Hydrogen sulfide attenuates myocardial ischemia-reperfusion injury by preservation of mitochondrial function

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Edited by Solomon H. Snyder, Johns Hopkins University School of Medicine, Baltimore, MD, and approved August 7, 2007 (received for review June 26, 2007)

The recent discovery that hydrogen sulfide (H_2S) is an endogenously produced gaseous second messenger capable of modulating many physiological processes, much like nitric oxide, prompted us to investigate the potential of H_2S as a cardioprotective agent. In the current study, we demonstrate that the delivery of H_2S at the time of reperfusion limits infarct size and preserves left ventricular (LV) function in an *in vivo* model of myocardial ischemia-reperfusion (MI-R). This observed cytoprotection is associated with an inhibition of myocardial inflammation and a preservation of both mitochondrial structure and function after I-R injury. Additionally, we show that modulation of endogenously produced H_2S by cardiac-specific overexpression of cystathionine γ-lyase (α-MHC-CGL-Tg mouse) significantly limits the extent of injury. These findings demonstrate that H_2S may be of value in cytoprotection during the evolution of myocardial infarction and that either administration of H_2S or the modulation of endogenous production may be of clinical benefit in ischemic disorders.

Hydrogen sulfide (H_2S) represents the most recently identified endogenously produced gaseous messenger (1). Although long considered a noxious gas with wide-ranging cytotoxic effects (2), there is now an accumulation of scientific evidence that H_2S plays a prominent role in cellular signaling. The production of H_2S in mammalian systems has been attributed to two key enzymes in the cysteine biosynthesis pathway, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CGL). CBS and CGL are heme-containing enzymes whose activity depends on the cofactor pyridoxal 5′-phosphate (PLP). CBS is capable of catalyzing the reaction of cysteine with free thiols to generate H_2S; and likewise thiocysteine generated by CGL can interact with thiols to generate H_2S (3).

The endogenous production of H_2S was initially described in the brain and attributed to CBS (1). Emerging evidence suggests that H_2S is a regulator of the *N*-methyl-D-aspartate receptor and may be central in long-term-potentiation of neuronal circuitry (4, 5). In addition, H_2S is known to be produced in the vasculature by CGL where it is proposed to mediate smooth muscle relaxation and subsequent vasodilation independent of the GC/cGMP pathway (6).

A great deal of scientific focus over the last two decades has been aimed at elucidating the role of nitric oxide (NO) and carbon monoxide in physiology and pathophysiology (3). The critical function of nitric oxide was made apparent by the finding that it was central in smooth muscle relaxation and vasodilation (7, 8) and necessary for the maintenance of vascular homeostasis (9). In recent years the translation of these basic science findings into the clinical setting demonstrate the therapeutic potential of gaseous signaling molecules (10). The discovery that hydrogen sulfide is an endogenously produced gaseous second messenger capable of modulating many physiological processes (11) including vasodilation (6, 12), much like NO, prompted us to investigate the potential of H_2S as a cardioprotective agent.

In the current manuscript, we establish that H_2S administered at the time of reperfusion decreases infarct size and preserves left ventricular function in an *in vivo* model of myocardial ischemia-reperfusion (MI-R). The observed protection is associated with reduced myocardial inflammation, preserved mitochondrial function and reduced cardiomyocyte apoptosis after infarction. Additionally, we demonstrate that overexpression of the enzyme CGL and subsequent increase in myocardial H_2S production, likewise limits the extent of MI-R injury. The current results suggest that H_2S is cytoprotective during the evolution of myocardial infarction and that either direct H_2S administration or the modulation of endogenous H_2S production may be of clinical importance.

Results

H_2S Dose-Dependently Limits Myocardial Infarct Size and Preserves Left Ventricular (LV) Structure and Function. In initial studies, mice were subjected to 30 min of LV ischemia and 24 h reperfusion. H_2S donor was administered into the LV lumen at the time of reperfusion at doses ranging from (10–500 μg/kg). Evaluation of infarct size revealed a dose-response curve with 50 μg/kg displaying the most significant cytoprotection as assessed by 2,3,5-triphenyltetrazolium chloride (TTC) staining (Fig. 1 *A* and *B*). Mice receiving 50 μg/kg displayed an infarct size per area-at-risk (INF/AAR) of (13.4 $\pm$ 1.4%) as compared with vehicle treated mice (47.9 $\pm$ 2.9%), representing a 72% reduction in infarct size. Representative mid-ventricular cross-sections of vehicle and H_2S (50 μg/kg) treated mice are shown in Fig. 1*A*. Additionally, all groups displayed similar AAR/LV (Fig. 1*B*). This optimal dose of 50 μg/kg was therefore used in all subsequent *in vivo* studies.

Circulating plasma levels of the cardiac-specific isoform of troponin-I (cTnI) were evaluated as an additional marker of myocardial injury (Fig. 1*C*). Serum samples from mice subjected to

Author contributions: J.W.E., J.W.C., and D.J.L. designed research; J.W.E., J.W.C., J.M., J.E.D., D.W.K., L.T., X.J., R.S., L.K., and C.S. performed research; J.W.E., J.E.D., D.W.K., H.K., and C.-W.C. contributed new reagents/analytic tools; J.W.E., J.W.C., and D.J.L. analyzed data; and J.W.E. and D.J.L. wrote the paper.

