Sodium Sulfide Attenuates Ischemic-Induced Heart Failure by Enhancing Proteasomal Function in an Nrf2-Dependent Manner

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Background—Therapeutic strategies aimed at increasing hydrogen sulfide (H₂S) levels exert cytoprotective effects in various models of cardiovascular injury. However, the underlying mechanism(s) responsible for this protection remain to be fully elucidated. Nuclear factor E2–related factor 2 (Nrf2) is a cellular target of H₂S and facilitator of H₂S-mediated cardioprotection after acute myocardial infarction. Here, we tested the hypothesis that Nrf2 mediates the cardioprotective effects of H₂S therapy in the setting of heart failure.

Methods and Results—Mice (12 weeks of age) deficient in Nrf2 (Nrf2 KO; C57BL/6J background) and wild-type littermates were subjected to ischemic-induced heart failure. Wild-type mice treated with H₂S in the form of sodium sulfide (Na₂S) displayed enhanced Nrf2 signaling, improved left ventricular function, and less cardiac hypertrophy after the induction of heart failure. In contrast, Na₂S therapy failed to provide protection against heart failure in Nrf2 KO mice. Studies aimed at evaluating the underlying cardioprotective mechanisms found that Na₂S increased the expression of proteasome subunits, resulting in an increased proteasome activity and a reduction in the accumulation of damaged proteins. In contrast, Na₂S therapy failed to enhance the proteasome and failed to attenuate the accumulation of damaged proteins in Nrf2 KO mice. Additionally, Na₂S failed to improve cardiac function when the proteasome was inhibited.

Conclusions—These findings indicate that Na₂S therapy enhances proteasomal activity and function during the development of heart failure in an Nrf2-dependent manner and that this enhancement leads to attenuation in cardiac dysfunction.

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Key Words: heart failure □ hydrogen sulfide □ myocardial infarction □ Nrf2 □ proteasome

Hydrogen sulfide (H₂S), an endogenously produced gaseous signaling molecule, is critical for the regulation of cardiovascular homeostasis.¹ Recently, therapeutic strategies aimed at increasing H₂S levels have shown cardioprotective actions in models of acute myocardial ischemia–reperfusion (I/R) injury and heart failure.²⁻⁶ These studies have provided insights into several mechanistic actions, demonstrating inhibition of apoptosis, augmentation of endogenous antioxidants, and stimulation of angiogenesis. Several years ago, we identified nuclear factor E2–related factor 2 (Nrf2) as a major cellular target for H₂S and a regulator of the acute cardioprotective effects induced by H₂S.⁷ Nrf2, a member of the NF-E2 family of transcription factors, regulates the gene expression of enzymes that serve to detoxify pro-oxidative stressors. This regulation is mediated by Nrf2 binding to the antioxidant responsive element (ARE) found in the promoter region of genes, such as heme oxygenase 1 and NADPH:quinone oxidoreductase 1.

See Clinical Perspective

The proteome is more complex than the genome and transcriptome in terms of informational content.⁸ Therefore, regulating the quality of this information is essential for cell survival and function.⁹,¹⁰ This is particularly true for organs like the heart that possess a limited ability to regenerate. As such, the protein quality control system, consisting of chaperone proteins, autophagy, and the ubiquitin–proteasome system, is critically important to maintain the fidelity of the heart under both physiological and pathological conditions.¹¹ The ubiquitin–proteasome system is the primary effector of the protein quality control system, protecting cardiomyocytes from the accumulation of aberrant and misfolded proteins,
which disrupt intracellular signaling and induce cell death. Studies have noted changes in the activity of the proteasome in the setting of heart disease. Inevitably, increases and decreases have been reported in animal models and in diseased human hearts. This is not surprising given the heterogeneity of heart disease. However, there is consistent data suggesting that proteasome function is decreased in response to myocardial ischemia and likely contributes to the progression of ischemic-induced heart failure. Therefore, strategies aimed at promoting proteasome activity may have a therapeutic benefit in this setting. However, little is known about how the proteasome is regulated in response to myocardial ischemia. The 26S proteasome is the cellular machinery responsible for the degradation of polyubiquitinated and oxidized proteins. It is composed of the 20S proteolytic core and the 19S activation particles, as well as other axillary components. The 20S core particle is made up of 4 heptameric 20S rings. Unfolded proteins are degraded in the cavity of the 20S core particle by the chymotrypsin-like, trypsin-like, and caspase-like activities of the β5, β2, and β1 subunits, respectively. The promoter of several proteasomal subunits contains an ARE, and there is evidence that Nrf2 activators increase the expression and activity of the β1, β2, and β5 proteasomal subunits.

Currently, it is not known if H2S induces Nrf2-related signaling or modulates the expression/function of the cardiac proteasome in the setting of heart failure. Therefore, a major goal of this study was to determine if H2S affords protection in the setting of ischemic-induced heart failure by modulating the cardiac proteasome in an Nrf2-dependent manner.

