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Overexpressing Superoxide Dismutase 2 Induces a Supernormal Cardiac Function by Enhancing Redox-dependent Mitochondrial Function and Metabolic Dilation*

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Abstract

During heightened cardiac work, O₂ consumption by the heart benefits energy production via mitochondria. However, some electrons leak from the respiratory chain and yield superoxide, which is rapidly metabolized into H₂O₂ by SOD2. To understand the systemic effects of the metabolic dilator, H₂O₂, we studied mice with cardiac-specific SOD2 overexpression (SOD2-tg), which increases the H₂O₂ produced by cardiac mitochondria. Contrast echocardiography was employed to evaluate cardiac function, indicating that SOD2-tg had a significantly greater ejection fraction and a lower mean arterial pressure (MAP) that was partially normalized by intravenous injection of catalase. Norepinephrine-mediated myocardial blood flow (MBF) was significantly enhanced in SOD2-tg mice. Coupling of MBF to the double product (Heart Rate × MAP) was increased in SOD2-tg mice, indicating that the metabolic dilator, “spilled” over, inducing systemic vasodilation. The hypothesis that SOD2 overexpression effectively enhances mitochondrial function was further evaluated. Mitochondria of SOD2-tg mice had a decreased state 3 oxygen consumption rate, but maintained the same ATP production flux under the basal and L-NAME treatment conditions, indicating a higher bioenergetic efficiency. SOD2-tg mitochondria produced less superoxide, and had lower redox activity in converting cyclic hydroxylamine to stable nitroxide, and a lower GSSG concentration. EPR analysis of the isolated mitochondria showed a significant decrease in semiquinones at the SOD2-tg Q_i site. These results support a more reductive physiological setting in the SOD2-tg murine heart. Cardiac mitochondria exhibited no significant differences in the respiratory control index between WT and SOD2-tg. We conclude that SOD2 overexpression in myocytes enhances mitochondrial function and metabolic vasodilation, leading to a phenotype of supernormal cardiac function.

Keywords

superoxide dismutase 2 (SOD2); mitochondria; redox regulation; metabolic dilation; cardiac function; bioenergetics; transgenic mice

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DISCLOSURES

None

1. INTRODUCTION

Manganese superoxide dismutase (MnSOD or SOD2) is one of the primary mitochondrial antioxidants in a network of detoxification enzymes that neutralizes the highly reactive superoxide anion radical ($\text{O}_2^{\bullet-}$) to a less reactive hydrogen peroxide (H_2O_2), allowing its free diffusion through hydrophobic inner membrane. The metabolic H_2O_2 of mitochondria under state 3 and state 4 respiration may induce modest mitochondrial oxidative stress, or diffuse to the cytosol, acting as a signaling molecule to trigger important physiological responses, such as the metabolic dilator that mediates shear or acetylcholine-induced smooth muscle relaxation in small arteries [1-3]. Therefore, the H_2O_2 generated at low to moderate concentrations under normal physiological conditions is easily tolerated by the cell, and has been recognized as a non-toxic by-product of cellular metabolism that can mediate physiological signaling.

In cardiac myocytes, it has also been demonstrated that H_2O metabolized from $\text{O}_2^{\bullet-}$ regulates collateral blood flow via diffusion to the microvascular system of the heart [4]. This provides an important physiological linkage between mitochondrial H_2O_2 and metabolic flow regulation, coupling the production of mitochondrial H_2O_2 as an endothelium-derived hyperpolarizing factor (EDHF) to the myocardial metabolism. Further studies have indicated that mitochondrial H_2O_2 is the vasoactive product of metabolically active cardiac myocytes, functioning as a coronary metabolic dilator, coupling myocardial oxygen consumption to coronary blood flow in a redox-dependent manner [5, 6].

The balance between the mitochondrial antioxidant system and reactive oxygen species (ROS) is finely maintained, and low levels of mitochondrial ROS are required by cells to modulate normal redox signaling networks. Excessive amounts of ROS are believed to shorten life span and induce age-associated pathological conditions. In mice, the $\text{MnSOD}^{-/-}$ gene is neonatal lethal with dilated cardiomyopathy [7], and the mouse model of $\text{MnSOD}^{+/-}$ displays increased cardiac oxidative injury and heart failure at day 10 after birth [8]. Mutations in SOD2 are associated with human age-related cardiovascular disorders, such as cardiomyopathy. These results clearly demonstrate the significant role of aberrant regulation of SOD2 in heart-related illnesses.

In vitro/vivo studies have established that overexpressing SOD2 normalizes endothelial dysfunction induced by high glucose-mediated diabetic complications [9-11] and chronic infusion of angiotension II [12], supporting the important causal role of mitochondria-derived ROS in vascular injury. Furthermore, cardiac-specific overexpression of SOD2 has been shown to effectively protect cardiac myocytes and mitochondria from the disease complications of diabetic cardiomyopathy [10]. Increased levels of SOD2 have also been shown to be protective against acute myocardial ischemia and reperfusion injury and doxorubicin-induced cardiotoxicity [13-15]. Thus the hypothesis that SOD2 overexpression improves cardiovascular function has been proven true in a number of disease models. However, there has been little investigation directed towards understanding the fundamental mechanism by which SOD2 overexpression enhances cardiac function under basal or normal physiological conditions. Here we explore the fundamental mechanism by which cardiac-

specific SOD2 overexpression induces supernormal cardiac function by testing the following hypotheses: 1) overexpressing SOD2 enhances metabolic dilation, 2) overexpressing SOD2 enhances mitochondrial function, and 3) overexpressing SOD2 modulates the redox status of the myocardium. We present evidence for higher mitochondrial ATP-producing efficiency and a more reducing physiological setting in the myocardium in a transgenic murine model with cardiac-specific SOD2 overexpression. When combined with a phenotype of increased myocardial blood flow, these attributes mediate a supernormal ventricular function characterized in the murine model of cardiac-specific SOD2 overexpression (SOD2-tg).

2. EXPERIMENTAL PROCEDURES

2.1 Animals

The heart-specific SOD2 transgenic mice (*SOD2-tg*, strain: FVB-Tg (Myh6-SOD2, Tyr) 3Pne/J, age of 12-13 weeks) were obtained from the Jackson Laboratory. All procedures were performed with the approval (protocol no. 12-031) of the Institutional Animal Care and Use Committee at Northeast Ohio Medical University (Rootstown, OH) and conformed to the Guide for the Care and Use of Laboratory Animals.

2.2 Reagents

Glutathione (GSH), diethylenetriaminepentaacetic acid (DTPA), ubiquinone-1 (Q_1), ubiquinone-2 (Q_2), sodium cholate, deoxycholic acid, rotenone, and β -nicotinamide adenine dinucleotide (reduced form, NADH), were purchased from Sigma Chemical Company (St. Louis, MO) and used as received. The antibodies against SOD2, the subunit I of complex IV, and the iron-sulfur protein of complex III were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX). The spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine.HCl (CMH) was purchased from Enzo Life Sciences Inc. (Farmingdale, NY). The DMPO spin trap was purchased from Dojindo Molecular Technologies, Inc. (Rockville, MD), and stored under argon at -80°C until needed.

2.3 Jugular and femoral artery catheterization and measurement of mouse blood pressure

Mice received a surgical plane of inhaled anesthesia from 1.5–2.5% sevoflurane gas with supplemental oxygen, using a veterinary anesthesia and monitoring device. Animals were placed on a controlled heating table and maintained at 37°C with core temperature measured via a rectal probe. Mice were secured in the supine position and placed under a dissecting microscope. The right jugular vein was cannulated with PE-50 polyethylene tubing (Becton Dickinson, Oakville, ON) containing heparin (50 U/ml in Dulbecco's PBS) in saline for intravenous drug infusions. The catalase infusion was following reported literature protocols [16-18]. Next, a midline incision was made on the ventral right thigh region and the femoral nerve was isolated and pulled aside. The distal and proximal ends of the femoral artery were held with surgical sutures for temporary control of bleeding, and the distal end of the femoral artery was tied off. The femoral artery was isolated and cannulated with a 1.4-Fr (SPR-1,000; Millar Instruments) high-fidelity microtip transducer catheter connected to a data acquisition system (PowerLab ML820; ADInstrument, Colorado Springs, CO) through a pressure interface unit (Millar Instrument, Transducer Balance, TCB 600) designed to

invasively measure systolic, diastolic, and pulse pressure, mean arterial blood pressure (MAP), and heart rate (HR). A microtip catheter was advanced into the femoral artery, and aortic blood pressure was recorded.

