Hydrogen Sulfide Mediates Cardioprotection Through Nrf2 Signaling

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Rationale: The recent emergence of hydrogen sulfide (H\textsubscript{2}S) as a potent cardioprotective signaling molecule necessitates the elucidation of its cytoprotective mechanisms.

Objective: The present study evaluated potential mechanisms of H\textsubscript{2}S-mediated cardioprotection using an in vivo model of pharmacological preconditioning.

Methods and Results: H\textsubscript{2}S (100 \mu g/kg) or vehicle was administered to mice via an intravenous injection 24 hours before myocardial ischemia. Treated and untreated mice were then subjected to 45 minutes of myocardial ischemia followed by reperfusion for up to 24 hours, during which time the extent of myocardial infarction was evaluated, circulating troponin I levels were measured, and the degree of oxidative stress was evaluated. In separate studies, myocardial tissue was collected from treated and untreated mice during the early (30 minutes and 2 hours) and late (24 hours) preconditioning periods to evaluate potential cellular targets of H\textsubscript{2}S. Initial studies revealed that H\textsubscript{2}S provided profound protection against ischemic injury as evidenced by significant decreases in infarct size, circulating troponin I levels, and oxidative stress. During the early preconditioning period, H\textsubscript{2}S increased the nuclear localization of Nrf2, a transcription factor that regulates the gene expression of a number of antioxidants and increased the phosphorylation of protein kinase C\textalpha and STAT-3. During the late preconditioning period, H\textsubscript{2}S increased the expression of antioxidants (heme oxygenase-1 and thioredoxin 1), increased the expression of heat shock protein 90, heat shock protein 70, Bcl-2, Bcl-xL, and cycloxygenase-2 and also inactivated the proapoptotic Bad.

Conclusions: These results reveal that the cardioprotective effects of H\textsubscript{2}S are mediated in large part by a combination of antioxidant and antiapoptotic signaling. (Circ Res. 2009;105:365-374.)

Key Words: hydrogen sulfide ▪ cardioprotection ▪ antioxidant signaling ▪ myocardial infarction ▪ Nrf2

Hydrogen sulfide (H\textsubscript{2}S) is an endogenously produced gaseous signaling molecule with a diverse physiological profile. Its production in mammalian systems has been attributed to 2 key enzymes in the cysteine biosynthesis pathway, cystathionine \beta-synthase (CBS) and cystathionine \gamma-lyase (CGL). The rate of H\textsubscript{2}S production in tissue homogenates is in the range of 1 to 10 pmol per second per milligram of protein, resulting in low micromolar extracellular concentrations.\textsuperscript{1,2} It is at these physiological concentrations that H\textsubscript{2}S is cytoprotective in various models of cellular injury.\textsuperscript{3,4} The reported cardioprotective effects of H\textsubscript{2}S are partially related to its ability to neutralize reactive oxygen species (ROS), to inhibit leukocyte-endothelial cell interactions, to promote vascular smooth muscle relaxation, to reduce apoptotic signaling, and to reversibly modulate mitochondrial respiration.\textsuperscript{5} Pretreatment with NaHS has been reported to reduce the number and duration of arrhythmias in isolated hearts subjected to global ischemia/reperfusion (I/R)\textsuperscript{6} and to enhance the viability of isolated rat ventricular myocytes exposed to glucose deprivation and 2-deoxy-glucose.\textsuperscript{4} Recently, Elrod et al\textsuperscript{7} reported that the administration of H\textsubscript{2}S at the time of reperfusion decreased infarct (INF) size and preserved left ventricular (LV) function in an in vivo model of myocardial I/R. Additional findings from this study also demonstrated that cardiac-specific overexpression of CGL likewise limited the extent of myocardial I/R injury. The findings of these studies and others suggest that H\textsubscript{2}S is cytoprotective during myocardial I/R injury and that either direct H\textsubscript{2}S administration or the modulation of endogenous H\textsubscript{2}S production may be of clinical importance.

Although the physiological and cardioprotective effects of H\textsubscript{2}S have previously been documented, the signaling mech-
anisms that mediate these effects have not been fully evaluated. Moreover, the signaling mechanisms that have been attributed to the cardioprotective effects of H₂S have predominantly been studied in in vitro model systems, with very few studies actually exploring the protective effects in in vivo systems. Therefore, the purpose of the present study was to evaluate signaling mechanisms triggered by H₂S treatment using an in vivo model of pharmacological preconditioning (PC). This model was chosen as a model for which early and late phase protection could be identified. Additionally, a cardiac-specific transgenic mouse that overexpresses CGL was used to evaluate signaling mechanisms mediated by endogenous H₂S.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Animals**

Male C57BL/6J mice, 8 to 10 weeks of age, were used (The Jackson Laboratory, Bar Harbor, Me). The generation of cardiac-specific transgenic mice overexpressing CGL (αMHC-CGL-Tg; FVB background),7 as well as the generation of Nrf2-deficient mice (Nrf2 knockout [KO]; ICR background),8 has been described previously. All experimental mouse procedures were approved by the Institute for Animal Care and Use Committee at Albert Einstein College of Medicine and Emory University and conformed to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 86-23, Revised 1996) and with federal and state regulations.

