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COMPREHENSIVE INVITED REVIEW

Hydrogen Sulfide in the Mammalian Cardiovascular System

Yi-Hong Liu,[1] Ming Lu,[1] Li-Fang Hu,[1] Peter T.-H. Wong,[1] George D. Webb,[2] and Jin-Song Bian[1]

Abstract

For more than a century, hydrogen sulfide (H_2S) has been regarded as a toxic gas. This review surveys the growing recognition of the role of H_2S as an endogenous signaling molecule in mammals, with emphasis on its physiological and pathological pathways in the cardiovascular system. In biological fluids, H_2S gas is a weak acid that exists as about 15% H_2S, 85% HS^-, and a trace of S^{2-}. Here, we use "H_2S" to refer to this mixture. H_2S has been found to influence heart contractile functions and may serve as a cardioprotectant for treating ischemic heart diseases and heart failure. Alterations of the endogenous H_2S level have been found in animal models with various pathological conditions such as myocardial ischemia, spontaneous hypertension, and hypoxic pulmonary hypertension. In the vascular system, H_2S exerts biphasic regulation of a vascular tone with varying effects based on its concentration and in the presence of nitric oxide. Over the past decade, several H_2S-releasing compounds (NaHS, Na_2S, GYY4137, *etc*.) have been utilized to test the effect of exogenous H_2S under different physiological and pathological situations *in vivo* and *in vitro*. H_2S has been found to promote angiogenesis and to protect against atherosclerosis and hypertension, while excess H_2S may promote inflammation in septic or hemorrhagic shock. H_2S-releasing compounds and inhibitors of H_2S synthesis hold promise in alleviating specific disease conditions. This comprehensive review covers in detail the effects of H_2S on the cardiovascular system, especially in disease situations, and also the various underlying mechanisms. *Antioxid. Redox Signal.* 17, 141–185.

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[1]Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore.
[2]Department of Molecular Physiology and Biophysics, College of Medicine, University of Vermont, Burlington, Vermont.

I. Introduction

Hydrogen sulfide (H_2S) has long been known as a toxic gas, and many reports on fatal intoxication by H_2S have been documented (27, 106, 229). Humans can smell < 0.1 ppm of H_2S in the air, and at 3–10 ppm, it has a very unpleasant odor (203). Above 50 ppm, H_2S irritates the eyes and respiratory tract, and mice inhaling 80 ppm H_2S at a low environmental temperature go into a reversible hibernation-like state with reduced metabolism and breathing rate (19). This effect is species dependent, as 80 ppm H_2S has no effect on 6 kg piglets (131), while 100 ppm of it kills canaries and guinea pigs (203). Above 500 ppm, H_2S may cause unconsciousness and death in humans (203). H_2S intoxication is often attributed to its potent, reversible inhibition of cytochrome *c* oxidase, thus blocking oxidative phosphorylation (12, 58, 203). The inhibition of other enzymes, such as carbonic anhydrase (178), monoamine oxidase (266), Na^+/K^+-ATPase, and cholinesterase (203), also contributes toward its toxicity.

The physiological importance of H_2S became recognized in the last one and a half decades; starting when Abe and Kimura reported in 1996 that H_2S acts as a novel neuromodulator (1). Now, H_2S is accepted as the third "gasotransmitter" after nitric oxide (NO) and carbon monoxide (CO) (257). Similar to NO and CO, H_2S is endogenously generated by several enzymes and has been demonstrated as influencing a wide range of physiological and pathological processes, including blood vessel relaxation (3, 47, 91, 116, 268, 288, 308, 309), arterial contraction (3, 116, 141, 268), neurotransmission (1), regulation of inflammation (95, 132), cardioprotection (61, 104), neuroprotection, neurotoxicity (93, 112), and insulin release (4). The role of H_2S has been demonstrated in the arteries of many species, including humans (268).

The molecular mechanisms underlying the biological actions of H_2S have remained elusive, but a recent article published in *Science Signaling* suggests that one of the key mechanisms may be that H_2S S-sulfhydrates proteins by converting cysteine-SH groups to −SSH (173). This S-sulfhydration occurs in many different proteins due to the action of endogenously produced H_2S, and it results in modifying the physiological functions of the proteins. Thus, post-translational modification by H_2S such as S-sulfhydration, similar to S-nitrosylation induced by NO, may be an important signaling mechanism underlying its diverse effects on the cardiovascular system (173). Several molecules have been proposed as being the potential targets of H_2S action, such as the adenonsine triphosphate (ATP)-sensitive potassium (K_{ATP}) channels (309), adenylyl cyclase (AC) (1, 113), mitogen-activated protein kinases (MAPKs) (95), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (136, 313).

There have been many reviews on the physiological and pathological effects of H_2S in the past decade (15, 34, 57, 63, 93, 114, 126, 132, 134, 135, 149, 150, 153, 171, 195, 196, 199, 240, 244, 257, 273, 275, 276). In addition, two Forum issues (9 and 10) of volume 12 of ARS (2010) were devoted to many aspects of the biology of H_2S, including eight experimental reports and seven reviews (16, 34, 115, 142, 183, 253, 258). Our review will focus on recent studies conducted on the role of H_2S actions in the cardiovascular system, with special emphasis on heart and vascular diseases.

II. Biochemistry of H_2S

A. Physical and chemical properties of H_2S and its free and stored concentrations in mammals

H_2S is a colorless, flammable, and water-soluble gas with a strong characteristic rotten egg smell. In water, H_2S is a weak acid that dissociates to form H^+, HS^-, and S^{2-} (203). Figure 1 shows the origins and disposal routes for H_2S and illustrates how free sulfide exists as H_2S, HS^-, and S^{2-} in body fluids. Earlier papers have often suggested that at pH 7.4, about one third of "H_2S" exists as the dissolved gas, H_2S, while the other two thirds are HS^- plus a trace of S^{2-}. This was calculated from the pK_{a1} of 7.05 for the reaction $H_2S \leftrightarrow H^+ + HS^-$ value at 25°C in pure water (140). At a mammalian body temperature of 37°C, the pK_{a1} for $H_2S \leftrightarrow H^+ + HS^-$ is 6.76 (57) in water and 6.6 in 140 m*M* NaCl (277). For $pK_{a1} = 6.6$, the Henderson–Hasselbach equation predicts that if H_2S gas, or HS^- (*e.g.*, NaHS), or S^{2-} (*e.g.*, Na_2S) is dissolved in an aqueous 140 m*M* NaCl solution at 37°C and pH 7.4, then 14% of the free sulfide will be H_2S gas and 86% will be HS^-, plus there will be a trace of S^{2-}. There will be only a trace of S^{2-}, because pK_{a2} is greater than 12 (57, 76, 97). These dissociation reactions for the weak acid H_2S are shown in Figure 1, along with the sources and disposal routes for sulfides. Since all three species of sulfide are always present in aqueous solutions, it has not been possible to determine which of these species is biologically active. Thus, the terminology of "the H_2S concentration" usually refers to the sum of H_2S, HS^-, and S^{2-}, although "sulfide concentration" is more accurate. In this review, we follow the common convention of calling the sum of all free sulfide species "H_2S concentration."

One important property of the H_2S gas is that it is lipophilic: It easily partitions into the hydrophobic core of the cell membrane similar to O_2 and CO_2, and, thus, rapidly diffuses into or out of cells (161). Since the H_2S gas is also very volatile, it rapidly diffuses out of the blood into the lungs (170), or out of organ baths or cell culture media into the air. For example, when a 2 mm deep pool of culture medium containing 100 μM NaHS (*i.e.*, *ca.* 14 μM H_2S gas and 86 μM HS^-) was exposed to

the air, the H_2S concentration ($H_2S + HS^-$) decayed exponentially with a half time of about 6 min as the H_2S gas escaped into the air (69). This is an important point to note, especially for *in vitro* experiments. As H_2S escaped, the H^+ in the buffered medium quickly combined with HS^- to keep the H_2S concentration at 14% in accordance with the pK_a for $H_2S \leftrightarrow HS^-$ of 6.6 in 140 m*M* NaCl at 37°C (277).

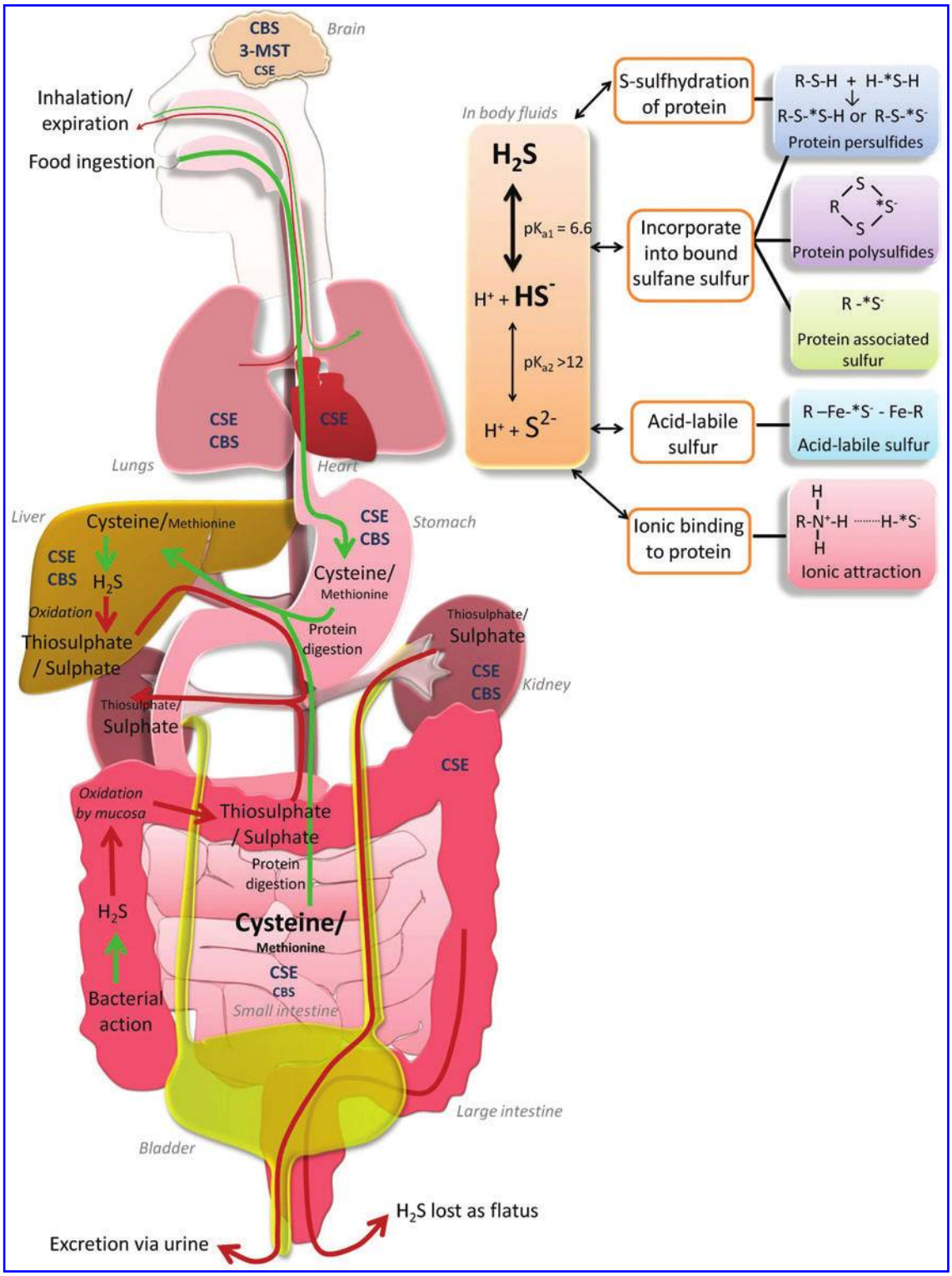
The human nose is a very sensitive H_2S gas detector. When sufficient NaHS or Na_2S is added to buffered physiological salt solution to make 10 μM sulfides, our nose detects a very strong H_2S odor. However, fresh blood and tissues are odorless despite being reported as having H_2S concentrations above 35 μM (79, 208, 264, 309). Current evidence has shown that endogenously generated H_2S is rapidly oxidized to sulfate, or can be stored in proteins (Figs. 1 and 2) where it may be released on a physiological stimulus (98). Free H_2S concentration in blood and tissues is generally only 14 or 15 n*M*, as shown by gas chromatography (GC) (66) or the polarographic sensor (56, 277). The great discrepancies in reported H_2S concentration can be explained by the various H_2S detection methods employed, some of which release bound and stored sulfide (Fig. 3) (43, 56, 107, 130, 169, 247).

Earlier publications that reported H_2S concentrations above 35 μM in fresh blood or plasma (46, 309, 315) have generally employed a strong acid or a strong base in their H_2S determination methods, both of which can release a sulfide that is bound to proteins (277). For example, the utilization of a strong acid in the methylene blue method (converts HS^- to H_2S) releases sulfides from acid-labile sulfur (98, 277). On the other hand, the strong base (converts H_2S and HS^- to S^{2-}) contained in the antioxidant buffer, as a part of the sulfide-sensitive electrode detection method, releases some of the protein-bound sulfide and can cause protein desulfuration (releasing sulfide from the constituent cysteine and methionine) (111, 277). This discrepancy between the methylene blue and sulfide electrode results, and the polarograhic sensor and GC results has been reviewed by Olson (180). In this review, we include a discussion of recently reported results with monobromobimane (MBB) and with several fluorescent probes.

The exclusion of a strong acid or a strong base in H_2S measurements has led to the detection of a significantly lowered range of free sulfides. In 1992, Togawa *et al.* reported 1.3 μM bound sulfide in human plasma utilizing the MBB measurement with dithiothreitol (DTT) (pH 8) (247). It was assumed that the DTT together with pH 8 would release bound sulfide. In a mildly basic medium, MBB reacts with HS^- to form sulfide dibimane, which can be separated from MBB by HPLC and detected by its fluorescence properties. Recently, the MBB method was employed without using DTT, to measure "available H_2S" in rat blood (278) and in mouse plasma (218). Wintner *et al.* used pH 8 and found 0.7 μM H_2S in rat blood (278), while Shen *et al.* used pH 9.5 and found 1.7 μM H_2S in mouse plasma (218). Possibly, the pH 9.5 used by Shen *et al.* was alkaline enough to release some bound sulfide. We hypothesize that as MBB removes free HS^-, more HS^- that is ionically associated with cationic sites on proteins will be released into the free solution, especially in mildly basic solutions that will neutralize some of the protein-NH_3^+ groups, allowing MBB to sequester the HS^- released. Thus, the MBB method finds higher H_2S concentrations than the polarographic sensor method (277), which measures free H_2S gas concentration without sequestering it. In addition to the MBB method, Wintner *et al.* also used a polarographic sensor (at physiological pH) and found free H_2S concentrations in the low nanomolar range in rat blood (278), in agreement with earlier measurements by Whitfield *et al.* using the polarographic sensor (277). Levitt *et al.* recently determined that the H_2S concentration in mouse blood is $\sim$15 n*M*, using a new sensitive GC method (130). Olson has reviewed the evidence that H_2S may not be a circulating gasotransmitter, due to the extremely low concentration of free H_2S in blood (180), contrary to previous reports.

Although the concentration of free H_2S in body fluids may be low, in intracellular locations where H_2S synthesizing enzymes are highly concentrated, H_2S might temporarily become highly concentrated in the micro environment before it has time to diffuse away, be bound, or be oxidized. For example, Levitt *et al.* have shown that free H_2S concentration in freshly homogenized mouse aorta is 20 to 200 times more concentrated than in various other tissues they measured with the same method (130), probably due to the higher concentration of cystathionine γ-lyase (CSE) in arteries. Moreover, under the right physiological conditions or on physiological stimuli, free H_2S may be released from sulfur stores to raise the free H_2S concentration in a micro environment (98). In rat brain, for example, it was demonstrated that bound sulfur can

FIG. 1. Handling of H_2S in the mammalian biological system. The main source of H_2S in the body starts with the digestion of proteins into amino acids. The cysteine and methionine may be metabolized to H_2S *via* the action of endogenous enzymes found in many of the body's cells. Bacteria in the lower gut also contribute another source of H_2S. The inhalation of H_2S from air may be an exogenous source of H_2S, but this is minimal under normal conditions. Due to the lipophilic, hydrophilic, and volatile properties of H_2S, it penetrates the cell membranes freely and can easily pass to and from the air, blood, and tissues. In an aqueous environment, H_2S is a weak acid that exists in three free states, H_2S gas, HS^-, and a trace of S^{2-}. The tissue and circulation level of free H_2S is in the nanomolar range. H_2S is ultimately oxidized to sulfate and excreted by the kidneys in order to maintain the sulfur balance: output (*red arrows*) should equal input (*green arrows*). Some H_2S is stored as sulfides by the formation of protein persulfides, or it becomes otherwise bound to proteins and exists as bound sulfane sulfur or acid-labile sulfur. Due to the negative charge on HS^-, it is attracted to positively charged amino groups on proteins; we postulate that this pulls some HS^- out of the pool of free H_2S, contributing toward the low concentration of free H_2S detected in blood and tissues. While H_2S may be lost from the body *via* flatus or exhalation, the large majority of H_2S is eliminated from the body *via* oxidation and excretion as sulfate in the urine. The main H_2S synthesizing enzymes are as follows: cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), mercaptopyruvate sulfurtransferase (3-MST), and cysteine aminotransferase (CAT) in specific organs are indicated in blue. Some organs express mainly one type of enzyme (*e.g.*, heart), whereas others might express multiple types (*e.g.*, liver). (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars). H_2S, hydrogen sulfide.



be released as free sulfide from astrocytes when nearby neurons are active, thus raising extracellular K^+, which activates the Na^+/HCO_3^- cotransporter and alkalinizes the astrocytes, which together with the reducing activity of the glutathione (GSH) and cysteine usually present, causes the release of bound H_2S (98). The brain has been reported as containing 61 μM "bound sulfur" (265). The H_2S released from stored sulfide in the brain as just described can act as a modulator of synaptic activity (1). Possible mechanisms similar to those described in the brain by Ishigami *et al.* (98) may occur in other

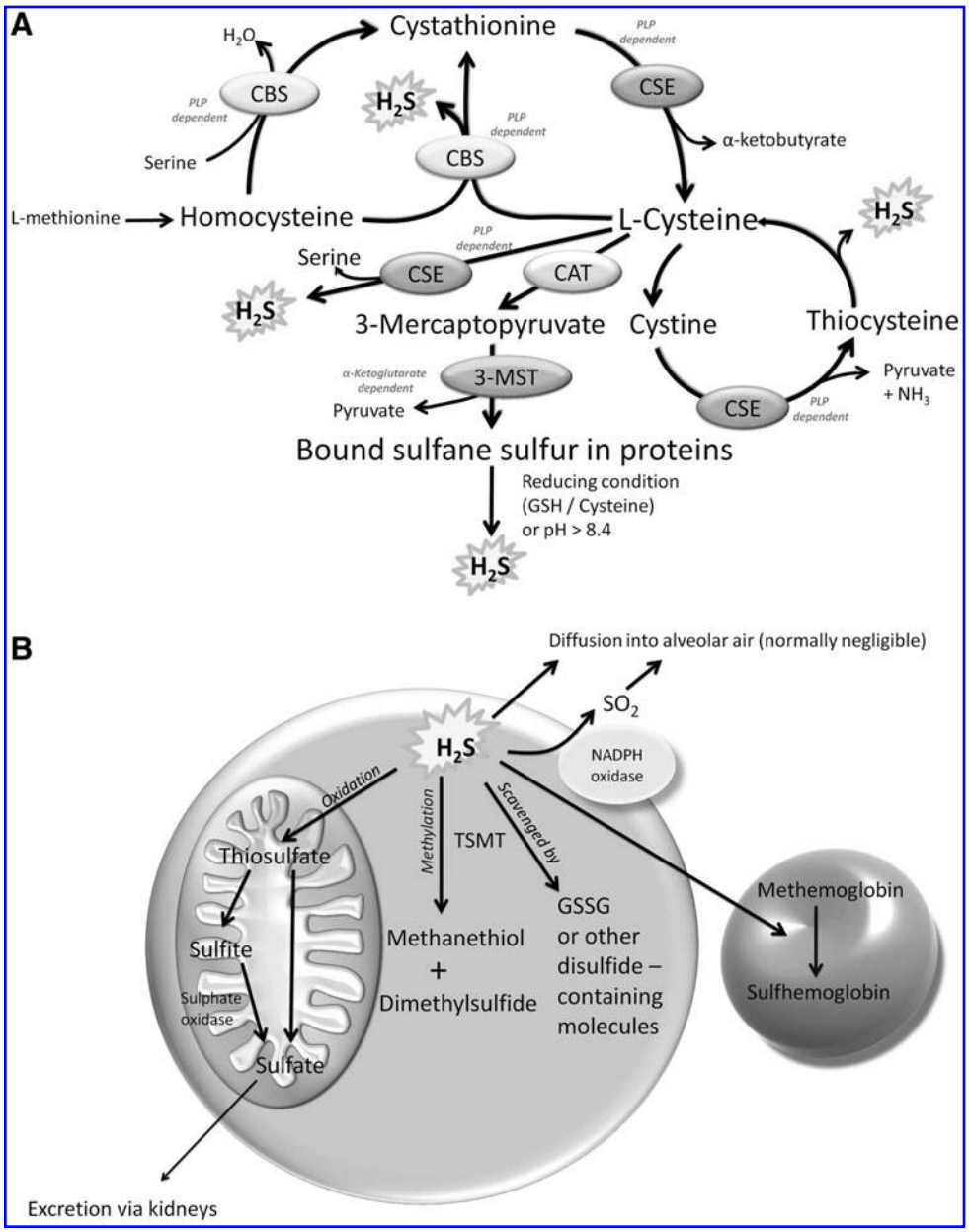


FIG. 2. Biosynthesis and catabolism of H_2S in mammals. A outlines the biosynthesis of H_2S in mammalian cells. There are four endogenous H_2S synthesizing enzymes: CBS, CSE, 3-MST, and CAT. The main H_2S precursor is L-cysteine. The other sulfur-containing amino acid, L-methionine, is a precursor of L-homocysteine, which can be metabolized to cysteines. CSE is expressed in the liver, heart, blood vessels, and other organs. CBS is expressed in the liver, brain, and other organs. CAT and 3-MST have been reported in the brain and aorta. **B** outlines the breakdown of H_2S at the cellular level. H_2S is mainly oxidized in mitochondria to thiosulfate, which is further converted to sulfite and, finally, sulfate. Sulfite can be converted to sulfate by sulfate oxidase. H_2S may also undergo methylation catalyzed by cytosolic S-methyltransferase (TSMT). H_2S may be scavenged by either methemoglobin or metallo-/disulfide-containing molecules. Potentially, H_2S can be exhaled as free H_2S gas or SO_2 gas after being metabolized by NADPH, but usually, this loss is negligible. NADPH, nicotinamide adenine dinucleotide phosphate.

organs or tissues. Physiological mechanisms, as yet poorly understood, may add to or remove the sulfide carried in plasma proteins. This may explain why the methylene blue and sulfide-sensitive electrode methods have shown that H_2S in plasma increases or decreases in some human diseases or animal disease models, and that the inhibitors of H_2S synthesizing enzymes in animal models cause the measured plasma H_2S (*i.e.*, stored sulfide) to decrease, while also changing physiological parameters such as blood pressure (BP) in parallel.

We postulate that experiments demonstrating the physiological effects of higher concentrations of H_2S than those that occur in mammalian macro environments may be uncovering the effects of H_2S concentrations that occur physiologically in micro environments near reservoirs of sulfide bound to proteins or near concentrations of CSE. The development of microelectrodes that are specific for detecting H_2S or HS^- may some day find such H_2S "hot spots." Another promising method that may be able to find H_2S "hot spots" in living cells is the use of intracellular fluorescent probes which are selective for H_2S. Just recently, at least four new fluorescent probes that are selective for H_2S have been described by different research groups (143, 144, 192, 198, 200).

The ability of H_2S to act as a reducing agent is often employed as the chemical basis of the probe. Lippert *et al.* utilized the reducing property of H_2S in the conversion of azides to fluorescent rhodamines to develop two fluorescent probes—Sulfidefluor-1 (SF1) and −2 (SF2) (143). Both probes have similar absorption and emission wavelengths (maximum absorption: ~490 nm; emission: 525 nm). Under *in vitro* conditions, the detection limits were shown to be 5–10 μM. At 100 μM NaHS, these probes showed ~50-fold greater selectivity than thiols and ~3-fold more selectivity than a variety of reactive sulfur, oxygen, and nitrogen species.