**Methods**

An expanded Materials and Methods section is available in the Data Supplement.

**Animals**

Male mice (12 weeks of age) with a global deficiency in Nrf2 (Nrf2 KO; kind gift from Thomas Kensler) and wild-type (WT) littermates (C57BL/6J background) were used in all experiments. All experimental 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**

H2S was administered as sodium sulfide (Na2S; Sigma Aldrich). Na2S was dissolved in saline and administered at a dose of 100 μg/kg (final volume of 50 μL) as an injection into the left ventricular (LV) lumen once at reperfusion followed by daily tail vein injections for the first week of reperfusion. Saline was administered in the same manner for the vehicle groups. Groups of mice were also treated with the proteasome inhibitor bortezomib (0.2 mg/kg; Millipore) either alone or together with Na2S.

**Heart Failure Protocol**

Ischemic-induced heart failure was produced by 60 minutes of left coronary artery occlusion followed by reperfusion for 4 weeks. All mice were randomly allocated to the treatment groups. Echocardiography, hemodynamics, and wheat germ agglutinin staining were performed as previously described.

**Protein Extraction and Western Blot Analysis**

Whole cell homogenates were obtained as previously described. Nuclear fractions were obtained using the Subcellular Protein Fractionation Kit for Tissue (87790, Thermo Scientific). Proteasome were collected as previously described. Western Blot analysis was performed as described previously.

**Immunoprecipitation**

Heart homogenates were immunoprecipitated with an antibody to the β2 subunit using the Dynabeads Protein G Immunoprecipitation Kit according to manufacturer’s instructions. Samples were then subjected to standard Western blot techniques.

**Proteasome Activity**

The caspase-, trypsin-, and chymotrypsin-like activities of proteasomes were assayed using the fluorescently tagged substrates.

**Cellular Soluble Oligomers of Misfolded Proteins and Oxidized Proteins**

Heart homogenates were dot-blotted onto polyvinylidene difluoride (PVDF) membranes, blocked, and probed overnight with antisoluble oligomer A11 antibody. Oxidized proteins were measured using an Oxyblot kit according to manufacturer’s instruction (Millipore).

**8-Isoprostane Assay**

Concentrations of 8-isoprostane were determined by an ELISA kit according to manufacturer’s kit according to manufacturer’s instruction (Active Motif).

**ARE Binding Assay**

ARE binding activity was evaluated using the TransAM-Nrf2 ELISA kit according to manufacturer’s instruction (Active Motif).

**Quantitative Polymerase Chain Reaction**

RNA isolation, reverse transcription, and Taqman quantitative polymerase chain reaction were performed as previously described.

**Apoptosis Signal–Regulating Kinase-1 Activity**

Apoptosis signal–regulating kinase-1 activity was measured using an assay buffer containing myelin basic protein (apoptosis signal–regulating kinase-1 target). The rate of ADP formed from the incorporation of ATP was measured with the ADP-Glo Kinase Assay kit (Promega) according to the manufacturer’s instructions.

**Statistical Analysis**

All data are expressed as mean±standard error of mean. Group comparisons were performed by 1- or 2-way analysis of variance or unpaired Student’s t test, as appropriate. Please see Data Supplement for detailed description of analysis. A value of P<0.05 denoted statistical significance, and P values were 2-sided. All statistical analyses were performed using Prism 5 (GraphPad Software Inc).

**Results**

**Na2S Therapy Enhances Nuclear Localization and Activation of Nrf2**

Nrf2 signaling is regulated on multiple levels. For instance, the phosphorylation of Nrf2 influences its nuclear translocation. A recent study found that polysulfides induced the phosphorylation of Nrf2 in an Akt-dependent manner. Here, we found that Na2S enhanced the phosphorylation of Akt, as well as the phosphorylation and nuclear accumulation of Nrf2 when compared with vehicle-treated hearts (Figure 1A–1E...
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Additionally, Bach1 directly competes with Nrf2 in binding to the promoter of ARE-related genes, leading to the negative regulation of Nrf2 signaling. We recently found that Na2S therapy activated Nrf2 signaling in the diabetic heart by inducing the nuclear export of Bach1 in an Erk-dependent manner.22 Here, we found that Na2S therapy increased the phosphorylation of Erk, reduced the nuclear levels of Bach1, and enhanced the ARE-binding activity of Nrf2 when compared with vehicle-treated hearts (Figure 1F and Figure I in the Data Supplement).

