Supporting Information

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SI Text

Western Blot Analysis of NOS. Western blot analysis was performed as described in detail (1). Myocardial or hepatic tissue lysates were transferred to PVDF membranes and incubated with mouse anti-eNOS, -iNOS, or -nNOS, or α-tubulin (loading control) in 5% BSA-Tris-Buffered Saline Tween-20 (TBST) overnight at 4°C. Because of the abundance of eNOS in the myocardial CS-eNOS-Tg samples, loading was at 1/5th that of WT lyaste (5 μg vs. 25 μg). Membranes were then reacted with HRP-linked anti-mouse secondary antibody (Amersham) at 1:2,000 in 5% BSA-TBST, incubated with ECL reagents (Amersham), and then exposed to film. Relative optical density was measured by using Quantity One software (Bio-Rad).

Assessment of Murine NOS mRNA Levels in the Heart and Liver. mRNA levels were assessed by using quantitative real-time RT-PCR (qPCR). Total RNA was extracted from organ homogenates by using RNeasy Protect Midi Kit (Qiagen) with DNase digestion. Purified RNA was quantified and cDNA was synthesized by using QuantiTect reverse transcriptase (Qiagen). QPCR was performed by using the DNA Master Plus SYBR Green I kit (Roche Applied Science) with a LightCycler thermocycler system (Roche Applied Science). All samples were probed by using primers for GAPDH, eNOS, iNOS, and nNOS in the same run. All data were assessed by using Lightcycler 4.0 software and evaluated by using the $\Delta\Delta$CT method with GAPDH correction.

Nitric Oxide Synthase Activity Assay. Heart and liver NOS activity was assessed with a commercially available kit (L-arginine to L-citrulline conversion assay (Cayman Chemical). Briefly, tissue was homogenized in buffer (250 mM Tris·HCl, pH 7.4; 10 mM EDTA; 10 mM EGTA) and centrifuged at 10,000 $\times$ *g* for 15 min at 4°C. An aliquot of the resulting supernatant was then incubated in a reaction mixture (50 mM Tris·HCl, pH 7.4, 0.006 mM tetrahydrobiopterin, 0.002 mM flavin adenine dinucleotide, 0.002 mM flavin adenine monucleotide, 10 μCi [^{3}H] arginine, 1.25 mM NADPH, and 0.75 mM $CaCl_2$) for 30 min at room temperature. The reaction was stopped with the addition of stop buffer (50 mM Hepes, pH 5.5 and 5 mM EDTA). Equilibrated resin was then added to the sample reactions, and the sample was added to a spin column and centrifuged to remove all arginine. NOS activity was then quantitated by measuring the radioactivity of the eluate by using a scintillation counter. Activity was expressed as nM citrulline per minute per milligram of tissue.

NOx Analysis of Cardiac and Hepatic Tissue and Plasma. Nitrite and nitrate concentrations were quantified by ion chromatography (ENO20 Analyzer, Eicom). Tissue nitroso compounds were quantified by using group-specific reductive denitrosation by iodine-iodide with subsequent detection of the liberated NO by using gas-phase chemiluminescence. S-nitrosothiol levels were detected by preincubation with 2% mercuric chloride followed by acidified sulfanilamide. Nitrite/nitroso levels were determined by the addition of acidified sulfanilamide alone. 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 described in detail (2).

Myocardial Ischemia/Reperfusion Protocol. All surgical procedures were performed by using aseptic techniques as described in detail (1). Briefly, mice were anesthetized via an i.p. injection of sodium pentobarbital (50 mg/kg) and ketamine (60 mg/kg). Mice were orally intubated with a PE-60 tube and ventilated with 100% oxygen by a rodent ventilator (HugoSachs, Minivent, Model 845) at a rate of 110 strokes/min, 230 μL tidal volume. A thoracotomy was performed, and the left coronary artery was visually identified and ligated with a 7-0 silk suture rendering the left ventricle ischemic.

Myocardial Infarct Size Determination. At 24 h of reperfusion, infarct size was determined as described in ref. 1. Briefly, Evan's blue dye (1 ml of a 5% solution) was injected into a carotid artery catheter to delineate the ischemic zone from the nonischemic zone. The heart was rapidly excised and cross-sectioned into (3, 4) 1-mm thick sections, which were then incubated in 1.0% 2,3,5-triphenyltetrazolium chloride for 5 min to demarcate the viable and nonviable myocardium within the risk zone. Images of each side of every 1 mm heart section were acquired and weighed. The areas of infarction, risk, and nonischemic left ventricular were assessed in a blinded fashion by using computer-assisted planimetry (National Institutes of Health Image 1.57).

