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Hydrogen sulfide-mediated myocardial pre- and post-conditioning

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Abstract

Coronary artery disease is a major cause of morbidity and mortality in the Western world. Acute myocardial infarction, resulting from coronary artery atherosclerosis, is a serious and often fatal consequence of coronary artery disease, resulting in cell death in the myocardium. Pre- and post-conditioning of the myocardium are two treatment strategies that reduce the amount of cell death significantly. Hydrogen sulfide has recently been identified as a potent cardioprotective signaling molecule, which is a highly effective pre- and post-conditioning agent. The cardioprotective signaling pathways involved in hydrogen sulfide-based pre- and post-conditioning will be explored in this article.

Keywords

acute myocardial infarction; apoptosis; ischemia/reperfusion injury; myocardial protection; oxidative stress

Acute myocardial infarction (AMI) is a serious and often fatal consequence of coronary artery disease. With AMI, the best course of action to limit myocardial infarct size is a timely restoration of blood flow to the ischemic myocardium by either thrombolysis or percutaneous angioplasty [1,2]. Unfortunately, the blockage is rarely recanalized before a considerable amount of damage is done to the myocardium, subsequently leading to persistent heart failure in a large percentage of patients [1,2].

Pre- and post-conditioning and pharmacological treatments to mimic these interventions have been shown to be effective in limiting infarct size in the setting of myocardial ischemia/reperfusion (MI/R) injury in both animals and humans [3]. When coupled with timely reperfusion, pre- and post-conditioning attenuate myocardial infarct size and postischemic left ventricular dysfunction more than reperfusion alone [3]. Both pre- and post-conditioning can be achieved in numerous ways to induce physiological changes that result in increased cytoprotection after reperfusion of the ischemic heart [3].

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This article focuses on the novel signaling molecule hydrogen sulfide (H₂S) as a pre- and post-conditioning agent to limit infarct size following AMI. H₂S exhibits many cytoprotective effects. This article will discuss the use of H₂S prior to ischemia to precondition and protect the myocardium during AMI, as well as H₂S therapy following the reperfusion of the ischemic myocardium to postcondition and protect the heart against reperfusion injury.

Preconditioning

Myocardial preconditioning is a phenomenon by which the myocardium is protected from ischemia/reperfusion damage by physiological or pharmacological means prior to an ischemic event (i.e., coronary artery occlusion). Therefore, when reperfusion is attained, the myocardium has a greater capacity to resist oxidative and myocardial cell death signals that result from the rapid reintroduction of oxygen to the ischemic tissue. The efficacy of the conferred protection is typically measured by a reduction in myocardial infarct size, as well as a minimized decline in myocardial contractile function.

Ischemic preconditioning (IPC) was first discovered over 20 years ago by Murry *et al.* [4]. In this seminal study, four rounds of 5 min of nonlethal coronary artery occlusion followed by 5 min of reperfusion was administered prior to the prolonged ischemic bout [4]. They found that these brief periods of ischemia significantly delayed myocardial cell death during a 40-min coronary artery ischemia, thereby demonstrating that they “could exploit ischemia to protect the heart from ischemia” [4]. Since the publication of this seminal work, a considerable amount of research has been published exploring the mechanisms behind IPC and the implementation of pharmacological strategies.

Ischemic preconditioning has been shown to protect the heart across all animal species investigated and is considered to be the most protective intervention against MI/R injury to date [5,6]. Some of the key signaling pathways and mediators involved in IPC are: PI3K, Akt, nitric oxide (NO), PKG, mitochondrial ATP-sensitive potassium channels (K_{ATP}), adenosine, oxygen-derived free radicals and the mitochondrial permeability transition pore (MPTP) [7]. Additionally, adenosine, bradykinin and opioids, which are all released during IPC, also serve to protect the myocardium when pharmacologically administered before an ischemic event [8–10]. Pharmacological preconditioning is distinct from IPC, as no ischemia is induced to obtain the cardioprotective benefits; rather, administration of cardioprotective agents mimic the signaling induced by IPC using exogenous pharmacological application.

Ischemic preconditioning also exhibits two phases of cardioprotection: an early phase, 1–3 h after preconditioning [4], and a late phase, 18–24 h later, which can persist for up to 72 h [11]. The major distinction between these two phases is that the early phase triggers the modification of existing proteins in the myocardium, while the late phase triggers the induction of numerous cytoprotective proteins [5]. Additionally, while early-phase IPC protects against myocardial infarction, it fails to limit myocardial contractile dysfunction or stunning, while late-phase IPC protects against myocardial infarction and preserves left ventricular function [5]. Therefore, late-phase IPC is considered to be more clinically beneficial, as it confers greater cardioprotection and has a longer duration of protective effects [3].