**Non-standard Abbreviations and Acronyms**

<table>
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<tr>
<th>Acronym</th>
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<tr>
<td>AAR</td>
<td>area at risk</td>
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<td>CBS</td>
<td>cystathionine β-synthase</td>
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<td>CGL</td>
<td>cystathionine γ-lyase</td>
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<td>E₀</td>
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<td>GSH</td>
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<td>H₂S</td>
<td>hydrogen sulfide</td>
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<td>HO</td>
<td>heme oxygenase</td>
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<td>INF</td>
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<tr>
<td>I/R</td>
<td>ischemia/reperfusion</td>
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<td>Keap1</td>
<td>Kelch ECH associating protein 1</td>
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<td>KO</td>
<td>knockout</td>
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<tr>
<td>LCA</td>
<td>left coronary artery</td>
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<td>LV</td>
<td>left ventricular</td>
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<td>Na₂S</td>
<td>sodium sulfide</td>
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<td>N-Tg</td>
<td>nontransgenic</td>
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<td>Nrf2</td>
<td>nuclear factor-E2–related factor</td>
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<td>PC</td>
<td>preconditioning</td>
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<td>PKC</td>
<td>protein kinase C</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>STAT-3</td>
<td>signal transducers and activators of transcription 3</td>
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<td>Tg</td>
<td>transgenic</td>
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**Materials**

H₂S was administered in the form of sodium sulfide (Na₂S). Na₂S was produced by Ikaria Holdings Inc (Seattle, Wash) using H₂S gas (Matheson, Newark, Calif) as a starting material and was formulated to pH neutrality, and isoosmolality. Na₂S (stock solution at 0.55 mg/mL and 7.1 mmol/L) was diluted in normal (0.9%) saline to the desired concentration in a rapid fashion, immediately before administration. For all experiments, normal saline (100 μL) or Na₂S (100 μg/kg) in a final volume of 100 μL was injected intravenously into the femoral vein using a 32-gauge needle. For this study, H₂S denotes Na₂S.

**Myocardial I/R Protocol and Myocardial Injury Assessment**

Surgical ligation of the left coronary artery (LCA), myocardial INF size determination, troponin I measurements, and LV echocardiography were performed similarly to methods described previously.9

**Glutathione and Lipid Hydroperoxide Assays**

Cardiac glutathione and lipid hydroperoxides were measured in heart tissue collected from mice subjected to 45 minutes of myocardial ischemia and 1, 4, or 24 hours of reperfusion using commercially available kits (Cayman Chemicals) as previously described.8 Glutathione (GSH) and GSH disulfide (GSSG) values were used to calculate the steady-state redox potential using the Nernst equation, as described previously.10

**Tissue Collection for Western Blot Analysis**

For the evaluation of cellular targets during early and late phase preconditioning, mice were administered H₂S as described above and then euthanized 30 minutes, 2 hours, and 24 hours after the injection. The hearts were rapidly excised and the LV was isolated and snap frozen in liquid nitrogen. The samples were then stored at −80°C. In a separate group of mice, heart samples were obtained following 45 minutes of myocardial ischemia and 4 hours of reperfusion.

**Western Blot Analysis**

Whole cell, cytosolic, membranous, nuclear, and mitochondrial fractions were prepared as described previously.11 Equal amounts of protein were loaded into lanes of polyacrylamide–sodium dodecyl sulfate gels and Western blot analysis was performed as previously described.9

**TUNEL Staining**

Following 45 minutes of ischemia and 4 hours of reperfusion hearts from sham-, vehicle (Veh)-, and H₂S PC–treated mice were rapidly excised, cross-sectioned into 3 sections, and fixed in 10% buffered formalin. Fixed tissue was then paraffin-embedded and sectioned in a standard fashion. TUNEL staining was conducted using a kit according to the instructions of the manufacturer (ApopTag HRP kit, DBA). The number of TUNEL-positive nuclei and the total number of nuclei per high-powered field were counted in a minimum of 10 fields from the area at risk (AAR) portion of the myocardium for each section (a total of 30 fields per heart).

**Statistical Analysis**

All data in this study are expressed as means±SEM. Differences in data between the groups were compared using Prism 4 (GraphPad Software Inc), with Student’s paired 2-tailed t test, 1-way ANOVA with post hoc Tukey test, or 2-way ANOVA with post hoc Bonferroni analysis where appropriate. A probability value of <0.05 was considered significant.

**Results**

H₂S PC Limited the Extent of Myocardial Injury Following I/R

Initial experiments were conducted to investigate if H₂S PC could limit myocardial I/R injury. For these experiments,
mice were subjected to 45 minutes of LCA ischemia followed by 24 hours of reperfusion. H2S (Na2S; 100 μg/kg) or Veh was administered 24 hours before ischemia via an intravenous injection. The extent of myocardial infarction was then evaluated at 24 hours of reperfusion. Representative photomicrographs of midventricular cross sections of 2,3,5-triphenyl-tetrazolium chloride–stained hearts taken from Veh- and H2S–treated mice are shown in Figure 1A. The AAR per LV was similar (P=NS) in all of the groups (Figure 1B). H2S PC decreased the INF relative to the AAR (INF/AAR) by 46% (48 ± 3 versus 26 ± 3%, P<0.001) and the INF relative to the entire LV (INF/LV) by 48% (29 ± 2.5 versus 15 ± 2%, P<0.001) when compared to Veh-treated mice. Circulating levels of troponin I were evaluated as an additional marker of myocardial injury at 24 hours of reperfusion (Figure 1C). Following myocardial I/R, circulating levels of troponin I rose from undetectable levels in sham-operated mice to 75 ± 9 ng/mL in the Veh-treated group (P<0.001 versus sham). H2S PC significantly (P=0.026) attenuated the rise in circulating troponin I by 69% (75 ± 9 versus 23 ± 7 ng/mL), thus confirming the cardioprotective effects of H2S PC.

