Effects of 4-h ischemia and 1-h reperfusion on rat muscle sarcoplasmic reticulum function

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The central events resulting in cellular injury from periods of IR appear to occur primarily during reperfusion (32). In fact, skeletal muscle injury is normally greatest in areas with the highest degree of blood flow during reperfusion (46). It is thought that a major mechanism leading to IR injury in both skeletal and cardiac muscle is an increased production of oxygen (O2) free radicals (40, 44). Because the potential sources of O2 free radical production during reperfusion are greater compared with ischemia alone, this may explain the unexpected deleterious effects associated with reperfusion after acute ischemia.

With the loss of ATP and total adenine nucleotides during ischemia (4, 7, 21, 22, 35, 43, 52), the primary source of O2 free radical production during the ischemic period is the enzyme xanthine oxidase. During reperfusion, potential sources of O2 free radical production include, but are not limited to, the muscle cell (xanthine oxidase, mitochondria, nitric oxide synthase (NOS) pathway), the capillary endothelial cell (xanthine oxidase, NOS pathway), and the neutrophil (NADPH oxidase, myeloperoxidase) (31, 44, 46). Consequently, there is a greater variety of molecular oxygen species generated during reperfusion than during ischemia alone and, therefore, more potential target sites of attack.

One potential site of attack by O2 free radicals that are produced during IR is the sarcoplasmic reticulum (SR) Ca2+-ATPase (27). Reductions in SR Ca2+ uptake with myocardial IR are well documented (34). Both short and more-prolonged periods of myocardial ischemia result in a decreased SR Ca2+ uptake, whereas reperfusion after only prolonged periods of ischemia (>30 min) leads to further reductions in SR Ca2+ uptake (34). Reperfusion after short periods of ischemia (<30 min) normalizes SR Ca2+ uptake to control levels despite sustained contractile dysfunction (23, 34). For this reason, it is thought that impaired SR function is not necessarily related to IR-induced contractile dysfunction or myocardial stunning (23).

Surprisingly little research has been completed addressing the role of IR on SR function in skeletal muscle. We have shown that prolonged ischemia alone

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results in a time-dependent increase in maximal SR Ca\(^{2+}\)-ATPase activity measured in homogenates prepared from rat skeletal muscle (14). Moreover, we have shown that reperfusion of skeletal muscle after ischemia leads to a reduction in SR Ca\(^{2+}\)-ATPase activity, the magnitude of which depends on the length of the ischemic period (1–3 h) (15). Interestingly, recovery of both muscle mechanical and metabolic function also appeared to depend on the duration of ischemia (4). Although substantial recovery of both properties was observed during reperfusion with short-duration ischemia, such was not the case when the ischemic period was extended. The impaired mechanical recovery during reperfusion could be due directly to an inhibiting effect of metabolic accumulation on the myofibrillar proteins (6) or to an impairment in SR Ca\(^{2+}\) cycling, either as a consequence of metabolic inhibition (59) or as structural alterations to specific SR proteins (10, 34). In our earlier study, changes in SR during IR were limited to determinations of maximal Ca\(^{2+}\) ATPase activity in crude homogenates (15). Consequently, it is unclear whether SR Ca\(^{2+}\) uptake and SR Ca\(^{2+}\) release were also altered and whether similar effects would be observed in crude homogenates and SR-enriched fractions. Reports have been published indicating that ischemia may result in a differential yield of the SR function (41, 56), potentially obscuring determination of the actual effects of ischemia.

It has also been shown that pretreatment with oxygen free radical scavengers superoxide dismutase (SOD) and catalase (CAT) maintained higher Ca\(^{2+}\) uptake by the SR of skeletal muscle after 3 h of ischemia and 19 h of reperfusion in rat hindlimb (30). Thus free radical formation is likely a mechanism for impaired SR function with IR in skeletal muscle. In a recent study, we observed a reduction in maximal SR Ca\(^{2+}\)-ATPase activity after 4 h of ischemia in rat skeletal muscle (54). The reduction in Ca\(^{2+}\)-ATPase, which was associated with a reduction in fluorescein isothiocyanate (FITC) binding, is apparently due to oxidation of one or more cysteine residues within the nucleotide binding domain (54).

In the present study, our major objective was to determine the effects of reperfusion after prolonged ischemia on SR Ca\(^{2+}\) uptake, Ca\(^{2+}\) release, and Ca\(^{2+}\)-ATPase function in homogenates and isolated SR fractions from rat skeletal muscle. On the basis of previous work, we expected that SR function would be impaired even further with reperfusion due to more potential sources of O\(_2\) free radical production generated during reperfusion. As a secondary objective, we examined treatment with oxygen free radical scavengers (SOD + CAT) just before reperfusion, hypothesizing that SOD and CAT would improve SR function compared with IR alone by attenuating the structural changes observed in the nucleotide binding site of SR Ca\(^{2+}\)-ATPase as measured with FITC binding. The efficacy of SOD + CAT treatment for improving force recovery during reperfusion was also tested in a separate group of animals by monitoring peak twitch force output of the gastrocnemius-plantaris-soleus complex during ischemia and reperfusion.

METHODS

Animal Description and Care

Adult female Sprague-Dawley rats weighing 290 ± 3.5 g (means ± SE) were housed in an environmentally controlled room (temperature 22–24°C, 40–60% relative humidity) with reversed light-dark cycles. Animals were fed ad libitum on laboratory chow and water until the time of the experiment. All experiments were initiated at approximately the same time each day to avoid large diurnal variations in muscle glycogen. Experimental protocols were approved by the Animal Care Committee of the University of Waterloo.

