Hydrogen Sulfide Preconditions the db/db Diabetic Mouse Heart
Against Ischemia-Reperfusion Injury by Activating Nrf2 Signaling in an Erk-Dependent Manner

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Running Title: H2S protects T2DM hearts from MI/R injury
Abstract

Hydrogen sulfide (H$_2$S) therapy protects non-diabetic animals in various models of myocardial injury, including acute myocardial infarction and heart failure. Here, we sought to examine if H$_2$S therapy provides cardioprotection in the setting of Type-2 diabetes. H$_2$S therapy in the form of sodium sulfide (Na$_2$S) beginning 24 hours or 7 days prior to myocardial ischemia significantly decreased myocardial injury in db/db diabetic mice (12 weeks of age). In an effort to evaluate the signaling mechanism responsible for the observed cardioprotection, we focused on the role of Nrf2 signaling. Our results indicate that diabetes does not alter the ability of H$_2$S to increase the nuclear localization of Nrf2, but does impair aspects of Nrf2 signaling. Specifically, the expression of NADPH;quinine oxidoreductase 1 (NQO1) was increased after the acute treatment, whereas the expression of heme-oxygenase-1 (HO-1) was only increased after 7 days of treatment. This discrepancy was found to be the result of an increased nuclear expression of Bach1, a known repressor of HO-1 transcription, which blocked the binding of Nrf2 to the HO-1 promoter. Further analysis, revealed that 7 days of Na$_2$S treatment overcame this impairment by removing Bach1 from the nucleus in an Erk1/2-dependent manner. Our findings demonstrate for the first time that exogenous administration of Na$_2$S attenuates myocardial ischemia-reperfusion injury in db/db mice suggesting the potential therapeutic effects of H$_2$S in treating a heart attack in the setting of Type-2 diabetes.

Keywords: Hydrogen Sulfide; Type-2 diabetes; Nrf2; Myocardial Infarction
Introduction

The endogenously produced gasotransmitter, hydrogen sulfide (H2S), exerts cytoprotective effects in various models of cardiac injury. Specifically, treatment with H2S before, during or after myocardial ischemia decrease injury (3, 6, 25). Furthermore, chronic H2S therapy improves survival and protects against ischemic-induced heart failure (2). These cytoprotective effects have been attributed to H2S' ability to upregulate antioxidant defenses and to reduce apoptosis, inflammation, and mitochondrial injury (22). Increasing evidence suggests that hyperglycemia-induced ROS production contributes to oxidative stress, resulting in myocardial damage (1). Thus, the reported antioxidant effects of H2S may be of critical importance in the diabetic myocardium. H2S upregulates endogenous antioxidant defenses by activating nuclear factor E2-related factor (Nrf2), a member of the NF-E2 family of nuclear basic leucine zipper transcription factors (3). Nrf2 regulates the gene expression of a number of enzymes that serve to detoxify pro-oxidative stressors (7), including heme-oxygenase-1 (HO-1) and NADPH:quinine oxidoreductase 1 (NQO1), by binding to the antioxidant response element (ARE) found in the gene’s promoter region (30, 38).

H2S is produced enzymatically in mammalian species via the action of three enzymes in the cysteine biosynthesis pathway: cystathionine-γ-lyase (CSE), cystathionine-β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3-MST). Several studies suggest that streptozotocin (STZ)-induced diabetes (model of Type 1 diabetes) increases the expression of CSE and CBS in the pancreas, kidney and liver (36, 37). In contrast, additional studies found that...
STZ does not alter the expression of either enzyme in the aorta, liver, kidney or heart (4, 27). Despite this contrasting evidence regarding the affects of STZ-induced diabetes on the expression of CSE and CBS, there is clear evidence that circulating levels of H₂S are decreased in animal models of diabetes (12, 27, 37) and may even contribute to the pathophysiology of diabetes (28). More importantly, lower circulating H₂S levels have been detected in plasma samples taken from patients with T2DM (12, 32).

Recently, therapeutic strategies aimed at increasing the levels of H₂S have been shown to provide protection against cardiomyopathy and vascular dysfunction in models of STZ-induced diabetes (5, 27). However, it has yet to be determined if it has similar effects in the setting of T2DM. Therefore, the purpose of this study was to investigate the potential cardioprotective effects of H₂S therapy in the setting of T2DM using a well-established in vivo mouse model of MI/R injury.
Materials and Methods

Animals. Male non-diabetic (C57BLKS/J) and diabetic (BKS.Cg-Dock7M+/+Leprdb/J mice; Jackson Labs, Bar Harbor, ME) were utilized at 12 weeks of age. All experimental mouse procedures were approved by the Institute for Animal Care and Use Committee at Emory University School of Medicine 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; Sigma Aldrich. USA; catalog# 407410) was dissolved in saline and administered using a 32-gauge needle at a dose of 0.1 mg/kg in a final volume of 50 µL via a tail vein injection. Mice received Na$_2$S either 24 hours prior to experimentation or as a daily injection for 7 days. Saline was administered in the same manner for the respective vehicle groups. In all cases, Na$_2$S was prepared just prior to use. Groups of mice also received 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene (U0126; 0.1 mg/kg).

Blood Glucose Determination. Blood obtained via a tail snip was screened using a Xtra glucose-monitoring system (Precision).

