Superoxide Dismutase 2 Overexpression Attenuates Effects of Ischemia Reperfusion-Induced Mitochondrial Dysfunction

A Thesis

Presented in partial fulfillment for the requirements for the degree of Masters of Science in the College of Graduate Studies of Northeast Ohio Medical University

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B.S

Integrated Pharmaceutical Medicine
Northeast Ohio Medical University
2017

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Abstract

Myocardial ischemia reperfusion (IR) injury has been shown to cause mitochondrial dysfunction. The electron transport chain (ETC) is a major source of superoxide and other superoxide derived reactive oxygen species during ischemia and reperfusion. Previous studies suggest that the downregulation of ETC, Krebs cycle, and antioxidant enzymes in the mitochondria occur as a result of increased oxidative stress. SOD2 is one of the primary antioxidants in the mitochondrial matrix. It is capable of scavenging superoxide into hydrogen peroxide. To test the therapeutic potential of increased superoxide scavenging in murine models, we subjected cardiac specific human SOD2 overexpressing (SOD2TG) murine hearts to 30 minutes of ischemia and 45 minutes of reperfusion using the Langendorff isolated heart system. SOD2TG hearts were relatively protected from impairment of electron transport activity in complex I-IV as well as downregulation of Krebs cycle enzyme activities. However, SOD2 overexpressing hearts had selectively decreased basal respiratory enzyme kinetics without indication of impairment of respiratory coupling and hydrogen peroxide scavenging.

Acknowledgements

I would like to thank my advisor, Dr. Yeong-Renn Chen for guiding me through my academic career at Northeast Ohio Medical University. I am also grateful for Chwen-Lih Chen and Dr. Patrick Kang for mentoring me on crucial lab techniques required for my development as a student and researcher.

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Field of Study

Major Field: Integrated Pharmaceutical Medicine

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Introduction

According to the World Health Organization, 17.5 million people die from cardiovascular diseases every year, accounting for 31% of all deaths worldwide. Up to 80% of Cardiovascular deaths are caused by heart attacks and strokes. ("Cardiovascular diseases (CVDs)", n.d). While detrimental to overall global health, cardiovascular diseases disproportionately affect low income individuals in the U.S. It is estimated by the CDC Foundation that nearly 800,000 Americans die from stroke and heart disease every year. Costs of treatments in the U.S due to preventable cardiovascular diseases exert hefty consequences on the American healthcare system; the costs extend to productivity loss in the workforce. Annual direct medical costs are projected to rise to $818 billion by 2030 while indirect losses are projected to exceed $275 billion annually ("Heart Disease and Stroke Cost America Nearly $1 Billion a Day in Medical Costs, Lost Productivity", 2015). The increasing prevalence of myocardial infractions must be addressed in a clinical sense. Patients who suffer from myocardial infarctions have been shown to have increased risk of recurrence of infarctions for at least 7 years after the first incidence (Smolina et al., 2012). Although antiplatelet and antithrombotic treatments are widely used to restore blood flow to infracted areas, there are no effective therapeutic options for injury that occurs during reperfusion after the reestablishment of blood flow

(Hausenloy & Yellon, 2013). More studies are required to fully understand the mechanisms of pathologies associated with ischemia reperfusion injury.

Ischemia reperfusion injuries occur subsequent to myocardial infarctions. The reintroduction of oxygen during reperfusion is required for survival of ischemic cells. Although it may seem paradoxical that the necessary reintroduction of oxygen is detrimental to cell survival, it is well documented that myocardial dysfunction and cellular apoptosis/necrosis persists or even worsens during reperfusion. Reperfusion injury was observed by earlier studies when rat hearts that were re-oxygenated after ischemia suffered massive release of myocardial enzymes (Hearse et al., 1973). Other studies observed contractile dysfunction and irreversible cell death. Calcium loading, cell swelling, and impaired vascular reactivity have all been proposed as mechanisms for myocardial dysfunction (Hansen, 1995; Zweier & Talukder, 2006). It is widely suggested that the myocardial pathologies from ischemia reperfusion injury results from the overproduction of reactive oxygen species or ROS (McCord, 1985). Mitochondrial dysfunction along with myocardial damage coincides with the large bursts of superoxide and other forms of reactive oxygen species during ischemia reperfusion (Zweier et al., 1987; Chouchani et al., 2016). Small amounts superoxides are formed during oxidative phosphorylation in mitochondria under physiological conditions when molecular oxygen is partially reduced by an electron (Murphy, 2009). The proteolyic/lipolytic nature of free oxygen radicals comes from the unpaired electron on molecular oxygen capable of oxidizing biomolecules in the cell. Oxygen derived free radicals largely contribute to cellular dysfunction when the antioxidant system is

overwhelmed by the sudden overproduction of radicals during IR. Electron transport induced leakage of electrons to molecular oxygen occurs largely at complex I to generate superoxide (Turrens & Bolveris, 1980). The increased flux of superoxide occurs when $NADH/NAD^+$ ratios are high and the FMN prosthetic groups are highly reduced (Kussmaul & Hirst, 2006). It has also been shown that high Δp in combination with highly reduced uniquinol supports reverse electron transport (Chance & Hollunger 1961; Chouchani et al., 2016). Another significant source of superoxide production occurs at complex III in the presence of ubiquinol and antimycin, however, complex III superoxide production may be insignificant compared to the amount produced by complex I during reverse electron transport (Murphy, 2009).

Oxidative stress induced by the overproduction of superoxide and other ROS species also impair electron transport chain complexes as observed in hearts subjected to IR. Complex I activity impairment occurs early during the onset of ischemia. Irreversible damage to distal electron transport complexes may occur after 30-40 minutes of ischemia (Lesnefsky et al., 2001). It is speculated that ROS induced cardiolipin loss contributes to the impairment of NADH dehydrogenase activity in complex I (Paradies et al., 2002). However, complex activity loss may also be due to post translational modifications. For example, 4HE protein modifications that reduce mitochondrial respiratory activity have been shown to be related with reperfusion injury and aging (Lucas & Szweda, 1998). Complex I impairment may also be attributed to NADH mediated calcium inhibition and subsequent modifications on sulfhydryl groups on cysteine residues, which may be prevented by superoxide dismutase (Sadek et al., 2004). Depressed GSH/GSSG ratio and

s-glutathionylation in pro-oxidant eNOS knockout myocardium have also resulted in decreased activities in complex I-IV (Kang et al., 2015a).

Downregulation of electron transport activities may impair oxidative phosphorylation, which is essential for maintaining sufficient ATP production for myocardial contracture and ion homeostasis within membrane compartments. The pathological increase in calcium levels results in mitochondrial calcium overload, leading to the opening the MPTP (Carreira et al., 2011; Crompton, 1999). ROS and calcium overload both contribute to mitochondria dysfunction causing apoptosis and necrosis through the induction of a variety of caspases and calpains. In addition to inhibiting the electron transport chain, changes in Krebs cycle enzyme activities after ischemia and reperfusion have also been documented in cardiac tissue. Since NADH linked respiration is widely known to be suppressed after IR injury, it may be possible that decreased NADH generation from the Krebs cycle reduces NADH linked oxygen consumption. Studies show that α-ketoglutarate dehydrogenase and aconitase activity are decreased after reperfusion (Sadek et al., 2002). Decreased ADP linked state 3 oxygen consumption and increased state 4 "leak" respiration are both associated with impairment of oxidative phosphorylation (Xu et al., 2016). Increased state 4 respiration suggests uncoupling of oxygen consumption and inability to maintain Δp between mitochondrial membranes. Mitochondrial dysfunction may be clearly demonstrated with the impairment of the respiratory control index and ATP production after IR (Xu et al., 2016). Declines in state 3 respiration are not rate limited by electron transport activity, but is suggested to be limited by the NADH generation as a result of the decline

in α-ketoglutarate dehydrogenase activity (Lucas & Szweda, 1999). In addition to Krebs cycle enzyme downregulation, $NADP^+$ dependent isocitrate dehydrogenase, an isoform of NAD^+ dependent isocitrate dehydrogenase has also been reported to have decreased activity in the presence of superoxide anions. $NADP^+$ dependent isocitrate dehydrogenase plays a role in regenerating NADPH for producing reduced glutathione via glutathione reductase, an essential antioxidant against oxidative stress (Lee et al., 2001).

Superoxide dismutase 2 is a major antioxidant enzyme present in the mitochondrial matrix capable of scavenging superoxide into less reactive H_2O_2. Overexpression of SOD2 or MnSOD has been shown to protect cardiac function from ischemia as well as diabetes induced oxidative stress (Suzuki et al., 1999; Shen et al., 2006). SOD2 overexpression has also been observed to improve LVDP and decrease infarct sizes after IR injury (Chen et al., 1998). Efforts have been made to increase SOD2 levels and activity in multiple organs such as liver, brain, and heart via pharmacological intervention (Robb et al., 2008). While complete SOD2 inactivation is lethal in mice, $SOD2^{+/-}$ show reduced mitochondrial respiration. NADH-oxidoreductase and aconitase both showed significant reduction, indicative of oxidative stress (Van Remmen et al., 2001). $SOD2^{+/-}$ mice have increased lipid peroxidation and impaired physiological functions after IR injury (Asimakis et al., 2001). This study will focus on cardiac specific overexpression of SOD2 in murine models in attempt to attenuate ROS induced mitochondrial dysfunction caused by ischemia reperfusion.

Methods

Animals

The heart specific SOD2 transgenic mice FVB-SOD2TG (Myh6-SOD2, Tyr 3Pne/J, age of 12–19 weeks) and FVB/NJ (control) were obtained from Jackson Laboratory. Procedures (protocol 15-028) were approved by Institutional Animal Care and Use Committee at Northeast Ohio Medical University (Rootstown, OH) and complied with the Guide for the Care and Use of Laboratory Animals.

Langendorff Heart Preparation

Murine heart aortas were cannulated with a 21g needle and received retrograde perfusion at 100 mmhg pressure at 37 °C with Krebs-Henseleit buffer containing 118 mM NaCl, 11.1 mM Glucose, 1.8 mM $CaCl_2$, 24 mM $NaHCO_3$, 1.2 mM $MgSO_4$, and 4.7 mM KCl, 1.2 mM KH_2PO_4 bubbled with 95%/5% O_2/CO_2. Hearts were equilibrated for 20 minutes for baseline conditions under described conditions. Ischemia reperfusion (IR45) consisted of 30 minutes of stopped buffer flow and 45 minutes of resumed perfusion of Krebs Henseleit buffer. The constant pressure recirculating Langendorff apparatus was purchased from Radnoti (Monrovia, Ca 91016 USA).

