Downregulation of Myoglobin Limits the Cardioprotective Effects of Exercise by Diminishing the Nitrite Reductase Capacity of the Heart

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Detailed Methods

Animals. Six strains of mice were utilized in this study: (1) Male C57BL6/J mice (Jackson Labs, Bar Harbor, ME; 8-10 weeks of age); (2) Male NFAT-luciferase reporter mice (10-12 weeks of age); (3) Male Calcineurin Aβ deficient, (4) Male NFATc2 deficient; (5) Male NFATc4 deficient; and (6) Male NFATc2/NFATc4 deficient mice. The generation of NFAT-luciferase reporter mice [1] has been described previously. Calcineurin Aβ [2], NFATc2 [3], NFATc4 [4], and NFATc2/NFATc4 [5] global deficient have been described previously. 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. DETA/NO, 2,2′ -(Hydroxynitrosohydrazino)bis-ethanamine (DETANO) was purchased from EMD Millipore. DETANO was dissolved in saline and administered using a 32-gauge needle at a dose of 0.5 mg/kg (final volume of 50 μL) as an injection into the LV lumen at the time of reperfusion. Saline was administered in the same manner for the vehicle groups.

Voluntary Exercise Protocol. Mice were placed in custom designed cages fitted with running wheels (Mini Mitter, Bend, OR) for a period up to 4 weeks. Running distances were monitored daily. After the exercise-training period, the running wheel was removed from the cage and the mice were allowed to rest for a 24-hour, 1-week, or 4-week period before further experimentation was conducted.

Myocardial Ischemia-Reperfusion (I/R) Protocol. Prior to any surgical procedure, mice were anesthetized with intraperitoneal injections of ketamine (60 mg/kg) and sodium pentobarbital (20 mg/kg). Mice also received 200 Units/kg of sodium heparin via intraperitoneal injection before surgery to prevent clot formation and allow for consistent and complete reperfusion postligation. The mice were then attached to a surgical board with their ventral side up and orally intubated with polyethylene-60 (PE-60) tubing connected via loose junction to a rodent ventilator (MiniVent Type 845, Hugo-Sachs Elektronik) set at a tidal volume 240 μL of and a rate of 110 breaths per minute and supplemented with 100% oxygen (0.1-0.2 liters/minute flow rate) via a side port on the ventilator. Effective ventilation was visually confirmed by vapor condensation in the endotracheal tube and rhythmic rising of the chest. Mice were maintained at a constant temperature of 37°C with a heat pad warmed by a circulating water bath. Temperature was monitored via a rectal probe connected to a Digisense K-Type digital thermometer. Hair remover (i.e., Nair®) was placed on the chest with a cotton swab and then removed along with the chest hair. The exposed regions were wiped with alcohol and betadine solution. A midline incision was then made along the sternum exposing the ribcage. Next, a median sternotomy was performed and the wound edges were cauterized with an electrocautery device.

The proximal left coronary artery (LCA) was visually identified with the aid of an Olympus stereomicroscope with a fiber optic light source. The LCA was ligated with a 7-0 silk suture passed with a tapered BV-1 needle. A short segment of PE-10 tubing was placed between the LCA and the 7-0 silk suture to minimized damage to the coronary artery and allow for complete reperfusion following the ischemic period. During the ischemic period the incision was covered with parafilm creating an effective barrier against desiccation and dehydration. Following 45 minutes of LCA occlusion, the ligature was removed, and reperfusion was visually confirmed. The chest wall and skin incision was carefully closed in layers with a 4-0 BIOSYN suture (CV-23 tapered needle). Animal recovery was supplemented by 100% oxygen and butorphanol (0.15 mg/kg) analgesia as well as a single dose of the antibiotic Cefazolin (80 mg/kg) to prevent infection. In the surgical recovery area, a heat pad warmed by a circulating water bath was utilized to maintain the appropriate body temperature of the mice. In addition, food and water were made available immediately and for the remainder of the first 24 hours of recovery.