Myocardial Ischemia-Reperfusion Protocol and Myocardial Injury Assessment. Surgical ligation of the left coronary artery (LCA) myocardial infarct size determination, and Troponin-I measurements were performed similar to methods described previously (3).
Oxidative Stress. The degree of lipid peroxidation was determined by evaluating the levels of malondialdehyde (MDA) in heart tissue using a commercially available thiobarbituric acid reactive substances (TBARS) assay kit according to the manufacture’s instructions (Enzo Life Sciences; catalog# ALX-850-287-KI01). Concentrations of 8-isoprostane in heart were determined by 8-isoprostane EIA kit according to manufacture’s instruction (Cayman Chemicals; catalog# 516351).

Subcellular Fractionation and Western Blot Analysis. Samples of the heart were homogenized and fractionated into whole cell and nuclear fractions as described previously (3). Equal amounts of protein were loaded into lanes of polyacrylamide-SDS gels and Western blot analysis was performed as previously described (3).

Chromatin Immunoprecipitation. Chromatin Immunoprecipitation studies were performed with Imprint ChIP kit (Sigma-CHP1) according to the manufacture’s instruction. Briefly, heart samples (40 mg) were cross-linked with 1% formaldehyde, homogenized, and sonicated to shear DNA. A portion of the precleared chromatin was stored and labeled as “input DNA.” Equal amounts of chromatin were immunoprecipitated with the following antibodies: Nrf2 (Santa Cruz), Bach1 (Santa Cruz), and control IgG. The chromatin was washed and the crosslink’s were hydrolyzed. The DNA was then purified through DNA binding column and subjected to qPCR analysis using SYBER Green-based detection according to the manufacturer’s instructions (Applied Biosystems). PCR primers were designed to amplify the fragment of the HO-1 and NQO1 promoter region containing the Nrf2 or Bach1 binding motif (ARE). The primers are as follows:
HO-1 forward, 5'-AGG TAC ACA TCC AAG CCG AGA A-3'; HO-1 reverse, 5'-CTC TGG ACA CCT GAC CCT TCT G-3'; NQO1 forward, 5'-GCA GTT TCT AAG AGC AGA ACG-3'; NQO1 reverse, 5'-GTA GAT TAG TCC TCA CTC AGC CG-3'. Results were calculated according the manufacture’s recommendations and presented as fold enrichment to the input DNA.

Measurement of Hydrogen Sulfide and Sulfane Sulfur. Hydrogen sulfide and sulfane sulfur levels were measured in tissue and blood according to previously described methods (23). Fresh tissue was homogenized in 5 volumes of PBS (pH 7.4). For measurement of hydrogen sulfide, 0.2 mL of the sample homogenate was placed in a small glass vial (5182-0553, Agilent Technologies, Santa Clara, CA, USA) along with 0.4 mL of 1 M sodium citrate buffer, pH 6.0, and sealed. The mixture was incubated at 37°C for 10 minutes with shaking at 125 rpm on a rotary shaker (Fisher Scientific) to facilitate the release of H₂S gas from the aqueous phase. After shaking, 0.1 mL of head-space gas was applied to a gas chromatograph (7890A GC System, Agilent) equipped with a dual plasma controller and chemiluminescence sulfur detector (355, Agilent) and a data processor. The carrier gas was helium with a flow rate of 2.4 mL/min. For the measurement of H₂S released from bound sulfane sulfur, 0.1 mL of the sample homogenates and 0.1 mL of 15 mM DTT in 0.1 mM Tris/HCl, pH 9.0, were placed in a in a small glass vial, sealed, and incubated at 37°C for 50 minutes. After the incubation, 0.4 mL of 1 M sodium citrate buffer was injected through the rubber stopper and the mixture was incubated at 37°C for 10 minutes
with shaking at 125 rpm on a rotary shaker to facilitate the release of H₂S gas from the aqueous phase. After shaking, 0.1 mL of head-space gas was applied to a gas chromatograph as detailed above. For the measurement of H₂S and sulfane sulfur in blood, 0.1 mL and 0.05 mL of whole blood was used for each measurement, respectively. The concentrations of H₂S in the samples were calculated using a standard curve of Na₂S as a source of H₂S. Chromatographs were captured and analyzed with Agilent ChemStation software (B.04.03). For tissue, the amount of H₂S is reported as nmole/mg wet weight. For the blood, the amount of H₂S is reported as µM.

**Enzymatic Production of Hydrogen Sulfide.** Enzyme reactions to determine the production of H₂S from 3-MST were performed as described previously (24). Briefly, heart tissue was homogenized in buffer containing 100 mM potassium phosphate, pH 7.4, 1 mM dithiothreitol (DTT), and protease inhibitor cocktail “complete”. For enzyme reactions, 0.2 mL of the homogenate was incubated with 0.022 mL of substrate mix (10 mM L-cysteine and 0.5 mM \(\alpha\)-ketoglutarate) in a sealed vial at 37°C for 50 minutes. After adding 0.444 ml of 1 M sodium citrate buffer (pH 6.0), the mixtures were incubated at 37°C for 10 min with shaking on a rotary shaker to facilitate a release of H₂S gas from the aqueous phase. After shaking, 0.1 mL of head-space gas was applied to a gas chromatograph as described above.

