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

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Abstract

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 following acute myocardial infarction. Here, we tested the hypothesis that Nrf2 mediates the cardioprotective effects of H2S therapy in the setting of heart failure (HF).

Methods and Results—Mice (12 weeks of age) deficient in Nrf2 (Nrf2 KO; C57BL/6J background) and wild-type (WT) littermates were subjected to ischemic-induced HF. WT mice treated with H2S in the form of sodium sulfide (Na2S) displayed enhanced Nrf2 signaling, improved left-ventricular function, and less cardiac hypertrophy following the induction of HF. In contrast, Na2S therapy failed to provide protection against HF in Nrf2 KO mice. Studies aimed at evaluating the underlying cardioprotective mechanisms found that Na2S increased the expression of proteasome subunits, resulting in an increase 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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Disclosures
None.
Keywords
hydrogen sulfide; heart failure; Nrf2; proteasome

Hydrogen sulfide (H\textsubscript{2}S), an endogenously produced gaseous signaling molecule, is critical for the regulation of cardiovascular homeostasis.\textsuperscript{1} Recently, therapeutic strategies aimed at increasing H\textsubscript{2}S levels have shown cardioprotective actions in models of acute myocardial ischemia-reperfusion (MI/R) injury and heart failure.\textsuperscript{2–6} These studies have provided insights into a number of mechanistic actions, demonstrating inhibition of apoptosis, augmentation of endogenous antioxidants, and stimulation of angiogenesis. Several years ago, we identified nuclear factor E2-related factor (Nrf2) as a major cellular target for H\textsubscript{2}S and a regulator of the acute cardioprotective effects induced by H\textsubscript{2}S.\textsuperscript{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 (HO-1) and NADPH:quinone oxidoreductase 1 (NQO1).

The proteome is more complex than the genome and transcriptome in terms of informational content.\textsuperscript{8} Therefore, regulating the quality of this information is essential for cell survival and function.\textsuperscript{9,10} This is particularly true for organs like the heart that possess a limited ability to regenerate. As such, the protein quality control (PQC) system, consisting of chaperone proteins, autophagy, and the ubiquitin-proteasome system (UPS), is critically important to maintain the fidelity of the heart under both physiological and pathological conditions.\textsuperscript{11} The UPS is the primary effector of the PQC system, protecting cardiomyocytes from the accumulation of aberrant and misfolded proteins, which disrupt intracellular signaling and induce cell death.\textsuperscript{12} Studies have noted changes in the activity of the proteasome in the setting of heart disease.\textsuperscript{11,13} Inevitably, increases and decreases have been reported in animal models and in diseased human hearts. This isn’t surprising given the heterogeneity of heart disease.\textsuperscript{10} 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.\textsuperscript{11,14} Therefore, strategies aimed at promoting proteasome activity may have a therapeutic benefit in this setting. However, very 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 polyubiquinated and oxidized proteins.\textsuperscript{15} 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 four heptameric (2α and 2β) rings.\textsuperscript{8} 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.\textsuperscript{15} The promoter of several proteasome subunits contains an ARE and there is evidence that Nrf2 activators increase the expression and activity of the β1, β2, and β5 proteasomal subunits.\textsuperscript{16–18}

Currently, it is not known if H\textsubscript{2}S 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 online data supplement

**Animals**

Male mice (12 weeks of age) with a global deficiency in Nrf2 (Nrf2 KO; kind gift from Thomas Kensler)¹⁹ 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 LV lumen once at reperfusion followed by daily tail vein injections for the first week of reperfusion.² 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 up to 4 weeks.² All mice were randomly allocated to the treatment groups. Echocardiography, hemodynamics, and wheat germ agglutinin staining were performed as previously described.³

**Protein extraction and Western Blot Analysis**

Whole cell homogenates were obtained as previously described.³ Nuclear fractions were obtained using the Subcellular Protein Fractionation Kit for Tissue (87790, Thermo Scientific). Proteasome were collected as previously described.²⁰ Western Blot analysis was performed as described previously.³

**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.²⁰
Cellular Soluble Oligomers of Misfolded Proteins and Oxidized Proteins

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

8-Isoprostane Assay

Concentrations of 8-isoprostane were determined by an ELISA kit according to manufacture’s instruction (Cayman Chemicals).

