Ischemia-reperfusion induced calpain activation and SERCA2a degradation are attenuated by exercise training and calpain inhibition


Department of Applied Physiology and Kinesiology, Center for Exercise Science
Departments of Psychiatry and Neuroscience, Center for Traumatic Brain Injury Studies
University of Florida. Gainesville, FL 32611

Supported by NIH HL072789 (Scott K. Powers), AHA 0415135B (Joel P. French).

Running title: Ischemia-reperfusion, calpain, antioxidants, oxidative stress, cardioprotection, and exercise training

Contact information:
S.K. Powers
Dept. of Applied Physiology and Kinesiology
Center for Exercise Science
University of Florida
Gainesville, FL 32611
Phone: (352)392-9575; fax (352)392-0316
E-mail: spowers@hhp.ufl.edu
ABSTRACT

The calcium-activated protease calpain has been shown to play a deleterious role in the heart during ischemia-reperfusion (IR). We tested the hypothesis that exercise training would minimize IR-induced calpain activation and provide cardioprotection against IR-induced injury. Hearts from adult male rats were isolated in a working heart preparation and myocardial injury was induced with 25 minutes of global ischemia followed by 45 minutes of reperfusion. In sedentary control rats, IR significantly increased calpain activity and impaired cardiac performance (cardiac work during reperfusion = 24% of baseline). Compared to sedentary animals, exercise training prevented the IR-induced rise in calpain activity and improved cardiac work (recovery = 80% of baseline). Similar to exercise, pharmacological inhibition of calpain activity resulted in comparable cardioprotection against IR injury (recovery = 86% of baseline). The exercise-induced protection against IR-induced calpain activation was not due to altered myocardial protein levels of calpain or calpastatin. However, exercise training was associated with increased myocardial antioxidant enzyme activity (Mn-superoxide dismutase, catalase) and a reduction in oxidative stress. Importantly, exercise training also prevented the IR-induced degradation of the calcium ATPase (SERCA2a). These findings suggest that increases in endogenous antioxidants may diminish the free radical mediated damage and/or degradation of Ca^{2+} handling proteins (such as SERCA2a), typically observed following IR. In conclusion, these results support the concept that calpain activation is an important component of IR-induced injury and that exercise training provides cardioprotection against IR injury, at least in part, by attenuating IR-induced calpain activation.
INTRODUCTION

Myocardial ischemia-reperfusion (IR) injury is a prevalent consequence of cardiovascular disease. Factors that contribute to myocardial IR injury include: oxidative stress due to the production of reactive oxygen species (ROS), disruption of Ca$^{2+}$ homeostasis, activation of proteases, and neutrophil infiltration/activation (1,10). Although each of these factors may contribute to cellular injury, growing evidence suggests that increased ROS production and cytosolic free Ca$^{2+}$ overload, either independently or cooperatively, are major contributors to IR-induced injury (1,20,22).

Increases in myocardial cytosolic Ca$^{2+}$ levels have been observed during both ischemia and reperfusion (19,30). 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 (11). 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$^{2+}$ levels (11). Importantly, increased calpain activity can contribute to IR-induced myocardial injury. Moreover, calpains’ deleterious role in IR injury is supported by strong evidence indicating that calpain inhibition significantly attenuates myocardial infarction (15,33,34,38).

Recent work has linked IR-induced ROS production to oxidative modification of Ca$^{2+}$ handling proteins, such as the sarcoplasmic/endoplasmic reticulum Ca$^{2+}$ ATPase (SERCA2a) (16,28,32). This is significant because damage to these proteins can lead to a disruption in cellular Ca$^{2+}$ homeostasis. Additionally, antioxidant treatment significantly attenuates I-R-induced damage to Ca$^{2+}$ handling proteins, maintaining Ca$^{2+}$ homeostasis as well as contractile function (32). Based on these findings, there is evidence to suggest that an increase in endogenous antioxidants may provide cardioprotection, at least in part, via regulation of free cytosolic Ca$^{2+}$ during IR.

Muscular exercise (i.e. bouts of endurance) is a well established means of inducing cardioprotection against IR-induced injury. Specifically, work from numerous laboratories has consistently demonstrated exercise-induced cardioprotection against IR insults of varying severities, ranging from minor injury to infarction (17,18,24,31). Although the mechanism(s) responsible for exercise-induced cardioprotection are not fully understood, growing evidence suggests that endurance exercise may provide protection, at least in part, by up-regulating endogenous antioxidants, such as manganese superoxide dismutase (Mn-SOD) (13,25,37). Moreover, antioxidant studies, using transgenic animals that over-express antioxidants as well as exogenous antioxidants delivered to the heart, have provided additional evidence that antioxidants can protect the heart against IR injury (6,7,12).

