


Summer 2006

Extracellular Superoxide Dismutase Protects Cardiomyocytes During Hyperoxia

Mervat Elsayed Ali
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Doctor of Philosophy (PhD), Dissertation, , Old Dominion University, DOI: 10.25777/wkqh-9s92
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**EXTRACELLULAR SUPEROXIDE DISMUTASE PROTECTS
CARDIOMYOCYTES DURING HYPEROXIA**

By

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M.D. June 1987, Suez Canal University, Egypt

A Dissertation Submitted to the Faculty of
Old Dominion University in Partial Fulfillment of the
Requirement for the Degree of

DOCTOR OF PHILOSOPHY

BIOMEDICAL SCIENCES

OLD DOMINION UNIVERSITY
and
EASTERN VIRGINIA MEDICAL SCHOOL

August 2006

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ABSTRACT

SUPEROXIDE DISMUTASE PROTECTS CARDIOMYOCYTES DURING HYPEROXIA

Mervat Elsayed Ali
Old Dominion University, 2006
Director: Dr. Frank J. Castora

Reactive oxygen species are implicated in tissue damage in many cardiovascular diseases. The current study was designed to test the hypothesis that exposure to high inspired oxygen concentrations (100%) damages cardiac mitochondria and that a biogenic response is needed for cell survival. The study was done in mice with a transgene (TG) for extracellular superoxide dismutase (EC-SOD) or with the EC-SOD gene knockout (KO). Adult EC-SOD mice and their wild type littermates and EC-SOD KO mice and their wild type littermates were exposed to 100% oxygen for 6 hours and for 72 hours, and the hearts were subsequently removed under deep anesthesia. The mortality rate in KO mice after 72 hours (25%) was significantly higher than wild type littermates (10%) and TG mice (4.2%). In the EC-SOD TG mice, 6 hours of exposure to hyperoxia activated the transcription of nuclear genes important for mitochondrial biogenesis- nuclear respiratory factors (NRF-1 and NRF-2) and their co-regulator PGC-1 α -leading to expression of mitochondrial transcription factor A (Tfam), which regulates mitochondrial transcription and replication. Hyperoxia also induced activation of NRF-1 and NRF-2 in EC-SOD KO mice after 6 hours but down-regulated Tfam and PGC-1 α transcripts. But after 72 hours, mRNA expression for NRF-2, PGC-1 α and Tfam increased in these mice.

The protein expression of the pro-survival Protein Kinase B (Akt) was significantly increased by hyperoxia in the TG hearts after 6 hours ($p<0.05$) and significantly decreased in KO mice ($p<0.05$). The protection of mitochondrial DNA (mtDNA) deletion was significantly higher in KO (23%) than in the TG hearts (3.4%). The transcription of mitochondrial 12S rRNA and nuclear COXIV increased significantly in TG hearts and decreased significantly in KO hearts ($p<0.05$). The pro-apoptosis markers, p-Bad and p-GSK-3 β , which are phosphorylated by Akt, increased significantly in TG cardiomyocytes and decreased significantly in KO cardiomyocytes.

In conclusion, Systemic hyperoxia consistently increases mitochondrial biogenesis markers in the mouse heart, most prominently in EC-SOD TG mice, and co-incidentally with increases in Akt, p-Bad and p-GSK-3 β . This suggests that extracellular reactive oxygen products activate mitochondrial biogenesis and the pro-survival pathway in TG mice. Also, hyperoxia decreased the activation of Tfam and PGC-1 α transcription after 6 hours and increased it again after 72 hours in KO mice. This was associated with a decrease in expression of Akt, p-Bad and p-GSK-3 β and an increase in p38, indicating that either hyperoxia activated mitochondrial biogenesis and pro-survival pathways through p38 or that hyperoxia produced more oxidative damage to the mitochondria in the KO. In the latter case, the increase in p38 may be a signal for activation of apoptotic pathways.

Co-Directors of advisory committee:

Dr. Claude A. Piantadosi
Dr. Hajer B. Suliman

*To my mother who supported me throughout my life and during
my doctoral training and who passed away recently,
and to my beloved family*

ACKNOWLEDGMENTS

I would like to thank all the members of my committee for all the help they have provided during my doctoral training. Specifically, I would like to thank Dr. Claude A. Piantadosi, the director of the Center for Hyperbaric Medicine and Environmental Physiology at Duke University Medical Center, for giving me the chance to do my research in his laboratory, for his guidance and support during my research training and for his careful reviewing my thesis. I would like to thank Dr. Hagir B. Suliman my mentor at Duke University Medical Center, for her close supervision and guidance during my training, for discussing and sharing with me the development of the successful and unsuccessful results I have made, and for her careful reviewing my thesis. I would also like to thank Dr. Frank Castora, my supervisor at Eastern Virginia Medical School for helping me to start my Ph.D. at Eastern Virginia Medical School, also for his continuous support and help during my Ph.D. I would like to thank Dr. Miriam Rosenthal, Dr. Howard White, and Dr. Peter Blackmore for their stimulating conversation and for carefully reviewing my dissertation. I also want to thank all the members in Dr. Piantadosi laboratory and Hyperbaric Center for helping me to get this work done; Dr. Martha sue Carraway for helping proofreading my thesis, Dr. Barry Allen for his help with organizing my reference list; Susan fields and Griag Marshal for helping with the mice and laboratory supplies; Owen Doar for helping with hyperoxia exposure, and Lynn Tatro for working out many technical problems encountered in the lab. I would like to thank my family for their support, patience and help especially during the last period of my work. Finally, I would like to thank Dr.

Taher I. Al-Serafi, the Chairman of the Biochemistry Department at the Medical School of Suez Canal University, and the Egyptian Ministry of High Education for giving me the Scholarship to do my doctorate in the United State of America.

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LIST OF ABBREVIATIONS

12S rRNA	12S mitochondrial ribosomal ribonucleic acid
AIF	Apoptosis Initiation factor
Akt	Protein kinase B
ARE	Antioxidant response element
AP-1	Activator protein-1 complex
Atox-1	Antioxidant-1 protein
CAMKIV	Cardiac Ca ²⁺ /calmodulin kinase isoform 4
cGTP	Cyclic guanosine triphosphate
COXIV	Nuclear cytochrome oxidase subunit 4
CREB	Cyclic AMP Response Element Binding Protein
CuZn-SOD	Copper zinc superoxide dismutase
DEPC	Diethylprocarbonate
DTT	Dithiothreitol
EC-SOD	Extracellular superoxide dismutase
EC-SOD KO	Superoxide dismutase knockout gene
EC-SOD TG	Superoxide dismutase transgene
EDTA	Ethylene diamine tetra acetic acid
EGTA	Ethylene guanidine tetra acetic acid
ES	Embryonic Stem
ETC	Electron Transport Chain
FGF	Fibroblast growth factor

GSK-3 β	Glycogen synthase kinase isoform β
GAPDH	Glyceraldehyde phosphate dehydrogenase
HRP	Horse radish peroxidase
I-CAD	Inhibitor of caspase-activated deoxyribonuclease
IFN- γ	Interferon gamma
IL-1	Interleukin-1
INF-alpha	Tumour Necrosis factor-alpha
KO	Knockout
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MAPKp38	Mitogen activator kinase protein 38
Mn-SOD	Manganese superoxide dismutase
MPT	Mitochondrial Permeability Transition
mtDNA	Mitochondrial deoxyribonucleic acid
mTFB1	Mitochondrial transcriptional factor B1
mTFB2	Mitochondrial transcriptional factor B2
NO	Nitric oxide
NRF-1	Nuclear respiratory factor -1
NRF-2	Nuclear respiratory factor -2
OXPHOS	Oxidative phosphorylation
PCR	Polymerase chain reaction
PDGF	Platelet derive growth factor
PGC-1 α	Peroxisome proliferator-activated receptor gamma co-activator-1 alpha

PKB	Protein kinase B
PKC	Protein Kinase C
PH	Pleckstrin homology
PMSF	Phenylmethyl sulfonyl fluoride
PPAP	Peroxisome proliferators activated receptor
PTEN	Phosphatase and tensin homologous on chromosome 10
PtdIns	Phosphatidylinositol
PtdIns (4,5) P ₂	Phosphatidylinositol 4,5 bisphosphate
PtdIns (3,4,5) P ₃	Phosphatidylinositol 3, 4,5 trisphosphate
ROS	Reactive oxygen species
RT	Reverse Transcriptase
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SERCA	Sarcoendoplasmic reticulum calcium ATPase
SQPCR	Semiquantitative PCR
TBS	Tris buffered saline
TEA	Tris-EDTA-Acetate
Tfam	Mitochondrial transcription factor A
TR	Thyroxin receptor
Tunel	Terminal deoxynucleotidyl transferase-mediated dUTP biotin nick-end label
UCP-1	Mitochondrial uncoupling protein-1
VSMC	Vascular smooth muscle cells
WT	Wild type

CHAPTER I

INTRODUCTION

Oxygen therapy is one of the most frequently used treatments in clinical medicine. It is administered to decrease and prevent tissue hypoxia in patients with acute and chronic cardiovascular and pulmonary diseases and also for premature infants with respiratory distress syndrome. Oxygen therapy is also important for many patients after heart surgeries such as coronary artery bypass. Ironically, oxygen at sufficiently high concentrations is toxic to all cells because of its propensity to accept electrons, resulting in incompletely reduced chemical species known as reactive oxygen species (ROS). The degree of oxygen toxicity is proportional to the oxygen concentration and the duration of exposure. Therefore, the administration of oxygen at concentrations higher than 60% at sea level can be toxic to the lungs, and in the newborn, to the eyes [1]. Pure oxygen concentrations higher than atmospheric, known as hyperbaric oxygenation, markedly accelerates oxygen toxicity in many organs including the lungs, the brain, and the heart.

Excessive ROS are scavenged by endogenous antioxidant defenses that protect the tissues from oxidative damage. The balance between ROS production and antioxidant capacity is very important, and its disruption contributes to the pathophysiology of numerous diseases. ROS are proposed to contribute to the deterioration of cardiac function in patients with heart diseases. It has been reported that ROS are increased in the failing heart, and are involved in atherosclerosis and myocardial ischemia/perfusion injury. Antioxidant enzymes are decreased in the decompensated heart, depressing

The journal model for this dissertation is *Current Biology*.

the defenses against oxidative stress. Overexpression of antioxidant enzymes, such as the extracellular isoform of superoxide dismutase (EC-SOD) has been found to protect the lungs from oxidative damage due to hyperoxia. The effect of increased expression of the EC-SOD gene or its deficiency in the heart after high level oxygen concentration is unknown. In this dissertation, I studied the effects of hyperoxia (100% oxygen) exposure on heart mitochondria in mice and the role of EC-SOD in protection from high oxygen damage.

Hyperoxia and Production of Reactive Oxygen Species (ROS)

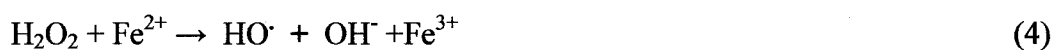
Molecular oxygen is an excellent terminal electron acceptor. In mitochondria, the high affinity of oxygen for electrons provides a large thermodynamic driving force for oxidative phosphorylation (OXPHOS). The transfer of four electrons leads to safe products (two molecules of H₂O), but partial reduction generates hazardous reactive oxygen species (ROS). The first ROS produced in the univalent reduction pathway of O₂ to water is the superoxide anion radical (.O₂⁻). In particular, superoxide anion, is a potentially destructive compound formed by the transfer of a single electron to O₂.



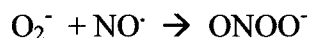
In the normoxic environment only 0.1-1.0% of the electrons transferred to O₂ result in .O₂⁻ production in most cells, but the production of .O₂⁻ is markedly accentuated under hyperoxic conditions. Spontaneous or enzymatic [via superoxide dismutase (SOD)] dismutation of .O₂⁻ produces hydrogen peroxide (H₂O₂):



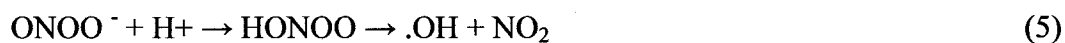
Hydrogen peroxide can also be produced directly from O_2 via enzymatic bivalent reduction. Superoxide can act as either an oxidant or a reductant, but under the intracellular reducing environment, it functions primarily as an oxidant. For example, superoxide produced by cellular reductants and autoxidation of respiratory chain electron carriers inactivates iron-sulfur-containing dehydratases including 6-phosphogluconate dehydratase and the citric acid cycle enzyme aconitase. Oxidation of $[4Fe-4S]^{2+}$ clusters in dehydratases such as aconitase inactivates the enzymes by releasing Fe^{2+} from the cluster. Reactivation is achieved by iron-sulfur cluster reduction and Fe^{2+} insertion. Inactivation-reactivation is a dynamic however Fe^{2+} , can reduce H_2O_2 in a Fenton-type reaction producing hydroxyl radical (OH^\cdot):



In addition, $.O_2^-$ reacts with nitric oxide (NO^\cdot) at a diffusion-limited rate producing peroxynitrite ($ONOO^-$):



Peroxynitrite is directly toxic to molecules containing sulfhydryl groups. It also can be protonated to peroxynitrous acid, which spontaneously decomposes to produce an OH^\cdot -like species and NO_2 . HO^\cdot and $ONOO^-$ are among the most potent oxidants in biological systems, and they are capable of oxidizing DNA, proteins and lipids leading to tissue injury [2].



Superoxide does not diffuse across biological membranes readily because of its negative charge. Thus, it is compartmentalized where it is produced. In the cytoplasm,

O_2^- can be generated either non-enzymatically via autoxidation of small molecules such as thiols, hydroquinones and catecholamines, or enzymatically by various oxidases and dehydrogenases such as xanthine oxidase and flavoprotein dehydrogenase.

The mitochondrial electron transport chain is a major site of $\cdot O_2^-$ production mainly via autoxidation of ubisemiquinone. The microsomal electron transport system present in the endoplasmic reticulum is also a source of $\cdot O_2^-$. In phagocytic cells such as neutrophils and macrophages, the NADPH oxidase system present in the plasma membrane releases large amounts of $\cdot O_2^-$ into the phagosomes and extracellular space when activated. Moreover, the peroxisomes are an important source of intracellular H_2O_2 .

Persistent exposure to high oxygen concentration (hyperoxia) increases the formation of more reactive oxygen species that induce damage to most of the cellular organelles.

ROS and Mitochondria

ROS can damage various cellular components such as proteins, lipids, and nucleic acids [3, 4]. ROS also mediate mtDNA damage, alterations of gene expression, and mitochondrial dysfunction [5]. Mitochondrial injury is evidenced by mitochondrial DNA (mtDNA) damage, as well as by a decline in mitochondrial RNA (mtRNA) transcription, protein synthesis, and general function. MtDNA is located within the matrix space of the mitochondria in close proximity to the electron transport chain. Due to this location, and its low repair capabilities, and lack of protective histone-like proteins, mtDNA is probably more vulnerable to oxidative damage than nuclear DNA [6, 7]. MtDNA deletion and mutation may prevent its replication and expression. Further, ROS may induce

progressive destruction of mtDNA, and this type of damage can lead to a decline in mtRNA transcription as well as a loss of function [8].

The mitochondrial respiratory chain Complexes I and III have been shown to be a major source of ROS *in vitro* [9]. ROS production increases in patients with mitochondrial cardiomyopathy [8] and recent studies have suggested a definite link between excessive generation of ROS and the development of myocardial remodeling and damage [5, 10]. Additionally, the amount of ROS generated, as determined by electron spin resonance spectroscopy, increases in the murine model of myocardial infarction [10]. Furthermore, mitochondrial electron transport has been shown to be a possible site for $\cdot\text{O}_2^-$ generation during heart failure.

Mitochondria Oxidation and Biogenesis

Mitochondria contain a closed, circular, double-stranded DNA of about 16.5 kb and 16.35 kb in human and mouse, respectively. The mitochondrial genome is similar to the bacterial genome in that both the heavy and light strands are transcribed. The mitochondrial genome encodes thirteen polypeptides involved in oxidative phosphorylation, including seven subunits of NADH-ubiquinone oxidoreductase (Complex I) (ND1, ND2, ND3, ND4, ND4L, ND5, and ND6), 1 subunit (cytochrome b) of ubiquinol-cytochrome c oxidoreductase (Complex III), 3 subunits (COI, COII, and COIII) of cytochrome c oxidase (Complex IV), and 2 subunits (ATPases 6 and 8) of Complex V along with 22 tRNA and 2 rRNA (12S and 16S) subunits (Figure 1.1).

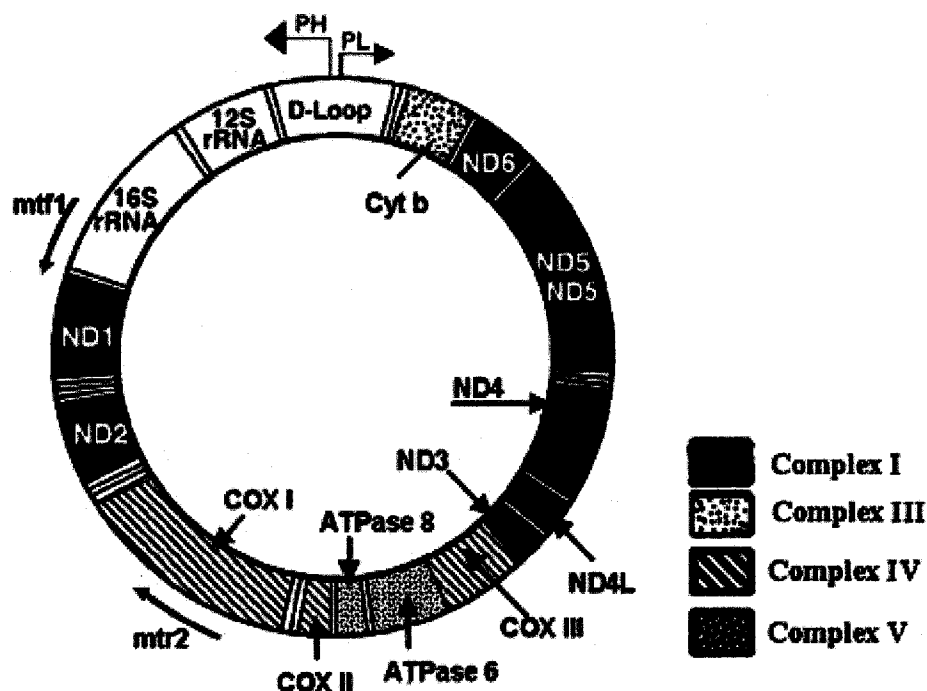


Figure 1.1. Illustration Map of Mouse Mitochondrial Genome. The 16.35 Kb mouse mitochondrial genome which includes 13 genes of the OXPHOS system 2 rRNA (12S and 16S) and 22 tRNA. PH and PL represent the promoters of the heavy and light strands. The figure also shows the site of the mtDNA deletion primers (mtf1 and mtr2) used for the deletion analysis in the study.