The effects of H2S PC on LV structure and function following myocardial I/R were evaluated in separate groups of mice using in vivo transthoracic echocardiography. For these experiments, mice were subjected to 45 minutes of myocardial ischemia and 7 days of reperfusion. Myocardial I/R increased LV end-diastolic diameter (P=NS) and LV end-systolic diameter (LVESD, P<0.05) in both groups compared to their respective baseline readings (Online Figure I). However, the increase in LV end-diastolic diameter (P=0.12 versus I/R+Veh) and LVESD (P=0.03 versus I/R+Veh) was attenuated in mice pretreated with H2S. Following myocardial I/R, LV ejection fraction (Figure 1D) decreased in both groups (P<0.001 versus baseline). H2S PC did, however, significantly improve ejection fraction by 87% (P=0.006 versus I/R+Veh). Together, these results suggest that H2S PC limits the extent of damage to the myocardium following I/R injury.

H2S PC Reduced Oxidative Stress and Apoptosis Following I/R

There is considerable evidence that implicates the production of ROS as an initial cause of injury to the myocardium following I/R.12 To evaluate the effects of H2S PC on cellular oxidative stress, lipid hydroperoxide levels were measured in tissue isolated from the hearts of Veh and H2S PC treated mice. In response to myocardial I/R injury, the redox potential (Em) (Figure 2A) significantly increased in the hearts of Veh-treated mice. This change in redox potential was accom-
panied by a significant rise in lipid hydroperoxide levels (Figure 2B, \( P \leq 0.001 \) versus sham). In contrast, the redox potential was preserved in the H2S PC treated hearts (\( P \leq 0.05 \) versus I/R + Veh) and a significant less rise in lipid hydroperoxide levels was also evident (\( P \leq 0.05 \) versus I/R + Veh). To investigate the effects of H2S PC on apoptosis, the expression of uncleaved caspase-3, cleaved caspase-3, cytosolic cytochrome c, and mitochondrial cytochrome c from the hearts of sham controls and Veh- and H2S PC–treated mice at 4 hours of reperfusion following myocardial ischemia. COX indicates cytochrome c oxidase. D, Ratio of cleaved caspase-3 to uncleaved caspase-3. E, Ratio of cytosolic cytochrome c to mitochondrial cytochrome c. F, Number of TUNEL-positive cells (percentage of total nuclei) from the hearts of sham controls and Veh- and H2S PC–treated mice at 4 hours of reperfusion following myocardial ischemia. Values are means ± SEM. Numbers inside bars indicate the number of animals that were investigated in each group. *\( P \leq 0.05 \), **\( P \leq 0.01 \), ***\( P \leq 0.001 \) vs sham.

Figure 2. H2S PC reduced oxidative stress and apoptotic cell death following myocardial ischemia and reperfusion. Cardiac redox state (\( E_h \)) for GSH and GSSG (A) and lipid hydroperoxide levels (\( \mu \text{mol/L} \)) (B) from sham controls and Veh- and H2S PC–treated mice at 1 to 24 hours of reperfusion following myocardial ischemia. C, Representative immunoblots of uncleaved caspase-3, cleaved caspase-3, cytosolic cytochrome c, and mitochondrial cytochrome c from the hearts of sham controls and Veh- and H2S PC–treated mice at 4 hours of reperfusion following myocardial ischemia. COX indicates cytochrome c oxidase. D, Ratio of cleaved caspase-3 to uncleaved caspase-3. E, Ratio of cytosolic cytochrome c to mitochondrial cytochrome c. F, Number of TUNEL-positive cells (percentage of total nuclei) from the hearts of sham controls and Veh- and H2S PC–treated mice at 4 hours of reperfusion following myocardial ischemia.
H$_2$S Increased the Nuclear Accumulation of Nrf2 and Increased the Protein Expression of Thioredoxin and Heme Oxygenase-1

Nuclear factor E2–related factor (Nrf2) is a key transcription factor that regulates antioxidant genes as an adaptive response to oxidative stress or pharmacological stimuli. To investigate whether H$_2$S induced Nrf2 signaling, H$_2$S was administered to mice via an intravenous injection and heart tissue was excised at different times following this administration. As early as 30 minutes following the administration of H$_2$S, Nrf2 accumulated in the nucleus of cardiac tissue and remained at an elevated level for at least 2 hours (Figure 3A and 3B). Subsequently, the protein expression (Figure 3C and 3D) of 2 downstream targets of Nrf2, thioredoxin (Trx)1 and heme oxygenase (HO)-1, were elevated 24 hours following the administration of H$_2$S. H$_2$S did not increase the protein expression of either copper zinc superoxide dismutase or manganese superoxide dismutase (CuZnSOD) and manganese superoxide dismutase (MnSOD) at this latter time point.

To determine whether H$_2$S upregulated HO-1 and Trx1 in an Nrf2-dependent manner, subsequent experiments were performed with mice deficient in Nrf2 (Nrf2 KO). For these experiments, H$_2$S was administered as noted above and heart tissue was excised 24 hours following the administration of H$_2$S. H$_2$S did not increase the protein expression of either copper zinc superoxide dismutase or manganese superoxide dismutase (Figure 3C and 3D) at this latter time point.