Postconditioning

Myocardial postconditioning is a phenomenon whereby the myocardium is protected from ischemia/reperfusion damage by physiological or pharmacological interventions following ischemia at the time of reperfusion. Postconditioning, similar to pre-conditioning, was first reported using a modulation of ischemia to protect against ischemia. In 1992, Zhao and

colleagues conducted unpublished studies on postconditioning in the Vinten-Johansen laboratory in an anesthetized rabbit model of coronary artery occlusion–reperfusion, using 5 min perfusion–ischemia intervals repeated at the onset of reperfusion [3]. These studies were halted owing to a lack of efficacy of the postconditioning treatment, which paralleled treatment performed in preconditioning [3]. Later, a report in 1996 by Na *et al.* showed that transient ischemia at the onset of arrhythmias during reperfusion suppressed the incidence of ventricular fibrillation in anesthetized cats, and used the term ‘postconditioning’ to describe this phenomenon [12]. Then, again in 2001, the Vinten-Johansen laboratory resumed postconditioning studies, using compressed 30-s cycles to account for the rapid time course of reperfusion injury [3]. Following this, in 2003, Zhao *et al.* reported that postconditioning, by cycling reperfusion for three 30-s intervals before prolonged reperfusion, significantly reduced infarct size in a dog model of coronary artery occlusion [13].

Ischemic postconditioning (mechanical manipulation of reperfusion blood flow) was initially performed by sequentially releasing and reapplying an external ligature around the coronary artery [13,14]. More recent studies have used fluoroscopically guided angioplasty balloon catheters to administer the sequential reperfusion in a closed-chest model [15], while cell culture models have been used to simulate the intervals of ischemia and reperfusion induced by mechanical manipulation of reperfusion of the myocardium by altering culture media of cultured cardiac myocytes via hypoxia/reoxygenation of the media or acidic–neutral pH perfusion [16].

Postconditioning has been shown to reduce infarct size by reducing apoptosis and necrosis, both of which presumably contribute to increased infarct size [17,18]. Postconditioning can attenuate superoxide generation in the myocardial area at risk after 3 h of reperfusion [16,17], induce antioxidant mechanisms and inhibit neutrophil adherence and accumulation in general [3]. Studies have also revealed that hypoxic postconditioning results in less intracellular and intramitochondrial Ca^{2+} accumulation, suggesting that triggers of MPTP opening are reduced. The MPTP opens during early reperfusion in response to oxidant and Ca^{2+} accumulation [19,20], initiating a cascade that results in either apoptosis or necrosis when ATP is present or depleted, respectively.

Postconditioning can also be mimicked pharmacologically by administering agents, such as inhalational anesthetics [21,22], bradykinin [23], cyclosporin A [23], erythropoietin [24], NO [25] and NO donors [26], adenosine [27], and H_2S [28], at the time of reperfusion. Most of these pharmacological agents activate the RISK pathway, ultimately inhibiting MPTP opening and increasing cell survival. MPTP opening causes mitochondrial membrane potential ($\Delta\Psi_m$) to depolarize [29], resulting in a rapid impairment of mitochondrial function that leads to apoptotic cell death. Not surprisingly, ischemic postconditioning coupled with pharmacological postconditioning serves to reduce infarct size even more than ischemic postconditioning alone. Jiang *et al.* coupled postconditioning and a protease-activated receptor-2 agonist at the onset of reperfusion, and observed that infarct size was reduced to a greater extent than with either intervention alone.

Mechanisms of H_2S cardioprotection in pre- & post-conditioning

Hydrogen sulfide is a gas signaling molecule capable of physiological actions, first described as a physiological mediator in the brain in 1996 by Abe and Kimura [30] and in the cardiovascular system the following year by Hosoki, Matsuki and Kimura [31]. H_2S is rapidly emerging as a novel, cytoprotective signaling molecule with potent antioxidant, anti-inflammatory and antiapoptotic effects (Figure 1). However, at supraphysiological concentrations, H_2S can be toxic and potentially lethal. H_2S can reversibly inhibit the electron-transport chain [32–34], open the mitochondrial permeability transition pore

[35,36], increase production of reactive oxygen species (ROS) [35–38] and outcompete oxygen for hemoglobin binding [39–41].

Hydrogen sulfide is a colorless, water-soluble gas, and has the characteristic smell of rotten eggs. In mammals, H₂S is produced enzymatically via the cysteine metabolic enzymes cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) [30,31] and the combined actions of cysteine aminotransferase (CAT) and 3-mercapto pyruvate sulfurtransferase (3-MST) [42,43], all of which are present in the heart and vasculature [43,44]. There are several sources of H₂S that are currently utilized to deliver H₂S to biological systems: there is H₂S gas, which can be bubbled directly into physiological saline, and two commonly used sources of H₂S – sodium sulfide (Na₂S) and sodium hydrosulfide (NaHS). Ikaria Holdings, Inc. (NJ, USA) has also formulated an injectable form of H₂S, (i.e., IK-1001), which is pure, pH neutral and stable at room temperature.

Several studies have initially demonstrated the cardioprotective action of H₂S in the setting of MI/R injury. Pretreatment with NaHS, a source of H₂S, reduced the number and duration of arrhythmias in isolated rat hearts subjected to global MI/R [45]. NaHS pretreatment 10 min before coronary artery occlusion also attenuated MI/R injury in isolated, perfused rat hearts subjected to 30 min of ischemia and 2 h of reperfusion [46]. An *in vivo*, murine model of MI/R showed drastic reduction (56%) in infarct size after treatment with Na₂S [47], while H₂S treatment in a rat model of MI/R injury showed a 25% reduction in infarct size in rats pretreated with NaHS [48]. A week-long treatment of rats with NaHS showed a reduction in infarct size, as well as protection of vascular smooth muscle cells against hypoxia/reoxygenation injury [49].

