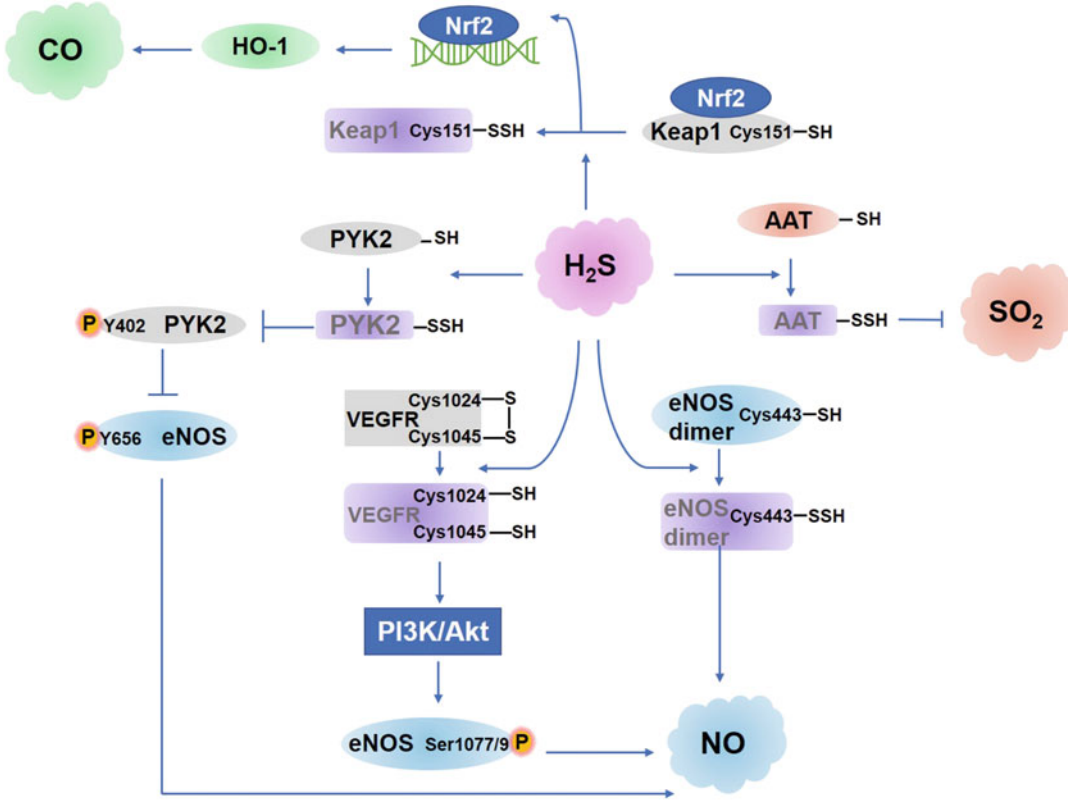


**Fig. 3** $H_2S$ regulates the generation of NO, CO, and $SO_2$ via sulfhydration. ↓ represents facilitate, ⊥ represents suppress