Myocardial Infarct Size Determination. At 24 hours of reperfusion, the mice were fully anesthetized as before, intubated, and connected to a rodent ventilator. A catheter (PE-10 tubing) was placed in the common carotid artery to allow for Evans Blue dye injection. A median sternotomy was performed and the LCA was re-ligated in the same location as before. Evans Blue dye (1.25 mL of a 7.0% solution, Sigma) was injected into the carotid artery catheter into the heart to delineate the ischemic zone from the non-ischemic zone. The heart was rapidly excised and serially sectioned along the long axis in five, 1 mm thick sections that were then incubated in 1.0% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) for 4 minutes at 37°C to demarcate the viable and nonviable myocardium within the risk zone. Each of the five, 1 mm thick myocardial slices were weighed and the areas of infarction, risk, and non-ischemic left ventricle were assessed by a blinded observer using computer-assisted planimetry (NIH Image 1.57). All of the procedures for the left ventricular area-at-risk and infarct size determination have been previously described.[6]

Troponin I. A blood sample (500 µL) was collected from mice prior to the Evans blue dye injection. Serum was obtained and the levels of the cardiac-specific isoform of Troponin-I (ng/mL) were assessed using an ELISA kit from Life Diagnostics (West Chester, PA).

Protein Extraction. Whole cell fractions were prepared as described previously[7, 8]. Briefly, frozen LV samples were powdered under liquid nitrogen with mortar and pestle prior to homogenization in 1 ml of ice-cold RIPA lysis buffer (Cell Signaling). Homogenates were then centrifuged at 1,300 g to remove any cellular debris. The pellet was discarded, and the supernatant was again centrifuged at 16,000 g for 30 min at 4°C. The resultant supernatant was collected. Protein concentrations of all cellular fractions were measured with the DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

Western blot analysis. Western blot analysis was performed as described previously.[6] 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. 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 as previously described.[6]

NO Metabolite Measurements. Nitrite concentrations were quantified by ion chromatography (ENO20 Analyzer, Eicom). Tissue nitrosothiol compounds were quantified using group specific reductive denitrosation by iodine-iodide with subsequent detection of the NO liberated by gas-phase chemiluminescence. Nitrosyl–heme (NO-heme) levels were determined by parallel injection of replicate aliquots of tissue homogenates into a solution of 0.05M ferricyanide in PBS at pH 7.5 and 37°C. This method employs one-electron oxidation rather than reduction to achieve denitrosation, with the liberated NO quantified by gas-phase chemiluminescence. All NO analysis procedures have been previously described in detail [9].

Nitrite Reductase Activity Measurements. Tissue was homogenized in PBS. An aliquot of the homogenate was then placed in a reaction vessel connected to a NO chemiluminescence detector and purged with helium. NO generation was then measured in the presence and absence of nitrite (200 μM) as described previously [10]. In separate experiments, samples were incubated with myoglobin (25 μM) prior to placement in the reaction vessel. NO generation was then measured after the addition of nitrite.

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 myoglobin 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.).

Calcineurin Activity Assay. Calcineurin activity was measured in heart samples with a commercially available kit (Calcineurin Cellular Activity Assay Kit, Calbiochem) according to the manufacturer's instructions as previously described [11]. Calcineurin activity was measured as the dephosphorylation rate of a synthetic phosphopeptide substrate (RII peptide).

***GSK-3β Activity*.** The activity of GSK-3β was measured in nuclear fractions prepared from heart tissue. The samples were first immunoprecipitated with a specific anti-GSK-3β antibody (Cell Signaling). An aliquot of the immunoprecipitated samples were incubated in a reaction buffer containing 12.5 mM Tris-HCl (pH 7.5), 2.5 mM β-glycerophosphate, 1 mM dithiothreitol, 0.05 mM Na_3VO_4, 5 mM MgCl2, 0.0625 mM ATP, and 10 μg of GSM (GSK-3β synthetic substrate peptide). 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 picomoles) per minute per milligram of protein.

***Xanthine Oxidase Activity*.** The activity of xanthine oxidase was measured in whole cell homogenates with a commercially available kit (Xanthine Oxidase Fluorometric Assay Kit, Cayman Chemical) according to the manufacturer's instructions.

