Heart Failure

Genetic and Pharmacologic Hydrogen Sulfide Therapy Attenuates Ischemia-Induced Heart Failure in Mice

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Background—Hydrogen sulfide (H\textsubscript{2}S) is an endogenous signaling molecule with potent cytoprotective effects. The present study evaluated the therapeutic potential of H\textsubscript{2}S in murine models of heart failure.

Methods and Results—Heart failure was induced by subjecting mice either to permanent ligation of the left coronary artery for 4 weeks or to 60 minutes of left coronary artery occlusion followed by reperfusion for 4 weeks. Transgenic mice with cardiac-restricted overexpression of the H\textsubscript{2}S-generating enzyme cystathionine \(\gamma\)-lyase (\(\alpha\)MHC-CGL-Tg\textsuperscript{+/-}) displayed a clear protection against left ventricular structural and functional impairment as assessed by echocardiography in response to ischemia-induced heart failure, as well as improved survival in response to permanent myocardial ischemia. Exogenous H\textsubscript{2}S therapy (Na\textsubscript{2}S; 100 \(\mu\)g/kg) administered at the time of reperfusion (intracardiac) and then daily (intravenous) for the first 7 days after myocardial ischemia also protected against the structural and functional deterioration of the left ventricle by attenuating oxidative stress and mitochondrial dysfunction. Additional experiments aimed at elucidating some of the protective mechanisms of H\textsubscript{2}S therapy found that 7 days of H\textsubscript{2}S therapy increased the phosphorylation of Akt and increased the nuclear localization of 2 transcription factors, nuclear respiratory factor 1 and nuclear factor-E2-related factor (Nrf2), that are involved in increasing the levels of endogenous antioxidants, attenuating apoptosis, and increasing mitochondrial biogenesis.

Conclusions—The results of the present study suggest that either the administration of exogenous H\textsubscript{2}S or the modulation of endogenous H\textsubscript{2}S production may be of therapeutic benefit in the treatment of ischemia-induced heart failure. (Circulation. 2010;122:11-19.)

Key Words: cystathionine \(\gamma\)-Lyase ♦ heart failure ♦ hydrogen sulfide ♦ ischemia ♦ myocardial infarction

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Heart failure continues to be a major health problem in the United States, especially in the elderly population.\textsuperscript{1,2} Unfortunately, current treatments for heart failure are insufficient, and the availability of hearts for transplantation is severely inadequate.\textsuperscript{3} Therefore, adjunct pharmacotherapies designed to coincide with the standard means of care are needed to decrease the extent of injury leading to the development of heart failure. Small gaseous signaling molecules are labile biological mediators that are able to freely diffuse through cell membranes to invoke cellular signaling, thus alleviating the need for membrane receptors and second messengers. Hydrogen sulfide (H\textsubscript{2}S), a recently classified small molecule effector,\textsuperscript{4} is produced in the body by the enzymes cystathionine \(\gamma\)-lyase (CGL; cystathione, CTH), cystathionine \(\beta\)-synthase, and 3-mercaptoppyruvate sulfurtransferase. H\textsubscript{2}S has been reported to provide cardioprotection in various models of cardiac injury through its ability to preserve mitochondrial function and to reduce cardiomyocyte apoptosis.\textsuperscript{5,6} Although the cytoprotective effects of H\textsubscript{2}S have been demonstrated in models of acute cardiac injury, the effects of H\textsubscript{2}S therapy on cardiac function in the setting of chronic heart failure are currently unknown. Therefore, the purpose of the present study was to investigate the potential cardioprotective effects of endogenous and exogenous H\textsubscript{2}S on survival and cardiac function in 2 murine models of ischemia-induced heart failure.

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Methods

Animals

Male C57BL6/J mice, 8 to 10 weeks of age, were used (Jackson Laboratories, Bar Harbor, Me). The generation of cardiac-specific transgenic mice overexpressing CGL (\(\alpha\)MHC-CGL-Tg\textsuperscript{+/-}; FVB background) has been described previously.\textsuperscript{6} \(\alpha\)MHC-CGL-Tg\textsuperscript{+/-} and nontransgenic littermates were bred and used at 8 to 10 weeks of age. All experimental mouse procedures were approved by the Institute

Received November 3, 2009; accepted May 10, 2010.

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The online-only Data Supplement is available with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.109.920991/DC1.

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Circulation is available at http://circ.ahajournals.org

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DOI: 10.1161/CIRCULATIONAHA.109.920991
for Animal Care and Use Committee at 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 to federal and state regulations.

Materials
Sodium sulfide (Na2S) was produced by Ikaria Holdings, Inc (Seattle, Wash) by using H2S gas (Matheson, Newark, Calif) as a starting material as previously described. Na2S (100 μg/kg) was administered with a 32-gauge needle in a final volume of 50 μL as an intracardiac injection once at the time of reperfusion (Na2S) or once at the time of reperfusion followed by daily tail vein (intravenous) injections for the first 7 days of reperfusion (Na2S 7 days). This dose of Na2S was selected on the basis of our previous experience investigating Na2S in murine models of cardiac ischemia/reperfusion injury.7,9 saline was administered in the same manner for the respective vehicle groups.

