

EXERCISE TRAINING PROTECTS MYOCARDIAL MITOCHONDRIA AGAINST
ISCHEMIA REPERFUSION-INDUCED INJURY

By

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To the numerous teachers and professors who contributed to my education; and to my wife,
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By

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Coronary artery disease (CAD) is a major contributor to morbidity and mortality. The primary pathological manifestation of CAD is myocardial injury due to ischemia-reperfusion (IR). Given the worldwide prevalence of CAD and the associated IR-induced cardiac injury, understanding the mechanisms of myocardial IR injury as well as developing countermeasures to provide cardioprotection against IR-induced damage is important. In this regard, regular bouts of endurance exercise training (ExTr) protect the heart against IR-induced injury. However, it is unclear whether ExTr-induced cardioprotection against IR injury is mediated through mitochondrial adaptations. These experiments tested the hypothesis that exercise training protects cardiac mitochondria against IR-induced injury. To test this hypothesis, hearts were isolated from exercise-trained and sedentary control animals. Subsequently, hearts were exposed to either IR or continuous perfusion using an *in vitro* isolated working heart system, and cardiac contractile profiles were measured. At the end of IR or continuous perfusion, subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM) were isolated from the hearts to assess mitochondrial respiratory function and ROS generation. Our results reveal that ExTr protects the heart against IR injury as shown by higher levels of contractile performance during recovery from an IR insult. Moreover, ExTr protects mitochondria, reflected in a higher respiratory

control ratio in both SSM and IFM. Additionally, ExTr prevents IR-induced elevation of mitochondrial ROS production and the release of proapoptotic proteins (i.e., cytochrome c and AIF) during an IR insult. Moreover, ExTr increases mitochondrial antioxidant capacity and protects cardiac mitochondria against IR-induced oxidative damage. Collectively, these novel findings reveal that ExTr protects IR-induced mitochondrial injury and provide new insight into the mechanism responsible for exercise-induced cardioprotection.

CHAPTER 1 INTRODUCTION

Coronary artery disease remains a major cause of death in the Western world. The primary pathological manifestation of coronary artery disease is myocardial damage due to ischemia-reperfusion (IR) injury. The level of IR-induced myocardial injury can range from a small insult resulting in limited myocardial damage to a large injury culminating in myocyte death.

Although IR-induced myocardial injury occurs due to the complex interaction of many factors, elevated production of reactive oxygen species (ROS) plays an important role in IR-induced myocardial injury (2, 13, 34, 102, 115).

In this regard, mitochondria are targets and sources of increased ROS generation and thus induce oxidative damage and cell death during IR (15, 30, 31, 36, 88). Moreover, mitochondria have been shown as the mediators of life and death because damaged mitochondria release proapoptotic proteins that initiate cell death (32, 49, 60, 70, 90). Additionally, it has been demonstrated that an *in vitro* anoxia-reoxygenation challenge to mitochondria increases oxidative modification of mitochondrial proteins and lipids (9). Collectively, it appears that IR-induced mitochondrial damage leads to cardiac contractile dysfunction, cellular damage and cell death. For this reason, developing countermeasures to protect mitochondria against IR injury is important to attenuate myocardial injury.

Currently, the only practical strategy to protect the heart against IR injury is endurance exercise training (ExTr). Indeed, regular exercise training has been known to protect the heart against all levels of IR-induced injury including myocardial infarction (16, 42, 43, 47, 65, 66, 117, 118, 121, 123, 135, 143). Although the detailed mechanisms responsible for exercise-induced cardioprotection remain unclear, numerous potentially cardioprotective candidates exist: increased myocardial levels of heat shock protein 72 (HSP72), increased endoplasmic reticulum

(ER) stress proteins, elevated cyclooxygenase-2 (COX-2) activity, increased sarcolemmal potassium channels (sarcoK_{ATP}), increased mitochondrial potassium channels (mitoK_{ATP}), and improvements in cardiac antioxidant capacity. Of these proposed cardioprotective candidates, studies suggest that HSP 72, ER proteins, and COX-2 activity are not essential to acquire exercise-induced cardioprotection (65, 108, 122, 123, 135).

In contrast, recent evidence suggests that ExTr-mediated cardioprotection is associated with improvement of myocardial antioxidant capacity (42, 43, 47, 121). Specifically, these studies suggest that an ExTr-mediated increase in myocardial manganese superoxide dismutase (MnSOD), an endogenous mitochondrial antioxidant enzyme, plays a crucial role in cardioprotection against IR injury (66, 143). For example, these studies demonstrate that when exercise-mediated increase in MnSOD is inhibited, exercise-induced cardioprotection against IR insult is significantly reduced (66, 143). Considering the localization of MnSOD in mitochondria, it appears that ExTr may confer mitochondrial protection against IR-induced oxidative injury, which may lead to cardioprotection. Currently, it is unknown whether ExTr provides mitochondrial protection against an IR insult. Furthermore, it has not been investigated if ExTr protects either or both of the two morphologically and biochemically different subpopulations of mitochondria (i.e., subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM)). The purpose of the current experiments was to investigate whether ExTr confers mitochondrial (both SSM and IFM) protection against IR injury. Our hypothesis was that ExTr would protect myocardial mitochondria (SSM and IFM) against IR-induced respiratory dysfunction and oxidative injury and retard IR-induced mitochondrial ROS generation and the release of proapoptotic proteins. We also postulated that if our hypothesis was correct, ExTr-mediated

mitochondrial protection would be associated with exercise-induced elevation of mitochondrial antioxidants.

CHAPTER 2 REVIEW OF RELATED LITERATURE

Introduction

Coronary artery disease (CAD) remains a major cause of death in the Western world and industrialized nations. The primary pathological consequence of CAD is myocardial damage due to ischemia-reperfusion (IR) injury (124). The level of IR-induced myocardial injury can range from a small insult resulting in limited myocardial damage to a large injury culminating in myocardial cell death. Given the worldwide prevalence of CAD and the associated IR-induced cardiac injury, understanding the mechanisms of myocardial IR injury as well as developing countermeasures to provide cardioprotection against IR-induced damage is important.

In this regard, numerous approaches to achieve cardioprotection against IR injury have been investigated. To date the only practical and sustainable strategy capable of providing cardioprotection is regular bouts of endurance exercise. Specifically, human epidemiological studies demonstrate that regular exercise reduces the risk of death during myocardial IR insult (73). Moreover, animal studies confirm that regular bouts of aerobic exercise (i.e., treadmill running or swimming) protect the heart from IR-induced injury (9, 17, 19, 42, 43, 47, 66, 67, 83, 84, 96, 130, 131, 135, 136, 143). The objectives of this review are two-fold. The mechanisms responsible for IR-induced myocardial injury are reviewed, and subsequently the current knowledge about the effects of exercise training in providing cardioprotection against IR-induced injury is reviewed.

Myocardial Ischemia-Reperfusion Injury

Levels of Injury

Depending upon the duration of ischemia, three levels of IR-induced myocardial injury have been described (Figure 2-1). In general, reperfusion after 1-5 minutes of ischemia can result

in ventricular tachycardia or fibrillation without cell death or a deficit in ventricular contractile performance (44). Reperfusion after an ischemic period of 5-20 minutes results in the second level of myocardial injury known as “myocardial stunning”(13, 44). Myocardial stunning is characterized by a deficit in myocardial contractility that occurs without myocardial cell death. Typically, IR-induced myocardial stunning results in ventricular contractile deficits lasting 24-72 hours after the IR event (13). The third and highest level of IR injury occurs when ischemia is extended beyond 20 minutes. In these circumstances, cardiac myocytes become irreversibly damaged, resulting in cell death (i.e., myocardial infarction)(44). It is now clear that IR-induced cardiac myocyte cell death occurs due to both apoptosis and necrosis and that mitochondrial injury plays a major role in both forms of cell death (52, 70, 110).

Events Leading to Myocardial Ischemia-Reperfusion Injury

Despite the complexity in the mechanism(s) responsible for the IR-induced myocardial damage, essential factors leading to IR-induced cellular injury have been described (Figure 2-2). Evidence indicates that several intertwined factors, including a decrease in cellular ATP levels, production of reactive oxygen species (ROS), accumulation of hydrogen ions, generation of reactive nitrogen species (RNS), calcium overload, and calpain activation contribute to IR injury (51, 57, 69, 129, 149, 151). Collectively, these factors promote cellular injury and subsequent cardiac myocyte death.

Importantly, the oxyradical theory of IR-induced myocardial injury was introduced in 1985 (102). This theory proposed that the elevation of ROS and free radical (i.e. superoxide anion, hydrogen peroxide, and hydroxyl radical) production during both ischemia and reperfusion contributes to myocardial injury. It has been also confirmed by using electron paramagnetic resonance that ROS production is highly elevated during IR (6, 148, 150). Furthermore, ROS production in re-oxygenated cells is increased and the amount of ROS depends upon the duration

of both anoxia and reoxygenation (10). A detailed discussion of ROS-induced oxidative stress follows.

Ischemia Reperfusion-induced Cellular Oxidative Stress: An Overview

Radicals are chemically reactive molecules due to an unpaired electron. Superoxide anions are oxygen-derived radicals and can be produced during IR from the one electron reduction of molecular oxygen (102). Superoxide production can lead to the formation of many other ROS/RNS including the hydroxyl radical, hydrogen peroxide, and peroxynitrite (14, 44, 50, 63, 94, 102, 145, 147). Although radicals and other ROS can promote several types of cellular damage, two classes of biomolecules that are targets for oxidant-induced damage are proteins and lipids. Radical-mediated oxidation of amino acids can impair cellular protein function by altering their biochemical structure. Further, oxidized proteins become targets for proteolytic degradation; hence, IR-induced oxidative injury to proteins within cardiac myocytes is associated with accelerated protein breakdown (26, 39, 40, 137). Lipid damage in cells can occur by radical species reacting with polyunsaturated fatty acids in membranes which results in propagation reactions and the formation of new radicals (63, 144). Lipid peroxidation of the cellular membrane can result in altered fluidity and increased membrane permeability (63, 145).

Experimental evidence for the involvement of ROS/RNS in myocardial IR injury includes detection of protein oxidation, lipid peroxidation, and protein nitration products in reperfused hearts (1, 42, 67, 121, 149). Furthermore, the importance of ROS-mediated damage to the heart after an IR insult has been confirmed by studies indicating that antioxidants can provide myocardial protection against IR-induced injury (66, 67, 143). Interestingly, it has been recently reported that ROS-mediated oxidative stress also contributes to the calcium overload observed during IR (22) indicating that many of the injurious effects of ROS on the myocardium reflect activation of the calcium dependent protease, calpain (26, 75, 128).

Myocardial protein oxidation

Many cardiac proteins can be oxidatively modified by free radicals in the heart during IR. Included in the list of “target proteins” are enzymes, structural proteins, contractile proteins, and membrane-bound proteins (64, 68, 126, 127). Often, the damage caused by free radical interaction is irreparable. In addition, many proteins which are oxidatively modified become more susceptible to cellular proteolytic pathway by way of calpain and proteosome (111). One example of oxidative modification to cellular proteins is the formation of carbonyl groups which can then be measured and provide an indirect indication of oxidative stress within the cell (20).

Myocardial lipid peroxidation

Polyunsaturated fatty acids are highly susceptible to free radical modification at their unsaturated sites (4). ROS pull out electrons or hydrogen atoms from the methylene groups of fatty acids, and this reaction initiates a chain reaction where one modified fatty acid chain reacts with a neighboring chain. This eventually leads to the damage of the lipid membrane resulting in altered cell membrane permeability and fluidity. In many cases, this damage to the cell membrane leads to cellular death through necrosis and/or apoptosis. Lipid peroxidation by-products can be measured and used as a marker of oxidative stress within the cell (64). One such by-product is the formation of the reactive aldehyde, 4-hydroxy-2-nonenal (HNE). The omega-6 acyl groups of polyunsaturated fatty acids (i.e., linoleic and arachidonic acids) produce HNE as a result of free radical attack. HNE is a highly reactive compound and can react with several functional groups on biological material, particularly sulfhydryl groups, to form thioester adduct and then hemiacetals. HNE may also react with histidine and lysine residues of proteins to form stable α,β -unsaturated carbonyl compounds. In addition, HNE-modification of proteins may impair biological functions. A discussion of the primary sources of ROS in cardiac myocytes during IR follows.

Sources of ROS

There are several potential sources of free radical production in the heart during IR. Major sources include: electron leak from the mitochondrial respiratory complexes, xanthine oxidase, enzymatic arachadonic acid oxygenation, the synthesis of nitric oxide, catecholamine oxidation and oxidative burst from neutrophils (12, 24, 27, 44). Although each may play a role in IR damage, a large volume of evidence implicates mitochondrial production of radicals as the primary source of oxidants during both ischemia and reperfusion (30, 32, 34, 70, 86, 87) . Therefore, a detailed discussion of mitochondrial ROS production follows.

Mitochondria constitute about 30% of cardiomyocyte volume and act as the cell's powerhouses. They predominantly supply the energy (i.e., adenosine triphosphate (ATP) and phosphocreatine) required for cardiac muscle contraction and relaxation. Mitochondrial oxidative phosphorylation is the major ATP synthetic pathway in eukaryotes. During this process, electrons liberated from reduced forms of nicotine amide dinucleotides (NADH) and flavin amide dinucleotides (FADH) are delivered to oxygen via electron transport chain (ETC). The ETC is composed of five complexes and creates an H^+ gradient across the inner mitochondrial membrane. The electrochemical energy of this gradient generated between intermembrane space and matrix is then utilized to drive ATP synthesis by complex V (ATP synthase). In these processes, it has been estimated that approximately 1-2% of the oxygen consumed by mitochondria is altered to superoxide due to the escape of electrons from the chain (54). More specifically, it is believed that the majority of the superoxide is produced by combining electrons escaped from complex I and complex III with oxygen (89). Importantly, the levels of superoxide from mitochondria are significantly elevated during IR (3, 32). For this reason, mitochondria are considered as a major source of ROS production during IR.