Subsequent studies have started to examine the signaling mechanisms responsible for the cardioprotective actions of H₂S. The presently known mechanisms of cardioprotective H₂S signaling include ion channel activation and upregulation of antioxidant, antiapoptotic and anti-inflammatory signaling pathways. Each of these signaling mechanisms will be addressed in further detail in subsequent sections.

Ion channel activation & regulation

Hydrogen sulfide activates K_{ATP} channels, and this activation is responsible for a number of its physiological actions [50–54]. K_{ATP} channels are heteromeric protein complexes consisting of four pore-forming subunits that belong to the family of inwardly rectifying potassium channels (Kir), which are inhibited by ATP [55]. Kir6.2 is the major pore-forming subunit in cardiac cells. The Kir tetramer is also associated with four sulfonylurea receptors (SURx) that belong to the ATP-binding cassette (ABC) family of proteins [56,57]. K_{ATP} can be inhibited through these SURx by sulfonylureas, and activated by diazoxide (SUR1) and pinacidil (SUR2) [58].

K_{ATP} channels in the heart have long been known to play a pivotal role in the cardioprotective mechanisms of IPC, and there has been a focus on the mitochondrial K_{ATP} as well as sarcolemmal K_{ATP} [59–65]. Sivarajah *et al.* investigated the role of H₂S in both IPC and endotoxin-mediated preconditioning using a Wistar rat model of MI/R [48]. They found a protective effect of NaHS application 15 min prior to MI/R, reducing infarct size significantly. Blockade of endogenous H₂S production by the CSE inhibitor propargylglycine (PAG) increased infarct size, as did specific inhibition of the mitochondrial K_{ATP} using 5-hydroxydecanoic acid [48], indicating that endogenous cardioprotection by H₂S may be mediated through mitochondrial K_{ATP}.

Sarcolemmal K_{ATP} channels also play a role in H₂S-mediated cardioprotection. Opening of sarcolemmal K_{ATP} leads to potassium efflux, cell membrane depolarization and shortening

of the action potential duration [66,67]. These events will reduce calcium influx, leading to a reduction of mechanical contraction and resulting in energy sparing in the myocardium during early reperfusion injury [68]. Pan *et al.* used isolated cardiac myocytes to investigate K_{ATP} activation and signaling in H_2S preconditioning [68]. They found that 30 min of NaHS preconditioning conferred protection approximately 1 h and 16–28 h after NaHS application when cardiac myocytes were exposed to hypoxia/reoxygenation injury, demonstrating that H_2S has two windows of protection, similar to IPC [68]. Bian *et al.* also demonstrated the importance of sarcolemmal K_{ATP} channels in H_2S -mediated cardioprotection in an isolated cardiac myocyte model [45]. When the sarcolemmal K_{ATP} inhibitor HMR-1098 was applied to isolated cardiac myocytes, the effects of H_2S preconditioning were lost, while blockade of mitochondrial K_{ATP} with 5-hydroxydecanoic acid had no effect on the cardioprotection on H_2S preconditioning [45].

Antioxidant signaling mechanisms

The antioxidant effects of H_2S were first demonstrated in neuronal cells. H_2S increased the transport of cysteine (substrate for glutathione [GSH] production) and cystine (dimeric cysteine), subsequently increasing the levels of GSH [69]. In HT22 neuronal cells, H_2S also acts through K_{ATP} and chloride channels, in addition to GSH and cysteine regulation, to exert its antioxidant function [70]. Furthermore, H_2S leads to the redistribution of GSH to the mitochondria, while direct production of H_2S by CAT/3-MST may directly suppress oxidative stress [71]. Finally, H_2S may also act as an antioxidant itself, as it can scavenge ROS *in vitro* [72].

Hydrogen sulfide preconditioning has been demonstrated by Calvert *et al.* to induce the translocation of the nuclear transcription factor nuclear factor E2-related factor 2 (Nrf-2) from the cytosol to the nucleus in cardiac tissue, resulting in an increase in antioxidants [73]. Nrf-2 is normally bound to Keap1 in the cytosol, but during oxidative stress, Nrf-2 is released for translocation to the nucleus. Once in the nucleus, Nrf-2 binds to the antioxidant response element (ARE), which is found in the promoter region of a number of antioxidant genes. Calvert *et al.* demonstrated the translocation of Nrf-2 through analysis of cytosolic and nuclear protein fractions in mouse hearts collected following intravenous injection of 100 $\mu\text{g/kg}$ Na_2S [73]. The authors also demonstrated upregulation of heme oxygenase-1 (HO-1) and thioredoxin (Trx)-1 and -2 at 24 h postinjection of Na_2S [73]. The activation of Nrf-2 by H_2S and downstream upregulation of antioxidant enzymes clearly contribute to the late phase of preconditioning.