Heart Failure Protocols
Heart failure was induced either by permanent ligation of the left coronary artery (LCA) or by subjecting mice to 60 minutes of LCA occlusion followed by reperfusion for up to 4 weeks as described previously.8 All mice were randomly allocated to treatment groups. Myocardial infarct size assessment, echocardiographic assessment of left ventricular (LV) structure and function, and histological analysis of infarct scar were all performed as previously described.7,9

Lipid Hydroperoxide Assay
Quantification of lipid peroxidation was performed to assess the extent of cardiac oxidative stress as described previously.7

Quantitative Real-Time Polymerase Chain Reaction for Mitochondrial DNA
Mitochondrial DNA content was quantified by real-time reverse-transcription polymerase chain reaction with cardiac DNA as described previously.10

Cardiac Mitochondria Isolation, Mitochondrial Respiratory Rate, and ATP Synthesis
Cardiac mitochondria were isolated from the following groups of mice: sham-operated, vehicle-treated, and Na2S-treated mice. Oxygen consumption and ATP synthesis rates were determined as previously described.8

Western Blot Analysis
Western blot analysis was performed as described previously.7

Statistical Analysis
All data in this study are expressed as mean±SEM. Means were compared by use of Prism 4 (GraphPad Software Inc) with a Student unpaired 2-tailed t test (Western blot analysis), 1-way ANOVA (ratio of heart to body weight, lipid hydroperoxidation [LPO] data, mitochondrial DNA, and mitochondrial respiration data), or 2-way ANOVA (echocardiography data) when appropriate. For the ANOVA, if a significant result was found, the Tukey (1-way ANOVA) or Bonferroni (2-way ANOVA) test was used as the posthoc analysis. Survival curves were compared by use of a log-rank (Mantel-Cox) test. For all data, a value of P<0.05 was considered significant.

Results
Endogenous Overexpression of the H2S-Generating Enzyme CGL Improves Survival After Permanent LCA Occlusion
The effects of H2S on heart failure were first evaluated in mice that overexpress the H2S-generating enzyme CGL (αMHC-CGL-Tg+). These cardiac-specific transgenic mice have an ∼15-fold overexpression of CGL in their hearts, which results in a 2-fold increase in cardiac H2S production.6 For these experiments, αMHC-CGL-Tg+ and nontransgenic mice were subjected to permanent occlusion of the LCA. At 4 weeks after myocardial ischemia, both groups of mice exhibited significant mortality (Figure 1). The αMHC-CGL-Tg+ mice exhibited an overall survival rate of 67% (22 of 33) during the 4-week follow-up; the nontransgenic mice exhibited an overall survival rate of 40% (14 of 35). Comparisons between survival curves were made with a log-rank (Mantel-Cox) test.

Endogenous Overexpression of CGL Reduces LV Dilatation and Cardiac Hypertrophy but Does Not Improve Function After Permanent LCA Occlusion
At the end of the 4-week follow-up period, the surviving mice were subjected to 2-dimensional, high-resolution echocardiography to determine the degree of LV dilatation and LV dysfunction. Analysis revealed that the LV end-diastolic (LVEDD) and end-systolic (LVESD) diameter of both the αMHC-CGL-Tg+ and nontransgenic mice were significantly higher than their respective baseline readings (P<0.001), suggesting that LV dilatation had occurred (Figure 2A and 2B). However, the hearts of αMHC-CGL-Tg+ mice had significantly smaller increases in both LVEDD (P<0.05 versus nontransgenic) and LVEDD (P<0.01 versus nontransgenic). Cardiac hypertrophy was also analyzed by determining the ratios of heart to body weight (Figure 2C). Both αMHC-CGL-Tg+ and nontransgenic mice displayed cardiac hypertrophy 4 weeks after myocardial ischemia compared with sham-operated animals (P<0.05), but αMHC-CGL-Tg+ mice displayed significantly less hypertrophy (P<0.01 versus nontransgenic). Despite these significant reductions in LV dilatation and cardiac hypertrophy, no improvement in LV ejection fraction was evident in the αMHC-CGL-Tg+ mice compared with the nontransgenic mice (Figure 2D). Additionally, the heart rate of the 2 groups of mice was evaluated at baseline and 4 weeks after myocardial ischemia (Figure 1A in the online-only Data Supplement). No differences at
baseline were observed, and both groups of mice exhibited an elevated heart rate 4 weeks after myocardial ischemia.

We also measured the infarct area relative to the entire LV at 4 weeks after infarction (Figure IIA in the online-only Data Supplement). Analysis revealed that the nontransgenic mice displayed a 22±3% infarct area/LV and the αMHC-CGL-Tg$^+$ mice displayed a 25±2% infarct area/LV. These findings suggest that overexpression of CGL improves survival after permanent LCA occlusion and that this survival benefit is independent of any effect on infarct size.

