Thioredoxin 1 Is Essential for Sodium Sulfide–Mediated Cardioprotection in the Setting of Heart Failure

Chad K. Nicholson, Jonathan P. Lambert, Jeffery D. Molkentin, Junichi Sadoshima, John W. Calvert

Objective—The aim of this study was to determine whether thioredoxin 1 (Trx1) mediates the cardioprotective effects of hydrogen sulfide (H$_2$S) in a model of ischemic-induced heart failure (HF).

Approach and Results—Mice with a cardiac-specific overexpression of a dominant negative mutant of Trx1 and wild-type littermates were subjected to ischemic-induced HF. Treatment with H$_2$S as sodium sulfide (Na$_2$S) not only increased the gene and protein expression of Trx1 in the absence of ischemia but also augmented the HF-induced increase in both. Wild-type mice treated with Na$_2$S experienced less left-ventricular dilatation, improved left-ventricular function, and less cardiac hypertrophy after the induction of HF. In contrast, Na$_2$S therapy failed to improve any of these parameters in the dominant negative mutant of Trx1 mice. Studies aimed at evaluating the underlying cardioprotective mechanisms found that Na$_2$S therapy inhibited HF-induced apoptosis signaling kinase-1 signaling and nuclear export of histone deacetylase 4 in a Trx1-dependent manner.

Conclusions—These findings provide novel information that the upregulation of Trx1 by Na$_2$S therapy in the setting of HF sets into motion events, such as the inhibition of apoptosis signaling kinase-1 signaling and histone deacetylase 4 nuclear export, which ultimately leads to the attenuation of left-ventricular remodeling. (Arterioscler Thromb Vasc Biol. 2013;33:744-751.)

Key Words: apoptosis signaling kinase-1 ■ histone deacetylase 4 ■ nuclear factor of activated T cells ■ thioredoxin 1 ■ heart failure ■ hydrogen sulfide ■ left ventricular remodeling

In response to myocardial injury, the geometry, mass, volume, and function of the left ventricle (LV) change during a process referred to as ventricular remodeling. Initially, this process is considered to be adaptive. However, in response to continuous stimuli after events, such as myocardial infarction, LV remodeling becomes maladaptive leading to the development of heart failure (HF). Moreover, the morphological and functional changes that accompany LV remodeling serve as predictors of morbidity and mortality. Therefore, it is necessary to elucidate cellular and molecular mechanisms that underlie the development of HF, so that pharmacotherapies designed to coincide with the standard means of care can be implemented to improve the prognosis of patients suffering from this debilitating disease.

In this regard, therapeutic strategies aimed at increasing the levels of hydrogen sulfide (H$_2$S) have come to be a focus of interest, given their ability to exert cytoprotective effects in various models of injury. In the heart, treatment with exogenous H$_2$S or modulation of the endogenous production of H$_2$S through the cardiac-specific overexpression of the H$_2$S-generating enzyme, cystathionine γ-lyase, promotes cardioprotection against acute myocardial ischemia–reperfusion injury and HF. In contrast, the pharmacological inhibition or genetic deficiency of cystathionine γ-lyase results in an exacerbation of myocardial injury. These and other studies demonstrate that H$_2$S uses a variety of effects to counter ischemic injury, including its ability to attenuate oxidative stress, inhibit apoptosis, and reduce inflammation. Although these studies provide important insights in the cardioprotective actions of H$_2$S, they have not fully investigated the cellular mechanisms that underlie these cytoprotective effects.

Thioredoxin 1 (Trx1) is an oxidoreductase enzyme that acts as an antioxidant by facilitating the reduction of other proteins by cysteine thiol–disulfide exchange. Through its redox activity, Trx1 regulates apoptosis signal-regulating kinase-1 (ASK1), nuclear factor of activated T cells, Ras, and Akt. Patients with acute coronary syndrome and dilated cardiomyopathy show elevated serum levels of Trx1, suggesting a possible association between Trx1 and the severity of HF. Experimental studies demonstrate that Trx1 plays a prosurvival role in response to myocardial injury. This is attributed to its ability to reduce cardiac hypertrophy in models of HF and to reduce...
apoptosis in models of HF\textsuperscript{13} and ischemia–reperfusion injury.\textsuperscript{14} Therefore, Trx1 is an ideal cellular target for impeding the progression of HF.