Since recent evidence suggests the possibility of ROS-mediated disturbances in cytosolic Ca$^{2+}$ homeostasis, it seems possible that exercise-induced increases in myocardial antioxidants can retard IR-induced calcium disturbances and consequently, prevent or attenuate IR-induced calpain activation. Therefore, this study addressed the question “does endurance exercise training provide cardioprotection by diminishing IR-induced calpain activation in the heart”? Based on work indicating that exercise protects against IR-induced oxidative stress, we hypothesized that exercise training would
attenuate IR–induced calpain activation in the heart. Our results supported this hypothesis and the objectives of our study were expanded to investigate the potential mechanisms responsible for this exercise-induced attenuation of calpain activation. Specifically, we determined if exercise training decreased myocardial calpain levels and/or increased cardiac levels of calpain’s endogenous inhibitor, calpastatin. Further, we ascertained if exercise was associated with a reduction in IR-induced oxidative stress, as well as the preservation of intact SERCA2a and phospholamban (PLB) protein in the heart following IR.

METHODS

Experimental design

These experiments were approved by the University of Florida Animal Care and Use Committee and followed guidelines established by the American Physiological Society for the use of animals in research. To investigate the relationship between exercise and IR-induced calpain activation in the heart, adult male rats (6 months old) were randomly assigned to one of seven experimental groups: Non-perfused control (n=11), Non-perfused trained (n=9), Perfused control (n=11), Control IR (n=11), Trained IR (n=12), Calpain inhibited IR (n=6), and Vehicle IR (n=6). Non-perfused sedentary and exercise-trained animal groups were included to determine the effects of exercise training on the un-stressed heart. Additionally, a sedentary perfused control group was included to serve as a control for any possible effects of perfusion prior to IR. Note that an exercise-trained, perfused group was not included in our experimental design because preliminary experiments in our laboratory have revealed no differences in cardiac performance between trained and untrained rat hearts prior to ischemia.

Throughout the experimental period, all animals were housed on a 12:12-h light-dark cycle and provided food (AIN93 diet) and water ad libitum.

Exercise Training Protocol

Animals assigned to the exercise-training groups were habituated to treadmill exercise for 5 consecutive days. This habituation period involved a gradual increase in running time beginning with 10 minutes per day and ending with 50 minutes per day. Following 2 days of rest, the animals then performed 3 consecutive days of treadmill exercise for 60 minutes per day at 30m/min, 0% grade [estimated work rate of 70% maximum oxygen consumption (VO2max)] (9). All hearts were excised 24 hours following the final exercise bout.

In vitro isolated perfused working heart protocol

To investigate myocardial function before and after an IR insult, we selected the in vitro isolated perfused 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 isolated perfused 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 (14).
Complete details of our isolated working heart preparation have been described earlier (17,18,26).

**Ischemia-reperfusion protocol**

Hearts were perfused with a modified Krebs-Henseleit perfusion buffer containing (in mM) 1.25 CaCl2, 130 NaCl, 5.4 KCl, 11 glucose, .5 MgCl2, .5 NaH2PO4, and 25 NaHCO3, and aerated with 95% O2 – 5% CO2. The simultaneous clamping of aortic and atrial lines induced global 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 perfusion with the atrial cannula open) and 25 minutes of normal reperfusion.

**Cardiac performance measurements**

Cardiac performance measurements were made every 5 minutes prior to ischemia and during reperfusion. Measurements included timed collections of aortic flow (AF) during working heart mode and coronary flow (CF) during both working heart and retrograde perfusion modes. Cardiac output (CO) was defined as the sum of these flows (CO = AF + CF). Peak systolic pressure, diastolic pressure, and heart rate were measured via a pressure transducer (Harvard Instruments) connected to the aortic cannula. Data were recorded using a customized computer data-acquisition system.

**Calpain inhibition**

To determine the effects of IR-induced calpain activation on myocardial function, calpain was inhibited (*in vitro*) using the selective inhibitor MDL 28170 (CI3) (EMD Biosciences, La Jolla, CA). The efficacy of CI3 as a selective inhibition of calpain I and II has been well established (4,5,34,36). 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. In addition, because DMSO has antioxidant properties, a vehicle-control group (Vehicle IR) was also included to demonstrate that our concentration of DMSO, without CI3, was not cardioprotective.

**Tissue Preparation**

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.

**IR-induced LDH release**

LDH activity in the coronary effluent was used as an indication of IR-induced myocardial injury. LDH activity was measured in triplicate as described previously (8,17,18). Measurements were made in coronary effluent collected prior to and following
ischemia. LDH activities were then normalized to heart wet weight and expressed as the percent difference between pre-ischemic and post-ischemic values.

**Biochemical analysis of endogenous antioxidant enzyme activity**

To assess the effect of exercise training on myocardial antioxidant capacity, a section of left ventricular free wall from the Non-perfused Control and/or Non-perfused Trained groups was homogenized in cold 100mM phosphate buffer with 0.5% bovine serum albumin (pH 7.4). Homogenates were centrifuged at 400xg for 10 minutes at 4°C. The resulting supernatant was used to determine protein content in addition to enzyme activities for superoxide dismutase (SOD) and catalase (CAT). Protein content was determined using the methods of Bradford (2). Total SOD activity as well as MnSOD and CuZnSOD activities, and CAT activity, were measured as described previously (17,18). Both biochemical assays were performed on the same day at 25°C to avoid inter-assay variation. The coefficients of variation for SOD and CAT assays were 4% and 5%, respectively.