**Endogenous Overexpression of CGL Improves LV Structure and Function After Ischemia-Induced Heart Failure**

To study the effects of H$_2$S in a more clinically relevant model of heart failure that mimics the effects of coronary revascularization therapy, αMHC-CGL-Tg$^+$ and nontransgenic mice were subjected to 60 minutes of LCA occlusion followed by 4 weeks of reperfusion. Myocardial infarction was evaluated in 2 different groups of mice at 24 hours and 4 weeks of reperfusion with the Evans blue/triphenyltetrazolium chloride method and histologically, respectively. Following 24 hours of reperfusion, the area at risk per LV was similar (P=NS) in both groups, and the αMHC-CGL-Tg$^+$ mice (n=7) displayed a 19% reduction (52±2% versus 42±4%; P<0.05) in infarct area relative to the area at risk and a 30% reduction (33±3% versus 23±2%; P<0.05) in infarct area/LV compared with the nontransgenic mice (n=10). Following 4 weeks of reperfusion, analysis revealed that the nontransgenic mice displayed an 11.8±1.2% infarct area/LV and the αMHC-CGL-Tg$^+$ mice displayed a 7.3±1% infarct area/LV at 4 weeks of reperfusion, which corresponded to a 38% reduction in infarct area (P<0.01 versus nontransgenic; Figure IIB in the online-only Data Supplement). Following 4 weeks of reperfusion, LV dilatation, cardiac hypertrophy, and LV dysfunction were all prevalent in both groups of mice (Figure 3). However, αMHC-CGL-Tg$^+$ mice displayed significantly smaller increases in LVEDD (P<0.05), LVESD (P<0.05), and ratio of heart to body weight compared with vehicle-treated mice (P<0.001) compared with nontransgenic mice. In addition, both groups of mice exhibited an elevated heart rate 4 weeks after myocardial I/R, but only the nontransgenic mice had a significant increase from baseline (P<0.05; Figure IB in the online-only Data Supplement). These findings suggest that increased production of H$_2$S during the reperfusion phase has a positive impact on LV structure and function after ischemia-induced heart failure.

**Single Injection of Na$_2$S Reduces Infarct Size but Does Not Improve LV Structure and Function**

In an effort to translate these findings to a more clinically relevant model we next utilized pharmacologic administration of H$_2$S (Figure 4). In these experiments, C57BL/6J mice were subjected to 60 minutes of LCA occlusion followed by 4 weeks of reperfusion. H$_2$S (Na$_2$S; 100 μg/kg) or vehicle (saline) was administered at the time of reperfusion (intracardiac). Again, myocardial infarction was evaluated in 2 different groups of mice at 24 hours and 4 weeks of reperfusion. Following 24 hours of reperfusion, Na$_2$S (n=9) decreased infarct area/area at risk by 14% (69±2% versus 59±2%; P<0.05) and decreased infarct area/LV by 20% (41±2% versus 33±2%; P<0.05) compared with vehicle-treated mice (n=8). At 4 weeks of reperfusion, analysis revealed a similar 25% reduction in infarct area/LV (12±1% versus 9±1%; P<0.05) in the mice treated with Na$_2$S compared with the vehicle-treated mice (Figure IIC in the online-only Data Supplement). However, following 4 weeks of reperfusion, the Na$_2$S-treated mice did not show any improvements in LVEDD, LVESD, ratio of heart to body weight, LV ejection fraction, or heart rate compared with the vehicle-treated group (Figure 4 and Figure IC in the online-only Data Supplement).
This finding suggests that a single administration of H2S at reperfusion is not sufficient to improve LV function at 4 weeks, even though a single administration of H2S reduces infarct size.

**Daily Injections of Na2S During the First 7 Days of Reperfusion Improve LV Structure and Function**

Subsequent experiments evaluated the effectiveness of daily administrations of H2S during the first 7 days of reperfusion (Figure 5). In these experiments, C57BL/6J mice were subjected to 60 minutes of LCA occlusion followed by 4 weeks of reperfusion. Analysis at 4 weeks of reperfusion revealed that treatment during the first 7 days of the reperfusion period led to a decrease in LV dilatation, a decrease in cardiac hypertrophy, and an improvement in cardiac function. No differences in heart rates were observed at baseline, and both groups of mice exhibited an elevated heart rate 4 weeks after myocardial ischemia (Figure ID in the online-only Data Supplement). To determine whether the 7-day treatment of Na2S had any additional effects on infarct size reduction, the area of infarction was evaluated at 4 weeks of reperfusion. Analysis revealed that the vehicle-treated mice displayed a 12±1% infarct area/LV and the Na2S-treated mice displayed a 9±1% infarct area/LV at 4 weeks of reperfusion, which corresponded to a 25% reduction in infarct area (P<0.01 vs sham).

Figure 3. Overexpression of CGL reduces LV dilatation, reduces cardiac hypertrophy, and improves LV function after myocardial ischemia and reperfusion. LVEDD (A), LVESD (B), ratio of heart to body weight (C), and LV ejection fraction (D) for αMHC-CGL-Tg+ and nontransgenic mice 4 weeks after 60 minutes of LCA occlusion and reperfusion (Post). Values are mean±SEM. Means for the echocardiography data were compared by use of a 2-way ANOVA with a Bonferroni test as the posthoc analysis. Means for the ratios of heart to body weight were compared by use of a 1-way ANOVA with a Tukey test as the posthoc analysis. ***P<0.001 vs baseline (Base) or sham; *P<0.05 vs sham.

Figure 4. Single administration of Na2S does not attenuate the development of ischemia-induced heart failure. LVEDD (A), LVESD (B), ratio of heart to body weight (C), and LV ejection fraction (D) for Na2S- and vehicle-treated mice 4 weeks after 60 minutes of LCA occlusion and reperfusion. (Post) Mice were treated with 100 μg/kg Na2S or vehicle at the time of reperfusion. Values are mean±SEM. Means for the echocardiography data were compared by use of a 2-way ANOVA with a Bonferroni test as the posthoc analysis. Means for the ratios of heart to body weight were compared by use of a 1-way ANOVA with a Tukey test as the posthoc analysis. ***P<0.001 vs baseline (Base); **P<0.01 vs sham.
versus vehicle; Figure IID in the online-only Data Supplement). These results suggest that treatment with exogenous H2S during the first 7 days of reperfusion is critical for sustained improvements in LV structure and function.