Mitochondria Isolation

Mitochondria were prepared from murine myocardium via differential centrifugation established from a previous protocol (Kang et al., 2015b). Hearts were homogenated with 1 unit of nagarse (Sigma-Aldrich, St. Louise, MO) 1 mL mannitol buffer containing 230 mM mannitol, sucrose 70 mM, EDTA 1 mM, EGTA 1 mM, and Trizma 5 mM, pH 7.4. Complete protease inhibitor cocktail (1×, Roche Life Science, Indianapolis, IN) and 1mM PMSF (phenylmethanesulfonylfluoride) were added after 5 minutes incubation on ice. The homogenate was centrifuged for 5 minutes at 500g. The resulting supernatant was then extracted and centrifuged for 10 min at 20,000g to yield mitochondrial pellet. The supernatant was removed and the mitochondrial pellet was suspended with mannitol buffer with 1x Complete protease inhibitor cocktail. The protein concentration of isolated mitochondria preparation was determined via Lowry method with bovine serum albumin as the standard.

Measurement of Oxygen Consumption Rate (OCR) and ATP/O Ratio Assay

Mitochondrial respiration was measured using a Clark-type oxygen electrode (Oxytherm, Hansatech Instruments, Norfolk, England) at 30 °C and reported in nmol O_2 consumed/mg protein/min using NADH-linked respiration buffer containing glutamate and malate (140 mM potassium glutamate, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 5 mM malate, 1 mM Trizma, 2.5 mM phosphate, 0.01 mM cytochrome c, pH 7.4). Isolated mitochondria (0.6 mg protein/mL) was added to the respiration buffer. State 3 oxygen consumption was stimulated by the addition of 0.2 mM ADP. State 4 was then measured

in the presence of 0.2 μM oligomycin**.** Uncoupled oxygen consumption was initiated by 2.5 μM FCCP. State 3 mitochondrial ATP flux was measured using ATP Bioluminescent Assay Kit (Sigma-Aldrich, St. Louis, MO) following the manufacturer's protocol as previous described (Kang et al., 2015b). Isolated mitochondria (0.6 mg/mL) was added into respiration buffer containing 1 mM ADP. State 3 oxygen consumption was measured across a 60 second interval. 5 μL samples were taken at 20 seconds, 40 seconds, and 60 seconds from the reaction chamber into aliquots of 495 μL preheated 70 °C ATP assay buffer. The bioluminescent signal was recorded on the OrionL microplate luminometer. The oxygen consumption and ATP production were compared to corresponding time points from the samples. The ratio was expressed as nmol ATP produced/nmol O2 consumed.

Measurement of Electron Transport Chain Activity

The electron transfer activities of Complex I - IV from the heart mitochondria preparation were determined using Shimadzu 2401 UV-visible spectrophotometer at 25 °C (Kang et al., 2015b). Complex I (NADH-ubiquinone reductase) activity assay was determined by ubiquinone-stimulated NADH oxidation measured by the decreasing absorbance at 340 nm ($\varepsilon = 6.22\ mM^{-1}cm^{-1}$). 500 μL of NADH ubiquinone reductase (NQR) buffer (20 mM potassium phosphate buffer, 2 mM NaN_3, 0.15 mM NADH, pH 8.0) were mixed with 0.1 mM ubiquinone-1 (Q_1). The reaction was initiated by the addition of 8 μL of mitochondria preparation (5mg/mL) and the change in absorbance for NADH was measured over 2 minutes. Complex I activity was expressed as rate of oxidation of

NADH to NAD^+ (nmol NADH oxidized /mg protein/min). Complex II (succinate-ubiquinone reductase) activity assay was determined by ubiquinone-2 (Q_2) stimulated dichlorophenolindophenol (DCPIP) reduction 50μM Q_2 was mixed with 500 μL ml succinate-ubiquinone reductase (SQR) assay buffer (50 mM phosphate buffer, 20 mM sodium succinate, 0.1 mM EDTA, 75 μM DCPIP, pH 7.4). The reaction was initiated by adding 2 μL of mitochondrial preparation. (5mg/ml) and the decrease of absorbance at 600 nm (ε = 21 $mM^{-1}cm^{-1}$) was measured. Complex II enzyme activity was expressed as the reduction of DCPIP (nmol DCPIP reduced/mg protein/min). Complex III (ubiquinol-cytochrome c reductase) activity assay was determined by ubiquinol-mediated ferricytochrome c reduction in the presence of isolated mitochondria. 1 μL of mitochondria preparation (5mg/mL) was added to 1 mL of ubiquinol-cytochrome c reductase (QCR) assay buffer (50 mM phosphate buffer, 1 mM EDTA, 50 μM cytochrome c, 0.1 mM KCN and 25 μM ubiquinol-2, pH 7.0) to initiate the reaction. The increase of absorbance at 550 nm (ε = 18.5 $mM^{-1}cm^{-1}$) was measured over 2 minutes. Complex III activity was expressed as the reduction rate of ferricytochrome c (nmol cytochrome c reduced/mg protein/min). Complex IV (cytochrome c oxidase) activity assay was analyzed by the oxidation of ferrocytochrome c in the presence of mitochondria preperation. 1 μL of mitochondrial preparation (2.5mg/mL) was added to 1 mL cytochrome c oxidase (CcO) assay buffer (50 mM phosphate buffer, 60 μM ferrocytochrome c, pH 7.4). Complex IV enzyme activity was determined by the decrease in absorbance at 550 nm (ε = 18.5 $mM^{-1}cm^{-1}$). The Complex IV enzyme activity

was expressed as the oxidation rate of reduced ferrocytochrome c (nmol cytochrome c oxidized/mg protein /min).

Measurement of IDH2 and Krebs Cycle Enzyme Activity

Isolated mitochondria were permeabilized with 25 μg/mL of alamethicin. A Shimadzu 2401 UV-visible spectrophotometer and quartz cuvette were used to determine enzymatic rates at 37 °C. $NADP^+$ dependent isocitrate dehydrogenase (IDH2) activity was calculated by reduction of $NADP^+$ to NADPH (Martinez-Rivas & Vega, 1998). 1 μL of mitochondria (5mg/mL) was added to 500 μL 50 mM tris-HCl buffer containing 1 mM $NADP^+$, 1 mM $MnCl_2$, 4 mM isocitrate, pH 7.4. $NADP^+$ reduction was determined by the increase in absorbance at 340 nm ($\varepsilon = 6.22\ mM^{-1}cm^{-1}$). IDH2 activity was expressed as nmol NADPH produced/mg protein/min. Aconitase activity was determined by reduction of NAD^+ to NADH in the presence of IDH2 (Kang et al., 2015a). 5 μL of mitochondria (5mg/mL) was added into 1 mL of 50 mM tris-HCl buffer containing 0.8 mM citric acid, 0.54 mM $NADP^+$, 1.3 mM $MnCl_2$ and 1 unit of $ICDH_2$, pH 7.4. Enzymatic activity was calculated by absorbance at 340 nm ($\varepsilon = 6.22\ mM^{-1}cm^{-1}$) expressed in NADPH produced/mg protein/min. α-ketoglutarate dehydrogenase activity was measured by the formation of NADH determined by the increase in absorbance at 340 nm ($\varepsilon = 6.22\ mM^{-1}cm^{-1}$) (Yarian et al., 2006). Isolated mitochondria was added to 50 mM potassium phosphate buffer pH 7.4, 0.1 mM coenzyme a, 0.2 mM thiamine pyrophosphate, 1 mM NAD^+, 0.5 mM EDTA, 5 mM $MgCl_2$, 40 μM rotenone, 0.1% triton x-100, 2.5 mM α-ketoglutarate. Malate dehydrogenase activity was

determined by the oxidation of NADH to NAD^+. 1 μL of mitochondria (5mg/mL) was added to 500 μL 50 mM tris-HCl buffer containing 0.48 mM Oxaloacetate, 0 .13mM NADH, pH 7.4. Malate dehydrogenase mediated NADH oxidation rates were determined by subtracting NADH oxidation rates in absence of oxaloacetate. Enzymatic activity was calculated by absorbance at 340 nm (ε = 6.22 $mM^{-1}cm^{-1}$) expressed in NADH oxidized/mg protein/min (Yarian et al., 2006). Citrate synthase activity was measured in 50mM Tris-HCl pH 8.0 buffer containing 170 μM oxaloacetate, 300 μM acetyl-coA, .24% triton x-100, and 100 μM DTNB (Ellman's Reagent). 8 μL of mitochondria was added into the mixture and absorbance was recorded at 412 nm(ε = 13.6 $mM^{-1}cm^{-1}$) at 30°C.

Measurement of Glutathione Peroxidase Activity

Isolated mitochondria were permeabilized with 25 μg/mL of alamethicin. A Shimadzu 2401 UV-visible spectrophotometer and quartz cuvette were used to determine enzymatic rates at 37 °C (Kang et al., 2015b). 6 μL of mitochondrial sample was added to 500 μL of 50mM Tris-HCl pH 7.5 buffer containing 5 mM EDTA, 4.4 μg/mL Glutathione reductase, 1 mM reduced glutathione, and 200 μM NADPH. After 2 min incubation, final concentration of 0.002% t-butyl-hydroperoxide was added to initiate the reaction. Indirect enzymatic activity was calculated by the absorbance at 340 nm (ε = 6.22 $mM^{-1}cm^{-1}$).

Measurement of Catalase Activity

Isolated mitochondria were permeabilized with 25 μg/mL of alamethicin. A Shimadzu 2401 UV-visible spectrophotometer and quartz cuvette were used to determine enzymatic rates at 37 °C (Kang et al., 2015b). Isolated mitochondrial samples were added to 50mM potassium phosphate buffer pH7.4 with 0.036% hydrogen peroxide. Enzymatic activity was calculated by the absorbance at 240 nm (ε = 43.6 $mM^{-1}cm^{-1}$)

Tetrazolium Chloride Staining

Langendorff treated murine hearts were frozen in -20 C for 25 minutes and sectioned. Heart sections were incubated in 2% TTC staining solution in 1x PBS at 37 C for 20 minutes. The heart sections were then imaged under visible light then preserved in formalin at 4 C.

