Supporting Information (SI)

Depletion of NADP(H) Due to CD38 Activation Triggers Endothelial Dysfunction in the Postischemic Heart

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Supporting Methods:

Detection of pyridine nucleotides by HPLC in whole hearts. Hearts were homogenized in ice-cold buffer consisting of 0.2 M KCN, 0.06 M KOH and 1 mM DTPA. Reaction of KCN with oxidized pyridine nucleotides (NAD^+ and $NADP^+$) allowed for rapid addition of CN at the 4 position of the nicotinamide ring. To limit interactions with free metals in the homogenization buffer (e.g. iron from hemoglobin) DTPA was added to the mixture. Homogenates were centrifuged at 16,000 x g (2 times; 5 min) with chloroform. The KOH supernatant was collected and further purified using Costar Spin-X 0.45 µm pore size. Just prior to injecting effluents onto the TSKgel column ODS-80TM (25 cm x 4.6 m) (Supelco, St. Louis, MO), the effluents were first diluted 1:5 with mobile phase (0.2 mM Ammonium Acetate/ 4 % Methanol, pH 5.8). This dilution destabilizes the KCN-$NAD(P)^+$ addition, however it is necessary to accommodate the operating pH of the columns. Errors from increased pH can be minimized through rapid injection onto the column. Separation was achieved with a flow rate of 1.0 mL/min and methanol gradient (0.2% per minute for 25 minutes). Analytes were detected via fluorescence spectroscopy (wavelength ex. 330 nm; em. 460 nm).

HPLC Measurement of 2'-P-ADPR. Hearts were homogenized using a Polytron tissue grinder (Brinkmann Instruments, Westbury, NY) (5x; 10sec) in ice-cold buffer (HEPES 20 mM; pH 7.3, Sucrose 250 mM, EDTA 0.1 mM, β-Mercaptoethanol (β-ME) 2.5 mM), after which, samples were kept on ice for 30 min for protein solubilization. Homogenates of injured and non-injured isolated hearts were first run through a HiTrap™ desalting column removing all substrates of the enzyme, then 25 µL of the homogenate was added to the reaction mixture (Tris buffer 50 mM; pH 7.4 with 100 µM $NADP^+$), and allowed to run for 30 minutes in 37˚C water bath (Fisher Scientific, Pittsburgh, PA). Upon reaction completion, samples were quickly removed and centrifuged for 3 minutes using Costar Spin-X 0.45 µm pore size, separating enzyme from reaction substrates/products which pass through membrane. The effluent from Costar Spin-X tubes was diluted 1:3 with mobile phase (0.04 M Sodium Phosphate/1% methanol; pH 7.0) and injected onto a TSKgel column ODS-80TM (25 cm x 4.6 mm) (Supelco, St. Louis, MO). Separation was achieved with a flow rate of 1.0 mL/min with buffer A (0.04M Sodium Phosphate) and buffer B (0.04M Sodium Phosphate/Methanol 1:1) gradient as follows: 1 min, 0%; 2.5 min, 0.5%; 5 min, 3%; 7 min, 5%; 8 min, 12%; 10 min, 20%; 12 min, 30%; 20 min, 50%. 2'-P-ADPR and other analytes of interest were detected via UV/Vis detection (ESA model 520, Chelmsford, MA) at wavelength 254 nm. Standards for 2'-P-ADPR were prepared by overnight incubation of 100 µM $NADP^+$ with ADP-ribosyl cyclase from *Californica apylsia* in a 50 mM phosphate buffer (pH 7.4). The reaction was followed with HPLC injection (as described above) every 4 hours until $NADP^+$ reached nominal levels and reaction was complete.

Heart tissue homogenate preparation for NOS activity measurements. Excised hearts were immediately frozen in liquid nitrogen, finely ground while still frozen with a mortar and pestle, and suspended in 3 ml of ice-cold homogenizing buffer consisting of 50 mM Tris, pH 7.4, containing 0.1 mM EDTA, 0.1 mM EGTA, 2.5 mM β-mercaptoethanol, and the protease inhibitors 2 mM phenylmethylsulfonylfluoride and 4 μM leupeptin. The suspension was homogenized and centrifuged at 100,000 *g* for 60 min at 5°C. The supernatant was discarded and the particulate fraction washed in 3 ml of ice-cold homogenizing buffer containing 1 M KCl for 5 min. The homogenate was then centrifuged at 100,000 *g* for 30 min at 5°C. The supernatant was discarded and the pellet rinsed several times with Tris buffer to remove excess KCl. Finally, the pellet was resuspended in homogenizing buffer containing calmodulin (330 nM) and tetrahydrobiopterin (10 μM). Calmodulin and tetrahydrobiopterin were added to the supernatant to achieve the same concentrations as the pellet.

