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## **Hydrogen Sulfide: A Potential Novel Therapy for the Treatment of Ischemia**

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## **ABSTRACT**

Hydrogen sulfide (H<sub>2</sub>S) is a novel signaling molecule most recently found to be of fundamental importance in cellular function as a regulator of apoptosis, inflammation, and perfusion. Mechanisms of endogenous H<sub>2</sub>S signaling are poorly understood, however, signal transmission is thought to occur via persulfidation at reactive cysteine residues on proteins. Although much has been discovered about how H<sub>2</sub>S is synthesized in the body, less is known about how it is metabolized. Recent studies have discovered a multitude of different targets for H<sub>2</sub>S therapy, including those related to protein modification, intracellular signaling, and ion channel depolarization. The most difficult part of studying hydrogen sulfide has been finding a way to accurately and reproducibly measure it. The purpose of this review is to: 1) elaborate on the biosynthesis and catabolism of H<sub>2</sub>S in the human body, 2) review current knowledge of the mechanisms of action of this gas in relation to ischemic injury, 3) define strategies for physiological measurement of H<sub>2</sub>S in biological systems, and 4) review potential novel therapies that use H<sub>2</sub>S for treatment.

**KEY WORDS:** hydrogen sulfide, NaHS, ischemia, persulfidation, polysulfide

## **BACKGROUND**

Hydrogen sulfide (H<sub>2</sub>S) is a colorless, flammable, malodorous gas that portends the smell of rotten eggs. It has previously been appreciated for its toxic effects, but more recently, has been found to facilitate a number of beneficial biological activities. It has been dubbed the “third gasotransmitter” alongside carbon monoxide (CO) and nitric oxide (NO). However, most literature suggests that it does not signal in its gaseous state but rather as a persulfide or polysulfide. Endogenously created, these signaling molecules travel both intracellularly and intercellularly. Pathways of these gasotransmitters may interact with and impact each other and have been implicated in regulation of many physiological processes(1). The effects of H<sub>2</sub>S have been studied on various physiological platforms, most notably in the regulation of cellular metabolism, inflammation, the immunological response, ischemia and cardiovascular biology(1, 2).

With the recent discovery of H<sub>2</sub>S as a physiological signaling molecule, its' role in end organ protection following ischemia has come into question. H<sub>2</sub>S could prove to be a novel drug for utilization in a variety of ischemic disorders, including those of the intestine, heart, and brain(3-5). Through this review we aim to: 1) elaborate on the biosynthesis and catabolism of H<sub>2</sub>S in the human body, 2) review current knowledge of the mechanisms of action of this gas in relation to ischemic injury, 3) define strategies for physiological measurement of H<sub>2</sub>S in biological systems, and 4) review potential novel therapeutic agents that use H<sub>2</sub>S therapy for treatment.

## **BIOSYNTHESIS OF H<sub>2</sub>S**

### **Enzymatic Synthesis**

Hydrogen sulfide is endogenously produced in mammalian tissues by both enzymatic and non-enzymatic pathways (i.e., reduction of thiols and thiol containing molecules). The enzymes involved in synthesis of H<sub>2</sub>S include: cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MPST) in collaboration with cysteine aminotransferase (CAT). These enzymes convert cysteine and homocysteine to H<sub>2</sub>S gas and specific byproducts such as serine, pyruvate, and cystathionine (Figure 1)(6). They also play a significant role in transsulfuration and reverse transsulfuration pathways, the formation of persulfides, and protein persulfidation.

The majority of H<sub>2</sub>S is produced by the two pyridoxal-5'phosphate (PLP) dependent enzymes, CBS and CSE in which homocysteine is metabolized to cysteine. CBS is a predominant enzyme in the central nervous system but can also be found in the liver, kidney, pancreas, lungs, endothelial cells and gastrointestinal tract(7-9).CSE is expressed in the liver, uterus, placenta, pancreatic islets, brain, gastrointestinal, cardiovascular, renal and pulmonary systems (7, 10, 11). Both of these enzymes are located in the cytosol but can traverse to the mitochondria during stress (12, 13). Specifically when exposed to hypoxic stress, an increase in intracellular levels of calcium triggers CSE translocation from the cytosol to the mitochondria to sustain mitochondrial ATP production (13). In the mitochondria, cysteine is available at

triple the concentration of that in the cytosol. Cysteine is metabolized by CSE to produce H<sub>2</sub>S and mitochondrial ATP production is increased in the presence of H<sub>2</sub>S in hypoxic conditions(13).

3-MPST, is a zinc-dependent enzyme located in both the mitochondria and cytosol, with majority located in mitochondria (12). Regarding the 3-MPST pathway, CAT, a PLP-dependent enzyme, converts L-cysteine and 2-ketoglutarate to 3-mercaptopyruvate (3-MP) and glutamate respectively. The reaction of 3-MP with 3-MPST forms a polysulfide on the enzyme. Production of H<sub>2</sub>S from 3-MPST requires the endogenous reducing cofactors thioredoxin and dihydrolipoic acid. Through the reaction of 3-MP with 3-MPST, 3-MP is converted to H<sub>2</sub>S, pyruvate and NH<sub>3</sub> (Figure 1). Additionally, 3-MPST has been found to also convert D-cysteine to H<sub>2</sub>S in the presence of D-amino acid oxidase (14). CAT and MPST are found in multiple organ systems most notably the cardiovascular, renal, liver, lung, thymus, testis, brain and thoracic aorta (8). MPST and CAT are both detected in vascular endothelium and vascular smooth muscle cells (VMSCs).

### Intracellular Sulfur Stores

H<sub>2</sub>S may also be formed from bound sulfane sulfur (intracellular sulfur stores) and has demonstrated potential importance in certain cells and certain cellular conditions(15, 16) (Table 1). H<sub>2</sub>S is carried on proteins attached to another sulfur as a persulfide or trisulfide. Carrier proteins of protein-bound sulfane sulfur in tissues include rhodanese, CBS, CSE, MPST, albumin, cytochrome, and ferredoxin(15). The sulfur is labile and has the distinctive ability to reversibly attach to other sulfur atoms and create elemental sulfur, persulfides, polysulfides, thiosulfate, and polythionates(16).

Rhodanese, CSE and MPST are the most commonly described carrier proteins and are widely distributed within animal tissues. These carrier proteins allow transport of sulfur to various acceptors and are of physiologic importance. If labeled cysteine (precursor of H<sub>2</sub>S), is injected into an animal, labeled bound sulfane sulfur is rapidly detected(17). Bound sulfur can be stored or transported in a less labile, nongaseous state to be released later through this reversible binding process. When H<sub>2</sub>S oxidizes cysteine thiols to forms stable persulfides which under reducing conditions can release stored H<sub>2</sub>S (18). Liver and kidney tissues have both demonstrated high amounts of bound sulfur(18).

### Sulfide Signaling – Is it H<sub>2</sub>S or other sulfide mediators?

Evidence in literature suggests that H<sub>2</sub>S signaling may be mediated endogenously by polysulfides (RS<sub>n</sub>R, RS<sub>n</sub>H, H<sub>2</sub>S<sub>n</sub>; R=radical group, n>2) and/or persulfides (n=2)(19). Persulfides and/or polysulfides bind to reactive cysteine residues on proteins causing persulfidation (aka S-sulphydration; changing –SH group of cysteine to an –SSH or persulfide) and thereby changing the functional properties of specific proteins. Over fifteen sulphydrated proteins have been identified (20). While the significance of polysulfides in H<sub>2</sub>S signaling has only recently been recognized, physiologic regulation by these sulphydrated proteins has been observed in the brain with activation of transient receptor potential ankyrin 1 (TRPA1) channels,

tensin homolog activity, and persulfidation of Kelch-like ECH-associated protein 1 allowing translocation of nuclear factor erythroid 2-related factor 2 to the nucleus (21). Polysulfides are potential signaling molecules modulating the activity of enzymes, channels and receptors through protein persulfidation.

