Emergence of Hydrogen Sulfide as an Endogenous Gaseous Signaling Molecule in Cardiovascular Disease

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Abstract: Long recognized as a malodorous and highly toxic gas, recent experimental studies have revealed that hydrogen sulfide (H₂S) is produced enzymatically in all mammalian species including man and exerts several critical actions to promote cardiovascular homeostasis and health. During the past 15 years, scientists have determined that H₂S is produced by 3 endogenous enzymes and exerts powerful effects on endothelial cells, smooth muscle cells, inflammatory cells, mitochondria, endoplasmic reticulum, and nuclear transcription factors. These effects have been reported in multiple organ systems, and the majority of data clearly indicate that H₂S produced by the endogenous enzymes exerts cytoprotective actions. Recent preclinical studies investigating cardiovascular diseases have demonstrated that the administration of physiological or pharmacological levels of H₂S attenuates myocardial injury, protects blood vessels, limits inflammation, and regulates blood pressure. H₂S has emerged as a critical cardiovascular signaling molecule similar to nitric oxide and carbon monoxide with a profound effect on the heart and circulation. Our improved understanding of how H₂S elicits protective actions, coupled with the rapid development of novel H₂S-releasing agents, has resulted in heightened enthusiasm for the clinical translation of this ephemeral gaseous molecule. This review will examine our current state of knowledge about the actions of H₂S within the cardiovascular system with an emphasis on the therapeutic potential and molecular cross talk between H₂S, nitric oxide, and carbon monoxide. (Circ Res. 2014;114:730-737.)

Key Words: carbon monoxide ■ gasotransmitters ■ heart failure ■ nitric oxide

Hydrogen sulfide (H₂S) has traditionally been viewed as a highly toxic gas devoid of any biological or physiological function. Dating back >250 million years, H₂S poisoning resulting from upwelling euxinix bottom water is postulated to have caused the sudden mass extinction of the Ediacaran fauna.¹ In the 18th century AD, detailed examination of environmental factors and workplace chemical exposures by Bernardino Ramazzini revealed that cesspit workers exposed to abnormally high levels of H₂S commonly acquired eye inflammation that led to secondary bacterial infection and blindness.² H₂S was eventually measured in the brain in 1989, and it quickly emerged as a critically important signaling molecule with widespread physiological actions.³,⁴ The existence of H₂S in the brain suggested physiological purpose. Cystathionine β-synthase (CBS) is believed to be the critical enzyme that produces H₂S, resulting in the modulation of neurological function.⁵ H₂S generated by cystathionine γ-lyase (CSE) was next discovered as an important modulator of vasorelaxation in smooth muscle.⁶ After the discovery of H₂S as a potential neurological and vasorelaxant signaling molecule, the number of publications pertaining to the physiology of H₂S drastically spiked. It was no longer regarded as a toxic modulator of cell death but a physiologically important and potentially highly salubrious molecule with diverse signaling actions.

Endogenous Synthesis of H₂S in Mammals

H₂S is produced endogenously via enzymatic activity, nonenzymatic pathways (such as reduction of thiol-containing molecules), and is also released from intracellular sulfur stores ( sulfane sulfur).⁷ In most tissue, CBS and CSE are primarily responsible for the production of H₂S. They separately coordinate with L-cysteine to produce H₂S, L-serine, and ammonium.⁸ Although found throughout the body, the discovery of CBS in the brain led to a consensus that it was the primary H₂S-producing enzyme affecting neurological signaling. However, CBS has been identified in tissues throughout the body and is thought to modulate global H₂S generation. More recently, it has been reported that 3-mercaptopuruvate sulfurtransferase (3-MST) is responsible for ≈90% of H₂S produced in the brain.⁹ 3-MST, primarily located in the mitochondria, enzymatically produces H₂S from α-ketoglutarate and L-cysteine via metabolic interactions with cysteine aminotransferase.⁹ Although 3-MST in the neurons is responsible for much of the brain’s H₂S production, CBS is localized in astrocytes, suggesting that a portion of the H₂S signaling may be a result of the actions of CBS.¹⁰
Vascular Actions of Endogenous H$_2$S

