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Hydrogen Sulfide and Ischemia - Reperfusion Injury

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Abstract

Gasotransmitters are lipid soluble, endogenously produced gaseous signaling molecules that freely permeate the plasma membrane of a cell to directly activate intracellular targets, thus alleviating the need for membrane-bound receptors. The gasotransmitter family consists of three members: nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S). H_2S is the latest gasotransmitter to be identified and characterized and like the other members of the gasotransmitter family, H_2S was historically considered to be a toxic gas and an environmental/occupational hazard. However with the discovery of its presence and enzymatic production in mammalian tissues, H_2S has gained much attention as a physiological signaling molecule. Also, much like NO and CO, H_2S's role in ischemia/reperfusion (I/R) injury has recently begun to be elucidated. As such, modulation of endogenous H_2S and administration of exogenous H_2S has now been demonstrated to be cytoprotective in various organ systems through diverse signaling mechanisms. This review will provide a detailed description of the role H_2S plays in different model systems of I/R injury and will also detail some of the mechanisms involved with its cytoprotection.

1. Overview of Gasotransmitters

Cellular signaling often involves complex systems, whereby interactions between membrane-bound proteins and signaling molecules lead to the activation of intracellular molecules. These intracellular molecules act as secondary messengers, which then relay a signal to a specific destination. A set of endogenous gaseous molecules called gasotransmitters possesses similar signaling capabilities as other signaling molecules but does not require the regular string of regulatory mechanisms to transmit a signal [1]. Gasotransmitters are lipid soluble, endogenously produced, and freely permeate the plasma membrane of a cell to pass the message directly to an intracellular target [2]. Nitric oxide (NO) was the first gasotransmitter to be recognized as a signaling molecule, when it was identified as both a smooth muscle relaxer through the actions of acetylcholine [3] and an activator of macrophages [4]. NO is synthesized by the enzyme nitric oxide synthase (NOS) from the oxidation of the guanidine group of L-arginine [5]. There are three known isoforms of NOS that have been characterized, purified and cloned: neuronal nitric oxide synthase (nNOS), involved with neuronal signal transmission [6]; inducible nitric oxide synthase

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(iNOS), responsible for macrophage activation [7]; and endothelial nitric oxide synthase (eNOS), plays a role in vasorelaxation [8]. Calcium-calmodulin pathways regulate the eNOS and nNOS isoforms whereas iNOS is independent of these actions [9]. When NO is produced it diffuses through the endothelial cell membrane into smooth muscle cells (SMCs) where it activates guanylyl cyclase to produce cyclic guanosine monophospate (cGMP) [10]. Since these discoveries it has been shown that increasing NO by the administration of NO donors [11], inhaled gas therapy [12], or overexpression of eNOS protects against ischemic injury (heart, brain, liver, etc.). Preconditioning with NO has also been shown to be protective in cerebral [13], intestinal [14], and hepatic [15] ischemia.

Following the discovery of NO as a gasotransmitter, carbon monoxide (CO) was found to have similar roles. CO is formed endogenously by the enzyme heme oxygenase (HO) through the degradation of heme. There are three known isoforms of HO: HO-1, inducible under cellular stress; HO-2, homeostatic form; and HO-3, recently found in rat brain but no gene for HO-3 has yet to been found [16]. HO-1 is responsible for degrading heme into biliverdin and CO. Biliverdin is quickly reduced in the cell to bilirubin, which is a very important antioxidant. Induction of HO-1 through pharmacological agents has been shown to significantly reduce myocardial infarct size *in vivo* [17]. Yet *et al*. [18] demonstrated that a cardiac specific HO-1 transgenic mouse is also protected in myocardial ischemia/reperfusion (MI/R) injury [18]. CO, like NO, is also a known vasodilator through the activation of guanylyl cyclase [19]. However, some evidence suggests calcium-activated potassium channels are targets of CO in the context of vasorelaxation [20]. The cytoprotective effects of CO are also not limited to the heart, as it has been shown that CO contributes to central nervous system mediated blood pressure regulation [21], protects against pulmonary [22] and renovascular hypertension [23], modulates atherosclerosis [24], improves both allograft and xenograft survival following organ transplantation [25], and exerts a restorative effect on the pathologic remodeling response after balloon angioplasty [26].

2. Emergence of H_2S as the Third Gasotransmitter

The third member of the gasotransmitter family to be identified was hydrogen sulfide (H_2S), a fetid smelling molecule long thought of as a toxic gas. Warenycia *et al* [27] first reported that H_2S was produced in very low concentrations endogenously when they were investigating acute H_2S poisoning in the brain. Soon after this discovery, evidence of a physiological role of H_2S began to unravel. Skrajny *et al* [28] found increased levels of serotonin and reduced levels of norepinephrine in the frontal cortex of a rat when chronically exposed to 20 ppm of H_2S and in 1996, Abe *et al* [29] suggested that H_2S was an endogenous neuromodulator, as they showed that physiological concentrations of H_2S enhanced NMDA receptor-mediated responses and aided in the induction of hippocampal long-term potentiation. Shortly after, Hosoki *et al* [30] reported that an enzyme, which produces H_2S, is present in the ileum, portal vein, and thoracic aorta and proposed that H_2S may be an endogenous smooth muscle relaxant. Accompanying these discoveries was an interest in the physiological role of H_2S in biological systems.

