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


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Ischemia-reperfusion-induced calpain activation and SERCA2a degradation are attenuated by exercise training and calpain inhibition. AM J Physiol Heart Circ Physiol 290: H128–H136, 2006. First published September 9, 2005; doi:10.1152/ajpheart.00739.2005.— The Ca\textsuperscript{2+}-activated protease calpain has been shown to play a deleterious role in the heart during ischemia-reperfusion (I/R). We tested the hypothesis that exercise training would minimize I/R-induced calpain activation and provide cardioprotection against I/R-induced injury. Hearts from adult male rats were isolated in a working heart preparation, and myocardial injury was induced with 25 min of global ischemia followed by 45 min of reperfusion. In sedentary control rats, I/R significantly increased calpain activity and impaired cardiac performance (cardiac work during reperfusion = 24% of baseline). Compared with sedentary animals, exercise training prevented the I/R-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 I/R injury (recovery = 86% of baseline). The exercise-induced protection against I/R-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-SOD, catalase) and a reduction in oxidative stress. Importantly, exercise training also prevented the I/R-induced degradation of sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA)2a. These findings suggest that increases in endogenous antioxidants may diminish the free radical-mediated damage and/or degradation of Ca\textsuperscript{2+} handling proteins (such as SERCA2a) typically observed after I/R. In conclusion, these results support the concept that calpain activation is an important component of I/R-induced injury and that exercise training provokes cardioprotection against I/R injury, at least in part, by attenuating I/R-induced calpain activation.

exercise training; myocardial ischemia-reperfusion; SERCA2a; antioxidative stress; cardioprotection; calpains; ROS

MYOCARDIAL ISCHEMIA-REPERFUSION (I/R) injury is a prevalent consequence of cardiovascular disease. Factors that contribute to myocardial I/R injury include oxidative stress due to the production of reactive oxygen species (ROS), disruption of Ca\textsuperscript{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\textsuperscript{2+} overload, either independently or cooperatively, are major contributors to I/R-induced injury (1, 20, 22).

Increases in myocardial cytosolic Ca\textsuperscript{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\textsuperscript{2+} in the pathogenesis of I/R-induced myocardial injury is through activation of the Ca\textsuperscript{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\textsuperscript{2+} required for their activation in vitro. Both calpain isoforms are activated by prolonged exposure to elevated cytosolic Ca\textsuperscript{2+} levels (11). Importantly, increased calpain activity can contribute to I/R-induced myocardial injury. Moreover, calpain’s deleterious role in I/R injury is supported by strong evidence indicating that calpain inhibition significantly attenuates myocardial infarction (15, 33, 34, 38).

Recent work has linked I/R-induced ROS production to oxidative modification of Ca\textsuperscript{2+} handling proteins such as sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA)2a (16, 28, 32). This is significant because damage to these proteins can lead to a disruption in cellular Ca\textsuperscript{2+} homeostasis. Additionally, antioxidant treatment significantly attenuates I/R-induced damage to Ca\textsuperscript{2+} handling proteins, maintaining Ca\textsuperscript{2+} homeostasis as well as contractile function (32). On the basis of 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\textsuperscript{2+} during I/R.

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

Because recent evidence suggests the possibility of ROS-mediated disturbances in cytosolic Ca\textsuperscript{2+} homeostasis, it seems possible that exercise-induced increases in myocardial antioxidants can retard I/R-induced Ca\textsuperscript{2+} disturbances and, consequently, prevent or attenuate I/R-induced calpain activation.

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Therefore, this study addressed the question “Does endurance exercise training provide cardioprotection by diminishing I/R-induced calpain activation in the heart?” On the basis of work indicating that exercise protects against I/R-induced oxidative stress, we hypothesized that exercise training would attenuate I/R-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 whether exercise training decreased myocardial calpain levels and/or increased cardiac levels of calpain’s endogenous inhibitor, calpastatin. Furthermore, we ascertained whether exercise was associated with a reduction in I/R-induced oxidative stress, as well as the preservation of intact SERCA2a and phospholamban (PLB) protein in the heart after I/R.

