Online Data supplement

Primary Culture of Neonatal Rat Ventricular Myocytes

Primary cultures of ventricular cardiac myocytes were prepared from 1-day-old Crl:(WI) BR-Wistar rats (Harlan). A cardiac myocyte-rich fraction was obtained by centrifugation through a discontinuous Percoll gradient as described.[1]

Ischemia/Reperfusion in Vivo

Mice were housed in a temperature-controlled environment with 12 h light/dark cycles where they received food and water *ad libitum*. Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). A rodent ventilator (model 683; Harvard Apparatus Inc) was used with 65% oxygen. The animals were kept warm with heat lamps. Rectal temperature was monitored and maintained between 36°C and 37°C. The chest was opened by a horizontal incision at the third intercostal space. I/R was achieved by ligating the anterior descending branch of the left coronary artery with an 8-0 Prolene suture, with silicon tubing (1 mm outer diameter) placed on top of the left anterior descending coronary artery, 2 mm below the border between the left atrium and left ventricle (LV). Ischemia was confirmed by ECG change (ST elevation). After 30 min of occlusion, the silicon tubing was removed to achieve reperfusion, and the rib space and overlying muscles were closed. When recovered from anesthesia, the mice were extubated and returned to their cages. They were housed in a climate-controlled environment. Twenty-four hours after reperfusion, the animals were reanesthetized and intubated, and the chest was opened. After the heart was arrested at the diastolic phase by KCl injection, the ascending aorta was cannulated and perfused with saline to wash out blood. The left anterior descending coronary artery was occluded with the same suture, which had been left at the site of

the ligation. To demarcate the ischemic area at risk (AAR), Alcian blue dye (1%) was perfused into the aorta and coronary arteries. Hearts were excised, and LVs were sliced into 1-mm-thick cross sections. The heart sections were then incubated with a 1% triphenyltetrazolium chloride (TTC) solution at 37°C for 15 min. The infarct area (pale), the AAR (not blue), and the total LV area from both sides of each section were measured using Adobe Photoshop (Adobe Systems Inc), and the values obtained were averaged. The percentages of area of infarction and AAR of each section were multiplied by the weight of the section and then totaled from all sections. AAR/LV and infarct size/AAR were expressed as percentages.[2]

Langendorff-Perfused Mouse Heart Model of Global I/R

Mice were anesthetized with pentobarbital (65 mg/kg IP) and treated intraperitoneally with 50 U of heparin. The heart was quickly removed and catheterized with a 22-gauge needle. The hearts were mounted on a Langendorff-type isolated heart perfusion system and subjected to retrograde coronary artery reperfusion with 37°C oxygenated Krebs-Henseleit bicarbonate buffer (mmol/L: NaCl 120, glucose 17, $NaHCO_3$ 25, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2.5, EDTA 0.5), pH 7.4, at a constant pressure of 80 mm Hg. A balloon filled with water was introduced into the LV through the mitral valve orifice and connected to a pressure transducer via a plastic tube primed with water. LV pressures and LV dP/dt were recorded with a strip chart recorder (Astro-Med Inc). The LV end-diastolic pressure was set at 4 to 10 mm Hg at the beginning of perfusion by adjusting the volume of the balloon in the LV, and the volume was kept constant throughout the experiment. After a 30 min equilibration period, the heart was subjected to 30 min of global ischemia (at 37°C) followed by 60 min of reperfusion.[3]

Evaluation of Apoptosis in Tissue Sections

DNA fragmentation was detected *in situ* with the use of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), as described. Nuclear density was determined by manual counting of DAPI-stained nuclei in 6 fields for each animal with the 40x objective, and the number of TUNEL-positive nuclei was counted by examining the entire section with the same power objective.[3]

Capillary Density

Capillary density was evaluated as previously described.[4] Heart samples were embedded in OCT compound (Miles, Elkhart, IN) and snap-frozen in liquid nitrogen. Frozen sections (5 μm in thickness) were stained for alkaline phosphatase using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate, and then counterstained with eosin. The capillary density was calculated as capillaries/1000 μm^2.

Immunoblot Analysis

Heart homogenates and cardiac myocyte lysates were prepared in a RIPA lysis buffer containing 50 mmol/L Tris (pH7.5), 150 mmol/L NaCl, 1% IGEPAL CA-630, 0.1% SDS, 0.5% deoxycholic acid, 10 mmol/L $Na_4P_2O_7$, 5 mmol/L EDTA, 0.1 mmol/L Na_3VO_4, 1 mmol/L NaF, 0.5 mmol/L 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 0.5 μg/ml aprotinin, and 0.5 μg/ml leupeptin. Samples were subjected to SDS-PAGE. Proteins were transferred onto polyvinylidene fluoride microporous membranes (Bio Rad) and probed with primary antibodies. The antibodies used in this study include anti-Nox4, raised as previously described [5], and anti-Nox1, anti-Nox2, anti-Nox3, and anti-nitric oxide synthase antibodies (Abcam), anti-HIF-1α and anti-COX IV antibodies (Cell Signaling Technology), anti-PDK4

antibody (BD Biosciences), anti-PPARα and anti-MCAD antibodies (Cayman), anti-GLUT4 and Hexokinase antibodies (Santa Cruz Biotechnology Inc), anti-actinin and anti-tubulin antibodies (Sigma), and xanthine oxidase antibody (Novus).

Adenoviruses

Adenovirus vectors harboring Nox4 (Ad-Nox4), DN-Nox (Ad-DN-Nox), shRNA-Nox4 (Ad-sh-Nox4), or shRNA-Nox2 (Ad-sh-Nox2) were generated using the AdMax system (Microbix) as described [6]. In brief, the recombinant adenoviruses were generated in HEK293 cells by co-transfection with a cosmid (pBHGloxΔE1,3Cre) containing the adenovirus type 5 genome (devoid of E1 and E3) and pDC316, a shuttle vector, containing a gene of interest. Adenovirus vectors harboring LacZ (Ad-LacZ) and Scramble shRNA (Ad-sh-Scr) were used as controls. Adenovirus vector harboring shRNA-HIF-1α (Ad-sh-HIF-1α) was kindly gifted by Dr. M. Abdellatif (University of Medicine and Dentistry of New Jersey).

Viability of the Cells

Viability of the cells was measured by Cell Titer Blue (CTB) assays (Promega). In brief, cardiac myocytes (1 $X10^5$ per 100 μl) were seeded onto 96-well dishes. The cells were preincubated with the indicated adenovirus for 48 h. Viable cell numbers were measured before hypoxia and after 12 h of hypoxia (<0.3% O_2 and 5% CO_2, 37°C) followed by 1, 6, and 24 h of reoxygenation (21% O_2 and 5% CO_2, 37°C) under conditions of serum deprivation. The CTB assays were performed according to the supplier's protocol.

Luciferase Assay

Transfection of plasmids into myocytes was performed with Fugene 6 (Roche).

Luciferase activity was measured with a luciferase assay system (Promega). The method of *in vivo* reporter gene assays has been described.[7]

Fractionation

To isolate crude nuclear fractions, we used procedures previously described [8]. Briefly, cultured neonatal rat myocytes were suspended in hypotonic lysis buffer (10 mmol/L K-HEPES (pH 7.9), 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 1 mmol/L DTT, 0.2 mmol/L Na_3VO_4, 1% Protease Inhibitor Cocktail), incubated for 15 min on ice and homogenized. Whole cell lysates were centrifuged at 60x*g* for 5 min to collect unbroken cells. The supernatants were collected and the homogenization and centrifugation were repeated on the pellets. The total homogenates were centrifuged at 1,200x*g* to separate crude nuclei and unbroken cells (pellet) from cell membrane and cytosolic proteins (supernatant). The supernatants of the total homogenates were centrifuged at 3,500x*g* for 20 min to separate mitochondrial fractions (pelleted in tube) from cytosolic and microsomal fractions (supernatant). The supernatants were then further centrifuged at 100,000x*g* for 60 min to separate microsomal fractions (pelleted in tube) from cytosolic fractions (supernatant).

Lucigenin Chemiluminescence Assay

Mouse whole heart homogenates, or mitochondrial or microsomal fractions, were suspended in 200 μL of an assay buffer composed of 100 mmol/L potassium phosphate (pH 7.0), 10 μmol/L flavin adenine dinucleotide (FAD), 1 mmol/L NaN_3, and 1 mmol/L EGTA. After preincubation with 5 μmol/L lucigenin, NADH or NADPH was added to a final concentration of 500 μmol/L.[9] The chemiluminescence was continuously monitored using a luminometer. The

reaction was terminated by the addition of SOD (100 μg/mL) [6].

H_2O_2 Measurement

H_2O_2 production was measured with an Amplex Red H_2O_2 assay kit (Molecular Probes; Invitrogen) according to the manufacturer's instructions. In brief, left ventricular blocks (30–50 mg) were incubated with Amplex Red (100 μmol/L) and horseradish peroxidase (1 U/mL) for 30 min at 37°C in Krebs–HEPES buffer protected from light. The supernatant was then transferred to a 96-well plate, and absorbance was measured (560 nm). Background fluorescence, determined in a control reaction without sample, was subtracted from each value. H_2O_2 release was calculated using H_2O_2 standards and expressed as micromoles per milligram of dry tissue.

DHE Staining

After harvest, heart tissues were immediately embedded in an optimum cutting temperature (OCT) compound in ethanol–dry ice and stored at −80°C. Unfixed frozen samples were cut into 5 μm-thick sections and placed on glass slides. DHE (10 μmol/L) was applied to each tissue section, and then the sections were coverslipped. The slides were incubated in a light-protected humidified chamber at 37°C for 30 min. Ethidium fluorescence (excitation at 490 nm, emission at 610 nm) was examined by fluorescence microscopy.

