Published in final edited form as:
Circ Res. 2014 February 14; 114(4): 730–737. doi:10.1161/CIRCRESAHA.114.300505.

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

David J. Polhemus, B.A. and **David J. Lefer, Ph.D.**
LSU Health Sciences Center-New Orleans, Department of Pharmacology and the LSU Cardiovascular Center of Excellence, 1901 Perdido Street, New Orleans, Louisiana, 70112

Abstract

Long recognized as a malodorous and highly toxic gas, recent experimental studies have revealed that hydrogen sulfide (H_2S) is produced enzymatically in all mammalian species including man and exerts a number of critical actions to promote cardiovascular homeostasis and health. During the past 15 years, scientists have determined that H_2S is produced by three 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 vast majority of data clearly indicate that H_2S 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 H_2S attenuates myocardial injury, protects blood vessels, limits inflammation, and regulates blood pressure. H_2S has emerged as a critical cardiovascular signaling molecule similar to nitric oxide (NO) and carbon monoxide (CO) with a profound impact on the heart and circulation (Figure 1). Our improved understanding of how H_2S elicits protective actions, coupled with the very rapid development of novel H_2S 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 regarding the actions of H_2S within the cardiovascular system with an emphasis on the therapeutic potential and molecular crosstalk between H_2S, NO, and CO.

Keywords

Nitric oxide (NO); Carbon monoxide (CO); gasotransmitter; cardioprotection; heart failure; acute myocardial infarction; endothelial nitric oxide synthase (eNOS)

Introduction

Hydrogen Sulfide (H_2S) has traditionally been viewed as an odorous and highly toxic gas devoid of any biological or physiological function. Dating back over 250 million years, H_2S poisoning due to 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 toxic levels of H_2S commonly acquired eye inflammation which led to secondary bacterial infection and blindness[2]. H_2S was eventually measured in the brain in 1989 and it quickly emerged as a critically important signaling molecule with

Address for Correspondence: David J. Lefer, Ph.D., Cardiovascular Center of Excellence, LSU Health Sciences Center-New Orleans, 533 Bolivar St., Suite 408, New Orleans, LA 70112, Phone: (504) 568-2109, FAX: (504) 568-2361, dlefe1@lsuhsc.edu.

Disclosures: D.J.L is a founder of and scientific advisor for Sulfagenix, Inc. Sulfagenix is currently developing hydrogen sulfide-based therapeutics for the treatment of cardiovascular disease.

widespread physiological actions[3, 4]. The existence of H_2S in the brain suggested physiological purpose. Cystathionine beta synthase (CBS) is believed to be the critical enzyme that produces H_2S resulting in the modulation of neurological function[5]. H_2S generated by cystathionine gamma lyase (CSE) was next discovered as an important modulator of vasorelaxation in smooth muscle[6]. Following the discovery of H_2S as a potential neurological signaling molecule and a vasorelaxant molecule, the number of publications pertaining to the physiology of H_2S 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 Hydrogen Sulfide in Mammals

H_2S is produced endogenously via enzymatic activity, non-enzymatic 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 H_2S. They separately coordinate with L-cysteine to produce H_2S, L-serine, and ammonium[8]. Although found throughout the body, the discovery in of CBS in the brain led to a consensus that is was the primary H_2S producing enzyme impacting neurological signaling. However, CBS has been identified in tissues throughout the body and is thought to modulate global H_2S generation. More recently, it has been reported that 3-mercaptopyruvate sulfurtransferase (3-MST) is responsible for roughly 90% of H_2S produced in the brain[9]. 3-MST, primarily located in the mitochondria, enzymatically produces H_2S from α-ketoglutarate and L-cysteine via metabolic interactions with cysteine aminotransferase (CAT)[9]. Although 3-MST in the neurons is responsible for much of the brain's H_2S production, CBS is localized in astrocytes suggesting that a portion of the H_2S signaling may be a result of the actions of CBS[10].

Under physiological conditions, H_2S can undergo several catabolic fates. Once deprotonated, HS^- is rapidly oxidized in the mitochondria to form thiosulfate (non-enzymatic conversion), which is ultimately converted to sulfite and sulfate[11]. H_2S can also be methylated by thiol S-methyltransferase (TMST) to form dimethylsulfide and methanethiol, or it can react with methemoglobin to form sulfermoglobin[12]. Similar to the other two gaseous signaling molecules, nitric oxide (NO) and carbon monoxide (CO), H_2S has a very high affinity for hemoglobin, resulting in profound scavenging. One of the major challenges to the study of H_2S under *in vivo* conditions is the extremely short half-life of this ephemeral molecule; estimated to be between seconds to minutes[8, 13] (between 12 and 37 hours in air[14])[8].

Vascular Actions of Endogenous Hydrogen Sulfide

One of the first proposed beneficial physiological effects of H_2S that was reported was its action on vascular tone (i.e., blood pressure regulation) and inflammation[15]. H_2S has been widely considered as a potent anti-inflammatory molecule with modest vasodilator actions. One of these effects is its capacity to hinder leukocyte adhesion by inhibition of leukocyte "rolling" and firm adhesion to the endothelium. H_2S has been shown to significantly inhibit the expression of leukocyte adhesion molecules[16]. Additionally, H_2S signaling promotes anti-inflammatory action by preventing tissue edema. This finding was shown in rats whereby the administration of an H_2S inhibitor led to edema formation[17]. The anti-inflammatory response of H_2S may also be dependent upon 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_2S to modulate adhesion may be dependent on the signaling of this channel[16]. H_2S 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[18].

A somewhat controversial action of H_2S 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 while others report vasoconstrictor effects. Mice with a genetic deletion of CSE, and consequently deficient H_2S production, displayed significant hypertension and diminished endothelial vasorelaxation[19]. Other studies reveal that exogenous administration of H_2S can cause vasoconstriction. The discrepancy in these findings appears to depend on the concentration of H_2S, the vascular bed that is studied, and the oxygen tension of the tissue or blood vessel under investigation. When H_2S is held above trace levels, it has been shown to be an effective vasodilator[20]. Interestingly, it exerted vasodilator effects at an oxygen partial pressure of 30 mmHg, yet acted as a vasoconstrictor at an elevated partial pressure of oxygen of 150 mmHg[21]. It has been suggested that the vasodilator actions of H_2S may be a result of eNOS generated NO promoted by H_2S signaling.

H_2S also has been shown to exert potent pro-angiogenic effect in vascular endothelial cells in the setting of chronic ischemia, while promoting extracellular kinase pathways that promote vessel growth[22]. Multiple groups have shown that H_2S stimulates endothelial cell proliferation and migration by either further developing current cells or by developing primary endothelial cells[23, 24]. H_2S participates in vascular endothelial growth factor (VEGF) signaling. $CSE^{-/-}$ mice exhibited significant reductions in H_2S and growth of endothelial cells *in vitro*[24]. However, all pro-angiogenic signaling is not H_2S dependent, since fibroblast growth factor levels were not attenuated in $CSE^{-/-}$ mice[22]. The signaling pathways for H_2S-mediated angiogenesis effects are somewhat complex. Exogenous H_2S donors have been shown to activate the AKT pathway, which in turn promotes angiogenesis and tumor development, and enhance phosphorylation of the mitogen activated protein kinase (MAPK) pathway (ERK1/2 and p38)[24]. These pathways have been shown to regulate RF/6A cells and human umbilical vein endothelial cells, respectively[22].