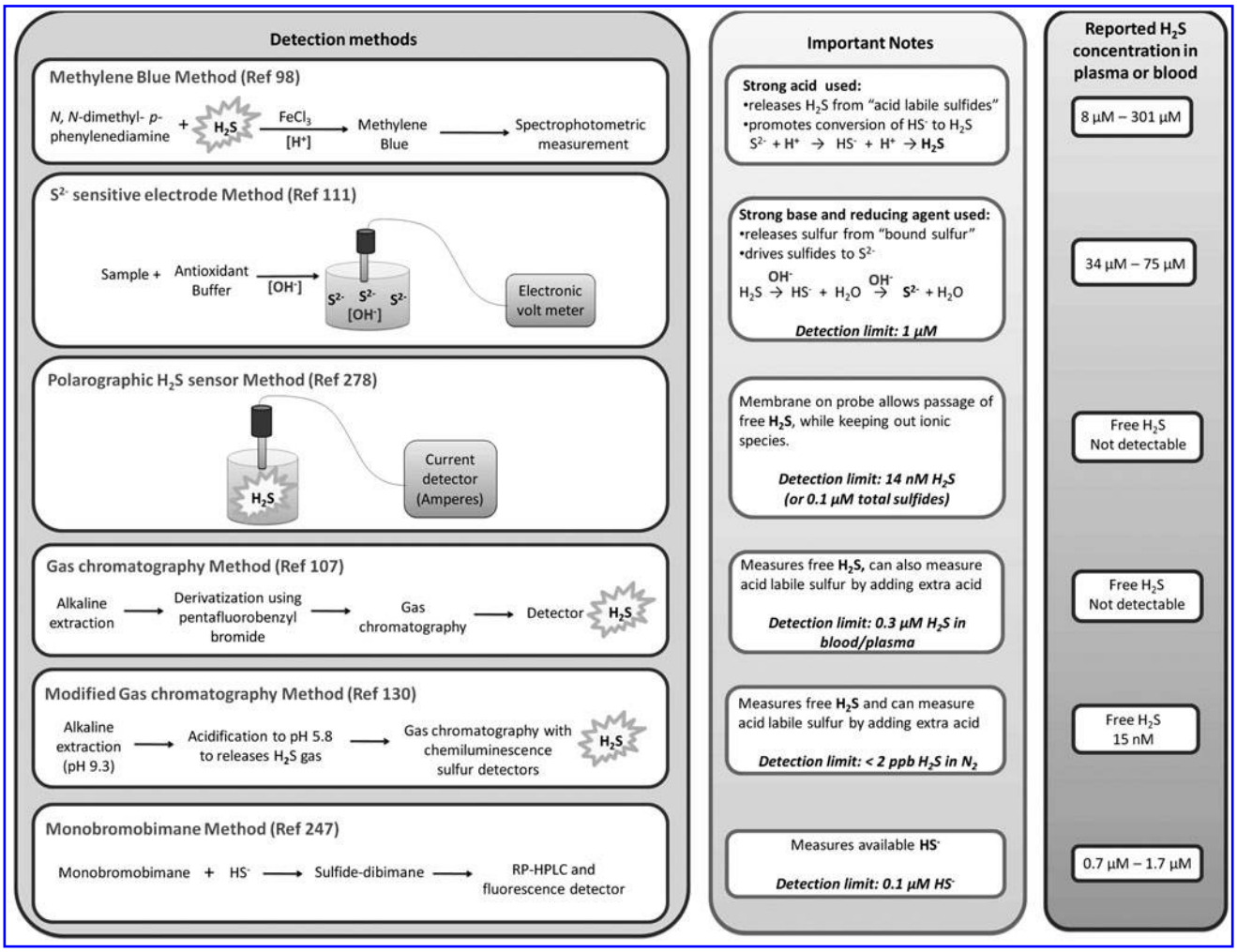


FIG. 3. Methods used to detect H_2S in biological samples. In the last 10 years, the two most commonly used methods for measuring the H_2S concentration have been the methylene blue and the sulfide-sensitive electrode. The reported concentration of H_2S in the blood or plasma of several species, including rat, human, or mouse, have ranged from 8 to 301 μM using these two methods. These relatively high concentrations would make fresh blood smell similar to rotten eggs if they represented free H_2S. The polarographic H_2S sensor and the gas chromatography can accurately measure free H_2S concentrations down to as low as 14 nM, without releasing stored sulfide. These methods find that the free H_2S concentration is either not detectable or within the nanomolar range of $\sim$15 nM. The sixth method measures the available HS^-, which may include ionically bound HS^- in addition to the free HS^-. This method finds about 1 μM HS^-.

Peng *et al.* also utilized H_2S-mediated reduction to synthesize dansyl azide (DNS-Az), a sulfonamide converted from sulfonyl azide (192). Under *in vitro* conditions, it was reported to have a detection limit of 1 μM sulfide and a signal-to-noise ratio of 3:1. The effects of 17 other anions with some reducing ability were investigated, and the fluorescence intensities were far less than those induced by HS^-. Interestingly, using this fluorescent chemoprobe, the concentration of H_2S in the blood of C57BL6/J mice was found to be $\sim$32 μM, a value much higher than the recently reported nanomolar range of free sulfides (130, 277, 278), but similar to the concentrations found with the methylene blue method. A possible explanation of this might be that the high level of GSH (>4 mM) present in the blood plasma could have interfered with the DNS-Az probe assay, as Peng *et al.* did not test the sensitivity of their probe for GSH or cysteine, two potent reducing agents present in the blood (192).

All three probes (SF-1, SF-2, and DNS-Ax) reflect the H_2S concentration by indirectly measuring redox status. High fluorescence intensity depicts a large extent of reduction, suggesting a high concentration of H_2S. It should be noted, therefore, that chemicals which strongly affect redox status should not be used along with these probes, as they will severely interfere with the assay.

The fluorescent probe (compound 1) created by Liu *et al.* made use of the ability of H_2S to undergo nucleophilic reactions twice, whereas most other biological nucleophiles and thiols undergo nucleophilic reactions only once (144). H_2S, but not cysteine or GSH, leads to increments in fluorescence *in vitro* with maximum intensity reached in 1 h. When this probe was added to bovine plasma, very weak fluorescence was observed, in contrast to the strong fluorescence seen with Peng *et al*'s DNS-Az. Most importantly, when researchers applied exogenous NaHS (10–600 μM) to bovine plasma,

intensities of the fluorescent probe were weaker than with equimolar concentration of H_2S in buffer solutions, in line with the postulation that H_2S rapidly binds to proteins or is oxidized in plasma (144, 277).

Qian *et al.* synthesized two sulfide-selective fluorescent probes (SFP-1 and SFP-2) that not only detect sulfides under *in vitro* conditions, but also monitor cell-based sulfides using live-cell imaging with minimal toxic effects on cell viability (198). SFP-1 emits blue fluorescence with an emission maximum at 391 nm (excitation: 300 nm), whereas SPF-2 emits green fluorescence with an emission maximum at 510 nm (excitation: 465 nm). Under *in vitro* conditions, SFP-1 produced a fluorescence signal in a concentration-dependent manner, starting from 10 μM Na_2S and reaching saturation at 50 μM. SFP-2 responded to Na_2S (5–100 μM) in a concentration-dependent manner, with a greater selectivity toward sulfides over thiols than SFP-1 (>150-fold *vs.* 50–100-fold). However, under *in vivo* conditions, the specificity was not as good. When tested in HeLa cells, for example, SFP-2 produced a similar extent of fluorescence using equimolar concentrations (100 μM) of cysteine, GSH, or Na_2S (198).

Other than DNS-Az, all other fluorescence probes were also tested in cultured cells for their potential use to monitor cell-based sulfides. Generally, no signs of toxicity on cell viability were observed when these probes were utilized. It is worthy to note that all these probes showed little or no fluorescence at basal levels and only emit strong fluorescence when high micromolar concentrations of H_2S donors (250 μM NaHS or Na_2S) are added to the cells (143, 144). In fact, SFP-1 and SFP-2 showed a reduced sensitivity for sulfide detection *in vivo* as compared with *in vitro* experiments. As mentioned earlier, *in vivo* GSH and cysteine may cause major false-positive results. This may limit their use to monitor endogenous H_2S production that is thought to be within the nanomolar range, far below the detection limits of these probes. Furthermore, the high H_2S concentrations applied in these experiments are probably beyond the physiological, and the micromolar detection limit might pose a major limitation of these probes in studying endogenous free sulfides.

It should also be noted that blood emits autofluorescence at 400 nm (160), whereas other endogenous fluorophores such as NaDH, FAD, and tryptophan also commonly display excitation/emission wavelengths in the range of 300–500 nm (312). As such, fluorescence probes with excitation/emission wavelengths falling within this range may face problems during H_2S assay dealing with blood samples. In view of this, Quek *et al.* synthesized a mixed-valence diruthenium complex that they abbreviated as $[Ru_2]^+$, a dye which can detect H_2S with an absorbance in the near infrared range (200). $[Ru_2]^+$ absorbs at 789 nm, but H_2S reduces it to $[Ru_2]$, which absorbs at 895 nm. The change over from 789 to 895 nm absorbance correlates linearly with NaHS concentration, with a detection limit of 1.35 to 16 μM. Though this method is highly specific for detecting HS^- as compared with many other anions and reducing agents, cysteine and ascorbic acid cause some reduction of $[RU_2]^+$ (200). For now, it is useful in measuring the rate of release of H_2S by slow release donors under *in vitro* conditions.

The most common H_2S donor utilized by most publications is sodium hydrosulfite (NaHS), because it is readily available, inexpensive, and easy to work with. However, the purity of commercially available NaHS hardly exceeds 70%. The 30% or more uncontrolled impurities may underlie some of the inconsistencies observed among different laboratories. Other H_2S donors such as sodium sulfide (Na_2S) or its liquid solution form, IK-1001 (Ikaria Holdings, Inc.), should be sought as purer alternatives, while slow H_2S-releasing compounds (*e.g.*, GYY4137) (128, 137, 138, 300) and H_2S-donating hybrids (*e.g.*, ACS14, ACS15, ACS21, ACS 84, S-diclofenac) (151, 206, 304) are under intensive research as promising therapeutics for disease conditions.

Given the factors just mentioned, organ bath or cell culture experiments utilizing < 100 μM H_2S could be of physiological relevance, in agreement with Furne *et al.*'s proposal (66). The development of new methods that measure H_2S in microenvironments is warranted for a better understanding of the intracellular H_2S levels and actions. According to Henry's Law, breathing in a lethal amount of 500 ppm H_2S gas will produce a blood concentration of 227 μM H_2S (277). With our present knowledge, we hypothesize that physiological H_2S concentrations in microenvironments and blood circulation do not exceed 100 μM. It is, therefore, noteworthy that *in vitro* experiments involving H_2S concentrations above 100 μM H_2S are not likely to depict physiological effects.

Another important factor affecting the final H_2S concentration in circulation and tissues of interest for *in vivo* experiments is the route of drug administration. While intravenous (i.v.) administration of H_2S results in greater bioavailability of H_2S, intraperitoneal (i.p.) or subcutaneous administration leads to relatively slower absorption and lower bioavailability. In some studies, researchers administered H_2S *via* oral gavage. This H_2S has to travel from the gastinointestinal tract to the liver, where it is metabolized before it reaches possible target organs. The eventual H_2S concentration in systemic circulation and target organs will be drastically reduced. This may justify the administration of higher than physiological concentrations of H_2S in some *in vivo* experiments. Nevertheless, caution should still be taken when we interpret data generated using high micromolar ranges of H_2S concentration for their physiological relevance.

B. H_2S biosynthesis

The concentration of free sulfides (H_2S, HS^-) in mammalian blood and most tissues is very low (< 100 nM) (277), although it is reported to be higher (1 μM) in aorta (130). Sulfides are also bound to proteins in blood and tissues; for example, the concentration of acid-labile sulfur in the heart was reported to be about 300 μM (130). Free and bound sulfide originates from the action of enzymes that synthesize H_2S. The four most important mammalian enzymes that synthesize H_2S are cystathionine β-synthase (CBS, EC 4.2.1.22), CSE (cystathionase, CSE, EC 4.4.1.1), and cysteine aminotransferase (CAT, EC 2.6.1.3) in conjunction with mercaptopyruvate sulfurtransferase (3-MST, EC 2.8.1.2). The biochemistry of these four enzymes is summarized in Figure 2A, and the details may be found in the reviews by Kabil and Banerjee (106) and Singh and Banerjee (225).

The expression of CBS and CSE has been detected in a broad variety of cell types, including those from the liver, kidney, heart, vasculature, brain, skin fibroblasts, and lymphocytes. In some tissues, both CBS and CSE contribute to the local generation of H_2S (such as in liver and kidneys) (282),

whereas in others, one enzyme predominates. For example, CSE is the main H_2S-generating enzyme in the cardiovascular system (17, 309). CSE mRNA expression was detected in the myocardium with 24.4% higher expression than that in the thoracic aorta (75). CSE is present in arteries in relatively large amounts and is expressed in the endothelial cells (ECs) (288) and smooth muscle cells (SMCs) (309). $CSE^{-/-}$ mice were reported to develop hypertension spontaneously (288), whereas a later study failed to reproduce this finding (99). Nevertheless, the significance of CSE in the cardiovascular system should not be disregarded, as $CSE^{-/-}$ mice developed lethal myopathy and were susceptible to oxidative injury with a diet deficient in cysteine (99).

It was conventionally regarded that CBS is the predominant H_2S synthase in the brain and nervous system (1). However, Shibuya *et al.* later discovered that brain homogenates of $CBS^{-/-}$ mice produce H_2S at levels similar to those of wild-type mice (221). They also showed that 3-MST is expressed in neurons in the brain. Along with CAT, 3-MST produces H_2S using both L-cysteine and α-ketoglutarate as substrates. Their experiments suggest that 3-MST and CAT contribute to H_2S formation in both the brain (201) and the vascular endothelium (18, 220, 221). A recent paper has shown that CAT and 3-MST can produce H_2S only in alkaline conditions and in the presence of DTT, a strong reducing agent (225). Therefore, the physiological relevance of 3-MST as a source of H_2S formation in the brain remains to be elucidated in the future.

Stearcy and Lee demonstrated the reduction of exogenous S_8 in producing H_2S by human erythrocytes using reducing equivalents from glucose oxidation (214). In addition, they also found a slower production of H_2S without adding S_8, suggesting an endogenous source of sulfur in red blood cells (214). Thus, inorganic synthesis of H_2S may contribute toward endogenous H_2S formation *in vivo*, though its implication is yet to be discovered.

C. H_2S catabolism

Figure 2B summarizes the cellular mechanisms that dispose of H_2S. The vast majority of H_2S is oxidized to sulfate, which leaves the body *via* the kidneys (52, 66, 67, 158). The primary site for this oxidation is in the liver, but all cells in the body can oxidize H_2S (66, 67, 277). The half time for disappearance of 30 μM free sulfides is 51 s for cow blood, and 191 s for 5% bovine serum albumin (277); hence, even plasma can oxidize H_2S, while blood does it more rapidly. It has been suggested that a major portion of the ability of plasma or blood to rapidly consume sulfide added *in vitro* is due to the binding of the sulfide to proteins (52).

Usually, most or all of the H_2S produced by CSE and CBS in the liver, from the cysteine and methionine derived from digested proteins, is oxidized (or bound) before it can escape into the hepatic veins that carry the blood to the inferior vena cava (66). Mitochondria are very effective in oxidizing sulfides; however, if the supply of sulfide exceeds the ability of the mitochondrial enzymes to oxidize all of it, then the H_2S can poison the mitochondrial enzymes (80). No H_2S is released when the homogenates of liver are incubated with the physiological concentrations of 1 or 0.1 mM cysteine. Anerobically, the same experiments show a huge release of H_2S with 10 mM cysteine, but still none with 1 or 0.1 mM cysteine (66). The results suggest that oxidation, especially in the liver, is an important way by which the body rids itself of endogenously produced H_2S. Even without oxygen, however, the liver can absorb all the H_2S produced with physiological levels of cysteine, probably due to the absorption by proteins and to methylation.

Endogenous H_2S may be metabolized *in vivo* by different routes (Fig. 2B). As a readily diffusible gas, it can be metabolized in mitochondria by oxidation to thiosulfate, which is further converted to sulfite and sulfate by sulfate oxidase (67). Finally, the end products, sulfates, are excreted in the urine as either free or conjugated sulfate (12, 52). Another metabolic pathway involves the methylation of sulfides by cytosolic S-methyltransferase to methanethiol and dimethylsulfide (67). H_2S can also be scavenged by methemoglobin (12) or metallo- or disulfide-containing molecules such as oxidized GSH (228). Hemoglobin may act as a common sink for vasoactive gases (CO, NO, and H_2S), and these three gases compete with oxygen for binding, thus contributing to their toxicity on high exposure.

Mammalian lungs may occasionally provide an escape route for H_2S (Fig. 1), possibly during septic shock, hemorrhagic shock, or pancreatitis when larger than normal amounts of H_2S may be generated. In healthy individuals, however, very little H_2S is lost *via* the lungs, because metabolic disposal keeps the free level of H_2S in blood very low (66). Alveolar air (end expiration) usually contains only 25–50 ppb H_2S (172, 237) in healthy subjects (not enough to smell); thus, the normal daily loss of H_2S *via* the lungs is very small compared with the loss of sulfate in the urine.

D. Subcellular compartmentalization of H_2S

Subcellular compartmentalization of NO synthase (NOS) is an important regulatory mechanism for NO signaling (8). Subcellular compartmentalization of H_2S is also suggested in eukaryotic cells, as a substantial amount of H_2S-oxidizing activity was observed in mitochondria while sulfur-reducing activity was observed in the cytoplasm (213, 214). The localization of H_2S synthesizing enzymes, sulfur stores, and metabolic routes provides insights to such an understanding. Both CBS and CSE are localized in the cytosol (5), and their activities are absent in mitochondria (5). 3-MST and CAT, on the other hand, are mainly localized in the mitochondria (123, 222). Since different cell types express different types of enzymes, the localization of H_2S synthesis may account for the differential effects of H_2S in different tissues. Interestingly, bound sulfane sulfur is localized primarily in the cytoplasm, whereas acid-labile sulfur occurs mainly in iron-sulfur enzymes found in mitochondria (179). Compartmentalization of sulfur stores may facilitate differential H_2S release for specialized purposes. Furthermore, bound sulfane sulfur is released as H_2S under reducing conditions or at pH >8.4 (249), while acid-labile sulfur is released at pH <5.4 (98), further contrasting the mechanism of release of H_2S from these stores. The metabolism of H_2S, as mentioned earlier, mainly occurs in the mitochondria. Subcellular compartmentalization of H_2S may have important physiological implications that are worthy of further studies and in-depth research. For example, crosstalk between H_2S and NO could be highly regulated by their compartmentalization, accounting for the differential effects observed in different systems.

III. Effects of H_2S on the Heart

A. H_2S effects on heart function

In the isolated rat heart, perfusion with 100 μM or less NaHS had no significant effect on heart rate (165, 314). However, 10–1000 μM NaHS decreased the amplitudes of myocyte twitch and electrically induced calcium transients in isolated rat ventricular myocytes treated with isoproterenol (ISO) (298). In the isolated heart, perfusion with NaHS (1–1000 μM) inhibited maximal/minimal left ventricular (LV) pressure development ($\pm LVdp/dt_{max}$) (75). The administration of NaHS (2.8 μmol/kg body weight) *in vivo via* the femoral vein produced a similar effect on cardiodynamics and a transient decrease in mean arterial pressure (MAP) in anesthetized rats (75). Since the high concentrations of NaHS used were well above the physiological H_2S concentrations of <100 μM, it is still unclear whether H_2S plays a physiological role in heart function.

B. Heart electrophysiology

The effects of H_2S on heart electrophysiology and intracellular signaling are illustrated in Figure 4.

1. Intracellular calcium regulation. During a cardiomyocyte action potential, depolarization of the sarcolemma due to an initial brief increase in Na^+ permeability, followed by a longer lasting opening of L-type Ca^{2+} channels (LTCC), results in inward Ca^{2+} currents. Sun *et al.* found that NaHS (25–400 μM) reduced the peak current of LTCC ($I_{Ca,\ L}$) in a concentration-dependent manner (239). It should be noted, however, that the effects observed at H_2S concentrations above 100 μM probably do not suggest physiological relevance. Whether or not LTCC is the primary action site of H_2S still remains to be determined. The possibility that the effect of H_2S on LTCC is secondary to other signaling pathways may not be fully excluded. For instance, the reduction of the Ca^{2+} current through LTCC may also result from hyperpolarization caused by the opening of K_{ATP} channels (102, 245) or the suppressed cAMP/protein kinase A [PKA] pathway (298) (Fig. 4). More work, such as single-channel recording, is needed to determine conclusively whether or not H_2S is a direct LTCC blocker using near-physiological H_2S concentration.

2. K_{ATP} channels. K_{ATP} channels are widely distributed in the myocardium. The opening of K_{ATP} channels generates outward currents, causing hyperpolarization. This reduces calcium influx *via* LTCC and prevents Ca^{2+} overload. Thus, the K_{ATP} channel opening is an important endogenous cardioprotective mechanism, and it is crucially involved in cardiac ischemia preconditioning.

Evidence that NaHS (100 μM) opens the K_{ATP} channels was obtained by Wang and coworkers (102, 245). This is discussed in detail in Mechanisms of H_2S-induced vasorelaxation section. Furthermore, H_2S may also indirectly activate the K_{ATP} channels by inducing intracellular acidosis (14, 51, 119, 127). By activation of the K_{ATP} channels, H_2S shortens action potential duration (APD) (2) and produces cardioprotective effects (17, 104, 188, 226, 306). This is further discussed in Mechanisms for H_2S-induced cardioprotection and Mechanisms for the cardioprotection of SPreC sections.

3. Chloride channels. Chloride channels play important roles in diverse processes such as BP regulation, cell cycle and apoptosis, muscle tone, volume regulation, synaptic transmission, and cellular excitability (164). Malekova *et al.* investigated the effect of H_2S on single-channel currents of chloride channels using the patch clamp technique (159). It was found that 20 to 200 μM NaHS inhibited the chloride channels by decreasing the channel open probability in a concentration-dependent manner. Therefore, the inhibitory effect of H_2S on the chloride channels may be involved in the biological actions of H_2S in the heart (159).

4. Action potential. H_2S has no significant effect on the amplitude of action potential and resting potential (239). However, NaHS at concentrations of 100–500 μM markedly reduced APD and decelerated the sinus rhythm. As just mentioned, the effect of H_2S on APD is attributed to the

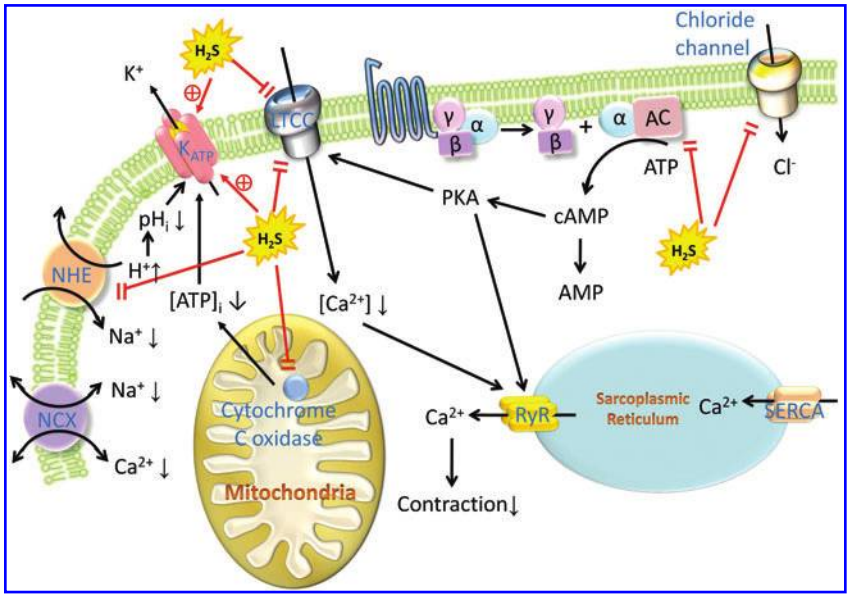


FIG. 4. Effect of H_2S on electrophysiology of the heart. H_2S inhibits heart contractions mainly by the suppression of adenylyl cyclase (AC), activation of ATP-sensitive potassium (K_{ATP}) channels, and blockade of L-type Ca^{2+} (LTCC) channels. H_2S may also inhibit the activity of chloride (Cl^-) channels, cytochrome *c* oxidase and Na^+/H^+ exchanger (NHE), and the resultant decrease in intracellular pH and ATP, further implicating the activity of the K_{ATP} channels (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars).

opening of the K_{ATP} channels (2). However, the significance of this finding is yet to be determined, as the concentration used is likely to be more than physiological.