H\textsubscript{2}S has previously been shown to increase the protein expression of Trx1.\textsuperscript{4} On the basis of this evidence and the evidence that Trx1 plays a protective role in the heart, one can speculate that Trx1 contributes to the cardioprotective mechanisms of H\textsubscript{2}S. Therefore, a major goal of this study was to determine whether Trx1 mediates the cardioprotective effects of H\textsubscript{2}S in a model of ischemic-induced HF.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**Na\textsubscript{2}S Treatment Limited the Extent of Myocardial Injury After HF**

Initial experiments were conducted to investigate the extent of myocardial injury and the effects of H\textsubscript{2}S in the ischemic HF model. For these experiments, mice were subjected to 60 minutes of left coronary artery ischemia followed by 4 weeks of reperfusion. H\textsubscript{2}S, administered in the form of Na\textsubscript{2}S (100 µg/kg), or vehicle was administered at reperfusion and then for the first 7 days of reperfusion. HF increased left-ventricular end-diastolic diameter and left-ventricular end systolic diameter in both groups (Figure 1A and 1B; \(P<0.01\) versus baseline). However, the increase in LV dimensions was attenuated in wild-type (WT) mice treated with Na\textsubscript{2}S (WT Na\textsubscript{2}S HF) compared with vehicle-treated mice (WT Veh HF; \(P<0.001\)). After HF, LV ejection fraction and LV fractional shortening decreased in both groups (Figure 1C and 1D; \(P<0.001\) versus baseline). Na\textsubscript{2}S treatment, however, significantly improved LV function (\(P<0.001\) versus WT Veh HF). Along with the improvements in LV dimensions and function, Na\textsubscript{2}S-treated mice displayed better contractility and relaxation after the induction of HF when compared with the vehicle-treated mice, as evidenced by the improvements in LV dp/dt max, dp/dt min, and the relaxation time constant \(\tau\) (Figure 1 in the online-only Data Supplement), as well as Max LV pressure, and circumferential stress (Figure 1E and 1F; \(P<0.05\) versus WT Veh HF).

Additional experiments were then conducted to determine the circulating and cardiac levels of sulfide after treatment with Na\textsubscript{2}S in the presence and absence of HF. For the first set of experiments, mice were treated with Na\textsubscript{2}S for 7 days (daily tail vein injections) and then different groups were euthanized at 1 week, 2 weeks, and 4 weeks after the start of treatment. At the end of the treatment period (1 week), free H\textsubscript{2}S and sulfane sulfur (bound sulfide) levels were significantly increased in the blood when compared with baseline levels (Figure 2A).

**Figure 1.** Na\textsubscript{2}S therapy failed to improve left-ventricular (LV) structure and function in thioredoxin 1 dominant negative transgenic mice after heart failure. (A) Left-ventricular end-diastolic diameter (LVEDD), (B) left-ventricular end systolic diameter (LVESD), (C) LV ejection fraction, (D) LV fractional shortening, (E) max LV pressure, and (F) circumferential stress for wild-type (WT) and Trx1 dominant negative transgenic (Tg-DN-Trx1) mice treated with vehicle (WT Veh HF and Tg-DN-Trx1 Veh HF) or Na\textsubscript{2}S (100 µg/ kg for 7 days; WT Na\textsubscript{2}S HF; and Tg-DN-Trx1 Na\textsubscript{2}S HF) 4 weeks after myocardial ischemia. Numbers inside bars indicate sample size. Values are means±SEM. *\(P<0.05\), **\(P<0.01\), and ***\(P<0.001\) vs baseline or WT Veh HF. \(\phi\) \(P<0.05\) and \(\psi\) \(P<0.01\) vs WT Veh HF.
However, these levels returned to baseline levels by 1 week after the end of the treatment period (2-week time point). In the heart, Na$_2$S treatment did not significantly increase free H$_2$S levels at any time point investigated (Figure 2B). Cardiac sulfane sulfur levels were increased at the end of the treatment period and then declined to baseline levels by 1 week after the end treatment (2-week time point). Experiments then were conducted to evaluate the circulating and cardiac levels of sulfide in the setting of HF. For these experiments, mice were subjected to HF and Na$_2$S treatment as described above. Different groups were then euthanized at 1, 2, and 4 weeks after reperfusion. Circulating levels of free H$_2$S and sulfane sulfur were not altered in the Veh HF group at any time point investigated (Figure 2C and 2D). In contrast, free H$_2$S levels trended ($P_N$S) to be higher in the Na$_2$S group, and sulfane levels were significantly higher at both 1 week ($P<0.05$ versus Veh HF and $P<0.01$ versus baseline) and 2 weeks ($P<0.05$ versus baseline) of reperfusion. In the heart, free H$_2$S and sulfane sulfur levels rose slightly higher than baseline levels at 1 week of reperfusion in the Veh HF group before significantly falling to levels below baseline at both 2 weeks and 4 weeks of reperfusion (Figure 2E and 2F). Cardiac levels of sulfide in the Na$_2$S HF group displayed similar trends with the Veh HF group. However, the levels in the Na$_2$S HF group were higher than the Veh HF levels at all time points evaluated, especially at 2 weeks after reperfusion ($P<0.05$ versus Veh HF).