2.1 Physiological Role of H_2S in Biological Systems

There are three known enzymes that produce H_2S endogenously in mammalian tissue: cystathionine β-synthase (CBS), cystathionine γ-lyase (CGL or CSE), and 3-mercaptopyruvate sulfur transferase (3MST). In most tissues, CBS and CSE are responsible for catalyzing the production of H_2S. CBS and CSE are both pyridoxal-5'-phosphate-dependent enzymes that utilize L-cysteine and homocysteine as substrates to liberate ammonium, pyruvate, and H_2S [1]. It was originally believed that CBS was responsible for H_2S production in the brain through the activation of the Ca^{2+}/calmodulin pathway [31], but

the discovery that approximately 90% of H_2S in the brain is produced by 3MST [32] has changed the perceived role of CBS in H_2S production. H_2S is produced by 3MST from L-cysteine and α-ketoglutarate through the metabolism with cysteine aminotransferase (CAT) [32]. Although 3MST is responsible for the majority of H_2S production in the brain, it is localized mainly in neurons, whereas CBS is located in astrocytes, suggesting that some H_2S signaling in the brain may require CBS.

Under physiological conditions, two thirds of H_2S is dissociated into H^+ and HS^- and the remaining one third is in its undissociated form ($H_2S \Leftrightarrow HS^- + H^+$, pKa = 6.9) [33]. There are three major fates of H_2S in the body. First, most of the H_2S produced in the body is oxidized in the mitochondria to an end product of sulfate [34]. The remaining H_2S is either methylated by thiol S-methyltransferase (TSMT) to methanethiol and dimethylsulfide [35] or binds to methemoglobin to form sulfhemoglobin [36]. Sulfate and thiosulfate are the major end product of H_2S and are excreted in urine [34].

3. Cytoprotective Effects of Endogenous/Exogenous H_2S

The physiological actions of H_2S make this gas ideally suited to protect the heart, brain, liver, kidney, and lungs against injury during ischemia/reperfusion (I/R). In recent years, the cytoprotective effects of endogenous and exogenous H_2S have been investigated in models of *in vitro* [37,38] and *in vivo* [39–45] ischemic injury (summarized in the Table). The effects of endogenous H_2S have primarily been studied by pharmacologically inhibiting CGL and by genetically targeting CGL in mice, whereas the effects of exogenous H_2S have been studied through the administration of H_2S in the form of sodium hydrosulfide (NaHS), sodium sulfide (Na_2S), or H_2S gas.

3.1 Cardioprotective Effects of H_2S

A number of studies have demonstrated the cytoprotective effects of H_2S in myocardial I/R (MI/R) injury [37,39–41,46–50]. The first study to show cytoprotection against MI/R injury was Johansen *et al* [46] who examined the hypothesis that the protective actions of H_2S are mediated by ATP-sensitive potassium channel (K_{ATP}) opening. In this study, the authors performed a dose response study using the Lagendorff hanging heart model with rat hearts. They observed an approximate 20% reduction in infarct size when treatment with NaHS (1 μM) was started 10 minutes prior to reperfusion and maintained until 10 min of reperfusion. Using K_{ATP} blockers they also provide evidence to support the involvement of K_{ATP} channel opening as the mechanism of action. Another study executed by Bian *et al* [37] examined the effect of endogenous H_2S and exogenous application of NaHS on cardiac rhythm in isolated rat hearts subjected to low-flow ischemia. In this study, NaHS was administered prior to ischemia at a concentration of 100 μM in the perfusate (3 min/each cycle separated by 5 min of recovery). The hearts were then subjected to 30 min of low-flow ischemia followed by 10 min of reperfusion. They found that the NaHS-treated group had a significantly decreased duration and severity of I/R-induced arrhythmias. Isolated rat cardiac myocytes were also investigated using a simulated ischemia solution [i.e., glucose-free Krebs buffer containing 10 mM 2-deoxy-D-glucose (2-DOG) and 10 mM sodium dithionite ($Na_2S_2O_4$)] and were found to have a significantly increased viability and cell shape when treated with NaHS (10–100 μM). Bliksoen *et al*. [41] examined the protective effects of H_2S in global I/R in the heart, the mechanistic role of H_2S in ischemic preconditioning, and the involvement of protein kinase phosphorylation with H_2S. In this study, the authors blocked H_2S production from CSE by using D,L-propargylglycine (PAG) in a Lagendorff perfusion model. A 38% increase in myocardial infarct size was observed after 40 min of low flow ischemia in the PAG-treated (prior to ischemia) hearts when compared to the control hearts. The dramatic increase in infarct size indicates that endogenous H_2S plays a key role in protecting the heart from MI/R injury. Evidence supporting cardioprotection of both