In addition to polysulfides and persulfides, thiosulfate ( $S_2O_3^{2-}$ ), an intermediate in oxidative  $H_2S$  metabolism has also been touted as a contributing member of  $H_2S$  signaling under hypoxic conditions as an oxygen sensor. Olson et al. has demonstrated that while  $H_2S$  is usually oxidized to form thiosulfate,  $H_2S$  metabolism can alternatively cause the regeneration of  $H_2S$  via thiol reduction in hypoxic conditions (22). Lastly, sulfite ( $SO_2^{3-}$ ) has also been reported to play a role with increased production via oxidation of  $H_2S$  by LPS-activated neutrophils as a result of oxidative stress and may play a key role in the setting of inflammation or hypoxia(23). All of this emerging evidence of other sulfide mediators demonstrates that further work is required to delineate this fine balance and the intricacy of  $H_2S$  mediated pathways.

### Toxicity of Hydrogen Sulfide

It is thought that the dose response relationship between toxic and physiologic effects of  $H_2S$  is very steep.  $H_2S$  has been found to have dual regulation in the mitochondria exhibiting both inhibitory and stimulatory effects. At lower concentrations  $H_2S$  acts as a mitochondrial substrate, similar to NADH or FADH<sub>2</sub>, donating electrons at sulfide quinone oxidoreductase (SQR) and stimulating the production of ATP and thus increasing metabolism(24). However, at high concentrations,  $H_2S$  has been found to reversibly and competitively bind to cytochrome c oxidase; inhibiting the binding of oxygen. With this inhibition, the electron transport chain is no longer able to move electrons and ATP production comes to a halt. By inhibiting mitochondrial respiration, a positive feedback loop results with increasing  $H_2S$  accumulation and a decreased oxidation rate that accelerates  $H_2S$  accumulation, leading to further suppression of the mitochondrial function.

$H_2S$  toxicity is measured in parts per million (ppm) and it has been found that 300ppm causes pulmonary edema with pulmonary vasoconstriction and systemic vasodilation(25), while higher concentrations nearing 1000ppm cause immediate death. Higher levels of  $H_2S$  result in free radical generation, glutathione depletion, intracellular iron release, and pro-apoptotic action through the death receptor and mitochondrial pathways (8).

The steady state of  $H_2S$  concentrations within tissues is very low because it is rapidly metabolized in mitochondria. Overall, cytoprotective effects have been found in nanomolar (nM) and micromolar ( $\mu M$ ) concentrations of  $H_2S$  while higher millimolar (mM)  $H_2S$  exposure tends to be cytotoxic (26). The effective concentration ( $EC_{50}$ ) values for  $H_2S$  have been studied in a few tissues and appear to be in the  $\mu M$  range. For example, in intestinal muscle, the  $H_2S$ -donor, sodium hydrosulfide (NaHS), was found to induce concentration-dependent muscle relaxation and  $EC_{50}$  values reported in the literature have ranged from 31-

514  $\mu\text{M}$  (27, 28). Vascular tissues have been slightly more sensitive to NaHS with  $\text{EC}_{50}$  values ranging between 104 – 136  $\mu\text{M}$  (27).

Lastly, the concentration-dependent benefit vs. harm of  $\text{H}_2\text{S}$  becomes even more fascinating in the sub-toxic concentrations of  $\text{H}_2\text{S}$ . With intermediate levels of  $\text{H}_2\text{S}$ , there is competitive inhibition of cytochrome c oxidase with oxygen consumption declining due to this inhibition. A study by Blackstone et al. utilized gaseous  $\text{H}_2\text{S}$  to induce ‘suspended animation’ in mice in which mice were placed in a hibernation-like state causing hypometabolic effects(29). In this study, they demonstrated a concentration-dependent reduction in core body temperature, total-body  $\text{CO}_2$  production and  $\text{O}_2$  consumption secondary to inhaled  $\text{H}_2\text{S}$  therapy. Similarly, this hypometabolism was also observed in a porcine model of aortic occlusion with intravenous administration of  $\text{Na}_2\text{S}$ . There was a markedly reduced utilization of catecholamines, decreased  $\text{CO}_2$  production and  $\text{O}_2$  uptake (30). Blackstone et al. found this sulfide-mediated reduction in metabolism was also found to be protective in the setting of lethal hypoxia(31).  $\text{H}_2\text{S}$ -induced ‘suspended animation’ mice could survive hypoxia (5%  $\text{O}_2$ ) for up to 6 hours while all animals in the control group died within 17 minutes (31). This  $\text{H}_2\text{S}$ -induced hypometabolism may be highly protective in different clinical scenarios of hypoxia and ischemia.

### **CATABOLISM OF $\text{H}_2\text{S}$**

Hydrogen sulfide catabolism occurs through 1) expiration and excretion, 2) oxidation, 3) methylation, and 4) scavenging (12). Urinary excretion represents a major metabolic and excretory pathway of  $\text{H}_2\text{S}$  with over 50% of orally administered ( $^{35}\text{S}$ )-barium sulfide in rats being excreted as sulfate or ethereal sulfate within 24 hours(32).  $\text{H}_2\text{S}$  has also been found to be excreted in feces and flatus as free sulfide. However this method only accounts for 8-10% of doses recovered in studies examining excretion (32, 33). Secretion of  $\text{H}_2\text{S}$  via exhalation has also been demonstrated after intravenous sodium sulfide ( $\text{Na}_2\text{S}$ ) administration(33). Sodium sulfide, when administered, dissociates to sodium ( $\text{Na}^+$ ) and the hydrosulfide anion ( $\text{HS}^-$ ), which then forms  $\text{H}_2\text{S}$  gas that may be expired during exhalation.

In the mitochondria,  $\text{H}_2\text{S}$  degradation occurs through rapid oxidation to thiosulfate and is further converted to sulfite and sulfate(19). Sulfur oxidation begins with catabolism of  $\text{H}_2\text{S}$  by SQR, a mitochondrial membrane flavoprotein, which oxidizes  $\text{H}_2\text{S}$  to sulfane sulfur forming a persulfide ( $\text{SQRS-S}$ )(19). Ubiquinone accepts the electrons in this reaction and transfers them to complex III and IV of the electron transport chain moving electrons to a terminal electron acceptor ( $\text{O}_2$ ) via a series of redox reaction resulting in a transmembrane proton gradient used to make ATP via ATP synthase. Further steps in  $\text{H}_2\text{S}$  catabolism occur via persulfide dioxygenase, rhodanese, and sulfite oxidase, which creates thiosulfate ( $\text{SSO}_3^{2-}$ ) and sulfate ( $\text{SO}_4^{2-}$ ) as final end products. Oxidation of  $\text{H}_2\text{S}$  to thiosulfate can be affected by heme compounds, ferritin, and metalloprotein complexes(34).

While oxidation occurs in the mitochondria, H<sub>2</sub>S is catabolized via methylation in the cytosol. H<sub>2</sub>S can also undergo cytosolic methylation by thiol-S-methyltransferase to yield methanethiol and dimethylsulfide(35). Lastly, H<sub>2</sub>S may be scavenged by methemoglobin to yield sulfhemoglobin or by metallo- or disulfide containing molecules.

## **MECHANISMS OF ACTION**

H<sub>2</sub>S has a number of beneficial properties that can be useful in the treatment of end organ ischemia. It targets proteins within endothelium, epithelium, and smooth muscle. Known effects of H<sub>2</sub>S include 1) vascular relaxation, 2) vascular angiogenesis, 3) modulation of cell death and apoptosis, 4) reduction of inflammatory processes, and 5) cytoprotection (Figure 2).

### **Vascular Relaxation**

Hydrogen sulfide has been found to be a key modulator of both vascular relaxation and vascular angiogenesis, both of which contribute to increased blood flow following intestinal ischemic injury. In VMSCs, H<sub>2</sub>S has been shown to modulate vasorelaxation via opening of potassium-dependent ATP (K<sub>ATP</sub>) channels in a number of vessels including the aorta, mesenteric arteries, hepatic artery and portal vein (36-38). Persulfidation of Cys<sup>34</sup> on the Kir6.1 subunit of the K<sub>ATP</sub> channel in smooth muscle cells induces vasodilation by preventing association of ATP with the channel(39). Phosphatidylinositol-4, 5-bisphosphate is consequently able to bind to the channel leading to channel opening, K<sup>+</sup> influx, and smooth muscle relaxation. H<sub>2</sub>S was also observed to upregulate K<sub>ATP</sub> channel expression, thereby enhancing vasorelaxation in spontaneously hypertensive rats (40). Additionally, angiotensin-converting enzyme, a zinc-containing enzyme that normally causes vasoconstriction, may be inhibited in a dose-dependent manner by H<sub>2</sub>S interaction with zinc. This effect may add to the vasodilatory influence of K<sub>ATP</sub> channels.