ARE binding Assay

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

qPCR

RNA isolation, reverse transcription and Taqman qPCR were performed as previously described.3

ASK1 Activity

ASK1 activity was measured using an assay buffer containing myelin basic protein (MBP, ASK1 target). The rate of ADP formed from the incorporation of ATP was the 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 (SEM). Group comparisons were performed by 1- or 2-way ANOVA, or unpaired Student t-test, as appropriate. Please see online supplement for detailed description of analysis. 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).

Results

Na$_2$S 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 Na$_2$S enhanced the phosphorylation of Akt, as well as the phosphorylation and nuclear accumulation of Nrf2 when compared to vehicle-treated hearts (Figure 1A–E and Supplemental Figure I).

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 Na$_2$S therapy activated Nrf2 signaling in the diabetic heart by inducing the nuclear export of Bach1 in and Erk-dependent manner.22 Here, we found that Na$_2$S therapy increased the phosphorylation of Erk, reduced the nuclear levels of Bach1, and enhanced the ARE-binding activity of Nrf2 when compared to vehicle-treated hearts (Figure 1F and Supplemental Figure I).
**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 significant increased left ventricular end-diastolic diameter (LVEDD) and LV end-systolic diameter (LVESD) in all groups of mice following I/R (Figure 2A–B). The increase in dimensions was significantly higher in both groups of Nrf2 KO mice compared to the WT Veh HF group. I/R injury also reduced LV ejection fraction (EF) in all groups of mice with the observed changes lower in both groups of Nrf2 KO mice (Figure 2C). Whereas, 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 following I/R injury when compared to vehicle-treated mice (Supplemental Figure II). WT mice treated with Na₂S also displayed less cardiac hypertrophy (Figure 2D–F and Supplemental Figure II). 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, as Na₂S therapy did not improve any of these changes in Nrf2 KO mice.

**Na₂S Therapy Attenuates Oxidative Stress in a Nrf2-Dependent Manner**

Compared with uninjured hearts, I/R injury increased the expression of HO-1 and NQO1 in samples collected from WT mice, but did not alter the expression of either in the hearts of Nrf2 KO mice (Figure 3A–C). 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 to vehicle-treated mice (Figure 3D–F). 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. Studies were conducted to determine if 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 (Supplemental Figure III), 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–F). 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 Na₂S therapy. Subsequent analysis revealed increased activities of the β₁ (caspase-like) and β₅ (chymotrypsin-like) subunits by Na₂S in an Nrf2-dependent manner (Figure 4G&I).

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. Na₂S 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–C). Whether changes in the pools of proteasome subunits translated into altered proteasome assembly was analyzed by coimmunoprecipitation using the β2 subunit as bait (Supplemental Figure IV). 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 Na2S-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&F). WT mice treated with Na2S displayed enhanced activities of the β1 and β5 subunits when compared to vehicle-treated mice. However, Na2S 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 if the observed changes in proteasomal activity affected its function. Myocardial I/R injury induced the accumulation of ubiquitinated proteins and misfolded protein in hearts of WT and Nrf2 KO mice (Figure 5G–I). In both cases, the levels were higher in Nrf2 KO hearts. Na2S 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 β1 and β5 subunits, as well as a decrease in the activity of the β2 (trypsin-like) subunit in both WT and Nrf2 KO mice treated with Veh and Na2S (Supplemental Figure V). In all groups, this was associated with a further increase in the accumulation of misfolded proteins. Na2S 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.

**Na2S Therapy Attenuates ER Stress in a Nrf2-Dependent Manner**

The accumulation of misfolded proteins interferes with the function of the endoplasmic reticulum (ER), resulting in the development of ER stress and the activation of the unfolded protein response (UPR). While the acute activation of the ER stress/UPR is protective, prolonged activation triggers an apoptotic-signaling cascade resulting in cell death. Therefore, we sought to determine if the observed changes in proteasomal activity and resultant accumulation of misfolded proteins lead to the activation of the ER stress response. For these studies, we focused on the inositol-requiring enzyme-1 (IRE1; Supplemental Figure VI) and PKR-like ER kinase (PERK; Supplemental Figure VII) arms of the ER stress/UPR. Myocardial I/R injury increased the protein expression of IRE1α, phosphorylated PERK, cleaved caspase-12, activating transcription factor-4 (ATF4), and CCAAT/enhancer-binding protein homologous protein (CHOP), as well as the activity of apoptosis signal-regulating kinase-1 (ASK1) in hearts of WT and Nrf2 KO mice. In all cases, the levels were higher in Nrf2 KO hearts. Na2S therapy attenuated the ER stress/UPR response WT hearts, but failed to do so in Nrf2 KO hearts.