exogenous and endogenous H_2S has also been observed *in vivo*. Sivarajah *et al*. [40] were the first to show that endogenous H_2S could be protective in myocardial injury. In this study mice were subjected to 25 min of regional myocardial ischemia followed by 2 hr of reperfusion. H_2S was administered as NaHS (3 mg/kg) 15 min prior to ischemia. A 26% reduction in infarct size was observed in the treated group when compared to the vehicle. Shortly after, Elrod *et al*. [39] investigated the potential of H_2S as a cardioprotective agent when given at the time of reperfusion. In this study mice subjected to 30 min of left coronary artery (LCA) ischemia and 24 hr reperfusion displayed a 72% reduction in infarct size when administered H_2S in the form of Na_2S (50 μg/kg) at the time of reperfusion. Elrod *et al*. also tested the cardioprotective effects of endogenous H_2S by subjecting mice with a cardiac-specific over expression of CGL (about a 2 fold increase in myocardial H_2S) to MI/R. After 45 min of left coronary artery occlusion and 72 hr reperfusion, the CGL transgenic mice displayed a 47% reduction in infarct size. This implies that increasing the production of endogenous H_2S can have similar cardioprotective effects as exogenous H_2S. Intriguingly inhibition of the endogenous production of H_2S has also been shown to cause hypertension and diminish endothelium-dependent vasodilatation [51] as well as exacerbates MI/R injury in mice [52]. These *in vitro*, *ex vivo*, and *in vivo* studies clearly show that exogenous and endogenous H_2S have cardioprotective effects and provide evidence that H_2S may be a potential therapeutic agent for the treatment of cardiovascular disease.

3.2 Neuroprotective Effects of H_2S

It has been reported that oxidative stress associated with I/R injury causes major damage to neurons [53]. An *in vitro* study of the efficacy of H_2S protection against oxidative stress in neurons was performed using glutamate (1mM) and demonstrated that NaHS (100 μM) protects neurons from glutamate toxicity [38]. Kimura *et al* [54] also suggested that H_2S may not itself function as an antioxidant in neuronal cells, but instead induces the production of the potent antioxidant glutathione (GSH). Another study on the neuroprotective role of H_2S was completed on a clonal hippocampal nerve cell line, HT22, using a model of oxidative stress [55]. HT22 cells exposed to glutamate (5 mM) that were treated with NaHS (10–300 μM) showed improved survival over untreated cells. Furthermore, Tyagi *et al* [56] investigated the effects of H_2S on methionine (Met)-induced oxidative stress in mouse brain endothelial cells (bEnd3). They found that pre-treatment with NaHS (0.05 and 0.1mM) attenuated cell death as well as peroxynitrite and superoxide anion formation in cells treated with Met (1.14 mM). Further *in vivo* studies have revealed additional roles of H_2S in the brain. For example, an elevated level of one of the cerebral substrates of H_2S, plasma homocysteine (Hcy), is a known risk factor for acute stroke [57]. Qu *et al*. [58] tested the effect of H_2S on the development of infarction in rats using a focal cerebral ischemic stroke model by inducing a permanent unilateral occlusion of the left middle cerebral artery (MCA) and found that administration of NaHS (0.18 mmol/kg) 10 min prior to the occlusion increased the infarct volume by approximately 150% compared to the control. Additionally, inhibition of the H_2S producing enzymes CBS and CSE using pharmacological inhibitors (in order of potency: aminooxyacetic acid [AOAA], hydroxylamine [HA], PAG, β-cyanoalanine [β-CNA]) significantly reduced infarct volume. However more recent studies have shown a protective role for H_2S *in vivo* at much lower concentrations. Florian *et al* [42] examined the effects of H_2S administered after cerebral ischemia by exposing rats to 2 days of H_2S gas (80 ppm H_2S in 19.5% O_2). The exposure of H_2S increased the concentration of H_2S in the brain from 1.12 ± 4.6 μg/g to 2.70 ± 5.3 μg/g, induced a state of hypothermia (30.8 ± 0.7 °C) and reduced infarct size by 50%. Kimura *et al* [59] studied the effect of H_2S on GSH levels in the brains of fetal mice using an intrauterine I/R (5 min/24 hr) model. Intrauterine I/R caused a 24% decrease in GSH levels. However, mice treated with NaHS (0.4375 μmol/kg, i.p.) 15 min before fetal ischemia showed improved fetal brain levels of GSH compared to the untreated mice, suggesting that H_2S protects by reinstating GSH

levels that were lowered during intrauterine I/R. Minamishima *et al* [60] used a murine model to study the neuroprotective effects of H_2S (Na_2S, 0.55 mg/kg) after cardiac arrest/cardiopulmonary resuscitation (CA/CPR). They observed improved neurological function, decreased activation of the apoptotic protein caspase-3 in the hippocampus, and enhanced phosphorylation (activation) of the anti-apoptotic protein GSK-3β in the brain cortex in mice injected with Na_2S via a femoral venous line 1 min prior to CPR.

The above studies reveal a variety of neuroprotective roles of H_2S. *In vitro and in vivo* studies demonstrate that low concentrations of H_2S can mediate oxidative stress by restoring GSH levels in the brain, whereas higher concentrations of H_2S have been shown to have effects that range from magnifying cerebral damage in the ischemic stoke model to inducing a state of neuroprotective. Studies aimed at investigating the role of 3MST in cerebral I/R injury could potentially elucidate the role H_2S plays in modulating cerebral ischemia.