Role of Hydrogen Sulfide in Cardiovascular Physiology and Pathophysiology

Ischemia-Reperfusion Injury in Heart and Brain

H_2S has been extensively examined 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 reported thus far have reported beneficial actions of H_2S when administered at physiological or pharmacological concentrations. In the setting of I/R injury, the cytoprotective actions are thought to result from anti-apoptotic, anti-inflammatory, antioxidant, and mitochondrial actions of H_2S. Diallyl trisulfide (DATS), a stable H_2S donor, was administered to mice following acute myocardial ischemia and markedly protected the myocardium[25]. DATS significantly decreased infarct size and troponin I levels, and improved mitochondrial coupling. Additionally in diabetic mice, H_2S therapy was shown to precondition the myocardium against I/R injury by activating the antioxidant-signaling molecule, Nfr2[26]. It should be noted that the effective therapeutic range for H_2S releasing agents studied thus far is relatively narrow and the administration of supra-pharmacological levels of H_2S clearly fails to protect and may even exacerbate I/R injury[27]. In this regard, H_2S therapy is very similar to NO therapy in the setting of I/R injury[28].

H_2S has also been reported to be a potent neuroprotective agent. Kimura et al. reported that H_2S protects neurons from oxidative stress by bolstering glutathione levels following I/R in

vitro[29]. To study a more severe cerebral injury model, mice were subjected to 30 minutes of ischemia, and NaHS was administered following 24 hours of reperfusion[30]. Pro-inflammatory markers, TNF-α and MCP-1, were significantly attenuated, while the anti-inflammatory marker, BcL-2, was enhanced[30]. In another cerebral ischemic study, H_2S protected against neuronal apoptosis by diminishing infarct volume and activation of caspase-3 in murine neurons[31].

Heart Failure

Heat failure prevalence has drastically increased, as it has become the primary discharge diagnosis in patients aged 65 years or older[32]. Following myocardial insult or injury, the left ventricle (LV) undergoes adaptive changes that ultimately transition the ventricle from a compensatory to a decompensated state. LV remodeling includes reactions such as apoptosis, inflammation, oxidative stress, and the development of fibrosis[33]. H_2S therapy has recently been shown to ameliorate ischemic-induced heart failure in a murine model system[34]. Genetic overexpression of CSE in mice resulted in increased H_2S levels and improved left ventricular performance and survival in the setting of ischemic heart failure[34]. In a hypertension induced heart failure model, it has been clearly demonstrated that H_2S decelerated progression to adverse remodeling of the LV and induced angiogenesis in the myocardium[35]. Administration of H_2S in the diet during heart failure significantly decreased adverse LV remodeling as compared to 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 (MMP-2), which promoted VEGF synthesis and angiogenesis and suppressed anti-angiogenic factors such as MMP-9 and tissue inhibitor of matric metalloproteinase (TIMP-3)[38]. Increasing myocardial vascularity and perfusion in concert with cardiac myocyte growth is critical to preventing the progression of heart failure and H_2S appears to be a potent pro-angiogenic 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 H_2S endogenously and that lipopolysaccharide (LPS), an inflammatory endotoxin, stimulates CSE production of H_2S in macrophages[39]. Moreover, the H_2S donor, NaHS, inhibited pro-atherogenic oxidized low-density lipoprotein (oxLDL) induced foam cell formation in macrophages[40]. Leukocyte velocity, attachment, and infiltration along the endothelium are key atherosclerotic factors and they were examined following H_2S therapy. Na_2S and NaHS inhibited aspirin and the chemotactic peptide N-formyl-L-leucyl-L-phenylalanine (fMLP) leukocyte adherence in a dose-dependent manner[16]. In a further study, deficiency of H_2S (i.e. $CSE^{-/-}$ mice) promoted leukocyte adhesion and decreased leukocyte velocity and exacerbated leukocyte infiltration, while administration of H_2S donors suppressed leukocyte penetration[16].

Molecular Signaling via Endogenous Hydrogen Sulfide

Similar to NO and CO, the effects of H_2S on the cardiovascular system are mediated via a very diverse array of cellular and molecular signals (Figure 2). Mitochondria are critical for cell survival and energy production. When mitochondrial function is compromised due to hypoxia or an increase reactive oxygen species (ROS), H_2S has been shown to protect mitochondria and ultimately improve respiration and promote biogenesis. This was shown when endogenous stimulation of H_2S production (10-100nM) enhanced mitochondrial electron transport and cellular bioenergetics[41]. However, at high concentrations, H_2S 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 aa_3 into the low spin form[42, 43]. Isolated murine cardiac mitochondria exposed to 10 μM H_2S were shown to have improved recovery of post-hypoxic respiration rate following 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 H_2S during ischemic states may be a result of potent actions on mitochondria[44]. *In vitro,* H_2S was shown to attenuate apoptosis in an adenocarcinoma cell line specific to colon cancer by preventing beta-phenyethyl isothiocyanate from inducing cell death[45]. Furthermore, H_2S protects against high-glucose induced cardiomyocyte apoptosis by altering regulatory gene expression[46]. Moreover, following myocardial infarction injury in a murine model, H_2S treated mice displayed significant reductions in apoptosis as evidenced by a decrease in caspase-3 activity TUNEL positive nuclei count[27]. Another mechanism by which mitochondria modulate cell death (i.e., necrosis) is by the induction of the mitochondrial permeability transition pore (MPTP) in response to oxidative stress, free radicals, and elevated matrix Ca^{2+} count commonly generated in ischemia and reperfusion[47]. 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 MPTP, aside from known inhibitors such as cyclosporine A, could be via potent inhibition of mitochondrial oxidative stress. H_2S has emerged as a very potent anti-oxidant molecule via both direct and indirect actions. Oxidative stress was studied following the introduction of the H_2S donor, sodium hydrosulfide (NaHS), *in vitro*. In an extracellular cysteine dependent manner, H_2S protected cultured neurons from oxidative stress by increasing glutathione levels instead of acting directly as an antioxidant[48]. Also, Nrf2, a transcription factor, regulates oxidative stress by impacting gene expression of several key enzymes[49]. As Nrf2 breaks its interaction with the repressor cytoplasmic protein Keap-1, Nrf2 translocates to the nucleus and promotes the expression of detoxifying genes such as heme oxygenase 1 (HO-1), superoxide dismutase 1, and catalase[50, 51]. Daily administration of Na_2S for 7 days increased Nrf2 expression in both cytosolic and nuclear fractions, indicating further antioxidant signaling by H_2S[52]. Furthermore, H_2S 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 was significantly decreased in the H_2S compared to vehicle treated mice[53]. H_2S ability to scavenge for oxidants, like hydroperoxide and ROS may also be contributing to its anti-inflammatory actions mentioned earlier.

Crosstalk Between Hydrogen Sulfide and Other Gaseous Signaling Molecules

NO and H_2S 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 endothelial nitric oxide synthase (eNOS) and initiates downstream signaling with guanylyl cyclase (GC) to form the second messenger cyclic guanosine 5'-monophosphate (cGMP). Although H_2S and NO exhibit independent signaling, it appears that there is crosstalk between these two molecules in a manner that modulates multiple pathways (Figures 3 and 4). eNOS function is tightly regulated 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 H_2S donor significantly increased phosphorylation of activation site, eNOS-$P^{Ser1177}$ compared to the control group[36]. This increase in eNOS phosphorylation was accompanied by increased NO

production. Mice treated with the H_2S donor, DATS, showed marked increases in plasma nitrite, nitrate, and RXNO levels 30 minutes following injection[25]. Furthermore, NO can also impact H_2S 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 crosstalk between H_2S and NO. For example, one group found that eNOS deficiency prevented the ability of H_2S to induce angiogenesis *in vivo* or in *in vitro*, suggesting that NO is required for H_2S to have vascular effects[57]. Yet, another group proposed that the pro-angiogenic effect of H_2S 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 regarding the crosstalk between H_2S and carbon monoxide (CO). Endogenously, CO is derived from the breakdown of heme by heme oxygenase (HO) and it can also activate GC which causes an increase in cGMP[59, 60]. CO also shares many of the same biological effects of NO and H_2S including its apoptotic and anti-inflammatory mechanisms. Zhang et al. demonstrated that exogenous H_2S upregulates the CO system in pulmonary arteries of hypoxic rats[61]. More recently, a long lasting H_2S donor was shown to inhibit oxidative stress and increase Nrf2, HO-1, and p-AKT levels more so than its short-acting counterpart[62], providing some evidence for for H_2S-CO crosstalk.