**Na2S Therapy Requires the Cardiac Proteasome to Attenuate the Development of Heart Failure**

To further evaluate if Na2S attenuates the development of ischemic-induced heart failure by enhancing the function of the proteasome, we administered the proteasome inhibitor,
bortezomib\textsuperscript{11}, to groups of mice treated with and without Na\textsubscript{2}S. Bortezomib was delivered with Na\textsubscript{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–E). Na\textsubscript{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 to vehicle-treated animals (Figure 6G–I). Importantly, Na\textsubscript{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 (i.e. Alzheimer’s), its role in cardiovascular disease is only beginning to gain attention.\textsuperscript{24} Genetic models in which the desmin $\alpha\beta$-crystallin is mutated provide evidence that protein aggregate-induced proteasome impairments is sufficient to induce cardiac dysfunction.\textsuperscript{25,26} Likewise, chronic administration of a proteasome inhibitor leads to functional and structural alterations of the heart consistent with hypertrophic-restrictive cardiomyopathy.\textsuperscript{27} Recent experimental evidence supports the idea that proteasomal dysfunction contributes to myocardial I/R injury and the progression of ischemic-induced heart failure.\textsuperscript{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.\textsuperscript{11,26} Coupled with evidence for decreased proteasomal activity in the myocardium of heart failure patients\textsuperscript{11,24}, it can be suggested that PFI is a common phenomenon of cardiac pathogenesis.\textsuperscript{14} The findings of the current study provide further evidence to support this notion. Specifically, we found that the activity of the $\beta_1$ and $\beta_5$ 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.\textsuperscript{14} Following the onset of myocardial I/R injury there is a robust increase in reactive oxygen species (ROS), which induces the accumulation of oxidized and misfolded proteins.\textsuperscript{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 ROS.\textsuperscript{9} It has, therefore, been postulated that enhancing proteasomal function should break this viscous cycle.\textsuperscript{9} Indeed, the genetic overexpression of PA28$\alpha$ enhanced proteasomal function and protected against myocardial I/R injury.\textsuperscript{14} While this 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 following 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 employed inhibitors.

Nrf2 is a master regulator of stress signaling through its ability to regulate the basal and inducible expression of a number of 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 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 Nrf2 signaling. Keap1 represses the ability of Nrf2 to induce endogenous antioxidants by binding very tightly to Nrf2 and anchoring it in the cytoplasm. Upon 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 to not 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 Na$_2$S 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 Na$_2$S therapy. We then turned our attention to the ability of Na$_2$S therapy to target the cardiac proteasome. First, we found that treatment with Na$_2$S increased the expression of subunits that form the 26S proteasome complex, as well as increased the activity of two of the beta catalytic subunits. Second, we found that Na$_2$S therapy increased the expression, assembly, and activity of the proteasome following 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 following Na$_2$S treatment were absent in Nrf2 KO mice, indicating a dependence on Nrf2. Additionally, we noted that Na$_2$S failed to improve cardiac function when the proteasome was inhibited; suggesting that diminishing PFI is key for Na$_2$S to attenuate the development of heart failure. Together these findings provide a novel mechanism of action for Na$_2$S therapy and support our hypothesis that Nrf2 plays a role in mediating the cardioprotective effects of Na$_2$S therapy.

There are a few caveats to note. First, the PQC system consists of components other than the proteasome, such as the autophagy machinery and chaperone proteins. H$_2$S can protect against myocardial I/R injury by restoring autophagic flux and we have previously reported that Na$_2$S 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 Na$_2$S-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 HO-1 and NQO1 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 attenuated PFI. Third, post-infarction remodeling involves a number of maladaptive and reparative processes. Given that Nrf2 regulates a broad range of targets that can influence aspects of these processes (i.e. 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, Na$_2$S potentially improve proteasome activity via indirect mechanisms. Alternatively, the injury observed in the Nrf2 deficient mice and the protective effects of Na$_2$S could also be due to mechanisms distinct from proteasome impairments. Therefore, future studies are warranted to fully investigate (1) the mechanisms by which Na$_2$S therapy regulates the PQC system in the setting of heart failure, and (2) mechanisms by which Nrf2 mediates the protective effects of Na$_2$S.