Mitochondria and IR Damage

Mitochondria are both targets and sources of oxidative damage. As illustrated in figure 2-2, mitochondrial damage is induced by two major factors. For example, an IR insult can damage mitochondria via calcium overload and overproduction of ROS. Indeed, it is believed that mitochondria are associated with calcium homeostasis and therefore are strategically located near the Ca^{2+} release channel (i.e., L-type Ca^{2+} channel) and endo/sarcoplasmic reticulum of cardiomyocytes. However, over-influx of Ca^{2+} into the mitochondria (e.g., IR) leads to the opening of mitochondrial permeability transition pore (mPTP) and dissipates membrane potential (41, 49, 82, 105, 107). As a result, potential oxidants such as superoxide, hydrogen peroxide, hydroxyl radicals, peroxy radicals, and peroxynitrite are generated via a series of chemical reactions (14, 44, 50, 63). Subsequently, these oxidants contribute to further impairing the ETC and a component of mPTP, cyclophilin D (11). The resulting damage initiates apoptosis by releasing pro-apoptotic proteins (11, 59). Indeed, it has been demonstrated that mitochondria after an IR insult increase ROS production, reduce oxidative phosphorylation, and release proapoptotic proteins (i.e. cytochrome c, apoptosis inducing factor (AIF)) into the cytosol (32, 56, 77, 88, 90).

Under normal conditions, cytochrome c is confined at the inner membrane of mitochondria by nonionic/electrostatic interactions, and AIF is localized in the intermembrane space of mitochondria. However, mitochondrial membrane damage or opening of mPTP due to an IR insult causes the release of cytochrome c and AIF into the cytosol from mitochondria initiating genomic DNA fragmentation and propagating cell death (52, 55). The cell death in which mitochondrial proapoptotic proteins are involved include both caspase-dependent and caspase-independent pathways. Activation of the caspase-dependent pathway occurs due to the translocation of cytochrome c to the cytosol activating caspase-9. Activated caspase-9 then

activates caspase-3. Activated caspase-3 then translocates to nucleus where it cleaves the DNA repairing enzyme, poly (ADP-ribose) polymerase (PARP) and activates endonucleases which cleave DNA. These events finally lead to cell death. On the other hand, the caspase-independent cell death pathway is initiated by AIF. For example, AIF released from damaged mitochondria translocates to the nucleus and initiates apoptosis by cleaving chromatin and inducing large-scale DNA (~50 kbp) fragmentation in the absence of caspase activation (21, 38, 133). Collectively, given the mitochondrial vulnerability to oxidative stress and Ca^{2+} overload and initiation of cell death signals during IR insult, mitochondrial damage appears to be a prime causative factor in myocardial injury and infarction (cell death) (32, 37, 52, 53, 55, 56, 88-91, 93). Although elevated ROS production from mitochondria is considered as a key factor in myocardial injury, mitochondria contain endogenous antioxidants that react against ROS. Therefore, the following section discusses mitochondrial antioxidants.

Mitochondrial Antioxidants

An antioxidant is characterized as a molecule capable of delaying or preventing oxidation of other molecules (62). A multitude of antioxidants exists in mitochondria to provide a protective network against ROS injury (Figure 2-3). Primary enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase, peroxiredoxin 3 (Prx3), thioredoxin 2 (Trx2), and thioredoxin reductase 2 (TrxR2). In addition, the non enzymatic antioxidants glutathione exists in mitochondria. The following discussion addresses the roles and function of individual antioxidants of mitochondria.

Superoxide Dismutase (SOD)

SOD was discovered in 1969 by McCord and Fridovich (103). SOD is characterized as an oxidoreductase and is a metalloenzyme containing metal ions in addition to amino acids. Two isoforms of SOD exist in mitochondria: manganese SOD (MnSOD) and copper-zinc SOD

(CuZnSOD). MnSOD is located in the matrix, and CuZnSOD resides in intermembrane space (132). In mitochondria, SOD is to dismutates superoxide into hydrogen peroxide which is less reactive and non-radical. Therefore, both MnSOD and CuZnSOD reduce mitochondrial free radical damage. The chemical reaction of SOD with superoxide is outlined below:



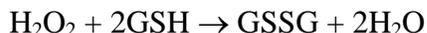
Catalase

Catalase is an antioxidant enzyme composed of four polypeptide chains containing four porphyrin heme groups that permit the enzyme to decompose hydrogen peroxide (139). The reaction of catalase in detoxification of hydrogen peroxide is



Glutathione Peroxidase (GPx)

Glutathione peroxidase is a selenium-containing tetrameric glycoprotein that catalyzes the decomposition of hydrogen peroxide to two molecules of water. Although GPx has a much lower K_m than catalase, GPx is more ubiquitous such that it also plays a critical role in reducing oxidative stress in mitochondria. The reaction relies on the oxidation of reduced glutathione (GSH), producing oxidized glutathione (GSSG). A reaction in which GPx catalyzes the decomposition of hydrogen peroxide is



Glutathione (GSH)

GSH is a tri-peptide containing a thiol group linked by cysteine and glutamate side chain. GSH exists in reduced and oxidized forms. GSH reduces any disulfide bonds formed by oxidative stress by donating electron from the thiol group. The thiol group of glutathione can be kept in a reduced form by a glutathione reductase that is constitutively active and inducible upon

oxidative stress. In the reduced state, the thiol group of cysteine donates a proton and an electron to other oxidized molecules. Importantly, since very little CAT resides within the mitochondria, GSH, in conjunction with Gpx, is the major means of decomposition of hydrogen peroxide in the mitochondria (104).

Peroxiredoxin 3 (Prx3)

Prx3 is a mitochondrial-specific antioxidant enzyme localized in the mitochondrial matrix that functions as an antioxidant enzyme removing mitochondrial H₂O₂ (23, 25). Indeed, the high abundance of Prx3 (~30 times more abundant than GPx) in mitochondria supports the importance of Prx3 in mitochondrial H₂O₂ detoxification (25). This reaction relies on the oxidation of reduced thioredoxin 2 (Trx2 red.) to oxidized thioredoxin 2 (Trx2 oxi.). A reaction where Prx3 catalyzes decomposition of hydrogen peroxide is



Thioredoxin 2 (Trx2)

Trx2 is an antioxidant protein containing a dithiol-disulfide active site. This enzyme facilitates the reduction of other proteins linked by a disulfide bond by donating an electron (oxidation). Trx2 in conjunction with Prx3 plays an important role in removing hydrogen peroxide in mitochondria.

Thioredoxin Reductase 2 (TrxR2)

Maintaining reduced state of Trx2 is important for Trx2 to continue to work as an antioxidant under the oxidative stress phase. Therefore, reduced Trx2 should be regenerated from oxidized Trx2. TrxR2 is the only known enzyme that reduces oxidized Trx2 in mitochondria via NADPH-dependent reaction (109). In this regard, TrxR2 contributes to reducing the accumulation of hydrogen peroxide in mitochondria.

Mitochondrial Antioxidant Network

It is clear that mitochondrial antioxidants work as a unit to remove ROS precursors (i.e. $O_2^{\cdot -}$ and H_2O_2) (Figure 2-3) before they form more reactive radicals (i.e. hydroxyl radical (HO \cdot) and peroxy radical (HOO \cdot)). Namely, $O_2^{\cdot -}$ molecules are dismutated by SOD to yield hydrogen peroxide and oxygen. The resulting hydrogen peroxide can be decomposed by actions of catalase, GPx, and Prx3 at the expense of GSH and Trx2, respectively. With this antioxidative pathway, mitochondrial redox balance is maintained.

Two Subpopulations of Mitochondria Exist in Cardiomyocytes

Mitochondria of cardiomyocytes are composed of two spatially and functionally distinct populations. One is located under the sarcolemma, referred to as subsarcolemmal mitochondria (SSM), and the other is interspaced in contractile muscle fibers, defined as interfibrillar mitochondria (IFM). Morphological, biochemical, and functional differences between SSM and IFM have been reported (32, 89, 125). It has also been demonstrated that SSM and IFM respond differently to an *in vitro* IR insult (32) and aging (45). Indeed, it is reported that IFM has an about 1.5 fold higher respiratory capacity (oxidative phosphorylation) and specific enzyme activities compared with SSM (114).

It has been recently proposed with the use of advanced electron microscopic techniques that the structural difference in cristae may confer distinct biochemical differences. For example, the more compact tubular structure of cristae in IFM may contribute to facilitating higher concentrations of H^+ resulting in higher oxidative phosphorylation. It is also assumed that differences in the electron transport chain complexes may be involved in the biochemical difference between SSM and IFM (125).

Exercise-Induced Cardioprotection against IR Injury

It is well established that regular bouts of endurance exercise training (ExTr) is cardioprotective against IR insult (17, 19, 42, 43, 47, 65-67, 71, 83-85, 118-121). Indeed, ExTR protects the heart against IR-induced oxidative injury, calcium overload and cell death (47, 121). Interestingly, short-term exercise training (i.e. 3-5 consecutive days) provides the same level of cardiac protection as that observed following long-term training (i.e.10 weeks) (42, 43, 47, 83, 121). Recent findings indicate that ExTR-induced upregulation of antioxidants and heat shock proteins are associated with cardioprotection (19, 42, 43, 72, 106, 143). In addition, a recent study from our laboratory demonstrated that short term ExTR reduces calcium overload-induced cellular damage (47). Moreover, it has been reported that ExTR protects the heart against IR-induced apoptosis as evidenced by the observation that ExTR decreases the level of caspase-3 activity and TUNEL (terminal deoxynucleotidyl transferase nick end labeling) positive stained nuclei. Nevertheless, the pathways responsible for ExTR-mediated cardioprotection against IR remain unclear. In this regard, the following sections will address possible mechanisms for exercise-mediated cardioprotection.

Endoplasmic Reticulum (ER) Proteins

Emerging evidence suggests that IR induces ER stress resulting in ER dysfunction. IR-induced ER dysfunction is involved in mitochondria-dependent and independent cell death due to disturbances in calcium homeostasis (142). Two proteins (GRP 78 and GRP 94) that are localized in ER and regulated by glucose are reported to protect the heart against IR-induced cell death (101). Therefore, it is possible that an ExTr-induced upregulation of GRP 78 and GRP 94 could induce cardioprotection. However, a recent study reveals that ExTr (short-term) does not elevate the ER proteins (108). Therefore, it appears that ER proteins are not required to attain ExTr-induced cardioprotection.

Cyclooxygenase-2 (COX-2) Activity

COX-2 is the rate-limiting enzyme converting arachidonic acid to prostanoid. The beneficial actions of COX-2 on the heart appear to stem from prostaglandin (PGE₂) and/or prostacyclin (PGI₂) production. For example, the elevated PGE₂ and PGI₂ production protects cardiomyocytes against oxidative stress (1). Nonetheless, a recent study reveals that ExTr does not elevate COX-2 levels in the rat heart (122). Therefore, it appears that increases in cardiac levels of COX2 are not required to attain ExTr-induced cardioprotection against IR injury.

Elevated Myocardial Heat Shock Proteins

Heat shock proteins (HSP) are a multifunctional group of proteins which can be induced by a variety of stimuli such as heat, oxidative stress, calcium overload, exercise training, and low pH. Once active, these proteins serve several functions within the cell, including chaperoning and/or transporting proteins, folding and refolding proteins, scavenging free radicals, and even facilitating protein synthesis (80, 97). Evidence exists that the elevation of HSP can confer cardioprotection against IR injury (99, 116). In addition, the cardioprotective properties of various HSP have been demonstrated. However, the importance of HSP to exercise-mediated cardioprotection has been controversial. For example, exercise training in a warm 22°C environment elevated myocardial levels of HSP 90, HSP 72, and HSP 40 and concomitantly provided cardioprotection against IR injury. However, recent studies reveal that exercise training in a cold environment (5°C) where exercise-induced increases in HSP were prevented provides cardioprotection against an *in vitro* IR insult without an elevation of myocardial HSP (43, 123, 135). These results suggest that an elevation in myocardial HSP (HSP 10, HSP 27, HSP 40, HSP 60, HSP 72, HSP 73 or HSP 90) is not essential for exercise-mediated cardioprotection. In this

regard, following section will discuss other possible mechanisms of exercise-mediated cardioprotection.

Sarcolemmal ATP-dependent Potassium Channel (sarcoK_{ATP})

SarcoK_{ATP} is localized in the sarcolemma of cardiac myocytes. Emerging evidence indicates that opening of sarcoK_{ATP} during IR protects the heart against an IR insult by reducing cellular calcium overload (58, 79). Nonetheless, the role that sarcoK_{ATP} plays in ExTr-induced cardioprotection remains to be investigated. To date, only one study reveals that ExTr increases sarcoK_{ATP} and when it was inhibited by a pharmacological inhibitor, ExTr-induced reduction in cell death (i.e., infarct size) was compromised (19). A recent study using cultured cardiomyocytes suggests that a sarcoK_{ATP}-induced protective mechanism against oxidative stress is accompanied by mitochondrial protection (i.e., inhibition of mPTP opening) (100).

Exercise Protects the Heart against IR-induced Calpain Activation

It has been reported that IR leads to activation of the calcium-activated protease calpain, resulting in myocardial damage (78). On the other hand, ExTr has been shown to decrease calpain activation following IR (47, 121). These studies suggest that exercise may confer cardioprotection through the regulation of IR-induced calpain activation. However, the mechanisms responsible for exercise-induced reduction in calpain activation during IR remain unclear. One possibility is that exercise-induced increases in antioxidative capacity may reduce oxidative damage to Ca²⁺-handling proteins resulting in the maintenance of intracellular Ca²⁺ homeostasis and regulating calpain activation (French et al, accepted in FASEB journal, 2008).

Improved Antioxidant Capacity

Recent evidence indicates that over-expression of myocardial antioxidants (i.e. MnSOD) via transgenic animal generation or gene therapy attenuates IR-induced myocardial infarction (34, 95, 141). In addition, a recent study demonstrated that using a mitochondria-targeted antioxidant

(Mito Q) results in cardioprotection against an IR insult (2). These results demonstrate that oxidative injury is a significant contributor to myocardial IR injury. In this regard, growing evidence suggests that endurance exercise (both short-term and long-term) provides cardioprotection by improving myocardial antioxidant capacity. Specifically, studies indicate that exercise elevates myocardial levels of GSH and activities of MnSOD and catalase (43, 117, 118).

As discussed earlier, MnSOD may play an important cardioprotective role in the heart during IR. Its localization to the mitochondrial matrix and ability to prevent oxidative stress induced by mitochondrial superoxide production indicates the importance of mitochondrial antioxidant capacity. Indeed, several studies have demonstrated the cardioprotective effects of exercise-mediated improvement of MnSOD. For example, Yamashita et al. (34, 143) demonstrated that the prevention of exercise-mediated increases in MnSOD (using an antisense oligodeoxyribonucleotide to MnSOD; AS-ODN-MnSOD) reduced exercise-induced cardioprotection. Further, Hamilton et al. (66) also showed that the prevention of exercise-mediated increase in MnSOD (using AS-ODN-MnSOD) attenuated the protection against IR-induced arrhythmia. It is unknown whether other antioxidants (i.e., Prx3, Trx2 and TrxR2) are upregulated by ExTr and if they are associated with ExTr-induced cardioprotection against an IR injury.