Conflict of interest statement: Funding for this study was provided by Ikaria, Inc. (Seattle, WA), and the hydrogen sulfide donor was also provided by Ikaria. D.J.L. is currently serving as a paid consultant to Ikaria, and C.S. is currently employed by Ikaria.

This article is a PNAS Direct Submission.

Abbreviations: CBS, cystathionine β-synthase; CGL, cystathionine γ-lyase; MI-R, myocardial ischemia-reperfusion; LV, left ventricular; INF/AAR, infarct size per area-at-risk; LCA, left coronary artery; MPO, myeloperoxidase; TTC, 2,3,5-triphenyltetrazolium chloride.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0705891104/DC1.

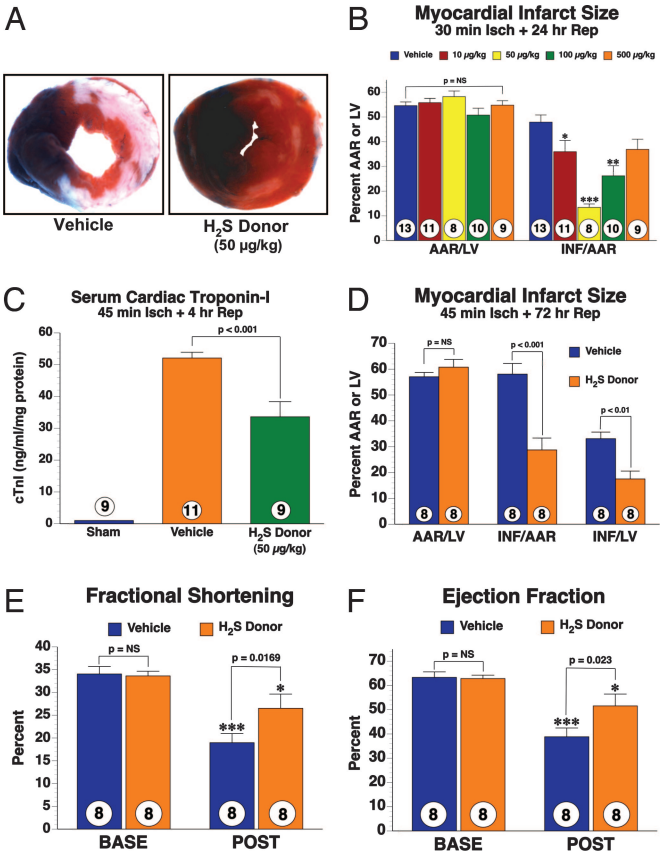

Fig. 1. H_2S donor therapy in MI-R. (*A*) Representative mid-myocardial cross sections of TTC-stained hearts for vehicle and 50 μg/kg H_2S donor. Dark blue area (i.e., Evan's blue-stained), nonischemic zone; remaining area, AAR; white area, infracted tissue; red (i.e., TTC-positive), viable myocardium. (*B*) Doses of (10–500 μg/kg) or vehicle were injected i.v. at the time of reperfusion. INF/AAR revealed a U-shaped dose–response curve. (*C*) Serum cardiac troponin-I (cTnI) in sham-, vehicle-, and H_2S (50 μg/kg)-treated mice. (*D*) Myocardial infarct size after 45-min LCA ischemia and 72-h reperfusion. H_2S-treated mice displayed a significant reduction in INF/AAR as well as INF/LV. (*E*) Percent fractional shortening (%FS) after myocardial infarction. Postinfarction H_2S-treated mice displayed a significant improvement in %FS as compared with vehicle-treated mice. (*F*) Percent ejection fraction (%EF) after myocardial infarction. Postinfarction H_2S-treated mice displayed a significant improvement in %EF as compared with vehicle-treated mice (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ vs. BASE). (Circles inside bars denote *n* per group.)

45 min of left coronary artery (LCA) ischemia and 4 h reperfusion receiving either vehicle or 50 μg/kg H_2S donor were found to contain (52.1 ± 1.8 vs. 33.6 ± 4.8 ng/ml per mg protein), thus confirming the cytoprotective effect of H_2S.

Mice underwent LV echocardiographic analysis one week before 45 min of LCA ischemia and again after 72 h of reperfusion. Confirming the earlier findings mice receiving 50 μg/kg at the time of reperfusion were found to have a 51% reduction in INF/AAR as compared with vehicle treated mice (Fig. 1*D*).

H_2S-treated mice displayed no significant increase in post-MI LV dimensions [left ventricular end-diastolic dimension (LVEDD) and left ventricular end-systolic dimension (LVESD)] as compared with vehicle treated mice which displayed significantly increased LVESD and LVEDD after MI (Table 1). Additionally, mice receiving the H_2S donor were found to have a 21% decrease in fractional shortening 72 h after MI as compared with mice receiving vehicle, which displayed a 44% reduction in fractional shortening (Fig. 1*E*). In parallel, H_2S-treated mice displayed a minimal 18% reduction in ejection fraction post-MI as compared with mice receiving vehicle that displayed a 39% reduction (Fig. 1*F*). There were no significant differences in heart rate at baseline or post-MI between the groups (Table 1).