All measured variables were continuously recorded and stored on an iMac computer that used the PowerLab system (AD Instruments; Castle Hill, Australia). The blood pressure data were collected and analyzed using AD Instruments Chart v5.1.2 software. To correct HR measurements by the ML820 for movement artifacts, an ECG monitor was connected throughout the experiments. All mice were euthanized following the experimental protocol with a lethal dose of pentobarbital sodium (50 µg/kg).

2.4 Preparation of contrast agent for myocardial perfusion imaging

Lipid-shelled microbubbles were prepared by sonication of an aqueous lipid dispersion of polyoxyethylene-40-stearate and distearoyl phosphatidylcholine saturated with decafluorobutane gas. Contrast agent prepared before the experiments. Microbubble concentration and size distribution were determined by Zetasizer Nano-ZS90 (Malvern Instruments). Prior to particle measurements, the nanoparticle suspension was diluted (1: 5 v/v) with filtered water (0.22 µm filter, Nalgene International) to ensure that light scattering signals are within the sensitivity of the instrument.

2.5 Contrast echocardiography MBF measurements

For myocardial contrast echocardiography (MCE), animals were prepared as above for transthoracic echocardiography. Left ventricle mass (LV mass) was assessed and calculated by echocardiography according to published methods [19-22]. Myocardial perfusion imaging was performed with a linear-array transducer (15L8) interface with an ultrasound system (Sequoia 512, Siemens medical System, Mountain View, CA). Long axis images were obtained and for perfusion imaging. Mice were simultaneously infused with norepinephrine (NE) and a non-targeted contrast agent (prepared as described before, diluted 1/10 in sterile saline). In all mice, the contrast agent was infused intravenously (jugular vein) at 20 µl/min (1×10^5 bubbles/min). MCE was performed using an ACUSON SEQUOIA 512 Ultrasound System (Siemens) with low-MI (0.2-0.25) imaging, and high-MI (1.9) destruction frames was used to detect the nonlinear fundamental component of the microbubbles signal at frequency of 7 MHz. All settings for processing were adapted and optimized for each animal: penetration depth was ~2–2.5 cm, near field was focused on the middle of the left ventricle (long axis view), and gains were adjusted to obtain images with no signal from the myocardium and then held constant. Regions of interest were positioned within the anterolateral and posterolateral walls in the short axis view. A curve of signal intensity over time was obtained in each region of interest and fitted to an exponential function: $y = A(1 - e^{-\beta t})$, where y is the signal intensity at any given time, A is the signal intensity corresponding to the microvascular cross sectional area, and β is the initial slope of the curve, which corresponds to the blood volume exchange frequency [23]. Relative blood volume (RBV) was calculated as the ratio of myocardial to cavity signal intensity ($RBV = A/A_{LV}$). A_{LV} corresponds to the signal intensity for the LV cavity. Color coded parametric images were used to outline region of interest (region of the left ventricle). MBF was estimated as the product $RBV \times \beta$. MAP and HR were recorded using a Millar probe

located in the abdominal aorta, for measurement of the double product ($\text{MAP} \times \text{HR}$) as a surrogate for cardiac work.

2.6 Analytical Methods

Optical spectra were measured on a Shimadzu 2600 UV/VIS recording spectrophotometer. The concentrations of Q_1 and Q_2 were determined by absorbance spectra from NaBH_4 reduction using a millimolar extinction coefficient $\epsilon_{(275\text{nm}-290\text{nm})} = 12.25 \text{ mM}^{-1} \text{ cm}^{-1}$ [24]. The electron transfer activities of Complexes I-IV from the heart mitochondrial preparations were assayed by published methods [25, 26]. The activity of catalase was analyzed in an assay mixture of 50 mM potassium phosphate buffer, pH 7.0, containing 0.036% (w/v) H_2O_2 , and an aliquot of mitochondrial or cytosolic preparation was added at 25 °C to initiate H_2O consumption by monitoring at $\text{OD}_{240\text{nm}}$ ($\epsilon = 43.6 \text{ mM}^{-1} \text{ cm}^{-1}$) or O_2 evolved rate measured by oxygen polarography. The activity of glutathione peroxidase (GPx2) in the mitochondria was measured in an assay mixture of 50 mM Tris-Cl, pH 7.5, containing 5 mM EDTA, 200 mM GSH, yeast GR (glutathione reductase, 0.8 unit), NADPH (0.2 mM), and an aliquot of mitochondria at 37 °C. The consumption of NADPH was initiated by *tert*-butyl hydroperoxide (0.002%), and monitored at 340 nm.

2.7 Preparation of mitochondria

Mitochondria were prepared from the murine myocardium by the differential centrifugation method according to our published protocol for use with rat hearts, with minor modifications [26-29]. Briefly, the mouse heart was homogenized in 1 mL of M-buffer (in mM, mannitol 230, sucrose 70, EDTA 1, EGTA 1, and Trizma 5, pH 7.4) containing 1 unit of nagarse (Sigma-Aldrich, St. Louise, MO) on ice. The cOmplete protease inhibitor cocktail (1 $\times$, Roche Life Science, Indianapolis, IN) and PMSF (phenylmethanesulfonylfluoride, 1 mM in DMSO) were added to the homogenate after a 5-min incubation. The resulting homogenate was subsequently centrifuged $500 \times g$ for 10 min to remove nucleus and debris, and a second centrifugation of supernatant at $20,000 \times g$ for 10 min yielded mitochondria sediment and the supernatant of cytosolic compartment containing sarcoplasmic reticulum and peroxisome. The mitochondrial pellet was re-suspended in M-buffer and the protein concentration was determined by the Lowry method using bovine serum albumin as a standard. The minimal cytosolic fraction contamination of the mitochondrial pellet was confirmed by immunoblotting the mitochondrial preparations using a monoclonal antibody (1:500) against a housekeeping cytosolic protein, glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH, Santa Cruz Biotechnology, Inc., Dallas, TX); blotting of cytosolic preparations served as a positive control as shown in the top panel of Fig. 1 [28].

2.8 Measurement of Oxygen Consumption Rate (OCR) of Mitochondrial Preparations

Mitochondrial respiration was measured by the polarographic method using a Clark-type oxygen electrode (Oxytherm, Hansatech Instruments, Norfolk, England) under the optimal kinetics mediated by ETC at 30 °C and reported in nmol/min [28, 29]. The NADH-linked respiration buffer contained malate/glutamate (in mM, potassium glutamate 140, NaCl 10, MgCl_2 1, EGTA 1, malate 5, Trizma 1, phosphate 2.5, and cytochrome *c* 0.01; adjusted to pH 7.4). Substrate combination of glutamate and malate activates malate-aspartate shuttle

and dehydrogenases of Krebs cycle with reduction of NAD^+ to NADH, then feeding electrons into complex I and down the thermodynamic cascade through complex III to complex IV to O_2 . The mitochondrial preparations from mouse hearts were added to the respiration buffer to a final concentration of 0.6 mg/ml. The oxygen consumption rate (OCR) was measured as follows: state 3, OCR stimulated by ADP (0.2 mM); state 4, OCR after the addition of oligomycin (2 $\mu\text{g}/\text{mL}$) following ADP addition; uncoupled respiration, OCR after the addition of FCCP (2.5 μM) following oligomycin. The oxygen electrode was calibrated at 1 atm by using the concentration of O_2 in the respiration buffer at 30 °C to be 230 μM .

2.9 Measurement of the mitochondrial State 3 ATP generation flux

The combination of oxygen electrode and ATP assay to simultaneously monitor mitochondrial state 3 ATP generation flux and OCR has been well documented and extensively reviewed [30]. The mitochondrial ATP flux (accumulated ATP within 1 min) was measured using ATP Bioluminescent Assay Kit (Sigma-Aldrich, St. Louis, MO) following manufacturer's protocol. The mitochondrial state 3 oxygen consumption rate was simultaneously measured on the Oxytherm under the conditions as previous described. Briefly, the mitochondrial preparation (to a final of 0.6 mg/mL) was added into the NADH-linked respiration buffer, and ADP (to a final of 1 mM) was subsequently added. 5 μL of reaction mixture was withdrawn from the reaction chamber and immediately added into 495 μL of pre-heated ATP-assay buffer (70 °C, 10 min) as initial ATP concentration. Another 5 μL of reaction mixture was sampled 20-sec, 40-sec, and 60-sec after the initial sampling as the final ATP concentration. The linear oxygen consumption rate during the ATP sampling time span was ensured by the simultaneous Oxytherm polarography monitoring. The bioluminescent signal was recorded on the OrionL microplate luminometer (Titertek-Berthold Detection Systems GmbH, Pforzheim, Germany).