One of the first proposed beneficial physiological effects of H$_2$S was its action on vascular tone (ie, blood pressure regulation) and inflammation.\textsuperscript{15} H$_2$S has been considered widely as a potent anti-inflammatory molecule with modest vasodilator actions. One of these mechanisms is its capacity to hinder leukocyte adhesion by inhibition of leukocyte rolling and firm adhesion to the endothelium. H$_2$S has been shown to inhibit significantly the expression of leukocyte adhesion molecules.\textsuperscript{16} In addition, H$_2$S signaling promotes anti-inflammatory action by preventing tissue edema. This finding was shown in rats, whereby the administration of an H$_2$S inhibitor led to edema formation.\textsuperscript{17} The anti-inflammatory response of H$_2$S may also be dependent on the activation of vascular K$_{ATP}$ channels. Rats treated with a specific K$_{ATP}$ channel antagonist did not show a reduction in leukocyte adhesion, suggesting that the ability of H$_2$S to modulate adhesion may be dependent on the signaling of this channel.\textsuperscript{18} H$_2$S activates K$_{ATP}$ channels, specifically in the smooth muscle, by increasing whole-cell K$_{ATP}$ currents to hyperpolarize membrane potentials and increases single-channel activity by enhancing permeability of single K$_{ATP}$ channels.\textsuperscript{18}

A somewhat controversial action of H$_2$S in the circulation is related to the role of the gaseous signaling molecule on vasodilation and blood pressure regulation. There are mixed results in the literature with some studies reporting vasodilatory actions, whereas others report vasoconstrictor effects. Mice with a genetic deletion of CSE, and consequently deficient H$_2$S production, displayed significant hypertension and diminished endothelial vasorelaxation.\textsuperscript{19} Other studies reveal that exogenous administration of H$_2$S can cause vasoconstriction. The discrepancy in these findings seems to depend on the concentration of H$_2$S, the vascular bed that is studied, and the oxygen tension of the tissue or blood vessel under investigation. When H$_2$S is held above trace levels, it has been shown to be an effective vasodilator.\textsuperscript{20} Interestingly, it exerted vasodilator effects at an oxygen partial pressure of 30 mm Hg, yet acted as a vasoconstrictor at an elevated partial pressure of oxygen of 150 mm Hg.\textsuperscript{21} It has been suggested that the vasodilator actions of H$_2$S may be a result of endothelial NO synthase (eNOS)–generated NO promoted by H$_2$S signaling.

H$_2$S also has been shown to exert potent proangiogenic effect in vascular endothelial cells in the setting of chronic ischemia while activating extracellular kinase pathways that promote vessel growth.\textsuperscript{22} Multiple groups have shown that H$_2$S stimulates endothelial cell proliferation and migration by either further enhancing current cell growth or by developing primary endothelial cells.\textsuperscript{23,24} H$_2$S participates in vascular endothelial growth factor signaling. CSE–/– mice exhibited significant reductions in H$_2$S and growth of endothelial cells in vitro.\textsuperscript{25} However, all proangiogenic signaling is not H$_2$S dependent because fibroblast growth factor levels were not attenuated in CSE–/– mice.\textsuperscript{22} The signaling pathways for H$_2$S-mediated angiogenesis effects are somewhat complex. Exogenous H$_2$S donors have been shown to activate the protein kinase B pathway, which in turn promotes angiogenesis and tumor development, and enhance phosphorylation of the mitogen-activated protein kinase pathway (extracellular-signal-regulated kinases and p38).\textsuperscript{24} These pathways have been shown to regulate RF/6A cells and human umbilical vein endothelial cells, respectively.\textsuperscript{26}