To determine whether H$_2$S upregulated HO-1 and Trx1 in an Nrf2-dependent manner, subsequent experiments were performed with mice deficient in Nrf2 (Nrf2 KO). For these experiments, H$_2$S was administered as noted above and heart tissue was excised 24 hours following the administration of H$_2$S. H$_2$S slightly increased the expression of Trx1 ($P<0.05$ versus sham) but failed to increase the expression of HO-1 in the hearts of Nrf2 KO mice. Subsequent experiments were then conducted to determine whether HO-1 was critical for the cardioprotective actions of H$_2$S PC. For these experiments, H$_2$S (Na$_2$S; 100 µg/kg) or Veh was administered 24 hours before ischemia via an intravenous injection to Nrf2 KO mice and wild-type littermates. H$_2$S PC reduced INF/AAR and reduced circulating troponin I levels when it was administered to wild-type mice. Myocardial injury following I/R was found to be exacerbated in Nrf2 KO mice compared with wild-type mice (Figure 4C and 4D), as evidenced by an increase in INF/AAR (44±5 versus 25±2, $P<0.01$) and circulating troponin I levels (52.4±9.0 versus 26±5.2, $P<0.01$). H$_2$S failed to provide protection in the Nrf2 KO mice, suggesting that Nrf2 plays a role in the cardioprotection actions mediated by H$_2$S. The smaller INF size reported here for the wild-type mice in these experiments as compared to the size reported in Figure 1 is indicative of the background strain of these mice (ICR), because past studies have reported the different susceptibility of various strains of mice to myocardial injury.

H$_2$S Activated a Protein Kinase C$_{p44/42}$-p44/42-STAT-3 Prosurvival Signaling Pathway

H$_2$S has been reported to induce protein kinase (PK)C activation in isolated rat cardiomyocytes. To investigate whether H$_2$S induced PKC$e$ activation in vivo, the translocation of PKC$e$ from the cytosolic fraction to the membranous fraction was evaluated after a single administration of H$_2$S. A marked increase in the phosphorylated form of PKC$e$ at serine residue 729 (PKC$e$-P$^{729}$) was evident in the membranous fraction ($P<0.05$ versus sham, Figure 5A) 30 minutes and 2 hours following the administration of H$_2$S. The total membrane levels of PKC$e$ were also increased during this time period ($P<0.05$ versus sham.), indicating translocation of PKC$e$ to the membrane. In addition, H$_2$S increased the phosphorylation of p44/42, a downstream effector of PKC$e$ ($P<0.05$ versus sham, Figure 5B). H$_2$S did not change the total levels of PKC$e$ or alter the phosphorylation state of PKC$e$ in the cytosolic fraction and did not change the total levels of p44/42.

The activation of prosurvival kinases, such as PKC$e$ and p44/42, has been demonstrated to confer cardioprotection...
through an upregulation of antiapoptotic signaling mediated in part by signal transducers and activators of transcription-3 (STAT-3). In the present study, H₂S increased the translocation of STAT-3 to the nucleus (Figure 5C), as evidenced by a decrease in the total cytosolic levels of STAT-3 (P < 0.01 versus sham) and an increase in the total nuclear levels of STAT-3 (P < 0.05 versus sham) from 30 minutes to 2 hours after its administration. A marked increase in the phosphorylated form of STAT-3 (P < 0.01 and P < 0.05 versus sham) at serine residue 727 (STAT-3Pser727) was also evident in both

Figure 4. Nrf2 mediates the cardioprotective effects of H₂S. A and B, Representative immunoblots (A) and densitometric analysis (B) of cardiac Trx1 and HO-1 from the hearts of Nrf2 deficient (Nrf2 KO) mice with or without H₂S. C and D, Myocardial INF size (C) and circulating levels of troponin I (D) were measured 24 hours after LCA ischemia in wild-type (WT) and Nrf2 KO mice receiving either Veh or H₂S PC (100 μg/kg) treatment. Nrf2 KO mice experienced exacerbated myocardial injury when compared to wild-type mice. However, no differences in myocardial INF size or circulating troponin I levels were observed in Nrf2 KO following H₂S PC treatment. Numbers inside bars indicate the number of animals that were investigated in each group. *P < 0.05, **P < 0.01 vs sham or WT.

Figure 5. H₂S activated PKCε-p44/42-STAT-3 signaling. Representative immunoblots and densitometric analysis of phosphorylated PKCε at serine residue 729 (PKCεPser729) and total PKCε (cytosolic and membranous fractions) (A), phosphorylated p44/42 and total p44/42 (cytosolic fraction) (B), and phosphorylated STAT-3 at serine residue 727 (STAT-3Pser727) and total STAT-3 (cytosolic and nuclear fractions) (C) 30 minutes and 2 hours following the administration of H₂S. Values are means ± SEM for n = 4 to 5 animals for each group. *P < 0.05, **P < 0.01 vs sham.
the cytosolic fraction and nuclear fraction of H2S-treated hearts during this time period. H2S also increased the phosphorylation of Bad at serine residue 112 (Bad-PSer112) in both the mitochondrial (P<0.05 versus sham) and the cytosolic fraction (P<0.05 versus sham) 24 hours after its administration. As a result of this phosphorylation, H2S induced the translocation of Bad from the mitochondria to the cytosol, as evidenced by a significant increase in the total cytosolic levels of Bad (P<0.05 versus sham). Further analysis revealed that H2S increased the protein expression of heat shock protein (HSP)90, HSP70, Bcl-2, Bcl-xL, and cyclooxygenase-2 (COX-2) 24 hours following the administration of H2S. Values are means±SEM for n=4 to 5 animals for each group. *P<0.05, **P<0.01 vs sham.