H_2S Therapeutic Agents and Mutant Mouse Models

Exogenous administration of H_2S or genetic modulation of CSE, CBS, or 3-MST levels are effective means by which the cardiovascular actions of H_2S can be investigated. Numerous H_2S donors with varying chemical and pharmacological properties have emerged as potential therapeutics. Na_2S and NaHS were among the first H_2S releasing agents studied in the cardiovascular system[27, 48]. These inorganic salts have the advantage of rapidly increasing H_2S concentration within seconds, but they also rapidly decline within tissue and could exert adverse side effects due to rapid increases in H_2S at high concentrations[63]. Additionally, many of the commercially available formulations of NaHS and Na_2S are highly impure and the impurities elicit toxic effects. Naturally occurring H_2S donors such as diallyl trisulfide (DATS), a polysulfide derived from garlic, have been shown to augment H_2S levels for extended periods of time[64]. Synthetic H_2S releasing compounds have also been developed. SG-1002[36] and penicillamine-based donors[65] are examples of synthesized H_2S donors whose release is more precisely controlled. As novel H_2S releasing agents or H_2S 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 H_2S production has also been investigated in cardiovascular disease. H_2S enzyme antagonists have been explored to reach the same goal. DL-propargylglycine (PAG), an inhibitor of CSE, exerted a dose-dependent inhibition of sulfide production[66]. However, the inhibition came at the cost of unrealistically high dosages (i.e., 50 mg/kg) and non-specific effects. There remain very 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 (i.e., 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 regarding genetic deficiency of 3-MST in cardiovascular disease at present. In addition, very little is currently known regarding genetic overexpression of either CBS or 3-MST due to a lack of these transgenic mouse models. However, $CSE^{-/-}$ and CSE

overexpressing transgenic mice have been developed and have been fairly well characterized in terms of cardiovascular disease states. Global $CSE^{-/-}$ mice show significant reduction in H_2S bioavailability in serum, heart, aorta, and several other tissues[19]. Global $CSE^{-/-}$ mice exhibit pronounced hypertension and reduced vasodilation, indicating CSE derived H_2S is an important mediator of vascular reactivity and blood pressure[19]. $CSE^{-/-}$ mice subjected to myocardial I/R injury[67] experience a 48% increase in infarct size compared to wild-type mice. Conversely, CSE overexpressing transgenic mice, following myocardial I/R, display a marked reduction in infarction compared to wild-type mice[27]. In a pressure overload heart failure model, $CSE^{-/-}$ exhibited exacerbated LV dysfunction, while CSE transgenic mice promoted cardiac structure and function compared to wild-type mice. Cardiac mitochondria isolated from $CSE^{-/-}$ mice exhibit profound mitochondrial dysfunction[36]. These data provide clear evidence for the cytoprotective actions of CSE-derived H_2S in various cardiovascular pathologies.

Challenges for the Hydrogen Sulfide Research Field

There are several difficulties that researchers face when studying H_2S in physiological or pathological *in vivo* systems. It is critical to accurately measure H_2S levels in blood and tissue samples from patients that suffer cardiovascular diseases. The first challenge is the measurement of H_2S 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[68]. Another common mode for measuring H_2S, with sensitivity in the nanomolar range, uses monobromobimane (MBB). This method includes nucleophilic substitution reactions to give a fluorescent sulfide dibrimane (SDB) whose emitted wavelengths can be detected in visible light range detected with HPLC. The limit of detection with this method is 2nM and the SDB product is very stable over time[69]. Likely a more precise measurement of H_2S and sulfane sulfur is through use of a combined gas chromatography-chemiluminescence approach[70, 71]. 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[72]. 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_2S field is the development of clinically relevant therapeutic agents to treat CV diseases. Aside from the aforementioned importance of a long acting donor with controlled H_2S 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_2S delivery to myocardial microvasculature was achieved using ultrasound to release encapsulated H_2S from per flourocarbon filled microbubbles (Wu et al. *Circulation* 2012;126:A10756 ABSTRACT). Additionally, mitochondria-targeted H_2S donors are in development and contain a mitochondria-targeting moiety aimed to mediate oxidative stress and cell injury (Le Trionnaire et al. *Nitric Oxide* 2013;31:S57 ABSTRACT). Mastering these issues would drastically advance H_2S research and further translate it into clinical relevance.

Future Directions and Clinical Translation

H_2S has very rapidly emerged as an exciting signaling molecule. This gaseous molecule impacts cells and cellular organelles throughout the body and freely diffuses across cellular membranes resulting in a universal biological impression. Yet, before H_2S therapies can be fully translated to a clinical setting, much more must be accomplished. Mechanistic discovery is underway and much has been accomplished in regards to aforementioned antioxidant and anti-apoptotic 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_2S 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 impacted and what pathological conditions are most responsive to H_2S therapy.

Exploring the relationship and interactions of H_2S with other endogenous gases, specifically NO, could improve clinical translation. H_2S therapy in conjunction with NO donors may augment outcome and bolster cardiovascular response and cellular function. Endogenous production of H_2S was shown to significantly increase the vasorelaxant effect of an NO donor (sodium nitroprusside)[56]. Also, exogenously administered H_2S increased eNOS activation and NO bioavailability[35, 36]. This indicates that H_2S enhances NO actions in the vasculature. The cooperative or competitive actions of H_2S and NO as they simultaneously interact with proteins in S-sulfhydration and S-nitrosylation reactions are also unknown[14].

Lastly, before making a complete transition to human testing (there are currently 2 cardiovascular H_2S trials on clinicaltrials.gov), well-established, large animal models of cardiovascular disease should be thoroughly investigated as the vast 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_2S actions in an animal model with similar cardiovascular characteristics as humans suffering from CV disease would help verify the safety and efficacy of the drug.

Acknowledgments

Sources of Funding: This work was supported by grants from the National Heart, Lung, and Blood Institute (National Institutes of Health; 1R01 HL092141, 1R01 HL093579, 1U24 HL 094373, and 1P20 HL113452. We are also grateful for the generous funding support from TEVA USA Scholars Program, the Carlyle Fraser Heart Center of Emory University Hospital Midtown, and the LSU Medical School Alumni Association.