**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 I/R-induced calpain activation in the heart, adult male rats (6 mo old) were randomly assigned to one of seven experimental groups: nonperfused control (n = 11), nonperfused trained (n = 9), perfused control (n = 11), control I/R (n = 11), trained I/R (n = 12), calpain-inhibited I/R (n = 6), and vehicle I/R (n = 6). Nonperfused sedentary and exercise-trained animal groups were included to determine the effects of exercise training on the unstressed heart. Additionally, a sedentary perfused control group was included to serve as a control for any possible effects of perfusion before I/R. It should be noted that an exercise-trained, perfused group was not included in our experimental design because preliminary experiments in our laboratory revealed no differences in cardiac performance between trained and untrained rat hearts before 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 min/day and ending with 50 min/day. After 2 days of rest, the animals then performed 3 consecutive days of treadmill exercise for 60 min/day at 30 m/min. 0% grade (estimated work rate of 70% maximum O2 consumption) (9). All hearts were excised 24 h after the final exercise bout.

**In vitro isolated, perfused working heart protocol.** To investigate myocardial function before and after an I/R insult, we selected the in

### Table 1. Animal characteristics

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body Wt, g</th>
<th>Heart Wt, g</th>
<th>Heart Wt-to-Body Wt Ratio, mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonperfused control</td>
<td>11</td>
<td>360±4.2</td>
<td>1.20±0.02</td>
<td>3.34±0.07</td>
</tr>
<tr>
<td>Nonperfused trained</td>
<td>9</td>
<td>339±5.8</td>
<td>1.21±0.03</td>
<td>3.57±0.10</td>
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<tr>
<td>Perfused Control</td>
<td>11</td>
<td>354±4.4</td>
<td>1.16±0.03</td>
<td>3.28±0.09</td>
</tr>
<tr>
<td>Control-I/R</td>
<td>11</td>
<td>347±8.4</td>
<td>1.22±0.03</td>
<td>3.53±0.11</td>
</tr>
<tr>
<td>Trained-I/R</td>
<td>12</td>
<td>322±4.2</td>
<td>1.17±0.02</td>
<td>3.65±0.08</td>
</tr>
<tr>
<td>Calpain inhibited I/R</td>
<td>6</td>
<td>374±7.9</td>
<td>1.28±0.08</td>
<td>3.41±0.20</td>
</tr>
<tr>
<td>Vehicle I/R</td>
<td>6</td>
<td>401±8.0</td>
<td>1.25±0.04</td>
<td>3.13±0.06</td>
</tr>
</tbody>
</table>

Values are means ± SE. *Significantly different from nonperfused control (P < 0.05). Note that nonperfused trained, trained-ischemia-reperfusion, and vehicle-ischemia-reperfusion (I/R) groups had significantly different body weights and heart-to-body weight ratios compared with nonperfused controls, although heart weight did not differ.

### Table 2. Cardiac performance data

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Perfused Control</th>
<th>Control-I/R</th>
<th>Trained-I/R</th>
<th>Inhibited-I/R</th>
<th>Vehicle-I/R</th>
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<tbody>
<tr>
<td><strong>Preischemia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CF, ml/min−1·g 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>
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<tr>
<td>CO, ml/min−1·g 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>
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<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 × CO</td>
<td>4,629±177</td>
<td>4,856±137</td>
<td>4,474±195</td>
<td>5,448±260†</td>
<td>4,690±384</td>
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<tr>
<td>RPP, HR × SP</td>
<td>24,899±562</td>
<td>23,812±2,398</td>
<td>26,201±401</td>
<td>26,913±1,062</td>
<td>26,699±1,625</td>
</tr>
</tbody>
</table>