C. Suppression of the AC/cAMP pathway

Yong *et al.* examined the action site of H_2S in the β-adrenoceptor system with pharmacological manipulation (298). It was found that the amplitudes of electrically induced $[Ca^{2+}]_i$ and myocyte twitch were significantly increased by the stimulation of AC with forskolin, activation of PKA with 8B-cAMP, or the opening of LTCC with Bay K-8644. NaHS (100 μM) treatment significantly attenuated the effects of forskolin, but not those caused by 8B-cAMP and Bay K8644 (298). These data suggest that H_2S may act on AC or its upstream signaling pathways, but not on PKA and LTCC. To confirm this, Yong *et al.* determined the effect of H_2S on AC activity and found that NaHS (100 μM) significantly decreased AC activity (298). These findings suggest that H_2S has an inhibitory effect on AC activity, which may account for the decreased cAMP production.

D. Interaction among gasotransmitters in the heart

Accumulating evidence suggests that interactions of biologically active gases (*i.e.*, H_2S, NO, and CO) may influence the biological effects of one another (65, 108, 110, 133, 181). Interactions between H_2S and NO have long been speculated, as several reports have demonstrated that H_2S and NO may influence each other's production (74, 121, 148, 283, 285, 307). Moreover, others have found evidence that interactions between H_2S and NO may alter the physiological response to either of these molecules alone (3, 274). Recently, Yong *et al.* found that a mixture of NO and H_2S (100 μM) produces an opposing effect in the heart as compared with either gas alone. This effect could be abolished by thiols, suggesting that the two molecules interact to form a third molecule that is thiol sensitive. Yong *et al.* proposed that nitroxyl anion (HNO) could be one of the possible candidates (296), based on the important reducing capability of H_2S (240, 257, 267) and the structural and pharmacological properties of HNO (296). Please refer to Figure 5 for greater details.

Experiments carried out in the liver suggest that CBS may act as an *in vivo* CO sensor (108, 223). It has also been observed that CBS activity can be directly inhibited by NO and CO (197). Although CBS plays a minor role in H_2S synthesis in the cardiovascular system, more work has to be done to unveil any possible physiological roles of CBS or of the interactions between H_2S and CO in the cardiovascular system.

IV. Potential Therapeutic Effects of H_2S in Heart Diseases

Cardiovascular diseases (CVD) are collectively a leading cause of death worldwide. The most common manifestations of CVD are coronary artery disease (CAD), chronic heart failure (CHF), and hypertrophy. The protective effects of H_2S in these pathological conditions are discussed next.

A. Ischemic heart diseases: Is H_2S a biomarker for ischemic heart diseases?

Both the endogenous H_2S level in ventricular myocytes (17) and CSE activity in the isolated heart (297) were found to be suppressed under ischemic conditions. An *in vivo* study showed that an injection of ISO that produces "infarct-like" myocardial necrosis reduces H_2S levels in the myocardium (73). In the ISO-treated heart model, CSE mRNA gene expression in the myocardium was reported by Geng *et al.* (73) to be upregulated, but by Zhu *et al.* (315), to be decreased. The discrepancy between the two groups may be secondary to the different models used and the different timings for tissue collection. Nevertheless, current evidence collectively indicates that endogenous H_2S production in the ischemic heart is likely to be reduced.

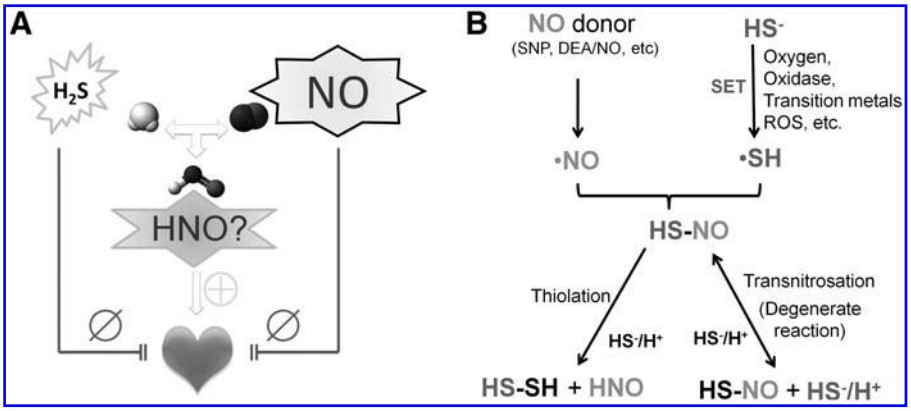


FIG. 5. Interaction of H_2S and NO in the heart. (A) H_2S and NO, each of which produces a negative inotropic effect on the heart, produce a positive inotropic effect when added together. Hence, H_2S may interact with NO to form a novel compound exhibiting distinctive characteristics. Under experimental conditions, one possible candidate for such interactions could be the nitroxyl anion (HNO). **(B)** Proposed mechanism of HNO formation as a result of H_2S-NO interaction. In the presence of cellular oxidants (such as molecular oxygen), ROS (such as H_2O_2), and oxidases, HS^- can be oxidized to give thiyl radical ($HS^{\cdot}$). The NO released from the NO donor will then quickly combine with the HS radical to give nitrosylthiol. In the presence of HS^-, there are two possible reactions with similar activation energy according to a model system studied by computational chemistry. The transnitrosation reaction with another molecule of HS^- will lead to HS exchange, and the reaction is just a degenerate reaction without the formation of a new product. The other possible reaction between HSNO and HS^- is the thiolation reaction, which leads to the formation of hydrogen disulfide (H_2S_2) and HNO. NO, nitric oxide.

Geng *et al.* also reported that the plasma H_2S level decreased by 66% (from 60 to 20 μM) in an ISO-induced myocardial ischemia rat model (73). Consistent with this, a clinical observational study has shown that the plasma concentration of H_2S in patients with coronary diseases is significantly lowered in comparison with that in normal control subjects (26 *vs.* 52 μM), suggesting that the decreased plasma H_2S levels may correlate with the severity of coronary diseases (103). These observations lead to the suggestion that the plasma H_2S level has the potential to be a biomarker for ischemic heart diseases. However, a good diagnostic or prognostic marker should change well before any clinical symptoms or vary according to the severity of the specific disease. It has been reported that the plasma H_2S concentration also decreases in association with other diseases, for example, hypertension (43), Alzheimer's disease (147), stable asthma (280), and pneumonia (46). As discussed earlier in the introduction, however, the methods of H_2S detection used in these studies involved harsh conditions that release bound sulfide; hence, caution should be exercised when these data are interpreted.

1. Cardioprotective effects of H_2S against ischemia-reperfusion-induced injury. The reduced endogenous H_2S production in the ischemic heart implies that the ischemic heart injury might, at least in part, result from the impaired endogenous production of H_2S. The cardioprotective effects of H_2S were, therefore, studied by several groups of scientists. Their results are summarized in Table 1.

Myocardial infarction (MI) is the most common presentation of ischemic heart disease; hence, it is also the gold standard in the study of cardioprotection. Johansen *et al.* reported that the perfusion of NaHS at 0.1–1 μM reduced MI size in the Langendorff-perfused heart after 30 min of left main coronary artery occlusion and 120 min of reperfusion (104). In *in vivo* studies, NaHS (0.1–14 μmol/kg) treatment also reduced MI size in rats (187, 315), mice (61), and pigs (186, 230, 231). Conversely, the administration of propargylglycine (PAG), a CSE inhibitor, significantly increased the infarct size caused by myocardial ischemia (20, 226). Moreover, elevation of endogenously produced H_2S by cardiac-specific overexpression of CSE (in α-MHC-CGL-Tg mouse) significantly limited the infarct size caused by left coronary artery (LCA) occlusion for 45 min followed by reperfusion for 72 h (61). This confirms that endogenous H_2S may play an important role in maintaining cell function in the ischemic heart. Detailed information is provided in Table 1.

In addition to the reduction of infarct size, NaHS (40–100 μM) treatment also significantly decreased the severity and duration of ischemia/reperfusion (I/R)-induced arrhythmias (17, 306) (Fig. 6) and improved myocardial contractile function in the ISO-induced ischemic rat heart (73) and in the I/R-induced ischemic porcine heart (230). In the isolated perfused heart, H_2S-releasing derivative compounds (ACS14 and 21) produced improvement in heart mechanics after I/R injury (206).

Yong *et al.* investigated the effect of rapid intermittent supply of H_2S in the early phase of reperfusion, termed H_2S postconditioning (SPostC), on heart contractile function (297). In the SPostC group, hearts received six cycles of 10 s reperfusion and 10 s NaHS (100 μM) infusion after ischemia, while NaHS was given for 2 min continuously after ischemia in the SPostC2 group. Both SPostC and SPostC2 treatments significantly improved the cardiodynamics, including LV end diastolic pressure, LV developed pressure, and $\pm dP/dt$ during reperfusion after ischemia.

Olson and colleagues reported that oxygen is critical for the catabolism of H_2S *via* mitochondrial oxidation. During hypoxia when intracellular oxygen is low, the catabolism of H_2S was found to be greatly reduced (183), resulting in an increased concentration of H_2S. This seems to contradict the results of most studies which have found that the H_2S concentration (measured with methylene blue method) was reduced during ischemia/hypoxia in the ischemic heart (see Ischemic heart diseases section). As just discussed, the methylene blue method is not a good assay for the detection of free H_2S. It is, therefore, not known whether endogenous free-form H_2S is decreased or increased. For these reasons, whether there is an inverse relationship between oxygen and H_2S in the heart cannot be concluded. Without a good method to measure endogenous free-form H_2S intracellularly, it is, therefore, difficult to explain why the inhibition of endogenous H_2S production with PAG resulted in increased infarct size (20, 226). The harmful effects of PAG may come from a further decrease in the H_2S level due to suppression of CSE or an attenuated elevation of the H_2S level if the level is increased due to inhibition of its catabolism in ischemia. Importantly, it should be noted with caution that PAG is a nonselective inhibitor of pyridoxal 5′-phosphate-dependent enzymes, and could have exerted its effects on other enzymes besides CSE (273), contributing toward its detrimental effects. Nonetheless, both ischemic preconditioning (IPreC) (17) and postconditioning (297) have been demonstrated as elevating endogenous H_2S levels in the heart. This implies that brief episodes of ischemia may increase the endogenous H_2S level irrespective of whether it is from the stimulation of CSE or the inhibition of H_2S catabolism.

a. Mechanisms for H_2S-induced cardioprotection. Ischemic injury is a complex process involving the action and interaction of many factors (Fig. 6). On the interruption of oxygen supply, mitochondrial oxidative phosphorylation rapidly stops, resulting in depletion of high-energy phosphate compounds such as ATP and creatine phosphate (89). Anerobic glycolysis increases to compensate for decreased aerobic ATP production, resulting in the accumulation of H^+ and lactate (25). The resultant intracellular acidosis causes alterations in ion transport in the sarcolemma and organelle membranes (26, 246). Initially, there is increased K^+ efflux related to an increased osmotic load caused by the accumulation of metabolites and inorganic phosphate. With a substantial reduction in [ATP], Na^+/K^+-ATPase activity declines, resulting in a decrease in $[K^+]_i$ and an increase in $[Na^+]_i$. Intracellular acidosis also activates the sarcolemmal Na^+/H^+ exchanger (NHE) (109, 292), which facilitates H^+ extrusion in exchange for Na^+. The accumulated Na^+, in turn, activates the reverse mode of the Na^+/Ca^{2+} exchanger (NCX), which extrudes Na^+ in exchange for Ca^{2+} entry. The resultant cytosolic loading of Ca^{2+} brings the progression of ischemic injury to an advanced stage by inducing (a) sustained impairment of contractile function; (b) cell membrane damage by activating Ca^{2+}-dependent phospholipase, which generates reactive oxygen species (ROS) from myocytes, ECs, and activated leukocytes;

Table 1. Comparison of the Cardioprotective Effects of Sodium Hydrosulfide Against Ischemic/Reperfusion-Induced Injury

Treatment	*I/R protocol*	*Species/tissue*	*Effects of NaHS*	*Mechanism*	*Ref.*
NaHS (0.1 and 1 μM perfusion 10 min prior to LAD occlusion till 10 min reperfusion	I (30 min)/R (120 min)	Rats/Langendorff heart	MI (↓)	K_{ATP} channel	(104)
NaHS (40 μM) throughout the experiment	I (40 min)/R (120 min)	Rats/Langendorff heart	MI (↔)	_	(20)
PAG	I (40 min)/R (120 min)	Rats/Langendorff heart	MI (↑)		
NaHS (40 μM) perfusion during reperfusion	I (30 min)/R (30 min)	Rats/Langendorff heart	Antiarrhythmias, improve contractile function	K_{ATP} channel	(306)
NaHS (14 μmol/kg/day) i.p. from 7 days before to 2 days after MI surgery	Permanent ligation w/o reperfusion	Rats/*in vivo*	MI (↓), mortality (↓)	_	(315)
NaHS (0.1, 1, 10 μmol/kg/day) i.p. for 3 days after MI surgery	Permanent ligation w/o reperfusion	Rats/*in vivo*	MI (↓), internal diameter (↓), Anterior wall thickness (↑)	_	(187)
NaHS (10–500 μg/kg) administered into LV lumen at the time of reperfusion; CSE overexpression	I (30 min)/R (24 h)	Male C57BL6/J mice or CSE transgenic mice/*in vivo*	MI (↓), apoptosis (↓), inflammation (↓)	Preserve mitochondrial function, improve recovery of respiration rate, antiapoptosis, Anti-inflammation	(61)
Bolus: NaHS (0.2 mg/kg) over 10 s at the onset of ischemia; Infusion: NaHS (2 mg/kg/h) during I/R period	I(60 min)/R (120 min)	Swine/*in vivo*	Bolus: no effect Infusion: MI (↓),	Hsp27, αB-crystallin, phosphor-glcogen synthase kinase-3 β, antiapoptosis	(186)
Na_2S: bolus (NaHS, 100 μg/kg) + infusin (NaHS, 1 mg/kg)	I(60 min)/R (120 min)	Swine/*in vivo*	MI (↓), improve contractile function and coronary microvascular reactivity	Anti-inflammation	(231)
NaHS: 100 μM perfusion 10 min before and during ischemia in the isolated heart	I (30 min)/R (60 min)	Rat/Langendorff heart	Improve contractile function and increase cell viability	Inhibition of NHE	(92)
Na_2S: 10 min prior to and throught reperfusion	I(60 min)/R (120 min)	Swine/*in vivo*	MI (↓)	Antiapoptosis	(230)
NaHS: 3 mg/kg, i.v.	I(25 min)/R (120 min)	Rat/*in vivo*	MI (↓)	K_{ATP}	(226)
PAG: 50 mg/kg, i.v.	I(15 min)/R (120 min)	Rat/*in vivo*	MI (↑)	_	

CSE, cystathionine γ-lyase; i.p., intraperitoneal; i.v., intravenous; I/R, ischemia/reperfusion; K_{ATP}, ATP-sensitive potassium; LAD, left anterior descending; LV, left ventricle; MI, myocardial infarction; NaHS, sodium hydrosulfide (H_2S donor); NHE, Na^+/H^+ exchanger; PAG, propargylglycine.

and (c) disruption of cellular scaffolds by activating Ca^{2+}-dependent proteases that cleave cytoskeletal filaments. Collectively, these Ca^{2+}-induced changes lead to a loss of membrane integrity and terminally demolished cellular structure (25).

The re-establishment of coronary blood flow to the infarcted area results in reperfusion injury, mainly mediated by ROS, overloaded Ca^{2+}, and neutrophils (38, 81, 191). Single-electron reduction of oxygen in injured myoctyes, ECs, and activated neutrophils generates the superoxide anion (O_2^-) (54), which goes on to generate other ROS. These free radicals exacerbate membrane damage and stimulate vasoconstriction, which, in severe cases, results in a "no flow" phenomenon. The Ca^{2+} overload induces maximum contraction of the myofibrils on reperfusion, resulting in contraction band necrosis (252). An increase in mitochondrial [Ca^{2+}] triggers the opening of mitochondrial permeability transition pores (mPTP), causing the release of cytochrome C and other proapoptotic factors that initiate the apoptotic cascade (85). Reperfusion is a potent stimulus for neutrophil activation and accumulation, which, in turn, enhances ROS production (44). In myocardium subjected to I/R, reoxygenated ECs express adhesion proteins, release cytokines, and reduce the production of NO, which promotes adherence, activation, and accumulation of neutrophils (62, 105). These activated neutrophils will release not only ROS but also proteolytic enzymes that can damage myocytes and vascular cells. Proinflammatory

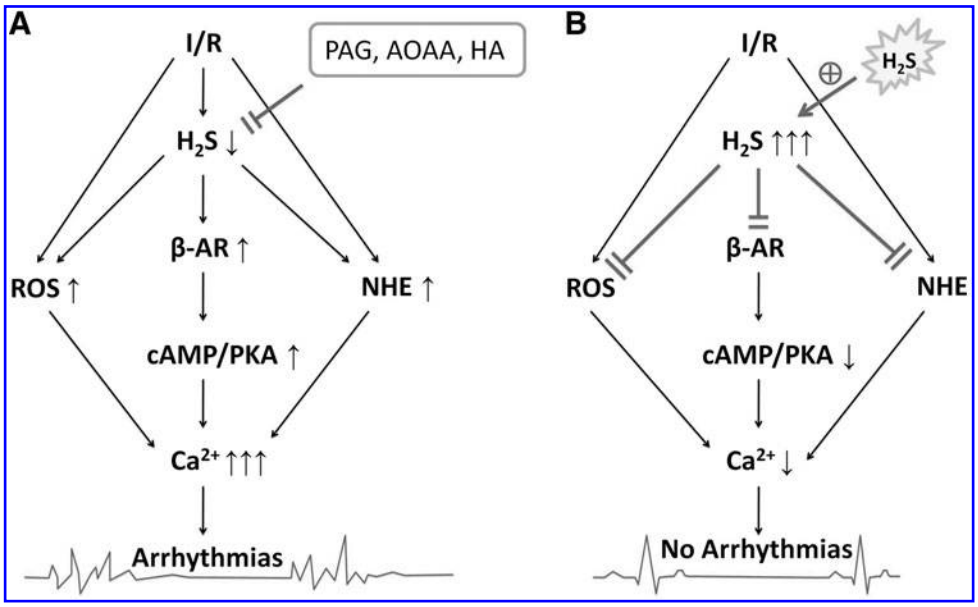


FIG. 6. Role of H_2S in ischemia/reperfusion (I/R)-induced arrhythmias. (A) On subjecting the myocardium to I/R, endogenous H_2S synthesis was found to be reduced. This leads to augmented β-adrenoceptor signaling, elevated reactive oxygen species (ROS) production, and enhanced action of NHE. Collectively, these events brought about the elevation of intracellular calcium and resulted in arrhythmia. When the inhibitors of endogenous H_2S synthesis [*e.g.*, DL-propargylglycine (PAG; a CSE inhibitor), amino-oxyacetate (AOAA; a CBS inhibitor) and hydroxylamine (HA; an inhibitor of both CBS and CSE)], were utilized, the extent of arrhythmia worsened. **(B)** Exogenously applied H_2S could reverse the effect of I/R on arrhythmias.

lipid metabolites released from neutrophils and other white blood cells enhance the expression and production of a proinflammatory cytokine cascade involving interleukin (IL)-1 and tumor necrosis factor-α (TNFα) (49). These cytokines then lead to the activation and infiltration of leukocytes. Proinflammatory molecules (such as IL-6, IL-8) and anti-inflammatory factors (including IL-4 and IL-10) may also be produced and act as negative feedbacks on the cascade (105).

(1). Antioxidative action. I/R-induced arrhythmias may develop as a result of ROS production and accumulation in the myocardium during reperfusion. Since H_2S production is markedly decreased during ischemia (17, 73, 103, 297, 298), ROS may, therefore, be increased. Excessive free radicals can react with proteins, lipids, and nucleic acids, thereby disrupting myocardium functions. The application of H_2S may produce protective effects *via* its antioxidative effects (232). In cultured H9c2 myoblasts, Na_2S (30–100 μM) was shown to protect against free-radical (H_2O_2)-induced cell death (242). However, unlike GSH, which is present at a 1–10 mM concentration, H_2S is present at relatively low concentrations ($\sim$15 nM) and it is also a poorer reductant (redox potential of +0.17 V *vs.* −0.25 V for the other two thiols) (106). Hence, the physiological relevance of H_2S as an endogenous antioxidant is still a question. The effect of H_2S on GSH activity or level has been previously reviewed (93). Heme oxygenase (HO)-1, the rate-limiting enzyme involved in heme degradation, has been implicated in both pro- and anti oxidative activities. H_2S infusion in porcine LCA occlusion was found to enhance HO-1 expression (186). The significance of this effect is yet to be understood.

(2). Suppression of β*-adrenergic function.* Overactivation of the sympathetic system is closely linked with the progression of cell injury and the incidence of ventricular arrhythmias during myocardial ischemia (211, 212). Excessive norepinephrine release and accumulation in the heart may cause the depletion of ATP and the accumulation of intracellular Ca^{2+} and Na^+ (39) that can lead to ventricular arrhythmias (185). Yong *et al.* found that NaHS (100 μM) negatively modulated β-adrenergic function (298) and the lowered H_2S production during ischemia may cause overstimulation of the β-adrenergic function. Therefore, exogenous application of H_2S may antagonize the negative consequences of sympathetic overactivation during ischemia by generating negative feedback to cAMP production. In this regard, H_2S replacement therapy may be a significant cardioprotective and antiarrhythmic intervention for those patients with chronic ischemic heart disease whose plasma H_2S level is reduced (103) (Fig. 7).

(3). Inhibition of NHE. Intracellular pH (pH_i) is an important endogenous modulator of cardiac function. The inhibition of NHE-1 protects the heart by preventing Ca^{2+} overload during I/R. H_2S was reported to produce cardioprotection. Hu *et al.* recently reported that NaHS (100 μM) may induce intracellular acidosis *via* suppression of NHE-1 and further produce cardioprotection. This effect was mediated by the activation of phosphoinositide 3-kinase (PI3K)/Akt/protein kinase G (PKG) (92). This action further offers cardioprotective effects against I/R-induced injury.

(4). Opening of K_{ATP} *channels and/or blockade of* Ca^{2+} *channels.* The opening of sarcolemmal and/or putative mitochondrial K_{ATP} channels has been extensively documented as mediating the protection against I/R injury. Johansen *et al.* first tested the involvement of K_{ATP} channels in the cardioprotection of NaHS (104). The injury-limiting action

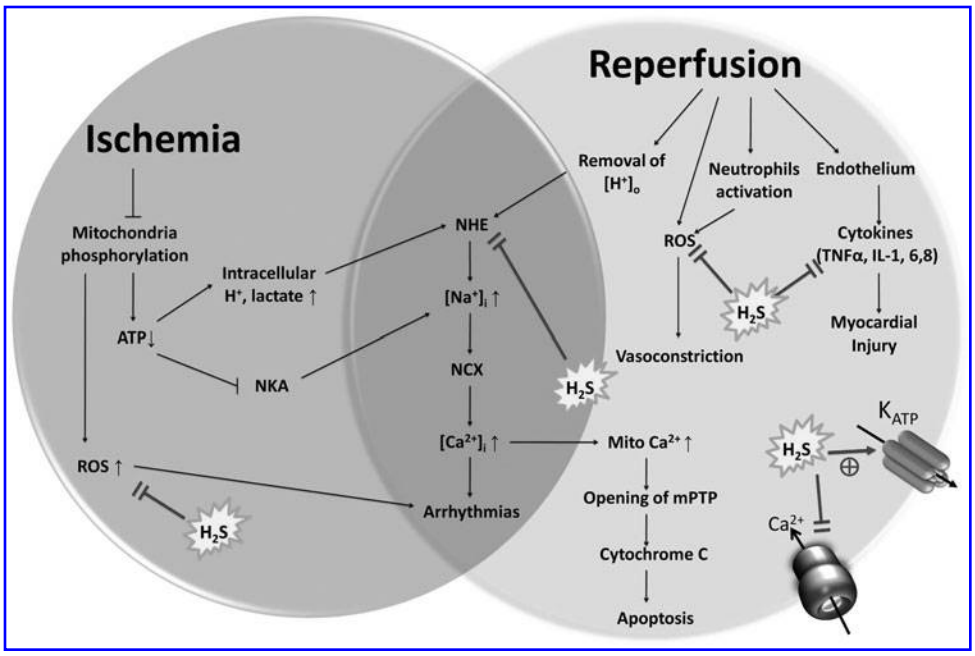


FIG. 7. H_2S-induced protection against I/R injuries. By inhibiting Na^+/H^+ exchanger (NHE) activity, H_2S brings about a decrease in intracellular Ca^{2+} and the attenuation of arrhythmias induced by both ischemia and reperfusion. Furthermore, H_2S may further protect against ischemia- and reperfusion-induced injury by attenuating the accumulation of oxidative stress. H_2S was also found to inhibit the cytokines that are responsible for reperfusion-induced myocardial injury. H_2S actions on the K_{ATP} channels, and the Ca^{2+} channels further protect the myocardium against reperfusion-induced injuries.

of NaHS was abolished by glibenclamide, a general nonselective blocker of K_{ATP}, or 5-hydroxydecanoate (5-HD, a selective mitochondrial K_{ATP} channel blocker), suggesting that the K_{ATP} channels play a central role in the cytoprotective action of H_2S (104). Activation of the K_{ATP} channels induces K^+ efflux, which causes hyperpolarization. This may close LTCC. LTCC can also be closed by the H_2S-suppressed cAMP/PKA pathway (298). Alternatively, H_2S may directly block LTCC (239). All these may further reduce Ca^{2+} influx and shorten APD to produce an antiarrhythmic effect (Fig. 4).