Na2S Therapy Failed to Attenuate the Development of Heart Failure in the Absence of Nrf2

WT and Nrf2 KO mice were next subjected to I/R injury and Na2S treatment. Echocardiography revealed a significantly increased LV end-diastolic diameter and LV end-systolic diameter in all groups of mice after I/R (Figure 2A and 2B). The increase in dimensions was significantly higher in both groups of Nrf2 KO mice compared with the WT vehicle-treated heart failure group. I/R injury also reduced LV ejection fraction in all groups of mice with the observed changes lower in both groups of Nrf2 KO mice (Figure 2C). Although Na2S therapy attenuated the changes in LV dimensions and function in WT mice, it failed to provide any improvements in Nrf2 KO mice. Along with the improvements in LV dimensions and function, WT mice treated with Na2S displayed better contractility and relaxation after I/R injury when compared with vehicle-treated mice (Figure II in the Data Supplement). WT mice treated with Na2S also displayed less cardiac hypertrophy (Figure 2D–2F and Figure II in the Data Supplement). The failure of Na2S therapy to attenuate the development of heart failure in the absence of Nrf2 was further confirmed with the hemodynamic and hypertrophy measurements because Na2S therapy did not improve any of these changes in Nrf2 KO mice.

Na2S Therapy Attenuates Oxidative Stress in an Nrf2-Dependent Manner

Compared with uninjured hearts, I/R injury increased the expression of heme oxygenase 1 and NADPH quinone oxidoreductase 1 in samples collected from WT mice, but did not alter the expression of either in the hearts of Nrf2 KO mice (Figure 3A–3C). Na2S therapy significantly enhanced the expression of both in hearts of WT mice, but failed to do so in hearts collected from Nrf2 KO mice. Hearts from WT mice treated with Na2S also displayed less oxidative stress and less of an accumulation of oxidized proteins compared with vehicle-treated mice (Figure 3D–3F). However, Na2S failed to alter oxidative stress in hearts of Nrf2 KO mice.

Na2S Therapy Enhances the Cardiac Proteasome in an Nrf2-Dependent Manner

Nrf2 has been reported to regulate the expression of several components of the proteasome.16–18 Studies were conducted to determine whether we could recapitulate these findings in the heart. WT mice were treated with Na2S for 7 days in the absence of myocardial I/R injury. Na2S therapy increased the nuclear accumulation of Nrf2 and increased the ARE-binding activity of Nrf2 (Figure III in the Data Supplement), suggesting that Nrf2 signaling was induced in these hearts. We then turned our attention to the ability of Na2S therapy to increase components of the 26S proteasome. Compared with heart samples collected from vehicle-treated mice, Na2S increased the gene and protein expression of the β1 and β5 subunits, as well as the gene and protein expression of PA28α and α4 in hearts from WT mice (Figure 4A–4F). Na2S failed to increase the expression of these subunits in heart samples collected from Nrf2 KO mice, indicating that the observed changes were dependent on Nrf2. Interestingly, the gene and protein expression of the β2 subunit remained unchanged in
the presence of Na$_2$S therapy. Subsequent analysis revealed increased activities of the β1 (caspase-like) and β5 (chymotrypsin-like) subunits by Na$_2$S in an Nrf2-dependent manner (Figure 4G and 4I).

Next we evaluated if the expression of these proteasomal subunits and the activity of the proteasome was altered in 1-week post myocardial I/R hearts. Na$_2$S therapy significantly enhanced the expression of the β1, β5, PA28α, and α4 subunits in WT mice, but failed to do so in hearts collected from Nrf2 KO mice (Figure 5A–5C). Whether changes in the pools of proteasome subunits translated into altered proteasome assembly was analyzed by coimmunoprecipitation using the β2 subunit as bait (Figure IV in the Data Supplement). Cardiac proteasome assembly was unaffected in vehicle-treated hearts. In contrast, an increase in the association of the PA28α, α4, β1, and β5 subunits with the β2 subunit was observed in Na$_2$S-treated hearts.

The activities of the β1 and β5 subunits were decreased after myocardial I/R injury in hearts of WT mice treated with vehicle (Figure 5D and 5F). WT mice treated with Na$_2$S displayed enhanced activities of the β1 and β5 subunits when compared with vehicle-treated mice. However, Na$_2$S failed to alter proteasome activity in Nrf2 KO hearts. The cardiac proteasome functions to prevent the accumulation of damaged proteins. Therefore, we sought to determine whether the observed changes in proteasomal activity affected its function.