*Postischemia*  

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Perfused Control</th>
<th>Control-I/R</th>
<th>Trained-I/R</th>
<th>Inhibited-I/R</th>
<th>Vehicle-I/R</th>
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<tbody>
<tr>
<td>CF, ml/min−1·g wet wt−1</td>
<td>NA</td>
<td>7±1.1</td>
<td>10±5.4†</td>
<td>12±1.8†</td>
<td>8±1.3</td>
</tr>
<tr>
<td>CO, ml/min−1·g 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 × CO)</td>
<td>NA</td>
<td>1,615±295†</td>
<td>3,468±332†</td>
<td>4,635±285†</td>
<td>1,980±613*</td>
</tr>
<tr>
<td>RPP (HR × SP)</td>
<td>NA</td>
<td>19,615±1,461</td>
<td>23,661±614†</td>
<td>26,699±1,626†</td>
<td>2,005±3,606</td>
</tr>
</tbody>
</table>

Values are means ± SE. Note that most postischemic measurements (after 25 min of ischemia) were significantly decreased compared with preischemic measurements, indicating myocardial dysfunction. However, both exercise training and calpain inhibition resulted in significantly improved postischemia 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 with control-I/R animals. NA, not applicable. *Significantly different from preischemic values (P < 0.05), †significantly different from control-I/R (P < 0.05).
vitro isolated, perfused working heart model. This model is a highly reproducible preparation for examination of cardiac performance, as cardiac preload and afterload pressures are maintained constant. Furthermore, an advantage of the isolated, perfused working heart model vs. an in vivo I/R 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 were described previously (17, 18, 26).

I/R protocol. Hearts were perfused with a modified Krebs-Henseleit perfusion buffer containing (in mM) 1.25 CaCl₂, 130 NaCl, 5.4 KCl, 11 glucose, 0.5 MgCl₂, 0.5 NaH₂PO₄, and 25 NaHCO₃ and aerated with 95% O₂-5% CO₂. The simultaneous clamping of aortic and atrial lines induced global ischemia. After 30 min of perfusion during the preischemic protocol, ischemia was maintained for 25 min, followed by 45 min of reperfusion. During ischemia, the heart was enclosed in a sealed, water-jacketed chamber maintained at 37°C. After the ischemic period, the heart was switched to the retrograde perfusion mode for 10 min, followed by 10 min of assist mode (retrograde perfusion with the atrial cannula open) and 25 min of normal reperfusion.

Cardiac performance measurements. Cardiac performance measurements were made every 5 min before 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 with a customized computer data-acquisition system.

Calpain inhibition. To determine the effects of I/R-induced calpain activation on myocardial function, calpain was inhibited (in vitro) with the selective inhibitor MDL 28170 or calpain inhibitor three (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 DMSO and added to the perfusion buffer before heart perfusion at a concentration of 10⁻⁶ M. 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 I/R) was also included to demonstrate that our concentration of DMSO, without CI3, was not cardioprotective.

Fig. 2. Effects of I/R injury, exercise and calpain inhibition on lactate dehydrogenase (LDH) release. LDH activity measurements were made in coronary effluent collected before and after ischemia. Activity was normalized to heart wet weight and expressed as % difference between preischemic and postischemic values. Importantly, LDH release was increased significantly after ischemic injury. However, both exercise training and calpain inhibition attenuated the I/R-induced increase in LDH release. *Significantly different from control-I/R (P < 0.05).

Fig. 3. Analysis of calpain-cleaved talin. A: representative Western blot illustrating calpain-mediated cleavage of talin after experimental treatments. Intact talin (225 kDa) and breakdown product (BDP; 190 kDa) are as indicated. B: semiquantitative analysis of % calpain-cleaved talin protein present. Note that calpain activation was elevated with I/R (control-I/R and vehicle-I/R groups); however, both exercise (trained-I/R) and calpain inhibition (inhibited-I/R) attenuated the I/R-associated increase in calpain activation. Data are integrated from multiple Western blots. *Significantly different from nonperfused control (P < 0.05).