Assays for Measurement of Oxidative Stress

Cardiac tissue homogenates were assessed for MDA+4HAE content (LPO-586; Oxis International Inc.) according to the manufacturer's instructions.

NADP/NADPH Ratio

NADP/NADPH ratio of lysates from mouse hearts was measured using the EnzyChrom NADP/NADPH Assay Kit according to the protocol of the manufacturer (ECNP-100, Bioassay Systems, Hayward, Calif).

Antioxidant Capacity

Antioxidant capacity of the lysates from mouse hearts was measured using an Antioxidant Assay Kit according to the protocol of the manufacturer (Cayman).

Measurement of ATP Content

ATP content was measured with an ATP Bioluminescent Assay kit (Sigma). In brief, the ischemic region of the mouse heart was suspended and homogenized with homogenate buffer (0.25 mol/L Sucrose, 10 mmol/L HEPES pH 7.8). After centrifuging at 10,000x*g* for 10 min, the supernatant was added to the ATP Assay Mix solution. Luciferase activity was monitored using a luminometer.

Evaluation of mitochondrial membrane potential

In order to evaluate mitochondrial membrane potential, staining of cultured cardiac myocytes with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanin iodide (JC-1) was conducted using MitoTP JC-1 (ImmunoChemistry Technologies), according to the manufacture's instructions.

Mitochondrial Swelling Assay

Fifty μg of isolated mouse heart mitochondria from from WT, cNox4 KO, or Nox2 KO

mice subjected to sham operation or I/R were suspended in a swelling buffer (250 mmol/L sucrose, 10 mmol/L MOPS, 5 μmol/L EGTA, 2 mmol/L $MgCl_2$, 5 mmol/L KH_2PO_4, 5 mmol/L pyruvate, and 5 mmol/L malate) and incubated with 150 μmol/L calcium chloride ($CaCl_2$) in a final volume of 200 μL in a 96-well plate for 20 min. Absorbance was read at 520 nm.

Myocardial Triglyceride Content

Myocardial triglyceride content was assayed as described.[10] Briefly, left ventricular tissues were homogenized with an ice-cold chloroform/methanol/water mixture (2:1:0.8) for 2 min. Additional chloroform and water were added to separate the organic and aqueous layers. After centrifugation, the aqueous layer was removed, the chloroform layer was decanted and the mixture was evaporated at 70°C. The residue was dissolved in 0.5 ml of isopropanol, and triglycerides were assayed with a triglyceride measurement kit from Sigma.

Oil Red O Staining

Left ventricular tissues were used for frozen sections and slides from the ischemic area were stained with oil red O staining as previously described.[10]

Quantitative RT-PCR

Total RNA was prepared from left ventricles or cultured cardiac myocytes using the RNeasy Fibrous Tissue Mini Kit (QIAGEN), and then cDNA was generated using M-MLV Reverse transcriptase (Promega). Real-time RT-PCR was performed using Maxima SYBR Green qPCR master mix (Fermentas). β-actin was used as an internal control. PCRs were carried out using the following oligonucleotide primers:

Pcg1a 5’-ATGAATGCAGCGGTCTTAGCACTC-3’

5’-TTGCTGTTGACAAATGCTCTTCGCTTTA-3’

Ppara 5’-CCTGGCCTTCTAAACATAGG-3’

5’-TCCCTGCTCTCGTGTATGGG-3’

Pdk4 5’-AGGTTATGGGACAGACGCTATCATCTACTT-3’

5’-GTTCTTCGGTTCCCTGCTTGGG-3’

Acox1 5’-ATGAATCCCGATCTGCGCAA-3’

5’-TTCTCGATTTCTCGACGGCG-3’

Cd36 5’-GAATCTGAAGAGACCTTACATTGTACC-3’

5’-CACTCCAATCCCAAGTAAGGCCAT-3’

Cpt1b 5’-TTTGGGAACCACATCCGCCAA-3’

5’-TTATGCCTGTGAGCTGGCCAC-3’

Mcad 5’-GAAGCTGASTGAGGGACGCCA-3’

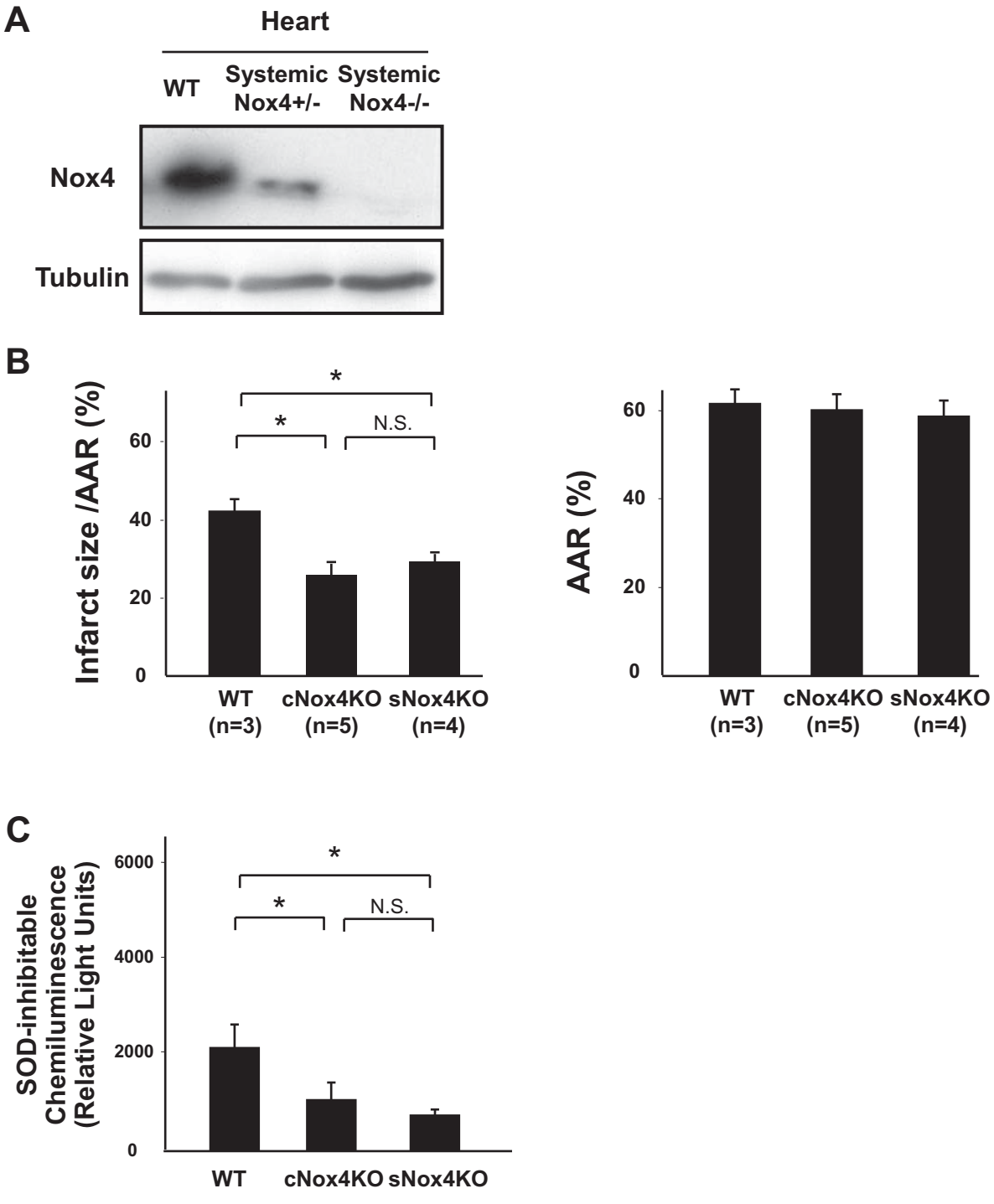
5’-GCTTGGAGCTTAGTTACACGAGG-3’

References

1. Morisco C, Zebrowski D, Condorelli G, Tsichlis P, Vatner SF, Sadoshima J. The akt-glycogen synthase kinase 3beta pathway regulates transcription of atrial natriuretic factor induced by beta-adrenergic receptor stimulation in cardiac myocytes. *J Biol Chem*. 2000;275:14466-14475
2. Yamamoto S, Yang G, Zablocki D, Liu J, Hong C, Kim SJ, Soler S, Odashima M, Thaisz J, Yehia G, Molina CA, Yatani A, Vatner DE, Vatner SF, Sadoshima J. Activation of mst1 causes dilated cardiomyopathy by stimulating apoptosis without compensatory ventricular myocyte hypertrophy. *J Clin Invest*. 2003;111:1463-1474
3. Hsu CP, Zhai P, Yamamoto T, Maejima Y, Matsushima S, Hariharan N, Shao D, Takagi H, Oka S, Sadoshima J. Silent information regulator 1 protects the heart from ischemia/reperfusion. *Circulation*. 2010;122:2170-2182
4. Kureishi Y, Luo Z, Shiojima I, Bialik A, Fulton D, Lefer DJ, Sessa WC, Walsh K. The hmg-coa reductase inhibitor simvastatin activates the protein kinase akt and promotes angiogenesis in normocholesterolemic animals. *Nat Med*. 2000;6:1004-1010
5. Ago T, Kuroda J, Pain J, Fu C, Li H, Sadoshima J. Upregulation of nox4 by hypertrophic stimuli promotes apoptosis and mitochondrial dysfunction in cardiac myocytes. *Circ Res*. 2010;106:1253-1264
6. Kuroda J, Ago T, Matsushima S, Zhai P, Schneider MD, Sadoshima J. Nadph oxidase 4 (nox4) is a major source of oxidative stress in the failing heart. *Proc Natl Acad Sci U S A*. 2010;107:15565-15570
7. Morisco C, Zebrowski DC, Vatner DE, Vatner SF, Sadoshima J. Beta-adrenergic cardiac hypertrophy is mediated primarily by the beta(1)-subtype in the rat heart. *J Mol Cell Cardiol*. 2001;33:561-573
8. Wright CD, Chen Q, Baye NL, Huang Y, Healy CL, Kasinathan S, O'Connell TD. Nuclear alpha1-adrenergic receptors signal activated erk localization to caveolae in adult cardiac myocytes. *Circ Res*. 2008;103:992-1000
9. Ago T, Kitazono T, Ooboshi H, Iyama T, Han YH, Takada J, Wakisaka M, Ibayashi S, Utsumi H, Iida M. Nox4 as the major catalytic component of an endothelial nad(p)h oxidase. *Circulation*. 2004;109:227-233
10. Cheng L, Ding G, Qin Q, Huang Y, Lewis W, He N, Evans RM, Schneider MD, Brako FA, Xiao Y, Chen YE, Yang Q. Cardiomyocyte-restricted peroxisome proliferator-activated

