SUPPLEMENTAL MATERIAL

Exercise Protects the Heart Against Ischemia-Reperfusion injury via stimulation of β_3-Adrenergic Receptors and Increased Cardiac Storage of Nitrite and Nitrosothiols

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Detailed Methods

Animals. Male C57BL6/J mice (Jackson Labs, Bar Harbor, ME), 8-10 weeks of age were utilized. eNOS deficient mice (eNOS$^{-/-}$; C57BL6/J background)[1] and β_3 adrenergic receptor deficient (β_3-AR$^{-/-}$; 8-10 weeks of age) mice[2] as well as littermate controls were also utilized. The β_3-AR$^{-/-}$ mice were developed on a FVB background and backcrossed 9 generations to a C57BL6/J background. 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.

Subjects and blood collection. All procedures were approved by the Institutional Review Board (Human Research Committee) of the University of Colorado at Boulder. The nature, benefits, and risks of the study were explained to the volunteers, and their written informed consent was obtained before participation. A total of 23 healthy young (aged 18-31 years) men were studied: 16 non-exercise trained individuals and 7 endurance athletes. The non-exercise trained subjects had performed no regular exercise for $\geq$2 years, whereas the trained endurance athletes performed >3 sessions/week of vigorous aerobic-endurance exercise (at least 45 minutes) for $\geq$2 years. All of the men had plasma LDL-cholesterol <160mg/dl, resting blood pressure <140/90 mm Hg, were non-smokers, non-diabetic (fasting blood glucose <126 mg/dl), non-obese (body mass index<30kg/m^2) and free of clinical diseases as assessed by medical history, physical examination, blood chemistry and resting ECG. Subjects were not taking medications and had not taken antioxidants (e.g., vitamins C and E) within 6 week of the study. Venous blood was collected into a 10mL EDTA Tube at the University of Colorado at Boulder Clinical Translational Research Center after a 12-hour fast and a 24-hr abstention from alcohol and physical activity. Blood was centrifuged at 400g for 20 minutes at 23°C. Plasma was aliquoted into 0.65 mL eppendorf tubes and stored in -80°C until analysis.

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.[3]

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[4,5]. 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.[3] 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 CuZnSOD, Cell Signaling; anti-rabbit AMPK-P^{Thr172}, Cell Signaling; anti-rabbit AMPK, Cell Signaling; anti-rabbit HSP70, Cell Signaling; anti-mouse eNOS, BD Transduction Laboratories; anti-rabbit eNOS^{Ser1177}, Cell Signaling; anti-rabbit eNOS^{Thr495}, Cell Signaling; anti-mouse iNOS, BD Transduction Laboratories; anti-mouse nNOS, BD Transduction Laboratories; anti-rabbit β_1-adrenergic receptor, Abcam; anti-rabbit β_2-adrenergic receptor, Abcam; anti-chicken β_3-adrenergic receptor, Abcam. Immunoblots were

next processed with secondary antibodies (anti-rabbit, anti-chicken, or anti-mouse, 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 are presented as the ratio of the expression of phosphorylated protein to total protein. 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 (SED) or vehicle group as previously described.[3]

Analysis of Nitrite, Nitrate, Nitrosothiols, and NO-Heme. Nitrite and nitrate concentrations were quantified by ion chromatography (ENO20 Analyzer, Eicom). Tissue nitrosothiol compounds (RXNO) were quantified using group specific reductive denitrosation by iodine-iodide with subsequent detection of the NO liberated by gas-phase chemiluminescence. S-nitrosothiol levels were detected by preincubation with 2% mercuric chloride followed by acidified sulfanilamide. The addition of mercuric chloride allows for the differentiation between mercury sensitive (RNNO) and mercury insensitive nitrosothiols (RSNO). All NO analysis procedures have been previously described in detail.[6,7]

Analysis of catecholamine levels. Catecholamines were measured in blood samples taken from mice using the Bi-CAT Elisa (ALPCO, Salem, NH) according the manufactor's instructions.

Statistical Analysis. All the data in this study are expressed as mean ± standard error (SEM). Differences in data between the groups were compared using Prism 4 (GraphPad Software, Inc) with Student's paired 2-tailed t-test, one-way analysis of variance (ANOVA), or two-way ANOVA where appropriate. For the ANOVA, if a significant variance was found, the Tukey or Bonferroni test was used as the post hoc analysis. A p value less than 0.05 was considered significant.