**Figure 2.** Left ventricular (LV) end-diastolic diameter (LVEDD; A), LV end-systolic diameter (LVESD; B), and LV ejection fraction (C) from wild-type (WT) and nuclear factor E2–related factor 2 (Nrf2)–deficient mice (Nrf2 KO). D, Ratios of heart to body weight (HW:BW) and heart weight to tibia length (HW:TL) were used as a measure of cardiac hypertrophy. E, Representative photomicrographs of wheat germ agglutinin–stained hearts from the experimental groups. F, Summary of myocyte cell surface area and cross-sectional area measurements of wheat germ agglutinin–stained hearts. Scale bar equals 100 μm. All measurements were performed in samples collected after 60 minute of ischemia and 4 weeks of reperfusion. Values are means±SEM. **P<0.01 and ***P<0.001 vs Sham or Baseline. ϕP<0.001 vs WT Veh. Two-way ANOVA. Veh indicates vehicle.
Myocardial I/R injury induced the accumulation of ubiquitinated proteins and misfolded protein in hearts of WT and Nrf2 KO mice (Figure 5G–5I). In both cases, the levels were higher in Nrf2 KO hearts. Na$_2$S therapy reduced the accumulation of these proteins in hearts from WT mice, but failed to do so in hearts of Nrf2 KO. Additional analysis at 4 weeks of reperfusion revealed a further decrease in the activities of the $\beta_1$ and $\beta_5$ subunits, as well as a decrease in the activity of the $\beta_2$ (trypsin-like) subunit in both WT and Nrf2 KO mice treated with vehicle and Na$_2$S (Figure V in the Data Supplement). In all groups, this was associated with a further increase in the accumulation of misfolded proteins. Na$_2$S therapy enhanced the activities of the subunits and decreased the accumulation of misfolded proteins in WT hearts but failed to do so in Nrf2 KO hearts.

Na$_2$S Therapy Requires the Cardiac Proteasome to Attenuate the Development of Heart Failure

To further evaluate if Na$_2$S attenuates the development of ischemic-induced heart failure by enhancing the function of the proteasome, we administered the proteasome inhibitor, bortezomib, to groups of mice treated with and without Na$_2$S. Bortezomib was delivered with Na$_2$S therapy starting at reperfusion and continuing for the first 7 days of reperfusion. Analysis of heart tissue collected at 1 week of reperfusion revealed that bortezomib significantly decreased the activities of the $\beta_1$, $\beta_2$, and $\beta_5$ subunits, resulting in a concomitant increase in the accumulation of misfolded proteins (Figure 6A–6E). Na$_2$S therapy failed to enhance the activity of the proteasome and failed to attenuate the accumulation of misfolded proteins when administered with bortezomib. Similar findings were observed with tissue collected at 4 weeks of reperfusion. Echocardiography analysis revealed that bortezomib exacerbated cardiac dilatation and LV dysfunction when compared with vehicle-treated animals (Figure 6G–6I). Importantly, Na$_2$S therapy failed to attenuate the changes in LV dimensions and function when administered with bortezomib.

Discussion

Despite the recognition that proteasomal functional insufficiency (PFI) contributes to the progression of several diseases (ie, Alzheimer’s), its role in cardiovascular disease is only beginning to gain attention. Genetic models in which the
desmin αβ-crystallin is mutated provide evidence that protein aggregate–induced proteasome impairments is sufficient to induce cardiac dysfunction.25,26 Likewise, chronic administration of a proteasome inhibitor leads to functional and structural alterations of the heart, consistent with hypertrophic-restrictive cardiomyopathy.27 Recent experimental evidence supports the idea that proteasomal dysfunction contributes to myocardial I/R injury and the progression of ischemic-induced heart failure.14 There is also growing evidence for the accumulation of ubiquitinated proteins and soluble protein aggregates in the myocardium of patients with end-stage heart failure stemming from both dilated cardiomyopathy and ischemic heart disease.11,26 Coupled with evidence for decreased proteasomal activity in the myocardium of heart failure patients,11,24 it can be suggested that PFI is a common phenomenon of cardiac pathogenesis.14 The findings of the current study provide further evidence to support this notion. Specifically, we found that the activity of the β1 and β5 proteasome subunits was decreased as early as 1 week after the onset of myocardial I/R injury. This dysfunction was associated with the accumulation of ubiquitinated proteins and misfolded protein. These alterations were worse by 4 weeks of reperfusion and were accompanied by cardiac function.

PFI occurs when the functional capacity of the proteasome is surpassed by demand.14 After the onset of myocardial I/R injury, there is a robust increase in reactive oxygen species, which induces the accumulation of oxidized and misfolded proteins.25 In response to a mild incidence of myocardial I/R injury, the proteasome is likely able to handle the accumulation of these aberrant proteins. However, in response to severe myocardial I/R injury, the proteasome is unable to keep pace. This leads to a viscous cycle whereby the accumulation of aberrant proteins contributes to proteasomal inhibition via the production of even more reactive oxygen species.9 It has, therefore, been postulated that enhancing proteasomal function should break this viscous cycle.9 Indeed, the genetic overexpression of PA28α enhanced proteasomal function and protected against myocardial I/R injury.14 Although this