Although mitochondria have their own genetic system, the protein encoding of mtDNA is limited to only the 13 most hydrophobic subunits of the respiratory apparatus mentioned above. Thus, mitochondria rely on nuclear genes to provide the majority of proteins and factors required for OXPHOS. Mitochondrial replication and transcription depend on nuclear proteins such as DNA polymerase γ , RNA polymerase II and mitochondrial

transcription factor A (Tfam). Tfam stimulates the transcription from bidirectional promoters within the D-loop region, and it is also essential for mtDNA replication.

Regulation of mitochondrial biogenesis largely depends on the expression of nuclear respiratory factors (NRF-1 and -2). Nuclear peroxisomal proliferator-activated receptor gamma co-activator α (PGC-1 α) has been suggested as a master regulator of the mitochondrial biogenesis and has a major impact on the NRF system. When introduced into muscle cells it dramatically induces gene expression for NRF-1, NRF-2 and Tfam [11] (Figure 1.2). Furthermore, PGC-1 α physically interacts with NRF-1 and co-activates its transcriptional activity. A dominant negative allele of NRF-1 completely blocks the ability of PGC-1 α to induce mitochondrial proliferation, indicating the importance of the association of the transcriptional factor/cofactor pair [12]. Furthermore, mutations that affect PGC-1 α expression lead to loss of heart mitochondria and down regulation of mitochondrial enzyme [13]. Interestingly, PGC-1 α has also been shown to be induced in brown fat cells upon cold stimulation, which suggests that PGC-1 α strongly co-activates several nuclear receptors (PPAR gamma, PPAR alpha, RAR and TR) that bind to mitochondrial uncoupling protein-1 (UCP-1) enhancer [12]. In the heart PGC-1 α was found to activate the gene expression of UCP-2 and UCP-3 after LPS stimulation [14].

In contrast to nuclear-encoded genes, mitochondrial-encoded gene expression is primarily regulated by mtDNA copy number [15]. A recent study in our laboratory [14] showed that lipopolysaccharide (LPS) administration to rats resulted in mtDNA deletion in the heart which decreased mtDNA copy number and decreased mtDNA gene transcription. Defective mtDNA generates more ROS by defective OXPHOS, which leads to more mitochondrial damage. Recovery of mitochondria after oxidation by LPS

was shown to involve increased mitochondrial transcriptional factor A (Tfam) and DNA polymerase γ expression which are important for mitochondrial biogenesis. The transcription of other mitochondrial biogenesis markers; PGC-1 α and the nuclear respiratory factors (NRF-1 and NRF-2) is also increased after the oxidative stimulation by LPS (Figure 1.2).

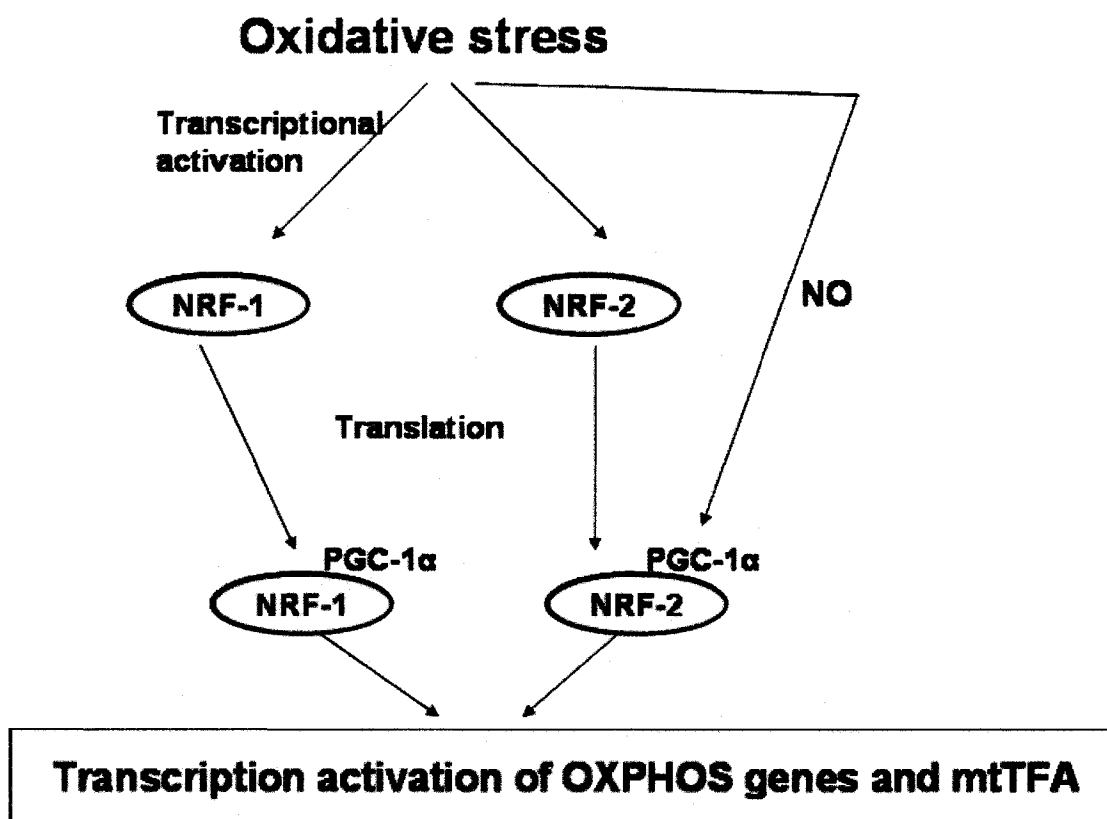


Figure 1.2. Oxidative Stress and Activation of Mitochondrial Biogenesis Markers. Oxidative stress stimulates transcriptional activation of NRF-1 and NRF-2 and in the presence of the co-activator PGC-1 α they activate the transcription of OXPHOX genes and mitochondrial transcription factor A (mtTFA).

ROS and Pro-Survival Pathways

Biogenic factors (cold, exercise, infection and oxidative stress) stimulate the activation of mitochondrial biogenesis. Changes in the intracellular Ca^{2+} concentration and ATP/AMP ratio activate Ca^{2+} /calmodulin-dependent protein kinase (CAMK), and protein kinase C (PKC) has been implicated in the regulation of expression of respiratory genes in the skeletal muscle. Constitutive expression of CAMKIV shows an increase in the level of PGC-1 α and increased mitochondrial biogenesis [16].

It is also reported that PGC-1 α can be activated by a p38 MAPK-sensitive pathway. MAPK p38 is a stress-activated kinase that responds to extracellular stimuli such as UV light, heat, osmotic shock, inflammatory cytokines (TNF- α and IL-1) and growth factors [17-20]. Regulation of respiratory gene expression via p38 MAPK dependent pathways and induction of mitochondrial proliferation by extrinsic factors such as cytokines has been reported. The involvement of p38 MAPK-dependent pathways in oxidative stress-induced increase in mitochondrial biogenesis is not well established.

The pro-survival phosphatidylinositol 3 kinase (PI3K) –Akt pathway has been shown to activate mitochondrial biogenesis in hepatic rat mitochondria after LPS stimulation [21]. Akt is also stimulated by growth and survival factors [22]. There are three isoforms of Akt, known as Akt1 (PKB α), Akt2 (PKB β), and Akt3 (PKB γ). Akt1 is ubiquitously expressed with high expression in heart, brain and lung [23]. All three isoforms of Akt share three domain structures consisting of an N-terminal pleckstrin homology (PH) domain, followed by a kinase domain related to protein kinases A and C (containing Thr³⁰⁸ in Akt1) and a C terminal regulatory domain (containing Ser⁴⁷³ in

Akt1). PH domains have high-affinity recognition of phosphoinositide head groups. Activation of PI3- kinase leads to phosphorylation of membrane phosphatidylinositol-(4, 5)-bisphosphate [PtdIns (4, 5) P2] generating PtdIns (3, 4, 5) P3. Accumulated PtdIns (3,4)P2 and PtdIns (3,4,5) P3 recruit Akt containing the PH domain to the plasma membrane [24]. There Akt is phosphorylated at Thr³⁰⁸ and Ser⁴⁷³ [25, 26]. Phosphoinositide-dependent protein kinase-1(PDK1) and integrin-linked kinase (ILK) phosphorylate Thr³⁰⁸ Ser⁴⁷³ respectively [27, 28].

Activation of Akt induces activation of downstream pathways important for cell survival and inhibition of apoptosis. Akt phosphorylates the pro-apoptotic protein Bad, a member of the Bcl-2 family which promotes cell survival. A second downstream pro-survival and anti-apoptotic mechanism is through phosphorylation of the glycogen synthase kinase (GSK-3 β). Phosphorylation of GSK-3 β leads to inactivation of the enzyme and reduces apoptosis [29, 30]. In addition, several studies indicate that activation of PI-3K/Akt suppresses caspase-3 activation and DNA fragmentation in a variety of cell lines [31-33].

ROS and Apoptosis

ROS function as signal transduction intermediates to induce transcription factor activation, gene expression, and cell growth; they also can induce apoptosis [34]. When the capacity of the antioxidant system is compromised, exposure of living tissue to higher oxidative stress will result in increased levels of defective mitochondria and mutated mtDNA, and thus create a cycle of increased ROS production and oxidative damage. When ROS increase above a threshold, mitochondrial damage irreversibly activates cell

death. Increased ROS leads to conformational changes in the adenine nucleotide translocator, the voltage gated anion channel protein (porin), the peripheral benzodiazepine receptor, and cyclophilin that are all involved in a pore complex between the outer and inner mitochondrial membrane. This protein pore can open to allow dissipation of the mitochondrial membrane potential and pH gradient. This is referred to as the mitochondrial permeability transition (MPT), or opening of the mitochondrial transition pore [35, 36]. Excess production of peroxides also oxidizes the glutathione pool and may also allow the formation of dithiols on the mitochondrial permeability transition (MPT) pore, which triggers pore opening [37], leads to the release of cytochrome c (cyt c), apoptosis initiation factor (AIF), and mitochondrial protective factors from mitochondria.

In the cytoplasm, cytochrome c interacts with Apaf-1 and caspase 9 to activate caspase 3. Caspase 3 has a cleavage effect on pre-PARP, ICAD, and pre-Acinus which together with AIF is also released from the mitochondria. The assembly of an apoptosome (cytochrome c, caspase 9 and AIF-1) is directed at the nucleus leading to chromatin cleavage and nuclear condensation [36]. Moreover, increased ROS may induce the peroxidation of cardiolipin in the mitochondrial inner membrane, which triggers dissociation of cytochrome c from cardiolipin [38]. The release of cytochrome c can also be regulated by the effect of translocation of pre-apoptotic proteins (Bax and Bad) from the cytosol to the mitochondrial membrane in response to increases in ROS production in cardiomyocytes [39]. Upon translocation to the mitochondria Bax/Bad associates with the antiapoptotic protein Bcl-2 to form a heterodimer. Activation of caspases and nucleases leads to mtDNA degradations decreased mitochondrial transmembrane potential,

uncoupling of electron transport from ATP production, increased cytosolic calcium and generation of more reactive oxygen species. Bad has been found to be a substrate for the pro-survival kinase, Akt [36]. Activation of Akt increases phosphorylation of Bad and prevents its association with Bcl-2 protein and prevents the initiation of apoptosis.

Akt phosphorylates another pro-apoptotic kinase, glycogen synthase kinase-3 β (GSK-3 β). GSK-3 β associates with the MPT, and phosphorylation of this enzyme on its serine residue number 9 results in its inactivation, and decreases opening of the mitochondrial MPT pores [40]. Activation of Akt/GSK-3 β also inhibits caspase activation and decreases apoptosis (Figure 1.3).

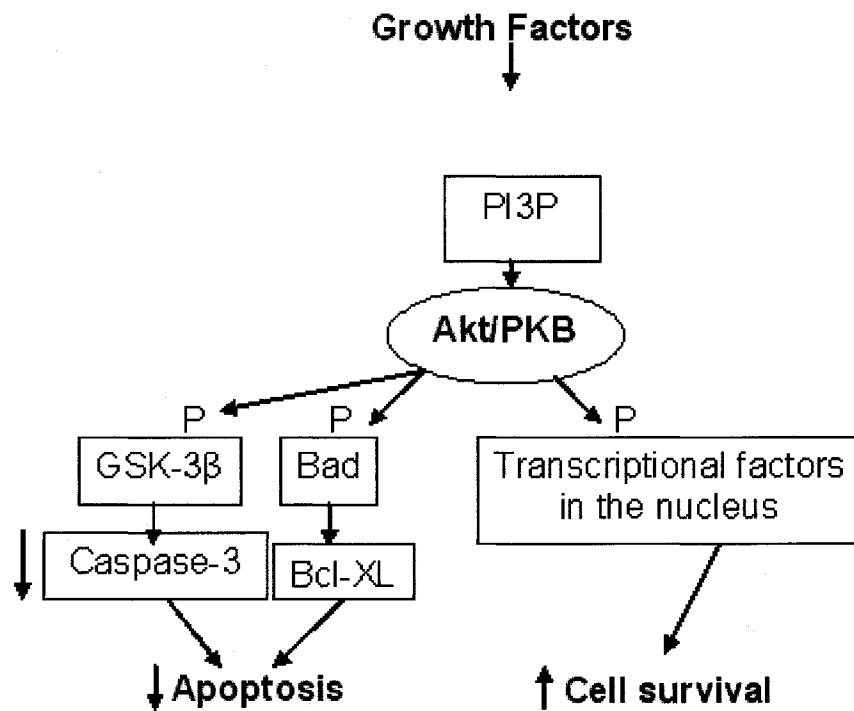


Figure 1.3. Illustration Showing the Activation of Akt through PI3P and its Downstream Pathway. Activation of Akt through PI3P leads to activation of transcription factors in the nucleus and increase cell survival. In the other hand activation of anti-apoptotic proteins (Bad and GSK-3 β) inhibit apoptosis.

Antioxidant Systems

Cellular defense against ROS-mediated injury includes both enzymatic and non-enzymatic antioxidant systems. While non-enzymatic antioxidants such as reduced glutathione (GSH), α -tocopherol (vitamin E), and ascorbic acid (vitamin C) serve important role in cellular antioxidant defense, the enzymatic antioxidant system plays the major roles in the cellular defense against oxygen toxicity. The superoxide dismutases, catalase, and the glutathione peroxidases constitute the major enzymatic antioxidant systems.

Superoxide Dismutases (SOD)

Superoxide dismutases are the most important class of antioxidant enzymes; they are metalloenzymes that catalyze the dismutation of two superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2) and oxygen (O_2) [41]. H_2O_2 can be further metabolized to water by catalase or other peroxidases. In 1969, the first antioxidant enzyme, Cu,Zn superoxide dismutase (SOD), was identified in the erythrocyte by McCord and Fridovich, who found it to catalyze the rapid dismutation of superoxide radical to hydrogen peroxide and oxygen (dissociation constant (K_d) $\sim 3 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$) [42]. Three isoforms of SOD have now been identified in mammals. The Cu,Zn isoform (SOD1) is the major intracellular SOD, and is found primarily in the cytosol, but may also appear in the nucleus, in lysosomes and in the space between the inner and outer mitochondrial membranes. Manganese SOD (SOD2), found in the mitochondria, has Mn in the active site. MnSOD plays an important role in protecting mitochondria from superoxide anions produced as a byproduct of electron transport required for oxidative phosphorylation.

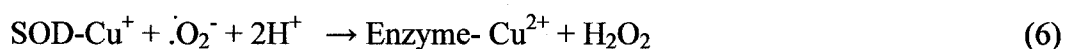
Extracellular superoxide dismutase (SOD3) is present mainly in the extracellular matrix. EC-SOD is considered the principal antioxidant enzyme in the extracellular spaces which protects the extracellular space from oxidant stress. The Cu and Zn containing SOD, CuZnSOD and EC-SOD, constitutes approximately 85-90% of the total SOD activity in the mouse tissues, while MnSOD constitutes approximately 10-15% of total SOD activity.

Molecular and Biological Characters of EC-SOD

The existence of extracellular SOD was suggested in 1980 by Stephan Marklund [43]. Examination of human serum and cerebrospinal fluid by gel chromatography revealed that extracellular fluid contains SOD activity that was immunologically distinct from the intracellular CuZn SOD [44].

The high molecular weight SOD was subsequently purified from human lungs where it is highly expressed and named extracellular superoxide dismutase (EC-SOD) [45]. EC-SOD is a hydrophobic glycoprotein with molecular weight of approximately 135 kd with minor species-specific differences. In most species, EC-SOD is a tetramer of identical 30 kd subunits, although it is occasionally found as a dimer. The tetramer is comprised of two dimers linked through disulfide bridges [46, 47]. The enzyme contains one copper and one zinc ion coordinated to the side chain of a histidine residue in the active site that catalyzes dismutation of the superoxide anion with a rate constant of $10^9 \text{ M}^{-1} \text{ s}^{-1}$. The copper ions are catalytically active in the dismutation reaction. The Zn does not function in the catalytic cycle, but stabilizes the enzyme during the reaction. As a copper-containing enzyme, the activity of EC-SOD is regulated by copper availability.

