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].

Adenoviruses

Adenovirus vectors harboring HA-tagged Nox4 (Ad-Nox4), Nox2 (Ad-Nox2), a catalytically inactive form of Nox4 (Ad-Nox4-P437H), or shRNA-Nox4 (Ad-shRNA-Nox4) were generated using the AdMax system (Clontech). 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. An adenovirus vector harboring LacZ (Ad-LacZ) was used as control.

For preparation of shRNA-Nox4, a hairpin-forming pair of oligonucleotides (GACCTGGCCAGTATATTATTTCAAGAGAATAATATACTGGCCAGGTCTTTTTT and AGCTAAAAAAGACCTGGCCAGTATATTATTCTCTTGAAATAATATACTGGCCAGGTC GGCC) were synthesized, annealed, and subcloned into pDC-silencer (Ambion). A recombinant shRNA-Nox4 adenovirus was also generated in HEK293 cells by co-transfection with pBHGloxΔE1,3Cre and the shuttle vector harboring shRNA-Nox4. Adenovirus harboring Bcl-xL has been described [2].

Assays for apoptosis

TUNEL staining was conducted as described [3, 4]. Deparaffinized sections were incubated with proteinase K, and DNA fragments were labeled with fluorescein-conjugated dUTP using TdT (Roche Molecular Biochemicals). Nuclear density was determined by manual counting of DAPI-stained nuclei in six fields of each animal using the 40 x objective. TUNEL-positive nuclei in the entire section were identified and counted using the same power objective. Histone-associated DNA fragments were quantified by the Cell Death ELISA (Roche) according to the manufacturer's instruction [3]. The mitochondrial-free cytosolic fraction was prepared as described previously [3].

Echocardiography

Mice were anesthetized using 12 μL/g BW of 2.5% avertin (Sigma-Aldrich), and echocardiography was performed using ultrasonography (Acuson Sequoia C256; Siemens Medical Solutions). A 13-MHz linear ultrasound transducer was used. M-mode measurements of left ventricular (LV) internal diameter were taken from more than three beats and averaged. LV end-diastolic diameter (LVEDD) was measured at the time of the apparent maximal LV diastolic dimension, while LV end-systolic diameter (LVESD) was measured at the time of the most anterior systolic excursion of the posterior wall. LV ejection fraction (LVEF) and percent fractional shortening (%FS) were calculated as follows: $LVEF = [(LVEDD)^3 – (LVESD)^3]/(LVEDD)^3$; $\%FS = (LVEDD – LVESD)/LVEDD \times 100$ [5].

Aortic banding

The method to impose pressure overload in mice has been described. Mice were anesthetized with a mixture of ketamine (0.065 mg/g), xylazine (0.013 mg/g), and acepromazine (0.002 mg/g) and mechanically ventilated. The left chest was opened at the second intercostal space. Aortic constriction was performed by ligation of the

transverse thoracic aorta between the innominate artery and left common carotid artery with a 28-gauge needle using a 7-0 braided polyester suture. Sham operation was performed without constricting the aorta. To measure arterial pressure gradients, high-fidelity micronanometer catheters (1.4 French; Millar Instruments Inc., Houston, Texas, USA) were used [5].

Continuous infusion of angiotensin II and phenylephrine

Continuous infusion of hypertrophic agonists or vehicle control was conducted with osmotic mini-pumps (model 2002, Alza Corp., Palo Alto, CA, USA) as described previously [6]. Control mice received pumps filled with 0.9% sodium chloride.

Immunoblot analyses

Heart homogenates and cardiac myocyte lysates were prepared in RIPA lysis buffer containing 50 mmol/L Tris (pH 7.5), 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. These include monoclonal antibodies raised against Nox4 (see above), anti-GAPDH (Sigma), anti-actin (Sigma), anti-cytochrome c (Santa Cruz), and polyclonal antibodies raised against $p22^{phox}$ (Santa Cruz) and anti-BiP (Cell Signaling Technology).

Immunostaining

Neonatal rat cardiac myocytes grown on chamber slides (Lab-Tek®) were washed three times with PBS. The cells were fixed with 4% paraformaldehyde, and washed four times with PBS containing 0.1% Triton X-100. The cells were boiled for 10 min with a pressure-cooker to allow the antigen to be better exposed to the antibody. Then the cells were blocked with PBS containing 5% normal goat serum for 60 min and stained with antibodies as indicated.