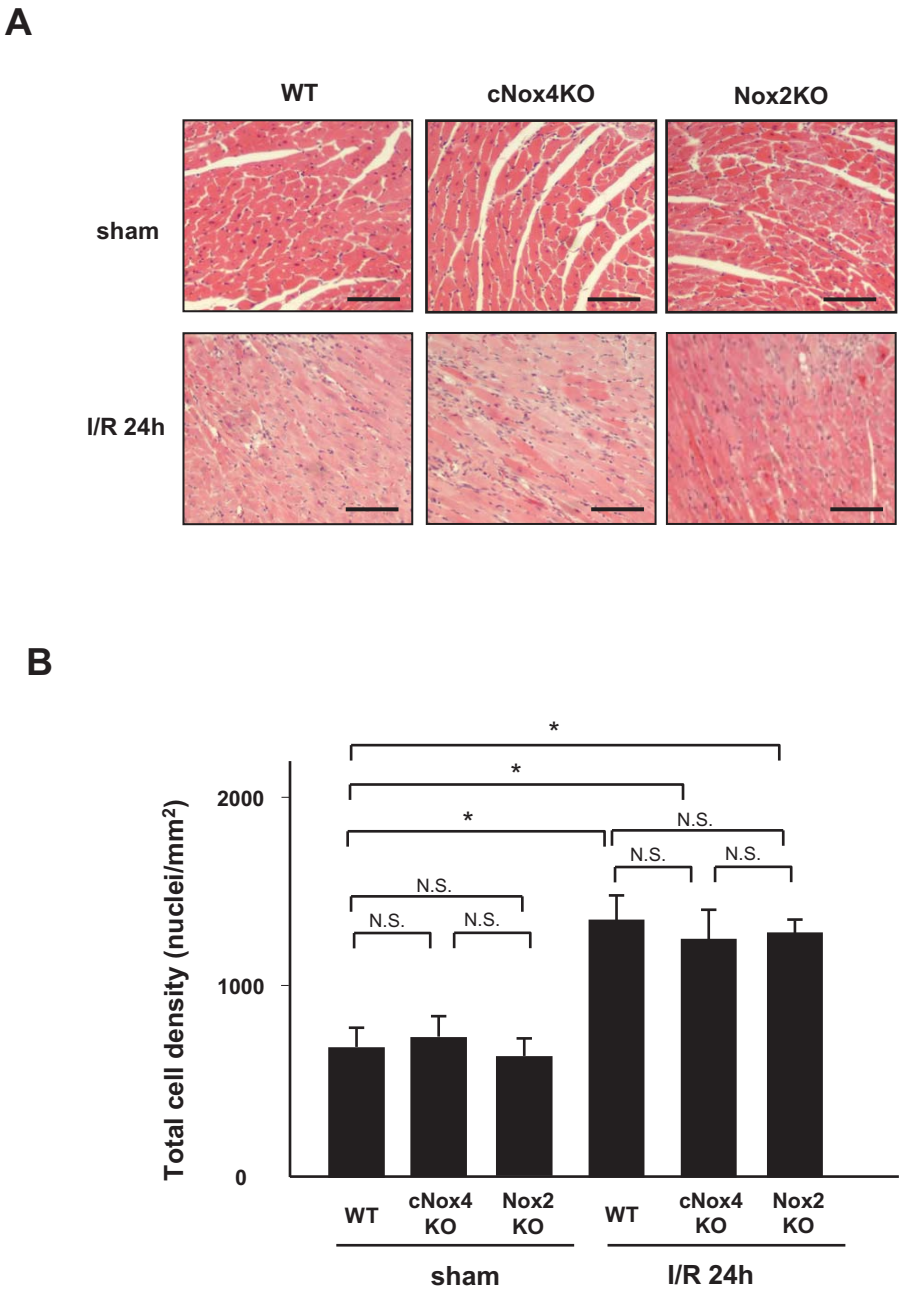
receptor-delta deletion perturbs myocardial fatty acid oxidation and leads to cardiomyopathy. *Nat Med*. 2004;10:1245-1250

Online Figure I



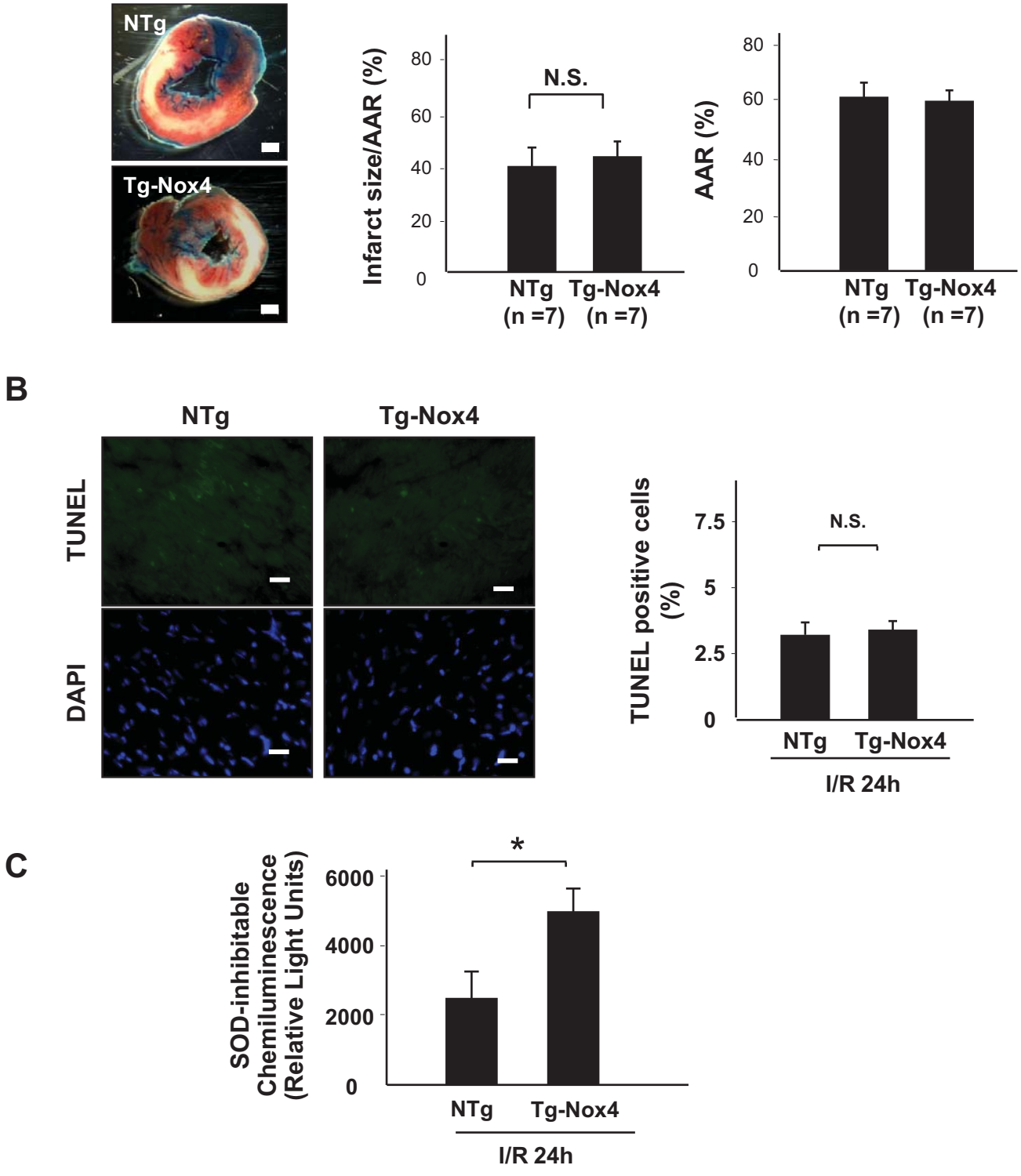
Online Figure I Systemic Nox4 KO inhibits I/R injury. **A.** Expression levels of Nox4 and tubulin in the LVs of WT and systemic Nox4 KO mice at baseline were evaluated by immunoblotting. **B.** WT, cardiac-specific Nox4 KO, and systemic Nox4 KO mice were subjected to ischemia (30 min) and reperfusion (24 h). Infarct size was evaluated by TTC and Alcian blue staining. *P<0.05, N.S. not significant. **C.** O_2^- production in LV sections from the indicated mice was evaluated by SOD-inhibitable chemiluminescence 24 h after reperfusion. *P<0.05, N.S. not significant.