**Na$_2$S Therapy Increases the Gene and Protein Expression of Trx1**

One of the potential mechanisms by which it is hypothesized that Na$_2$S provides cytoprotection during the development of HF is through an increase in Trx1 signaling. Therefore, experiments were conducted to evaluate whether Na$_2$S therapy altered the expression of Trx1 in the setting of HF. For these experiments, mice were divided into sham or HF groups. The mice in the sham groups were administered vehicle (Sham) or Na$_2$S (Na$_2$S Sham) for 7 days. The mice in the HF groups were subjected to myocardial ischemia–reperfusion injury and received either vehicle (Veh HF) or Na$_2$S (Na$_2$S HF) at reperfusion and then daily for 7 days after reperfusion. Mice were then euthanized at this time and the hearts were processed to evaluate changes in the gene and protein expression of Trx1. Polymerase chain reaction analysis revealed an increase in the gene expression of Trx1 in the Na$_2$S Sham mice (Figure 3A; $P<0.05$ versus Sham). HF also significantly increased the gene expression of Trx1 when compared with the sham hearts ($P<0.001$). Importantly, the Trx levels were found to be the highest in the hearts from the Na$_2$S HF mice ($P<0.01$ versus Sham and $P<0.001$ versus Veh HF). Western blot analysis confirmed the increase in Trx1 protein levels with similar trends (Figure 3B). Additionally, Trx1 activity was significantly increased after HF in both groups (Figure 3C; $P<0.001$ versus Sham). Again,
Trx activity was found to be the highest in the hearts from the Na$_2$S HF mice ($P<0.01$ versus Veh HF).

**Na$_2$S Therapy Failed to Attenuate the Development of HF Without Functional Trx1**

Experiments were then conducted to investigate whether Trx1 was critical for the cardioprotection afforded by Na$_2$S therapy. Dominant negative mutant Trx1 (Tg-DN-Trx1) mice were subjected to HF and Na$_2$S treatment as described above. Echocardiography at 4 weeks of reperfusion revealed that HF significantly increased left-ventricular end-diastolic diameter and left-ventricular end systolic diameter in the Tg-DN-Trx1 mice receiving vehicle (Tg-DN-Trx1 Veh HF) or Na$_2$S (Tg-DN-Trx1 Na$_2$S HF; Figure 1A and 1B; $P<0.05$ versus baseline). The increase in left-ventricular end systolic diameter was significantly higher in both groups of Tg-DN-Trx1 mice when compared with the WT Veh HF group ($P<0.05$).

HF also reduced LV ejection fraction and LV fractional shortening in both groups (Figure 1C and 1D; $P<0.001$ versus baseline). Again the decrease in both LV ejection fraction and LV fractional shortening was significantly lower in both groups of Tg-DN-Trx1 mice when compared with the WT Veh HF group ($P<0.05$). Importantly, Na$_2$S therapy failed to attenuate the development of HF without functional Trx1. The failure of Na$_2$S therapy to attenuate the development of HF without functional Trx1 was further confirmed with hemodynamic measurements. Na$_2$S therapy failed to improve any of these measurements in the Tg-DN-Trx1 mice (Figure 1E and 1F in the online-only Data Supplement).