Hepatic Ischemia/Reperfusion Protocol. The hepatic ischemia/reperfusion protocol has been described (5). Mice were anesthetized with ketamine (100 mg/kg) and xylazine (8 mg/kg). The left lateral and median lobes of the liver were rendered ischemic by complete occlusion of the hepatic artery and the portal vein by using a microaneurysm clamp. The duration of hepatic ischemia was 60 min, and reperfusion time was 5 h. After this time, mice were completely exsanguinated, and serum samples were analyzed for alanine aminotransferase and aspartate aminotransferase by using a spectrophotometric method (Thermo Electron) and reported as units/liter. A subset of mice were allowed to reperfuse for 24 h at which time hepatic tissue was fixed and sectioned for H&E staining for the assessment of histology. In experiments evaluating the role of nitrosylation, vehicle, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (0.5 mg/kg), or *N*-ethylamide (2 mg/kg) was injected i.p. 30 min before ischemia. In subsequent experiments, WT mice were administered nitrite and S-nitrosoglutathione just before reperfusion via an i.v. injection (femoral vein) in a final volume of 100 μl.

Statistical Analysis. All values are reported as means ± SEM. Student's *t test* or one-way ANOVA with Tukey's post-hoc analysis was performed when appropriate by using JMP-IN software (SAS). Values >2 standard deviations outside the mean were considered outliers. Probability values <0.05 were considered statistically significant.

1. Bryan NS, *et al.* (2007) Dietary nitrite supplementation protects against myocardial ischemia-reperfusion injury. *Proc Natl Acad Sci USA* 104:19144–19149.
2. McKnight GM, *et al.* (1997) Chemical synthesis of nitric oxide in the stomach from dietary nitrate in humans. *Gut* 40:211–214.
3. Han TH, *et al.* (2003) Regulation of nitric oxide consumption by hypoxic red blood cells. *Proc Natl Acad Sci USA* 100:12504–12509.
4. Thomas DD, Liu X, Kantrow SP, Lancaster JR, Jr. (2001) The biological lifetime of nitric oxide: Implications for the perivascular dynamics of NO and O2. *Proc Natl Acad Sci USA* 98:355–360.
5. Cannon RO, III, *et al.* (2001) Effects of inhaled nitric oxide on regional blood flow are consistent with intravascular nitric oxide delivery. *J Clin Invest* 108:279–287.

Table S1. Myocardial murine NOS expression

		Myocardial RNA NOS Expression (fold Δ WT)		
Group	*n*	eNOS	iNOS	nNOS
WT	4	1.00 ± 0.03	1.00 ± 0.04	1.00 ± 0.19
CS-eNOS-Tg	4	0.80 ± 0.09	0.91 ± 0.07	0.97 ± 0.18

Myocardial RNA was isolated and purified from WT and cardiac-specific eNOS transgenic (CS-eNOS-Tg) mice. Real-time PCR (qPCR) analysis revealed no difference in all murine nitric oxide synthase (NOS) isoforms eNOS, iNOS, or nNOS message, corrected by the housekeeping gene GAPDH, in CS-eNOS-Tg mice compared with WT littermates. The ΔΔCT method was used for all qPCR analyses and data expressed as mean ± SEM fold-change from WT.

Table S2. Hepatic murine NO synthase RNA expression (fold Δ WT)

Group	*n*	eNOS	iNOS	nNOS
WT	5	1.00 ± 0.14	1.00 ± 0.24	1.00 ± 0.18
CS-eNOS-Tg	4	0.96 ± 0.09	1.07 ± 0.31	0.97 ± 0.26

Hepatic RNA was isolated and purified from WT and cardiac-specific eNOS transgenic (CS-eNOS-Tg) mice. Real-time PCR (qPCR) analysis revealed no differences in all murine NOS isoforms eNOS, iNOS, or nNOS message, corrected by the housekeeping gene GAPDH. The ΔΔCT analysis method was used for all qPCR analyses.