Antiapoptotic signaling mechanisms & mitochondrial preservation

Apoptotic cell death during reperfusion is an important contributor to reperfusion-induced injury [74]. Activation of the pro-survival kinases of the RISK pathway, including PI3K, Akt, PKC ϵ and Erk1/2, before or at the time of reperfusion, confers cardioprotection against MI/R through an inhibition of apoptosis [75]. H_2S has recently been shown to modulate components of the RISK pathway [73]. Activation of the RISK pathway inhibits the opening of the MPTP, which plays a fundamental role in determining cellular survival during MI-R injury through various signaling mechanisms downstream of RISK pathway activation [76].

PI3K, Akt and PKC ϵ signal to activate Erk1/2. Erk1/2 can then phosphorylate and inactivate the proapoptotic factor Bad through the actions of p90RSK [77]. Bad phosphorylation leads to preservation of mitochondrial membrane potential. Erk1/2 also upregulates Bcl-2 and Bcl-xL, as well as heat-shock proteins (HSP90 and HSP70) through signal transducers and activators of transcription-3 (STAT-3) [78,79]. These also function to preserve the mitochondrial membrane potential, decreasing apoptotic cell death.

Calvert *et al.* have demonstrated that H₂S upregulates multiple components of the RISK pathway in a murine model [73]. When C57BL/6J mice were injected with Na₂S (100 µg/kg) and a time course was followed from 30 min, 2 h to 24 h, PKCε and Erk1/2 were upregulated 30 min to 2 h after H₂S treatment [73], while the downstream effectors of Erk1/2, STAT-3, HSP90, HSP70, Bcl-2, Bcl-xL and phosphorylated-Bad, were all upregulated for 24 h following H₂S treatment [73].

A study in Yorkshire pigs by Osipov *et al.* investigating bolus or infusion of H₂S at reperfusion after 1 h of ischemia demonstrated that both methods of application increased antiapoptotic signaling mechanisms, and that infusion was more effective than a bolus [80]. A bolus of H₂S upregulated Erk1/2, increased phosphorylated-Bad at Ser 136 (proapoptotic, inactivated form) and decreased cleaved caspase 3 (proapoptotic, activated form) [80]. Infusion of H₂S downregulated apoptosis-inducing factor (AIF), and both bolus and infusion increased phosphorylated GSK-3β [80]. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining for apoptotic cells showed a significant decrease in apoptotic cells in both the bolus and the infusion groups compared with control, although the infusion group showed 3.3-fold less apoptotic cells than the bolus group [80].

Both studies by Calvert *et al.* and Osipov *et al.* implicate that H₂S pre- and post-conditioning may involve activation of the RISK pathway. Owing to the short and long time courses involved in the signaling, the RISK pathway may be involved in both early and late phases of preconditioning. It also appears that bolus injection and infusion of H₂S have differential effects on conditioning, which may be the result of varying pathway activation by acute or chronic H₂S application.

Anti-inflammatory signaling mechanisms

Hydrogen sulfide reduces proinflammatory cytokines to attenuate the inflammatory response in MI/R injury, including NF-κB, IL-6, IL-8 and TNF-α [81,82]. IL-6 is released in response to MI/R injury and can depress myocardial function [83,84], while IL-8 is released after MI/R injury and increases the inflammatory response, as well as neutrophil adhesion [85]. TNF-α has multiple effects exacerbating MI/R injury, including inducing endothelial adhesion molecules, enhancing neutrophil infiltration and increasing the inflammatory response [86]. TNF-α also increases the production of ROS [87], and has myocardial-depressant and apoptotic actions [88].

Some of the anti-inflammatory actions of H₂S are mediated through K_{ATP} channels. H₂S has been shown to inhibit leukocyte-mediated inflammation by acting upon the K_{ATP} channels [89,90]. Leukocyte infiltration through the endothelial cells into the underlying tissue is a relatively early event in the inflammatory response. Leukocytes can then generate free radicals and proteases, which are damaging to the myocardium. H₂S treatment 10 min before reperfusion, and then throughout reperfusion in a swine model of MI/R, also reduced myeloperoxidase activity [81], which is a marker of leukocyte infiltration and activity. H₂S donors also reduced carrageenan-induced paw edema in a dose-dependant manner that was inhibited by glibenclamide (a nonselective K_{ATP} channel blocker) and mimicked by pinacidil (a K_{ATP} agonist), suggesting that the action was through K_{ATP} channels [89].

The anti-inflammatory actions of H₂S may be greatly beneficial in the setting of MI/R by maintaining coronary microvascular perfusion. Dysfunction in the microcirculation is thought to be, in part, a result of reduction in the bioavailability of NO, which is scavenged by free radicals released in the setting of MI/R injury [91]. By inhibiting neutrophil and leukocyte infiltration and repressing proinflammatory cytokines from generating excessive ROS, H₂S may serve as a potent anti-inflammatory agent, helping to maintain microvascular function and myocardial blood flow following MI/R injury.