References

1. Wille M, Nagler TF, Lehmann B, Schroder S, Kramers JD. Hydrogen sulphide release to surface waters at the precambrian/cambrian boundary. Nature. 2008; 453:767–769. [PubMed: 18509331]
2. Ramazzini B. De morbis artificum; diatriba. Romae: Typ C Columbi. 1943
3. Goodwin LR, Francom D, Dieken FP, Taylor JD, Warenycia MW, Reiffenstein RJ, Dowling G. Determination of sulfide in brain tissue by gas dialysis/ion chromatography: Postmortem studies and two case reports. Journal of analytical toxicology. 1989; 13:105–109. [PubMed: 2733387]
4. Savage JC, Gould DH. Determination of sulfide in brain tissue and rumen fluid by ion-interaction reversed-phase high-performance liquid chromatography. Journal of chromatography. 1990; 526:540–545. [PubMed: 2361993]
5. Abe K, Kimura H. The possible role of hydrogen sulfide as an endogenous neuromodulator. The Journal of neuroscience : the official journal of the Society for Neuroscience. 1996; 16:1066–1071. [PubMed: 8558235]

6. Hosoki R, Matsuki N, Kimura H. The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. Biochemical and biophysical research communications. 1997; 237:527–531. [PubMed: 9299397]
7. Li L, Rose P, Moore PK. Hydrogen sulfide and cell signaling. Annual review of pharmacology and toxicology. 2011; 51:169–187.
8. Wang R. Two's company, three's a crowd: Can h2s be the third endogenous gaseous transmitter? FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2002; 16:1792–1798. [PubMed: 12409322]
9. Shibuya N, Tanaka M, Yoshida M, Ogasawara Y, Togawa T, Ishii K, Kimura H. 3-mercaptopyruvate sulfurtransferase produces hydrogen sulfide and bound sulfane sulfur in the brain. Antioxidants & redox signaling. 2009; 11:703–714. [PubMed: 18855522]
10. Nicholson CK, Calvert JW. Hydrogen sulfide and ischemia-reperfusion injury. Pharmacological research : the official journal of the Italian Pharmacological Society. 2010; 62:289–297. [PubMed: 20542117]
11. Lowicka E, Beltowski J. Hydrogen sulfide (h2s) - the third gas of interest for pharmacologists. Pharmacological reports : PR. 2007; 59:4–24. [PubMed: 17377202]
12. Beauchamp RO Jr, Bus JS, Popp JA, Boreiko CJ, Andjelkovich DA. A critical review of the literature on hydrogen sulfide toxicity. Critical reviews in toxicology. 1984; 13:25–97. [PubMed: 6378532]
13. Insko MA, Deckwerth TL, Hill P, Toombs CF, Szabo C. Detection of exhaled hydrogen sulphide gas in rats exposed to intravenous sodium sulphide. British journal of pharmacology. 2009; 157:944–951. [PubMed: 19422378]
14. Wang R. Physiological implications of hydrogen sulfide: A whiff exploration that blossomed. Physiological reviews. 2012; 92:791–896. [PubMed: 22535897]
15. Wang ZT, Lau CW, Chan FL, Yao X, Chen ZY, He ZD, Huang Y. Vasorelaxant effects of cardamonin and alpinetin from alpinia henryi k. Schum. Journal of cardiovascular pharmacology. 2001; 37:596–606. [PubMed: 11336110]
16. Zanardo RC, Brancaleone V, Distrutti E, Fiorucci S, Cirino G, Wallace JL. Hydrogen sulfide is an endogenous modulator of leukocyte-mediated inflammation. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2006; 20:2118–2120. [PubMed: 16912151]
17. Wallace JL, Caliendo G, Santagada V, Cirino G, Fiorucci S. Gastrointestinal safety and anti-inflammatory effects of a hydrogen sulfide-releasing diclofenac derivative in the rat. Gastroenterology. 2007; 132:261–271. [PubMed: 17241876]
18. Tang G, Wu L, Liang W, Wang R. Direct stimulation of k(atp) channels by exogenous and endogenous hydrogen sulfide in vascular smooth muscle cells. Molecular pharmacology. 2005; 68:1757–1764. [PubMed: 16150926]
19. Yang G, Wu L, Jiang B, Yang W, Qi J, Cao K, Meng Q, Mustafa AK, Mu W, Zhang S, Snyder SH, Wang R. H2s as a physiologic vasorelaxant: Hypertension in mice with deletion of cystathionine gamma-lyase. Science. 2008; 322:587–590. [PubMed: 18948540]
20. Bhatia M. Hydrogen sulfide as a vasodilator. IUBMB life. 2005; 57:603–606. [PubMed: 16203678]
21. Koenitzer JR, Isbell TS, Patel HD, Benavides GA, Dickinson DA, Patel RP, Darley-Usmar VM, Lancaster JR Jr, Doeller JE, Kraus DW. Hydrogen sulfide mediates vasoactivity in an o2-dependent manner. American journal of physiology Heart and circulatory physiology. 2007; 292:H1953–1960. [PubMed: 17237242]
22. Szabo C, Papapetropoulos A. Hydrogen sulphide and angiogenesis: Mechanisms and applications. British journal of pharmacology. 2011; 164:853–865. [PubMed: 21198548]
23. Cai WJ, Wang MJ, Moore PK, Jin HM, Yao T, Zhu YC. The novel proangiogenic effect of hydrogen sulfide is dependent on akt phosphorylation. Cardiovascular research. 2007; 76:29–40. [PubMed: 17631873]
24. Papapetropoulos A, Pyriochou A, Altaany Z, Yang G, Marazioti A, Zhou Z, Jeschke MG, Branski LK, Herndon DN, Wang R, Szabo C. Hydrogen sulfide is an endogenous stimulator of

angiogenesis. Proceedings of the National Academy of Sciences of the United States of America. 2009; 106:21972–21977. [PubMed: 19955410]