2.10 Measurement of mitochondrial $\cdot\text{O}_2^-$ production by EPR spin trapping

EPR measurements of $\cdot\text{O}_2^-$ generation by isolated mitochondria were carried out on a Bruker EMX Micro spectrometer operating at 9.43 GHz with 100 kHz modulation frequency at room temperature [28, 29, 31]. The reaction mixture containing the NADH-linked respiration buffer supplemented with DTPA (1 mM) /DMPO (90 mM) was mixed with the mitochondrial preparation (to a final concentration of 0.5 mg protein/mL) at 30 °C for 4 min. The reaction mixture was then transferred into a 50- μL capillary (Drummond Wiretrol, Broomall, PA), sealed, loaded into the EPR resonator (HS cavity, Bruker Instrument, Billerica, MA), equilibrated to 298 K, and tuned within 2 min. The scan of the EPR spectrum was started at exactly 6 min after the initial reaction. Parameters: center field 3360 G, sweep width 100 G, power 20 mW, receiver gain 1×10^5 , modulation amplitude 1 G, conversion time 40.96 ms, time constant 163.84 ms, number of scans: 5. The spectral simulations were performed using the WinSim program developed at NIEHS by Duling [32].

2.11 Cell Culture and Fluorescence Microscopy

Murine myocytes (HL-1 cell line) were grown and maintained in Claycomb medium (Sigma-Aldrich, St. Louise, MO) supplemented with 10% fetal bovine serum, 0.01%

penicillin/streptomycin antibiotic, 2 mM glutamine, and 100 μ M norepinephrine in 35 mm polystyrene tissue culture flasks at 37°C in the presence of 5% CO₂ as previously described [29, 33]. Confluent cells with ~ 40% viability were used for plasmid pBI-eGFP-SOD2 (Addgene, Cambridge MA) transfection to overexpress human SOD2 in HL-1 cells.

After transfection, HL-1 myocytes were cultured on sterile coverslips in 35-mm dishes for 40 h at 37 °C. Cells were then subjected to GFP detection using fluorescence microscopy and to EPR spin trapping of $\cdot$ O₂⁻ induced by antimycin A. Nuclei were stained with blue fluorescence DAPI (2 μ M).

2.12 Measurement of OCR of Living Myocytes

The OCR of myocytes was measured with a Seahorse Bioscience extracellular flux analyzer (model XF24, North Billerica, MA) and presented in pmol/min. HL-1 myocytes were seeded overnight at a density of 3×10^4 cells per well on the fibronectin pre-coated XF24 V7 cell culture microplate. After pBI-eGFP-SOD2 transfection, myocytes were equilibrated with DMEM containing glucose (10 mM) and sodium pyruvate (0.2 mM), and lacking bicarbonate, at 37 °C without CO₂ for 1 hr. Oligomycin (2 μ g/mL), FCCP (0.3 μ M), and antimycin A (10 μ M) were sequentially injected into the microplate for the readout of ATP-linked OCR and the calculation of reserve capacity.

2.13 Assays of mitochondrial electron transport chain complex activity

Enzymatic activities of electron transport chain (ETC) components in the as-isolated mitochondria were assayed according to previously published methods [25, 26]. Complex I activity in mitochondrial preparation was assayed by Q₁-stimulated NADH oxidation. Complex II activity was analyzed by Q₂-stimulated dichlorophenolindophenol (DCPIP) reduction. Complex III activity was evaluated by ubiquinol (Q₂H₂)-mediated ferricytochrome *c* reduction. The activity of SCR supercomplex containing complex II and complex III was assayed by succinate-mediated ferricytochrome *c* reduction. Complex IV activity was measured by oxygen-mediated ferocytocrome *c* oxidation.

2.14 Measurement of metabolic H₂O₂ by isolated mitochondria of murine heart

The mitochondrial H₂O₂ production rate was measured using Amplex Red Hydrogen Peroxide Assay Kit (Life Technologies, Grand Island, NY) following manufacturer's protocol. Briefly, the mitochondrial preparation (0.08 mg/mL) was suspended in the NADH-linked respiration buffer containing glutamate/malate (in mM, potassium glutamate 140, NaCl 10, MgCl₂ 1, EGTA 1, malate 5, Trizma 1, phosphate 2.5, cytochrome *c* 0.01, BSA 0.1% w/w, adjusted to pH 7.4) supplemented with AmplexRed (50 μ M) and HRP (0.1 Unit/mL) at 30 °C. The reaction buffer also contained ADP (1 mM) for the state 3 respiration settings, and it contained ADP (1 mM)/oligomycin (20 μ g/mL) for the state 4 respirations. Standard H₂O₂ (supplied with the assay kit) and heat denatured mitochondrial preparation (70 °C, 10 min) were used as standard regression curve calculation. The accumulation of signal was detected by Synergy 4 fluorescence microplate reader (BioTek, Winooski, VT) in kinetic mode (3-min interval).

2.15 Glutathione recycling method

The amount of reduced and oxidized glutathione (GSH and GSSG) in mitochondria was determined by the glutathione recycling method [34, 35]. In this method, the reaction rates of excess glutathione reductase (GR), NADPH, and DTNB were limited by the presence of GSH and GSSG. Isolated mitochondria, as well as a series of known concentrations of GSH and GSSG, were mixed with 5-sulfosalicylic acid (5-SSA, 5%) in the presence of Triton X-100 (0.3%). The protein mixture was subjected to precipitation by centrifugation (12,000 rpm $\times$ 2 min at 4 °C). The supernatant was divided into two portions: one portion (room temperature, RT) for additional GSH-quenching and another portion (sitting on ice) ready for total [GSH+GSSG] determination. For the GSH-quenching, an excess amount of 2-vinylpyridine (2-VP, to a final concentration of 2%) was added to the mixture after adjusting the pH to 6~7 with triethanolamine. The mixture was incubated at RT for 2 hr. The reduced GSH was thus blocked by 2-vinylpyridine and the remaining oxidized GSSG was ready for measurement. Standard concentrations of GSH and GSSG undergoing the same 5-SSA/2-VP treatment were used to create a linear regression standard curve. The above standards/samples were added into glutathione reaction buffer (in mM, potassium phosphate 50, pH 7.5, EDTA 1, DTNB 0.6, NADPH 0.1, 30 °C), and the reaction was started by the addition of GR enzyme (2 units/mL for the detection of [GSH + GSSG], and 0.4 units/mL for the detection of [GSSG]). The assay was monitored by absorbance increase at 412 nm following the reduction of DTNB ($\epsilon_{412\text{nm}} = 13.6 \text{ mM}^{-1} \text{ cm}^{-1}$). The concentrations of GSH + GSSG as well as GSSG were calculated by interpolation from the standard curves.

2.16 Immunoblotting Analysis

For immunoblotting, the reaction mixture was mixed with Laemmli sample buffer at a ratio of 4:1 (v/v), incubated at 70 °C for 10 min, and then immediately loaded onto a 4-12 % Bis-Tris polyacrylamide gradient gel. Samples were run at room temperature for 55 min at 190 V. Protein bands were electrophoretically transferred to a nitrocellulose membrane in 25 mM Bis-Tris, 25 mM Bicine, 0.029% (w/v) EDTA, and 10% methanol. Membranes were blocked for 1 h at room temperature in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TTBS) and 5% dry milk (BioRad, Hercules, CA). The blots were then incubated overnight with the primary antibody at 4 °C. Blots were then washed 3 times in TTBS and incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG in TTBS at RT. The blots were again washed twice in TTBS and twice in TBS, and then visualized using ECL Western Blotting Detection Reagents (GE Healthcare Life Sciences, Fairfield, CT).

2.17 Data Analysis

Statistical analysis was performed using Origin 9.1 data analysis software. Results were presented as mean $\pm$ SEM, followed by group number (*n*), and *p* value. Comparisons between two groups (the data sets of Figs. 3, 4, 5, 6, 7, 8, 9, and Tables I-II) were assessed by student's *t*-test to analyze the significance of differences. Comparisons among multiple groups (the data set of three groups in Fig. 4D) were assessed by one-way ANOVA followed by Tukey's post hoc tests. A probability value of *p* < 0.05 was used to establish statistical significance.