Other cytoprotective H₂S signaling mechanisms

COX-2 and NO may also mediate cardioprotection induced by H₂S. Hu *et al.* exposed isolated rat cardiac myocytes to 100 μ mol/l H₂S for 30 min, and then cultured them for 20 h before insulting them with hypoxia/reoxygenation injury [92]. H₂S significantly increased cell viability, and this was ameliorated when inhibitors of COX-2 (NS-398 and Celecoxib) were administered [92]. H₂S induced COX-2 expression and prostaglandin E₂ (PGE₂) synthesis in the cardiac myocytes, and these actions were blocked by glibenclamide and chelerythrine (a PKC inhibitor), suggesting that K_{ATP} and PKC are required for the stimulation of COX-2 [92]. Furthermore, they also found that L-NG nitroarginine methyl ester (L-NAME) application inhibited COX-2 activation and PGE₂ production [92]. Therefore, NO may also be involved in the H₂S-mediated activation of COX-2 [92]. Calvert *et al.* also showed an increase in COX-2 in their *in vivo* model of H₂S preconditioning [73]. A 100- μ g/kg dose of Na₂S increased COX-2 expression after 24 h [73]. Similarly, Pan *et al.* found that H₂S may induce NO release to confer its cardio protective effects in their isolated rat cardiac myocyte model of ischemia/reperfusion injury [68]. Inhibition of NO production by L-NAME attenuated the cardioprotective effects of NaHS administration [68]. Furthermore, H₂S can activate Akt in the setting of angiogenesis [93] and pre-conditioning [94]. Akt can also phosphorylate endothelial NO synthase (eNOS) to enhance the production of NO [95].

A new method of covalent protein modification by H₂S, S-sulfhydration, has been described by Mustafa *et al.* [96]. S-sulfhydration is the process by which H₂S covalently modifies cysteine residues, creating a R-SSH group. Using a modified biotinylated switch method, Mustafa *et al.* showed a decrease in basal S-sulfhydration in CSE^{-/-}-knockout mice when compared with wild-type controls [96]. This indicates that basal H₂S production by CSE is important in maintaining S-sulfhydration levels, and that it potentially acts as a critical pro-survival signaling event [96]. This has already been linked to a regulatory role of glyceraldehyde 3-phosphate dehydrogenase, actin and tubulin, as well as the interaction of H₂S with the subunits of K_{ATP} channels [96]. Since S-sulfhydration may act on proteins already expressed in the cell to change their activity, it is likely that S-sulfhydration accounts for at least some of the signaling in H₂S-mediated pre- as well as post-conditioning signaling. Exploring this new mechanism of signaling may lead to further advances in understanding H₂S signaling in pre- and post-conditioning.

Hydrogen sulfide is also biologically stored as bound sulfane sulfur, potentially for later release and physiological action/cytoprotection [97]. Bound sulfane sulfur is comprised of two sulfur atoms covalently bonded to each other. Examples of bound sulfane sulfur are the outer sulfur atoms of the persulfides and the inner chain atoms of polysulfides. Cysteine is metabolized via the actions of CSE, CBS and 3-MST to form H₂S, which can then be incorporated into a pool of bound sulfane sulfur [98]. Exogenously applied H₂S can also be absorbed into this bound sulfane sulfur pool [99].

Bound sulfane sulfur can release H₂S under reducing conditions and is one of two known sulfur stores in the cell, the other being acid-labile sulfur [100,101]. Acid-labile sulfur comprises sulfur atoms in iron-sulfur complexes, which are responsible for a variety of redox reactions that occur in the mitochondrial respiratory complex enzymes. Acid-labile sulfur releases H₂S below a pH of 5.4 [99]. Since the mitochondrial pH is between 7 and 8, acid-labile sulfur may not release H₂S under physiologic conditions [97]. During ischemia/reperfusion injury, the myocardium will be under oxidative stress, and the required reducing conditions for the release of H₂S from bound sulfane sulfur may not be met. However, during H₂S-mediated pre- or post-conditioning, the upregulation of antioxidant enzymes and GSH synthesis by exogenous H₂S may lead to additional H₂S release from these bound

sulfane sulfur stores, perpetuating the pre- and post-conditioning effects, and potentially contributing to the late phase of protection observed during preconditioning.

Connection between H₂S & ischemic conditioning

Hydrogen sulfide-mediated pre- and post-conditioning are highly effective phenomena that share many similarities with their ischemic counterparts (i.e., IPC and ischemic postconditioning), including the time course and some cytoprotective signaling pathways. However, very little is currently known of the mechanisms by which H₂S conditions the myocardium, and it remains to be seen how far ischemic conditioning and H₂S overlap in their signaling. For example, IPC may work, in part, through H₂S signaling. The concept that H₂S works depending on the local oxygen tension has been proposed in several papers by Olson *et al.* and Whitfield *et al.* [102–105]. If this is the case, then ischemic preconditioning may induce increases in local H₂S concentrations by transiently creating a low-oxygen environment that favors the formation of H₂S. This increase in local H₂S may mimic the effect of exogenous application. So far, investigation into H₂S concentration in a non-mammalian, vertebrate heart using a polarographic, H₂S-specific electrode revealed an increase in H₂S during exposure to hypoxic conditions [105]. While this remains to be validated and expanded in mammalian models, some studies have already begun to link H₂S and IPC in mammalian systems [48,68]. Additionally, the use of DL-propargylglycine and β-cyano-L-alanine, inhibitors of H₂S synthesis, during metabolic ischemic preconditioning significantly reduced the effectiveness of the preconditioning protocol, which supports the idea that endogenous H₂S contributes to preconditioning [68].