Exercise and Mitochondrial Protection

It is well established that mitochondrial respiratory dysfunction and damage due to an IR insult lead to cardiac injury (32, 88, 91). In contrast, recent evidence suggests that ExTr attenuates the damaging effects of *in vitro* anoxia-reoxygenation, calcium overload, and apoptotic stimuli on isolated cardiac mitochondria (8, 9, 76, 130). These studies demonstrated that ExTr reduces H₂O₂ production and the release of proapoptotic proteins and sustains

respiratory function of cardiac mitochondria. Hence, it is feasible that ExTr-induced cardioprotection may be attained by mitochondrial protection against IR injury.

Summary

Myocardial IR injury resulting from coronary artery disease remains a primary cause of morbidity and mortality in industrialized nations. Given the worldwide prevalence of IR-induced cardiac injury, understanding the mechanisms of myocardial IR injury as well as developing countermeasures to provide cardioprotection against IR-induced damage is important. Despite the complexity in the mechanism(s) responsible for the IR-induced myocardial damage, recent evidence suggests that overproduction of ROS and calcium overload play a critical role in IR-induced myocardial injury. In this regard, it is well documented that mitochondrial damage and dysfunction due to IR result in elevation of ROS production and contribute to IR-mediated myocardial oxidative injury and cell death. Additionally, it is notable that calcium overload in mitochondria plays an important role in mediating the production of ROS and initiating mitochondria-mediated apoptosis.

Currently, the only practical strategy to protect the heart against IR-induced myocardial injury is ExTr. Indeed, it has been well documented that ExTr protects the heart against all levels of IR induced injury including myocardial infarction. Although the detailed mechanisms responsible for exercise-induced cardioprotection remain unclear, numerous potentially cardioprotective candidates exist: HSP72, ER stress proteins, COX-2 activity, elevated sarcoK_{ATP} and improvements in cardiac antioxidant capacity. Of these proposed cardioprotective candidates, studies suggest that HSP 72, ER proteins, and COX-2 activity are not essential to acquire exercise-induced cardioprotection. ExTr-induced increase in sarcoK_{ATP} and reduction in calpain activation are reported to contribute to cardioprotection. Importantly, recent evidence suggests that ExTr-mediated improvement of myocardial antioxidant capacity plays a key role in

cardioprotection. Specifically, these studies suggest that the ExTr-mediated increase in myocardial MnSOD is a crucial factor inducing cardioprotection against IR injury including myocardial infarction. Collectively, these positive potential mechanisms appear to confer mitochondrial tolerance or adaptation to IR-induced oxidative injury, which may result in cardioprotection. Currently, it is unknown whether ExTr provides mitochondrial protection against an IR insult, which may be a probable cardioprotective pathway. Furthermore, it has not been investigated whether ExTr protects either one or both of the subpopulations of mitochondria.

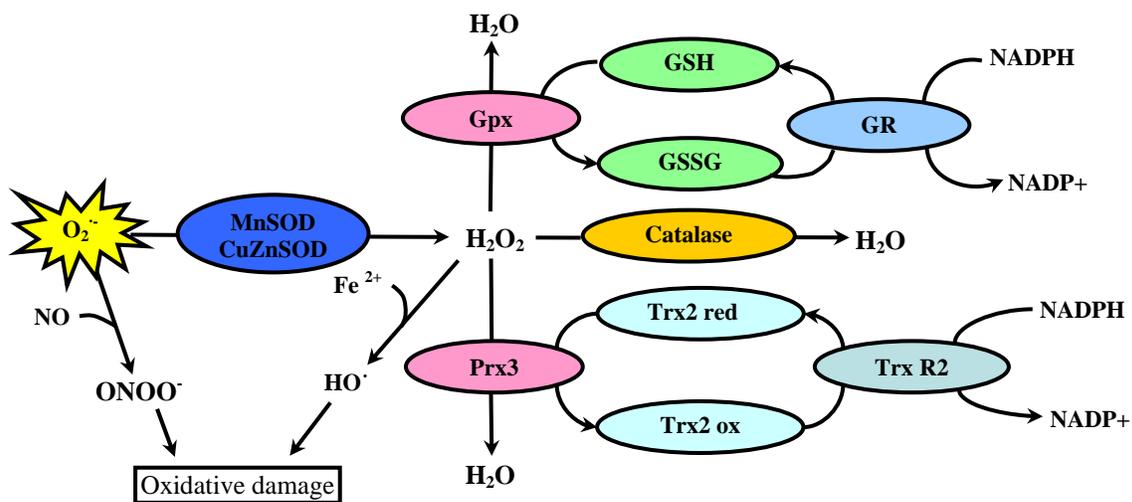


Figure 2-3. Illustration of interaction of endogenous antioxidant systems in the mitochondria of cardiac myocytes.

CHAPTER 3 METHODS

These experiments tested the hypothesis that endurance exercise training will attenuate IR-induced mitochondrial injury in cardiac myocytes. This hypothesis was addressed by the experiments outlined in this section. This section is subdivided into two segments. The first segment will describe the animal model and experimental design. Note that dependent measures will be mentioned by name only. A general methods section will follow to provide methodological details.

Experimental Overview

Animal Model Justification

Adult (3-5 months old) male Sprague-Dawley (SD) rats were used for these experiments. The SD rats were chosen for several reasons: first, the invasive nature of these experiments precludes the use of human subjects. Second, the SD rat model is a well-accepted model for the study of myocardial ischemia reperfusion injury (17, 74, 81, 113, 117). Third, the SD rat does not display large inter-animal variation in measures of cardiac contractility and collateral circulation. In addition, we chose to study male rats to avoid the possibly confounding effects of varying estrogen levels across the estrus cycle (117).

Animal Housing and Diet

All animals were housed at the University of Florida Animal Care Service Center. Animals were housed on a 12:12 hour reverse light-dark cycle and provided food (AIN93 diet) and water *ad libitum* throughout the experimental protocol.

Experimental Design and Primary Dependent Measures

The experimental design is illustrated (Figure 3-1). Briefly, 32 young male Sprague-Dawley rats were randomly assigned to either an exercise training group or a sedentary control

group. The sedentary and exercise training groups were further sub-divided into IR groups and IR sham groups of isolated working heart treatments (n=8). Animal sample size was determined based upon statistical power analysis using data from our laboratory.

The IR sham groups received continuous perfusion (85 minutes) without an IR insult. IR groups underwent 40 minutes of global ischemia and 45 minutes of reperfusion. During perfusion or an IR insult, working heart variables (i.e., cardiac output, cardiac work) were measured. After perfusion or an IR insult, two subpopulations of mitochondria were isolated. Then mitochondrial variables (i.e., oxygen consumption, ROS production, mitochondrial proapoptotic proteins, oxidation of proteins and lipids, and antioxidant enzymes) were measured.

General Methods

Exercise Training Protocol

The animals assigned to the exercise training groups were habituated to treadmill running for five days. Treadmill habituation of the first day was started with 10 minutes of treadmill running at an intensity of ~ 70% of VO_2 max, and from the second day exercise, exercise time was increased by 15 minutes each day, ending in 45 minutes of running on the fourth and fifth day. Following habituation, animals were rested for 2 days and then performed 5 consecutive days of treadmill running at an intensity of approximately 70% of VO_2 max (60 min at 30 m/min) (43, 84). The sedentary control animals were placed in the non-moving treadmill for the duration of their matched exercise training animals running to eliminate the possibility that handling or confinement may be a factor that induces cardioprotection.

***In Vitro* Isolated Working Heart Preparation**

To investigate myocardial contractile function before and after an IR insult, we selected the in vitro working heart model. This model is a highly reproducible preparation for examination of cardiac contractile performance, as cardiac preload and after-load pressures are maintained

constant. Further, an advantage of in vitro working heart model versus an in vivo IR model is the elimination of the confounding influence of other organ systems, systemic circulation, and peripheral complications. This preparation has been successfully used by our laboratory for over five years (83, 117, 121). Briefly, the animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (100mg/kg) and when a surgical plane of anesthesia was reached (an absence of foot and eye reflexes), 100 IU heparin was injected into the hepatic vein through the opened abdominal area. The hearts were excised and quickly placed in cold 0.9% NaCl and then weighed. The aorta was secured on a cannula and perfused in a retrograde mode at 80 cmH₂O with a modified Krebs-Henseleit buffer, containing 1.25mM CaCl₂, 130mM NaCl, 5.4mM KCl, 11 mM glucose, 0.5mM MgCl₂, 0.5mM NaH₂PO₄, 25mM NaHCO₃, aerated with 95% O₂-5% CO₂ gas at 37°C. Then the left atrium was cannulated through the pulmonary vein. Following 10 minutes of retrograde perfusion and 5 minutes of assist mode perfusion (retrograde perfusion with the atrial cannula open), the perfusion was switched to working heart mode where the preischemic-function measurements were set at 13 cmH₂O atrial filling pressure (preload) and 80 cmH₂O of aortic column (after load).

***In Vitro* Ischemia-Reperfusion Protocol**

After 15 minutes of stabilization in working heart mode during which pre-ischemic cardiac performance was measured, global, normo-thermic, no flow ischemia was induced by simultaneously clamping atrial and aortic lines. Ischemia was maintained for 40 minutes in an environment where the heart was enclosed in a water-jacketed, sealed glass chamber maintained at 37°C. Following 40 minutes of ischemia, the heart was reperfused for 10 minutes via retrograde mode (with only the aortic cannula open). Then 25 minutes of reperfusion was continued via the working heart mode in which post-ischemic cardiac performance

measurements were measured every 5 minutes for 20 minutes. At the end of IR or timed perfusion, the heart was cut and immediately transferred to cold saline to stop the heart beat.

Cardiac Performance Measurements

Cardiac contractile performance measurements were recorded every 5 minutes for 15 minutes prior to ischemia and every 5 minutes for 20 minutes during reperfusion. The same measurements were recorded for timed control groups. Measurements included: cardiac output, cardiac work, systolic/diastolic pressure, left ventricular developed pressure (LVDP), the rates of pressure development (+dp/dt) and decline (-dp/dt), and heart rate. These variables were measured via a calibrated pressure transducer (Harvard Instruments) connected to the aortic cannula and collecting both coronary and aortic effluent. Data was recorded and stored using a customized computer data-acquisition system (Labview).

Isolation of Subsarcolemmal Mitochondria (SSM) and Interfibrillar Mitochondria (IFM)

Cardiac mitochondria were isolated using the procedure of Palmer (114) with slight modification. Trypsin was used as the protease (32) in order to isolate IFM. At the end of either IR or timed perfusion, hearts were removed from the cannula and placed into saline at 4°C. The hearts were transferred into isolation buffer (100 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM MgSO₄·7 H₂O, 1 mM ATP, and 0.2% fatty acid free bovine serum albumin, pH 7.4) at 4°C. Cardiac tissue was finely minced and homogenized with a polytron tissue processor (Virtis, Gardiner, NY) for 7 seconds at a setting of 50. The homogenate was centrifuged at 500g for 10 minutes. The supernatant was saved for isolation of SSM. The remaining pellet was resuspended in isolation buffer and homogenized with the same polytron tissue processor for 5 seconds. Then the homogenate was incubated with 5 mg/g (wet weight) trypsin for 10 minutes at 4°C. The same amount of isolation buffer (10 ml/g wet weight) was added to deactivate trypsin activity. The homogenate was centrifuged at 500g for 10 minutes. The supernatant was saved for isolation of

IFM. The supernatants saved for isolation of SSM and IFM were centrifuged at 3,000g for 10 minutes to sediment SSM and IFM. The supernatant collected after isolation of SSM was further centrifuged at 20,000g for 30 minutes at 4°C. The resulting supernatant was used as the soluble cytosolic fraction.

The pellets of SSM and IFM were washed twice and the final pellets were resuspended using a dounce homogenizer in resuspension buffer (220 mM manitol, 70 mM sucrose, 2 mM Tris base, and 20 mM HEPES, pH 7.4) at 4 °C. Mitochondrial proteins and soluble cytosolic proteins were measured by the Bradford protein assay method (18), using fatty acid free BSA as a standard.

Mitochondrial Respiration (Oxidative Phosphorylation)

Mitochondrial oxygen consumption was measured at 37°C by polarography using a Clark type electrode (Oxygraph, Hansatech, Norfolk, UK). Experiments began with the addition of ~0.2 mg mitochondria in 1 ml of respiration buffer (in mM; 100 KCL, 5 KH₂PO₄, 1 EGTA, 50 MOPS, 10 MgCl₂) containing 0.2% BSA. Since IR-induced mitochondrial damage occurs primarily in complex I and III within the ETC, we studied mitochondrial respiration using selective respiratory substrates. Specifically, 2mM pyruvate and 2 mM malate (final concentration) were used as a complex I respiratory substrate. 5 mM succinate with 5 µM rotenone (to prevent electron backflow to complex I) was used as a complex II respiratory substrate. The maximal respiration (state 3), defined as the rate of respiration in the presence of ADP was initiated by adding 0.25 mM ADP to the respiration chamber containing mitochondria and respiratory substrates. State 4 (resting respiration) was obtained when the complete conversion (phosphorylation) of ADP to ATP was reached. The respiratory control ratio (RCR) commonly referred to as an index of mitochondrial integrity (coupled respiration) was calculated from the ratio of state 3 to state 4 respiration.

In order to examine the efficiency of ATP synthesis coupled to mitochondria, the P/O ratio were calculated from the ratio of ADP utilized to O₂ consumed during state 3 respiration.

Mitochondrial H₂O₂ Production

Mitochondrial H₂O₂ production was measured with the Amplex red-horseradish peroxidase (HRP) method (Molecular Probes, Eugene, OR) in freshly isolated mitochondria. HRP catalyzes the H₂O₂-dependent oxidation of nonfluorescent Amplex red (146). CuZnSOD was added to convert all superoxide into H₂O₂ because otherwise superoxide reacts very rapidly with HRP, resulting in underestimation of the actual rate of H₂O₂ production. Therefore, our results reflect the sum of both superoxide and H₂O₂ production are referred to as ROS rather than H₂O₂ production per se. More importantly, a submaximal respiration rate conditions was used with a phosphocreatine (PCr)/creatine (Cr) ratio of 0.5 to generate a free ADP concentration of about 67 μM. This ADP concentration was determined by progressive creatine kinase clamp experiment. This condition gives a steady state respiration rate of roughly 50% of state 3 respiration. Importantly, this method allows us to resolve one of the very valid criticisms about conventional *in vitro* mitochondrial ROS assays. For example, many people only look at either state 4 (“rest”) or state 3 (“all out”) rates of ROS production. In an *in vivo* environment, mitochondria obviously operate at a rate intermediate to these two extremes. Therefore, H₂O₂ production measured by this proposed technique allowed us to evaluate more physiological levels of mitochondrial H₂O₂ production.