Online Figure II



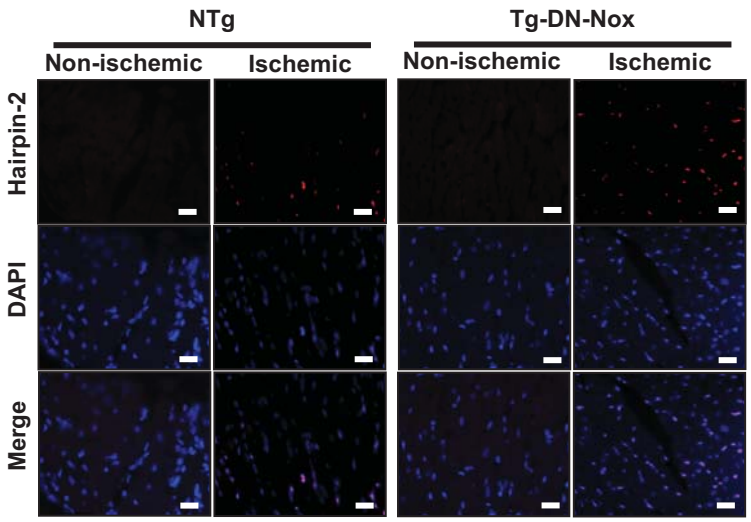
Online Figure II The cell density in the ischemic area after I/R. **A and B.** The cell density in the ischemic area was evaluated by HE staining. *P<0.05, N.S. not significant. Bar=30 μm.

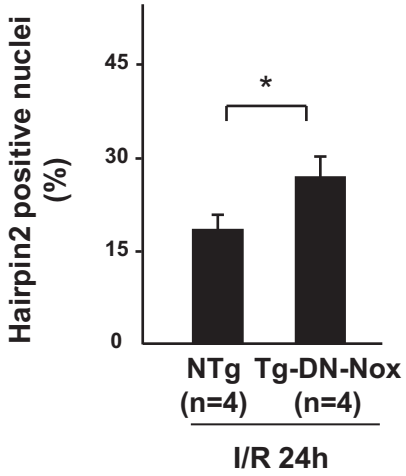
Online Figure III



Online Figure III Cardiac specific overexpression of Nox4 does not exacerbate I/R injury. **A.** NTg and Tg-Nox4 mice were subjected to ischemia (30 min) and reperfusion (24 h). Infarct size was evaluated by TTC and Alcian blue staining. N.S. not significant. Bar=1 mm. **B.** Apoptotic cells in the ischemic area were evaluated by TUNEL staining 24 h after reperfusion. N.S. not significant. Bar=10 μm. **C.** O_2^- production in LV sections was evaluated by SOD-inhibitable chemiluminescence 24 h after reperfusion. *P<0.05.

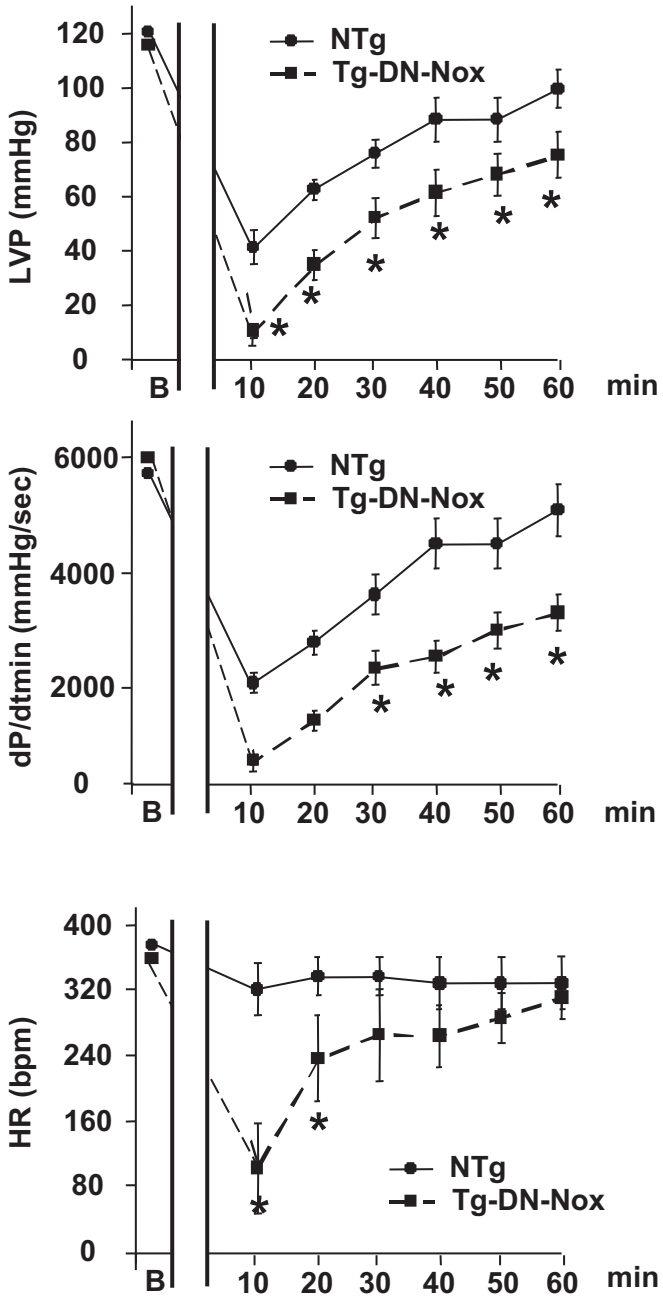
Online Figure IV





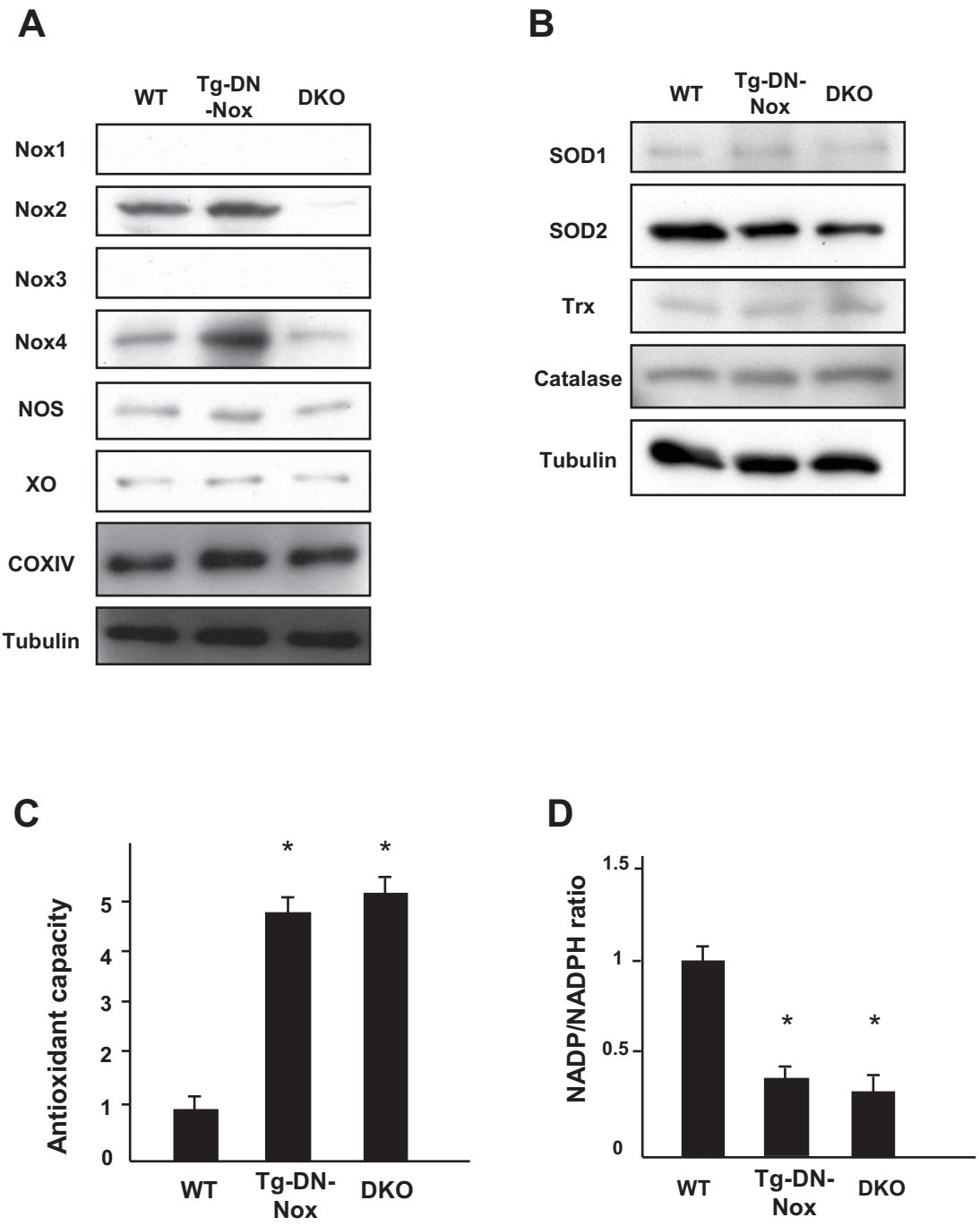
Online Figure IV Necrotic cells were evaluated by hairpin2 staining in NTg and Tg-DN-Nox mice. The number of hairpin2-positive nuclei in the ischemic area was increased in Tg-DN-Nox mice compared to in NTg mice. *P<0.05. Bar=10 μm.