References

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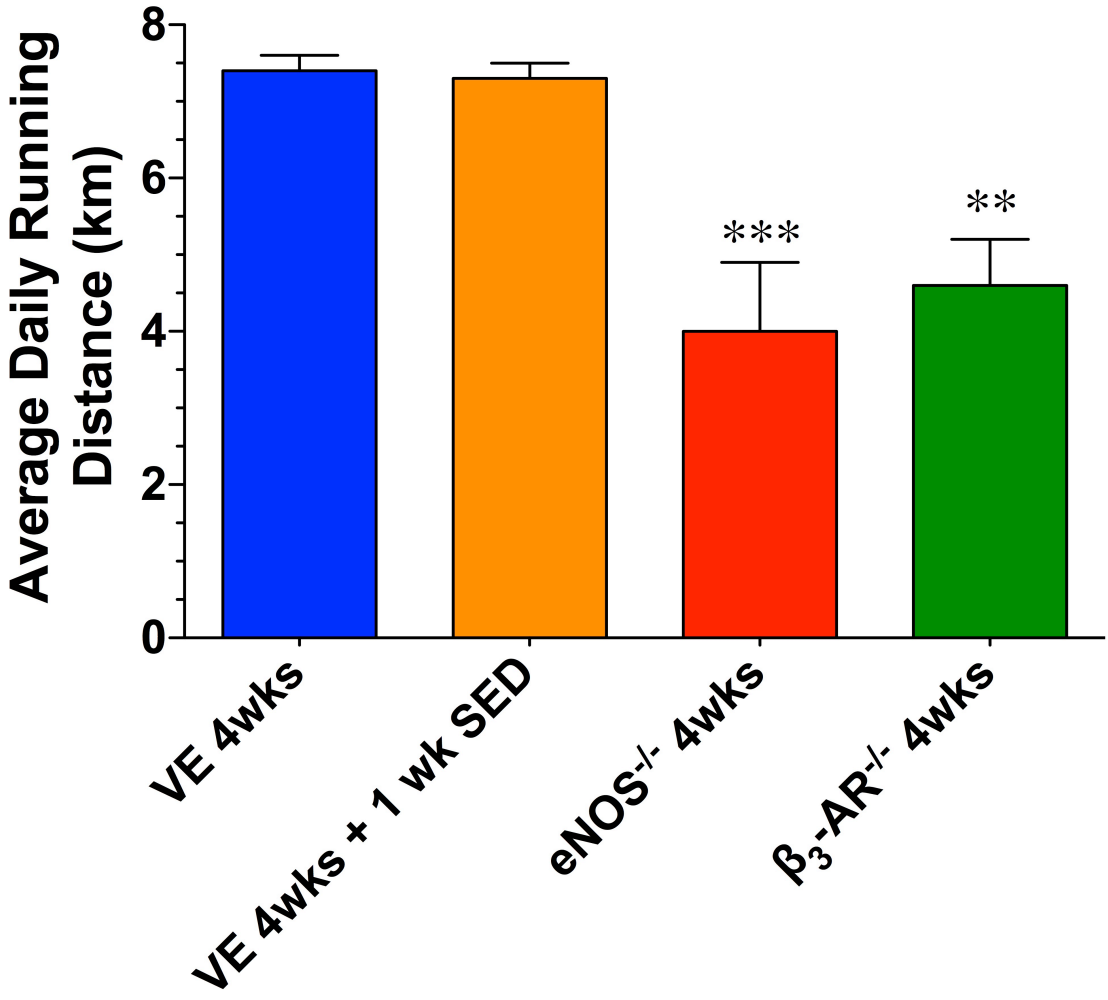
Online Table I. Subject Characteristics

Subject Characteristics	Non-trained individuals	Trained endurance athletes
n	16	7
Age (years)	27±1	24±2
Body mass (kg)	81±2	75±3
BMI (kg/m^2)	25±1	23±1
Systolic BP (mmHg)	118±2	125±3
Diastolic BP (mmHg)	66±2	62±2
Total Cholesterol (mg/dL)	163±7	155±16
LDL-C (mg/dL)	98±6	87±14
HDL-C (mg/dL)	41±2	48±4
Triglycerides (mg/dL)	120±11	103±21
Glucose (mg/dL)	89±1	91±2
VO_2 max (ml/kg/min)	47±2	59±2*

