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

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**Materials and Methods**

**Animals.** Two different strains of mice were utilized: (1) Male mice (10-12 weeks of age) with a cardiac-specific overexpression of a dominant negative redox-inactive mutant of Trx1 (Tg-DN-Trx1) and wild-type (WT) littermates (FVB background), and (2) Male NFAT-luciferase reporter mice (10-12 weeks of age). The generation of cardiac-specific Trx1 dnTg mice\(^1\) and NFAT-luciferase reporter mice\(^2\) have been described previously. All experimental mouse procedures were approved by the Institute for Animal Care and Use Committee at Emory University School of Medicine and conformed to the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health (NIH Publication No. 86-23, Revised 1996) and with federal and state regulations. The number of animals used for each experiment is depicted on each figure.

**Materials.** \(\text{H}_2\text{S}\) was administered as sodium sulfide (\(\text{Na}_2\text{S}; \text{Sigma Aldrich}\)). \(\text{Na}_2\text{S}\) was dissolved in saline and administered using a 32-gauge needle at a dose of 100 \(\mu\text{g/kg}\) (final volume of 50 \(\mu\text{L}\)) 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 \(\text{Na}_2\text{S}\) was selected based on our previous experience investigating \(\text{Na}_2\text{S}\) in murine models of cardiac I/R injury.\(^3\) Saline was administered in the same manner for the vehicle groups. \(\text{Na}_2\text{S}\) was always prepared just prior to use.

**Heart Failure Protocol.** Ischemic-induced heart failure was produced by subjecting mice to 60 minutes of left coronary artery (LCA) occlusion followed by reperfusion for up to 4 weeks as previously described.\(^3\) All mice were randomly allocated to the treatment groups.

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

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

**Isolation of mRNA and Taqman qPCR.** RNA was isolated using the RiboPure kit according to manufacturer’s instructions (Ambion). Reverse transcription was performed in a standard fashion with QuantiTect Reverse Transcription Kit (QIAGEN) supplemented with DNase treatment. Taqman qPCR was carried out according to the manufacturer’s instructions using probe sets for Trx1 and 18S. Analysis was carried out using the ΔΔ-CT method with 18S correction and reported as relative fold change versus sham.

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

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

**Hydrogen Sulfide Measurements.** Hydrogen sulfide and sulfane sulfur levels were measured in tissue and blood according to previously described methods. Fresh tissue was homogenized in 5 volumes of PBS (pH 7.4). For measurement of H₂S, 0.2 mL of the sample homogenate was placed in a small glass vial (5182-0553, Agilent Technologies, Santa Clara, CA, USA) along with 0.4 mL of 1 M sodium citrate buffer, pH 6.0, and sealed. The mixture was incubated at 37°C for 10 minutes with shaking at 125 rpm on a rotary shaker (Fisher Scientific) to facilitate the release of H₂S gas from the aqueous phase. After shaking, 0.1 mL of head-space gas was applied to a gas chromatograph (7890A GC System, Agilent) equipped with a dual plasma controller and chemiluminescence sulfur detector (355, Agilent) and a data processor. The carrier gas was helium with a flow rate of 2.4 mL/min. For the measurement of H₂S released from bound sulfane sulfur, 0.1 mL of the sample homogenates and 0.1 mL of 15 mM DTT in 0.1 mM Tris/HCl, pH 9.0, were placed in a in a small glass vial, sealed, and incubated at 37°C for 50 minutes. After the incubation, 0.4 mL of 1 M sodium
Citrate buffer was injected through the rubber stopper and the mixture was incubated at 37°C for 10 minutes with shaking at 125 rpm on a rotary shaker to facilitate the release of H₂S gas from the aqueous phase. After shaking, 0.1 mL of head-space gas was applied to a gas chromatograph as detailed above. For the measurement of H₂S and sulfane sulfur in blood, 0.1 mL and 0.05 mL of whole blood was used for each measurement, respectively. For the measurement of H₂S and sulfane sulfur in urine, 0.025 mL and 0.0125 mL of urine was used for each measurement, respectively. The concentrations of H₂S in the samples were calculated using a standard curve of Na₂S as a source of H₂S. Chromatographs were captured and analyzed with Agilent ChemStation software (B.04.03). For tissue, the amount of H₂S is reported as nmole/mg wet weight. For the blood and urine, the amount of H₂S is reported as µM.

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

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