Measurement of NOS activity. NOS activity was measured from the conversion rate of L-[^{14}C]arginine to L-[^{14}C]citrulline in heart tissue subcellular preparations in a manner similar to that previously reported (1-3). The reaction mixture contained either no additional NADPH or 1.0 mM NADPH, 200 μM $CaCl_2$, 30 μM EDTA, 30 μM EGTA, 100 nM calmodulin, and 3 μM tetrahydrobiopterin in Tris buffer. The reaction was initiated by the addition of purified L-[^{14}C]arginine (317 mCi/mmol) to produce a 10 μM final concentration and carried out for 8 min at 37 °C. The reaction was quenched with 3 ml of ice-cold stop buffer (20 mM HEPES and 2 mM EDTA, pH 5.5). Experiments were also performed in the presence of L-NAME (1 mM). L-[^{14}C]Citrulline content was determined by liquid scintillation counting after separation from the reaction mixture by passage through a column of the cation exchange resin Dowex AG 50WX-8 (500 μl of the Na^+ form). Samples of buffer containing L-[^{14}C]arginine in the absence of heart tissue were added to the Dowex resin column to determine background counts, which were subtracted from all other measurements. eNOS activity was determined by subtracting total counts from L-NAME inhibited counts and normalized for protein content (measured by the Bradford method) and conversion time.

EPR spin trapping of NO and $O_2^{\bullet-}$. Spin trapping measurements of NO formation from the heart were performed using the Fe^{2+}-*N*-methyl-D-glucamine (Fe-MGD) complex. Fresh stock solutions of Fe-MGD (1:5) were prepared by adding ferrous sulfate (0.5 mM) to aqueous solutions of MGD (2.5 mM) in perfusion buffer with 10 mg/ml bovine serum albumin. Sidearms in the perfusion line allowed direct infusion of perfusion buffer containing the spin trap and acetylcholine. Acetylcholine 100 nmoles were administered as a bolus, and then after onset of vasodilation spin trap solution was infused by a Harvard pump at 1.6 mL/min (~ 1/10 of the basal coronary flow), delivering 0.5 mM Fe-MGD. EPR spectra of NO trapped by Fe-MGD were recorded at room temperature with a Bruker EMX spectrometer operating at X-band, ~9.77 GHz, with 100-kHz modulation frequency and a HS resonator, with a microwave power of 40 mW and a modulation amplitude 3.0 G. Spin trap containing effluent was collected every 15 s for 2 minutes. The effluent samples were immediately measured or frozen in liquid nitrogen and thawed for EPR measurement. Spin trapping measurements of $O_2^{\bullet-}$ were performed using 50 mM DMPO in perfusate immediately prepared and infused by a Harvard pump at 1.6 mL/min.

The DMPO-infused samples were collected into tubes containing methyl-β-cyclodextrin (final concentration of 100 mM) to enhance spin adduct stability. EPR measurements were performed in a quartz flat cell and spectra obtained at room temperature at a microwave frequency of 9.77 GHz using 20 mW of microwave power and 1.0 G modulation amplitude. For both sets of measurements, pre-ischemic control and I/R with and without pre-ischemic α–NAD treatment were performed. In the DMPO measurements, the effects of L-NAME and SOD1 were also measured, and the SOD1 spectra were used for background subtraction from all other DMPO spectra. L-NAME was administered at 1 mM concentration and SOD1 at 200 U/mL.

Stable Knockdown of CD38 in RAECs. Lipofectamine 2000 (Invitrogen Corporation, CA) was added to serum and antibiotic-free opti-MEM medium and incubated at room temperature. 2 μg of CD38 shRNA plasmid or empty plasmid were mixed with the Lipofectamine-medium mixture, incubated at room temperature and added to a culture well containing exponentially growing RAECs in serum and antibiotic-free opti-MEM medium. RAECs were incubated at 37°C in a 5% CO_2 humidified incubator for 7 hours and the serum and antibiotic-free opti-MEM medium was changed to growth medium containing 20% serum. At 48 hours post transfection, selection medium containing puromycin was added and cells allowed to grow and expand. CD38 expression in CD38-transfected or empty plasmid control RAECs was evaluated by Western blotting.