Enzyme reactions to determine the production of H₂S from CBS and CSE were performed as described previously (37) with slight modification. Briefly, heart
tissue was homogenized in buffer containing 100 mM potassium phosphate, pH 7.4. For enzyme reactions, 0.172 mL of homogenate was incubated with 0.028 mL of substrate mix (10 mM L-cysteine, 2 mM pyridoxal 5’-phosphate) in a sealed vial at 37°C for 30 minutes. After adding 0.400 ml of 1 M sodium citrate buffer (pH 6.0), the mixtures were incubated at 37°C for 10 min with shaking on a rotary shaker to facilitate a release of H₂S gas from the aqueous phase. After shaking, 0.1 mL of head-space gas was applied to a gas chromatograph as described above. For both reactions, the H₂S concentration of each sample was calculated against a calibration curve of Na₂S and results are expressed as nanomole H₂S formed per milligram of soluble protein (determined using the DC protein assay, Bio-Rad).

**Isolation of mRNA and Taqman qPCR.** RNA was isolated using the RiboPure kit according to manufacturer’s instructions (Ambion). Reverse transcription was performed in a standard fashion with QuantiTect Reverse Transcription Kit (QIAGEN) supplemented with DNase treatment. Taqman qPCR was carried out according to the manufacturer’s instructions using probe sets for Cbs, Cth, and Mpst and 18S (Applied Biosystems). Analysis was carried out using the ΔΔ-CT method with 18S correction and reported as relative fold change versus Non-diabetic controls.

**Statistical Analysis.** All data in this study are expressed as mean ± standard error (SEM). Differences in data between the groups were compared using Prism 4 (GraphPad Software, Inc) with Student’s paired 2-tailed t test or one-way
analysis of variance (ANOVA) where appropriate. For the one-way ANOVA, if a significant variance was found, the Tukey test was used as the post hoc analysis. A p value less than 0.05 was considered significant.
Results

**Sulfide levels are decreased in diabetic mice.**

Diabetic mice exhibited the typical characteristics of a severe diabetic phenotype when compared to non-diabetic mice, including marked obesity and hyperglycemia (**Table 1**). Initial studies examined the effects of diabetes on the gene and protein expression of the three known H$_2$S-producing enzymes, as well as the levels of circulating and myocardial sulfide. qPCR analysis revealed that the gene expression of all three enzymes were elevated in the diabetic heart compared to the non-diabetic heart (**Fig. 1A**). The protein expression of CBS and CSE were unaltered in the diabetic heart compared to the non-diabetic heart (**Fig. 1B**). However, the expression of 3-MST was significantly upregulated (p<0.001 vs. Non-Diabetic). Further studies revealed that the biosynthesis of H$_2$S from the pyridoxal-5'-phosphate (PLP)-dependent enzymes, CBS and CSE, as well as from 3-MST was decreased in the diabetic heart (**Fig. 1D**; p<0.05 vs. Non-Diabetic). Finally, free H$_2$S and sulfane sulfur levels were significantly lower in the blood and heart of diabetic mice compared to non-diabetic mice (**Fig. 1E-F**; p<0.05).

**Pretreatment with Na$_2$S therapy reduces myocardial injury after I/R.**

Given that diabetes is a condition in which preconditioning strategies may have clinical relevance, studies were conducted to determine if Na$_2$S therapy could increase sulfide levels and provide protection against acute myocardial I/R injury.
For these studies, diabetic mice received either a single tail vein injection of Na$_2$S the day before experimentation (Na$_2$S PC; 0.1 mg/kg) or Na$_2$S for 7 days prior to experimentation (Na$_2$S 7d PC) or Vehicle. Free H$_2$S levels were not different between the groups (Fig. 2A). However, sulfane sulfide levels were slightly higher in the hearts of the Na$_2$S PC mice (p=NS) and significantly increased in the hearts of Na$_2$S 7d PC mice (Fig. 2B; p<0.001 vs. Vehicle and Na$_2$S PC).

Additional groups of mice were subjected to 30 min of ischemia and 2 hr of reperfusion. Representative mid-ventricular photomicrographs of hearts from the different groups of mice are shown in Fig. 2C. Pretreatment with Na$_2$S for 24 hours significantly decreased infarct size relative the area-at-risk (INF/AAR) by 17% and INF relative to the left ventricle (INF/LV) by 19% compared to Vehicle-treated mice (Fig. 2D; p<0.001). Pretreatment with Na$_2$S for 7 days significantly decreased INF/AAR by 35% and decreased INF/LV by 33% when compared to Vehicle-treated mice (Fig. 2D; p<0.001). Comparing the infarct size lowering effects between the two therapeutic approaches reveals that the 7-day pretreatment strategy was 51% more effective in reducing INF/AAR than the acute strategy (p<0.05). Likewise, both strategies significantly decreased circulating Troponin-I levels when compared to the Vehicle-treated mice (Fig. 2E; p<0.05). These changes were independent of any effects on body weight or blood glucose levels (Table 1).
Na$_2$S therapy reduced myocardial I/R-induced oxidative stress and apoptosis.