Table 1. MI-R echocardiographic data

Group		*n*	LVEDD, mm	LVESD, mm	HR, bpm
Base	Vehicle	8	3.69 ± 0.14	2.46 ± 0.11	423 ± 18
	H_2S	8	3.69 ± 0.11	2.45 ± 0.10	410 ± 16
Post	Vehicle	8	4.62 ± 0.15*	3.75 ± 0.20*	521 ± 11*
	H_2S	8	3.90 ± 0.17†	2.90 ± 0.24†	529 ± 12*

Mice underwent echocardiographic analysis 1 wk before (Base) 45 min. ischemia and following 72 h reperfusion (Post). Mice received either vehicle or H_2S donor (50 μg/kg i.v.) at reperfusion. *n*, number of animals per group; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; HR, heart rate. *, $P < 0.01$ vs. baseline; †, $P < 0.01$ vs. Vehicle Post.

H_2S Reduces Myocardial Inflammation After MI-R. Blinded histological analysis of heart sections stained with H&E were scored 24 h after reperfusion (Fig. 2 *A* and *B*). Mice receiving H_2S donor displayed a reduced degree of myocardial neutrophilic infiltrate, necrosis, hemorrhage, and spindle-shaped interstitial cells as compared with mice receiving vehicle.

To quantify neutrophilic infiltrate myeloperoxidase (MPO) activity was analyzed 4 h after reperfusion. H_2S-treated mice were found to have a 26% reduction in MPO activity as compared with vehicle-treated mice (Fig. 2*C*).

ELISA analysis of key myocardial cytokines after 45 min ischemia and 4 h reperfusion revealed H_2S-treated mice to have no significant increase in IL-1β as compared with sham animals (206 ± 21 vs. 185 ± 26 pg/mg protein). In stark contrast, vehicle-treated mice were found to have a 2.1-fold increase in IL-1β [390 ± 90 pg/mg protein; supporting information (SI) Fig. 7*A*]. The assessment of TNF-α and IL-10 in cardiac lysate revealed no differences between any of the groups (SI Fig. 7 *B* and *C*).

Intravital microscopy of leukocyte rolling revealed that H_2S completely inhibited thrombin induced leukocyte-endothelial cell interactions (Fig. 2*D*). H_2S-treated mice displayed levels of leukocyte rolling similar to control levels at all time points analyzed.

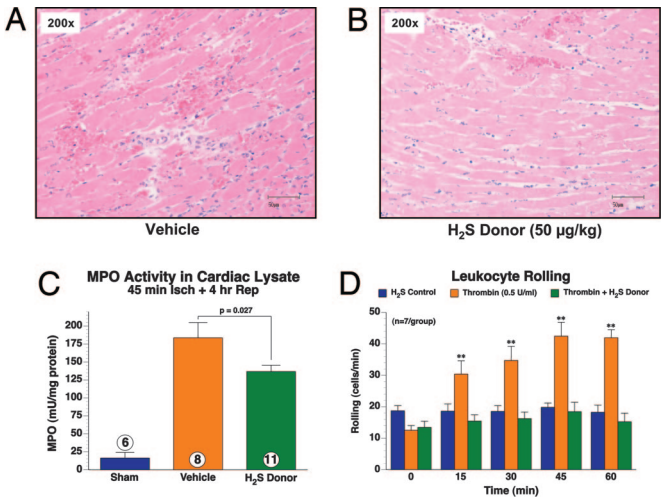

Fig. 2. H_2S therapy limits the extent of myocardial inflammation. Representative H&E-stained histological images after 45 min LCA ischemia and 24 h reperfusion. (*A*) Vehicle-treated mice displayed a high degree of hemorrhage and infiltrating leukocytes within the ischemic zone. (*B*) Histopathology was attenuated in the myocardial sections of mice treated with H_2S donor (50 μg/kg). (*C*) MPO activity was significantly decreased in mice treated with H_2S donor 4 h after reperfusion. (*D*) Leukocyte rolling was reduced down to control levels in mice receiving thrombin plus H_2S donor at all time points (**, $P < 0.01$ vs. all other groups, $n = 7$/group; circles inside bars denote *n* per group).

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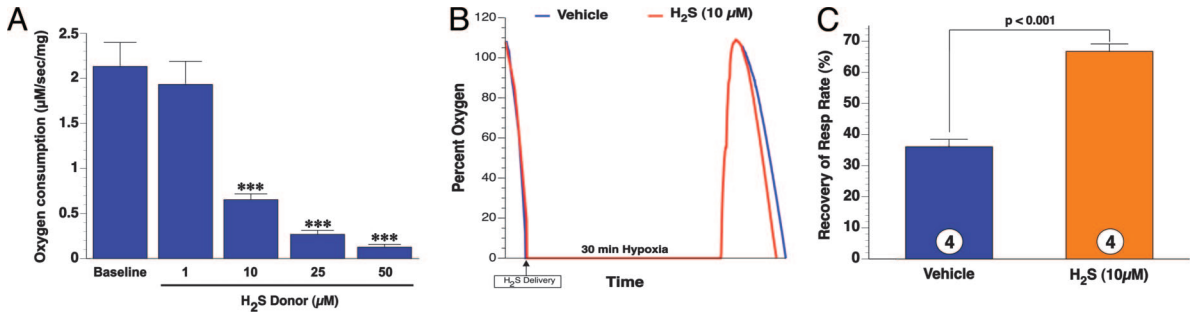