Furthermore, there are both direct and indirect mechanisms by which H₂S can act as a cardioprotectant. While direct actions of H₂S on ROS or leukocyte–endothelial cell interactions, for example, contribute to cardioprotection, they may not necessarily be responsible for the pre- or post-conditioning induced by H₂S. Activation of the RISK pathway or Nrf-2 translocation may serve to precondition the heart, since these mechanisms require more time to become fully activated. The concentration(s) of H₂S at which these direct actions and conditioning actions occur should also be considered. While a specific study on this has not been conducted, we know there is activation of the RISK pathway and Nrf-2 translocation at doses of Na₂S 100 µg/kg [73]. However, significant cardioprotection can be produced at doses of Na₂S 50 µg/kg [44].

Endogenous H₂S & cardiovascular disease

Decreases in H₂S are associated with the pathology of a number of cardiovascular and related diseases, including hypertension and diabetes. H₂S has also been implicated in vasodilation of the corpus cavernosum [106–108] and the dysregulation of H₂S has been linked to erectile dysfunction [109].

The dysregulation of H₂S that occurs in these pathologies is predominantly a decrease in endogenous H₂S, implying that a basal level of H₂S is very important for cardiovascular homeostasis. Spontaneously hypertensive rats also have reduced H₂S levels in their blood, and *CSE*^{−/−} mice display an increase in blood pressure as they age [110,111]. In nonobese diabetic mice, endogenous H₂S levels decrease and endogenous production is impaired with an increase in severity of the disease pathology [112]. This study also suggests that NO may be involved in the enzymatic conversion of L-cysteine to H₂S in the cardiovascular system [112]. CSE contains 12 cysteine residues that may be the target of S-nitrosylation, potentially enhancing its activity [113]. If this is the case, then diseases that involve endothelial dysfunction (resulting in decreased endogenous NO production) actually involve the functional loss of two key gasotransmitters (H₂S and NO). Furthermore, endogenous H₂S also declines in obese and diabetic humans [114,115].

The dysregulation of H₂S, particularly the loss of bio available H₂S or the capacity for endogenous production, has several impacts on cardiovascular disease. The endogenous production of H₂S is likely critical in maintaining basal antioxidant defenses and mitochondrial membrane potential and is instrumental in resisting inflammation and the tonic regulation of blood vessels (Figure 2). As the endogenous level of H₂S declines, all of these functions will become compromised. This will ultimately result in an increased susceptibility to cell death following reperfusion of an ischemic heart and an increase in morbidity and mortality. This dysregulation may be exacerbated by age, as there is evidence that H₂S bioavailability, like NO, decreases with age [116].

Clinical translation of H₂S

Clinical use of IPC for the heart

Even though IPC is the most potent and reproducible phenomenon in cardioprotection, it has not been readily translated to clinical use [117]. A major issue in IPC is that the timing of the main ischemic event must be known ahead of time for IPC to be effectively applied. Unfortunately, there is no way to predict when a myocardial infarction will occur and, therefore, no way to induce IPC just before the infarction. However, in scenarios when the ischemic event is known, such as elective coronary artery balloon angioplasty, coronary bypass surgery or heart transplantation, preconditioning can be effectively employed. Here, invasive measures can be taken to induce IPC via balloon inflation or cross clamping. Yellon *et al.* were the first to demonstrate the feasibility of IPC in the human heart during cardiac surgery [118]. In their study, to achieve IPC, the heart was subjected to two periods of crossclamping, and preserved myocardial ATP levels were used as an index of myocardial protection [118]. The two periods of crossclamping significantly preserved the myocardial ATP levels following a prolonged period of ischemia, suggesting that the hearts would exhibit a greater tolerance to MI/R injury [118].

A noninvasive form of IPC has been developed as remote ischemic preconditioning (RIP). RIP is based on the idea that one vascular bed may precondition another vascular bed. The initial study into RIP was carried out by Przyklenk *et al.* in 1993 [119]. Przyklenk *et al.* demonstrated in a canine model that preconditioning the opposite coronary bed reduced myocardial infarct size to the same extent as preconditioning in the same coronary bed [119]. This concept was followed by additional studies that suggested remote organ ischemia, such as the kidney or mesentery, could also confer protection to the myocardium [117]. Remote limb ischemia also effectively preconditions the myocardium. Birnbaum *et al.* published the first study in a rabbit model that suggested that transient limb ischemia could be used in RIP [120]. Subsequent studies in humans showed that transient limb ischemia protects against endothelial ischemic reperfusion injury of the opposite limb [121], and reduces myocardial ischemic damage during coronary artery bypass surgery [122] and during pediatric cardiac surgery [123].

Preconditioning mimetics can also be used to mimic IPC. These can be proactively applied in patients at risk for cardiovascular disease or in patients that will undergo elective surgical procedures, such as organ transplantation or cardiac bypass surgery, that involve a defined ischemia/reperfusion injury. H₂S may potentially fulfil this role as well, as there is accumulating evidence in animal models that it can be used as a preconditioning agent. However, this has yet to be demonstrated in a randomized clinical trial (see below).