Fig. 4. Analysis of calpain-cleaved αII-spectrin. A: representative Western blot illustrating calpain-mediated cleavage of αII-spectrin after experimental treatments. Intact αII-spectrin (260 kDa) and BDP (150 kDa) are as indicated. B: semiquantitative analysis of % calpain-cleaved αII-spectrin protein present. Note that calpain activation was elevated with I/R (control-I/R and vehicle-I/R groups); however, both exercise (trained-I/R) and calpain inhibition (inhibited-I/R) attenuated the I/R-associated increase in calpain activation. Data are integrated from multiple Western blots. *Significantly different from nonperfused control (P < 0.05).
Tissue preparation. On the conclusion of nonperfusion, perfusion, or I/R treatments, the left ventricular free wall was immediately sectioned into four strips cut from base to apex. Before storage, heart sections were rinsed in a cold antioxidant buffer (in mM: 50 NaHPO₄, 0.1 butylated hydroxytoluene, and 0.1 EDTA). These tissue sections were then rapidly frozen in liquid nitrogen and stored at −80°C until subsequent biochemical analysis.

I/R-induced lactate dehydrogenase release. Lactate dehydrogenase (LDH) activity in the coronary effluent was used as an indication of I/R-induced myocardial injury. LDH activity was measured in triplicate as described previously (8, 17, 18). Measurements were made in coronary effluent collected before and after ischemia. LDH activities were then normalized to heart wet weight and expressed as the percent difference between preischemic and postischemic 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 nonperfused control and/or nonperfused trained groups was homogenized in cold 100 mM phosphate buffer with 0.5% bovine serum albumin (pH 7.4). Homogenates were centrifuged at 400 g for 10 min at 4°C. The resulting supernatant was used to determine protein content in addition to enzyme activities for SOD and catalase (Cat). Protein content was determined with the methods of Bradford (2). Total SOD activity as well as Mn-SOD and Cu/Zn-SOD 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 interassay 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 with 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). After washing, an anti-mouse or anti-rabbit IgG-horseradish peroxidase (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 with 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 nonperfused control group.

Fig. 5. Analysis of calpain-cleaved calpastatin. A: representative Western blot illustrating cleavage of calpastatin after experimental treatments. B: semiquantitative analysis of % cleaved calpastatin protein present. Note that calpastatin cleavage was elevated with I/R (control-I/R and vehicle-I/R groups); however, both exercise (trained-I/R) and calpain inhibition (inhibited-I/R) attenuated the I/R-associated increase in calpastatin cleavage. Data are integrated from multiple Western blots. *Significantly different from nonperfused control (P < 0.05).

Fig. 6. Analysis of intact calpastatin protein. A: representative Western blot illustrating calpastatin protein from experimental groups. B: semiquantitative analysis of calpastatin protein present. Note that I/R resulted in a significant decrease in intact calpastatin protein. *Significantly different from nonperfused control (P < 0.05). Data are integrated from multiple Western blots.
Western blot analysis of intact calpain, calpastatin, SERCA2a, and PLB. Briefly, proteins were separated with 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 calpain I, calpain II (Chemicon International, Temecula, CA), calpastatin (Triple Point Biologics), SERCA2a (Affinity Bioreagents, Golden, CO), or PLB (Upstate, Lake Placid, NY). After primary antibody exposure, an anti-mouse or anti-rabbit IgG-HRP-conjugated secondary antibody (Amersham) was applied for chemiluminescence detection. Each blot was analyzed with a Kodak imaging system (Kodak Image Station 440) and normalized to β-actin to adjust for protein loading. Results were then expressed as a percentage of nonperfused control.