Briefly, experiments for the static condition began with the addition of 20 μg mitochondria in 180 μl of pre-warmed respiration buffer at 37°C (in mM; 100 KCL, 5 KH₂PO₄, 1 EGTA, 50 MOPS, 10 MgCl₂, 0.2% fatty acid free BSA, 50 μM Amplex Red, and 5 mM succinate) containing CuZnSOD (40 unit/ml) and HRP (10 units/ml). For submaximal respiration condition, 5 mM ATP, 10 mM creatine phosphate, 20 mM creatine were added to the same

respiration buffer. H₂O₂ generation was determined by measuring fluorescence (excitation at 544 nm emission at 590 nm) for 15 minutes at 37°C using a fluorometric device (Spectra Max, Molecular device). The sample values (nmol/mg/min) were calculated based on the values acquired from standard curve using H₂O₂ as a standard.

Assessment of Mitochondrial Protein Oxidative Damage

Proteins carbonyls, an index of oxidative modification of proteins in SSM and IFM, were detected using a protein oxidation detection kit (Oxyblot, Chemicon, U.S.A). Briefly, 20 µg of mitochondrial proteins were denatured by adding 12% SDS for a final concentration of 6% SDS. The protein samples were derivatized by adding 2,4-Dinitrophenylhydrazine (DNPH). The samples were incubated for 15 minutes at room temperature. Then the samples were neutralized by adding neutralization solution from the kit. Finally, 10 µg of the prepared protein was loaded into 4-20% gradient acrylamide Criterion gel (Bio Rad). SDS-PAGE was performed at constant 200 Volts for 1 hour. The separated proteins were transferred to a pure nitrocellulose membrane by using a Transblot unit (Bio Rad) at 45 V for 2 hours. Equal loading was confirmed by staining the proteins with 0.1% Ponceau solution before the membrane was incubated in blocking buffer containing 5% non-fat milk with 0.05% Tween-20. The membrane was incubated in the primary antibody solution overnight at 4°C with gentle shaking. The membrane was then rinsed with washing buffer and incubated with secondary antibody for 1 hour at room temperature. Finally, enhanced chemiluminescence detection reagents from Amersham (Amersham Pharmacia Biotech, Piscataway, NJ) were used to generate chemiluminescent signals and the bands were visualized by exposing the membrane to light-sensitive film. The blot was analyzed using Kodak 1D image analysis software (Eastman Kodak, Rochester, NY).

Assessment of Mitochondrial Lipidperoxidation

4-Hydroxynonenal (4-HNE or HNE), an index of lipid peroxidation chain reaction due to oxidative stress, was measured from SSM and IFM using a western blot technique. Equal amounts of mitochondrial protein were loaded and separated via SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to a nitrocellulose membrane. The membrane was blocked in PBS containing 0.05% Tween 20 and 5% nonfat milk and incubated overnight with primary antibodies targeted to 4-HNE (Ab 46545). The membranes was washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour. Finally, enhanced chemiluminescence detection reagents were used to generate chemiluminescent signals and the bands were visualized by exposing the membrane to light-sensitive film. The blot was analyzed using Kodak 1D image analysis software.

Assessment of the Release of Mitochondrial Proapoptotic Proteins

Cytosolic cytochrome c and AIF were measured by western blot techniques. Briefly, cytosolic proteins were separated using SDS-PAGE under denaturing conditions and then transferred to nitrocellulose membranes. The membranes were blocked in PBS containing 0.05% Tween 20 and 5% nonfat milk and incubated overnight with primary antibodies (cytochrome c: sc 8385; AIF: sc 9416). The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour. Finally, enhanced chemiluminescence detection reagents from Amersham were used to generate chemiluminescent signals and the bands were visualized by exposing the membrane to light-sensitive film. Blots were analyzed using Kodak 1D image analysis software.

Assessment of Mitochondrial Antioxidant Proteins

MnSOD, CuZn SOD, catalase, Prx3, Trx2, and TrxR2 protein contents were measured by Western blot techniques. Briefly, mitochondrial proteins were separated using SDS-PAGE

under denaturing conditions and then transferred to nitrocellulose membranes. The membranes were blocked in PBS containing 0.05% Tween 20 and 5% nonfat milk and incubated overnight with primary antibodies (MnSOD: sc 30080; CuZnSOD: sc 11407; catalase: ab 16731; Prx3: LF-PA0030; Trx2: sc 50336; TrxR2: LF-PA0024). The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour. Finally, enhanced chemiluminescence detection reagents were used to generate chemiluminescent signals and the bands were visualized by exposing the membrane to light-sensitive film. Blots were analyzed using Kodak 1D image analysis software.

Data Analysis

To test our hypothesis, we performed a one-way ANOVA. When significance was indicated, Fisher's LSD post hoc test was chosen to determine group differences. Significance was established at $P < 0.05$.

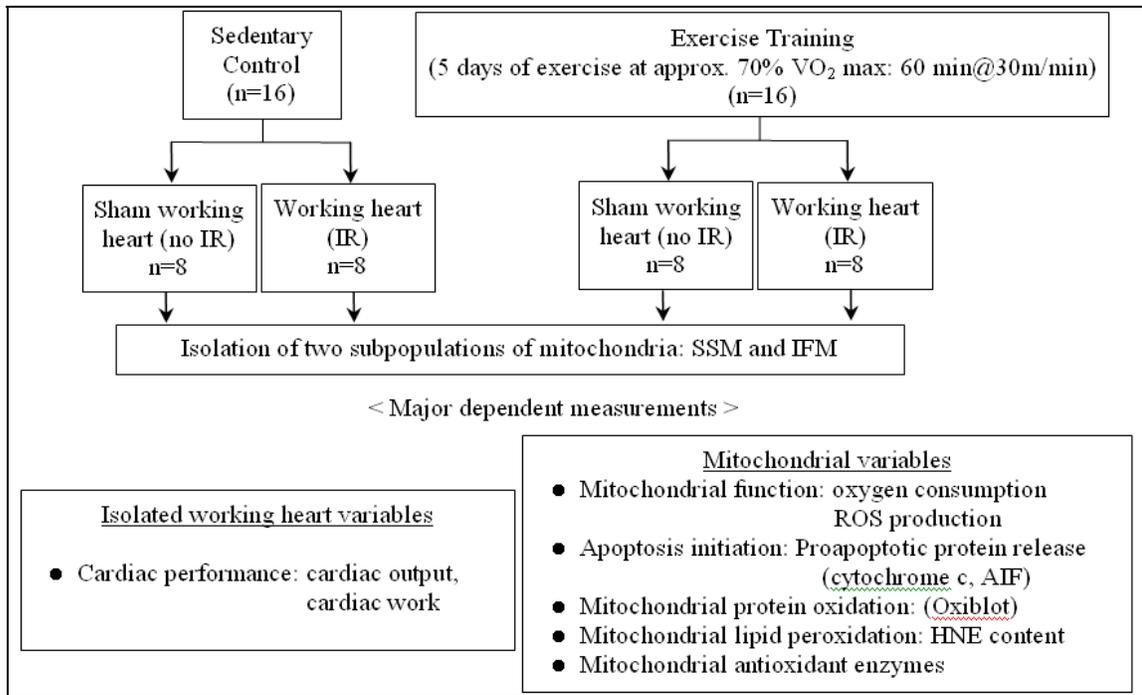


Figure 3-1. Experimental design examining the role of exercise training in mitochondrial protection following IR.

CHAPTER 4 RESULTS

Animal Characteristics

The physical characteristics for the animals in all experimental groups are presented in Table 4-1. No significant differences in animal body weight, heart weight, and heart/body weight ratio existed between experimental groups.

Myocardial Performance during IR

Myocardial Functional Characteristics

The functional characteristics of the isolated, perfused hearts are presented in Table 4-2. Coronary flow (CF), cardiac output (CO), systolic pressure (SP), heart rate (HR), % left ventricular developed pressure (% LVDP), and rate pressure product (RPP) were measured as indexes of myocardial function. Note that timed control groups (CP vs. EP) did not show any significant differences in myocardial function, indicating that perfusion time (85 minutes of isolated perfused-working heart) did not affect cardiac function. IR compromised the cardiac contractile function, but ExTr prevented IR-induced contractile dysfunction. For example, CF, CO, SP, % recovery of LVDP, and RPP were significantly reduced in CIR group compared to EIR groups.

Percent Recovery of Cardiac Work (CW: Systolic Pressure x Cardiac Output)

CW is commonly used in an *in vitro* working heart model as an index of myocardial function. This marker of contractile performance is important, as it normalizes each group to its baseline (pre-ischemia) values. As illustrated in Figure 4-1, CW was not different between CP and EP groups. However, % recovery of CW following IR was significantly lower in CIR group compared to EIR group.

Percent Recovery of +dp/dt and -dp/dt

The measurements of +dp/dt and -dp/dt are indicative of the rate of systolic pressure development (i.e., rate of ventricular contraction) and the rate of systolic pressure decline (i.e., rate of ventricular relaxation), respectively. Because myocardial contraction is regulated by the release and re-sequestration of Ca^{2+} within the sarcoplasmic reticulum, these measurements also reflect myocardial Ca^{2+} release and uptake kinetics. Further, by comparing +dp/dt and -dp/dt following IR, we can quantify the changes in myocardial contraction and relaxation rates and/or Ca^{2+} -handling kinetics. As illustrated in Figure 4-2 and 4-3, no differences were found between CP and EP groups. However, +dp/dt and -dp/dt were significantly reduced in CIR group compared to EIR group.

Mitochondrial Measurements

Subsarcolemmal and Interfibrillar Mitochondrial Protein Yield and Integrity

Mitochondrial protein yield is presented in Table 4-3. The protein yield of SSM in EP group was significantly increased compared to other groups. The protein yield of IFM of CIR group was significantly reduced compared to other groups. Additionally, the EIR group showed significant reduction in IFM protein compared with EP group. Total mitochondrial proteins (the sum of SSM and IFM) were not significantly different between CIR and EIR group.

The integrity of two subpopulations of isolated mitochondria was confirmed by images obtained with transmission electron microscopy (Figure 4-4). In addition, the integrity of isolated mitochondria was reconfirmed by measuring mitochondrial respiratory control ratio (RCR = state 3/state 4 respiration). Indeed, RCR from both SSM (~4.5) and IFM (~10) demonstrate that isolated mitochondria were intact and well coupled.

Mitochondrial Oxidative Phosphorylation (State 3 and State 4) and RCR of SSM

Changes in the rate of state 3 and state 4 are used as an index of mitochondrial damage. With pyruvate/malate as a substrate to donate reducing equivalents to complex I, no differences existed between CP and EP in the rates of state 3 and 4 respiration. The rates of state 3 respiration following IR were decreased following IR (Figure 4-5). The rates of state 4 respiration following IR were significantly increased. However, compared to CIR group, the rate of state 4 respiration was significantly lower in EIR group (Figure 4-6). Thus, respiratory control ratio (RCR) was significantly higher the EIR group compared to CIR group (Figure 4-7).

With succinate as a substrate to donate reducing equivalents to complex II, the rates of state 3 and 4 respiration were not different between CP and EP groups. The rates of state 3 respiration following IR were significantly reduced. However, EIR group maintained higher state 3 respiration rate compared to CIR group (Figure 4-8). The rates of state 4 respiration following IR were significantly elevated. However, EIR group sustained lower state 4 respiration rate compared to CIR group (Figure 4-9). As a result, the RCR was significantly higher in EIR group compared to CIR group (Figure 4-10).

Mitochondrial Oxidative Phosphorylation (State 3 and State 4) and RCR of IFM

With pyruvate/malate, no differences existed between CP, EP, and EIR groups in the rates of state 3 and 4 respiration. The rate of state 3 respiration in following IR was significant reduced in CIR group compared to other groups (Figure 4-11). Moreover, the rate of state 4 respiration following IR was significantly elevated in CIR group compared to other groups (Figure 4-12). Therefore, the RCR of CIR group was significantly lower compared to other groups (Figure 4-13).

With succinate, no differences existed in the rate of state 3 respiration between CP and EP groups. The rate of state 3 respiration following IR was significantly decreased in CIR group

compared to other groups (Figure 4-14). However, the rates of state 4 respiration were not different between groups (Figure 4-15). The RCR was significantly lower in CIR group compared to other groups (Figure 4-16).

P/O Ratio

Mitochondrial P/O ratios are presented in Table 4-4. With pyruvate/malate as a complex I substrate, P/O ratio in SSM of CIR group was significantly lower compared to EIR group. However, no difference was found in IFM between CIR and EIR group. No significant differences were found in P/O ratio between groups when succinate (complex II) was used as a substrate.

Mitochondrial H₂O₂ Production

Horseradish peroxidase and Amplex red do not penetrate intact mitochondria (33). Therefore, only H₂O₂ released from mitochondria is detected by this assay. Conventionally, H₂O₂ production is measured in a static condition (the presence of added substrate but not ADP). As shown in Table 4-5, the rate of H₂O₂ production was not different between groups when the static conditions were used. In contrast, in constantly active respiration condition where constant ADP concentration is maintained by creatine kinase (CK) clamp, the rate of H₂O₂ production in SSM was significantly higher in CIR group compared to EIR group (Figure 4-17). Similarly, the rate of H₂O₂ production in IFM was also significantly higher in CIR group compared to EIR group (Figure 4-18).

IR-induced Oxidative Modifications in Mitochondria

Carbonyl formations in proteins are one of the features of oxidatively modified proteins. Our results show that the level of oxidatively modified proteins in SSM was significantly increased in CIR group compared with CP and EP groups (Figure 4-19). No differences existed in the level of protein carbonyls in IFM between groups.

4-HNE is generated by the lipid peroxidation chain reaction due to oxidative stress. Therefore, elevated levels of this marker indicate an increase in lipid oxidation. Our results show that the level of 4-HNE products in SSM was significantly increased in CIR group compared to other groups (Figure 4-20). Moreover, the level of 4-HNE products in IFM was significantly elevated in CIR group compared to EP and EIR groups (Figure 4-21). Additionally, CP group showed higher levels of 4-HNE formation compared to EP group.