3. RESULTS

3.1 Cardiac-specific overexpression of SOD2 prevents the mitochondria from undergoing oxidative stress

Cardiac-specific SOD2 transgenic mice (SOD2-tg) were employed as the animal model for investigation. The SOD2-tg mice with hemizygotes exhibit a phenotype that is viable, fertile, and normal in size. Transgenic expression of SOD2 is specific to the heart and localized to the mitochondria. Western blot analysis confirmed no cytosolic contamination in the mitochondrial preparation (Fig. 1, top panel). Western blot analysis further indicated enhanced SOD2 expression in the mitochondria of murine hearts by 5.8 ± 0.8 fold ($n=6$) [Fig. 1, middle panel, with subunit I (70 kDa and FAD-binding protein, nuclear-encoded) of complex II and subunit I of complex IV (Cox I, mitochondrial DNA-encoded) as the loading control, $n=6$]. Mitochondria were isolated from the SOD2-tg mouse hearts and subjected to measurement of $\cdot\text{O}_2^-$ generation mediated by glutamate and malate (NADH-linked). EPR spin-trapping analysis showed no detectable $\cdot\text{O}_2^-$ generation (Fig. 1, lower panel, *b* vs *e*) and significantly lower sensitivity to antimycin A treatment (Fig. 1, lower panel, *c* vs *f*), confirming that SOD2 was functionally expressed in the mitochondria of mouse heart.

3.2 Cardiac-specific overexpression of SOD2 results in enhanced cardiac function

To test the hypothesis that SOD2 overexpression mediates an enhanced cardiac function, studies were performed by echocardiography to evaluate the physiological phenotype of the SOD2-tg murine heart (Fig. 2). As indicated in Table I, SOD2 overexpression significantly increased the parameters of ejection fraction (increased from $74.1 \pm 2.3\%$ to $89.3 \pm 1.6\%$, $n=6$) and fractional shortening (from $42.5 \pm 2.0\%$ to $58.9 \pm 2.4\%$, $n=6$), while it significantly decreased the left ventricular internal diameter at systole (LVIDs, decreased from 2.2 ± 0.1 to 1.4 ± 0.1 mm, $n=6$) and at diastole (LVIDd, decreased from 3.82 ± 0.04 to 3.36 ± 0.11 mm, $n=6$), indicating enhancement of cardiac contractility. Furthermore, SOD2-tg hearts exhibited a significant increase in mitral valve E/A ratio (from 1.27 ± 0.04 to 1.65 ± 0.1 mm, $n=6$), suggesting improving diastolic filling. No significant difference in the body weight (in gm) was detected between WT and SOD2-tg mice (male: 29.4 ± 1.1 vs 27.2 ± 0.6 , $n=7$, $p=0.08$; female: 23.4 ± 0.1 vs 22.8 ± 0.4 , $n=6$, $p=0.21$; age of 12-13 weeks). However, echocardiography also revealed a phenotype of physiological hypertrophy characterized by a larger relative wall thickness and a higher ratio of LV mass to body weight (Fig. 2 and Table I).

3.3 Cardiac-specific overexpression of SOD2 results in decreasing mean arterial pressure (MAP) and enhanced myocardial blood flow (MBF)

Contrast echocardiography was employed to evaluate hemodynamic effects mediated by SOD2 overexpression. Under the basal conditions, the mouse model of SOD2-tg exhibited a significantly lower heart rate (HR) and mean arterial pressure (MAP) (for HR, 388 ± 13 bpm vs 519 ± 18 bpm, Figs. 3A-3B); the norepinephrine (NE)-mediated increase in MBF was also significantly enhanced (Fig. 3C). When plotting MBF against the double product ($\text{HR} \times \text{MAP}$) for both wild type control and SOD2-tg mice, we observed that the coupling of MBF to the double product is leftward- and upward- shifted in the SOD2-tg mice compared to the

control mice (Fig. 3D), supporting the conclusion that the enhanced cardiac function resulted from a better efficacy of myocardial perfusion induced by overexpressing SOD2.

3.4 Enhancement of mitochondrial function in the myocardium of SOD2-tg mice

Age-matched wild-type mice were used as controls. Mitochondria were prepared from the murine myocardium by differential centrifugation, and then subjected to oxygen consumption rate (OCR) measurements [26, 28]. Fig. 4A shows a typical profile of oxygen consumption by the mitochondria from the myocardium of wild type and SOD2-tg mice. State 3 (ADP-dependent) and state 4 (ADP-independent) respiration of mitochondrial preparations was measured by oxygraph. The NADH-linked and ADP-stimulated O₂ consumption rate (state 3) declined from 155.8 ± 5.3 to 108.4 ± 3.6 (in nmol O₂/min/mg protein, wild-type vs SOD2-tg, $p < 0.001$, $n = 12$, in Fig. 4B). The ADP-independent OCR (state 4) decreased from 25.4 ± 1.6 to 19.8 ± 2.1 ($p < 0.05$, $n = 12$). Although the State 3 OCR was decreased, the respiratory control index (defined as the ratio of state 3 to state 4) remained the same (6.32 ± 0.37 for wild type and 6.02 ± 0.49 for SOD2-tg, $p = 0.38$, $n = 12$, in Fig. 4C), which indicated the overexpression of SOD2 did not compromise the mitochondrial integrity.

FCCP (2.5 μ M) was added to uncouple the mitochondria and maximize the respiratory capacity. We observed that NADH-linked and FCCP uncoupling of OCR was significantly decreased from 171.7 ± 12.0 to 101.2 ± 3.8 ($n = 12$, $p < 0.001$, Fig. 4B), implying that a down-regulation of the reserved bioenergetics capacity was induced by SOD2 overexpression in murine heart.

The ATP production flux mediated by isolated mitochondria was induced by glutamate and malate (NADH-linked) under state 3 conditions, and determined by luciferase assay. The ATP production flux remained the same in the isolated mitochondria (213.2 ± 7.9 vs. 198.0 ± 6.9 nmol ATP produced/min/mg protein, WT vs. SOD2-tg, $n = 10$, $p = 0.18$). Because the SOD2-tg consumed less O₂ in state 3, the ATP production/O₂ consumption ratio (nmol ATP produced/nmol O₂ consumed, and is abbreviated as ATP/O ratio) was significantly increased (1.32 ± 0.05 vs. 2.12 ± 0.12 , WT vs. SOD2-tg, $n = 6$, $p < 0.001$). The increased ATP/O ratio indicated an enhanced bioenergetic efficiency of mitochondria, which may lead to improved heart function.

Because the SOD2-tg mice exhibited a lower blood pressure phenotype, we thought of using a hypertensive model as a control of the above mitochondrial bioenergetics efficiency study. The nitric oxide synthase (NOS) inhibitor L-NAME is known to induce arterial hypertension and microvascular lesions linked to mitochondrial dysfunction, as well as impairing cardiac function in vivo [28, 36, 37]. The mouse model of L-NAME-treated wild type was thus served as a negative control. Oral administration of L-NAME (1 mg/ml in drinking water, 3 days) to wild type mice significantly decreased mitochondria-mediated ATP generation in response to NADH-linked O₂ consumption (ATP/O ratio, 1.32 ± 0.05 for WT vs 0.93 ± 0.09 for WT+L-NAME; $n = 6$; $p < 0.001$, Fig. 4D), indicating a reduction in bioenergetic efficiency. Interestingly, the L-NAME negatively impacted the ATP flux (141.8 ± 9.1 nmol ATP produced/min/mg protein, $n = 6$, $p < 0.001$; vs. WT), but not state 3 OCR (data not shown) which was consistent to previous report [28]. Whereas, L-NAME treatment had no

significant impact on the ATP flux (198.0 ± 6.9 vs 201.7 ± 11.2 , $n=5-10$, $p=0.76$) and state 3 OCR. Overexpressing SOD2 in myocardium significantly preserved the ATP/O ratio of mitochondria from the mouse heart of L-NAME-treated SOD2-tg as indicated in the Fig. 4D, thus reinforcing cardioprotective effect of SOD2. EPR spin trapping analysis further revealed no detectable superoxide generation mediated by the mitochondria of L-NAME-treated SOD2-tg, reinforcing overexpressing SOD2 suppressed ROS overproduction induced by L-NAME treatment.

Adenylate kinase 2 (AK2) resides in the mitochondrial inter membrane space and it supports ADP/ATP flux exchange [38]. We did not observe significant alteration in AK2 protein expression levels (Western blot using a polyclonal antibody against AK2 from Santa Cruz Biotechnology Inc, $n=7$, data not shown) between heart mitochondria of SOD2-tg and the WT. It has been shown that the Ap5A [P1,P5-di(adenosine-5') pentaphosphate], an AK2 inhibitor, has no effect on the mitochondria respiration rate [39], and we have confirmed the results ($n=3$, data not shown). After AK2 inhibition, both wild type and cardiac-specific overexpression of SOD2 exhibited an enhanced ATP production flux and ATP/O ratio of mitochondria (2.31 ± 0.15 vs. 3.01 ± 0.03 , WT vs. SOD2-tg, $n=6$, $p<0.01$, in Fig. 4D). Our findings indicated an improved bioenergetic efficiency of SOD2-tg, and adenylate kinase 2 could not be completely accounted for this enhancement.

