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Hydrogen sulfide cytoprotective signaling is endothelial nitric oxide synthase-nitric oxide dependent

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Previous studies have demonstrated that hydrogen sulfide (H₂S) protects against multiple cardiovascular disease states in a similar manner as nitric oxide (NO). H₂S therapy also has been shown to augment NO bioavailability and signaling. The purpose of this study was to investigate the impact of H₂S deficiency on endothelial NO synthase (eNOS) function, NO production, and ischemia/reperfusion (I/R) injury. We found that mice lacking the H₂S-producing enzyme cystathionine γ-lyase (CSE) exhibit elevated oxidative stress, dysfunctional eNOS, diminished NO levels, and accelerated myocardial and hepatic I/R injury. In CSE KO mice, acute H₂S therapy restored eNOS function and NO bioavailability and attenuated I/R injury. In addition, we found that H₂S therapy fails to protect against I/R in eNOS phosphomutant mice (S1179A). Our results suggest that H₂S-mediated cytoprotective signaling in the setting of I/R injury is dependent on eNOS function and NO generation.

H₂S therapy has been shown to exert potent antioxidant actions during ischemic conditions (10). To examine whether endogenous eNOS uncoupling | myocardial infarction | cystathionase | Cth | nitrite

H₂S therapy has been shown to attenuate ischemia/reperfusion injury (I/R) (7, 8). Similarly, NO therapy and eNOS gene overexpression are also protective in ischemic disease states (9). Given the potent antioxidant actions of H₂S (10, 11) and the effects of exogenous H₂S therapy on NO bioavailability (5, 8), we investigated the effects of genetic deletion of the cystathionase gene (Cth, i.e., CSE KO) on the regulation of eNOS function and NO bioavailability.

Significance

Physiological concentrations of hydrogen sulfide (H₂S) exert potent prosurvival actions. We demonstrate that the cytoprotective actions of H₂S are mediated in part via a second gaseous signaling molecule, nitric oxide (NO). We found that cystathionine γ-lyase (CSE) KO mice with reduced H₂S levels exhibit increased oxidative stress and an exacerbated response to myocardial ischemia/reperfusion injury. CSE KO mice also exhibit reduced levels of NO and reduced NO synthesis via endothelial NO synthase (eNOS). Both oxidative stress and myocardial injury in CSE KO mice were attenuated by exogenous H₂S therapy, with increased eNOS function and restoration of NO levels. These findings provide insight into H₂S-mediated cytoprotection and important information regarding the translation of H₂S therapy to the clinic.


Conflict of interest statement: D.J.L. is a participant in a pending US patent, filed through the National Institutes of Health (patent no. 60/511, 244), regarding the use of sodium hydrosulfide (Sulfagenix, a biotechnology company that is currently developing hydrogen sulfide-based therapeutics for human disease conditions. The conflicts are considered significant.

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insufficiency of H$_2$S promotes oxidative stress, we evaluated the extent of oxidative stress in two organ systems. Measurement of malondialdehyde (MDA), which is commonly used as an index for lipid peroxidation, revealed significantly higher baseline levels in both the heart (Fig. 2A) and liver (Fig. 2B) in CSE KO mice compared with WT controls. Levels of protein carbonyl groups, an indicator of the oxidative modification of proteins, were also significantly higher in the heart (Fig. 2C) and liver (Fig. 2D) in the CSE KO mice.

In vivo immune-spin trapping of biomolecular free radicals using the EPR nitronine spin trap 5,5-dimethyl-1-pyrroline N-oxide (DMPO) revealed enhanced immunodetectable, covalently bound, and CSE KO mice significantly higher in the heart (Fig. 2E) and liver (Fig. 2F) in CSE KO mice compared with WT mice (Fig. 2G and H). CSE KO mice, further confirming elevated oxidative stress in these tissues.