CM-H Spin Probe

CM-H oxidation was measured in isolated mitochondria under non-energetic conditions with a Bruker EMX Micro spectrometer operating at center field: 3359 G, modulation frequency 100 kHz, modulation amplitude 1 G, modulation phase 0 degrees, offset .8%, time constant of 163.84 seconds, conversion time 41 ms, and 298 K temperature. The NADH-linked reaction mixture containing 1 mM DTPA, 1 mM (1-Hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidin HCl (CM-H) , and 1 uL of 33.33 mM saturated Ebselen or 1 uL ethanol was mixed with isolated mitochondria to

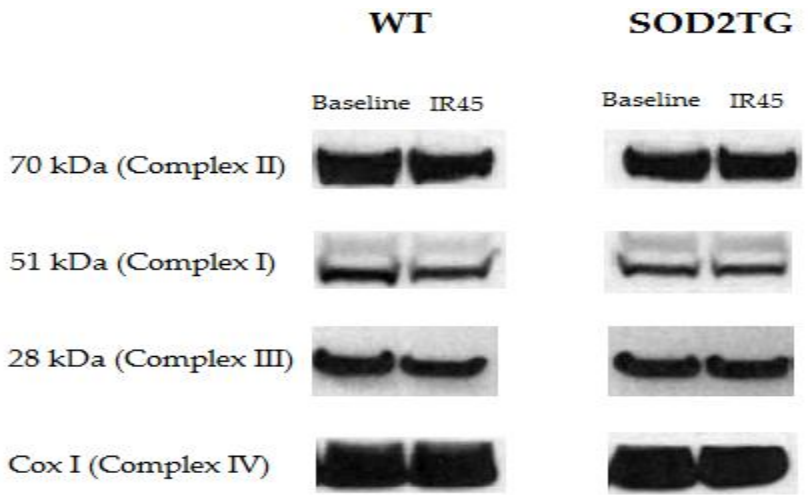
final concentration of 0.4 mg/mL. The mix was transferred to a 50 uL glass capillary (Drummond Wiretrol, Broomall, PA), sealed and deposited into the EPR resonator (HS cavity, Bruker Instrument, Billerica, MA). The spectrum was taken every minute and the units were calculated as nmol CM-H oxidized/mg protein/min calculated by WINSIM simulations. The protocol was similar to the one found in Kang et al., 2015b.

Immunoblotting Analysis

Western blotting with ~120 μg protein from isolated mitochondrial samples was loaded into NuPage 4-12% Bis-Tris gel. Complex I was blotted with anti-51 kDa antibody against flavin mononucleotide. Anti-70 kDa antibody was used against the FAD binding subunit of complex II. Complex III was blotted with monoclonal anti-Rieske or anti-FeS Ab. Complex IV was probed with anti-cox I subunit.

Results

1a.



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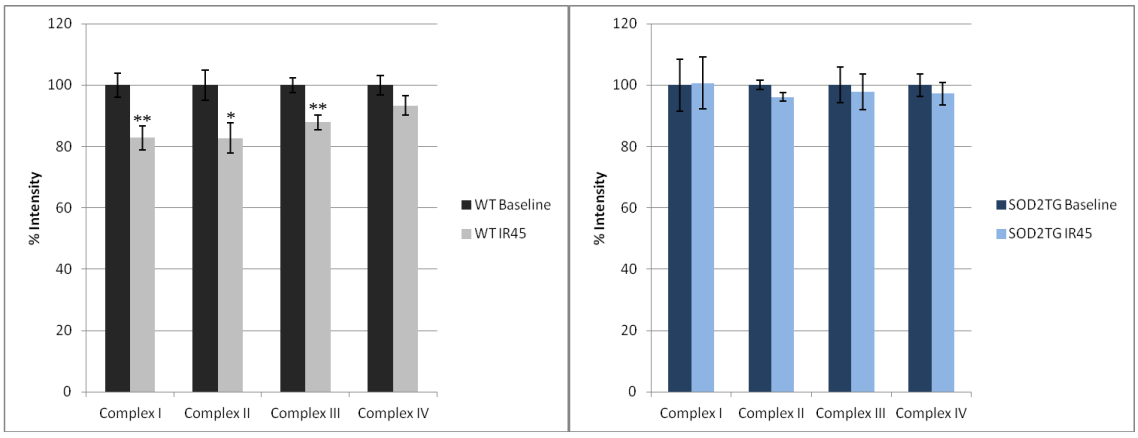

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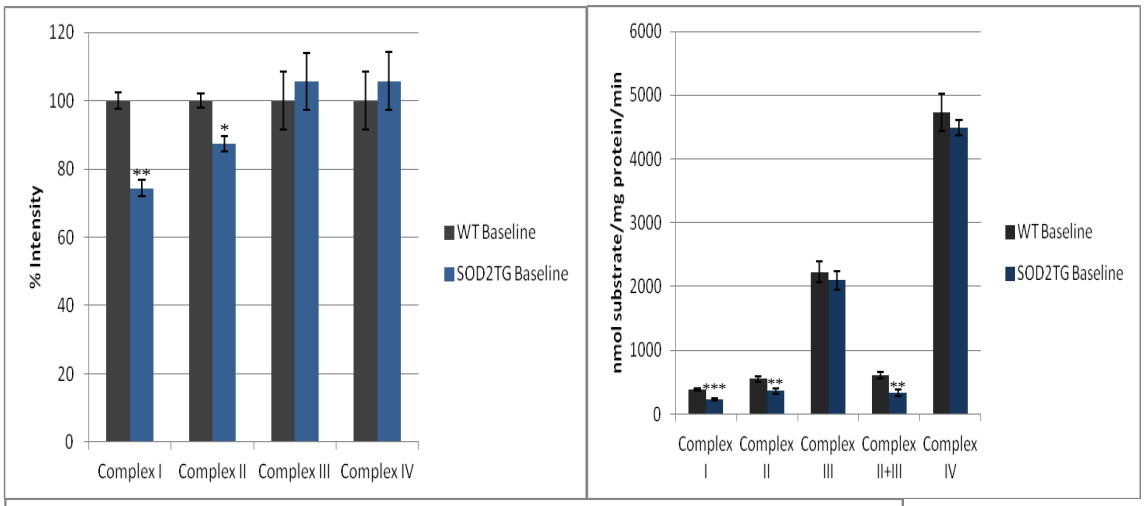

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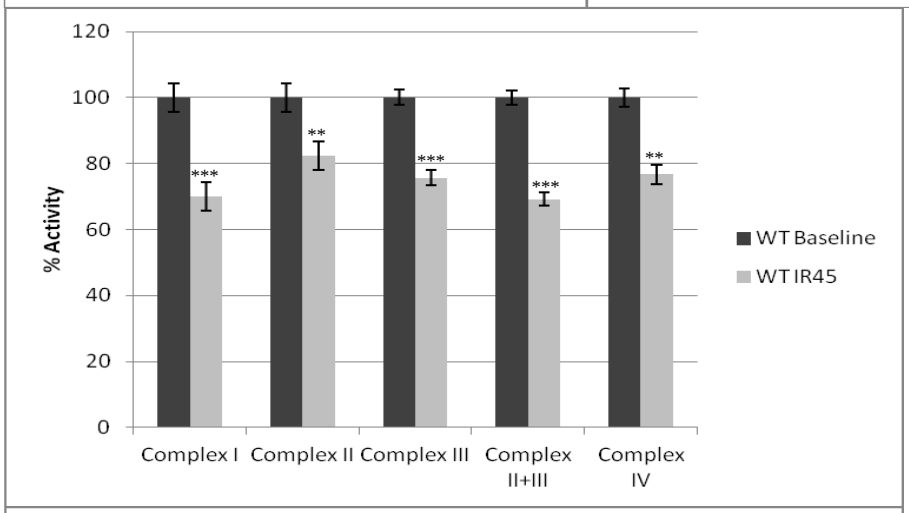

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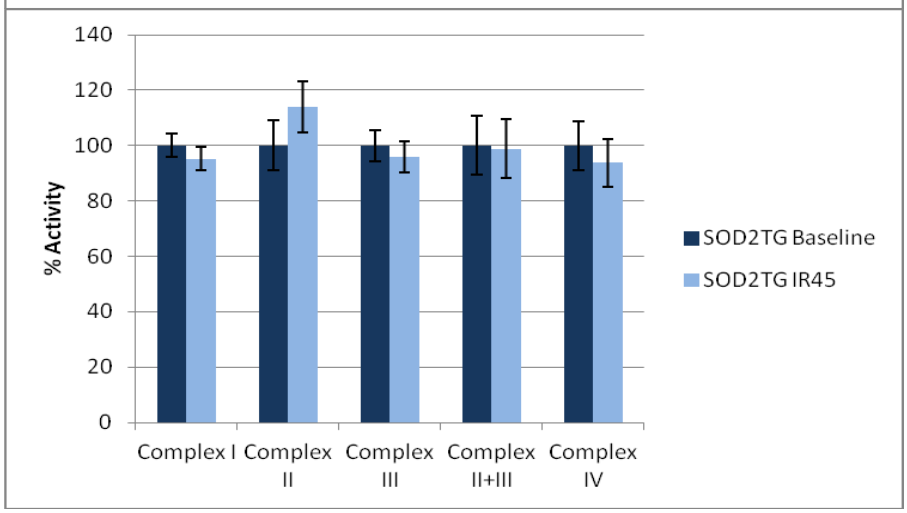


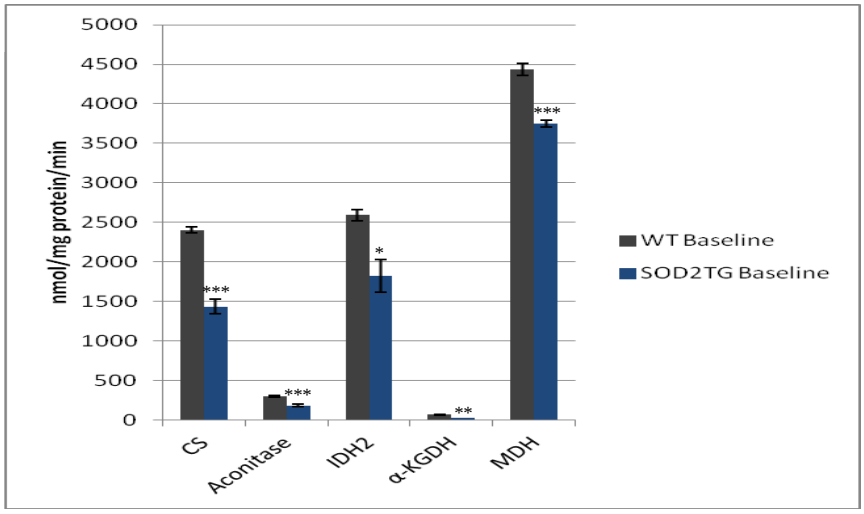
Fig. 1 Effects of IR injury on mitochondrial electron transport are mitigated by SOD2 overexpression. (**1a**) shows protein expression of electron transport complexes I-IV in isolated mitochondria from WT and SOD2TG hearts under baseline conditions vs 30 min ischemia and 45 min reperfusion injury (IR45). **(1b)** Western blot intensities were normalized to their respective baselines after IR45. (**1c)** Baseline values between WT and SOD2TG complex protein expression and complex activities were compared. (**1d and 1e**) Individual complex activities andsupercomplex II+III were measured spectrophotometrically between WT and SOD2TG hearts. Activities were normalized to their respective baselines after IR45.