The opening of K<sub>ATP</sub> channels also hyperpolarizes the cell membrane and inactivates voltage-dependent L-type Ca<sup>2+</sup> channels. This leads to cell relaxation and blood vessel dilation by decreasing intracellular Ca<sup>2+</sup>. Large conductance Ca<sup>2+</sup>-activated potassium (BK<sub>Ca</sub>) channels, also present in VMSCs, have demonstrated increased vasodilatory effects in the presence of NaHS (H<sub>2</sub>S donor; sulfide salt) in rat mesenteric rat arteries(41). This mechanism was inhibited by use of a ryanodine blocker, a BK<sub>Ca</sub> blocker, and by endothelial disruption.

Additionally, both CBS and CSE are vital and interdependent modulators of blood pressure and vasorelaxation. Rats given both aminooxyacetic acid (a CBS inhibitor) and DL-propargyl glycine (PAG; a CSE inhibitor) would develop hypertension. However, if only one of the inhibitors was administered, the other enzyme's activity was upregulated to maintain endogenous H<sub>2</sub>S levels (42). It has also been found that mesenteric artery cholinergic relaxation was reduced by 75-80% in mice with homozygous CSE deletion and by 50% in heterozygotes (43). CSE has been found to be highly localized in the endothelial layer of blood vessels. Studies using CSE knockout mice have demonstrated pronounced hypertension and diminished

endothelium-dependent vasorelaxation (43). Additionally, Cheng et al. observed that the presence or absence of the endothelium in the mesenteric artery bed played a significant role in the vasodilatory properties of H<sub>2</sub>S and that H<sub>2</sub>S-induced vasorelaxation of mesenteric artery beds is at least partially mediated by the endothelium via an endothelium derived hyperpolarizing factor related mechanism (37). These studies demonstrate that the ability to endogenously produce H<sub>2</sub>S within the vascular endothelium plays a vital role in vascular relaxation.

When specifically examining mesenteric artery beds (MAB), Cheng et al. found ~20% MAB relaxation with applications of < 1 μM H<sub>2</sub>S, and, with greater concentrations (>10 μM H<sub>2</sub>S) there was >90% relaxation(37). Additionally, when L-cysteine, a pre-cursor to H<sub>2</sub>S production was added, this was also found to produce vasodilation while the addition of a CSE inhibitor caused attenuation of the vasodilatory effects (37). In a pig model, the benefits of H<sub>2</sub>S on mesenteric perfusion have been observed with a two fold increase in mesenteric perfusion following ischemic injury with application of NaHS (44). Thus, with application of an H<sub>2</sub>S donor following intestinal ischemic injury, a direct vasorelaxant effect on mesenteric arteriolar smooth muscle may be observed, enhancing end organ mesenteric perfusion and preferentially distributing blood flow to the damaged intestinal mucosa(44).

### Vascular Angiogenesis

Hydrogen sulfide has been found to exhibit potent angiogenic properties. This concept was demonstrated with an observed dose-dependent increase in endothelial cell proliferation, enhanced capillary-like structure formation, increased motility, and increased length and number of vascular formations (45, 46). H<sub>2</sub>S promotes angiogenesis via pathways involving vascular endothelial growth factor (VEGF), protein kinase B (AKT) and p38. H<sub>2</sub>S activation of these pathways enhances endothelial migration (45). When p38 and AKT inhibitors are used, H<sub>2</sub>S-induced migration was respectively decreased or blocked completely (45). In another study, NaHS was observed to mediate AKT phosphorylation. However, there was no effect on p38. This may have been due to lower experimental levels of NaHS administration (10 μM vs. 60 μM NaHS in previous study) (46). Both of these studies demonstrate the intricate biological pathways of H<sub>2</sub>S modulation of neovascularization. Additionally, if further demonstrates the critical dose response relationship seen with therapeutic H<sub>2</sub>S application.

VEGF receptor 2 (VEGFR2), a mediator of most of the biological effects of VEGF, has also been found to be modified by H<sub>2</sub>S to facilitate its activation after ligand binding (47). H<sub>2</sub>S promotes increased binding VEGF to VEGFR2, thereby enhancing migration and growth of vascular endothelial cells. In CBS deficient endothelial cells, there was decreased H<sub>2</sub>S production and significant transcriptional down regulation of VEGFR2 and neuropilin 1. This down-regulation was mediated by specificity protein 1 which has been found to be regulated via persulfidation of Cys<sup>68</sup> and Cys<sup>755</sup>. When H<sub>2</sub>S was administered,



specificity protein 1 levels were restored, and there was an increase in both VEGFR2 and NRP-1 expression(9).

In addition to endothelial cells, H<sub>2</sub>S also regulates VSMC proliferation and apoptosis. This regulation is vital and key to vascular remodeling. If CSE is overexpressed to produce higher levels of endogenous H<sub>2</sub>S or if H<sub>2</sub>S is exogenously applied, there is inhibition of proliferation and induction of apoptosis in aortic VSMCs via MAPK pathway activation of ERK and caspase 3 (48). Additionally, in the development of balloon injury-induced neointimal hyperplasia there is decreased CSE expression and H<sub>2</sub>S production. However treatment with NaHS induces vasorelaxation and significantly inhibits neointima formation by decreasing VSMC proliferation(49). These findings demonstrate the importance of H<sub>2</sub>S in VSMC modulation.

Various ischemia and reperfusion models have shown increased angiogenesis with the application of H<sub>2</sub>S. In hind limb ischemia, NaHS has been observed to increase regional blood flow to ischemic tissues after femoral artery ligation, to promote collateral vessel growth and to cause an increase in capillary density(50). In murine myocardial infarction models, mice that underwent ligation of the left anterior descending artery and administration of NaHS were found to have increased expression of angiogenic factors (VEGF, flk-1, flt-1), and decreased levels of anti-angiogenic factors (endostatin, angiostatin, parstatin)(51). Improved angiogenesis was also visualized by radiographic and Doppler blood flow measurements(51).

The pro-angiogenic effects of H<sub>2</sub>S converge on the same downstream molecular target as NO. The angiogenesis and wound healing effects of H<sub>2</sub>S were completely absent in endothelial nitric oxide (eNOS) knock out mice. If H<sub>2</sub>S production was reduced through CSE inhibition, NO-stimulated angiogenesis was significantly decreased(52). NO and H<sub>2</sub>S are mutually dependent and converge at cyclic guanosine 5'-monophosphate (cGMP). NO activates soluble guanylyl cyclase to generate cGMP whereas H<sub>2</sub>S inhibits phosphodiesterase type 5 inhibitor to slow down the degradation of cGMP(52). With an increase in cGMP there is increased activation of protein kinase G with its subsequent downstream effects.

### Cell Death and Apoptosis

In addition to the direct stimulatory effects of H<sub>2</sub>S on vasodilation, cell growth, and angiogenesis, it also plays a role in modulating cell death and apoptosis. The endothelium covers the inner surface of the cardiovascular system and protects the vascular system from inflammatory damage, provides a permeability barrier to control blood volume and osmotic homeostasis. The endothelium is also where the earliest signs of vascular inflammation are detected with propagation of pro-inflammatory cytokines, upregulated expression of adhesion molecules, adherence of leukocytes to the vascular endothelium, and subsequent extravasation and migration to sites of injury. In previous studies, H<sub>2</sub>S has demonstrated the ability to inhibit vascular inflammation through multiple different pathways, including inhibition of p38 and nuclear factor k light-

chain enhancer of activated B cells (NF- $\kappa$ B), activation of  $K_{ATP}$  and  $BK_{Ca}$  channels as well as heme oxygenase 1 expression (53).  $H_2S$  is also known to decrease reactive oxygen species levels in endothelial cells through scavenging reactive oxygen species. Many of the antioxidant enzymes including catalase, superoxide dismutase, glutathione peroxidase, and glutathione-S-transferase are upregulated in the presence of  $H_2S$ . Reduced glutathione production is also increased with  $H_2S$  accounting for additional cytoprotective effects seen in endothelial cells.