Experimental Groups

To investigate the effects of complete ischemia followed by reperfusion on SR function, animals were randomly assigned to control control (CC; n = 10) and two ischemic experimental (n = 20) groups. Experimental groups were divided on the basis of the type of treatment they received. In the treatment (T) group, a single dose of SOD at 60,000 U/kg plus catalase (CAT) at 500,000 U/kg, dissolved in 2 ml of sterile saline, was administered intravenously through a tail vein 10–20 min before reperfusion. The other experimental (I) group was given 2 ml of sterile saline before reperfusion. The CC group did not receive any treatment. For each experimental animal, 4 h of total ischemia and 1 h of reperfusion were randomly assigned to one hindlimb (I or IT), whereas the contralateral limb served as a control limb (C or CT). Due to tissue requirements for the isolation procedure used to obtain SR vesicles, experiments were conducted on one CC, two I, and two IT animals each day.

Experimental Protocol

Before the induction of ischemia, the rats were weighed and anesthetized. Anesthesia was initially accomplished using an intraperitoneal injection of pentobarbital sodium (6 mg/100 g body wt) and then was maintained using supplementary intraperitoneal injections as required. Throughout the ischemic periods, body temperature was maintained between 37 and 39°C by having the rats lie in a prone position on a warm heating pad. Ischemia was induced by placing a tourniquet around the upper hindlimb and proximal to the knee joint. To ensure total occlusion of blood flow to the hindlimb, a 350-mmHg pressure was employed (12). Total ischemia was confirmed on the basis of almost total depletion of muscle phosphocreatine (PCr) and ATP after 4 h of ischemia (unpublished results). Reperfusion consisted of simply deflating and removing the tourniquet. After removal of the tourniquet, the area directly under the tourniquet was also massaged for ~2 min to help support blood flow (30).

At the end of the 1-h reperfusion period, a small piece of white gastrocnemius muscle was rapidly sampled from each of the I and T limbs and frozen in liquid nitrogen for later analysis of muscle metabolites. The remainder of the gastrocnemius muscle (both red and white portions), along with the entire tibialis anterior (TA) muscle, was excised and placed in ice-cold buffer to be used for SR isolation by differential centrifugation. The gastrocnemius and TA muscles from each limb of the CC animal were sampled in the same manner immediately after anesthetization.
**Sample Preparation for SR Assessment In Vitro**

All muscles were prepared according to Heilman et al. (20). Mixed gastrocnemius and TA muscles were diuted ~1:5 (wt/vol) in homogenizing buffer containing (in mM) HEPES (pH 7.5), 250 sucrose, 0.2 phenylmethylsulfonyl fluoride [no dithiothreitol (DTT)], and 0.2% sodium azide and mechanically homogenized with a polytron homogenizer (PT 3100) at 16,500 rpm, for 2 × 30-s bursts. Properties of the SR were examined in both homogenates and SR membrane fractions. To obtain an enriched SR membrane fraction, a combination of two SR isolation protocols was used (8, 20). The homogenate was centrifuged at 5,500 g for 10 min to remove cellular debris, and the supernatant was filtered through four layers of gauze to remove as much fat as possible. The supernatant was then transferred to clean tubes and centrifuged at 12,500 g for 18 min. These pellets were discarded, and the spin was repeated. Again, the supernatant was transferred to clean tubes and centrifuged at 50,000 g for 52 min. These pellets were resuspended in 10 ml of homogenizing buffer plus 600 mM KCl and allowed to incubate at 4°C for 30 min. This suspension was then centrifuged at 15,000 g for 10 min to pellet nearly all of the mitochondria. The supernatant was centrifuged at 50,000 g for 52 min. The final pellet, enriched in SR membranes (no sucrose cushion), was resuspended in homogenizing buffer at a protein concentration of 2–6 mg/ml. SR isolation was carried out by differential centrifugation with the use of a Beckmann Ultracentrifuge with a 70.1 Ti fixed-angle rotor.

**Analytical Procedures**

**Muscle metabolite analysis.** To determine the effects of ischemia on muscle metabolic behavior, metabolites of the high-energy phosphate system [PCr, creatine (Cr), P, glycogen (Gly), and lactate (Lac)] were measured. Metabolites were measured in freeze-dried tissue after perchloric acid (PCA) extraction by fluorometric procedures, as previously reported (16). The muscle Gly concentration was determined from the pellet of the PCA extract (acid-insoluble fraction). All concentrations were expressed as micromoles per gram of dry weight.

**SR Ca2+ uptake measurements.** Oxalate-supported Ca2+ uptake was measured in muscle homogenates and purified SR by means of the Ca2+-fluorescent dye Indo-1 according to methods of O’Brien (37) and O’Brien et al. (39), with minor modifications. Fluorescence measurements were made on a spectrofluorometer (RatioMaster system, Photon Technology International) equipped with dual emission monochromators. The measurement of free calcium ([Ca2+]f) using the Indo-1 procedure is based on the difference in the maximal emission wavelengths between the Ca2+-bound form of Indo-1 and the Ca2+-free form. The excitation wavelength was 355 nm, and the emission maxima were 485 and 405 nm for Ca2+-free (G) and Ca2+-bound (F) Indo-1, respectively. Photon counts per second were recorded simultaneously for both emission wavelengths. The Ca2+-independent [background] fluorescence was measured in the reaction medium (without Indo-1) at each emission wavelength before the experiment was started. Background fluorescence was automatically corrected before the start of each assay by use of the Felix software (Photon Technology International).

