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By

Joel P. French
To my family: Asimina, Janet, John and Jena—their unwavering love and support have enabled every major accomplishment in my life.
ACKNOWLEDGMENTS

This work would not have been possible without the guidance and support of several important people. First, I would like to express my appreciation to my mentor and committee chairp, Dr. Scott Powers. You have taught me everything I know about research, grant and manuscript writing, teaching and presenting. Through example, you have also instilled in me a drive to succeed. I believe that you have given me all of the skills I need to be successful in this field and I hope that my future accomplishments will reflect the extremely high quality of your mentoring. I would also like to thank my doctoral committee, Dr. Stephen Dodd, Dr. David Criswell and Dr. Nihal Tumer for their patience and expertise throughout this project.

Importantly, thank you to Dr. John Quindry for his guidance and friendship. I appreciate everything that you taught me and more importantly, your ability to make the lab such an enjoyable environment to work in.

Additionally, thanks to everyone in the lab who contributed to this project: Dr. Karyn Hamilton, Patrick Upchurch, Dr. Jessica Staib and Darin Falk. Thanks also to the rest of the team in the lab: Youngil Lee, Joe McClung, Zsolt Murlasits, Melissa Deering, Keith Deruisseau, and Darin Van Gammeren.

Finally, and most importantly I would like to thank my family; Mina, Janet, John and Jena. Without your love and support I never could have come this far. I love you all.
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MECHANISMS OF PROTECTION AGAINST MYOCARDIAL ISCHEMIA-REPERFUSION INJURY

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December 2006

Chair: Scott K. Powers
Major: Health and Human Performance

Myocardial ischemia-reperfusion (IR) is characterized by an increase in reactive oxygen species (ROS) generation as well as increased free cytosolic Ca\(^{2+}\) (Ca\(^{2+}\)-overload) resulting in myocardial contractile dysfunction and injury.

We have previously demonstrated that both exercise training and inhibition of the Ca\(^{2+}\)-activated protease calpain, protect the heart against IR injury. Additionally, we have shown that exercise regulates IR-induced calpain activation. However, the mechanisms involved in exercise-induced calpain regulation and calpain-mediated injury are not completely understood. We hypothesized that the oxidation and calpain-mediated degradation of critical Ca\(^{2+}\)-handling proteins was an important mechanism of IR injury and exercise-induced cardio-protection.

Therefore, we conducted two separate experiments to examine the relationships between IR, calpain activation, exercise training and the oxidation and degradation of myocardial Ca\(^{2+}\)-handling proteins.

Our first set of experiments looked at the effects of calpain inhibition on myocardial function, Ca\(^{2+}\)-handling protein oxidation and degradation following IR. We found that IR resulted in impaired LVDP, +dp/dt and –dp/dt and increased oxidative modification and
degradation of several Ca\textsuperscript{2+}-handling proteins (LTCC, SERCA2a, PLB, NCX). In addition, we found that pharmacological inhibition of calpain prevented contractile dysfunction as well as the degradation of these Ca\textsuperscript{2+}-handling proteins.

Our second set of experiments looked at the mechanisms of exercise-induced cardio-protection against IR injury. We found that short-term exercise training attenuated calpain activation as well as the oxidative modification and degradation of Ca\textsuperscript{2+}-handling proteins. In addition, when the exercise-induced over-expression of the endogenous antioxidant enzyme MnSOD was prevented, using an antisense oligonucleotide, the protective effects of exercise training were lost.

Therefore, we propose a series of events during IR, which are initiated by ROS-mediated oxidative modification of critical Ca\textsuperscript{2+}-handling proteins, resulting in increased free cytosolic Ca\textsuperscript{2+} and calpain activation. Once active, calpain can cleave Ca\textsuperscript{2+}-handling proteins, facilitating their degradation, exacerbating Ca\textsuperscript{2+}-overload, ROS generation and IR injury.

Exercise appears to provide protection against these events by over-expressing MnSOD, which attenuates IR-induced Ca\textsuperscript{2+}-handling protein oxidation, calpain activation and Ca\textsuperscript{2+}-handling protein degradation.
CHAPTER 1
INTRODUCTION

Rationale

Myocardial ischemia-reperfusion (IR) injury is a prevalent consequence of cardiovascular disease. Although there are many factors leading to myocardial dysfunction following IR, growing evidence suggests that increased reactive oxygen species (ROS) production and cytosolic free Ca\(^{2+}\)-overload, either independently or cooperatively, are major contributors to IR-induced injury (10, 80, 89).

ROS generated during IR can have several targets within the myocardium (including proteins, lipids, and DNA), impairing their function and/or promoting their degradation, leading to contractile dysfunction, cellular damage and cell death. Additionally, ROS can interfere with intracellular Ca\(^{2+}\) homeostasis, further exacerbating the deleterious effects of IR (9, 64).

Increases in myocardial cytosolic Ca\(^{2+}\) levels have been observed during both ischemia and reperfusion. In this regard, it has been hypothesized that one role of cytosolic Ca\(^{2+}\) in the pathogenesis of IR-induced myocardial injury is through the activation of the Ca\(^{2+}\)-dependent protease, calpain. Calpain exists in myocytes in two primary isoforms, micro (calpain I) and milli (calpain II), named for the respective amounts of Ca\(^{2+}\) required for their activation in vitro. Both calpain isoforms are activated by prolonged exposure to elevated cytosolic Ca\(^{2+}\) and it is well documented that calpain activation occurs in the heart during IR (55, 120, 122, 128). This is significant because calpain can injure cardiac myocytes via several different pathways. For example, calpains cleave several structural proteins leading to the release of myofilaments, facilitating their degradation by the proteosome. In addition, calpains may contribute to apoptosis, through cleavage of Bid, mediating cytochrome c release from the mitochondria. Also, calpains increase the expression of cell adhesion molecules, leading to an increase in
neutrophil-mediated oxidative damage. Each of these pathways has been shown to significantly contribute to IR-associated injury. Moreover, calpains’ deleterious role in IR injury is supported by strong evidence indicating that calpain inhibition significantly attenuates myocardial contractile dysfunction, cellular injury, and cell death (55, 120, 122, 128).

Recent evidence suggests an additional role for calpain as a link between the oxyradical and Ca\(^{2+}\)-overload theories of IR-induced myocardial injury. This research suggests that ROS may oxidatively modify Ca\(^{2+}\)-handling proteins, impairing their function and possibly leading to their cleavage by calpain (64, 126, 129, 130). Degradation and/or functional impairment of these proteins would lead to an increase in free cytosolic Ca\(^{2+}\), further exacerbating IR-induced calpain activation, ROS production and myocardial dysfunction (Figure 1-1). In support of this postulate, work from our laboratory has described an IR-induced increase in oxidative stress (protein carbonyls) as well as an increase in calpain-mediated cleavage of the Ca\(^{2+}\)-handling protein, SERCA2a. Indeed, this suggests that some Ca\(^{2+}\)-handling proteins are cleaved by calpain, and degraded during IR. Therefore, the first goal of these experiments was to determine the effects of IR on the oxidative modification and degradation of several key Ca\(^{2+}\)-handling proteins. The proteins studied were: the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA2a), phospholamban (PLB), the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) and L-type calcium channels (LCC). We also determined if those proteins/protein complexes, which were oxidatively modified, had a higher incidence of degradation. Additionally, we determined the effects of \textit{in-vitro} calpain inhibition on the degradation of Ca\(^{2+}\)-handling proteins and myocardial function following IR.

Endurance exercise training is an established means of inducing cardioprotection against IR-induced injury (71, 72, 93, 96, 113, 114). Although the mechanisms of exercise-induced
cardioprotection are unknown, work from our laboratory indicates that exercise may provide
cardioprotection against IR injury, at least in part, through the regulation of calpain (30). This
work reveals that exercise training completely attenuated the IR-induced increase in calpain
activation and was associated with improved myocardial contractile function following IR.
Additionally, exercise trained hearts showed an increase in the endogenous antioxidant MnSOD
and a decrease in oxidative stress (protein carbonyl formation). We hypothesize that endurance
exercise may regulate calpain activation through an up-regulation of endogenous antioxidants,
such as Mn-SOD.

Superoxide production is believed to be a major source of oxidative stress during IR.
Mitochondrial produced superoxide is thought to play an important role in myocardial IR injury
due to the aerobic nature of the heart. Superoxide production has been shown to dramatically
increase following ischemia and the use of superoxide scavengers has been shown to improve
contractile function following IR. Because of the detrimental impact of superoxide generation
during IR, we hypothesize that an exercise-induced increase in MnSOD may provide protection
against oxidative modification of key cellular proteins, including critical Ca\textsuperscript{2+}-handling proteins
associated with both the SR and plasma membrane. Reducing oxidative damage to calcium
handling proteins may result in improved Ca\textsuperscript{2+}-handling and reduced calpain activation during IR
and, therefore reduce myocardial dysfunction and injury. Therefore, the second goal of these
experiments was to investigate the effects of exercise training on oxidative modification and
degradation of Ca\textsuperscript{2+}-handling proteins following IR. Additionally, we determined if the exercise-
induced reduction in calpain activity and preservation of Ca\textsuperscript{2+}-handling proteins during IR was
dependant on the exercise-induced over-expression of MnSOD in the heart.
Specific Aims

The goal of these experiments was to address two separate but related hypotheses involving myocardial IR injury, oxidative stress, and calpain activation.

Hypothesis one: IR will result in increased oxidative modification (carbonyl and HNE formation) and/or calpain-mediated degradation of myocardial calcium handling proteins. Further, calpain inhibition will attenuate the IR-induced degradation of Ca$^{2+}$-handling proteins. This hypothesis will be tested by achieving the following specific aims.

Aim (A): To determine if IR results in increased carbonyl and/or HNE formation to Ca$^{2+}$-handling proteins within the heart.

Aim (B): To ascertain if oxidative modification to calcium handling proteins is associated with an increase in the degradation of these proteins.

Aim (C): To discern if calpain inhibition attenuates the degradation of key Ca$^{2+}$-handling proteins in the heart following IR.

Hypothesis two: Exercise training will provide protection against IR-induced oxidative modification (carbonyl and HNE formation) and degradation of myocardial Ca$^{2+}$-handling proteins via an increase in the endogenous antioxidant, MnSOD. This hypothesis will be rigorously tested by achieving the following specific aims.

Aim (A): To determine if exercise training attenuates IR-induced carbonyl and/or HNE formation on key Ca$^{2+}$-handling proteins and/or degradation of these proteins within the heart.

Aim (B): To ascertain if exercise-induced protection against IR-induced oxidative modification and degradation of Ca$^{2+}$-handling proteins is dependent on an increase in myocardial MnSOD.
Figure 1-1. Proposed mechanisms underlying IR-induced calpain activation and myocardial dysfunction.
CHAPTER 2
REVIEW OF RELATED LITERATURE

Introduction

Coronary heart disease (CHD) is the number one cause of death for both men and women in the United States, as well as most other industrialized nations. In 2002, CHD was responsible for 927,448 deaths in the U.S., roughly one in every three deaths (34.2%). Additionally, health care costs related to CHD in 2005 were estimated at $393.5 billion within the U.S. alone (6). Because CHD typically results in periods of myocardial ischemia, often leading to terminal infarction, understanding the mechanisms of myocardial IR injury as well as possible mechanisms of protection against IR injury is important in the treatment and management of patients with CHD.

Many factors can contribute to IR-induced myocardial IR injury. The first goal of this review will be to provide an overview of IR-induced cellular injury and to discuss the mechanisms responsible for IR-induced cellular injury. Although several factors contribute to IR injury, this review will focus primarily on the two dominant theories of myocardial IR injury, the oxyradical theory and the Ca\textsuperscript{2+} overload theory, as well as possible interaction between the two.