Histological analyses

The LV accompanied by the septum was cut into base, mid portion, and apex, fixed with 10% formalin, embedded in paraffin, and sectioned at 6 μm thickness. The sections were incubated in 3% H_2O_2 in PBS to prevent endogenous peroxidation and blocked with 5% BSA in PBS. Anti–8-hydroxy-2′ -deoxyguanosine (anti–8-OHdG) antibody (Oxis International Inc.) was diluted to 7.5 μg/ml in PBS and applied to the sections for 1 hour at 37°C. After washing, biotinylated secondary antibody (anti-mouse IgG; BD Pharmingen) was applied for 1 hour, followed by streptavidin-HRP (BD Pharmingen) for 30 minutes at room temperature. Myocyte cross-sectional area was measured from images captured from sections stained with anti-wheat germ agglutinin (WGA) antibody as previously described [5]. The outlines of 100–200 myocytes were traced in each section. Interstitial fibrosis was evaluated by Masson Trichrome staining [5]. Confocal microscopic analyses were conducted as described previously [5].

Dihydroethidium (DHE) staining

After harvest, heart tissues were immediately embedded in 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 minutes. Ethidium fluorescence (excitation at 490 nm,

emission at 610 nm) was examined by fluorescent microscopy [7]. The level of O_2^- in the mitochondria was also evaluated with Mitosox™ Red (Invitrogen) according to the manufacturer's instructions.

Lucigenin assays

Cytosol, mitochondria and microsome fractions of the heart 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 [7]. The chemiluminescence was continuously monitored using a luminometer. The reaction was terminated by the addition of superoxide dismutase (SOD) (100 µg/ml).

Evaluation of mitochondrial membrane potential/integrity

In order to evaluate mitochondrial membrane potential/integrity, staining of cultured cardiac myocytes with tetramethylrhodamine ethyl ester (TMRE) and 5,5',6,6'-tetrachloro-1,1' 3,3'-tetraethylbenzimidazolocarbocyanine iodide (JC-1) was conducted using MitoPT® TMRE and MitoPT® JC-1 (ImmunoChemistry Technologies), respectively, according to the manufacturer's instructions.

Mitochondrial function

A mitochondrial fraction was prepared from mouse hearts, as previously described [8]. In brief, isolated mouse hearts were homogenized in 10 volumes of ice-cold Buffer A (200 mmol/L mannitol, 50 mmol/L sucrose, 10 mmol/L KCl, 1 mmol/L EDTA, 10 mmol/L Hepes-KOH (pH 7.4), 0.1% bovine serum albumin, and a mixture of protease inhibitors). Homogenates were centrifuged at 600 *g* for 5 min at 4°C. Supernatants were then centrifuged at 3,500 *g* for 15 min at 4°C. The pellets were resuspended in Buffer A, and centrifuged at 1,500 *g* for 5 min. The supernatants were centrifuged at 5,500 *g* for 10 min at 4°C, and then the pellets were suspended as the mitochondrial fraction in 100 µl of CelLytic M Lysis Reagent (Sigma-Aldrich, St. Louis, MO) for immunoblot analyses or in PBS containing protease inhibitors for lucigenin assays. Lysates containing equal amounts of proteins were assessed for citrate synthase (citrate synthase assay kit, Sigma-Aldrich) and cytochrome *c* oxidase (cytochrome *c* oxidase assay kit, Sigma-Aldrich) activity.

Iodoacetamide-biotin labeling experiments

The heart was freshly removed from each mouse and homogenized in RIPA buffer (50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1% NP-40, 0.1% SDS, 0.5% deoxycholic acid, 10 mmol/L $Na_4P_2O_7$, 5 mmol/L EDTA, 1 mmol/L Na_3VO_4, 1 mmol/L NaF, and protease inhibitors) containing 200 µmol/L biotinylated polyethyleneoxide iodoacetamide (Sigma). The homogenate was centrifuged at 10,000 x g for 10 min. The supernatant was incubated with streptavidin-beads (Sigma) for 2 hours with rotation at 4 °C. After the beads were washed with RIPA buffer three times, iodoacetamide-labeled proteins were eluted with sample buffer (100 mmol/L Tris, pH 6.8, 2% SDS, 5% glycerol, 2% 2-mercaptoethanol, 0.05% bromophenol blue) by heating at 95 °C for 5 min, separated by SDS-PAGE, and then transferred to Hybond™-P-polyvinylidene difluoride membranes. Immunoblot analyses were performed using antibodies raised against aconitase-2 and ANT-1 (SantaCruz Biotechnology).

Quantitative real-time PCR reaction for mitochondrial DNA

Mitochondrial DNA (mtDNA) content was quantified by real-time PCR of cardiac DNA.

Primer sequences for cytochrome *b* and β-actin are as follows: CCACTTCATCTTACCATTTATTATCGC (forward primer) and TTTTATCTGCATCTGAGTTTAA (reverse primer) for cytochrome *b*; CTGCCTGACGGCCAGG (forward primer) and CTATGGCCTCAGGAGTTTTGTC (reverse primer) for genomic β-actin.