Cardiac protein carbonyl levels. Protein carbonyls are formed by a variety of oxidative mechanisms and are sensitive indexes of oxidative injury (3). Carbonyl formation was determined in left ventricle homogenates from all experimental groups with 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 I/R, 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 among 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 in vitro I/R protocol was successfully performed on 6–12 hearts from each experimental group. The preischemia and postreperfusion cardiac performance data are presented in Table 2. As
expected, compared with preischemia, most indexes of cardiac performance were significantly depressed after I/R. It should be noted that postreperfusion cardiac output, heart rate, cardiac work, and rate-pressure product were preserved in exercise-trained and calpain-inhibited animals compared with sedentary controls. Cardiac work measurements for each group are also displayed, expressed as % recovery, in Fig. 1. Importantly, exercise-trained and calpain-inhibited hearts both recovered significantly better than control hearts in response to I/R. Additionally, LDH activity was measured in coronary effluent before and after ischemia to assess myocardial damage. These data, presented in Fig. 2, demonstrate an increase in LDH release during reperfusion after 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 I/R injury.

Calpain activity. In vivo calpain activity in the left ventricle was assessed via the proteolytic cleavage of three calpain substrates, talin, α1-spectrin, and calpastatin, illustrated in Figs. 3–6. Compared with perfused control hearts, I/R induced a significant increase in calpain cleavage of all three substrates (Figs. 3–5) as well as a decrease in intact calpastatin (Fig. 6). Additionally, the I/R-associated increase in calpain substrate cleavage was attenuated in exercised and calpain-inhibited hearts exposed to I/R. This indicates that our calpain inhibitor (CI3) successfully inhibited activation of calpain, and, similarly, exercise, through unknown mechanisms, also attenuated the I/R-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 Figs. 6–8, reveal that neither calpain nor calpastatin protein contents were altered with exercise training before I/R. 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-treated animals and are 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 after I/R, which was attenuated in exercise-trained animals (Fig. 9).

SERCA2a and PLB protein. Intact SERCA2a and PLB protein were measured by Western blotting. The results indicate a significant decrease in both proteins resulting from I/R, which was attenuated by both exercise training and calpain inhibition (Figs. 10 and 11).

DISCUSSION

These experiments tested the hypothesis that endurance exercise training would provide cardioprotection against I/R-induced injury, at least in part, by attenuating the I/R-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 I/R-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 I/R-induced degradation of the Ca\(^{2+}\)-ATPase SERCA2a along with the Ca\(^{2+}\) regulatory protein PLB, which was prevented by exercise training. Collectively, these findings advance our understanding of the mechanism(s) responsible for both I/R-induced injury and cardioprotection and are discussed in the following paragraphs.

Calpain inhibition attenuates I/R-induced contractile dysfunction. It is well established that myocardial I/R results in elevated cytosolic levels of Ca\(^{2+}\) that lead to the activation of the Ca\(^{2+}\)-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,

![Fig. 9. Effects of I/R injury and exercise on the formation of protein carbonyls. An increase in oxidative stress, measured by protein carbonyl formation, was observed after I/R. Importantly, exercise training significantly attenuated oxidative stress after I/R. Values are means ± SE. *Significantly different from perfused control (P < 0.05).](http://ajpheart.physiology.org/)
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 I/R-associated injury.

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

Exercise retards I/R-induced calpain activation. Although it is well established that exercise provides protection against I/R-induced cardiac injury, the mechanism(s) responsible for this form of cardioprotection remains unknown. We postulated that exercise-induced cardioprotection against I/R injury could be due, in part, to diminished calpain activation during the I/R insult. Our calpain activity data (Figs. 3–6) were consistent with this hypothesis. Therefore, we also investigated several potential mechanisms responsible for exercise-induced protection against I/R-induced calpain activation. Theoretically, exercise-induced protection against I/R-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 Ca^{2+}/H^{+} homeostasis in the heart during I/R.

