SUPPLEMENTAL MATERIAL

Expanded Materials and Methods

Animals. Two different stains of mice were utilized in this study: (1) Male C57BL6/J mice, 8-10 weeks of age (Jackson Labs, Bar Harbor, ME) and (2) Male mice (8-10 weeks of age) with a cardiac-specific overexpression of CGL (αMHC-CGL-Tg) and non-transgenic littermates (FVB background). The generation of cardiac-specific transgenic mice overexpressing CGL (αMHC-CGL-Tg^{+}) has been described previously.[1] All experimental mouse procedures were approved by the Institute for Animal Care and Use committee at Albert Einstein College of Medicine and Emory University 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. Sodium Sulfide (Na_2S) was produced by Ikaria Inc. (Seattle, WA) by using H_2S gas (Matheson, Newark, CA) as a starting material. Na_2S was formulated to pH neutrality, and iso-osmolarity. Na_2S (stock solution at 0.55 mg/ml and 7.1 mM) was diluted in normal (0.9%) saline to the desired concentration in a rapid fashion immediately before administration. Na_2S (100 μg/kg) was administered using a 32-gauge needle in a final volume of 50 μL as an intracardiac (i.c.) injection once at the time of reperfusion (Na_2S) or once at the time of reperfusion followed by daily tail vein (i.v.) injections for the first 7 days of reperfusion (Na_2S 7d). This dose of Na_2S was selected based on our previous experience investigating Na_2S in murine models of

cardiac ischemia-reperfusion injury.[2] Saline was administered in the same way for the respective vehicle groups.

Heart failure Protocols. Heart failure was induced by either permanent ligation of the LCA or by subjecting the mice to 60 min of LCA occlusion followed by reperfusion for up to 4 weeks. Surgical ligation of the LCA was performed according to methods described previously.[3] All mice were randomly allocated to the treatment groups.

Myocardial Area-at-Risk and Infarct Size Determination. Left ventricular area-at-risk (AAR) and infarct size (INF) determination was performed using Evans blue dye and 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) staining method. All of the procedures for the AAR and INF determination have been previously described.[4]

Echocardiograhic Assessment of Left Ventricular Structure and Function. Baseline echocardiography images were obtained one week prior to LCA ischemia to avoid any anesthetic effects as previously described.[5] The mice were lightly anesthetized with isoflourane in 100% O_2 and *in vivo* transthoracic echocardiography of the left ventricle (LV) using a 30-MHz RMV scanhead interfaced with a Vevo 770 (Visualsonics) was used to obtain high-resolution two-dimensional ECG based kilohertz visualization (EKV) B mode images acquired at the rate of 1000 frames/sec over 7 minutes. These images were used to measure LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD) and ejection fraction (EF). Echocardiography images were obtained and analyzed again 4 wk following the induction of myocardial ischemia.

Histological Analysis of Infarct Size. After the post myocardial infarction echocardiographic assessment, the mice were re-anesthetized, intubated, and connected to a rodent ventilator as previously described [2]. A median sternotomy was performed and the heart was rapidly excised and fixed in conventional fixing solutions (4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer). After 12 hours in 4% paraformaldehyde, the heart was cut into 1 mm thick as detailed above. The slices were section with a cryostat and then stained with hematoxylin and eosin (H&E). Digital images of the slides were then captured and analyzed using computer-assisted planimetry with NIH ImageJ 1.37 software to measure the area of infarct or scar relative to the left ventricle. For each heart, we analyzed multiple sections taken from the mid-ventricle and then averaged these numbers to obtain a single INF/LV measurement for each animal.

***Lipid hydroperoxide assay*.** Quantification of lipid peroxidation was performed to assess the extent of cardiac oxidative stress as described previously.[6] Lipid peroxidation results in the formation of highly unstable and reactive hydroperoxides of both saturated and unsaturated lipids. Cardiac tissue was collected at 1 and 4 weeks of reperfusion. Lipid hydroperoxides were measured using a commercially available kit (Cayman Chemicals) according to the manufacturer's recommendations. The assay is based on the principle that hydroperoxides are highly unstable and react with ferrous ions readily to produce ferric ions. The resulting ferric ions are detected using thiocynate as the chromogen. Myocardial lipid hydroperoxide (LPO) is reported in micromolars.