H/R Model. RAECs were washed with PBS and kept in serum-free DMEM in a hypoxic environment created by placing the flasks containing cells at confluence into a Billups-Rothenberg incubator chamber flushed with a 95 % N_2/5 % CO_2 gas. The O_2 level was monitored with an O_2 electrode placed inside the incubator chamber interfaced to an Apollo 4000 control unit (World Precision Instruments, Inc., Sarasota, FL). Cells were kept inside the chamber at 37 °C for 24 h with an O_2 concentration in the medium of ~4 Torr, followed by reoxygenation for 1 h by replacing the hypoxic medium with normoxic PBS with calcium, magnesium, 1 g/L d-glucose, and 36 mg/L sodium pyruvate. Cell viability was checked by trypan blue exclusion. Cells were stained with 0.02% trypan blue in PBS, and cell counts were performed with a Zeiss Laboratory light microscope. H/R of cultured endothelial cells is commonly used as a mimic of I/R (4).

Isolated heart studies in rats and mice. Isolated hearts from male, Sprague-Dawley rats (275-300 grams), or C57BL/6J mice (WT and $eNOS^{-/-}$) age 6 to 8 months were prepared as described previously (10). $eNOS^{-/-}$ mice, strain B6.129P2-*Nos3*^{tm1Unc}/J, were purchased from The Jackson Laboratory. Hearts were excised, aorta cannulated, and perfused retrograde with Krebs buffer (119 mM NaCl, 17 mM Glucose, 25 mM $NaHCO_3$, 5.9 mM KCl, 1.2 mM $MgCl_2$, 2.5mM $CaCl_2$, 0.5 mM EDTA, and 2 mM pyruvate (mice only)). A balloon connected to a pressure transducer was placed in the left ventricle (LV) to measure contractile function. Basal values of contractile function in mouse hearts expressed as mean ± SE were 101.4 ± 3.1 mmHg for LV developed pressure (LVDP), 3089 ± 109 mmHg/sec for dP/dt, 369.1 ± 9.1 beats/ min for heart rate, and 37.40 ± 1.39 x 10^3mmHg/min for rate pressure product with similar values in WT and $eNOS^{-/-}$ hearts.

An in-line flow probe and flowmeter (Transonic, Ithaca, NY) were used to measured coronary flow (CF).

Myocardial infarction was measured by 2,3,5-triphenyltetrazolium chloride (TTC; Sigma) staining of heart sections, as reported previously (5). Briefly, the heart was immediately removed after I/R, wrapped in polyethylene wrap, and frozen for 10 min for hardening. Then the heart was serially sectioned into transverse slices (1 mm) by a heart slicer (Zivic Laboratories) and was incubated in 1.5% TTC in PBS for 15 min at room temperature to demarcate the viable (brick red) and infarcted (pale) myocardium. Heart slices were then fixed overnight in 10% neutral-buffered formaldehyde for better color contrast and were digitally imaged. Computerized planimetry (with image-analysis software Metamorph, v. 6.0) of each section was used to determine percent infarction from the total cross-sectional area of the LV.