The reaction buffer for muscle homogenates and for SR fractions (in parentheses) contained 200 (100) mM KCl, 20 mM HEPES, 10 mM NaN3, 5 µM N,N,N’,N’-tetakis(2-pyridylmethyl)-ethylenediamine, 5 mM oxalate, 15 (10) mM MgCl2 and 10 mM phosphoenolpyruvate, pH 7.0. Before emission spectra were collected, 18 U/ml each of lactate dehydrogenase and pyruvate kinase and 1.5 µM Indo-1 were added to a cuvette containing 2 ml of reaction buffer. Also, 3 µl of CaCl2 (10 mM) were added in each trial to achieve an initial [Ca2+]f, before the reaction was started, of ~2.5 µM with isolated SR and 2.0 µM with homogenates. Immediately after data collection was initiated, 40 µl of homogenate (20 µl SR) were added to the cuvette. Shortly after the addition of homogenate or SR, 5 mM ATP were added to initiate Ca2+ uptake.

As Ca2+ decreases because of active SR Ca2+ uptake, F decreases, G increases, and the ratio of F to G decreases. The ratio (R) is used to calculate [Ca2+]f. With the use of Felix software, the ionized Ca2+ concentration was calculated by the following equation (18)

\[
[Ca^{2+}]_f = K_d \cdot \frac{G_{\text{max}}}{\text{G}_{\text{min}}} \left( \frac{\text{R}_{\text{min}} - \text{R}}{\text{R}_{\text{max}} - \text{R}} \right) \tag{1}
\]

where \(K_d\) is the equilibrium constant for the interaction between Ca2+ and Indo-1, \(\text{R}_{\text{min}}\) is the minimum value of R at an addition of 250 µM EGTA, \(G_{\text{max}}\) is the maximum value of G at addition of 250 µM EGTA, \(G_{\text{min}}\) is the minimum value of G at addition of 1 mM CaCl2, and \(\text{R}_{\text{max}}\) is the maximum value of R at addition of 1 mM CaCl2. The \(K_d\) value for the Ca2+-dye complex is 250 and 135 mM for muscle homogenates and purified SR vesicles, respectively (18). For all Ca2+ uptake trials, \(\text{R}_{\text{min}}\) and \(\text{R}_{\text{max}}\) were not determined until Ca2+ uptake had plateaued, which occurs at ~100 nM [Ca2+]f.

Before the rate of Ca2+ uptake was calculated, the generated curve from Eq. 1, [Ca2+]f vs. time, was smoothed over 21 points by use of the Savitsky-Golay algorithm. The rate of Ca2+ uptake was then analyzed at four separate [Ca2+]f concentrations (0.5, 1.0, 1.5, and 2.0 µM) for isolated SR and only the lowest three [Ca2+]f concentrations for muscle homogenates. First, linear regression was done on a range of values 100 nM above and below the desired [Ca2+]f. The rate of Ca2+ uptake was then determined by differentiating the linear-fit curve and expressed in µmoles·g protein−1·min−1.

**SR Ca2+ release measurements.** Ca2+ release was measured on both muscle homogenates and SR vesicles according to the methods of Ruell et al. (45), with minor modifications. Ca2+ release assays were conducted as for Ca2+ uptake, except that when the [Ca2+]f declined to a plateau, 3 µl of AgNO3 were added to give a final concentration of 141 µM. The reaction was then allowed to proceed for ~3 min. With the addition of AgNO3, Ca2+ release consistently proceeded in two distinct phases. There was an initial rapid rate of release (phase 1), followed by a slower, more prolonged rate of release (phase 2). As for Ca2+ uptake, the generated curve from Eq. 1 was smoothed over 21 points and differentiated. The maximal rate of Ca2+ release was calculated by taking the maximum positive derivative for each phase and was expressed in micromoles per gram per minute.

**SR Ca2+-ATPase activity measurements.** Spectropho-

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**SR Ca2+-ATPase activity measurements.** Spectrophotometric (Schimadzu UV 160U) measurement of SR Ca2+-ATPase activity was performed on homogenates (51) and SR samples (29) with minor modifications. Total (Mg2+-activated) ATPase activity was measured in the presence of the Ca2+-ionophore A23187 across a range of CaCl2 concentrations. Basal activity was measured in the presence of 40 µM cyclopiazonic acid, which completely inhibits SR Ca2+-ATPase activity (49). The difference between total and basal activities represents the Ca2+-activated ATPase activity. Maximal activity and the Ca2+-dependency of Ca2+-ATPase activity were assessed by adding 1–11 µl of 100 mM CaCl2 in 0.5-µl additions. Ca2+-ATPase activity increases with [Ca2+]f until a plateau occurs once maximal activity is reached. The [Ca2+]f corresponding to each CaCl2 addition was assessed separately, on a different SR aliquot, by use of
dual-wavelength spectrofluorometry and the Ca\(^{2+}\)-fluorescent dye Indo-1. Maximal activity occurred at a [Ca\(^{2+}\)]\(_{\text{r}}\) of 6–10 \(\mu\)M for all groups. Ca\(^{2+}\)-ATPase activity was then plotted against the negative logarithm of [Ca\(^{2+}\)]\(_{\text{r}}\) (pCa), and the Hill coefficient, along with the [Ca\(^{2+}\)]\(_{\text{r}}\), which gives half-maximal activity (Ca\(_{50}\)), was determined using the equation

\[
Y = Y_{\text{bot}} + Y_{\text{top}} - Y_{\text{bot}}/1 + 10^{\log\text{Ca}^{2+} - z} \cdot n_H
\]

where \(Y_{\text{bot}}\) is the value at the bottom of the plateau, \(Y_{\text{top}}\) the value at the top of the plateau, log Ca\(_{50}\) is the logarithm of Ca\(_{50}\), the concentration that gives a response halfway to \(Y_{\text{bot}}\) and \(Y_{\text{top}}\), and \(n_H\) is the Hill slope or Hill coefficient. For calculation of the properties, only a portion of the curve that corresponded to a value between 20 and 80% of maximal activity was used. For SR Ca\(^{2+}\) uptake, uptake, release, and ATPase assays, protein was determined by the method of Lowry, as modified by Schacterle and Pollock (47). On a given day, an equal number of samples from each condition was analyzed in duplicate.