In response to myocardial I/R injury, lipid peroxidation and 8-isoprostane levels increased in all of the groups (Figure 3A-B). Lipid peroxidation levels were significantly lower in the Na$_2$S PC mice compared to the Vehicle-treated mice (p<0.05). However, both markers of oxidative stress were significantly lower in the Na$_2$S 7d PC mice (p<0.001 vs. Vehicle). Furthermore, lipid peroxidation levels were significantly lower in the Na$_2$S 7d PC mice compared to the Na$_2$S PC mice (p<0.01), whereas 8-isoprostane levels trended lower. MI/R also increased the expression of cleaved caspase-3 in the hearts of all the groups (Fig. 3C-D; p<0.001 vs. Sham). The hearts of both groups of mice treated with Na$_2$S exhibited a significant reduction in cleaved caspase-3 expression compared to Vehicle-treated mice (p<0.05 for Na$_2$S PC and p<0.01 for Na$_2$S 7d PC). Additionally, the Na$_2$S 7d PC mice displayed a lower expression compared to the Na$_2$S PC mice (p<0.05).

Na$_2$S treatment strategies affect the expression of NQO1 and HO-1 differently.

Experiments were then conducted to elucidate potential mechanisms responsible for the cardioprotective effects of Na$_2$S pretreatment. As an initial measure, it was determined if diabetes altered the expression of Nrf2. For these experiments, non-diabetic and db/db diabetic mice were sacrificed at 12 weeks of age. Western
blot analysis revealed no difference in the whole cell or nuclear expression of Nrf2 between non-diabetic and diabetic mice (Fig. 4A). Next, experiments were conducted to determine if Na$_2$S pretreatment induced Nrf2 signaling in the diabetic heart. For these experiments, both the acute and 7 day strategies were investigated. For the acute strategy (Na$_2$S Acute), Na$_2$S was administered to diabetic mice and heart tissue was collected 1 hour and 24 hours later. One hour after the administration of Na$_2$S, the nuclear expression of Nrf2 was increased (Fig. 4B; p<0.001 vs. Sham). Analysis at 24 hours revealed that NQO1 levels were significantly increased (Fig. 4C; p<0.001 vs. Sham). In contrast, HO-1 levels were not increased at this time point. Analysis revealed that the nuclear expression of Nrf2 remained elevated after 7 days treatment (Na$_2$S 7d; Fig. 4B; p<0.001 vs. Sham). Furthermore, NQO1 levels also remained elevated in the hearts of mice treated with Na$_2$S for 7 days (Fig. 4C; p<0.001 vs. Sham). Importantly, 7 days of Na$_2$S treatment increased HO-1 levels in the hearts of diabetic mice (p<0.05 vs. Sham).

Next, chromatin immunoprecipitation (ChIP) assays were performed to examine the binding of Nrf2 to the ARE in the NQO1 and HO-1 promoters. As shown in Fig. 4D, Na$_2$S therapy enhanced the binding of Nrf2 to the NQO1 promoter after both a single administration and 7 days of treatment (p<0.05 vs. Sham). In contrast, a single administration of Na$_2$S did not increase the binding of Nrf2 to the HO-1 promoter. However, after 7 days of Na$_2$S treatment, an increase in binding was observed (p<0.05 vs. Sham).
Pretreatment with Na$_2$S does not alter the nuclear expression of Keap1 or Fyn in the Diabetic Heart but does reduce the expression of Bach1.

In an effort to determine the possible mechanisms underlying the diminished binding of Nrf2 to the ARE in the HO-1 promoter, the expression of 3 proteins known to influence the signaling or activation of Nrf2 were evaluated: Kelch-like ECH-associated protein 1 (Keap1), Fyn, and Bach1. The expression of each was first evaluated in the nuclear fraction of heart tissue taken from non-diabetic and diabetic mice and then evaluated in the nuclear fraction of heart tissue taken from Vehicle, Na$_2$S PC and Na$_2$S 7d PC treated mice. The nuclear expression of Keap1 was similar in the hearts of non-diabetic and diabetic Sham mice (Fig. 5A) and no significant changes were observed in either group of mice treated with Na$_2$S (Fig. 5B). The expression of Fyn kinase, a key regulator of Nrf2 nuclear export (11), was then evaluated. The nuclear expression of Fyn was significantly elevated in the hearts of diabetic mice when compared to non-diabetic mice (Fig. 5A; p<0.001). However, neither pretreatment strategy of Na$_2$S altered the nuclear expression of Fyn (Fig. 5B). Experiments then focused on Bach1, a stress responsive transcription factor that has been reported to repress the transcription of HO-1 by competing with Nrf2 in binding to the promoter of HO-1 (15). As with Fyn, the nuclear expression of Bach1 was significantly elevated in the hearts of diabetic mice compared to non-diabetic mice (Fig. 5A; p<0.05). Importantly, the expression of Bach1 trended lower in the Na$_2$S PC mice (Fig. 5B; p=NS vs. Sham) and was markedly decreased in the hearts of Na$_2$S 7d PC (p<0.05 vs. Sham). Further experiments were then conducted to determine if
the changes in the nuclear levels of Bach1 were accompanied by a decrease in
the binding of Bach1 to the NQO1 and HO-1 promoters (Fig. 5C). ChIP analysis
revealed that significantly less Bach1 was bound to the NQO1 promoter in the
hearts of Na₂S PC and Na₂S 7d PC (p<0.05 vs. Sham). In contrast, a single
administration of Na₂S did not result in less Bach1 bound to the HO-1 promoter
in the diabetic heart. However, after 7 days of Na₂S treatment, significant
decrease was observed (p<0.05 vs. Sham).

Pretreatment with Na₂S for 7 days removed Bach1 from the nucleus by
activating Erk.