The Overexpression of CGL Increased Nrf2 Nuclear Accumulation and Activated PKCe, p44/42, and STAT-3

Previously, the cardiac-specific overexpression of CGL was shown to increase H2S production in the heart and reduce the degree of injury following myocardial I/R.7 Therefore, the next series of experiments were conducted to evaluate if endogenous H2S activated the same cardioprotective signaling pathways as exogenous H2S. The overexpression of CGL resulted in the accumulation of Nrf2 in the nucleus of hearts isolated from transgenic mice (P<0.01 versus nontransgenic [Non-Tg], Figure 7A). Although there was a trend for a decline, cytosolic Nrf2 levels were not significantly different between the groups of mice. A marked increase in PKCe-PSer729 was evident in both the cytosolic (P<0.05 versus Non-Tg, Figure 7B) and membranous fractions (P<0.01 versus Non-Tg) of hearts isolated from CGL-Tg+ mice. The total membrane levels of PKCe were also increased in the hearts of CGL-Tg+ mice (P<0.05 versus Non-Tg), indicating translocation of PKCe to the membrane. CGL overexpression also induced the translocation of STAT-3 to the nucleus, as evidenced by an increase in the total nuclear levels of STAT-3 (P<0.01 versus Non-Tg, Figure 7C). A marked increase in STAT-3PSer727 was also evident in the cytosolic (P<0.01 versus Non-Tg) and nuclear fractions (P<0.05 versus Non-Tg) of CGL-Tg+ hearts. Further analysis revealed that hearts from CGL-Tg+ mice have increased protein expressions of HSP90, HO-1, Trx-1, and Bcl-2 (P<0.05 and P<0.01 versus Non-Tg, Figure 7D).

Discussion

As a gaseous signaling molecule, H2S is able to freely diffuse across cell membranes in a receptor-independent manner and activate various cellular targets. This distinct ability makes H2S an attractive pharmacological agent for the treatment of cardiovascular disease. The present study highlights 2 signaling cascades which lead to the upregulation of antioxidants and antiapoptogens and provides evidence that pharmacological preconditioning with H2S results in profound protection against myocardial I/R injury, as evidenced by a significant
decrease in INF size and a preservation of LV geometry and cardiac function.

Under physiological conditions, small amounts of ROS produced in cells are quenched by cellular antioxidant defense systems. Antioxidants act by scavenging oxidative species and their precursors, inhibiting their formation, and enhancing endogenous antioxidant defenses. There is considerable evidence that implicates the production of ROS and subsequent related cellular damage as an initial cause of injury to the myocardium following I/R injury. Therefore, the capacity of cardiac myocytes to maintain homeostasis during periods of oxidative stress resides in the ability to

Figure 7. Cardiac-specific overexpression of CGL increased Nrf2 nuclear accumulation and PKCe-STAT-3 signaling. Representative immunoblots and densitometric analysis of Nrf2 (cytosolic and nuclear fractions) (A), phosphorylated PKCe<sup>Ser729</sup> and total PKCe (cytosolic and membranous fractions) (B), phosphorylated STAT-3<sup>Ser727</sup> and total STAT-3 (cytosolic and nuclear fractions) (C), Trx1, HO-1, HSP90, and Bcl-2 (D) from the hearts of CGL-Tg<sup>+</sup> (n=4) and Non-Tg (n=4) mice. Values are means±SEM *P<0.05, **P<0.01 vs Non-Tg.
activate or induce protective enzymes. However, during I/R the activity of many of the endogenous antioxidant enzyme systems are compromised or even abolished, suggesting that increasing the activity of cellular antioxidant enzymes may protect tissues against reperfusion damage. Previous studies have reported that H2S protects various cell types, including myocytes, from oxidative stress. There is some debate, however, regarding the nature in which H2S reduces oxidative stress. H2S acts as a direct scavenger of ROS and upregulates endogenous antioxidant defenses. Kimura and Kimura demonstrated that H2S protects neurons from cell death by increasing GSH levels through an enhancement of γ-glutamylcysteine synthetase activity and an upregulation of cystine transport. A major finding of the present study supports the latter notion of H2S inducing a signaling mechanism to combat oxidative stress, as evidenced by the ability of H2S to upregulate cellular antioxidants in the heart in a Nrf2-dependent manner. Nrf2, a member of the NF-E2 family of nuclear basic leucine zipper transcription factors, regulates Nrf2-dependent manner. Nrf2, a member of the NF-E2 family of nuclear basic leucine zipper transcription factors, regulates the gene expression of a number of enzymes that serve to detoxify prooxidative stressors. This regulation is mediated by Nrf2 binding to the antioxidant responsive element, a cis-acting regulatory element or enhancer sequence found in the promoter region of certain genes, including HO-1 and Trx1. In the present study, H2S was shown for the first time to induce the nuclear accumulation of Nrf2 very rapidly after its administration and to subsequently increase the protein expression of HO-1 and Trx1. Additionally, this is the first study to report that Nrf2 deficient mice experienced an exacerbated injury in response to myocardial I/R, suggesting that Nrf2 is an important endogenous cardioprotective signal that protects against oxidant-mediated injury. This is further supported by previous reports indicating that Nrf2 deficiency is associated with enhanced oxidative stress and cell death.