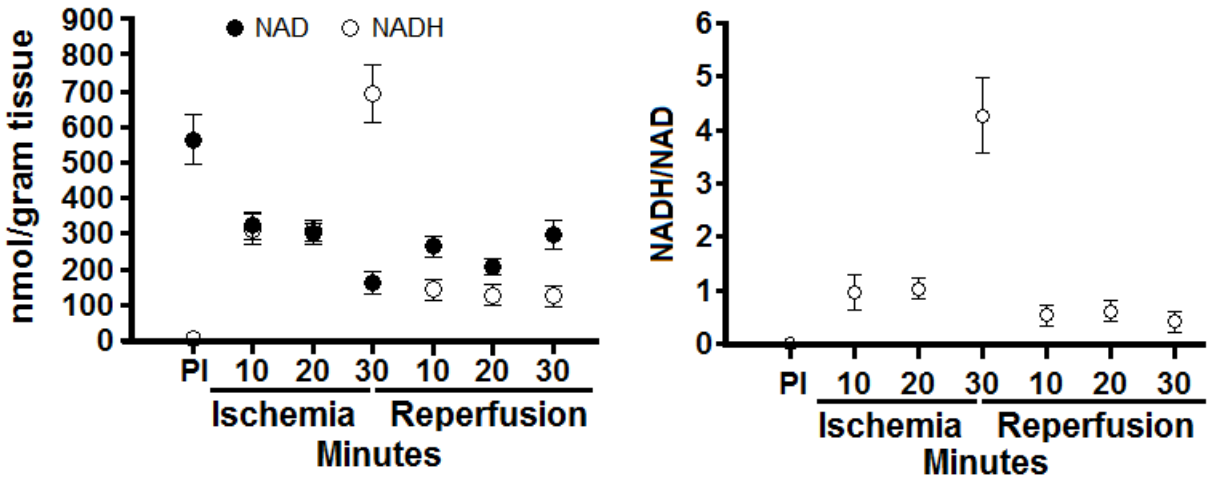

Figure S1. Whole heart levels of NAD(H) during I/R. (Left) Levels of the non-phosphorylated pyridine nucleotides are predominately in the oxidized form in the non-injured heart. Levels of NAD^+ are depleted during ischemia ~50%; however, there is a concurrent rise in NADH levels, suggesting interconversion within the pool. During reperfusion, levels of NAD(H) return toward, but never reach pre-ischemic levels. (Right) During ischemia there is a large shift towards the reduced form; however, this is not sustained upon reperfusion (mean ± SEM; n = 5-7).

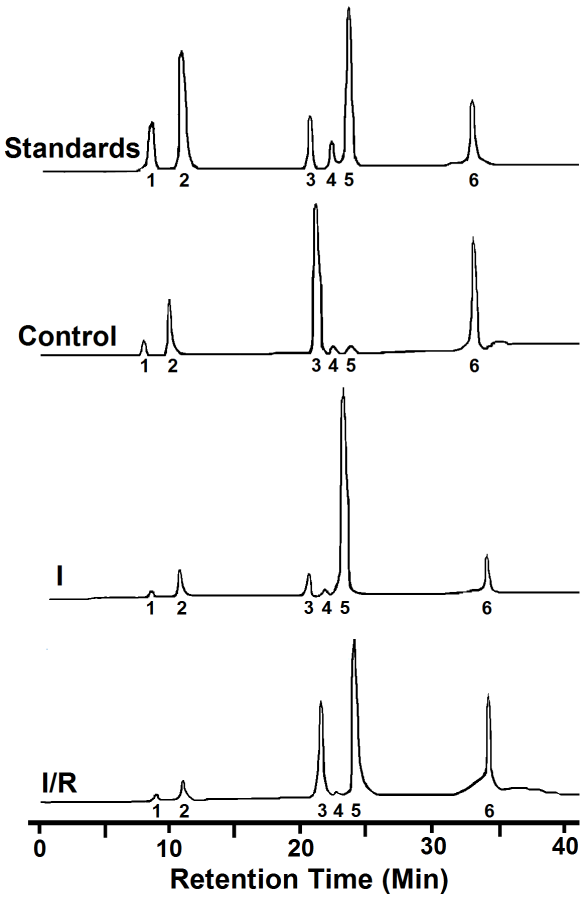

Figure S2. Complete chromatograms for pyridine nucleotides in standard and heart samples. Samples were reacted with KCN which resulted in the addition of CN to the oxidized pyridine nucleotides. Fluorescence for all pyridine nucleotides could be detected at the wavelength ex. 330; em. 460. Peaks 2 and 5 correspond to NADPH and NADH, respectively. Peaks 1 and 3 correspond to $NADP^+$; a doublet peak is due to the addition of CN in the ortho/para position on the nicotinamide ring. Peaks 4 and 6 correspond to NAD^+. Again the peak is a doublet due to CN addition. Standards are 25 μM for all pyridine nucleotides. Control- normal perfused heart, I- 30 min ischemia, and I/R- 30 min ischemia/30 min reperfusion.