**Western blot analysis of calpain cleavage products**

To assess calpain activity, calpain-specific cleavage products of three proteins (αII-spectrin, talin, and calpastatin) were analyzed. Briefly, proteins were separated using standard SDS-PAGE techniques on a 4%-20% polyacrylamide gel. Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes and exposed to a mouse monoclonal primary antibody to αII-spectrin (Biomol, Plymouth Meeting, PA), talin (Sigma, St. Louis, MO), or calpastatin (Triple Point Biologics, Forest Grove, OR). Following washing, an anti-mouse or anti-rabbit IgG-HRP-conjugated secondary antibody was applied for chemiluminescence detection (Amersham, Piscataway, NJ). Both intact and calpain-cleaved fragments of each of the three proteins were analyzed using a Kodak imaging system (Kodak Image Station 440, Rochester, NY). The cleaved bands were then expressed as a percentage of the intact bands and finally normalized as a percentage of the Non-perfused Control group.

**Western blot analysis of intact calpain, calpastatin SERCA2a, and PLB**

Briefly, proteins were separated using standard SDS-PAGE techniques on a 4%-20% polyacrylamide gel. Proteins were then transferred to PVDF membranes and exposed to a mouse monoclonal primary antibody to either calpain I, calpain II (Chemicon International, Temacula, CA), calpastatin (Triple Point Biologics, Forest Grove, OR), SERCA2a (Affinity Bioreagents, Golden, CO), or PLB (Upstate, Lake Placid, NY). Following primary antibody exposure, an anti-mouse, or anti-rabbit IgG-HRP-conjugated secondary antibody (Amersham, Piscataway, NJ) was applied for chemiluminescence detection. Each blot was analyzed using a Kodak imaging system (Kodak Image Station 440, Rochester, NY) and normalized to β-actin in order to adjust for protein loading. Results were then expressed as a percent of Non-perfused Control.

**Cardiac protein carbonyl levels**

Protein carbonyls are formed by a variety of oxidative mechanisms and are sensitive indices of oxidative injury (3). Carbonyl formation was determined in left ventricle homogenates from all experimental groups using a sensitive and reliable, commercially available ELISA (Zentech Technology, Dunedin, NZ).
Data analysis

To test our hypothesis, a one-way ANOVA was 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 a priori at \( P < 0.05 \).

RESULTS

Animal characteristics

The physical characteristics for the animals in all experimental groups are presented in Table 1. Although body mass differed between several of the experimental groups, heart weights and heart to body weight ratios were not significantly different between any of the experimental groups.

Myocardial performance and damage during I-R

The \textit{in vitro} ischemia-reperfusion protocol was successfully performed on 6-12 hearts from each experimental group. The pre-ischemia and post-reperfusion cardiac performance data are presented in Table 2. As expected, compared to pre-ischemia, most indices of cardiac performance were significantly depressed following IR. Note that post-reperfusion cardiac output, heart rate, cardiac work and rate pressure product were preserved in exercise-trained and calpain-inhibited animals compared to sedentary controls. Cardiac work measurements for each group are also displayed, expressed as \% recovery, in Figure 1. Importantly, exercise-trained and calpain-inhibited hearts both recovered significantly better than control hearts in response to IR. Additionally, LDH activity was measured in coronary effluent before and after ischemia in order to assess myocardial damage. These data, presented in Figure 2, demonstrate an increase in LDH release during reperfusion following ischemia, which is attenuated by both exercise training and calpain inhibition. Combined with the measurements of cardiac work, these data indicate that both exercise and calpain inhibition provided cardioprotection against IR injury.

Calpain activity

In vivo calpain activity in the left ventricle was assessed via the proteolytic cleavage of three calpain substrates; talin, \( \alpha \)-spectrin, and calpastatin, illustrated in Figures 3-6. Compared to perfused control hearts, IR induced a significant increase in calpain-cleavage of all three substrates (Figures 3-5) as well as a decrease in intact calpastatin (Figure 6). Additionally, the IR-associated increase in calpain substrate cleavage was attenuated in exercised and calpain-inhibited hearts exposed to IR. This indicates that our calpain inhibitor (CI3) successfully inhibited activation of calpain, and, similarly, exercise, through unknown mechanisms, also attenuated the IR-induced increase in calpain activation. Collectively, these observations suggest that calpain regulation plays a critical role in exercise-induced cardioprotection.
Calpain and calpastatin protein levels

Intact calpastatin, calpain II, and calpain I protein levels were measured via Western blot analysis. The results, displayed in Figures (6-8), reveal that neither calpain, nor calpastatin protein contents were altered with exercise training prior to IR. These data indicate that exercise does not regulate calpain activity through alterations in protein levels of calpain or its inhibitor, calpastatin.