Clinical use of postconditioning for the heart

Postconditioning is easier to apply clinically, as the time of ischemia does not have to be known ahead of time since the conditioning begins after reperfusion. Clinical use of postconditioning thus far has largely been done in several smaller proof-of-concept trials.

However, the results are very encouraging [3]. Seven prospective randomized trials have investigated postconditioning in percutaneous coronary intervention, totaling 353 patients [124–130].

These clinical studies have used varying intervals of postconditioning, ranging from two to four cycles of 30 to 90 s per cycle [124–130]. All of the studies consistently showed a protective effect of postconditioning, even though their conditioning protocols varied [124–130]. Various markers of improvement were measured and showed protection: infarct size was decreased [127], ejection fraction increased by postconditioning [127,128], ST-segment resolution was increased in those who underwent postconditioning [124,129] and serum markers of cardiac damage, such as creatine kinase and troponin-I, were also decreased in postconditioning groups [125–128] in addition to the proapoptotic serum marker sFasL [130].

Translation of H₂S to the clinic

There are three clinical trials currently ongoing that involve H₂S and H₂S therapy. An observational study at University Medical Center in Ljubljana, Slovenia, is investigating H₂S as a prognostic factor in shock by measuring serum levels of H₂S upon admission in the intensive care unit. The aim is to link the serum levels of H₂S with a higher mortality in patients with shock. Ikaria Holdings, Inc. has two ongoing clinical trials. Both of these trials will use IK-1001 as a source of injectable H₂S. The first study, taking place in the USA, is a Phase I study to assess the pharmacokinetics of IK-1001 in healthy subjects and in those with renal impairment of various degrees (mild–severe). All subjects will receive the drug as a single intravenous infusion. The second study, taking place at several locations in Australia and Canada, is a Phase II clinical trial that will investigate the safety and effectiveness of IK-1001 when used in coronary artery bypass graft (CABG) patients to reduce the damage done to the heart during surgery. The randomly assigned subjects will also receive IK-1001 as an intravenous infusion while they are undergoing CABG surgery.

Use of H₂S therapy in cardiac surgery

Hydrogen sulfide is beginning to be translated to a surgical setting. However, the chemical properties of H₂S, namely high reactivity and potential toxicity, pose a challenge when administering H₂S *in vitro* or *in vivo*. IK-1001 has shown promising effects in preconditioning and in reducing infarct size after MI/R in murine models [44,73]. However, H₂S generated from solutions of Na₂S can be rapidly oxidized once liberated in the presence of molecular oxygen and trace metals [38], conditions that are ubiquitous in most experimental models and in the human body. Therefore, a stable source of H₂S that can be released at the intended site of action would be desired. A H₂S-releasing derivative of diclofenac (*S*-diclofenac) has been shown recently to have cardioprotective actions in an isolated rabbit heart model [131]. Rossoni *et al.* showed that *S*-diclofenac achieved a dose-dependant normalization of coronary perfusion pressure, as well as reduced levels of creatine kinase and lactate dehydrogenase, and increased levels of GSH in the perfusate [131]. Additionally, there are several currently available options to fill this role, such as organic polysulfides, molecules with chains of sulfur atoms between two functional groups. Diallyl disulfide and diallyl trisulfide are garlic-derived organic polysulfides that act as stable H₂S donors when they react with biological thiols, such as GSH [132,133].

Hydrogen sulfide donors may play an important role in cardiac surgery, where damage to the heart and ischemia happen at a largely predictable time. H₂S donors could be administered 24 h preoperatively to invoke delayed or late preconditioning cytoprotective signaling cascades if the surgery was not time critical, or right before or during ischemia to invoke the early pre- and post-conditioning signaling cascades. It will be exciting to see the

results of the Ikaria trial in CABG patients, which may pave the way for future clinical trials using H₂S as a pre- and postconditioning agent during other procedures, such as balloon angioplasty, stenting or transplantation.

Summary

It is clear that H₂S can be used as an effective preconditioning and postconditioning agent. However, it is currently difficult to define the precise mechanisms for this protection. A summary of the known mechanisms by which H₂S-induced pre- and post-conditioning may work is shown in Figure 3.

In the early phase of preconditioning, H₂S can activate Nrf-2, and subsequently induce antioxidant defenses in the late phase. H₂S also activates the RISK pathway, leading to antiapoptogen and antioxidant increases in the late phase. H₂S may also act through S-sulfhydration, activating several molecular targets already present in the myocardium, which may lead to early-phase protection. H₂S leads to increased eNOS activity and NO production, as well as increased COX and PGE₂ production, which may contribute to the late phase. In postconditioning, H₂S can readily scavenge ROS produced during reperfusion and reduce neutrophil–endothelial cell interactions. H₂S will also activate Nrf-2 translocation and the RISK pathway, and may act through S-sulfhydration of proteins. During later reperfusion, H₂S preconditioning will result in increased NO bioavailability and antioxidant defenses, such as GSH, Trx-1 and -2 and HO-1. There is a resulting reduction in oxidative stress, as well as in myocardial necrosis and apoptosis, which ultimately results in improved left ventricular function.

