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

Short Title: Shimizu-H$_2$S Attenuates Heart Failure via Nrf2

Yuuki Shimizu, M.D., Ph.D.$^1$, Chad K. Nicholson, B.S.$^1$, Jonathan P. Lambert, B.S.$^1$, Larry A. Barr, Ph.D.$^1$, Nicholas Kuek$^1$, David Herszenhaut$^1$, Lin Tan, Ph.D.$^2$, Toyoaki Murohara, M.D., Ph.D.$^3$, Jason M. Hansen, Ph.D.$^4$, Ahsan Husain, Ph.D.$^2$, Nawazish Naqvi, Ph.D.$^2$, John W. Calvert, Ph.D.$^1$

$^1$Department of Surgery, Division of Cardiothoracic Surgery, Carlyle Fraser Heart Center, Emory University School of Medicine, Atlanta GA USA

$^2$Department of Medicine, Division of Cardiology, Emory University School of Medicine, Atlanta GA USA

$^3$Department of Cardiology, Nagoya University Graduate School of Medicine, Nagoya, Japan

$^4$Department of Pediatrics, Emory University School of Medicine, Atlanta GA USA

Correspondence:
John W. Calvert, Ph.D.
Department of Surgery
Division of Cardiothoracic Surgery
Carlyle Fraser Heart Center
Emory University School of Medicine
380 Northyards Boulevard, Suite B
Atlanta, GA 30313
Phone: 404-251-0663
jcalver@emory.edu
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 \(\mu\)g/kg (final volume of 50 \(\mu\)L) 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.\(^1\) 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. 4

**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. 6 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-isoprostane 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 manufacture’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 (Na₂S 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 Na₂S 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 1 α (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

B

C

D

E

F

Sham VEH HF Na₂S HF

p-Akt
63 kDa
63 kDa
37 kDa

t-Akt
63 kDa

GAPDH
37 kDa

0.0
1.0
1.5

Sham Veh HF Na₂S HF

t-Akt/GAPDH (Relative Intensity)

0.0
1.0
1.5
2.0
2.5

Sham Veh HF Na₂S HF

t-Akt/GAPDH (Relative Intensity)

Sham Veh HF Na₂S HF

p-Akt
63 kDa
63 kDa
37 kDa

t-Akt
63 kDa

GAPDH
37 kDa

0.0
1.0
1.5

Sham Veh HF Na₂S HF

t-Akt/GAPDH (Relative Intensity)

0.0
1.0
1.5
2.0
2.5

Sham Veh HF Na₂S HF

t-Akt/GAPDH (Relative Intensity)

Sham Veh HF Na₂S HF

p-Akt
63 kDa
63 kDa
37 kDa

t-Akt
63 kDa

GAPDH
37 kDa

0.0
1.0
1.5

Sham Veh HF Na₂S HF

t-Akt/GAPDH (Relative Intensity)

0.0
1.0
1.5
2.0
2.5

Sham Veh HF Na₂S HF

t-Akt/GAPDH (Relative Intensity)

Sham Veh HF Na₂S HF

p-Akt
63 kDa
63 kDa
37 kDa

t-Akt
63 kDa

GAPDH
37 kDa

0.0
1.0
1.5

Sham Veh HF Na₂S HF

t-Akt/GAPDH (Relative Intensity)

0.0
1.0
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Sham Veh HF Na₂S HF

t-Akt/GAPDH (Relative Intensity)

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p-Akt
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63 kDa
37 kDa

t-Akt
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GAPDH
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Sham Veh HF Na₂S HF

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t-Akt/GAPDH (Relative Intensity)

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63 kDa
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t-Akt
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GAPDH
37 kDa

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1.5

Sham Veh HF Na₂S HF

t-Akt/GAPDH (Relative Intensity)

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Sham Veh HF Na₂S HF

t-Akt/GAPDH (Relative Intensity)

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37 kDa

t-Akt
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GAPDH
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1.5

Sham Veh HF Na₂S HF

t-Akt/GAPDH (Relative Intensity)

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t-Akt
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GAPDH
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GAPDH
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Sham Veh HF Na₂S HF

t-Akt/GAPDH (Relative Intensity)

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2.5

Sham Veh HF Na₂S HF

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t-Akt
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GAPDH
37 kDa

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1.0
1.5

Sham Veh HF Na₂S HF

t-Akt/GAPDH (Relative Intensity)

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2.5

Sham Veh HF Na₂S HF

t-Akt/GAPDH (Relative Intensity)

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GAPDH
37 kDa

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1.0
1.5

Sham Veh HF Na₂S HF

t-Akt/GAPDH (Relative Intensity)