References

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Supplemental Figure I

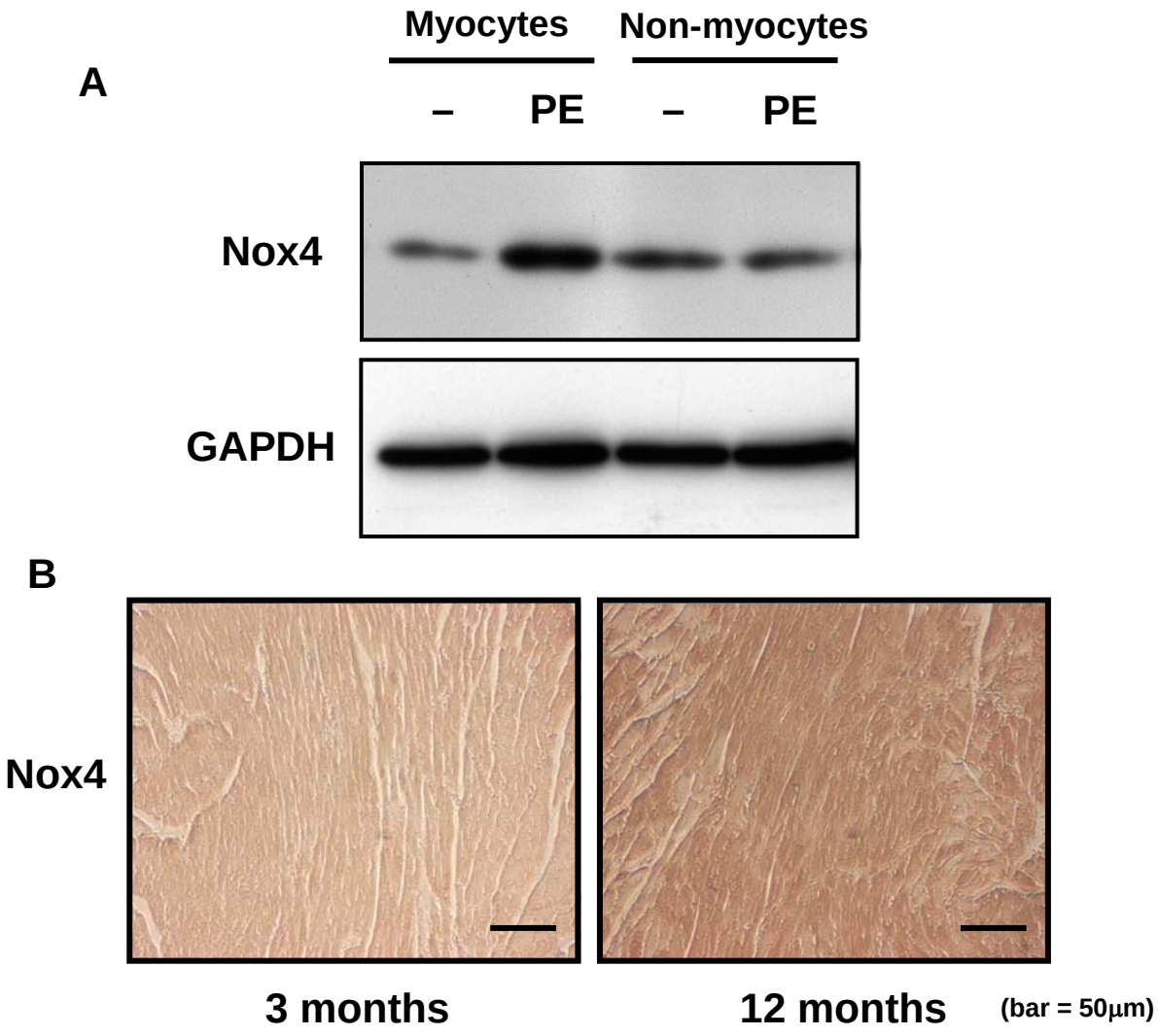

Figure I Expression of Nox4 in cardiac myocytes. A) Cardiac myocyte-rich and non-myocyte-rich cultures were treated with phenylephrine (PE, 20 μM) for 48 hours. Cells were harvested and cell lysates were subjected to immunoblotting with specific anti-Nox4 and anti-GAPDH antibodies. B) Immunostaining of the heart tissue from mice at 3 or 12 months of age with the specific anti-Nox4 antibody.