GAPDH, a known regulator of a cell death cascade can be sulfhydrated by a polysulfide at Cys<sup>150</sup> causing a dramatic increase in its enzymatic activity(54). The anti-apoptotic transcription factor, NF-  $\kappa$ B has also been observed to be sulfhydrated on Cys<sup>38</sup> of the p65 subunit. The addition of the sulfur moiety promotes NF-  $\kappa$ B binding to co-activator ribosomal protein S3 to facilitate its further binding to promoters of anti-apoptotic genes (55).

Cell death during ischemia and following reperfusion are fundamental to the pathology of ischemic injury. By decreasing cell death and apoptosis,  $H_2S$  has the capacity to lessen tissue injury and accelerate recovery and repair following ischemia. Understanding how to manipulate this gasotransmitter to reduce cell death is paramount for treatment strategies.

### Inflammation and Sepsis

Literature regarding the role of  $H_2S$  in inflammation and sepsis has been somewhat controversial and contradicting. While some research in the literature has noted  $H_2S$ ' ability to decrease the production of inflammatory cytokines, others have demonstrated an up regulation of apoptosis and  $H_2S$  formation following inflammation. However, similar debates have arisen in the literature regarding two other inflammatory mediators; nitric oxide and prostaglandins. To some extent the differences in observations are dose-related, drug and model dependent, and related to setting. The contradictory findings largely reflect variations in dose-response relationships with low, physiologic concentrations being anti-inflammatory, while higher concentrations promote inflammation as also seen with NO and CO(56, 57).

Studies implicating  $H_2S$  as a pro-inflammatory mediator include that of Zhang et al., who used a mouse model of cecal ligation and puncture (CLP) induced sepsis (58). Mice were treated with either PAG (50mg/kg; intraperitoneal), a CSE inhibitor, or NaHS (10 mg/kg; intraperitoneal) (58). With the induction of sepsis, CSE mRNA production in the liver was significantly upregulated. They also found a significant down regulation of inflammation after administration of PAG (IL-1 $\beta$ , IL-6, TNF- $\alpha$ , monocyte chemotactic protein-1 and macrophage inflammatory protein 2). Additionally, PAG reduced myeloperoxidase (MPO) activity and histological changes in the lung and liver. However, in contrast, there was an upregulation of pro-inflammatory markers with administration of NaHS. This  $H_2S$  mediated systemic inflammation was thought to occur through a mechanism involving activation of ERK pathway and upregulation of NF- $\kappa$ B activity.

Similarly, in a study by Li et al., mice underwent intraperitoneal *E. coli* LPS administration and demonstrated a dose-dependent increase in plasma H<sub>2</sub>S concentration and an increase in CSE gene expression in both kidney and liver tissues (59). This same pro-inflammatory effect was confirmed with administration of NaHS which similarly increased MPO activity and increased plasma TNF- $\alpha$  concentration. Similarly, when PAG was administered there was a decrease in MPO activity in the lung and liver (59).

In contrast to the literature noting the pro-inflammatory effects of H<sub>2</sub>S, a more recent article of CLP sepsis demonstrated opposite effects, and noted attenuation of pro-inflammatory markers (IL-1 $\beta$ , IL-5, IL-6, TNF- $\alpha$ , HMGB1) following NaHS administration (60). In this particular study, survival and end-organ blood flow was increased when NaHS (3mg/kg) was administered via intraperitoneal injection 24 hours post-CLP (72). This dose was a lower concentration than the previous studies demonstrating pro-inflammatory effects (3mg/kg vs 10 mg/kg), thereby suggesting a bimodal dose response. In a study using a rats to model gastric I/R injury, administration of NaHS caused the pro-inflammatory cytokines, IL-1B and TNF-a, to be significantly decreased in a dose dependent manner(61). Additionally, the highest amount of area with mucosal lesions was seen in rats treated with PAG (61).

In other studies examining inflammatory properties, H<sub>2</sub>S has been found to be an important endogenous regulator of leukocyte activation and trafficking during the inflammatory response, and similarly to above studies, pro- or anti-inflammatory effects vary dependent on dose. Zanardo et al. demonstrated that H<sub>2</sub>S donors (NaHS 1-150  $\mu$ mol/kg) suppress leukocyte adherence to vascular endothelium and reduce leukocyte infiltration and edema formation (53). Additionally, when inhibited by CSE inhibitor, there was enhanced leukocyte adhesion, leukocyte infiltration and edema formation mediated via K<sub>ATP</sub> channels(53). Conversely, in a study done by Zhang, et al. when PAG was given to block endogenous production of NaHS in a septic mouse, there was reduced leukocyte rolling and adhesion in mesenteric venules along with decreased ICAM-1, P-selectin and E-selectin production (62). Additionally, when exogenous NaHS (10 mg/kg) was administered to a septic mouse, there was increased leukocyte rolling and adhesion along with increased levels of ICAM-1, P-selectin and E-selectin (62). This difference in pro-inflammatory and anti-inflammatory effects is most notably dose-dependent.

It is unclear from the current body of literature if H<sub>2</sub>S is a pro-inflammatory or anti-inflammatory mediator. Although its effects are likely dose dependent, it is unclear at this point what the most optimal dose for therapy is. Further studies are needed to quantify the dose response curve in various models of end organ ischemia to provide an appropriate dose that inhibits the inflammatory response.

### Cytoprotection

The majority of research into the cytoprotective effects of H<sub>2</sub>S in ischemia-reperfusion injury has been done in both renal and cardiac injury models. In cardiac I/R injury, the administration of NaHS has been found to enhance the activity of CSE, reduce ischemic injury, and attenuate the injury to organelles

(mitochondria, nucleus and myofilaments). This has been demonstrated by a reduction of lactate dehydrogenase activity, decreased mitochondrial malondialdehyde, and an increase in the activities of reactive oxygen species scavengers (superoxide dismutase and glutathione peroxidase) (63). Xiao et al. found that H<sub>2</sub>S decreased cell injury in cardiomyocytes following hypoxic injury by regulating autophagy pathways and mTOR activation (64). Additionally in a study by Sivarajah et al. the administration of NaHS following myocardial ischemic insult caused a significant decrease in both apoptosis and caspase 9 activity while increasing the production of anti-apoptotic protein Bcl-2 (65). The benefit of this NaHS therapy was abolished with the application of a mitochondrial K<sub>ATP</sub> blocker suggesting mediation of anti-apoptotic effects through this pathway (65). Multiple other studies have also confirmed the ability of H<sub>2</sub>S to provide protection to cardiomyocytes following ischemia and reperfusion injury(66, 67).

H<sub>2</sub>S has also been shown to be protective in a number of renal ischemia and reperfusion models. In an *in vitro* model of hypoxia/oxidative stress injury, glucose oxidase stimulation led to mitochondrial dysfunction and decreased intracellular ATP, and, at higher concentrations, necrosis and increased intracellular oxidant formation (68). However, when the mitochondrially-targeted H<sub>2</sub>S donor A39 was administered, there was a dose-dependent decrease in the observed injury. This was translated into an *in vivo* rat model of renal I/R injury and A39 was found to provide dose-dependent protection against all known signs of oxidative stress. Additionally, there was decreased neutrophil infiltration and BUN and creatinine levels were decreased, thus, attenuating the inflammatory and oxidative processes(68).

Han et al. demonstrated similar findings with the use of NaHS following renal ischemia-reperfusion injury. In their study, NaHS was found to accelerate recovery of renal function and tubular morphology and to decrease the production of reactive oxygen species(69). Furthermore, when PAG was added they found delayed recovery following injury(69). More importantly, with their study, they also found a resultant decrease in CSE and CBS expression/activity following ischemia that did not return until 8 days after ischemia. This would suggest that during ischemia, the ability to produce adequate endogenous H<sub>2</sub>S is significantly reduced, and therefore, exogenous applications are required to restore homeostasis. Similarly, in a study by Stenzel et al., the ability to preserve renal function and high creatinine clearance following renal I/R was found to be correlated with the ability to maintain high expression of CSE(70). When examining the benefits of pre-treatment with an H<sub>2</sub>S donor prior to aortic occlusion in a porcine model, Simon et al. observed similar benefits with attenuated kidney dysfunction, decreased inflammation, and decreased iNOS expression and NO release (71). These findings support the notion that novel exogenous H<sub>2</sub>S therapy could change management and potential outcomes following ischemic injuries.