Online Figure V



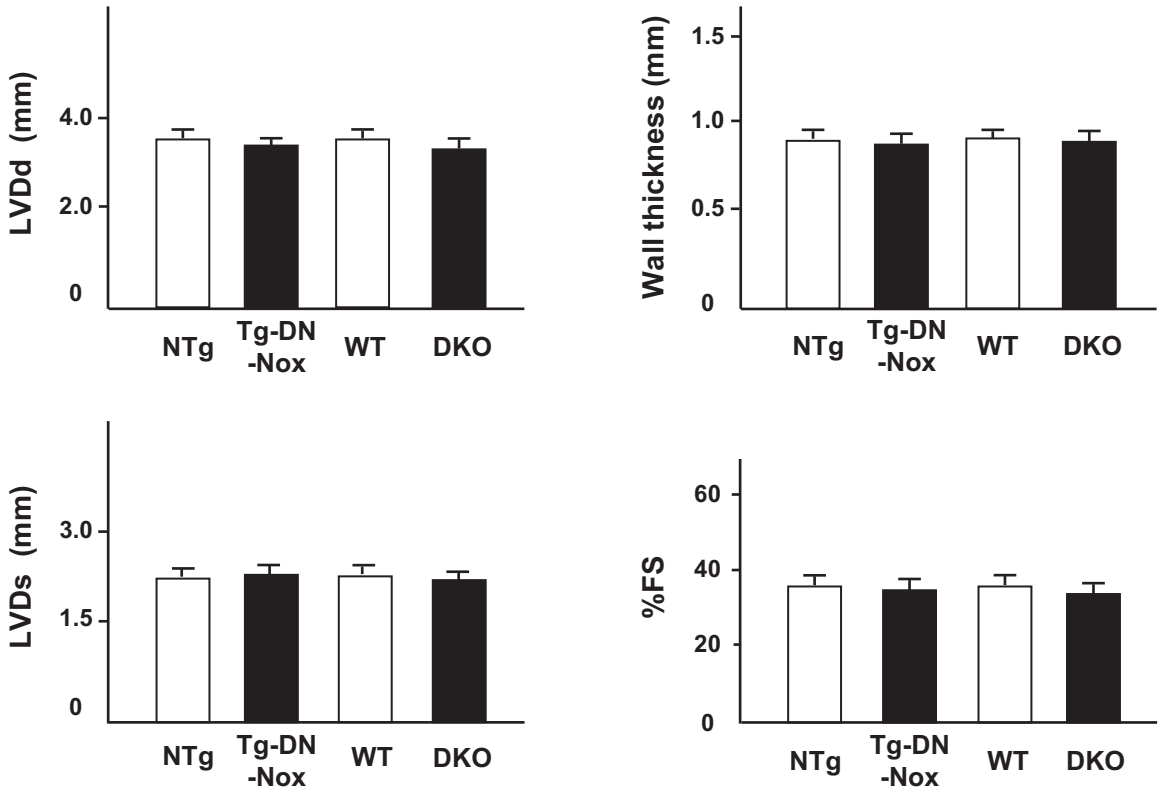
Online Figure V Functional recovery during reperfusion is attenuated in Tg-DN-Nox mouse hearts. Cardiac function in NTg and Tg-DN-Nox mice was evaluated by isolated perfused heart assay using the Langendorff system. *P<0.05 vs. NTg at same time point. LVP: left ventricular pressure, HR: Heart rate.

Online Figure VI



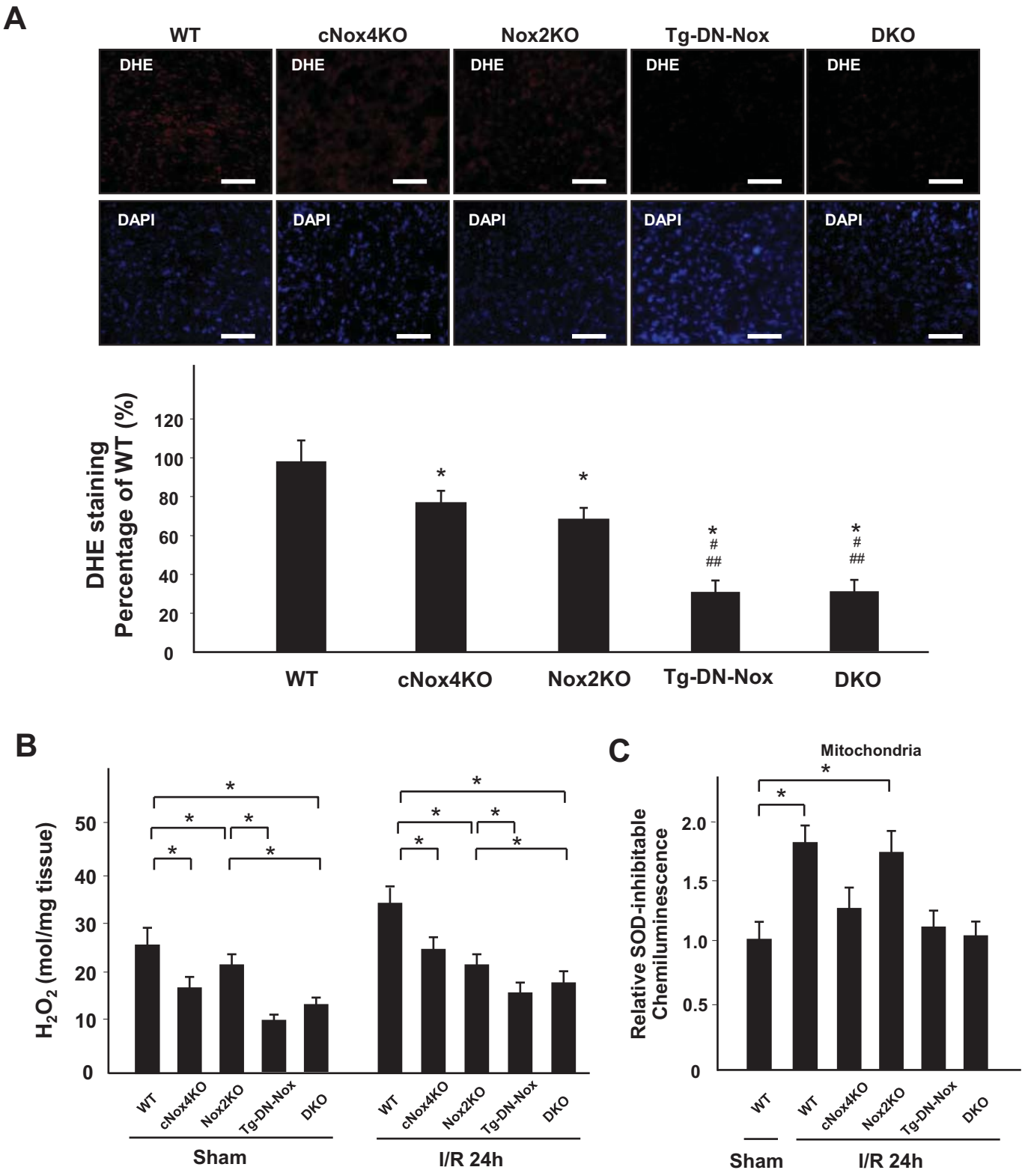
Online Figure VI The effect of broad suppression of Nox on the sources of ROS and antioxidants. **A.** Expression levels of Nox1, Nox2, Nox3, Nox4, NOS, XO, COX IV, and tubulin in the LVs of WT, Tg-DN-Nox, and DKO mice were evaluated by immunoblotting. **B.** Expression levels of SOD1, SOD2, Trx, catalase, and tubulin in the LVs of WT, Tg-DN-Nox, and DKO were evaluated by immunoblotting. **C.** Antioxidant capacity was measured in the LVs of WT, Tg-DN-Nox, and DKO. *P<0.05 vs. WT. **D.** NADP/NADPH ratio was measured in the LVs of WT, Tg-DN-Nox, and DKO mice. *P<0.05 vs WT.

Online Figure VII



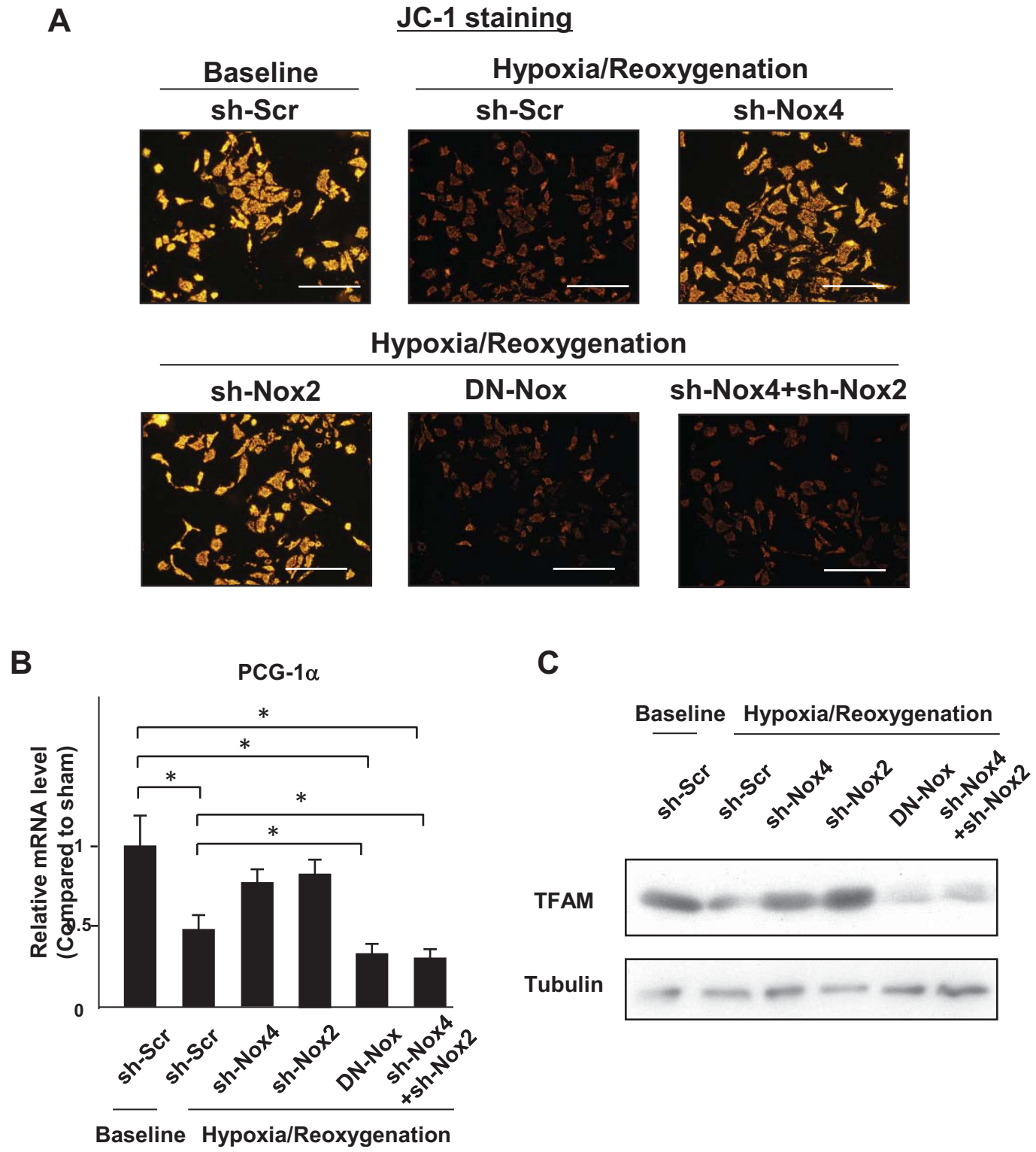
Online Figure VII Echocardiographic data at baseline in NTg, Tg-DN-Nox, WT, and DKO mice. LVDs: systolic left ventricular dimension, LVDd: diastolic left ventricular dimension, FS: Fractional shortening.