The Release of Mitochondrial Proapoptotic Proteins

The release of pro-apoptotic proteins (i.e., cytochrome c and AIF) from mitochondria into the cytosol is an index of mitochondrial damage and cell death. As illustrated in Figure 4-22, our results show that the level of cytochrome c released from mitochondria was significantly higher in CIR group compared to the other groups. Furthermore, the level of AIF released from mitochondria was also significantly higher in the CIR group compared to other groups (Figure 4-23).

Mitochondrial Antioxidant Proteins

Mitochondrial antioxidant proteins in both SSM and IFM were measured to determine whether exercise-mediated mitochondrial protection is associated with an increase in mitochondrial antioxidant capacity. Overproduction of superoxide anions from mitochondria during IR causes oxidative damage. In this regard, MnSOD and CuZnSOD dismutate superoxide anions to a less reactive oxidant (i.e., H₂O₂). Our results show that both MnSOD (Figure 4-24) and CuZnSOD (Figure 4-25) in SSM were significantly elevated in EP group compared with CP and CIR groups. In terms of IFM, the level of MnSOD proteins in IFM was significantly reduced in CIR group compared to EP group (Figure 4-28). The level of CuZnSOD proteins was significantly reduced in CIR group compared to other groups (Figure 4-29).

Trx2 is a protein containing dithiol groups and thus acts as an antioxidant. It is specifically involved in detoxifying H₂O₂. We measured the levels of Trx2 protein contents utilizing immunoblotting techniques to examine if exercise training increases this protein and thus reduces oxidative stress. Our results indicate that the level of Trx2 proteins in SSM was significantly higher in EP and EIR groups compared with CP group. In addition, Trx2 in EIR group was significant higher than CIR group (Figure 4-26).

Maintaining the reduced state of Trx2 is important for Trx2 to act as an antioxidant. In this regard, TrxR2 is the only known enzyme to reduce Trx2 and works as a unit with Trx2 to remove H₂O₂. The level of Trx2R proteins was significantly reduced in CIR group compared to other groups (Figure 4-27). In regard to IFM, The level of TrxR2 was significantly lower in CIR group compared to EP and EIR groups (4-30).

No significant differences were found in Prx3 and catalase of SSM between groups (data not shown). In addition, no differences were found in Trx2, Prx3, and catalase in IFM between groups (data not shown).

Table 4-1. Animal body and heart weights.

Group	Number	Body Weight (g)	Heart Weight (g)	Heart / Body Weight Ratio (mg/g)
CP	8	422 ± 8.2	1.82 ± 0.06	4.31 ± 0.19
EP	8	412 ± 11.5	1.68 ± 0.04	4.10 ± 0.16
CIR	8	437 ± 8.6	1.79 ± 0.03	4.10 ± 0.10
EIR	8	423 ± 9.6	1.76 ± 0.07	4.15 ± 0.12

Values are mean ± SE. No differences existed between the experimental groups in any variables ($P < 0.05$). CP= control-perfused; EP= exercise-perfused; CIR= control-ischemia reperfusion; EIR= exercise-ischemia-reperfusion.

Table 4-2. Functional Characteristics of isolated, perfused hearts during IR.

Parameters	CP	EP	CIR	EIR
CF (ml/min/g wet. wt.)	11.41 ± 0.39	12.21 ± 0.68	7.054 ± 0.46 ***	10.48 ± 0.66 *
CO (ml/min/g wet. wt.)	38.76 ± 1.36	42.38 ± 1.20	11.71 ± 3.51 ***	30.05 ± 1.99 **
SP (mmHg)	93.13 ± 3.34	87.50 ± 1.59	69.88 ± 1.48 ***	80.75 ± 1.22 **
HR (beats/min)	317 ± 12	339 ± 7	292 ± 23 *	324 ± 10
% recovery of LVDP	101 ± 3.6	95 ± 3.5	21 ± 9 ***	70 ± 4 **
RPP (HR x SP)	29310 ± 335	29680 ± 546	20600 ± 1800 ***	26160 ± 494 **

Values are mean ± SE. *** Significantly different from CP, EP, and EIR groups, P < 0.05. ** Significantly different from CP and EP groups, P < 0.05. * Significantly different from EP group, P < 0.05. CP= control-perfused; EP= exercise-perfused; CIR= control ischemia reperfusion; EIR= exercise ischemia-reperfusion.

Table 4-3. Mitochondrial protein yield.

Parameters	CP	EP	CIR	EIR
SSM (mg/g.w.wt)	4.16 ± 0.17	4.74 ± 0.22 §	3.96 ± 0.25	3.95 ± 0.15
IFM (mg/g.w.wt)	9.48 ± 0.25	9.96 ± 0.28	7.92 ± 0.51 ***	8.59 ± 0.26 *
Total mitochondria (mg/g.w.wt)	13.64 ± 0.27	14.70 ± 0.37	11.89 ± 0.67 **	12.54 ± 0.39 *

Values are mean ± SE. *** Significantly different from CP, EP, and EIR groups, P < 0.05. ** Significantly different from CP and EP groups, P < 0.05. * Significantly different from EP group, P < 0.05. § Significantly different from CP, CIR and EIR groups, P < 0.05. CP= control-perfused; EP= exercise-perfused; CIR= control ischemia-reperfusion; EIR= exercise ischemia-reperfusion.

Table 4-4. P/O ratio.

	SSM				IFM			
	CP	EP	CIR	EIR	CP	EP	CIR	EIR
Pyr/mal	4.2±0.1	4.3±0.1	3.8±0.2 ***	4.3±0.2	4.3±0.1	4.4±0.1	4.0±0.2 *	4.1±0.1
Succinate	2.0±0.1	2.0±0.1	1.9±0.2	1.8±0.1	2.3±0.1	2.3±0.1	2.20±0.1	2.1±0.1

Values are mean ± SE. *** Significantly different from CP, EP, and EIR groups, P < 0.05. * Significantly different from EP group, P < 0.05. CP= control-perfused; EP= exercise-perfused; CIR= control ischemia reperfusion; EIR= exercise ischemia-reperfusion.

Table 4-5. Mitochondrial ROS production during state 4 respiration.

	SSM				IFM			
	CP	EP	CIR	EIR	CP	EP	CIR	EIR
Static	373± 91	331± 81	472±55	461±79	372± 91	306±70	457± 52	391± 59

Values are mean ± SE. CP= control-perfused; EP= exercise-perfused; CIR= control ischemia reperfusion; EIR= exercise ischemia-reperfusion. No significant differences were found in any of the variables measured (P < 0.05).

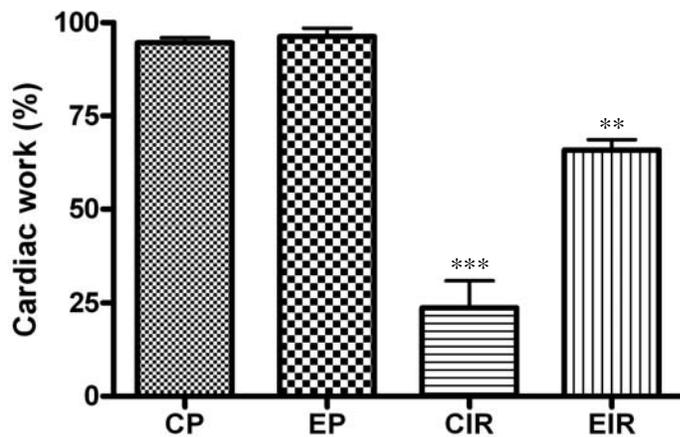


Figure 4-1. Percent recovery of cardiac work (systolic pressure x cardiac output). Values are mean \pm SE. *** Significantly different from CP, EP, and EIR groups, $P < 0.05$. ** Significantly different from CP and EP group, $P < 0.05$. CP=control-perfused; EP=exercise-perfused; CIR=control ischemia-reperfusion; EIR= exercise ischemia-reperfusion.

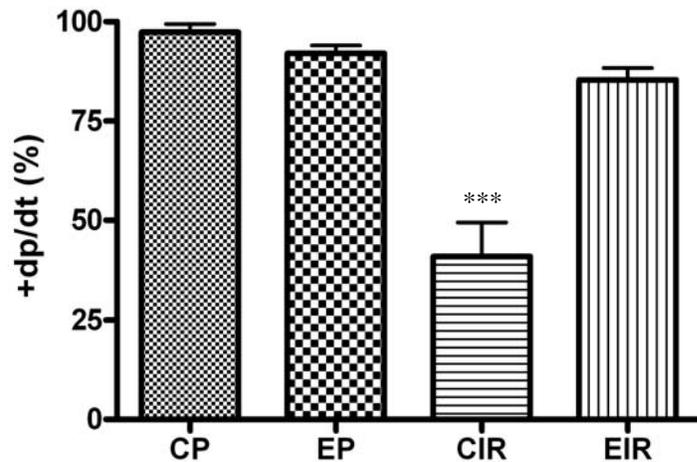


Figure 4-2. Percent recovery rate of +dp/dt (left ventricular systolic function). Values are mean \pm SE. *** Significantly different from CP, EP, and EIR groups, $P < 0.05$. CP=control-perfused; EP=exercise-perfused; CIR=control ischemia-reperfusion; EIR= exercise ischemia-reperfusion.

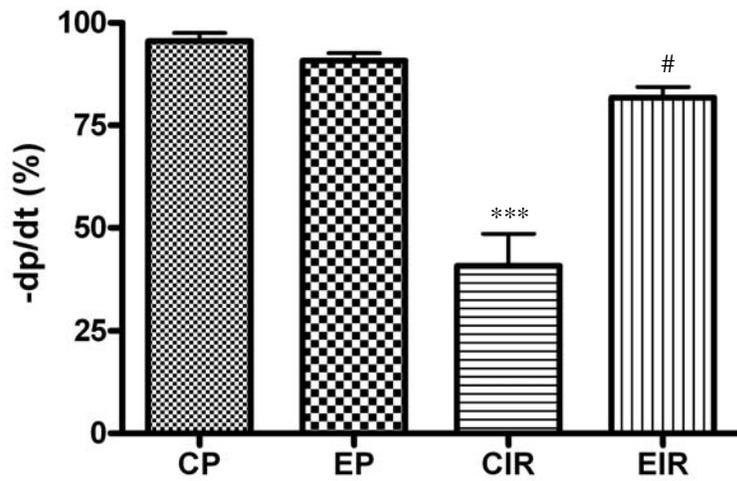


Figure 4-3. Percent recovery rate of $-dp/dt$ (left ventricular diastolic function). Values are mean \pm SE. *** Significantly different from CP, EP, and EIR groups, $P < 0.05$. # Significantly different from CP group, $P < 0.05$. CP=control-perfused; EP=exercise-perfused; CIR=control ischemia-reperfusion; EIR= exercise ischemia-reperfusion.

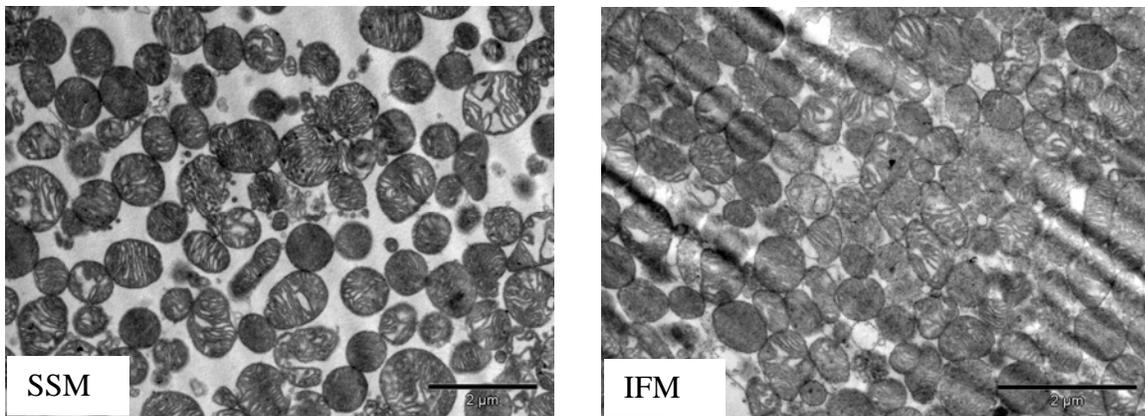


Figure 4-4. Electron microscopic photographs of subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM) isolated from a rat heart.

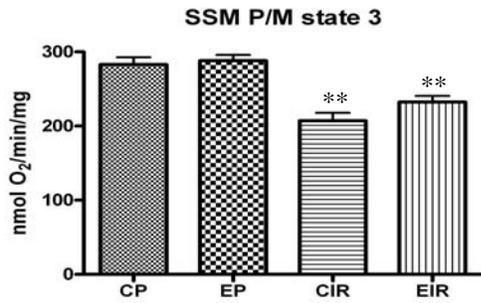


Figure 4-5. The rate of state 3 respiration of SSM using pyruvate/malate. Values are mean \pm SE. ** Significantly different from CP and EP groups, $P < 0.05$. P/M= pyruvate/malate; CP= control-perfused; EP= exercise-perfused; CIR= control ischemia reperfusion; EIR= exercise ischemia-reperfusion.

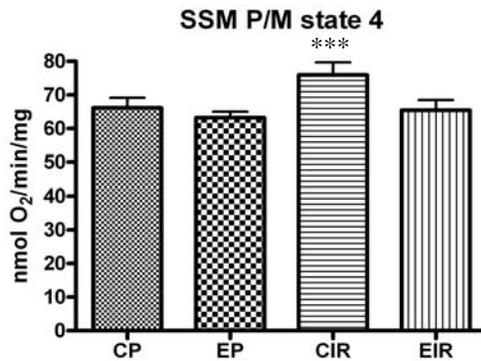


Figure 4-6. The rate of state 4 respiration of SSM using pyruvate/malate. Values are mean \pm SE. *** Significantly different from CP, EP and EIR groups, $P < 0.05$. P/M=pyruvate/malate; CP= control-perfused; EP= exercise-perfused; CIR= control ischemia reperfusion; EIR= exercise ischemia-reperfusion.