Quantitative Real-Time Polymerase Chain Reaction for Mitochondrial DNA Mitochondrial DNA (mtDNA) content was quantified by real-time reverse-transcription polymerase chain reaction with cardiac DNA as described previously.[7] Briefly, DNA was extracted from frozen heart tissue of sham operated, vehicle and Na_2S-treated mice by trizole (Sigma, St Louis, MO)/ cloroform extraction followed by on column purification and elution (RNeasy mini kit, Quiagen). Total RNA concentration was determined with a spectrometer. Five nanograms of genomic DNA were assayed in triplicate with Sybrgreen core reagents (Applied Biosystems, Foster City, Calif) and cytochrome b (mitochondrial) or β-actin (nuclear) and a Mini Opticon Detector (Biorad). mtDNA per nuclear genome was calculated as the ratio of cytochrome b DNA to β -actin DNA quantity.

Cardiac Mitochondria Isolation. Cardiac mitochondria were isolated from the following groups of mice: sham operated, vehicle and Na_2S-treated mice. Briefly, the heart was quickly excised and washed in buffer containing 200 mM sucrose, 20 mM Tris, 2 mM EGTA, pH 7.4 at 4°C. After changes of buffer, the cardiac samples were cut into small pieces and homogenized. The samples were centrifuged at 450 X g for 3 min to remove debris, and mitochondria were obtained by a differential centrifugation technique as previously described.[1] All isolated mitochondria were kept on ice and used within 3 h of isolation.

Mitochondrial Respiratory Rate and ATP synthesis. Oxygen consumption of cardiac mitochondria was measured in a sealed chamber magnetically stirred at 37°C by using calibrated Clark-type electrodes in the presence of succinate (8 mmol/L) and glycerol-3-phosphate (4 mmol/L) as previously described.[1] Maximal (ADP-stimulated) respiration

was measured after the addition of ADP (1 mmol/L). Additionally, respiration in the absence of ADP phosphorylation was determined in the presence of 1 mg/ml oligomycin. Respiratory control ratios were determined as the ratio of oligomycin to state 3 respirations. To evaluate ATP synthesis, aliquots were taken from the respiration chamber over a 1-minute period after the addition of ADP. ATP was then quantified with a bioluminescence assay using an ATP determination kit (A-22066; Molecular Probes, Eugene, OR). The ATP/O_2 ratio was calculated with the state 3 respiratory rate for each sample.[7]

Western blot analysis. Western blot analysis was performed as described previously.[2] 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. The following primary antibodies were used: anti-rabbit Nrf2 (1:3000; abcam, Cambridge, MA); anti-rabbit Akt (1:5000; Cell Signaling, Danvers, MA); anti-rabbit phosphorylated Ser473 Akt (1:2000; Cell Signaling); anti-rabbit NRF-1 (1:3000; abcam). Immunoblots were next processed with secondary antibodies (anti-rabbit; Cell Signaling) for 1 hr at room temperature. Immunoblots were then probed with an ECL+Plus chemiluminescence reagent kit (GE Healthcare) 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. The membranes were incubated with the phospho-specific antibody first.

Membranes were then stripped and incubated with the total-specific antibody. Results were presented as the ratio of the expression of phosphorylated protein to total protein.