The second goal of this review will be to discuss calpain and its role in IR injury. Increases in both free Ca\textsuperscript{2+} and ROS have been shown to increase the cellular activity of the Ca\textsuperscript{2+}-activated protease calpain (8, 12, 19). Further, calpain activation has been shown to play a deleterious role in the heart. Calpain may play a critical role in IR injury by linking the theories of Ca\textsuperscript{2+} overload and ROS production. Calpain may cleave Ca\textsuperscript{2+}-handling proteins, which have been oxidatively modified by ROS, leading to a further increase in the levels of free Ca\textsuperscript{2+} and active calpain, exacerbating myocardial damage and dysfunction.
The third goal of this review will be to describe exercise-induced cardioprotection and the role of the antioxidant, manganese superoxide dismutase (MnSOD), as a potential mediator of exercise-induced cardioprotection. Endurance exercise training has been shown to provide protection against IR-induced elevations in calpain activity, myocardial injury and contractile dysfunction, however, the precise mechanisms of this cardio-protection have not been elucidated (95, 96). One possible explanation is that exercise provides cardioprotection through an increase in the endogenous antioxidant MnSOD. Increasing MnSOD protein and/or activity would result in a reduction in IR-induced ROS damage. This could lead to a reduction in the oxidative modification of Ca^{2+}-handling proteins, as well as their degradation by calpain, reducing not only ROS-related injury but disturbances in Ca^{2+} homeostasis and calpain-related pathology as well.

**Myocardial Ischemia-Reperfusion Injury: Characteristics and Mechanisms**

Myocardial ischemia is defined as the reduction or cessation of blood flow to myocardial tissue, below the metabolic requirements of that tissue. In addition to being a clinical manifestation of coronary artery disease, the latest clinical treatments for this disease (i.e. coronary bypass surgery, balloon angioplasty) subject the heart to episodes of ischemia and subsequent restoration of blood flow (reperfusion). Following a period of ischemia and reperfusion, the heart can undergo temporary or permanent injury, depending on the duration of the ischemia (10). Brief periods of ischemia, less than 5 minutes, will generally result in arrhythmias. However this length of ischemia is not associated with any long-term loss in heart function or cell death. A period of ischemia lasting approximately 5-20 minutes results in ventricular contractile dysfunction and is referred to as “myocardial stunning.” This ischemic duration will typically result in temporary contractile function without causing any permanent damage (necrosis or apoptosis) to the heart (10). The most severe form of IR injury, “myocardial
infarction”, occurs when the duration of ischemia exceeds 20 minutes. Infarction results in loss of contractile function as well as cellular death, through pathways of both necrosis and apoptosis (7).

There are many mechanistic factors that may contribute to myocardial dysfunction and cellular death (7). These factors include, but are not limited to: ROS production, Ca^{2+}-overload, increased proteolytic activity, and platelet / inflammatory cell infiltration. Although there are many factors leading to myocardial dysfunction and cell death following IR, growing evidence suggests that increased free cytosolic Ca^{2+} and/or ROS production, either independently or cooperatively, are two of the major contributors to IR-induced injury (9). A brief discussion of the roles of both ROS and free cytosolic Ca^{2+} in IR-induced injury follows.

**The Oxyradical Hypothesis**

The oxyradical hypothesis of IR injury was first proposed in 1985 when it was postulated that the generation of reactive oxygen and free radical species such as the superoxide anion (O_2^•⁻), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^•) during reperfusion contributed to myocardial injury (86). Reactive oxygen species (ROS) are derived from the reduction of molecular oxygen (78). Some ROS (such as O_2^•⁻ and OH^•) are known as free radicals because they contain one or more unpaired electrons in their outer most orbital, making them highly unstable and reactive (42). For example, when an O_2 molecule accepts a single free electron, the product is superoxide (O_2^•⁻). When a second free electron is accepted, hydrogen peroxide (H_2O_2) is formed. In the presence of an iron (ferrous) salt, the O-O bond can be broken resulting in the formation of two hydroxyl radicals (OH^•). Importantly, the OH^• produced is one of the most highly reactive free radicals, capable of reacting with almost every component of the cell (76). Free radicals produced via these pathways have been implicated in damage to several structures.
within the cell, including cellular proteins, lipids, and DNA (104). Supporting the deleterious role of ROS, ischemia-reperfusion experiments using iron chelators and/or various antioxidants (free radical scavengers) have demonstrated a reduction in free radical generation and protection against IR-induced injury (4, 9, 28, 86, 106).

**ROS related damage**

Damage caused by free radicals has been identified during both periods of ischemia and reperfusion in the heart, although it is currently believed that the majority of the radicals are produced during the first few minutes of reperfusion (4, 11, 13, 28, 57, 74, 119). Once generated, free radicals have several targets within the cell, which will be discussed in the following sections.

**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 (41, 43, 49, 100, 101). Often, the damage caused by free radical interaction is irreparable. In addition, many proteins, which are oxidatively modified, become more susceptible to proteolytic cleavage by calpain and degradation by the proteosome. One example of oxidative modification to cellular proteins is the formation of carbonyl groups. This carbonyl formation can then be measured, providing an indirect indication of oxidative stress within the cell (15).

**Myocardial lipid peroxidation.** Polyunsaturated fatty acids are highly susceptible to free radical modification at their unsaturated sites (3). Once ROS extract electrons, or hydrogen atoms, from the methylene groups of fatty acids, a chain reaction is initiated where one “modified” fatty acid chain reacts with a neighboring chain, and so on. 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. There are examples of lipid peroxidation by-products which can be measured in order to indirectly quantify oxidative stress within the cell (43). One such by-product is the formation of the reactive aldehyde, HNE (4-hydroxy-2-nonenal). The w-6-family (linoleic and arachidonic acids) of polyunsaturated fatty acids produce HNE as a result of free radical attack. HNE is a highly reactive compound and it can react with several functional groups on biological material, particularly sulphydryl groups, to form thioester adduct and then hemiacetals. HNE may also react with histidine and lysine residues of proteins to form stable Michael addition-type of adducts. In addition, HNE-modification of proteins may impair biological functions.

**DNA damage.** ROS have also been reported to damage DNA, preventing the translation and transcription of new cellular proteins by stimulating the degradation of DNA and oligonucleosomal fragments (50). More specifically, ROS can cause permanent or transient damage to nucleic acids within the cells, leading to such events as DNA strand breakage and disruption of Ca\(^{2+}\) metabolism. Additionally, a high rate of oxidative damage to mammalian DNA has been demonstrated by measuring oxidized DNA bases excreted in urine following DNA repair. Further, the rate of oxidative DNA damage has been found to be directly related to metabolic rate and inversely related to life span.

**Sources of myocardial ROS**

There are several potential sources of free radical production in the heart during ischemia-reperfusion. 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 (9, 17, 19, 27). Nonetheless, a large volume of evidence implicates mitochondrial production of radicals as the primary source of oxidants during both ischemia and reperfusion. Therefore, a detailed discussion of mitochondrial ROS production follows.
Mitochondrial ROS production during IR. Again, the mitochondrial respiratory chain has been identified as one of the largest contributors of IR-induced free radical production (9). Mitochondrial oxidative phosphorylation is the major ATP synthetic pathway in eukaryotes. During this process, electrons liberated from reducing substrates are delivered to oxygen via a chain of respiratory H⁺ pumps. These pumps (complexes I-IV) establish a H⁺ gradient across the inner mitochondrial membrane. The electrochemical energy of this gradient is then used to drive ATP synthesis by complex V (ATP synthase). During this process, it has been estimated that approximately 1-2% of the oxygen present is reduced to form some sort of ROS or free radical (36). The primary radical made by the mitochondria is superoxide (O₂⁻). It is believed that the majority of the superoxide generated originates from electrons “leaked” from ubisemiquinone, located at complex III. Once generated, superoxide can be converted to a less reactive oxygen species, hydrogen peroxide (H₂O₂) by the antioxidant enzyme superoxide dismutase (SOD), or converted to a more reactive hydroxyl (OH⁻) radical, in the presence of iron.

Confirming the importance of mitochondrial free radical production, studies using antioxidants targeted specifically to the mitochondria have demonstrated a significant reduction in oxidant-related damage within the myocardium, as well as improved myocardial function (4, 11, 28, 86).

Antioxidant defenses against IR-induced ROS production

Fortunately, the cell has several antioxidant defense mechanisms against the increase in free radicals typically seen during IR. An antioxidant has been defined as “any substance that significantly delays or prevents the oxidation of that substrate” (42). The cell contains a variety of enzymatic and non-enzymatic antioxidants, located in various strategic locations and specifically targeted to different ROS.
**Superoxide dismutase (SOD).** Superoxide dismutase (SOD) was discovered in 1969 by McCord and Fridovich (81). SOD is an enzyme that catalyzes the reduction of superoxide to hydrogen peroxide, thus forming a less reactive, non-radical species.

\[ \text{O}_2^{*-} + \text{O}_2^{*-} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \]

SOD exists in three isoforms: manganese superoxide dismutase (MnSOD), copper-zinc superoxide dismutase (CuZnSOD) and extracellular superoxide dismutases (ECSOD). Although each isoform catalyzes the reaction of superoxide to hydrogen peroxide, they each reside in different locations within the cell. MnSOD is located exclusively within the mitochondria, CuZnSOD is found predominantly within the cytosol and, as its name implies, ECSOD is found in extracellular fluids such as plasma, as well as in the extracellular matrix of tissues. Because superoxide generation has been identified as a major contributing factor to IR injury, the regulation of SOD plays a critical role in the heart.

**Catalase (CAT).** The enzyme catalase (CAT) catalyzes the breakdown of hydrogen peroxide to water and oxygen.

\[ \text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \]

Catalase is found predominantly within the peroxisome along with several other enzymes, which can generate hydrogen peroxide such as urate oxidase, glycolate oxidase, and flavenoid dehydrogenases, involved in beta-oxidation of fatty acids (81).

**Glutathione peroxidase (GPx).** The antioxidant enzyme glutathione peroxidase (GPx) also catalyzes the breakdown hydrogen peroxide to two molecules of water. However, this reaction depends on the concomitant oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG).
H₂O₂ + 2GSH → GSSG + 2H₂O

Because of the dependant relationship between the oxidation of glutathione and the reduction of H₂O₂, the measurement of the ratio of GSH to GSSG is often used to assess the amount of hydrogen peroxide production, as well as GPx activity within the cell. Both GPx and CAT work in tandem to remove hydrogen peroxide from the cell. CAT has a much higher Kₘ than GPx, responding very quickly to increases in hydrogen peroxide. GPx has a much lower Kₘ but is more ubiquitous, breaking down the majority of hydrogen peroxide generated from the mitochondria and sarcoplasmic reticulum (81).

Glutathione (GSH). Glutathione (GSH) is an intracellular thiol-containing tripeptide, which is produced inside cells. Importantly, since very little CAT resides within the mitochondria, GSH, in conjunction with Gpx, is the major means of hydrogen peroxide breakdown (82). The importance of GSH to the mitochondria is illustrated by the fact that when cytosolic GSH levels begin to fall, the mitochondria reduce GSH release in order to conserve their own reserves.