Data are mean ± SE. BMI indicates body mass index; BP, blood pressure; LDL-C, low-density-lipoprotein cholesterol; HDL, high-density-lipoprotein cholesterol; VO_2 max, maximal oxygen consumption. *$p<0.05$ vs non-trained individuals

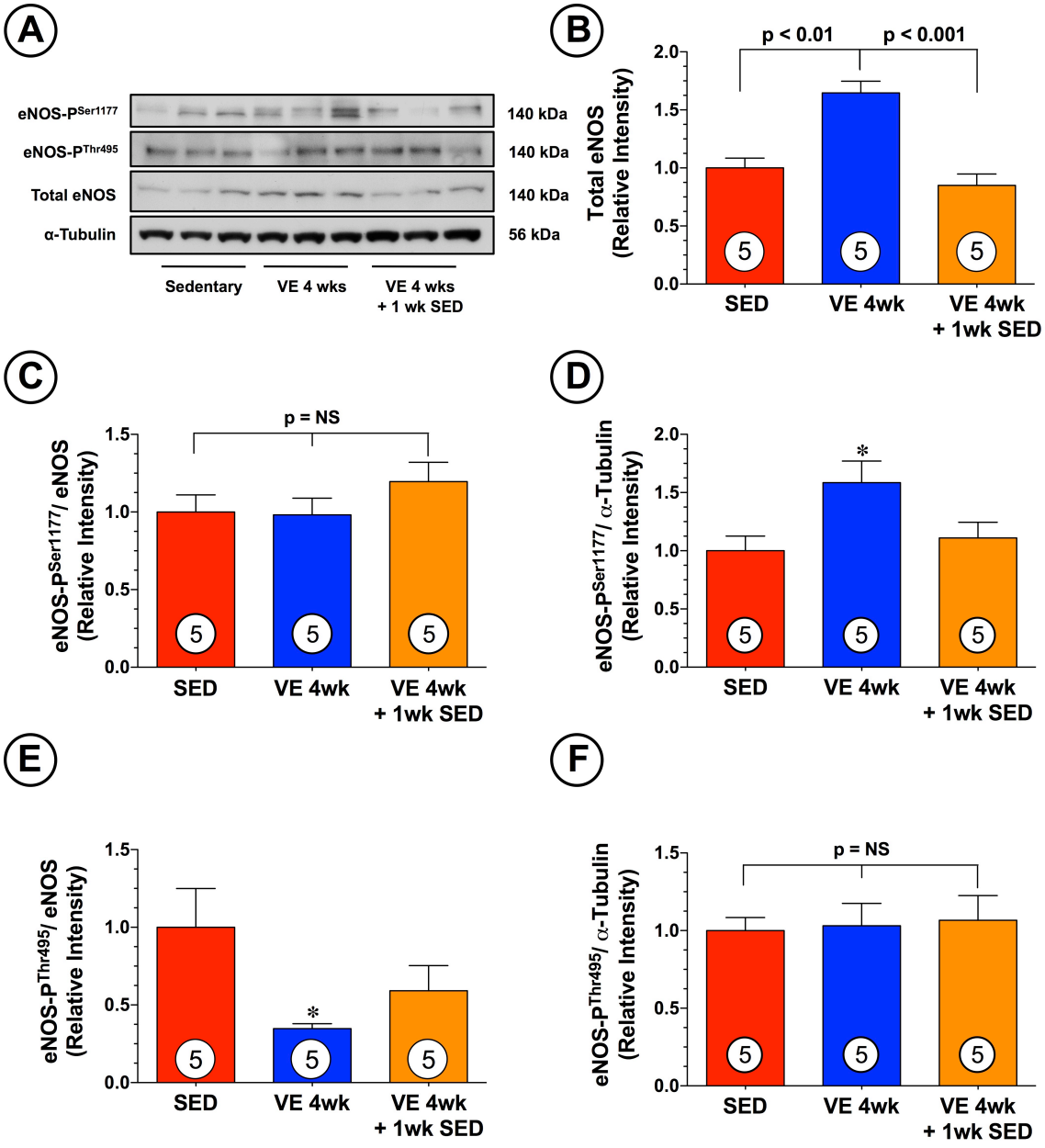
Supplemental Figures

Online Figure I



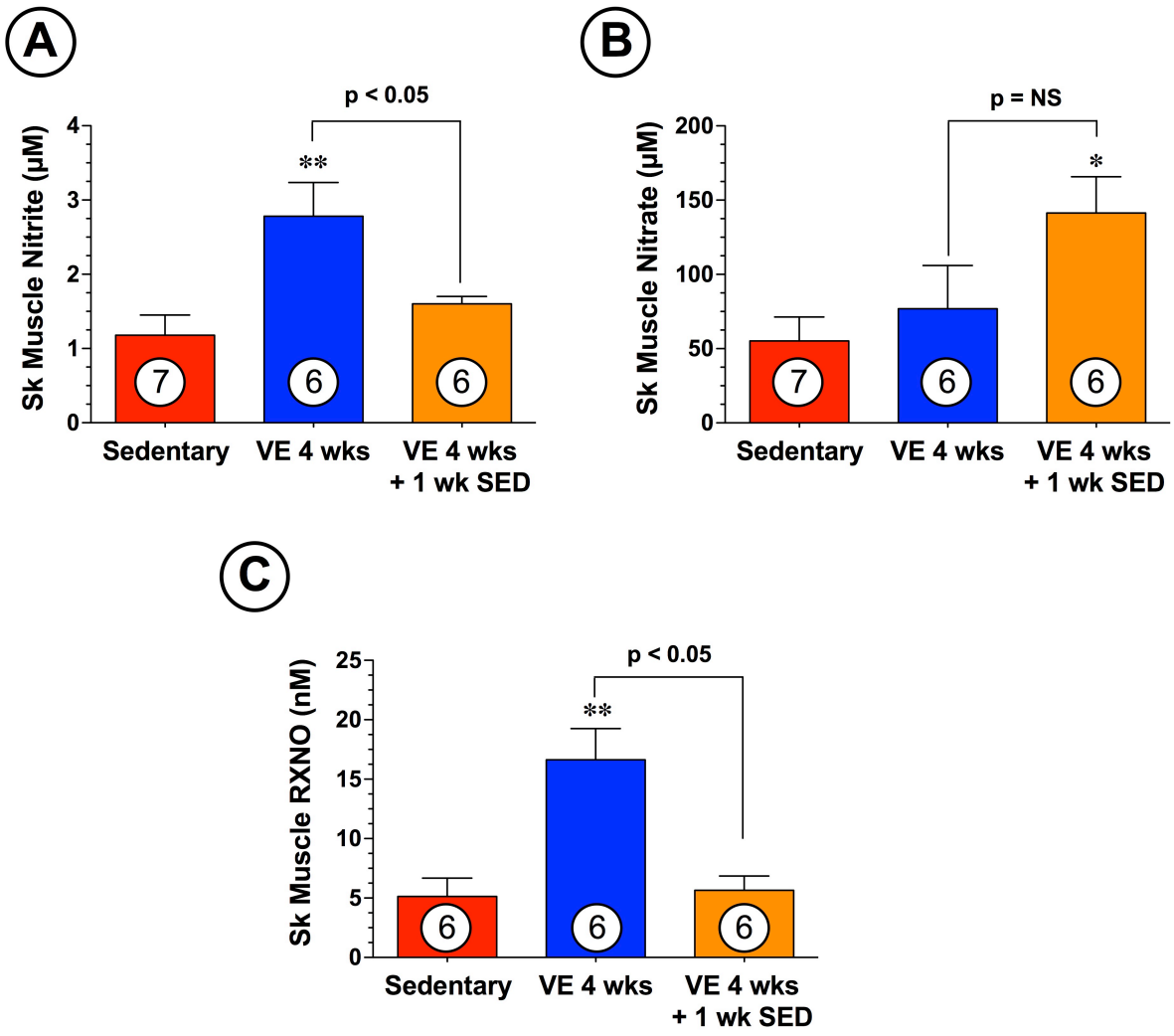
Online Figure I. Average daily running distances for C57BL/6J mice subjected to 4 weeks of voluntary exercise (VE) training, 4 wks of VE training followed by 1 week of rest, eNOS deficient mice ($eNOS^{-/-}$) subjected to 4 weeks of VE training, and β_3-Adrenergic Receptor deficient mice (β_3-$AR^{-/-}$) subjected to 4 weeks of VE training. Values are means ± S.E.M. **p<0.01 and ***p<0.001 vs. VE 4 wks.