Free intracellular copper is extremely limited [48], and soluble carrier proteins or “copper chaperones” are used for copper trafficking to specific copper-containing proteins. The copper chaperone that delivers copper to EC-SOD and regulates its activity is called antioxidant-1 protein (Atox1) [49]. The copper ions appear to function in the dismutation reaction by undergoing alternate oxidation and reduction as summarized in the following equations:



The net reaction of the above 2 reactions is:



Analysis of cDNA isolated from a human placental cDNA library revealed that EC-SOD contains 240 amino acids including an 18 amino acid signal peptide for secretion [50]. The remaining 222 amino acids make up the mature protein. The first 95 amino acids showed no sequence homology with other proteins, but a possible N-glycosylation site was identified at Asn-89. 50% sequence homology with other eukaryotic CuZn SOD's is present in residues 96-193 of EC-SOD. Amino acids 194-222 are hydrophilic and contain nine positively charged amino acids, which were believed to contribute to EC-SOD's affinity to heparin and heparin sulfate (Figure 1.4).

The mature human EC-SOD contains six cysteine residues [50], and occurs naturally in two different forms with evident disulfide bridges [51]. One form is active (aEC-SOD) and has a free cysteine residue in position 195. The intrasubunit disulfide bridge formed by Cys107 and Cys189 is necessary for the SOD activity [51]. The other form has a free cysteine residue at position 45 and is inactive (iEC-SOD). EC-SOD is

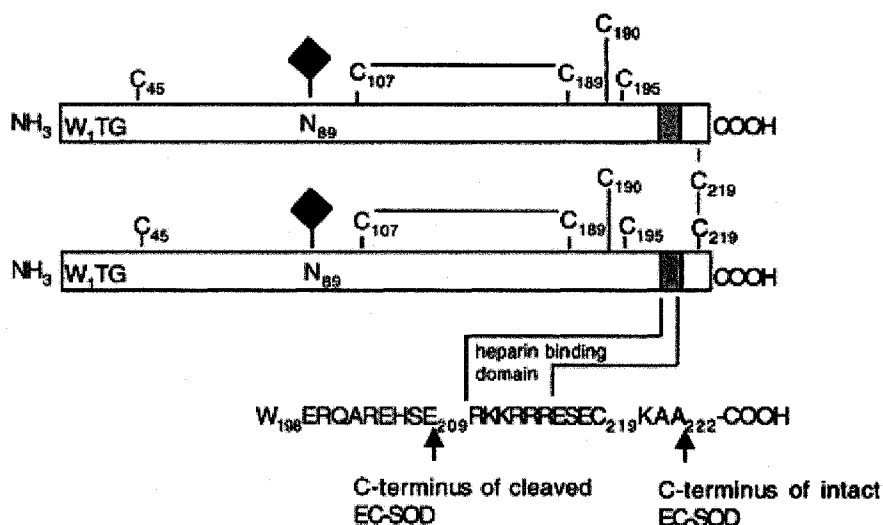


Figure 1.4. Schematic Showing the Structure of the EC-SOD Dimer. The N-terminal amino acid residues and the position of the single *N*-linked glycan at Asn⁸⁹ are indicated. The putative intrachain disulfide bridge between Cys¹⁰⁷ and Cys¹⁸⁹ or possible Cys¹⁹⁰ is shown based on the homology to Cu/Zn-SOD. The C-terminal amino acid residues include the heparin binding domain.

unusually resistant to high temperatures, extreme pH, and high urea concentrations, although it can be inhibited by a variety of agents including azide and cyanide and inactivated by diethyldithiocarbamate and hydrogen peroxide.

The C-terminal region is highly rich in basic amino acid residues [50]. This polybasic region is involved in the binding to heparin/heparan sulfate [52] and type I collagen [53], and is referred to as the extracellular matrix (ECM)-binding region [53]. The heparin-binding domains of two EC-SOD subunits are linked by a disulfide bond to form a cluster of 12 positively charged amino acid residues (Figure 1.2) [47]. These residues provide the basis for the electrostatic interactions with heparin sulfate which anchors EC-SOD to specific regions of the extracellular matrix and on the cell surface.

The ECM region is removed proteolytically just before secretion. This produces both intact and cleaved subunits of EC-SOD. Hence, the enzyme is heterogeneous in its affinity for heparin three subtypes of EC-SOD exist; type A has no affinity, type B, with intermediate affinity, and type C, with high affinity for heparin [54]. The heparin-binding domain has been proposed to act as a nuclear localization signal in culture cells (3T3-L1) [55], suggesting that the role of EC-SOD is to protect the genome DNA from damage by reactive oxygen species and /or the transcriptional regulation of redox-sensitive gene expression [56].

A natural mutation located in the center of the carboxyl-terminal cluster of positively charged amino acid residues, which defines the heparin-binding domain, has been described for human EC-SOD where ArgGly (R213G). The effect of this polymorphism, which is found in 4% of Swedish, 3% of Australian and 6% of Japanese people, increases the concentration of EC-SOD in the plasma of homozygous individuals 10 to 30-fold [57, 58]. This increase in EC-SOD blood concentration, which reflects decreased tissue antioxidant levels, has now been shown to increase the risk of ischemic heart disease [58].

The genomic structure and chromosomal localization of the EC-SOD gene has been mapped in humans to chromosome 4q21, and in mouse to chromosome 5. The human EC-SOD gene consists of three exons and two introns, while the mouse has two exons separated by a 4 kb intron. The entire human EC-SOD coding region (720-bp) is located within exon 3 [59]. The promoter regions of human and mouse EC-SOD apparently lack classical TATA or CCAAT boxes. In humans, the promoter region of the gene contains various regulatory elements including two potential antioxidant response

elements (ARE), AP-1 binding sites, xenobiotic response elements, NF-B motifs, and C/EBP β -binding sites [50, 60, 61].

Regulation of EC-SOD Transcription and Protein Expression

The expression of EC-SOD mRNA and protein, unlike, SOD1 and SOD2, is cell- and tissue-specific and is prominent in heart, lung, blood vessels, placenta, and kidney. High levels of EC-SOD mRNA and protein are present in alveolar type II cells, proximal renal tubular cells, lung macrophages, vascular smooth muscle cells, some fibroblast lines, glial cells, and endothelial cells. Despite detailed promoter analysis that reveals multiple potential regulatory sequences, as described above, mechanisms regulating this cell-specific enzyme expression are not yet known.

In vitro studies in skin fibroblasts indicate that heparin and heparan sulfate induce both EC-SOD mRNA and protein expression. In vascular smooth muscle cells (VSMC) and lung alveolar type 2 cells, inflammatory cytokines such as TNF- α and IFN- γ are a potent combination for induction of EC-SOD mRNA and protein expression. Angiotensin II strongly induces EC-SOD activity in mouse aorta and in cultured human smooth muscle cells [62] through transcriptional activation and stabilization of the mRNA. This effect is mainly due to activation of ERK (p42/p44 MAP kinases). Exercise training, which increases production of nitric oxide in mouse vessel endothelial cells, in turn up-regulates expression of EC-SOD in adjacent smooth muscle cells [63]. In this study, nitric oxide exerted its effect through the MAP kinase p38 pathway. This is an example of the important relationship between EC-SOD and endothelial nitric oxide synthesis. While certain stimuli induce EC-SOD expression, a variety of growth factors repress EC-

SOD mRNA expression. These include TGF- β in human fibroblasts [64] and PDGF and FGF in vascular smooth muscle cells [62].

In a recent study, plasma EC-SOD levels were measured in type 2 diabetic patients and found to be significantly and inversely related to insulin resistance. Interestingly, the authors concurrently reported that in cultured fibroblasts, the C/EBP β enhancer, prolactin, which regulates genes associated with insulin resistance, significantly induced EC-SOD transcription and protein expression [65]. Understanding the complex regulation of EC-SOD will facilitate further studies to define the role of EC-SOD in different disease states.

Biological Role of EC-SOD

EC-SOD is the only known antioxidant enzyme that scavenges superoxide specifically in the extracellular compartment. Superoxide has only a few direct targets, but it does maintain iron in the reduced state, which is then available to participate in Fenton chemistry to produce hydroxyl radical, a highly reactive and toxic ROS. In addition, superoxide reacts rapidly with nitric oxide ($K_d \sim 6.7 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$) to deplete nitric oxide bioactivity and form a strong oxidant, peroxynitrite. EC-SOD limits these toxic extracellular reactions. Numerous studies utilized EC-SOD transgenic and knock-out mouse models to show its role in protecting or amplifying tissue injury.

EC-SOD and Diseases

Disruption of EC-SOD localization or activity may contribute to increased ROS and impaired nitric oxide-mediated vasodilation in cardiovascular diseases [66]. EC-SOD

protects against oxidation of LDL, a major contributor to atherosclerosis, and EC-SOD activity is reduced in patients with coronary artery disease. In diabetes, glycation of EC-SOD is associated with a decrease in heparin affinity and impaired vascular function [64]. Furthermore, in cardiac ischemia/reperfusion, over-expression of EC-SOD in transgenic mice or treatment with recombinant EC-SOD preserves cardiac function and decreases infarct size. A protective effect for EC-SOD has also been reported in ischemia/reperfusion injury in the brain and kidney. As EC-SOD promotes nitric oxide-mediated vasodilation, [67] so it is suggested that antioxidant treatment improves coronary and peripheral endothelial function in patients with coronary artery disease or coronary risk factors [68]. Previous studies showed that overexpression of EC-SOD in new born mice protected lung development in hyperoxia-exposed newborn mice [69]. Also overexpression of EC-SOD in the airways of the transgenic mice had been shown to reduce inflammation and attenuates lung toxicity [70].

The effect of hyperoxia exposure on the heart of the transgenic EC-SOD or the EC-SOD knockout mice has not been studied before. In this study, I investigated the effect of exposure to 100% oxygen at sea level (hyperoxia) on cardiac mitochondria of the transgenic and knockout EC-SOD mice and also the effect of hyperoxia on the activation of mitochondrial biogenesis and cell survival in these mice.

General Hypothesis and Research Objectives

General hypothesis: Extracellular superoxide dismutase (EC-SOD) plays an important role in scavenging superoxide radicals in the extracellular milieu, and it also decreases the oxidative damage to the intracellular compartment especially to

macromolecules such as DNA and RNA. Based on these actions of EC-SOD, I hypothesized that EC-SOD functions to protect cardiac mitochondria from oxidative damage after exposure to hyperoxia (100% oxygen), and improves cell survival through activation of mitochondrial biogenesis. This hypothesis was tested in mice in which levels of EC-SOD were genetically altered, which allowed me to investigate the research objectives.

Specific Aims:

- 1- Study the effect of overexpression and lack of expression of EC-SOD on activation of cardiac mitochondrial biogenesis after hyperoxia exposure.
- 2- Study the effect of hyperoxia in inducing damage to mtDNA, and mitochondrial RNA transcription in genetic altered EC-SOD mice.
- 3- Test the hypothesis that the overexpression of EC-SOD will protect cardiomyocytes from apoptosis which leads to cellular death.

CHAPTER II

MATERIAL AND METHODS

Animal Protocol

The protocol was approved by The Duke University Animal Care and Use Committee.

The study was carried out in two separate groups - the EC-SOD transgenic (TG) group and their wild type (C57/BL6-C3) control and EC-SOD knockout (KO) and their wild type (C57/BL6-J) control group. Both male and female mice were equally represented in each group and they were between the ages of 9-12 months old.

Transgenic mice (TG) used in this study were generated by Oury et al., (1992) [71] using an expression vector comprised of the human β -actin promoter coupled to a full-length cDNA encoding for human EC-SOD. This linearized transgene construct was microinjected into the pronuclei of fertilized single-cell mouse embryos, which were re-implanted into the oviduct of adult female mouse. Offspring were screened for the presence of the transgene by Southern blot and/or polymerase chain reaction analysis of DNA. A founder mouse was then bred with Bl6-C3 (F1) mice.

Knockout mice (KO) were generated by Carlsson et al., (1995) [60]. The mouse EC-SOD coding region (exon 2) was replaced with the gene encoding the neomycin-resistance gene by homologous recombination in embryonic stem (ES) cells. The blastocysts were isolated from C57/BL6-J mice on day 3.5 of pregnancy, and then ES cells were injected into each blastocoele cavity. The blastocysts were introduced into the uterine horns of

pesudopregnant (C57/BL6-J X CBA) F1 mice. Offspring were screened for the presence of the EC-SOD null by Southern blot. The null mice were bred with C57BL/6 mice and homozygous animals were generated (Figure 2.1).

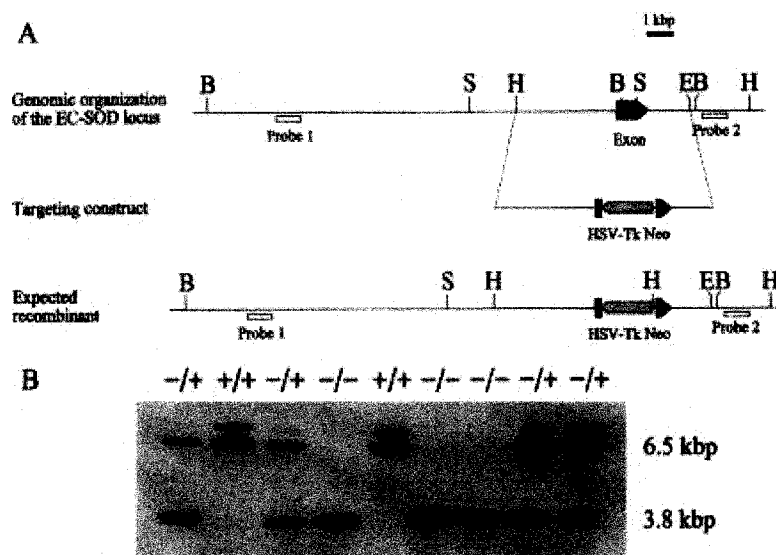


Figure 2.1. Illustration Map of of EC-SOD Knockout Gene. A, Restriction map of the native mouse EC-SOD gene and the disruption of the EC-SOD exon with the neomycin gene. B, Southern Blot analysis, the wild type gene was recognized as a 6.5 kbp fragment, whereas the EC-SOD knockout gene was recognized as 3.8 kbp fragment [57].

Exposure to Hyperoxia

Mice were randomly assigned and placed in small-animal chambers with an airtight oxygen flow rate of 10 L/min. The concentration of oxygen in the chambers was monitored continuously with a Servomex O₂ analyzer (model 572, Sybron, Norwood, Massachusetts, USA) and maintained at > 99%, except for 10 min/day when the cages were opened to room air during periods of cleaning and general maintenance. The CO₂

concentration was held below 0.1%, the temperature between 25°C and 26°C, and the humidity was < 40%. The mice were provided *ad libitum* access to food and water and frequently observed for signs of discomfort or O₂ toxicity. We exposed two groups of mice for each experiment, one group for 6 hours and the second group for 72 hours. We chose these two early (6 hours) and late (72 hours) time periods because at 6 hours significant changes in the expression of mitochondrial biogenesis markers were observed and 72 hours was the maximum time that oxidative damages after exposure to hyperoxia were observed (personal communication with Dr. Piantadosi). Control mice were kept in identical chambers and exposed only to room air.

Harvest the Mouse Heart

After 6 hours and 72 hours exposure the mice were euthanized using Halothane, the thoracic cage was opened and the hearts were harvested and snap frozen in liquid nitrogen and stored in -80°C until use. Mice were taken out of the hyperoxia chamber individually at the time of sacrificing because acute drops in oxygen concentration (100% to normal air 21%) caused death in mice that had been exposed to O₂ for 72 hours.

Total RNA Isolation

All RNA work was done in a specific area designated for RNA extraction and analysis. All equipment and glassware were cleaned using RNAase ZAP. TRIZOL reagent (Life Technologies) was used according to the manufacturer's instructions:

1-The mouse heart was weighed. For each 100 mg of heart tissue 1ml of TRIZOL Reagent was added on ice.

2- The heart was homogenized using a Polytron (Power Gen 125, Fisher Scientific). The Polytron was washed with DEPC water (RNAase free water) before each sample. The homogenization was carried out for 10 seconds twice with an interval of 15 seconds. After homogenization, the sample was left for 5 minutes at room temperature for complete dissociation of nucleoprotein complexes.

3- Two hundred microliters of chloroform per 1 ml of TRIZOL Reagent were added and the sample was shaken vigorously by hand for 15 seconds followed by incubation at room temperature for 2-3 minutes.

4-The sample was centrifuged in a cold microcentrifuge at 4°C at 12,000 x g for 15 minutes to separate the homogenate into an aqueous phase and lower red phenol chloroform phase (RNA is in the aqueous phase). Three hundred microliters of the aqueous phase was transferred into a clean 1.5 microcentrifuge tube and 500 µl of isopropyl alcohol was added to each sample. The sample was gently mixed and left at room temperature for 10-15 minutes to precipitate the RNA.

5-The sample was centrifuged at 12,000 x g at 4°C for 10 minutes. The RNA pellet was washed with 75% ethanol in DEPC water (1ml ethanol: 1 ml of TRIZOL).

Next, the sample was centrifuged at 7500 x g for 5 minutes at 4°C. The RNA pellet was left to air dry for 5 minutes and resuspended in 50 µl of DEPC water and incubated at 55°C for 5 minutes to completely dissolve the RNA pellet.