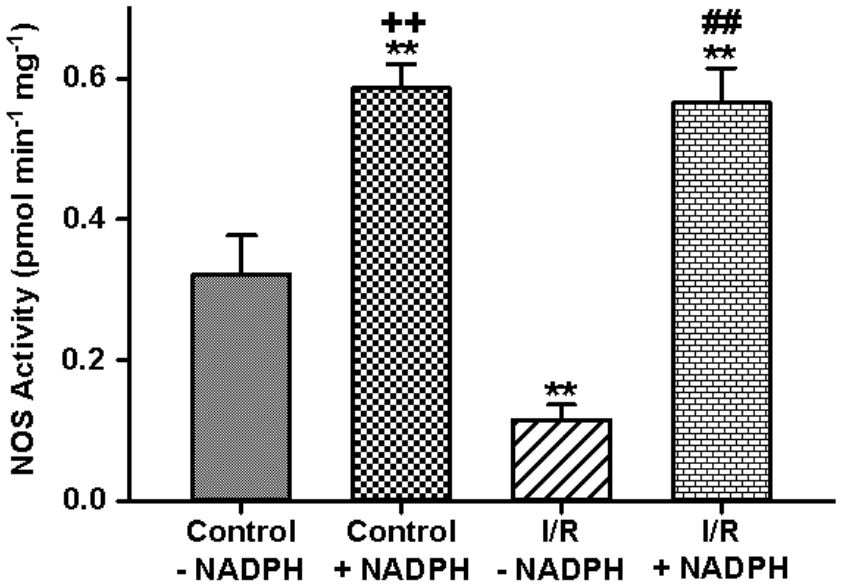

Figure S3. Effect of ischemia and reperfusion (I/R) on eNOS activity in the heart. Hearts were homogenized at the end of control perfusion (Control) or after 30 min ischemia and 30 min of reperfusion (I/R), and eNOS activity was measured by L-[^{14}C]arginine to L-[^{14}C]citrulline conversion. eNOS activity without NADPH added is significantly lower in hearts undergoing I/R compared to control hearts. In I/R hearts, NADPH addition greatly increased eNOS activity, indicating that NADPH limits eNOS activity. A lesser increase was also seen in non-ischemic control. **P<0.01 versus Control-NADPH, ++P<0.01 versus Control-NADPH, ##P<0.001 versus I/R-NADPH (mean ± SEM; n = 5-7).

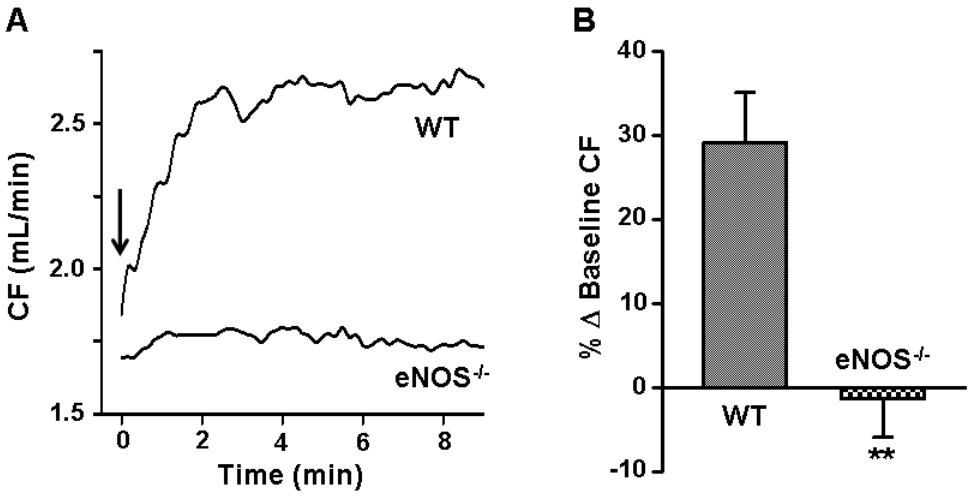

Figure S4. Effect of NADPH repletion on coronary flow (CF) in WT and eNOS$^{-/-}$ mouse hearts after I/R. A. Representative tracings showing change in coronary flow after infusion of 175 μM liposomal NADPH with start of administration at time denoted by arrow. **B.** Graph of the mean changes in CF. While in WT hearts, NADPH elicited a dramatic increase in CF, in eNOS$^{-/-}$ hearts, there was no significant increase in CF. Thus, the increase in CF was shown to be eNOS-dependent. **P<0.01 versus WT (mean ± SEM; n = 4).