Online Figure VIII



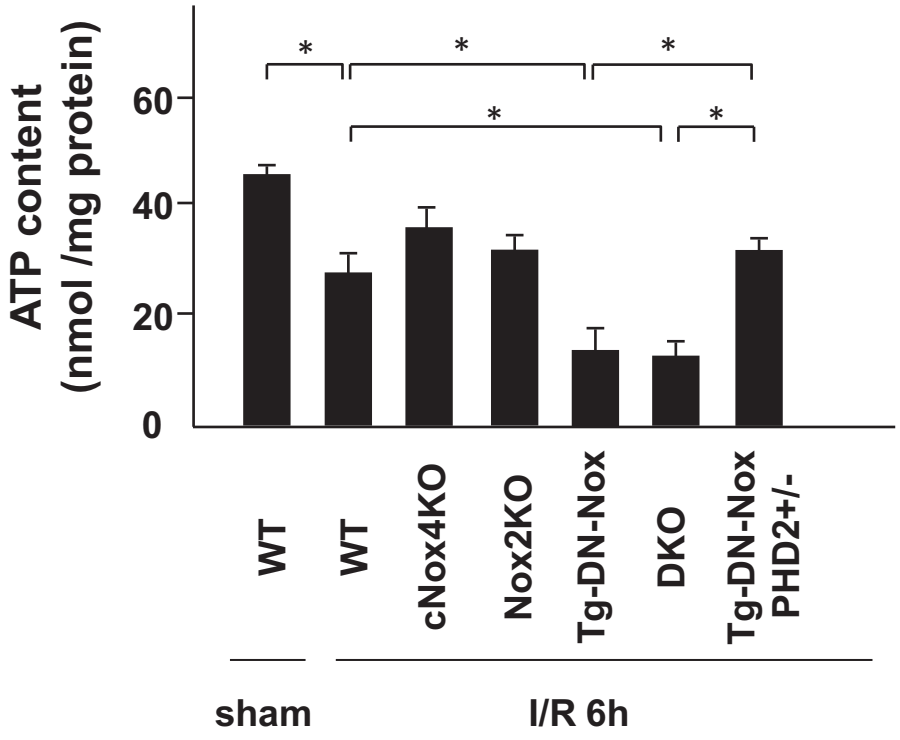
Online Figure VIII Superoxide production at baseline is mediated through Noxs in the heart. **A.** Superoxide production in the LV sections was evaluated with DHE staining at baseline. Bar=20 μm. *P<0.05 vs. WT, #P<0.05 vs. cNox4KO, ## P<0.05 vs. Nox2KO. **B.** H_2O_2 production in LV sections was evaluated with the Amplex Red Assay. **C.** O_2^--producing activity of mitochondrial fraction from hearts of the indicated mice was evaluated with lucigenin chemiluminescence assays. *P<0.05

Online Figure IX



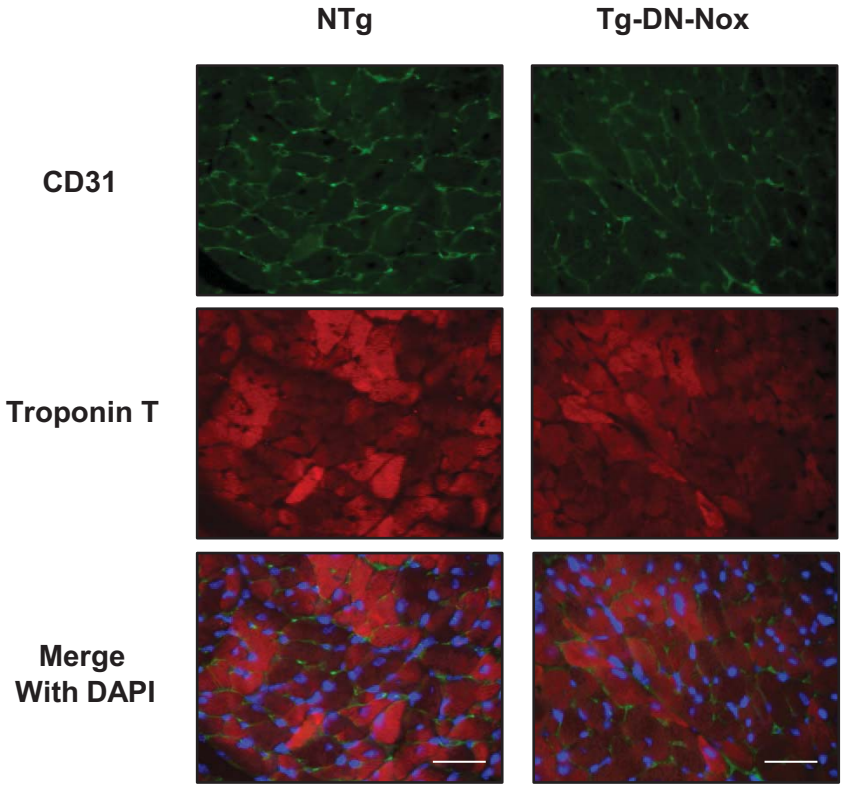
Online Figure IX **A.** Mitochondrial membrane potential of myocytes treated with the indicated adenoviruses was evaluated with JC-1 staining after 12 h of hypoxia and 24 h of reoxygenation. Bar=50μm. *P<0.05, N.S. not significant. **B.** Expression of mRNA for PGC-1α was evaluated with quantitative RT-PCR. *P<0.05. **C.** Expression levels of TFAM and tubulin were evaluated by immunoblotting.

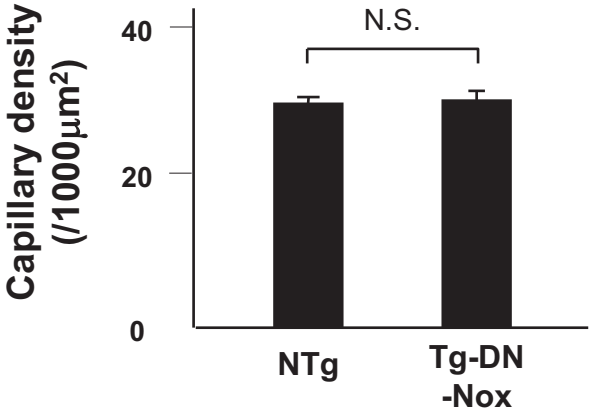
Online Figure X



Online Figure X ATP content in the ischemic area was evaluated in the indicated mice 6h after reperfusion. *P<0.05.