Figure 4. Relative gene (A) and protein expression (B and C) of the β1 (psmb1), β2 (psmb2), and β5 (psmb5) proteasome subunits. Relative gene (D) and protein expression (E and F) of the PA28α (psme) and α4 (psma4) proteasome subunits. Activities of the β1 (caspase-like; G), β2 (trypsin-like; H), and β5 (chymotrypsin-like; I) proteasome subunits. All samples were collected from the hearts of wild-type (WT) and nuclear factor E2–related factor 2 (Nrf2)–deficient mice (Nrf2 KO) mice treated with saline (Veh) or Na2S for 1 week. Because GAPDH is somewhat removed from the proteasome, we first evaluated the expression of each subunit relative to total protein load (Coomassie blue–stained membranes). Our analysis revealed that the expression of the β2 subunit did not change when normalized to the total protein load. Therefore, we used the β2 subunit as the loading control for all of the other subunits. Values are means±SEM. *P<0.05, **P<0.01, and ***P<0.001 vs WT vehicle. Kruskal–Wallis ANOVA.
provides a proof-of-concept that enhancing proteasomal function protects the heart against I/R injury, it does not offer insights into how proteasomal function is regulated by myocardial ischemia. A recent study found that activation of the PKCβII isozyme in response to myocardial ischemia inhibited proteasomal function. Importantly, the authors demonstrated that targeting PKCβII with a specific inhibitor restored proteasomal function and attenuated myocardial I/R injury. As such, the results of the Ferreira study provided mechanistic insights into the regulation of the cardiac proteasome after myocardial ischemia and provided evidence that enhancing proteasomal function pharmacologically afforded cardioprotection. The latter is of importance given that most, if not all, studies using pharmacological agents to target the proteasome used inhibitors.

Nrf2 is a master regulator of stress signaling through its ability to regulate the basal and inducible expression of several antioxidant genes and other cytoprotective phase II detoxifying enzymes. As such, Nrf2 signaling augments a wide range of cell defense processes, which enhance cell’s capacity to detoxify harmful substances or adapt to stressful stimuli. Emerging evidence indicates the important roles that Nrf2 and its downstream gene targets play in protecting the heart from ischemic injury, as well as from maladaptive remodeling and cardiac dysfunction. For instance, Nrf2 KO mice display exacerbated cardiac injury in response to acute myocardial I/R injury. Additionally, Nrf2 signaling protects cardiac cells from oxidative stress in vitro and protects against cardiac remodeling induced by pressure overload or angiotensin II. Evidence also suggests that Nrf2 signaling regulates the expression of subunits that form the 26S proteasome complex. Specifically, the induction of Nrf2 in cultured cells delays senescence and contributes to the adaptation to oxidative stress by enhancing the expression and activity of the proteasome. Moreover, a deficiency of Nrf2 is associated with the augmentation of protein aggregation and neuronal death in a mouse model of Parkinson’s disease.

Several years ago, we identified Nrf2 as a major cellular target for H₂S and as a regulator of the acute cardioprotective effects induced by H₂S. However, it was not known if H₂S could induce Nrf2-related signaling in the setting of heart failure. Here, we found that treatment with the Na₂S enhanced

Figure 5. A, Representative immunoblots and densitometric analysis of (B and C) the β1, β2, β5, α4, and PA28α proteasome subunits. Activities of β1 (D), β2 (E), and β5 (F) proteasome subunits. G, Representative immunoblots and densitometric analysis of (H) ubiquitinated proteins and (I) misfolded proteins. All samples were collected from hearts of the experimental groups at 1 week of reperfusion. Values are means±SEM. *P<0.05, **P<0.01, and ***P<0.001 vs WT Sham. Kruskal–Wallis ANOVA. Veh indicates vehicle.
Nrf2 signaling. Keap1 represses the ability of Nrf2 to induce endogenous antioxidants by binding tightly to Nrf2 and anchoring it in the cytoplasm. On stimulation, Nrf2 dissociates from Keap1 and translocates to the nucleus. H2S has recently been shown to sulfhydrate Keap1, which results in the release of Nrf2. Additionally, several studies have reported that the phosphorylation of Nrf2 in either a PKC-, Akt-, or MAPK-dependent manner stabilizes Nrf2, allowing it to translocate to the nucleus. However, the phosphorylation of Nrf2 seems not to be a requirement for its nuclear accumulation or activation of ARE genes. This has led to the suggestion that the phosphorylation of Nrf2 occurs in response to certain stimuli or may serve as a regulatory mechanism to activate certain genes. However, this idea has not been fully investigated. We have previously reported that Na2S induces the phosphorylation of PKC. Here, we found that Na2S increased Akt and Erk phosphorylation and removed the Nrf2 repressor Bach1 from the nucleus. Therefore, it can be hypothesized that Nrf2 activation by Na2S occurs via multiple levels: modification of Keap1, phosphorylation of Nrf2, and removal of Bach1 from the nucleus. However, we currently do not know the mechanism(s) by which Na2S phosphorylates Nrf2, nor do we fully understand the relevance of Nrf2 phosphorylation in regards to the ability of Na2S to induce Nrf2-related targets. Therefore, further work is needed to evaluate these questions.