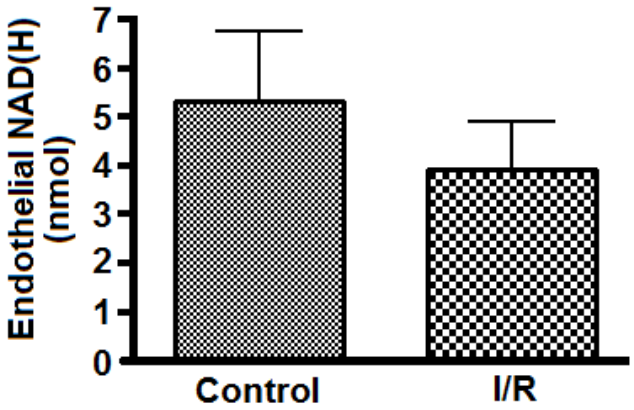

Figure S5. Endothelial NAD(H) levels. Levels of endothelial NAD(H) trended downwards after I/R in Triton X-100 treated isolated hearts. However, this depletion is not as severe when compared to the phosphorylated pool (mean ± SEM; n = 5-7).

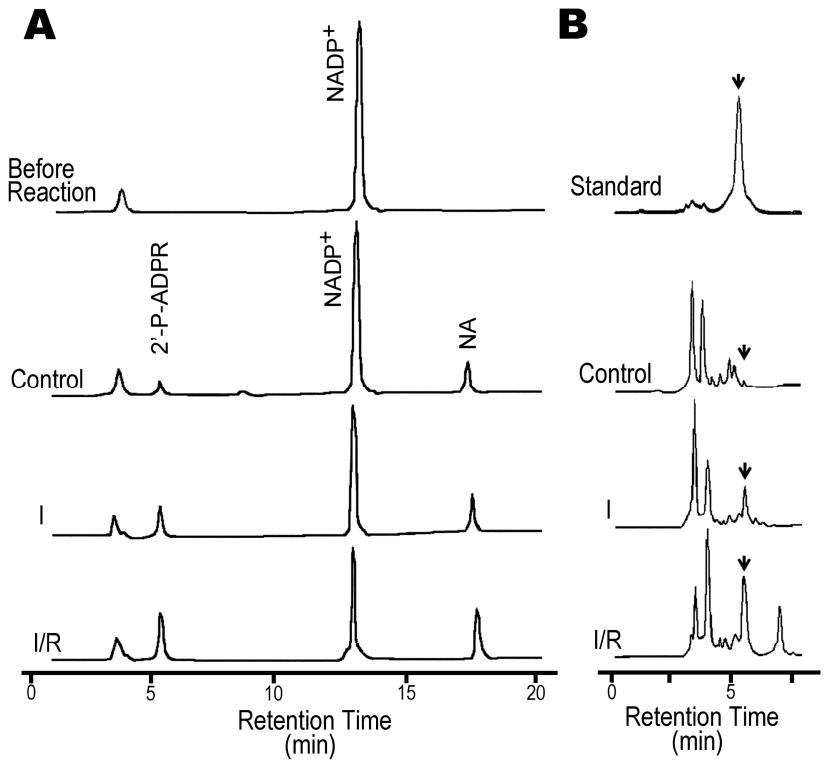

Figure S6. Chromatograms of CD38 activity, showing 2'-P-ADPR, $NADP^+$ and Nicotinamide (NA). A. As $NADP^+$ is consumed by CD38, both NA and 2'-P-ADPR increase. **B.** Chromatograms of 2'-P-ADPR (denoted by arrow ↓), a product of CD38 activity, when $NADP^+$ is used as a substrate (observed with UV/VIS detection at 254 nm). Measurements were performed in control hearts (Control), hearts subjected to 30 min ischemia (I), or 30 min ischemia and 30 min reperfusion (I/R).