6-The RNA concentration was measured by diluting 3 µl of sample in 147µl of TE buffer (Tris- EDTA). The RNA concentration was assayed by spectrophotometer at wavelength 260 nm. We also assayed the absorbance at 280 nm (to check for protein contamination

in the sample). The 260/280 ratio was 1.8-2 in all samples. The RNA concentration was calculated according to the equation:

$$\text{RNA concentration} = \text{OD value at 260} \times 50 \text{ (the dilution factor)} \times 40.$$

RNA Gel Electrophoresis

The extracted RNA was separated on 1 % agarose gels in 1X TEA Buffer in sterile water to check the yield of the extraction and the integrity of the RNA. Three microliters of RNA were diluted with 4 μl of DEPC water and 3 μl of RNA loading buffer (Sigma) and loaded into the gel. The separation was performed at 70 volts using a Bio RAD power supply (Model 200/2.0).

Mitochondrial and Nuclear mRNA Expression

One microliter of RNA from each sample was reverse transcribed in a total volume of 25 μl using Moloney murine leukemia virus reverse transcriptase (180 units, Promega) in a reaction buffer containing dNTPs (2 μl), 0.5 μl Oligo dT primers and random primers in a ratio 3:2, DTT (2 μl) and ribonuclease inhibitor RNasin (1 μl) (Promega). First, 1 μg of RNA was brought up to 13.5 μl with sterile water and incubated in a heat block at 70°C for 5 minutes to remove the secondary structures. Then, the samples were returned to ice and 11.5 μl of RT mix was added and incubated in the oven at 42°C for 90 minutes. 1 μl of the cDNA was amplified using PCR with the different oligonucleotide primers as in (Table 2.1) to measure the expression level of mRNA of PGC-1 α , NRF-1, NRF-2, Tfam, 12S rRNA, COX IV, MnSOD and GAPDH. The number of PCR cycles was optimized during the exponential phase of the PCR by titration of visible products on Gel Star-

stained agarose gels. Quantification of amplified mRNA signal was done by densitometry normalized to GAPDH mRNA signal density for each sample using image analysis software (Bio-Rad).

Protein Expression

Preparation of the sample. Protein expression was assayed by Western immunoblot analysis. Frozen mouse heart tissue was homogenized in Tris phosphate buffer at pH 7.5 with antiproteases in a ratio 1:10 respectively. The phosphate buffer contains 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1mM B-glycerophosphate, 1 mM Na_3VO_4 , 1 $\mu\text{g/ml}$ Leupeptin and 1mM PMSF. The sample was homogenized using Polytron on ice 3 times for 15 seconds each and cooled on ice for 30 seconds between homogenizations. Next, the sample was centrifuged in a cold centrifuge (RC-5 Superspeed refrigerated centrifuge, Sorvall) at 4°C at 10,000 x g for 10 minutes to pellet down the nuclei and cellular debris. The supernatant was decanted to a new tube. An aliquot was saved for protein assay and the rest of the sample was mixed directly with an equal volume of (2x) SDS (Laemmli buffer) 125 mM Tris-HCl, 4 % Sodium Dodecyl Sulfate (SDS), 20% Glycerol, 100 mM Dithiothreitol (DDT) and 0.02% Bromophenol blue. The sample was then boiled on a heating block at 95°C for 5 minutes to denature the protein.

Protein Assay

Protein concentration by the Bicinchoninic Acid Method. Reagent A which contains 1% Bicinchoninic Acid and Reagent B which contains 4% Cupper Sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$)

Table 2.1. Primer Sequences Used to Amplify Mitochondrial and Nuclear mRNA

Gene Name	Sequence	
MnSOD	Sense:	5' GGCCAAGGGAGATGTTACAA 3'
	Antisense:	5' GCTTGATAGCCTCCAGCAAC 3'
Tfam	Sense:	5' GGAAGAGCAGATGGCTGAAG 3'
	Antisense:	5' CCAATGACAACCTCCGTCTT 3'
COX IV	Sense:	5' GTCACACGAGACAGATGA 3'
	Antisense:	5' CATCGAAGGGAGTTTACA 3'
PGC1α	Sense:	5' AGCCAGCAGTTTGGTTCTTG 3'
	Antisense:	5' CCACTTTGCCTTCCACAAAT 3'
NRF-1	Sense:	5' CCACGTTGGATGAGTACACG 3'
	Antisense:	5' CTGAGCCTGGGTCATTTTGT 3'
NRF-2	Sense:	5' CCGCTACACCGACTACGATT 3'
	Antisense:	5' ACCTTCATCACCAACCCAAG 3'
12S rRNA	Sense:	5' GAATTACGGCTCCTGCTCA 3'
	Antisense:	5' GGAGGCAAGCATAAGACTG 3'
GAPDH	Sense:	5' TTCACCACCATGGAGAAGGC 3'
	Antisense:	5' GGCATGGACTGTGGTCATGA 3'

All the primers are synthesized using mouse mitochondrial genome from the gene bank (accession # NC006914) by Invitrogen.

were mixed together to make a working solution in a ratio 49:1. Bovine Serum Albumin was used to make standard curve from a stock concentration of 1mg/ml. Serial dilution of the standard was made; 5 µg, 20µg, 40µg, 80 µg, and 160 µg. One ml of the working solution was added to each tube of the standard and the samples and 5 µl of the protein homogenate was added to each sample tube run in triplicate. 160 µl of distilled water was added to each sample tube to make the final volume equal to the volume of the standard. Both the sample and the standard tubes were incubated in a water bath at 60°C for 30 minutes. Then, 3 ml of distilled water was added to each tube to dilute the concentrated purple color. Next, the OD was measured at wavelength 562 nm (Spectrophotometer, Hitachi, U-2000). A standard curve was plotted using computer software and actual protein values of the samples were obtained.

Gel Electrophoresis for the Protein Sample

The samples were separated on 4-20% gradient polyacrylamide, 15 wells minicassette gels (Invitrogen). 20 µg of the protein sample was loaded on the gel from each diluted SDS sample. Color protein Marker (Sigma) was used to identify the bands of interest in each experiment. Electrophoresis Tris glycine (10 mM) running buffer was used and the samples were separated at 125 Volts and a constant current of 36 Amps for 90 minutes using a power supply (Bio-Rad).

Transfer Protein Sample to Immobilon Membrane

The protein bands that were separated on the polyacrylamide gels were electro-transferred to an Immobilon membrane with a pore size of 0.45µm (Millipore).

Corporation). The Immobilon membrane was pre-washed in methanol for 2 minutes and then in transfer buffer for 10 minutes before the transfer. The transfer was done at constant current (500 Amps) for one-hour using transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol in distilled water at pH 8.3). After the transfer, the membrane was blocked in 5 % non-fat dry milk for 30 minutes followed by a wash with Tris Buffered Saline with Tween 20 (TBS-Tween) (Sigma) three times for 10 minutes each.

Immunoblotting the Immobilon Membrane

1-The membranes were incubated with different primary antibodies (Akt, pAkt, p38, p-p38, p-Bad, pGSK-3B, CAMKIV, alpha-actinin and tubulin antibodies) (Table 2.2) in 5% non-fat dry milk overnight at 4°C on the shaker.

2- The membrane was washed with TBS-Tween buffer (Sigma) for 10 minutes 3 times and incubated with HRP-labeled secondary antibodies with dilution 1: 2000 in 5 % milk for one hour at room temperature.

3-The membrane was washed 3X with TBS-Tween to remove unbound secondary antibody.

4-Enhanced Chemilluminescence Detection Reagents (Amersham) were used to detect the protein signal. The HRP/hydrogen peroxide present in the secondary antibody catalyzes the oxidation of luminol in alkaline conditions. After oxidation, the luminol is in an excited state that then decays to a ground state by emitting light. The light reaches a peak after 5-20 minutes and decays slowly with a half life of approximately 60 minutes. Maximum light emission is detected at 428 nm.

5-The membrane was exposed to x-ray film and developed in a Philips x-ray developer.

Table 2.2. Primary Antibodies used to detect the Level of Protein Expression in the Mouse Heart in the Study

Antibodies	Type of Antibody	Dilution	Size of the band	Cataloge#	Source
p-P38	Polyclonal	1:800	38 KD	sc-17852	Santa Cruz
P38	Polyclonal	1:1000	38 KD	sc-535	Santa Cruz
pAkt	Polyclonal	1:1000	60 KD	sc-16646	Santa Cruz
Akt	Polyclonal	1:1000	60 KD	sc-8312	Santa Cruz
p-Bad	Polyclonal	1:1000	45 KD	5284	Cell Signaling
p-GSK-3 β	Polyclonal	1:1000	30 KD	sc-9166	Santa Cruz
α -Actinin	Monoclonal	1:1000	100 KD	sc-17829	Santa Cruz
CAMK-IV	Monoclonal	1:800	59 KD	sc-1545	Santa Cruz
β -Tubulin	Monoclonal	1:1000	45 KD	T-4026	Sigma

Phosphorylated (p) and total primary antibodies were used to detect the expression level of the activated (phosphorylated) protein and the level of the total protein.

Mitochondrial DNA Deletion

MtDNA was analyzed for a deletion between direct repeats (direct repeat 16 corresponding to bp 1095 – 4905 of mouse mtDNA) (Figure 2.2) by polymerase chain reaction (PCR) using mouse mtDNA deletion primers as shown below in Table 2.3 [72] and Takara Ex Taq Kit (Takara BIO INC.). The total DNA was extracted by Gen Elute Mammalian Genomic DNA kit (Sigma), as follows:

- 1-Frozen heart tissue from mice exposed to hyperoxia and non-exposed (air) mice were allowed to thaw on ice and then minced into small pieces to enable more efficient lysis.
- 2- 180 µl of STL Buffer was added to about 50 mg of heart tissue. Twenty microliter of proteinase K (5%) was added and the samples were mixed by vortexing and incubated at 55°C for 4 hours until the tissues were completely digested.
- 3- After complete digestion 200 µl of SL Buffer was added to the samples and vortexed thoroughly for 15 seconds followed by incubation at 70°C for 10 minutes.
- 4- Two hundred microliters of ethanol (95-100%) was added to the sample, and mixed thoroughly by vortexing for 5-10 seconds. The contents of the tube were transferred into a pre-assembled binding column.
- 5-The sample was centrifuged at 6500 x g for 1 minute. The collection tube was discarded and 500 µl of 1x SW Buffer was added to the binding column to wash and centrifuged at 6500 x g for 1 minute. Afterwards, the collection was discarded. The wash step was repeated with centrifugation for 3 minutes at maximum speed (12,000-16,000 x g) to dry the binding column and to be sure it was free of ethanol.
- 6- Two hundred microliters of SE Buffer was pipetted into the center of the binding column and incubated for 5 minutes and then centrifuged for 1 minute at 6500 x g to

elute the DNA. The eluted DNA was stored at 4°C and used for detection of mtDNA deletion in the WT, TG, and KO mouse hearts.

7- The DNA sample was amplified using the SQPCR and the mtDNA deletion primers.

The PCR conditions were: first cycle 96 °C for 2 minutes, second cycle 96 °C for 50 seconds, annealing at 50 °C for 40 seconds and extension at 72 °C for 1 minute for 40 cycles with final extension at 72 °C for 6 minutes. The number of PCR cycles was optimized during the exponential phase of the PCR by titration of visible products on Gel Star-stained agarose gels.

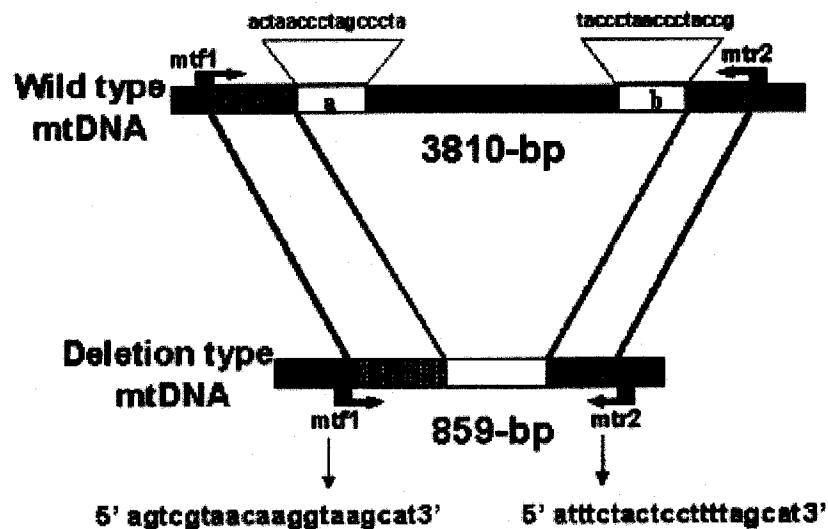


Figure 2.2. Illustration of mtDNA Deletion Primers. The expected mtDNA deletion occurs between two 16-bp direct repeat sequences 5' act aac cct age cct a 3' (1095–1110) and 5' tac cct aac cct acc g 3' (4890–4905). The 16-bp direct repeats are represented by boxes labeled (a and b). Primers 1 and 2 amplify the 859-bp fragment in the presence of the 3810-bp deletion in the mtDNA. The genes in this region include ND1, ND2, COX1, 16S rRNA and tRNA for six amino acids.

Table 2.3. Mouse Mitochondrial DNA Deletion Primers Sequences

Primer	Nucleotide Position	Sequence
mtf1	982-1001 bp	AGTCGTAAGGTAAGCAT
mtr2	5632-5651 bp	ATTCTACTCCTTTTAGCAT

Mitochondrial DNA forward primer (mtf1) and reverse primer (mtr1) designed to detect deletion in mouse heart mitochondrial DNA.

EC-SOD Activity Assay

EC-SOD activity was measured by inhibition of cytochrome *c* reduction as described by Crapo (1978) [73]. Four heart tissues from control and transgenic mice in air and after 72 hours exposure to hyperoxia (100% oxygen) were weighed and homogenized in 10 volumes of ice-cold 50 mM potassium phosphate buffer (pH 7.4) with 0.3 M KBr, and a set of antiproteolytic agents (0.5 mM phenyl methylsulfonyl fluoride, 3 mM diethylenetriaminepentaacetic acid, 90 mg of aprotinin per liter, 10 mg of pepstatin per liter, 10 mg of chymostatin per liter, and 10 mg of leupeptin per liter). The homogenate was then sonicated, and separation of EC-SOD from CuZn-SOD and Mn-SOD was accomplished by passage over a concanavalin A–Sephrose column as described previously [74]. The EC-SOD activity was measured by inhibition of cytochrome *c* reduction at pH 10. One unit of SOD activity is defined as the enzyme activity needed to inhibit a 50% reduction in cytochrome *c*.

Statistical Analysis

Grouped data were expressed as mean \pm SD. Group sizes were $n=4$ or 5 for all studies. Statistics were performed using two-way ANOVA (analysis of variance) followed by Tukey's *post hoc* comparison using Sigma Plot computer software. A *p* value of 0.05 or less was accepted as significant.

CHAPTER III

RESULTS

Because the EC-SOD TG was developed in a strain (C57/Bl6-C3) different than the one used for EC-SOD KO (C57/Bl6-J), we used two different wildtype strains that match with the respective mouse strain as reference controls. The results from the transgenic and the knockout mice are separated in the following two parts. The difference of mitochondrial and nuclear gene expression between each animal group and its control at the base line (air) was summarized in Table 3.1. Also, the effect of hyperoxia for 6 hours and 72 hours on the expression of mitochondrial and nuclear genes in the EC-TG and KO mice is summarized in (Table 3.2 and Table 3.3).

Part I: Effect of Hyperoxia in EC-SOD Overexpressing Mice (TG)

Hyperoxia and Mortality

On exposure to 100% oxygen: after 24 hours the mice started to huddle together and there were no signs of respiratory distress but prolonged exposure for 72 hours was lethal to some mice. Hyperoxia was lethal to 12.5% of the exposed WT mice (3/24), while the mortality rate was lower in the TG mice 4.2% of the exposed mice (1/24), Table 3.4.

Effect of Hyperoxia on Cardiac mtDNA

Hyperoxia increases reactive oxygen species (ROS), which can be deleterious to macromolecules such as DNA and RNA. Because mtDNA is sensitive to damage by ROS, the integrity of mtDNA was assessed by semiquantitative PCR to detect deletion of a specific GC-rich region vulnerable to oxidation [72]. The PCR product of the intact

wild type (WT) mtDNA is 4669 bp, while the size of the deletion between direct repeats is 3810-bp, producing a PCR product of 859 bp. This deletion was detectable in both WT and EC-SOD transgenic (TG) mice after oxygen exposure for 72 hours but it was absent in their littermate air-control mice (Figure 3.1). The amount of deletion was higher in the WT (4.8%) group than the TG mice (3.4%) but the difference was not statistically significant.

Hyperoxia and Mitochondrial RNA Transcription

To further evaluate the damage to mtDNA after hyperoxia, the efficiency of mitochondrial gene transcription was measured using RT-PCR. The level of expression of 12S rRNA was not different between WT and TG mice at room air (Figure 3.2). However after 72 hours there was significant decrease in the expression in the WT than in the TG mice ($p<0.05$). However, 72 hours of hyperoxia induced a significant decrease in 12S rRNA expression in the WT compared to their littermate air-control mice ($p<0.05$).

Hyperoxia and Nuclear Gene Transcription

To investigate the possibility that hyperoxia induced oxidative damage to the nuclear genome essential for mitochondrial function, the expression level of COX IV mRNA was measured. COX IV is a mitochondrial ETC (OXPHOX) subunit protein of Complex IV encoded by the nuclear genome. At baseline the cardiac transcripts of COX IV were significantly higher in WT than TG control mice ($p<0.05$). After hyperoxia the WT

COX IV mRNA level did not change significantly but COX IV mRNA level in TG heart increased significantly (about 40%, $p < 0.05$) (Figure 3.3). This result indicates that oxidative stress in EC-SOD TG mice might up-regulate transcription of nuclear genes essential for mitochondrial respiration.