3.3 H_2S and Hepatoprotection

The cytoprotective effects of H_2S can also be seen in the liver. Jha *et al* [43] tested the hepatoprotective effects of H_2S in a murine model of hepatic I/R by subjecting mice to 60 min of ischemia followed by 5 hr of reperfusion. Na_2S was administered intravenously 5 min before reperfusion and serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured to quantify liver injury. Na_2S treatments reduced AST levels by 71% and ALT levels by 69% suggesting that H_2S attenuates hepatic I/R injury. The authors also demonstrated that the hepatoprotective effects of H_2S were mediated by a decrease in lipid peroxidation levels, an increase in antioxidant signaling, and an increase in anti-apoptotic signaling. While this study provides evidence for exogenous H_2S therapy it does not address the ability of endogenous H_2S to affect hepatic injury. Therefore additional studies are warranted to fully elucidate the hepatoprotective effects of H_2S.

3.4 H_2S and Renal Ischemia

There has been a collection of studies that suggest exogenous and endogenous H_2S is protective against renal I/R injury. Tripatara *et al* [44] investigated the effects of endogenous and exogenous H_2S in renal I/R. They treated rats with PAG (1ml/kg, i.p.) 1 hr before ischemia to inhibit CSE and found that animals displayed increased serum creatine levels signifying a decrease in renal function. Next they performed a bilateral renal occlusion, this time treating the mice with NaHS topically on the kidneys 15 prior to ischemia. A significant increase in renal function was observed, indicating that H_2S plays a key role in protection against renal ischemia. Xu *et al*. [45] examined if renal injury was caused by the reduction in activity of CBS in the kidney during I/R by measuring H_2S and creatine levels after 45 min of renal ischemia and 6 hr of reperfusion. A significant decrease of H_2S and an increase in plasma creatine levels were observed in the rats subjected to the ischemia, indicating that during ischemia, H_2S levels drop along with kidney function. The activity of CBS and CGL were also measured in the rats after ischemia; CBS activity levels were significantly decreased while CGL activity was not significantly changed. These data suggest that not only does H_2S play a role in renal ischemia, but also a major enzyme responsible for H_2S production is inhibited. Moreover, because CBS is being inhibited, there is an accumulation of Hcy causing a worsening in renal I/R injury [61].

3.5 H_2S and Pulmonary Ischemia

Only a small number of studies have investigated the role of H_2S in pulmonary I/R injury. Fu *et al* [62] explored the potential of H_2S as a lung protective agent. They measured endogenous H_2S generation in the rat lung under I/R injury and found that ischemia caused a 46% increase in CSE activity resulting in a 54% increase in H_2S levels. They also perfused

the rat lungs with H_2S gas (50 μmol/l, 100 μmol/l) 5 min prior to ischemia and found that the pretreated mice displayed pulmonary protection as assessed by lung morphology. H_2S also decreased I/R-induced lung histological injury, increased lung perfusion flow rate, lowered lung wet/dry weight ratio, and improved lung compliance. Malondialdehyde (MDA) levels were also measured to determine the amount of oxidation in the cells and it was found that H_2S had significantly lowered MDA levels, suggesting that H_2S attenuated oxidative stress in the lung.

3.6 Limitations of Pharmacological Inhibitors of H_2S Producing Enzymes

Although multiple pharmacological inhibitors of H_2S biosynthesis are available, the potency, selectivity, and permeability of the compounds could be challenging when using these inhibitors to suppress a particular H_2S producing enzyme. PAG and β-CNA are commonly used to inhibit CSE. Unfortunately, they have low potency (25–100 mg/kg) and selectivity in most organs with limited cell membrane permeability [63]. Also, AOAA and HA frequently used inhibitors of CBS, target the pyridoxal-binding site of the enzyme and therefore have the potential of affecting other enzymes in biological systems [64]. Lastly, hydrogen peroxide and tetrathionate are used to inhibit 3MST by interfering with its catalytic cysteine residue. However, many enzymes use cysteines in the catalytic sites and would thus be inhibited by these chemicals [65]. Therefore, there is much room for the development of more specific and potent inhibitors of H_2S producing enzymes in which could aid in expanding the field's understanding of the endogenous role of H_2S.

4. Mechanisms of Action and the Diverse Physiological Profile of H_2S

It is shown above that H_2S is protective in many biological systems. The mechanisms through which H_2S can protect these systems are important to understand in order to utilize its effectiveness as a cytoprotection agent. H_2S has a wide range of physiological roles in mammalian tissue. In the nervous system, H_2S has been shown to function as a neuromodulator [29], modulate NMDA receptors (NMDAR) by inducing the production of cyclic-adenosine monophosphate (cAMP) [66], and act as an inhibitor of peroxynitrite ($ONOO^-$) [67]. In the cardiovascular system, numerous roles for H_2S have been identified: vasorelaxant [68] and antiapoptotic [69] properties by opening of K_{ATP} channels, proangiogenic factor through the phosphorylation of Akt [70], modulator of leukocyte-mediated inflammation [71], upregulator of antioxidant signaling [43], and involved in cytoprotection through the preservation of mitochondrial function [39].