Daily Injections of Na2S Induce the Nuclear Localization of Nrf2 and NRF-1 and Increase the Phosphorylation of Akt

H2S has a diverse physiological profile, which may account for the cardioprotection observed in the current models of heart failure. Recently, nuclear factor-E2–related factor (Nrf2) has been identified as an important cellular target of H2S.7 Nrf2 is a key transcription factor that regulates antioxidant genes as an adaptive response to oxidative stress11–13 and regulates mitochondrial biogenesis through an upregulation of nuclear respiratory factor 1 (NRF-1).14 Therefore, experiments were conducted to evaluate Nrf2 signaling after H2S treatment. For these experiments, Na2S was administered to mice for 7 days (intravenous), at which time hearts were excised and processed for Western blot analysis (Figure 6). Because Nrf2 is a transcription factor, its protein expression was evaluated in both cytosolic and nuclear fractions. Analysis revealed that Nrf2 protein levels were increased (P<0.05) in both the cytosolic and nuclear fractions in the hearts treated with Na2S compared with the sham hearts (Figure 6A). Subsequently, the nuclear expression, but not the cytosolic expression, of NRF-1 was elevated in the hearts of mice treated with Na2S. Daily injections of Na2S attenuate the development of ischemia-induced heart failure. LVEDD (A), LVESD (B), ratio of heart to body weight (C), and LV ejection fraction (D) for Na2S- and vehicle-treated mice 4 weeks after 60 minutes of LCA occlusion and reperfusion (Post). Mice were treated with 100 μg/kg Na2S at the time of reperfusion and then daily for the first 7 days of reperfusion. Values are mean±SEM. Means for the echocardiography data were compared by use of a 2-way ANOVA with a Bonferroni test as the posthoc analysis. Means for the ratios of heart to body weight were compared by use of a 1-way ANOVA with a Tukey test as the posthoc analysis. ***P<0.001 vs baseline (Base); *P<0.05 vs sham.

To determine whether Na2S could alter the expression levels of Nrf2, NRF-1 and Akt in nonvascular tissue, additional studies were performed using hepatic tissue taken from mice administered Na2S for 7 days. These studies revealed that Na2S therapy increased the nuclear expression of both Nrf2 and NRF-1 (P<0.05 versus vehicle) but did not increase the levels of Akt or alter its phosphorylation status (Figure III in the online-only Data Supplement), suggesting that the activation of Nrf2 and NRF-1 by Na2S was not restricted to the heart. Additionally, we have previously reported that hearts from αMHC-CGL-Tg mice have an increased nuclear expression of Nrf2.7 Further analysis in the present study revealed that hearts from αMHC-CGL-Tg mice had an increased nuclear expression of NRF-1 (P<0.01 versus nontransgenic) and an increased expression of Akt (P<0.05 versus nontransgenic) but no changes in Akt-PSer473 (Figure IV in the online-only Data Supplement). No changes in Nrf2, NRF-1, and Akt were observed in the livers of αMHC-CGL-Tg mice (Figure V in the online-only Data Supplement), which confirms our previous findings that the increased generation of H2S is confined to the heart.6

Daily Injections of Na2S Attenuate Oxidative Stress

Lipid hydroperoxidation (LPO) was used as a measure of cardiac oxidative stress during the development of heart failure. In these experiments (Figure 7A), 2 groups of C57BL/6j mice were subjected to 60 minutes of LCA occlusion followed by 1 and 4 weeks of reperfusion. At 1 week of reperfusion, both the vehicle-treated (P<0.001) and 7-day Na2S-treated (P<0.05) mice exhibited significantly higher levels of LPO compared with sham-operated controls. However, the Na2S-treated mice displayed significantly lower levels of LPO compared with the vehicle-treated mice (P<0.05). LPO levels remained elevated above sham levels.
in both groups of mice at 4 weeks of reperfusion \((P<0.01)\), and although not statistically significant, there was a trend for lower LPO levels in the hearts of mice treated with \(\text{Na}_2\text{S}\) compared with the vehicle-treated mice. These findings suggest that treatment with \(\text{H}_2\text{S}\) during the first 7 days of reperfusion reduces oxidative stress associated with heart failure.

**Daily Injections of \(\text{Na}_2\text{S}\) Did Not Increase Mitochondrial Biogenesis but Did Improve Mitochondrial Respiration and ATP Synthesis**

\(\text{NRF-1}\) regulates the expression of several genes responsible for mitochondrial biogenesis.\(^{14,16}\) Because \(\text{H}_2\text{S}\) increased the nuclear accumulation of \(\text{NRF-1}\), the next series of experiments evaluated mitochondrial biogenesis. For these experiments, mice were subjected to 60 minutes of LCA occlusion followed by 4 weeks of reperfusion. \(\text{Na}_2\text{S}\) or vehicle was administered at the time of reperfusion (intracardiac) and then daily for 7 days (intravenous). At 4 weeks of reperfusion, the hearts from all groups of mice were found to have similar ratios of cytochrome b DNA to \(\beta\)-actin DNA quantity, suggesting that \(\text{H}_2\text{S}\) did not induce mitochondrial biogenesis (Figure 7B).