Other Antioxidants. The cell contains several other non-enzymatic antioxidants that contribute to maintaining redox balance. Many of these non-enzymatic antioxidants are consumed in the diet, such as vitamin E, vitamin C, lipoic acid, carotenoids, and flavenoids to name a few. In addition other endogenous antioxidants such as heat shock proteins and ubiquinones also play a role in maintaining cellular redox balance. Because of the extensive number of antioxidants, complexity of their function, as well as relevance to these experiments, these antioxidants will not be discussed in this proposal.
The Calcium-Overload Hypothesis

The Ca$^{2+}$ overload hypothesis predicts that myocardial IR injury results from a disturbance in cellular Ca$^{2+}$ homeostasis. Intracellular cytosolic Ca$^{2+}$ levels are typically maintained at a low level (approximately 0.1 μM) while total cellular Ca$^{2+}$ is considerably higher. Much of the cellular Ca$^{2+}$ is stored within the sarcoplasmic reticulum (SR) and the mitochondria. Numerous studies have demonstrated a dramatic increase in cytosolic Ca$^{2+}$ levels during ischemia (60, 68, 78, 110). This elevation in free Ca$^{2+}$ persists during the early stages of reperfusion, finally returning to normal levels during late reperfusion (65, 78). The Ca$^{2+}$-overload hypothesis was first described in detail by Grinwald (37), who proposed the following mechanisms to attempt to explain this increase in free Ca$^{2+}$, or Ca$^{2+}$-overload. During ischemia, intracellular sodium accumulates due to energy depletion, and Na+/Ca$^{2+}$ exchange is inhibited by the concomitant acidosis. Upon reperfusion, the rapid reversal of acidosis reactivates Na$^+$/Ca$^{2+}$ exchange at a time when sodium overload has not yet been resolved, driving Ca$^{2+}$ into the cells. The damaging effects of the Ca$^{2+}$-overload were later documented by Kusuoka (66) who discovered that hearts which were reperfused with a low Ca$^{2+}$ solution showed a marked decrease in IR-induced injury. In fact, a transient Ca$^{2+}$-overload, even in the absence of ischemia, has been shown to cause myocardial dysfunction and injury (63).

Increased free Ca$^{2+}$ can contribute to the pathology of the heart cell through several mechanistic pathways. In fact, since the early work of Grinwald and Kusuoka (38, 67), the calcium hypothesis has evolved to incorporate several distinct mechanisms, which attempt to explain the means through which Ca$^{2+}$ may lead to myocardial dysfunction and/or injury. The proposed mechanisms include: increased ROS production, excitation-contraction (E-C) uncoupling due to decreased Ca$^{2+}$ responsiveness and increased protease activity (12).
Calcium induced ROS production

Several researchers have suggested that IR-induced increases in free Ca$^{2+}$ may lead to increased ROS generation. For example, Seno et al. found that Ca$^{2+}$ stimulated increased radical production from NADPH oxidase (103). Further, Gottlieb et al. proposed a model through which Ca$^{2+}$ could increase mitochondrial ROS production via several pathways (36). First, Ca$^{2+}$ stimulates KREBS cycle activation which results in increased electron flow into the respiratory chain and therefore, increased “leaking” of electrons from the respiratory complexes to oxygen, forming the superoxide radical. In addition, Ca$^{2+}$ stimulates nitric oxide production, which can inhibit electron flow into the mitochondria through complex IV as well as complex I, resulting in increased ROS production.

E-C uncoupling and decreased calcium sensitivity

It is well accepted that IR results in a decrease in myocardial contractile function. One possible explanation is a decrease in E-C coupling. Early work by Kusuoka et al. described a decline in maximal Ca$^{2+}$-activated force production in the heart following IR (65). Because electrical activation was not impaired in the heart following IR (47), the explanation for the IR-induced reduction in E-C uncoupling must lie in either of two mechanisms: a reduction in Ca$^{2+}$ availability within the cell or a decrease in calcium responsiveness of the contractile machinery. As discussed earlier, several groups have shown an increase in intracellular Ca$^{2+}$ following IR (38, 67). Therefore, a reduction in calcium availability is not a likely explanation for the observed decrease in E-C coupling. This leaves the possibility of a reduced Ca$^{2+}$ sensitivity of the contractile machinery within the cell.

The idea that myocardial Ca$^{2+}$ sensitivity is reduced following IR originated from the observation that although contractile function was significantly impaired following IR, Ca$^{2+}$ levels were actually elevated. Since Ca$^{2+}$ stimulates muscular contraction, it was postulated that
the Ca\(^{2+}\) sensitivity of the contractile proteins must be reduced. Although several studies have confirmed these findings, the exact mechanisms responsible for the IR-induced reduction in Ca\(^{2+}\) responsiveness are not completely understood. Most of the work completed to date has implicated the structural modification of one or more of the myofibrillar proteins (65). Studies using skinned fibers have suggested that IR results in modification of the myofilaments (51). This may be an additional point of interaction between the Ca\(^{2+}\) overload and oxyradical theories because ROS have been shown to modify cellular proteins, impairing their function. Among their many possible targets, radicals may modify myofibrillar proteins, by oxidizing thiol groups, resulting in impaired Ca\(^{2+}\) responsiveness (150, 92). Using immunohistochemistry, Matsumura observed degradation of the myofilament-associated scaffolding protein α-actin following IR (79). In addition, Gao documented a decrease in the thin-filament regulatory protein troponin-I following IR (32, 33). Further, Gao prevented the degradation of troponin-I by altering the reperfusate in such a way to mitigate Ca\(^{2+}\)-overload in the heart following ischemia. These observations are particularly important given the crucial role of troponin-I as an intermediary between Ca\(^{2+}\) activation and cross-bridge cycling. This degradation of troponin-I may explain much of the depression in myocardial contractile function following IR. This idea becomes even more pertinent to the experiments proposed in this manuscript when considering the fact that troponin-I is also cleaved by the Ca\(^{2+}\)-activated protease calpain.

**Calcium-activated proteases**

**Cathepsins.** The cathepsins are a group of lysosomal proteases, which are found in innate immune cells such as neutrophils and macrophages. Therefore these lysosomal enzymes are frequently found in areas of inflammation and injury. For example, increased levels of cathepsin B and D are frequently observed in patients with heart disease and other chronic
inflammatory conditions (84). Although cathepsins clearly play a role in chronic inflammatory conditions, their contribution to acute IR injury is unlikely to be important given the fact that reperfusion times of almost two hours are required for significant neutrophil infiltration into myocardial cells.

**Calpain.** Increases in free Ca\(^{2+}\) during IR can lead to increased activation of Ca\(^{2+}\)-activated cysteine proteases, such as calpain. Calpain exists in myocytes in two primary isoforms, micro (calpain I) and milli (calpain II), named for the respective amounts of Ca\(^{2+}\) required for their activation *in vitro*. Both calpain isoforms are heterodimers made up of a large (80 kDa) and small (28-30 kDa) regulatory subunit (35). Calpain has several Ca\(^{2+}\) binding domains similar to calmodulin. Ca\(^{2+}\) binding causes a shift in the structure of the protein, exposing a site for interaction with various substrates (35). Although the two calpain isoforms are named for Ca\(^{2+}\) concentrations needed for activation *in vitro* there is evidence that calpain II can be activated by far less than millimolar Ca\(^{2+}\) concentrations *in vivo*, increasing its relevance to myocardial IR injury (35). Regardless, once activated, calpain migrates in the cytosol toward the SR and/or plasma membrane where the majority of its substrates are located. These substrates include structural and contractile proteins as well as Ca\(^{2+}\) handling proteins, to name a few. The exact number of calpain-targeted proteins is currently unknown, however, a recent review by Goll et al. (35) reported over 100 different proteins that serve as calpain substrates. Note that this review only discussed cytoskeletal proteins, kinases and phosphateses, just a few categories of potential calpain substrates (35). Hence, it is likely that calpain cleaves many more than 100 proteins in cells.

Although both calpain I and II have similar substrates, there is some evidence to suggest slightly different roles for the two isoforms *in vivo*. For example, work in skeletal and cardiac
tissues have documented an initial increase in both calpain I and II activities in response to various modes of tissue injury followed by a later, second increase in calpain I activity. This evidence has led some to believe that while both isoforms clearly contribute to cellular injury in response to various stressors (IR, hypoxemia, hydrogen peroxide, calcium overload) calpain I may also play a more “productive” role in the removal of damaged proteins, etc. following injury. In addition, long-term inhibition of both isoforms typically results in the death of the animal suggesting that calpain plays a role in normal homeostatic cellular pathways. However, in the current review, the primary interest in calpain is directed toward its contributing role in cellular injury following a stressor, such as IR.

**Calpain and IR Injury**

The IR-induced increase in calpain activation has been well documented in the heart (9, 19, 35, 55, 115, 122, 128). This increase in calpain activity has long been known to play a deleterious role in myocardial IR-induced injury. Once activated through binding with calcium, calpain can injure cardiac myocytes via several different pathways. Calpain cleaves several structural proteins leading to the release of myofilaments, facilitating their degradation by the proteosome (35, 83, 99, 124, 125). Moreover, calpains may contribute to apoptosis, through cleavage of Bid, mediating cytochrome c release from the mitochondria (18, 19, 35). Also, calpains increase the expression of cell adhesion molecules, leading to an increase in neutrophil-mediated oxidative damage (91, 108). Each of these pathways has been shown to significantly contribute to IR-associated injury. In support of this postulate, several studies have demonstrated cardioprotection through the use of calpain inhibitors prior to IR (55, 120, 122, 128). Recent work from our laboratory supports these findings, demonstrating almost complete cardioprotection against IR-induced contractile function and injury using the calpain inhibitor MDL-28170 (30). These results provide physiological support to the notion that calpain
activation plays a significant role in IR-induced myocardial injury. In fact, calpain may play a unique role in linking the two predominant mechanistic theories of myocardial IR injury, the Ca\(^{2+}\)-overload hypothesis and the oxyradical hypothesis.

**Calpain: Linking the Oxyradical and Calcium-Overload Theories**

Although several independent theories have been proposed in order to explain myocardial injury and dysfunction following IR, the pathogenesis of IR most likely involves a complex interaction between the oxyradical and Ca\(^{2+}\)-overload hypotheses. Several mechanisms have been proposed linking the two theories. One possibility is that free radicals generated during IR could interact with various SR proteins, causing SR dysfunction and damage (58, 61, 64, 102). The Impairment in SR function would likely result in increased free cytosolic Ca\(^{2+}\), and calpain activation leading to further cellular damage. Supporting this postulate, cell culture studies administering antioxidants prior to reoxygenation have observed a significant attenuation in Ca\(^{2+}\)-overload (85). In addition studies using both *in vitro* working heart and langendorf IR models have described an attenuation in IR-induced calpain activation through the use of various antioxidants (107). Because calpain is activated by Ca\(^{2+}\) this indirectly indicates a reduction in IR-induced Ca\(^{2+}\)-overload (35). When also taking into consideration the possibility that oxidative modification of SR proteins by free radicals may increase the likelihood of their cleavage by calpain, it appears that calpain may play a critical role in IR-induced injury, linking the oxyradical and Ca\(^{2+}\)-overload theories.

**Calpain and Calcium-Handling Proteins.**

**Regulation of Free Cytosolic Calcium**

The bulk of the Ca\(^{2+}\) released within the cell comes from calsequestrin-bound Ca\(^{2+}\) stores within the sarcoplasmic reticulum (SR). Ca\(^{2+}\) is normally released by the process of Ca\(^{2+}\)-induced Ca\(^{2+}\) release in which the entry of a small amount of Ca\(^{2+}\) across the plasma membrane
(sarcolemma) triggers the release of much more from the SR. This mechanism depends on the fact that Ca^{2+} entry from the extracellular fluid, via the dihydropyridine receptor (DHPR) and L-type Ca^{2+} channels, and to a lesser extent the sodium-calcium exchanger (NCX), increases the probability that the SR Ca^{2+} release channel (ryanodine receptor, RyR) is open. The greater the probability that the RyR is open the greater the release of Ca^{2+} from the SR. Once cytosolic calcium levels are elevated, the calcium must either be moved back into the SR, or removed from the cell completely via the plasma membrane.

There are two primary means of calcium removal from the cytosol: the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA), and the sodium-calcium transporter (NCX). The SERCA is an ATP-dependant pump located in the SR, which removes calcium from the cytosol, returning to the SR where it is stored bound to the protein calsequestrin for later release. SERCA activity is regulated by the protein phospholamban (PLB), as well as ATP levels (77). Calcium can also be removed via the plasma membrane by the NCX. The NCX couples the transport of three Na^+ molecules to one Ca^{2+} molecule in the opposite direction in two consecutive steps. Together, these proteins/protein complexes play a critical role in the regulation of free cytosolic Ca^{2+} levels and therefore, calpain regulation, during IR. Importantly, all of these proteins/protein complexes are targets for oxidative modification by ROS during IR as well (64).