4.1 K_{atp} Channel – Vasodilation or Calcium Handling

K_{ATP} channels are located on the sarcolemma [72], the inner mitochondrial membrane [73], and nuclear membrane [74] and play an important role in glucose metabolism in the cell by membrane hyperpolarization. K_{ATP} channels are composed of two types of subunits: four inwardly rectifying potassium channels $K_{ir}6$ ($K_{ir}6.1$, $K_{ir}6.2$) and four sulfonylurea receptors (SUR1, SUR2A, SUR2B) [75]. $K_{ir}6$ subunits form the pore of the channel and are capable of opening and closing to regulate ion flow [73]. SUR receptors are a high affinity receptor sensitive to [ATP/ADP] that facilitates the opening/closing of the $K_{ir}6$ subunits [76] and are the target of many pharmacological compounds. It has been proposed that H_2S-stimulated vasorelaxation occurs through the opening of the K_{ATP} channel [68]. Zhao *et al* [68] attempted to assess the physiological role of H_2S in the regulation of vascular contractility, the modulation of H_2S production in vascular tissues, and the underlying mechanism involved. To achieve this, Zhao *et al*. gave an intravenous bolus injection of NaHS (2.8 and 14 μmol/kg) to rats and observed a decrease in mean arterial blood pressure similar to an injection of pinacidil (2.8 μmol/kg), a known K_{ATP} channel opener. Supporting this hypothesis, H_2S has also been shown to increase K_{ATP} channel currents in isolated smooth

muscle cells [77]. Cardioprotection, however, may be limited only to specific K_{ATP} channels. In an *in vitro* study, Bian *et al*. [37] used the mitochondrial K_{ATP} blocker 5-hydroxydeconoate (5-HD) to demonstrate that mitochondrial K_{ATP} channels were not responsible for the cardioprotective effects of H_2S. However, they found that inhibition of the sarcolemmal K_{ATP} channels took away the cardioprotective effects of H_2S. Sivarajah *et al*. [69] recently performed an *in vivo* MI/R study to investigate the effect NaHS on infarct size and apoptosis caused by ischemia (25 min) and reperfusion (2 hr). Sivarajah *et al*. inhibited the mitochondrial K_{ATP} channel with 5-HD and found, contradictory to Bian *et al*., that H_2S did not protect the heart when the mitochondrial channel blocker was applied intravenously. These results suggests a new method in studying mitochondrial K_{ATP} channels must be developed to obtain more consistent results before any conclusions can be taken from these experiments.

In MI/R models, Ca^{2+} handling is critical in controlling cellular damage. During ischemia, excess Ca^{2+} and Na^{+} builds up in the cytosol of the cell [78]. Due to the large [Ca^{2+}] in the cell at the onset of reperfusion, the reoxygenation of the cell causes myofibrils to generate a large amount of force, called hypercontraction [79]. The excessive force irreparably damages the cytoskeleton. Therefore, Ca^{2+} handling is essential in controlling reperfusion damage in the cell. Protein kinase C (PKC) has been shown to be involved with Ca^{2+} handling and protects against I/R damage. There are three isoforms of PKC in the heart [80]: PKCα, PKCε, and PKCδ. Recently, Pan *et al*. [81] examined the regulatory effect of PKC on intracellular Ca^{2+} handling in H_2S-preconditioning and demonstrated that H_2S preconditioning in rat myocytes activates all three isoforms of PKC, lowers intracellular [Ca^{2+}] and protects the cardiomyocytes from damage. However an *in vivo* model of H_2S and PKC activation during I/R should be completed to solidify their roles in mediating intracellular [Ca^{2+}].

4.2 H_2S and Apoptosis

The mechanism behind the cardioprotective effects of H_2S is not limited to modulation of K_{ATP} channels and Ca^{2+} handling. There is much evidence to suggest that H_2S also has anti-apoptotic roles in the cell during M/IR. The activation of two important cell survival pathways, extracellular signal-regulated kinase (ERK1/2)/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI-3-kinase), can inactivate pro-apoptotic pathways through interaction with PKCε [82] or inhibition of Bad and caspase-9 [83], respectively. The activation of the ERK1/2 and PI-3-kinase pathways has been found to be influenced by H_2S [84,85]. Additionally, activation of the PI-3-kinase/Akt pathway increases the production of the cardioprotective gasotransmitter NO [86]. In an attempt to elucidate the signaling mechanism of H_2S in the ERK1/2 pathway during cardioprotection, Hu *et al* [85] found that preconditioning rat myocytes with NaHS (1–100 μM) not only increased cell viability, but that the blockade of ERK1/2 or Akt during preconditioning or ischemia significantly decreased the cardioprotection of H_2S. Western blot analysis also revealed that H_2S preconditioning increased the phosphorylation status of ERK1/2 and Akt, further activating the protein's pro-survival mechanisms. Elrod *et al* [39] performed an *in vitro* experiment with isolated adult myocytes, which were subjected to 6 hr of hypoxia and 12 hr reoxygenation to further examine the role of H_2S on apoptotic pathways. They found that myocytes treated with NaHS displayed a decreased activation of caspase-3 and a decrease in the number of nuclei with fragmented DNA (a result of apoptotic signaling cascades). These data suggest that H_2S inhibited the progression of apoptosis after MI/R injury. Additionally, Sivarajah *et al* [69] demonstrated that regional MI/R on rat hearts increased the phosphorylation of p38, MAPK, and JNK1/2, pro-apoptotic proteins in the heart, but the administration of NaHS 15 min prior to ischemia significantly reduced the phosphorylation of p38, MAPK, and JNK caused by I/R injury. Cytochrome C, another pro-