Because oxidative stress is associated with mitochondrial dysfunction, we examined mitochondrial respiration in WT, CSE KO, and CSE KO + H$_2$S donor-treated mice. Heart mitochondria were isolated and used for respiration measurements (Fig. SL1A). The CSE KO mice displayed a marked reduction in state 3 respiration compared with the WT mice, which was restored with H$_2$S therapy (Fig. SL1A). State 3 respiration indicates electron transport efficiency and subsequently, the rate of ATP synthesis. In a similar fashion, the respiratory control ratio (RCR; i.e., state 3/state 4) was reduced in CSE KO mice, but restored with an H$_2$S donor (Fig. SL1B). The RCR is an index of the efficiency of the electron transport in regards to ATP generation (higher RCR = increased coupling of oxidative phosphorylation). We also evaluated calcium-induced mitochondrial swelling, and found no difference in either rate of swelling or time to swelling between the CSE KO and WT mice (Fig. SI C and D).

CSE KO Mice Display Impaired eNOS Function. We investigated whether H$_2$S deficiency alters eNOS function under basal conditions. Myocardial eNOS expression and phosphorylation was determined by Western blot analysis in CSE KO and WT mice. CSE KO mice demonstrated significantly lower phosphorylation of the eNOS activation site, P-eNOS(S1177), compared with WT mice (Fig. 3A and B). Moreover, the eNOS inhibitory site, P-eNOS(T1495), was phosphorylated to a significantly greater degree in CSE KO mice (Fig. 3A and C). There was no difference in total eNOS expression (Fig. 3A and D), and also no significant difference in iNOS or nNOS myocardial expression (Fig. S2).

We next measured cardiac levels of the eNOS cofactor tetrahydrobiopterin (BH$_4$) by HPLC, and found significantly lower cardiac BH$_4$ levels in the CSE KO mice compared with the WT mice (Fig. 3E-G). Our findings of no significant differences in inducible nitric oxide synthase (iNOS), neuronal nitric oxide synthase (nNOS), protein kinase B (AKT), and phosphorylated protein kinase B (P-AKT) expression between CSE KO mice and WT mice (Fig. S2) suggest an alteration in eNOS activity is most likely the key mechanism accounting for alterations in NO bioavailability.

H$_2$S Deficiency Reduces NO Bioavailability and cGMP Levels. We measured NO metabolites (nitrite and nitrosylated protein, RXNO) in plasma and myocardial tissue to evaluate NO bioavailability. We observed a significant reduction in circulating and myocardial nitrite levels in CSE KO mice compared with WT mice (Fig. 4A and B). Similarly, RXNO measurements in plasma and heart tissue revealed a threefold reduction in CSE KO mice (Fig. 4 C and D). After generation by eNOS, NO activates soluble guanylyl cyclase (sGC) to form cyclic guanosine 5'-monophosphate (cGMP). Circulating cGMP levels were significantly diminished in CSE KO mice (Fig. 4E).

H$_2$S Therapy Restores eNOS Function and NO Bioavailability in CSE KO Mice. To determine whether exogenous H$_2$S could restore eNOS function and NO bioavailability in CSE KO mice, we utilized the H$_2$S donor, diallyl trisulfide (DATS) in CSE KO mice. Treatment with DATS restored eNOS P-eNOS(S1177) to near-WT levels (Fig. 5C). After administration of DATS, cardiac nitrite levels increased significantly in CSE KO mice (Fig. 5F). Further evidence for increased NO bioavailability resulting from H$_2$S therapy was provided by a 6.5-fold increase in circulating RXNO levels (Fig. 5G) and a 6-fold increase in myocardial RXNO levels (Fig. 5H).

H$_2$S Therapy Protects Against Exacerbated I/R Injury in CSE KO Mice. We next subjected CSE KO mice to cardiac and hepatic I/R injury. Mice were subjected to myocardial ischemia for 45 min, followed by 24 h of reperfusion. The CSE KO mice displayed a significant (P < 0.01) increase in myocardial infarct size (INF) per area-at-risk (AAR) compared with the WT mice (62 ± 2% vs. 42 ± 3%) (Fig. 6A). This exacerbation of myocardial I/R injury in CSE KO mice was completely reversed to WT levels with acute treatment with DATS (Fig. 6B). The RCR is an index of the efficiency of the electron transport in regards to ATP generation (higher RCR = increased coupling of oxidative phosphorylation). We also evaluated calcium-induced mitochondrial swelling, and found no difference in either rate of swelling or time to swelling between the CSE KO and WT mice (Fig. SI C and D).