***Echocardiography*.** Echocardiography was performed with a Vevo 2100 (Visualsonics) as previously described [12]. Baseline echocardiography images were obtained one week prior to myocardial ischemia to avoid any anesthetic effects. The mice were lightly anesthetized with isoflourane (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 1 week following myocardial ischemia and reperfusion.

Statistics. All the data in this study are expressed as mean ± standard error (SEM). Differences in data between the groups were compared using Prism 5 (GraphPad Software, Inc) with Student's paired 2-tailed t-test or one-way analysis of variance (ANOVA. For the one-way ANOVA, if a significant variance was found, the Tukey or Dunnett test was used as the post hoc analysis. For the echocardiography data, a 2-way repeated measures ANOVA with a Bonferroni test as the posthoc analysis was used. The p-value for these evaluations was

adjusted by applying the Bonferroni correction for multiple comparisons. A p value less than 0.05 was considered statistically significant and p-values were two-sided.

References

[1] Braz JC, Bueno OF, Liang Q, Wilkins BJ, Dai YS, Parsons S, et al. Targeted inhibition of p38 MAPK promotes hypertrophic cardiomyopathy through upregulation of calcineurin-NFAT signaling. J Clin Invest 2003;111:1475-86.

[2] Bueno OF, Brandt EB, Rothenberg ME, Molkentin JD. Defective T cell development and function in calcineurin A beta -deficient mice. Proc Natl Acad Sci U S A 2002;99:9398-403.

[3] Xanthoudakis S, Viola JP, Shaw KT, Luo C, Wallace JD, Bozza PT, et al. An enhanced immune response in mice lacking the transcription factor NFAT1. Science 1996;272:892-5.

[4] Graef IA, Chen F, Chen L, Kuo A, Crabtree GR. Signals transduced by Ca(2+)/calcineurin and NFATc3/c4 pattern the developing vasculature. Cell 2001;105:863-75.

[5] Yang TT, Suk HY, Yang X, Olabisi O, Yu RY, Durand J, et al. Role of transcription factor NFAT in glucose and insulin homeostasis. Mol Cell Biol 2006;26:7372-87.

[6] Calvert JW, Jha S, Gundewar S, Elrod JW, Ramachandran A, Pattillo CB, et al. Hydrogen sulfide mediates cardioprotection through Nrf2 signaling. Circ Res 2009;105:365-74.

[7] Calvert JW, Gundewar S, Jha S, Greer JJ, Bestermann WH, Tian R, et al. Acute metformin therapy confers cardioprotection against myocardial infarction via AMPK-eNOS-mediated signaling. Diabetes 2008;57:696-705.

[8] Hunter JC, Kostyak JC, Novotny JL, Simpson AM, Korzick DH. Estrogen deficiency decreases ischemic tolerance in the aged rat heart: Roles of PKCdelta, PKCepsilon, Akt, and GSK3beta. Am J Physiol Regul Integr Comp Physiol 2007;292:R800-9.

[9] Bryan NS, Calvert JW, Elrod JW, Gundewar S, Ji SY, Lefer DJ. Dietary nitrite supplementation protects against myocardial ischemia-reperfusion injury. Proc Natl Acad Sci U S A 2007;104:19144-9.

[10] Bryan NS, Calvert JW, Gundewar S, Lefer DJ. Dietary nitrite restores NO homeostasis and is cardioprotective in endothelial nitric oxide synthase-deficient mice. Free Radic Biol Med 2008;45:468-74.

[11] van Rooij E, Doevendans PA, Crijns HJ, Heeneman S, Lips DJ, van Bilsen M, et al. MCIP1 overexpression suppresses left ventricular remodeling and sustains cardiac function after myocardial infarction. Circ Res 2004;94:e18-26.

[12] Nicholson CK, Lambert JP, Molkentin JD, Sadoshima J, Calvert JW. Thioredoxin 1 is essential for sodium sulfide-mediated cardioprotection in the setting of heart failure. Arterioscler Thromb Vasc Biol 2013;33:744-51.