Online Figure II



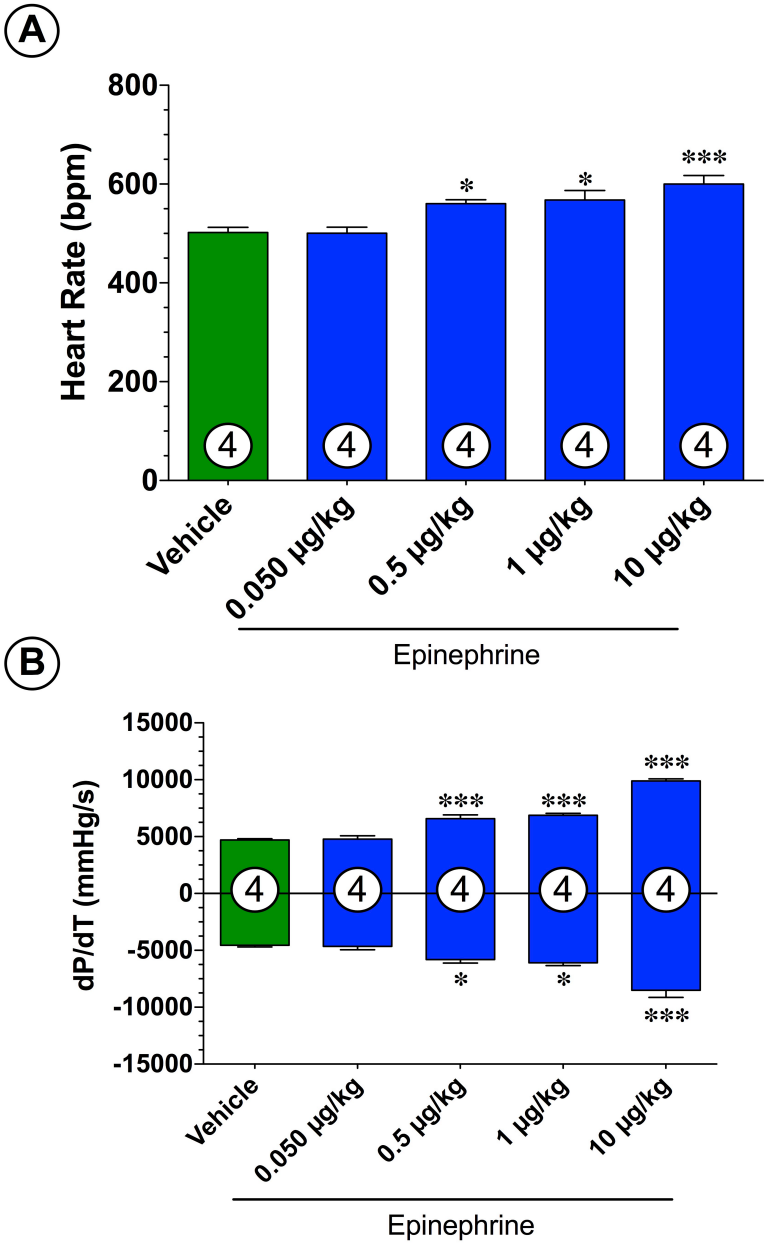
Online Figure II. VE training increased the expression and altered the phosphorylation status of skeletal muscle eNOS. (A) Representative immunoblots and densitometric analysis of (B) total eNOS, (C-D) phosphorylated eNOS at serine residue 1177 (eNOS-$P^{Ser1177}$), (E-F) phosphorylated eNOS at threonine residue 495 (eNOS-P^{Thr495}). The expression of phosphorylated eNOS was normalized to the expression of total eNOS and α-tubulin since the total levels of eNOS increased after VE training. Values are means ± S.E.M. Numbers inside the bars are the number of animals investigated. *p<0.05 vs. SED.