**Fluorescence measurements.** Fluorescence measurements were made on an SLM-4800S spectrofluorometer (SLM Instruments, Urbana, IL). FITC (Sigma) and N-cyclohexyl-N'-dimethylaminocyclohexylcarbodiimide (NCD-4; Molecular Probes) were stored at a concentration of 5 mM in ethanol at \(-20^\circ\)C. FITC emission spectra (490–550 nm) were collected by exciting samples at 490 nm. FITC labeling was done by washing the SR samples once in wash buffer with no dithiothreitol (DTT) and then resuspending the samples in FITC labeling buffer (wash buffer + 2.5 \(\mu\)M FITC, pH 8.8) and vortexing gently in darkness for 20 min at 25°C. The SR samples were then washed again in ordinary wash buffer to remove unbound label. NCD-4 emission spectra were collected by exciting samples at 340 nm and scanning the emission intensity from 400 to 430 nM at 1-nm increments. NCD-4 labeling was done by washing the SR samples once in wash buffer with no DTT and then resuspending in NCD-4 labeling buffer (wash buffer + 150 \(\mu\)M NCD-4, pH 6.2) and incubating in darkness for 3 h at 25°C. As before, the sample was washed to remove unbound label. These procedures were essentially as previously reported (28).

**Muscle force measurements.** In a separate group of I and IT animals (\(n=5\)), the effects of IR on electrically evoked muscle twitch force were assessed for the gastrocnemius-plantaris-soleus complex from each hindlimb. Surgical preparation and the animal stimulation apparatus have been described in detail elsewhere (4, 5). For all animals, peak twitch force (\(P_t\)) was measured at rest and at 15, 30, 45, 60, 120, 180, and 240 min of ischemia and at 5, 10, 15, 30, 45, and 60 min of reperfusion. Twitch force was obtained via direct muscle stimulation, with the use of stainless steel electrodes and with a single 0.2-ms pulse at 70 V. Muscle stimulation was performed using a Grass S48 Stimulator, and force data were collected on-line and analyzed using the WatSCOPE Data Acquisition Unit and Software (Northern Digital). An independent shunt calibration was performed daily for each force transducer. A shunt calibration was employed to avoid problems associated with mechanical calibration. Before induction of ischemia, optimal length (\(L_o\)) for maximal \(P_t\) and maximum voltage for optimum muscle twitch force were established. In determining \(L_o\), muscle Twitches were separated by 30 s to avoid a fatigue or potentiation effect. After \(L_o\) and supramaximal voltage were established, the muscle was allowed to equilibrate for 5 min before ischemia was induced. Twitch force was expressed relative to the initial resting value and was calculated as a percentage of initial force.

**Statistical Analysis**

For all measurements, a one-way ANOVA was used to test for differences between means. Where significant differences were found, Tukey’s post hoc tests were used to compare specific means. For all comparisons, statistical significance was accepted at \(P<0.05\). All data are expressed as means \(\pm\) SE.

**RESULTS**

**Muscle Metabolites**

Muscle metabolites for each group are shown in Table 1. With the exception of muscle Gly, there were no differences between CC, C, and CT for any of the metabolites measured. Although muscle Gly was \(~10\%\) lower (\(P<0.05\)) in both C and CT compared with CC, this difference was considerably less than the 86% reduction (\(P<0.05\)) in Gly measured in I and IT, compared with CC. As expected, muscle ATP and PCr were nearly completely depleted in I and IT. Muscle Cr was \(~1.5\)-fold higher (\(P<0.05\)) in both I and IT compared with CC, C, and CT. Similarly, \(P_l\) was \(~2.8\)-fold higher (\(P<0.05\)), and Lac was \(~5.7\)-fold higher (\(P<0.05\)) in I and IT, compared with CC, C, and CT.

**Ca\(^{2+}\)**-ATPase Activity

Surprisingly, there were no significant differences between groups in maximal SR Ca\(^{2+}\)-ATPase activity measured in both muscle homogenates and isolated SR vesicles between groups (Table 2). Moreover, kinetic analysis of the Ca\(^{2+}\)-ATPase activity-pCa curves showed that both the Hill coefficient and the Ca\(_{50}\) were not different between groups in either muscle homogenates or isolated SR vesicles (Table 2).

Table 1. Effects of ischemia and reperfusion on muscle metabolite concentrations

<table>
<thead>
<tr>
<th></th>
<th>CC</th>
<th>C</th>
<th>I</th>
<th>CT</th>
<th>IT</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>28.2 (\pm) 0.6</td>
<td>28.3 (\pm) 0.3</td>
<td>21.1 (\pm) 0.6*</td>
<td>27.6 (\pm) 0.5</td>
<td>2.4 (\pm) 1.1*</td>
</tr>
<tr>
<td>PCr</td>
<td>64.3 (\pm) 8.2</td>
<td>57.9 (\pm) 9.3</td>
<td>5.7 (\pm) 1.3*</td>
<td>64.7 (\pm) 3.2</td>
<td>5.5 (\pm) 2.7*</td>
</tr>
<tr>
<td>Cr</td>
<td>102.6 (\pm) 8.5</td>
<td>107 (\pm) 9.5</td>
<td>158 (\pm) 8.4*</td>
<td>102 (\pm) 4.5</td>
<td>157 (\pm) 8.4*</td>
</tr>
<tr>
<td>P_l</td>
<td>63.5 (\pm) 8.6</td>
<td>62.6 (\pm) 9.9</td>
<td>175 (\pm) 5.9*</td>
<td>50.1 (\pm) 1.9</td>
<td>176 (\pm) 8.1*</td>
</tr>
<tr>
<td>Lac</td>
<td>33.6 (\pm) 7.8</td>
<td>39.4 (\pm) 9.3</td>
<td>189 (\pm) 15.7*</td>
<td>30.6 (\pm) 3.1</td>
<td>206 (\pm) 17.2*</td>
</tr>
<tr>
<td>Gly</td>
<td>132 (\pm) 6.2</td>
<td>119 (\pm) 5.4*</td>
<td>19.1 (\pm) 3.1</td>
<td>116 (\pm) 4.5*</td>
<td>17.5 (\pm) 2.7*</td>
</tr>
</tbody>
</table>