Antioxidant enzyme activities

The activities of key antioxidant enzymes were determined in hearts from sham animals and reported in Table 3. Endurance exercise training resulted in a significant increase in myocardial Mn-SOD and CAT activities.

Oxidative Stress

Oxidative stress was assessed through the measurement of protein carbonyl formation. There was a significant increase in myocardial carbonyl levels following IR, which was attenuated in exercise-trained animals (Figure 9).

SERCA2a and phospholamban protein

Intact SERCA2a and phospholamban protein were measured using Western blotting. The results indicate a significant decrease in both proteins resulting from IR, which was attenuated by both exercise training and calpain inhibition (Figures 10 and 11).

DISCUSSION

These experiments tested the hypothesis that endurance exercise training would provide cardioprotection against IR-induced injury, at least in part, by attenuating the IR-associated increase in myocardial calpain activity. Our findings clearly supported this hypothesis. Moreover, our results suggest that the mechanism explaining the exercise-induced reduction in IR-induced calpain activation in the heart was not due to diminished calpain protein levels or increased levels of the endogenous calpain inhibitor, calpastatin. However, we did observe an increase in the IR-induced degradation of the Ca\(^{2+}\)ATPase SERCA2a along with the calcium regulatory protein PLB, which was prevented by exercise training. Collectively, these findings advance our understanding of the mechanism(s) responsible for both IR-induced injury and cardioprotection, and are discussed in the following paragraphs.

Calpain inhibition attenuates IR-induced contractile dysfunction

It is well established that myocardial IR results in elevated cytosolic levels of calcium which lead to the activation of the calcium-regulated protease, calpain (1,5,11,15,32,34,38). This is significant because calpain activation promotes the degradation of key cardiac proteins leading to myocardial contractile dysfunction and cell death. Indeed, activation of 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 (11,21,27,35,36). Moreover, calpains may contribute to apoptosis, through cleavage of Bid, mediating cytochrome c release from the mitochondria (5,11). Also, calpains increase the expression of cell adhesion molecules, leading to an increase in neutrophil-mediated
oxidative damage (23,29). Each of these pathways has been shown to significantly contribute to IR-associated injury.

Our findings confirmed the damaging impact of calpain activation on IR-induced cardiac injury as indicated by the observation that a selective calpain inhibitor (MDL 28170) provided cardioprotection against IR-induced injury (Figure 1). These results provide physiological support to the notion that calpain activation plays a significant role in IR-induced myocardial injury and are consistent with previous work on this topic (15,33,34,38).

**Exercise retards IR-induced calpain activation**

Although it is well established that exercise provides protection against IR-induced cardiac injury, the mechanism(s) responsible for this form of cardioprotection remain unknown. We postulated that exercise-induced cardioprotection against IR injury could be due, in part, to diminished calpain activation during the IR insult. Our calpain activity data (Figures 3-6) was consistent with this hypothesis. Therefore, we also investigated several potential mechanisms responsible for exercise-induced protection against IR-induced calpain activation. Theoretically, exercise-induced protection against IR-induced calpain activation could result from one or more of the following possibilities: 1) decreased cardiac levels of calpain I or calpain II; 2) increased cellular levels of the calpain inhibitor, calpastatin; and/or 3) improved maintenance of calcium homeostasis in the heart during IR.

The ratio of calpain to calpastatin in cells is physiologically important because this ratio greatly influences the ability of calcium to activate calpain. That is, an increased calpain to calpastatin ratio would favor calpain activation whereas a decreased calpain to calpastatin ratio would favor calpain inhibition. Although it is possible that exercise training could decrease IR-induced calpain activation by promoting a decrease in cardiac levels of calpain and/or an increase in calpastatin, our results indicate that exercise did not alter the levels of calpain (I and II) or calpastatin in the unstressed heart (Figures 6-8). Therefore, by process of elimination, we hypothesize that exercise diminishes IR-induced calpain activation in the heart by improving maintenance of calcium homeostasis. This postulate is supported by the knowledge that calpain is activated by prolonged exposure to elevated Ca^{2+} levels (11); hence, improved maintenance of myocyte Ca^{2+} homeostasis would minimize IR-induced calpain activation. The question remains, however, how does exercise training retard IR-induced disturbances in myocardial Ca^{2+} homeostasis?