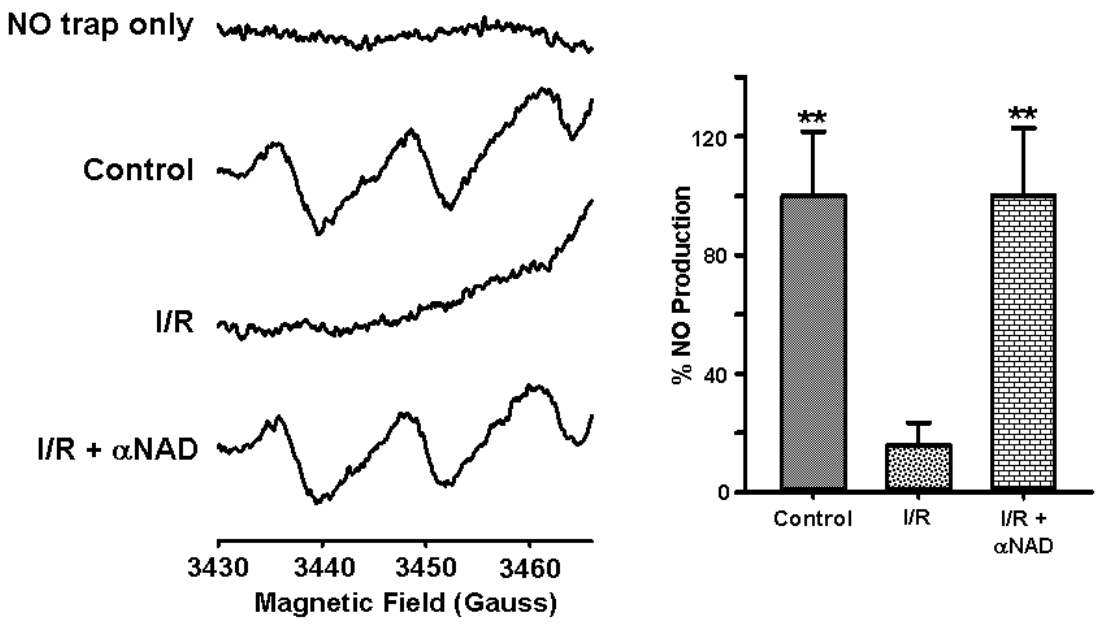

Figure S7. EPR spin trapping measurements of NO. Hearts were perfused with NO spin-trap Fe-MGD (0.5 mM) and coronary effluent was collected prior to the onset of I/R (Control) and after 30 min ischemia and 30 min reflow (I/R) with and without pre-ischemic treatment with α–NAD. Measurements of NO were made in the effluent. NO was greatly decreased following I/R, but pre-ischemic treatment with α–NAD restored NO production to control levels. EPR spectra of NO trapped by Fe-MGD were recorded at room temperature with a Bruker EMX spectrometer operating at X-band, 9.77 GHz, with 100-kHz modulation frequency and a HS resonator, with a microwave power of 40 mW and a modulation amplitude 3.0 G. **P<0.01 versus I/R (mean ± SEM; n = 5-7).