\(\text{H}_2\text{S}\) can preserve mitochondrial function after acute myocardial ischemia/reperfusion injury.\(^6\) Therefore, we investigated the effects of \(\text{H}_2\text{S}\) on mitochondrial function during the development of heart failure (Figure 7C and 7D). Mitochondria isolated from the hearts of vehicle-treated mice were found to have a 61\% reduction \((P<0.001\text{ versus sham})\) in maximal ADP-stimulated (state 3) oxygen consumption, a slightly increased oligomycin-inhibited respiration, and reduced respiratory control ratio \((P<0.001\text{ versus sham})\), suggestive of uncoupling. ATP synthesis rates and the ATP/oxygen consumption ratios in the mitochondria from vehicle-treated mice were significantly \((P<0.001)\) reduced compared with sham-operated mice (Figure 7D). Mitochondria isolated from the hearts of \(\text{Na}_2\text{S}\)-treated mice were found to have slightly higher rates of ADP-stimulated oxygen consumption compared with vehicle-treated mice \((P=0.07)\). Despite similar rates of oligomycin-inhibited respiration, the mitochondria isolated from \(\text{Na}_2\text{S}\)-treated mice displayed higher respiratory control ratios \((P<0.05)\), greater ATP synthesis rates \((P<0.05)\), and slightly higher ATP/oxygen consumption ratios compared with vehicle-treated mice. These data indicate that the respiration of cardiac mitochondria during heart failure was inefficient, likely a result of uncoupled respiration, and that \(\text{H}_2\text{S}\) treatment is able to limit this dysfunction.

**Discussion**

In recent years, the cardioprotective effects of \(\text{H}_2\text{S}\) have been demonstrated in various models of myocardial injury.\(^{17}\) These studies have provided important mechanistic insights into its
cardioprotective actions and important information on dosage, timing, and route of administration. For instance, a single administration of H2S before, during, or after myocardial ischemia decreases myocardial infarct size and attenuates LV dysfunction in both rodents and pigs.6,7,18 Although these studies provide strong evidence for the cardioprotective effects of short-term H2S therapy (ie, single treatment, short follow-up), these studies do not offer any insights into the long-term effects (ie, daily administration, long follow-up) of H2S therapy. Thus, the present study provides the first evidence that H2S therapy can provide long-term protection against myocardial injury. Importantly, the present study demonstrates that although a single administration of H2S at the time of reperfusion is beneficial in attenuating infarct size, this alone is not sufficient to cause a significant improvement in cardiac function. On the other hand, daily H2S therapy initiated at the time of reperfusion and continued for the first 7 days of reperfusion provided significant improvements in cardiac function and LV dimensions despite not providing any additional infarct-sparing benefits over a single treatment of H2S. This suggests that the first 7 days of reperfusion is a critical period for the development of heart failure in this murine model and that initiating therapeutic interventions during this time is paramount for improvements in outcome. This is further supported by the additional findings that genetic overexpression of a critical H2S-producing enzyme, CGL, results in increased endogenous H2S production6 and a profound protection against ischemia-induced heart failure and decreased mortality. Together, these results suggest that H2S treatment could potentially be initiated at the time of coronary artery reperfusion and then continued daily to achieve a long-term improvement in cardiac function and to decrease the morbidity and mortality resulting from heart failure.

H2S possesses a diverse physiological profile that contributes to its cardioprotective actions.19 Of the reported physiological effects of H2S, several could provide protection during the development of heart failure. First, it has become evident that H2S itself serves over the short term as a potent antioxidant20,21 and under more long-term conditions upregulates antioxidant defenses20,22 through the activation of the transcription factor Nrf2.7 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 pro-oxidative stressors.11 This regulation is mediated by Nrf2 binding to the antioxidant responsive element found in the promoter region of genes12 such as heme oxygenase-1, thioredoxin, thioredoxin reductase, glutathione reductase, glutathione peroxidase, glutathione S-transferase, and catalase.13,23,24 The reported antioxidant effects of H2S may be of critical importance in the setting of heart failure because oxidative stress plays a prominent role in the development of LV remodeling and dysfunction associated with heart failure,25 suggesting that increasing the activity of cellular antioxidant enzymes should protect the failing myocardium.26 The results of the present study support the previous finding that the cardioprotective effects of H2S are related to a reduction in oxidative stress because it was observed that H2S reduced LPO levels at both 1 and 4 weeks of reperfusion. The results of the present study also support a
role for Nrf2 in mediating the antioxidant effects of H2S because H2S treatment was observed to induce the nuclear localization of Nrf2. Together, these findings indicate that H2S therapy creates an environment in the heart that is resistant to the oxidative stress associated with the development of heart failure.

Another physiological characteristic of H2S that could provide protection in the failing heart relates to the evidence that H2S can alter the metabolic state of organisms by modulating mitochondrial function.27 In heart failure, there is a decrease in the activity of the complexes of the respiratory chain and Krebs cycle enzymes. The reduced expression of mitochondrial proteins results in decreased mitochondrial respiration efficiency and limited ATP synthesis capacity and myocardial energy production.28 The decreased oxidative capacity of the failing myocardium therefore limits the ability of the heart to meet hemodynamic demands and leads to symptoms of heart failure. Mitochondria are essential for cell survival because of their roles as metabolic energy producers and regulators of programmed cell death.29 Mitochondria rely on an intrinsic genome that is replicated and transcribed semiautomonously but whose maintenance requires nuclear factors such as NRF-1. Recently, the promoter region of NRF-1 was found to contain 4 antioxidant responsive elements that, when bound by Nrf2, led to an increase in NRF-1 protein levels and an increase in gene activation responsible for mitochondrial biogenesis.14 Additionally, NRF-1 transcriptional activity was reported to be regulated by Akt.15 Despite increasing the phosphorylation of Akt and the nuclear accumulation of both Nrf2 and NRF-1, H2S therapy failed to increase mitochondrial biogenesis 4 weeks after myocardial infarction. H2S therapy also failed to provide significant improvements in mitochondrial function, although slight improvements in ATP synthesis were noted. These slight improvements suggest that the effects of H2S on the mitochondria were not direct. Rather, the slight improvements are more likely attributed to the ability of H2S to reduce oxidative stress, suggesting that, in this model of heart failure, the antioxidant effects of H2S may play a more prominent role in mediating its cardioprotective actions.