Effect of Hyperoxia on Cardiac SOD Activity

Total SOD activity was significantly higher in heart homogenate in TG compared to WT mice (Figure 3.4A) and was mainly due to increases in the EC-SOD isoenzyme, which was about 2-fold higher (Figure 3.4B). Exposure to hyperoxia decreased WT EC-SOD activity in WT mice but did not affect the enzyme activity of the TG animals (Figure 3.4B).

Hyperoxia and Mitochondrial Defenses Against Oxidative Stress

MnSOD mRNA Expression.

Since mitochondria are a major source of ROS and free radicals during the exposure to hyperoxia, we sought to determine whether cardiac cells had responded to the oxidative stress by inducing the expression of mitochondrial SOD antioxidant enzyme (MnSOD) (SOD2). We assayed the expression of Mn SOD mRNA, which at baseline (in air) was already significantly higher in TG mice than in WT mice. Upon stimulation with 100% O₂ both WT and TG mice showed a significant increase in MnSOD level. With prolonged stimulation for 72 hours the expression of MnSOD dropped in both WT and TG but the decrease was more in WT (~ 2.6- fold) than in TG mice (~1.6- fold) (Figure 3.5).

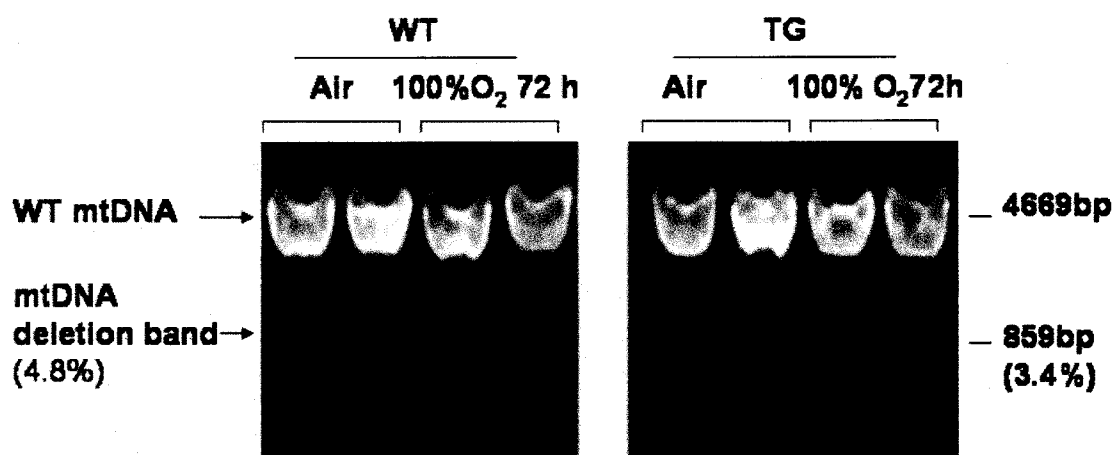


Figure 3.1. Identification of mtDNA Deletion in TG Mouse Hearts. SQPCR amplification from heart mtDNA of wildtype (WT) and EC-SOD transgene (TG) mice exposed to air and hyperoxia (100% oxygen) for 72 hours. The upper band (4669 bp) represents the normal mtDNA that amplified by the deletion primers. The lower band (859 bp) represents deletion of 3810 bp of mtDNA that was amplified. The deletion was detected more in WT (4.8%) than the TG (3.4%) mice after exposure to hyperoxia but the difference was not statistically significant.

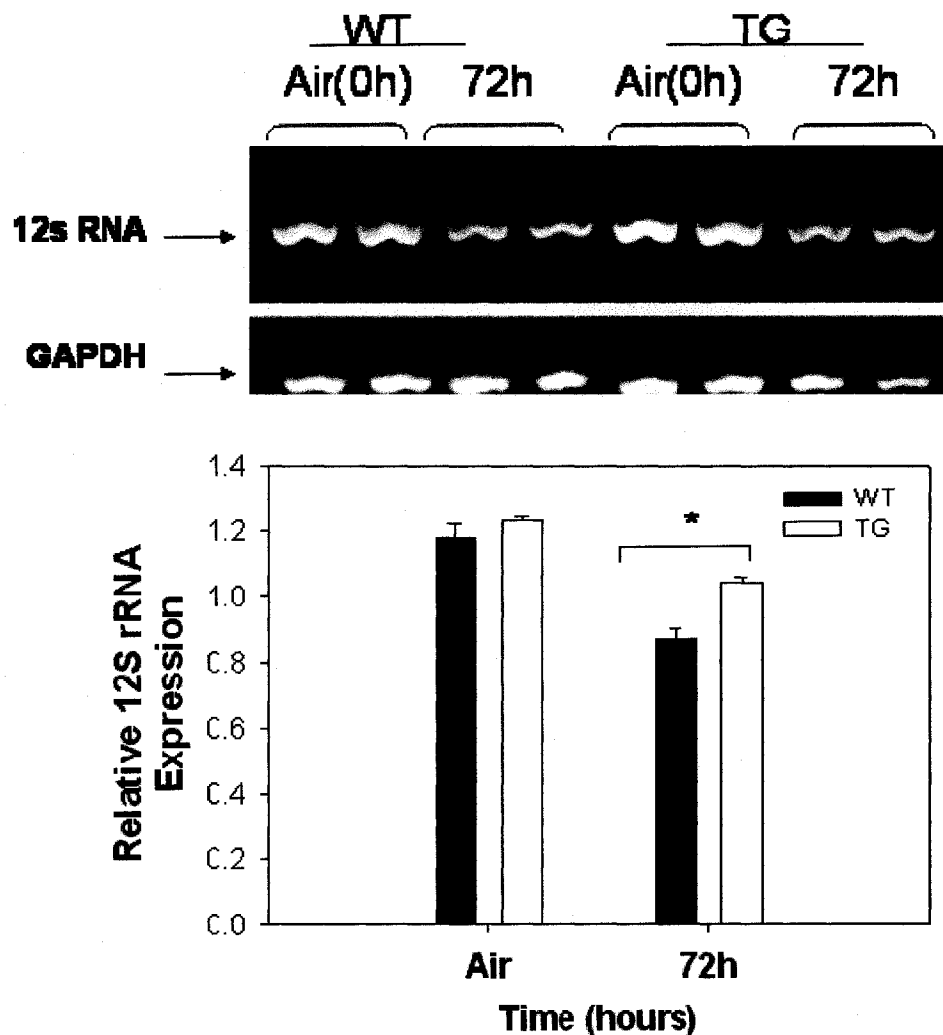


Figure 3.2. Mitochondrial Expression of 12S rRNA Transcripts in WT and TG Mouse Hearts. *Top*: 2% agarose gels of RT-PCR. Total RNA was prepared from mice hearts in air and after exposure to 100% oxygen for 72 hours. The RNA was reverse transcribed with gene specific oligonucleotide primers for mouse mitochondrial 12S rRNA. GAPDH mRNA was used as a control for RNA loading and efficiency of RT-PCR. *Bottom*: Densitometry values showing the relative expression of 12S rRNA expressed as mean \pm SEM in air (0 h) and after 72 h exposure to hyperoxia ($n = 4$ per group $*p < 0.05$).

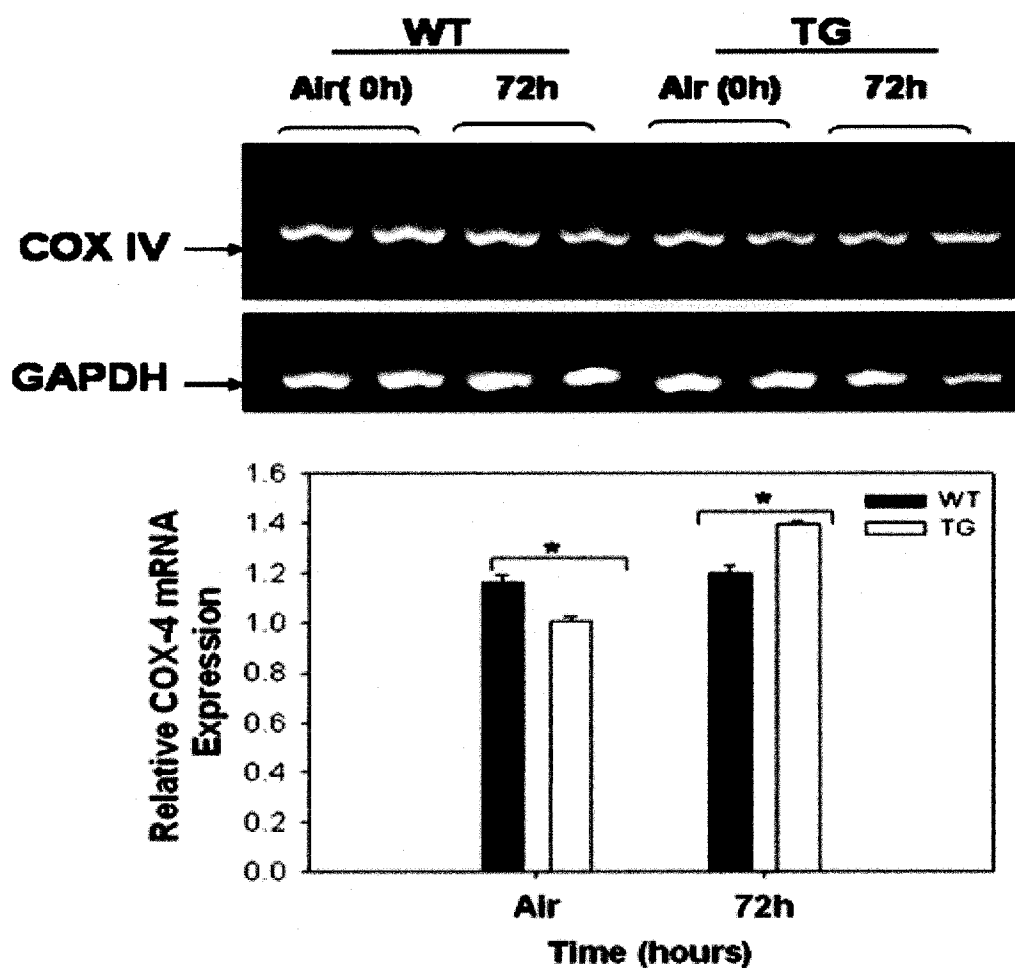


Figure 3.3. Nuclear Expression of COX IV mRNA in WT and TG Mouse Hearts. *Top*: 2% agarose gels demonstrating RT-PCR products of nuclear COX IV transcripts in WT and TG hearts in air (0 h) and after exposure to 100% oxygen for 72 hours. Total RNA was reverse transcribed with gene specific oligonucleotide primers for mouse nuclear COX IV. *Bottom*. Histogram shows the densitometry of COX IV mRNA expression normalized to GAPDH from a representative experiment. Values are expressed as means \pm SD of 4 animals/group (* p <0.05).

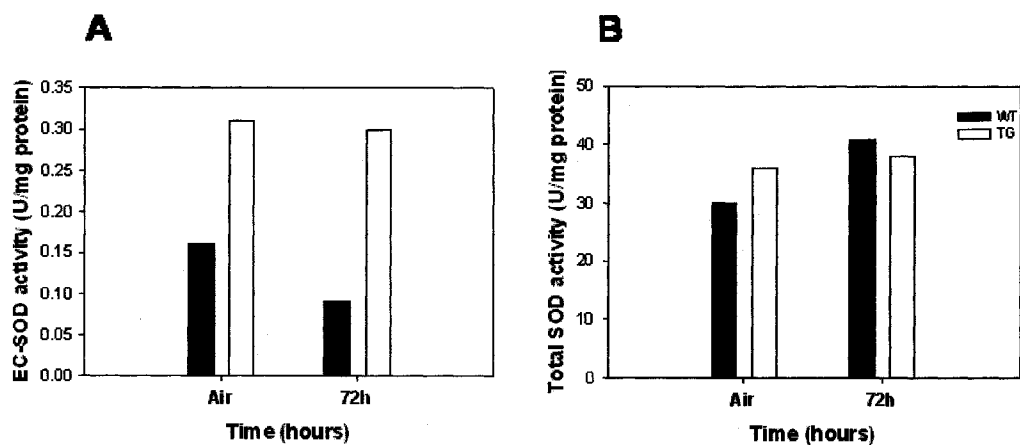


Figure 3.4. SOD activity in wildtype and transgenic mouse hearts. (A) The activity of EC-SOD in both wild type (WT) and in transgenic mice (TG). (B) The activity of Total EC-SOD in wildtype and transgenic mice (B). The activity was measured by inhibition of cytochrome *c* oxidase reduction at pH10 in air and after 72 hours exposure to hyperoxia (100% O₂) in 4 mouse hearts pooled /group. One unit of SOD activity is defined as the enzyme activity needed to inhibit a 50% reduction in cytochrome *c* oxidase.

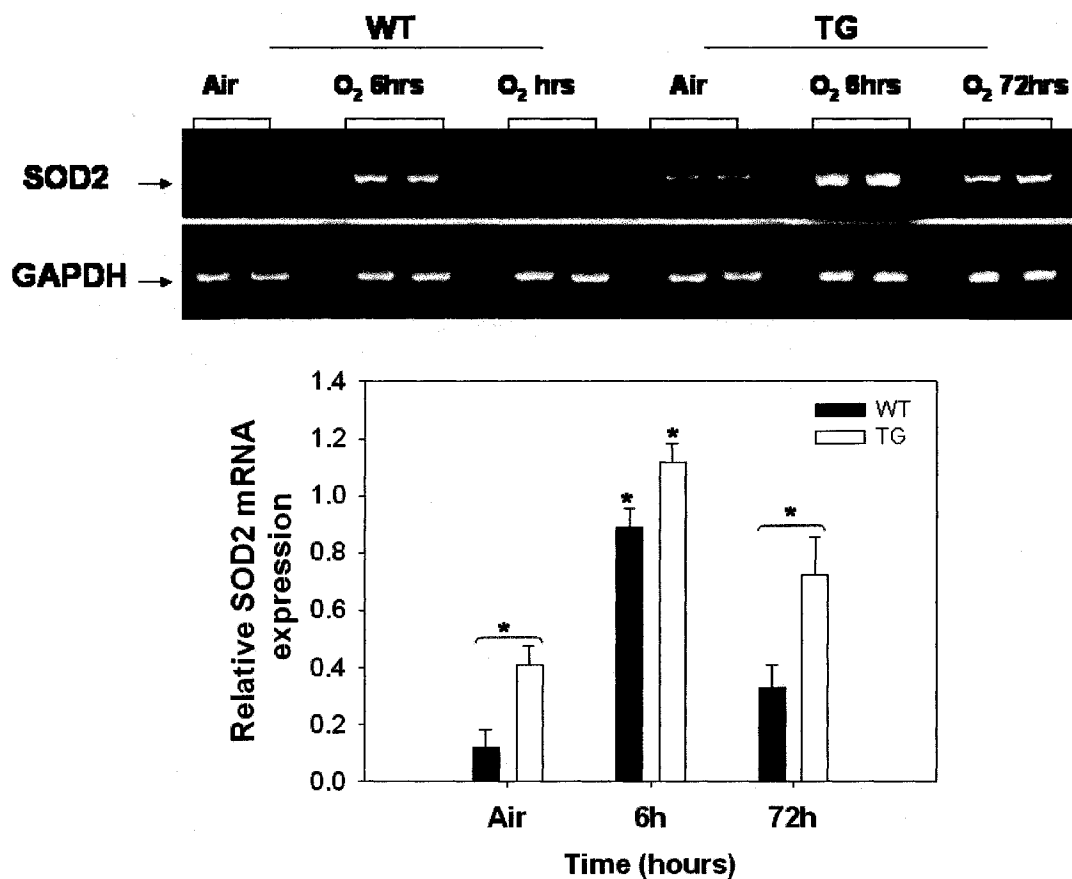


Figure 3.5. Expression of Mitochondrial SOD (SOD2) mRNA in WT and TG Hearts.
Top: 2% agarose gel electrophoresis using specific oligonucleotide primers for SOD2 mRNA in EC-SOD TG mice in air and after 6 and 72 hours exposure to 100% oxygen.
Bottom: Histogram shows densitometry of the optical density of SOD2 mRNA expression normalized to GAPDH mRNA. Values are expressed as means \pm SD of 4 animals/group (* $p < 0.05$).

Effect of Hyperoxia on Mitochondrial Biogenesis Markers

To test the effect of hyperoxia exposure on stimulating mitochondrial biogenesis, the transcription of nuclear respiratory factors NRF-1 and NRF-2 and their transcriptional co-activator (PGC-1 α) was measured. Nuclear respiratory factors are encoded in the nucleus and control synthesis of ETC components as well as the transcription of mitochondrial transcription factor A (Tfam) which regulates transcription and replication of the mitochondrial genome.

NRF-1 mRNA expression

The expression level of NRF-1 mRNA was measured using RT-PCR. The message was normalized to the nuclear encoded gene GAPDH. The expression of NRF-1 in air-control mice between WT and TG groups was similar. On exposure to oxygen for 6 hours, both WT and TG mice showed an increase in NRF-1 but the expression was most significant in TG mice (79%, ~ 1.8-fold, $p < 0.05$). With prolonged O₂ stimulation for 72 hours the expression level in TG mice remained elevated, while in WT mice it decreased by 64% compared to air- control (Figure 3.6A).

NRF-2 mRNA expression

The relative mRNA expression for NRF-2 in air-control groups was about two-fold higher in TG mice than in WT animals. After 6 hours of exposure to hyperoxia the expression increased by 80% in WT but in TG mice the increase in mRNA was not statistically significant. However after 72 hours of exposure the expression was significantly increased in TG mice by 64% (~ 1.64-fold) compared to the WT mice and

this difference in NRF-1 expression between the TG and the WT groups was highly significant ($p < 0.05$). This result showed that the TG mice have a higher basal level of NRF-2 expression than the WT and the cardiac expression of NRF-2 can be highly stimulated in the TG mice by O₂ exposure (Figure 3.6B).