**Oxidative Modification and Degradation of Calcium-Handling Proteins**

There is a significant body of work detailing the oxidative modification of Ca^{2+}-handling proteins within the cell (64). Following is a very brief review of pertinent studies, which deal with oxidative stress and the Ca^{2+}-handling proteins mentioned earlier.
SERCA and PLB

SERCA, and its regulatory protein PLB, play a critical role in the removal of Ca^{2+} from the cytosol, returning it to the SR. Any impairment in function could lead to elevations in free cytosolic Ca^{2+}. Importantly, Favero et al. (29) found that hydrogen peroxide inhibited SERCA activity within the myocardium. In addition, Grover et al. (39) studied the interaction of ROS with smooth muscle SERCA and found that hydrogen peroxide-induced damage to SERCA diminished the SR Ca^{2+} pool as well as the smooth muscle response to Angiotensin II. Grover also reported similar results using superoxide (39). In addition, Suzuki and Ford (111) reported that ROS induced concentration-dependant inhibition of SERCA. Collectively, these studies indicate that ROS can impair SERCA function and impair Ca^{2+} uptake into the SR.

NCX

The NCX is the primary means of removing Ca^{2+} from the cell via the plasma membrane. A reduction in NCX function could elevate levels of free cytosolic calcium. There is evidence suggesting that this exchanger is a tetramer linked by disulfide bonds, and therefore, is susceptible to modification by ROS (16, 59, 90). Supporting this theory, Coetzee et al. (21) reported NCX inhibition in guinea pig cardiac myocytes following a hypoxanthine / xanthine oxidase treatment (i.e., superoxide generating system). Kato et al. (59) also observed similar results in isolated SR vesicles from bovine hearts. In addition, DiPolo and Beauge (26) proposed that NCX inhibition is due to a ROS-induced reduction in the calcium sensitivity of the exchanger. Hence, similar to ROS damage to SERCA, it also appears that ROS can modify and damage membrane Ca^{2+} transport as well.

L-type calcium channels and the DHPR

Several groups have documented a decrease in L-type Ca^{2+} channel current by ROS. For example, Tokube et al. (118) found that hypoxanthine / xanthine oxidase treatments, as well as
hydrogen peroxide treatments inhibited the L-type Ca\textsuperscript{2+} channel current in cardiac myocytes. Further, this inhibition was prevented by SOD. Guerra et al. (40) observed almost identical findings, using dihydroxyfumaric acid (DHF). In addition, Coeteez et al. (22) described a decrease in the peak Ca\textsuperscript{2+} current of L-type Ca\textsuperscript{2+} channels following hypoxanthine / xanthine oxidase treatments.

**Summary: Calpain and Calcium-Handling Proteins**

There is recent evidence suggesting that calpain may cleave key SR Ca\textsuperscript{2+}-handling proteins during IR (64). Degradation of these proteins would lead to a disruption in Ca\textsuperscript{2+} transport within the cell, further exacerbating the IR-induced increase in free cytosolic Ca\textsuperscript{2+}. Importantly, there is reason to believe that Ca\textsuperscript{2+}-handling proteins within the SR may become targets for calpain cleavage after they are oxidatively modified by ROS. This may provide a very important link between the two predominant mechanistic theories of IR injury, the Ca\textsuperscript{2+}-overload theory and the oxyradical theory. Additionally, this would present an interesting scenario in which calpain can could regulate its own activation in a feed-forward mechanism. Therefore, the first goal of this these experiments will be determine the relationships between IR-induced ROS production, the oxidative modification of Ca\textsuperscript{2+}-handling proteins, calpain activation, and the calpain-mediated cleavage of Ca\textsuperscript{2+}-handling proteins.

**Antioxidants and SR Dysfunction**

Because of the possible link between the oxidation of Ca\textsuperscript{2+}-handling proteins and their subsequent degradation by calpain, alterations in the antioxidant status within the cell may play a critical role in reducing calpain activation, Ca\textsuperscript{2+}-overload, and the associated deleterious effects within the myocardium. As discussed earlier, the addition of antioxidants has been shown to significantly reduce Ca\textsuperscript{2+}-overload in cardiac myocyte cultures and the intact heart following IR (58, 61, 64, 102). Additionally, antioxidant treatments can also preserve Ca\textsuperscript{2+}-handling protein
function in cells following oxidative stress. However, which antioxidant(s) are most effective in providing protection to Ca^{2+}-handling proteins in vivo are currently unknown. Nonetheless, an antioxidant that is thought to play a critical role in protecting the myocardium against IR injury is MnSOD (45, 93, 127). As discussed earlier, MnSOD may play a very important role in protecting the heart against ROS because of its location within the mitochondria, a major source of myocardial radical production. It is possible that an increase in myocardial MnSOD activity may reduce the oxidative modification of critical SR and plasma membrane Ca^{2+}-handling proteins, maintaining their function and reducing the likelihood of their degradation by calpain. This would in turn serve to attenuate any further IR-induced increase in free cytosolic calcium and calpain activation. One well established model of increasing MnSOD protein content and enzyme activity is endurance exercise training (55, 120, 122, 128).

**Exercise-Induced Cardio-Protection Against IR Injury**

Regular bouts of muscular exercise (e.g., 60 minutes of endurance exercise) is a well-established means of inducing cardio-protection against IR-induced injury (71, 72, 93, 113, 114). Work from numerous laboratories has consistently demonstrated exercise-induced cardio-protection against IR insults of varying severities, ranging from minor injury to infarction. Additionally, work from our laboratory has determined that three days of exercise training provides the same degree of cardio-protection as long-term (weeks) training (14, 25, 44, 93). Although there is little debate concerning the protective effects of exercise training, the mechanisms through which it provides cardio-protection are not completely understood. Several potential mechanisms to explain exercise-induced cardio-protection exist, including increases in myocardial heat shock proteins (HSP’s), increased antioxidant capacity, and reduced calpain activation. The following sections will address each of these possibilities in greater detail.
Increased Myocardial Heat Shock Proteins

Heat shock proteins are a multifunctional group of proteins, which are stimulated by a variety of stimuli such as heat, oxidative stress, Ca\(^{2+}\)-overload, exercise training, and low pH. Once active, these proteins serve several functions within the cell, including: chaperoning and/or translocating proteins, folding and refolding proteins, scavenging free radicals, and even facilitating protein synthesis. However, although the cardio-protective properties of various HSPs have been demonstrated, the importance of HSPs to exercise-induced cardio-protection is somewhat controversial. For example, work from our laboratory has suggested that an elevation in HSPs is not essential for exercise induced cardio-protection (46). In these studies, hearts from animals, which were exercise trained in a cold environment (4°C), were compared to the hearts from animals that were trained at room temperature. Hearts from the cold-trained animals demonstrated a similar level of cardio-protection against IR injury compared to the warm-trained animals, even without the exercise-induced increase in HSP protein content. These results have been confirmed by other groups as well (114). Therefore, it appears that an elevation in myocardial HSPs is not essential for exercise-induced cardio-protection. Another possible mechanism of exercise-induced cardio-protection is through an up-regulation of endogenous antioxidants.

Increased Myocardial Antioxidant Capacity

As discussed earlier, the cell contains several antioxidant defenses against IR-induced ROS production. The primary antioxidant defenses are thought to include GSH, GPX, CAT, and SOD. Although there is an abundance of research demonstrating the cytoprotective effects of these antioxidants during IR, the question of which antioxidants may play a critical role in exercise-induced cardio-protection is yet unanswered. Importantly, protein levels and activities of only a few antioxidant enzymes have been shown to increase consistently following exercise
training (95, 96). For example, GSH content has been shown to be elevated following long-term (8-10 weeks) exercise training ((52, 53, 56, 98) whereas short-term training has been found to increase GSH protein content (75), decrease it (69), or result in no change (105). Therefore it can be concluded that long-term exercise training elevates GSH but the conflicting evidence in the literature does not permit a firm conclusion about the impact of short-term training on cardiac levels of GSH. Moreover, most studies have concluded that exercise training does not elevate GPX levels in the heart (25, 92, 94). The effect of exercise on CAT activity is also somewhat unclear with some studies reporting increases and others reporting no change following training (25, 44, 46, 109). In contrast, it is widely agreed that exercise elevates myocardial MnSOD protein content and activity (25, 44, 45).

**Increased MnSOD activity.** Growing evidence suggests that endurance exercise may provide protection, at least in part, by up-regulating the endogenous antioxidant MnSOD. As discussed earlier, MnSOD may play an important cardio-protective role in the heart during IR due to its localization in the mitochondria and ability to prevent oxidative stress induced by mitochondrial superoxide production. Several studies have documented the protective effects of MnSOD. For example, Chen et al. (20) demonstrated that MnSOD over-expression reduced infarct size following IR injury. Further, Abunasra et al. (1) observed cytoprotection against IR injury using adenoviral gene transfer of MnSOD. In addition, recent studies using a MnSOD mimetic, which was directed almost exclusively into the mitochondria, observed that hearts from animals which were given the mimetic showed a significant improvement in myocardial function and a reduction in myocardial injury following IR (2).

**Exercise-Induced Regulation of Calpain Activation**

Recent work from our laboratory has revealed an exercise-induced decrease in calpain activation following IR (30, 97). This work also demonstrated a decrease in calpain-mediated
cleavage and degradation of the Ca\textsuperscript{2+}-handling protein SERCA2a (30). This suggests that exercise may provide cardio-protection, at least in part, through the regulation of IR-induced calpain activation. However, the mechanisms of exercise-induced calpain regulation are not currently known. One possibility is that exercise-induced increases in MnSOD may reduce oxidative modification to Ca\textsuperscript{2+}-handling proteins, attenuating the IR-induced increase in free Ca\textsuperscript{2+} and calpain activation. Therefore, the second goal of these experiments will be to determine the relationships between MnSOD, oxidative modification of Ca\textsuperscript{2+}-handling proteins, calpain activation, and calpain-mediated degradation of Ca\textsuperscript{2+}-handling proteins.

**Summary**

Myocardial IR injury is a complex problem involving both the generation of free radical species (the oxyradical theory), as well as increases in free cytosolic Ca\textsuperscript{2+} (the Ca\textsuperscript{2+}-overload theory), resulting in loss of myocardial function, damage and degradation of cellular proteins and lipids, and cell death. Increased activation of the Ca\textsuperscript{2+}-dependant protease, calpain during IR may provide an important link between these two theories by preferentially cleaving Ca\textsuperscript{2+}-handling proteins which have been modified by free radicals, thus exacerbating the problem of Ca\textsuperscript{2+}-overload and calpain-mediated injury within the myocardium.

It is believed that much of the ROS production during IR originates from the mitochondrial respiratory chain. The antioxidant MnSOD is localized in the mitochondria and can reduce the generation of the free radical superoxide. Additionally, exercise training has been consistently shown to provide cardio-protection against myocardial IR injury and is also associated with an increase in MnSOD and a decrease in calpain activation. This increase in MnSOD may reduce the oxidative modification of calcium handling proteins thus reducing calpain activation and maintaining Ca\textsuperscript{2+} homeostasis.
CHAPTER 3
METHODS

The methods segment of this proposal will be divided into five sections with the first providing details about the experimental animals, the second outlining the experimental designs, the third and fourth detailing the general methods and dependant measurements used in these experiments and the fifth describing statistical analyses.

Experimental Animals

Animal Model Justification

Adult (3–5 month old) male Sprague-Dawley (SD) rats were used for these experiments. The animals were 3–5 months of age (young adult) at the time of sacrifice. The SD rat was chosen for several reasons: first, the invasive nature of these experiments precludes the use of human subjects. Second, the SD model is a well accepted model for the study of myocardial ischemia reperfusion injury (14, 56, 70, 88, 92). Third, the SD rat does not display large inter-animal variation in measures of cardiac contractility and/or collateral circulation. In addition, we chose to study male rats to avoid the possibly confounding effects of varying estrogen levels across the estrus cycle (116).