(5). Anti- and proinflammatory factors. Cytokines mediate the development of ischemic injury in the heart (194). IL-6 and IL-8 are released on myocardial I/R damage. The former depresses myocardial function (90), whereas the latter increases neutrophil adhesion and inflammatory responses (122). TNF-α may play multiple roles in the pathogenesis of myocardial I/R injury by inducing EC adhesion molecules, allowing for neutrophil infiltration (55), amplifying the inflammatory response, increasing the production of ROS, and having direct myocardial depressant and apoptotic actions.

The effect of H_2S on inflammation is dubious. Whiteman and Winyard reviewed 14 studies showing an anti-inflammatory effect of H_2S, and 15 studies showing a proinflammatory effect of H_2S (276). It has been reported that H_2S may increase intercellular adhesion molecule-1 (ICAM-1) level in blood vessels (305) and stimulate the production of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) *via* activation of the extracellular signal-regulated kinase (ERK)-NF-κB signaling pathway in human monocytes (313). In animal experiments, the biosynthesis of H_2S was found to be increased along with inflammation-associated septic shock, hemorrhagic shock, and pancreatitis, while the inhibition of H_2S biosynthesis alleviated such inflammation (132).

On the other hand, anti-inflammatory effects of H_2S have also been displayed by its ability to interfere with neutrophil activity, suppress granulocyte adherence to the endothelium and infiltration, and suppress the expression of TNF-α (232, 272). In myocardial I/R experiments, Elrod *et al.* have demonstrated that at the time of heart reperfusion, H_2S decreased the number of leukocytes within the ischemic zone (61). Quantitative myeloperoxidase (MPO) analysis further confirmed a significant decrease in neutrophils within the myocardial tissue after I/R. Intravital microscopy demonstrated that H_2S was a potent inhibitor of *in vivo* leukocyte-EC interactions. The valuation of inflammatory cytokines revealed that H_2S decreased myocardial IL-1β (61). Using the ischemic porcine heart, Sodha *et al.* found that NaHS treatment decreased TNF-α, IL-6, and IL-8 levels. Therefore, inhibition of leukocyte transmigration and inhibition of cytokine release are possible mechanisms by which H_2S restrains the extent of inflammation, thereby limiting the extent of MI (231). Wallace *et al.* recently reviewed the role of endogenous H_2S in resolving inflammation and injury (254). The authors suggest that H_2S-releasing agents may be clinically useful.

However, since the effects of H_2S on inflammation are conflicting, more work has to be done to fully understand its biology and implications.

(6). Preservation of mitochondrial function. In dogs subjected to 90 min of cardiopulmonary bypass (CPB) and 60 min of cardiac arrest, Na_2S (1 mg/kg/h infusion) improved ventricular functions by preserving cardiac ATP pools (242). In addition to being the site of energy production, mitochondria are a central locus in the regulation of cell death. The maintenance of oxidative phosphorylation for preventing myocyte death after ischemic injury has long been recognized as a critical event after MI. Elrod and colleagues found a dose-dependent reduction in mitochondrial oxygen consumption followed by a complete recovery to baseline level after the administration of H_2S (1–50 μM) (61). H_2S preserves mitochondrial function 24 h after reperfusion as noted by increased complex I and II efficiency (6). Moreover, electron microscopy revealed a striking reduction in mitochondrial swelling and increased matrix density in H_2S treated mice, further suggesting a prominent role of H_2S in the preservation

of mitochondrial function in the observed cytoprotection (61). H_2S may also protect mitochondrial function by inhibiting respiration, thus limiting the generation of ROS and diminishing the degree of mitochondrial uncoupling, leading to decreased infarct size and preserved function (61). Intriguingly, reports demonstrated that a low concentration of H_2S ($<20\,\mu M$) stimulates oxygen consumption and increases membrane potential (80, 299). The endogenous role of H_2S *in vivo*, therefore, is yet to be fully understood.

(7). Antiapoptosis. The preservation of mitochondrial function and structure is linked to the inhibition of cell apoptosis. Early in apoptotic signaling, the antiapoptotic B-cell lymphoma 2 (Bcl-2) and proapoptotic Bcl-2-associated death promoter (Bad) are in opposition. A shift in favor of the proapoptotic proteins subsequently results in increased mitochondrial pore permeability, releasing cytochrome C and apoptosis-inducing factor (AIF), which can activate caspase-3, and facilitate DNA fragmentation. On cleavage of terminal caspases, cell death is generally thought to be inevitable.

It was found that NaHS (10–500 μg/kg) treatment suppressed the activation of caspase-3, poly (ADP-ribose) polymerase and/or terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive nuclei in both mice (61) and swine (230), suggesting that H_2S was capable of inhibiting the progression of apoptosis after I/R injury.

However, Osipov *et al.* found that infusion of H_2S (IK-1001, 2 mg/kg/h), while exerting cardioprotective effects, failed to significantly alter the activation of caspase-3, Bcl-2, and Bad. A single bolus injection of IK-1001 just before infusion led to increased Bcl-2 and Bad, though the elevation did not reach statistical significance among six pigs per group being examined. These results indicate that H_2S administration by bolus injection may trigger Bcl-2 and Bad, but these do not underlie the protective effects seen in H_2S infusion alone (186). AIF and Bnip-3 (Bcl-2/adenovirus E1B 19 kDa-interacting protein), on the other hand, were significantly reduced on H_2S treatments in both infusion alone and bolus injection before infusion groups, suggesting that H_2S more likely attenuates the expression of proapoptotic proteins *via* caspase-independent cell death (186).

Survivin is an antiapoptotic gene implicated in the initiation of mitochondrial-dependent apoptosis. In an *in vivo* ischemic reperfusion rat model, the administration of NaHS (14 μmol/kg/day) for 6 days before surgery significantly upregulated survivin mRNA and protein expressions by 3.4-fold and ~1.7-fold, repectively (317), suggesting another route of action for H_2S-induced cardioprotection.

Glycogen synthase kinase-3 (GSK-3β) has been proposed as a viable target in the ischemic injury of the heart. Its activity has been associated with both apoptosis and cell survival. Osipov *et al.* found that H_2S infusion leads to a higher expression of the phosphorlyated form of GSK-3β (186). Similarly, Yao *et al.* also demonstrated that NaHS (30 μmol/kg) increased the phosphorylation of GSK-3β (Ser9) and, thus, inhibited the opening of mPTP, preventing apoptosis and protecting the heart against ischemic damage (291).

(8). Preservation of endothelial function. In the canine CPB model, Na_2S (1 mg/kg/h) infusion restored the sensitivity of coronary arteries to acetylcholine (Ach)-induced vasorelaxation, suggesting that H_2S improves conditions of endothelial dysfunction during cardiac injury (242). Similarly, in a porcine CPB model, H_2S treatment (IK-1001 0.2 mg/kg bolus injection and infusion 2 mg/kg/h) improved endothelium-dependent coronary arteriole relaxation (186).

(9). Attenuation of endoplasmic reticulum stress. Hyperhomocysteineamia (HHcy) is an independent risk factor for CVD (21, 40, 50, 162, 216, 234). HHcy is a medical condition characterized by an abnormally high level of homocysteine in circulation, and homocysteine at a higher level was reported to inhibit CSE activity (290), causing a reduction in H_2S production *via* substrate inhibition, as homocysteines contribute to H_2S synthesis only to a small extent as compared with cysteines (41, 235). Wei *et al.* showed that NaHS (14 μmol/kg/day) protected the heart against HHcy-induced cardiomyotic injury *via* attenuation of cardiomyocytic endoplasmic reticulum stress-associated proteins such as glucose-regulated protein 78, C/EBP homologous protein, and caspase-12 (269).

2. Cardioprotection caused by H_2S preconditioning. IPreC is a powerful natural cardioprotective mechanism, and was later reported to offer similar protective effects in various other systems (*e.g.*, liver, kidney, brain, and ECs). Bian *et al.* first reported that H_2S preconditioning (SPreC) protects the heart against I/R-induced arrhythmias, cell injuries and death, contractile dysfunction, and MI (17, 94, 96, 187–189). Subsequently, Calvert and colleagues confirmed that SPreC produces cardioprotection against MI-induced injury in mice (36). A more detailed summary is presented in Table 2.

Pan *et al.* compared the cardioprotective effects caused by SPreC and postischemic treatment. The infarct per area at risk in the SPreC group was significantly lower than those in the postischemic H_2S treatment group (187). In addition, it was found that H_2S direct treatment had no significant effect on sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) function (298), whereas SPreC significantly stimulated SERCA activity (189). These findings suggest that apart from the mechanisms discussed in Mechanisms for H_2S-induced cardioprotection section, SPreC may stimulate additional pathways that produce stronger protective effects.

The beneficial effects of postischemic H_2S treatment may rely mainly on the ability of sulfide to reduce inflammatory responses (302) and to neutralize cytotoxic reactive species such as peroxynitrite ($ONOO^-$) (272), which may partly relieve the oxidative stress but is not likely to rescue already infarcted myocardium (See mechanisms discussed in Cardioprotective effects of H_2S against ischemia-reperfusion-induced injury section). However, SPreC is more likely to protect by switching the heart to a defensive state against ischemic insult. For example, the activation of protein kinases (*e.g.*, PKC, MAPK, and Akt) would lead to the phosphorylation of many cardioprotective proteins (*e.g.*, heat shock proteins [HSPs], cyclooxygenase [COX]-2, and Bcl-2) (Fig. 8).

a. Mechanisms for the cardioprotection of SPreC. A series of experiments was conducted to probe the signaling mechanism(s) involved in the late cardioprotection of SPreC. The mechanisms are discussed next.

(1). K_{ATP} channels. The involvement of the K_{ATP} channels in the cardioprotection of preishcemic H_2S treatment was first demonstrated by Pan *et al.* (188). Opening of the K_{ATP} channels affords protection by shortening APD and by

Table 2. Cardioprotection of Hydrogen Sulfide Preconditioning Against Ischemic Injury

H_2S Preconditioning	*I/R protocol*	*Species/tissue*	*Effects of NaHS*	*Mechanism*	*Ref.*
Late: After preconditioning with NaHS (100 μM) for 30 min, cells were cultured in normal medium for 20 h	I (5 min)/R (10 min)	Rats/ cardiomyocytes	Cell viability (↑), LDH (↓), improvement of calcium handling	K_{ATP}, NO	(188)
Early: Three cycles (NaHS 100 μM for 3 min each cycle separated by 5 min of recovery)	I (30 min)/R (10 min)	Rats/ cardiomyocytes	Antiarrhythmias, Cell viability (↑), improvement of $[Ca^{2+}]_i$ handling	K_{ATP}	(17)
Late: After preconditioning with NaHS (100 μM) for 30 min, cells were cultured in normal medium for 20 h	I (5 min)/R (10 min)	Rats/ cardiomyocytes	Cell viability (↑), LDH (↓), improvement of contractile function	COX-2/PGE2	(94)
Late: After preconditioning with NaHS (100 μM) for 30 min, cells were cultured in normal medium for 20 h	I (5 min)/R (10 min)	Rats/ cardiomyocytes	Cell viability (↑), improvement of $[Ca^{2+}]_i$ handling	PKC	(189)
Early: Three cycles (NaHS 100 μM for 3 min each cycle separated by 5 min of recovery)	I (35 min)/R (60 min)	Rats/ Langendorff hearts	Antiarrhythmias, Cell viability (↑), improvement of contractile function	ERK, Akt	(96)
Late: NaHS (0.1–1 μmol/kg i.p.) 1, 3 or 5 day before MI	Permanent MI	Rats/*in vivo*	MI (↓)	PKC	(187)
Early: Na2S (100 μg/kg i.v.) 30 min or 2 h before MI Late: Na_2S (100 μg/kg i.v.) 1 day before MI	I(45 min)/R (24 h)	mice/*in vivo*	MI (↓)	Early: Antioxidant (Nrf2), PKCε, STAT-3 Late: Antioxidants (Heme oxygenase-1 and thioredoxin 1), hsp90, 70, antiapoptosis, COX-2	(36)

COX, cyclooxygenase; H_2S, hydrogen sulphide; LDH, lactate dehydrogenase; NO, nitric oxide; Nrf2, nuclear factor-erythroid-derived 2 related factor 2; PKC, protein kinase C; STAT, signal transducer and activator of transcription.

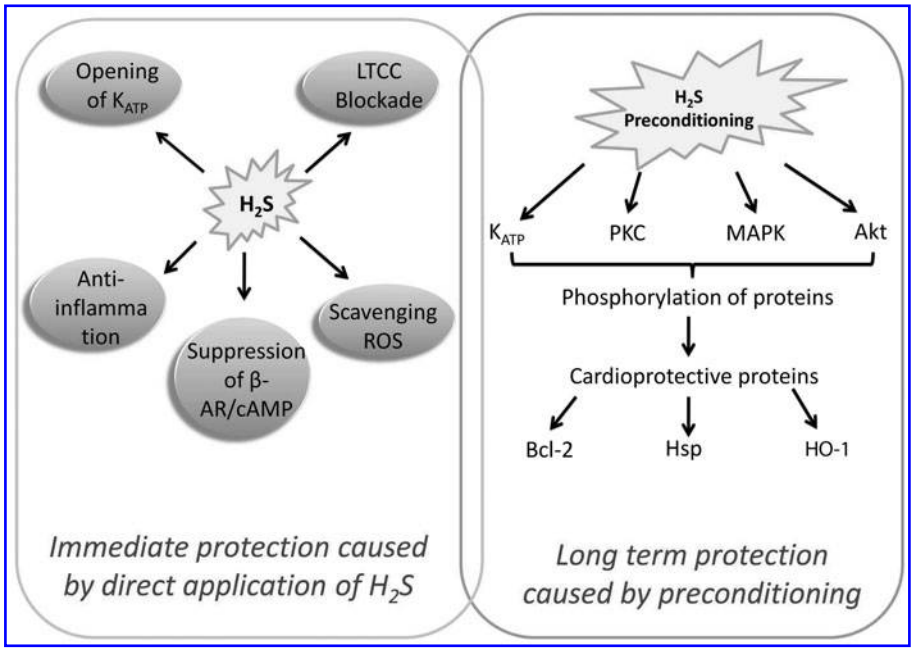


FIG. 8. Different mechanisms underlying the cardioprotection produced by direct application of H_2S and H_2S preconditioning. The acute effect of H_2S-induced cardioprotection involves the actions of the K_{ATP} channel opening, blockade of LTCC, reduction of ROS, inhibition of the β-adrenoceptor/cAMP pathway, and anti-inflammatory responses. In addition, H_2S offers chronic protection *via* numerous signaling mechanisms involving the protein kinase C (PKC) pathway, mitogen-activated protein kinase (MAPK) pathway, serine/threonine protein kinase Akt pathway, and the opening of K_{ATP} channels. A cascade of proteins would then be phosphorylated, and cardioprotective proteins, including B-cell lymphoma 2 (Bcl-2), heat shock proteins (Hsp), and heme oxygenase-1 (HO-1), would be regulated.

preventing calcium overload (83) as just discussed in the K_{ATP} channels section. However, the K_{ATP} channel opening also exerts a cardioprotective effect *via* more complicated crosstalk with other rescuing pathways. The opening of the K_{ATP} channels and activation of protein kinase C (PKC) have been shown to be co-dependent during IPreC (72, 139). The crosstalk among the K_{ATP} channels and protein kinases (see next section) suggests that, similar to other preconditioning events, SPreC may orchestrate a vast network of rescuing pathways that execute the prosurvival signals.

(2). Protein kinase C. The PKC family consists of at least 10 isoforms, of which PKC-α, ε, and δ are the prominent isoforms expressed in the heart (157). Pan *et al.* found that the SPreC induced the translocation of all three prominent isoforms of PKC from the cytosol to the membrane (189). Such translocation before an ischemic attack may offer the cells tolerance to the insults.

H_2S was reported as exerting a direct effect on the K_{ATP} channels (245, 309); hence, the effect of SPreC on PKC might only be secondary to the opening of the K_{ATP} channels. However, Pan and colleagues observed that blockade of the K_{ATP} channel by glibenclamide not only blocked SPreC-induced translocation of PKCε, but also failed to affect the translocation of PKCα and δ (189). Thus, the K_{ATP} channel opening may only activate PKCε translocation in the SPreC signaling pathway. PKC can also be activated by other signaling molecules such as NO or Ca^{2+} (168, 193). More studies are warranted to test whether SPreC induces activation of PKCα and PKCδ through provoking the release of these signaling molecules.

(3). Prevention of intracellular calcium overload and hypercontracture. A timely reduction of the elevated $[Ca^{2+}]_i$ during an ischemic challenge could be of therapeutic importance, as Ca^{2+} overload could induce irreversible injuries such as mitochondria dysfunction (166), membrane degradation, and contractile derangement (82). Pan *et al.* found that SPreC lowered the elevation of $[Ca^{2+}]_i$ during ischemia. This is mediated by the stimulatory effect of H_2S on SERCA2 and NCX (189).

Elevated $[Ca^{2+}]_i$ may also stimulate myocytes that cause hypercontracture as determined by cell length shortening a few minutes after the onset of reperfusion (224). Pan *et al.* found that SPreC significantly attenuated I/R-induced myocyte detrimental shortening (189). This protective effect of H_2S is PKC dependent (189).

(4). NO. In the past decade, many studies have revealed a critical role of NO in IPreC-induced cardioprotection (22). Importantly, NO alone is also sufficient to induce late cardioprotection against myocardial ischemia (243). Pan *et al.* found that the inhibition of NO production with N (G)-nitro-L-arginine methyl ester (L-NAME), a nonselective inhibitor of all NOSs, significantly attenuated the cardioprotective effects of SPreC, but not those of IPreC. Therefore, NO may well be the main reason that NaHS produced a stronger protective effect on cell viability in SPreC than that in IPreC (188).

The administration of NaHS (50 μmol/kg/day) 1 h before ISO-induced toxic cardiomyopathy produced cardioprotection along with significantly increased myocardial and serum NO levels (232). More evidently, the coadministraion of NOS inhibitor (L-NAME) reversed the protective effects of H_2S, indicating that H_2S-induced cardioprotection may be mediated by NO.

(5). ERK1/2-MAPK. ERK 1 and 2 belong to the MAPKs family, which includes ERK1/2 and stress-activated protein kinases containing Jun N-terminal Kinase 1/2 and p38-MAPK. It is commonly accepted that phosphorylation of ERK1/2 in cardiomyocytes during early reperfusion serves as a defense mechanism against ischemic stress stimuli (301). This defense mechanism can be magnified by IPreC and several forms of pharmacologically induced preconditioning (86, 248). Hu *et al.* observed that SPreC protected the heart against I/R injury by reducing myocardial injury (96). The blockade of ERK1/2 with PD98059 during either preconditioning or ischemia periods reversed these cardioprotective effects, suggesting that ERK1/2 may at least partly mediate the cardioprotection afforded by SPreC (96). This was further confirmed by the demonstrable phosphorylation of ERK1/2 (96).

(6). PI3K/Akt. The activation of the PI3K/Akt pathway has been demonstrated as playing a key role in both early and delayed myocardial preconditioning (86–88). Hu *et al.* found that SPreC induced a significant phosphorylation of Akt in the isolated rat hearts, and, more importantly, the cardioprotective effects of SPreC were greatly attenuated by inhibition of PI3K and Akt during preconditioning and ischemia (96). These data suggest that, similar to ERK1/2, PI3K/Akt may serve as a trigger and mediator in SPreC-induced cardioprotection.

(7). COX-2/PGE2 pathway. COX, the rate-limiting enzyme in prostaglandin synthesis, catalyzes the conversion of arachidonic acid (AA) to prostaglandin H_2. Two distinct COX isoforms have been characterized. COX-1 is constitutive and COX-2 is usually induced in response to stress and inflammation. There is accumulating evidence that COX-2 plays an essential role in mediating the late phase, but not the early phase, of IPreC-induced cardioprotection (261, 293).

Hu *et al.* found that the blockade of COX-2 with two selective inhibitors, NS-398 and celebrex, attenuated SPreC-induced cardioprotection (94). Moreover, SPreC significantly increased PGE_2 formation at the end of lethal ischemia, indicating that PGE_2 is likely to be one of the mediators of COX-2-dependent protection produced by SPreC (94). The administration of another selective COX-2 inhibitor, Celecoxib, abolished the protective effects of NaHS (50 μmol/kg/day) pretreatment in ISO-induced MI (232). Collectively, the current evidence implies that COX-2 may mediate the cardioprotective effects of H_2S.

(8). Nuclear factor-erythroid-derived 2 related factor 2/antioxidant. Nuclear factor-erythroid-derived 2 (NF-E2) related factor 2 (Nrf2), a member of the NF-E2 family of nuclear basic leucine zipper transcription factors, regulates the gene expression of a number of enzymes that serve to detoxify prooxidative stressors. This regulation is mediated by Nrf2 binding to the antioxidant responsive element, a *cis*-acting regulatory element, or enhancer sequence, found in the promoter region of certain genes, including HO-1 and thioredoxin-1 (Trx1). During I/R, the activities of many endogenous antioxidant enzyme systems are compromised or even abolished. Calvert *et al.* found that H_2S (100 μg/kg) induced

the nuclear accumulation of Nrf2 very rapidly after its administration and subsequently increased the protein expression of HO-1 and Trx1 (36). These results suggest that H_2S therapy may enhance the endogenous antioxidant defenses of myocytes and create an environment resistant to the oxidative stress associated with myocardial I/R injury, as evidenced by the preservation of redox state and a reduction in lipid peroxidation.

The ability of a cell to counteract stressful conditions, known as cellular stress response (30, 33), requires the activation of prosurvival pathways. These pathways, under the control of protective genes called vitagenes (31), result in the production of various molecules (*e.g.*, HSPs, GSH, and bilirubin) endowed with antioxidant and antiapoptotic activities (32). The vitagene network relevant to cytoprotection could be a potential target for the cardioprotective effects of H_2S.

(9). Antiapoptosis. Myocardial I/R induces apoptosis and necrosis. Calvert *et al.* found that SPreC preserved uncleaved caspase-3, decreased cleaved caspase-3, inhibited the translocation of cytochrome C, and decreased the number of TUNEL-positive nuclei (36). The antiapoptotic actions were mediated in part by the phosphorylation and inhibition of the proapoptotic factor Bad, an upregulation of the prosurvival factors Bcl-2 and Bcl-xL, and an upregulation of HSPs. During the early preconditioning period (30 min and 2 h), H_2S activated PKCε, ERK1/2-MAPK, and signal transducer and activator of transcription (STAT)-3. The STAT pathway has been shown to be an integral part of the response of the myocardium to various cardiac insults, including MI (9).

(10). Heat shock proteins. HSPs have also been demonstrated as providing cardioprotection in the setting of I/R. In particular, HSP70 suppresses apoptosis in a caspase-dependent (207) and caspase-independent manner (202). During the late preconditioning period (24 h), SPreC increased the expression of HSP90 and HSP70 (36). These data suggest that SPreC not only reduces apoptosis through a reduction in oxidative stress, but also promotes direct antiapoptotic signaling.

In contrast, a bolus injection of Na_2S (IK-1001, Ikaria Inc) before CPB together with continuous IK-1001 infusion resulted in reduced expressions of HSP70, enhanced expression of HO-1, and no significant difference in HSP90 expression (186). The controversies in findings, therefore, warrant further investigation and in-depth studies.

Most concentrations used in SPreC in the *in vitro* studies just mentioned were within 10–100 μM. Interestingly, it was found that with concentrations higher than 100 μM, the cardioprotection induced by SPreC may be weaker. The possible explanation for the phenomenon just mentioned might be the involvement of hormesis (30), which refers to generally favorable biological responses to low exposures to many stressors (29). H_2S showing hormesis, thus, has the opposite effect in small doses as in large doses. The biochemical mechanisms for hormesis are still not well understood. It is conjectured that low doses of H_2S or other stressors might activate the repair mechanisms of the body. The repair process fixes not only the damage caused by the toxin, but also other low-level damage that might have accumulated before the repair mechanism was triggered.