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

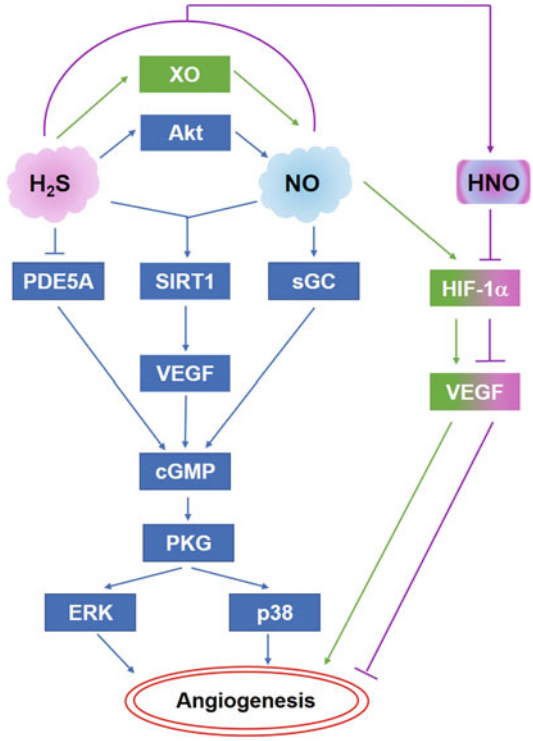


**Fig. 4** Effect of $H_2S$–NO crosstalk on angiogenesis. ↓ represents facilitate, ⊥ represents suppress

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

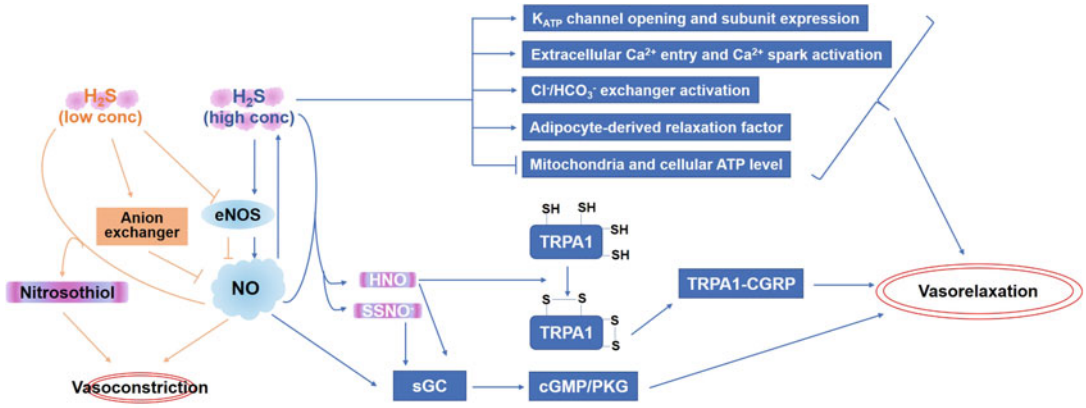


**Fig. 5** Effect of $H_2S$–NO crosstalk on vascular tone regulation. ↓ represents facilitate, ⊥ represents suppress. Conc, concentration

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

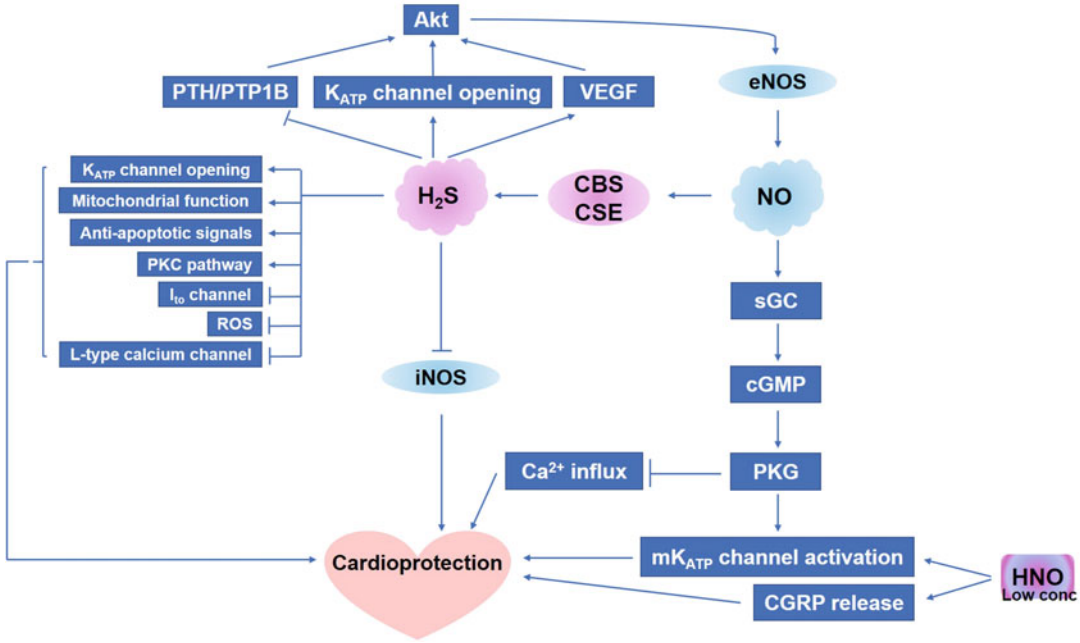


**Fig. 6** Effect of $H_2S$–NO crosstalk on cardioprotection. ↓ represents facilitate, ⊥ represents suppress. Conc, concentration; $mK_{ATP}$, mitochondrial $K_{ATP}$; PTH, phosphatase and tensin homolog; PTP1B, protein tyrosine phosphatase 1B; $I_{to}$, transient outward potassium current

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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