Fig. 3. H_2S donor inhibits cardiac mitochondrial respiration and preserves mitochondrial function in vitro. (*A*) Cardiac mitochondrial respiratory rate. Mitochondria isolated from WT mouse hearts were analyzed for oxygen consumption in the presence of H_2S. H_2S (1–50 μM) dose-dependently reduced mitochondrial respiration. (***, $P < 0.001$, $n = 4$ per group). (*B*) Representative tracings of O_2 consumption in vehicle- and H_2S-treated (10 μM) mitochondria. Whereas both groups had similar rates of O_2 consumption before hypoxia, H_2S-treated mitochondria displayed a significantly greater rate of O_2 consumption 30 min after hypoxia as evident by a steeper slope of the red line. (*C*) Mitochondrial recovery 30 min after hypoxia. Percentage recovery of respiration rate was significantly greater in mitochondria treated with H_2S donor. Circles inside bars denote *n* per group.

H_2S Dose-Dependently Inhibits Cardiac Mitochondrial Respiration and Preserves Mitochondrial Function *in Vitro*. Oxygen consumption during state 3 respiration was measured in mitochondria isolated from murine hearts. H_2S (1–50 μM) was introduced into a sealed chamber after steady-state respiration and O_2 was monitored with a Clark-type electrode. A dose dependent reduction of oxygen consumption compared with baseline was observed: (1 μM = 7%, 10 μM = 63%, 25 μM = 86%, 50 μM = 95%, Fig. 3*A*). Freshly isolated cardiac mitochondria were then subjected to an *in vitro* hypoxic assay. Mitochondria in the presence of 10 μM H_2S were found to have a significantly improved recovery of respiration rate after 30 min of hypoxia as seen by the steeper slope in Fig. 3*B*. The numeric results can be seen in Fig. 3*C* with a 36 ± 2% recovery in the vehicle-treated group as compared with a 67 ± 2% recovery in H_2S-treated mitochondria.

H_2S Preserves Mitochondrial Function and Membrane Integrity After *in Vivo* MI-R Injury. Cardiac mitochondria were isolated from mice subjected to 45 min ischemia and 24 h reperfusion for assessment of respiratory rate. Mitochondria isolated from vehicle-treated mice were found to have a 3.4-fold decrease in the rate of O_2 consumption (complex I substrate) as compared with sham-operated animals. Mitochondria from mice receiving 50 μg/kg H_2S at reperfusion were found to have a significantly greater rate of O_2 consumption as compared with mice receiving vehicle (Fig. 4*A*). Mitochondria from the same groups were also analyzed for complex II efficiency. There was a 3.2-fold decrease in the rate of O_2 consumption in mitochondria isolated from vehicle-treated mice. The respiration rate in mitochondria, isolated from H_2S-treated animals, was increased 2.2-fold over mitochondria isolated from vehicle-treated animals (Fig. 4*B*).

After 45 min of ischemia and 24 h reperfusion myocardial samples were qualitatively assessed by transmission electron microscopy for structural mitochondrial changes (Fig. 4*C*). Longitudinal sections of vehicle-treated hearts displayed uniform mitochondrial swelling with disorganized cristae and decreased matrix density. The presence of amorphous matrix densities or granular dense bodies, a distinctive feature of irreversible myocardial cell injury after reperfusion, can also be seen in a number of mitochondria in vehicle-treated samples. H_2S donor-treated hearts displayed little change in mitochondrial structure. In general, mitochondria appeared highly dense with well organized cristae.