In the present study, H2S modestly increased the expression of Trx1, but failed to increase the expression of HO-1 in the hearts of Nrf2 KO mice. This is an important finding because the increase in Trx1 was found to be only 58% of the increase observed in wild-type mice (1.4±0.1 versus 3.3±0.7), suggesting that H2S upregulates HO-1 in an Nrf2-dependent manner but only partially upregulates Trx1 in an Nrf2-dependent manner. This indicates that H2S regulates Trx1 expression through an additional unidentified mechanism. Interestingly, the modest increase in Trx1 levels in the Nrf2 KO hearts was not sufficient to induce protection, because H2S failed to reduce myocardial injury following I/R in these mice. Our data indicate that Nrf2 plays an important role in mediating the cardioprotective effects of H2S and provides important evidence linking Nrf2 and its downstream effectors to the antioxidant effects of H2S. Moreover, these results suggest that H2S therapy enhances the endogenous antioxidant defenses of myocytes and create an environment resistant to the oxidative stress associated with myocardial I/R injury, as evidenced by the preservation of redox state and a reduction in lipid peroxidation.

Another major finding of the present study relates to H2S-mediated activation of a PKCe-STAT-3 signaling cascade. Although, there is in vitro evidence in the literature suggesting that H2S activates PKC, the present study is the first to report that H2S activates PKCe in vivo. The activation of this prosurvival signaling cascade has previously been shown to confer cardioprotection against myocardial I/R through inhibition of apoptotic cell death and activation of cyclooxygenase-2 and has been shown to play a prominent role in the cardioprotective signaling of ischemic PC. The antiapoptotic actions of this pathway are mediated in part by the phosphorylation and inhibition of the proapoptotic factor Bad, upregulation of the prosurvival factors Bcl-2 and Bcl-xL, and upregulation of HSPs. H2S promotes the phosphorylation of Bad through the actions of p90RSK, whereas it upregulates Bcl-2, Bcl-xL, and HSPs through STAT-3. The STAT pathway has recently been shown to be an integral part of the response of the myocardium to various cardiac insults, including myocardial infarction. In particular, the overexpression of STAT-3 results in cardioprotection, whereas cardiac-specific deficiency of STAT-3 exacerbates cardiac injury. H2S have also been demonstrated to provide cardioprotection in the setting of I/R. In particular, HSP70 suppresses apoptosis in a caspase-dependent and caspase-independent manner. The findings of the present study suggest that H2S therapy does not simply reduce apoptotic cell death following myocardial I/R through a reduction in oxidative stress, but actually promotes direct antiapoptotic signaling. The activation of multiple pathways by H2S in the present study highlights the diversity of this gasotransmitter. Unlike other pharmacological agents that rely on receptor-mediated signaling, H2S activates multiple pathways simultaneously. In addition to the pathways studied here, H2S could promote cardioprotection via the activation of KATP channels and by inhibiting leukocyte-endothelial interactions and subsequent inflammation. An important question that remains unanswered relates to the mechanism by which H2S induces the nuclear accumulation of Nrf2. Under basal conditions, Keap1 (Kelch ECH associating protein 1) represses the ability of Nrf2 to induce endogenous antioxidants by binding very tightly to Nrf2, anchoring it in the cytoplasm, and targeting it for ubiquitination and proteasome degradation. Only when this association is disrupted can Nrf2 translocate to the nucleus and bind antioxidant responsive elements. It is thought that several critical cysteine residues in Keap1 serve as the primary sensor for stress signals (ie, ROS) and that their modification leads to conformational changes in Keap1, which results in the release of Nrf2. In addition to Keap1 being a target, Nrf2 can also be directly modified by kinases, such as PKC, to induce its release. Whether H2S alters Keap1 and/or Nrf2 directly or through upstream signaling (ie, PKC) remains an important unanswered question that requires further study.

Together, the findings of the present study indicate that the cardioprotective effects of H2S are mediated in large part by a combination of antioxidant and antiapoptotic signaling and highlight a novel signaling cascade involving Nrf2. Furthermore, this study suggests that either the administration of H2S donors or the modulation of the endogenous production of H2S may be of therapeutic benefit in the setting of myocardial I/R.

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Expanded Materials and Methods

Animals. Three different stains of mice were utilized: (1) Male C57BL6/J mice, 8-10 weeks of age (Jackson Labs, Bar Harbor, ME), (2) Male mice (8-10 weeks of age) with a cardiac-specific overexpression of CGL (αMHC-CGL-Tg) and non-transgenic littermates. (3) Male mice deficient in Nrf2 (Nrf2 KO) and wild-type littermates. The αMHC-CGL-Tg mice were generated on an FVB background. Nrf2 knockout mice were originally generated on an ICR/129SV background and then backcrossed onto an ICR background. All experimental mouse procedures were approved by the Institute for Animal Care and Use Committee at Albert Einstein College of Medicine and Emory University and conformed to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 86-23, Revised 1996) and with federal and state regulations.