Signaling through Erk has been reported to regulate the nuclear localization of
Bach1 (9). Analysis revealed that the expression of phosphorylated Erk was not
increased in the hearts of Na₂S PC treated mice (Fig. 6A). However, Erk
phosphorylation was significantly increased in the hearts of Na₂S 7d PC mice
(p<0.001 vs. Sham and p<0.01 vs. Na₂S PC). Experiments then focused on the
importance of Erk in mediating the Na₂S-induced removal of Bach1 from the
nucleus. Since, the nuclear expression of Bach1 was only significantly decrease
in the heart of the Na₂S 7d PC treated mice, these additional studies only
focused on 7 days of pretreatment. For these experiments, mice received
vehicle, the Erk1/2 signaling inhibitor U0126 (U0126), or U0126 and Na₂S
(U0126+Na₂S) for 7 days. In the presence of U0126, Na₂S pretreatment failed to
increase the phosphorylation of Erk (Fig. 6B). Na₂S pretreatment did, however,
increase the nuclear expression of Nrf2 in the presence of U0126 (Fig. 6C;
p<0.05 vs. Sham and U0126), but failed to reduce the nuclear expression of
Bach1 (*Fig. 6C*) and failed to increase the expressions of NQO1 or HO-1 (*Fig. 6D*). Additionally, Na$_2$S pretreatment failed to provide protection against myocardial I/R injury when administered in the presence of U0126 (*Fig. 7*).  

**Discussion**

Our main findings in this study are (1) Na$_2$S therapy attenuates MI/R injury in the setting of T2DM; (2) the activity of the three H$_2$S-producing enzymes is decreased in the hearts of db/db mice; (3) Nrf2 signaling induced by Na$_2$S is partially impaired in the setting of T2DM by Bach1; (4) Na$_2$S therapy for 7 days overcomes this impairment by removing Bach1 from the nucleus in a Erk-dependent manner.

A main finding of our study relates to the effects of T2DM on the activity and expression of the H$_2$S-producing enzymes. Similar to previous reports using Type-1 diabetic models, we found that T2DM increased the gene expression of *Cbs* and *Cth*. More importantly, for the first time we report that the gene expression of *Mpst* was upregulated. While the increase in the gene expression of *Cbs* and *Cth* did not result in an increase in protein levels of these two enzymes, we did find that the protein expression of 3-MST was significantly upregulated. This suggests that 3-MST could be regulated differently than the other two enzymes in the setting of T2DM. However, the mechanism(s) behind this regulation remain to be elucidated. We also report here for the first time that T2DM lowers cardiac sulfide levels. Suzuki et al (27) suggest that the elevated
levels of glucose seen in diabetes may cause tissues to increase their consumption of H$_2$S resulting in lower levels. While this postulated mechanism could contribute to the lower circulating and cardiac levels seen in the current study, we provide novel evidence that the enzymatic activity of the pyridoxal-5’-phosphate (PLP)-dependent enzymes (CBS and CSE) and 3-MST were decreased in the diabetic heart. Therefore, we suggest that the lower levels of H$_2$S seen in the setting of diabetes are caused by the decreased production of endogenous H$_2$S and/or the increased tissue consumption of the available H$_2$S.

Currently, the mechanism(s) by which T2DM alters the expression and activity of the enzymes is unknown. There is evidence that the transcription factor, specificity protein 1 (Sp1) regulates the hyperglycemia-induced upregulation of CSE gene expression in pancreatic β-cells (29). However, it is not known if Sp1 regulates the cardiac gene expression of CSE in the setting of diabetes/hyperglycemia or if Sp1 regulates the expression of CBS or 3-MST under any conditions. In regards to enzymatic activity, the activity of 3-MST is inhibited by oxidative stress (21). Additionally, thioredoxin interacts with and regulates the H$_2$S-producing activity of 3-MST (20, 21). Recent work also demonstrates that hyperglycemia associated with STZ-induced diabetes increases the susceptibility of the heart to I/R injury by enhancing the nitrative inactivation of thioredoxin (35). Therefore, the oxidative stress associated with T2DM may potentially alter the activity of the enzymes. However, further work is needed to test this hypothesis and to determine if thioredoxin can regulate CBS and CSE in a manner similar to 3-MST.
Another main finding of the current study relates to the cardioprotective effects of H$_2$S in the setting of T2DM. H$_2$S therapy provides protection against several models of myocardial injury in the setting of Type-1 diabetes by alleviating apoptosis and oxidative stress (5, 8, 27). In agreement, the current study also found that H$_2$S attenuates apoptosis and oxidative stress in the setting of diabetes. More importantly, it provides evidence for the first time that H$_2$S therapy reduces myocardial injury in the T2DM heart. This is an important observation given that T2DM encompasses roughly 90% of diabetic patients (13). Our study also highlights the complexity of therapeutic intervention for the diabetic heart following ischemia, as the robust cardioprotective effects of H$_2$S that have previously been reported in the non-diabetic state (3) were diminished in the diabetic heart. Finally, our study revealed that the 7 days of treatment significantly increased the levels of sulfane sulfur in the heart suggesting that correcting the diabetes-induced deficit of cardiac H$_2$S levels may be responsible for the enhanced protection. Sulfane sulfur or bound sulfur has been suggested to be an important storage pool that regulates the amount of bioavailable free H$_2$S (17, 33). Moreover, there is evidence to suggest that free H$_2$S is released from bound sources under acidic conditions (19). Therefore, during myocardial ischemia it is likely that bound sulfane sulfur becomes a source of free H$_2$S, which can then serve as a signaling molecule to protect the heart against ischemic injury.
Another major finding of our study relates to aspects of Nrf2 signaling that were impaired in the diabetic heart. This is based on the findings that the two Na$_2$S treatment strategies affected the expression of NQO1 and HO-1 differently. The inability of a single injection of Na$_2$S to upregulate the expression of HO-1 is in contrast to our previous findings in the non-diabetic heart (3) and may provide an explanation as to why the 7-day treatment strategy lowers oxidative stress and infarct size more than the acute. Therefore, we can speculate that NQO1 is sufficient to provide the moderate antioxidant observed after the acute treatment of Na$_2$S, however both NQO1 and HO-1 are able to provide the robust effects induced by the 7-day treatment. Importantly our study revealed that the impairment in Nrf2 signaling as it relates to the regulation of HO-1 was initially caused by the inability of Nrf2 to bind to the ARE in the promoter of HO-1. This suggests two things: 1) although Nrf2 is present in the nucleus after a single injection of Na$_2$S something prevents it from binding to the HO-1 promoter and 2) Na$_2$S therapy for 7 days overcomes this impairment.