The following antibodies were used as loading controls: anti-fibrillarin served as the subcellular marker for the nuclear fraction (1:5000; Cell Signaling) and anti-α-tubulin served as the subcellular marker for the cytosolic fraction (1:40000; Santa Cruz, Santa Cruz, CA). All experiments were performed in triplicate. For each membrane the relative intensity of each band was normalized to the value of the weakest band (smallest intensity). The values for each individual sample were averaged to obtain one value for each sample. The values for each group were then averaged and subsequently normalized to the mean of the control group (Sham) as previously described.[2]

Statistical Analysis. All the data in this study are expressed as mean ± standard error (SEM). Means were compared using Prism 4 (GraphPad Software, Inc) with Student's unpaired 2-tailed t-test (Western Blot analysis), one-way analysis of variance (ANOVA; heart to body weight ratios, LPO data, mitochondrial DNA and mitochondrial respiration data), or two-way ANOVA (echocardiography data) where appropriate. For the ANOVA, if a significant result was found, the Tukey (one-way ANOVA) or Bonferroni (two-way ANOVA) test was used as the post hoc analysis. The survival curves were compared with a Log-rank (Mantel-Cox) Test. For all data, a p value less than 0.05 was considered significant.

Online Figure Legends

Supplemental Figure 1. Alterations in heart rates following myocardial ischemia. Heart rates were evaluated with a Visualsonics Vevo 770 echocardiography machine in Non-transgenic (Non-Tg) and αMHC-CGL-Tg mice at baseline and 4 weeks following (A) permanent left coronary artery (LCA) occlusion and (B) 60 minutes of LCA occlusion. Heart rates were also evaluated at baseline and 4 weeks after 60 minutes of LCA occlusion in C57BL/6J mice administered (C) a single injection of Na_2S (100 μg/kg) or (D) an injection of Na_2S (100 μg/kg) once daily for the first 7 days of reperfusion. Numbers inside bars indicate the number of animals that were investigated in each group. *p<0.05, **p<0.01, and ***p<0.001 vs. Base. Base, baseline. Post, 4 wk post myocardial ischemia. Means were compared with a two-way ANOVA using a Bonferroni test as the post-hoc analysis.

Supplemental Figure 2. Infarct Area following myocardial ischemia. The infarct area (INF) relative to the entire left ventricle (LV) was evaluated at 4 wk following myocardial ischemia in Non-transgenic (Non-Tg) and αMHC-CGL-Tg mice subjected to (A) permanent left coronary artery (LCA) occlusion and (B) 60 minutes of LCA occlusion. INF area was also evaluated 4 wks after 60 minutes of LCA occlusion in C57BL/6J mice administered (C) a single injection of Na_2S (100 μg/kg) or (D) an injection of Na_2S (100 μg/kg) once daily for the first 7 days of reperfusion. For each heart, we analyzed multiple sections taken from the mid-ventricle and then averaged these numbers to obtain a single INF/LV measurement for each animal. Numbers inside bars indicate the

number of animals that were investigated in each group. *p<0.05 vs. Vehicle and **p<0.01 vs. Non-Tg. Means for all data were compared with an unpaired t-test.

Supplemental Figure 3. Daily administrations of Na_2S alter the expression of Nrf2 and NRF1 in the liver. (A) Representative immunoblots and densitometric analysis of hepatic Nrf2 and NRF-1 in the cytosolic and nuclear fractions following 1 week of Na_2S treatment. (B) Representative immunoblots and densitometric analysis of phosphorylated Akt at serine residue 473 (Akt^{Ser473}) and total Akt following 1 week of Na_2S treatment. Values are means ± S.E.M. *p<0.05 vs. Vehicle. Means for all data were compared with an unpaired t-test.

Supplemental Figure 4. Cardiac-Specific overexpression of CGL alters the cardiac expression of NRF1 and Akt. (A) Representative immunoblots and densitometric analysis of NRF-1 in the cytosolic and nuclear fractions of hearts from αMHC-CGL-Tg^+ and Non-Tg mice. (B) Representative immunoblots and densitometric analysis of phosphorylated Akt at serine residue 473 (Akt^{Ser473}) and total Akt from the hearts of αMHC-CGL-Tg^+ and Non-Tg mice. Values are means ± S.E.M. *p<0.05 and **p<0.01 vs. Non-Tg. Means for all data were compared with an unpaired t-test.