3.5 Overexpression of SOD2 in the HL-1 myocytes

We further evaluated the above results in a model of isolated myocytes, the HL-1 cell line, using an extracellular flux analyzer (Seahorse Bioscience, Billerica, MA). We hypothesized that SOD2 overexpression in the HL-1 cell line could recapitulate the bioenergetic effect of the mitochondria from cardiac-specific SOD2-tg mice. Transfection of the HL-1 culture with pBI-eGFP-SOD2 resulted in overexpression of green fluorescent protein (GFP) and human SOD2 by 2.3-fold in mitochondria (Fig. 5A, upper panel). The scramble plasmid pGFP (from CloneTech Laboratory, Inc.) was employed as an additional control to express only GFP in HL-1 cells. The pBI-eGFP-SOD2 transfection resulted in a dramatic reduction in antimycin A-stimulated O_2^- production by living HL-1 cells, as detected by EPR spin-trapping (Fig. 5A, lower panel), confirming the functional expression of human SOD2 in HL-1 cells. A decrease in the FCCP uncoupling OCR and reserve bioenergetic capacity in HL-1 was further indicated as a result of overexpressing SOD2 in mitochondria as shown by extracellular flux analysis (Fig. 5B). The parallel HL-1 data thus corroborated our findings in studies using mitochondria from the mouse heart of SOD2-tg (Fig. 4B). Further extracellular flux analysis using palmitate as the substrate showed a similar result, indicating significant reduction of fatty acid oxidation in HL-1 cells with an enhanced SOD2 expression by transfection of pBI-eGFP-SOD2 (Supplementary Figure 1).

3.6 The enzymatic activities of ETC and antimycin A-sensitive semiquinone in the mitochondria of SOD2-tg myocardium

Analysis of ETC activities in the mitochondria indicated that overexpressing SOD2 significantly decreased complex I activity (by $34.8 \pm 3.3\%$, $n=6$; in Fig. 6A), which was correlated to down-regulation of the protein expression of complex I in mitochondria (Fig. 6B). A stable semiquinone radical formed at the Q_i site of mitochondrial complex III is EPR

detectable and sensitive to antimycin A. When the mitochondria isolated from SOD2-tg myocardium were subjected to low temperature EPR measurement at 100 K, a significant decrease (by $60.6 \pm 11.5\%$, $n=3$, Fig. 6C) in the antimycin A-sensitive semiquinone ($g=2.008$) was detected. However, no significant changes between wild type and SOD2-tg mice were detected in the absolute electron transport activity and protein expression (data not shown) of complex III (enzymatic activity of QCR in Fig. 6A) in mitochondria. Further analysis revealed no significant change in complex II activity (SQR) (Fig. 6A). However, a decrease (by $34.8 \pm 8.8\%$, $n=6$) in the enzymatic activity of the supercomplex (SCR) containing complex II and complex III (Fig. 6A) was detected, suggesting that a reduction of Q_i^- weakened the protein-protein interaction between complex II and complex III in the mitochondria of SOD2-tg mice.

To further confirm the role of ubiquinone in the partial function of mitochondrial SCR from the SOD2-tg murine heart, Q_2 was incubated with mitochondria at 0°C prior to the assay of SCR activity. The activity of mitochondrial SCR was restored to the level of wild type mitochondria when mitochondrial preparation of SOD2-tg mice was externally reconstituted with ubiquinone-2 (Q_2) (Fig. 6A, *inset in gray box*), presumably due to reestablishing interaction between complex II and complex III.

3.7 Overexpression of SOD2 enhances generation of metabolic H_2O_2 by the isolated mitochondria from the myocardium

The generation of *metabolic* H_2O_2 (induced by glutamate plus malate; NADH-linked) mediated by mitochondria was measured by Amplex® Red/HRP. Mitochondria-derived H_2O_2 production was significantly higher under state 4 conditions. The amount of H_2O_2 produced by the mitochondria of SOD2-tg was increased to 2.88 ± 0.13 (in nmol/min/mg; 0.98 ± 0.10 for wild type control; $p < 0.001$, $n=5$) under state 3 conditions, and to 4.78 ± 0.21 (1.76 ± 0.09 for wild type; $p < 0.001$, $n=5$) under state 4 conditions (Fig. 7).

3.8 Differential regulation of catalase activity in the murine heart of SOD2-tg

Catalase was found to be present in murine and rat cardiac mitochondria [40, 41]. The enzymatic activities of catalase in the mitochondrial preparation and cytosolic fraction containing peroxisome were assessed by measuring H_2O_2 consumption or oxygen evolved rate. Overexpressing SOD2 in myocytes marginally enhanced protein expression of catalase in mitochondria (by $15.9 \pm 3.3\%$, $n=4$, probed with anti-catalase Ab and 70 kDa subunit of complex II as a loading control in supplementary Figure 2A), and its activity from 10.9 ± 0.4 to 12.9 ± 0.2 (in nmol H_2O_2 consumption/min/mg protein, $p < 0.01$, $n=4$ in Fig. 8A), which was confirmed by an enhanced oxygen evolved rate mediated by SOD2-tg mitochondria (shown in Fig. 8B). Whereas we have further detected a down-regulation in the protein expression of catalase (by $22.6 \pm 5.2\%$, $n=6$, probed with anti-catalase Ab in supplementary Figure 2B) and catalase activity (from 17.4 ± 0.3 to 14.1 ± 0.4 , $p < 0.001$, $n=7$, Fig. 8C) from the cytosolic fraction of SOD2-tg murine heart, indicating a differential regulation of catalase activity between the compartments of mitochondria and cytosol in the murine heart of SOD2-tg.

3.9 Effect of catalase infusion on the mean arterial pressure of SOD2-tg mice

We then evaluated whether increased H_2O_2 metabolized by mitochondria was responsible for the decreased MAP observed in the mouse model of SOD2-tg (Fig. 3B). Infusion of catalase has been widely used to reduce H_2O_2 in vivo [16-18]. Intravenous infusion of catalase (15,000 units/kg and 35,000 units/kg) partially increased the MAP of SOD2-tg mouse to the normal level observed in WT at the dosage 35,000 units/kg (shown in the Fig. 8D), suggesting that H_2O_2 metabolized by the mitochondria of the myocardium functions as the metabolic dilator.

3.10 Redox status in the myocardium of SOD2-tg mice

The redox status of the myocardium was subjected to analysis of the redox activity of oxidizing CM-H (1-Hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine. HCl, a spin probe of cyclic hydroxylamine) to a stable nitroxide with EPR. Under non-energized conditions (without external NADH or succinate stimulation), the redox activity of a wild type myocardium is 1.76 ± 0.15 nmol nitroxide produced/min/mg protein of tissue homogenate (based on spin quantitation, $n=6$). We found that the redox status of the myocardium from SOD2-tg mice was more reduced and less oxidized, based on their lower activity of conversion of CM-H to stable nitroxide (decreased to 0.97 ± 0.07 , $n=6$ and $p < 0.001$, Fig. 9).

It has been documented that the oxidation of cyclic hydroxylamine is often dependent on $\cdot\text{O}_2^-$ [42]. Ex vivo addition of polyethylene glycol-linked superoxide dismutase (PEG-SOD) significantly decreased the redox activity (in oxidation of CM-H) of WT myocardium by 27.8 % under the basal/non-energized conditions (decreased to 1.27 ± 0.13 , $n=3$, $p < 0.05$, Fig. 9). Whereas, the presence of PEG-SOD did not affect the redox activity of SOD2-tg myocardium (Fig. 9), confirming that SOD2 overexpression in vivo eliminated excess mitochondrial $\cdot\text{O}_2^-$; mediating a more reduced physiological setting of myocardium. This result basically corroborates the data from the EPR spin trapping with DMPO shown in the Fig. 1.