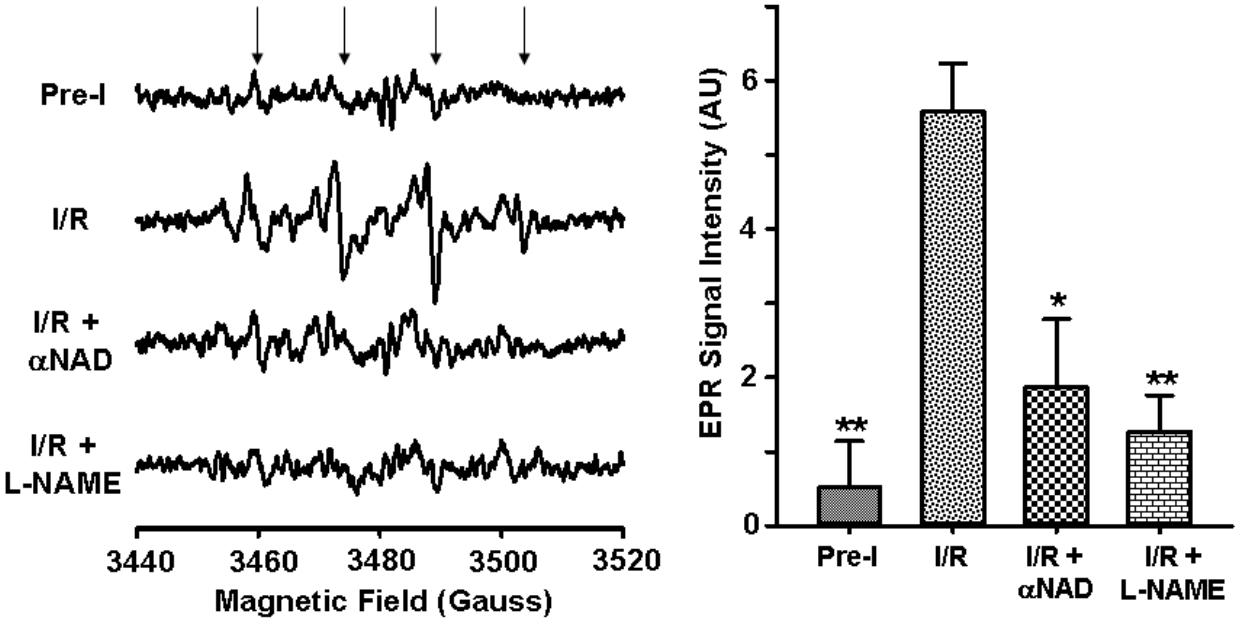

Figure S8. EPR spin trapping experiments of $O_2^{\bullet-}$. Hearts were perfused with $O_2^{\bullet-}$ spin-trap DMPO (50 mM) and coronary effluent was collected under non-ischemic conditions (Control) and after I/R with and without pre-ischemic treatment with α–NAD. Levels of $O_2^{\bullet-}$ were increased after I/R compared to pre-ischemic control (Pre-I), and this increase was blunted both by α–NAD inhibition of CD38, and by L-NAME inhibition of NOS. Spectra were obtained at room temperature at a microwave frequency of 9.77 GHz using 20 mW of microwave power and 1.0 G modulation amplitude. *P<0.05; **P<0.01 versus I/R (mean ± SEM; n = 5-7). The arrows show the magnetic field location of the 4 peaks of the DMPO-OH adduct derived from $O_2^{\bullet-}$.

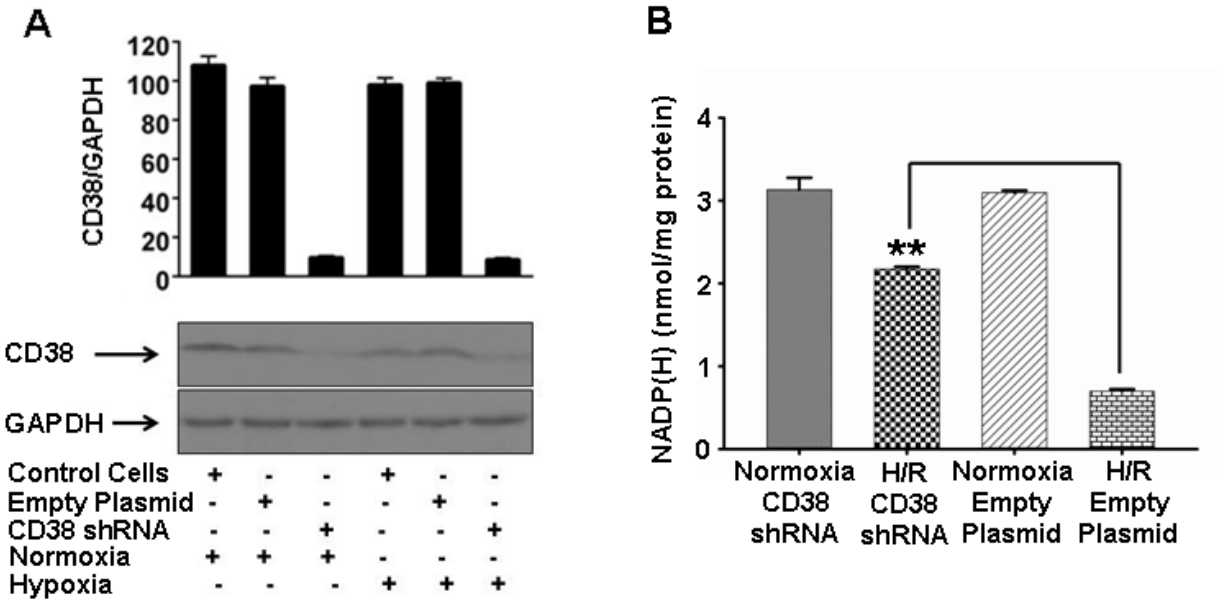

Figure S9. CD38 knockdown by shRNA protects NADP(H) levels in endothelial cells subjected to hypoxia/reoxygenation (H/R). H/R treatment significantly depleted the levels of NADP(H) compared to normoxic treatment in cultured RAECs. To test the contribution of CD38 activation to this depletion, RAECs were given CD38 shRNA to knockdown CD38 expression. With CD38 knockdown, NADP(H) was protected after H/R treatment compared to cells without CD38 knockdown. **P<0.01 versus H/R Empty Plasmid (mean ± SEM; n = 4).

References

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