The Tg-DN-Trx1 mice possess a cardiac-specific overexpression of a dominant negative mutant of Trx1, which results in diminished activity of endogenous Trx. Because this is not a true knockout model, experiments were conducted to determine how HF and Na$_2$S treatment affect the expression and activity of endogenous Trx1 in the Tg-DN-Trx1 mice. First, using an antibody from Santa Cruz Biotechnology (sc-18215) that recognizes both human and mouse Trx1 and an antibody from Cell Signaling Technology (2298S) that only recognizes mouse and rat Trx1, it was determined that the cardiac expression of endogenous Trx1 was similar between Tg-DN-Trx1 and WT mice (Figure IIA and IIB in the online-only Data Supplement). HF significantly increased the expression of endogenous Trx1 in both groups of Tg-DN-Trx1 mice (Figure 3D; $P<0.05$ versus Sham). The increase in expression was not as high as the increase observed in the WT mice, and Na$_2$S treatment did not further increase Trx1 levels in the Tg-DN-Trx1 mice. Additionally, the activity of Trx1 was not increased in either group of mice after the induction of HF (Figure 3C). Together, these data suggest that HF does increase the expression of endogenous Trx1 in the Tg-DN-Trx1 mice, but the activity of Trx1 does not change.

**Na$_2$S Therapy Failed to Decrease Cardiac Hypertrophy Without Functional Trx1**

Cardiac hypertrophy was analyzed by determining heart weight/body weight ratios, heart weight/tibia length ratios, and myocardial cell surface area in both WT and Tg-DN-Trx1 mice (Figure 4). Analysis at 4 weeks of reperfusion revealed that HF significantly increased heart weight/body weight and heart weight/tibia length ratios, as well as myocardial cell surface area in both WT and Tg-DN-Trx1 mice receiving vehicle or Na$_2$S treatment. When compared with the WT Veh HF group, the Tg-DN-Trx1 Veh HF and Tg-DN-Trx1 Na$_2$S HF groups displayed more of an increase in cardiac hypertrophy based on the heart weight/tibia length ratios and myocardial cell surface area measurements (Figure 4B and 4D; $P<0.01$). Treatment
with Na₂S reduced cardiac hypertrophy in the WT mice but failed to reduce hypertrophy in the Tg-DN-Trx1 mice.

**Na₂S Therapy Decreased the HF-Induced Activation of the ASK1-Jnk/p38 Signaling Cascade in a Trx1-Dependent Manner**

Experiments were then conducted to investigate potential mechanisms by which Trx1 mediates the protective effects of Na₂S therapy. In the heart, Trx1 plays a prosurvival role in response to stress by inhibiting ASK1 and modulating the nuclear localization of class II histone deacetylases (HDACs). Therefore, subsequent experiments focused on these 2 downstream targets of Trx1.

ASK1 regulates cardiac remodeling in response to different HF stimuli via the activation of p38 mitogen-activated protein kinase and Jun N-terminal kinase (Jnk). Experiments were therefore conducted to examine whether the activation of ASK1 signaling accompanied the development of ischemic-induced HF and whether Na₂S therapy could alter any changes. Analysis of heart homogenates from WT mice revealed that 1 week after the induction of HF, there was a significant increase (Figure 5A and 5B; *P* <0.001 versus WT Sham) in the

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**Figure 4.** Na₂S therapy failed to improve cardiac hypertrophy without functional thioredoxin 1 (Trx1). (A) Ratios of heart/body weight (HW:BW) and (B) heart weight/tibia length (HW:TL) were used as a measure of cardiac hypertrophy in wild-type (WT) and dominant negative mutant Trx1 (Tg-DN-Trx1) mice 4 weeks after the induction of heart failure. C, Representative photomicrographs of wheat germ agglutinin stained hearts from WT Sham, WT Veh HF, WT Na₂S HF, Tg-DN-Trx1 Sham, Tg-DN-Trx1 Veh HF, and Tg-DN-Trx1 Na₂S HF mice. D, Summary of myocyte cell surface area measurements of wheat germ agglutinin stained hearts. Scale bar equals 100 μm. Values are means±SEM. **P*<0.01 and ***P*<0.001 vs Sham. ψP*<0.01 vs WT Veh HF.