B. Heart failure and other related heart diseases

CHF is a growing health concern. In the United States, for example, it affects 5.2 million Americans with more than 400,000 new cases being diagnosed each year (204). In 2007, it has been associated with one in every nine deaths (204). However, existing treatments are far from adequate, and the search for new treatment strategies is crucial and urgently needed.

The main pathological mechanism for CHF appears to be oxidative stress (Fig. 9). When the local levels of ROS are high, they tend to react with numerous protein centers, DNA, cell membranes, and other molecules, causing considerable cellular damage as well as generating other more reactive radicals (215). At lower concentrations, however, local targeted ROS serves as a second messenger system that transmits biological information through the highly specific modulation of small intracellular molecules and proteins (215). Redox signaling processes are involved in the activation of many signal transduction protein kinases and transcription factors, the stimulation of DNA synthesis, and the expression of growth-related genes. These effects are relevant not only to CHF but also to its predisposing conditions, such as LV hypertrophy and adverse remodeling after MI (215) (Fig. 9). H_2S is a ROS scavenger and an antioxidant activator (167). The application of H_2S donors may, therefore, be potentially useful for treating heart failure (HF) Table 3.

1. Endogenous H_2S production in CHF. MI is one of the leading causes for HF. Wang *et al.* found that the plasma H_2S level decreased from 65 to 53 μM in an MI-induced CHF model (259). In addition, endogenous H_2S synthesis in the heart was also found to be lowered in hearts of arteriovenous fistula (AVF)-induced CHF model (167, 217) and adriamycin (ADR)-induced cardiomyopathy model (236). This was further supported by transgenic mice overexpressing CSE, as excessive H_2S production protects against HF injuries in both the permanent LCA ligation model and the LCA I/R model (35).

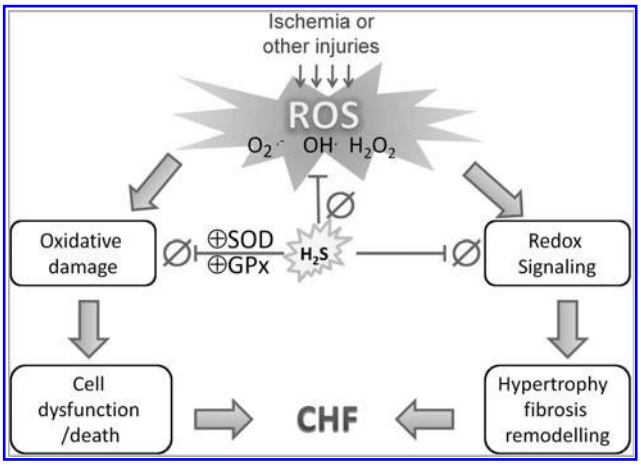


FIG. 9. Effect of H_2S on the pathogenesis of heart failure. H_2S was shown to protect against chronic heart failure (CHF) by scavenging ROS, inhibiting redox signaling, and attenuating oxidative damages. The action of H_2S in reducing oxidative damage is partially mediated by the activation of superoxide dismutase (SOD) and glutathione peroxidase (GPx).

Table 3. Protective Effects of Hydrogen Sulfide Against Heart Failure

Experimental model	*Species*	*H_2S treatment*	*Results*	*Conclusions*	*Proposed mechanism(s)*	*Ref.*
Permanent ligation of the LCA	CSE overexpression transgenic mice (MHC-CGL-Tg^+) *vs.* C57BL6/J mice	NA	Transgenic mice displayed: 68%↑in survival rate smaller↑in LVEDD, LVESD and heart to body weight ratio	CSE overexpression reduced LV dilatation and cardiac hypertrophy	Transgenic mice hearts expressed: ↑Nrf2 and NRF-1 ↑Akt	(35)
60 min of LCA occlusion followed by 4 weeks of reperfusion			Transgenic mice displayed: 38%↓in infarct area smaller↑in LVEDD, LVESD, and heart to body weight ratiobetter LV ejection fraction	↑production of H_2S during reperfusion has positive impact on LV structure and function		
	C57BL/6J mice	Single bolus of Na_2S at reperfusion (100 μg/kg, i.c)	24 h reperfusion: 14%↓in infarct area/area at risk 20%↓in infarct area/LV 4 weeks reperfusion: 25%↓in infarct area/LV No change in LVEDD, LVESD, heart:body weight ratio, LV ejection fraction, or heart rate	Single administration of H_2S at reperfusion improves infarct size, but not sufficient to improve LV function at 4 weeks	↑nuclear localization of Nrf2 and NRF-1 ↑Akt phosphorylation in heart at serine residue 473 Attenuation of oxidative stress ↑mitochondrial respiration and ATP synthesis, but no effect on mitochondrial biogenesis	
		Na_2S (100 μg/kg, i.v.) during first 7 days of reperfusion	Na_2S treatment: 25%↓in infarct area, ↓in LV dilatation and cardiac hypertrophy improved cardiac function	H_2S during first 7 days of reperfusion is critical for sustained improvements in LV structure and function		
Arteriovenous fistula (AVF)—volume overload	C57BL/6J mice	NaHS; 30 μM in drinking water	H_2S treatment: ↓heart weight ↓collagen, ↓fibrosis ↓caspase-3 and apoptosis ↓nitrotyrosine formation ↓MMP-9 and MMP-2 activation ↑TIMP-4, ↓TIMP-1 and TIMP-3 ↑β1-integrin, ↓ADAM-12	H_2S ↓oxidative and proteolytic stresses improved cardiac histology by ↓fibrosis and apoptosis	↓oxidative and nitrosative stresses Reversed altered expression of MMPs, TIMPs, β1 and ADAM-12	(167)
Aortic banding (AB)—pressure overload	C57BL/6J mice	NaHS; 30 μM in drinking water	H_2S treatment: ↓in LV chamber diameters restored hemodynamics parameters of heart-EF, EDP, ESP, dP/dt max and SV ↑expression of MMP-2, CD31 and VEGF ↓expression of MMP-9, endostatin, angiostatin, TIMP-3	H_2S ↓dilatation of heart ↑LV functional status promote angiogenic inhibit antiangiogenic factors	↑MMP-2 activation to promote VEGF synthesis and angiogenesis ↓MMP-9, TIMP-3 levels and antiangiogenic factors	(77)
Ligation of left anterior descending coronary artery	Sprague-Dawley rats, male	NaHS (3.136 mg/kg/day)	H_2S treatment: ↑survival rate by 15% ↑LVSP ↓LVEDP ↑LV± dp/dt ↓lung:body weight ratio ↓fibrosis area/total LV area ↑CSE, Bcl-2 expression ↓Bax expression ↓mitochondrial:cytoplasm cytochrome C and caspase-3 activation	H_2S improve cardiac functions ↓pulmonary oedema ↓fibrosis ↓cardiac apoptosis	↓leakage of cytochrome c protein from mitochondrial to cytoplasm to improve mitochondrial derangements ↑Bcl-2 protein and mRNA expression ↓Bax and caspase-3 protein and mRNA expression	(259)

ADAM-12, disintegrin and metalloproteinase domain-containing protein 12; Bcl-2, B-cell lymphoma 2; LCA, left coronary artery; LVEDP, LV end diastolic pressure; NRF-1, nuclear respiratory factor 1; TIMP, tissue inhibitor of matrix metalloproteinases; VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinases. NA, not applicable.

2. Effect of H_2S on HF, hypertrophy, and cardiomyopathy. Cardiac hypertrophy as a result of sustained overload can lead to progression of HF. H_2S has been reported as inhibiting hypertrophy (145) as well as its transition to HF (77). In rat primary cultures, NaHS (100 μM) pretreatment prevented cardiomyocyte hypertrophy by lowering intracellular ROS, upregulating microRNA-133a, and suppressing microRNA-21 (145). Transgenic mice with cardiac-restricted overexpression of CSE displayed a clear protection against LV structural and functional impairment as assessed by echocardiography in response to ischemia-induced HF (35). The endogenous overexpression of CSE reduced LV dilation and cardiac hypertrophy. The exogenous application of NaHS (100 μg/kg or 30 μM in drinking water) also produced similar cardioprotective effects in different HF models (35, 167, 219, 259) and attenuated the development of ADR-induced cardiomyopathy (236) and hypertrophy in spontaneously hypertensive rats (SHR) (219).

The induction of antioxidative responses is an important mechanism for the protective effect of H_2S. Na_2S (100 μg/kg) treatment significantly protected the heart against ischemia-induced lipid hydroperoxidation (LPO) in HF (35). Nrf2 is a key transcription factor involved in the regulation of antioxidant genes as well as mitochondrial biogenesis through the upregulation of nuclear respiratory factor 1 (NRF-1). Calvert *et al.* found that treatment with H_2S for 7 days stimulated Akt and nuclear localization of NRF-1 and Nrf2. However, H_2S therapy failed to increase mitochondrial biogenesis and did not significantly improve mitochondrial function 4 weeks after MI, although ATP synthesis was slightly improved. The slight improvements are more likely attributed to the ability of H_2S to reduce oxidative stress, suggesting that in this model of HF, the antioxidant effects of H_2S may play a more prominent role in mediating its cardioprotective actions (35).The protective effects of H_2S in the cardiomyopathy model were also attributed to its antioxidant effects (236). NaHS (100 μg/kg) treatment inhibited LPO and increased superoxide dismutase (SOD) and GSH peroxidase activities. Therefore, treatment with NaHS stimulates the activity of antioxidant enzymes. The protective effects against ischemia-induced injury are also mediated by the antiapoptotic effects of H_2S (259). In an ischemia-induced HF model, NaHS reduced the number of apoptotic cells through promoting the expression of antiapoptotic factor Bcl-2 while suppressing the expressions of proapoptotic factors Bax and caspase-3. NaHS (3.136 mg/kg/day) treatment also significantly reduced the release of cytochrome c from the mitochondria and, therefore, protected the heart against ischemic injury during HF (259).

H_2S may also protect against HF *via* promoting angiogensis. One group of researchers reported that NaHS (30 μM in drinking water) reversed the alteration of various matrix metalloproteinases and tissue inhibitor of matrix metalloproteinases (TIMP) in response to cardiac insults, and these factors resulted in enhanced angiogensis in H_2S-treated animals (77, 167). However, it should be taken into consideration that H_2S is known to be very volatile, has a very short half life in a solution when exposed to air, and may escape readily within minutes. Furthermore, NaHS at 30 μM produces strong irritating odor, and its dissociation ions (HS^- or S^{2-}) may change the taste of water. The amount of water intake by H_2S-treated animals may differ from those in other groups.

Gap junction channels are specialized intercellular connections between the cytoplasm of two cells, controlling the passage of molecules and ions. A gap junction channel is made up of two connexons, each composed of six connexin proteins. The administration of NaHS (50 μmol/kg/day) and H_2S-donating S-diclofenac (25 and 50 μmol/kg/day) enhanced mRNA and protein expressions of connexin 43 and 45 in doxorubicin-induced cardiomyopathy (304), indicating that H_2S may also protect the heart against HF *via* inhibiting pathogenic gap junction remodeling in the heart.

V. Physiological Function of H_2S in Vascular Tissue

It was first demonstrated by Hosoki *et al.* in 1997 that arteries and veins express CSE and generate H_2S (91). NaHS at concentrations above 100 μM may induce the relaxation of precontracted isolated rat artery (3, 91, 309). Furthermore, perfusion of the rat mesenteric arterial bed with the H_2S precursor, 1 mM cysteine, increased endogenous release of H_2S and relaxed the arterial bed (47). In contrast, NaHS at concentrations below 100 μM may induce further contraction of precontracted isolated vessels (3, 141, 148). Figure 10A and B show the mechanisms accounting for the relaxant and constrictive effects seen with H_2S, respectively. The response of blood vessels to H_2S varies according to the type of vessel: large conductance vessels *versus* small resistance vessels; systemic *versus* pulmonary; the condition of endothelium (intact *vs.* denuded); the precontraction agonist used (*e.g.*, potassium chloride *vs.* phenylephrine); the method of H_2S administration (single *vs.* cumulative application); and the duration, concentration, and rate of change in the concentration of the H_2S administered. The administration of slow-release H_2S donors probably more closely resembles endogenous H_2S release. The concentration of H_2S in blood has been reported to be altered in several pathological states, including patients suffering from CAD (103), hypertension (43), and diabetes (100). Although these changes in H_2S levels reflect changes in the amounts of stored sulfide (due to the methods used to measure blood concentrations), the H_2S concentrations of stored sulfide probably reflect the status of H_2S activity. Whether such changes in the H_2S level are the causes or consequences of these diseases warrants further investigations.

A. H_2S-induced vasorelaxation

As just mentioned, Hosoki *et al.* first found that H_2S can relax portal vein and thoracic aorta (91). The vasodilatory effect of H_2S was later observed in several types of arteries, including mesenteric (47), pulmonary (263), hepatic (63), and tail arteries (309). H_2S-induced vasorelaxation is mainly brought about by the opening of the K_{ATP} channels (47, 121, 309) and partially mediated by endothelium-dependent mechanism(s) (309). Other signaling mechanisms may involve intracellular acidosis (127) and depletion of intracellular ATP levels in aortic rings (116, 240, 268) (Fig. 10A). Since vasorelaxant responses are only observed in *in vitro* organ bath studies at NaHS concentrations above 100 μM, the significance of this effect and its mechanisms are yet to be fully understood *in vivo*.

1. Mechanisms of H_2S-induced vasorelaxation

a. Opening of K_{ATP} channels. The K_{ATP} channel is an inward rectifying channel composed of two types of subunits.

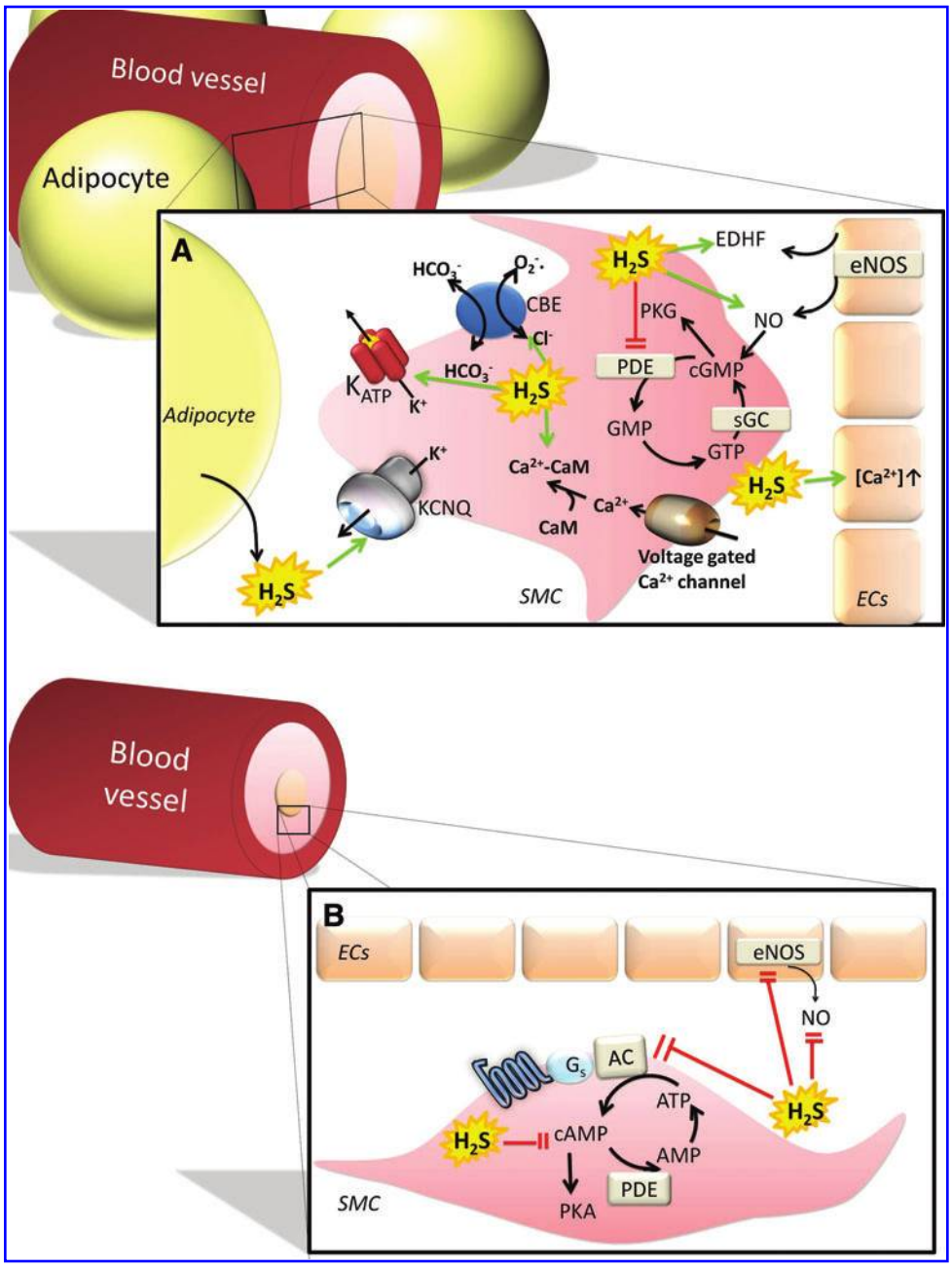


FIG. 10. Mechanisms for H_2S-induced vascular responses. (A) H_2S may induce vasorelaxant responses by the activation of the K_{ATP} channels and Cl^-/HCO_3^- (CBE) channels. H_2S has been implicated as an adipoctye-derived relaxing factor that causes the relaxation of smooth muscle cells (SMCs) by acting on $K_v7.x$ (KCNQ) channels. Other mechanisms of H_2S-induced relaxation include binding of the Ca^{2+}-calmodulin complex; interaction with NO and/or endothelium-derived hyperpolarizing factor (EDHF); inhibition of phosphodiesterase (PDE); and elevation of intracellular Ca^{2+} in endothelial cells (ECs). **(B)** H_2S may induce vasoconstrictive responses by the inhibition of AC, eNOS, and scavenging of NO (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars). eNOS, endothelial NOS.

The smaller pore-forming subunits are the inwardly rectifying potassium channel subunits (Kir6.1 or Kir6.2), while the larger regulatory subunits are the sulfonylurea receptors (SUR1, SUR2A, and SUR2B). The K_{ATP} channel isoforms come from differing combinations of subunits. For example, Kir6.1/SUR2B is found in vascular smooth muscle cells (VSMCs), whereas Kir6.2/SUR2B is found in nonvascular SMCs (64). The K_{ATP} channels exist in the sarcolemmal, mitochondrial, and nuclear membranes of various tissues (316), and are characterized by their inhibition by micromolar concentrations of intracellular ATP (7). Hence, an increase in intracellular ATP closes these channels, leading to cell depolarization. The K_{ATP} channels participate in the control of membrane potentials and the regulation of cellular activities such as energy metabolism, apoptosis, and gene expression.

Zhao *et al.* first reported that glibenclamide, a K_{ATP} channel antagonist, significantly attenuated the vasodilatory effect of H_2S (600 μM), whereas pinacidil, a K_{ATP} channel opener, mimicked the effect of H_2S (300 μM) in a concentration-dependent manner (309). Using the patch clamp technique in single VSMC isolated from the aorta, H_2S induced K^+ currents and led to membrane hyperpolarization, inhibitable by glibenclamide (309). In another study, H_2S elevated whole-cell K_{ATP} currents and membrane hyperpolarization in the VSMCs of rat mesenteric arteries with an EC_{50} value of 116 ± 8.3 μM (245). Moreover, whole-cell K_{ATP} currents could be attenuated by the inhibition of endogenous H_2S production, indicative of basal K_{ATP} stimulation by endogenous H_2S (245). In addition, H_2S (200 μM) enhanced single-channel activity of K_{ATP} channels by increasing the open probability without any effect on single-channel conductance (245).

When the K_{ATP} subunits (*i.e.*, rvKir6.1 and rvSUR1) were heterologously expressed in HEK-293 cells and studied using whole-cell patch-clamp technique (102), it was observed that although both rvKir6.1 alone and rvKir6.1/rvSUR1 co-expressed channels conduct K_{ATP} channel currents, H_2S only elevated K_{ATP} currents generated by the latter (102). This strongly indicates that H_2S acts on SUR1 but not Kir6.1 subunit, of the K_{ATP} channel. It was also reported that expressions of SUR2B were higher in the aorta than in the pulmonary artery, suggesting that the greater K_{ATP} channel density in the aorta may underlie its greater extent of relaxation than that in pulmonary arteries (238).

To localize the target of H_2S action on the K_{ATP} channel, the channel was chemically modified by N-ethylmaleimide (NEM, a cysteine-specific sulfhydryl alkylating agent) or chloramine T (CLT, an agent for oxidation of sulfhydryl groups cysteine and methionine) (102). Both drugs could abolish the stimulatory effect of H_2S on K_{ATP} currents completely. While NEM did not alter rvKir6.1/rvSUR1 currents, CLT inhibited rvKir6.1/rvSUR1 currents by $\sim$45%, suggesting that its high concentration could have oxidized methionine residues besides the intended cysteine sulfhydryl groups. Using site-directed mutagenesis, extracellular cysteine residues on rvSUR1 subunit were replaced with structurally similar serine residues. Single-site mutants on cysteine-6 and cysteine-26, but not cysteine-1051 and cysteine-1057, completely abolished the H_2S effect on rvKir6.1/rvSUR1. Hence, extracellular cysteine residues cysteine-6 and cysteine-26 of rvSUR1 subunit are most likely the targets of H_2S actions (102). In the most recent report published by the same group, the researchers found evidence that H_2S covalently sulfhydrates cysteine-43 on Kir 6.1 to bring about the hyperpolarization which underlies H_2S-induced vasorelaxation (174). Furthermore, H_2S has been shown as enhancing the binding of phosphatidylinositol-4,5-bisphosphate to Kir 6.1 to elicit K_{ATP} activation. Conversely, H_2S attenuated the binding of the inhibitor, ATP, to Kir6.1 (174).

Despite numerous reports and the widely accepted notion that the K_{ATP} channel is the main underlying mediator of most H_2S-induced vascular responses, H_2S-induced vascular relaxation is often only partially inhibited by glibenclamide, and, in some cases, no inhibition can be achieved (42, 116, 268). Cheang *et al.* recently reported that the K_{ATP} channels may not be the underlying mechanism of H_2S effects in rat coronary arteries, suggesting that the effect of H_2S on K_{ATP} channels could be tissue specific (42). There is also no evidence that supports a role for the K_{ATP} channels in the vasoconstrictive action of H_2S (3, 141, 268, 274).

b. Endothelium-dependent mechanism. The endothelium is the production site of NO and perhaps other unidentified endothelium-derived relaxing factors in blood vessels. NO is a potent vasorelaxant, acting in the nanomolar range. Hosoki *et al.* first reported the synergistic effect between NO and H_2S where NaHS was observed as inducing a significantly greater concentration-dependent relaxant response in the presence of an NO donor (sodium nitroprusside [SNP] or morpholinosydnonimine). Interestingly, NaHS at a low concentration (30 μM) that induces vasoconstriction by itself enhanced the NO-induced relaxant effect by up to 13-fold (91). Conversely, blocking endogenous NO production with L-NAME, or physically removing the endothelium, attenuated H_2S-induced relaxation (309). The co-administration of charybdotoxin and apamin to inhibit the classical pathway of endothelium-derived hyperpolarizing factor (EDHF) in endothelium intact vessels also attenuated H_2S-induced relaxation (309). In line with these synergistic effects, the incubation of homogenized rat vascular tissues with SNP increased H_2S production in a concentration-dependent manner (307, 309), an effect that could be abolished by a soluble guanylate cyclase inhibitor (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one). Similarly, the NO donor (diethylamine NONOate) also increased H_2S production rate (307). Moreover, incubation of cultured VSMCs with another NO donor (S-nitroso-N-acetylpenicillamine [SNAP]) increased the transcription level of CSE (309). However, the relationship between NO and H_2S is complicated. Mechanisms of H_2S-induced vasoconstrictions section describes the antagonizing effects of these gasotransmitters.

c. Acidification. The level of pH_i has been shown as regulating vascular tone. A change in pH_i alters $[Ca^{2+}]_i$ in VSMC, which may subsequently lead to changes in force generation *via* modulation of various ion channels and actin/myosin crossbridge activity in a concerted manner (279). In most vascular tissues, it is widely regarded that alkalinization causes contraction, whereas acidification causes relaxation.