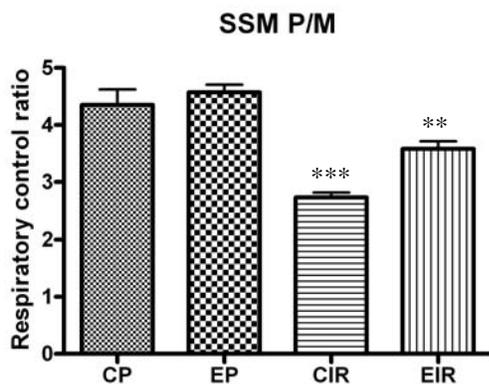


Figure 4-7. Respiratory control ratio of SSM, using pyruvate/malate. Values are mean \pm SE. *** Significantly different from CP, EP, and EIR groups, $P < 0.05$. ** Significantly different from CP and EP groups, $P < 0.05$. P/M=pyruvate/malate; CP=control-perfused; EP=exercise-perfused; CIR=control ischemia-reperfusion; EIR= exercise ischemia-reperfusion.

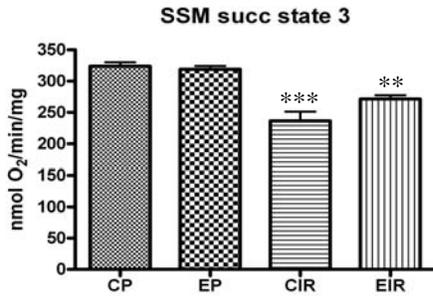


Figure 4-8. The rate of state 3 respiration of SSM using succinate. Values are mean \pm SE. *** Significantly different from CP, EP, and EIR groups, $P < 0.05$. ** Significantly different from CP and EP groups, $P < 0.05$. succ=succinate; CP= control-perfused; EP= exercise-perfused; CIR= control ischemia reperfusion; EIR= exercise ischemia-reperfusion.

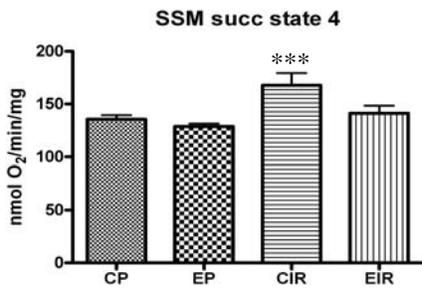


Figure 4-9. The rate of state 4 respiration of SSM using succinate. Values are mean \pm SE. *** Significantly different from CP, EP, and EIR groups, $P < 0.05$. succ=succinate; CP= control-perfused; EP= exercise-perfused; CIR= control ischemia reperfusion; EIR= exercise ischemia-reperfusion.

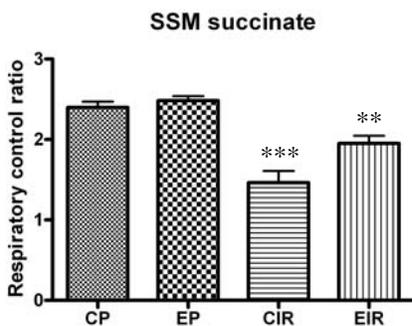


Figure 4-10. Respiratory control ratio of SSM, using succinate. Values are mean \pm SE. *** Significantly different from CP, EP, and EIR group, $P < 0.05$. ** Significantly different from CP and EP group, $P < 0.05$. CP=control-perfused; EP=exercise-perfused; CIR=control ischemia-reperfusion; EIR= exercise ischemia-reperfusion.

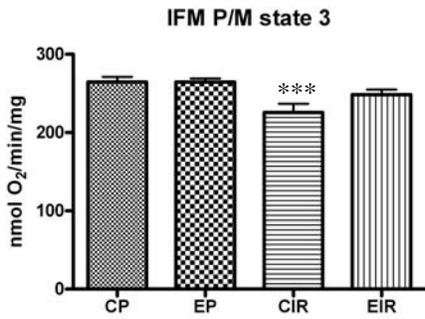


Figure 4-11. The rate of state 3 respiration of IFM using pyruvate/malate. Values are mean \pm SE. *** Significantly different from CP, EP, and EIR groups, $P < 0.05$.

P/M=pyruvate/malate; CP= control-perfused; EP= exercise-perfused; CIR= control ischemia reperfusion; EIR= exercise ischemia-reperfusion.

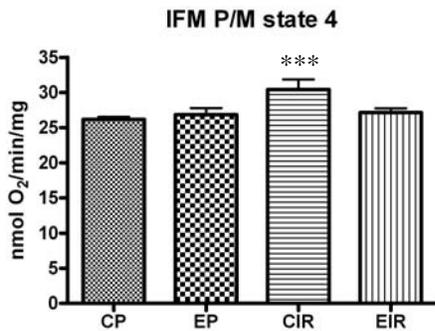


Figure 4-12. The rate of state 4 respiration of IFM using pyruvate/malate. Values are mean \pm SE.

*** Significantly different from CP, EP, and EIR groups, $P < 0.05$. P/M= pyruvate/malate; CP= control-perfused; EP= exercise-perfused; CIR= control ischemia reperfusion; EIR= exercise ischemia-reperfusion.

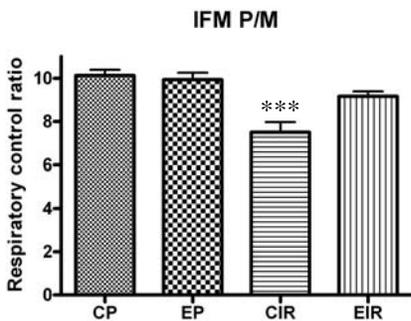


Figure 4-13. Respiratory control ratio of IFM, using pyruvate/malate. Values are mean \pm SE. *** Significantly different from CP, EP, and EIR groups, $P < 0.05$. P/M= pyruvate/malate; CP=control-perfused; EP=exercise-perfused; CIR=control ischemia-reperfusion; EIR= exercise ischemia-reperfusion.

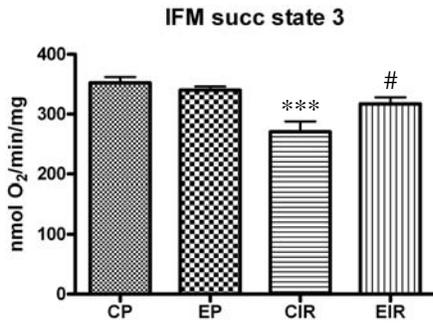


Figure 4-14. The rate of state 3 respiration of IFM using succinate. Values are mean \pm SE. *** Significantly different from CP, EP, and EIR groups, $P < 0.05$. # Significantly different from CP, $P < 0.05$. succ=succinate; CP= control-perfused; EP= exercise-perfused; CIR= control ischemia reperfusion; EIR= exercise ischemia-reperfusion.

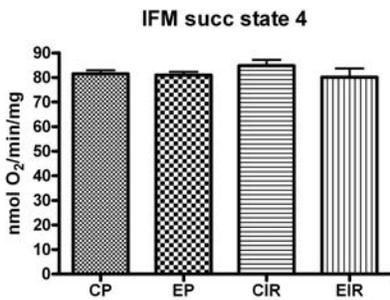


Figure 4-15. The rate of state 4 respiration of IFM using succinate. Values are mean \pm SE. No significant differences exist. succ=succinate; CP= control-perfused; EP= exercise-perfused; CIR= control ischemia reperfusion; EIR= exercise ischemia-reperfusion.

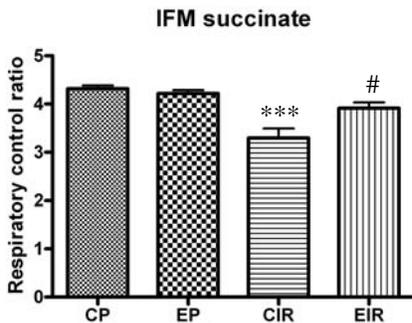


Figure 4-16. Respiratory control ratio of IFM, using succinate. Values are mean \pm SE. *** Significantly different from CP, EP, and EIR group, $P < 0.05$. # Significantly different from CP group, $P < 0.05$. CP=control-perfused; EP=exercise-perfused; CIR=control ischemia-reperfusion; EIR= exercise ischemia-reperfusion.

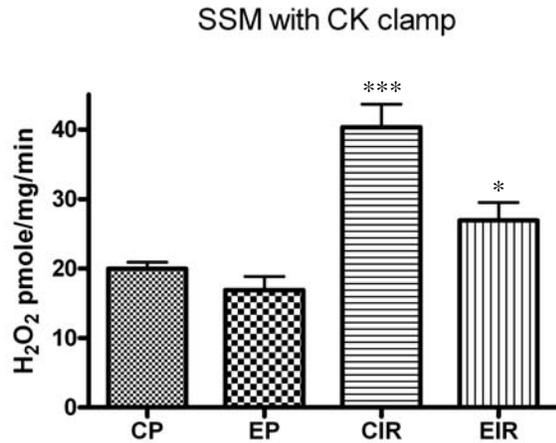


Figure 4-17. The levels of H₂O₂ production from SSM, using CK clamp. Values are mean ± SE. *** Significantly different from CP, EP, and EIR group, P < 0.05. * Significantly different from EP, P < 0.05. SSM=subsarcolemmal mitochondria; CK= creatine kinase; CP=control-perfused; EP=exercise-perfused; CIR=control ischemia-reperfusion; EIR= exercise ischemia-reperfusion.

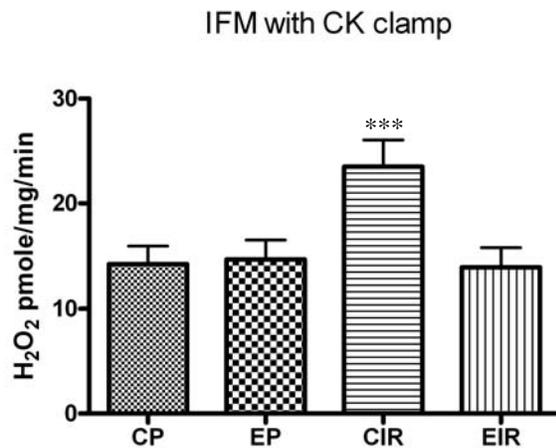


Figure 4-18. The levels of H₂O₂ production from IFM, using CK clamp. Values are mean ± SE. *** Significantly different from CP, EP, and EIR groups, P < 0.05. IFM=interfibrillar mitochondria; CK= creatine kinase; CP=control-perfused; EP=exercise-perfused; CIR=control ischemia-reperfusion; EIR= exercise ischemia-reperfusion

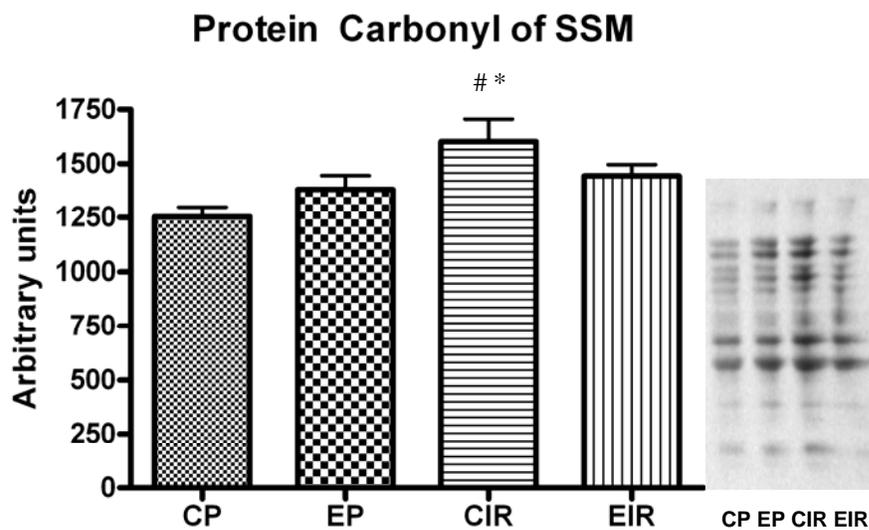


Figure 4-19. The levels of protein carbonyl formation in SSM. Values are mean \pm SE. # Significantly different from CP group, $P < 0.05$. * Significantly different from EP group, $P < 0.05$. SSM=subsarcolemmal mitochondria; CP=control-perfused; EP=exercise-perfused; CIR=control ischemia-reperfusion; EIR= exercise ischemia-reperfusion.

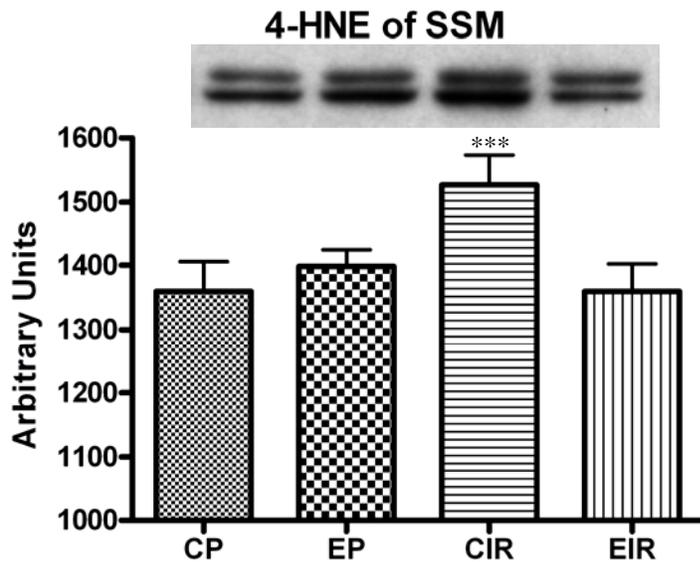


Figure 4-20. The levels of 4-HNE products of IFM following IR. Values are mean \pm SE. *** Significantly different from CP, EP and EIR groups, $P < 0.05$. SSM=subsarcolemmal mitochondria; CP=control-perfused; EP=exercise-perfused; CIR=control ischemia-reperfusion; EIR= exercise ischemia-reperfusion.