Tfam mRNA expression

Because Tfam plays an important role in regulating mitochondrial replication and transcription, the level of Tfam transcripts was measured. The expression of Tfam mRNA in TG mice in air (normal oxygen) was significantly increased by 20% compared with the WT group. On stimulation with 100% oxygen for 6 hours the mRNA expression in both WT and TG mice decreased but only the decline in TG mice (by 61%) was significant. With prolonged O₂ stimulation for 72 hours the Tfam expression in TG mice increased back to normal levels, (Figure 3.6C). The persistent decrease in Tfam expression in WT mice indicates that oxidative stress was more pronounced in the WT mice than the TG mice and that oxidative stress affects nuclear genes essential for mitochondrial biogenesis.

PGC-1 alpha mRNA expression

Because PGC-1 α is a master regulator of mitochondrial biogenesis through co-activation of nuclear transcription factors (NRF-1 and 2), we wanted to know if the increase in expression of NRF-1 and NRF-2 after exposure to hyperoxia was associated with an increase in expression of PGC-1 α . The expression of PGC-1 α mRNA increased by ~1.8-fold (78%) in WT and ~2-fold in TG mice after O₂ exposure for 6 hours compared with

air-control group ($p<0.05$). With prolonged hyperoxia exposure for 72 hours, PGC-1 α mRNA expression was increased by 60% in TG mice but decreased dramatically in WT ~ 2.6-fold (Figure 3.6D). The increased transcription of PGC-1 α with coordinate expression of the downstream genes, NRF-1 and NRF-2, is consistent with the role PGC-1 α as an important nuclear regulator of respiratory gene expression.

Effect of Hyperoxia on Mitochondrial Biogenesis and Cell Survival

Akt protein expression

Because PGC-1 α expression was up regulated in EC-SOD- TG mice after hyperoxia, as was expression of NRF-1 and NRF-2, we were interested in finding the signal that could induce the expression of NRF-1, NRF-2 and PGC-1 α . Because the murine NRF-1 peptide sequence has an Akt phosphorylation motif (RXRXX (S/T) at position 104-109, we assessed Akt activation by immunoblot analysis using anti-Akt and anti-phospho-Akt antibodies. For animals exposed to normal air, the expression level of a 60 KD protein band of pAkt in TG mice was ~1.46 fold ($p<0.05$) higher than WT mice. After 6 hours of hyperoxia, p-Akt expression was increased in both WT and TG, but the expression was marginally increased in TG than the WT. After 72 hours, both WT and TG Akt level dropped below air-control levels but the level in TG mice was still higher than in WT (Figure 3.7). These results showed that Akt was activated early after oxidative stress in the mouse heart and may be the activator for transcription of NRF-1 after hyperoxia exposure.

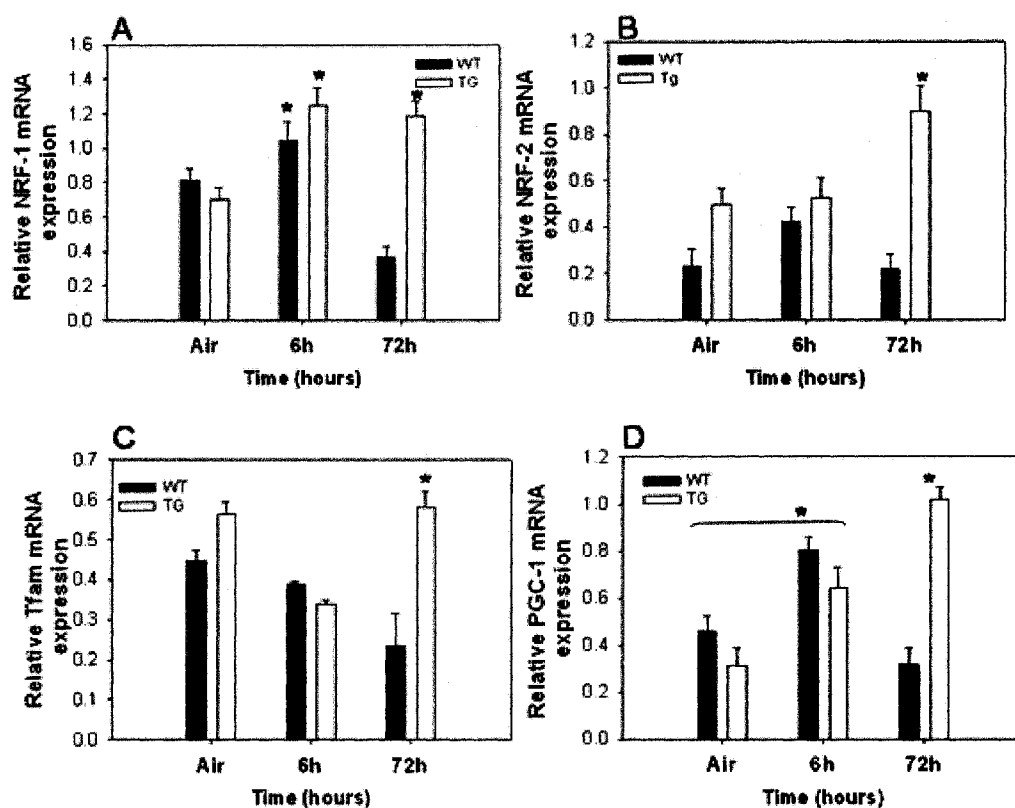


Figure 3.6. Nuclear Markers for Mitochondrial Biogenesis in WT and TG Mouse Hearts. The histogram shows the relative mRNA expression of mitochondrial biogenesis markers; nuclear respiratory factors (NRF-1 and NRF-2 (A&B)), mitochondrial master regulator PGC-1 α (C) and mitochondrial transcription factor A, Tfam in (D), in WT and EC-SOD TG mice hearts. The mice groups were exposed to room air (0 h) and after 6 and 72 hours to 100% oxygen. The densitometry values normalized to GAPDH mRNA expression. Values are expressed as means \pm SD of 4 animals/group (* $p < 0.05$).

MAPK p38 protein expression

The activation of p38 by cytokines has been reported to activate PGC-1 α [75]. Therefore we were interested to determine the effect of hyperoxia on p38 in TG mouse heart. The activation of p38 was measured by Western blot using antibodies to phosphorylated-p38 (p-p38). The p-p38 expression was normalized to total p38. At baseline p-p38 protein expression was significantly higher in TG hearts than in WT mice (100%). After 6 hours of exposure to 100% oxygen, WT mice showed a 2.5 fold increase in p-p38 expression but the TG mice showed no significant change. With prolonged O₂ stimulation, the p-p38 expression level dropped by 50% in TG and WT hearts (Figure 3.8). This result showed that cardiac p38 was not activated in TG mice heart by stress with hyperoxia. In contrast, the WT mice had higher p38 activation after 6 hours, suggesting that the cells could be more likely to undergo apoptosis after prolonged oxygen exposures.

CAMKIV expression

Because Ca²⁺/calmodulin kinase (CAMK) is known to increase expression of the respiratory genes and PGC-1 α and therefore mitochondrial biogenesis, we investigated the effect of exposure to hyperoxia on the activation of CAMK in TG mice cardiomyocytes. The protein expression of CAMKIV was assessed by Western blot analysis. We found that expression of cardiac CAMKIV was significantly higher in TG mice hearts than in WT in the basal line (air control) and after short term and long term exposure to hyperoxia ($p < 0.05$) (Figure 3.9).

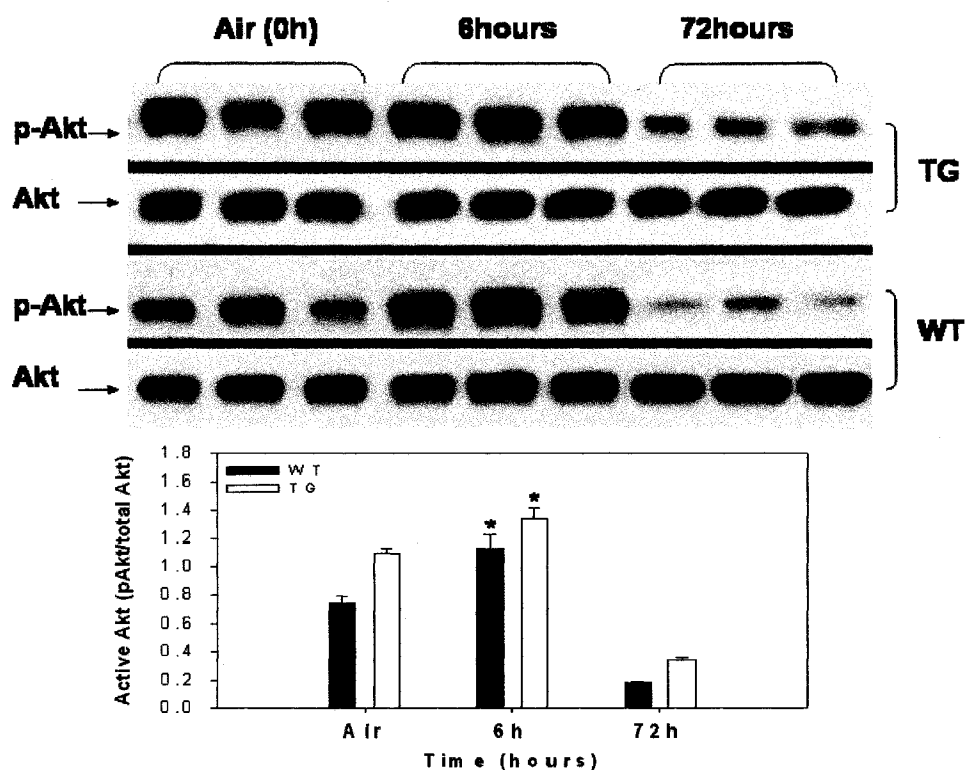


Figure 3.7. Western Blot Analysis of Cardiac p-Akt in TG and WT Mouse Hearts. Western blot were performed using 20 μ g of total heart cellular protein of TG (top) and WT (bottom) and antibody to p-Akt. Total Akt antibody was used as internal control. The protein expression was measured in air (0 h), after 6 hours, and after 72 hours exposure to hyperoxia (100% O_2). Histogram shows densitometry values for p-Akt/Akt as means \pm SD of 5 animals/group (* $p < 0.05$).

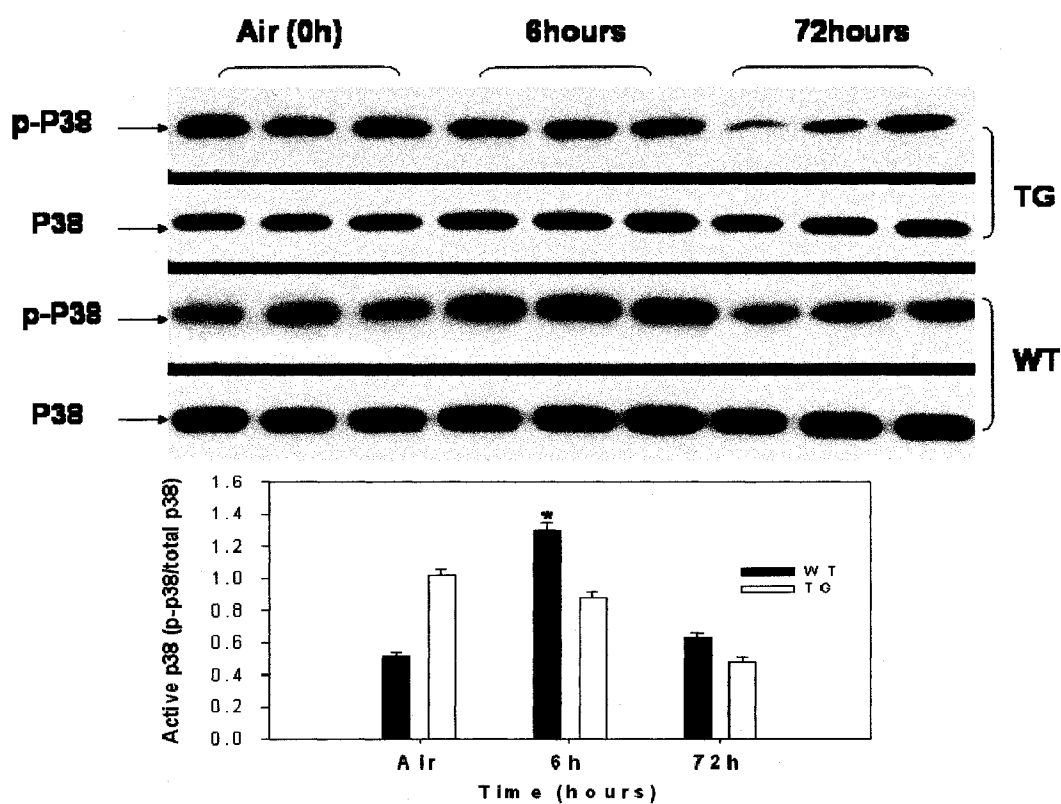


Figure 3.8: Western Blot Analysis of Cardiac p38 in TG and WT. *Top*, immunoblot shows protein expression of phosphorylated p38 in EC-SOD TG and WT mice hearts in air (0h), and after 6h and 72h exposure to 100% oxygen using specific p-p38 antibody. Phosphorylated-p38 was normalized to the expression of total p38. *Bottom*, Histogram showing densitometry data expressed as mean \pm SED of 3 animal/group (* $p < 0.05$).

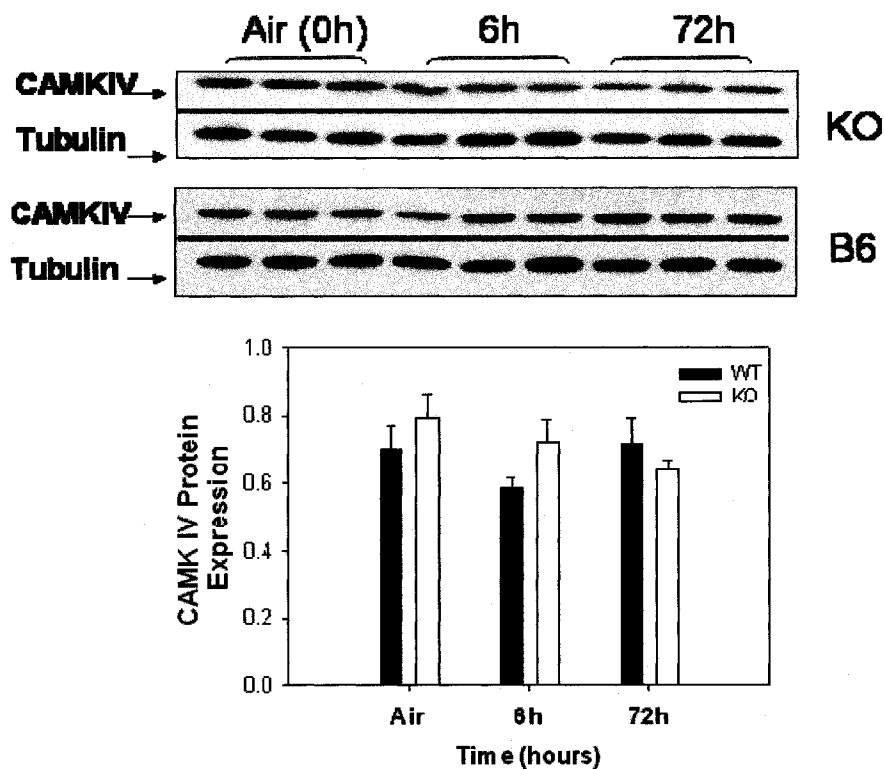


Figure 3.9. Western Blot Analysis of Cardiac CAMKIV Expression in TG and WT Mouse Hearts. *Top*, Immunoblot shows protein expression of cardiac CAMKIV in EC-SOD TG and WT mouse hearts in air (0 h), after 6 h and after 72 h exposure to hyperoxia. The expression level was normalized to tubulin protein expression. *Bottom*, Densitometry data was expressed as mean \pm SD of 5 animals/group ($*p < 0.05$).

Effect of Hyperoxia on Cardiac Muscle Survival

Alpha actinin (α -actinin) is the major structural components of sarcomeric Z-lines of striated muscle. To define if the damage by oxidative stress affects cardiac muscle integrity, α -actinin protein expression was measured by Western blot. Expression of a 100 KDa band was detected and analyzed by densitometry and normalized to tubulin protein. The α -actinin expression was higher in TG cardiac mice in air control compared with the wild type mice. After 72 hours exposure to 100% O₂, α -actinin expression increased in both TG and WT cardiac muscle and the increase was significant in both groups ($p < 0.05$) (Figure 3.10).

p-Bad protein expression.

We investigated whether Bad protein is activated after exposure to hyperoxia. The expression of 45 KDa phospho-Bad (p-Bad) was assayed by Western blot. The expression level was analyzed by densitometry and normalized to tubulin protein. The expression of p-Bad in TG hearts in air-control mice was significantly higher than in WT air-control mice. After oxygen exposure for 72 hours, cardiac p-Bad expression increased by 8-fold in WT mice, but only by 1.3 fold in TG mice compared to the air-control group (Figure 3.11). This result showed that EC-SOD TG mouse hearts have a high continuous expression of p-Bad at the baseline and that it responds to stimulation by oxygen. This could explain why these mice are better protected than WT mice from oxidative stress that leads to apoptosis.

p-GSK-3 β protein expression

We found no significant difference in the expression of p-GSK-3 β between TG mice in air-control and after exposure to hyperoxia. However, WT mice showed a 3.7 fold increase in p-GSK-3 β expression after exposure to hyperoxia. The expression was also highly significant between the WT and TG mice after hyperoxia ($p < 0.05$) (Figure 3.12).