Role of H$_2$S in Cardiovascular Physiology and Pathophysiology

Ischemia/Reperfusion Injury in Heart and Brain

H$_2$S has been examined extensively as a potential therapeutic in the setting of ischemia/reperfusion (I/R) injury in the heart, brain, lungs, and liver. The majority of in vitro and in vivo studies thus far have reported beneficial actions of H$_2$S when administered at physiological or pharmacological concentrations. In the setting of I/R injury, the cytoprotective actions are thought to result from antiapoptotic, anti-inflammatory, antioxidant, and mitochondrial actions of H$_2$S. Diallyl trisulfide, a stable H$_2$S donor, was administered to mice after acute myocardial ischemia and markedly protected the myocardium.\textsuperscript{27} Diallyl trisulfide significantly decreased infarct size and troponin I levels and improved mitochondrial coupling. Additionally in diabetic mice, H$_2$S therapy was shown to precondition the myocardium against I/R injury by activating the antioxidant-signaling molecule, nuclear factor E2-related factor (Nrf2).\textsuperscript{28} It should be noted that the effective therapeutic range for H$_2$S-releasing agents studied thus far is relatively narrow, and the administration of suprapharmacological levels of H$_2$S clearly fails to protect and may even exacerbate I/R injury.\textsuperscript{27} In this regard, H$_2$S therapy is similar to NO therapy in the setting of I/R injury.\textsuperscript{27,28}

H$_2$S has also been reported to be a potent neuroprotective agent. Kimura et al\textsuperscript{29} reported that H$_2$S protects neurons from oxidative stress by bolstering glutathione levels after I/R in vitro. To study a more severe cerebral injury model, mice were subjected to 30 minutes of ischemia, and sodium hydrosulfide (NaHS) was administered after 24 hours of reperfusion.\textsuperscript{30}
H2S therapy has recently been shown to ameliorate ischemic heart failure.34 Genetic overexpression of CSE in mice resulted in increased H2S levels and improved LV performance and survival in the setting of ischemic heart failure.34 In a hypertension-induced heart failure model system,34 Genetic overexpression of CSE in mice resulted in increased H2S levels and improved LV performance and survival in the setting of ischemic heart failure.34 In a hypertension-induced heart failure model, it has been demonstrated clearly that H2S delayed progression to adverse remodeling of the LV and induced angiogenesis in the myocardium.35 Administration of H2S in the diet during heart failure significantly decreased adverse LV remodeling compared with the control group.36 The transition to decompensated heart failure progresses with a decline in vascular growth.37 In a similar heart failure model, NaHS-treated mice induced matrix metalloproteinase-2, which promoted vascular endothelial growth factor synthesis and angiogenesis and suppressed antiangiogenic factors such as matrix metalloproteinase-9 and tissue inhibitor of matrix metalloproteinase-3.38 Increasing myocardial vascularity and perfusion in concert with cardiac myocyte growth are critical in the regulation of cell death and apoptosis, and much of the cytoprotective actions of H2S seem to be a potent proangiogenic agent for this indication.

Atherosclerosis
Atherosclerosis is characterized by several pathological events that include endothelial dysfunction, monocyte penetration and conversion into macrophage foam cells, and mired leukocyte rolling along the endothelium. Recent studies have shown that macrophages produce H2S endogenously and that lipopolysaccharide, an inflammatory endotoxin, stimulates CSE production of H2S in macrophages.39 Moreover, the H2S donor, NaHS, inhibited proatherogenic-oxidized low-density lipoprotein–induced foam cell formation in macrophages.40 Leukocyte velocity, attachment, and infiltration along the endothelium are key atherosclerotic factors, and they were examined after H2S therapy. Sodium sulfide (Na2S) and NaHS inhibited aspirin and the chemotactic peptide N-formyl-L-leucyl-L-phenylalanine leukocyte adherence in a dose-dependent manner.16 In a further study, deficiency of H2S (ie, CSE−/− mice) promoted leukocyte adhesion and decreased leukocyte velocity and exacerbated leukocyte infiltration, whereas administration of H2S donors suppressed leukocyte penetration.16