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H$_2$S therapy (Na$_2$S, 100 μg/kg at 5 min before reperfusion), evident by a significant reduction in myocardial infarct size and plasma troponin-I levels (Fig. 6 A and B). We next subjected another cohort of mice to 45 min of hepatic ischemia and 5 h of reperfusion, then measured liver transaminases, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), as markers of hepatic injury. AST and ALT levels were significantly increased in hepatic I/R injury in CSE KO mice compared with WT mice (Fig. 6C). This exacerbated hepatic injury was largely reversed after H$_2$S treatment with Na$_2$S (500 μg/kg) administered i.v. 5 min before reperfusion (Fig. 6C). Hepatic 8-isoprostane measurements indicated that oxidative stress was increased in CSE KO mice after I/R and corrected to WT levels with H$_2$S therapy (Fig. 6D).

H$_2$S Therapy Fails to Reduce Myocardial I/R Injury in eNOS Phospho-Dead Mutant Mice. Exogenous H$_2$S therapy has been demonstrated to attenuate myocardial I/R injury in mice (7, 8, 12). To determine whether the protective mechanism is dependent on eNOS, we first used a mouse model with global genetic ablation of eNOS. Again, mice were subjected to 45 min of ischemia, followed by 24 h of reperfusion. The H$_2$S donor, Na$_2$S (100 μg/kg), or vehicle (0.9% NaCl) was administered 5 min before reperfusion. There was no significant change in infarct size in the H$_2$S-treated group compared with the vehicle-treated group (Fig. 7A). We then examined whether H$_2$S therapy could protect against myocardial I/R injury in a mutant mouse model expressing a transgene encoding non-phosphorylatable eNOS (S1179A) on the eNOS$^{+/+}$ background. S1179A mutant mice and WT control mice were subjected to the same myocardial I/R protocol, and the H$_2$S donor, DATS (200 μg/kg), was administered 5 min before reperfusion. Similarly, H$_2$S was unable to reduce injury in eNOS phospho-dead mice (Fig. 7B). These results indicate that the cardioprotective actions of H$_2$S are dependent on eNOS phosphorylation.

Discussion

It is well established that both H$_2$S and NO exert potent cytoprotective effects in the setting of cardiovascular disease in various animal model systems (5, 7, 13–15). In addition, H$_2$S has been reported to be cytoprotective in some organ systems, such as the central nervous system and gastrointestinal tract, independent of NO (3, 11, 16–18). Although originally considered separate signaling pathways, recent evidence suggests cross-talk between H$_2$S and NO signaling in the cardiovascular system. Previous studies have reported that vascular function, inflammation, angiogenesis, and ischemic injury are regulated by cross-talk between the H$_2$S and NO signaling pathways (8, 19–21). We postulate that the cross-talk between H$_2$S and NO is mediated via the phosphorylation of eNOS (enzymatic production of NO).

In mammalian tissues, CSE is the dominant enzyme for H$_2$S formation (22). Yang et al. (23) reported that CSE genetic ablation in mice results in significant depletion of H$_2$S levels in peripheral tissue and blood. The loss of CSE-derived H$_2$S in these mice also resulted in an age-dependent hypertension (23). In the present study, we used the same CSE KO mouse to evaluate cross-talk between CSE and eNOS. Consistent with findings of Yang et al. (23), loss of the CSE enzyme resulted in marked depletion of H$_2$S and sulfane sulfur, a storage intermediate of H$_2$S, in the circulation and the heart. We previously showed that treatment with exogenous H$_2$S or genetic over-expression of CSE in cardiomyocytes results in increased endogenous H$_2$S production, and profound protection against ischemia-induced heart failure and myocardial I/R injury (14). A recent clinical study reported lower circulating sulfide levels in patients suffering from congestive heart failure (24). Using a mouse model lacking CSE, we have demonstrated that a deficiency in endogenous H$_2$S results in increased myocardial injury. The administration of an H$_2$S donor immediately before reperfusion in CSE KO mice reversed injury to WT levels. Similarly, CSE ablation in a model of hepatic I/R resulted in markedly elevated levels of the circulating liver enzymes AST and ALT, indicating significant liver injury, which was attenuated after acute administration of H$_2$S.