Part 2: Effect of Hyperoxia in EC-SOD Knockout Mice (KO)

The EC-SOD KO mice were bred on a C57/BL6-J background strain. Therefore the wild type C57/BL6-J strain was used as control group for the KO mice experiments. EC-SOD KO mice are phenotypically the same as the wild type mice and they lived to the same age as the wild type mice (~18 months). On exposure to hyperoxia (100% oxygen), they behaved differently: after 24 hours the mice started to huddle together, and after 48 hours developed signs of respiratory distress, and some died during the exposure.

Hyperoxia and mortality

Hyperoxia was lethal to 10% of the exposed WT mice (2/20), and was more lethal to the exposed KO mice (6/24, 25%). The mortality occurred between 48 and 72 hours of exposure, Table 3.4. Exposure to 100% oxygen increases formation of reactive oxygen species which may cause oxidative damage to the organs. The lung and heart are especially sensitive and may progress to cardiac and pulmonary failure.

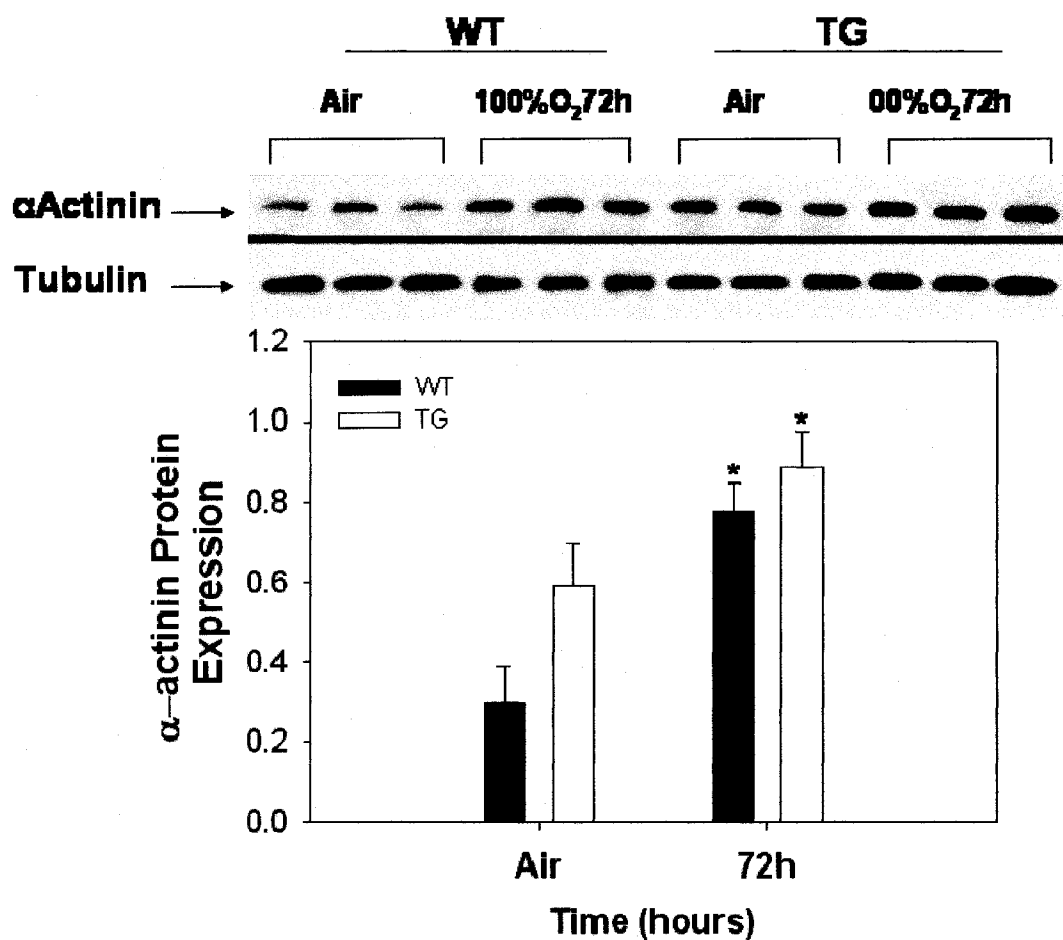


Figure 3.10. Alpha-Actinin Protein Expression in TG Mouse Hearts. α -actinin is a marker for cardiac muscle integrity. Expression of α -actinin in EC-SOD TG and WT mouse hearts was measured by Western blot analysis using specific α -actinin antibody. The relative expression of α -actinin was normalized to tubulin protein expression. The histogram shows the densitometry data of α -actinin /tubulin expressed as mean \pm SD of 5 animals/group (* $p < 0.05$).

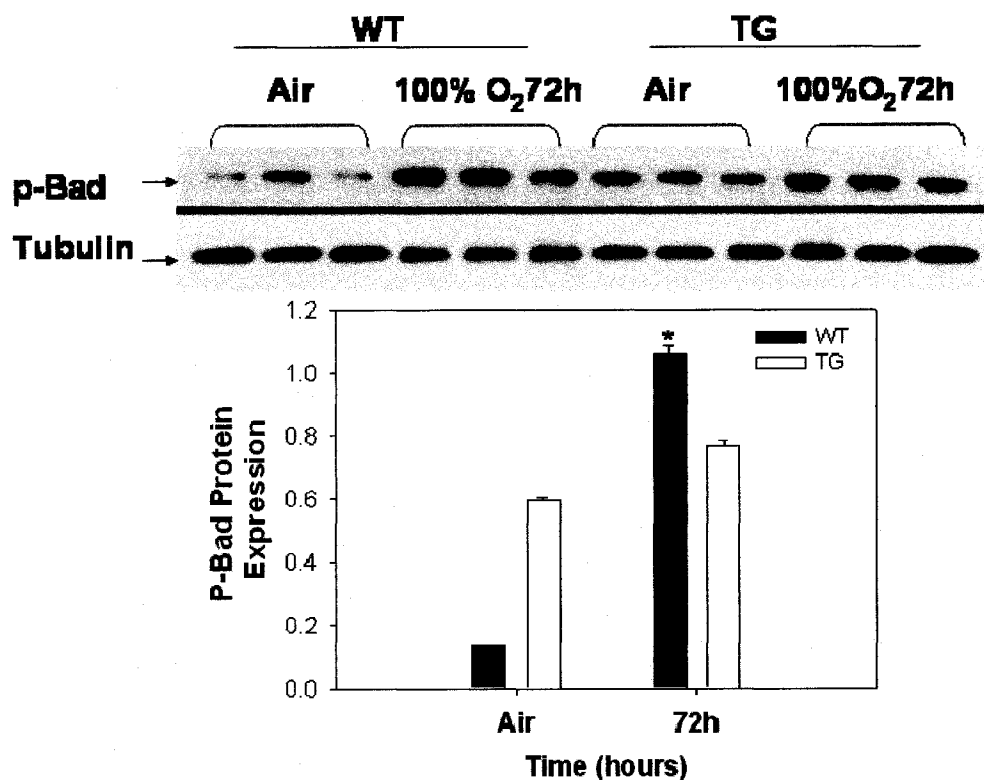


Figure 3.11. Protein Expression of p-Bad in TG Mouse Hearts. Bad is a marker for apoptosis, protein expression for phosphorylated Bad (p-Bad) in EC-SOD TG and WT mouse hearts was measured by Western blot analysis using p-Bad antibody. The expression was measured in basal line (air), and after 72 h exposure to hyperoxia. The protein expression was normalized to tubulin expression. Densitometry data of p-Bad/tubulin was expressed as mean \pm SD of 5 animals/group (* $p < 0.05$).

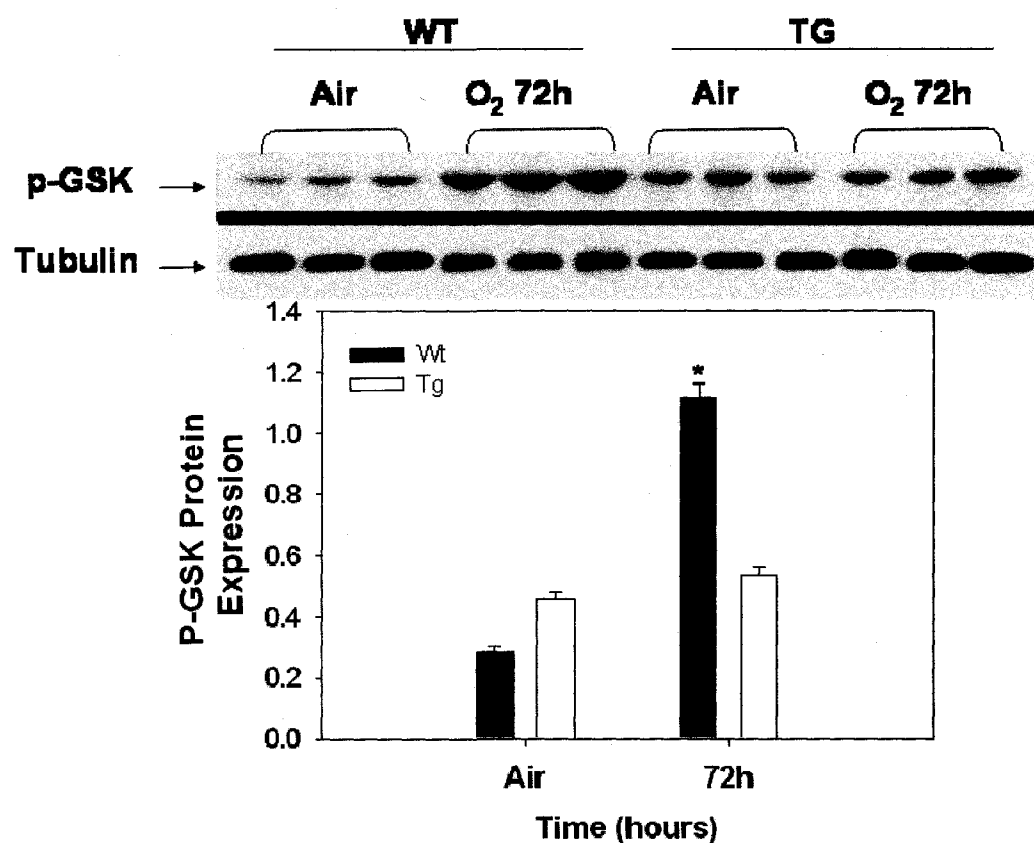


Figure 3.12. Protein Expression p-GSK-3 β in TG Mouse Hearts. Glycogen synthase kinase-3 β (GSK-3 β) is a pro-apoptotic protein. Expression of p-GSK-3 β in EC-SOD TG and WT mouse hearts was measured by Western blot using p-GSK-3 β antibody. The expression was measured in air and after 72 hours exposure to hyperoxia. P-GSK-3 β protein expression was normalized to tubulin protein expression. Densitometry data of p-GSK-3 β /tubulin was expressed as mean \pm SD of 5 animals/group (* $p < 0.05$).

CHAPTER IV

DISCUSSION AND CONCLUSION

The discussion is divided into three parts: Part 1 discusses and interprets the studies on EC-TG mice; Part 2 does the same for the EC-SOD KO mice data; Part 3 is a general discussion and integration of both the TG and the KO mouse data sets. Both sets of experiments used hyperoxia (100% O₂ by inhalation) at early and late (6 and 72 h) time points. There were, however, initial differences between the two strains of genetically-altered mice relative to the appropriate wild type strains that resulted in some important differences in baseline cardiac gene expression. These differences had never been characterized previously and probably contributed to the observed strain differences in survival in hyperoxia (see again Table 3.1).

Part 1: Discussion for EC-SOD TG Mice Data

This study demonstrated for the first time that prolonged exposure to 100% O₂ by inhalation causes changes in gene expression that indicate mitochondrial oxidative stress. Most notable was the presence of a small amount of 3810 bp deletion in the mtDNA of the mouse heart. The amount of deleted cardiac mtDNA (3.4%) in the TG mouse was less prominent than that in WT hearts (4.8%). This oxidant- sensitive mtDNA deletion has been reported earlier in the rat liver and heart after exposure to LPS and hemin [21, 72, 76]. The large deletion (3810 bp) in the mitochondrial genome includes codons for NADH dehydrogenase (ND1 and ND2), cytochrome *c* oxidase (COX I), 16S rRNA and six tRNAs for amino acids. Mitochondria carrying this deletion in sufficient copy number

would have both impaired entry of reducing equivalents into the respiratory chain at Complex I and alterations in the capacity to reduce O_2 at Complex IV. This deletion is mostly accompanied by the accumulation of substantial 8-hydroxyguanine [72] initiated by oxidation of DNA bases by H_2O_2 -catalyzed hydroxylation and other strong oxidants [77-79]. Because mitochondrial DNA is a naked double-stranded circular DNA molecule and because of its low proofreading efficiency and insufficient repair during replication, it is vulnerable to oxidative damage and mutations [80].

Overexpression of an extra copy of EC-SOD in TG mice increased the activity of EC-SOD in TG mice hearts by 2-fold (Figure 3.4) over WT. Moreover, the primary antioxidant enzyme in mitochondria, SOD2 was also increased ≈ 2 fold in TG mice compared to the WT mice after hyperoxia exposure. The reason for the SOD2 response is unclear and is unexpected. However, the increase in SOD2 and SOD3 activity in the TG mice provide an explanation for the attenuated damage to the mitochondria after hyperoxia exposure compared to WT mice. Thus, the link between extracellular and mitochondrial defensive mechanisms could be directly involved in the protection of cells and organelles from oxidative damage. The increase in EC-SOD and MnSOD expression in TG are most responsible for elimination of hyperoxia induced ROS production and decreased formation of superoxide and peroxynitrite anion ($ONOO^-$).

The significant decrease (29%) in mitochondrial expression of 12S rRNA in WT hearts after hyperoxia compared to TG mice suggests that either mtDNA in TG mice is relatively undamaged compared with WT hearts and /or the transcriptional machinery or the transcriptosome in the TG cardiac mitochondria is not affected by exposure to hyperoxia. Because Tfam is an essential component of the mitochondrial transcriptosome

as well as necessary for mtDNA replication and genome maintenance, we investigated cardiac mRNA expression levels for Tfam before and after oxygen exposure. Tfam mRNA expression was significantly increased in TG hearts with long term exposure by 1.6-fold but decreased in WT hearts, which is consistent with the decrease in 12S rRNA expression. This finding supports the notion that low Tfam levels stimulate mtDNA replication while higher levels stimulate transcription [81]. In addition, these data provide significant evidence that increases in the extracellular antioxidant EC-SOD can influence mitochondrial function and biogenesis in response to direct oxidative insults such as hyperoxia. The increase in the nuclear expression of cytochrome c oxidase (COX IV) mRNA after exposure to hyperoxia in TG hearts by 40% with no change in WT indicates that the nucleus in the TG mice has responded positively to the oxidative damage that targets the mitochondria. Additionally, an earlier study showed that COX IV expression in cardiomyocytes of WT mice is an early event that peaked by 6 hours and goes down to 40- 50% by 72 hours (Suliman, unpublished data). This data may explain the blunted response of COX IV expression in WT hearts after 72 hours of hyperoxia.

The recovery of mitochondrial damage by biogenesis requires coordinated activation of transcription factors and binding to promoter consensus sequences in nuclear genes encoding for mitochondrial proteins [82]. NRF-1, NRF-2, SP1 and PGC-1 α , Tfam, mTFB1 and mTFB2 are the relevant transcription factors and cofactors involved in mitochondrial biogenesis that have been identified to date [83]. In response to biogenesis-inducing stimuli, transcription for these proteins changes before or coincident with increases in the expression of their target genes [82]. The data presented here showed that hyperoxia increased mRNA expression of PGC-1 α , NRF-1 and NRF-2 as

well as Tfam expression concurrently in TG hearts. However, in the WT control group, although there was an early increase in the mRNA expression of PGC-1 α , NRF-1 and NRF-2, and this response did not persist with prolonged hyperoxia as in TG mice. Because the promoter region of Tfam has consensus motifs recognized by NRF-1 and or NRF-2 upstream of the transcription machinery [84], the decrease in NRF-1 and NRF-2 may explain the down regulation of Tfam transcripts in the WT hearts. Cooperation among DNA-binding proteins and promoter diversity among nuclear genes for mitochondria regulate biogenesis [85]. PGC-1 α has a major impact on the NRF system, and transfection of PGC-1 α in muscle cells dramatically induces gene expression for NRF-1, NRF-2, and Tfam. Furthermore, PGC-1 α physically interacts with NRF-1 and co-activates its transcription activity [11]. Because PGC-1 α promoters have multiple sites for redox-sensitive transcription factors, e.g., CREB and AP1 [86, 87], oxidant regulation of PGC-1 α should co-activate NRF-1 and NRF-2 simultaneously, which are also oxidant-sensitive transcription factors. Furthermore, mutations that affect PGC-1 α expression lead to loss of heart mitochondria and down regulation of mitochondrial enzymes [13]. Interestingly, PGC-1 α has been also shown to be induced in brown fat cells on cold stimulation, suggesting that PGC-1 α strongly co-activates several nuclear receptors (PPAR gamma, PPAR alpha, RAR and TR) that bind to mitochondrial uncoupling protein-1(UCP-1) enhancer [12]. So PGC-1 α in cardiac cells regulates several genes of the electron transport chain, mitochondrial biogenesis, and fatty acid β -oxidation. Effective cardioprotection would require not only cardiomyocyte survival but also preservation of function. Acute activation of PI3-kinase or Akt is sufficient to inhibit cardiomyocyte apoptosis [88], but more importantly, acute activation of Akt also preserves

function in surviving cardiomyocytes [89]. In cardiomyocytes, IGF-1 activates PI 3-kinase /Akt signaling pathway, which appears largely necessary to reduce hypoxia-induced apoptosis in cardiomyocytes in vitro [88]. In this study, acute activation of Akt (at 6 hours) by hyperoxia in both WT and TG hearts provides direct evidence that the PI 3-k/Akt pathway was a protective response induced by high oxygen exposure (100% O₂). The exact mechanism that activates PI3k/Akt pathway in hyperoxia is not yet delineated. However, the increase in activity of EC-SOD may contribute to increased dismutation of $\cdot\text{O}_2$ to H₂O₂ which could mediate the activation of the PI3k/Akt pathway [90]. Greater Akt activation in EC-SOD TG mice than in WT mice after hyperoxia may indicate either that Akt activation is stimulated by the production of more H₂O₂ by extra EC-SOD enzyme or that TG mice have less oxidative damage so the activation of the pro-survival pathway is more evident.