Lee *et al.* found that H_2S may induce intracellular acidosis by stimulating Cl^-/HCO_3^- exchanger (CBE) activity (127). Intracellular acidosis can stimulate K_{ATP} channels and, thus, cause vasodilation (227, 260); H_2S may, therefore, induce vasodilation *via* the intracellular acidosis/K_{ATP} pathway. In addition, angiotensin II (Ang II) has been reported as exhibiting a similar pH_i-lowering effect in cat papillary muscles *via* the activation of cardiac CBE in a PKC-dependent manner (37). Thus, more studies are warranted to examine the involvement of PKC in the pH regulatory role of H_2S.

d. Metabolic inhibition. H_2S (320 μM) significantly decreased ATP levels in vascular tissues 10 s after its administration, with an even greater reduction after 3 min. The extent of H_2S-induced relaxation and the time course of H_2S effects on ATP were similar to those induced by hydrogen cyanide (complex IV inhibitor) and 2,4-dinitrophenol (mitochondrial uncoupler) (116). The physiological significance of this finding warrants further investigations using lower H_2S concentration. It was also reported that H_2S-induced relaxation was more pronounced at a low ambient oxygen level, whereas vasoconstriction was enhanced by a high oxygen level (116, 117), probably due to the competition between sulfide and oxygen at the level of cytochrome c (116). Since sustained contraction of blood vessels requires ATP, it was thought that H_2S may induce relaxation *via* metabolic inhibition in vascular tissues, thus reducing ATP and oxygen consumption (116), in line with its cardioprotective effect in the heart (61, 188). Intriguingly, it should be noted that NaHS below 20 μM has shown a stimulatory effect on oxygen consumption (80, 299). As such, the role of H_2S under physiological conditions is still unclear and worthy of in-depth research.

e. Opening of voltage-dependent K^+ channels. The involvement of voltage-dependent K^+ (K_v) channels in the vascular function of H_2S is somewhat controversial. Zhao *et al.* first reported that 4-aminopyridine (4-AP, a specific K_v inhibitor)

failed to affect H_2S-induced vasorelaxation in rat aorta (309). A more recent study using denuded rat coronary artery, however, reported that 4-AP reduced NaHS-induced vasorelaxation when the tissue was precontracted with U46619 (42). Results obtained from these studies suggest that the action of H_2S varies among the type of vascular tissue being examined. While H_2S activates K_{ATP} channels in rat mesenteric arteries (47) and the aorta (309), it appears to activate the K_v channels that induce hyperpolarization in the rat coronary artery (42).

In another study, H_2S was implicated as an adipoctye-derived relaxing factor (ADRF) *via* its action on the KCNQ channels (210). The KCNQ channels are a type of K_v channel residing in many types of vascular tissues. Perivascular adipose tissue has been reported to secrete ADRF in a paracrine fashion that reduces vascular tone (60, 78, 152, 251). The presence of perivascular fat in mesenteric arteries [(+)fat] significantly attenuated 5-HT-induced contraction when compared with fat-free [(−)fat] vessels. This anticontractile effect of perivascular fat can be reversed by 4-AP (K_v channel blocker) or XE991 (KCNQ channel blocker), but not glibenclamide, suggesting that the K_v channels, not the K_{ATP} channels, underlie the differential observations in fat-mediated contractile responses. The inhibition of endogenous H_2S production by incubating aortic rings with CSE inhibitors, β-cyano-L-Alanine (BCA) or PAG, has no effect on (-)fat vessels, but significantly reverses the anticontractile effect of (+)fat vessels. Hence, H_2S could be an endogenous ADRF released by perivascular adipose tissues acting on the vascular tissues to exert its relaxant effects. More direct evidence was provided when (-)fat aortic tissue was preincubated with XE991, which prevented NaHS evoked concentration-dependent vasorelaxations, indicating that KCNQ channels play a role in mediating control of vascular tone by H_2S (210).

f. Increase in cyclic guanosine monophosphate levels. Cyclic guanosine monophosphate (cGMP)/PKG facilitates the dephosphorylation of myosin light chain (MLC), preventing the association of myosin with actin. Bucci *et al.* recently reported that H_2S elevates cGMP levels by inhibiting phosphodiesterase (PDE) activity (24). The incubation of cultured rat aortic SMC with exogenous NaHS (10–200 μM) increased cGMP levels in a concentration-dependent manner. More importantly, CSE overexpression (by CSE cDNA transfection) and CSE gene silencing (using small interfering RNA [siRNA] approach) greatly enhanced and attenuated cGMP levels, respectively, suggesting an endogenous role of H_2S in regulating cGMP levels (24). There is a need, therefore, to re-evaluate the conventional view that H_2S has no effect on cGMP levels (245, 308, 309), and to clarify the inconsistency in observations.

g. Modulation of Ca^{2+} signaling. Both calcium-free bath solution and nifedipine (a Ca^{2+} channel blocker) attenuated H_2S-induced vasorelaxation of rat aortic tissue, implying that it may partly be dependent on Ca^{2+} influx (308). However, in human saphenous vein ECs, NaHS significantly increases $[Ca^{2+}]_i$ with maximal responses observed at 200–500 μM (11). This concentration range, however, is probably beyond a physiological level. Nevertheless, the chelation of extracellular Ca^{2+} with EDTA failed to block this $[Ca^{2+}]_i$ elevation, r evealing that the mobilization of Ca^{2+} came from intracellular sources. In fact, in the absence of extracellular Ca^{2+}, depletion of Ca^{2+} stores by exposing cells to either ATP (to activate P2Y receptors and generate IP_3) or 4-chloro-3-ethylphenol (4-CEP, to activate ryanodine receptors [RyRs]) diminished H_2S-induced $[Ca^{2+}]_i$ rise. Store depletion with SERCA inhibitors, thapsigargin, or cyclopiazonic acid produced similar effects. H_2S probably mobilizes the same pool of intracellular Ca^{2+} store as IP_3 receptors (IP_3Rs) or RyRs (11). The regulatory effect of H_2S on $[Ca^{2+}]_i$ is not mediated by NCX, Ca^{2+}-ATPase, or cyclic ADP ribose (cADPR), as the blockade of NCX with its two structurally distinct inhibitors, SEA0400 or bepidril, Ca^{2+} ATPase with carboxyeosin or cADPR with 8-Br-cADPR (a cADPR antagonist) or nicotinamide (an ADP ribosyl cyclase inhibitor), failed to attenuate the effect of NaHS (11). In some but not all the cells, re-exposure to extracellular Ca^{2+} after the addition and removal of H_2S activated capacitative Ca^{2+} entry (11). Taken together, these data suggest that H_2S modulates endothelial $[Ca^{2+}]_i$ *via* multiple mechanisms.

h. AA cascade. AA is the precursor of eicosanoids such as prostaglandins and leukotrienes, present in abundance in muscles. AA implicated in cell signaling is derived by the action of a phosphatidylcholine-specific cytosolic PLA_2 ($cPLA_2$). NaHS (100 μM–1 mM)-induced relaxation in mesenteric artery beds could be greatly attenuated by 4-(4-Octadecylphenyl)-4-oxobutenoic acid) (OBAA, PLA_2 inhibitor) (53). Moreover, NaHS (1 mM) induced a greater extent of $cPLA_2$ translocation from the cytoplasm to the nucleus, indicative of increased activation of AA synthesis in response to H_2S (53). These authors further demonstrated that H_2S-induced relaxation could also be significantly reduced in the presence of proadifen (PRD, CYP inhibitor), suggestive of the involvement of AA metabolites in mediating H_2S-induced vasorelaxation (53). It should be noted, however, that the concentration of NaHS used in this study was way above the physiological range and, hence, probably lacks relevance to *in vivo* situations. Furthermore, several other studies have reported that prostaglandins and COX have little role in the vascular effects of H_2S (42, 116, 309), hence suggesting probable species or cell specificity for such effects. More studies are needed to further understand the interaction of H_2S with AA and its metabolites.

i. Release of EDHF. EDHF causes vasodilatation by hyperpolarizing VSMCs, particularly in small-resistance arteries. EDHF-induced vasorelaxation is mainly generated by the activation of small and intermediate conductance calcium-activated potassium channels (SK_{Ca}/K_{Ca}2.3 and IK_{Ca}/K_{Ca}3.1). H_2S has been proposed as exhibiting properties that resemble EDHF in several reports (53, 150, 174, 288). As discussed earlier, H_2S-induced vascular responses are endothelium dependent, and it was suggested that its relaxant responses in small-resistance arteries such as mesenteric arteries or arterioles may be greater than in large conduit arteries such as the aorta (47, 174). Furthermore, both Zhao *et al.* and Bianca *et al.* reported that coapplication of charybdotoxin (IK_{Ca} blocker) and apamin (SK_{Ca} blocker) reduced the extent of the H_2S-induced vasorelaxation (53, 308), while charybdotoxin and iberiotoxin (BK_{Ca} blocker) alone had no such effect (309). In addition, coapplication of charybdotoxin and apamin significantly attenuated H_2S-induced vasorelaxation in the endothelium intact mesenteric artery bed, but not that in the endothelium-denuded counterpart (47). These findings suggest that H_2S may act by releasing EDHF from the endothelium.

The strongest evidence that supports the role of H_2S as an EDHF probably comes from the recent findings of Mustafa *et al.* (174). They found that coapplication of the K_{ATP} blocker (glibenclamide) with IK_{Ca}/SK_{Ca} blockers (charybdotoxin/apamin) abolished all H_2S-mediated vasorelaxation in rat mesenteric arteries. By measuring membrane potentials of primary mouse aortic ECs, they found that CSE-knockout mice failed to respond to Ach-induced hyperpolarization, unlike wild-type mice. The coapplication of charybdotoxin/apamin completely attenuated hyperpolarization in wild-type mice, suggesting that IK_{Ca}/SK_{Ca} channels underlie H_2S effects (174). In separate experiments, the researchers further proved that IK_{Ca}, but not K_{ATP} and BK_{Ca} channels, mediate H_2S-induced hyperpolarization in cultured human aortic ECs (174). Collectively, these data suggest that H_2S may play an important role in mediating the vascular responses of small and intermediate resistance vessels.

B. H_2S-induced vasoconstriction

NaHS induces concentration-dependent vasoconstriction at concentrations of 10–100 μM, lower than those that cause vasorelaxation (3, 121, 141) (Fig. 10B). Unlike H_2S-induced vasorelaxation, the K_{ATP} channels are unlikely to be involved in H_2S-induced vasoconstriction (121, 141).

1. Mechanisms of H_2S-induced vasoconstrictions

a. NO-H_2S interaction. While NO and H_2S act synergistically in vasorelaxation (previous section on Endothelium-Dependent Mechanism), H_2S-induced vasoconstriction is more likely a result of NO depletion. Zhao *et al.* observed that H_2S (60 μM) pretreatment inhibited SNP-induced vasorelaxation with a rightward shift of the concentration-response curve and elevated the IC_{50} value (308). Ali *et al.* showed that H_2S, at relatively low concentrations of 10–100 μM, had a vasoconstrictive effect in endothelium-intact vessels but not in endothelium-denuded vessels (3), suggesting probable quenching of endogenous NO by H_2S. Moreover, NaHS (50–200 μM) reversed Ach- or histamine-induced relaxations (both are endothelium dependent) but not ISO-induced relaxation (endothelium independent), showing that H_2S-induced constriction is endothelium dependent (3). Furthermore, mixing NO donors (SNP, SIN-1, or SNAP) with NaHS (100 μM) reduced the extent of vasorelaxation compared with the relaxation with NO donors alone, further indicating the inactivation of NO by H_2S (3). The authors ascribed these observations to the formation of a nitrosothiol compound, as the administration of copper sulfate, which converts nitrosothiol to nitrite and nitrates, abolished NaHS (50–200 μM)-induced vasoconstriction (3). This is in line with their earlier report that utilized a combination of biochemical assays for NO_2^-, NO amperometric detection and electron paramagnetic resonance that determines nitrosothiol formation between NO and H_2S (274). Since the identity and physiological significance of the nitrosothiol compound is unknown, and the methods employed are indirect, more studies such as mass spectrometry or high-performance liquid chromatography are warranted to confirm this interaction.

In vivo, a bolus injection of SNP (16.5 nmol/kg) or NaHS (5 μmol/kg) alone resulted in a 35 mm Hg or a $<$10 mm Hg drop in the MAP of anesthetized rats, respectively, but an injection of their mixture resulted in a loss of MAP reducing ability (3). Interestingly, a slow infusion of NaHS (10 μmol/kg/min) significantly increased MAP, an effect that could be reversed by the infusion of L-NAME (3). This implies that a low concentration of H_2S may scavenge endogenously produced NO. The sustained effect of NaHS infusion on MAP is inconsistent with the transience or absence of vasodepression reported by others (refer to Effect of H_2S on BP of normotensive animals section), probably due to the different route of H_2S administration.

b. Downregulation of endothelial NOS. H_2S can exert inhibitory effects on endothelial NOS (eNOS) activity and, thus, reduce NO synthesis in the endothelium (121). Kubo *et al.* first observed that NaHS (30–3000 μM) concentration dependently inhibited the activity of recombinant eNOS (121). Geng *et al.* further demonstrated that H_2S could inhibit NO production, downregulate eNOS activity, reduce eNOS transcript abundance, and decrease L-[^{3}H] Arg uptake in aortic tissues (74). Using human umbilical vein ECs (HUVECs), NaHS (50 μM) decreased the catalytic efficiency of eNOS but not its affinity for L-arginine (eNOS substrate). NaHS (14 μmol/kg, i.p.) administration into rats resulted in a decreased plasma level of NO metabolites, aortic eNOS activity, and L-arginine uptake (74), suggesting the downregulation of L-Arginine/eNOS/NO pathway *in vivo*. iNOS and nNOS, on the other hand, are not targets of H_2S action (74). However, the significance of these results warrants further investigation, as NaHS above 100 μM probably does not bespeak physiological relevance.

c. Decrease in cAMP level. H_2S-induced vasocontriction was not completely abolished in the presence of NOS inhibitor, L-NAME, or removal of endothelium, suggesting the involvement of an NO-independent mechanism. One possibility is the downregulation of the cAMP level in VSMCs (141). A decrease in cAMP brought about by H_2S induces vasoconstriction by upregulating the activation of MLC kinase, the enzyme responsible for triggering the interaction between actin and myosin.

Lim *et al.* reported that NaHS (at concentrations between 10 and 100 μM) could inhibit relaxations induced by isoprenaline, salbutamol (two β-adrenoceptor agonists), and forskolin (an AC activator), implying that H_2S at lower concentrations can counteract the relaxant effects brought about by vasodilators *via* vasoconstrictive effects (141). More importantly, NaHS (5–100 μM) administration significantly inhibited forskolin-induced cAMP accumulation in rat aortic SMC (141). Since the low concentration of NaHS utilized is near physiological, this could be a potential underlying signaling mechanism *in vivo*.

d. Reactive oxygen species. O_2^- is implicated in the etiology of a variety of vascular diseases, such as atherosclerosis (294), diabetic angiopathy (84), and hypertension (13). It generally induces vasoconstriction, probably due to the quenching of NO. The incubation of human VSMCs with NaHS was found to concentration dependently inhibit O_2^- formation, nicotinamide adenine dinucleotide phosphate oxidase expression, and Rac_1 activity (175). Importantly, O_2^- was found to mediate H_2S-induced vascular responses. Tiron (an SOD mimetic) significantly enhanced NaHS-induced vasorelaxation, but greatly inhibited NaHS-induced vasoconstriction (318). The transport of O_2^- across the cellular plasma membrane *via* anion exchanger (CBE) was found to be upregulated in the presence of H_2S (127, 318), and the rise in

extracellular O_2^- depletes NO, resulting in a reduction in relaxation response and an enhancement of constrictive response (318). $ONOO^-$ derived from O_2^- and NO interaction might be removed by H_2S acting as an $ONOO^-$ scavenger as reported in human neuroblastoma SH-SY5Y cells (272). As mentioned in the previous Acidification section, H_2S-induced stimulation of CBE at a concentration $>100\,\mu M$ results in intracellular acidosis and a relaxant response, masking this vasoconstrictive effect. Nevertheless, the inhibition of CBE resulted in a stronger degree of H_2S-induced vasorelaxation (148), evident of the NO depletion phenomenon.

VI. Other Roles of H_2S in Blood Vessels

A. Angiogenesis

Angiogenesis is a physiological process responsible for the growth of new blood vessels from existing ones. It is a complex biological process characterized by extracellular matrix remodeling and alterations in the behavior of ECs. Cell proliferation, adhesion, and migration, and assembly into capillary structures (development of vessel lumen) are all key events in blood vessel formation. The dysregulation of angiogenesis may result in tumor growth, psoriasis, arthritis, neurodegeneration, wound healing defects, and hair loss (190).

1. Effect of H_2S on angiogenesis. In blood vessels, ECs are both targets and sources of H_2S (288). Current evidence suggests that H_2S promotes angiogenesis and cell growth (Fig. 11), or apoptosis and cell death according to its concentration (28, 241, 256). For example, the expression of survivin (an inhibitor of apoptosis) was significantly upregulated at low concentrations of NaHS (1–10 μM), while its expression at a high concentration of 200 μM NaHS was significantly attenuated (28), suggestive of its physiological importance.

a. In vitro *EC growth and migration.* In *in vitro* experiments with different types of cells such as RF/6A ECs (28) and HUVECs (190), H_2S has been shown as enhancing growth and proliferation. In addition, either exogenous or endogenous H_2S enhances cell migration in ECs using both the transwell migration assay and the *in vitro* scratch wound healing assay (28, 190). EC adhesion was observed only at low concentrations of NaHS (10 and 20 μM) but not at concentrations above 50 μM, indicating that H_2S is proangiogenic only at lower concentrations (28).

The initial phase of angiogenesis involves the organization of ECs into a three-dimensional tube-like structure. The treatment of RF/6A ECs with NaHS (10–20 μM) or H_2S solution (10 μM) increased the tube length and branching points of a microvessel formed on Matrigel after 16 h of culture (28). Similarly, Papapetropoulos *et al.* also observed a 34% increase in the formation of a capillary-like structure of ECs cultured on reduced-growth factor Matrigel in response to H_2S (60 μM) stimulation (190).

Liu *et al.* found that cobalt chloride ($CoCl_2$) (to mimic hypoxia) treatment of cultured VSMCs increased hypoxia inducible factor (HIF)-1α and vascular endothelial growth

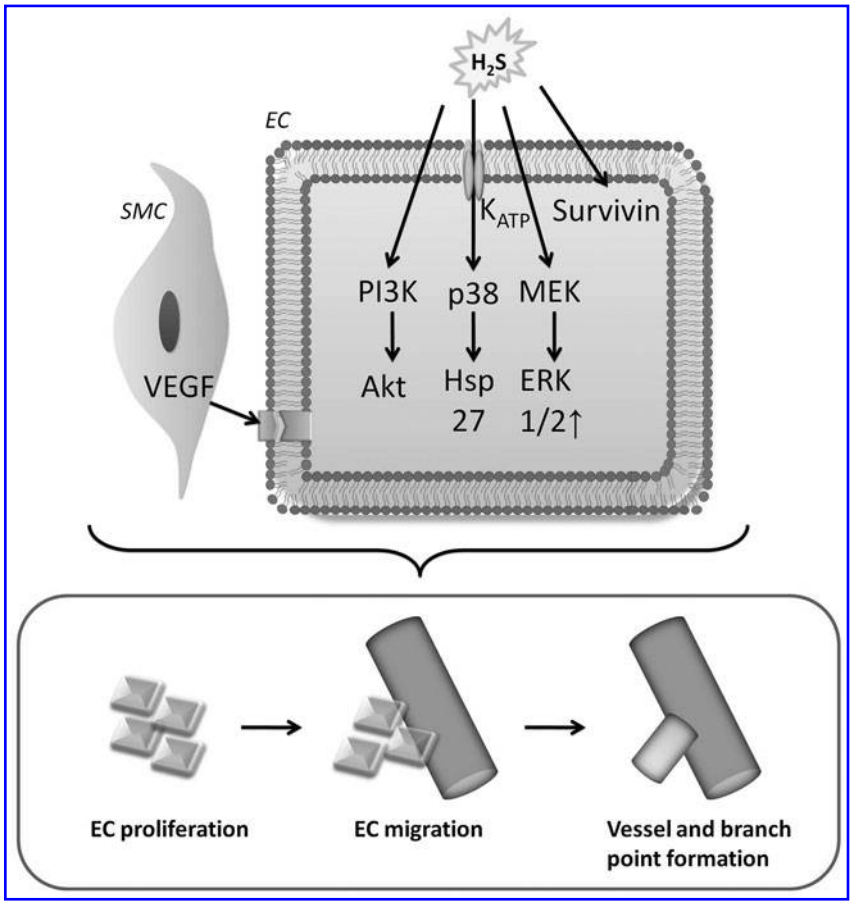


FIG. 11. Schematic illustration of possible signal transduction pathways that may underly H_2S induced endothelial cell (EC) proliferation, migration, and vessel/branch point formation.

factor (VEGF) mRNAs and proteins expression. Furthermore, the co-administration of NaHS (300 μM) with $CoCl_2$ further enhanced the expressions of HIF-1α and VEGF (146). In separate experiments, cultured ECs exposed to conditioned media from $CoCl_2$-treated VSMC cultures showed decreased EC cell numbers than cultured ECs exposed to media from untreated VSMCs. However, ECs that had been exposed to media from VSMCs co-treated with NaHS (300 μM) and $CoCl_2$ showed increased proliferation and migration (146). Hence, under hypoxic conditions, H_2S-induced angiogenesis is probably HIF-1α/VEGF dependent. The physiological relevance of this study, however, requires further in-depth study, because 300 μM NaHS is nonphysiological.

b. In vivo *vascular network formation.* In an *in vivo* model utilizing Matrigel plug in mice, histological analysis revealed that injections of NaHS (10–50 μmol/kg/day, i.p.) for 7 days significantly enhanced EC infiltration into the Matrigel embedded under the abdominal skin. A significantly increased level of hemoglobin content further demonstrated a higher degree of neovascularization. However, the proangiogenic effect of H_2S *in vivo* was lost when the dose was 200 μmol/kg/day NaHS (28).

The administration of H_2S (0.24–240 pmol/egg) to fertilized White Leghorn chicken eggs dose dependently increased the total length of the vascular network in the chick chorioallantoic membrane (190). Conversely, the administration of CSE inhibitors (PAG or BCA) dose dependently reduced the network length and vessel branching, effects that could be reversed by exogenously applied H_2S (190). These results suggest that endogenous H_2S is positively involved in the basal vascular network formation *in vivo*.

H_2S also promotes vascular network formation in pathological situations. A hindlimb ischemic model was established in rats that had been subjected to unilateral femoral artery ligation. Chronic administration with NaHS (50 μmol/kg/day) for 4 weeks promoted collateral vessel growth in ischemic hindlimbs, along with increased regional blood flow and increased capillary density, both indicative of microvessel growth. Furthermore, NaHS was also shown to enhance the growth of ECs and myocytes in ischemic muscles (255). Nevertheless, it is noteworthy that NaHS at a higher dose (200 μmol/kg/day) failed to produce any significant proangiogenic effect (255). Taken together, these studies suggest that H_2S may promote vascular network formation *in vivo* at a near physiological concentration.

c. In vivo *wound healing.* The effect of H_2S on angiogenesis was also tested with burn wound assay (30% of the total body surface area) in rats. Daily topical administration of H_2S (300 μg/kg) enhanced wound healing along with a significant augmentation in re-epithelialization. When comparing CSE-knockout mice against wild-type controls, the sizes of the wound areas were consistently larger in CSE-knockout mice, suggesting that endogenous H_2S generation contributes toward wound healing (190).

2. Mechanisms of the proangiogenic effects of H_2S

a. Effect of H_2S on proangiogenic factors. NaHS (1–200 μM) has no effects on the levels of VEGF, basic fibroblast growth factor (bFGF), and angiopoietin-1 in the culture medium of RF/6A ECs (28). Subsequently, Papapetropoulos *et al.* also showed that endogenous H_2S synthesis or action does not affect bFGF-induced migration (190). However, Zhu *et al.* reported that NaHS (20–50 μmol/kg/day) administration induced VEGF biosynthesis and protein expression in skeletal muscle cells, suggesting that H_2S may exert its proangiogenic effect on neighboring ECs in a paracrine fashion (255). VEGF receptor 2 (VEGFR2) is the main receptor type involved in mediating the proangiogenic effect of VEGF, and these receptors are mainly localized in the vascular ECs (255). The downregulation of VEGFR2 during ischemia was reversed by NaHS (50 μmol/kg/day) treatment, with specific phosphorylation at Tyr 996 of the receptor (255). This further strengthens the idea that H_2S-induced VEGF in SMCs may act on ECs to promote angiogenesis.

b. Signaling mechanisms

(1). MAPK/ERK kinase/ERK pathway. Cai *et al.* first noted that NaHS (10 μM) increased Akt phosphorylation by about twofold, but not ERK and p38 activation (28). However, it was later discovered that H_2S (60 μM) elevates the phosphorylation of ERK1/2, p38 and Akt with differing kinetics: ERK1/2 activation was rapid and sustained; p38 activation was rapid and transient; and Akt activation was slow but sustained (190). In addition, MAPK/ERK kinase (MEK), a tyrosine/threonine kinase that phosphorylates and activates ERK, inhibitors (PD098059 or U0126) attenuated the H_2S-elicited EC migration (190), suggesting that MEK lies upstream of ERK in mediating the angiogenic effect of H_2S at a low concentration.