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2.0
2.5

Sham Veh HF Na₂S HF

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2.0
2.5

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63 kDa
63 kDa
37 kDa

t-Akt
63 kDa

GAPDH
37 kDa

0.0
1.0
1.5

Sham Veh HF Na₂S HF

t-Akt/GAPDH (Relative Intensity)

0.0
1.0
1.5
2.0
2.5

Sham Veh HF Na₂S HF

t-Akt/GAPDH (Relative Intensity)
Supplemental Figure III

A

Nrf2
Fibrillarin

57 kDa
37 kDa

Vehicle
Na₂S

B

Nrf2 Nuclear Expression (Relative Intensity)

0.0
0.5
1.0
1.5
2.0
2.5

Vehicle
Na₂S Sham

C

Nrf2 Activation (Relative to Sham)

0.0
1.0
1.5
2.0
2.5

Vehicle
Na₂S Sham

Nrf2
Fibrillarin

57 kDa
37 kDa

Vehicle
Na₂S

Supplemental Figure III

A

Nrf2
Fibrillarin

57 kDa
37 kDa

Vehicle
Na₂S

B

Nrf2 Nuclear Expression (Relative Intensity)

0.0
0.5
1.0
1.5
2.0
2.5

Vehicle
Na₂S Sham

C

Nrf2 Activation (Relative to Sham)

0.0
1.0
1.5
2.0
2.5

Vehicle
Na₂S Sham

A

Nrf2
Fibrillarin

57 kDa
37 kDa

Vehicle
Na₂S

B

Nrf2 Nuclear Expression (Relative Intensity)

0.0
0.5
1.0
1.5
2.0
2.5

Vehicle
Na₂S Sham

C

Nrf2 Activation (Relative to Sham)

0.0
1.0
1.5
2.0
2.5

Vehicle
Na₂S Sham

Supplemental Figure III

A

Nrf2
Fibrillarin

57 kDa
37 kDa

Vehicle
Na₂S

B

Nrf2 Nuclear Expression (Relative Intensity)

0.0
0.5
1.0
1.5
2.0
2.5

Vehicle
Na₂S Sham

C

Nrf2 Activation (Relative to Sham)

0.0
1.0
1.5
2.0
2.5

Vehicle
Na₂S Sham

A

Nrf2
Fibrillarin

57 kDa
37 kDa

Vehicle
Na₂S

B

Nrf2 Nuclear Expression (Relative Intensity)

0.0
0.5
1.0
1.5
2.0
2.5

Vehicle
Na₂S Sham

C

Nrf2 Activation (Relative to Sham)

0.0
1.0
1.5
2.0
2.5

Vehicle
Na₂S Sham

Supplemental Figure III

A

Nrf2
Fibrillarin

57 kDa
37 kDa

Vehicle
Na₂S

B

Nrf2 Nuclear Expression (Relative Intensity)

0.0
0.5
1.0
1.5
2.0
2.5

Vehicle
Na₂S Sham

C

Nrf2 Activation (Relative to Sham)

0.0
1.0
1.5
2.0
2.5

Vehicle
Na₂S Sham
Supplemental Figure V

A. Caspase-Like Activity (U/min/mg) for Wild-Type and Nrf2 KO mice treated with Sham, Veh HF, and Na$_2$S HF. 

B. Trypsin-Like Activity (U/min/mg) for Wild-Type and Nrf2 KO mice treated with Sham, Veh HF, and Na$_2$S HF. 

C. Chymotripsin-Like Activity (U/min/mg) for Wild-Type and Nrf2 KO mice treated with Sham, Veh HF, and Na$_2$S HF. 

D. Western blot analysis of A11 and GAPDH for Wild-Type and Nrf2 KO mice treated with Sham, Veh HF, and Na$_2$S HF. 

E. Soluble Oligomer (Relative Intensity) for Wild-Type and Nrf2 KO mice treated with Sham, Veh HF, and Na$_2$S HF.
**Supplemental Figure VI**

(A) Western blot analysis showing IRE1α, cleaved Caspase-12, and GAPDH. The molecular weights are indicated: 130 kDa, 42 kDa, and 37 kDa. The blots are compared between Wild-Type and Nrf2 KO groups.

(B) Bar graph showing the relative intensity of IRE1α/GAPDH in Wild-Type and Nrf2 KO groups. The p-values are indicated for each group comparison.

(C) Bar graph showing ASK1 activity in Wild-Type and Nrf2 KO groups. The p-values are indicated for each comparison.

(D) Bar graph showing cleaved Caspase-12/GAPDH in Wild-Type and Nrf2 KO groups. The p-values are indicated for each comparison.