Values are means \(\pm\) SE in micromoles per gram dry weight except for glycogen (Gly), which is in micromolar glucosyl units per gram dry weight. CC, control control; C, control; I, ischemia; CT, control treatment; IT, ischemia treatment. PCr, phosphocreatine; Cr, creatine; P_l, inorganic phosphate; Lac, lactate. For each group, \(n=9\). *Significantly different (\(P<0.05\)) from CC, C, and CT; †significantly different (\(P<0.05\)) from CC.
Table 2. Kinetic properties of the Ca$^{2+}$-ATPase activity-pCa curves in homogenates and isolated SR vesicles

<table>
<thead>
<tr>
<th></th>
<th>CC</th>
<th>C</th>
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<th>CT</th>
<th>IT</th>
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<tr>
<td>Homogenates</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>783 ± 63</td>
<td>790 ± 73</td>
<td>842 ± 67</td>
<td>779 ± 46</td>
<td>799 ± 36</td>
</tr>
<tr>
<td>Hill coefficient</td>
<td>1.4 ± 0.3</td>
<td>1.3 ± 0.3</td>
<td>1.3 ± 0.1</td>
<td>1.5 ± 0.5</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>Ca$_{50}$</td>
<td>6.3 ± 0.1</td>
<td>6.2 ± 0.1</td>
<td>6.2 ± 0.1</td>
<td>6.4 ± 0.1</td>
<td>6.3 ± 0.1</td>
</tr>
<tr>
<td>SR vesicles</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>11,296 ± 899</td>
<td>10,642 ± 1130</td>
<td>10,303 ± 1052</td>
<td>11,096 ± 723</td>
<td>9,847 ± 668</td>
</tr>
<tr>
<td>Hill coefficient</td>
<td>1.1 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>1.1 ± 0.1</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Ca$_{50}$</td>
<td>6.0 ± 0.1</td>
<td>6.1 ± 0.1</td>
<td>6.0 ± 0.1</td>
<td>6.1 ± 0.1</td>
<td>6.1 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n = 10$ per group. $V_{\text{max}}$, maximal Ca$^{2+}$-ATPase activity in micromoles per gram of protein per minute; SR, sarcoplasmic reticulum. Hill coefficient was obtained from Hill plots of the section of the Ca$^{2+}$ activity vs. free calcium concentration ([Ca$^{2+}$]$_f$) corresponding to 20–80% of normal activity. Ca$_{50}$ is defined as the calcium concentration (pCa) at half-normal activity. Maximal SR Ca$^{2+}$-ATPase activity occurred at a [Ca$^{2+}$]$_f$ at −6–10 μM in all groups.

**Fluorescence Measurements**

FITC and NCD-4 binding were used as a method to assess the structure of the nucleotide binding domain and the Ca$^{2+}$ binding sites of the Ca$^{2+}$-ATPase, respectively. There were no differences among any of the groups in either FITC (Fig. 1A) or NCD-4 (Fig. 1B) binding.

**Ca$^{2+}$ Uptake**

Ca$^{2+}$ uptake was assessed at three and four different [Ca$^{2+}$]$_f$ concentrations in muscle homogenates and purified SR vesicles, respectively. In muscle homogenates, Ca$^{2+}$ uptake was 20–25% lower ($P < 0.05$) in C compared with CC, measured at both 0.5 and 1.0 μM [Ca$^{2+}$]$_f$ compared with CC (Fig. 2). Ca$^{2+}$ uptake was also 15–17% lower ($P < 0.05$) in C compared with CT at 0.5 and 1.5 μM [Ca$^{2+}$]$_f$ with a strong trend ($P = 0.08$) toward lower Ca$^{2+}$ uptake in C compared with CT at 1.0 μM [Ca$^{2+}$]$_f$. There was also a strong trend ($P = 0.08$) toward lower Ca$^{2+}$ uptake in IT compared with CC, measured at both 0.5 and 1.0 μM [Ca$^{2+}$]$_f$. There were no differences among CC, CT, I, and IT at any [Ca$^{2+}$]$_f$. In purified SR vesicles, Ca$^{2+}$ uptake was lower by 30–38% ($P < 0.05$) in both I and IT across all [Ca$^{2+}$]$_f$ compared with CC (Fig. 3). Ca$^{2+}$ uptake was also ~25% lower ($P < 0.05$) in IT compared with CT across all [Ca$^{2+}$]$_f$. There were no differences among CC, C, and CT in Ca$^{2+}$ uptake; however, at 1.0 μM [Ca$^{2+}$]$_f$, there was a trend ($P = 0.09$) toward lower Ca$^{2+}$ uptake in C compared with CC.