The acute increase in Akt was coincident with an increase in PGC-1 α , and NRF-1 mRNA expression, suggesting that PGC-1 α and NRF-1 are activated by Akt in our mice after hyperoxia. This stimulation of mitochondrial biogenesis markers confirms that Akt has an important role in promoting cell survival. Besides its effect as a pro-survival factor, Akt has also been found to restore cardiac function in normal and failing heart most probably by increasing SERCA phosphorylation by Akt which enhances calcium mobilization in the heart [91]. Additional molecular work will be needed to determine if Akt activation by oxygen leads to phosphorylation and nuclear translocation of NRF-1 and PGC-1 α .

Akt also plays an important role in inhibiting apoptosis: adenoviral expression of constitutively active mutants of either PI 3-k or Akt reduces hyperoxia-induced apoptosis

in cardiomyocytes in vitro [88]. Multiple anti-apoptotic downstream effectors of Akt have been identified. Phosphorylation of the pro-apoptotic Bcl family protein, Bad, has been reported to mediate anti-apoptotic signaling [33, 92]. Also phosphorylation of caspase -9 by Akt inhibits its activity [93]. Phosphorylation of the Forkhead transcription factors, FOXO3a, by Akt induces nuclei exclusion and reduces transcription of specific pro-apoptotic molecules [94]. In our study, a concomitant increase of p-Akt and pBad in the TG hearts at base line in air-control group (~ 4-fold in pBad) substantiates the Akt/Bad pathway activation in these TG mice and implies that they are better protected from apoptotic cell death. Also in our study, increased phosphorylation of glycogen synthase kinase-3 β (GSK-3 β) in TG mice in the air control and after hyperoxia groups is an indication that Akt/GSK signaling pathway is activated. Phosphorylation of GSK-3 β by Akt inactivates GSK-3 β and leads to inhibition or delayed activation of the mitochondrial permeability transition, a key regulator of apoptosis. Also, it is reported that overexpression of catalytically active GSK-3 β induces apoptosis, whereas expression of a dominant-negative prevents apoptosis after inhibition of PI-3K [95].

Activation of the Akt signaling pathway by adrenomedullin (a potent vasoactive peptide) protects against cardiomyocyte apoptosis induced by ischemia/reperfusion injury through the Akt-GSK caspase signaling pathway [96]. It is also reported that increased Akt activity inactivates GSK-3 β and downregulates caspase 3- activity and decreases apoptosis in cardiomyocytes. Other studies have shown drugs that inhibit GSK-3 β are cardioprotective [97, 98] and appear to mediate cardioprotection by phosphorylation of GSK-3 β [40]. These studies support our finding that increased phosphorylated GSK-3 β in EC-SOD mice hearts mediate cardioprotection.

Negative regulation of Akt in the heart is important, because continuous activation of Akt is sufficient to induce substantial cardiac hypertrophy [99, 100]. Akt is inactivated either through inhibition by PTEN, which inhibits PI3-k /Akt signaling pathway[101-103] or through dephosphorylation of Akt itself by phosphatase, PP2A [104]. Such negative regulation might explain the decrease in the expression of Akt after 72 hours exposure to hyperoxia that we found in TG mice. This will require additional work.

Because p38 is a stress kinase that is cell type and stimulus dependent, its signaling has been shown to promote cell death in some cell lines, while in other cell lines it enhance survival, cell growth and differentiation. In the heart, activation of p38 is accompanied by cardiomyocyte hypertrophy. Also because p38 functions upstream and downstream of caspases in apoptosis its activation can promote apoptosis [105, 106]. Activation of p38 in this study by hyperoxia was significantly increased in the WT mice hearts after only 6 hours of exposure. While the MAPK p38 pathway did not change in the TG mice after 6h of hyperoxia, it decreased afterwards. Decreases in p38 activation in TG hearts after hyperoxia exposure indicates that p38 apoptotic pathway is not activated, and these mice should be protected from apoptosis.

This study provides the first evidence that overexpression of extracellular antioxidant enzyme EC-SOD protects an intracellular organelle, the mitochondria, from oxidative stress. Also TG mice respond to oxidative stress induced by hyperoxia through activation of the cell survival pathway mediated by PI3k/Akt signaling pathway. Moreover, the important role of Akt in promoting cell survival appears to be mediated via

activation of all or part of the mitochondrial biogenesis pathways and inhibition of pro-apoptotic pathways.

Part 2: Discussion for EC-SOD KO Mice Data

Mitochondrial DNA (mtDNA) deletions have been shown to increase with aging and ischemia as a result of oxidative stress, and are suggested to contribute to myocardial dysfunction [14, 107]. In this study we demonstrated that hyperoxia induces mitochondrial DNA deletion in KO mouse hearts. Accumulation of ROS during hyperoxia exposure in these KO mice may be the direct cause of this mtDNA deletion. The deleted part of the mitochondrial genome (3810 bp) constitutes about 23% of the total mtDNA genome (16459 bp). Because the 3810 bp deleted portion of mitochondrial genome includes codons for NADH dehydrogenase (ND1 and ND2), cytochrome *c* oxidase (COX I), 16S rRNA and six tRNAs mitochondria carrying this deletion in sufficient copy number will have changes in the OXPHOS system, which further amplifies ROS formation and diminishes production, and may predispose to heart failure. The high mortality rate (25%) among the EC-SOD KO mice after prolonged continuous hyperoxia may be due to lung or cardiac damage or both, caused by excessive ROS formation. Oxygen itself is implicated because the heart mitochondria manifested an increased mtDNA deletion in cardiomyocytes (24%), especially in EC-SOD KO mice. In other words, the absence of EC-SOD probably led to accumulation of overwhelming levels of ROS ($\cdot\text{O}_2^-$) after exposure to hyperoxia that influenced not only the extracellular milieu but also the mitochondrial compartment, either directly or indirectly, causing appreciable oxidative damage to mtDNA. Deletion of mtDNA had been found in tissues

of coronary artery disease patients [108]. The increased oxidative stress in cardiovascular disease especially from transient episodes of ischemia may be responsible for the accumulation of mtDNA damage in these patients.

When mitochondrial gene transcription was measured using RT-PCR, we found that the transcript for 12S rRNA in KO compared to the WT hearts was decreased at baseline in air control mice. Thus, lack of EC-SOD not only affects the mitochondrial compartment during hyperoxia but also under normal conditions. This decrease in 12S rRNA in EC-SOD KO mice is most probably due to oxidative damage to mtDNA which affects transcription of its genes. In contrast, expression of nuclear gene COX IV was similar between the KO and WT mice at baseline. These findings confirm that the mtDNA genome is more sensitive to oxidative damage than the nuclear genome in the heart, especially in the absence of EC-SOD.

Expression of the mitochondria antioxidant SOD2 (MnSOD) was also affected during hyperoxia. At baseline the expression was higher in the KO mice compared to the WT, perhaps to compensate for the absence of EC-SOD. However, SOD2 expression decreased dramatically (~ 4.5 fold) after a 6 hour exposure to hyperoxia in both the WT and KO mice. The exact mechanism responsible for this finding is not yet identified. However, this phenomenon might be due to the increased CpG islands in the promoter region of MnSOD and the susceptibility of these islands to oxidative stress induced methylation, thus silencing of the gene. The CpG is a C+G rich area span >3.5 Kb near the 5' edge of the SOD2 promoter region and extends into intron 2. Increased cytosine methylation in this area induces epigenetic silencing of MnSOD gene expression. This mutation has been found in many tumor cell lines, e.g myeloma cell line KAS 6/1[109].

Increased ROS formation in malignant cells or in hyperoxia exposure may lead to this methylation. Enforced expression of SOD2 in many transformed cell has been associated with suppression of the malignant phenotype of the transformed cells, suggesting that SOD2 is also a tumor suppressor gene via protection of the mitochondrial compartment [110].

In this study we also showed that nuclear respiratory factors, NRF-1 and NRF-2, which regulate mitochondrial biogenesis, are stimulated early by hyperoxia in KO mouse hearts but not the WT. The cardiac mRNA expression of NRF-1 and NRF-2 in WT mice was inversely related to prolonged exposure to hyperoxia compared with that in the EC-SOD KO mouse hearts. Excessive ROS production in KO mice may be the signal to increase expression of these nuclear respiratory factors. Unexpectedly, the increase in mRNA of NRF-1 and NRF-2 in KO mice after 6 hours exposure to hyperoxia was not associated with an increase in PGC-1 α protein expression, a known nuclear receptor and co-activator of NRF-1 [111]. Moreover, in addition to the decrease in PGC-1, the decrease in activated Akt, phosphorylated Bad and GSK proteins in the KO mice after hyperoxia imply that early expression of NRF-1 and NRF-2 in the KO mice was not due to activation of Akt and not associated with PGC-1. The decrease in Tfam expression after exposure for 6 hours in both KO and WT may be also due to methylation of the NRF-1 binding site of the Tfam promoter, therefore suppressing Tfam expression and decreasing mitochondrial biogenesis, as was reported by another group [112].

There are many links between Ca²⁺ and respiratory gene expression mediated by Ca²⁺-dependent regulatory enzymes, Ca²⁺/calmodulin-dependent protein kinase (CAMK) and protein kinase C (PKC). It was reported that calcium ionophores lead to

increase of cytochrome *c* gene expression through a Ca^{2+} –sensitive and PKC-dependent pathway [113]. Activation of CAMKIV due to changes in intracellular Ca^{2+} [16] and activation of AMP kinases due to change in ATP/AMP ratio [114] can lead to an increase in mitochondrial abundance. Our data on CAMKIV protein showed no significant change in the expression in the KO or in the WT mice before or after exposure to hyperoxia, which disqualifies the involvement of this pathway in activation of NRF-1 and NRF-2 in this study.

The mitogen-activated protein kinase p38 is activated by cellular stressors in the heart, including ischemia, hypoxia, and hypertrophic growth stimuli. The role of p38 – MAPK in hyperoxia is not yet well understood and its role in myocardial ischemia-reperfusion injury is controversial. Several lines of evidence suggest that the activation of p38-MAPK/MAPKMAPK2/Hsp-27 axis contributes to ischemic preconditioning. However, others have concluded that p38-MAPK inactivation might be cardioprotective [115]. The different results might be due to the existence of two p38-MAPK isoforms (α and β) in the heart: while p38 α transmits pro-apoptotic signals, p38 β triggers a hypertrophic response and promotes survival [116]. Our findings showed that p38 was activated in KO mice after hyperoxia, which is consistent with the activation of NRF-1 and NRF-2. Activation of the nuclear respiratory factors here may suggest that the p38 β is activated and this stimulated the pro-survival pathway. P38 was also activated in WT mice after 6 hours exposure to hyperoxia but had decreased significantly by 72 hours. In the heart, activation of p38 had been implicated in increased mitochondrial biogenesis and energy levels in the cell through phosphorylation of peroxisome proliferative-activated receptor alpha (PPAR α) and its co-activator PGC-1 α [117]. The p38 isoforms

need to be further defined in these KO and WT mouse hearts in order to discern the effect of p38 on cell survival during hyperoxia.

The decrease of p-Bad and pGSK-3 β expression after hyperoxia was more prominent in the EC-SOD KO mice, consistent with decreased activation of Akt. However, Akt was activated in the WT after 6 hours and was accompanied by phosphorylation of BAD and no significant change in GSK-3 β . The responses to hyperoxia in these two groups were different, as was the subsequent activation of cell signaling pathways.

Our results indicate that EC-SOD KO mice are more susceptible to oxidative mtDNA damage and mtDNA deletion than the WT control. In the absence of EC-SOD, the activation of mitochondrial biogenesis in response to hyperoxia most probably happens not through Akt, but may involve the activation of p38 MAPK. However, the exact outline of the p38 activation pathway can not yet be drawn, but there may be excessive oxidative stress which leads to decreased mitochondrial energy production. This could decrease the ATP/AMP ratio and thereby stimulate cAMP, which would also activate p38. This possibility will require further study. Also the decreased expression of p-Bad and p-GSK-3 β in KO mice after hyperoxia may suggest that the hearts of these mice are more likely to undergo apoptosis, which needs to be evaluated specifically, using cell death analysis in a future study.

Part 3: General Discussion, Conclusions and Further Directions

This study has four main new findings. First, EC-SOD TG mice have a higher tolerance to hyperoxia exposure than KO mice. Oxygen toxicity was manifest as increased respiratory distress and an increased mortality rate in the KO mice. Second, EC-SOD KO mice were more sensitive than TG mice to cardiac oxidative damage which appeared as an increase in the extent of mtDNA deletions (23%) vs (3.4%). Third, transcription of mitochondrial and nuclear genes decreased after exposure to hyperoxia in KO mice in the some genes (12S rRNA, COX IV, SOD2, PGC-1, and Tfam) in contrast to TG mice where the transcription increased after hyperoxia exposure. Fourth, there were basal differences in cardiac mitochondrial gene expression in the genetically altered EC-SOD mouse strains.

The absence of the EC-SOD enzyme in KO mice may increase formation of reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical and peroxynitrite. ROS oxidatively damages mtDNA, and also affects mitochondrial and nuclear mRNA expression. Because ROS are produced locally in the mitochondria by leakage from electron transport and because mtDNA repair mechanisms are less efficient than nuclear DNA repair mechanisms, a significant deletion or mutation in mtDNA can affect mitochondrial respiration, and ATP production rates. This would decrease cardiomyocyte function, which may lead to cardiac failure. A number of recent clinical studies have reported reduced levels of human extracellular superoxide dismutase in patients with cellular artery disease and myocardial infarction [118-120]. In Denmark, 2-3% of the population carries a mutation in the EC-SOD gene (R213G) which results in increased plasma concentration of EC-SOD enzyme and reduced arterial wall EC-SOD

concentration, and is associated with elevated risk for ischemic heart disease. [58].

Moreover, experience with EC-SOD gene therapy has been reported to protect the myocardium against experimental episodes of ischemia/reperfusion injury [121, 122].

Mitochondrial biogenesis markers NRF-1, NRF-2, Tfam, and PGC-1 α were up-regulated in TG mice after short term exposure to hyperoxia. In KO mice, the NRF-1 and NRF-2 increased after short term exposure to hyperoxia but Tfam and PGC-1 expression increased after long term exposure to hyperoxia. The upstream signal for activation of mitochondrial biogenesis most probably was through Akt activation in TG mice but this could not be supported in the KO mice. Akt was not activated in the KO but the stimulation of the nuclear respiratory factors was, and possibly through activation of p38. Because we did not study p38 isoforms, it is not known for sure that activation of p38 found in the heart was either the signal for survival (p38 β) or apoptosis (p38 α). However, a decrease in pBad expression and pGSK-3 β in KO mice after hyperoxia exposure suggests that these mice are more likely to have cardiomyocyte apoptosis during oxidative stress.

Conclusions: Systemic hyperoxia consistently increases mitochondrial biogenesis markers in the mouse heart, most prominently in EC-SOD TG mice, and co-incidentally with increases in Akt, CAMK, p-Bad and p-GSK-3 β . This suggests that extracellular ROS, most likely hydrogen peroxide, activate mitochondrial biogenesis and the pro-survival pathway in TG mice. On the other hand, the presence of the transgene may allow augmentation of nitric oxide (NO bioactivity) to stimulate biogenesis, e.g. through cyclic GMP-mediated effects. This possibility was not explored. Also, hyperoxia decreased the activation of Tfam and PGC-1 α transcription after 6 hours and increased it again after 72

hours in KO mice. This was associated with decreased expression of Akt, CAMK, p-Bad and p-GSK-3 β and an increase in p38, indicating that either hyperoxia activated mitochondrial biogenesis and pro-survival pathways through p38 or that hyperoxia produced more oxidative damage to the mitochondria in the KO. In the latter case, the increase in p38 may be a signal for activation of apoptotic pathways. These unexpected findings in KO mice need further exploration in the future.

Further Directions:

The following studies should help further elucidate the mechanisms by which EC-SOD protects the heart from hyperoxia and other types of extracellular oxidative stress.

- 1-Examine the TG and KO mouse heart tissues for evidence of apoptosis by TUNEL and DNA laddering.
- 2- Measure caspase -3 and cytochrome c activity to assess mitochondrial initiation of apoptosis.
- 3- Measure the expression of p38 isoforms in the hearts in the TG and KO mice to evaluate the possible protective activation of this signaling pathway during hyperoxia stress.
- 4- Measure ANF as a marker for cardiac hypertrophy and functional impairment.

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Publications:

- 1- PETER J. MOLER, JEAN Schott, Anthony O. Gramolini, Keith W. Dilly, Silvia Guatimosim, William H. dubell, Long—sheng Song, Karine Haurogene, Florence Kyndt, **Mervat E. Ali**, Terry B. Rogers, W.J. lederer, denis Escands, herve le marec, and Vann Bennett. Ankyrin-B mutation causes type 4 long Qt cardiac arrhythmia and sudden cardiac death. *Nature vol. 421 6 Februray 2003.*
- 2- Hagir B. Suliman, **Mervat E. Ali**, and Claude A. Piantadosi, "**Superoxide dismutase-3 promotes full expression of the EPO response to hypoxia**". *Blood, 1 July 2004, Vol. 104, No. 1, pp. 43-50.*
- 3- **Mervat E. Ali**, Hajer B. Suliman, and Claude A. Piantadosi, "**Superoxide dismutase-3 protects cardiomyocytes after exposure to hyperoxia**". *In preparation.*