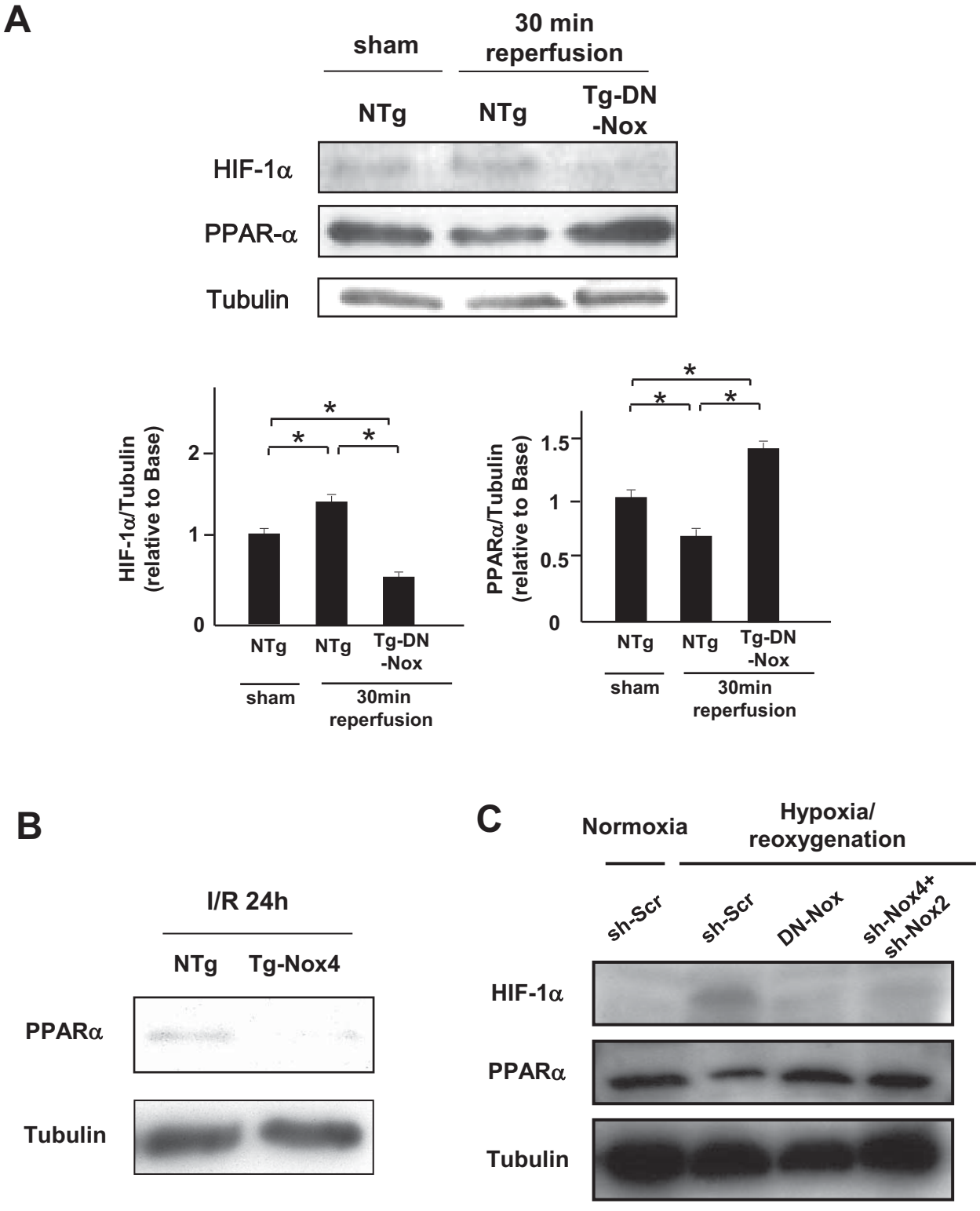
Online Figure XI





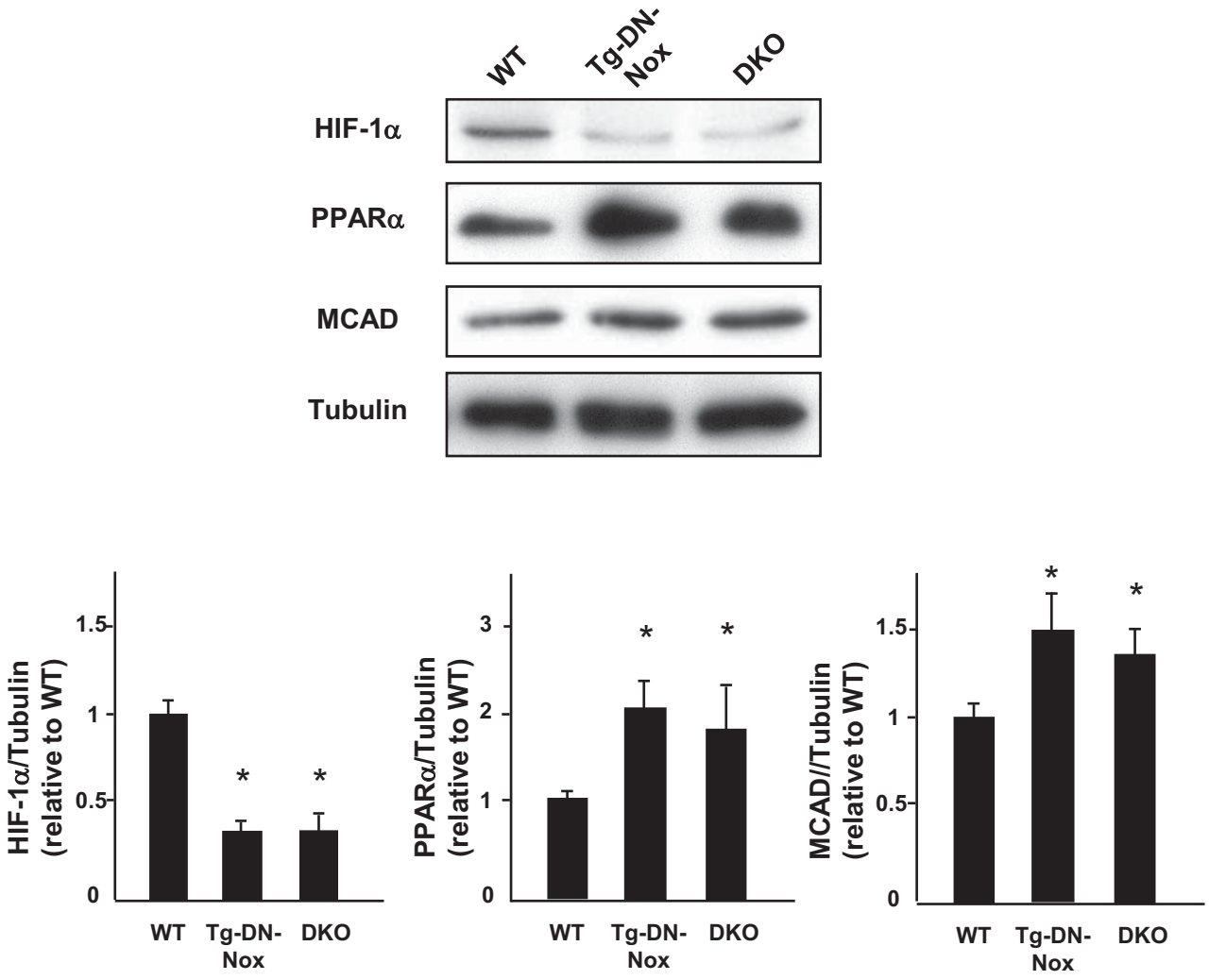
Online Figure XI Capillary density in the LV at baseline in NTg and Tg-DN-Nox4 mice was evaluated by immunostaining with CD31 and Troponin T antibody. Nuclei were stained with DAPI. N.S. not significant. Bar=20 μm.

Online Figure XII



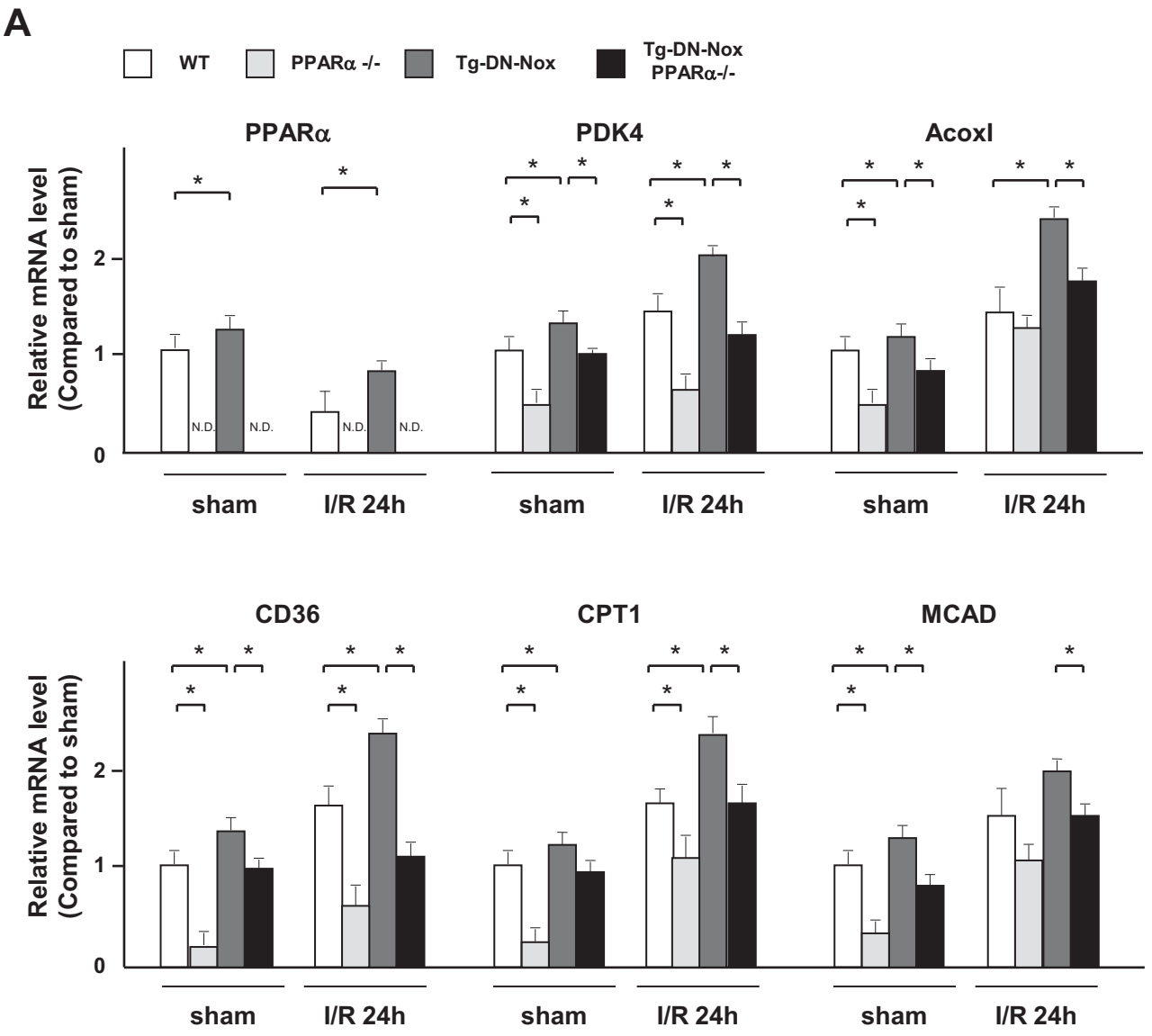
Online Figure XII Expression of HIF-1α and PPARα during ischemia/reperfusion and hypoxia/reoxygenation. **A**. Expression of HIF-1α, PPARα, and tubulin in NTg and Tg-DN-Nox mice 30 min after reperfusion was evaluated by immunoblotting. **B** Expression levels of PPARα and tubulin in the LVs of NTg and Tg-Nox4 mice with I/R were evaluated by immunoblotting. **C.** Expression levels of HIF-1α, PPARα, and tubulin in myocytes transduced with the indicated adenoviruses with or without hypoxia/reoxygenation were evaluated by immunoblotting.