Under basal conditions, Keap1 represses the ability of Nrf2 to induce endogenous antioxidants by binding very tightly to Nrf2, anchoring it in the cytoplasm, and targeting it for ubiquitination and proteasome degradation (31). Upon stimulation, Nrf2 dissociates from Keap1 and translocates to the nucleus (16). H$_2$S has recently been shown to sulfhydrate Keap1, which results in the release and translocation of Nrf2 to the nucleus (10, 34). Recent findings also indicate that Keap1 acts as a shuttle protein suggesting that it has a functional role not only in the cytoplasm but also in the nucleus (18, 26). Therefore, it
appears that under certain conditions Keap1 travels into the nucleus, dissociates Nrf2 from the ARE sequence and escorts it out of the nucleus in an effort to turn the Nrf2 signaling pathway off (18). The results of the current study indicate that diabetes does not alter the ability of Na$_2$S to disrupt the interaction between Keap1 and Nrf2, as evidenced by the findings Na$_2$S therapy increased the nuclear levels of Nrf2. Our analysis also found that nuclear Keap1 levels were not altered in the diabetic heart and that Na$_2$S did not alter the expression of Keap1.

Although Keap1 is the major regulator of Nrf2 activation, there is evidence indicating multiple levels of Nrf2 regulation. For instance, Bach1 directly competes with Nrf2 in binding to the promoter of ARE-related genes leading to the negative regulation of Nrf2 signaling (14, 15). Our initial analysis found that the nuclear expression of Bach1 was increased in the diabetic heart, suggesting that it may contribute to the impairment of Nrf2 signaling in response to Na$_2$S as it related to HO-1. We found that an acute treatment of Na$_2$S slightly reduced its nuclear expression, whereas 7 days of Na$_2$S treatment significantly reduced the nuclear expression of Bach1. Given that this coincided with increased HO-1 expression, it can be suggested that 1) Bach1 potentially causes the disruption of Nrf2/HO-1 signaling in the diabetic heart and 2) removal of Bach1 from the nucleus of the diabetic heart allows for Nrf2 to bind to the ARE in the HO-1 promoter. The latter was confirmed by ChIP analysis, which demonstrated that 7 days of Na$_2$S treatment increased the binding of Nrf2 to the HO-1 promoter while at the same time reducing the binding of Bach1. Interestingly, the acute
treatment of Na$_2$S decreased the binding of Bach1 to the NQO1 promoter. This provides an explanation as to why NQO1 was increased after the acute treatment and suggests that the binding of Bach1 to the promoter of distinct Nrf2 target genes is differently regulated. It also suggests that in the heart binding of Bach1 to the NQO1 promoter is not as tightly regulated as binding to the HO-1 promoter. Additionally, inhibition of Erk1/2 signaling prevented Na$_2$S therapy from removing Bach1 from the nucleus and subsequently prevented Na$_2$S therapy from increasing the expression of NQO1 and HO-1. Inhibition of Erk1/2 signaling also prevented Na$_2$S therapy from reducing myocardial injury following I/R, indicating that Erk1/2-mediated activation of Nrf2 signaling in part contributes to the cardioprotective effects of Na$_2$S in the db/db diabetic heart. Currently, it is not known how H$_2$S increases the phosphorylation of Erk. Previous studies suggest that it could be dependent on PKC (3). Alternatively, H$_2$S could phosphorylate Erk in an PKC-independent manner. Therefore, further work is needed to determine the mechanisms by which H$_2$S activates both Nrf2 and Erk signaling in the heart.

In summary, our findings demonstrate for the first time that T2DM decreases the levels of H$_2$S in the heart and that exogenous administrations of Na$_2$S attenuate MI/R injury in the setting of T2DM. Our results also provide important insights into the mechanism responsible for this cardioprotection. Specifically, Na$_2$S treatment sets into motion events such as Erk phosphorylation, which ultimately lead to the removal of Bach1 from the nucleus and activation of Nrf2 signaling (Fig. 7C).