(2). K_{ATP}/p38/hsp27 pathway. As just mentioned, ECs show a rapid and transient activation of p38 when exposed to a low concentration of H_2S (60 μM). p38 inhibitors (SB203580 or SB239063) were found to completely abolish the H_2S-induced migration of EC (190), revealing an essential role of p38 in mediating H_2S-induced angiogenic effects.

Hsp27, a modifier of actin cytoskeleton, is also implicated in the regulation of cell migration. Knockdown of hsp27 using the siRNA method significantly reduced H_2S-induced migration of EC (190). Moreover, inhibition of p38 with SB203580 and SB239063 reduced hsp27 phosphorylation (190), suggesting that the p38 pathway is upstream of hsp27 in the H_2S signaling cascade.

H_2S-elicited EC migration was attenuated by a nonselective K_{ATP} channel blocker (glibenclamide) or a selective mitochondrial-K_{ATP} channel inhibitor (5-HD). Furthermore, glibenclamide attenuated H_2S-induced p38 and hsp27 phosphorylation, while the K_{ATP} opener (SG209) alone was capable of enhancing EC migration in a concentration-dependent manner, along with increased p38 phosphorylation (190). Taken together, these data suggest that the actions of H_2S on K_{ATP} channels are upstream of p38/hsp27.

(3). PI3K/Akt pathway. Cai *et al.* discovered that pretreatment with LY294002 (an inhibitor of PI3K) or transfection of dominant negative (kinase-inactive mutant Myr-Akt-K179M) Akt could greatly attenuate H_2S (10–20 μM)-induced wound-healing acceleration along with increments in tube length and branch points (28). The application of PI3K inhibitors (wortmannin or LY 294002) completely abolished NaHS (10–200 μM)-induced increase in Akt phosphorylation in RF/6A ECs, suggesting that PI3K is upstream of Akt in the

underlying H_2S signaling mechanism. In a hindlimb ischemia model, rats treated with NaHS (20–200 μmol/kg/day) injections also showed significantly increased Akt phosphorylation compared with control animals (255).

In direct opposition, Papapetropoulos *et al.* reported that the angiogenic effect of H_2S (60 μM) is independent of the PI3K/Akt pathway, as the PI3K inhibitor LY-2924002 failed to affect the migratory rate of ECs in response to H_2S (190). Therefore, the involvement of the PI3K/Akt pathway in the angiogenic effect of H_2S is yet to be confirmed.

B. H_2S as an oxygen sensor

In mammals, hypoxia has been known to relax the systemic vessels while constricting the pulmonary vessels (270). Olson and colleagues found many similarities between H_2S and hypoxia-induced pulmonary responses (182–184). Oxygen is critical for the catabolism of H_2S *via* mitochondrial oxidation, the main route of H_2S disposal. During hypoxia, when intracellular oxygen is low, the catabolism of H_2S was found to be greatly reduced (56), resulting in an increased concentration of H_2S. Conversely, during normoxia, much of the intracellular H_2S would be oxidized by available oxygen, resulting in a low intracellular level of H_2S. In addition to H_2S catabolism, the oxygen level was also found to affect H_2S production. At a low concentration of O_2 ($< 5\ \mu M$), H_2S production was detectable in various rat tissues that diminished in an air-equilibrated buffer with 200 μM O_2 (56).

Therefore, the tissue H_2S concentration is inversely correlated with the oxygen concentration (184), making it a likely candidate for an oxygen sensing/signal transduction cascade involved in hypoxic response. The balance between endogenous H_2S biosynthesis and its catabolism by available oxygen is considered the "oxygen sensor."

C. Atherosclerosis

Atherosclerosis, also known as arteriosclerotic vascular disease, is a chronic and complex condition that involves arterial wall thickening as a result of fat accumulation and plaque formation, mainly in large and medium-sized arteries. While the exact underlying mechanisms are not clearly understood, atherosclerotic plaque formation is thought to be mediated by a number of factors such as vascular inflammation, endothelial damage, smooth muscle proliferation and migration, macrophage and foam cell accumulation, and lipid deposition (68, 156). The consequential narrowing and stiffening of blood vessels impedes blood flow and increases plaque thrombogenicity. Current findings suggest that H_2S, while it exerts proliferative roles in ECs that enhance angiogenesis, induces apoptosis in SMCs that protects against atherosclerosis (Figs. 12 and 13).

1. Potential therapeutic effects of H_2S on atherosclerosis. CSE expression and activity and/or H_2S level were significantly reduced during the development of atherosclerosis in either vascular beds or plasma using a balloon injury model (163) and a vascular calcification model (281). However, in apolipoprotein E knockout ($apoE^{-/-}$) mice, CSE mRNA in aorta was found to be elevated, although plasma H_2S and aortic H_2S synthesis were lowered compared with wild-type mice (262). The discrepancy might be attributed to the existence of a positive compensatory feedback mechanism under *in vivo* conditions to salvage the low H_2S level by CSE activity upregulation. In $apoE^{-/-}$ mice, PAG administration induced marked reductions in plasma H_2S level and aortic H_2S synthesis, and increases in CSE mRNA expression and the size of atherosclerotic plaques in comparison to untreated $apoE^{-/-}$ mice (262).

Exogenously administered NaHS suppressed the development of neointima hyperplasia (163), decreased vascular calcium content, calcium overload, and alkaline phosphatase activity in calcified vessels (281); reduced atherosclerotic plaque size; and improved aortic ultrastructure (262).

2. Mechanisms underlying the effects of H_2S on atherosclerosis. The inhibitory effect of H_2S on atherosclerosis is a novel discovery reported only within recent years; hence, the underlying mechanisms are not completely understood. A myriad of factors, including inhibition of SMC proliferation, anti-inflammatory responses, and attenuation of low-density lipoprotein (LDL) modification, are known as preventing the development of atherosclerosis.

a. Proapoptotic/antiproliferative effects of H_2S on SMCs. The proliferation of VSMCs plays an important role in the structural remodeling of blood vessels. Many *in vitro* studies have been performed to investigate the involvement of the proapoptotic effect of H_2S in the treatment of atherosclerosis. Cultured human aorta SMCs (HASMCs) treated with exogenous NaHS (200–500 μM) showed a concentration-dependent increase in the number of apoptotic cells revealed by Hoechst 33258 staining and TUNEL assay, and further confirmed by internucleosomal DNA fragmentation (286). The physiological relevance of this finding is questionable, as H_2S concentration utilized was beyond physiological. Du *et al.* also reported an antiproliferative effect of H_2S on VSMCs by measuring the incorporation of [^{3}H]-thymidine into cells (59). S-diclofenac (10–100 μM), a slow releasing H_2S donor, also attenuated cell proliferation in both primary (A-10) SMCs and immortalized (SV-40 transformed) SMCs (10).

Interestingly, when endogenous synthesis of H_2S was decreased by inhibiting CSE with PAG, the increased cell apoptosis induced by exogenously administered H_2S (100 μM) was increased. However, PAG treatment alone failed to exert any proapoptotic effect (286). These authors explained this observation by proposing that endogenous H_2S acts in suppressing or desensitizing cells to apoptotic signaling. Therefore, PAG unveiled an increased response of HASMCs to an apoptosis-inducing concentration of H_2S (286). These researchers later established a CSE overexpression model by infecting HASMCs with a recombinant defective adenovirus containing the CSE gene (Ad-CSE) (289). Ad-CSE transfection resulted in a 12.3-fold increase in CSE activity and a 6.2-fold increase in endogenous H_2S production rate. CSE overexpression significantly reduced cell proliferation, shown by a cell growth curve and a cell viability assay. In line with this, CSE overexpression promoted cell apoptosis, as seen in Hoechst 33258 staining and TUNEL assay, together with the activation of caspase-3. In order to confirm that the proapoptotic effect of CSE overexpression is attributable to H_2S production but not products of other CSE-catalyzed reactions, CSE gene activity was knocked down by the RNA interference approach, and H_2S was then exogenously administered. As expected, H_2S (100 μM) promoted the apoptosis of

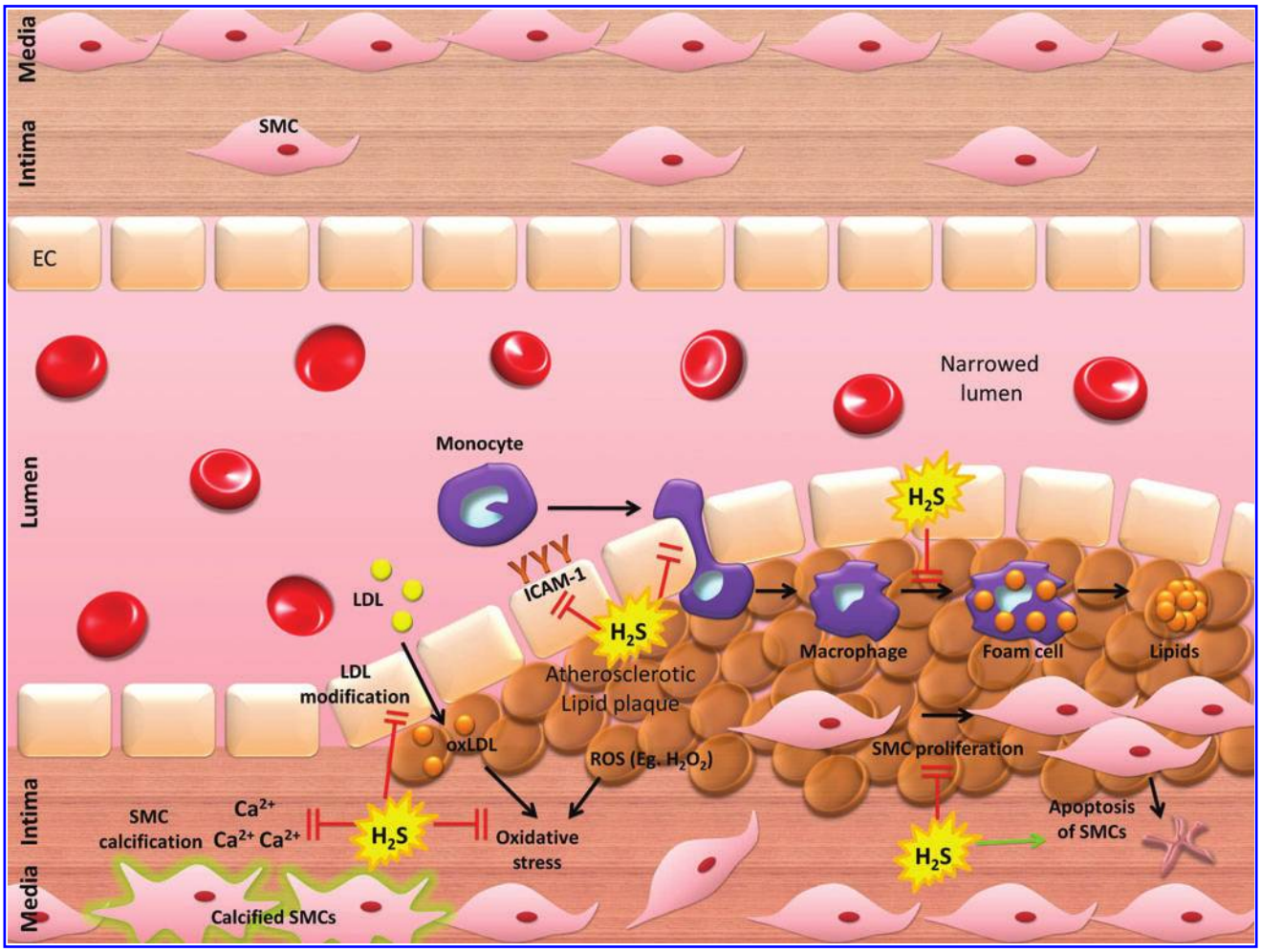


FIG. 12. H_2S inhibits atherosclerosis. H_2S suppresses (indicated by red line indicators) intercellular adhesion molecule-1 (ICAM-1) expression and prevents monocyte infiltration into atherosclerotic plaques that form macrophages. Furthermore, when the macrophages are exposed to oxidized low-density lipoprotein (oxLDL), foam cells are formed, which make up the atherosclerotic core. H_2S prevents the formation of atherosclerotic plaques by preventing the modification of LDL along with the inhibition of foam cell formation. Moreover, H_2S acts on SMCs that prevent SMC proliferation and calcification, while SMC apoptosis is enhanced (indicated by *green arrows*). H_2S is also responsible for scavenging ROS and reducing the oxidative stress of atherosclerotic tissues (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars).

HASMCs, while ammonium and pyruvate (two other products of a CSE-catalyzed reaction) exerted no induction effect on cell apoptosis (289).

The effect of endogenous H_2S on apoptosis in VSMCs was investigated in CSE-knockout mice (287). Consistent with observations in the CSE overexpression model, CSE-knockout mice exhibited SMC overproliferation in the media of the aorta. These data collectively suggest that the endogenous CSE/H_2S system plays a role in suppressing SMC proliferation while enhancing apoptosis.

(1). Signaling mechanisms. Exogenously administered H_2S (20–500 μM) activated ERK and p38 MAPK in a concentration-dependent manner (286). In line with this, H_2S produced endogenously *via* CSE overexpression with Ad-CSE transfection was also found to upregulate the activation of ERK and p38 MAPK (289). In CSE-knockout mice, a reduced level of ERK1/2 activation was observed as a result of decreased endogenous H_2S production (287). In contrast to these findings, Du *et al.* failed to observe MAPK activation when VSMCs were treated with NaHS at 50–100 μM in the absence of fetal bovine serum (FBS) or endothelin-1 (59). In fact, in the presence of FBS or endothelin-1, NaHS (50–500 or 50–100 μM, respectively) significantly reduced the activity of ERK1/2 in a concentration-dependent manner. Nevertheless, these results suggest that the H_2S-induced inhibitory effect on VSMC proliferation is, in part, mediated *via* the MAPK pathway (59).

The blockade of MEK was capable of attenuating a H_2S (500 μM)-elicited increase in ERK activation, caspase-3 activation, and the induction of apoptosis (286), suggesting that H_2S-induced apoptosis in VSMCs is mediated *via* the MEK/ERK/Caspase-3 pathway. On the other hand, the administration of SB203580, a powerful inhibitor of p38 phosphorlyation, failed to inhibit the effect of H_2S (500 μM) on caspase-3 activation and cell apoptosis. Moreover, the inhibition of caspase-3 has no influence on the H_2S-induced p38 activation (286). Thus, unlike

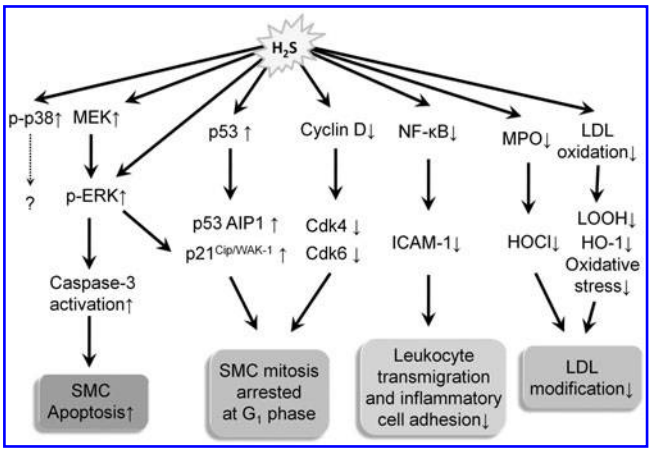


FIG. 13. Signaling mechanisms underlying H_2S inhibition on atherosclerosis. H_2S could elevate phosphorylation (prefix p-) of both extracellular signal-regulated kinases (ERK) and p38, but only the ERK pathway was capable of caspase-3 activation and induction of the proapoptotic effect on SMCs. H_2S may regulate cell-cycle progression *via* the inhibition of cyclin D1 expression and the upregulation of p53, p53 apoptosis-inducing protein (AIP) 1, and p21 Cip/WAK-1. H_2S inhibition of leukocyte transmigration and inflammatory cell adhesion is mediated by its inhibitory effect on nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and subsequent downregulation of ICAM-1. H_2S also prevents modifications of LDL by the inhibition of LDL oxidation, lowering heme oxygenase 1 (HO-1) expression, lipid hydroperoxide (LOOH) levels and reducing oxidative stress. The downregulation of the MPO/H_2O_2/Cl^- system by H_2S may also contribute toward the prevention of atherosclerotic plaque developments. MPO, myeloperoxidase.

MEK/ERK, the p38 pathway is probably not involved in H_2S-induced proapoptotic responses.

The involvement of these pathways warrants further research efforts using lower H_2S concentrations (below 100 μM), as concentrations beyond this range are probably not physiological.

(2). Cell-cycle regulators. A family of protein kinases, cyclin-dependent kinases (cdk), may associate with cyclins to form cyclin-Cdk complexes and trigger cell-cycle events. H_2S appears to interfere with cell-cycle regulation, as both NaHS (50 μM) and S-diclofenac (50 μM) were found to decrease the percentage of cells in the G1 phase after 24 and 48 h of treatment. Further studies showed that S-diclofenac altered the cell cycle and prevented the mitosis of asynchronized cells (G1 phase). Its effect was much less in synchronized cells (G_0 phase) (10).

Both endogenously produced H_2S (CSE overexpression) or exogenously applied H_2S (100 μM) suppressed the expression of cyclin D1 (an activator of cdk4 and cdk6) while elevating the expression of $p21^{Cip/WAK-1}$ (a cdk inhibitory protein) (289). An increase in p21 protein level was also induced by S-diclofenac, accompanied by a near fourfold increase in p53. Proapoptotic proteins such as p53 apoptosis-inducing protein (AIP) 1 and Bax were also time-dependently elevated by S-diclofenac (10). Intriguingly, it was previously reported that treatment of HASMCs with H_2S (20–500 μM) failed to alter the protein expression of Bax (286). Such a discrepancy warrants further studies. Nevertheless, the lack of Bcl-2 involvement in mediating H_2S-induced proapoptosis was consistent in both studies (10, 286).

Using both isolated SMCs and whole vascular tissues, Yang *et al.* demonstrated that the reduced level of endogenous H_2S in CSE-knockout mice correlates with lowered $p21^{Cip/WAF-1}$ expression but enhanced cyclin D1 expression when compared with wild-type mice (287). The exogenous administration of H_2S could increase the expression of $p21^{Cip/WAF-1}$ while attenuating the expression of cyclin D1 in both CSE-knockout and wild-type mice, but with a much greater effect in the former. In addition, the CSE-knockout mice expressed a higher level of CDK4, which could be attenuated by exogenously applied H_2S. Exogenous H_2S administration elevated the expression of $p15^{INK4B}$ and $p27^{Kip1}$ in the CSE-knockout mice, but not in wild-type mice. Surprisingly, SMCs of CSE wild-type, but not of CSE knockout, expressed an increased level of cyclin D3 in response to exogenous H_2S. The expression of $p16^{INK4A}$ increased by a similar extent in both CSE-knockout and wild-type mice. The gene expression using microarray analysis demonstrated that the CSE-knockout mice express a higher level of calcitonin receptor-like, integrin β1, heparin-binding epidermal growth factor-like growth factor in aortic tissues than that in their wild-type counterparts (287).

b. Anti-inflammation. ICAM-1 facilitates leukocyte transmigration and inflammation cell adhesion to the endothelium. The administration of NaHS suppressed ICAM expression in both the plasma and aorta of $apoE^{-/-}$ mice, whereas PAG enhanced the upregulation of ICAM expression. The inhibitory effect of NaHS (1–100 μM) on the ICAM-1 protein level was further reaffirmed in TNF-α-elicited HUVECs. These data suggest that H_2S may exert its protective effect against atherosclerosis formation *via* the downregulation of ICAM-1 under both *in vivo* and *in vitro* conditions (262).

NF-κB is a transcription factor involved in the stimulation of ICAM-1 expression in ECs. NaHS (100 μM) significantly inhibited NF-κB activation, indicated by an attenuated breakdown of the NF-κB inhibitor (IκBα) and nuclear translocation of NF-κB (262).

The formation of foam cells contributes toward the formation of atherosclerosis. Macrophages are converted into foam cells after being exposed to oxidized LDL (oxLDL). H_2S prevented oxLDL-induced foam cell formation in a concentration-dependent manner (262, 311) along with blunted oxLDL binding and uptake in macrophages (311). Studies unveiling the underlying mechanisms revealed that NaHS (50–200 μM) suppresses oxLDL-induced elevated expressions of scavenger receptor CD36, scavenger receptor A, and acyl-coenzyme A: cholesterol acyltransferase-1 mediated by the K_{ATP}/ERK1/2 signaling pathway (311).

c. Inhibition of LDL modification. The oxidative modification of LDL is implicated in the early development of arterogenesis. Hypochlorite (HOCl), the product of the activated myeloperoxidase/H_2O_2/chloride (MPO/H_2O_2/Cl^-) system, has been implicated as a trigger for LDL modification, and HOCl-modified LDL has been found in atherosclerotic plaques (125). The elevation of Apo B modifications (assessed by relative electrophoretic mobility and carbonyl formation) in LDL could be attenuated by either the exogenous administration of NaHS (750 μM–3 mM) or the endogenous precursors of H_2S (cysteine, homocysteine, or methionine) (125). The inactivation

of HOCl-induced LDL modification could be attributed to the scavenging property of H_2S, which was demonstrated using the 3,3′,5,5′-tetramethylbenzidine assay to quench equimolar concentrations of HOCl. Moreover, H_2S was further proved to inactivate MPO enzyme activity and decompose H_2O_2, suggesting that the antiatherosclerotic property of H_2S is mediated by downregulation of the $MPO/H_2O_2/Cl^-$ system that limits LDL modifications. The high H_2S concentrations used in these studies were far beyond the physiological and may be of little value to our understanding of H_2S biology *in vivo*.

In another study, the LDL oxidation model was established by hemin exposure as inducing EC damage either directly or *via* the induction of oxidative alterations in lipids and apolipoproteins to form cytotoxic oxidized products (101). The administration of NaHS (5–20 μM) during hemin-induced LDL modification resulted in the concentration-dependent inhibition of LDL oxidation, represented by the retarded formation of lipid peroxidation products such as conjugated dienes, lipid hydroperoxide (LOOH), and thiobarbituric acid-reactive substrates (TBARS) (101). In both HUVECs and atherosclerotic plaques, NaHS (25–200 μM) treatment, subsequent to the induction of LDL oxidation, lowered the LOOH level in a concentration-dependent manner, while not affecting conjugated dienes and TBARS. This phenomemon may account for the cytoprotection seen in ECs subjected to H_2S treatment, as elevated LOOH content is implicated in cytotoxicity (177). Moreover, the oxidized LDL was found to upregulate the expression of HO-1, and H_2S (25–200 μM) could concentration dependently reduce HO-1 mRNA and protein induction (101). H_2S may also exert its cytoprotective effects on ECs *via* its antioxidant property, as it was found to reduce oxidative stress elicited by H_2O_2 or oxidized LDL (101).

VII. Hypertension

Hypertension is the most common CVD and a leading risk factor for stroke or heart problems. In the United States, for example, hypertension has a prevalence rate of about 40% (204). Generally, H_2S is a vasodilator (in the short term, it relaxes vascular smooth muscles) and is able to produce an antihypertensive effect. Studies demonstrating the antihypertensive effects of H_2S are summarized in Table 4.