**Figure 5.** Na₂S therapy attenuates the heart failure–induced activation of the ASK1-Jnk/p38 signaling cascade in a thioredoxin 1 (Trx1)-dependent manner. (A) Representative immunoblots and densitometric analysis of (B) total and phosphorylated apoptosis signaling kinase-1 (ASK1) at threonine residue 845 (ASK1-P), (C) total and phosphorylated c-Jun N-terminal kinase (Jnk) at threonine residue 183 and tyrosine residue 185 (Jnk-P), and (D) total and phosphorylated p38 at threonine residue 180 and tyrosine residue 182 (p38-P) from the hearts of WT Sham, WT Veh HF, WT Na₂S HF, dominant negative mutant Trx1 (Tg-DN-Trx1) Sham, Tg-DN-Trx1 Veh HF, and Tg-DN-Trx1 Na₂S HF mice collected 1 week after the induction of heart failure. Values are means±SEM. **P*<0.01 and ***P*<0.001 vs Sham.
phosphorylation of ASK1 at threonine residue 845 (ASK1-P; phosphorylation here is indicative of activation). This was accompanied by a significant increase in the phosphorylation of Jnk (Jnk-P; threonine residue 183 and tyrosine residue 185), and p38 (p38-P; threonine residue 180 and tyrosine residue 182) (Figure 5A, 5C, and 5D; \( P < 0.001 \) and \( P < 0.01 \) versus WT Sham, respectively), suggesting that ASK1 signaling was activated in response to HF. However, treatment with Na\textsubscript{2}S attenuated the HF induction of ASK1 signaling, as evidenced by a significant decrease in the phosphorylation of ASK1, Jnk, and p38 (\( P < 0.05 \) versus WT Veh HF). These data suggest that Na\textsubscript{2}S therapy requires Trx1 to inhibit the ASK1-Jnk/p38 signaling cascade initiated by HF.

**Na\textsubscript{2}S Therapy Attenuated the HF-Induced Nuclear Export of HDAC4 in a Trx1-Dependent Manner**

HDACs regulate several biological processes, largely through their repressive influence on transcription.\textsuperscript{19} In particular, HDAC influences the activity of nuclear factor of activated T cells (NFAT), a procardiac hypertrophy transcription factor.\textsuperscript{20} Trx1 protects the heart from stress by modulating the nuclear export of HDAC4.\textsuperscript{16} Therefore, experiments were performed to determine whether the attenuation in cardiac hypertrophy observed after Na\textsubscript{2}S therapy was associated with changes in HDAC4 nuclear expression and NFAT activity. Analysis revealed that 1 week after the induction of HF, there was a significant decrease in the nuclear expression of HDAC4 (Figure 6A; \( P < 0.001 \) versus WT Sham). Importantly, Na\textsubscript{2}S therapy attenuated this HF-induced export of HDAC4 (\( P < 0.05 \) versus WT Veh HF). Further studies were then conducted to determine whether the changes in nuclear HDAC4 levels resulted in the activation of NFAT. For these studies, NFAT-luciferase reporter mice were subjected to HF and Na\textsubscript{2}S therapy. HF induced a significant increase in the activity of NFAT, as evidenced by an increase in the luciferase activity measured in the hearts of Veh HF and Na\textsubscript{2}S HF mice (Figure 6B; \( P < 0.001 \) versus WT Sham). However, the Na\textsubscript{2}S HF mice displayed significantly lower luciferase activity when compared with the Veh HF mice (\( P < 0.05 \)). These data suggest that the reduction in cardiac hypertrophy observed in the Na\textsubscript{2}S HF mice could possibly be mediated through the actions of HDAC4 on NFAT transcriptional activity.

To determine whether Trx1 was needed for Na\textsubscript{2}S therapy to prevent the HF-induced nuclear export of HDAC4, Tg-DN-Trx1 mice were subjected to HF and Na\textsubscript{2}S treatment as before. These experiments found that HF increased the nuclear export of HDAC4 and that Na\textsubscript{2}S therapy failed to decrease this export (Figure 6A), suggesting that Na\textsubscript{2}S therapy requires Trx1 to prevent the nuclear export of HDAC4 after the initiation of HF.

**Discussion**

The cytoprotective effects of H\textsubscript{2}S have been documented in numerous models of injury, including HF. For instance, Mishra et al\textsuperscript{21} reported that treatment with H\textsubscript{2}S in the drinking water of mice attenuated the adverse remodeling of the LV in an arteriovenous fistula model of chronic HF. Additionally, Givvimani et al\textsuperscript{22} found that treatment with H\textsubscript{2}S in the drinking water mitigated the transition from compensatory hypertrophy to HF in response to aortic banding. Finally, it was reported that both endogenous and exogenous H\textsubscript{2}S improved survival and attenuated the morphological and functional impairments of the LV in mice after the initiation of ischemic-induced HF.\textsuperscript{5} The present study further supports these previous findings and provides evidence that treatment with Na\textsubscript{2}S during the first 7 days of reperfusion not only improves LV dilatation...
and function during the development of ischemic-induced HF but also improves LV contractility and relaxation, as well as attenuates the development of cardiac hypertrophy. Together, these findings suggest that treatment with H$_2$S attenuates adverse LV remodeling in response to different HF stimuli.