In summary, we provide novel evidence that Na$_2$S therapy attenuates ischemic-induced heart failure in an Nrf2-dependent manner. Furthermore, these findings offer important information that the augmentation of Nrf2 signaling by Na$_2$S enhances the function of the cardiac proteasome. Finally, our study indicates that Na$_2$S-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.9

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**References**


Clinical Perspective

In recent years, great efforts have been devoted to unraveling the basic mechanisms of heart failure, which remains a major cause of morbidity and mortality worldwide. Some of this work has provided evidence to support the idea that proteasomal dysfunction contributes to myocardial ischemia-reperfusion injury and the progression of ischemic-induced heart failure. However, very little is known about how the proteasome is regulated in response to myocardial ischemia. In recent years, our group and others have reported on the beneficial effects of hydrogen sulfide (H₂S) therapy in various models of cardiac injury, including heart failure. These studies have provided insights into a number of mechanistic actions. The present study further supports these previous findings and provides evidence for the first time that H₂S therapy attenuates the development of proteasomal functional insufficiency (PFI) following the onset of ischemic-induced heart failure. Specifically, we found that H₂S therapy enhanced the proteasome and attenuated the development heart failure by activating the transcription factor Nuclear-factor-E2-related factor-2 (Nrf2). Our study also provided evidence that Nrf2 is a key regulator of cardiac proteasome expression and function during the development of heart failure. Specifically, Nrf2 does not appear to regulate the basal expression of subunits that form the 26S proteasome complex. Rather, it regulates the ischemic-induced upregulation. Therefore, targeting Nrf2 with H₂S or other pharmacological agents may provide viable treatment options in diseases where PFI is prevalent.
Figure 1.
(A–E) Representative immunoblots and densitometric analysis of phosphorylated Nrf2 at serine residue 40 (p-Nrf2) and total Nrf2 (t-Nrf2) in whole cell fractions and nuclear Nrf2. (F) Antioxidant response element binding activity of Nrf2. All samples were collected from hearts of sham mice and mice subjected to 60 minutes of ischemia and 1-week 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. Numbers in the bars represent sample size for each group. Values are means±SEM. *p<0.05, **p<0.01, and ***p<0.001 vs. Sham. 1-way ANOVA.
Figure 2.
(A) Left ventricular end-diastolic diameter (LVEDD), (B) LV end-systolic diameter (LVESD), and (C) LV ejection fraction from wild-type (WT) and 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 following 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. 2-way ANOVA.
Figure 3.
(A) Representative immunoblots and densitometric analysis of (B) heme oxygenase 1 (HO-1) and (C) NADPH:quinone oxidoreductase 1 (NQO1). (D) Cardiac 8-isoprostane levels. (E–F) Representative immunoblots and densitometric analysis of oxidized 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.
Figure 4.
(A) Relative gene and (B–C) protein expression of the β1 (psmb1), β2 (psmb2), and β5 (psmb5) proteasome subunits. (D) Relative gene and (E–F) protein expression of the PA28α (psme) and α4 (psma4) proteasome subunits. Activities of the (G) β1 (caspase-like), (H) β2 (trypsin-like) and (I) β5 (chymotrypsin-like) proteasome subunits. All samples were collected from the hearts of WT and Nrf2 KO mice treated with saline (Veh) or Na₂S for 1-week. Since 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.
Figure 5.
(A) Representative immunoblots and densitometric analysis of (B–C) the β1, β2, β5, α4, and PA28α proteasome subunits. Activities of (D) β1, (E) β2 and (F) β5 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.
Figure 6.
Activities of the (A) β1, (B) β2 and (C) β5 proteasome subunits from heart samples collected at 1 and 4-weeks of reperfusion. (D) Representative immunoblots and (E–F) densitometric analysis of misfolded proteins at 1-week and 4-weeks of reperfusion. (G) LVEDD, (H) LVESD, and (I) LV ejection fraction from subjected to 60 minutes of ischemia and 4-weeks of reperfusion. Mice received daily injections of DMSO (Veh HF), DMSO and Na$_2$S (Na$_2$S HF), Bortezomib (Bort HF; 200 µg/kg) or Na$_2$S and Bort for the first week of reperfusion. Values are means±SEM.*p<0.05, **p<0.01 and ***p<0.001 vs. Sham. ***p<0.001 vs. Sham or Baseline. 1-way or 2-way ANOVA.