A. Effect of H_2S on BP of normotensive animals

The blockade of endogenous H_2S production by hydroxylamine hydrochloride (0.5 mg/kg i.p.), a nonspecific inhibitor of both CSE and CBS, for over 4 weeks failed to influence systolic blood pressure (SBP) in rats (154). In contrast, Yan *et al.* found that the administration of PAG (37.5 mg/kg), an inhibitor of CSE, to rats for 5 weeks significantly elevated BP and increased the medial cross-sectional area of the thoracic aorta wall, indicating significant aortic structural remodeling (284). The discrepancy may arise from the different drugs used in the two studies, as well as from the different dosages and duration of treatment. It should be noted that existing

Table 4. Antihypertensive Effect of Hydrogen Sulfide in Different Hypertensive Animal Models

Animal model	*Endogenous H_2S level*	*Treatment*	*Effect of exogenous H_2S*	*Mechanisms*	*Ref.*
Genetic model-SHR	Plasma H_2S (↓), aortic CSE mRNA (↓) aortic CSE activity (↓)	NaHS 56 μmol/kg or PAG (37.5 mg/kg) i.p. for 5 weeks	NaHS treatment: Antihypertension, Plasma H_2S (↑), aortic CSE mRNA (↑) aortic CSE activity (↑) PAG treatment: hypertension development and vascular remodeling	Lessened aorta remodeling	(284, 310)
SHR	NA	NaHS (10, 30, 90 μmol/kg/day for 3 m)	Antihypertension and antihypertrophy	Antioxidant	(219)
SHR and L-NAME-induced hypertensive rats	NA	GYY (133 μmol/kg/day for 14 days, iv or ip)	Antihypertension	K_{ATP} channels	(138)
2K1C renin-dependent hypertensive rats	NA	NaHS (0.56, 1.68. 5.6 mg/kg/day for 4 m)	Antihypertension	Renin-inhibition	(154)
1K1C renin-independent hypertensive rats	NA	NaHS (0.56, 1.68. 5.6 mg/kg/day for 4 m)	No effect	NA	(154)
Aorta-inferior cava vein shunting pulmonary hypertsion	Plasma and lung H_2S (↓), CSE mRNA (↓)	NaHS (56 μmol/kg/day) for 11 weeks	Antipulmonary hypertension	NA	(283)
Hypoxia Pulmonary hypertension Rats	Plasma H_2S (↓), aortic CSE mRNA (↓) aortic CSE activity (↓)	NaHS 14 μmol/kg	Antipulmonary hypertension	Lessened vascular remodeling	(48)

1K1C, one-kidney-one-clip; 2K1C, two-kidney-one-clip; L-NAME, N (G)-nitro-L-arginine methyl ester; SHR, spontaneously hypertensive rats; NA, not applicable.

H_2S synthesizing enzyme inhibitors have low potency and specificity for their intended use, and the data obtained should be interpreted with caution.

A better alternative than using CSE inhibitors in animal experiments would be the use of genetically modified animals. CSE-knockout mice were reported to exhibit pronounced BP elevations compared with their wild-type counterparts (288), suggesting that the physiological production of H_2S by CSE may be critical for the regulation of basal BP. However, the possibility that hypertension in CSE-knockout animals developed as a result of hyperhomocysteinemia cannot be totally excluded. Although the researchers explained that male and female knockout mice have similar BP despite vastly different homocysteine levels, BP control mechanisms differ between genders, and factors such as sex hormones could have counteracted the effect of homocysteines. In a further attempt to exclude the contribution of hyperhomocysteinema, Yang *et al.* carried out a separate set of experiments. L-methionine was administered to wild-type mice *via* drinking water for 6 weeks to induce hyperhomocysteinemia, and no hypertension was observed in these animals (288). Unfortunately, plasma homocysteine levels in L-methionine-treated animals increased by merely 1.5 times, a huge disparity compared with the 18-fold difference between CSE-knockout and wild-type mice. In contrast to the findings of Yang *et al.*, the CSE-knockout mice developed by Ishii *et al.* did not exhibit hypertension (99), whereas L-methionine-induced hyperhomocysteinemia resulted in elevated BP (233). The great disparity in observations and the complexity of the science involved definitely warrant further research efforts that study these effects in greater detail.

The acute administration of H_2S causes transient dose-related falls in MAP without significant alterations in heart rate (3, 138, 288, 309). For example, Li and colleagues demonstrated that an i.v. bolus injection of NaHS (2.5 to 20 μM) caused an immediate fall in BP in anesthetized rats, and this transient effect lasted for only 10 to 30 s (138). However, the chronic administration of NaHS (5.6 mg/kg/day, i.p.) for 4 weeks failed to significantly affect SBP in conscious rats, measured using the tail-cuff method (154). Similarly, exogenous NaHS (56 μmol/kg, i.p.) administered for 5 weeks did not affect the SBP and aortic structure in normotensive rats (284). Therefore, it appears that H_2S does not significantly affect the BP in normotensive rats.

B. Effect of H_2S on BP in hypertensive animals

Several groups investigated the effect of H_2S on hypertension in different hypertensive models. Treatment with H_2S can significantly lower the BP of hypertensive animals (Fig. 14). We will first introduce the antihypertensive effects of H_2S in these models followed by discussing the underlying mechanisms.

1. H_2S in SHR. Yan *et al.* found that the plasma level of H_2S in SHR was significantly lower than that of the WKY controls (284). The expression of CSE mRNA was also found to be lower than that of control rats. These findings suggest that the hypertension in SHR involves a reduction in the production and function of H_2S (284). This is supported by the observation that chronic daily administration of NaHS (56 μmol/kg/day, i.p.) for 5 weeks significantly reduced the SBP by 25 mm Hg and inhibited aortic structural remodeling in the SHR (284). Consistently, Shi *et al.* reported significantly reduced SBP, diastolic blood pressure (DBP), and MAP of SHR to similar extents after NaHS (30 or 90 μmol/kg/day, i.p.) for 3 months. A lower dose (10 μmol/kg/day i.p.) also reduced DBP and MAP, but the decrease in SBP was not statistically significant (219). In addition, chronic treatment with GYY4137 (a slow-releasing H_2S donor, 133 μmol/kg, i.p.) also significantly reduced the SBP in SHR from day 2 after treatment (138). Therefore, H_2S has a profound antihypertensive effect in SHR.

2. H_2S in renovascular hypertension. The two-kidney-one-clip (2K1C, a clip constricting one renal artery) model is a good animal model of renovascular hypertension, which is a common type of secondary hypertension and the most prevalent form of curable hypertension (201). The removal of the unclipped kidney gives rise to the one-kidney-one-clip (1K1C) model. The mechanism underlying

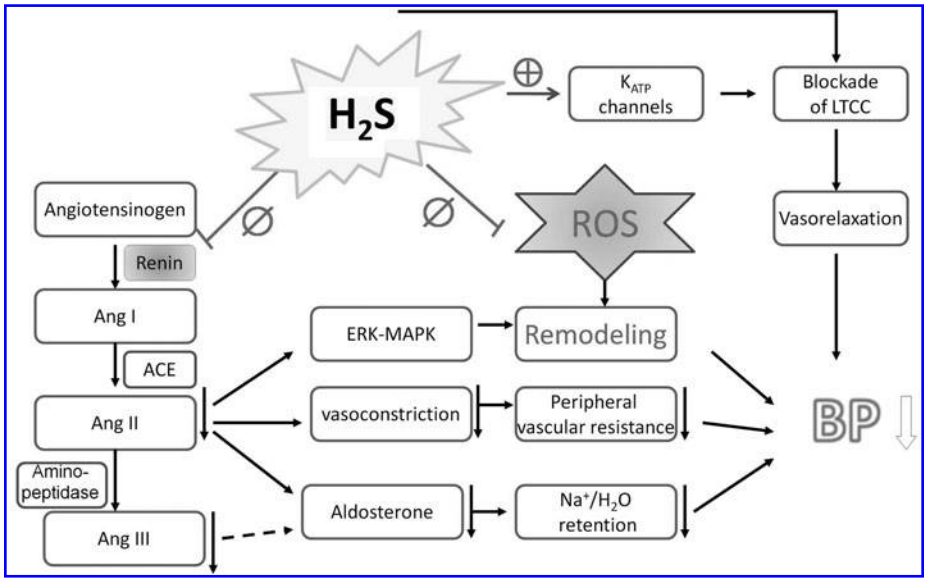


FIG. 14. Effect of H_2S on blood pressure (BP) of hypertensive animals. H_2S lowers the BP of hypertensive animals mainly *via* the inhibition of renin, as H_2S could significantly attenuate the rise in the BP of renin-dependent renal hypertension but not that of renin-independent mechanisms. Other contributing factors of H_2S may include the scavenging of ROS and the inhibition of the K_{ATP} channels.

the hypertensive effect of 2K1C and 1K1C models differs. Hypertension in the 2K1C model results from an increased release of renin and aldosterone (23, 118). In the 1K1C model, on the other hand, the renin level increased only transiently, and is considered a trigger for the development of hypertension, which is then maintained by Na^+/H_2O retention. (70, 71).

Lu *et al.* found that the administration of NaHS (5.6 mg/kg/day, i.p.) successfully attenuated the development of hypertension in the renin-dependent 2K1C model (154) (Fig. 15). Furthernore, NaHS treatment also reversed the high BP after hypertension had developed in the 2K1C rats. On the contrary, NaHS administration failed to affect the BP in the renin-independent 1K1C model (154). These data suggest that the antihypertensive effect of H_2S is mediated *via* a renin-dependent mechanism.

3. H_2S in pulmonary hypertension. Pulmonary hypertension (PH) is a common complication of congenital heart disease with a left-to-right shunt characterized by high pulmonary blood flow (283) in which blood from the arteries is mixed with that of the veins. Xiaohui *et al.* found that the H_2S level in lung tissue was decreased, while the mean pulmonary arterial pressure (mPAP) increased significantly 11 weeks after aorta-veno cava shunting, which was utilized as a rat model for PH. This suggests that a reduced level of H_2S may contribute to the detrimental effect of shunting (283).

Hypoxic pulmonary hypertension (HPH) is another important pathophysiological process that commonly occurs in several cardiac and pulmonary diseases. It is characterized by high pulmonary arterial pressure and high pulmonary vascular resistance, often resulting from a combination of hypoxic vasoconstriction and vascular structural remodeling (303). It was found that the CSE mRNA level, H_2S synthesis, and plasma level of H_2S were all significantly decreased in the rat HPH model. Conversely, the exogenous administration of NaHS completely abolished hypoxia-induced mPAP elevation, and greatly attenuated right ventricular hypertrophy (303). These findings suggest that the impaired H_2S production might contribute to the pathogenesis of HPH, and the exogenous application of H_2S may have therapeutic value in the treatment of HPH.

4. Mechanisms underlying antihypertensive effects of H_2S

a. Inhibition of the renin-angiotensin system. Renin-angiotensin system (RAS) plays an important role in regulating blood volume and systemic vascular resistance, which influence BP. Renin catalyzes the first and rate-limiting step in the

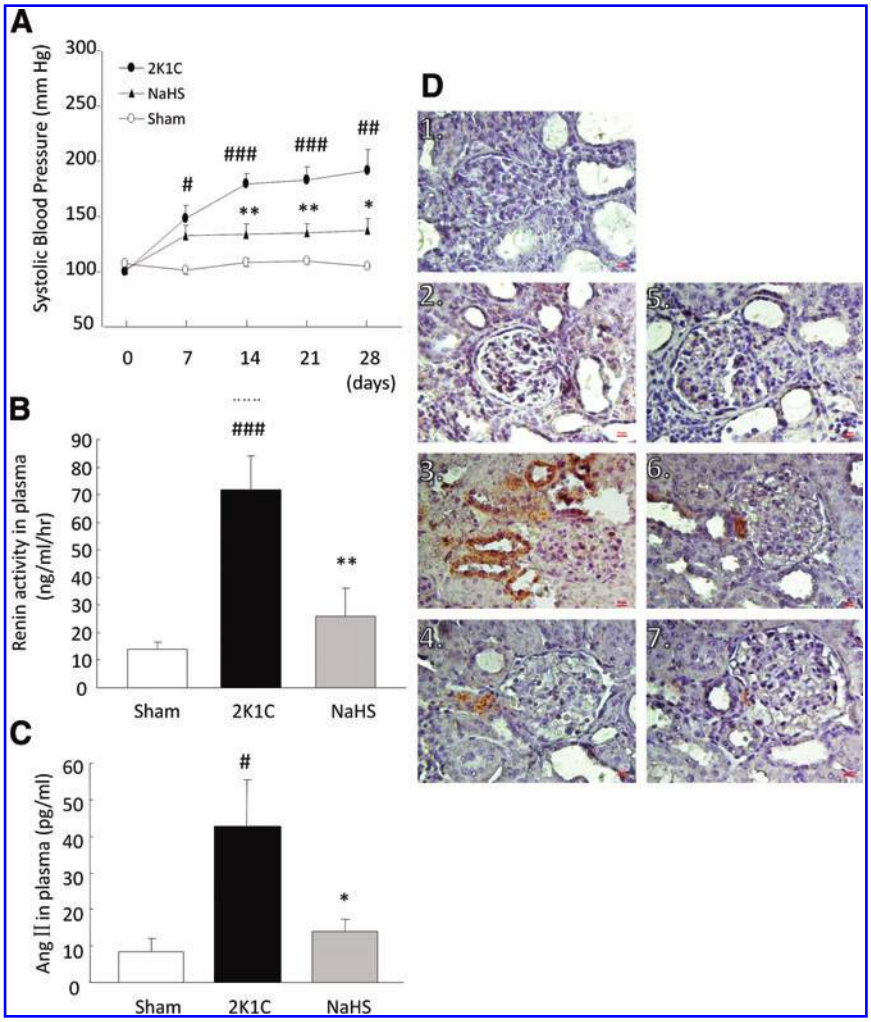


FIG. 15. Effect of H_2S on renin-dependent renal hypertension. (A). Time course for the development of renovascular hypertension in the presence and absence of NaHS treatment (5.6 mg/kg/day) in two-kidneys-one-clip (2K1C) rats over 4 weeks. NaHS treatment significantly reversed the BP elevations as compared with the 2K1C untreated group **(B** and **C)**. Elevations in renin **(B)** and angiotensin II **(C)** levels were significantly attenuated by NaHS treatment. Data are expressed as mean ± SEM, #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ *versus* sham rats. *$p < 0.05$ and **$p < 0.01$ *versus* 2K1C rats **(D)**. NaHS treatment decreased renin expression in the clipped kidneys. (1) Negative control stained by preimmune mouse lgG. Renin expression increased drastically in (3) clipped kidneys compared with those in (2) the sham group. (4) NaHS treatment markedly attenuated the upregulation of renin expression in the clipped kidneys (5 through 7). There was no significant change of renin expression in the unclipped kidneys of 2K1C rats along (5) sham, (6) vehicle, (7) and NaHS treatment groups.
The photos were taken at ×40 magnification [Adapted with permission from *Journal of the American Society of Nephrology* (Ref. 154)]. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars). NaHS, sodium hydrosulfide (H_2S donor).

production of Ang II. Lu *et al.* found that H_2S decreased plasma renin activity (PRA) in renin-dependent 2K1C rats, but had no significant effect on PRA in normotensive or renin-independent 1K1C rats (154). H_2S suppressed renin degranulation and release was further shown to be mediated by the attenuation of AC activity and lowered cAMP production (155). Although NaHS (1 m*M*) also exhibited an inhibitory effect on PDE activity, the high concentration of H_2S needed has limited physiological relevance and implications. The authors also reported that neither acute (up to 1 m*M*, 30 min) nor chronic (5.6 mg/kg/day for 4 weeks) treatment with H_2S significantly affected the angiotensin converting enzyme (ACE) activity in normal (basal ACE activity) or hypertensive (elevated ACE activity) rat aortic tissues (154). Laggner *et al.* however, found that H_2S (60 μM) inhibited ACE activity, but not its mRNA expression, on the surface of cultured HUVEC monolayers, *ex vivo* in umbilical veins and in HUVEC protein extracts (124). The inconsistent H_2S effect on ACE could be attributed to the different type of blood vessel used. Nevertheless, H_2S inhibition of renin or ACE would lead to significantly attenuated Ang II. For example, H_2S inhibited the fivefold elevation of Ang II level in the plasma of 2K1C rats through the inhibition of renin (154).

Zhao *et al.* also observed that H_2S can decrease the binding affinity of Ang II type 1 (AT1) receptors, thereby attenuating Ang II-induced AT1 activation. This would lead to a reduction in downstream signaling, including MAPK and ERK 1/2 activation (310). Collectively, these findings indicate that the inhibition of various RAS components plays a substantial role in the underlying H_2S-induced antihypertensive effects.

b. Inhibition of vascular remodeling. Vascular structural remodeling is one important factor in the development of hypertension. In addition, elevated BP itself promotes blood vessel remodeling. Therefore, structural remodeling of small arterioles is a target for the treatment of hypertension. Vascular remodeling is linked to RAS, as the interruption of the RAS with ACE inhibitors or AT1 receptor antagonists corrects vascular structure (209), and ERK1/2 signaling stimulated by Ang II is often associated with VSMC hypertrophy (120). H_2S also reversed the elevated hydroxyproline and collagen type I levels (310). Another mechanism for the preventive/therapeutic effects of NaHS on vascular structural remodeling may be its antioxidant effects. H_2S may reduce the tissue ROS level, which is important in vascular remodeling.

c. Activation of K_{ATP} channels. The administration of GYY4137, a water-soluble compound that slowly releases H_2S, can cause an increase in the plasma H_2S concentration for up to 180 min (138). This slow and sustained increase of the H_2S level may produce an antihypertensive effect *via* the activation of K_{ATP} channels, which hyperpolarizes and, therefore, relaxes vascular smooth muscle. However, it was observed that BP only returned to normal 7 days after the last injection, implying that other long-term regulatory mechanisms probably exist.

VIII. Concluding Remarks

Current evidence indicates that H_2S plays important roles in mediating cardiovascular functions under both physiological and pathological conditions. H_2S inhibits heart contractile functions *via* its inhibitory effects on the β-adrenergic system and intracellular calcium handling. These observations are further supported with discoveries that H_2S exerts inhibitory effects on LTCC and chloride channels, while it stimulates the K_{ATP} channels. In the vascular system, *in vitro* tissue contractility studies demonstrated that NaHS induces vasoconstriction at lower concentrations (10–100 μM) while exerting vasodilatory responses at concentrations above about 100 μM. Though uncertainties still persist in the determination of endogenous H_2S level in plasma and tissues, the latest consensus favors the notion that free H_2S exists in the nanomolar range. However, tissues may contain stored sulfide, probably near a low micromolar range, and this sulfide can be released under specific conditions. It is still thought by many that endogenous H_2S produces vasodilation under physiological conditions. Thus, local micro environments may contain much higher concentrations of free H_2S than those found in tissue samples.

It is important to note that the endogenous production of H_2S appears to be significantly reduced in many disease states, including myocardial ischemia; MI-induced or AVF-induced CHF; spontaneous, pulmonary, or hyperhomocysteinemia-induced hypertension; vascular calcification; or apoE$^{-/-}$ atherosclerotic models. Whether reduction of H_2S level is the cause or consequence of these pathological states is not well understood. However, these findings are clear evidence that support the involvement of endogenous H_2S in maintaining basal physiological functions.

Another exciting aspect of H_2S research will be the crosstalk among H_2S and other gaseous transmitters—NO and CO. Efforts have been made to understand the interaction between NO and H_2S. Though great disparities and complexities exist in research outcomes, these interactions have important potential implications for our understanding of the cardiovascular system and are of potential therapeutic value.

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Address correspondence to:
Dr. Jin-Song Bian
Department of Pharmacology
Yong Loo Lin School of Medicine
National University of Singapore
18 Medical Drive
Singapore 117597
Singapore

E-mail: phcbjs@nus.edu.sg

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Abbreviations Used

$[Ca^{2+}]_I$ = intracellular calcium
$[cAMP]_I$ = intracellular cAMP
$[K^+]_i$ = intracellular potassium
$[Na^+]_i$ = intracellular sodium+dl
dt = maximal velocity of cell shortening
1K1C = one-kidney-one-clip
2K1C = two-kidney-one-clip
3-MST = mercaptopyruvate sulphurtransferase
4-AP = 4-aminopyridine
5-HD = 5-hydroxydecanoate
AA = arachidonic acid
AC = adenyl cyclase
ACE = angiotensin converting enzyme
Ach = acetylcholine
ADAM-12 = disintegrin and metalloproteinase domain-containing protein 12
Ad-CSE = adenovirus containing CSE gene
ADR = adriamycin
ADRF = adipoctye-derived relaxing factor
AE = anion exchanger
AIF = apoptosis-inducing factor
AIP = apoptosis-inducing protein
Ang II = angiotensin II
AOAA = amino-oxyacetate
APD = action potential duration
apoE = apolipoprotein E
$apoE^{-/-}$ = apolipoprotein E knockout
AT1 = Ang II type 1
ATP = adenosine triphosphate
AVF = arteriovenous fistula
BCA = β-cyano-L-alanine
Bcl-2 = B-cell lymphoma 2
bFGF = basic fibroblast growth factor
BP = blood pressure
CAD = coronary artery disease
cADPR = cyclic ADP ribose
cAMP = cyclic adenosine monophosphate
CAT = cysteine aminotransferase
CBE = Cl^-/HCO_3^- exchangers
CBS = cystathionine β-synthase
cdk = cyclin-dependent kinase
cGMP = cyclic guanosine monophosphate
CHF = chronic heart failure
CLT = chloramine T
CO = carbon monoxide
$CoCl_2$ = cobalt chloride
COX = cyclooxygenase
CPB = cardiopulmonary bypass
CSE = cystathionine γ-lyase
CVD = cardiovascular diseases
DBP = diastolic blood pressure
DNS-Az = dansyl azide
DTT = dithiothreitol
ECs = endothelial cells
EDHF = endothelium-derived hyperpolarizing factor
eNOS = endothelial NO synthase
ERK = extracellular signal-regulated kinase
FBS = fetal bovine serum
GC = gas chromatography

Abbreviations Used (Cont.)

GSH = glutathione
GSK-3β = glycogen synthase kinase-3
HA = hydroxylamine
HASMCs = human aorta SMCs
HF = heart failure
HHcy = hyperhomocysteineamia
HIF = hypoxia inducible factor
HNO = nitroxyl anion
HO-1 = heme oxygenase-1
HOCl = hypochlorite
HPH = hypoxic pulmonary hypertension
H_2S = hydrogen sulfide
HSP = heat shock protein
HUVECs = human umbilical vein endothelial cells
i.p. = intraperitoneal
i.v. = intravenous
I/R = ischemia/reperfusion
ICAM-1 = intercellular adhesion molecule-1
IL = interleukin
IPreC = ischemic preconditioning
ISO = isoproterenol
K_{ATP} = ATP-sensitive potassium
K_v = voltage-dependent K^+
LAD = left anterior descending
LCA = left coronary artery
LDH = lactate dehydrogenase
LDL = low density lipoprotein
L-NAME = N (G)-nitro-L-arginine methyl ester
LOOH = lipid hydroperoxide
LPO = lipid hydroperoxidation
LTCC = L-type Ca^{2+} channels
LV = left ventricle
LVDP = left ventricular developed pressure
$LVdp/dt_{max}$ = maximal left ventricular pressure development
LVEDP = left ventricular end diastolic pressure
MAP = mean arterial pressure
MAPK = mitogen-activated protein kinase
MBB = monobromobimane
MEK = MAPK/ERK kinase
MI = myocardial infarction
MLC = myosin light chain
MMP = matrix metalloproteinases
mPAP = mean pulmonary arterial pressure
MPO = myeloperoxidase
mPTP = mitochondrial permeability transition pore
NADPH = nicotinamide adenine dinucleotide phosphate
NaHS = sodium hydrosulfide (H_2S donor)
NCX = Na^+/Ca^{2+} exchanger
NEM = N-ethylmaleimide
NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells
NHE = Na^+/H^+ exchanger
NO = nitric oxide
NOS = NO synthase
NRF-1 = nuclear respiratory factor 1
Nrf2 = nuclear factor-erythroid-derived 2 related factor 2
O_2^- = superoxide anion
$ONOO^-$ = peroxynitrite
oxLDL = oxidized low-density lipoprotein
PAG = propargylglycine
PDE = phosphodiesterase
PH = pulmonary hypertension
pHi = intracellular pH
PI3K = phosphoinositide 3-kinase
PKA = protein kinase A
PKC = protein kinase C
PRA = plasma renin activity
ROS = reactive oxygen species
rvSUR1 = rat vascular sulfonylurea receptor 1
RyR = ryanodine receptor
SBP = systolic blood pressure
SERCA = sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase
SF = sulfidefluor
SFP = selective fluorescent probe
SHR = spontaneously hypertensive rats
siRNA = small interfering RNA
SMC = smooth muscle cell
SNAP = S-nitroso-N-acetylpenicillamine
SNP = sodium nitroprusside
SOD = superoxide dismutase
SPostC = H_2S postconditioning
SPreC = H_2S preconditioning
STAT = signal transducer and activator of transcription
TBARS = thiobarbituric acid-reactive substrates
TIMP = tissue inhibitor of matrix metalloproteinases
TNFα = tumor necrosis factor-α
Trx1 = thioredoxin-1
TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGF = vascular endothelial growth factor
VEGFR2 = VEGF receptor 2
VSMC = vascular smooth muscle cell
WKY = Wistar-Kyoto rats
XE991 = 10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone

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