H$_2$S has the ability to attenuate many of the processes that lead to LV remodeling. In terms of cellular targets, Na$_2$S therapy increases the transcriptional activity of nuclear-factor-E2-related factor-2, increases the phosphorylation of Akt, suppresses matrix metalloproteinases, and increases vascular endothelial growth factor synthesis. The activation/inhibition of these proteins certainly contributes to the protective effects observed in the current study. However, given the nature of H$_2$S as a gasotransmitter to activate multiple signaling pathways at the same time, it can be hypothesized that other cellular targets are involved. Therefore, the main goal of the current study was to evaluate the role that Trx1 plays in mediating the cardioprotective effects of Na$_2$S therapy. Trx1, a small redox-active multifunctional protein, acts as a potent antioxidant and a redox-regulator of many cellular processes. Trx1 plays a prosurvival role in response to myocardial injury by reducing cardiac hypertrophy and apoptosis. Trx1 modulates the development of cardiac hypertrophy by regulating the nucleocytoplasmic shuttling of HDAC4. ASK1, enabling it to phosphorylate Jnk and p38, which ultimately leads to pathological LV structural and functional remodeling. The control of histone deacetylation by HDACs has been reported to be the central point for the control of cardiac growth and gene expression in response to acute and chronic stress stimuli. In response to stress stimuli, the expression of class II HDACs does not change. Rather, HDACs are shuttled from the nucleus to the cytosol, where they can no longer suppress target transcription factors. In the heart, pathophysiological stress signals associated with HF stimulate the nuclear export of HDAC4. This in turn elicits the activation of NFAT, which ultimately leads to the development of cardiac hypertrophy.

In the original description of the Tg-DN-Trx1 mouse, it was postulated that Trx1 could potentially mediate the protective effects of Na$_2$S therapy in the setting of ischemic-induced HF because of these reported cardioprotective effects and because it was previously found that the protein expression of Trx1 was upregulated by a single injection of Na$_2$S. However, it was not known whether multiple injections of Na$_2$S could maintain the elevated levels of Trx1. As such, the current study is the first to provide evidence that 7 days of Na$_2$S therapy not only increases the gene and protein expression of Trx1 in the absence of ischemia but also that it augments the HF-induced increase in both, as well as increases Trx1 activity. More importantly, the current study provides direct evidence to support the hypothesis that Trx1 mediates the cardioprotective effects of Na$_2$S therapy, as evidenced by the findings that Na$_2$S therapy failed to improve cardiac dilatation, dysfunction, or hypertrophy in Tg-DN-Trx1 mice.

Another major finding of the current study relates to the mechanism that Na$_2$S therapy signals through Trx1 to attenuate the adverse remodeling of LV during HF. Specifically, the current study provides important insights into the effects of Na$_2$S therapy on ASK1-mediated signaling and HDAC nuclear expression. ASK1 is strongly activated in various cell types in response to stimuli, such as oxidative stress and tumor necrosis factor-α. In the heart, ASK1 has emerged as a kinase of essential importance, given its prevailing role in regulating cell death and cardiac remodeling via the activation of p38 mitogen-activated protein kinase and Jnk. Numerous proteins interact with ASK1 through protein–protein interactions to regulate its activity. In particular, Trx1 was identified in a yeast 2-hybrid screen as a negative regulator of the ASK1-Jnk/p38 pathway. Under normal conditions, ASK1 constantly forms an inactive complex with Trx1. However, in response to the oxidative stress after myocardial infarction, Trx1 releases ASK1 enabling it to phosphorylate Jnk and p38, which ultimately leads to pathological LV structural and functional remodeling. The findings of the current study support the hypothesis that activation of ASK1 contributes to the development of HF after myocardial infarction, as evidenced by the finding that the improvements in LV dilatation and function in response to Na$_2$S therapy are associated with a suppression of the ASK1-Jnk/p38 pathway. More importantly, the current study provides evidence for the first time that Na$_2$S therapy inhibits ASK1-Jnk/p38 signaling in a Trx1-dependent manner, as evidenced by the findings that Na$_2$S therapy fails to reduce the phosphorylation of ASK1, Jnk, or p38 in Tg-DN-Trx1 mice.