Animal Housing and Diet

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

Experimental Design

Experimental Design: Hypothesis One

Animals were randomly assigned to one of five experimental groups (Figure 3-1). The Control group (hearts were quickly removed from anesthetized animals, i.e. no in-vitro...
perfusion) served as a control for all dependant measures. A Control-perfused group was also included to control for any dependant variable changes that result from perfusion on the isolated working heart preparation. To investigate the effects of IR, three experimental groups (Control-IR, Calpain-Inhibited-IR, and Vehicle-IR) were subjected to an in-vitro IR stress (i.e., no flow ischemia, followed by reperfusion). At the conclusion of each experiment, segments of the left ventricle were rapidly frozen in liquid nitrogen and stored at -80°C until assay. Samples were subsequently assayed to determine the levels of selected biochemical dependent measures.

Figure 3-1. Experimental design for Hypothesis One.

**Experimental Design: Hypothesis Two**

Animals were randomly assigned to one of six experimental groups. Four groups were exercise trained, as detailed below, while the other two groups remained sedentary (Figure 3-2). To elucidate the role that MnSOD plays in exercise-induced cardio-protection, one exercise trained experimental group received an antisense oligonucleotide against MnSOD following each exercise training session. Importantly, our experience with this antisense oligonucleotide is that this treatment consistently attenuates the exercise-induced increase in myocardial MnSOD.
activity associated with exercise training without reducing MnSOD activity and/or protein levels below those of control animals (45, 73). In addition, an oligonucleotide mismatch group of trained animals was included to account for any extraneous effects of the oligonucleotide.

All experimental groups underwent either a sham surgery or an *in vivo* IR surgery, also described below. The Control-Sham group served as a control for all dependant measures. At the conclusion of each experiment, segments of the left ventricle were assayed to assess the levels of numerous biochemical dependant measures.

![Experimental design for Hypothesis Two.](image)

**General Methods**

**The Isolated Working Heart Preparation/IR Protocol (Hypothesis One)**

To investigate myocardial 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 performance, as cardiac preload and after-load pressures are maintained constant. Further, an
advantage of the 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 (30, 71-73, 92, 97).

**In Vitro Ischemia-Reperfusion Protocol (Hypothesis One)**

Simultaneous clamping of aortic and atrial lines was used to induce global, normothermic, no flow ischemia. Following 30 minutes of perfusion during the pre-ischemic protocol, ischemia was maintained for 25 minutes followed by 45 minutes of reperfusion. During ischemia, the heart was enclosed in a sealed, water-jacketed chamber maintained at 37°C. Following the ischemic period, the heart was switched to the retrograde perfusion mode for 10 minutes followed by 10 minutes of assist mode (retrograde perfused with the atrial cannula open) and 25 minutes of normal reperfusion. Upon the conclusion of non-perfusion, perfusion, or IR treatments, the left ventricular free wall was immediately sectioned into four strips cut from base to apex. Prior to storage, heart sections were rinsed in a cold antioxidant buffer (50mM NaHPO4, 0.1mM butylated hydroxytoluene, and 0.1mM EDTA). These tissue sections were then rapidly frozen in liquid nitrogen, and stored at -80°C until subsequent biochemical analysis.

**Cardiac Performance Measurements (Hypothesis One)**

Cardiac performance measurements were recorded every 5 minutes prior to ischemia and during reperfusion. Measurements included: 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. Data was recorded and stored using a customized computer data-acquisition system.
Calpain Inhibition (Hypothesis One)

To determine the effects of IR-induced calpain activation on oxidation and degradation of myocardial calcium handling proteins, calpain was inhibited (in vitro) using the selective inhibitor MDL 28170, also known as “calpain inhibitor three” or (CI3) (EMD Biosciences, La Jolla, CA). The efficacy of CI3 as a selective inhibition of calpain I and II has been well established (18, 19, 30, 122, 125). The inhibitor was dissolved in Dimethyl Sulfoxide (DMSO) and added to the perfusion buffer prior to heart perfusion at a concentration of 10μM. In preliminary experiments, this concentration of CI3 was shown to inhibit calpain I and II without inhibiting the proteosome.

Exercise Training Protocol (Both Hypotheses)

Exercise trained animals began by performing 5 consecutive days of gradual habituation to treadmill running. Treadmill habituation initiated with 10 minutes of training the first day and was increased by 10 minutes each day, ending in 50 minutes of running on the fifth day. Following habituation, exercise trained animals performed 3 consecutive days of treadmill running (60 minutes/day) at an intensity of approximately 60-70% of VO₂max.

In Vivo Ischemia-Reperfusion Protocol (Hypothesis Two)

The in vivo model of coronary artery ligation has been used successfully by our laboratory for over 12 years. In our hands, ligation of the left main coronary artery (close to its origin) using this in vivo preparation consistently results in ischemia in 60% of the ventricular free wall. Rats were anesthetized (80 mg/kg sodium pentobarbital i.p.) and ventilated (Harvard Apparatus, Holliston, MA) with room air via a tracheostomy tube. A saline-filled catheter attached to a pressure transducer was placed in the carotid artery and interfaced with a computerized heart performance analyzer for continuous monitoring of arterial blood pressures (Digi-Med, Louisville, KY). Arterial blood (<100 μl) samples were obtained prior to ischemia to assess
blood gases and pH using a calibrated electronic blood gas analyzer (Instrumentation Laboratories, Lexington, MA). When indicated, the tidal volume delivered by the ventilator was adjusted to correct blood gas abnormalities. An additional catheter was placed in the jugular vein for delivery of sodium pentobarbital (20 mg·kg−1) as needed. Following a left thoracotomy, a ligature was placed around the left anterior descending coronary artery (LCA), close to its origin. In sham surgery, the hearts were removed without occlusion of the LCA. For the IR surgery, a soft piece of polyethylene tubing was threaded through the ligature, pressed on the surface of the LCA, and secured with a small hemostat. Coronary occlusion was maintained for 50 min followed by 120 min of reperfusion. Electrocardiographic activity was continuously monitored and recorded via an interfaced customized data acquisition program with data points recorded every millisecond. Following reperfusion, hearts were removed, rinsed in a cold antioxidant buffer (50 mM NaHPO4, 0.1 mM BHT, 0.1 mM diethylenetriaminepentaacetic acid, pH 7.4), and quickly frozen in liquid nitrogen for later analysis.

**Inhibition of MnSOD Protein Translation (Hypothesis Two)**

Antisense oligonucleotides (AS-ODN) are single-stranded synthetic DNA that typically contain a backbone with modification to a specific sequence to hybridize to a specific messenger RNA. Hybridization of the ODN to mRNA inhibits the mRNA from initiating translation. To block the translation of MnSOD protein, animals were injected (i.p.) with a 22-mer phosphorothioate derivative of the AS-ODN (5’-CACGCCGCCGACACAAACATTG-3’) immediately post-exercise at a dose of 10 mg/kg. The injection time and dose of this specific AS-ODN have been shown to provide optimal experimental conditions to inhibit the exercise-induced increase of MnSOD activity in myocardial tissue (45, 73). This has been confirmed by previous experiments from our laboratory. In addition, a mismatch control group (MM-ODN (CAC TCC TCC CAG CAC AAC AGTC)) was included in these experiments to verify that the
inhibition of MnSOD translation was due to the specific AS-ODN sequence, as well as accounting for any extraneous factors resulting from the delivery of the ODN.

**Dependant Measures (Both Hypotheses)**

**Measurement of Calpain Activation**

To assess calpain activity, calpain-specific cleavage product of the protein αII-spectrin was analyzed. Briefly, proteins were separated using standard SDS-PAGE techniques on a 4%-20% polyacrylamide gel. Proteins were then transferred to nitrocellulose membranes and exposed to a mouse monoclonal primary antibody to αII-spectrin (SIGMA, St. Louise, MO). Following washing, an anti-mouse IgG-HRP-conjugated secondary antibody was applied for chemiluminescence detection (Amersham, Piscataway, NJ). Both αII-spectrin intact and calpain-cleaved fragments were analyzed using a Kodak imaging system. The cleaved band was expressed as a percentage of the intact band and finally expressed as a percentage of the Perfused Control group.

**Western Blots for Calcium-Handling Proteins**

Western blots were used to determine protein levels of the following Ca\(^{2+}\)-handling proteins: SERCA2a, phospholamban, L-type Ca\(^{2+}\) channels and the Na\(^+\)/Ca\(^{2+}\) exchanger. These measurements were used to determine which Ca\(^{2+}\)-handling proteins were degraded following IR, as well which proteins were degraded specifically by calpain during IR (Hypothesis one). Briefly, proteins were separated using standard SDS-PAGE techniques on 4%-20% polyacrylamide gels. Proteins were then transferred to Polyvinylidene Difluoride (PVDF) membranes and exposed to a monoclonal primary antibody. Following primary antibody exposure, an anti-mouse, or anti-rabbit 800 (green) or 680 (red) infrared secondary antibody (Li-Cor, Lincoln, Nebraska) was applied for infrared detection. Each blot was then analyzed using an Odyssey infrared imaging system (Li-Cor, Lincoln, Nebraska) and normalized to a comassie
blue protein stain in order to adjust for protein loading. Results were then expressed as a percent of either Control-Perfused (Hypothesis one), or Control-Sham (Hypothesis two).

**Immunoprecipitation of Calcium-Handling Proteins**

To determine the effects of exercise, IR and calpain inhibition on the oxidation of Ca\(^{2+}\)-handling proteins, each of the following proteins (SERCA2a, phospholamban, L-type Ca\(^{2+}\) channels and the Na\(^{+}\)/Ca\(^{2+}\) exchanger) were first isolated by immunoprecipitation. Briefly, heart tissue was homogenized at a 1:10 dilution factor in a 100mM KPO\(_4\) buffer containing 1 μm lactacystine, 1 μm MG-132 (SIGMA, St. Louise, MO), pH 7.4. The homogenate was then centrifuged at 1000 g for 20 minutes to clear cellular debris. Approximately 1000 μg of protein was then transferred to a new tube and exposed to 10 μl of primary antibody to the protein of interest. Following an overnight incubation on a Fisher rocker, 40 μl of protein A/G PLUS-agrose (Santa Cruz Biotechnology, Santa Cruz, CA) were added and incubated overnight. Four centrifugations (2500 rpm for 10 minutes) were then used to separate the agrose-bound antibody/protein complex. Following each spin, the pelleted complex was suspended in 118 μl of KPO\(_4\) buffer. Finally, the Bradford protein assay was run to determine final protein concentration and the samples were normalized to approximately 2 mg of protein / ml.

**Measurement of Protein Carbonyl Formation on Calcium-Handling Proteins**

Protein carbonyls are formed by a variety of oxidative mechanisms and are sensitive indices of oxidative injury (15). Proteins, isolated via immunoprecipitation, were examined for carbonyl formation using a commercially available Western Blot kit from Chemicon International (Chemicon International, Temecula, CA). This allowed for the determination of the level of oxidative modification to specific calcium-handling proteins.
Measurement of HNE Formation on Calcium-Handling Proteins

HNE (4-hydroxy-2-nonenal) formation is a major product of endogenous lipid peroxidation. The w-6-family (linoleic and arachidonic acids) of polyunsaturated fatty acids produce HNE as a result of free radical-induced lipid peroxidation. HNE is a highly reactive aldehyde and can react with several functional groups on biological material, particularly sulfydryl groups to form thioester adduct and then hemiacetals. HNE may also react with histidine and lysine residues of proteins to form stable Michael addition-type of adducts. In addition, HNE-modification of proteins may impair biological functions. Proteins, isolated via immunoprecipitation, were examined for HNE formation using a commercially available Western Blot kit from Calbiochem (SanDiego, Ca). This allowed for the determination of the level of oxidative modification to specific Ca\(^{2+}\) handling proteins.