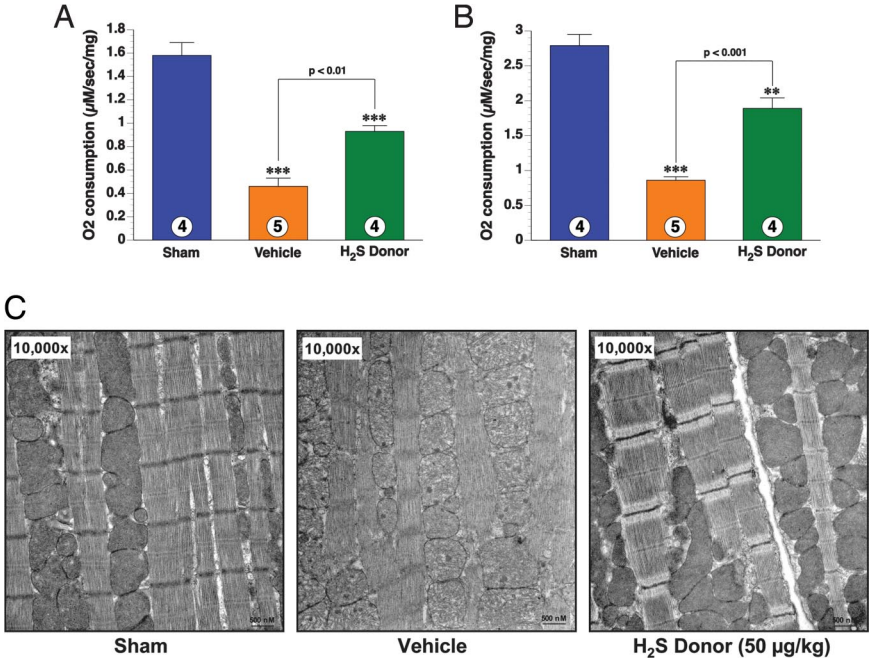

Fig. 4. H_2S preserves mitochondrial function and structure after *in vivo* MI-R. Mitochondria were isolated from hearts after 45 min LCA ischemia and 24 h reperfusion. (*A*) Mice treated with H_2S at the time of reperfusion displayed a significant improvement in mitochondrial complex I efficiency as determined by using pyruvate and malate as substrate. (*B*) The efficiency of complex II was assessed by using succinate and G3P as substrate and inhibiting complex I with rotenone. The same H_2S donor-treated mice displayed significantly greater rate of O_2 consumption after MI-R (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. sham). Circles inside bars denote *n* per group. (*C*) After 45 min of ischemia and 24 h reperfusion, myocardial samples were qualitatively assessed by transmission electron microscopy for structural mitochondrial changes. Longitudinal sections of vehicle-treated hearts displayed uniform mitochondrial swelling with disorganized cristae and decreased matrix density. The presence of amorphous matrix densities or granular dense bodies, a distinctive feature of irreversible myocardial cell injury after reperfusion, can also be seen in a number of mitochondria in vehicle-treated samples. H_2S donor-treated hearts displayed little change in mitochondrial structure. Generally, mitochondria seemed to be highly dense with well organized cristae with little distinguishable differences from sham samples.

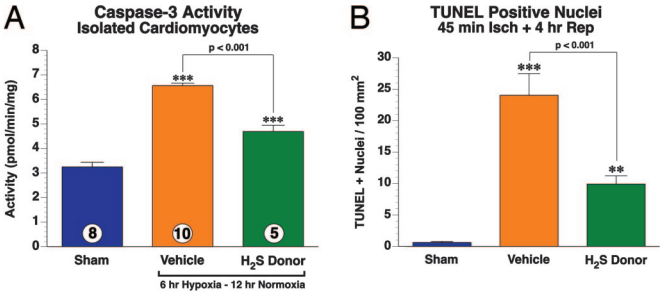

Fig. 5. H_2S reduces cardiomyocyte apoptosis *in vitro* and *in vivo* after MI/R. (*A*) Isolated adult cardiomyocytes were subjected to 6 h hypoxia and 12 h reoxygenation. Groups receiving the H_2S donor (100 μM) at the time of reoxygenation displayed a significant reduction in caspase-3 activity (circles inside bars denote *n* per group). (*B*) After 45 min LCA ischemia and 4 h reperfusion, mouse heart samples from sham, vehicle, and H_2S donor-treated mice were evaluated for TUNEL-positive nuclei. H_2S donor-treated mice were found to have a 59% reduction in the number of TUNEL-positive nuclei as compared with vehicle-treated animals ($P < 0.001$, $n = 6$ per group).

H_2S Reduces Cardiomyocyte Apoptosis *in vitro* and *in vivo* After MI-R. Isolated adult cardiomyocytes were subjected to 6 h hypoxia and 12 h reoxygenation. Groups receiving the H_2S donor at the time of reoxygenation displayed a significant reduction in caspase-3 activity as compared with vehicle-treated myocytes (Fig. 5*A*).

After 45 min LCA ischemia and 4 h reperfusion mouse heart samples from sham, vehicle and H_2S donor-treated mice were evaluated for TUNEL positive nuclei. H_2S donor-treated mice were found to have a 59% reduction in the number of TUNEL positive nuclei as compared with vehicle-treated animals (Fig. 5*B*).

Hemodynamics After H_2S Donor Administration. Mice were implanted with radiotelemeters and subjected to femoral vein injection of H_2S. H_2S had no effect on mean arterial blood pressure (MABP) at doses ranging from 10–500 μg/kg, as compared with vehicle-treated mice (SI Table 2). H_2S (1 mg/kg) did significantly reduce MABP from (102.4 ± 2.3 to 64.6 ± 6.3 mmHg), representing a 37% decrease that lasted for an average of 7.4 seconds followed by a period of hypertension. Assessment of heart rate revealed no significant alteration after doses ranging from 10–100 μg/kg as compared with vehicle-treated mice (SI Table 3).

Mice with Cardiac-Restricted Overexpression of Cystathionine γ-Lyase (αMHC-CGL-Tg) Are Protected Against MI-R Injury. Transgenic mice were generated by using a construct consisting of the αMHC promoter driving and restricting expression of the murine CGL gene to cardiomyocytes (Fig. 6*A*). Incorporation of the transgene was identified by RT-PCR analysis of genomic DNA (data not shown). αMHC-CGL-Tg mice and their WT littermates were analyzed for increased myocardial CGL message. Tg mice were found to have a significant increase in CGL mRNA as corrected by the housekeeping gene GAPDH (Fig. 6*B*). Additionally, no compensatory alterations in the myocardial expression of cystathionine β-synthase (CBS) were observed. This increase in CGL message translated to increased protein expression as can be seen in the immunoblot and calculated optical density in Fig. 6*C*. Importantly, this increase in CGL enzyme resulted in an ≈2-fold increase in H_2S production by myocardial homogenates of αMHC-CGL-Tg mice as assessed by an H_2S specific electrode (Fig. 6*D*).