Finally, given that H$_2$S plays multiple protective roles in both the vasculature and the heart, it can be suggested that lower circulating and tissue H$_2$S levels may
contribute to the pathophysiology of diabetes (28) and strategies aimed at correcting this decline may serve as an effective therapeutic option in the treatment of heart disease in the setting of diabetes.

Grants

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Disclosures

No potential conflicts of interest relevant to this article were reported.

Author Contributions

REFERENCES


Figure Legends

**Fig. 1. Diabetes reduces sulfide levels.** (A) mRNA expression of the genes that encode for cystathionine-β-synthase (Cbs), cystathionine-γ-lyase (Cth) and 3-mercaptopyruvate sulfotransferase (Mps) in the hearts of non-diabetic and diabetic mice. (B-C) Representative immunoblots and densitometric analysis of CSE, CBS, and 3-MST proteins in the hearts of non-diabetic and diabetic mice. (D) Biosynthesis of H$_2$S from the pyridoxal-5'-phosphate (PLP)-dependent enzymes (CBS and CSE) and from 3-MST. (E) Circulating and (F) myocardial free hydrogen sulfide (H$_2$S) and sulfane sulfur levels in non-diabetic and diabetic mice. Values are mean ± SEM. Numbers inside of the bars indicate the number of animals that were investigated in each group. Data was compared with a student’s T-test. *p<0.05, **p<0.01, and ***p<0.001 vs. Non-Diabetic.

**Fig. 2. Na$_2$S pretreatment reduces the extent of myocardial injury in diabetic mice following ischemia and reperfusion.** (A) Myocardial free H$_2$S and sulfane sulfur levels from diabetic mice treated with Vehicle or a single tail vein injection of sodium sulfide (Na$_2$S) the day before experimentation (Na$_2$S PC; 0.1 mg/kg) or Na$_2$S for 7 days prior to experimentation (Na$_2$S 7d PC). (C) Representative midventricular photomicrographs of hearts, (D) myocardial infarct size relative to the area-at-risk (INF/AAR) and INF relative to the left ventricle (INF/LV), and (E) circulating Troponin-I levels from the experimental groups following 30 minutes of ischemia and 2 hours of reperfusion. Values are mean ± SEM. Data was compared through the use of a 1-way ANOVA with a Tukey test as the posthoc analysis. *p<0.05, **p<0.01, and ***p<0.001 vs. Vehicle.
Fig. 3. Na$_2$S pretreatment reduces oxidative stress and apoptosis following myocardial ischemia and reperfusion. (A) Lipid hydroperoxide, (B) 8-isoprostane levels and (C-D) representative immunoblots and densitometric analysis of cleaved caspase-3 expression from hearts of sham, vehicle, Na$_2$S PC, and Na$_2$S 7d PC mice following 30 minutes of ischemia and 1 hour of reperfusion. Values are mean ± SEM. Data was compared through the use of a 1-way ANOVA with a Tukey test as the posthoc analysis. *p<0.05 and ***p<0.001 vs. Sham.

Fig. 4. Na$_2$S increases the nuclear expression of Nrf2. (A) Representative immunoblots and densitometric analysis of whole cell and nuclear Nrf2 from the hearts of non-diabetic and diabetic mice. (B) Representative immunoblots and densitometric analysis of nuclear Nrf2 in the hearts of diabetic mice treated with either a single injection of Na$_2$S (Na$_2$S Acute) or with daily injections for 7 days (Na$_2$S 7d). (C) Representative immunoblots and densitometric analysis of NQO1 and HO-1 in the hearts of sham, Na$_2$S Acute, and Na$_2$S 7d mice. (D) ChIP analysis of Nrf2 binding to the NQO1 or HO-1 promoter in the hearts of Sham, Na$_2$S Acute, and Na$_2$S 7d mice. A parallel ChIP assay was performed with IgG as a ChIP assay control. Data in panel A was compared with a student’s T-test. All other data was compared through the use of a 1-way ANOVA with a Tukey test as the posthoc analysis. *p<0.05, **p<0.01, and ***p<0.001 vs. Sham.

Fig. 5. Na$_2$S treatment for 7 days increases the phosphorylation of Erk and decreases the nuclear expression of Bach1. (A) Representative immunoblots and densitometric analysis of Bach1, Keap1 and Fyn kinase in the nuclear
fractions of hearts taken from non-diabetic and diabetic mice. (B) Representative immunoblots and densitometric analysis of Bach1, Keap1 and Fyn kinase in the nuclear fractions of hearts taken from sham, Na$_2$S Acute, and Na$_2$S 7d mice. (C) ChIP analysis of Bach1 binding to the NQO1 or HO-1 promoter in the hearts of Sham, Na$_2$S Acute, and Na$_2$S 7d mice. Values are mean ± SEM. Data in panel A was compared with a student’s T-test. All other data was compared through the use of a 1-way ANOVA with a Tukey test as the posthoc analysis. *p<0.05, **p<0.01, and ***p<0.001 vs. Sham or Non-Diabetic.

Fig. 6. Na$_2$S treatment for 7 days fails to decrease the nuclear expression of Bach1 when Erk is inhibited. (A) Representative immunoblots and densitometric analysis of total Erk and phosphorylated Erk in the hearts of sham, Na$_2$S Acute, and Na$_2$S 7d mice. Representative immunoblots and densitometric analysis of (B) total Erk and phosphorylated Erk, (C) nuclear Bach1 and Nrf2, and (D) NQO1 and HO-1 in the hearts of diabetic mice treated with vehicle, U0126, or U0126 and Na$_2$S (U0126+Na$_2$S) for 7 days. Values are mean ± SEM. Data was compared through the use of a 1-way ANOVA with a Tukey test as the posthoc analysis. *p<0.05, ***p<0.001 vs. Sham or Vehicle.