apoptotic protein, is an essential part of the electron transport chain, but when subjected to pro-apoptotic stimuli, it transports out of the mitochondria into the cytosol. There it activates other apoptotic proteins, including caspase-3, an activator of apoptosis [87]. To determine the effect of H_2S on the cytochrome C apoptotic pathway during I/R injury, Calvert *et al* [47] performed a study where mice were given either saline (vehicle) or Na_2S 24 hr prior to ischemia. After 45 min of ischemia and 24 hr of reperfusion, the tissue isolated from the vehicle treated mice hearts, when compared to sham operated mice, had a significant decrease in the expression of uncleaved caspase-3 (inactive) and a significant increase in the expression of cleaved caspase-3 (active), as well as an induced translocation of cytochrome C from the mitochondria to the cytosol. The mice treated with Na_2S exhibited a preservation of uncleaved caspase-3, a reduction in cleaved caspase-3, and a reduction in the translocation of cytochrome C to the cytosol (compared to the vehicle treated mice). Therefore, Na_2S protects the heart tissue from undergoing apoptotic signaling during MI/R.

The Signal Transducers and Activators of Transcription (STAT) pathway has also been shown to be an important part of the myocardium response to myocardial infarction [88] and an overexpression of the STAT isoform STAT-3 has been shown to be cardioprotective [89]. Inhibition of STAT-3 has also been shown to increase caspase-3 expression in the heart, thereby increasing I/R injury [90]. Calvert *et al* demonstrated *in vivo* that H_2S activates PKCε and STAT-3 by altering their phosphorylation, inhibiting the proapoptotic factor Bad, upregulating pro-survival factors Bcl-2 and Bcl-xL, and upregulating heat shock proteins (HSPs) [47]. Therefore, evidence suggests H_2S has many anti-apoptotic roles in MI/R.

4.3 H_2S and Mitochondrial Protection

The life of a cell is dependent on the degree of mitochondrial functionality. During I/R, mitochondria are subjected to oxygen deprivation, reactive oxygen species (ROS) overproduction, and mitochondrial membrane potential ($\Delta\Psi_m$) depolarization [91]. Roth *et al* [92] have shown that H_2S at high levels (80 ppm) can induce a state of hypothermia in mice by inhibiting cytochrome c oxidase, in turn decreasing their metabolic rate and core body temperature. They also showed that inducing this “suspended animation” state can prevent ischemic damage to cells. Elrod *et al* [39] evaluated potential mechanisms of H_2S-mediated mitochondrial preservation using an *in vitro* model of Na_2S treatment. They found that isolated mitochondria, after 30 min of hypoxia, treated with Na_2S (10 μM) had a greater recovery of posthypoxic respiration rate. Similarly mice that were given Na_2S at reperfusion displayed a reduction in mitochondrial swelling and an increase in matrix density suggesting preservation in mitochondrial function.

Under myocardial ischemia, the production of ROS is accelerated and all of the cell’s antioxidants become depleted. H_2S is a cytochrome C oxidase inhibitor and therefore inhibits respiration [93]. Inhibition of respiration has been shown to decrease the production of ROS thereby maintaining $\Delta\Psi_m$ [94]. Thus H_2S, at low concentrations, can decrease the production of ROS and preserve mitochondrial function [52,95]. Another factor concerning cellular injury is Ca^{2+} influx. The Ca^{2+} uniporter is the main pathway for Ca^{2+} influx [96] during myocardial ischemia. Pan *et al* [81] demonstrated that H_2S activates PKC and lowers intracellular [Ca^{2+}] in cardiomyocytes protecting the cells from injury. H_2S also has the ability to activate other pathways. The PI3K-Akt and Erk 1/2 pro-survival kinase cascades (Reperfusion Injury Salvage Kinase, RISK pathway) are activated during I/R and launches anti-apoptotic responses [97]. A major target of the RISK pathway is the mitochondrial permeability transition pore (mPTP) [97]. Opening of the mPTP occurs during reperfusion and is a terminal cellular event contributing to myocardial injury [96]. H_2S can overactivate Akt, a major kinase in the RISK pathway [85]. Akt then activates eNOS which evidence

suggests inhibits the opening of mPTP [97]. Therefore, H_2S acts to preserve mitochondrial function, thereby imparting cytoprotection.