**Ca$^{2+}$ Release**

Due to the biphasic response of Ca$^{2+}$ release, we obtained two measures of maximal Ca$^{2+}$ release corresponding to each phase for each trial. In muscle ho-
mog enates, there were no differences in AgNO₃-induced Ca²⁺ release among any of the groups for either phase 1 or phase 2 (Fig. 4). However, in purified SR vesicles, maximal Ca²⁺ release was 31% higher (P < 0.05) in I compared with CC for phase 1, with no differences among any of the other groups (Fig. 5). No differences existed among any of the groups for phase 2 in purified SR vesicles.

**Force Measurements**

The changes in Pᵢ with IR are presented in Fig. 6. Just after 1 h of ischemia, the muscle was incapable of producing force and remained in this state for the duration of the ischemic protocol. Reperfusion for 1 h did not result in force recovery. Treatment with SOD + CAT just before reperfusion had no effect on force recovery in the gastrocnemius-plantaris-soleus complex after ischemia. Importantly, Pᵢ force output was maintained in C and CT throughout the IR protocol. There were no differences in Pᵢ between C and CT at any time point.

**DISCUSSION**

In this study, our major objective was to assess the effects of reperfusion after prolonged ischemia on skeletal muscle SR function in vitro. Previously, we had shown that prolonged ischemia alone for 4 h resulted in reduced Ca²⁺ release, Ca²⁺ uptake, and maximal Ca²⁺-ATPase activity in SR fractions, possibly due to increased O₂ free radical production (55). Because O₂ free radical production is believed to be even greater with reperfusion after acute ischemia, we hypothesized that SR function would be impaired even further with 4-h ischemia and 1-h reperfusion compared with 4-h ischemia alone. Moreover, we also postulated that treatment with free radical scavengers would attenuate the damage to the Ca²⁺-ATPase during IR, reducing the depression in activity.

Maximal Ca²⁺-ATPase activity and the kinetic properties, Hill coefficient, and Ca₅₀ were not different between IR and control groups in both muscle homogenates and isolated SR vesicles. Ca²⁺ uptake was ~30% lower in I compared with CC, however, only in isolated SR vesicles and not in homogenates. Somewhat unexpectedly, IR in one hindlimb led to reductions in homogenate Ca²⁺ uptake in the contralateral control limb compared with CC, with a similar trend observed in SR vesicles. Moreover, homogenate AgNO₃-induced Ca²⁺ release was not different among groups for both phases 1 and 2, whereas in isolated SR vesicles, phase 1 Ca²⁺ release was actually higher in I compared with CC. Although a similar trend was observed in homogenates, it was not significant.
different (P \leq 0.05) from C and CT and SE; n = 5 per group. *Significantly different (P < 0.05) from C and CT

Fig. 6. Changes in peak twitch tension (P_t) during ischemia and reperfusion (IR) of C, I, CT, and IT gastrocnemius-plantaris-soleus complex. Values are means ± SE; n = 5 per group. *Significantly different (P < 0.05) from C and CT

When these results in isolated SR fractions with the effects of ischemia alone are compared, it appears that SR function, including Ca^{2+}-ATPase activity, Ca^{2+} release, and Ca^{2+} uptake, recovers substantially with just 1 h of reperfusion in skeletal muscle. In the heart, SR function recovers after short periods of ischemia and reperfusion but is depressed even further with reperfusion after prolonged periods of ischemia (34). The absolute duration of the ischemic period is not comparable between skeletal muscle and cardiac muscle in terms of the time course and severity of injury that occur. However, a 4-h period of ischemia is comparable to prolonged ischemia in the heart in terms of the metabolic response (21, 22). Therefore, it is unclear why reperfusion after prolonged ischemia in skeletal muscle allows recovery of SR function, as occurred in this study, but not in the heart. In addition to tissue-specific differences, there are differences between the two preparations in the level of contractile activity. In the current study, skeletal muscles were generally inactive. However, the heart remains active. It is possible that the increased Ca^{2+} cycling and the elevated strain induced on the processes involved in Ca^{2+} uptake and Ca^{2+} release in the beating heart could exaggerate the reperfusion effects.

**Ca^{2+}-ATPase**

Numerous reports have shown that exposure of SR vesicles to O_2 free radicals in vitro leads to protein oxidation and a reduction in Ca^{2+}-ATPase activity (33). It is also well established that both O_2 free radicals and altered SR function play an important role in the etiology of IR injury in skeletal (44) and cardiac (34) muscle. Thus, clearly, we should have expected reductions in SR vesicle Ca^{2+}-ATPase activity in this study, as we had found with ischemia alone (54). It would appear that, during the reperfusion period, changes occur that allow restoration of SR Ca^{2+}-ATPase activity in SR fractions. A number of possible mechanisms exist to explain the effects of reperfusion.

It is possible that the reduction in Ca^{2+}-ATPase activity that occurs in vitro with ischemia alone is reversible with reperfusion or that recovery of Ca^{2+}-ATPase activity with reperfusion reflects recruitment of a latent pool of enzyme that is not activated even under optimal conditions and is unaffected by ischemia. With regard to the former possibility, considerable evidence exists to indicate that reductions in Ca^{2+}-ATPase activity occur as a result of structural alterations in the region of the nucleotide binding site (10). Because alterations of this nature do not appear to be readily reversed (24), this would not appear to be a viable mechanism in this study, given the length of the reperfusion period. If the latter is true, the latent pool of Ca^{2+} pumps would normally be oriented such that FITC would be unable to bind to Lys^{515}; otherwise, FITC binding should still have been reduced from ischemia (54). Interestingly, however, activation of the SR Ca^{2+}-ATPase above normal has been reported to occur during recovery from low-intensity exercise in rats (11).