3.11 Up-regulation of GSH/GSSG and down-regulation of GPx2 activity

Overexpression of SOD2 resulted in significantly enhanced reductive status of the GSH pool in the mitochondrial fraction of the myocardium. We determined GSH and GSSG levels quantitatively by the enzymatic recycling method, observing (i) a significant reduction of GSSG levels in the mitochondrial preparation from SOD2-tg murine hearts (from 0.100 ± 0.010 to 0.066 ± 0.007 nmol/mg protein, $p < 0.05$, $n=6$), and (ii) a significant up-regulation in the GSH/GSSG ratio of the mitochondria (from 41.94 ± 2.03 to 73.58 ± 10.57 , $p < 0.05$, $n=6$) as shown in Table II. However, in the cytosolic fraction of SOD2-tg murine hearts, the reductive status of the GSH pool was only increased modestly (Table II).

The enzymatic activity of glutathione peroxidase (GPx2) in the mitochondria was further analyzed by using *tert*-butyl hydroperoxide plus GSH as the substrates, and coupled with the NADPH consumption rate mediated by purified yeast glutathione reductase. As a result of SOD2 overexpression in the murine heart, the activity of GPx2 was down-regulated, decreasing from 18.6 ± 0.6 (in nmol/min/mg protein) to 11.4 ± 0.7 ($p < 0.001$, $n=9$). The result

indicated a decreased enzymatic conversion of GSH to GSSG, thus supporting up-regulation of GSH/GSSG in the murine heart of SOD2-tg (Table II).

DISCUSSION

4.1 Novelty

In this study we have shown that cardiac-specific overexpression of SOD2 mediates a superior heart function via improved mitochondrial function due to preserving mitochondrial integrity with an enhanced ATP/O ratio. We have also demonstrated that in a SOD2-tg mouse model, cardiac-specific SOD2 overexpression mediates mean arterial pressure and myocardial blood flow in a redox-dependent manner, leading to a phenotype of supernormal cardiac function.

4.2 Mitochondrial function in the SOD2-tg mouse heart

As indicated by respiratory analysis, a decline in state 3, state 4, and FCCP-uncoupling OCR suggested a reduction in the reserved respiratory capacity in the isolated mitochondria of SOD2-tg mouse hearts (Fig. 4B). This result was corroborated by our extracellular flux analysis (Fig. 5B); HL-1 cells transfected by pBI-eGFP-SOD2 exhibited decreased reserved capacity. It is likely that the down-regulation of state 3 and state 4 OCRs by mitochondria resulted from decreased electron transport to molecular oxygen in response to SOD2 overexpression. Overexpressing SOD2 decreased FCCP-mediated electron transport activity from NADH to O₂ (Fig. 4B) due to declined electron flow in the electron transport chain. Current results clearly indicated down-regulated electron transport activities of complex I and the supercomplex of SCR hosting complex II and complex III (Fig. 6A). Analysis of the individual ETC complex activity in mitochondria indicated decreased complex I activity, presumably due to down-regulated protein expression in SOD2-tg mitochondria (Fig. 6B), and diminished SCR activity was caused by a decrease in antimycin A-sensitive semiquinones in SOD2-tg mitochondria (Fig. 6C), and can be restored by external addition of Q₂ (Fig. 6A, *gray inset*).

Despite the decreased mitochondria-mediated OCR, it was intriguing that SOD2 overexpression was capable of maintaining mitochondrial integrity (as shown by the unchanged respiratory control index) and the ATP generation flux (Fig. 4C-4D). It should be noted that the concentration of ADP and ATP in a heart *in vivo* are tightly regulated [43, 44]. The energy produced by mitochondria is converted to phosphocreatine and dissipated. In isolated mitochondria, a surge of ADP was employed to stimulate the maximum oxygen consumption (state 3 in the Fig. 4, A-B). In the absence of cytosolic creatine phosphate shuttle, the accumulated ATP could therefore be measured (Fig. 4D).

The FCCP induced uncoupling and maximized OCR (Fig. 4 and Fig. 5). However, it should be noted that the maximized oxygen consumption does not necessarily translate into maximal ATP production. The reduced FCCP uncoupling OCR in the mitochondria of SOD2-tg myocardium was likely due to down-regulation of complex I (Fig. 6A-6B) and semiquinone (Fig. 6C) which would decrease OCR induced by electron transport and the OCR caused by [•]O₂⁻ production mediated by complex I, semiquinone, and complex III.

Because ATP production remained the same in the myocardial mitochondria of wild type and SOD2-tg mice, a better coupling of oxygen consumption to ATP production (higher ATP/O ratio in the Fig. 4D) can be concluded in the mitochondria of SOD-tg heart.

Adenylate kinase 2 (AK2) mediates a fully reversible reaction in converting ATP and AMP to ADP ($G^{0'} \approx 0$). The presence of AK2 inhibitor, Ap5A, enhances ATP generation by oxidative phosphorylation (Fig. 4D). Inhibition of AK2 thus restores and normalizes the ratio of ATP/O as mediated by the isolated mitochondria of murine heart. The measured Ap5A-normalized ATP/O ratio is consistent to the theoretical and experimental values of P/O ratio (≈ 2.3 –3) as reported and reviewed in the literatures [30, 45, 46].

4.3 Physiological redox setting in the murine heart of SOD2-tg

The results obtained from EPR using the spin probe cyclic hydroxylamine (CM-H) indicated overall a more reductive status detected in the SOD2-tg murine heart versus its wild type littermate (Fig. 9). No detectable $\cdot\text{O}_2^-$ -dependent redox activity mediating CM-H oxidation was observed in the myocardium of SOD2-tg (Fig. 9B). It is plausible that the redox alteration in the myocardium is the consequence of increased myocardial SOD2 expression. These results are consistent with the redox status of mitochondrial thiols and the quinone pool in the SOD2-tg murine heart, which showed elevated mitochondrial GSH/GSSG and cytosolic GSH/GSSG ratios (Table II), down-regulated enzymatic activity of GPx2, and decreased semiquinone anion radical in the mitochondria (Fig. 6C). A modest up-regulation of mitochondrial catalase that directly reduces H_2O_2 without involving in additional reducing power may further help maintaining high GSH/GSSG (Fig. 8A and Table II). Recently, we have reported that oxidative stress in vivo and in vitro can induce protein radicals of mitochondrial complex I [28, 31, 47]. We further demonstrated that a highly reductive *milieu* generated by SOD2 overexpression diminished complex I-derived protein thiyl radicals detected by immune-spin trapping technique in the murine heart of SOD2-tg [28]. The depression of complex I-derived thiyl radical(s) formation in the mitochondria of SOD2-tg murine hearts provides further evidence in support of a more reductive status. Thus, an increased functional expression of SOD2 conclusively shifted the redox balance in myocytes to a more reductive *milieu*, which may help counter oxidative stress induced by excess H_2O_2 (*vide infra*).

4.4 Supernormal cardiac function of SOD-tg mice

It is likely that the overexpression of SOD2 in mitochondria enhanced the ATP/O ratio and the efficacy of excitation-contraction coupling, and contributed to the detected supernormal phenotype of cardiac function. The heart requires a constant supply of energy in order to support contractile activity; therefore, maintaining the supernormal cardiac function has a higher energy demand. Our results suggest that an enhanced mitochondrial ATP/O ratio leading to a higher efficiency of oxidative phosphorylation should support and facilitate the bioenergetics of superior contractility as characterized in the murine heart of SOD2-tg using echocardiography (Fig. 2 and Table I). Furthermore, a more reductive *milieu* in the myocardium likely minimizes the electron leakage under the conditions of state 3 oxygen consumption, maximizes the efficiency of electron transport and ATP production, and potentiates the development of the observed supernormal phenotype.

The production of H_2O_2 metabolite mediated by the mitochondria of SOD2-tg mice was significantly enhanced compared with that of the wild type littermates (Fig. 7). EPR measurements indicated no detectable $\cdot\text{O}_2^-$ generated by the SOD2-tg mitochondria, which indicates high efficacy in converting $\cdot\text{O}_2^-$ to H_2O_2 (Fig. 1, e). Moreover, a down-regulation of GPx2 activity in mitochondria would synergistically facilitate the accumulation of H_2O_2 in mitochondria and in the extra-mitochondrial compartment. However, excess H_2O_2 in the SOD2-tg mitochondria was not expected to induce oxidative damage to impair mitochondrial function (Fig. 4), coronary blood flow (Fig. 3C), and cardiac work (coupling of MBF to $\text{HR} \times \text{MAP}$ in Fig. 3D). Instead, a marginal enhancement of mitochondrial catalase activity was expected to alleviate H_2O_2 -induced oxidative stress in mitochondria (Fig. 8A-8B), and a highly reductive *milieu* neutralized H_2O_2 -induced oxidative stress, and thus prevented oxidative injury in the myocardium of SOD2-tg mice.