I/R injury involves multiple pathological mechanisms, including free radical accumulation and reduced bioavailability of NO. The controlled regulation of NO synthesis by eNOS is essential for cardiovascular health. It is well established that eNOS can undergo posttranslational modifications, including multisite phosphorylation, which tightly regulates NO production (25–27). Specifically, phosphorylation of the amino acids S1177 and T495 regulates eNOS activity, thereby enhancing or inhibiting NO production (25–27). In the present study, evaluation of eNOS S1177 and T495 revealed that compared with WT mice, CSE KO mice exhibited markedly lower phosphorylation...
at the active site, eNOS<sup>S1177</sup>, and greater phosphorylation at the inhibitory site, eNOS<sup>T495</sup>. This altered phosphorylation of eNOS was coupled with concomitant reductions in both circulating and myocardial levels of nitrate, nitrosylated proteins, and plasma cGMP, corroborating decreased NO bioavailability and signaling. These results are consistent with the previous finding of marked reductions in cGMP in the plasma, aorta, and mesenteric artery in CSE KO mice (28). Diminished cGMP and reductions in protein kinase G activity are also likely to contribute to the exacerbated tissue injury in the heart and liver observed in CSE KO mice. When CSE KO mice were treated with the H<sub>S</sub> donor DATS for 7 d, phosphorylation at the active site of eNOS<sup>S1177</sup> was enhanced compared with controls. Treatment with DATS significantly enhanced cardiac nitrite and both plasma and cardiac levels of nitrosylated proteins in the CSE KO mice. Previous studies from our laboratory demonstrated that in a murine model of myocardial I/R, treatment with DATS increases eNOS phosphorylation at S1177 compared with vehicle. This resulted in elevated circulating nitrate and nitrite and promoted increased protection on the heart (8). Similarly, 12 wk of H<sub>S</sub> therapy during pressure overload heart failure in mice similarly enhanced eNOS phosphorylation at the active site S1177, increasing cardiac nitrite and ultimately preserving cardiac function (5).

In the present study, we examined the effect of H<sub>S</sub> in eNOS- and a phospho-dead mutant mouse model. There was no change in infarct size in either the total eNOS KO mice or the eNOS<sup>S1177A</sup> mice that received H<sub>S</sub>, compared with vehicle controls. Previous studies reveal not only that H<sub>S</sub> is cardioprotective in the setting of myocardial I/R (7, 8), but also that it activates eNOS and augments NO bioavailability (8, 29). These results further corroborate the regulation of H<sub>S</sub> cardioprotection through eNOS phosphorylation at the S1177 active site.

Previous studies have demonstrated that phosphorylation of S1177 is mediated through an Akt-dependent mechanism (30, 31). Moreover, numerous factors, including insulin (31), corticosteroids (32), bradykinin (33), and H<sub>S</sub> (5), stimulate NO production through Akt-induced phosphorylation of eNOS<sup>S1177</sup>. In the present study, examination of Akt phosphorylation revealed no difference between CSE KO and WT mice perhaps suggesting a mechanism independent of AKT. Tetrahydrobiopterin (BH<sub>4</sub>), an essential cofactor, has been shown to regulate eNOS activity. When BH<sub>4</sub> availability is limiting, electron transport within the active site becomes “uncoupled” from l-arginine oxidation, causing a reduction of oxygen to superoxide (34). Depletion in BH<sub>4</sub> and eNOS uncoupling has been correlated with cardiac dysfunction in murine models of hypertension and transverse aortic constriction (35, 36). Compared with WT mice, CSE KO mice had significantly lower cardiac BH<sub>4</sub> levels, and a trend toward higher dihydrobiopterin (BH<sub>4</sub>) levels. Moreover, CSE KO mice exhibited significantly elevated levels of MDA, protein carbonyl, and tissue free radicals (markers of oxidative stress) in the heart and marked increases of MDA and carbonyl in the liver. Oxidation of BH<sub>4</sub> can result in uncoupled eNOS, which can lead to increased oxidative stress (37). However, oxidative stress also may be related in part to changes in cellular respiration via impaired mitochondrial function. The elevated oxidative stress in CSE KO mice may be related in part to the absence of the enzyme responsible for the conversion of homocysteine to cystathionine. Although mitochondrial function and eNOS function were restored with H<sub>S</sub> therapy, the elevated homocysteine levels may be partially responsible for the increased oxidative stress in CSE KO mice.