It should also be noted that recovery of SR vesicle Ca^{2+}-ATPase activity during reperfusion would have to involve a mechanism independent of muscle metabolism. When measured in vitro, maximal SR Ca^{2+}-ATPase activity is reduced after 4-h ischemia in rat skeletal muscle despite being measured under supposedly optimal conditions (54). When the severe metabolic alterations that occur with 4-h ischemia in rat skeletal muscle are considered, it is possible that sustained incubation of the SR Ca^{2+}-ATPase in vivo, in the presence of one or more metabolic byproducts, alters enzyme structure, reducing SR Ca^{2+}-ATPase activity in vitro. However, because reperfusion restored Ca^{2+}-ATPase activity but resulted in minimal metabolic recovery in this study, it is unlikely that the metabolic response to ischemia alone or to IR has an effect on SR Ca^{2+}-ATPase activity measured in vitro. It should be noted, however, that, in vivo, under the extreme metabolic perturbations that occur with IR, large depressions in SR Ca^{2+}-ATPase activity would result (25, 59).

It has been shown that heat shock and expression of heat shock protein (HSP) 72 before IR in the heart are associated with an increase in maximal SR Ca^{2+}-ATPase activity (38). Unfortunately, few studies have
examined the expression and localization of HSP 72 with IR in skeletal muscle. However, it is well known that oxidative stress, leading to protein destabilization and exposure of hydrophobic residues, induces the synthesis of HSPs (for review see Ref. 13). One study has shown that ischemia alone and IR in the heart increase the expression of HSP 72, with much higher levels found with IR compared with ischemia alone (58). Collectively, these results establish the possibility that HSP elevations with reperfusion may normalize SR Ca\(^{2+}\)-ATPase activity, measured in vitro, after ischemia.

Another possibility to explain the normalization of maximal Ca\(^{2+}\)-ATPase activity in isolated SR fractions with reperfusion involves the character of the SR obtained during the isolation procedure and not to specific changes in the enzyme. It has been observed previously (41, 56) that, after ischemia in the heart, isolated SR displays considerably more damage than the unfractionated homogenate, ostensibly as a result of a differential SR yield. Such may be the case in the current study, because after reperfusion we could find no change in homogenate determinations of maximal Ca\(^{2+}\)-ATPase activity. It has previously been reported that, at least in ischemic hearts, reductions in vesicle SR activity occur as a result of a selective discard of undamaged SR during the isolation procedure (41). In previous work (15), employing \(\approx 3\) h of ischemia, after 2 h of reperfusion we found that homogenate-based determinations of maximal Ca\(^{2+}\)-ATPase activity in skeletal muscle are lower than in the contralateral control. This is in contrast to the increase in Ca\(^{2+}\)-ATPase activity observed over 3 h of ischemia (14). Although we cannot explain the reasons for the difference between the current studies and our previous work, differences in the ischemia and reperfusion models may be important as found in the heart (34). In addition, in our previous work, we stimulated the muscle every 15 min to assess contractile changes. This was done only in separate experiments in our current work. The effect of the contractile activity during the experimental period needs to be examined.

**Ca\(^{2+}\) Uptake**

Despite normalization of maximal Ca\(^{2+}\)-ATPase activity in both muscle homogenates and isolated SR vesicles with reperfusion after ischemia, Ca\(^{2+}\) uptake remained depressed, at least in isolated SR vesicles. Thus the efficiency of Ca\(^{2+}\) transport is reduced with prolonged IR in skeletal muscle. A number of possibilities exist to explain a reduction in efficiency of SR Ca\(^{2+}\) transport, including 1) a change in Ca\(^{2+}\) binding properties of the Ca\(^{2+}\)-ATPase, 2) SR Ca\(^{2+}\) channel activation and increased passive Ca\(^{2+}\) release, and 3) an increase in membrane permeability to Ca\(^{2+}\) and excessive Ca\(^{2+}\) leakage. In this current study, no measures of membrane integrity were performed.

Our results show that the Ca\(^{2+}\) binding properties of the Ca\(^{2+}\)-ATPase were not affected by IR, because there were no changes in either NCD-4 binding or the Hill coefficient for Ca\(^{2+}\)-ATPase activity. Due to limited sample, we did not measure Ca\(^{2+}\) uptake in the presence of ryanodine to assess the activation state of the Ca\(^{2+}\) release channel at rest. The reduction in Ca\(^{2+}\) uptake that occurs after 4 h of ischemia alone is not affected by ryanodine and thus is not due to activation of the SR Ca\(^{2+}\) release channel (55). It is probable that activation of the Ca\(^{2+}\) release channel and increased passive Ca\(^{2+}\) release do not contribute to the reduced efficiency of Ca\(^{2+}\) transport observed in this study.

The remaining possibility is that SR membrane permeability to Ca\(^{2+}\) was increased as a result of oxidative damage. An impressive number of studies have reported various levels of oxidative membrane damage after IR in skeletal muscle (9, 17, 50) and the heart (33, 34). Moreover, the effects of O\(_2\) free radicals on membrane permeability and ion transport in vitro are well documented (26). Consequently, SR membrane damage involving lipid peroxidation may explain the reduction in Ca\(^{2+}\) uptake after reperfusion, in contrast to the isolated SR fraction, may well reflect reperfusion-induced effects on regional SR yield. In this regard, a limitation in our measurements of Ca\(^{2+}\) uptake must be acknowledged. We did not ensure that Ca\(^{2+}\) release was inhibited during the Ca\(^{2+}\) uptake procedure. It is possible that Ca\(^{2+}\) uptake values would be more depressed in SR vesicles if some release from the channel occurred. Unfortunately, tissue limitations precluded additional measurements.