Ischemia Reperfusion Injury on Electron Transport Chain

Mice mitochondria isolated from hearts after ischemia and reperfusion treatments were subjected to an assay of individual respiratory complex activity normalized to total mitochondrial protein. Protein expression was probed by western blot with 120 μg of mitochondrial protein loaded per well. In WT mitochondria, 30 min ischemia and 45 reperfusion reduced complex I expression by 17.18% ± 3.97% (n=6, $p<0.01$) while SOD2TG complex I did not show decrease in protein band intensity (n=4, $p\approx0.94$) when probed with Ab against 51 kDa subunit in complex I. Complex II expression in WT hearts dropped by 17.24% ± 4.87% (n=6, $p<0.05$) when probed with anti-70 kDa Ab. SOD2TG complex II expression showed little or insignificant decrease 3.84% ± 1.86% (n=4, $p\approx0.08$). Complex III (anti-RISP Ab) in WT decreased by 12.14% ± 2.38% after IR (n=6, $p<0.01$) while SOD2TG hearts did not see significant decrease (n=4, $p\approx0.72$). As for complex IV (anti-cox I subunit Ab) WT and SOD2TG hearts saw little or insignificant decrease 6.63% ± 3.17% (n=6, $p\approx0.09$) and 2.74% ± 3.66% (n=4, $p\approx0.51$) respectively. SOD2TG (n=4) hearts had lower complex I and II protein expression compared to WT (n=6) 25.61% ± 2.49 $p<0.01$ and 12.62% ± 2.17% $p<0.05$, respectively. Cox I of complex IV was used as loading control since of the expression consistency between strains and preservation of protein band intensity after IR. Ischemia reperfusion injury on WT heart mitochondria reduced complex I (NQR) activity by 29.83% ± 4.34% (n=6, $p=0.001$) measured in nmol NADH oxidized/mg protein/min. No significant change was observed in SODTG2 complex I activity after reperfusion (n=6, $p\approx0.30$). WT complex II (SQR) showed a 17.61% ± 4.21% decrease in nmol DCPIP reduced/mg protein/min (n=6,

p<0.01). SOD2TG complex II did not show significant change after reperfusion (n=6, p≈0.18). WT complex III (QCR) activity decreased by 24.32% ± 2.31% measured in cytochrome c reduced/mg protein/min (n=6, p<0.001). SOD2TG complex III activity did not significantly decrease (n=6, p≈0.49). WT complex II+III (SCR) dropped 30.76% ± 2.03% cytochrome c reduced/mg protein/min (n=6, p<0.001). While SOD2TG SCR activity after IR was not significantly different from its respective baseline value (n=6, p=0.92). WT complex IV (CCO) activity decreased by 23.31% ± 2.80% measured in nmol cytochrome c oxidized/mg protein/min (n=4, p<0.01). SOD2TG complex IV showed insignificant decrease (n=4, p=0.52). SOD2TG mice exhibited lower baseline complex I activity compared to WT 227.03 ± 11.56 vs. 378.90 ± 17.90 nmol NADH oxidized/mg protein/min (n=6, p<0.001). For complex II, SOD2TG baseline activity was also lower 360.80 ± 38.86 vs. 552.14 ± 43.95 nmol DCPIP reduced/mg protein/min in WT (n=6, p<0.01). Complex II+III activity was lower 330.47 ± 46.01 vs. 609.37 ± 55.61 cytochrome c reduced/mg protein/min (n=6, p<0.01). However, SOD2TG hearts complex III (n=6) and complex IV (n=4) baseline values did not show difference between the two groups (p≈0.54 and p≈0.49 respectively). The wild type myocardium showed a decrease across all complexes after reperfusion while the SOD2 transgenic mice did not see significant decrease in any complexes after IR45, consistent with FCCP induced uncoupled oxygen consumption rates.

2a.



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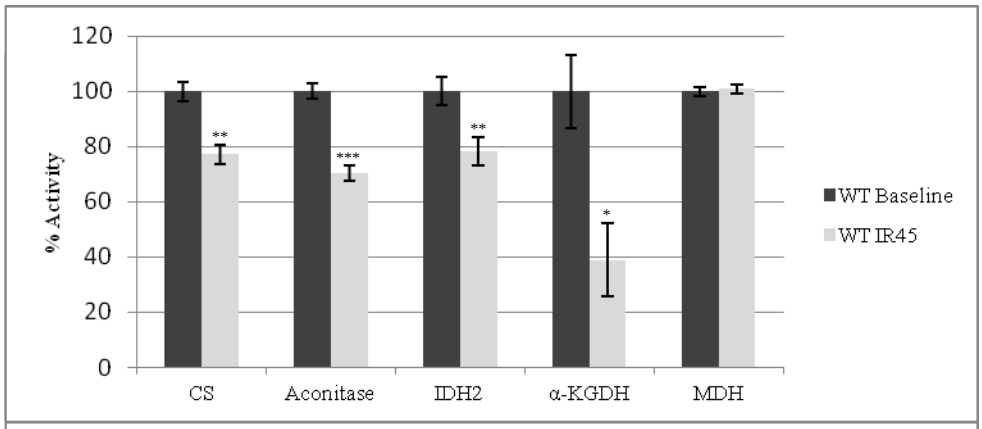

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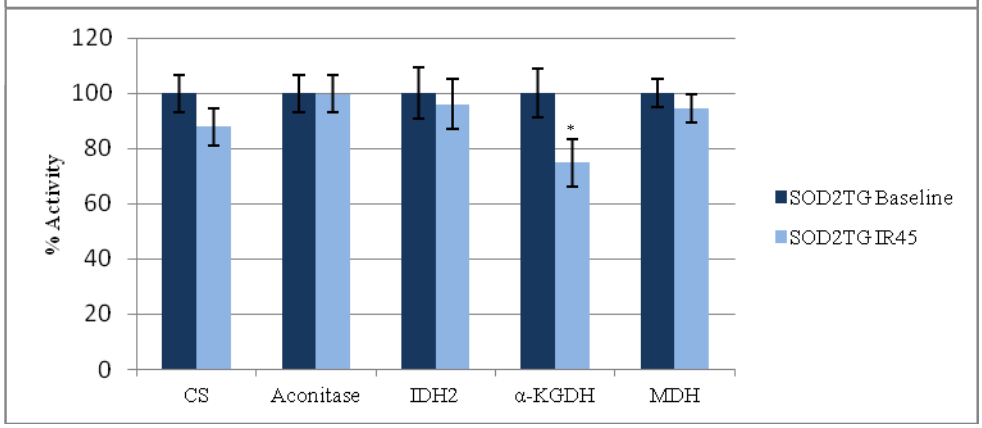


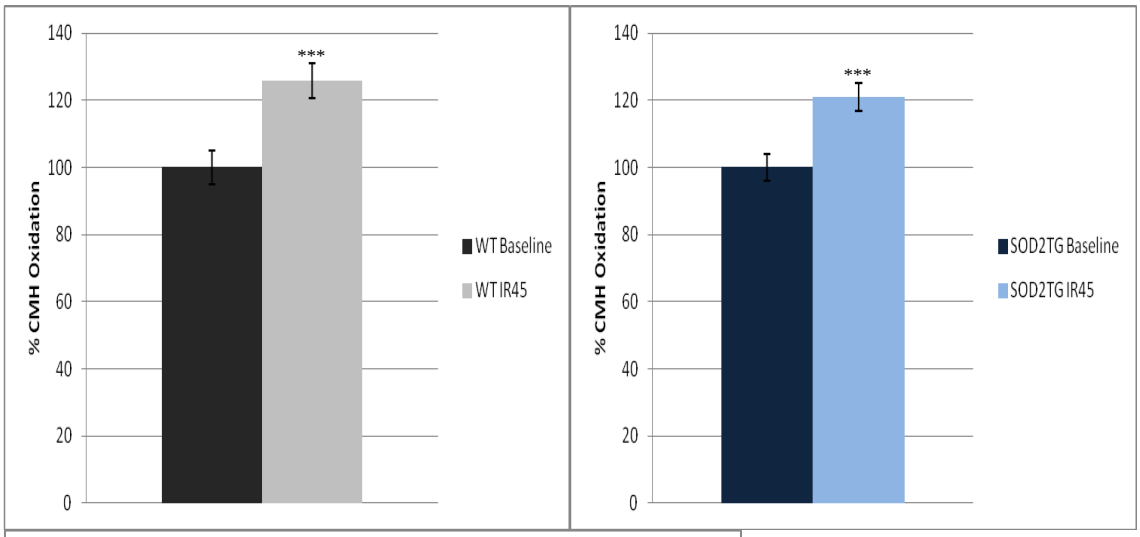
Fig 2. SOD2 overexpression prevented impairment of mitochondrial matrix enzyme activities. Citrate Synthase (CS), aconitase, isocitrate dehydrogenase 2 (IDH2), α-ketoglutarate dehydrogenase (α-KGDH), and malate dehydrogenase (MDH) activity from isolated heart mitochondria were measured spectrophotometrically. Aconitase, α-ketoglutarate dehydrogenase, and isocitrate dehydrogenase 2 activities are represented by nmol NADPH or NADH formed/mg protein/min while malate dehydrogenase was measured by the reverse reaction of nmol NADH oxidized/mg protein/min. Citrate synthase was measured by nmol DTNB reduced/mg protein/min. (**2a**) compares the baseline enzymatic values between WT and S OD2TG. (**2b and 2c**) show WT and SOD2TG activities after ischemia reperfusion (IR45) normalized to their respective baseline values.