Online Figure III



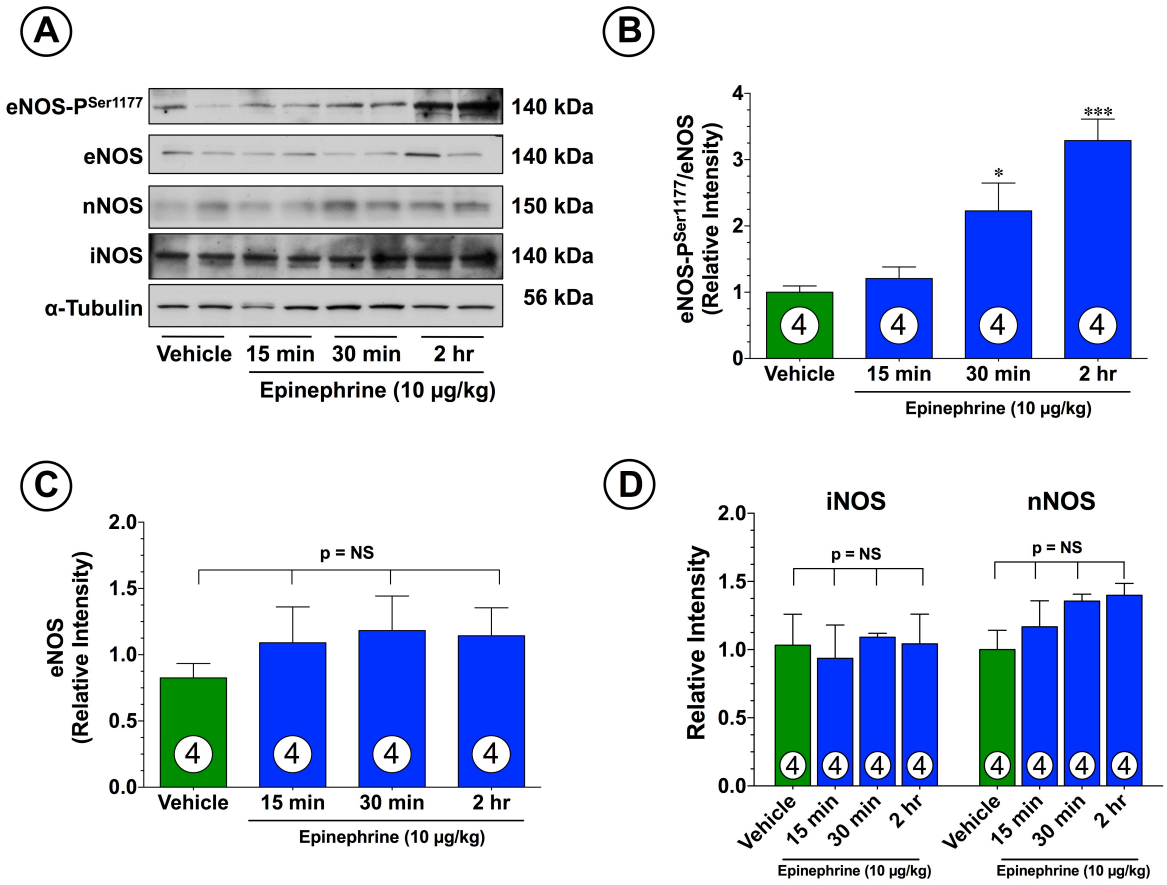
Online Figure III. VE training increased the levels of NO metabolites in skeletal muscle. Levels of (A) nitrite, (B) nitrate, and (C) nitrosothiols (RXNO) were measured in the skeletal muscle following 4 weeks of VE training. Values are means ± S.E.M. *p<0.05, and **p<0.01 vs. SED.