Expert commentary

Hydrogen sulfide has great potential as a pre- and post-conditioning agent. While the exact mechanisms behind its cardioprotective effects are not entirely known, thus far, it has been efficacious in small-animal research and some pioneering large-animal work. Current clinical trials and future translational research will shed light on the efficacy of H₂S as a cytoprotective agent in humans during ischemic injury, while more basic science research will be needed to uncover the H₂S signaling pathways that confer cytoprotection in the ischemic myocardium in order to fully realize its therapeutic potential. Additionally, to aid in clinical translation and address human complexity, research needs to include models of comorbid conditions that may modify the cardioprotective signaling, such as dyslipidemia, atherosclerosis, diabetes and age.

Five-year view

The future looks bright for H₂S as a pre- and post-conditioning agent. There are several drugs and donor agents that are available for research purposes, and some are even undergoing clinical trials. Ideally, several stable donors of H₂S would be available in the next 5 years for use in clinical research trials, particularly in cardiac surgery and other surgical applications where ischemia/reperfusion injury is prevalent, such as organ transplantation. There have already been multiple patents filed on potential H₂S-releasing drugs [134,135]. New, stable H₂S donors will also allow further elucidation of the many signaling mechanisms involved in the cardioprotective actions of H₂S in experimental models, which can then be translated to the clinic.

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Figure 1. Major known mechanisms of hydrogen sulfide-mediated cardioprotection

H₂S acts on sarcolemmal and mitochondrial ATP-sensitive potassium channels (K_{ATP}), reducing calcium influx into the myocardium and maintaining the mitochondrial permeability transition pore, preventing it from opening and preserving mitochondrial membrane potential, respectively. H₂S has antiapoptotic effects by activating the reperfusion injury salvage kinase pathway. H₂S has anti-inflammatory effects by reducing cytokine levels and preventing neutrophil infiltration. H₂S also has antioxidant effects, upregulating antioxidant production through Nrf-2. These combined actions of H₂S serve to make it a very cardioprotective molecule.

H₂S: Hydrogen sulfide; Nrf-2: Nuclear factor E2-related factor 2.

Figure 2. Endogenous H₂S signaling contributes to cardiovascular homeostasis

Endogenous H₂S is produced from CSE, CBS or 3-MST. Excess H₂S produced is stored in the cell as bound sulfane sulfur. H₂S may act through Akt to phosphorylate eNOS, in turn upregulating production of the cardioprotective gas, NO, from Arg. H₂S increases glutathione production, and can act on vascular smooth muscle K_{ATP} to cause SMC relaxation and a decrease in BP. H₂S also acts on the K_{ATP} channels of leukocytes (i.e., polymorphonuclear leukocytes and monocytes) to prevent rolling, adhesion and transmigration through the endothelial cell layer. H₂S can scavenge free radicals that are produced from the mitochondria, and activate mitochondrial K_{ATP} channels, which may contribute to maintenance of the mitochondrial membrane potential ($\Delta\Psi_m$) and cardioprotection. H₂S also decreases respiration by inhibiting the electron transport chain, which also leads to cardioprotection.

3-MST: 3-mercaptopyruvate sulfurtransferase; BP: Blood pressure; CBS: Cystathionine β -synthase; CSE: Cystathionine γ -lyase; eNOS: Endothelial nitric oxide synthase; ETC: Electron transport chain; GSH: Glutathione; H₂S: Hydrogen sulfide; K_{ATP}: ATP-sensitive potassium channels; L-Arg: L-arginine; NO: Nitric oxide; PMN: Polymorphonuclear leukocyte; RBC: Red blood cell; ROS: Reactive oxygen species; SMC: Smooth muscle cell.

Figure 3. Hydrogen sulfide as a pre- and post-conditioning agent

In the early phase of preconditioning, H₂S can activate the Nrf-2, and subsequently induce antioxidant defenses in the late phase. H₂S also activates the RISK pathway leading to antiapoptogen and antioxidant increases in the late phase. H₂S may also act through S-sulfhydration, activating several molecular targets already present in the myocardium, which may lead to early-phase protection. H₂S also leads to increased eNOS activity and NO production, as well as increased COX and PGE₂ production, which may contribute to the late phase. In postconditioning, H₂S can readily scavenge ROS produced during reperfusion and reduce neutrophil–endothelial cell interactions. H₂S will also activate Nrf-2 translocation and the RISK pathway, and may act through S-sulfhydration of proteins. During later reperfusion, H₂S preconditioning will result in increased NO bioavailability and antioxidant defenses, such as GSH, Trx-1 and -2 and HO-1. There is a resulting reduction in oxidative stress, as well as in myocardial necrosis and apoptosis, which, ultimately, results in improved left ventricular function.

eNOS: Endothelial nitric oxide synthase; GSH: Glutathione; H₂S: Hydrogen sulfide; HO-1: Heme oxygenase-1; NO: Nitric oxide; Nrf-2: Nuclear factor E2-related factor 2; PGE₂: Prostaglandin E₂; RISK: Reperfusion injury salvage kinase; ROS: Reactive oxygen species; Trx: Thioredoxin.