Fig. 7. Pretreatment with Na$_2$S for 7 days does not provide protection against myocardial ischemia and reperfusion injury when Erk is inhibited. (A) Myocardial INF/AAR and INF/LV and (B) circulating Troponin-I levels from following 30 minutes of ischemia and 2 hours of reperfusion. Mice were treated with vehicle, U0126, or U0126 and Na$_2$S (U0126+Na$_2$S) for 7 days prior to myocardial ischemia. (C) Schematic depicting the proposed signaling mechanism
by which H$_2$S activates Nrf2 signaling. Values are mean ± SEM. Data was
compared through the use of a 1-way ANOVA with a Tukey test as the posthoc
analysis.

### Table 1. Body weights and blood glucose levels.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body Weight (grams)</th>
<th>Blood Glucose (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-diabetic</td>
<td>5</td>
<td>25.7±0.6</td>
<td>199±19</td>
</tr>
<tr>
<td>Diabetic</td>
<td>5</td>
<td>44.4±0.4***</td>
<td>456±25***</td>
</tr>
<tr>
<td>Diabetic + Vehicle</td>
<td>8</td>
<td>46.7±0.9***</td>
<td>506±27***</td>
</tr>
<tr>
<td>Diabetic + Na$_2$S PC</td>
<td>8</td>
<td>46.9±1.8***</td>
<td>508±49***</td>
</tr>
<tr>
<td>Diabetic + Na$_2$S 7d</td>
<td>10</td>
<td>46.1±1.1***</td>
<td>490±29***</td>
</tr>
<tr>
<td>Diabetic + Vehicle 7d</td>
<td>8</td>
<td>43.5±1.1***</td>
<td>450±28***</td>
</tr>
<tr>
<td>Diabetic + U0126 7d</td>
<td>8</td>
<td>47.5±2.2***</td>
<td>523±21***</td>
</tr>
<tr>
<td>Diabetic + U0126 + Na$_2$S 7d</td>
<td>8</td>
<td>47.2±1.1***</td>
<td>448±32***</td>
</tr>
</tbody>
</table>

Values are means ± SEM. ***p<0.001 vs. Non-Diabetic
Figure 2

A. 

Free H₂S (nmol/mg wet weight) 

Vehicle  Na₂S PC  Na₂S 7d PC

B. 

Sulfane Sulfur (nmol/mg wet weight) 

Vehicle  Na₂S PC  Na₂S 7d PC

p < 0.001

C. 

Vehicle  Na₂S 24hr PC  Na₂S 7d PC

D. 

% AAR or LV 

AAR/LV  INF/AAR  INF/LV

p = NS  p < 0.001  p < 0.05

E. 

Troponin-I (ng/ml) 

Vehicle  Na₂S PC  Na₂S 7d PC

*  **
Figure 4

A. 

![Graph showing Nr2 and α-Tubulin expression in Non-Diabetic and Diabetic Whole Cell and Nuclear fractions.]

B. 

![Graph showing Fibrillarin expression in Sham, Na₂S Acute, and Na₂S 7d fractions.]

C. 

![Graph showing NQO1 and HO-1 expression in Sham, Na₂S Acute, and Na₂S 7d fractions.]

D. 

![Graph showing Relative Fold Enrichment of IP-Nrf2 and IP-IgG for NQO1 and HO-1. The annotations indicate statistical significance at p<0.01, p<0.001, and p<0.05.]
Figure 6

A. Erk-P

B. Erk-P

C. Erk

D. α-Tubulin

Sham Na₂S PC Na₂S 7d PC

Erk-P/Sham 44/42 kDa
Erk-P/Na₂S PC 44/42 kDa
Erk-P/Na₂S 7d PC 52 kDa

Vehicle U0126 U0126 + Na₂S

44/42 kDa
44/42 kDa
52 kDa

5 5 5
5 5 5
5 5 5

p > NS
p = NS
p = NS

Total Erk

Erk-P/Erk

p > NS
p > NS
p > NS

Vehicle U0126 U0126 + Na₂S

5 5 5
5 5 5
5 5 5

p = NS
p = NS
p = NS

Total Erk

Erk-P/Erk

p > NS
p > NS
p > NS

Vehicle U0126 U0126 + Na₂S

5 5 5
5 5 5
5 5 5

p > NS
p > NS
p > NS

Bach1

Nr2f

Fibrillarin

Vehicle U0126 U0126 + Na₂S

92 kDa
57 kDa
37 kDa

5 5 5
5 5 5
5 5 5

p > NS
p > NS
p > NS

Vehicle U0126 U0126 + Na₂S

5 5 5
5 5 5
5 5 5

p > NS
p > NS
p > NS

NQO1

HO-1

α-Tubulin

Vehicle U0126 U0126 + Na₂S

29 kDa
31 kDa
52 kDa

5 5 5
5 5 5
5 5 5

p > NS
p = NS
p = NS

Vehicle U0126 U0126 + Na₂S

5 5 5
5 5 5
5 5 5

p > NS
p = NS
p = NS

NQO1

HO-1