Supplemental Figure Legends

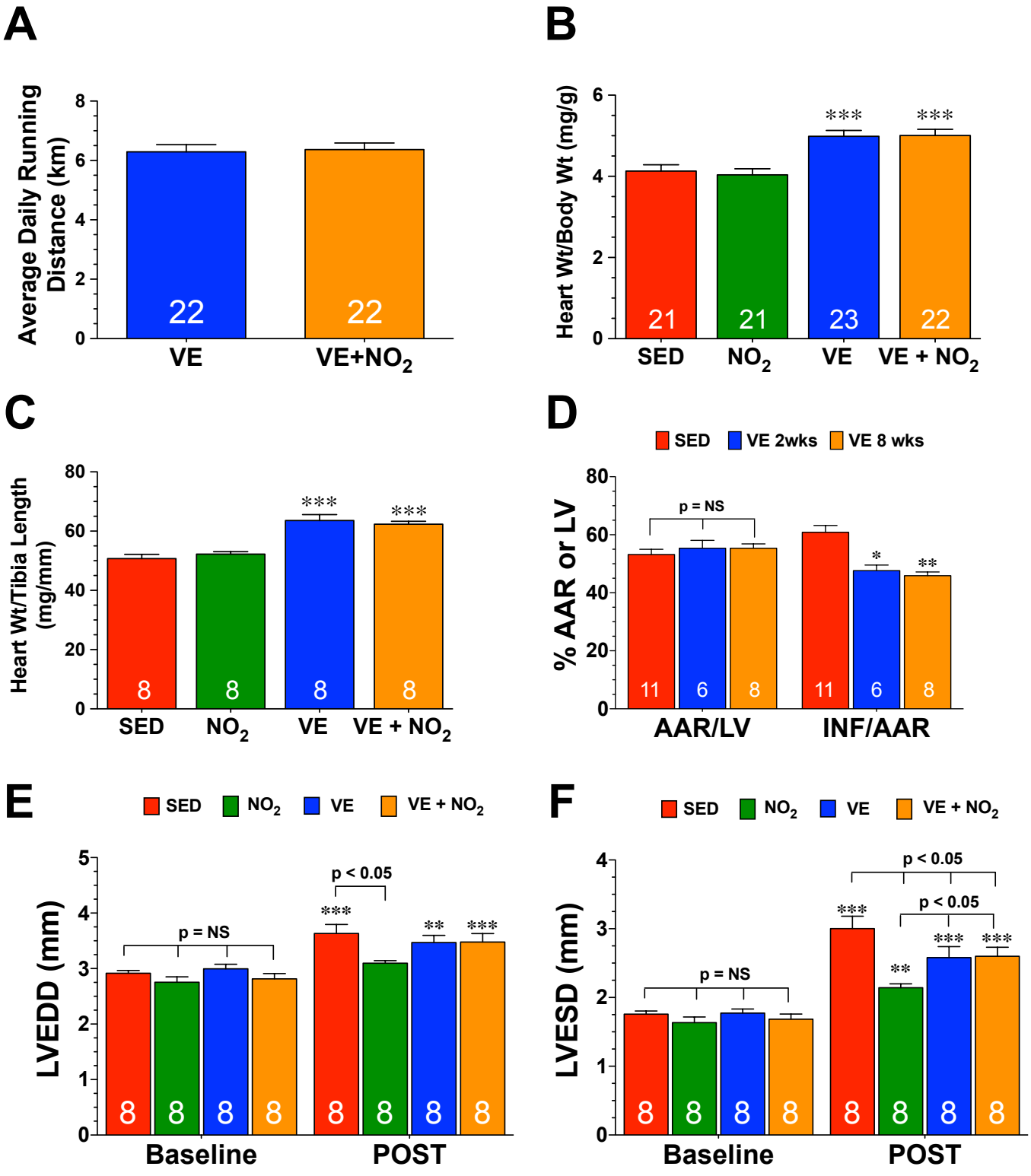
Supplemental Fig. I. Mice were housed in cages fitted with running wheels and allowed to exercise voluntarily for 4 weeks (VE). Control mice (sedentary, SED) were housed in cages without running wheels for the same durations as the VE mice. Groups of both SED and VE were also administered oral nitrite therapy (NO_2, 25 mg/L in drinking water; NO_2 and VE+NO_2). (**A**) Average daily running distances for the mice in the exercise groups. (**B**) Heart weight to body weight ratios for the experimental groups. (**C**) Heart weight to tibia length ratios for the experimental groups. (**D**) Myocardial area-at-risk (AAR) as a percentage (%) of total left ventricle (LV) and infarct size (INF) as a percentage of area-at-risk (AAR) for SED mice and mice subjected to 2 weeks (VE 2 wks) or 8 weeks (8 wks) of VE. (**E**) Left ventricular end-diastolic diameter (LVEDD, mm) and (**F**) Left ventricular end-systolic diameter (LVESD, mm) as determined by echocardiography at baseline and 1 week after 45 minutes of myocardial ischemia and reperfusion. Values in panels are means ± S.E.M. *p<0.05, **p<0.01, ***p<0.001 vs. SED or Baseline.