Molecular Signaling via Endogenous H2S
Similar to NO and CO, the effects of H2S on the cardiovascular system are mediated via a diverse array of cellular and molecular signals (Figures 1 and 2). Mitochondria are critical for cell survival and energy production. When mitochondrial function is compromised because of hypoxia or an increase in reactive oxygen species, H2S has been shown to protect mitochondria and ultimately improve respiration and promote biogenesis. This was shown when endogenous stimulation of H2S production (10–100 nmol/L) enhanced mitochondrial electron transport and cellular bioenergetics.41 However, at high concentrations, H2S is toxic, resulting in inhibition of mitochondrial respiration via direct inhibition of cytochrome c oxidase enzyme by rapid sulfide oxidation, oxygen uptake, and conversion of cytochrome aa3 into the low spin form.42,43 Isolated murine cardiac mitochondria exposed to 10 μmol/L H2S were shown to have improved recovery of posthypoxic respiration rate after 30 minutes of hypoxia.27 Mitochondria are unique in that they are critical in the regulation of cell death and apoptosis, and much of the cytoprotective actions of H2S during ischemic states may be a result of potent actions.
on mitochondria. In vitro, H$_2$S was shown to attenuate apoptosis in an adenocarcinoma cell line specific to colon cancer by preventing $\beta$-phenethyl isothiocyanate from inducing cell death. Furthermore, H$_2$S protects against high-glucose–induced cardiomyocyte apoptosis by altering regulatory gene expression. Moreover, after myocardial infarction injury in a murine model, H$_2$S-treated mice displayed significant reductions in apoptosis as evidenced by a decrease in caspase-3 activity terminal deoxynucleotidyl transferase dUTP nick end labeling positive nuclei count. Another mechanism by which mitochondria modulate cell death (ie, necrosis) is by the induction of the mitochondrial permeability transition pore in response to oxidative stress, free radicals, and elevated matrix Ca$^{2+}$ count commonly generated in ischemia and reperfusion. The activation of this pore leads to a halt in ATP production and a breakdown of the mitochondria. 

A potential mechanism to prevent the induction of mitochondrial permeability transition pore, aside from known inhibitors such as cyclosporine A, could be via potent inhibition of mitochondrial oxidative stress. H$_2$S has emerged as a potent antioxidant molecule via both direct and indirect actions. Oxidative stress was studied after the introduction of the H$_2$S donor, NaHS, in vitro. In an extracellular cysteine dependent manner, H$_2$S protected cultured neurons from oxidative stress by increasing glutathione levels instead of acting directly as an antioxidant. Also, Nrf2, a transcription factor, regulates oxidative stress by affecting gene expression of several key enzymes. Because Nrf2 breaks its interaction with the repressor cytoplasmic protein Kelch-like ECH-associated protein 1, Nrf2 translocates to the nucleus and promotes the expression of detoxifying genes such as heme oxygenase-1, superoxide dismutase 1, and catalase. Daily administration of Na$_2$S for 7 days increased Nrf2 expression in both cytosolic and nuclear fractions, indicating further antioxidant signaling by H$_2$S. Furthermore, H$_2$S was administered to mice exposed to 60 minutes of hepatic ischemia and 5 hours of reperfusion. At both 1-hour and 5-hour reperfusion time points, lipid hydroperoxide levels in hepatic tissue were decreased significantly in the H$_2$S compared with vehicle-treated mice. H$_2$S ability to scavenge for oxidants, such as hydroperoxide and reactive oxygen species, may also be contributing to its anti-inflammatory actions mentioned earlier.

**Cross Talk Between H$_2$S and Other Gaseous Signaling Molecules**

NO and H$_2$S share many of the same regulatory roles including vasodilation, promotion of angiogenesis, attenuation of apoptosis, and antioxidant actions. In endothelial cells, NO is synthesized by eNOS and initiates downstream signaling with guanylyl cyclase to form the second messenger cGMP.
Although H2S and NO exhibit independent signaling, it seems that there is cross talk between these 2 molecules in a manner that modulates multiple pathways (Figures 3 and 4). eNOS function is regulated tightly by post-translational modifications (such as the phosphorylation of amino acids such as Ser-1177 and Thr-495) that can enhance or thwart eNOS production of NO.54,55 In a pressure-overload murine heart failure, Kondo et al reported that mice treated with an H2S donor significantly increased phosphorylation of activation site, eNOS-P Ser1177 compared with the control group. This increase in eNOS phosphorylation was accompanied by increased NO production. Mice treated with the H2S donor, diallyl trisulfide, showed marked increases in plasma nitrite, nitrate, and nitrosylated protein (RXNO) levels 30 minutes after injection.25 Furthermore, NO can also effect H2S generation. NO donors have been shown to increase the expression of CSE in isolated aortic smooth muscles cells.56 There still remains some controversy over cross talk between H2S and NO. For example, 1 group found that eNOS deficiency prevented the ability of H2S to induce angiogenesis in vivo or in vitro, suggesting that NO is required for H2S to have vascular effects.57 Yet, another group proposed that the proangiogenic effect of H2S is regulated by both an NO-dependent and an independent manner.58 Once we better understand how these molecules work together, we can begin to build therapeutics that maximize the benefits of both signaling molecules.