#### Interactions and Interdependency between Gasotransmitters

When looking at differences between gasotransmitter signaling, it is apparent that different vascular beds have different thresholds for gasotransmitter mediated vascular relaxation. Therefore, NO and H<sub>2</sub>S

induced vasorelaxation via endothelium-dependent mechanisms may vary by blood vessel type. For example, H<sub>2</sub>S is 5 times more potent in rat mesenteric artery (EC<sub>50</sub> ~ 25 μM) compared to rat aorta (EC<sub>50</sub> ~125 μM)(72), and therefore has a greater role in mesenteric artery vasorelaxation, while the aorta displays more prominent NO-mediated relaxation(73). In the aorta, Coletta et al. reported that when aortic endothelial cells are exposed to H<sub>2</sub>S, intracellular cGMP, activated PKG and vasodilator-stimulated phosphoprotein all increase in an NO-dependent manner. When inhibitors of these cellular proteins are used, the angiogenic and vasodilatory properties of this pathway are not elicited demonstrating the unique requirement of NO in vascular H<sub>2</sub>S signaling within the aorta(52).

Gasotransmitter signaling may also be dependent on one another. H<sub>2</sub>S has been found to upregulate NO production through persulfidation of eNOS(74). Altaany et al. demonstrated that Cys<sup>443</sup> was the primary persulfidation site in eNOS and once sulfhyrated by H<sub>2</sub>S, dimerization of eNOS occurred activating the protein for NO production. Additionally, wild-type mice and CSE knockout mice were also compared and eNOS dimerization was predominately found in wild-type mice whereas eNOS monomers were prevalent in the CSE-knockout mice (only eNOS dimers produce NO) further confirming gasotransmitter interdependency. In a cardiac study of rats with metabolic syndrome (obesity, high triglycerides, fasting blood sugar and blood pressure) established by a 16-week high fat diet, both H<sub>2</sub>S and NO yielded synergistic effects in myocardial tissue. In these animals, there was an increase in the expression of both CSE and eNOS mRNA. When CSE was inhibited, nitric oxide levels increased, as did the expression of eNOS and iNOS. With use of an H<sub>2</sub>S donor, eNOS and iNOS were significantly decreased. In the myocardium, NO and H<sub>2</sub>S appear to work synergistically to provide cardiac protection (75). Sodium nitroprusside, a source of NO, reacts directly with H<sub>2</sub>S yielding a redox sibling of nitric oxide known as nitroxyl (76). Nitroxyl activates sensory chemoreceptor TRPA1 by formation of amino-terminal disulfide bonds (77). This transformation allows for sustained calcium influx, the release of calcitonin gene-related peptide, and ultimately, local and systemic vasodilatation within the cardiovascular system (76). This reaction between NO and H<sub>2</sub>S also produces polysulfides that might also play a role in vasodilation, however this has yet to be studied.

### **MEASURING H<sub>2</sub>S in PHYSIOLOGICAL SYSTEMS**

When at physiological pH of 7.4, dissolved H<sub>2</sub>S gas is a weak acid and in equilibrium  $H_2S \leftrightarrow HS^- \leftrightarrow S^{2-}$  (pK<sub>a1</sub> = 6.9, pK<sub>a2</sub> = >12) with one third as H<sub>2</sub>S and two thirds as HS<sup>-</sup> with very little S<sup>2-</sup> in extracellular fluids and plasma(78). Intracellularly, H<sub>2</sub>S exists as both the free form (with equal parts H<sub>2</sub>S and HS<sup>-</sup> at pH ~6.9) and in a variety of bound forms including persulfides (thiosulfate, hydrosulfides), thiosulfonates, polysulfides, polythionates and iron-sulfur clusters.

Many of the methods currently used to measure H<sub>2</sub>S were originally developed to measure and determine sulfide concentration in air and water samples and were subsequently applied to biological samples. Through experience we have learned that many of these methods provide inaccurate measurements

of physiologically active H<sub>2</sub>S. Therefore, finding a reliable, accurate, and reproducible method for *in vivo* and *in vitro* H<sub>2</sub>S measurement has continued to be exceedingly difficult. In addition to the multiple sources of H<sub>2</sub>S, there have also been multiple techniques and experimental conditions by which values in the literature have been obtained and reported(78). Due to its volatile nature and low steady state concentrations, obtaining reliable measures of H<sub>2</sub>S in biological samples continues to be a challenge. The methodologies that are most frequently used are discussed.

### Methylene Blue Assay

The most common method of detecting H<sub>2</sub>S is a colorimetric assay using spectrophotometry in which H<sub>2</sub>S is trapped with a metal (usually zinc) followed by acidification and a reaction with a dye, *N,N*-dimethyl-*p*-phenylenediamine, to form methylene blue (35). This technique, however, has multiple issues that make it unreliable for measuring biological levels including: 1) possible interference from other colored substances that would decrease reliability, 2) methylene blue dimer and trimer formation, 3) strong acid chemical pretreatment (releases sulfide from iron-sulfur centers that grossly inflate values) and 4) low sensitivity at low (<1 μM) H<sub>2</sub>S concentrations(it measures H<sub>2</sub>S as well as other sulfur-containing species such as hydrosulfide ions, sulfide, etc.) and 5) it cannot be used for continuous measurements of H<sub>2</sub>S under physiological conditions in real-time or with simultaneous measurement of O<sub>2</sub>, making this method less than optimal for measurement(35, 79, 80). With the strongly acidic conditions used for this technique, large sulfide concentrations in biological samples are liberated thus causing measurements to be inaccurately high.

### Monobromobimane Method

The monobromobimane (MBB) method coupled with reverse phase high-performance liquid chromatography measures H<sub>2</sub>S at physiological pH and hence provides smaller numbers (79, 80). Sulfide derivatization occurs via MBB to form sulfide-dibimane. This relatively new methodology is a useful and sensitive quantitative method to measure all biochemical forms of sulfide. Disadvantages to the MBB method include: 1) light sensitivity (MBB is light-sensitive reagent; derivatization should be performed in the dark), and 2) H<sub>2</sub>S binds to glass, therefore high-quality polypropylene plastic tubes need to be used for preparation of samples. Additionally, chemical derivatization reactions are heavily influenced by pH, time, oxygen tension and volatilization all of which affect sample H<sub>2</sub>S concentration. Also, due to irreversible sulfide binding or shifts in phase transition equilibria, these methods potentially can liberate loosely bound sulfide.

### Ion Selective Electrodes

Sulfide-ion electrodes have also been developed that measure S<sup>2-</sup> within biological samples. Measurement accuracy, however, is also not without error. To form S<sup>2-</sup>, a strong alkaline solution must be used (pH>11) as the antioxidant buffer to drive equilibrium of H<sub>2</sub>S, HS<sup>-</sup> and S<sup>2-</sup> to S<sup>2-</sup>. The buffer promotes hydroxyl replacement of cysteine sulfur producing erroneously high sulfide concentrations that increase over

time (81). Similarly, to other methods, measurement accuracy is dependent on maintaining a consistent and accurate pH and other biomolecules within the sample may interfere with measurement (such as thiols). Additionally, similarly to the methylene blue assay, it cannot be used for real-time analysis of H<sub>2</sub>S, with living tissue or for simultaneous measurement with O<sub>2</sub>.

### Gas Chromatography

Gas chromatography (GC) is another analytical method used to measure physiological sulfide levels. However, it may liberate loosely-bound sulfide during phase transition equilibrium and falsely elevate H<sub>2</sub>S measurements. It relies on equilibration of H<sub>2</sub>S between the liquid sample and gas phase measured following GC separation(82). Studies using GC have had variable results depending on methodology. Certain reports denote being able to detect sulfur at levels of 0.5 picograms with detectable ranges spanning four orders of magnitude(82). Similarly, to other current methods, limitations include instrumental parameters such as column type, injection volume and mode, as well as gas flow rate(82). While is it more sensitive than the methylene blue assay and ion selective electrodes, it is also unable to provide real-time measurements of H<sub>2</sub>S (80).

### Polarographic (Amperometric) Electrode

To overcome the interference from other biomolecules within the samples and the requirement for frequent reconditioning as was seen with the ion selective electrode, the amperometric electrode was developed to protect the cathode, anode and electrolytes from solution constituents and more accurately measure H<sub>2</sub>S. This H<sub>2</sub>S specific electrode operates similarly to the standard Clark O<sub>2</sub> electrode with an H<sub>2</sub>S gas specific membrane and polarizing voltage. The electrode is used with unadulterated samples and may be used for continuous monitoring of plasma H<sub>2</sub>S in unanesthetized animals(80). It directly measures the concentrations of dissolved H<sub>2</sub>S gas in real time with a detection limit of 10-20 nanomolar (nM) H<sub>2</sub>S gas or ~100-200 nM total sulfide(80). Disadvantages to use of the amperometric electrodes include: 1) its ability to consume sulfide causing inaccurate measurements of H<sub>2</sub>S, 2) pressure and temperature sensitivity, and 3) prone to drift requiring frequent calibration(81).