αMHC-CGL-Tg mice and their WT littermates were subjected to 45 min LCA occlusion and 72 h reperfusion. TTC analysis of infarct size revealed αMHC-CGL-Tg mice to have a significant reduction in infarct size (representative mid-myocardial sections can be seen in Fig. 6*E*). αMHC-CGL-Tg mice exhibited a 47% reduction in INF/AAR as compared with WT littermates (Fig. 6*F*).

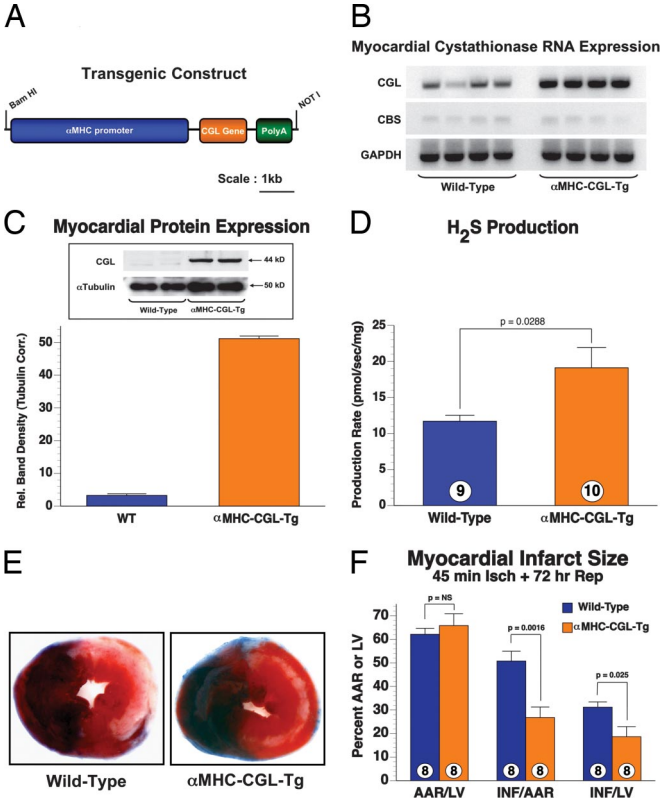

Fig. 6. αMHC-CGL-Tg mice are protected against MI-R injury. (*A*) Transgenic construct. (*B*) Myocardial cystathionase RNA expression. αMHC-CGL-Tg mice and their WT littermates were analyzed for increased myocardial CGL message. Tg mice were found to have a significant increase in CGL mRNA as corrected by the housekeeping gene GAPDH with no alteration in the expression of cystathionine β-synthase (CBS). (*C*) Myocardial protein expression. Increase in message translated to a significant increase in protein expression as can be seen in the immunoblot and calculated optical density. (*D*) H_2S production. The increase in CGL enzyme translated into ≈2-fold increase in H_2S production by myocardial homogenates of αMHC-CGL-Tg mice as assessed by an H_2S specific electrode (circles inside bars denote *n* per group). (*E*) Representative mid-myocardial cross sections of TTC-stained hearts for WT and αMHC-CGL-Tg mice after 45 min LCA ischemia and 72 h reperfusion. (*F*) Myocardial infarct size (45 min ischemia and 72 h reperfusion). Transgenic mice displayed a reduction in infarct size per area-at-risk (INF/AAR) as compared with WT littermates. Infarct per LV (INF/LV) was also significantly reduced.

Discussion

In the current study we show that administration of H_2S at the time of reperfusion limits the extent of myocardial infarction in an *in vivo* murine model. A dose–response study revealed that H_2S displayed a biphasic reduction in infarct size as has previously been reported by Johansen *et al.* (13). Importantly, the decrease in infarct size translated into reduced LV dilatation and improved LV function as measured by echocardiography. In an effort to further explore the role of H_2S in myocardial infarction, we generated cardiac-specific transgenic mice with overexpression of the H_2S producing enzyme CGL (αMHC-CGL-Tg), resulting in increased myocardial levels of H_2S. These mice were also found to have a reduction in infarct size after MI-R injury establishing that exogenous, or endogenous increases in H_2S protect against myocardial infarction. This finding that modulation of endogenous H_2S is cardioprotective is supported by the recent work of Sivarajah *et al.* (14) which showed that pharmacologic inhibition of CGL resulted in an increase in infarct size in a rat model of MI-R.

Given that H_2S has been reported (6) to act as a vasodilator we assessed whether H_2S modulated systemic hemodynamics. A dose–

response study in mice implanted with radiotelemetric pressure transducers revealed no effect on systemic blood pressure and heart rate in conscious animals until doses well outside the therapeutic range were administered. These findings ruled out the possibility that H_2S was altering afterload and thus impacting myocardial oxygen demand.