Myocardial Ischemia-Reperfusion (I/R) Protocol. Prior to any surgical procedure, mice were anesthetized with intraperitoneal injections of ketamine (60 mg/kg) and sodium pentobarbital (20 mg/kg). Mice also received 200 Units/kg of sodium heparin via intraperitoneal injection before surgery to prevent clot formation and allow for consistent and complete reperfusion postligation. The mice were then attached to a surgical board with their ventral side up and orally intubated with polyethylene-60 (PE-60) tubing connected via loose junction to a rodent ventilator (MiniVent Type 845, Hugo-Sachs Elektronik) set at a tidal volume 240 µL of and a rate of 110 breaths per minute and supplemented with 100% oxygen (0.1-0.2 liters/minute flow rate) via a side port on the ventilator. Effective ventilation was visually confirmed by vapor condensation in the endotracheal tube and rhythmic rising of the chest. Mice were maintained at a constant temperature of 37°C with a water-filled heating pad connected to a circulating water pump (Gaymar). Temperature was monitored via a rectal probe.
connected to a Digisense K-Type digital thermometer. Hair remover (i.e., Nair®) was placed on the chest with a cotton swab and then removed along with the chest hair. The exposed regions were wiped with alcohol and betadine solution. A midline incision was then made along the sternum exposing the ribcage. Next, a median sternotomy was performed and the wound edges were cauterized with an electrocautery device. The proximal left coronary artery (LCA) was visually identified with the aid of an Olympus stereomicroscope with a fiber optic light source. The LCA was ligated with a 7-0 silk suture passed with a tapered BV-1 needle in close approximation just under the coronary artery. A short segment of PE-10 tubing was placed between the LCA and the 7-0 silk suture to minimized damage to the coronary artery and allow for complete reperfusion following the ischemic period. Ischemia was visually confirmed by cyanosis of the affected left ventricle. During the ischemic period the incision was covered with parafilm creating an effective barrier against desiccation and dehydration. Following 45 minutes of LCA occlusion, the ligature was removed, and reperfusion was visually confirmed. The chest wall and skin incision was carefully closed in layers with a 4-0 BIOSYN suture (CV-23 tapered needle). Animal recovery was supplemented by 100% oxygen and butorphanol (0.15 mg/kg) analgesia as well as a single dose of the antibiotic Cefazolin (80 mg/kg) to prevent infection. In the surgical recovery area, a heat lamp was utilized to maintain the appropriate body temperature of the mice. In addition, food and water were made available immediately and for the remainder of the first 24 hours of recovery.

Myocardial Infarct Size Determination. At 24 hours of reperfusion, the mice were anesthetized, intubated, and connected to a rodent ventilator. A catheter (PE-10 tubing) was placed in the common carotid artery to allow for Evans Blue dye injection. A median sternotomy was performed and the LCA was re-ligated in the same location as before. Evans Blue dye (1.2 mL of a 7.0% solution, Sigma) was injected into the carotid artery catheter into the heart to delineate the ischemic zone from the non-ischemic zone. The heart was rapidly excised and serially sectioned along the long axis in five, 1 mm thick sections that were then
incubated in 1.0% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) for 5 minutes at 37°C to demarcate the viable and nonviable myocardium within the risk zone. Each of the five, 1 mm thick myocardial slices were weighed and the areas of infarction, risk, and non-ischemic left ventricle were assessed by a blinded observer using computer-assisted planimetry (NIH Image 1.57). All of the procedures for the left ventricular area-at-risk and infarct size determination have been previously described.4

**Troponin I.** A blood sample (500 µL) was collected from mice prior to the Evans blue dye injection. Serum was obtained and the levels of the cardiac-specific isoform of Troponin-I (ng/mL) were assessed using an ELISA kit from Life Diagnostics (West Chester, PA).

**Echocardiographic Assessment of Left Ventricular Structure and Function.** Baseline two-dimensional echocardiography images were obtained one week prior to LCA ischemia as previously described.4 The mice were lightly anesthetized with isoflurane (3% for induction and 2% for maintenance) in 100% O₂ and in vivo transthoracic echocardiography of the left ventricle (LV) using a 30-MHz RMV scanhead interfaced with a Vevo 770 (Visualsonics) was used to obtain high-resolution, two-dimensional ECG-based kilohertz visualization, B mode images acquired at the rate of 1,000 frames/sec over 7 min. These images were used to measure LV end-diastolic diameter (LVEDD) and LV end-systolic diameter (LVESD) and to calculate LV ejection fraction (EF). One week after the baseline images were acquired, the mice were subjected to 45 min of LCA occlusion followed by reperfusion as described above. At 1 week of reperfusion, post I/R echocardiographic images were obtained and analyzed.

**TUNEL Staining.** After 45 min of ischemia and 4 hr of reperfusion hearts from sham, vehicle, and H₂S PC treated mice were rapidly excised, cross-sectioned into 3 sections and fixed in 10% buffered formalin. Fixed tissue was then paraffin embedded and sectioned in a standard fashion. TUNEL staining was conducted
using a kit according to the manufacturer's instructions (ApopTag HRP kit, DBA). The number of TUNEL-positive nuclei and the total number of nuclei per high-powered field were counted in a minimum of 10 fields from the area-at-risk for each section (a total of 30 fields per heart).

**Subcellular Fractionation.** Whole cell, cytosolic, membranous, nuclear, and mitochondrial fractions were prepared as described previously\(^4,5\). Briefly, frozen LV samples were powdered under liquid nitrogen with mortar and pestle prior to homogenization in 10 volumes of buffer A (containing in mM: 250 sucrose; 20 Tris, pH 7.4; 2 EDTA, pH 7.15; phosphatase inhibitors; 5 µg/ml each of leupeptin and aprotinin; 0.5 µg/ml pepstatin A; 0.3 PMSF) and subjected to serial centrifugations of 1,000 g, 10,000 g, and 100,000 g. The 1,000 g pellet (nuclear fraction) and the 10,000 g pellet (mitochondrial fraction) were washed in buffer A and recentrifuged. The final pellets were resuspended in buffer B (containing in mM: 150 NaCl; 20 Tris·HCl, pH 7.4; 10 EDTA; phosphatase inhibitors; 5 µg/ml each of leupeptin and aprotinin; 0.5 µg/ml pepstatin A; 0.1 PMSF) and subjected to a 21,000 g centrifugation for 10 min. The resultant supernatants were defined as the nuclear and mitochondrial fractions. The 100,000 g supernatant was defined as the cytosolic fraction and the 100,000 g pellet was resuspended in buffer B and defined as the membranous fraction. For whole cell preparations, samples were homogenized in 1 ml of ice-cold RIPA lysis buffer (Cell Signaling). Homogenates were then centrifuged at 1,300 g to remove any cellular debris. The pellet was discarded, and the supernatant was again centrifuged at 16,000 g for 30 min at 4°C. The resultant supernatant was collected. Protein concentrations of all cellular fractions were measured with the DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