Effects of Ischemia Reperfusion on IDH2 and Krebs Cycle Enzymes

The enzymatic rates were measured independently for each enzyme spectrophotometrically. Ischemia reperfusion treatment in WT hearts decreased mitochondrial isocitrate dehydrogenase 2 (IDH2) activity by 21.76% ± 5.19% measured in NADPH produced/mg protein/min (n=6, $p<0.01$). The Krebs cycle provides a steady flux of reducing equivalents required for oxidative phosphorylation. Oxidative stress has been shown to reduce key enzymes for NADH linked respiration. (Sadek et al., 2002; Humphries & Szweda, 1998) As expected, aconitase activity for WT hearts decreased by 29.58% ± 2.67% nmol NADPH produced/mg protein/min when coupled with isolated isocitrate dehydrogenase 2 to generate absorbance of light at 340nm due to NAPDH production (n=6, $p<0.001$). Citrate synthase (CS) activity was lowered by 22.89% ± 3.52% DTNB reduced/mg protein/ min (n=6, $p<0.01$). α-ketoglutarate dehydrogenase (α-KGDH) activity for wild types decreased by 61.13% ± 13.30% nmol NADH formed/mg protein/min (n=4, $p<0.05$) after IR45. Malate dehydrogenase (MDH) activity in WT hearts was not significantly different from baseline values after IR45 (n=5, $p\approx0.60$). Activities after ischemia reperfusion in SOD2TG hearts did not significantly change IDH2 (n=4, $p\approx0.69$), CS (n=5, $p\approx0.14$), aconitase (n=5, $p\approx0.96$), nor MDH activities (n=4, $p\approx0.35$). However, SOD2TG α-KGDH activity was decreased by 25.23% ± 8.79% (n=6, $p<0.05$). The baseline values for CS, IDH2, aconitase, α-KGDH and MDH in SOD2TG mice were significantly lower compared to WT mice. Aconitase activity decreased from 299.89 ± 9.75 in WT hearts (n=6) to 182.73 ± 14.58 NADPH formed/mg protein/min in SOD2TG hearts (n=5, $p<0.001$). Citrate synthase decreased from 2403.45 ± 40.48 in WT (n=6) to 1432.59 ± 91.15 in SOD2TG hearts nmol DTNB reduced/mg protein/min (n=5, $p<0.001$). Baseline α-KGDH

activity was decreased from to 65.36 ± 6.75 in WT (n=4) to 28.54 ± 3.35 nmol NADH formed/mg protein/min in SOD2TG (n=6) p<0.01. Malate dehydrogenase activity decreased from 4438.42 ± 75.77 in WT (n=5) to 3748.19 ± 44.68 nmol NADH oxidized/mg protein/min in SOD2TG mice (n=4, p<0.001). Isocitrate dehydrogenase 2 activity dropped from 2590.90 ± 72.37 in WT (n=6) to 1825.67 ± 207.60 nmol NADPH/mg protein/min in SOD2TG mice (n=4, p<0.05).

3a.



3b.

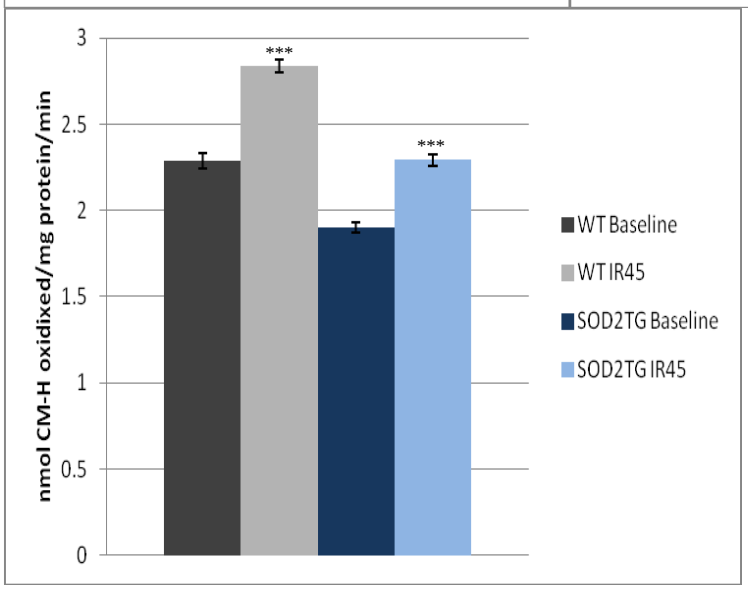
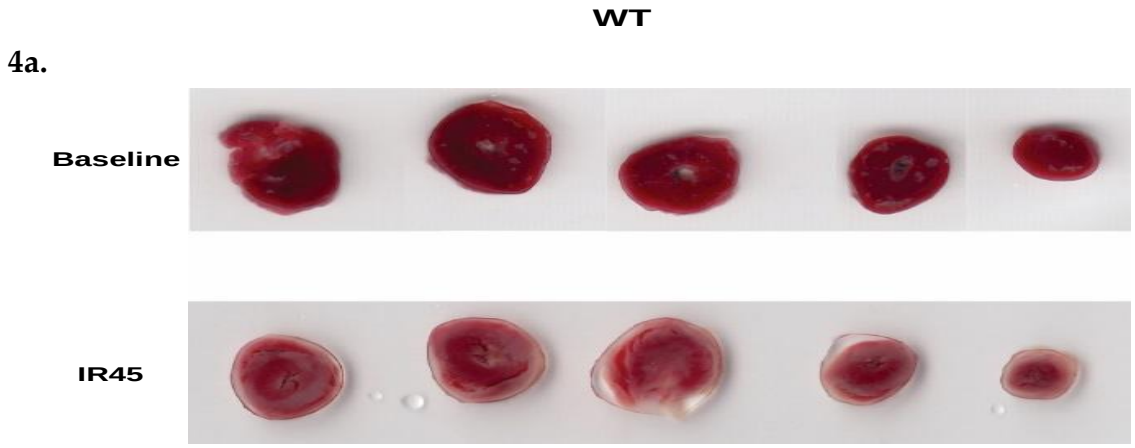


Fig.3 Oxidation of CM-H mediated by isolated mitochondria redox status measured by EPR. The production of stable nitroxide in isolated cardiac mitochondria was calculated under non-energized conditions as nmol CM-H oxidized/mg protein/min. (**3a)** CM-H values for WT IR45 and SOD2TG were normalized to their respective baselines. (**3b**) Absolute values for CM-H were measured for WT and SOD2TG groups after IR45.

Isolated Mitochondria Mediated CM-H Oxidation

CM-H (1-Hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine HCl, a spin probe of hydroxylamine) oxidation into nitroxide was measured in isolated murine cardiac mitochondria under non-energized conditions. More reduced myocardium results in less nitroxide production from CM-H. The rate of CM-H oxidation increased in WT heart mitochondria preparation after ischemia reperfusion by 25.85% ± 5.15% nmol CM-H oxidized/mg protein/min ($n=10$, $p<0.001$). SOD2TG mitochondria also saw a 20.98% ± 4.03% increase in CM-H oxidation rate after ischemia reperfusion ($n=10$, $p<0.001$). However, the SOD2TG myocardium was overall more reduced. SOD2TG hearts subjected to IR were not significantly different from baseline values of WT hearts in terms of oxidation 2.29 ± 0.13 for WT baseline vs. 2.29 ± 0.11 nmol CM-H oxidized/mg protein/min for the SOD2TG IR45 group ($n=10$, $p\approx0.98$).



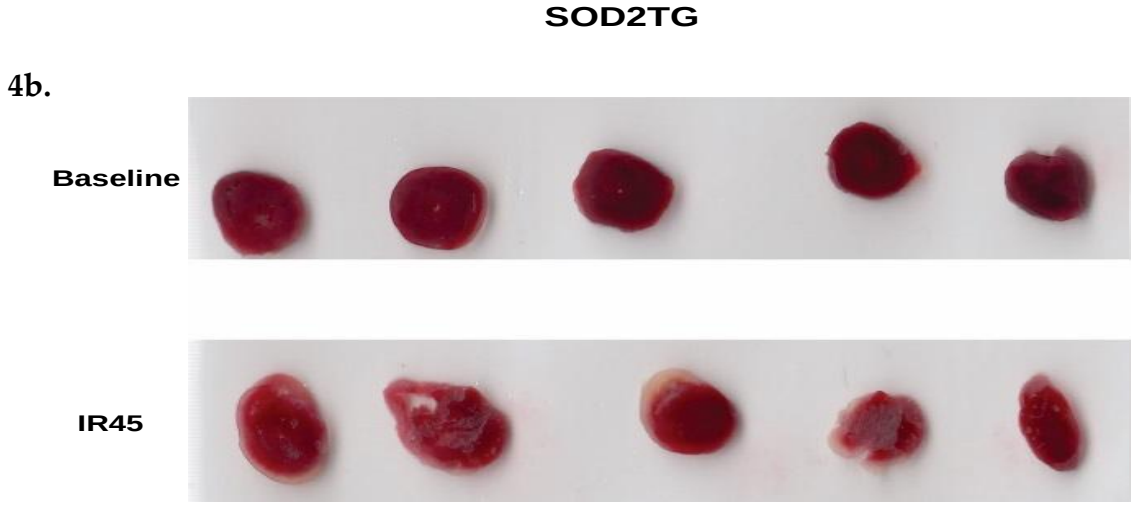


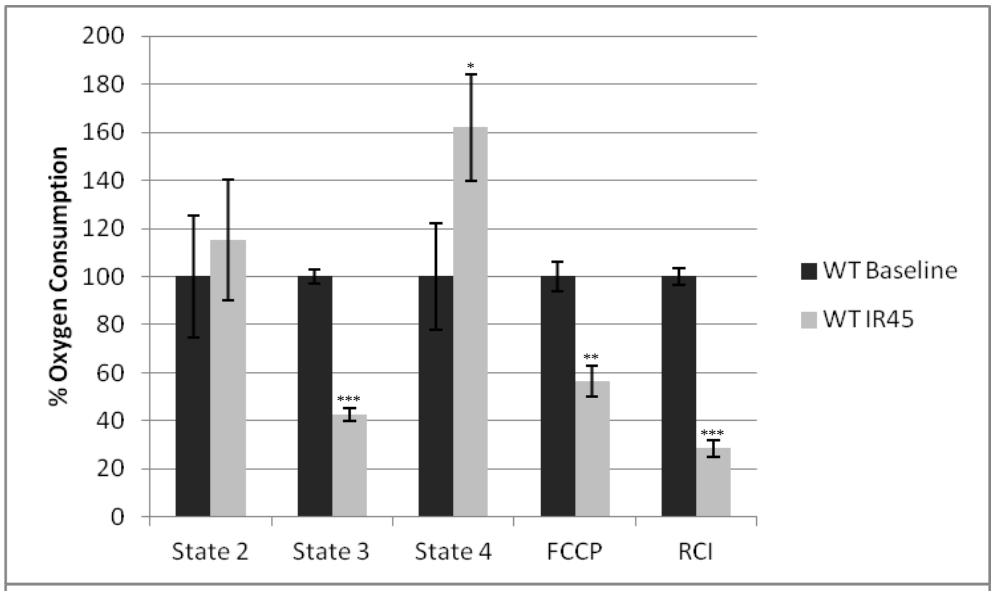
Fig.4 Whole heart tissue slices stained in 2% tetrazolium chloride (TTC) after IR45. (4a) WT hearts displayed pale areas of infarction due to unreduced TTC indicator**. (4b)** SOD2TG hearts showed less noticeable infracted tissue after IR treatment.