We live in the midst of the proclaimed epidemic of heart failure, as evidenced by a rise in the number of hospitalizations for heart failure, the number of deaths attributed to heart failure, and the costs associated with care.30,31 Couple this with the ever-increasing prevalence of diabetes mellitus and obesity, 2 of the main risk factors for the development of coronary artery disease, and it is readily apparent that treatment strategies aimed at combating the development and progression of heart failure are important and severely needed. The findings of the present study provide the first evidence that either the modulation of endogenous H2S production or direct H2S administration significantly attenuates the severity of ischemia-induced heart failure in mice by reducing oxidative stress and attenuating mitochondrial dysfunction. Therefore, these findings further support the emerging concept that H2S therapy may be of clinical importance in the treatment of cardiovascular disease and may have a practical clinical use after myocardial infarction to reduce the morbidity and mortality associated with ischemia-induced heart failure.

Acknowledgments

We thank P. Hill (Ikaria Holdings, Inc) for the formulation of the Na2S and thank D.B. Grinsfelder and J.P. Aragon for their invaluable assistance in the completion of this study.

Sources of Funding

This work was supported by grants from the American Diabetes Association (7-09-BS-26 to Dr Calvert) and the National Heart, Lung, and Blood Institute (National Institutes of Health; 2R01HL-060849-09, 5R01HL-092141-01, and 1R01HL093579-01 to Dr Lefer, 1R01HL098481-01 to Dr Calvert, and F32HL092737 to Dr Elrod). This work was also supported by funding from the Carlyle Fraser Heart Center of Emory University Hospital Midtown.

Disclosures

Ikaria Holdings, Inc provided the Na2S. The authors report no other conflicts.

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Heart failure continues to be a major health problem as evidenced by a rise in the number of hospitalizations for heart failure, the number of deaths attributed to heart failure, and the ever-increasing costs associated with care. Therapeutic strategies designed to coincide with the standard means of care are, therefore, needed to combat the development and progression of heart failure. Hydrogen sulfide (H$_2$S) is an endogenous gaseous signaling molecule with a diverse physiological profile that has recently been shown to be cardioprotective in various models of cardiac injury. In the present study, we found that either the modulation of endogenous H$_2$S production or direct pharmacologic H$_2$S administration significantly reduced mortality and attenuated the severity of ischemia-induced heart failure in mice. Importantly, the present study demonstrates that although a single administration of H$_2$S at the time of reperfusion is beneficial in attenuating infarct size, this alone is not sufficient to improve cardiac function significantly. On the other hand, daily H$_2$S therapy for the first 7 days of reperfusion or increased endogenous H$_2$S production provided significant improvements in cardiac function, suggesting that multiple therapeutic interventions are paramount for improvements in outcome. Together, these findings further support the emerging concept that H$_2$S therapy may be of clinical importance in the treatment of cardiovascular disease and may have a practical clinical use after myocardial infarction to reduce the morbidity and mortality associated with ischemia-induced heart failure.
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Circulation. 2010;122:11-19; originally published online June 21, 2010;
doi: 10.1161/CIRCULATIONAHA.109.920991

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/122/1/11

Data Supplement (unedited) at:
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Expanded Materials and Methods

**Animals.** Two different stains of mice were utilized in this study: (1) Male C57BL6/J mice, 8-10 weeks of age (Jackson Labs, Bar Harbor, ME) and (2) Male mice (8-10 weeks of age) with a cardiac-specific overexpression of CGL (αMHC-CGL-Tg) and non-transgenic littermates (FVB background). The generation of cardiac-specific transgenic mice overexpressing CGL (αMHC-CGL-Tg\(^+\)) has been described previously.\(^1\) 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.

**Materials.** Sodium Sulfide (Na\(_2\)S) was produced by Ikaria Inc. (Seattle, WA) by using H\(_2\)S gas (Matheson, Newark, CA) as a starting material. Na\(_2\)S was formulated to pH neutrality, and iso-osmolarity. Na\(_2\)S (stock solution at 0.55 mg/ml and 7.1 mM) was diluted in normal (0.9%) saline to the desired concentration in a rapid fashion immediately before administration. Na\(_2\)S (100 µg/kg) was administered using a 32-gauge needle in a final volume of 50 µL as an intracardiac (i.c.) injection once at the time of reperfusion (Na\(_2\)S) or once at the time of reperfusion followed by daily tail vein (i.v.) injections for the first 7 days of reperfusion (Na\(_2\)S 7d). This dose of Na\(_2\)S was selected based on our previous experience investigating Na\(_2\)S in murine models of
cardiac ischemia-reperfusion injury. Saline was administered in the same way for the respective vehicle groups.

**Heart failure Protocols.** Heart failure was induced by either permanent ligation of the LCA or by subjecting the mice to 60 min of LCA occlusion followed by reperfusion for up to 4 weeks. Surgical ligation of the LCA was performed according to methods described previously. All mice were randomly allocated to the treatment groups.