Supplemental Figure II

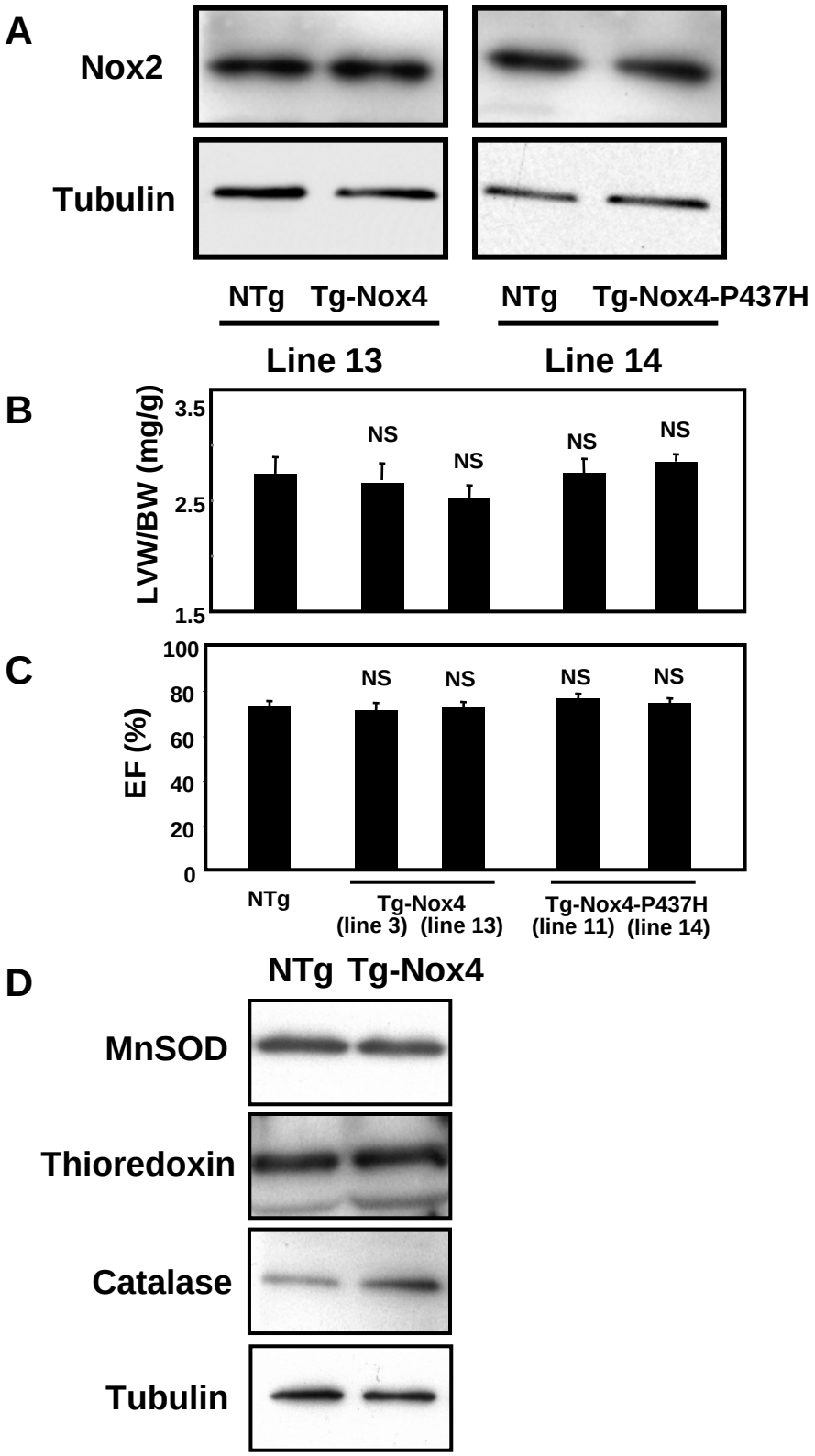

Figure II A) Heart homogenates were prepared from Tg-Nox4 (line 13) and Tg-Nox4-P437H and immunoblotted with anti-Nox2 and anti-tubulin antibody. B,C) Basal cardiac phenotype in Tg-Nox4 and Tg-Nox4-P437H mice. LVW/BW (upper) and LVEF (lower) in Tg-Nox4 (lines 3 and 13), Tg-Nox4-P437H (lines 11 and 14), and non-transgenic (NTg) controls at 3-4 months of age. LVEF was evaluated with echocardiographic measurements. Data were obtained from 6-8 mice. The data for NTg mice from all lines were combined. NS, not significant vs. NTg. D) Protein expression of antioxidants and tubulin (internal control) in NTg or Tg-Nox4 mouse hearts was determined with immunoblot analyses. n=3.