**Western blot analysis.** Western blot analysis was performed as described previously (16). Equal amounts of protein were loaded into lanes of polyacrylamide-SDS gels. The gels were electrophoresed, followed by transfer of the protein to a PVDF membrane. The membrane was then blocked and probed with primary antibodies overnight at 4°C. The following primary antibodies were
used: anti-rabbit Trx1 (1:3000; Cell Signaling Technology, Danvers, MA); anti-rabbit Nrf2 (1:3000; abcam, Cambridge, MA); anti-mouse heme oxygenase-1 (HO-1) (1:3000; abcam); anti-rabbit phospho-PKCε Ser729 (1:3000; Upstate, Lake Placid, NY); anti-rabbit PKCε (1:5000; Upstate, Lake Placid, NY); anti-rabbit phospho-STAT-3 Ser727 (1:1500; Cell Signaling); anti-rabbit STAT-3 (1:3000; Cell Signaling); anti-rabbit p44/42 (1:3000; Cell Signaling); anti-rabbit phospho-p44/42 (1:1500; Cell Signaling); anti-rabbit HSP70 (1:3000; Cell Signaling); anti-rabbit HSP90 (1:5000; Cell Signaling); anti-rabbit Caspase-3 (1:3000; Cell Signaling); anti-rabbit cleaved Caspase-3 Asp 175 (1:1500; Cell Signaling); anti-rabbit cytochrome C (1:5000; Cell Signaling); anti-rabbit fibrillarin (1:5000; Cell Signaling); anti-rabbit Na/K ATPase (1:5000; Cell Signaling); anti-rat α-tubulin (1:40,000; Santa Cruz Biotechnology Inc, Santa Cruz, CA); anti-rabbit phospho-Bad Ser112 (1:1000; Cell Signaling); anti-rabbit Bad (1:1000; Cell Signaling); anti-rabbit Bcl-2 (1:1500; Cell Signaling); anti-rabbit Bcl-xL (1:3000; Cell Signaling); anti-rabbit cyclooxygenase-2 (COX-2) (1:2000; Cell Signaling); anti-rabbit CuZnSOD (1:3000; Cell Signaling); anti-rabbit MnSOD (1:3000; Santa Cruz); anti-rabbit cytochrome c oxidase IV (1:20,000; Cell Signaling).

Immunoblots were next processed with secondary antibodies (anti-rabbit, anti-mouse, or anti-rat; Cell Signaling or Santa Cruz) for 1 hr at room temperature. Immunoblots were then probed with an ECL+Plus chemiluminescence reagent kit (GE Healthcare) to visualize signal, followed by exposure to X-ray film (Denville Scientific). The film was scanned to make a digital copy and densitometric analysis was performed to calculate relative intensity with ImageJ software from the National Institutes of Health (version 1.40g) using the Rodbard function. In all cases, the membranes were incubated with the phospho-specific antibody first. Membranes were then stripped and incubated with the total-specific antibody. Results were presented as the ratio of the expression of phosphorylated protein to total protein.

The following antibodies were used as loading controls: anti-fibrillarin served as the subcellular marker for the nuclear fraction; anti-α-tubulin served as the
subcellular marker for the cytosolic fraction; anti-cytochrome c oxidase served as the subcellular marker for the mitochondrial fraction; anti-Na/K-ATPase served as the subcellular marker for the membraneous fraction. All experiments were performed in triplicate. For each membrane the relative intensity of each band was normalized to the value of the weakest band (smallest intensity). The values for each individual sample were averaged to obtain one value for each sample. The values for each group were then averaged and subsequently normalized to the mean of the control group (Sham or Non-Tg group).

**Gluthathione and Lipid hydroperoxide assays.** Cardiac glutathione and lipid hydroperoxides were measured in heart tissue collected from mice subjected to 45 min of myocardial ischemia and 1, 4, or 24 hr of reperfusion using commercially available kits (Cayman Chemicals) as previously described.\(^6\) Reduced glutathione (GSH) and oxidized glutathione (GSSG) values were used to calculate the steady-state redox potential using the Nernst equation,

\[
E_h = E_0 + \frac{2.3 \times RT}{nF} \times \log\left(\frac{[\text{GSSG}]}{[\text{GSH}]^2}\right)
\]

where \(E_0 = -264\) at pH 7.4 and \(n=2\) as described previously.\(^7,8\)
Online Figure. Hydrogen sulfide preconditioning (H$_2$S PC) preserved left ventricular dimensions in mice following myocardial ischemia and reperfusion. (A) Left ventricular end-diastolic diameter (LVEDD) and (B) left ventricular end-systolic diameter (LVESD) were calculated using high-resolution, two-dimensional B-mode echocardiography images at baseline and 7 days following myocardial ischemia. Values are means ± S.E.M. Numbers inside bars indicate the number of animals that were investigated in each group. *p<0.05, ***p<0.001 vs. BASE. H$_2$S denotes Na$_2$S.
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