The ratio of calpain to calpastatin in cells is physiologically important because this ratio greatly influences the ability of Ca^{2+} 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 I/R-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 (Figs. 6–8). Therefore, by process of elimination, we hypothesize that exercise diminishes I/R-induced calpain activation in the heart by improving maintenance of Ca^{2+}/H^{+} 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 I/R-induced calpain activation. The question remains, however: How does exercise training retard I/R-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 I/R-induced oxidative stress, which in turn, retards oxidative stress-induced Ca^{2+} overload within cardiac myocytes. Indeed, our results reveal that exercise attenuated the I/R-induced increase in myocardial oxidative stress (Fig. 9), presumably
through an increase in myocardial antioxidant enzymes (Table 3). Specifically, the myocardial increase in the endogenous antioxidant Mn-SOD after several days of exercise training has been shown to play a protective role against I/R-induced oxidative injury (15, 33, 34, 38).

Although the relationships among ROS, Ca\(^{2+}\) homeostasis, and calpain activation during I/R are complex and not completely understood, oxidative stress can increase intracellular Ca\(^{2+}\) through interaction with the sarcoplasmic reticulum and/or plasma membrane (16, 32). Specifically, ROS have been shown to affect Ca\(^{2+}\) homeostasis in several possible ways, such as oxidation of sulfhydryl groups located on Ca\(^{2+}\) transport proteins, peroxidation of membrane lipids, and inhibition of membrane-bound regulatory enzymes (16). Accordingly, Ca\(^{2+}\)-ATPases are likely targets for free radicals during I/R. Superoxide, hydrogen peroxide, and the hydroxyl radical have also been implicated in Ca\(^{2+}\)-ATPase modification and inhibition (16). In addition, modification of Ca\(^{2+}\)-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\(^{2+}\) homeostasis, resulting in increased calpain activation. Hence, it seems plausible that the exercise-induced increases in myocardial antioxidants and the associated prevention of I/R-associated oxidative stress may serve to maintain free cytosolic Ca\(^{2+}\) 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 after I/R compared with hearts from sedentary control animals (Figs. 3–6 and 9).

**Exercise retards I/R-induced SERCA2a and PLB degradation.** To further investigate the idea of free radical interaction with Ca\(^{2+}\)-handling proteins, we measured protein levels of intact SERCA2a, the most abundant Ca\(^{2+}\)-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 after I/R (28, 32). Our present work supports these findings, demonstrating a 60% loss in SERCA2a protein and a 52% loss in PLB protein after I/R. Moreover, work by Temsah et al. (32) reveals that addition of the antioxidant SOD and Cat attenuated the I/R-induced loss of SERCA2a function and mRNA in the heart. These findings are also supported by the present study, which demonstrated an increase in myocardial Mn-SOD and Cat along with a preservation of SERCA2a and PLB protein in exercise-trained animals (Table 3, Figs. 10 and 11). Although, Temsah et al. (32) did not detect preservation of PLB protein after I/R with the administration of their antioxidant treatment, we observed that exercise training preserved PLB protein after I/R (Fig. 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 Cat. It is also possible that exercise training may prevent PLB degradation through another, unknown, mechanism.

Importantly, the present study is among the first to demonstrate that calpain inhibition prevents the I/R-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\(^{2+}\) through the cleavage of SERCA2a and/or PLB. Recent work by Sing et al. (28) also reported attenuation of SERCA2a cleavage with a calpain inhibitor, although the inhibitor used in their study, leupeptin, has also been found to have antioxidant properties. This could be confounding based on the possibility of interaction between free radicals and Ca\(^{2+}\)-ATPases.

In conclusion, this work confirms the deleterious role of I/R-induced calpain activation. Importantly, this is the first experiment to investigate the effects of endurance exercise on I/R-induced calpain activation in the heart. Our findings clearly support the hypothesis that endurance exercise training attenuates the I/R-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. Furthermore, our findings confirm that I/R-induced myocardial oxidative stress is attenuated by the improved myocardial antioxidant capacity of the exercise-trained animals. Finally, our findings are consistent with the concept that regulation of redox balance and calpain activation play a significant role in cardioprotection against I/R injury in the myocardium. Additional experiments are required to provide further insight into the specific mechanism(s) responsible for protection against I/R-induced calpain activation.

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**REFERENCES**

Hearse DJ and Sutherland FJ.