Supplemental Figure III

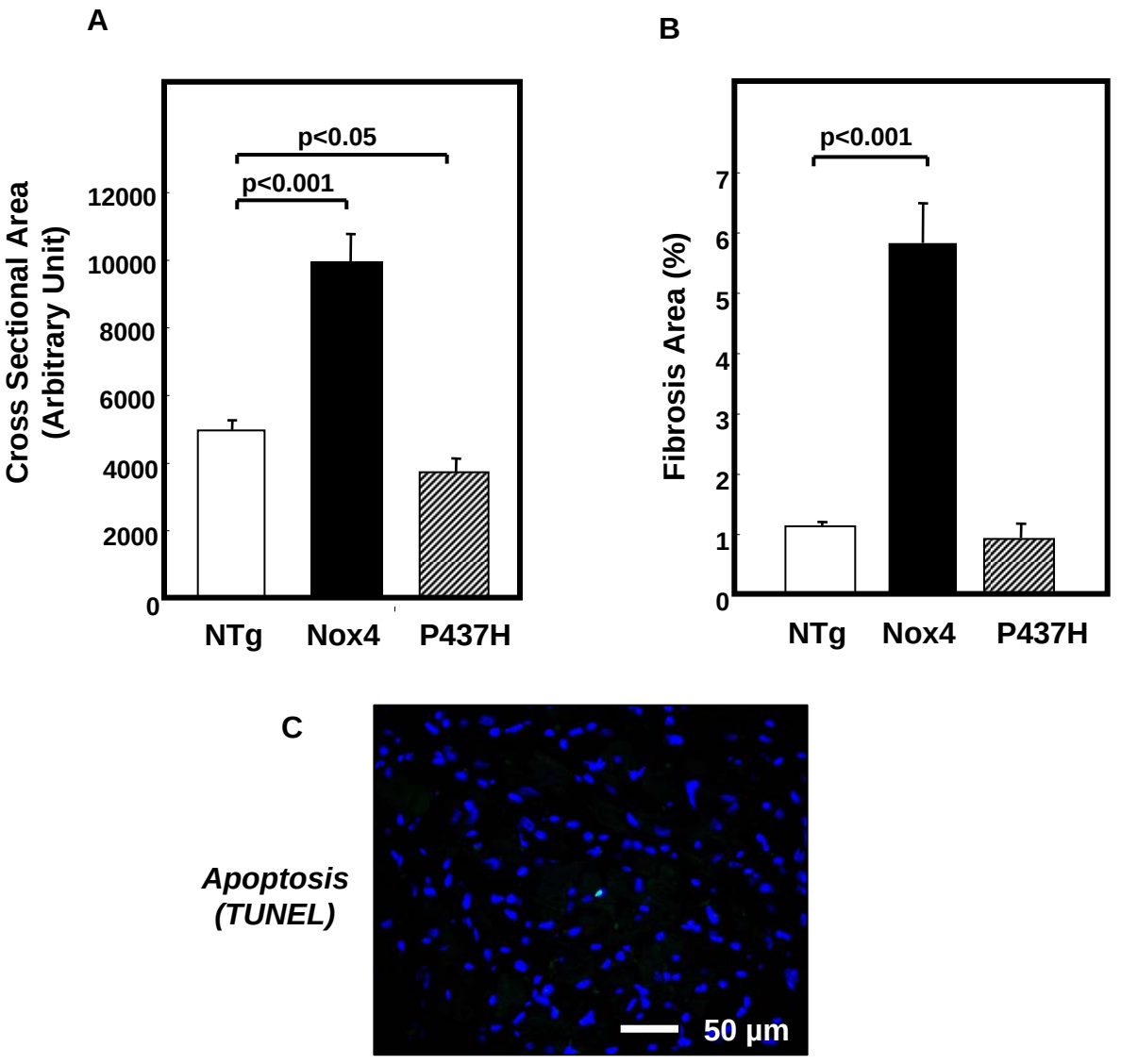

Figure III Histological findings in Tg-Nox4, Tg-DN-Nox4 and NTg at 13-14 month old.
A) LV myocyte cross sectional area as evaluated by wheat germ agglutinin (WGA) staining. The result of the quantitative analysis is shown. n=5. B) LV fibrosis as determined by Masson's Trichrome staining. The result of the quantitative analysis is shown. n=5. C) Apoptosis as determined by TUNEL staining. A representative picture from the Tg-Nox4 heart is shown.