TTC Stain of Reperfused Heart Slices

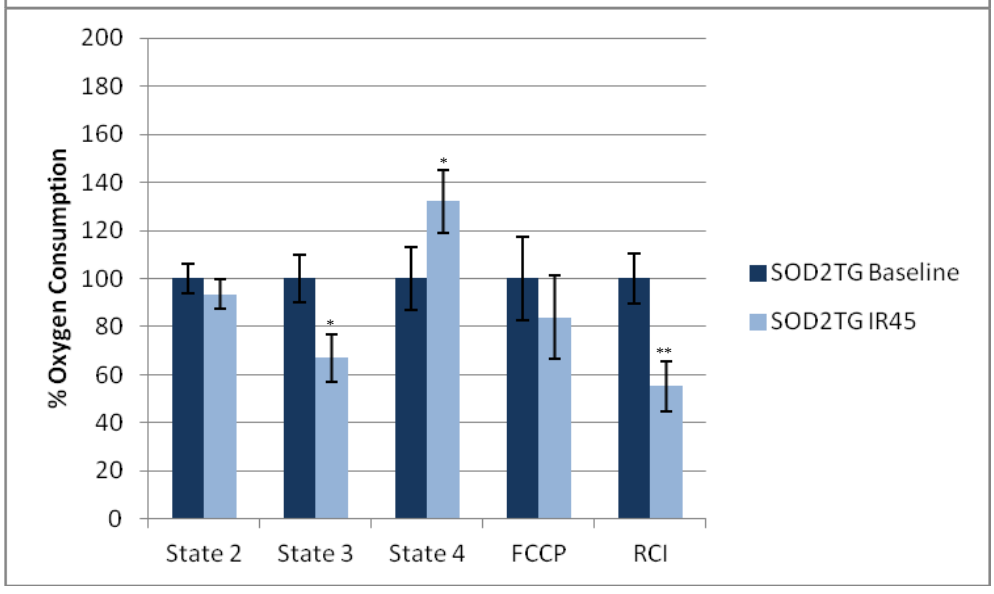
Whole baseline and IR treated heart tissue were frozen and sliced. The sliced tissue was submerged in 2% TTC stain in PBS. The baseline conditions for the WT and

SOD2TG hearts resulted in a deep red stain in the heart slices as shown in the top portions of Fig.4a and Fig. 4b. WT hearts exposed to ischemia reperfusion (IR45) yielded pale metabolically inactive or infarcted heart tissue as visualized in the bottom portion of fig. 4a. SOD2TG hearts exposed to ischemia reperfusion in fig. 4b resulted in slightly pale coloration. However, WT heart slices appear to yield larger area of infarction upon ischemia reperfusion treatment compared to the SOD2TG heart slices.

5a.



5b.



5c.

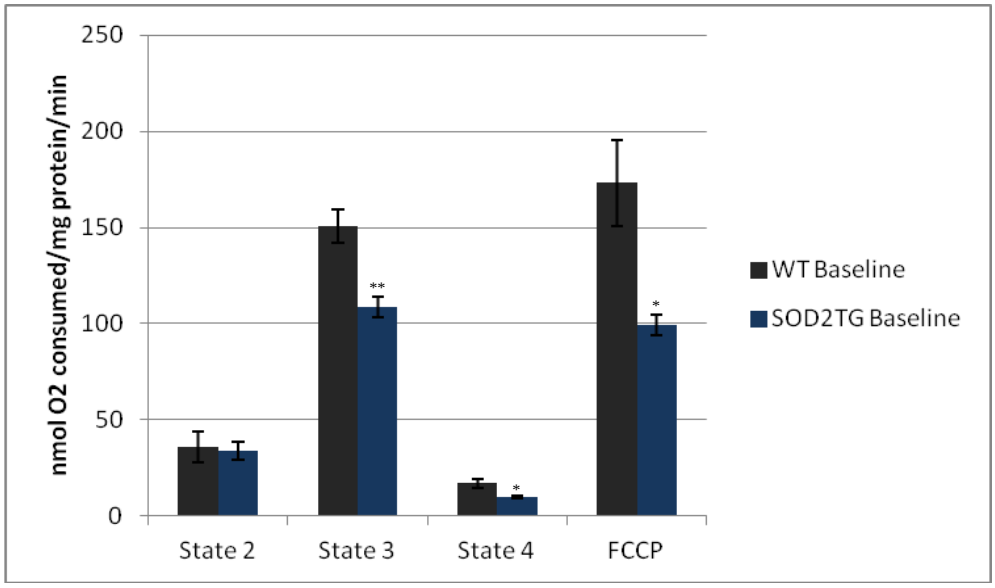
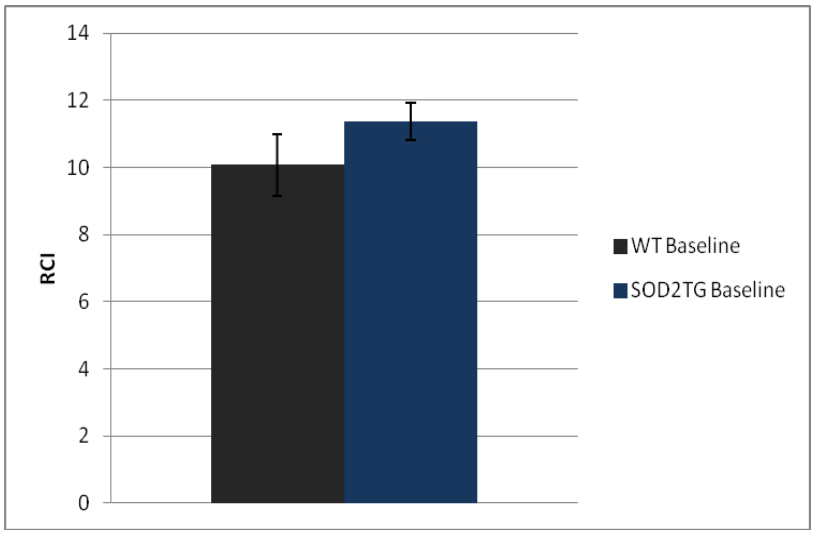


Fig. 5 Isolated heart mitochondria O_2 Consumption was measured with NADH linked substrates (glutamate and malate) under state 2, 3, 4 (with oligomycin), and FCCP. **(5a and 5b)** WT and SOD2TG IR45 groups' oxygen consumption were normalized to respective baseline values. **(5c)** The baseline consumption rates between WT and SOD2TG heart mitochondria were compared.

6.

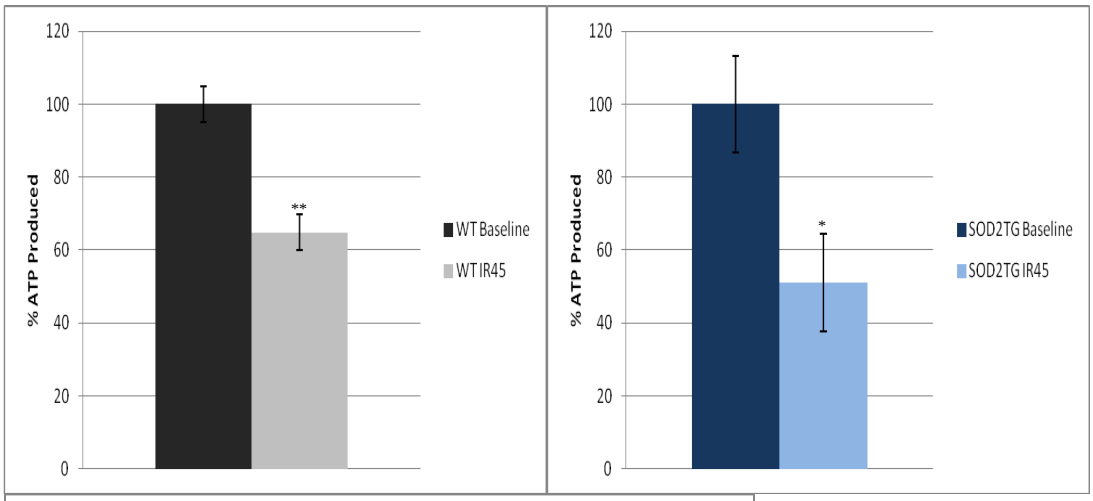


(6.) The RCI (state3/state4) oxygen consumption between WT and SOD2TG isolated heart mitochondria under baseline conditions.

Oxygen Consumption of Isolated Mitochondria after IR

Both WT and SOD2TG mitochondria displayed impaired oxygen consumption after ischemia reperfusion. State 2 conditions were not significantly different after IR in both groups (WT n=6, p≈0.57 and SOD2TG n=8, p≈0.32). Oxygen consumption under state 3 conditions decreased after IR45 for WT mitochondria by 57.27% ± 2.70% (n=6, $p<0.001$) while SOD2TG state 3 respiration decreased by 32.96% ± 9.94% (n=8, $p<0.05$) measured in nmol O_2 consumed/mg protein/min. Under state 4 conditions in the presence of oligomycin, WT mitochondria consumed 61.91% ± 21.93% more O_2 (n=6, $p<0.05$) after IR while SOD2TG mitochondria saw an increase of 32.05% ± 13.22% (n=8, $p<0.05$). The respiratory control index (RCI) decreased in WT after IR by 71.54% ± 3.50% (n=6, $p<0.001$) while the SOD2TG RCI showed a 44.77% ± 10.37% decrease (n=8, $p<0.01$). WT mitochondria had 43.49% ± 6.27% (n=6, $p<0.01$) decrease in FCCP mediated oxygen consumption after IR. SOD2TG mitochondria did not show significant decrease (n=8, p≈0.38). Baseline oxygen consumption rates were different between WT and SOD2TG groups. SOD2TG mitochondria (n=8) under state 3 conditions consumed 108.81 ± 5.46 while WT consumed 150.52 ± 8.61 nmol O_2/mg protein/min (n=6) $p<0.01$. Under state 4 conditions with oligomycin WT consumed 17.02 ± 2.42 (n=6) nmol O_2/mg protein/min compared to 9.81 ± 0.56 (n=8) by SOD2TG mitochondria $p<0.05$. FCCP mediated oxygen consumption for WT (n=6) was 173.03 ± 22.15 while SOD2TG consumed 99.02 ± 5.28 nmol O_2/mg protein/min (n=8) $p<0.05$. There was no significant difference in RCI between baseline values p≈ 0.68 or under state 2 conditions p≈0.85.

7a.



7b.