Cellular respiration is controlled by NO interacting with complexes I and IV of the electron transport chain (38). Examination of basal respiration revealed significantly lower state 3 respiration levels and RCR in CSE KO mice compared with WT mice, indicating mitochondrial dysfunction. Much of the cellular damage observed in cardiovascular disease is the result of mitochondrial events, such as Ca<sup>2+</sup> overload, which leads to overproduction of reactive oxygen species (ROS) (39, 40). In the presence of NO, produced enzymatically or directly via the reduction of nitrite, the inhibition of complex I and IV has been reported to be cytoprotective. In the present study, the loss of the CSE-derived H<sub>S</sub> resulted in a significant increase in ROS. Moreover, these mice exhibited a reduction in nitrite levels owing to lower phosphorylation of eNOS<sup>S1177</sup> and reduced eNOS activity compared with WT mice. This combination of insufficient NO bioavailability and increased ROS may explain in part why CSE KO mice are more susceptible to myocardial I/R injury.

In this study, we used various H<sub>S</sub>-releasing compounds to confirm that the effects of H<sub>S</sub> on eNOS were not limited to a particular H<sub>S</sub> donor compound. For example, polysulphide compounds

Fig. 5. H<sub>S</sub> therapy activates eNOS and augments NO bioavailability in CSE KO mice. (A) Representative immunooblots of eNOS. (B–D) Relative intensity of P-eNOS<sup>S1177</sup>, P-eNOS<sup>T495</sup>, and total eNOS (D) protein expression in CSE KO hearts ± H<sub>S</sub> donor. (E–H) Levels of plasma nitrate (E), cardiac nitrite (F), plasma RXNO (G), and cardiac RXNO (H). Circles inside bars denote the number of animals studied per group.
(i.e., DATS) have previously been shown to be antioxidants and modify thiol proteins (41, 42). It is possible that some of the effects of DATS that we observed are independent of H\textsubscript{2}S release and are a result of DATS-mediated protein modifications. In addition, H\textsubscript{2}S may alter the overall redox status, leading to increased eNOS coupling (phosphorylation at S1177).

The findings in this study reveal that cytoprotection elicited by CSE-derived H\textsubscript{2}S is eNOS/NO-dependent. Future studies will aim to more fully understand the mechanisms related to crosstalk between CSE and eNOS-derived NO in the context of clinical syndromes.

**Methods**

**Animals.** CSE-ablated (KO) mice (Sv129/C57 background) and eNOS KO (eNOS\textsuperscript{−/−}) mice were developed as described previously (5). The eNOS phospho-dead mutant mouse model expresses a bovine eNOS cDNA with a single-point mutation (S1179A) under the control of the human eNOS promoter. This transgenic mouse was then crossed into the eNOS\textsuperscript{−/−} background. This model has been previously described in detail (43). Male mice age 14–16 wk were used during the course of this present study. All animals received humane care in compliance with the National Society of Medical Research’s Principles of Laboratory Animal Care and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (44), and all protocols were approved by Emory University’s Institutional Animal Care and Use Committee.

**Mouse DMPO Protocol.** Between 14–16 wk of age, WT and CSE KO mice received DMPO (Dojindo) dissolved in pyrogen-free saline [1.5 g/kg i.p. total in three doses (0.5 g/kg) over 24 h]. At sacrifice, hearts and livers were perfused with PBS (pH 7.4), removed, and fixed for immunohistochemical analysis (see below).

**H\textsubscript{2}S Donors.** Na\textsubscript{2}S was produced and formulated to pH neutrality by Ikaria using H\textsubscript{2}S gas (Matheson) as a starting material. DATS was prepared and dosed as described previously (29).