Supplemental Figure IV

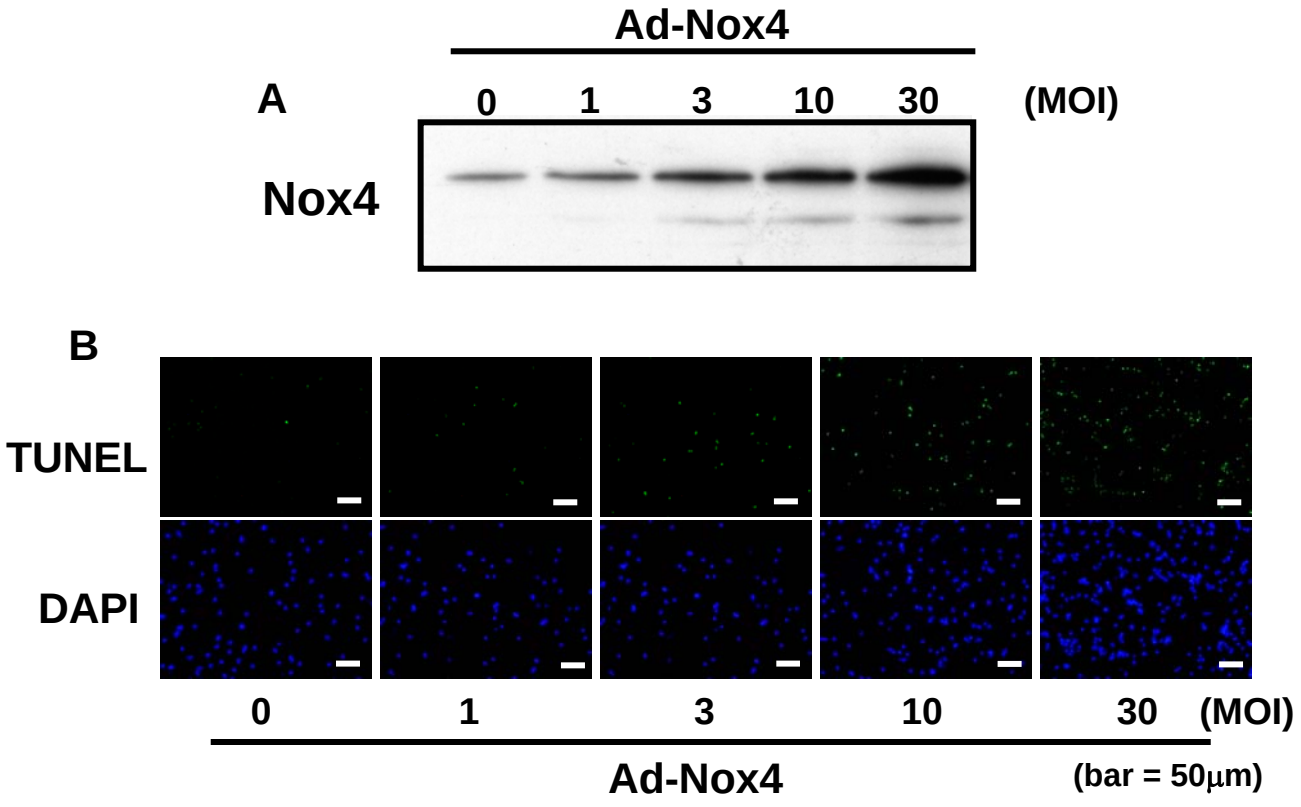

Figure IV Ad-Nox4 was transduced into cultured neonatal rat cardiac myocytes at various multiplicities of infection (MOI). A) Expression of Nox4 protein was evaluated with immunoblot analyses. n=3. B) The number of apoptotic cells was evaluated with TUNEL staining. Representative images are shown. n=4-6.