Supplemental Figure 5. Cardiac-Specific overexpression of CGL does not alter the hepatic expression of Nrf2, NRF1 and Akt. (A) Representative immunoblots and densitometric analysis of Nrf2 and NRF-1 in the cytosolic and nuclear fractions of livers

from αMHC-CGL-Tg^{+} and Non-Tg mice. (B) Representative immunoblots and densitometric analysis of phosphorylated Akt at serine residue 473 (Akt^{Ser473}) and total Akt from the livers of αMHC-CGL-Tg^{+} and Non-Tg mice. Values are means ± S.E.M. Means for all data were compared with an unpaired t-test.

References

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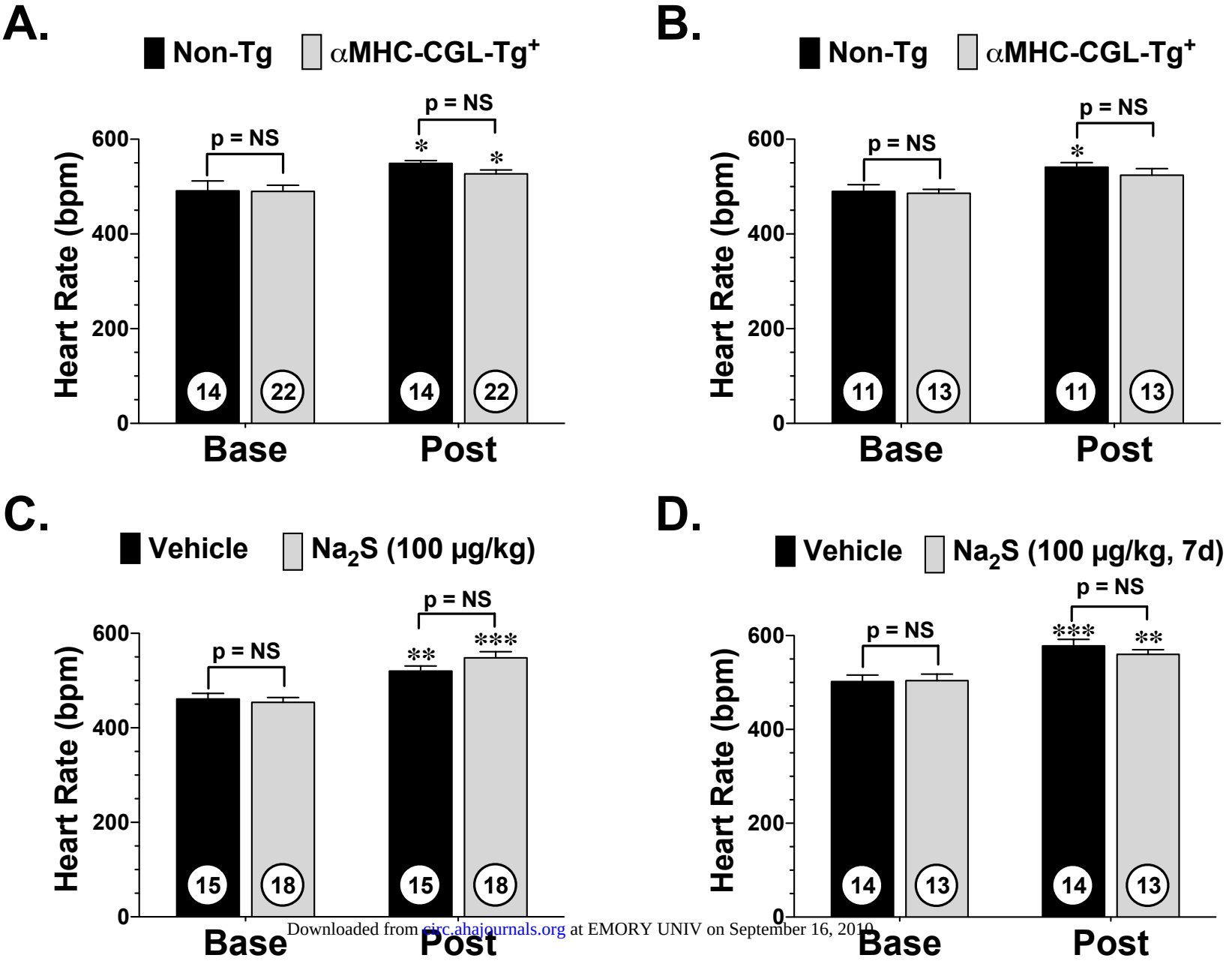
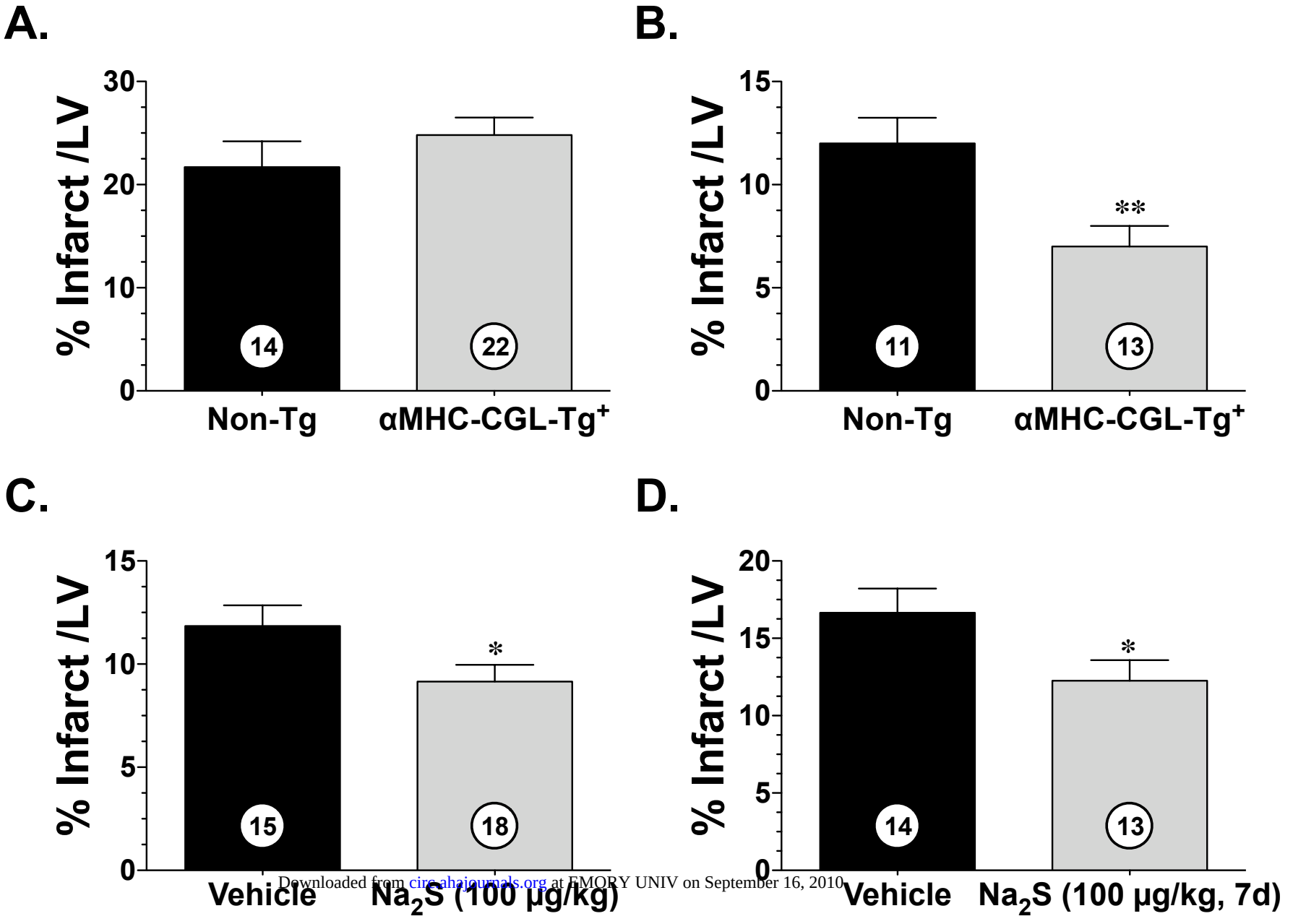
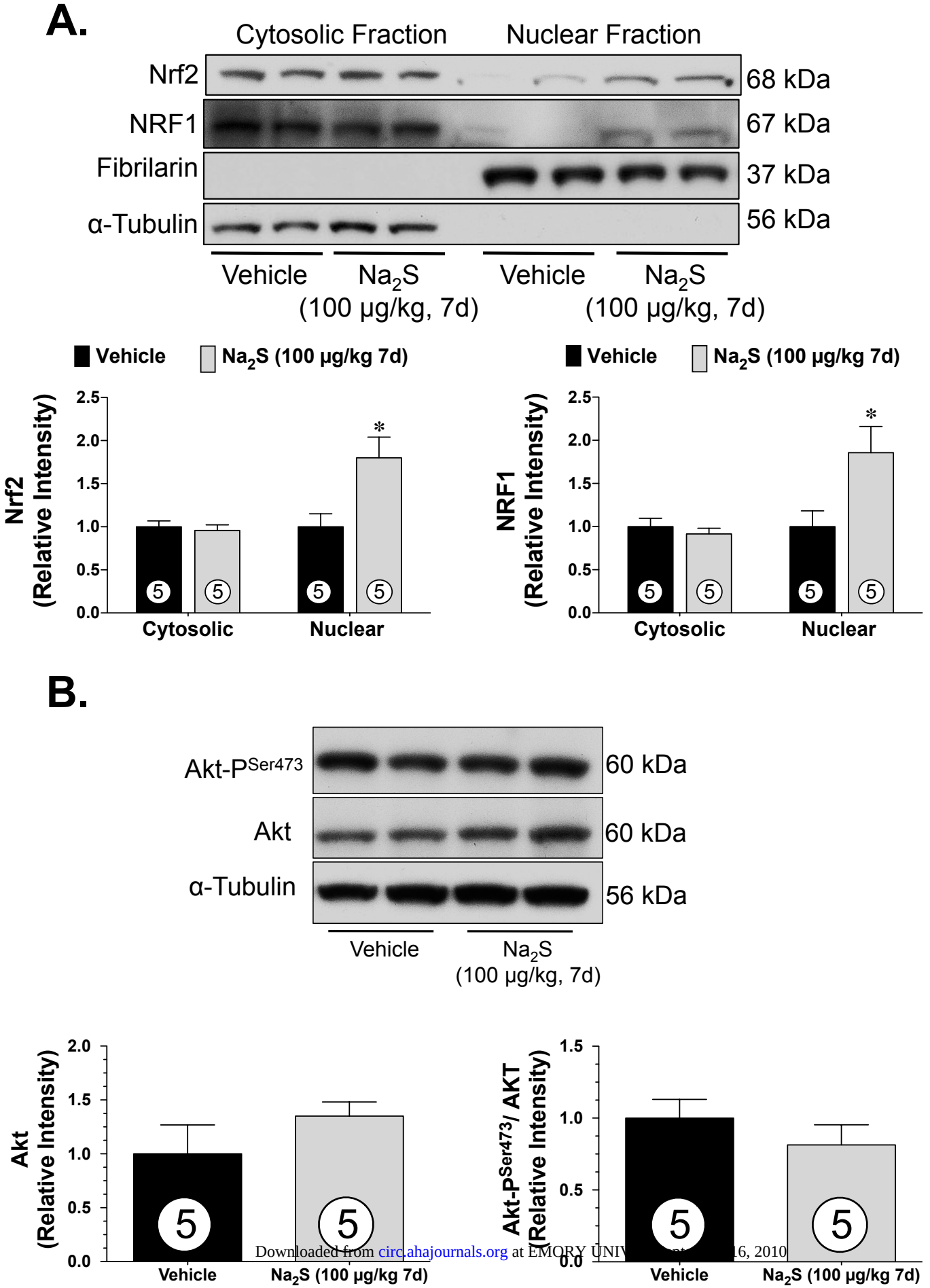
Supplemental Figure 1