### Fluorescent Probes

The most recent and novel tool to measure H<sub>2</sub>S has been the development of fluorescent sulfide probes. This new easy-to-use technology provides immediate H<sub>2</sub>S detection in both living cells and organ tissues(83). It has been found previously that H<sub>2</sub>S itself has reducing ability and is able to change from the oxidation state of sulfur of -2 (H<sub>2</sub>S) to 0 (S) via reducing azide and nitro groups(83). Using this knowledge, novel fluorescent probes have been developed. The first two probes developed were azide-caged rhodamine analogs. When these probes were exposed to H<sub>2</sub>S, the azide would be reduced to amine on the probe to generate a highly fluorescent rhodamine product(84). Other azide-based probes have also been developed to target specific organelles such as mitochondria(85) or lysosomes(86). Additionally, like the azide group,

nitro groups, nucleophilic reactions and metal-sulfide precipitations can also be reduced by H<sub>2</sub>S and used as part of fluorescent probes to determine H<sub>2</sub>S concentration(87). Potential down falls of this technology are that few can be applied to real biological detection due to slow reaction rate and/or low sensitivity. With these probes, it may take over an hour to achieve maximum fluorescence. Additionally, selectivity has been largely unaddressed. There may be unidentified compounds, such as polysulfides, that react with the fluorescent probes as well as or better than H<sub>2</sub>S that have yet to be identified in the screening process.

All of the above methodologies have their own strengths and weaknesses. With translating our H<sub>2</sub>S knowledge and applications from “bench to bedside”, it will become even more crucial to find a reliable, accurate, rapid and reproducible way to measure H<sub>2</sub>S within biological systems.

### **CLINICAL ADMINISTRATION OF H<sub>2</sub>S FOR TREATMENT OF ISCHEMIA**

Research surrounding H<sub>2</sub>S has become quite popular over the last decade, most notably due to its potential vasodilatory and cytoprotective properties. These effects make H<sub>2</sub>S a potential agent for therapy in ischemic diseases. In fact, many different H<sub>2</sub>S donor drugs have been created to harness the beneficial properties of the gas. These H<sub>2</sub>S donors aim to mimic H<sub>2</sub>S production under physiologic conditions or diseased states. Although H<sub>2</sub>S in these medications is biologically active, its function may be inconsistent depending on its concentration.

Problems with many H<sub>2</sub>S donors include an uncontrolled pattern of release, an unclear mechanism of action, and unknown by-product profiles. At present, it is difficult to define a universal best donor for all pathological applications. Difficulties with creating an optimal donor include considerations and physiological effects of H<sub>2</sub>S by-products. These effects are different based on donor, H<sub>2</sub>S releasing capabilities and cross-reactivity. An ideal therapeutic H<sub>2</sub>S donor for clinical application will have the ability to be easily measured, to work locally without systemic effects, to have controlled and sustained release, and will have a minimal toxic side effect profile. Herein, we discuss and review some of the most common currently available H<sub>2</sub>S donor medications (Table 1).

#### **Sulfide Salts**

Sodium sulfide and sodium hydrosulfide are two widely used inorganic sulfide salts in H<sub>2</sub>S research. Sulfide salts have the advantage of boosting H<sub>2</sub>S concentration fast. They are also “clean” donors, meaning that they do not produce by-products from H<sub>2</sub>S release. However, the major problem is that release is uncontrollable. Both inorganic substances release H<sub>2</sub>S immediately and completely as soon as they are dissolved in aqueous buffers. Furthermore, because H<sub>2</sub>S is a gaseous molecule, it can be quickly lost secondary to volatilization in an open stock solution. Previous studies have found the half-life of sulfide loss to be approximately 5 minutes (88). Lastly, there is a lack of understanding of the long term effects.

#### **Hydrogen Sulfide Gas**



H<sub>2</sub>S gas has been used to induce a suspended animation state in mice in which exposure to 80 ppm decreased metabolic rate via reduction of oxygen consumption and carbon dioxide output (29). This hibernation state revealed promising effects of H<sub>2</sub>S gas in mice but failed in large animals such as sheep and piglets(25, 89). Additionally, H<sub>2</sub>S gas has not been considered to be an ideal source due to difficulties in obtaining precisely controlled concentrations as well as potential toxic impacts of excess H<sub>2</sub>S gas exposure.

### Slow Releasing H<sub>2</sub>S Donor

There are many organic substances currently under investigation. Most notably is the slow-releasing, H<sub>2</sub>S donor, GYY4137, a derivative of Lawesson's reagent. It has sustained release in aqueous solution and releases H<sub>2</sub>S very slowly via what is likely to be hydrolysis. It has been found to be pH and temperature dependent with greater release in acidic environments and at higher temperatures. It induces vasorelaxation and decreased blood pressure(90). It has been found to reduce inflammation in LPS-stimulated macrophages, while limiting tissue damage, sepsis-induced hypotension, and production of pro-inflammatory markers in certain in vivo models (56, 91). Although it has been widely studied, the by-product profile has yet to be fully elucidated.

### 1,2-Dithiole-3-Thiones

1,2-Dithiole-3-thiones are novel H<sub>2</sub>S-releasing donors with an unclear mechanism of action. H<sub>2</sub>S is produced by hydrolysis when 1,2-Dithiole-3-thiones are heated (92). 1,2-Dithiole-3-thiones have been used to make hybrid drugs by coupling it with parent nonsteroidal anti-inflammatory drugs (NSAIDs). As such, many hybrid drugs (H<sub>2</sub>S-NSAIDs) have been made: ACS15 (Diclofenac), ATB-429 (Mesalamine), SH-SUL (Sulindac), HS-NAP (Naproxen), ACS14 (Aspirin), and HS-IBU (Ibuprofen)(93).

Side effect profiles of the hybrid drugs also appear to be better than the parent drugs, yielding potential promise for clinical translation. For example, studies found that seven days of aspirin therapy yielded significant hemorrhagic gastritis in rats given only aspirin. However, in rats given the H<sub>2</sub>S-releasing aspirin hybrid, ACS14 did not exhibit this gastritis (93). These findings were thought to occur as a result of down regulation of oxidative stress. Studies found an up-regulation of heme oxygenase 1 promoter activity with ACS14 while aspirin alone had no such activity. A similar study was performed with the diclofenac hybrid, ACS15, in which decreased gastric and intestinal mucosal damage was observed. In addition, greater inhibition of TNF- $\alpha$  expression and decreased leukocyte adherence to vascular endothelium was also observed with the use of ACS15 compared to diclofenac (93). Similarly, ACS15 was also found to decrease lung inflammation in the setting of acute pancreatitis through significantly reduced MPO activity (94). Finally, the H<sub>2</sub>S-mesalamine hybrid, ATB-429, was found to be more effective in decreasing the severity of colitis and the production of pro-inflammatory cytokines in mouse models of this disease.

Although 1,2-Dithiole-3-thiones are thought to be H<sub>2</sub>S donors, the extent of the effects actually related to H<sub>2</sub>S has not been fully elucidated. Other studies suggest that in vivo elevations of glutathione

found with use of 1,2-Dithiole-3-thiones could also be involved in the demonstrated effects (93). Additionally, they are known to participate in electrophilic reactions and the conversion of molecular oxygen to reactive oxygen radicals. Therefore, more information is needed on mechanisms of action of these drugs prior to widespread application.