25. Predmore BL, Kondo K, Bhushan S, Zlatopolsky MA, King AL, Aragon JP, Grinsfelder DB, Condit ME, Lefer DJ. The polysulfide diallyl trisulfide protects the ischemic myocardium by preservation of endogenous hydrogen sulfide and increasing nitric oxide bioavailability. American journal of physiology Heart and circulatory physiology. 2012; 302:H2410–2418. [PubMed: 22467307]
26. Peake BF, Nicholson CK, Lambert JP, Hood RL, Amin H, Amin S, Calvert JW. Hydrogen sulfide preconditions the db/db diabetic mouse heart against ischemia-reperfusion injury by activating nrf2 signaling in an erk-dependent manner. American journal of physiology Heart and circulatory physiology. 2013; 304:H1215–1224. [PubMed: 23479260]
27. Elrod JW, Calvert JW, Morrison J, Doeller JE, Kraus DW, Tao L, Jiao X, Scalia R, Kiss L, Szabo C, Kimura H, Chow CW, Lefer DJ. Hydrogen sulfide attenuates myocardial ischemia-reperfusion injury by preservation of mitochondrial function. Proceedings of the National Academy of Sciences of the United States of America. 2007; 104:15560–15565. [PubMed: 17878306]
28. Duranski MR, Greer JJ, Dejam A, Jaganmohan S, Hogg N, Langston W, Patel RP, Yet SF, Wang X, Kevil CG, Gladwin MT, Lefer DJ. Cytoprotective effects of nitrite during in vivo ischemia-reperfusion of the heart and liver. The Journal of clinical investigation. 2005; 115:1232–1240. [PubMed: 15841216]
29. Kimura Y, Goto Y, Kimura H. Hydrogen sulfide increases glutathione production and suppresses oxidative stress in mitochondria. Antioxidants & redox signaling. 2010; 12:1–13. [PubMed: 19852698]
30. Yin J, Tu C, Zhao J, Ou D, Chen G, Liu Y, Xiao X. Exogenous hydrogen sulfide protects against global cerebral ischemia/reperfusion injury via its anti-oxidative, anti-inflammatory and anti-apoptotic effects in rats. Brain research. 2013; 1491:188–196. [PubMed: 23123706]
31. Lin X, Yu S, Chen Y, Wu J, Zhao J, Zhao Y. Neuroprotective effects of diallyl sulfide against transient focal cerebral ischemia via anti-apoptosis in rats. Neurological research. 2012; 34:32–37. [PubMed: 22196859]
32. Cohen-Solal A, Beauvais F, Logeart D. Heart failure and diabetes mellitus: Epidemiology and management of an alarming association. Journal of cardiac failure. 2008; 14:615–625. [PubMed: 18722328]
33. Maack C, Kartes T, Kilter H, Schafers HJ, Nickenig G, Bohm M, Laufs U. Oxygen free radical release in human failing myocardium is associated with increased activity of rac1-gtpase and represents a target for statin treatment. Circulation. 2003; 108:1567–1574. [PubMed: 12963641]
34. Calvert JW, Elston M, Nicholson CK, Gundewar S, Jha S, Elrod JW, Ramachandran A, Lefer DJ. Genetic and pharmacologic hydrogen sulfide therapy attenuates ischemia-induced heart failure in mice. Circulation. 2010; 122:11–19. [PubMed: 20566952]
35. Polhemus D, Kondo K, Bhushan S, Bir SC, Kevil CG, Murohara T, Lefer DJ, Calvert JW. Hydrogen sulfide attenuates cardiac dysfunction following heart failure via induction of angiogenesis. Circulation Heart failure. 2013
36. Kondo K, Bhushan S, King AL, Prabhu SD, Hamid T, Koenig S, Murohara T, Predmore BL, Gojon G Sr, Gojon G Jr, Wang R, Karusula N, Nicholson CK, Calvert JW, Lefer DJ. H(2)s protects against pressure overload-induced heart failure via upregulation of endothelial nitric oxide synthase. Circulation. 2013; 127:1116–1127. [PubMed: 23393010]
37. Izumiya Y, Shiojima I, Sato K, Sawyer DB, Colucci WS, Walsh K. Vascular endothelial growth factor blockade promotes the transition from compensatory cardiac hypertrophy to failure in response to pressure overload. Hypertension. 2006; 47:887–893. [PubMed: 16567591]
38. Givvimani S, Munjal C, Gargoum R, Sen U, Tyagi N, Vacek JC, Tyagi SC. Hydrogen sulfide mitigates transition from compensatory hypertrophy to heart failure. Journal of applied physiology. 2011; 110:1093–1100. [PubMed: 21233344]
39. Zhu XY, Liu SJ, Liu YJ, Wang S, Ni X. Glucocorticoids suppress cystathionine gamma-lyase expression and h2s production in lipopolysaccharide-treated macrophages. Cellular and molecular life sciences : CMLS. 2010; 67:1119–1132. [PubMed: 20063035]

40. Wang Y, Zhao X, Jin H, Wei H, Li W, Bu D, Tang X, Ren Y, Tang C, Du J. Role of hydrogen sulfide in the development of atherosclerotic lesions in apolipoprotein e knockout mice. Arteriosclerosis, thrombosis, and vascular biology. 2009; 29:173–179.
41. Modis K, Coletta C, Erdelyi K, Papapetropoulos A, Szabo C. Intramitochondrial hydrogen sulfide production by 3-mercaptopyruvate sulfurtransferase maintains mitochondrial electron flow and supports cellular bioenergetics. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2013; 27:601–611. [PubMed: 23104984]
42. Hill BC, Woon TC, Nicholls P, Peterson J, Greenwood C, Thomson AJ. Interactions of sulphide and other ligands with cytochrome c oxidase. An electron-paramagnetic-resonance study. The Biochemical journal. 1984; 224:591–600. [PubMed: 6097224]
43. Nicholls P, Kim JK. Sulphide as an inhibitor and electron donor for the cytochrome c oxidase system. Canadian journal of biochemistry. 1982; 60:613–623. [PubMed: 6288202]
44. Murphy E, Steenbergen C. Preconditioning: The mitochondrial connection. Annual review of physiology. 2007; 69:51–67.
45. Rose P, Moore PK, Ming SH, Nam OC, Armstrong JS, Whiteman M. Hydrogen sulfide protects colon cancer cells from chemopreventative agent beta-phenylethyl isothiocyanate induced apoptosis. World journal of gastroenterology : WJG. 2005; 11:3990–3997. [PubMed: 15996021]
46. Zhou X, Lu X. Hydrogen sulfide inhibits high-glucose-induced apoptosis in neonatal rat cardiomyocytes. Experimental biology and medicine. 2013; 238:370–374. [PubMed: 23760002]
47. King AL, Lefer DJ. Cytoprotective actions of hydrogen sulfide in ischaemia-reperfusion injury. Exp Physiol. 2011; 96:840–846. [PubMed: 21666033]
48. Kimura Y, Kimura H. Hydrogen sulfide protects neurons from oxidative stress. Faseb Journal. 2004; 18:1165–+. [PubMed: 15155563]
49. Fisher CD, Augustine LM, Maher JM, Nelson DM, Slitt AL, Klaassen CD, Lehman-McKeeman LD, Cherrington NJ. Induction of drug-metabolizing enzymes by garlic and allyl sulfide compounds via activation of constitutive androstane receptor and nuclear factor e2-related factor 2. Drug Metab Dispos. 2007; 35:995–1000. [PubMed: 17353348]
50. Motohashi H, Yamamoto M. Nrf2-keap1 defines a physiologically important stress response mechanism. Trends in molecular medicine. 2004; 10:549–557. [PubMed: 15519281]
51. Chan K, Han XD, Kan YW. An important function of nrf2 in combating oxidative stress: Detoxification of acetaminophen. Proceedings of the National Academy of Sciences of the United States of America. 2001; 98:4611–4616. [PubMed: 11287661]
52. Calvert JW, Elston M, Nicholson CK, Gundewar S, Jha S, Elrod JW, Ramachandran A, Lefer DJ. Genetic and pharmacologic hydrogen sulfide therapy attenuates ischemia-induced heart failure in mice. Circulation. 2010; 122:11–U45. [PubMed: 20566952]
53. Jha S, Calvert JW, Duranski MR, Ramachandran A, Lefer DJ. Hydrogen sulfide attenuates hepatic ischemia-reperfusion injury: Role of antioxidant and antiapoptotic signaling. Am J Physiol-Heart C. 2008; 295:H801–H806.
54. Boo YC, Sorescu G, Boyd N, Shiojima I, Walsh K, Du J, Jo H. Shear stress stimulates phosphorylation of endothelial nitric-oxide synthase at ser1179 by akt-independent mechanisms: Role of protein kinase a. The Journal of biological chemistry. 2002; 277:3388–3396. [PubMed: 11729190]
55. Lin MI, Fulton D, Babbitt R, Fleming I, Busse R, Pritchard KA Jr, Sessa WC. Phosphorylation of threonine 497 in endothelial nitric-oxide synthase coordinates the coupling of l-arginine metabolism to efficient nitric oxide production. The Journal of biological chemistry. 2003; 278:44719–44726. [PubMed: 12952971]
56. Zhao W, Zhang J, Lu Y, Wang R. The vasorelaxant effect of h(2)s as a novel endogenous gaseous k(atp) channel opener. The EMBO journal. 2001; 20:6008–6016. [PubMed: 11689441]
57. Coletta C, Papapetropoulos A, Erdelyi K, Olah G, Modis K, Panopoulos P, Asimakopoulou A, Gero D, Sharina I, Martin E, Szabo C. Hydrogen sulfide and nitric oxide are mutually dependent in the regulation of angiogenesis and endothelium-dependent vasorelaxation. Proceedings of the National Academy of Sciences of the United States of America. 2012; 109:9161–9166. [PubMed: 22570497]