**Myocardial Area-at-Risk and Infarct Size Determination.** Left ventricular area-at-risk (AAR) and infarct size (INF) determination was performed using Evans blue dye and 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) staining method. All of the procedures for the AAR and INF determination have been previously described.

**Echocardiographic Assessment of Left Ventricular Structure and Function.** Baseline echocardiography images were obtained one week prior to LCA ischemia to avoid any anesthetic effects as previously described. The mice were lightly anesthetized with isoflurane 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 (EKV) B mode images acquired at the rate of 1000 frames/sec over 7 minutes. These images were used to measure LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD) and ejection fraction (EF). Echocardiography images were obtained and analyzed again 4 wk following the induction of myocardial ischemia.
**Histological Analysis of Infarct Size.** After the post myocardial infarction echocardiographic assessment, the mice were re-anesthetized, intubated, and connected to a rodent ventilator as previously described. A median sternotomy was performed and the heart was rapidly excised and fixed in conventional fixing solutions (4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer). After 12 hours in 4% paraformaldehyde, the heart was cut into 1 mm thick as detailed above. The slices were section with a cryostat and then stained with hematoxylin and eosin (H&E). Digital images of the slides were then captured and analyzed using computer-assisted planimetry with NIH ImageJ 1.37 software to measure the area of infarct or scar relative to the left ventricle. For each heart, we analyzed multiple sections taken from the mid-ventricle and then averaged these numbers to obtain a single INF/LV measurement for each animal.

**Lipid hydroperoxide assay.** Quantification of lipid peroxidation was performed to assess the extent of cardiac oxidative stress as described previously. Lipid peroxidation results in the formation of highly unstable and reactive hydroperoxides of both saturated and unsaturated lipids. Cardiac tissue was collected at 1 and 4 weeks of reperfusion. Lipid hydroperoxides were measured using a commercially available kit (Cayman Chemicals) according to the manufacturer's recommendations. The assay is based on the principle that hydroperoxides are highly unstable and react with ferrous ions readily to produce ferric ions. The resulting ferric ions are detected using thiocynate as the chromogen. Myocardial lipid hydroperoxide (LPO) is reported in micromolars.
Quantitative Real-Time Polymerase Chain Reaction for Mitochondrial DNA

Mitochondrial DNA (mtDNA) content was quantified by real-time reverse-transcription polymerase chain reaction with cardiac DNA as described previously. Briefly, DNA was extracted from frozen heart tissue of sham operated, vehicle and Na$_2$S-treated mice by trizole (Sigma, St Louis, MO)/ chloroform extraction followed by on column purification and elution (RNeasy mini kit, Quiagen). Total RNA concentration was determined with a spectrometer. Five nanograms of genomic DNA were assayed in triplicate with Sybrgreen core reagents (Applied Biosystems, Foster City, Calif) and cytochrome b (mitochondrial) or β-actin (nuclear) and a Mini Opticon Detector (Biorad). mtDNA per nuclear genome was calculated as the ratio of cytochrome b DNA to β-actin DNA quantity.

Cardiac Mitochondria Isolation. Cardiac mitochondria were isolated from the following groups of mice: sham operated, vehicle and Na$_2$S-treated mice. Briefly, the heart was quickly excised and washed in buffer containing 200 mM sucrose, 20 mM Tris, 2 mM EGTA, pH 7.4 at 4°C. After changes of buffer, the cardiac samples were cut into small pieces and homogenized. The samples were centrifuged at 450 X g for 3 min to remove debris, and mitochondria were obtained by a differential centrifugation technique as previously described. All isolated mitochondria were kept on ice and used within 3 h of isolation.

Mitochondrial Respiratory Rate and ATP synthesis. Oxygen consumption of cardiac mitochondria was measured in a sealed chamber magnetically stirred at 37°C by using calibrated Clark-type electrodes in the presence of succinate (8 mmol/L) and glycerol-3-phosphate (4 mmol/L) as previously described. Maximal (ADP-stimulated) respiration
was measured after the addition of ADP (1 mmol/L). Additionally, respiration in the absence of ADP phosphorylation was determined in the presence of 1 mg/ml oligomycin. Respiratory control ratios were determined as the ratio of oligomycin to state 3 respirations. To evaluate ATP synthesis, aliquots were taken from the respiration chamber over a 1-minute period after the addition of ADP. ATP was then quantified with a bioluminescence assay using an ATP determination kit (A-22066; Molecular Probes, Eugene, OR). The ATP/O₂ ratio was calculated with the state 3 respiratory rate for each sample.\(^7\)

**Western blot analysis.** Western blot analysis was performed as described previously.\(^2\) 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 Nrf2 (1:3000; abcam, Cambridge, MA); anti-rabbit Akt (1:5000; Cell Signaling, Danvers, MA); anti-rabbit phosphorylated Ser473 Akt (1:2000; Cell Signaling); anti-rabbit NRF1 (1:3000; abcam). Immunoblots were next processed with secondary antibodies (anti-rabbit; Cell Signaling) 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. 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 (1:5000; Cell Signaling) and anti-α-tubulin served as the subcellular marker for the cytosolic fraction (1:40000; Santa Cruz, Santa Cruz, CA). 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) as previously described.²

**Statistical Analysis.** All the data in this study are expressed as mean ± standard error (SEM). Means were compared using Prism 4 (GraphPad Software, Inc) with Student’s unpaired 2-tailed t-test (Western Blot analysis), one-way analysis of variance (ANOVA; heart to body weight ratios, LPO data, mitochondrial DNA and mitochondrial respiration data), or two-way ANOVA (echocardiography data) where appropriate. For the ANOVA, if a significant result was found, the Tukey (one-way ANOVA) or Bonferroni (two-way ANOVA) test was used as the post hoc analysis. The survival curves were compared with a Log-rank (Mantel-Cox) Test. For all data, a p value less than 0.05 was considered significant.
Online Figure Legends