Data Analysis

To test our hypotheses, one-way ANOVA’s were performed to assess IR, calpain inhibition, and exercise training differences for the primary dependent measures. A Tukey post hoc test was used to determine group differences when indicated. Significance was established \textit{a priori} at \(P < 0.05\). The relationship between the oxidative modification (carbonyl and HNE formation) of Ca\(^{2+}\)-handling proteins and the degradation of Ca\(^{2+}\)-handling proteins was assessed using a Pearson’s simple correlation.
CHAPTER 4
RESULTS

Hypothesis One

Animal Characteristics

The physical characteristics for the animals in all experimental groups are presented in Table 4-1. Although body mass differed between the experimental groups, heart weights and heart-to-body weight ratios were similar.

Table 4-1. Animal body and heart weights.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Body Weight (g)</th>
<th>Heart Weight (g)</th>
<th>Heart / Body Weight Ratio (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-perfused Control</td>
<td>11</td>
<td>360 ± 4.2 ♦</td>
<td>1.20 ± .02</td>
<td>3.34 ± .07</td>
</tr>
<tr>
<td>Non-perfused Trained</td>
<td>9</td>
<td>339 ± 5.8 •♦</td>
<td>1.21 ± .03</td>
<td>3.57 ± .10</td>
</tr>
<tr>
<td>Perfused Control</td>
<td>11</td>
<td>354 ± 4.4 •♦</td>
<td>1.16 ± .03</td>
<td>3.28 ± .09</td>
</tr>
<tr>
<td>Control-IR</td>
<td>11</td>
<td>347 ± 8.4 •♦</td>
<td>1.22 ± .03</td>
<td>3.53 ± .11</td>
</tr>
<tr>
<td>Trained-IR</td>
<td>12</td>
<td>322 ± 4.2 ♦</td>
<td>1.17 ± .02</td>
<td>3.65 ± .08</td>
</tr>
<tr>
<td>Calpain inhibited-IR</td>
<td>6</td>
<td>374 ± 7.9 •↑</td>
<td>1.28 ± .08</td>
<td>3.41 ± .20</td>
</tr>
<tr>
<td>Vehicle-IR</td>
<td>6</td>
<td>401 ± 8.0 •↑</td>
<td>1.25 ± .04</td>
<td>3.13 ± .06</td>
</tr>
</tbody>
</table>

Values are means ± SE. • Significantly different from Trained-IR, ♦ Significantly different from Vehicle-IR, * P < 0.05. Note that Non-perfused Trained and vehicle-IR groups had significantly different body weights and heart / body weight ratios compared to Non-perfused Controls, although heart weight did not differ.

Figure 4-1. % Recovery of left ventricular developed pressure (LVDP). Values are means ± SE.
* Significantly different from Control-IR, P < 0.05. Note that “inhibition” denotes the inhibition of calpain using the inhibitor CI3.
Cardiac Performance Measures

Percent recovery of left ventricular developed pressure (LVDP)

LVDP is commonly used as an index of myocardial function. By comparing LVDP prior to ischemia with post-ischemic LVDP we can quantify myocardial dysfunction. As expected, % recovery of LVDP was significantly depressed in the Control-IR group (Figure 4-1). However, both exercise training and calpain inhibition attenuated the loss of LVDP following IR. This demonstrates the cardio-protective effects of exercise as well as the deleterious effects of the Ca\(^{2+}\)-activated protease calpain.

Figure 4-2. Rate of systolic pressure increase (+dp/dt). Values are means ± SE. • Significantly different from Control-IR, \(P < 0.05\). Note that “inhibition” denotes the inhibition of calpain using the inhibitor CI3 and “vehicle” denotes vehicle treatment only without CI3.

Percent of pre-ischemic +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-sequestering 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 prior to and following ischemia, we can quantify the changes in myocardial contraction/relaxation rates and/or Ca\(^{2+}\)-handling kinetics.

As illustrated in Figures 4-2 and 4-3, both +dp/dt and –dp/dt decreased significantly following IR in both the control-IR and vehicle-IR experimental groups, suggesting a decrease in Ca\(^{2+}\)-handling efficiency. However, both exercise training and calpain inhibition nearly completely prevent this IR-induced dysfunction.

![Figure 4-3. Rate of systolic pressure decline (-dp/dt). Values are means ± SE. Significantly different from Control-IR, \(P < 0.05\). Note that “inhibition” denotes the inhibition of calpain using the inhibitor CI3 and “vehicle” denotes vehicle treatment only without CI3.](image)

**Oxidative Modification of Critical Calcium-Handling Proteins**

The increase in free cytosolic Ca\(^{2+}\) and corresponding increase in calpain activity have been shown to play a deleterious role in cardiac myocytes following IR. Because the oxidative modification of Ca\(^{2+}\)-handling proteins has been shown to lead to impaired Ca\(^{2+}\)-handling, we postulated that the oxidation of Ca\(^{2+}\)-handling proteins within the cardiac myocytes may impair
function of the proteins and thus lead to increases in free cytosolic Ca\(^{2+}\), the activation of calpain, myocardial damage and contractile dysfunction.

![Figure 4-4. Carbonyl formation on critical Ca\(^{2+}\)-handling proteins: L-type calcium channel (LTCC), Sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA2a), Phospholamban (PLB) and Sodium/calcium exchanger (NCX). Representative blots are displayed above. Values, below, are means ± SE. • Significantly different from respective Control-Perfused group, \(P < 0.05\). Note that “inhibition” denotes the inhibition of calpain using the inhibitor CI3 and “vehicle” denotes vehicle treatment only without CI3.]

We assessed oxidative modification of calcium handling proteins by measuring both carbonyl and HNE formation via Western Blotting of each individual Ca\(^{2+}\)-handling protein. Protein carbonyl levels of proteins are indicative of the magnitude of oxidative modification of proteins whereas HNE-protein interaction is taken as an indication of protein reactions with the reactive aldehyde, HNE. The measurement of both carbonyl and HNE formation are commonly used to assess oxidative stress to proteins and lipids within the cell.
**Carbonyl formation on calcium-handling proteins**

IR resulted in an increase in carbonyl formation to all four Ca\(^{2+}\)-handling proteins measured (LTCC, SERCA2a, PLB, and NCX). Importantly, exercise training attenuated the IR-associated increase in carbonyl formation in all four proteins (Figure 4-4). This suggests that all four proteins are subject to oxidative modification following IR.

**HNE formation on calcium-handling proteins**

An increase in HNE formation on SERCA2a and PLB was also observed following IR. However, the oxidative modification of these proteins was attenuated by exercise training.
(Figures 4-5). Note that no significant changes in HNE formation were detected on the NCX, and there were not detectable levels of HNE formation on the LTCC.

![Western blotting for intact Ca$^{2+}$-handling proteins: L-type calcium channel (LTCC), Sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA2a), Phospholamban (PLB) and Sodium/calcium exchanger (NCX). Representative blots are displayed above. Values below are means ± SE. ••• Significantly different from respective Control-Perfused group, P < 0.05. Note that “inhibition” denotes the inhibition of calpain using the inhibitor CI3 and “vehicle” denotes vehicle treatment without CI3.](image)

**Calpain-Mediated Degradation of Calcium-Handling Proteins**

We have previously demonstrated that IR results in increased calpain activation. Once active, calpain degrades many proteins/protein complexes within the cell. We hypothesized that calpain may cleave critical Ca$^{2+}$-handling proteins, which have been oxidatively modified,
exacerbating IR-induced Ca\textsuperscript{2+}-overload, calpain activation and myocardial dysfunction. To test this hypothesis we performed Western Blot analyses to determine protein content of each of the four Ca\textsuperscript{2+}-handling proteins with and without calpain inhibition. Our results demonstrated a decrease in intact LTCC, SERCA2a, PLB, and NCX following IR in both control-IR and vehicle-IR groups. However, both exercise training and calpain inhibition prevented this IR-induced degradation in all four proteins.

Table 4-2. Correlations Between the Oxidative Modification of Ca\textsuperscript{2+}-handling Proteins and Their Degradation Following IR

<table>
<thead>
<tr>
<th>Carbonyl formation / Intact protein (R\textsuperscript{2})</th>
<th>HNE formation / Intact protein (R\textsuperscript{2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTCC -.901</td>
<td>-</td>
</tr>
<tr>
<td>SERCA2a -.939</td>
<td>-.939</td>
</tr>
<tr>
<td>PLB -.827</td>
<td>-.955</td>
</tr>
<tr>
<td>NCX -.511</td>
<td>-.263</td>
</tr>
</tbody>
</table>

Pearson correlation R2 values depicting the relationship between oxidative modification (carbonyl formation and HNE formation) to critical Ca\textsuperscript{2+}-handling proteins and the total amount of intact protein, determined by Western Blot. All experimental groups were pooled for this analysis.

Our results indicate that all four of the measured Ca\textsuperscript{2+}-handling proteins, LTCC, SERCA2a, PLB and NCX, are degraded by calpain during IR (Figure 4-6). In addition, exercise training attenuated the degradation of these proteins, most likely through a reduction in IR-induced calpain activation. Consistent with this postulate, a strong negative correlation exists between the oxidative modification (carbonyl and HNE formation) and amount of intact protein of each of the Ca\textsuperscript{2+}-handling proteins (Table 4-2). This may suggest that oxidative modification makes these proteins more susceptible to calpain-mediated degradation.

**Hypothesis Two**

**Calpain Activation (Calpain-Cleaved αII-Spectrin)**

The deleterious effects of IR-induced calpain activation have been well documented (55, 120, 122, 128). Additionally, we have previously demonstrated that exercise training attenuates
calpain activation and provides cardioprotection against IR injury (71, 72, 93, 96, 113, 114). The mechanism(s) of this protection, however, are currently unknown.

![Western blotting for calpain-cleaved αII-Spectrin](image)

**Figure 4-7.** Western blotting for calpain-cleaved αII-Spectrin (an in-vivo calpain substrate). A representative blot, above, displaying intact (250 kD) and calpain-cleaved αII-Spectrin (145 kD). Values below are means ± SE. • Significantly different from Control-Sham group, P < 0.05.

We hypothesized that exercise may provide protection through up-regulation of myocardial MnSOD resulting in protection against IR-induced oxidative stress to Ca²⁺-handling proteins, Ca²⁺-overload and calpain activation. Therefore, we performed Western Blotting for αII-Spectrin, a well-characterized in-vivo calpain substrate, in order to determine the effects of MnSOD on IR-induced calpain activation.

As expected, IR resulted in an increase in calpain activation in control (sedentary animals), which was attenuated by exercise training (Figure 4-7). Importantly, exercise trained animals treated with the antisense oligonucleotide against MnSOD had similar levels of calpain
activation to control-IR animals. This finding is consistent with the hypothesis that MnSOD plays a critical role in regulating IR-induced calpain activation.

**Oxidative Modification of Critical Calcium-Handling Proteins**

The increase in free cytosolic Ca$^{2+}$ and corresponding increase in calpain activity have been shown to play a deleterious role in the heart following IR.

Because the oxidative modification of Ca$^{2+}$-handling proteins has been shown to lead to impaired Ca$^{2+}$-handling, we have hypothesized that the oxidation of Ca$^{2+}$-handling proteins within the myocardium may lead to increases in free cytosolic Ca$^{2+}$, the activation of calpain, myocardial damage and contractile dysfunction.

![Figure 4-8. Carbonyl formation on critical Ca$^{2+}$-handling proteins: L-type calcium channel (LTCC), Sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA2a), Phospholamban (PLB) and Sodium/calcium exchanger (NCX). Representative blots are displayed above. Values below are means ± SE. • Significantly different from respective Control-Sham group, $P < 0.05$.](image_url)
Carbonyl formation on calcium-handling proteins

IR resulted in an increase in carbonyl formation on all four Ca\textsuperscript{2+}-handling proteins measured in hearts from control (untrained animals): LTCC, SERCA2a, PLB, and NCX (illustrated in Figure 4-8). Exercise training attenuated the IR-associated increase in carbonyl formation in three of the four measured Ca\textsuperscript{2+}-handling proteins: LTCC, SERCA2a, and PLB. Importantly, exercise-induced protection against IR-induced carbonyl formation was abolished by the antisense oligonucleotide against MnSOD in both the LTCC and SERCA2a, but not in PLB. In addition, neither exercise training nor antisense treatment affected IR-induced carbonyl formation on the NCX.