Here, we found that Na2S therapy failed to attenuate the development of heart failure in Nrf2 KO mice, suggesting that Nrf2 plays a role in mediating the cardioprotective effects of Na2S therapy. We then turned our attention to the ability of Na2S therapy to target the cardiac proteasome. First, we found that treatment with Na2S increased the expression of subunits that form the 26S proteasome complex, as well as increased the activity of 2 of the beta catalytic subunits. Second, we found that Na2S therapy increased the expression, assembly, and activity of the proteasome after the onset of myocardial I/R injury. These changes were associated with improved proteasomal function as evidenced by a decrease in the accumulation of damaged proteins. Under both experimental conditions, the observed changes in the proteasome after Na2S treatment were absent in Nrf2 KO mice, indicating a dependence on Nrf2. Additionally, we noted that Na2S failed to improve cardiac function when the proteasome was inhibited, suggesting that diminishing PFI is key for Na2S to attenuate the development of heart failure. Together, these findings...
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provide a novel mechanism of action for Na2S therapy and support our hypothesis that Nrf2 plays a role in mediating the cardioprotective effects of Na2S therapy.

There are a few caveats to note. First, the protein quality control system consists of components other than the proteasome, such as the autophagy machinery and chaperone proteins. H2S can protect against myocardial I/R injury by restoring autophagic flux, and we have previously reported that Na2S increases the expression of heat shock proteins 70 and 90. Either of these mechanisms could contribute to the reduced accumulation of misfolded proteins observed in the Na2S-treated heart. Second, the activity of individual proteasomal subunits can be directly affected by oxidation. Therefore, the observed changes in the activity of the proteasome, especially in the Nrf2-deficient heart, could also be attributed to oxidative stress. As such, the lack of an increase in heme oxygenase 1 and NADPH:quione oxidoreductase 1 observed in the Nrf2 KO heart could also play a role in the diminished activity of the proteasomal subunits, suggesting that regulation of oxidative stress and replenishing of the proteasomal subunits is needed to attenuate PFI. Third, postinfarction remodeling involves several maladaptive and reparative processes. Given that Nrf2 regulates a broad range of targets that can influence aspects of these processes (ie, oxidative stress, apoptosis, autophagy, and inflammation), we would predict that all of these contribute to the injury observed in the Nrf2-deficient mice. As such, some aspects of each of these cascades would predictably contribute to cell injury and the accumulation of damaged proteins, which in turn could lead to impairments in the proteasome. As such, Na2S potentially improve proteasome activity via indirect mechanisms. Alternatively, the injury observed in the Nrf2-deficient mice and the protective effects of Na2S could also be because of mechanisms distinct from proteasome impairments. Therefore, future studies are warranted to fully investigate (1) the mechanisms by which Na2S therapy regulates the proteasome protein quality control system in the setting of heart failure and (2) mechanisms by which Nrf2 mediates the protective effects of Na2S.

In summary, we provide novel evidence that Na2S therapy attenuates ischemic-induced heart failure in an Nrf2-dependent manner. Furthermore, these findings offer important information that the augmentation of Nrf2 signaling by Na2S enhances the function of the cardiac proteasome. Finally, our study indicates that Na2S-mediated protection against ischemic-induced heart failure is dependent on its ability to restore proteasome function, which supports the emerging idea that enhancing proteasome function is a potential therapeutic strategy for the treatment of heart failure.

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Disclosures

None.

References


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Sodium Sulfide Attenuates Ischemic-Induced Heart Failure by Enhancing Proteasomal Function in an Nrf2-Dependent Manner

Short Title: Shimizu-H2S Attenuates Heart Failure via Nrf2

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Expanded Materials and Methods

**Animals.** Male mice (12 weeks of age) with a global deficiency in Nrf2 (Nrf2 KO)\(^1\) and wild-type (WT) littermates (C57BL/6J background) were used in all experiments. 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.** \(\text{H}_2\text{S}\) was administered as sodium sulfide (Na\(_2\)S; Sigma Aldrich). Na\(_2\)S was dissolved in saline and administered 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 Na\(_2\)S was selected based on our previous experience investigating Na\(_2\)S in murine models of cardiac I/R injury.\(^2\) Saline was administered in the same manner for the vehicle groups. Na\(_2\)S was always prepared just prior to use. In addition, groups of mice were treated similarly with the proteasome inhibitor bortezomib (0.2 mg/kg) either alone or together with Na\(_2\)S.

**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.\(^2\) 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 was 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.\(^3\)

**Protein extraction.** Proteins were extracted from heart samples taken from separate groups of mice. Whole cell homogenates were obtained as previously described.\(^4\) Nuclear fractions were obtained using the Subcellular Protein Fractionation Kit for Tissue (87790, Thermo Scientific). Proteasome were collected as previously described.\(^5\) Briefly, hearts were homogenized in buffer...
containing 20mmol/L HEPES (pH 7.5), 150mmol/L NaCl, 1mmol/L MgCl₂, 0.5mmol/L EDTA, 1mmol/L DTT. Proteasomes were collected in the supernatant after 1-hour centrifugation at 100,000xg.