**Ca\(^{2+}\) Release**

Membrane damage may also explain our finding that the early rapid rate of AgNO\(_3\)-induced Ca\(^{2+}\) release was slightly higher with IR in isolated SR vesicles. Alternatively, it is well known that O\(_2\) free radicals can trigger rapid Ca\(^{2+}\) release from the SR by modifying critical sulfhydryls in the Ca\(^{2+}\) release channel and increasing the open probability of the channel (for review see Refs. 1 and 10). However, we found that Ca\(^{2+}\) release was reduced with 4-h ischemia alone (55), and on the basis of pilot data, we assumed that the concentration of AgNO\(_3\) employed in this study (141 \(\mu\)M) maximally activates all Ca\(^{2+}\) release channels in each sample. It appears that the increase in Ca\(^{2+}\) release observed in isolated SR vesicles with IR was not a result of modifications to the open probability of the release channels. Instead, flux through the Ca\(^{2+}\) release channels could be increased with IR due to changes in some aspect of the complex control of Ca\(^{2+}\) release (i.e., phosphorylation-dephosphorylation of SR proteins), or increased flux through a leaky SR membrane could explain the increase in Ca\(^{2+}\) release with IR.

As discussed with the Ca\(^{2+}\)-ATPase and Ca\(^{2+}\) uptake, it is probable that the increase in Ca\(^{2+}\) release observed in SR but not in homogenates is due to differential selection of a population of SR vesicles during
the isolation procedure between IR and control muscle, as has been reported in the heart (41, 56). In other words, it is possible that vesicles more enriched in Ca\(^{2+}\) release channels were obtained after SR isolation from IR muscle compared with control muscle.

In this study, we also tested the efficacy of SOD + CAT treatment on improving SR function with IR. Numerous studies have shown that SOD + CAT treatment attenuates IR injury in skeletal (30, 50, 57) and cardiac muscle (36, 53). However, in one study, it was shown that SOD + CAT treatment had no effect on myocardial SR Ca\(^{2+}\) uptake after 15 min of ischemia and 15 min of reperfusion, because SR Ca\(^{2+}\) uptake recovered in hearts reperfused both with and without SOD + CAT (42). Given the general absence of an effect of IR on skeletal muscle SR function in this study, it was not surprising to find that SOD + CAT treatment was without effect in I on all SR parameters measured. Interestingly, however, homogenate Ca\(^{2+}\) uptake was higher in contralateral control muscle with SOD + CAT than with saline treatment alone. Also, in SR vesicles, Ca\(^{2+}\) uptake was higher in C compared with I after SOD + CAT treatment (i.e., CT > IT), with no difference between C and I in saline-treated animals. Collectively, these results suggest that SOD + CAT treatment protected against IR-induced reductions in Ca\(^{2+}\) uptake that normally occur with prolonged IR in skeletal muscle from the contralateral limb. From our results, it is not clear how much of the unconjugated SOD and CAT was taken up by the muscle cells. Whether conjugated or unconjugated forms should be used remains speculative (44).

In discussing possible mechanisms for the effects of IR in one hindlimb on skeletal muscle SR function in the contralateral limb, it is important to note that no change in SR function occurs in the contralateral limb after 4-h ischemia alone (55). Therefore, although it has been reported that myocardial ischemia increases sympathetic nervous system (SNS) activity and release of norepinephrine 1,000-fold (48) and that \(\beta\)-blockers protect against IR-induced changes in cardiac SR function (53), the reduction in Ca\(^{2+}\) uptake in the contralateral control limb observed in this study is not likely mediated via any IR-induced hormonal response.

Given that SOD + CAT treatment prevented the IR-induced reduction in Ca\(^{2+}\) uptake in the contralateral limb, the mechanism must have involved an O\(_2\) free radical-mediated process. Experimental evidence suggests that hindlimb IR may initiate a systemic inflammatory response, leading to injury of remote organs such as the liver (3). It is possible that this also occurs in skeletal muscle tissue. Depending on the degree of inflammation, infiltrating white blood cells may adhere to the endothelium and cause narrowing of the microvasculature, which, in combination with SNS-mediated vasoconstriction, may result in hypoxic tissue damage. Moreover, white blood cells (and neutrophils in particular) can produce O\(_2\) free radicals (46). The effectiveness of SOD + CAT treatment in protecting the contralateral limb from IR-induced changes in SR function corresponds to this mechanism.

The microcirculatory changes in IR parallel those seen in inflammation (46). It has been shown in skeletal muscle that <15% of the muscle is being perfused with blood 24 h post-IR (31). This phenomenon is referred to as “no-reflow” (2). The extent of no-reflow that occurred in this study was not determined, but such a mechanism could partially explain the lack of metabolic recovery with reperfusion observed in this study and, therefore, lack of force recovery. Significant no-reflow may also have prevented adequate delivery of SOD + CAT to the ischemic tissue, which may help explain the lack of improvement in muscle metabolism, SR Ca\(^{2+}\) uptake, and contractile function in ischemic tissue with SOD + CAT treatment. Finally, it should be noted that interpreting the effects of IR on SR function is problematic where significant no-reflow occurs, because reperfused tissue cannot be sampled with ease independently from nonperfused tissue.

In summary, 4-h ischemia and 1-h reperfusion in rat skeletal muscle lead to severe metabolic and contractile function perturbations but only minimal changes in SR function. There were no changes in SR Ca\(^{2+}\)-ATPase activity with IR, and both the increase in SR Ca\(^{2+}\) release and the reduction in SR Ca\(^{2+}\) uptake with IR were observed only in isolated SR vesicles and not in muscle homogenates. Apparently, however, SR Ca\(^{2+}\) uptake in the contralateral limb is reduced after IR, and this is prevented with SOD + CAT treatment. It is concluded that SOD + CAT treatment may protect against injury in nonischemic tissue after prolonged skeletal muscle IR in rats.

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