58. Altaany Z, Yang G, Wang R. Crosstalk between hydrogen sulfide and nitric oxide in endothelial cells. Journal of cellular and molecular medicine. 2013
59. Choi AM, Otterbein LE. Emerging role of carbon monoxide in physiologic and pathophysiologic states. Antioxidants & redox signaling. 2002; 4:227–228. [PubMed: 12006173]
60. Moody BF, Calvert JW. Emergent role of gasotransmitters in ischemia-reperfusion injury. Medical gas research. 2011; 1:3. [PubMed: 22146243]
61. Zhang QY, Du JB, Zhang CY, Tang CS. the regulation of carbon monoxide/heme oxygenase system by hydrogen sulfide in rats with hypoxic pulmonary hypertension. Zhonghua jie he he hu xi za zhi = Zhonghua jiehe he huxi zazhi = Chinese journal of tuberculosis and respiratory diseases. 2004; 27:659–663. [PubMed: 16200866]
62. Majid AS, Majid AM, Yin ZQ, Ji D. Slow regulated release of h2s inhibits oxidative stress induced cell death by influencing certain key signaling molecules. Neurochemical research. 2013; 38:1375–1393. [PubMed: 23585122]
63. Caliendo G, Cirino G, Santagada V, Wallace JL. Synthesis and biological effects of hydrogen sulfide (h2s): Development of h2s-releasing drugs as pharmaceuticals. Journal of medicinal chemistry. 2010; 53:6275–6286. [PubMed: 20462257]
64. Benavides GA, Squadrito GL, Mills RW, Patel HD, Isbell TS, Patel RP, Darley-Usmar VM, Doeller JE, Kraus DW. Hydrogen sulfide mediates the vasoactivity of garlic. Proceedings of the National Academy of Sciences of the United States of America. 2007; 104:17977–17982. [PubMed: 17951430]
65. Zhao Y, Bhushan S, Yang C, Otsuka H, Stein JD, Pacheco A, Peng B, Devarie-Baez NO, Aguilar HC, Lefer DJ, Xian M. Controllable hydrogen sulfide donors and their activity against myocardial ischemia-reperfusion injury. ACS chemical biology. 2013
66. Collin M, Anuar FB, Murch O, Bhatia M, Moore PK, Thiemermann C. Inhibition of endogenous hydrogen sulfide formation reduces the organ injury caused by endotoxemia. British journal of pharmacology. 2005; 146:498–505. [PubMed: 16100527]
67. Calvert JW, Coetzee WA, Lefer DJ. Novel insights into hydrogen sulfide--mediated cytoprotection. Antioxidants & redox signaling. 2010; 12:1203–1217. [PubMed: 19769484]
68. Nagy P, Palinkas Z, Nagy A, Budai B, Toth I, Vasas A. Chemical aspects of hydrogen sulfide measurements in physiological samples. Biochimica et biophysica acta. 2013
69. Shen X, Pattillo CB, Pardue S, Bir SC, Wang R, Kevil CG. Measurement of plasma hydrogen sulfide in vivo and in vitro. Free radical biology & medicine. 2011; 50:1021–1031. [PubMed: 21276849]
70. Furne J, Saeed A, Levitt MD. Whole tissue hydrogen sulfide concentrations are orders of magnitude lower than presently accepted values. American journal of physiology Regulatory, integrative and comparative physiology. 2008; 295:R1479–1485.
71. Ubuka T. Assay methods and biological roles of labile sulfur in animal tissues. Journal of chromatography B, Analytical technologies in the biomedical and life sciences. 2002; 781:227–249.
72. Olson KR. A practical look at the chemistry and biology of hydrogen sulfide. Antioxidants & redox signaling. 2012; 17:32–44. [PubMed: 22074253]
73. King AL, Lefer DJ. Cytoprotective actions of hydrogen sulfide in ischaemia-reperfusion injury. Experimental physiology. 2011; 96:840–846. [PubMed: 21666033]

Non-Standard Abbreviations and Acronyms

H_2S	hydrogen sulfide
NO	nitric oxide
CO	carbon monoxide
CBS	cystathionine beta synthase
CSE	cystathionine gamma lyase

3-MST	3-mercaptopyruvate sulfurtransferase
CAT	cysteine aminotransferase
TMST	S-methyltransferase
VEGF	vascular endothelial growth factor
MAPK	mitogen activated protein kinase
I/R	ischemia/reperfusion
DATS	diallyl trisulfide
TNF-α	tumor necrosis factor-alpha
MCP-1	monocyte chemotactic protein-1
Bcl-2	B-cell lymphoma 2
LV	left ventricle
MMP-2	matric metalloproteinase-2
TIMP-3	tissue inhibitor of metalloproteinases-3
oxLDL	oxidized low-density lipoprotein
fMLP	N-formyl-L-leucyl-L-phenylalanine
ROS	reactive oxygen species
MPTP	mitochondrial permeability transition pore
NaHS	sodium hydrosulfide
HO-1	heme oxygenase 1
hyc	homocysteine
eNOS	endothelial nitric oxide synthase
(s)GC	(soluble) guanylyl cyclase
cGMP	cyclic guanosine 5'-monophosphate
PAG	DL-propargylglycine
MBB	monobromobimane
SDB	sulfide dibrimane
BH_4	tetrahydrobiopterin