Metabolic dilation in the heart is partially mediated by H_2O_2 . H_2O_2 originating from mitochondrial $\cdot\text{O}_2^-$ in the vasculature has been described as a hyperpolarizing factor that acts as an intracellular signaling molecule to mediate shear- or acetylcholine-induced smooth muscle relaxation [1-3]. In cardiac myocytes, H_2O_2 derived from mitochondrial $\cdot\text{O}_2^-$ has also been proposed to regulate collateral blood flow via underlying diffusion to the microvascular system of the heart [4-6]. Therefore, enhanced mitochondrial H_2O_2 production coupled with a more reductive status in the cardiac myocytes of SOD2-tg would mediate metabolic flow regulation via a decreased rate pressure product and more effective myocardial blood flow (Fig. 3C-3D). In brief, metabolic H_2O_2 induced by overexpressing SOD2 functions as a coronary vasodilator to increase perfusion and enhance myocardial contractility. A down-regulation of cytosolic catalase activity of SOD2-tg murine heart may facilitate the above process (Fig. 8C). Evidence from this study has reinforced the contention that mitochondrial H_2O_2 is the vasoactive product of metabolically active cardiac myocytes, functioning as a coronary metabolic dilator coupling myocardial oxygen consumption to coronary blood flow. In the SOD2-tg mouse model, our results have been translated to the novel finding, showing that the coupling of myocardial oxygen consumption to myocardial blood flow can be effectively regulated by a more reductive physiological setting in the myocardium.

Clinically, overexpression of SOD2 can sensitize a variety of cancer cell lines to many anticancer drugs. In the case of anticancer drug, BCNU, studies have shown cancer cells can be sensitized to cell killing by BCNU-mediated inhibition of peroxide removal and raising peroxide level via gene delivery of SOD2. The findings of current studies thus supports that clinical use of increasing SOD2 expression may combine with chemotherapy to reduce the adverse effect of heart failure through reinforcing the mechanism of improved mitochondrial bioenergetics and myocardial blood flow. Our previous work has indicated mitochondrial integrity and systolic function were preserved in the mouse model of SOD2-tg subjected to BCNU treatment [29], thus supporting increased SOD2 expression as a potential treatment strategy for cancer patients who receives chemotherapy in alleviating systolic dysfunction and related cardiomyopathy.

5. Conclusion

The present studies provide insights into the mechanism by which SOD2 overexpression in vivo mediates development of a supernormal cardiac function. The underlying mechanism is characterized by preserved mitochondrial membrane integrity, increased ATP production flux in response to oxygen consumption, increased reductive status in the mitochondria of myocytes, enhanced H₂O₂-mediated rate pressure product, and associated myocardial blood flow as illustrated in the diagram of Fig. 10. In myocyte mitochondria, transgenic overexpression of SOD2 increases the efficacy of converting $\cdot\text{O}_2^-$ to H₂O₂, which serves as a metabolic vasodilator to persistently induce smooth muscle relaxation and enhance myocardial blood flow in the vasculature. Persistently improved myocardial perfusion would favor the development of supernormal cardiac function. The mechanism addressed here provides a useful concept for understanding the fundamental mechanism of mitochondria-mediated metabolic dilation in vivo. Recognition of this mechanism is valuable in understanding the fundamental basis by which overexpressing SOD2 regulates intracellular signaling in the cardiovascular system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

SOD2	manganese-containing superoxide dismutase
SOD2-tg	transgenic mice with cardiac-specific SOD2 overexpression
RPP	rate pressure product
MBF	myocardial blood flow
HR	heart rate
MAP	mean arterial pressure
EDHF	endothelium-derived hyperpolarizing factor
GPx2	mitochondrial glutathione peroxidase
NQR	NADH ubiquinone reductase, or mitochondrial Complex I
SCR	succinate cytochrome <i>c</i> reductase
$\cdot\text{O}_2^-$	superoxide anion radical
ROS	reactive oxygen species
ETC	electron transport chain
OCR	oxygen consumption rate

RCI	respiratory control index
Q₁	ubiquinone-1
Q₂	ubiquinone-2
DMPO	5,5-dimethyl pyrroline N-oxide
CM-H	1-Hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine•HCl
FMN	flavin mononucleotide
FCCP	carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
GSH	glutathione
L-NAME	L-nitro-arginine methyl ester
Ab	antibody
SDS-PAGE	SDS polyacrylamide gel electrophoresis
EPR	electron paramagnetic resonance
PBS	phosphate buffered saline
ATP/O	the ratio ATP production rate to oxygen consumption rate

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Fig. 1. Cardiac-specific SOD2 overexpression diminished O_2^- mediated by mitochondria
 Hearts were removed from cardiac-specific SOD2-tg and wild type mice, and subjected to mitochondrial and cytosolic preparations. In the top panel, mitochondria (mito., 100 μg loaded) and cytosol (100 μg loaded) were immunoblotted with anti-GAPDH monoclonal antibody (1:500). In the middle panel, mitochondria were immunoblotted using anti-SOD2 Ab (1:2000), an antibody against the 70 kDa subunit of complex II (1:30,000), and anti-Cox I Ab (1:3000). Lower panel, *b* and *e*: O_2^- generation mediated by mitochondria isolated

from murine hearts of wild type and SOD2-tg mice, respectively; *c* and *f*: the same as *b* and *e*, except that antimycin A (10 μ M) was included in the system.

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A. wild type mouse **B. SOD2-tg mouse**

Table I: Effect of cardiac function by conditional over expression of SOD2 in the myocardium as determined by echocardiography.

	Wild type	SOD2-tg
%, ejection fraction	74.1±2.3	89.3±1.6**
%, fractional shortening	42.5±2.0	58.9±2.4**
mm, LVID; diastolic	3.82±0.04	3.36±0.11**
mm, LVID; systolic	2.20±0.09	1.38±0.08***
μl, LV Vol.; diastolic	62.9±1.6	46.4±3.8**
μl, LV Vol.; systolic	16.3±1.6	4.9±0.7***
mm, relative wall thickness	0.46±0.01*	0.65±0.05**
ratio (mg/g), HW/BW	3.37±0.18	4.10±0.11*
ratio, MV E/A	1.27±0.04	1.65±0.10*

LVID, left ventricle internal dimension; LV, left ventricle; HW, heart weight; BW, body weight; MV E/A, mitral valve controlling the ratio of the early (E) to late (A) ventricular filling velocities, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, $n=6$.

Fig. 2. Cardiac structure and enhanced function are detected in SOD2-tg mice by echocardiography

A and B, Representative images taken using 12-week-old mice. The images demonstrate intact left ventricular (LV) wall integrity, greater wall thickness, and reduced chamber size in SOD2-tg hearts ($n=6$). Echocardiographic assessment of wild type and SOD2-tg mice (12 weeks old, $n=6$) were summarized in the Table I.

Fig. 3. Cardiac-specific SOD2 overexpression decreases heart rate in *A*, mean arterial pressure (MAP) in *B*, increases norepinephrine (NE)-mediated myocardial blood flow (MBF) in *C*, and enhances the efficacy of coupling RPP to MBF in *D*. * $p < 0.05$, ** $p < 0.01$ ($n = 6$)

Fig. 4. Mitochondria were prepared from the myocardia of wild type, SOD2-tg (*in A-D*) and L-NAME-treated wild type mice (*in D*), and then subjected to measurement of oxygen consumption rate (OCR) by oxygen polarography at 30 °C and NADH-linked ATP production as described under “Experimental Procedures”. **A-B**, state 3, state 4, and FCCP-mediated oxygen consumption rates by the isolated mitochondria. **C**, Respiratory control index obtained from the ratio of state 3 OCR to state 4 OCR. **D**, the ATP/O ratio is calculated from the ratio of ATP production rate to state 3 OCR. The effect of AK2 inhibitor was assessed by pre-incubation of as-isolated mitochondria with Ap5A (20 μM) prior to ATP production assay. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, assessed by student's *t*-test between WT control and SOD2-tg (*in B-C*, $n = 12$) or WT control and L-NAME-treated WT ($n = 6$) or L-NAME-treated WT and L-NAME-treated SOD2-tg ($n = 5$) or WT+Ap5A and SOD2-tg+Ap5A ($n = 6$) or L-NAME-treated WT+Ap5A and L-NAME-treated SOD2-tg+Ap5A ($n = 3$) (*in D*). Comparison among the three groups (WT control, SOD2-tg, and L-NAME-treated WT) in *D* was analyzed by one-way ANOVA followed by Tukey's post hoc test, indicating

that there is a significant difference among the three groups ($p < 0.001$). There is no significant difference between SOD2-tg and L-NAME-treated SOD2-tg or between SOD2-tg+Ap5A and L-NAME-treated SOD2-tg+Ap5A.