Supplemental Fig. II. (**A**) Representative immunoblots and densitometric analysis of **(B)** total eNOS and phosphorylated eNOS at serine residue 1177 (eNOS-$P^{Ser1177}$) **(C)**, nNOS and **(D)**, iNOS from the hearts of SED, NO_2, VE, and VE+NO_2 mice. Values in panels are means ± S.E.M. ***p<0.001 vs. SED.

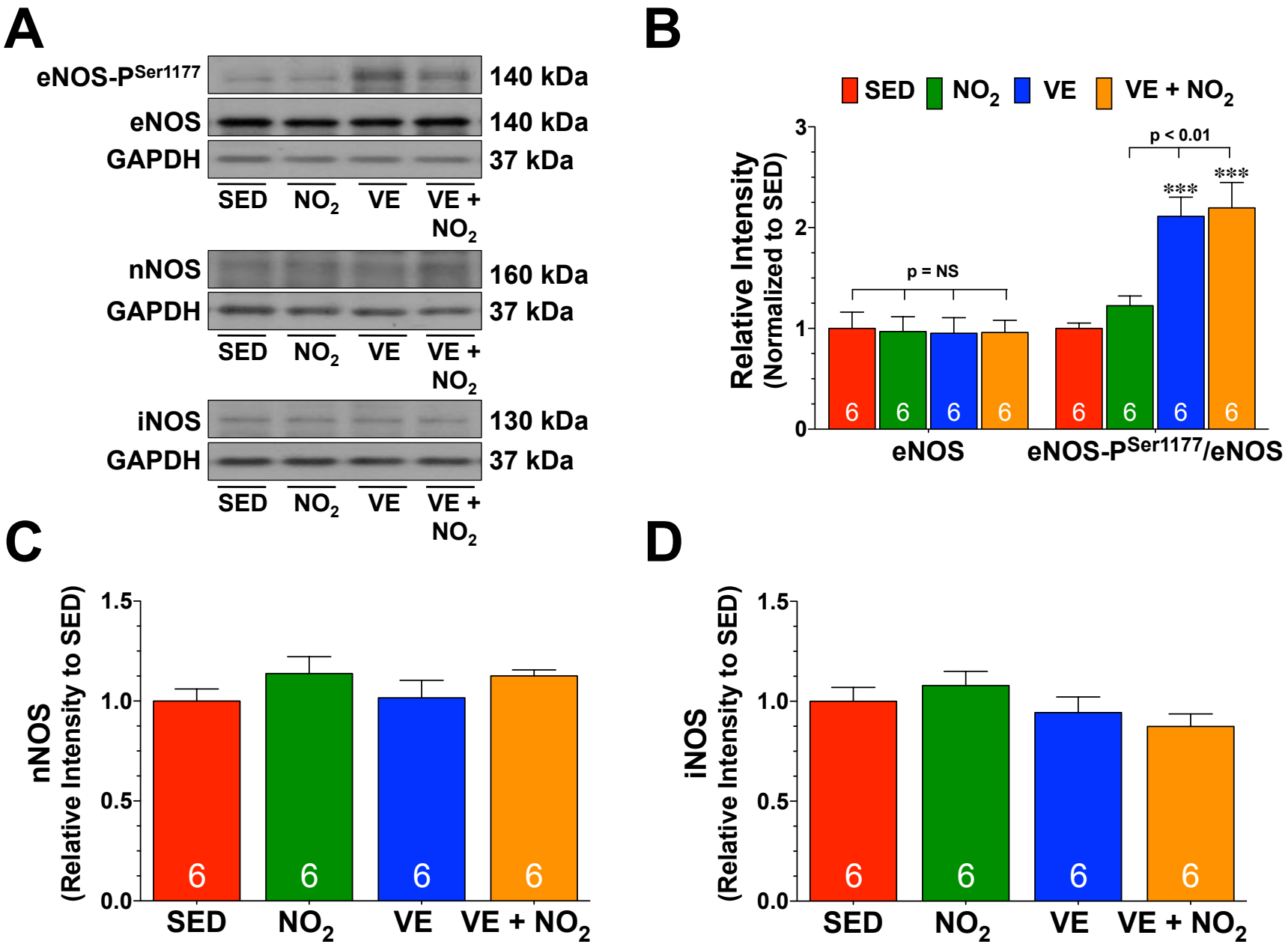
Supplemental Fig. III. (**A**) Representative immunoblots and densitometric analysis of xanthine oxidase and (**B**) xanthine oxidase activity from the hearts of SED, NO_2, VE, and VE+NO_2 mice. Values in panels are means ± S.E.M.