Supplemental Figure 1. Alterations in heart rates following myocardial ischemia. Heart rates were evaluated with a Visualsonics Vevo 770 echocardiography machine in Non-transgenic (Non-Tg) and αMHC-CGL-Tg mice at baseline and 4 weeks following (A) permanent left coronary artery (LCA) occlusion and (B) 60 minutes of LCA occlusion. Heart rates were also evaluated at baseline and 4 weeks after 60 minutes of LCA occlusion in C57BL/6J mice administered (C) a single injection of Na₂S (100 µg/kg) or (D) an injection of Na₂S (100 µg/kg) once daily for the first 7 days of reperfusion. Numbers inside bars indicate the number of animals that were investigated in each group. *p<0.05, **p<0.01, and ***p<0.001 vs. Base. Base, baseline. Post, 4 wk post myocardial ischemia. Means were compared with a two-way ANOVA using a Bonferroni test as the post-hoc analysis.

Supplemental Figure 2. Infarct Area following myocardial ischemia. The infarct area (INF) relative to the entire left ventricle (LV) was evaluated at 4 wk following myocardial ischemia in Non-transgenic (Non-Tg) and αMHC-CGL-Tg mice subjected to (A) permanent left coronary artery (LCA) occlusion and (B) 60 minutes of LCA occlusion. INF area was also evaluated 4 wks after 60 minutes of LCA occlusion in C57BL/6J mice administered (C) a single injection of Na₂S (100 µg/kg) or (D) an injection of Na₂S (100 µg/kg) once daily for the first 7 days of reperfusion. For each heart, we analyzed multiple sections taken from the mid-ventricle and then averaged these numbers to obtain a single INF/LV measurement for each animal. Numbers inside bars indicate the
number of animals that were investigated in each group. *p<0.05 vs. Vehicle and **p<0.01 vs. Non-Tg. Means for all data were compared with an unpaired t-test.

**Supplemental Figure 3.** Daily administrations of Na₂S alter the expression of Nrf2 and NRF1 in the liver. (A) Representative immunoblots and densitometric analysis of hepatic Nrf2 and NRF-1 in the cytosolic and nuclear fractions following 1 week of Na₂S treatment. (B) Representative immunoblots and densitometric analysis of phosphorylated Akt at serine residue 473 (AktSer473) and total Akt following 1 week of Na₂S treatment. Values are means ± S.E.M. *p<0.05 vs. Vehicle. Means for all data were compared with an unpaired t-test.

**Supplemental Figure 4.** Cardiac-Specific overexpression of CGL alters the cardiac expression of NRF1 and Akt. (A) Representative immunoblots and densitometric analysis of NRF-1 in the cytosolic and nuclear fractions of hearts from αMHC-CGL-Tg+ and Non-Tg mice. (B) Representative immunoblots and densitometric analysis of phosphorylated Akt at serine residue 473 (AktSer473) and total Akt from the hearts of αMHC-CGL-Tg+ and Non-Tg mice. Values are means ± S.E.M. *p<0.05 and **p<0.01 vs. Non-Tg. Means for all data were compared with an unpaired t-test.

**Supplemental Figure 5.** Cardiac-Specific overexpression of CGL does not alter the hepatic expression of Nrf2, NRF1 and Akt. (A) Representative immunoblots and densitometric analysis of Nrf2 and NRF-1 in the cytosolic and nuclear fractions of livers
SUPPLEMENTAL MATERIAL

from \( \alpha \text{MHC-CGL-Tg}^+ \) and Non-Tg mice. (B) Representative immunoblots and densitometric analysis of phosphorylated Akt at serine residue 473 (Akt\( ^{\text{Ser473}} \)) and total Akt from the livers of \( \alpha \text{MHC-CGL-Tg}^+ \) and Non-Tg mice. Values are means ± S.E.M. Means for all data were compared with an unpaired t-test.


Supplemental Figure 1

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Supplemental Figure 3

A. Cytosolic Fraction Nuclear Fraction

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Vehicle (100 µg/kg, 7d) Na₂S (100 µg/kg, 7d)

- Vehicle
- Na₂S (100 µg/kg 7d)

Nrf2 (Relative Intensity)

- Cytosolic
- Nuclear

NRF1 (Relative Intensity)

- Cytosolic
- Nuclear

B. Akt-P<sup>Ser473</sup>

- Vehicle
- Na₂S (100 µg/kg 7d)

Akt

- Vehicle
- Na₂S (100 µg/kg, 7d)

α-Tubulin

- Vehicle
- Na₂S (100 µg/kg, 7d)

Akt (Relative Intensity)

- Vehicle
- Na₂S (100 µg/kg 7d)

Akt-P<sup>Ser473</sup>/AKT

- Vehicle
- Na₂S (100 µg/kg 7d)
Supplemental Figure 4

A. Cytosolic Fraction | Nuclear Fraction
---|---
NRF1 | 67 kDa
Fibrilarin | 37 kDa
α-Tubulin | 56 kDa

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NRF1 (Relative Intensity)

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B. Akt-P<sub>Ser473</sub> | 60 kDa
Akt | 60 kDa
α-Tubulin | 56 kDa

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Akt (Relative Intensity)

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