4.4 H_2S and Inflammation

Inflammatory responses are an important part of early I/R injury [98]. Li *et al* [99] investigated the effects of a H_2S donor, S-diclofenac, on anti-inflammatory activity by administering the proinflammatory bacterial endotoxin lipopolysaccharide (LPS, 10 mg/kg intraperitoneally) to rats, with a group being treated with S-diclofenac (47.2 μmol/kg, i.p.) 60 min prior to LPS injection. They found that the H_2S donor significantly reduced the rise in plasma concentrations of proinflammatory interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) while also augmenting the rise in plasma concentrations of the anti-inflammatory cytokine interleukin-10 (IL-10). Other studies have investigated the pathways that may be involved in the anti-inflammatory effects of H_2S. After I/R, early inflammatory effects are derived from leukocyte adhesion to endothelial cells [100]. Zanardo *et al* [71] examined H_2S as an endogenous regulator of leukocyte-endothelial interactions. They pretreated rats intragastrically with Na_2S (1–100 μmol/kg) or NaHS (100 μmol/kg) 30 min before administering the leukocyte adhering aspirin and Formyl-Methionyl-Leucyl-Phenylalanine (fMLP). They found that rats treated with H_2S dose-dependly decreased leukocyte adherence to endothelial cells. Additionally, Zanardo *et al* revealed that the rats pretreated with the K_{ATP} channel antagonist glibenclamide did not have a decrease in leukocyte adherence suggesting that the anti-inflammatory effects of H_2S was through K_{ATP} channel activation. Yusof *et al* [101] studied the effects of preconditioning with H_2S on leukocyte rolling (2^{nd} step in leukocyte-endothelial interactions during ischemia [102]) and adhesion induced by ischemia. They injected NaHS (14 μmol/kg, i.p.) 24 hr before ischemia, 1 hr before ischemia, and at reperfusion in mice receiving 45 min of superior mesenteric artery ischemia and 60 min reperfusion. They observed that H_2S initiated late-phase preconditioning (tolerance to ischemia 12–24 hr after preconditioning stimulus) and through Western blot analysis revealed a significant increase in phosphorylation (activation) of eNOS and p38 MAPK. These data suggests that the anti-inflammatory effects of preconditioning with H_2S are eNOS and p38 MAPK pathway dependent since the protective roles of eNOS and p38 MAPK have already been established [103,104]. More mechanistic studies of H_2S in inflammation could provide insight into preventing inflammatory tissue damage after I/R.

5. Summary and Perspective concerning H_2S in Ischemic Disorders

Whether it is endogenous or exogenous, H_2S has a wide range of protective functions after I/R injury throughout the body. Mechanisms of protection include control of intracellular $[Ca^{2+}]$ by stimulating K_{ATP} channel opening, anti-apoptotic pathway (ERK1/2/MAPK, PI-3 kinase/Akt, JAK-STAT) activation, mitochondrial protection through preservation of $\Delta\Psi_m$ and inhibition of mPTP opening, and antiinflammatory effects by activation of eNOS and p38 MAPK. Since the discovery of the cytoprotective effects of H_2S, developing methods to up-regulate endogenous H_2S production or provide efficacious treatments of H_2S donors is becoming a focus of scientists and clinicians alike. Ikaria Holdings Incorporated has two clinical trials beginning based on an H_2S drug, IK-1001 (Na_2S). The first ongoing study is the reduction of I/R mediated cardiac injury in patients undergoing coronary artery bypass graft surgery (CABG) to evaluate the capability of IK-1001 as a robust treatment for abbreviating surgical injury. Patients undergoing surgery will receive intravenous infusion for six hours while having the CABG surgery and will be evaluated six months later. The other clinical trial by Ikaria Holdings Inc. that is beginning is a pharmokinetic assessment of Na_2S in patients with impaired renal function. In this study patients with healthy, mild, and moderately impaired renal function will be injected intravenously with IK-1001 (1.5 mg/kg/hr) for 3 hours while patients with severely impaired renal function will receive IK-1001

(1.0 mg/kg/hr) for 3 hours intravenously. The patients will then be followed over a seven-day period to investigate any restoration in renal function.

Though H_2S shows therapeutic potential in I/R injury, an underlying problem with the H_2S field is that the conventional H_2S donors, NaHS and Na_2S, are short acting and do not give a continuous release of H_2S. Recent studies are suggesting that garlic derivatives could provide an answer to this problem. Garlic has been used for prevention and treatment of atherosclerosis, hyperlipidemia, thrombosis, hypertension, and diabetes [105]. Garlic contains a number of sulfur rich chemical compounds including diallyl disulfide (DADS) and diallyl trisulfide (DATS). Benavides *et al* [106] conducted a study that demonstrated that DADS and DATS induced H_2S production and are responsible for the vasoactivity of garlic. However, exhaustive studies demonstrating the protective effects of DADS and DATS in I/R injury have yet to be published.