Supplemental Figure V

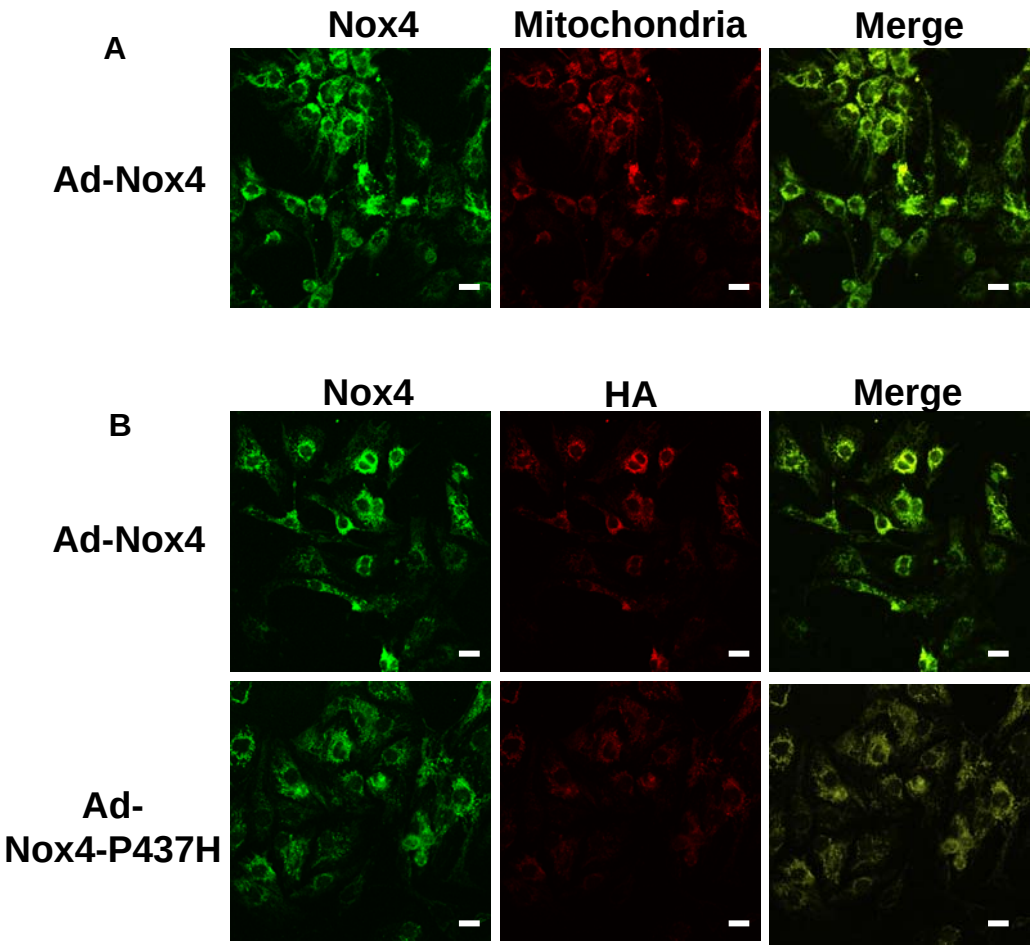

Figure V Subcellular localization of Nox4 and DN-Nox4.
Cultured ventricular cardiac myocytes were transduced with adenovirus harboring Nox4-HA or DN-Nox4-HA. A) Staining with anti-Nox4 antibody, that with Mitotracker and a merged image are shown. B) Staining with anti-Nox4 antibody, that with anti-HA antibody and a merged image are shown. Note that DN-Nox4-HA showed a staining pattern similar to Nox4-HA.

Supplemental Figure VI

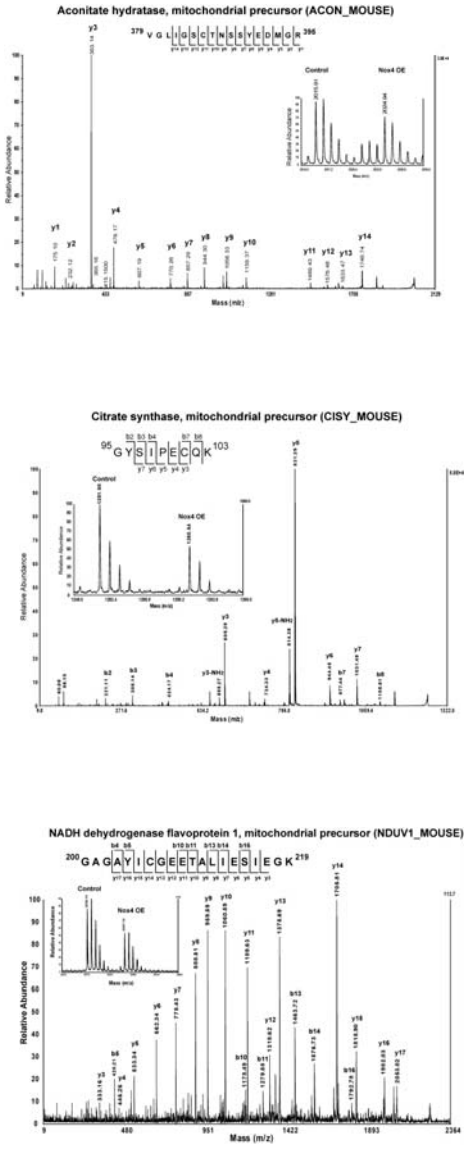

Figure VI Cysteine oxidation of mitochondrial proteins in Tg-Nox4 hearts.
Purified mitochondrial fraction was prepared from aging Tg-Nox4 or NTg mouse hearts and subjected to ICAT assays (Inset). Nox4 OE indicates signals from Nox4 overexpressed (Tg-Nox4) hearts. The spectra of MS/MS confirmed the identity of the peptide fragments. Representative ICAT and MS/MS patterns of aconitase (top), citrate synthase (middle) and NADH dehydrogenase flavoprotein 1 (bottom) are shown.