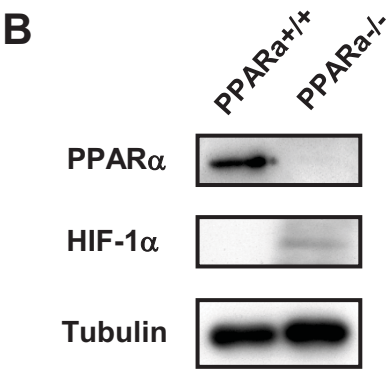
Online Figure XIII



Online Figure XIII Expression levels of HIF-1α PPARα, MCAD, and Tubulin at baseline were evaluated by immunoblotting in WT, Tg-DN-Nox, and DKO mice. *P<0.05.

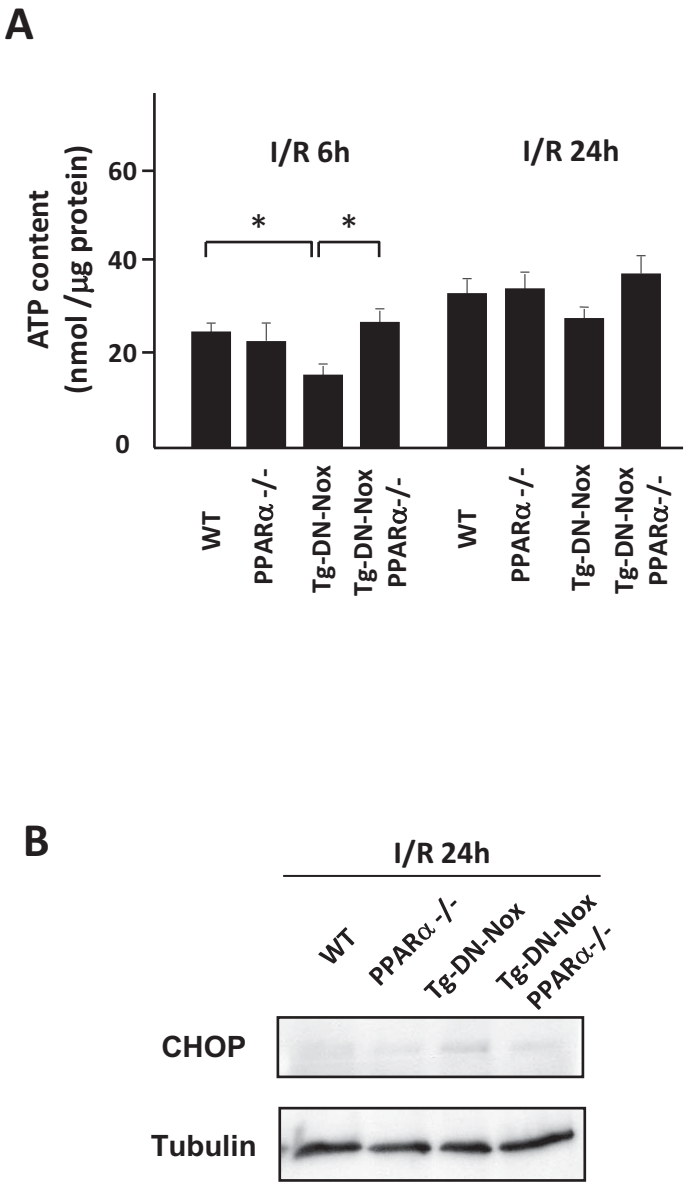
Online Figure XIV





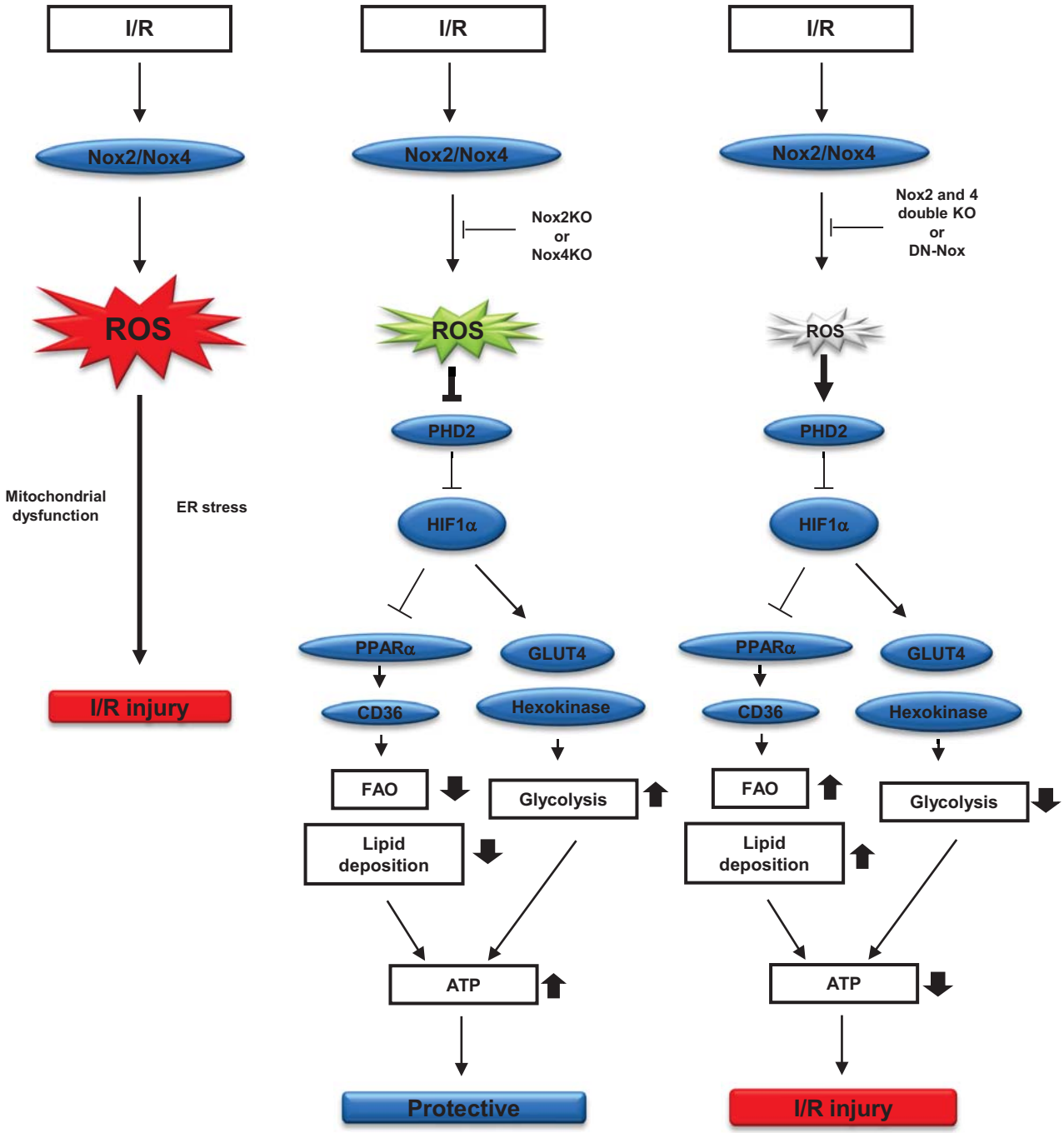
Online Figure XIV DN-Nox upregulates FAO-related genes through PPARα after I/R. **A.** Expression of mRNA for PPARα and FAO-related genes was measured by quantitative RT-PCR. *P<0.05, N.D. not detectable. **B.** Expression of PPARα, HIF-1α and tubulin in WT and PPARα -/- mice was evaluated by immunoblotting.

Online Figure XV



Online Figure XV **A.** ATP content in the ischemic area was evaluated in the indicated mice 6 and 24 h after reperfusion. *P<0.05. **B.** Expression levels of CHOP and tubulin in the indicated mice after I/R were evaluated by immunoblotting.

Online Figure XVI



Online Figure XVI A hypothetical model of the role of Nox2 and Nox4 in response to I/R. I/R induces ROS production by stimulating Nox2 and Nox4, leading to cytotoxicity via mitochondrial dysfunction and ER stress. Downregulation of Nox2 or Nox4 mildly suppresses ROS production and attenuates I/R injury. ROS from either Nox2 or Nox4 is essential for upregulation of HIF-1α and stimulation of glycolysis. Downregulation of both Nox2 and Nox4 strongly suppresses ROS production, and thereby downregulates HIF-1α and upregulates PPARα. A metabolic shift from glycolysis to FAO during I/R induces myocardial TG deposition, which leads to lipotoxicity. The presence of a physiological level of Nox-derived ROS protects the heart from I/R injury. PHD: prolyl hydroxylase, HIF-1α: hypoxia-inducible factor-1α, PPARα: peroxisome proliferator-activated receptor a, MCAD: Medium-chain Acyl-CoA Dehydrogenase