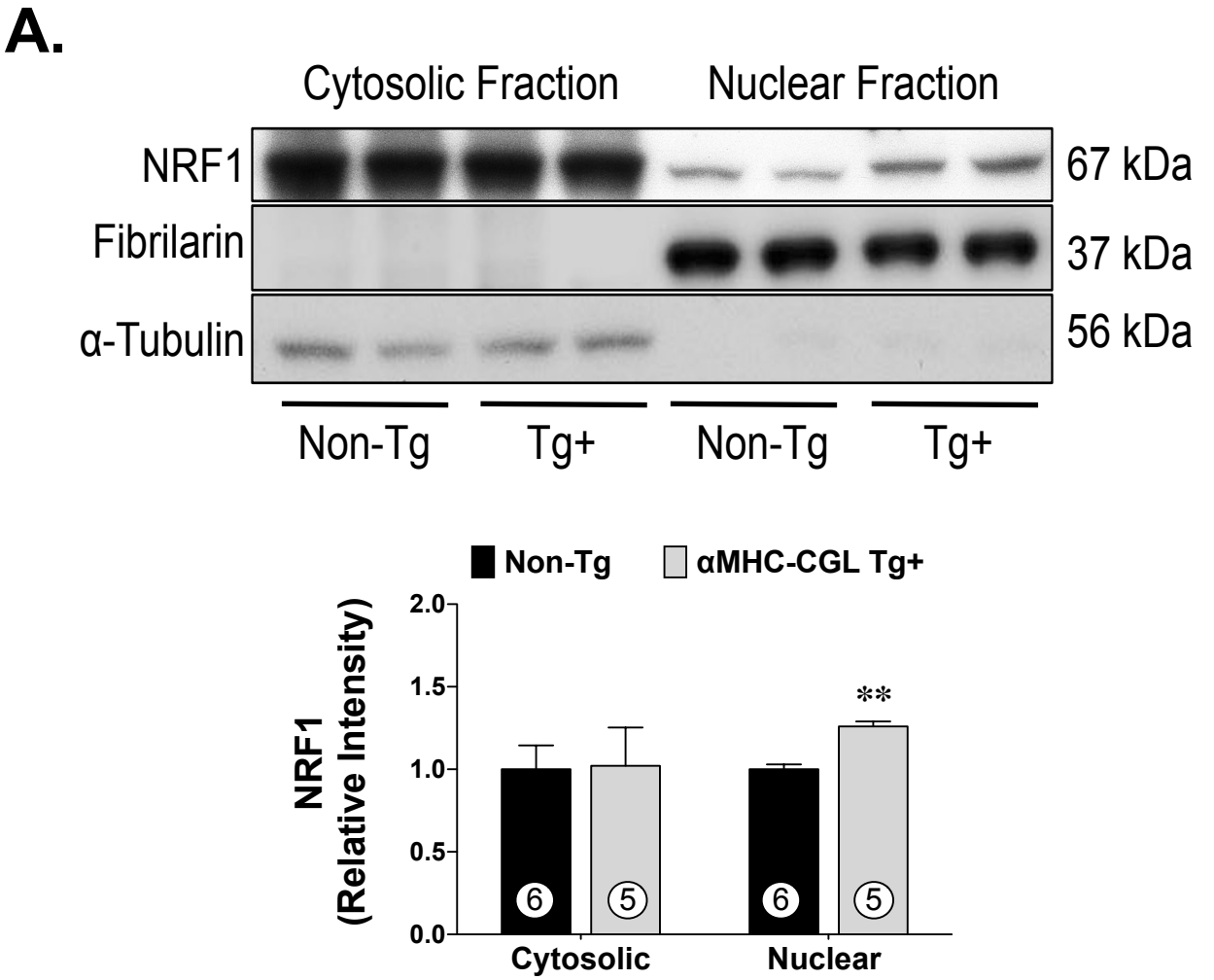
Supplemental Figure 2

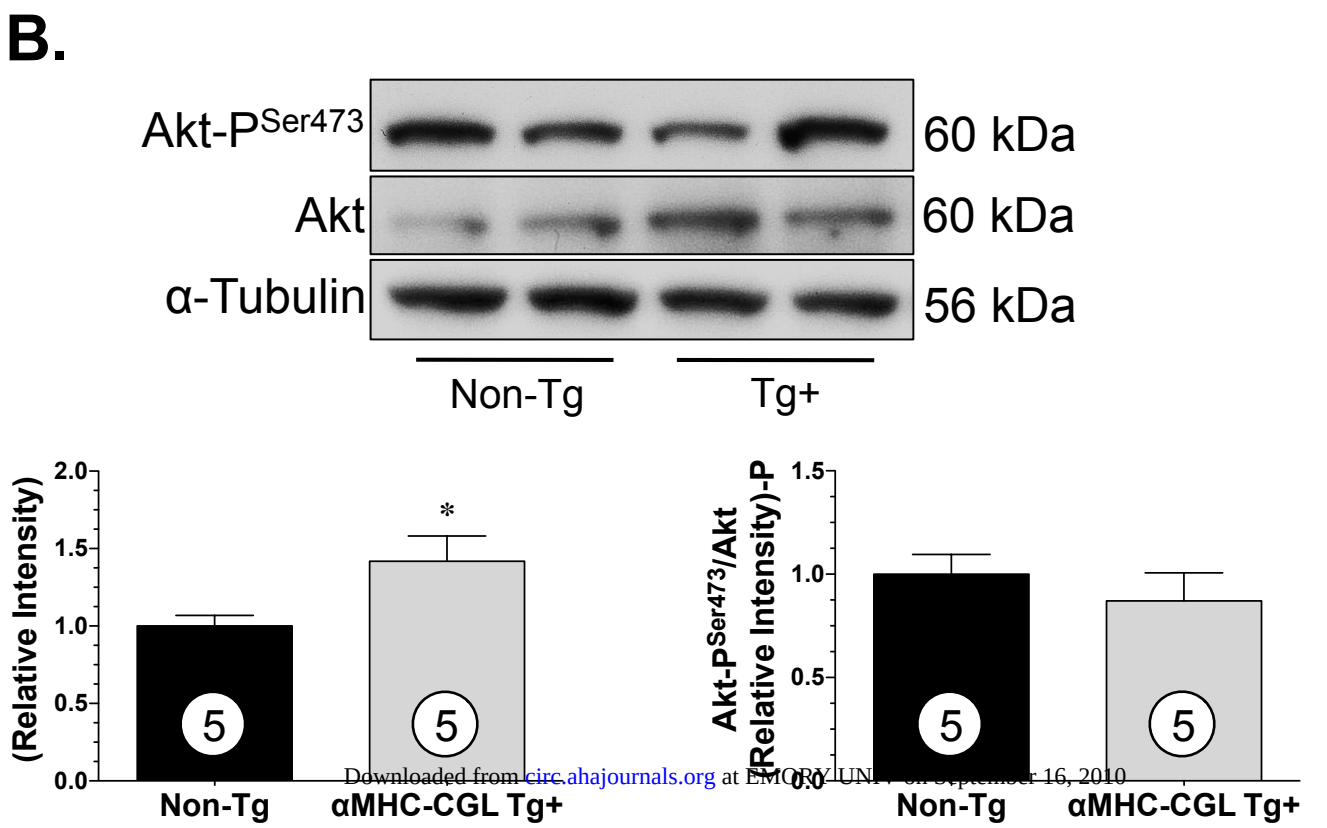


Supplemental Figure 3



Supplemental Figure 4





Supplemental Figure 5