The current study does not provide a definitive answer to this question. Nonetheless, an intriguing possibility is that exercise training elevates myocardial antioxidants and protects against IR-induced oxidative stress, which in turn, retards oxidative stress induced calcium overload within cardiac myocytes. Indeed, our results reveal that exercise attenuated the IR-induced increase in myocardial oxidative stress (Figure 9), presumably through an increase in myocardial antioxidant enzymes (Table 3). Specifically, the myocardial increase in the endogenous antioxidant Mn-SOD, following several days of exercise training, has been shown to play a protective role against IR-induced oxidative injury (15,33,34,38).
Although the relationships between ROS, Ca\textsuperscript{2+} homeostasis, and calpain activation during IR are complex and not completely understood, oxidative stress can increase intracellular Ca\textsuperscript{2+} through interaction with the SR and/or plasma membrane (16,32). Specifically, ROS have been shown to affect Ca\textsuperscript{2+} homeostasis in several possible ways, such as oxidation of sulfhydryl groups located on Ca\textsuperscript{2+} transport proteins, peroxidation of membrane lipids, and inhibition of membrane-bound regulatory enzymes (16). Accordingly, Ca\textsuperscript{2+}-ATPases are likely targets for free radicals during IR. Superoxide, hydrogen peroxide, and the hydroxyl radical have also been implicated in Ca\textsuperscript{2+}-ATPase modification and inhibition (16). In addition, modification of Ca\textsuperscript{2+}-ATPases by free radicals may increase the likelihood of their degradation by calpain (28). Regardless of the specific mechanisms, disruption of these ion transport mechanisms would lead to alterations in Ca\textsuperscript{2+} homeostasis, resulting in increased calpain activation. Hence, it seems plausible that the exercise-induced increases in myocardial antioxidants and the associated prevention of IR-associated oxidative stress may serve to maintain free cytosolic Ca\textsuperscript{2+} homeostasis and, therefore, attenuate calpain activation. Our results are consistent with this possibility as hearts from exercise-trained animals experienced less oxidative stress and calpain activation following IR compared to hearts from sedentary control animals (Fig. 3-6, 9).

**Exercise retards IR-induced SERCA2a and PLB degradation**

To further investigate the idea of free radical interaction with Ca\textsuperscript{2+} handling proteins, we measured protein levels of intact SERCA2a, the most abundant Ca\textsuperscript{2+}-ATPase within the myocardium, as well as PLB, a key SERCA regulatory protein. In this regard, two recent papers have described the loss of intact SERCA2a and PLB protein in the heart following IR (28,32). Our current work supports these findings, demonstrating a 60% loss in SERCA2a protein and a 52% loss in PLB protein following IR. Moreover, work by Temsah et. al. reveals that addition of the antioxidants superoxide dismutase and catalase attenuated the IR-induced loss of SERCA2a function and mRNA in the heart (32). These findings are also supported by the current study, which demonstrated an increase in myocardial Mn-SOD and catalase along with a preservation of SERCA2a and PLB protein in exercise-trained animals (Table 3, Figures 10,11). Although, Temsah et al. (32) did not detect preservation of PLB protein following IR with the administration of their antioxidant treatment, we observed that exercise training preserved PLB protein following IR (Figure 11). The explanation for this divergent finding is not clear, however, it is possible that exercise training provides a more potent endogenous antioxidant defense than supplementation of exogenous SOD and catalase. It is also possible that exercise training may prevent PLB degradation through another, unknown, mechanism.

Importantly, the current study is among the first to demonstrate that calpain inhibition prevents the IR-associated degradation of SERCA2a and PLB protein. Our work suggests that calpain may be in part responsible for cleavage of these proteins, raising the interesting possibility that calpain may regulate itself through a feed-forward mechanism by increasing levels of free cytosolic Ca\textsuperscript{2+} through the cleavage of SERCA2a and/or PLB. Recent work by Sing, et. al. also reported attenuation of SERCA2a cleavage using a calpain inhibitor, although the inhibitor used in their study, leupeptin, has also been found to have antioxidant properties (28). This could be confounding based on the possibility of interaction between free radicals and Ca\textsuperscript{2+}-ATPases.
In synopsis, we postulate that the IR-induced increase in free radicals may lead to increased oxidative modification of Ca\(^{2+}\) handling proteins, such as SERCA2a and PLB, making them targets for calpain cleavage. In addition, the cleavage of these proteins would logically lead to an increase in free cytosolic Ca\(^{2+}\) and, therefore, calpain activation. This hypothesis is supported by the fact that increasing antioxidants (either endogenously or exogenously), and/or inhibiting calpain both attenuate the IR-induced reduction in SERCA2a and PLB protein.

**Summary and Conclusions**

This work confirms the deleterious role of IR-induced calpain activation. Importantly, this is the first experiment to investigate the effects of endurance exercise on IR-induced calpain activation in the heart. Our findings clearly support the hypothesis that endurance exercise training attenuates the IR-associated increase in myocardial oxidative stress and calpain activity. Importantly, our results also reveal that the mechanism to explain the exercise-induced reduction in calpain activity is not due to diminished myocardial calpain levels or increased levels of the endogenous calpain inhibitor, calpastatin. Further, our findings confirm that IR-induced myocardial oxidative stress is attenuated by the improved myocardial antioxidant capacity of the exercise-trained animals. Finally, we show an attenuation of IR-associated SERCA2a and PLB degradation with both exercise training and calpain inhibition. Collectively, our findings are consistent with the concept that regulation of redox balance, and calpain activation play a significant role in cardioprotection against IR injury in the myocardium. Additional experiments are required to provide further insight into the specific mechanism(s) responsible for protection against IR-induced calpain activation.