Far less has been studied on the cross talk between H2S and CO. Endogenously, CO is derived from the breakdown of heme by heme oxygenase, and it can also activate guanylyl cyclase, which causes an increase in cGMP.59,60 CO also shares many of the same biological effects of NO and H2S including its apoptotic and anti-inflammatory mechanisms. Zhang et al demonstrated that exogenous H2S upregulates the CO system in pulmonary arteries of hypoxic rats. More recently, a long-lasting H2S donor was shown to inhibit oxidative stress and increase Nrf2, heme oxygenase-1, and p-protein kinase B levels more so than its short-acting counterpart,62 providing some evidence for H2S–CO cross talk.

**H2S Therapeutic Agents and Mutant Mouse Models**

Exogenous administration of H2S or genetic modulation of CSE, CBS, or 3-MST levels are effective means by which the cardiovascular actions of H2S can be investigated. Numerous H2S donors with varying chemical and pharmacological properties have emerged as potential therapeutics. Na2S and NaHS were among the first H2S-releasing agents studied in the cardiovascular system.27,49 These inorganic salts have the advantage of rapidly increasing H2S concentration within seconds, but they also rapidly decline within tissue and could exert adverse side effects because of rapid increases in H2S at high concentrations.63 In addition, many of the commercially available formulations of NaHS and Na2S are highly impure, and the impurities elicit toxic effects. Naturally occurring H2S donors such as diallyl trisulfide, a polysulfide derived from garlic, have been shown to augment H2S levels for extended periods of time.64 Synthetic H2S-releasing compounds have also been developed. SG-1002 and penicillamine-based donors are examples of synthesized H2S donors whose release is more precisely controlled. As novel H2S-releasing agents or H2S donors develop, these novel agents should ultimately address the clinically relevant issues such as sustained release/half-life, route of administration, tissue specificity, and low toxicity.

The effects of decreased endogenous H2S production have also been investigated in cardiovascular disease. H2S enzyme antagonists have been explored to reach the same goal. DL-propargylglycine, an inhibitor of CSE, exerted a dose-dependent inhibition of sulfide production.66 However, the inhibition came at the cost of unrealistically high dosages.
There remain few other targeting molecules with high potency and high selectivity but would be more valuable than knocking out an entire enzyme. Complete genetic deficiency of CBS (ie, homozygote knockout mouse) is lethal, and limited literature exists examining the genetic deficiency of CBS in heterozygote knockouts. In contrast, a global 3-MST knockout mouse has been developed, but there is a paucity of information about genetic deficiency of 3-MST in cardiovascular disease at present. In addition, little is currently known about genetic overexpression of either CBS or 3-MST because of a lack of these transgenic mouse models. However, CSE$^{-/-}$ and CSE-overexpressing transgenic mice have been developed and have been well characterized fairly in terms of cardiovascular disease states. Global CSE$^{-/-}$ mice show significant reduction in H$_2$S bioavailability in serum, heart, aorta, and several other tissues. Global CSE$^{-/-}$ mice exhibit pronounced hypertension and reduced vasodilation, indicating CSE-derived H$_2$S is an important mediator of vascular reactivity and blood pressure. CSE$^{-/-}$ mice subjected to myocardial I/R injury experience a 48% increase in infarct size compared with wild-type mice. Conversely, CSE overexpressing transgenic mice, after myocardial I/R, display a marked reduction in infarction compared with wild-type mice. In a pressure-overload heart failure model, CSE$^{-/-}$ exhibited exacerbated LV dysfunction, whereas CSE transgenic mice promoted cardiac structure and function compared with wild-type mice. Cardiac mitochondria isolated from CSE$^{-/-}$ mice exhibit profound mitochondrial dysfunction. These data provide clear evidence for the cytoprotective actions of CSE-derived H$_2$S in various cardiovascular pathologies.