Although the physiological and cytoprotective effects of H_2S have been examined, the mechanisms involved are not fully understood. In the past 20 years, H_2S has come from a reputation as a toxic gas to potentially a therapeutic drug for the treatment I/R injury. Recent research has revealed many beneficial roles for H_2S in ischemia for a variety of tissues. For example, H_2S has been shown to be cardioprotective through both endogenous and exogenous applications at or prior to ischemia, neuroprotective via H_2S-induced hypothermia, hepatoprotective by decreasing lipid peroxidation, increasing antioxidant and anti-apoptotic signaling, renal protective through the actions of CBS, and protective against pulmonary ischemia injury by the activity of CSE. Although much progress has been made in the H_2S field regarding its physiological roles in different organ systems, there is room still available for more advancement to be made. First, the enzymes responsible for the endogenous production of H_2S (CBS, CGL, and 3MST) need to be investigated further for other functions and regulatory mechanisms they may have inside biological systems. Knowing an enzyme's specific role and localization in each individual organ system will allow for the targeting of that enzyme to modify endogenous concentrations of H_2S suitable for the treatment of I/R injury in that tissue. Second, improvement in H_2S donors could allow for treatments to mimic the endogenous protective effects that H_2S has in the body. As a result, traditional treatments aimed at combating I/R injury could be challenged with the development of new therapeutic strategies designed capitalize on the potential protective effects of endogenous and exogenous H_2S.

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Table

Summary of H_2S in Ischemia/Reperfusion Injury

Organ	Experimental Model*	Treatment**	Effects	Ref.
Heart	Langendorff hanging heart model (30 min I/2 hr R)	NaHS (1 (μM) in perfusate 10 min prior to R	20% reduction in infarct size	46
	Perfused rat heart (30 min I/90 min R)	NaHS (1 μM) at onset of R	Significant decrease in myocardial infarct size	50
	Langendorff hanging heart model (30 min I/10 min R)	NaHS (100 μM) in perfusate prior to I	Decreased duration and severity of I/R-induced arrhythmias	37
	Isolated rat cardiac myocytes	NaHS (10–100 μM) with simulated I solution	Increased myocyte viability and shape	37
	Langendorff hanging heart model (40 min I/2 hr R)	PAG-treated (prior to I)	38% increase in myocardial infarct size	41
	in vivo (25 min I/2 hr R)	NaHS (3 mg/kg, r.j.v.) 15 min prior to I	26% reduction in myocardial infarct size	40
	in vivo (30 min I/24 hr R)	Na_2S (50 μg/kg, i.c.) at R	72% reduction in infarct size	39
	in vivo (45 min I/24 hr R)	Na_2S (100 μg/kg, i.v.) 24 hr before I	46% reduction in infarct; reduction of oxidative stress; decreased anti-apoptotic signaling	47
	in vivo (60 min I/2 hr R)	Na_2S (100 μg/kg, i.v.) 10 min before R	2.3-fold reduction in infarct size in porcine model	48
	in vivo (60 min I/2 hr R)	Na_2S (2 mg/kg per hr, i.v. infusion) at I	significantly decreased the area of necrosis; higher expression of cell survival proteins; decrease apoptosis	49
Brain	Primary cultures of cortical neurons	NaHS (100 μM) with glutamate	Protects neurons from glutamate toxicity	38
	Cultured hippocampal HT22 cells	NaHS (300 μM) with glutamate	Improved survival of HT22 cells	55
	Cultured brain endothelial cells	NaHS (0.05 and 0.1 mM) with methionine	Attenuated cell death and radical formation	56

Organ	Experimental Model*	Treatment**	Effects	Ref.
	in vivo (24 hr I of MCA)	NaHS (0.18 mmol/kg, i.p.) 10 min prior I	Increased infarct volume 150%	58
	in vivo (90 min I of MCA)	2 days of H_2S gas (80 ppm)	Reduced infarct size by 50%	42
	in utero (5 min I/24 hr R of BUA)	NaHS (O.4375 µmol/kg, i.p.)	Protects fetal brains by reinstating the GSH levels decreased by *in utero* I/R	59
	in vivo (8 min CA followed by CPR)	Na_2S (0.55 mg/kg f.v.) 1 min before CPR	Improved neurological function; decreased apoptotic proteins and activation of anti-apoptotic proteins	60
Liver	*in vivo* (60 min I/5 hr R)	Na_2S (1 mg/kg, i.v.) 5 min prior to R	Reduced AST and ALT levels, LPO levels, increase in antioxidant signaling and decrease in anti-apoptotic signaling	43
Kidney	*in vivo* (45 min I/72 hr R)	PAG (1 ml/kg, i.p.) NaHS (100 µmol/kg) topically on kidney	PAG decreased renal function; NaHS treatment increased renal function	44
	in vivo (45 min I/6 hr R)	--	Ischemia decreased CBS activity and H_2S levels	45
Lung	*in vivo* (45 min I/ 45 min R of PA)	H_2S gas (50, 100 µmol/l) 5 min prior to I	Pulmonary protecion; decreased histological injury; increased perfusion flow rate; lowered lung wet/dry ratio; improved lung compliance; lowered MDA levels	62

* I = Ischemia; R = Reperfusion; CA = Cardiac Arrest; CPR = Cardiopulmonary Resuscitation; MCA = Middle Cerebral Artery; BUA = Bilateral Uteroovarian Artery; PA = Pulmonary Artery; LV = left ventricle;

** r.j.v. = right jugular vein; i.c. = intracardiac; i.v. = intravenous; i.p. = intraperitoneal; f.v. = femoral venous