**Acknowledgements and Grants**

This work was supported by funding from the National Institute of Health (NIH HL072789 - Scott K. Powers), and the American Heart Association (AHA 0415135B - Joel P. French).
References


Figure Legends

**Figure 1.** Mean % recovery of cardiac work (cardiac output * systolic blood pressure (SBP)). Both exercise training and calpain inhibition significantly improved cardiac work following 25 minutes of ischemia. Values are means ± SE. * Significantly different from Control-IR, \( P < 0.05 \)

**Figure 2.** Effects of IR injury, exercise and calpain inhibition on LDH release. LDH activity measurements were made in coronary effluent collected prior to and following ischemia. Activity was normalized to heart wet weight and expressed as the percent difference between pre-ischemic and post-ischemic values. Importantly, LDH release was increased significantly following ischemic injury. However, both exercise training and calpain inhibition attenuated the IR-induced increase in LDH release. * Significantly different from Control-IR, \( P < 0.05 \).

**Figure 3.** Analysis of calpain-cleaved talin
(A) Representative western blot illustrating calpain-mediated cleavage of talin following experimental treatments. Intact talin (225 kDa) and breakdown product (BDP; 190 kDa) are as indicated.
(B) Semi-quantitative analysis of % calpain-cleaved talin protein present. Note that calpain activation was elevated with IR (Control-IR and Vehicle-IR groups), however, both exercise (Trained-IR) and calpain inhibition (Inhibited-IR) attenuated the IR-associated increase in calpain activation. Data are integrated from multiple western blots. * Significantly different from Non-perfused Control, \( P < 0.05 \).

**Figure 4.** Analysis of calpain-cleaved \( \alpha \)II-spectrin
(A) Representative western blot illustrating calpain-mediated cleavage of \( \alpha \)II-spectrin following experimental treatments. Intact \( \alpha \)II-spectrin (260 kDa) and breakdown product (BDP; 150 kDa) are as indicated.
(B) Semi-quantitative analysis of % calpain-cleaved \( \alpha \)II-spectrin protein present. Note that calpain activation was elevated with IR (Control-IR and Vehicle-IR groups), however, both exercise (Trained-IR) and calpain inhibition (Inhibited-IR) attenuated the IR-associated increase in calpain activation. Data are integrated from multiple western blots. * Significantly different from Non-perfused Control, \( P < 0.05 \).

**Figure 5.** Analysis of calpain-cleaved calpastatin.
(A) Representative western blot illustrating cleavage of calpastatin following experimental treatments.
(B) Semi-quantitative analysis of % cleaved calpastatin protein present. Note that calpastatin cleavage was elevated with IR (Control-IR and Vehicle-IR groups), however, both exercise (Trained-IR) and calpain inhibition (Inhibited-IR) attenuated the IR-associated increase in calpain activation. Data are integrated from multiple western blots. * Significantly different from Non-perfused Control, \( P < 0.05 \).

**Figure 6.** Analysis of intact calpastatin protein
(A) Representative western blot illustrating calpastatin protein from experimental groups.
(B) Semi-quantitative analysis of calpastatin protein present. Note that IR resulted in a significant decrease in intact calpastatin protein. * significantly different from Non-perfused Control, \( P < 0.05 \). Data are integrated from multiple western blots.

**Figure 7.** Analysis of calpain II protein
(A) Representative western blot illustrating calpain II protein from all experimental groups.
(B) Semi-quantitative analysis of calpain II protein present. Note that calpain II protein was not altered in any of the experimental groups. Data are integrated from multiple western blots.

**Figure 8.** Analysis of calpain I protein
(A) Representative western blot illustrating calpain I protein from all experimental groups.
(B) Semi-quantitative analysis of calpain II protein present. Note that calpain I protein was not altered in any of the experimental groups. Data are integrated from multiple western blots.

**Figure 9.** Effects of IR injury and exercise on the formation of protein carbonyls
An increase in oxidative stress, measured by protein carbonyl formation, was observed following IR. Importantly, exercise training significantly attenuated oxidative stress following IR. Values are means ± SE. Significantly different from Perfused-Control, \( P < 0.05 \)

**Figure 10.** Analysis of intact sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA2a) protein
(A) Representative western blot illustrating intact SERCA2a protein.
(B) Semi-quantitative analysis of intact SERCA2a protein present. Note that IR was associated with a decrease in intact SERCA2a protein (Control-IR and Vehicle-IR groups), however, both exercise training and calpain inhibition maintained SERCA2a protein following IR. Data are integrated from multiple western blots. * significantly different from Perfused-Control, \( P < 0.05 \). • significantly different from Non-perfused-Control, \( P < 0.05 \).