![Image of protein bands]

**Figure 4-9.** HNE formation on critical Ca\textsuperscript{2+}-handling proteins: Sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA2a), Phospholamban (PLB) and Sodium/calcium exchanger (NCX). Representative blots are displayed above. Values below are means ± SE. • Significantly different from respective Control-Sham group, $P < 0.05$. 

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**HNE formation on calcium-handling proteins**

An increase in HNE formation on SERCA2a and PLB in the heart was also observed following IR, which was attenuated by exercise training (Figures 4-9). In addition, exercise-induced protection against IR-induced HNE formation on SERCA2a and PLB was abolished by the antisense oligonucleotide against MnSOD. However, no significant changes in HNE formation were detected on the NCX, and there was no detectable amount of HNE formation on the LTCC.

![Western blotting for intact Ca\(^{2+}\)-handling proteins](image)

**Figure 4-10.** Western blotting for intact Ca\(^{2+}\)-handling proteins: L-type calcium channel (LTCC), Sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA2a), Phospholamban (PLB) and Sodium/calcium exchanger (NCX). Values are means ± SE. • Significantly different from respective Control-IR group, \(P < 0.05\).
Calpain-Mediated Degradation of Calcium-Handling Proteins

We and others (18, 19, 31, 97) have previously demonstrated that IR results in increased calpain activation in the heart. Once active, calpain degrades calpain substrates (proteins / protein complexes) within the cell. We hypothesized that calpain cleaves important Ca$^{2+}$-handling proteins, which have been oxidatively modified, exacerbating IR-induced Ca$^{2+}$-overload, calpain activation and myocardial injury. To test this hypothesis we performed Western Blot analyses to determine protein content of each of the four Ca$^{2+}$-handling proteins.

Our results demonstrated a decrease in myocardial levels of intact LTCC, SERCA2a, PLB, and NCX following IR, which was attenuated by exercise training. Moreover, MnSOD antisense oligonucleotide treatment abolished the exercise-induced cardioprotection against Ca$^{2+}$-handling protein degradation in all four proteins (Figure 4-10). These results are consistent with the notion that MnSOD plays a critical role in both the regulation of calpain activation as well as the preservation of Ca$^{2+}$-handling proteins during IR. Additionally, these results suggest that oxidative modification of these proteins leads to their degradation.

Table 4-3. Correlations between the oxidative modification of calcium-handling proteins and their degradation following IR

<table>
<thead>
<tr>
<th></th>
<th>Carbonyl formation / Intact (R$^2$)</th>
<th>HNE formation / Intact (R$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTCC</td>
<td>-.809</td>
<td>-</td>
</tr>
<tr>
<td>SERCA2a</td>
<td>-.802</td>
<td>-.789</td>
</tr>
<tr>
<td>PLB</td>
<td>-.722</td>
<td>-.823</td>
</tr>
<tr>
<td>NCX</td>
<td>-.810</td>
<td>-.792</td>
</tr>
</tbody>
</table>

Pearson correlation R2 values depicting the relationship between oxidative modification (carbonyl formation and HNE formation) to critical Ca$^{2+}$-handling proteins and the total amount of intact protein, determined by Western Blot. All experimental groups were pooled for this analysis.

Finally, there was a strong negative correlation between the oxidative modification and amount of intact protein of each of the Ca$^{2+}$-handling proteins (Table 4-3). This finding is
consistent with the concept that oxidative modification makes these proteins more susceptible to calpain-mediated degradation.
CHAPTER 5
DISCUSSION

Overview of Principal Findings

These experiments examined both the mechanistic role that calpain plays in IR-induced cardiac injury as well as the mechanism responsible for exercise-induced cardioprotection against IR injury via two separate groups of experiments.

Experiments conducted to determine the mechanism(s) responsible for calpain-induced cardiac injury (Hypothesis One) tested the following separate hypotheses: (1) IR promotes increased oxidative modification of important myocardial Ca\(^{2+}\)-handling proteins; (2) oxidative modification of Ca\(^{2+}\)-handling proteins is associated with increased degradation of these proteins; and (3) inhibition of calpain will attenuate the IR-induced degradation of myocardial Ca\(^{2+}\)-handling proteins. Our data confirm the deleterious effects of IR on the myocardium; including impaired Ca\(^{2+}\)-handling and contractile function, oxidation and degradation of critical Ca\(^{2+}\)-handling proteins, and increased calpain activation. In addition, we observed strong negative correlations between the degree of Ca\(^{2+}\)-handling protein oxidative modification and the amount of intact protein, suggesting a link between oxidative stress and protein degradation during IR. Importantly, our experiments established that inhibition of calpain protects the heart against IR-induced contractile dysfunction as well as the degradation of Ca\(^{2+}\)-handling proteins. Our results provide the first evidence that calpain cleaves the following critical Ca\(^{2+}\)-handling proteins (LTCC, NCX and PLB) in the intact heart during IR. In addition, our data confirms previous work describing the calpain-mediated cleavage of SERCA2a during IR.

Experiments completed to examine the mechanism responsible for exercise-induced cardioprotection against IR injury (Hypothesis Two) tested the following postulates: (1) exercise training attenuates IR-induced oxidation and degradation of critical myocardial Ca\(^{2+}\)-handling
proteins; and (2) exercise-induced cardioprotection is dependent on an exercise-induced over-expression of cardiac levels of MnSOD. Our results reveal that exercise attenuated IR-induced calpain activation, oxidative modification of critical Ca^{2+}-handling proteins and the degradation of these proteins. Importantly, when the exercise-induced over-expression of MnSOD was attenuated via gene silencing (i.e., antisense oligonucleotide against MnSOD), cardioprotection against Ca^{2+}-handling protein oxidation and degradation, as well as calpain activation was abolished. These results support the concept that MnSOD plays a critical role in exercise-induced cardio-protection against IR injury, at least in part, by preventing the IR-induced oxidation and degradation of Ca^{2+}-handling proteins and calpain activation. Moreover, these findings represent the first in-vivo data demonstrating that oxidatively modified Ca^{2+}-handling proteins in the heart are more susceptible to degradation during IR.

In combination, the results of experiments suggest a chain of events during IR beginning with an increase in oxidative damage of Ca^{2+}-handling proteins, leading to impaired Ca^{2+}- handling and calpain activation, in turn resulting in the calpain-mediated degradation of critical Ca^{2+}-handling proteins by calpain, exacerbating Ca^{2+}-overload, and myocardial injury. In contrast, exercise attenuates these events, due in a large part, to the up-regulation of MnSOD in the heart. Increased myocardial MnSOD reduces the oxidation of Ca^{2+}-handling proteins, and the deleterious chain of events that follow by dismutating superoxide produced in the mitochondria during IR.

**Hypothesis One: The Effects of IR and Calpain Inhibition on Myocardial Calcium-Handling Proteins**

The following paragraphs provide a detailed discussion of the findings of experiments designed to determine the role that calpain plays in IR-induced damage and removal of Ca^{2+}-handling proteins in the heart.
Calpain Inhibition Protects Against IR-induced Damage and Removal of Calcium-Handling Proteins.

Calpain can modify and damage the myocardium during IR via multiple pathways. For example, calpains cleave several structural proteins leading to the release of myofilaments, facilitating their degradation by the proteosome. In addition, calpains can also promote apoptosis, through cleavage of Bid, mediating cytochrome c release from the mitochondria. Moreover, calpains increase the expression of cell adhesion molecules, leading to an increase in neutrophil-mediated oxidative damage. Each of these pathways has been shown to significantly contribute to IR-induced injury (55, 120, 122, 128). Further supporting the deleterious role of calpain during IR, inhibition of calpain has been previously reported, by our group and others, to reduce many of the deleterious effects of IR including contractile dysfunction, infarct area and apoptosis (18, 19, 122, 123). Our experiments contribute to previous studies, demonstrating that calpain inhibition attenuates the decline in LVDP, +dp/dt and -dp/dt typically observed following IR (Figures 4-1, 4-2, 4-3). Decreased peak pressures (LVDP), as well as decreased rates of pressure development (+dp/dt) and relaxation (-dp/dt) typically occur following myocardial ischemia and are indicative of impaired Ca\(^{2+}\) transport within the myocardium. Therefore, our data suggests that calpain impairs myocardial Ca\(^{2+}\)-handling following ischemia. To elucidate a potential mechanism of calpain-induced impairments in Ca\(^{2+}\)-handling we measured the protein content of several critical myocardial Ca\(^{2+}\)-handling proteins following IR, with or without calpain inhibition.

Calpain Degrades Critical Calcium-Handling Proteins

We postulated that calpain degrades myocardial Ca\(^{2+}\)-handling proteins during IR, exacerbating Ca\(^{2+}\)-overload and IR injury. Further, we hypothesized that IR-induced oxidation
of Ca$^{2+}$-handling proteins would be associated with calpain-mediated degradation. We were lead
to these hypotheses by three key observations.

First, calpain cleaves Ca$^{2+}$-handling proteins. There is growing evidence from in-vitro
studies suggesting that calpain can cleave Ca$^{2+}$-handling proteins. For example, Belles et al. (8)
and De Jongh et al. (24) have reported that calpain degrades the LTCC *in-vitro*. Other *in-vitro*
experiments have demonstrated calpain-cleavage of the ryanodine receptor and the
sodium/potassium ATPase (126, 130). Our results demonstrated a decrease in intact Ca$^{2+}$-
handling proteins following IR, which was attenuated by calpain inhibition (Figure 4-6). These
findings indicate that calpain mediates the degradation of several critical myocardial Ca$^{2+}$-
handling proteins, including the LTCC, SERCA2a, PLB and NCX, during IR. Although calpain-
mediated cleavage of various Ca$^{2+}$-handling proteins has been demonstrated *in-vitro*, to our
knowledge, the present study is the first to investigate calpain-mediated degradation of these
Ca$^{2+}$-handling proteins in intact hearts.

Second, Ca$^{2+}$-handling proteins lose function when oxidized during IR. There is a strong
body of in-vitro evidence examining the effects of ROS on Ca$^{2+}$-handling protein function. This
work is summarized in review papers detailing the ROS-mediated loss of function in all of the
Ca$^{2+}$-handling proteins examined in these experiments (LTCC, NCX, SERCA, and PLB) (64,
129). In addition, several studies have demonstrated a loss in Ca$^{2+}$-handling protein function
following IR (48, 62). In combination with the knowledge that widespread protein damage
occurs during IR, we postulated that Ca$^{2+}$-handling proteins might also be oxidized during IR,
accounting for their loss of function. This postulate was supported by our findings that IR
resulted in increased oxidative modification of critical Ca$^{2+}$-handling proteins (Figures 4-4, 4-5).
In addition, physiological measures of myocardial $\text{Ca}^{2+}$-handling (+dp/dt, -dp/dt and LVDP) were depressed following ischemia (Figures 4-1, 4-2, 4-3).

Third, oxidative modification of proteins can lead to degradation. Several studies have demonstrated that oxidative modification predisposes cellular proteins for degradation. Recent work by Zolotarjova et al. (130) demonstrated that when oxidized, the sodium/potassium exchanger is increasingly prone to calpain-mediated degradation. Wu et al also found that oxidation of the RYR leads to an increased incidence of degradation by calpain (126).

Supporting this work, our results demonstrated strong negative correlations between the amount of oxidized calcium handling proteins (i.e., LTCC, NCX, SERCA, and PLB) and the level of intact protein (Table 4-2), suggesting a link between the oxidative modification of $\text{Ca}^{2+}$-handling proteins and their degradation. Nonetheless, this finding should be viewed with caution because, although strong correlations exist between the degree of $\text{Ca}^{2+}$-handling protein oxidation and degradation, a strong positive correlation alone does not confirm a causal relationship.