### Challenges for the H$_2$S Research Field

There are several difficulties that researchers face when studying H$_2$S in physiological or pathological in vivo systems. It is critical to measure H$_2$S levels accurately in blood and tissue samples from patients who experience cardiovascular diseases. The first challenge is the measurement of H$_2$S and quantifying its bioavailability in vivo. Besides free sulfide, molecule-bound sulfide is also present in biological systems and can be liberated and quantified. One of the most common measurements is by way of the methylene blue method. This assay is conducted under acidic conditions and measures sulfide concentrations in biological samples by releasing acid labile sulfide. A weakness of this method, as well as all colorimetric detection, is that it interferes with other chromophores, resulting in an artifactual signal. Another common mode for measuring H$_2$S, with sensitivity in the nanomolar range, uses monobromobimane. This method includes nucleophilic substitution reactions to give a fluorescent sulfide dibrimane whose emitted wavelengths can be detected in visible light range detected with high-performance liquid chromatography. The limit of detection with this method is 2 nmol/L, and the sulfide dibrimane product is stable over time. Likely a more precise measurement of H$_2$S and sulfane sulfur is through use of a combined gas chromatography–chemiluminescence approach. This method requires fresh tissue homogenate reacting with buffer for an extended incubation period that releases the gas into headspace. The disadvantage of measuring headspace concentrations that have been incubating for relatively long periods of time make real-time measurements problematic. Another key method of detection is the use of fluorescent probes, but it too faces the challenge of thiol interference that is present in most cellular compartments and biological fluids.

Another challenge for the H$_2$S field is the development of clinically relevant therapeutic agents to treat cardiovascular diseases. Aside from the aforementioned importance of a long-acting donor with controlled H$_2$S release, developing a drug that can specifically target a body system would alleviate unwanted side effects. The mechanisms of site-specific delivery remain challenging; however, targeted H$_2$S delivery to
myocardial microvasculature was achieved using ultrasound to release encapsulated H$_2$S from perfluorocarbon-filled microbubbles. In addition, mitochondria-targeted H$_2$S donors are in development and contain a mitochondria-targeting moiety aimed to mediate oxidative stress and cell injury. Mastering these issues would drastically advance H$_2$S research and further translate it into clinical relevance.

**Future Directions and Clinical Translation**

H$_2$S makes a universal biological impression by freely diffusing across cellular membranes and affecting cells and cellular organelles throughout the body. Yet, before H$_2$S therapies can be fully translated to a clinical setting, much more must be accomplished. Mechanistic discovery is underway in regard to aforementioned antioxidant and antiapoptotic signaling. However, a greater depth of knowledge is required to develop effective therapeutics. Specifically, function and signaling relating to the enzymes responsible for the endogenous production of H$_2$S are worthy of further study. Understanding location and activity of these enzymes in particular disease states would help direct gene therapy or localized drug delivery. Understanding these mechanisms would help identify what tissues can be effected and what pathological conditions are most responsive to H$_2$S therapy.

Exploring the relationship and interactions of H$_2$S with other endogenous gases, specifically NO, could improve clinical translation. H$_2$S therapy in conjunction with NO donors may augment outcome and bolster cardiovascular response and cellular function. Endogenous production of H$_2$S was shown to increase significantly the vasorelaxant effect of an NO donor (sodium nitroprusside). Also, exogenously administered H$_2$S increased eNOS activation and NO bioavailability. This indicates that H$_2$S enhances NO actions in the vasculature. The cooperative or competitive actions of H$_2$S and NO as they simultaneously interact with proteins in S-sulfhydration and S-nitrosylation reactions are also unknown.

Last, before making a complete transition to human testing (there are currently 2 cardiovascular H$_2$S trials on clinicaltrials.gov), well-established, large animal models of cardiovascular disease should be investigated thoroughly because the majority of cardiovascular studies have been performed in murine model systems. These murine model systems provide a good foundation but are lacking in clinical relevance. Examining H$_2$S actions in an animal model with similar cardiovascular characteristics as humans experiencing cardiovascular disease would help verify the safety and efficacy of the drug.

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**Disclosures**

D.J. Lefer is a founder of and scientific advisor for Sulfagenix Inc. Sulfagenix is currently developing hydrogen sulfide–based therapeutics for the treatment of cardiovascular disease. The other author reports no conflicts.

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