Together these findings suggest that an additional mechanism of action for Na$_2$S therapy attenuates cardiac hypertrophy in a Trx1-dependent manner and attenuated the HF-induced activation of NFAT. Together these findings suggest that an additional mechanism of action for Na$_2$S therapy attenuates cardiac hypertrophy in a Trx1-dependent manner and attenuated the HF-induced activation of NFAT. Although this was not observed in the current study, care should be taken when considering the effects of Trx1 activity and Na$_2$S treatment on the development of cardiac hypertrophy after ischemic-induced HF. Second, these experiments cannot completely rule out the contribution of Trx1-independent mechanisms. For example, Akt, which is activated by Na$_2$S, has been shown to inhibit ASK1 and could possibly contribute to the suppression of ASK1 signaling. Additionally, the activation of protein kinase C, which is also induced by Na$_2$S, can regulate the phosphorylation of Jnk and p38 in an ASK1-independent manner.

In summary, this study provides novel evidence that Na$_2$S therapy attenuates LV dilatation, LV dysfunction, and LV hypertrophy in the setting of ischemic-induced HF in a Trx1-dependent manner. Furthermore, these findings provide important information that the upregulation of cardiac Trx1 by Na$_2$S in the setting of HF sets into motion events, such as the inhibition of ASK1 signaling and HDAC4 nuclear export, which ultimately leads to an attenuation of LV remodeling. Because patients with major remodeling demonstrate progressive worsening of cardiac function, preventing, slowing, or reversing remodeling is a goal of any HF therapy.
Therefore, the findings of the current study continue to support the emerging concept that treatment strategies aimed at increasing the levels of H₂S may be of clinical importance in reducing the mortality and morbidity associated with HF.

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Disclosures

None.

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Significance

Therapeutic strategies aimed at increasing the levels of hydrogen sulfide (H₂S) have been shown to exert cytoprotective effects in various models of heart failure. The current study expanded on these initial findings and sought to investigate the role that thioredoxin 1 (Trx1) plays in mediating these effects. Specifically, this study provides novel evidence that treatment with H₂S in the form of sodium sulfide (Na₂S) attenuates left-ventricular dysfunction and hypertrophy in the setting of ischemic-induced heart failure in a Trx1-dependent manner. Furthermore, these findings provide important information that the upregulation of cardiac Trx1 by Na₂S sets into motion events, such as the inhibition of apoptosis signaling kinase-1 signaling and histone deacetylase 4 (HDAC4) nuclear export, which ultimately leads to an attenuation of left-ventricular remodeling. Together, these findings further support the emerging concept that H₂S therapy may be of clinical importance in the treatment of heart failure.
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Supplemental Figure Legends

Supplemental Figure I. (A) Max dP/dT and Min dP/dT, and (B) Tau were evaluated in WT Veh HF, WT Na\(_2\)S HF, Tg-DN-Trx1 Veh HF, and Tg-DN-Trx1 Na\(_2\)S HF 4 weeks after induction of heart failure. Values are means ±SEM. Means for all data were compared by use of a 1-way ANOVA with a Tukey Test as the posthoc analysis.*p<0.05 vs. WT Veh HF.

Supplemental Figure II. (A) Represetative immunoblots of Trx1 from the heart of wild-type and Tg-DN-Trx1 mice using an antibody that recognizes both human and mouse Trx1. (B) Represetative immunoblots of Trx1 from the heart of wild-type and Tg-DN-Trx1 mice using an antibody that recognizes only mouse Trx1.
Supplemental Figure II

A.

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Thioredoxin 1 is Essential for Sodium Sulfide-Mediated Cardioprotection in the Setting of Heart Failure

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Materials and Methods

Animals. Two different strains of mice were utilized: (1) Male mice (10-12 weeks of age) with a cardiac-specific overexpression of a dominant negative redox-inactive mutant of Trx1 (Tg-DN-Trx1) and wild-type (WT) littermates (FVB background), and (2) Male NFAT-luciferase reporter mice (10-12 weeks of age). The generation of cardiac-specific Trx1 dnTg mice and NFAT-luciferase reporter mice have been described previously. 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. The number of animals used for each experiment is depicted on each figure.

Materials. H₂S was administered as sodium sulfide (Na₂S; Sigma Aldrich). Na₂S was dissolved in saline and administered using a 32-gauge needle at a dose of 100 µg/kg (final volume of 50 µL) as an injection into the LV lumen once at the time of reperfusion followed by daily tail vein injections for the first 7 days of reperfusion. This dose of Na₂S was selected based on our previous experience investigating Na₂S in murine models of cardiac I/R injury. Saline was administered in the same manner for the vehicle groups. Na₂S was always prepared just prior to use.