Supplemental Figure VII

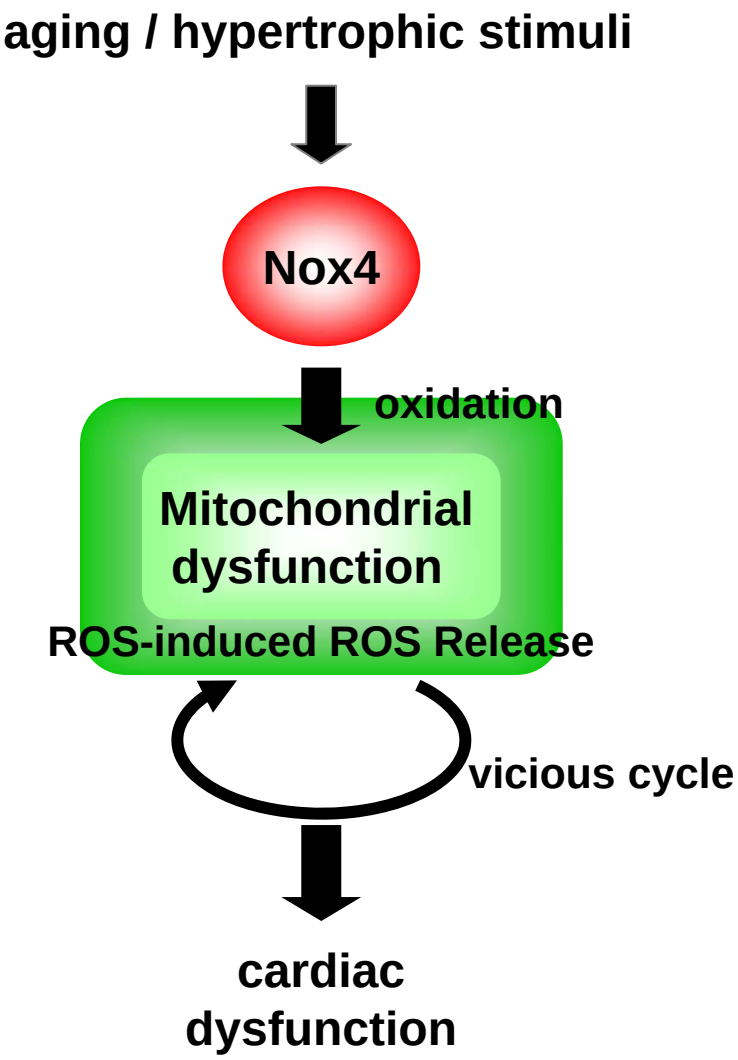

Figure VII Our hypothesis regarding cardiac function of Nox4.
Aging and hypertrophic stimuli upregulate Nox4 in cardiac myocytes. Upregulation of Nox4 in mitochondria enhances O2- production and oxidation/dysfunction of mitochondrial proteins, such as aconitase and NADH dehydrogenase flavoprotein I, which in trigger leakage of electron from mitochondria and increase oxidative stress. Increases in oxidative stress stimulates ROS-induced ROS release from the mitochondria, thereby promoting mitochondrial dysfunction and further increases in oxidative stress.

Online Table I

	NTg (n=7)	Tg-Nox4 (n=6)	Tg-Nox4-P437H (n=6)
Age (month)	13.8 ± 0.7	14.0 ± 0.6	13.8 ± 0.0
Body Weight (g)	32.3 ± 1.2	30.0 ± 0.7	31.6 ± 1.2
Heart Rate	450 ± 26	473 ± 38	421 ± 22
DSEP width (mm)	0.95 ± 0.02	**0.87 ± 0.01 ^{ns}/***	1.03 ± 0.05
LVEDD (mm)	3.71 ± 0.12	**3.84 ± 0.05 ^{ns}/***	3.47 ± 0.09
LVESD (mm)	2.44 ± 0.07	**2.69 ± 0.06 */****	2.29 ± 0.06
EF (%)	71.0 ± 0.8	**65.3 ± 1.9 */****	73.1 ± 1.4

ns not significant, *p < 0.05, **p < 0.01 vs. NTg / Tg-Nox4-P437H

Table I Cardiac dimensions and LV function in Tg-Nox4, Tg- Nox4-P437H and NTg mice at 13-14 months of age, as evaluated by echocardiographic analyses. Hypertrophy and LV dysfunction were observed in Tg-Nox4. DSEP diastolic septal wall thickness, LVEDD left ventricular end diastolic dimension, LVESD left ventricular end systolic dimension, EF ejection fraction.