### Garlic-Derived Hydrogen Sulfide

Garlic is known to be rich in sulfur-containing compounds and has demonstrated both anti-inflammatory and cardioprotective effects secondary to its H<sub>2</sub>S-releasing capability. Allicin (diallyl thiosulfinate) is the best characterized compound in garlic. In aqueous solution, it decomposes to diallyl sulfide, diallyl disulfide and diallyl trisulfide(95). Additionally, recent literature suggests that H<sub>2</sub>S release from garlic oil is thiol-mediated while polysulfides are readily detected in garlic oil alone (96). Administration of garlic oil rapidly increases intracellular polysulfide with minimal effects on H<sub>2</sub>S suggesting that polysulfides, not H<sub>2</sub>S, may be the actual mediator in physiological signaling. Human red blood cells are able to convert these garlic-derived organic polysulfides into H<sub>2</sub>S thus promoting vasodilation in blood vessels(97). Garlic is also well known to be protective against ischemic injury in many different organ systems (renal, hepatic, cardiac, cerebral and pulmonary)(96, 97). Garlic and garlic-derived sulfur compounds exhibit concentration-dependent vasorelaxation most likely due to the presence of polysulfides.

### FUTURE DIRECTIONS

This review of H<sub>2</sub>S suggests that H<sub>2</sub>S-related drugs may be a novel therapy to halt disease progression in the setting of ischemia. H<sub>2</sub>S is an endogenous gaseous mediator that plays a key role in a broad range of physiological functions including vasorelaxation, angiogenesis, cell protection, and inflammation. While many of these protective benefits have been attributed to the production of H<sub>2</sub>S, there is suggestion that some of the observed benefits could be related to the release of other sulfide mediators such as polysulfides or persulfides. Additionally, the possibility of H<sub>2</sub>S generation and metabolism via another pathway is possible and the physiologic triggers that induce production of H<sub>2</sub>S, persulfides and polysulfides endogenously to maintain local levels remains unknown. Understanding the mechanisms controlling the actual concentrations of H<sub>2</sub>S in circulation and in tissues becomes paramount. In this regard, further investigation into the role of other sulfide mediators in end-organ ischemia is warranted along with inquiry into additional H<sub>2</sub>S pathways.

In the setting of end-organ ischemia, an H<sub>2</sub>S-releasing drug could prove to be a novel therapy where current abilities to reverse damage are limited. A number of slow-releasing H<sub>2</sub>S drugs and hybrid H<sub>2</sub>S drugs have been developed and found to have effectiveness in various models. However, with many of these drugs, byproducts are still largely unknown. Additionally, the pharmacokinetics, pharmacodynamics, efficacy and toxicity profiles of many of these hybrids remain unanswered. Prior to H<sub>2</sub>S therapy being brought to the forefront of clinical use, it is essential to have precise control of H<sub>2</sub>S release so that we are able to emulate

endogenous H<sub>2</sub>S production. Additionally, it is also crucial to determine the most effective concentration and relevant route of administration. The ideal H<sub>2</sub>S-releasing drug should work locally without systemic effects, have controlled and sustained release, and limited side effects. Lastly, further development of these donors requires a way to accurately and consistently measure absolute concentrations of H<sub>2</sub>S in the circulation and in tissues.

While multiple avenues of H<sub>2</sub>S measurement have been utilized, all of them come with inherent flaws. Hydrogen sulfide's volatile nature makes measurement exceedingly difficult. Previous work has demonstrated that with intravenous administration, H<sub>2</sub>S is rapidly taken in and bound to other thiols or carbon and/or coordinated with metals(39). As we continue to make strides of improvement with advancing techniques for H<sub>2</sub>S measurement in biological samples, there is still no consensus on the most acceptable and reliable technique. Additionally, with the uncertainty of all known "key players" of H<sub>2</sub>S protection (namely free H<sub>2</sub>S gas versus sulfane sulfurs (persulfides, polysulfides, thiosulfate, etc.)); a way to improve accuracy and reliability in measurement of absolute concentrations of all H<sub>2</sub>S is paramount. Investigations into the potential biological activity of other sulfur containing molecules such as persulfides and others confirm that a way to more accurately and consistently measure H<sub>2</sub>S in the blood may be through measurement of total sulfide rather than the gas itself. As previously described by Shen et al. and as described above, using the monobromobimane method coupled with RP-HPLC (which involves liberation, trapping and derivatization of H<sub>2</sub>S), may allow for repeatable measurement of total sulfide concentrations in various biological specimens(98). Once a consensus is reached on the best way to measure endogenous levels of H<sub>2</sub>S, known benefits of H<sub>2</sub>S therapies may be translated into a clinical setting for further investigation.

## **CONCLUSION**

End-organ ischemia continues to be a complex pathophysiological process involving many factors including oxidative stress and systemic inflammation. The design of novel therapies to treat these conditions is therefore paramount. Hydrogen sulfide is one such potential option. As a potent vasodilatory and cytoprotective agent, H<sub>2</sub>S exhibits a number of biological properties that make it an attractive agent for therapy. However, prior to widespread clinical use, its safety profiles and mechanisms of action need to be further delineated, and an accurate and rapid method of assessment needs to be created. Further studies on these areas of interest will allow for more rapid translation to the care of critically ill patients with ischemia.

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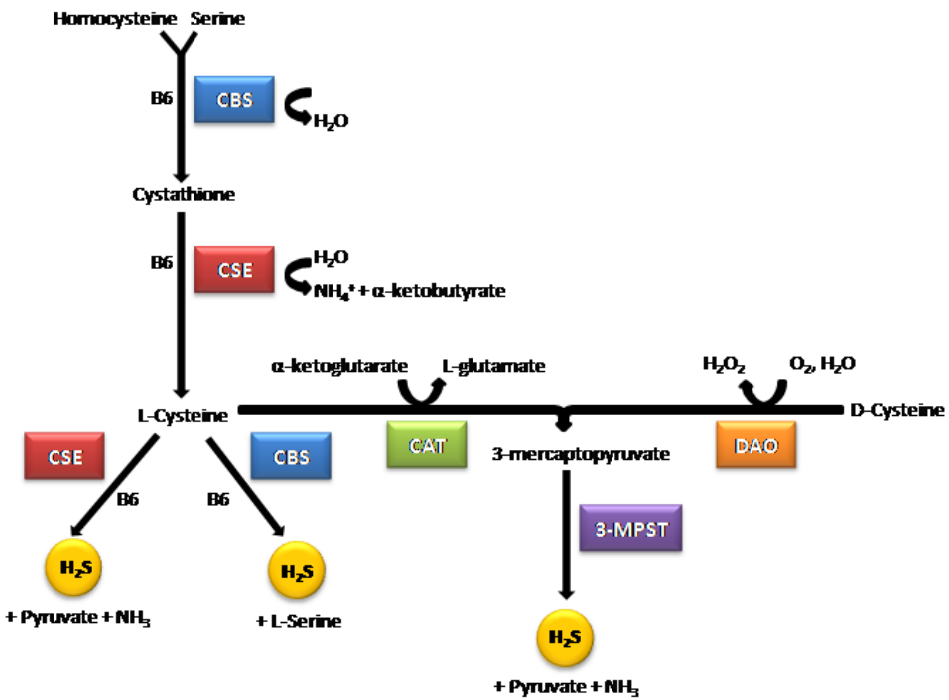
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Figure 1. Enzymatic production of H<sub>2</sub>S within mammalian cells. To produce H<sub>2</sub>S, CBS catalyzes the reaction between homocysteine and serine to form cystathionine and H<sub>2</sub>O. Cystathionine and H<sub>2</sub>O are subsequently catalyzed by CSE to form 2-ketobutyrate, cysteine, H<sub>2</sub>O and ammonia. CBS then converts L-cysteine to H<sub>2</sub>S and L-serine, while CSE converts L-cysteine to H<sub>2</sub>S, pyruvate and ammonia.