Heart Failure Protocol. Ischemic-induced heart failure was produced by subjecting mice to 60 minutes of left coronary artery (LCA) occlusion followed by reperfusion for up to 4 weeks as previously described. All mice were randomly allocated to the treatment groups.

Echocardiograph Analysis. Baseline echocardiography images were obtained one week prior to myocardial ischemia to avoid any anesthetic effects. The mice were lightly anesthetized with isoflurane (1-5% in 100% oxygen) and in vivo transthoracic echocardiography of the LV using a 38-MHz linear array scanhead interfaced with a Vevo 2100 (Visualsonics) was used to obtain high-resolution M-mode images. From these images LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), fractional shortening (FS), and ejection fraction (EF) were calculated. Echocardiography images were obtained and analyzed again 4 weeks following the induction of heart failure.

Hemodynamic Analysis. Following echocardiography analysis, mice were anesthetized with isoflurane (1-5% in 100% oxygen). LV hemodynamics were assessed by passing a 1.2F pressure catheter (Scisense) into the LV lumen via the right common carotid artery. The catheter was connected to a computer and data was collected with LabScribe2 software (Version 2.334, iWorx Systems, Inc). Circumferential stress was calculated as previously described.
Subcellular Fractionation and Western Blot Analysis. Subcellular fractionation and Western blot analysis was performed as described previously. Protein concentrations were measured with the DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were loaded into lanes of polyacrylamide-SDS gels. The gels were electrophoresed, followed by transfer of the protein to a PVDF membrane. The membrane was then blocked and probed with primary antibodies overnight at 4°C. Immunoblots were next processed with secondary antibodies (Cell Signaling) for 1 hr at room temperature. Immunoblots were then probed with a Super Signal West Dura kit (Thermo) to visualize signal, followed by exposure to X-ray film (Denville Scientific). The film was scanned to make a digital copy and densitometric analysis was performed to calculate relative intensity with ImageJ software from the National Institutes of Health (version 1.40g) using the Rodbard function.

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 Trx1 and 18S. Analysis was carried out using the ΔΔ-CT method with 18S correction and reported as relative fold change versus sham.

NFAT Luciferase activity. Luciferase enzymatic activity in heart extracts was measured with a commercially available kit (luciferase assay system, Promega Corp.).

Thioredoxin 1 Activity Assay. Thioredoxin 1 activity in heart extracts was measured with a commercially available kit according to the manufacturer’s instructions (Thioredoxin/Thioredoxin Reductase Mammalian Assay Kit, Cayman Chemical).

Hydrogen Sulfide Measurements. Hydrogen sulfide and sulfane sulfur levels were measured in tissue and blood according to previously described methods. Fresh tissue was homogenized in 5 volumes of PBS (pH 7.4). For measurement of H₂S, 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. For the measurement of H₂S and sulfane sulfur in urine, 0.025 mL and 0.0125 mL of urine 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 and urine, the amount of H₂S is reported as µM.

**Wheat germ agglutinin staining.** Cell surface area (µm²) was analyzed by staining cardiac cryosections with wheat germ agglutinin (WGA)-Texas Red-X conjugate (Life Technologies) as described previously to show myocyte membranes in histological sections. Cryosections were first washed in 1XPBS and then incubated in 10µg WGA-Texas Red-X conjugate for 1 hour at room temperature followed by additional washes in 1XPBS. Slides were mounted with Vectashield mounting medium (Vector Labs) and sealed. Digital images were captured and cell surface area was assessed with NIS Elements Imaging Software (version 3.22.11) in at least 5 animals per group with at least 3 randomly taken sections per heart and at least 100 myocytes were counted per animal.

**Statistical Analysis.** All the data are expressed as mean ± standard error (SEM). Means were compared using Prism 4 (GraphPad Software, Inc) with Student’s unpaired 2-tailed t-test, one-way analysis of variance (ANOVA), or two-way ANOVA where indicated. For the ANOVA, if a significant result was found, the Tukey (one-way ANOVA), Dunnett’s (one-way ANOVA), or Bonferroni (two-way ANOVA) test was used as the post hoc analysis. For all data, a p value less than 0.05 was considered significant.
References