Supplemental Fig. IV. (**A**) Gene expression and (**B**) representative immunoblots and densitometric analysis of myoglobin from the skeletal muscle of SED and VE mice. (**C**) Nitrite reductase activity from the skeletal muscle of SED and VE mice. Values are means ± S.E.M.

Supplemental Figure I

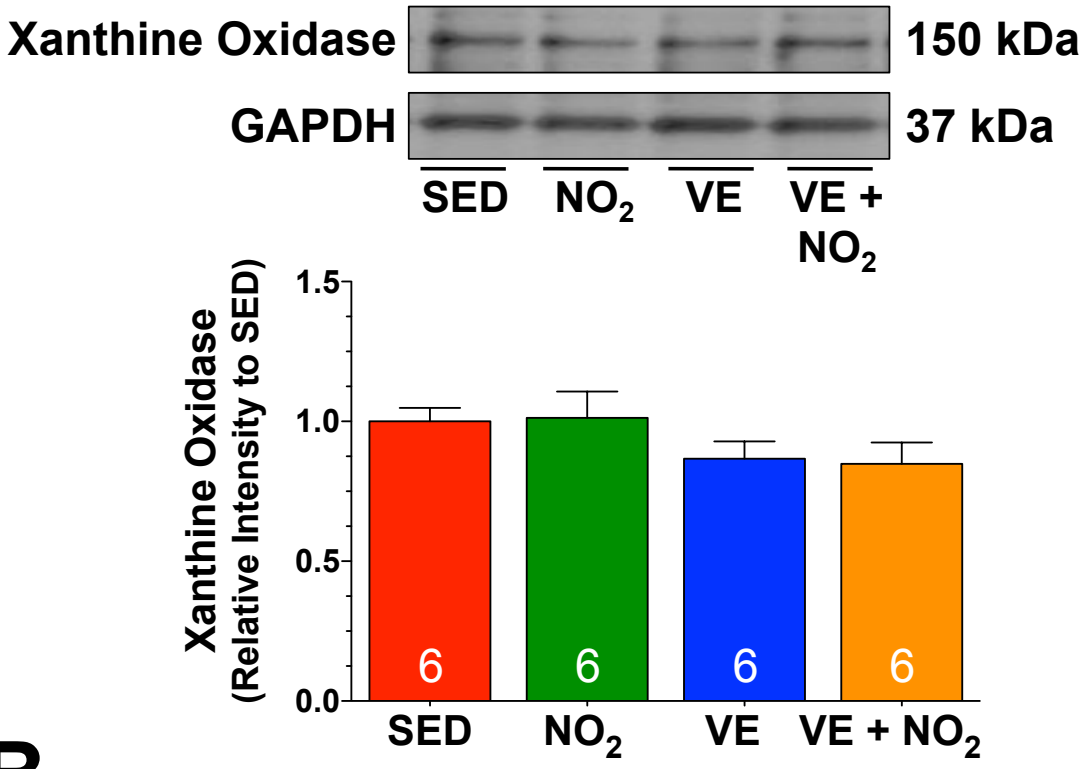


Supplemental Figure II