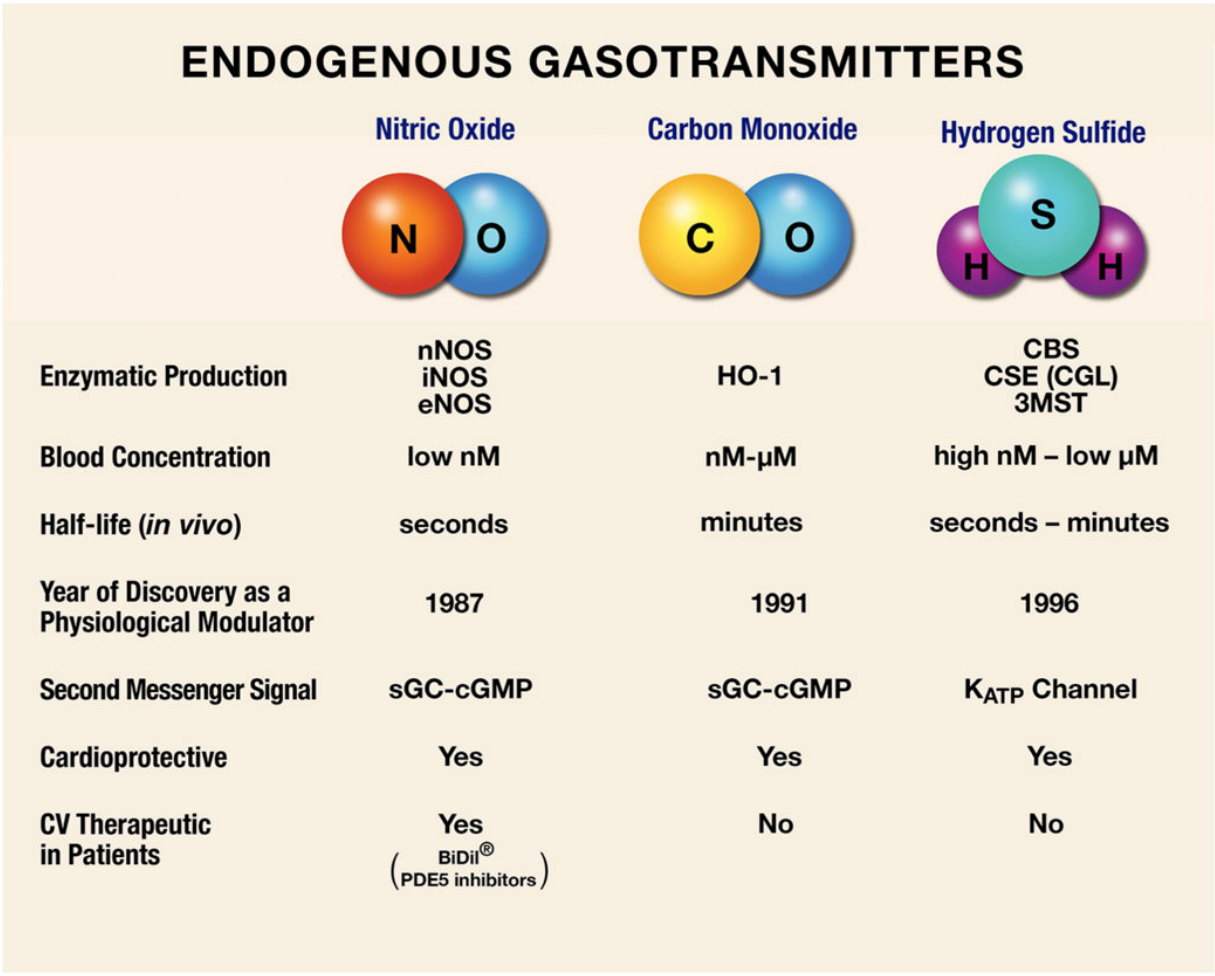

Figure 1. Currently Recognized Gasotransmitters

Nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S) are all produced endogenously via enzymes. NO is synthesized by neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS), and endothelial nitric oxide synthase (eNOS). CO is generated by the heme oxygenase (HO) family of enzymes (HO-1, HO-2, and HO-3). H_2S is synthesized via the actions of cystathionine beta synthase (CBS), cystathionine gamma lyase (CSE or CGL), and 3-mercaptopyruvate sulfur transferase (3MST). These gaseous molecules are produced in very low concentrations ranging from low nM to low μM and are very labile. Adapted and modified from Calvert et al.[67]

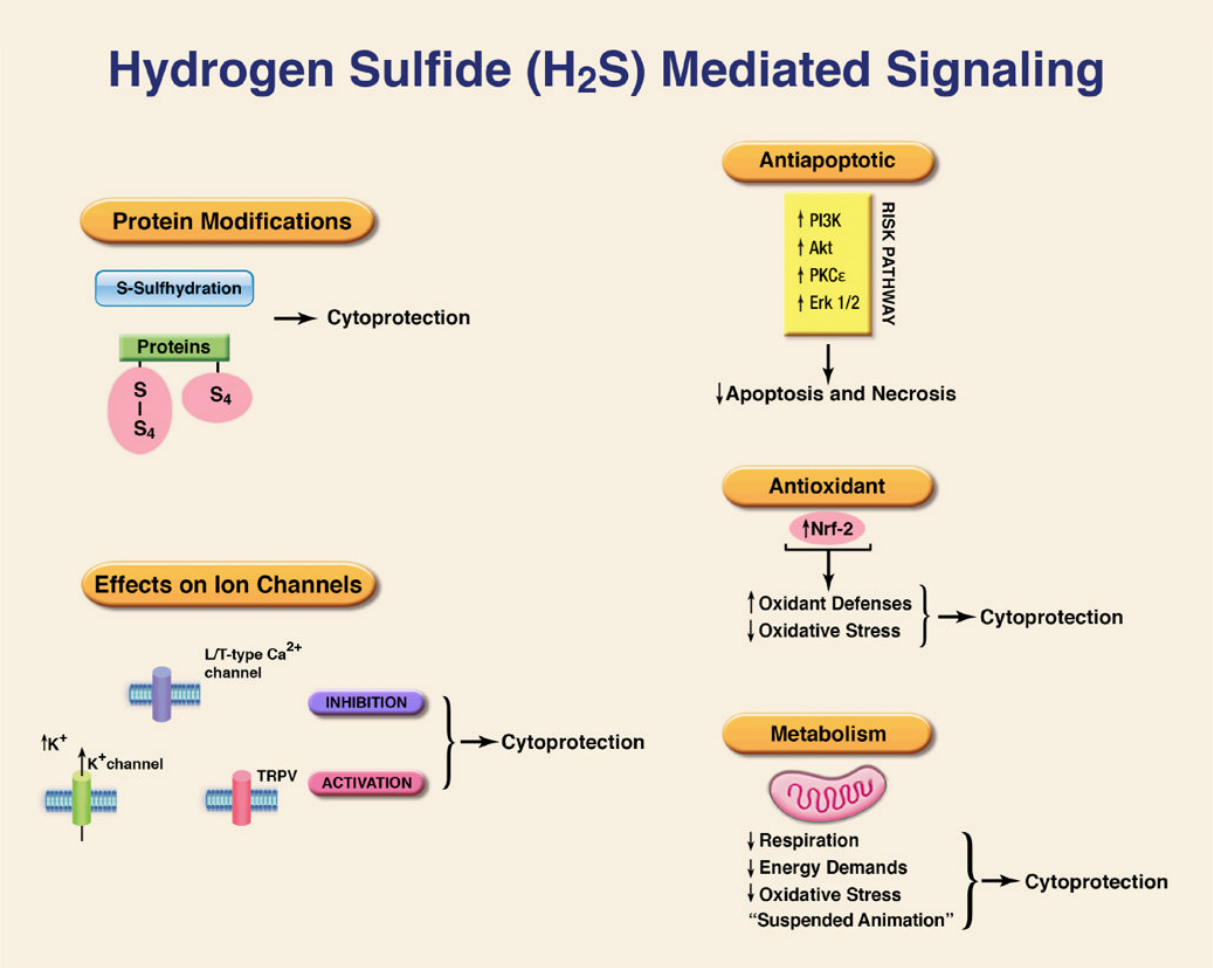

Figure 2. Hydrogen Sulfide Mediated Signaling

H_2S is known to modify proteins, modulate the function of various ion channels, attenuate apoptosis and oxidative stress, and to be a potent modulator of cellular metabolic function. Adapted and modified from King et al.[73]