We next assessed the impact of H_2S on myocardial inflammation after infarction. Histological analysis revealed a substantial decrease in hemorrhage and necrosis as well as a decrease in the number of leukocytes within the ischemic zone. MPO analysis quantitatively confirmed a significant decrease in neutrophils within the myocardial tissue after MI-R. The evaluation of inflammatory cytokines revealed myocardial levels of IL-1β to be markedly reduced to those observed in sham-operated mice. Additionally, H_2S was found to potently reduce *in vivo* leukocyte-endothelial cell interactions, as assessed by intravital microscopy. This finding is bolstered by a recent report by Zanardo *et al*. (15) showing that H_2S limited aspirin-induced inflammation by inhibiting leukocyte rolling, adhesion and subsequent diapedesis. Inhibition of leukocyte transmigration is one possible mechanism by which H_2S restrains the extent of inflammation and thereby limits the extent of myocardial infarction. A number of studies (16) have cited the influence of IL-1β on the development of ischemic injury in the heart and the pathogenic role of inflammatory cytokines in the recruitment and subsequent migration of inflammatory cells into the heart (17).

Another important area of study in myocardial cytoprotection is the preservation of mitochondrial function (18, 19). The mitochondria are unique in that not only are they the site of energy production but also a central locus in the regulation of cell death (20, 21). The maintenance of oxidative phosphorylation to forgo myocyte death in the face of ischemic injury has long been recognized as a critical event after myocardial infarction (22). To optimize administration of the H_2S donor for *in vitro* experiments we first performed a dose–response in isolated cardiac mitochondria to determine its effect on respiration. As has been previously reported in a placental cell line (23) we found a dose-dependent reduction in mitochondrial oxygen consumption followed by complete recovery to baseline. Based on these findings, we selected 10 μM, which reduced mitochondrial respiration rate by 65%, in a clear and reproducible manner. Mitochondria treated with 10 μM H_2S were found to have a significantly greater recovery of posthypoxic respiration rate. These experiments were followed by the discovery that mitochondria isolated from mice given H_2S at the time of reperfusion (*in vivo* MI-R) displayed preserved mitochondrial function 24 h following reperfusion as noted by increased complex I and II efficiency. Electron microscopy revealed a striking reduction in mitochondrial swelling and increased matrix density in mice receiving H_2S, further suggesting a prominent role for the preservation of mitochondrial function in the observed cytoprotection.

H_2S is known (24) to be a potent and reversible inhibitor of cytochrome *c* oxidase (complex IV of the mitochondrial electron transport chain) and the profound nature of H_2S ability to influence whole organism metabolism was recently demonstrated by its ability to induce a suspended animation-like state in mice (25). Another possible mechanism for H_2S protective action on mitochondrial function may lie in its ability to modulate cellular respiration during reperfusion. The inhibition of mitochondrial respiration has been shown (26, 27) to protect against MI-R injury by limiting the generation of reactive oxygen species and diminishing the degree of mitochondrial uncoupling leading to decreased infarct size and preserved function. The accumulation of evidence that mitochondrial function and structure was preserved after myocardial infarction in mice treated with H_2S was further corroborated by the decreased activation of caspase-3 and a decrease in the number of TUNEL positive nuclei, suggesting that H_2S was capable of inhibiting the progression of apoptosis after MI-R injury. The capacity of H_2S to modulate apoptosis has been controversial with some groups reporting H_2S mediating apoptosis (28–30) and others citing inhibition (31, 32). However, it is important to note that none of the studies to date have used *in vivo* model systems and have used varying concentrations of H_2S, some well outside the physiologically relevant range.

In conclusion, we have shown that either exogenous or endogenous (by means of Tg overexpression) increases in hydrogen sulfide at the time of reperfusion limits the extent of myocardial infarction. This protection is accompanied by a decrease in myocardial inflammation and a preservation of mitochondrial function. These results suggest that H_2S therapy may be a promising candidate for the treatment of acute myocardial infarction.

Materials and Methods

Detailed methods can be found in *SI Materials and Methods*.

H_2S Donor. Na_2S was produced and formulated to pH neutrality and iso-osmolarity by Ikaria Inc. (Seattle, WA) by using H_2S gas (Matheson, Newark, CA) as a starting material.

Mice. Male C57BL6/J mice 8 wk of age were purchased from The Jackson Laboratory (Bar Harbor, ME). αMHC-CGL-Tg mice were developed and bred in-house at Albert Einstein College of Medicine (AECOM). All experimental procedures complied with the *Guide for the Care and Use of Laboratory Animals* and were approved by the AECOM Animal Care and Use Committee.

MI-R Protocol. All surgical procedures were performed as previously described in detail (33).

Assessment of Infarct Size. After 24 or 72 h of reperfusion, infarct size was determined as previously described in detail (33).

Cardiac Troponin-I Assay. Mice were completely exsanguinated after 45 min of LCA ischemia and 4 h reperfusion. This time point was chosen after preliminary studies that revealed 4 h to be the time point for peak cardiac enzyme levels in the circulating plasma. Serum levels of the cardiac-specific isoform of troponin-I were assessed using an ELISA from Life Diagnostics (West Chester, PA).

Echocardiography. Detailed echocardiographic methods are described in *SI Materials and Methods*.

Hemodynamic Studies. Aortic blood pressures (systolic, diastolic, and mean) and heart rate were acquired in the conscious state by using radiotelemetry techniques as described in detail (33).

Myocardial Histology. After 45 min of ischemia and 24 h reperfusion hearts were rapidly excised, cross-sectioned and fixed in 10% buffered formalin. Fixed tissue was then paraffin embedded and sectioned in a standard fashion and stained with H&E. Slides were then assessed in a blinded fashion by a pathologist and scored for the following: percentage of LV involvement, myodegeneration, cardiomyocyte hydropic changes, neutrophilic infiltrate, hemorrhage, lymphohistiocytic infiltrate, and acute myocardial necrosis.