**Figure 11.** Analysis of intact phospholamban (PLB) protein
(A) Representative western blot illustrating PLB protein.
(B) Semi-quantitative analysis of PLB protein present. Note that IR was associated with a decrease in PLB protein (Control-IR and Vehicle-IR groups), however, both exercise training and calpain inhibition maintained PLB protein following IR. Data are integrated from multiple western blots. * significantly different from Perfused-Control, \( P < 0.05 \). • significantly different from Non-perfused-Control, \( P < 0.05 \).
Figures

Figure 1

Mean % recovery of cardiac work

% Cardiac work (CO x SBP)

Control-IR  Trained-IR  Vehicle-IR  Inhibited-IR

* *
Figure 2

LDH Activity

% Difference

Control-IR  Trained-IR  Vehicle-IR  Inhibited-IR
Figure 3

A

<table>
<thead>
<tr>
<th></th>
<th>Non-perf. Control</th>
<th>Non-perf. Trained</th>
<th>Perfused Control</th>
<th>Control IR</th>
<th>Trained IR</th>
<th>Vehicle IR</th>
<th>Inhibited IR</th>
</tr>
</thead>
</table>

![Image: Protein bands with markers 225 kDa and 190 kDa.]

B

% Calpain-cleaved talin

![Image: Bar graph showing % Calpain-cleaved talin with * indicating significant differences.]

Legend:
- Non-perf. Control
- Non-perf. Trained
- Perfused Control
- Control IR
- Trained IR
- Vehicle IR
- Inhibited IR

* Indicates significant differences compared to Non-perf. Control.
Figure 4

A

Non-perf. Control  Non-perf. Trained  Perfused Control  Control IR  Trained IR  Vehicle IR  Inhibited IR

B

% Calpain-cleaved spectrin

% of Non-perfused Control
Figure 5

A

<table>
<thead>
<tr>
<th></th>
<th>Non-perf. Control</th>
<th>Non-perf. Trained</th>
<th>Perfused Control</th>
<th>Control IR</th>
<th>Trained IR</th>
<th>Vehicle IR</th>
<th>Inhibited IR</th>
</tr>
</thead>
</table>

B

% Cleaved calpastatin (35kDa)

% of Non-perfused Control

Non-perf. Control | Non-perf. Trained | Perfused Control | Control IR | Trained IR | Vehicle IR | Inhibited IR

*
Figure 6

A

Non-perf. Control  Non-perf. Trained  Perfused Control  Control IR  Trained IR  Vehicle IR  Inhibited IR

B

Intact Calpastatin Protein (110kDa)

% of Non-perfused control

Non-perf. Control  Non-perf. Trained  Perfused Control  Control IR  Trained IR  Vehicle IR  Inhibited IR
Figure 7

A

<table>
<thead>
<tr>
<th></th>
<th>Non-perf.</th>
<th>Non-perf.</th>
<th>Perfused</th>
<th>Control</th>
<th>Trained</th>
<th>Vehicle</th>
<th>Inhibited</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>IR</td>
<td>IR</td>
<td>IR</td>
<td>IR</td>
<td>IR</td>
<td>IR</td>
<td></td>
</tr>
</tbody>
</table>

B

Calpain II protein

% of Non-perfused control

0  20  40  60  80  100  120  140  160  180

Non-perf. Control  Non-perf. Trained  Perfused Control  Control IR  Trained IR  Vehicle IR  Inhibited IR

80kDa
Figure 8

A

<table>
<thead>
<tr>
<th>Condition</th>
<th>Non-perf. Control</th>
<th>Non-perf. Trained</th>
<th>Perfused Control</th>
<th>Control IR</th>
<th>Trained IR</th>
<th>Vehicle IR</th>
<th>Inhibited IR</th>
</tr>
</thead>
</table>

Calpain I protein

B

![Calpain I protein graph](image_url)

% of Non-perfused Control

Calpain I protein

80kDa
Figure 9

Protein Carbonyl Formation (nmol/mg protein)

- Non-perf. Control
- Non-perf. Trained
- Perfused Control
- Control IR
- Trained IR
- Vehicle IR
- Inhibited IR

* Indicates significant difference.
Figure 10

A

<table>
<thead>
<tr>
<th>Condition</th>
<th>Non-perf. Control</th>
<th>Non-perf. Trained</th>
<th>Perfused Control</th>
<th>Control IR</th>
<th>Trained IR</th>
<th>Vehicle IR</th>
<th>Inhibited IR</th>
</tr>
</thead>
</table>

B

Intact SERCA2a protein

[Graph showing the percentage of intact SERCA2a protein for different conditions with error bars and asterisks indicating significant differences.]
Figure 11

A

Phospholamban Protein

B

% of Perfused control

Non-perf. Control  Non-perf. Trained  Perfused Control  Control IR  Trained IR  Vehicle IR  Inhibited IR

=13kDa

Phospholamban Protein
Table 1. Animal characteristics.