Supplemental Figure III

A



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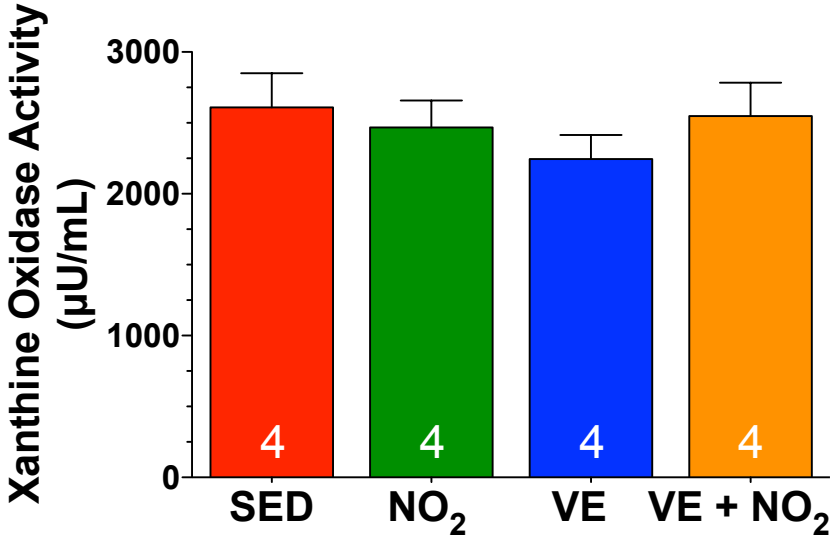

Supplemental Figure IV

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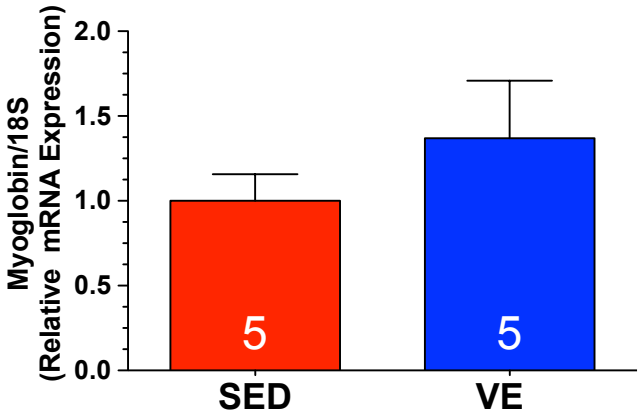

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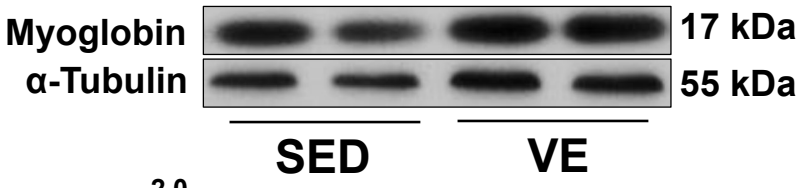

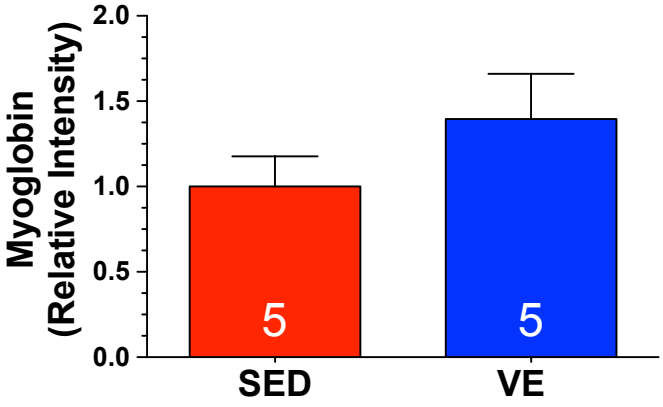

C