Online Figure IV



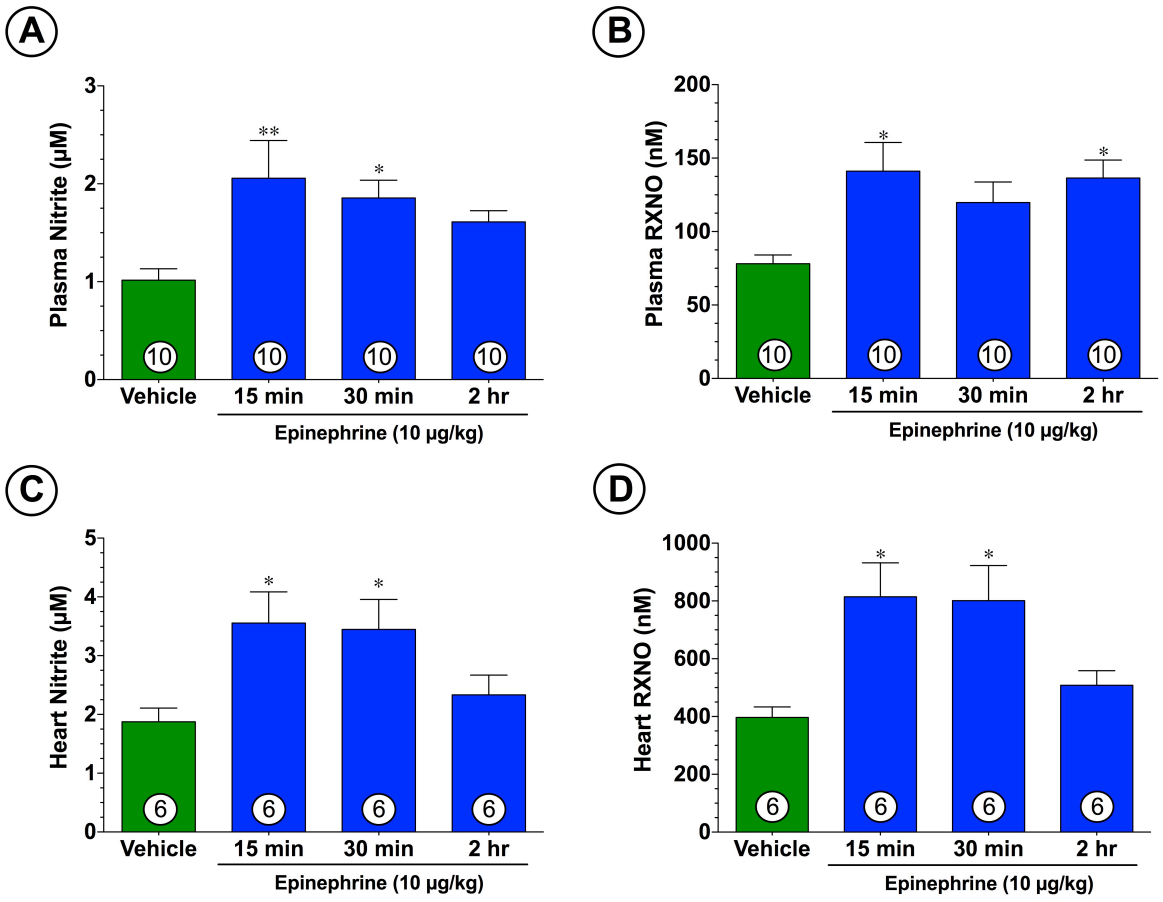
Online Figure IV. Single injection of epinephrine altered the phosphorylation status of cardiac eNOS and increased the bioavailability of NO. A single intravenous injection of epinephrine dose dependently increased the (A) heart rate and (B) cardiac contractility of mice at concentrations ranging from 0.050 µg/kg to 10 µg/kg. Values are means ± S.E.M. *p<0.05 and ***p<0.001 vs. Vehicle.

Online Figure V



Online Figure V. Single injection of epinephrine altered the phosphorylation status of cardiac eNOS. (A) Representative immunoblots and (B-D) densitometric analysis of the expression of eNOS, eNOS-P^{Ser1177}, iNOS and nNOS in heart samples taken from 15 minutes to 2 hours following a single injection of epinephrine (10 µg/kg). Values are means ± S.E.M. *p<0.05 and ***p<0.001 vs. Vehicle.

Online Figure VI



Online Figure VI. Single injection of epinephrine increased plasma and heart NO metabolite levels. Levels of nitrite and nitrosothiols were also measured in the (A-B) plasma and (C-D) heart from 15 minutes to 2 hours following a single injection of epinephrine (10 μg/kg). Values are means ± S.E.M. *p<0.05 and **p<0.01 vs. Vehicle.