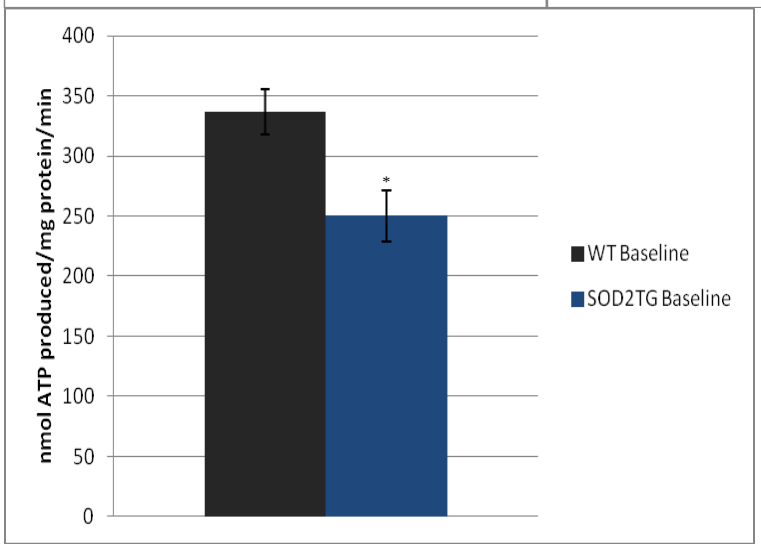


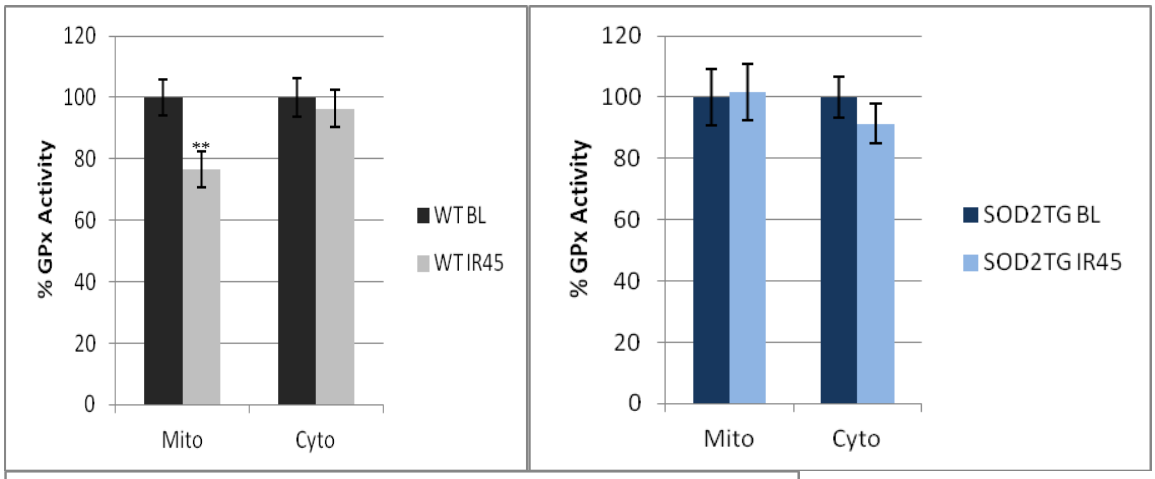
Fig. 7 shows the ATP production rate in isolated heart mitochondria under state 3 conditions using chemiluminescence luciferase assay. **(7a)** Relative ATP production after IR45 normalized to their respective baseline values for WT and SOD2TG mitochondria. **(7b)** Baselines values of ATP production for WT and SOD2TG were compared.

Ischemia Reperfusion Injury Downregulates ATP Production

ATP production was downregulated in both WT and SOD2TG mitochondria after IR45 treatment. WT IR45 showed 35.12% ± 4.89% decrease (n=5, $p<0.01$) and SOD2TG IR45 had 48.95% ± 13.27% decrease in ATP production (n=4, $p<0.05$) measured in nmol ATP produced/mg protein/min. The baseline values between WT and SOD2TG mitochondria were also significantly different $p<0.05$. WT (n=5) produced 336.41 ± 18.83

while SOD2TG (n=4) mitochondria produced 250.11 ± 21.60 nmol ATP/mg protein/min.

8a.



8b.

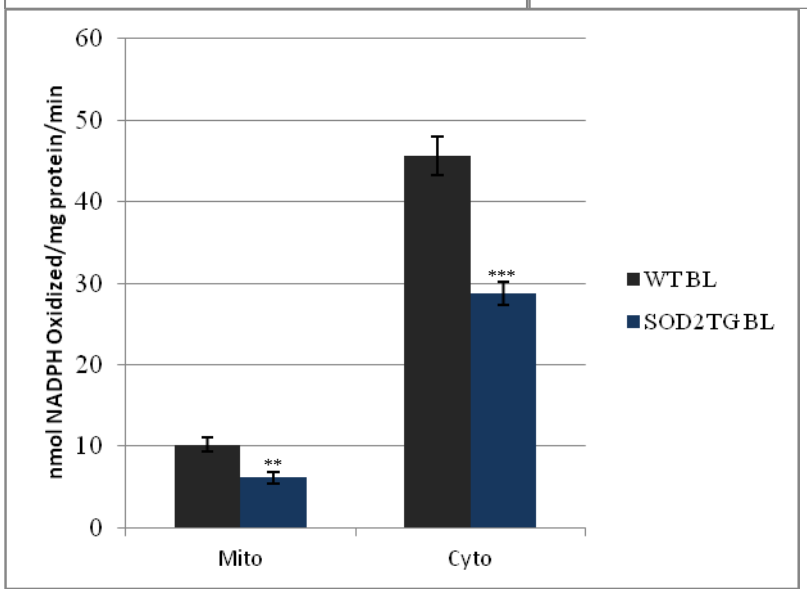


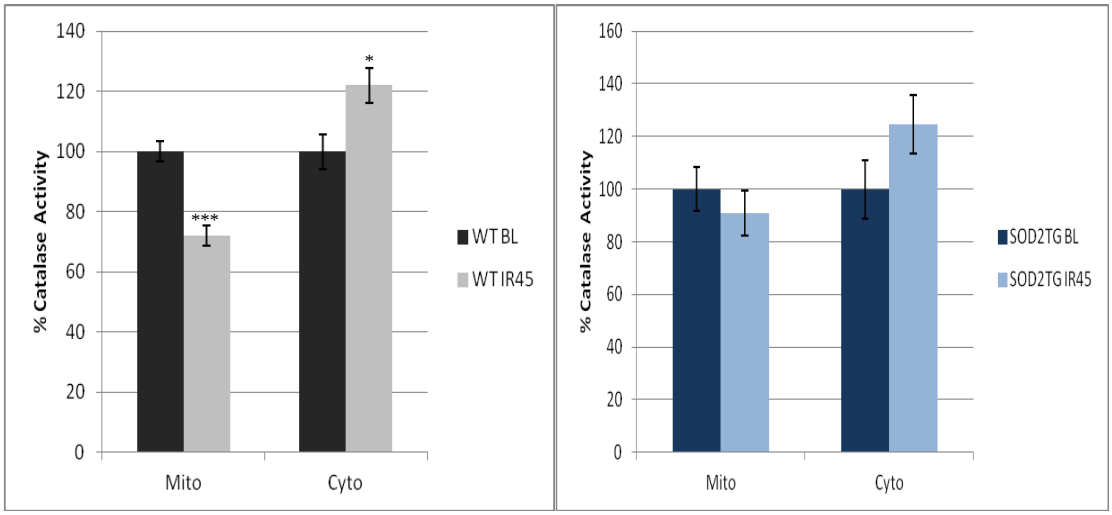
Fig. 8 Mitochondrial and Cytosolic Glutathione Peroxidase Activity (Gpx) was measured spectrophotometrically in WT and SOD2TG isolated heart mitochondrial and cytosolic fractions quantified by nmol NADPH oxidized/mg protein/min.**(8a)** GPx activities were normalized to their respective baselines after ischemia reperfusion treatment. **(8b)** Baseline Gpx values between WT and SOD2TG were compared.

Glutathione Peroxidase Activity after Ischemia Reperfusion

Glutathione peroxidase activity (GPx) was downregulated after ischemia reperfusion in WT heart mitochondria by 23.34% ± 5.71% (n=6, $p<0.01$) The SOD2TG hearts saw insignificant change in Gpx activity (n=7, $p\approx0.86$). Both groups did not see significant decrease in cytosolic GPx activity after ischemia reperfusion, WT (n=8, $p\approx0.57$) and SOD2TG (n=8, $p\approx0.22$). The SOD2TG hearts (n=7) had a lower baseline mitochondrial as well as cytosolic GPx activity of 6.12 ± 0.74 nmol NADPH oxidized/mg

protein/min compared to WT (n=6) baseline of 10.20 ± 0.94, p<0.01 measured in nmol NADPH oxidized/ mg protein/min mitochondrial fractions. The cytosolic fractions in WT (n=6) was 45.67 ± 2.37 compared to 28.78 ± 1.40 nmol NADPH oxidized/ mg protein/min in SOD2TG (n=7) p<0.001.

9a.



9b.

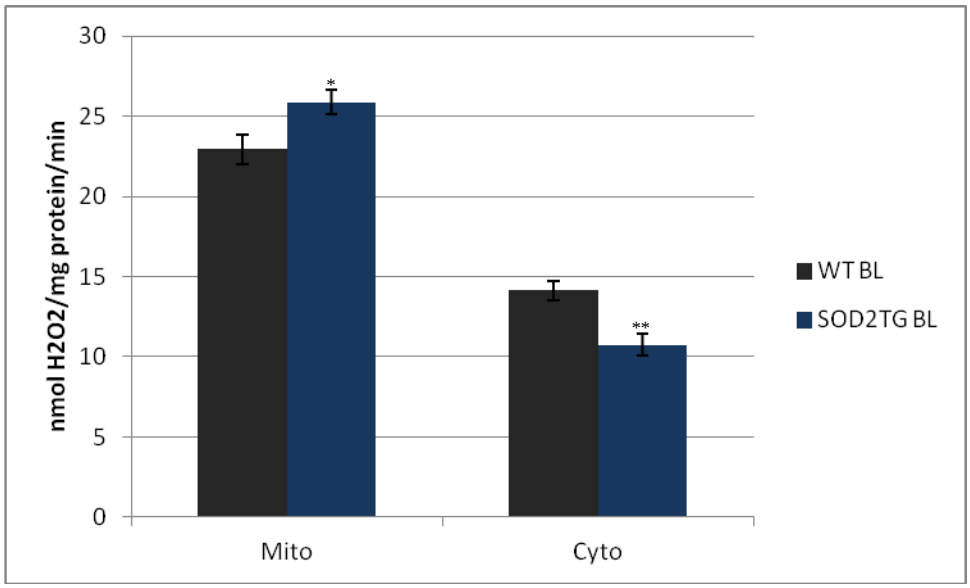


Fig. 8 Mitochondrial and Cytosolic Catalase Activity were measured spectrophotometrically in WT and SOD2TG isolated heart mitochondria quantified by nmol hydrogen peroxide reduced /mg protein/min**. (9a)** Catalase activities were normalized to their respective baselines in mitochondrial and cytosolic fractions after ischemia reperfusion treatment. **(9b)** Baseline values between WT and SOD2TG catalase activity were compared in both fractions.