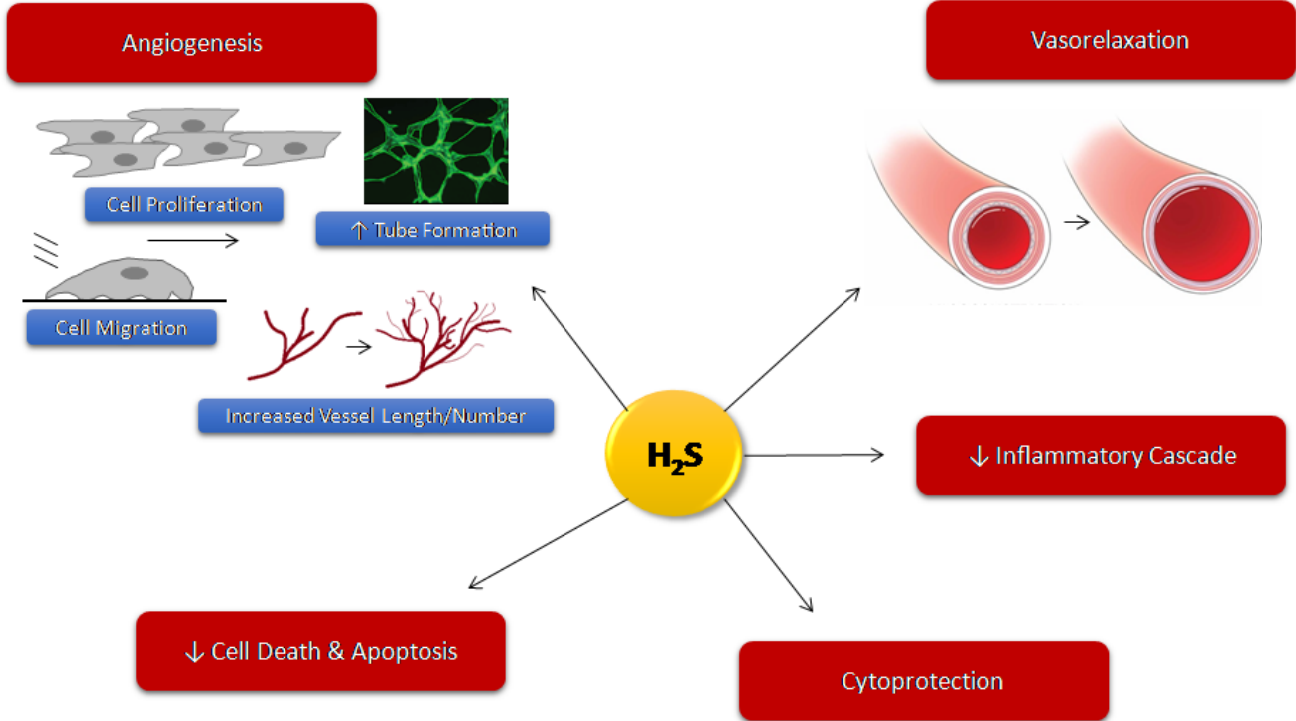
Figure 1.



AC

Figure 2. H<sub>2</sub>S has a number of beneficial properties in the setting of ischemia. It has been found to be a mediator of angiogenesis and vasorelaxation. Additionally, it has been found to decrease the inflammatory cascade, cell death, and apoptosis.

Figure 2.



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Table 1. Intracellular sulfane sulfur stores.

Sulfane Sulfur Stores	
Thiosulfate	$S_2O_3^{2-}$
Persulfides	R-S-SH
Thiosulfonates	R-S(O)-S-R'
Polysulfides	R-S <sub>n</sub> -R
Polythionates	$S_nO_6^{2-}$
Elemental sulfur	S <sup>0</sup>
Protein-associated sulfur	On Rhodanese, CSE, MPST, etc

Table 2. The benefits and disadvantages of the most commonly studied H<sub>2</sub>S donors

H <sub>2</sub> S Donors	H <sub>2</sub> S release mechanism	Benefits	Disadvantages	Species	Ischemia Model
<b>H<sub>2</sub>S Gas</b>	Authentic H <sub>2</sub> S resource	<ul style="list-style-type: none"> <li>• Authentic source of H<sub>2</sub>S</li> <li>• Found to induce suspended animation-like state in mice and reduce metabolic rate</li> </ul>	<ul style="list-style-type: none"> <li>• Difficult to obtain precisely controlled cellular concentrations of H<sub>2</sub>S over a prolonged period</li> <li>• Possible toxic impact of H<sub>2</sub>S excess</li> <li>• Hibernation studies have not been replicated in large animal models</li> </ul>	Mouse	Endotoxemia(99) Metabolism(100)
				Rat	Cerebral (101, 102) Retinal (103) Metabolism(100, 104)
				Rabbit	Cerebral(105)
				Pig	Metabolism(89)
				Sheep	Metabolism(25)
<b>Inorganic Sulfide Salts (NaHS, Na<sub>2</sub>S)</b>	Hydrolysis	<ul style="list-style-type: none"> <li>• Rapid boost of H<sub>2</sub>S concentration</li> </ul>	<ul style="list-style-type: none"> <li>• Uncontrollable release as soon as solution is prepared</li> <li>• Can be quickly lost due to volatilization, t<sub>1/2</sub> ~5min</li> <li>• Significant amount of impurities, polysulfide contamination</li> </ul>	Mouse	Cardiac(106) GI(107) Hepatic(108) Renal(69)
				Rat	Cerebral(109-111) Cardiac (63, 64, 112, 113) Hepatic(114, 115) Renal(116-118) Cerebral(109-111)
				Rabbit	Cardiac(119)
				Pig	Cardiac(120) Renal(71) Metabolism(30)
<b>Garlic-Derived Organic Sulfur Compounds (diallyl thiosulfinate → diallyl sulfide (DAS), diallyl disulfide (DADS),</b>	Thiol Activation	<ul style="list-style-type: none"> <li>• H<sub>2</sub>S release greatest from DATS &gt;DADS&gt;DAS</li> <li>• Natural source of H<sub>2</sub>S</li> </ul>	<ul style="list-style-type: none"> <li>• Diallyl thiosulfinate is unstable in aqueous solution and quickly decomposes to DAS, DADS, DATS and ajoene</li> <li>• DAS, DADS, and DATS only produce H<sub>2</sub>S when they react with GSH and NADPH is necessary to support the activity of GSH reductase</li> </ul>	Mouse	Cerebral(121) Cardiac(122)
				Rat	Cerebral (123-125) Cardiac(126) Hepatic(127) Renal (128-130)
				Rabbit	Cerebral (132) Cardiac(131)

<b>diallyl trisulfide (DATS), and ajoene)</b>			<ul style="list-style-type: none"> <li>• Rate of release of H<sub>2</sub>S from garlic-derived compounds still unknown</li> <li>• Unknown mechanism of action of H<sub>2</sub>S release, effects may be derived from polysulfides</li> </ul>							
<b>1,2-Dithiole-3-Thinones (H<sub>2</sub>S hybrid drugs w/ NSAIDS)</b>	Hydrolysis	<ul style="list-style-type: none"> <li>• Improvements in side-effects of NSAIDs with hybridization with H<sub>2</sub>S</li> <li>• Significant improvements in activity profile of NSAIDs as hybrids compared to NSAIDs alone</li> </ul>	<ul style="list-style-type: none"> <li>• Known to also significantly elevate <i>in vivo</i> GSH level, may contribute to pharmacological benefits</li> <li>• Known to also participate in other biological reactions including electrophilic reactions and conversion of molecular oxygen to ROS</li> </ul>	<table border="1"> <tr> <td data-bbox="1119 466 1219 534">Mou se</td> <td data-bbox="1235 466 1456 499">Cerebral(4, 133)</td> </tr> <tr> <td data-bbox="1119 534 1219 602">Rat</td> <td data-bbox="1235 534 1456 602">Cardiac (134, 135)</td> </tr> <tr> <td data-bbox="1119 602 1219 671">Rabbi t</td> <td data-bbox="1235 602 1456 636">Cardiac(136)</td> </tr> </table>	Mou se	Cerebral(4, 133)	Rat	Cardiac (134, 135)	Rabbi t	Cardiac(136)
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Rabbi t	Cardiac(136)									
<b>GY4137</b>	Hydrolysis	<ul style="list-style-type: none"> <li>• Slow-releasing H<sub>2</sub>S donor</li> <li>• H<sub>2</sub>S concentration reaches maximum value within 6-10 minutes but at a very low level</li> <li>• pH and temperature dependent with more release at acidic pH and less release at low temperatures</li> </ul>	<ul style="list-style-type: none"> <li>• Fixed slow release may not be of benefit in all disease models of ischemia</li> <li>• Byproducts produced through H<sub>2</sub>S release still unknown</li> </ul>	<table border="1"> <tr> <td data-bbox="1119 967 1219 1036">Mou se</td> <td data-bbox="1235 967 1456 1000">Cardiac(137)</td> </tr> <tr> <td data-bbox="1119 1036 1219 1104">Rat</td> <td data-bbox="1235 1036 1456 1104">Cardiac (138) GI (139)</td> </tr> </table>	Mou se	Cardiac(137)	Rat	Cardiac (138) GI (139)		
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