**Western Blot Analysis.** 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 hour 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 as described previously.

**Immunoprecipitation.** Heart homogenates were immunoprecipitated with an antibody to the β₂ subunit using the Dynabeads® Protein G Immunoprecipitation Kit according to manufacturer's instructions. Samples were then subjected to standard Western blot techniques.

**Proteasome Activity.** The caspase-, trypsin and chymotrypsin-like activities of proteasomes were assayed using the fluorescently tagged substrates Z-LLE-AMC, AC-RLR-AMC and AC-LLVY-AMC, respectively. An aliquot of the samples (25 µg) were incubated in a reaction buffer containing 20mmol/L HEPES (pH 7.5), 150 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 1mM DTT, and 50 µM ATP. Released AMC was measured using a Biotek Synergy 2 plate reader at an excitation wavelength of 390 nm and an emission wavelength of 460 nm for up to 90min. Each assay was conducted in the presence and absence of a specific proteasome inhibitor to subtract non-proteasomal proteolysis (10 µM epoxomicin for chymotrypsin-like, 20µM epoxomicin for trypsin-like, and 30µM MG-132 for caspase-like activity). Proteasome activity is expressed as relative fluorescence units (RFU)/minute/mg protein.

**Cellular Soluble Oligomers of Misfolded Proteins.** To assess the levels of soluble oligomers of misfolded proteins, heart homogenates were dot-blotted onto PVDF membranes (BioRad). The membranes were blocked for 1 hour with SuperBlock (Thermo Scientific). After blocking, the membranes were incubated overnight with anti-soluble oligomer A11 antibody, an antibody shown to recognize cellular soluble oligomers of misfolded proteins. This antibody does not recognize monomers of mature fibers of proteins or peptides. Blots were then incubated with a secondary anti-IgG rabbit antibody linked to horseradish peroxidase for 1 hour. Protein dots were visualized and quantified as in Western blot. Membranes were stripped and probed with GAPDH.

**Cellular Oxidized Proteins.** Oxidized proteins were measured using an Oxyblot kit according to manufacture’s instruction (Millipore).

**8-Isoprostane Assay.** Concentrations of 8-isoprostan in the heart were
determined by 8-isoprostane EIA kit according to manufacture’s instruction (Cayman Chemicals, Michigan).

**ARE binding Assay.** The ARE binding activity of Nrf2 was evaluated in heart homogenates using the TransAM Nrf2 ELISA kit according to manufacturer’s instruction (Active Motif).

**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 proteasomal subunits (β1, β2, β5, PA28α, α4) and 18S. Analysis was carried out using the ΔΔ-CT method with 18S correction and reported as relative fold change versus WT sham.

**Wheat germ agglutinin staining.** Cell surface area and cross sectional area (µm²) were analyzed by staining cardiac cryosections with wheat germ agglutinin (WGA)-Texas Red-X conjugate (Life Technologies) to show myocyte membranes in histological sections. Cryosections were first washed in 1XPBS and then incubated in 10 µg 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.

**ASK1 Activity.** The activity of ASK1 was measured in whole cells fractions prepared from heart tissue. The samples were first immunoprecipitated with a specific anti-ASK1 antibody (Biorbyt). An aliquot of the immunoprecipitated samples were incubated in a reaction buffer containing 4 mM MOPS (pH 7.2), 2.5 mM β-glycerophosphate, 1 mM EGTA, 0.4 mM EDTA, 0.6 mM BSA, 0.05 mM dithiothreitol, 4 mM MgCl2, 0.250 mM ATP, and 10 µg of *Myelin basic protein* (MBP, ASK1 target). The rate of ADP formed from the incorporation of ATP in the synthetic peptide was the measured with the ADP-Glo Kinase Assay kit (Promega) according to the manufacturer’s instructions. Activity was expressed as ADP generated (in nanomoles) per minute per milligram of protein.

**Statistical analysis.** All data are expressed as mean ± SEM. Statistical significance was evaluated as followed: (1) unpaired Student t-test for comparison between 2 means; (2) a 1-way ANOVA with a Bonferroni test as the posthoc analysis for comparison among 3 or more means from groups of the same strain; and (3) a Kruskal-Wallis ANOVA with a Dunn’s Multiple Comparison test as the posthoc analysis for comparison among the means from groups of both WT and Nrf2 KO mice. For the echocardiography data, a 2-way repeated measures ANOVA with a Bonferroni test as the posthoc analysis was used. The following comparisons were made separately: (1) baseline vs. post-baseline
measurements for each group, (2) differences between each groups baseline measurements, and (3) differences between each groups post-baseline measurements. The p-value for these evaluations was adjusted by applying the Bonferroni correction for multiple comparisons. A value of p<0.05 denoted statistical significance and p-values were two-sided. All statistical analysis was performed using Prism 5 (GraphPad Software Inc).