**Myocardial I/R Protocol and Myocardial Infarct Size Determination.** Mice were subjected to 45 min of myocardial ischemia and 24 h of reperfusion. Surgical procedures were performed as described previously (45).

**Liver I/R Protocol.** Mice were subjected to 45 min of regional hepatic ischemia and 5 h of reperfusion as described previously (46).

**AST and ALT Assay.** Blood from mice subjected to 45 min of hepatic ischemia and 5 h of reperfusion was collected and used to determine liver transaminase values as described previously (46).

**Cardiac Troponin-I Assay.** Serum was obtained from mice after 45 min of LCA ischemia and 24 h of reperfusion to measure the cardiac-specific isoform of troponin-I using a mouse-specific ELISA kit (Life Diagnostic).

**8-Isoprostane Assay.** After 45 min of hepatic ischemia and 5 h of reperfusion, mouse livers were rapidly excised and homogenized. 8-Isoprostane was assayed using an ELISA kit (Cell Biolabs) according to the manufacturer’s recommendations.

**Cardiac Mitochondrial Isolation.** Mitochondria were isolated and prepared as described previously (8).

**Mitochondrial Respiration Measurement.** Oxygen consumption and respiration were determined as described previously (8).

**Measurement of Hydrogen Sulfide and Sulfane Sulfur.** H\textsubscript{2}S and sulfane sulfur levels were measured in heart and blood by gas chromatography chemiluminescence (Agilent 7890 GC gas chromatography system and G660A Series chemiluminescence detector). Free H\textsubscript{2}S in fresh blood and tissue was liberated by incubating in 1 M sodium citrate solution at 37 °C for 10 min. Sulfane sulfur was released by incubating 100 μL of sample in an equal volume of 15 mM DTT for 50 min, followed by the addition of 400 μL of 1 M sodium citrate at 37 °C. The resultant headspace gases were analyzed using the GC system.

**Measurement of NO Metabolites.** Nitrite concentrations were quantified by ion chromatography (EN020 Analyzer; Elcom). RXNO levels were measured by chemiluminescence detection (CLD 88Y; Eco Physics) achieved by an acidified sulfanilamide reaction with the biological samples into a tri-iodide-containing mixture purged continuously with helium.

**cGMP RIA.** cGMP standards (Sigma-Aldrich) and samples were acetylated by adding 8 μL of 5 N KOH and 2 μL of acetic anhydride in a volume of 200 μL, followed by incubation at room temperature for 30 min. The tubes were then placed on ice to stop the reaction. All determinations were performed in duplicate. Each tube contained 50 μL of acetylated standards or samples, 50 μL of iodinated cGMP (14,000–16,000 cpm), and 50 μL of cGMP antibody (Sigma-Aldrich). The reaction mixture was incubated overnight at 4 °C, followed by precipitation of the cGMP-antibody complex by 12% PEG8000. Radioactivity was counted in a gamma counter.

**Western Blot Analysis.** Myocardial tissue was used for Western blot analysis, performed as described previously (5). The following primary antibodies were used: GAPDH, α-tubulin, P-eNOS\textsuperscript{T147/S1179}, iNOS, nNOS, total AKT, and P-AKT\textsuperscript{T308} (Cell Signaling), and total eNOS (BD Biosciences).

**HPLCy Analysis of BH\textsubscript{4} and BH\textsubscript{2}.** Cardiac BH\textsubscript{4} and BH\textsubscript{2} quantifications were determined as described previously (47).

**Determination of Protein Carboxyl Content.** Protein carboxyl content from heart and liver was measured as described previously (48).

**Measurement of MDA Levels.** MDA levels were measured in heart and liver protein as described previously (48).

**Immunohistochemistry.** Samples for immunohistochemistry analysis were prepared as described previously (49).

**Statistical Analysis.** All data in this study are expressed as mean ± SEM. Differences in data between groups were compared using Prism 4 (GraphPad Software) with the Student unpaired two-tailed t test when comparing two groups and one-way ANOVA when comparing three or more groups. If a significant difference was found on ANOVA, then Tukey’s multiple-comparison test was used for post hoc analysis. A P value of <0.05 was considered significant.

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