<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 Non-Perfused Control, $P < 0.05$. 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.
Table 2. Cardiac performance data

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Perfused Control</th>
<th>Control-IR</th>
<th>Trained-IR</th>
<th>Inhibited-IR</th>
<th>Vehicle-IR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre-ischemia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CF (ml/min⁻¹g wet wt⁻¹)</td>
<td>13 ± .77</td>
<td>11 ± .55</td>
<td>12 ± .58</td>
<td>14 ± 1.23</td>
<td>10 ± 1.02</td>
</tr>
<tr>
<td>CO (ml/min⁻¹g wet wt⁻¹)</td>
<td>51 ± 1.9</td>
<td>51 ± 1.9</td>
<td>51 ± 2.1</td>
<td>45 ± 4.7</td>
<td>45 ± 2.2</td>
</tr>
<tr>
<td>SP (mmHg)</td>
<td>79 ± 1.1</td>
<td>79 ± 1.5</td>
<td>76 ± 1.8</td>
<td>86 ± 3.1</td>
<td>82 ± 2.9</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>316 ± 6.5</td>
<td>332 ± 7.4</td>
<td>345 ± 10.8</td>
<td>315 ± 13.9</td>
<td>324 ± 11.7</td>
</tr>
<tr>
<td>CW (SP x CO)</td>
<td>4629 ± 177</td>
<td>4856 ± 137</td>
<td>4474 ± 195</td>
<td>5448 ± 269*</td>
<td>4690 ± 384</td>
</tr>
<tr>
<td>RPP (HR x SP)</td>
<td>24899 ± 562</td>
<td>23812 ± 2398</td>
<td>26201 ± 401</td>
<td>26913 ± 1062</td>
<td>26699 ± 1625</td>
</tr>
<tr>
<td><strong>Post-reperfusion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CF (ml/min⁻¹g wet wt⁻¹)</td>
<td>NA</td>
<td>7±1.1</td>
<td>10±.54*</td>
<td>12±1.8*</td>
<td>8±1.3</td>
</tr>
<tr>
<td>CO (ml/min⁻¹g wet wt⁻¹)</td>
<td>NA</td>
<td>11 ± 3.6●</td>
<td>39 ± 3.1 ●●</td>
<td>45 ± 4.7●</td>
<td>19 ± 5.4●</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>NA</td>
<td>71 ± 1.8●</td>
<td>76 ± 2.7</td>
<td>83 ± 3.8</td>
<td>81 ± 8.0</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>NA</td>
<td>261 ± 21.2●</td>
<td>315 ± 11.5 ●●</td>
<td>308 ± 14.6●</td>
<td>305 ± 21.5●</td>
</tr>
<tr>
<td>CW (SP x CO)</td>
<td>NA</td>
<td>1615 ± 295●</td>
<td>3468 ± 332● ●</td>
<td>4635 ± 285● ●</td>
<td>1980 ± 613●</td>
</tr>
<tr>
<td>RPP (HR x SP)</td>
<td>NA</td>
<td>19615 ± 1461</td>
<td>23661 ± 614● ●</td>
<td>26699 ± 1626●</td>
<td>20053 ± 3606</td>
</tr>
</tbody>
</table>

Note that most post-perfusion measurements (following 25 minutes of ischemia) were significantly decreased compared to pre-ischemic measurements, indicating myocardial dysfunction. However, both exercise training and calpain inhibition resulted in significantly improved post-perfusion values for coronary flow (CF), cardiac output (CO), heart rate (HR), cardiac work (CW), and rate pressure product (RPP), without altering systolic blood pressure (SBP) compared to control-IR animals. Values are means ± SE. ● Significantly different from pre-ischemic values, \( P < 0.05 \); ●● Significantly different from Control-IR, \( P < 0.05 \).
Table 3. Myocardial antioxidant enzyme activity

<table>
<thead>
<tr>
<th>Group</th>
<th>Non-perfused Control (n = 11)</th>
<th>Non-perfused Trained (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enzyme activity</strong> (Units / mg Protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total SOD, units</td>
<td>$55.1 \pm 1.1$</td>
<td>$65.9 \pm 3.7 \bullet$</td>
</tr>
<tr>
<td>MnSOD, units</td>
<td>$44.2 \pm 1.5$</td>
<td>$52.2 \pm 2.9 \bullet$</td>
</tr>
<tr>
<td>CuZnSOD, units</td>
<td>$10.9 \pm 1.1$</td>
<td>$13.7 \pm 1.5$</td>
</tr>
<tr>
<td>CAT, units</td>
<td>$1.1 \pm .05$</td>
<td>$1.3 \pm .03 \bullet$</td>
</tr>
</tbody>
</table>

Manganese superoxide dismutase (MnSOD), total SOD, and Catalase activities were significantly increased following exercise training. Values are means ± SE. • Significantly different from Non-perfused Control, $P < 0.05$