**Literature Cited**


Supplemental Figure Legends

**Supplemental Figure I.** (A-C) Representative immunoblots and densitometric analysis of phosphorylated and total Akt. (D) Representative immunoblots and densitometric (E) phosphorylated and total Akt and (F) nuclear Bach1. All samples were collected from the hearts of the experimental groups at 1 week of reperfusion. Numbers in the bars represent sample size for each group. Bars are means ± SEM. *p<0.05, **p<0.01 and ***p<0.001 vs. Sham.

**Supplemental Figure II.** (A) Max dP/dT and Min dP/dT, (B) relaxation constant Tau, (C) circumferential stress, and (D) circulating BNP levels from wild-type (WT) and Nrf2 deficient mice (Nrf2 KO) subjected to 60 minutes of ischemia and 4 weeks of reperfusion. Mice received daily injections of sodium sulfide (Na$_2$S HF; 100 µg/kg) or saline (Veh HF) for the first week of reperfusion. Bars are means ± SEM. **p<0.01 and ***p<0.001 vs. WT Veh HF or WT Sham.

**Supplemental Figure III.** (A-B) Representative immunoblots and densitometric analysis of nuclear Nrf2. (C) Antioxidant response element binding activity of Nrf2. All samples were collected from the hearts of WT and Nrf2 KO mice treated with saline (Vehicle) or Na$_2$S for 1 week. ***p<0.001 vs. Vehicle.

**Supplemental Figure IV.** Representative immunoblots and densitometric analysis of the assembly of the cardiac proteasome. The association of the PA28α, α4, β1, β2 and β5 subunits were analyzed by coimmunoprecipitation using the β2 subunit as bait. All samples were collected from the hearts of the experimental groups at 1 week of reperfusion. Bars are means ± SEM. *p<0.05, **p<0.01 and ***p<0.001 vs. Sham.

**Supplemental Figure V.** Activities of the (A) β1 (caspase-like), (B) β2 (trypsin-like) and (C) β5 (chymotrypsin-like) proteasome subunits. (D-E) Representative immunoblots and densitometric analysis of soluble oligomers of misfolded proteins. All samples were collected from the hearts of the experimental groups at 4 weeks of reperfusion. **p<0.01 and ***p<0.001 vs. WT Sham.
**Supplemental Figure VI.** (A) Representative immunoblots of inositol-requiring enzyme 1α (IRE1α) and cleaved caspase-12. (B) Densitometric analysis of IRE1α. (C) Apoptosis signal-regulating kinase 1 (ASK1) activity. (E) Densitometric analysis of cleaved caspase-12. All samples were collected from the hearts of the experimental groups at 1 week of reperfusion. **p<0.01 and ***p<0.001 vs. WT Sham.

**Supplemental Figure VII.** (A) Representative immunoblots and densitometric analysis of (B) phosphorylated PKR-like ER kinase, (C) activating transcription factor 4 (ATF4), and (D) CCAAT/enhancer-binding protein homologous protein (CHOP). All samples were collected from the hearts of the experimental groups at 1 week of reperfusion. *p<0.05, **p<0.01 and ***p<0.001 vs. WT Sham.
**Supplemental Figure I**

**A**

![Sham Veh HF Na\textsubscript{2}S HF p-Akt t-Akt GAPDH](image)

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**B**

![Graph showing t-Akt/GAPDH](image)

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**C**

![Graph showing t-Akt/GAPDH](image)

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**D**

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**E**

![Bar graph showing p-Akt/t-Akt](image)

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**F**

![Bar graph showing Bach1/Fibrillarin](image)

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*Significance levels:

- *p < 0.05
- **p < 0.01
- ***p < 0.001
Supplemental Figure III

A

Nrf2
Fibrillarin

Vehicle Na$_2$S Sham

57 kDa

37 kDa

B

Nrf2 Nuclear Expression (Relative Intensity)

Vehicle Na$_2$S Sham

C

Nrf2 Activation (Relative to Sham)

Vehicle Na$_2$S Sham

57 kDa

37 kDa

Nrf2

Fibrillarin
Supplemental Figure V

A. Caspase-Like Activity (U/min/mg)
B. Trypsin-Like Activity (U/min/mg)
C. Chymotripsin-Like Activity (U/min/mg)

D. A11/GAPDH Western Blot

E. Soluble Oligomer (Relative Intensity)

Wild-Type vs. Nrf2 KO
Sham vs. Veh HF vs. Na2S HF

Statistical significance:
- * p < 0.05
- ** p < 0.01
- *** p < 0.001