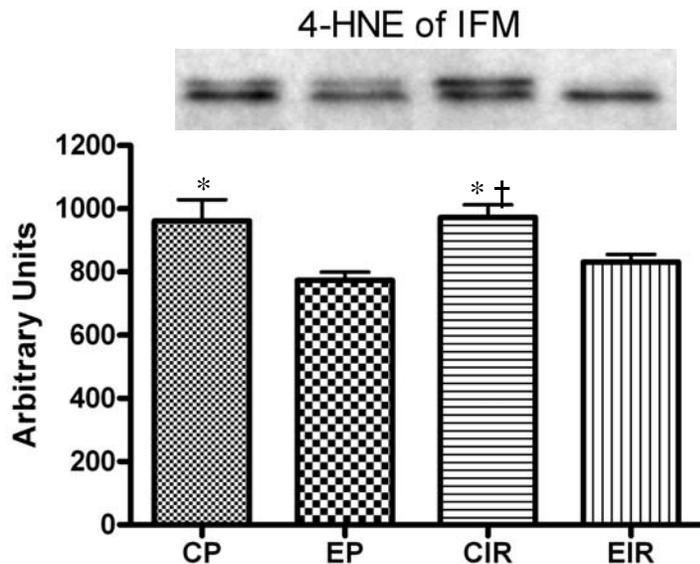


Figure 4-21. The levels of 4-HNE products in IFM following IR. Values are mean \pm SE. * Significantly different from EP group $P < 0.05$. † Significantly different from EIR group $P < 0.05$. IFM=interfibrillar mitochondria; CK= creatine kinase; CP=control-perfused; EP=exercise-perfused; CIR=control ischemia-reperfusion; EIR= exercise ischemia-reperfusion.

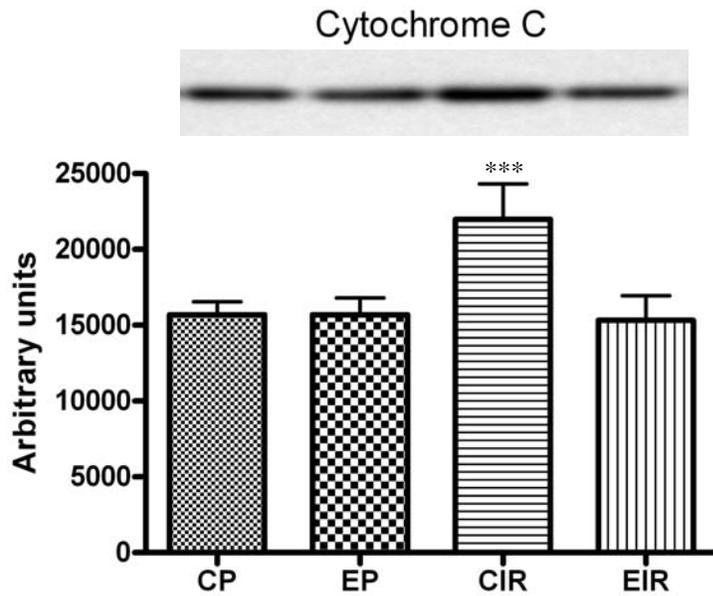


Figure 4-22. Levels of cytochrome c released from cardiac mitochondria. Values are mean \pm SE. *** Significantly different from CP, EP, and EIR group, $P < 0.05$. CP=control-perfused; EP=exercise-perfused; CIR=control ischemia-reperfusion; EIR= exercise ischemia-reperfusion.

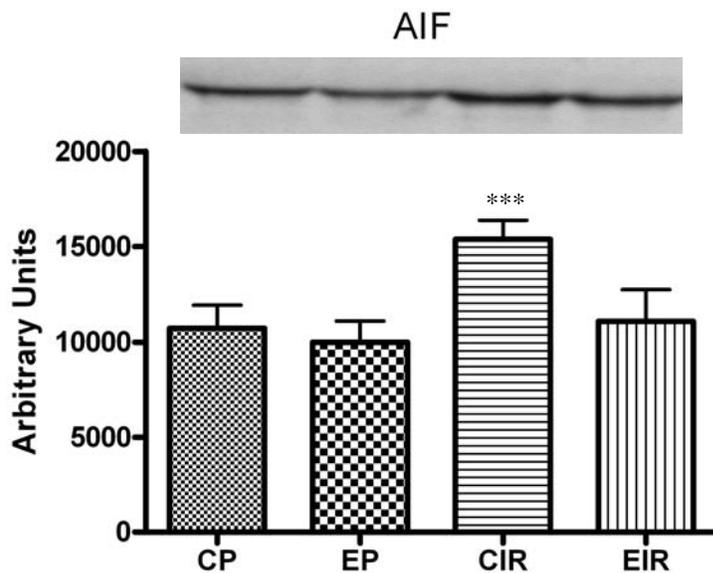


Figure 4-23. Levels of AIF released from cardiac mitochondria. Values are mean \pm SE. *** Significantly different from CP, EP, and EIR groups, $P < 0.05$. CP=control-perfused; EP=exercise-perfused; CIR=control ischemia-reperfusion; EIR= exercise ischemia-reperfusion.

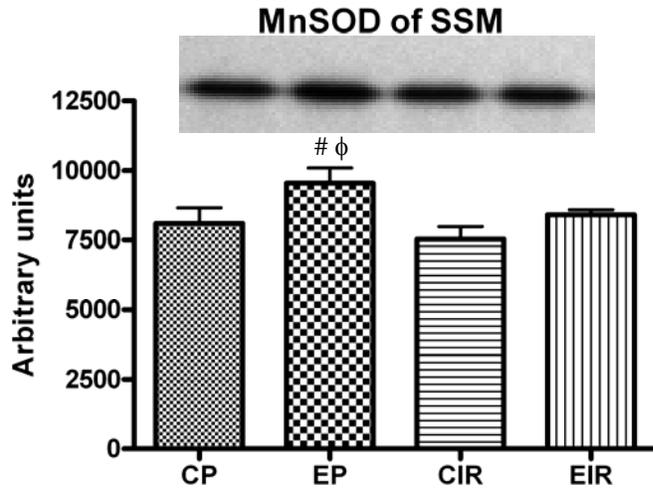


Figure 4-24. Protein levels of manganese superoxide dismutase (MnSOD) in SSM. Values are mean \pm SE. # Significantly different from CP group, $P < 0.05$. ϕ Significantly different from CIR group, $P < 0.05$. CP=control-perfused; EP=exercise-perfused; CIR=control ischemia-reperfusion; EIR= exercise ischemia-reperfusion.

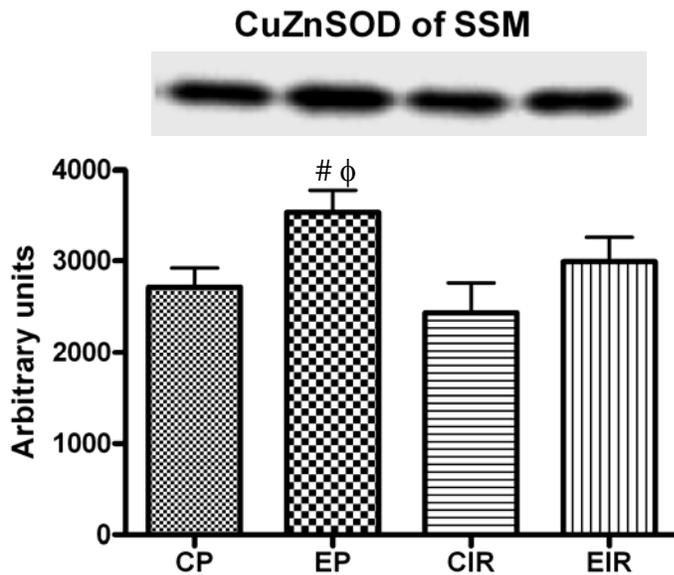


Figure 4-25. Protein levels of copper zinc superoxide dismutase (CuZnSOD) in SSM. Values are mean \pm SE. # Significantly different from CP group, $P < 0.05$. ϕ Significantly different from CIR group, $P < 0.05$. CP=control-perfused; EP=exercise-perfused; CIR=control ischemia-reperfusion; EIR= exercise ischemia-reperfusion.

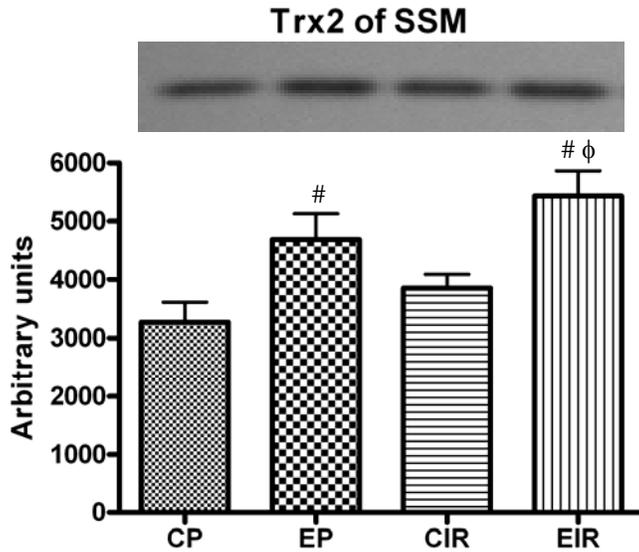


Figure 4-26. Protein levels of thioredoxin 2 (Trx2) in SSM. Values are mean \pm SE. # Significantly different from CP group, $P < 0.05$. ϕ Significantly different from CIR, $P < 0.05$. IFM=interfibrillar mitochondria; CK= creatine kinase; CP=control-perfused; EP=exercise-perfused; CIR=control ischemia-reperfusion; EIR= exercise ischemia-reperfusion.

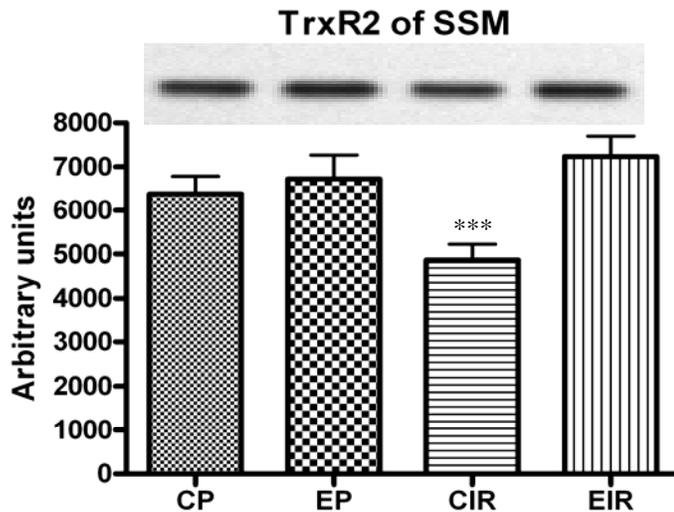


Figure 4-27. Protein levels of thioredoxin reductase 2 (TrxR2) in SSM. Values are mean \pm SE. *** Significantly different from CP, EP, and EIR groups, $P < 0.05$. CP=control-perfused; EP=exercise-perfused; CIR=control ischemia-reperfusion; EIR= exercise ischemia-reperfusion.

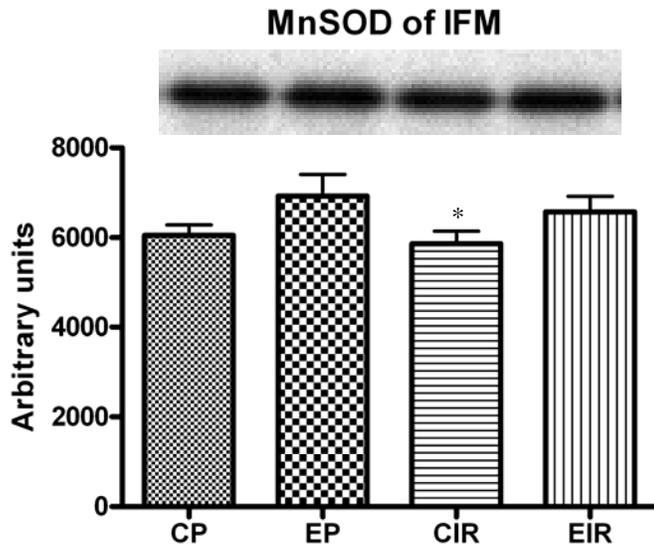


Figure 4-28. Protein levels of manganese superoxide dismutase (MnSOD) in IFM. Values are mean \pm SE. * Significantly different from EP group, < 0.05 . CP=control-perfused; EP=exercise-perfused; CIR=control ischemia-reperfusion; EIR= exercise ischemia-reperfusion.

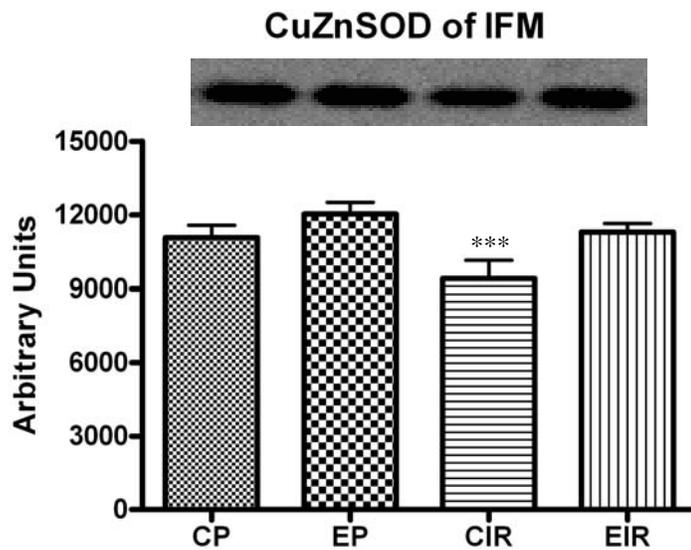


Figure 4-29. Protein levels of copper zinc superoxide dismutase (CuZnSOD) of IFM. Values are means \pm S.E.M. *** significantly different from CP, EP, and EIR groups, < 0.05 . CP=control-perfused; EP=exercise-perfused; CIR=control ischemia-reperfusion; EIR= exercise ischemia-reperfusion.

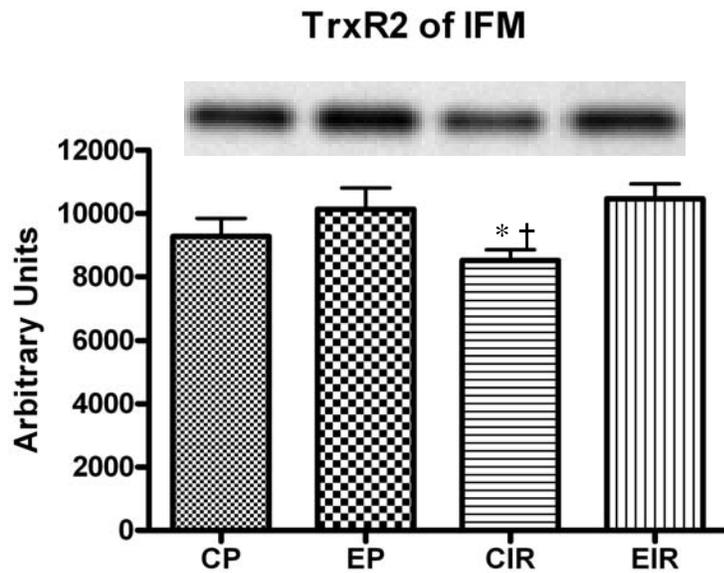


Figure 4-30. Protein levels of thioredoxin reductase 2 (TrxR2) in IFM. Values are mean \pm SE. * Significantly different from EP, $P < 0.05$. † Significantly different from EIR, $P < 0.05$. IFM=interfibrillar mitochondria; CK= creatine kinase; CP=control-perfused; EP=exercise-perfused; CIR=control ischemia-reperfusion; EIR= exercise ischemia-reperfusion.