Catalase Activity after Ischemia Reperfusion

Ischemia and reperfusion had a differential effect on catalase activity in mouse hearts. WT hearts saw a decrease in catalase activity in mitochondrial fractions 27.89% ± 3.36% ($n=6$, $p<0.001$) after IR45. The cytosolic catalase activity in WT increased by 21.93% ± 5.78% ($n=5$, $p<0.05$). Mitochondrial catalase activity in SOD2TG hearts insignificant decreased 9.07% ± 8.43% ($n=6$, $p\approx0.33$). The cytosolic fraction in SOD2TG hearts saw almost significant increase of 24.77% ± 11.14% ($n=6$, $p\approx0.08$). The SOD2TG hearts had slighter higher mitochondrial catalase activity 25.87 ± 0.74 compared to WT 22.95 ± 0.94 nmol H_2O_2/mg protein/min ($n=6$, $p<0.05$). However the wild types ($n=5$) had higher cytosolic catalase activity 14.12 ± 0.62 vs. 10.75 ± 0.68 nmol H_2O_2/mg protein/min in SOD2TG mice ($n=6$) $p<0.01$.

Discussion

Differential Effects on Electron Transport with Increased Superoxide Scavenging

The electron transport chain has been widely recognized to produce excessive amounts of reactive oxygen species after ischemia and reperfusion. Reactive oxygen species produced by the ETC impair electron transport rates across complexes I-IV. Superoxide generated by xanthine oxidase reaction with xanthine has also been shown to inactivate complex I activity by decreasing cardiolipin content (Paradies et al., 2002). Downregulation of complex activity has been documented during reperfusion as well as during ischemia alone (Lesnefsky et al., 2001). As previously described in the results, electron transport activity was significantly decreased after ischemia reperfusion injury across all measured complexes I-IV including the supercomplex of complex II and III in WT heart mitochondria. The decreased protein levels of 51 kDa, 70 kDa, and 28 kDA of complexes I-III may be due to activation of mitochondrial proteolytic systems that recognize and degrade oxidatively damaged/modified proteins (Marcillat et al., 1988). Superoxide and downstream oxygen derived radicals such as peroxynitrites are able to induce protein modifications such as nitration or thiol oxidation (Jassem & Heaton, 2004). The SOD2 transgenic mice had preserved electron transport activity and protein band intensity after ischemia reperfusion compared to their baseline values as shown in

Fig.1. However, baseline values of complexes I, II, and SCR activity seemed to be downregulated by the overexpression of SOD2. Baseline down regulation of complex I was attributed to decreased protein expression while decreased SCR activity was due to lower antimycin A sensitive semiquinone pool in SOD2 transgenic mitochondria as previously described our lab (Kang et al., 2015b). In this study we saw that complex II protein expression was also slightly decreased in the transgenic hearts. Other studies have found that NADH-oxidoreductase in $SOD2^{-/+}$ was downregulated, presumably due to oxidative stress (Van Remmen et al., 2001). Superoxide dismutase 2 removes superoxide generated by the electron transport chain that either directly impairs complex activities or prevents generation of downstream reactive oxygen species that also affect electron transport activities. Increased superoxide dismutation however results in increased production of H_2O_2 as seen in SOD2 transgenic hearts (Kang et al., 2015b). Theoretically, the SOD2 transgenic mice hearts would have an increased influx of H_2O_2 levels after ischemia reperfusion, however, no further decreases in complex activity was observed after IR. Other studies also observed insignificant effects of hydrogen peroxide on electron transport chain rates (Nulton-Persson & Szweda, 2001). The complex activity assays in this study were not affected decreased flux of reducing equivalents from impaired Krebs cycle enzymes due to the addition of excess amounts of substrates such as NADH to measure electron transport activity as part of the assay protocol.

Krebs Cycle Enzymes, Oxygen Consumption, and IR

The inactivation of aconitase during cardiac ischemia reperfusion injury and in the presence of high superoxide has also been widely observed. Aconitase activity is impaired when the [4Fe-4S] $^{2+}$ cluster reacts with superoxide to produce an inactive [3Fe-4S] $^{+}$ cluster (Sadek et al., 2002; Gardner et al., 1995). Other studies have found that SOD2 heterozygous knockouts had impaired aconitase activity (Van Remmen et al., 2001). As expected, aconitase activity in our study for WT mice was significantly lowered after ischemia reperfusion. The activity was relatively preserved in SOD2 overexpression after ischemia and reperfusion injury compared to baseline conditions. However, the baseline aconitase activity was significantly lower in SOD2 transgenic mice similarly with isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, and malate dehydrogenase baseline activities. Ischemia reperfusion also impaired NADP+ dependent isocitrate dehydrogenase activity, which may play a role in providing NADPH for reduced glutathione generation by glutathione reductase (Lee et al., 2001; Jo et al., 2001). Ischemia reperfusion decreased isocitrate dehydrogenase activity in WT hearts while SOD2 transgenic mice did not see significant change. This suggests that superoxide scavenging potentially plays a role in providing a reduced glutathione pool, enabling more effective glutathione mediated antioxidant capabilities. Although glutamate and malate induced NADH linked respiration was reduced after ischemia and reperfusion, malate dehydrogenase was not affected by ischemia reperfusion injury.

While state 3 oxygen consumption was significantly decreased for both WT and SOD2TG groups after IR45, SOD2 overexpression attenuated the drop in oxygen

consumption in state 3 conditions after IR. The downregulation of state 3 respiration may indicate changes in rates of substrate oxidation, electron transport (specifically NADH linked electron transport), uniquinone pool, or cytochrome c availability (Brand & Nicholls, 2011). Our results show that SOD2 overexpression preserved electron transport activity in complex I-IV after IR, which may imply uninhibited flow of electrons to molecular oxygen using NADH linked substrates. Other studies also showed that α-ketoglutarate dehydrogenase was also responsible for respiratory dysfunction (Lucas & Szweda, 1999). The massive impairment observed in α-ketoglutarate dehydrogenase activity in WT and a mild decrease in the transgenics coincided with the impairment of oxygen consumption in both groups. Ischemia reperfusion injury raised state 4 oxygen consumption in the presence of oligomycin. SOD2 overexpression had a lower increase in state 4 respiration, suggesting a greater ability to maintain proton motive force or decreased proton leak across the membrane. The RCI or ratio between state 3 and state 4 provides a sensitive measure of mitochondrial dysfunction by being able to detect changes in oxidative phosphorylation (Brand & Nicholls, 2011). Lowered RCI after reperfusion injury implies mitochondrial dysfunction. Although both WT and SOD2TG groups experienced decreased RCI after IR, SOD2TG mitochondria experienced a smaller drop in RCI as the WT mitochondria. However, increased superoxide scavenging did not protect the downregulation of ATP production after ischemia and reperfusion injury.

State of Glutathione Peroxidase and Catalase after IR

Mitochondria contain a network of antioxidant enzymes that are required to protect myocytes from protein oxidation, DNA damage, and lipid peroxidation. These enzymes include glutathione reductase, glutathione peroxidase, and SOD. The depletion of GSH pool has been observed after oxidative stress induced by ischemia and reperfusion (Dhalla et al., 2000). Glutathione peroxidase downregulation after ischemia and reperfusion remains trivial. Some groups observed impairment of activity under different reperfusion conditions in cerebral homogenates while other groups failed to observe significant decreases in glutathione peroxidase, SOD, nor glutathione reductase activity in heart homogenates after IR (Işlekel et al., 1999; Curello et al., 1985). We found that Glutathione peroxidase activity was downregulated after 30 minutes ischemia and 45 minutes reperfusion in isolated heart mitochondria. It may be possible that different isoforms of GPx present in whole tissue homogenates may be differentially affected by ischemia reperfusion. GPx4 knockouts have been shown to be embryonic lethal (Marí et al., 2009). Deficiency of GPx has also been correlated with increased myocyte injury characterized by increased infarct sizes, increased creatine kinase release, and decreased cardiac contractility (Dhalla et al., 2000). The SOD2TG mice had lower baseline of mitochondrial GPx activity, however our previous studies have shown that the SOD2TG hearts had increased contractility and enhanced cardiac function, uncharacteristic of GPx deficiency (Kang et al., 2015b). GPx activity for the SOD2TG hearts did not decrease after ischemia reperfusion and TTC staining showed attenuated infarct sizes in heart slices even with lower basal levels of GPx activity.

There are conflicting reports of how catalase activity is affected after ischemia reperfusion injury. It has been shown that isolated catalase is sensitive to superoxide induced inactivation using xanthine oxidase reaction. This downregulation of activity was preventable by the administration of MnSOD (Kono & Fridrovich, 1982). However, other studies saw no change or increased catalase activity in tissue homogenates after IR. (Haramaki et al., 1998; Işlekel et al.,1999). We found that catalase activity was decreased in mitochondrial fractions after ischemia reperfusion injury while the cytosolic fractions saw increases in activity. Other studies show that catalase may be regulated by c-Abl tyrosine kinase localization to mitochondria in oxidative environments. Both c-Abl and Arg are able to regulate catalase function as well as necrosis/apoptosis (Cheng et al., 2003; Kumar et al., 2001). The impairment mitochondrial catalase was mitigated by SOD2 overexpression. Superoxide and hydrogen peroxide in the presence of iron create hydroxyl radicals in the Haber-Weiss and Fenton reaction (Winterbourne, 1995). The extreme reactivity of hydroxyl radicals also contributes to a wide variety of oxidative stress pathologies (Kehrer, 2000). The SOD2 overexpression hearts had about ~13% higher baseline catalase activity compared to WT.

Downregulation of Basal Mitochondrial Respiration from SOD2 Overexpression

There is still conflicting evidence on the protective nature of SOD2 overexpression in the mitochondrial matrix. While this study shows that SOD2 overexpression was able to attenuate further impairment of mitochondrial enzyme kinetics, the SOD2TG hearts displayed lower basal activities for respiration and certain

mitochondrial enzymes such as complex I, complex II, citrate synthase, aconitase, IDH2, malate dehydrogenase and glutathione peroxidase. The increased influx of hydrogen peroxide as a result of increased superoxide dismutation from overexpression has been shown to dampen mitochondrial respiration, induce mtDNA damage, and ATP production by some studies (Ansenberger-Fricano et al., 2013). However, our study showed preserved respiratory coupling and higher ATP ratios even with lower basal respiratory enzyme activities and oxygen consumption (Kang et al., 2015b). A steady flux of hydrogen peroxide from MnSOD upregulation has been shown to downregulate basal electron transport complex expression and shift cells towards glycolysis via AMPK mediated pathways for ATP production (Hart et al., 2015).

Conclusion

It is well documented that cardiac ischemia and reperfusion injury results in mitochondrial dysfunction including impairment of oxidative phosphorylation, respiratory complexes, and Krebs enzymes. These effects may be attributed to superoxide and other reactive oxygen species that arise during reperfusion. This work demonstrates that SOD2 overexpression attenuates deficits in electron transport for complexes I-IV as well as other enzymatic activities in the Krebs cycle. Superoxide scavenging may also serve to protect mitochondrial antioxidant enzyme impairment after ischemia reperfusion injury.

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