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Fig. 5.

A, Upper panel, increased SOD2 expression in the HL-1 by pBI-eGFP-SOD2 transfection was measured by Western blotting (80 μg of cell lysates for each sample was loaded) using a polyclonal antibody against SOD2 (400 $\times$), and the 70 kDa FAD-binding subunit of Complex II (20,000 $\times$) was used as a loading control. **Lower panel,** the effect of SOD2 overexpression in HL-1 on the antimycin A-stimulated O_2^- production measured by EPR spin-trapping with DMPO. The pGFP was employed as a control scramble plasmid. 1.2×10^7 of HL-1 myocytes ($\sim 6.5 \text{ mg/ml}$) were used for EPR assay. **B, Upper panel,** the chart of

extracellular flux analysis showing representative measurements of the percentage decrease in oxygen consumption rates (OCR) and relative rates in control, peGFP (scramble plasmid) transfected, and pBI-eGFP-SOD2 transfected HL-1 myocytes in response to glucose (10 mM) and pyruvate (0.2 mM) as the substrates. *Lower panel*, the effect of overexpressing SOD2 in HL-1 through pBI-eGFP-SOD2 transfection on the OCR of HL-1 myocytes was measured by extracellular flux analyzer.

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Fig. 6.

Hearts were removed from wild type and SOD2-tg mice, and subjected to mitochondrial preparation. **A**, the activities of electron transport chain components in the as-isolated mitochondria were assayed by the methods described in *Experimental Procedure* ($n=6$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$). Inset in gray box, the mitochondrial preparation (5 mg/ml) was incubated with Q₂ (20 μ M) in ice for 30 min. An aliquots of Q₂-reconstituted SOD2-tg mitochondria (SOD2-tg mito. + Q₂) was then subjected to assay for SCR activity. **B**, Protein expression of complex I in the mitochondria and SMP was assessed by Western

blot using antibodies against the 51 kDa subunit (nuclear DNA-encoded, Ab51, 10,000 × and ND1 (mitochondrial DNA-encoded, 1,000 ×) of complex I [48]. The protein expression levels of subunit I (70 kDa flavoprotein, nuclear DNA-encoded, 20,000×) of complex II in mitochondria and subunit I (COXI, mitochondrial DNA-encoded, 4,000×) of complex IV in SMP were used as loading controls. The density ratio of the blotting signals was quantitated by Image J software ($n=6$ for 51 kDa/70 kDa in mitochondria, $p<0.001$; $n=4$ for ND1/COXI in SMP, $p<0.05$). *C*, A mitochondrial preparation (15 mg/ml in M-buffer) was frozen in liquid N₂, and then transferred to an HS cavity equipped with a cryostat operated at 100° K. *Left*: the spectra of antimycin A-sensitive semiquinone were obtained from the differential spectra of mitochondria with or without antimycin A treatment (50 μM). *Right*: the spin quantitation of ubisemiquinone was obtained by double integration of simulation spectrum ($n=3$, $*p<0.05$). EPR setting: center field, 3350 G, sweep width: 100 G, power: 20 mW, receiver gain: 5×10^4 , modulation amplitude: 0.8 G, time constant: 81.92 ms, conversion time: 838.8 ms/G, number of scan: 20.

Fig. 7. *A and B*, The kinetics of H₂O₂ generation mediated by mitochondria in the presence of glutamate and malate (NADH-linked) under state 3 and state 4 conditions was assessed by an HRP/Amplex Red assay ($n=5$). *C*, Rate of H₂O₂ generation mediated by the mitochondria of WT and SOD2-tg murine hearts ($n=5$, *** $p<0.001$).

Fig. 8.

A-C, Overexpressing SOD2 in myocytes enhanced the catalase activity in mitochondria (**A** and **B**, $n=4$, $**p < 0.01$), and decreased the activity of cytosolic catalase (**C**, $n=7$, $***p < 0.001$). The enzymatic activity of the catalase were measured at 25 ° C as described in “Experimental Procedures”. **D**, The effect of H₂O₂ on the mean arterial pressures of wild type and SOD2-tg mice was elevated by intravenous injection of catalase (15,000 and 35,000 units/kg). Mouse MAP was accessed by the approach of jugular and femoral characterization as described in the “Experimental Procedures”. The MAP of SOD2-tg was

significantly elevated and normalized to the level of wild type by infusion of catalase at a dosage of 35,000 units/kg ($n=4$, $*p<0.05$).

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Fig. 9. The redox activities of the myocardium from wild type and SOD2-tg mice were measured by EPR with the cyclic hydroxylamine spin probe CM-H

A, The tissue homogenates (0.5 mg/ml) of wild type or SOD2-tg myocardium in SHE buffer (250 mM sucrose, 10 mM HEPES, and 1 mM EDTA, pH 7.4) containing 1 mM DTPA and 1 mM CM-H were subjected to EPR measurement after converting CM-H to a stable nitroxide at 298° K. . The $\cdot\text{O}_2^-$ -dependent CM-H oxidation was assessed by pre-treatment of tissue homogenates with PEG-SOD (0.04 unit/ μl) prior to EPR measurement. **B**, The effect of PEG-SOD on the redox activity of converting CM-H to stable nitroxide is presented in bar graph ($n=3-6$, $**p<0.01$ and $*p<0.05$). The instrumental settings used for detecting the three-line spectrum of the nitroxide are: center field, 3360.3 G; sweep width, 60 G; microwave frequency, 9.43 GHz; power, 20 mW; receiver gain, 5.02×10^3 ; modulation frequency, 100kHz; modulation amplitude, 1 G; time constant, 163.84 ms; conversion time, 41 ms; sweep time, 42 sec; number of X-scans, 1. The parameters for the kinetic mode are: static field, 3360.3 G; receiver gain, 2×10^3 ; time constant, 2624.44 ms; conversion time, 1,000 ms; and sweep time, 300s; number of scan, 1.

Fig. 10. Diagram showing the mechanism by which transgenic overexpression of SOD2 *in vivo* mediates supernormal cardiac function by enhancing metabolic dilation and mitochondrial function

In comparison to basal conditions, overexpression of SOD2 increases conversion of $\cdot\text{O}_2^-$ to H_2O_2 (brick red coarse arrows). Accumulation of H_2O_2 in mitochondria is further facilitated by down-regulation of GPx2. Excess mitochondrial H_2O_2 diffusing to the vasculature serves as a metabolic dilator, mediating smooth muscle cell (SMC) relaxation, enhancing myocardial blood flow (MBF) and cardiac function. The process is also mediated by down-regulation of the catalase activity in the cytosol of myocytes. Oxidant stress induced by excess H_2O_2 is presumably neutralized by a more reductive physiological setting and up-regulation of catalase activity in mitochondria. SOD2 overexpression *in vivo* further down-regulates the function of complex I, complex III, and the SCR supercomplex to

minimize the electron leakage, preserve membrane integrity, and increase ATP production in response to oxygen consumption (*i.e.* increasing ATP/O ratio, indicated by *blue fine and coarse arrows*), thus enhancing mitochondrial function.

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Table I

Effect of cardiac function by conditional overexpression of SOD2 in the myocardium as measured by echocardiography

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LVID, left ventricle internal dimension; LV, left ventricle; HW, heart weight; BW, body weight; MV E/A, mitral valve controlling the ratio of early (E) to late (A) ventricular filling velocities

*
 $p < 0.05$

**
 $p < 0.01$

 $p < 0.001$; $n = 6$.

Table II

Level of GSH pool in the cytosolic compartment and mitochondria from the myocardium of wild type and SOD2-tg mice.

		[GSH]+[GSSG]	[GSSG]	[GSH]/[GSSG]
		(nmol/mg protein)	(nmol/mg protein)	
		Mean±SE	Mean±SE	Mean±SE
<i>n</i> = 6	WT-cytosol	16.22±0.69	0.374±0.044	45.54±4.13
<i>n</i> = 6	SOD2-tg-cytosol	17.31±0.42	0.300±0.049	67.74±13.2
<i>n</i> = 6	WT-mitochondria	4.14±0.30	0.100±0.010	41.94±2.03
<i>n</i> = 6	SOD2-tg-mitochondria	4.53±0.27	0.066±0.007 [*]	73.58±10.57 [*]

^{*}
 $p < 0.05$ compared with WT-mitochondria.