Quantitative Assessment of Neutrophil Accumulation. Hearts from mice subjected to 45 min of ischemia and 4 h reperfusion were assessed for MPO activity as a marker of neutrophil accumulation as described in detail (34).

Intravital Microscopy. Postcapillary venules (20-μm to 40-μm diameter) were observed under fluorescence microscopy, as described in detail (35).

Cardiac Mitochondria Isolation. Cardiac mitochondria were isolated from C57BL6/J mice 8 wk of age from the following groups: control, sham operated, vehicle-treated, or mice that received 50 μg/kg H_2S donor after 45 min ischemia and 24 h reperfusion. Briefly, the heart was quickly excised and washed in buffer containing 250 mM sucrose, 10 mM Tris, 1 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 3,000 $\times$ *g* for 3 min to remove debris, and mitochondria were obtained by a differential centrifugation technique (36). All isolated mitochondria were kept on ice and used within 3 h of isolation.

Mitochondrial Respiratory Rate. Oxygen consumption of cardiac mitochondria was measured in a sealed chamber magnetically stirred at 37°C by using calibrated Clark-type electrodes connected to an Instech amplifier and data recorded on a computer running Windaq software, method adapted from Shiva *et al.* (36).

***In Vitro* Mitochondria Hypoxia Assay.** State 3 respiration was initiated and the mitochondria were allowed to consume all oxygen until the chamber became anoxic. The mitochondria were left anoxic for 30 min in the sealed chamber. To reoxygenate, mitochondria were centrifuged and resuspended in oxygenated buffer containing fresh substrate and ADP for reestablishment of state 3 respiration. Post anoxic respiratory rate was expressed as a percentage of preanoxic rate and is referred to as recovery of mitochondrial respiration.

Transmission Electron Microscopy. After 45 min of ischemia and 24 h reperfusion, hearts were rapidly excised and fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, postfixed with 1% osmium tetroxide followed by 1% uranyl acetate, dehydrated through a graded series of ethanol and embedded in LX112 resin (LADD Research Industries). Ultrathin (80 nm) sections were cut on a Reichert Ultracut UCT, stained with uranyl acetate followed by lead citrate, and viewed on a JEOL 1200EX transmission electron microscope at 80 kV.

Caspase-3 Activity. Adult Cardiomyocytes were isolated as previously reported for determination of caspase-3 activity (37).

TUNEL Assay. TUNEL assay was conducted by using a kit according to the manufacturer's instructions (ApopTag HRP kit, DBA). The number of TUNEL-positive nuclei/high-power field was counted in a minimum of 20 fields for each section (n = 6/group).

Generation of CGL Transgenic Mice. Cardiac-specific CGL transgenic mice (αMHC-CGL-Tg) were generated by ligating the full-length *Mus musculus* cystathionine γ-lyase (CGL) cDNA (acquired from ATCC, gene bank no. BC019483) to the murine α-myosin heavy chain promoter (a kind gift of Dr. Jeffrey Robbins, Cincinnati Children's Hospital Medical Center) (38), followed by injection of the DNA into newly fertilized mouse embryos (FVB/n background, AECOM Transgenesis Core). Mice were genotyped by RT-PCR and used at 8–12 wk of age.

Western Blot Analysis. Protein analysis was performed as described in detail (39). The antibody was raised against the N terminus of CGL and has been described (40). Densitometric quantification of CGL and α-tubulin was performed using Quantity One (Bio-Rad).

RT-PCR of CGL mRNA. RNA isolation was carried out by using the RNeasy Protect Midi Kit (Qiagen, Valencia, CA). Reverse transcription was performed in standard fashion with QuantiTect Reverse Transcription Kit (Qiagen) supplemented with DNase treatment. PCRs were carried out on an MJ Mini Thermocycler (Bio-Rad) by using primers generated for GAPDH, cystathionine-γ-lyase (CGL) and cystathionine-β-synthase (CBS).

Polaragraphic Assessment of H_2S Production. The polaragraphic technique for assessment of H_2S production in tissue homogenates has been described in detail (41).

Statistical Analysis. All values are reported as mean ± SEM. Statistics were performed by using JMP 5.1 statistical software with the Student t test, one-way ANOVA, and Tukey post hoc tests when appropriate.

We thank Mark Duranski for surgical assistance and Iris Toedt-Pingel for technical assistance in these studies. Additionally, we thank Isao Ishii (National Institute of Neuroscience) for his contribution of the CGL antibody. The assistance of Asaf Stein is also noted for his contribution to H_2S production studies. The sodium sulfide solution was prepared by Paul Hill (Ikaria). These studies were supported by National Institutes of Health Grants 2RO1 HL060849-07 (to D.J.L.), 1F32 DK077380-01 (to J.W.C.), R01 DK064344 (to R.S.), and R21 GM73049-01 (to D.W.K.), American Diabetes Association Grant 7-04-RA-59 (to D.J.L.), and American Heart Association Grant in Aid 0455296B (to D.W.K.). Grant support was also received from Ikaria (D.J.L.).

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