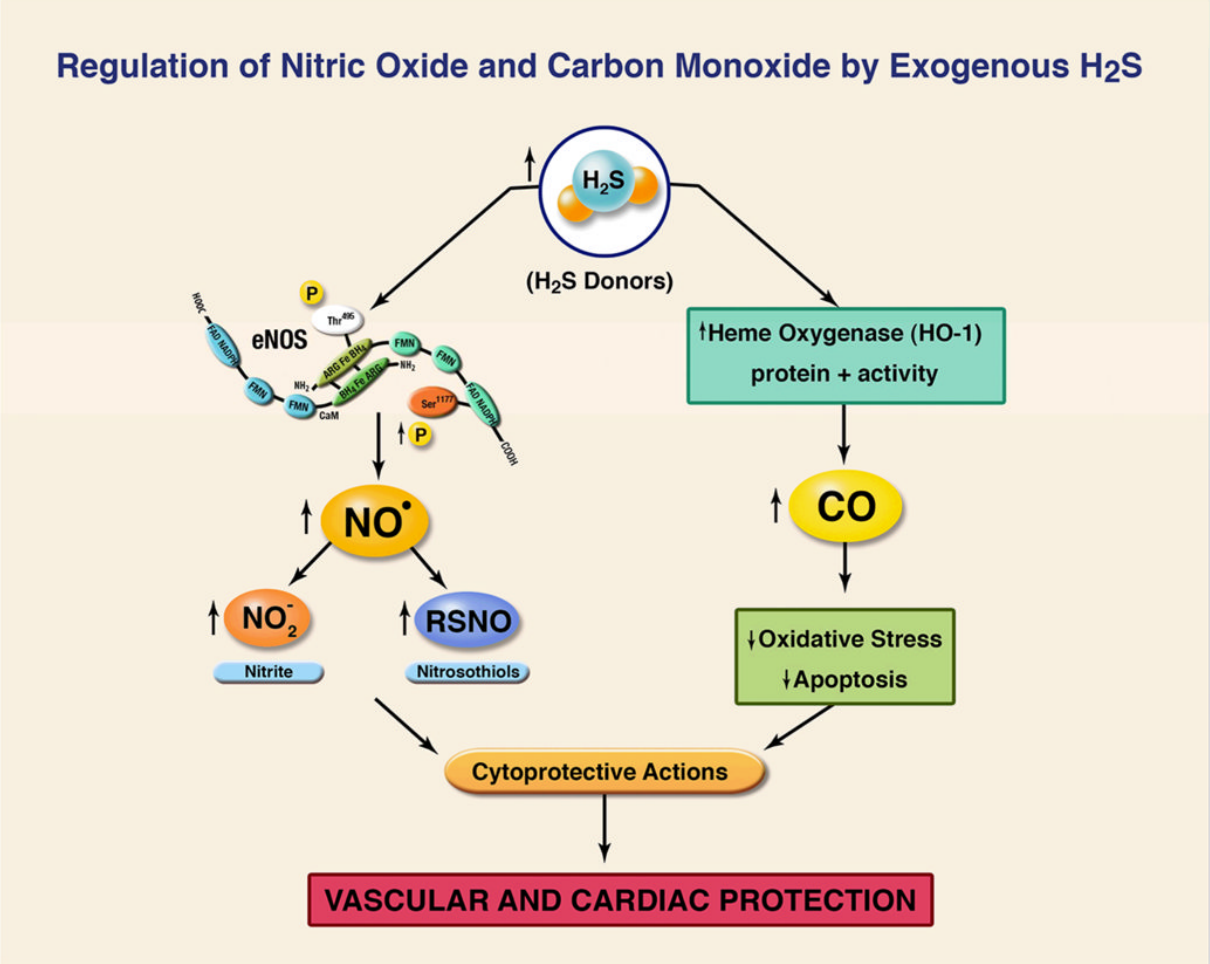

Figure 3. Crosstalk Between Exogenous Hydrogen Sulfide (H_2S), Nitric Oxide (NO), and Carbon Monoxide (CO)

Proposed interaction between H_2S and eNOS to increase nitric oxide generation. In addition, H_2S can activate heme oxygenase-1 via genetic upregulation and increased activity to enhance levels of carbon monoxide. Nitric oxide and carbon monoxide can ultimately synergize with H_2S to exert both vascular and cardiac protection during cardiovascular disease states.

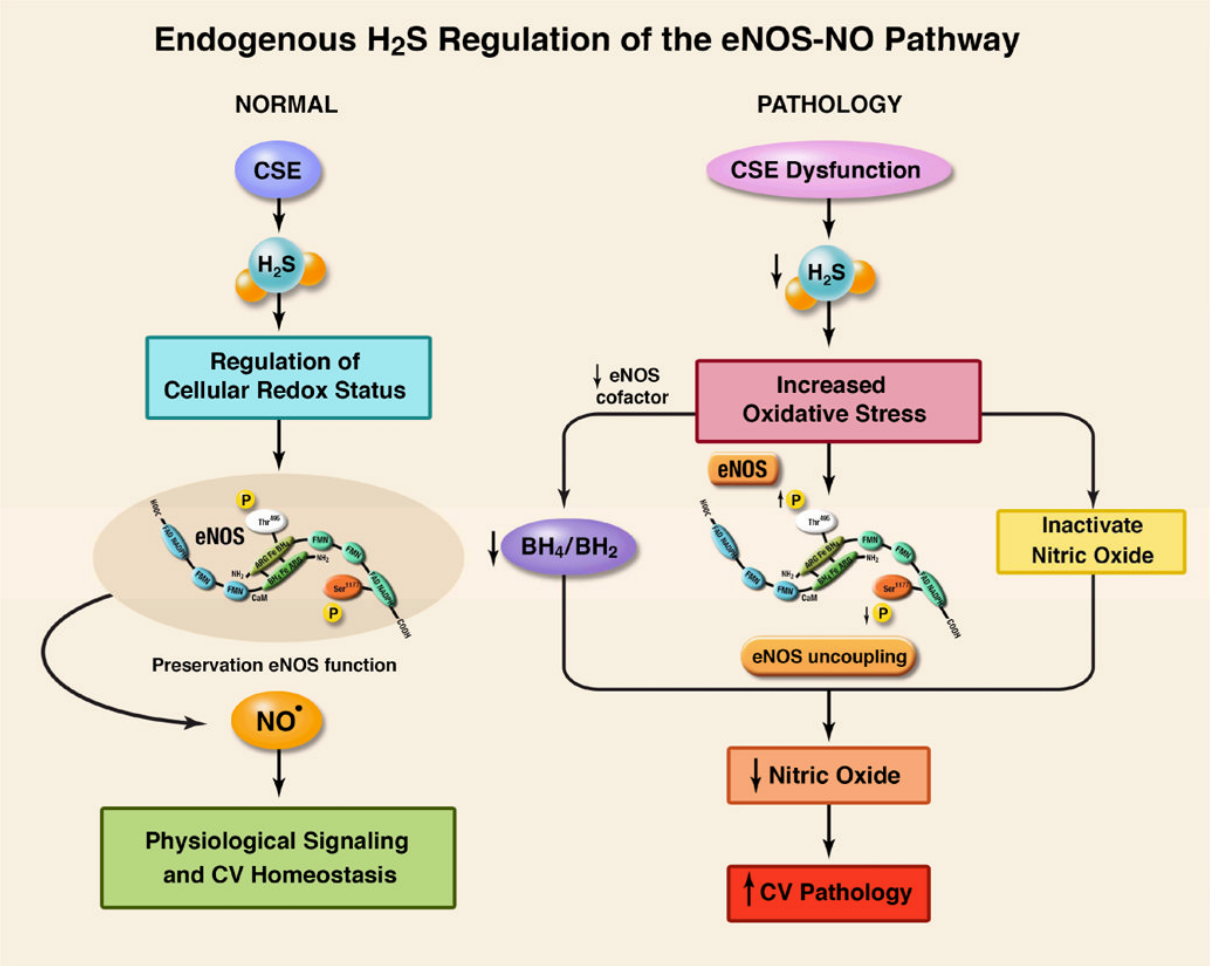

Figure 4. Endogenous H_2S Regulation of the eNOS-NO Pathway

Proposed actions of CSE derived H_2S under normal conditions and in the setting of cardiovascular disease. H_2S is a potent regulator of cellular redox status that limits oxidative stress thereby preserving eNOS function and promoting nitric oxide (NO) production. Diminished endogenous H_2S results in profound oxidative stress, reduced BH_4 levels and dysfunctional eNOS (i.e., eNOS uncoupling). Reduced nitric oxide levels exacerbate cardiovascular pathology.