CHAPTER 5 DISCUSSION

Overview of Principal Findings

This experiment investigated the ability of ExTr to protect the two populations of cardiac mitochondrial (SSM and IFM) against damage induced by an IR insult. Four major findings emerged from our study. First, ExTr protected both SSM and IFM against IR-induced respiratory dysfunction resulting from long duration global ischemia (40 minutes) followed by reperfusion (45 minutes). Second, ExTr prevented an IR-induced increase in mitochondrial ROS production in both SSM and IFM. Third, ExTr attenuated IR-induced oxidative damage to cardiac mitochondria. Finally, ExTr retarded the IR-induced release of proapoptotic proteins (cytochrome c and AIF) from cardiac mitochondria. Collectively, our results indicate that ExTr induces a mitochondrial phenotype that resists IR-induced damage; hence, ExTr-induced mitochondrial adaptation may be the key cellular alteration that explains exercise-induced cardioprotection. A detailed discussion of these and other related findings follows.

ExTr Provides Cardioprotection against an IR Insult

It is well established that regular ExTr provides myocardial protection against IR-induced arrhythmias, myocardial stunning, and cell death (infarction) (65-67, 84, 85, 118, 121, 123, 135, 136, 143). Interestingly, as few as 3-5 exercise sessions (i.e., 3-5 consecutive days) confers the same level of cardioprotection compared to chronic exercise training (i.e., weeks to months)(43). To confirm that our ExTr protocol was effective in providing cardioprotection, we evaluated cardiac mechanical performance before and after an *in vitro* IR insult in hearts from both control and ExTr animals. Our results indicate that, compared to hearts from untrained animals, ExTr improved post-ischemia recovery of coronary flow, cardiac output, and cardiac work. In addition, our findings indicate that ExTr improves post-ischemia recovery of left ventricular systolic

(+dp/dt) and diastolic function (-dp/dt). Collectively, these results substantiate that our ExTr protocol was successful in providing cardioprotection against an IR-insult.

Mitochondria are unique organelles that supply cellular energy and play a key role as the arbitrators of cellular life and death. Moreover, it is well established that IR-induced cardiac insult results in significant mitochondrial damage and respiratory dysfunction. Therefore, we reasoned that ExTr-induced mitochondrial adaptations could be a primary mechanism to explain why exercise training provides cardioprotection against IR injury. A detailed discussion of our findings related to exercise-induced mitochondrial protection during an IR insult follows.

ExTr Protects Mitochondrial Respiratory Function

Previous work indicates that ischemia alone or a combination of ischemia and reperfusion damages cardiac mitochondria (2, 32, 88). Specifically, it has been reported that cardiac ischemia damages the mitochondrial electron transport chain resulting in respiratory dysfunction due to a decrease in state 3 respiration and an increase in state 4 respiration (28, 32, 91, 92). As a result, mitochondria become uncoupled and the respiratory control ratio (RCR = state 3/state 4 respiration) is decreased. Prior to the current study, only one published report has addressed the impact of ExTr on protection of mitochondria against anoxia-reoxygenation injury. The earlier study indicated that ExTr provides protection to a combined pool of cardiac SSM and IFM against an *in vitro* anoxia-reoxygenation insult (9). While the report suggests that mitochondrial adaptation occurs with exercise training, the *in vitro* anoxia-reoxygenation protocol used in that study does not mimic the *in vivo* physiology of an IR insult that occurs within an intact heart. Indeed, many key elements that contribute to IR-induced mitochondrial damage are missing from the *in vitro* experimental model (e.g., cytosolic sources of ROS production, cytosolic calcium overload, etc.). Therefore, the current study significantly advances our knowledge in this

area by investigating the ability of exercise training to provide protection to both subpopulations of mitochondria in an intact heart following an IR insult.

Importantly, our results show that an IR insult applied to hearts of untrained (CIR) animals results in diminished mitochondrial state 3 respiration and an increased state 4 respiration when both complex I (pyruvate/malate) and complex II substrates (succinate) are used. In contrast, compared to CIR animals, ExTr provided significant protection against IR-mediated mitochondrial damage as indicated by a higher RCR in both SS and IFM. Collectively, these findings indicate that ExTr promotes a mitochondrial phenotype that resists IR-induced respiratory dysfunction.

ExTr Retards IR-induced ROS Production

It is well established that a myocardial IR insult promotes mitochondrial damage resulting in an increased production of ROS (31, 32, 140). In our experiments, the rate of mitochondrial ROS production was assessed using a novel experimental model that mimics *in vivo* mitochondrial function. This model, using a creatine clamp of respiration, permits the measurement of ROS production in respiring mitochondria at a fixed submaximal rate (i.e., ~60%) of state 3 respiration. In this way, this *in vitro* creatine clamp model mimics the *in vivo* respiratory function of mitochondria in the heart. Indeed, considering the fact that cardiac mitochondria are never exposed to state 2 respiration (without ADP) in normal physiology, the findings from the present study provide the first physiological assessment of cardiac mitochondrial ROS production following an IR insult.

Our findings reveal that ExTr significantly reduces IR-induced mitochondrial ROS production. More specifically, our results provide novel evidence that ExTr decreases ROS production in both SSM and IFM following IR, which indicates that ExTr protects both

subpopulations of mitochondria against IR-mediated damage. Similarly, Starnes et al. (130) reported that ExTr reduces mitochondrial H₂O₂ production during *in vitro* apoptotic challenges.

In theory, ROS can be produced in mitochondria at nine different sites (5). Nonetheless, most studies suggest that complex I and complex III are the major sites of electron leaks leading to the univalent reduction of oxygen and the production of superoxide (31, 33, 138). With regard to the mechanisms responsible for the IR-induced increase in mitochondrial ROS production, recent studies suggest that cytochrome c leakages from the mitochondria along with oxidative damage to complex I and III lead to this elevated ROS production (29, 32, 88). Our findings reveal that ExTr prevents IR-induced cytochrome c release from the mitochondria. This observation can explain, at least in part, why ExTr reduces the IR-induced increase in mitochondrial ROS production.

ExTr Attenuates IR-induced Oxidative Damage to Mitochondria

As mentioned earlier, ROS generated during an IR insult are implicated in mitochondrial dysfunction and damage. Using protein carbonyl formation as a marker of protein oxidation, our findings show that IR increases protein oxidation in SSM of sedentary animals. These results may be explained by the fact that SSM and IFM respond differently to an *in vitro* oxidative challenge as SSM mitochondria appear to be more susceptible to damage (76).

4-HNE (a reactive aldehyde) and lipid hydroperoxides are major by-products of ROS-mediated lipid peroxidation that occurs in cardiac mitochondria following IR (98, 140). Our results indicate that 4-HNE increases in mitochondria following an IR insult. Moreover, ExTr attenuates the increase in 4-HNE levels in both SSM and IFM. Although our results do not provide a definitive explanation for these results, our data suggest that two possible interacting mechanisms could contribute to the protection of cardiac mitochondria against IR-induced oxidative damage. First, the ExTr-induced mitochondrial protection against oxidative injury

could be mediated, at least in part, by ExTr-induced improvement in mitochondrial antioxidant capacity. For example, compared to cardiac mitochondria from the CIR group, both SSM and IMF from EIR animals contained a higher protein abundance of several key antioxidants (i.e., MnSOD, CuZnSOD, TrxR2, etc.). Further, our findings reveal that ExTr retards the IR-induced increase in mitochondrial ROS production. Hence, it is feasible that the mechanism to explain ExTr-induced mitochondrial protection against IR-induced oxidative damage is via a combination of increased mitochondrial antioxidants and a reduction in IR-induced mitochondrial ROS production.

ExTr Reduces the Release of Proapoptotic Proteins

IR-induced mitochondrial dysfunction and damage are closely linked to myocardial injury (2, 32, 46, 53-55, 61, 70, 77, 90, 91, 105). Studies indicate that mitochondria-mediated apoptosis is associated with IR-induced myocardial injury. Specifically, cytochrome c (2, 32) and/or AIF (77) release from mitochondria into the cytosol are known to be a critical initiating step of the apoptotic processes during IR. Recent studies report that ExTr attenuates myocardial apoptosis induced by an IR insult (121, 123). These studies show that ExTr decreases caspase-3 activity and the number of TUNEL positive nuclei. At present, it is unknown why ExTr prevents IR-induced caspase-3 activation and apoptosis in the heart. In this regard, our findings show that ExTr prevents cytochrome c and AIF release from the mitochondria following IR. These findings are novel and indicate that ExTr prevents a critical step in the initiation of both mitochondria-mediated caspase-dependent (i.e., cytochrome c) and independent (i.e., AIF) apoptosis. Furthermore, considering the fact that AIF is activated by mitochondrial calpain due to mitochondrial calcium overload (7, 48, 112), our findings are consistent with the notion that ExTr reduces mitochondrial calcium overload during IR. Indeed, this postulate is supported by the observation that cardiac mPTP of exercise-trained animal is more resistant to exogenous

calcium overload challenge (76). Taken together, our findings provide new evidence that regular bouts of ExTr prevent a critical step in initiating mitochondria-mediated apoptosis by inhibiting the release of key proapoptotic proteins during IR.

Potential Mechanisms Responsible for ExTr-induced Mitochondrial Protection

At present, the molecular mechanisms responsible for ExTr-induced mitochondrial protection against IR injury remain unknown. Given that IR-induced oxidative damages to mitochondria contribute to myocardial injury (30-33, 87, 91), and that ExTr improves antioxidant capacity (66, 76), we postulated that an ExTr-induced improvement in mitochondrial antioxidant capacity could protect mitochondria (both SSM and IFM) against an IR insult. Our results are consistent with this notion as ExTr increased the protein abundance of MnSOD, CuZnSOD and Trx2 in SSM. Moreover, ExTr prevented IR-induced TrxR2 degradation in SSM. Although ExTr did not significantly increase antioxidants within IFM of the EP group, the protein abundance of MnSOD, CuZnSOD, and TrxR2 was significantly higher in IFM from the EIR group compared to CIR. One interpretation of this finding is that an IR insult activates mitochondrial proteases that degrade antioxidants. Although it is unclear if IR-induced protease activation plays a key role in degrading antioxidant enzymes, numerous studies indicate that mitochondria contain endogenous proteases (i.e., mitochondrial calpain (7, 48, 112); HtrA2/Omi (134), etc.) that are activated by increased mitochondrial calcium levels and upon activation degrade oxidized proteins. If this is the case, activation of these proteases may explain our findings that, compared to EIR, lower levels of IFM antioxidants exist within the CIR group.

Importantly, our results reveal that ExTr promotes an increase in both Trx2 and TrxR2 within SS mitochondria. This novel finding is significant because an increase in these proteins would increase the ability of mitochondria to remove H₂O₂. Specifically, thioredoxins are a class of small 12-kDa proteins present in all eukaryotic cells. Although thioredoxins are known for a

variety of biological activities, thioredoxins play an important role in scavenging ROS and therefore can provide cellular protection against oxidative stress. In particular, Trx2 is a mitochondrial thioredoxin that provides reducing equivalents to the H₂O₂ scavenging antioxidant enzyme, peroxiredoxin3 (23, 35). Although TrxR2 is not directly involved in scavenging oxidants, this key molecule plays a critical role in the peroxiredoxin/Trx2 antioxidant couple by recycling reducing equivalents to Trx2. Hence, TrxR2 and Trx2 work as a unit to maintain peroxiredoxin3's H₂O₂ scavenging capacity. In summary, our collective findings indicate that ExTr-induced improvement of mitochondrial antioxidative capacity may be involved in ExTr-induced mitochondrial protection (both SSM and IFM) against IR-induced oxidative injury.

Conclusions and Future Directions

These experiments provide the first direct evidence that ExTr supplies protection to cardiac mitochondria against IR-induced damage. Specifically, our findings reveal that ExTr protects both SSM and IFM against IR-induced injury and respiratory dysfunction. We interpret these results as proof that mitochondrial adaptation is a key cellular event that contributes to exercise-induced cardioprotection.

Although our experiments do not provide a definitive explanation for the mechanism(s) responsible for ExTr-induced protection of mitochondria, our data suggest that two key possibilities exist: 1) ExTr prevents the IR-induced increase in mitochondrial ROS production; and 2) ExTr promotes an increase in mitochondrial antioxidants. Collectively, these ExTr induced changes in mitochondrial phenotype result in protection against IR-induced oxidative damage/respiratory dysfunction along with a retardation of the release of proapoptotic proteins.

Future experiments are required to define the definitive mechanism(s) responsible for ExTr-induced mitochondrial protection. These experiments could employ several strategies including siRNA to retard exercise-mediated expression of selected antioxidant proteins or

transgenic animals to define the role that one or more specific proteins play in mitochondrial protection against an IR injury. Delineating the mechanism(s) responsible for mitochondrial protection during IR could lead to the development of pharmacological or molecular interventions to protect the heart against IR injury. This is an exciting area for future research.

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BIOGRAPHICAL SKETCH

Youngil Lee was born in Seoul, Korea. He attained a Bachelor of Science degree from Korean National Sport University-Seoul. After graduation, he pursued a master's degree in exercise biochemistry and graduated from Korean National University-Seoul in 1997. After finishing the master's degree, he became a lecturer at Kyung-Gi University and taught several classes for two years. Deciding to advance his scientific knowledge, Youngil pursued his second master's degree in exercise physiology under the instruction of Dr. Roger P. Farrar and graduated from the University of Texas at Austin in 2002. Deciding to focus his career in basic science, Youngil began his doctoral work at the University of Florida in 2003 under the instruction of Dr. Scott K. Powers. Youngil focused his studies on the mechanisms responsible for exercise-induced cardioprotection against ischemia-reperfusion injury. He received his Ph.D. in 2008.