In combination, we feel that these data supports the idea that ROS produced during IR oxidatively modify critical myocardial $\text{Ca}^{2+}$-handling proteins, resulting in both a loss in $\text{Ca}^{2+}$-handling ability, as well as calpain-mediated degradation of $\text{Ca}^{2+}$-handling proteins, exacerbating IR injury. If this line of reasoning proves correct, a reduction in IR-induced oxidative stress would attenuate the oxidation and degradation of myocardial $\text{Ca}^{2+}$-handling proteins, thereby decreasing IR-induced $\text{Ca}^{2+}$ overload and calpain activation, attenuating myocardial injury.

Exercise training has been shown to reduce oxidative stress within the myocardium during IR, possibly through the over-expression of endogenous antioxidant enzymes such as MnSOD. We have also previously demonstrated that exercise reduces IR-induced calpain activation. We postulate that exercise regulates IR-induced calpain activation by reducing the oxidative
modification of myocardial Ca\(^{2+}\)-handling proteins. This postulate was tested by a second series of experiments and these results will be discussed in subsequent sections.

**Hypothesis Two: IR, Exercise, MnSOD and Calcium-Handling Proteins**

These experiments tested the hypothesis that exercise would provide cardio-protection against the IR-induced oxidative modification and degradation of critical Ca\(^{2+}\)-handling proteins as well as calpain activation. In addition, we postulated that exercise-induced over-expression of MnSOD is a critical component of this exercise-induced cardio-protection.

**Exercise Training Provides Cardio-Protection**

We and others have previously demonstrated that exercise protects the heart against IR injury (71, 72, 93, 96, 113, 114). In addition, we have reported that exercise training prevents IR-induced calpain activation (31). However, the mechanism(s) responsible for this protection have not been determined. Expanding on our previous work, the current experiments identified one possible mechanism through which exercise may regulate calpain activation and provide cardioprotection by the preservation of critical Ca\(^{2+}\)-handling proteins within the myocardium.

Our results reveal that exercise provides cardioprotection against both the IR-induced oxidation and degradation of Ca\(^{2+}\)-handling proteins (Figures 4-8, 4-9, 4-10). In addition, exercise attenuates IR-induced calpain activation (Figure 4-7), potentially through an improved regulation of free cytosolic Ca\(^{2+}\) in cardiac myocytes. Although the mechanism(s) responsible for these protective effects are not completely understood, we hypothesize that one possibility is that an exercise-induced over-expression of the antioxidant MnSOD may provide protection against ROS-mediated Ca\(^{2+}\)-handling protein degradation, calpain activation and Ca\(^{2+}\)-handling protein degradation. To further investigate this possibility, the exercise-induced over-expression of MnSOD was prevented via a gene silencing using an antisense oligonucleotide against MnSOD.
Exercise-Induced Over-Expression of MnSOD Prevents the Oxidation of Calcium-Handling Proteins.

As discussed previously, there is strong evidence that both ROS and free cytosolic Ca\(^{2+}\) increase dramatically during IR. Moreover, there is a biological rationale to support a causal relationship between the IR-induced increase in ROS production and Ca\(^{2+}\)-overload. In regard to the source of radical production during both ischemia and reperfusion, the mitochondrial respiratory chain has been identified as a major contributor to IR-induced free radical production (9). Further supporting the importance of mitochondrial free radical production, studies using antioxidants targeted specifically to the mitochondria have demonstrated a significant reduction in oxidant-related damage within the myocardium, as well as improved myocardial function (4, 11, 28, 86).

Results from our experiments demonstrated a reduction in the IR-induced oxidation of Ca\(^{2+}\)-handling proteins following exercise training (Figures 4-8, 4-9). Importantly, the exercise-induced reduction in Ca\(^{2+}\)-handling protein oxidation was abolished by the antisense oligonucleotide against MnSOD. This suggests that mitochondrial superoxide production during IR contributes to the oxidation of critical myocardial Ca\(^{2+}\)-handling proteins.

The mechanism through which mitochondria-produced superoxide affects Ca\(^{2+}\)-handling proteins located in the SR and/or plasma membrane is currently not understood. Mitochondria-produced superoxide has several potential fates including conversion to a less reactive oxygen species, such as hydrogen peroxide (H\(_2\)O\(_2\)) by the antioxidant enzyme superoxide dismutase (SOD), or conversion to a more reactive species such as the hydroxyl radical (OH\(^{•}\)), in the presence of iron, or peroxynitrite (ONOO\(^{•}\)), through reaction with nitric oxide. Although highly reactive, peroxynitrite and the hydroxyl radical have very short half-lives and therefore would not be likely to oxidize Ca\(^{2+}\)-handling proteins located in the SR and/or plasma membrane.
Hydrogen peroxide, however, has a longer half-life and could leave the mitochondria to directly interact with Ca\textsuperscript{2+}-handling proteins.

If hydrogen peroxide is responsible for oxidation of cytosolic calcium handling proteins during an IR insult, an important question emerges. That is, what is the fate of the excess hydrogen peroxide produced due to the exercise-induced over-expression of MnSOD? As discussed earlier, MnSOD converts superoxide to hydrogen peroxide. Therefore, it is probable that an exercise-induced over-expression of MnSOD would result in a marked increase in hydrogen peroxide, which has also been shown to exacerbate myocardial injury. Since we have consistently observed a decrease, not an increase, in IR-induced oxidative modification of proteins with exercise training, we reason that exercise must also over-express or up-regulated the activity of one or more hydrogen peroxide scavenging systems within the myocardium. One possibility is an elevation in either protein content and/or activity of the hydrogen peroxide scavenger, catalase (12, 31). Another possibility is that exercise training increases the hydrogen peroxide buffering capacity of the glutathione system through an increase in the amount of glutathione protein and/or an increase in the amount of glutathione reductase protein or activity. Any of these alterations would allow the cell to more effectively manage increased amounts of hydrogen peroxide. Nonetheless, previous experiments have failed to confirm that exercise training results in an increase in myocardial levels of catalase, glutathione peroxidase, or glutathione (44, 72, 87, 96, 117). Hence, it seems likely that another mechanism exists in cardiac myocytes to remove hydrogen peroxide. Two recently discovered molecules involved in the removal of hydrogen peroxide from the mitochondria include both periredoxin III and thioredoxin (5, 23, 34, 54, 112, 121). However, at present, it is unclear if one or both of these
antioxidants are exercise-induced in the heart. Clearly, this is an important topic for future research.

**Exercise-Induced Over-Expression of MnSOD Attenuates Calpain Activation**

In addition to protecting the heart against ROS-mediated damage to Ca\(^{2+}\)-handling proteins, exercise-induced over-expression of MnSOD also attenuated the IR-induced increase in calpain activation (Figure 4-7). This supports the idea that IR-induced calpain activation, is at least in part, due to ROS-mediated damage to Ca\(^{2+}\)-handling proteins. It is possible that the oxidative modification of Ca\(^{2+}\)-handling proteins contributes to IR-induced Ca\(^{2+}\)-overload, resulting in greater calpain activation.

**Exercise-Induced Over-Expression of MnSOD Prevents the Degradation of Calcium-Handling Proteins**

MnSOD also appears to play a role in the exercise-induced reduction of Ca\(^{2+}\)-handling protein degradation during IR (Figure 4-10). Indeed, prevention of exercise-induced increases in myocardial MnSOD via antisense oligonucleotides eliminated the exercise-induced protection against IR-induced degradation of these important proteins. This finding is consistent with the concept that the oxidation of these Ca\(^{2+}\)-handling proteins may make them more susceptible to cleavage by calpain.

**Degradation of Calcium-Handling Proteins is Associated with Oxidation**

As previously discussed, research links the IR-induced oxidation of Ca\(^{2+}\)-handling proteins with a loss of function both in-vitro as well as in the intact heart (62, 64, 129). Additionally there is evidence supporting the idea that oxidation of these proteins may facilitate their degradation by calpain (126, 130). Our experiments provide two lines of evidence to support these ideas. First, strong correlations were observed between the amount of protein oxidation and degradation of Ca\(^{2+}\)-handling proteins following IR, suggesting that oxidized Ca\(^{2+}\)-handling proteins...
proteins are more likely to be degraded (Tables 4-2, 4-3). Second, exercise-induced protection against both the oxidation and degradation of Ca\textsuperscript{2+}-handling proteins was almost completely prevented by MnSOD antisense oligonucleotide treatment. This finding is consistent with the notion that a causal relationship exists between the oxidative modification of Ca\textsuperscript{2+}-handling proteins and their degradation.

**Summary and Future Directions**

This project utilized two separate experiments to investigate the relationships between ROS, Ca\textsuperscript{2+}-handling protein oxidation and degradation as well as calpain activation during IR. Major findings include the following: (1) IR results in contractile dysfunction and impaired Ca\textsuperscript{2+}-handling, calpain activation and the oxidation and degradation of critical myocardial Ca\textsuperscript{2+}-handling proteins; (2) calpain degrades several important Ca\textsuperscript{2+}-handling proteins during IR including LTCC, SERCA2a, PLB, and NCX; (3) exercise training attenuates IR-induced oxidation of these calcium handling proteins and preserves their levels in the heart; (4) exercise-induced cardioprotection is critically dependant on an up-regulation of MnSOD. In combination, these findings are consistent with the mechanistic series of events, which is depicted in Figure 5-1. We postulate that IR results in an increase in mitochondrial superoxide production, which leads to the oxidation of critical Ca\textsuperscript{2+}-handling proteins, resulting in increased free cytosolic Ca\textsuperscript{2+} and calpain activation and finally the calpain-mediated degradation of critical Ca\textsuperscript{2+}-handling proteins.

The results of these experiments provide a unique contribution to the existing research describing the mechanisms of IR injury and the mechanisms of exercise-induced cardioprotection against IR injury. Indeed, these new data provide a mechanistic link connecting the relationship between IR-induced ROS and Ca\textsuperscript{2+}-overload. This work is also the first to demonstrate calpain-cleavage of several critical Ca\textsuperscript{2+}-handling proteins. Finally, our work
provides additional insight into the mechanisms of exercise-induced cardioprotection as well as the cardioprotective effects of MnSOD. In fact, these experiments may suggest a possible beneficial clinical role for the acute use of calpain inhibitors and/or mitochondria-targeted superoxide scavengers.

Future research is needed to determine if exercise training elevates mitochondrial or cytosolic antioxidants capable of removing hydrogen peroxide. Moreover, more work is required to clarify the oxidation pathway connecting mitochondrial superoxide production to the oxidative damage of Ca\(^{2+}\)-handling proteins. Finally, the sequence of events involving the oxidation and calpain-mediated degradation of Ca\(^{2+}\)-handling proteins is not yet completely understood and warrants additional research.

Figure 5-1. Proposed mechanisms underlying the IR-induced increase in calpain activity and myocardial dysfunction.
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BIOGRAPHICAL SKETCH

Joel French was born in Sioux Falls, South Dakota. He attained two bachelor degrees from the University of Sioux Falls (Exercise Science and Psychology) along with two minors in Computer Science and English Literature. During this time, Joel also taught Tae-kwon-do, started a personal training business and worked as a youth director at the Sioux Falls YMCA. Following graduation, he then worked for two years as a rehabilitation specialist at Central Plains Clinic and McKennan hospital in Sioux Falls. Joel graduated with his masters in Exercise Physiology from St. Cloud State University (St. Cloud, MN) in 1998. He then worked for a year at the US Olympic Training Center in Lake Placid, NY, returning to Minneapolis, MN for another two years working in cardiac and orthopedic rehabilitation. Finally, deciding to focus his career in basic science, Joel began his doctoral work at the University of Florida in 2001, studying the mechanisms of heart disease and protection against myocardial infarction.