Ischemia-induced structural change in SR Ca\(^{2+}\)-ATPase is associated with reduced enzyme activity in rat muscle

R. TUPLING,
H. GREEN,
G. SENISTERRA,
J. LEPOCK,
AND N. MCKEE

Departments of Kinesiology and Physics, University of Waterloo, Waterloo N2L 3G1; and Department of Surgery, University of Toronto, Toronto, Ontario, Canada M5S IAI

Received 7 December 2000; accepted in final form 29 June 2001.

Tupling, R., H. Green, G. Senisterra, J. Lepock, and N. McKee. Ischemia-induced structural change in SR Ca\(^{2+}\)-ATPase is associated with reduced enzyme activity in rat muscle. Am J Physiol Regulatory Integrative Comp Physiol 281: R1681–R1688, 2001.—In this study, we employed an in vivo model of prolonged ischemia in rat skeletal muscle to investigate the hypothesis that structural modifications to the sarcoplasmic reticulum (SR) Ca\(^{2+}\)-ATPase can explain the alterations in Ca\(^{2+}\)-ATPase activity that occur with ischemia. To induce total ischemia, a tourniquet was placed around the upper hindlimb in 27 female Sprague-Dawley rats weighing 256 ± 6.7 g (mean ± SE) and was inflated to 350 mmHg for 4 h. The contralateral limb served as control (C) to the ischemic limb (I), and the limbs of animals killed for 350 mmHg for 4 h. The contralateral limb served as control (C) to the ischemic limb (I), and the limbs of animals killed immediately after anesthetization served as a double control (CC) to the ischemic limb (I), and the limbs of animals killed immediately after anesthetization served as a double control (CC). Mixed gastrocnemius and tibialis anterior muscles were sampled and used for SR vesicle preparation. Maximal Ca\(^{2+}\)-ATPase activity (\(\mu\)mol·g protein\(^{-1}·\)min\(^{-1}\)) of C (15,802 ± 1,246) and I (11,609 ± 1,029) was 90 and 73% (\(P < 0.05\)) of CC (17,562 ± 1,682), respectively. No differences were found between groups in either the Hill coefficient or the free Ca\(^{2+}\) at half-maximal activity. The fluorescent probes, FITC and N-cyclohexyl-N′-(dimethylamino-alpha-naphthyl) carbodiimide, used to assess structural alterations in the regions of the ATP binding site and the Ca\(^{2+}\) binding sites of the Ca\(^{2+}\)-ATPase, respectively, indicated a 26% reduction (\(P < 0.05\)) in FITC binding capacity (absolute units) in I (0.22 ± 0.01) compared with CC (0.29 ± 0.02) and C (0.29 ± 0.03). Our results suggest that the reduction in maximal SR Ca\(^{2+}\)-ATPase activity in SR vesicles with ischemia is related to structural modification in the region of the nucleotide binding domain by mechanisms that are as yet unclear.

Address for reprint requests and other correspondence: H. J. Green, Dept. of Kinesiology, Univ. of Waterloo, Waterloo, ON, Canada N2L 3G1 (E-mail: green@healthy.uwaterloo.ca).

http://www.ajpregu.org

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Studies of ischemia-reperfusion in cardiac muscle, and more recently in skeletal muscle, have shown that impaired SR function and Ca\textsuperscript{2+} homeostasis may also be involved in the etiology of ischemia-reperfusion injury (9, 14, 19). In one study, pretreatment with the oxygen free radical scavengers superoxide dismutase and catalase maintained higher Ca\textsuperscript{2+} uptake by the SR of skeletal muscle after 3 h of ischemia and 19 h of reperfusion in rat hindlimb (19). Thus free radical formation is likely a mechanism for impaired SR function with ischemia and reperfusion.

Because measurement of SR function in ischemia studies is generally done in vitro, under optimal conditions, ischemia-induced reductions in Ca\textsuperscript{2+}-ATPase activity and Ca\textsuperscript{2+} uptake are probably due to structural alterations to the Ca\textsuperscript{2+}-ATPase protein and/or the SR membrane. However, the nature or precise location of altered structure on the SR Ca\textsuperscript{2+}-ATPase has not been determined in skeletal muscle samples harvested from tissue that was made ischemic in vivo.

In this study, we employed a 4-h hindlimb ischemia model in rats to characterize the alterations in SR Ca\textsuperscript{2+}-ATPase activity and structure that occur with prolonged periods of skeletal muscle ischemia. To assess the structural alterations to the Ca\textsuperscript{2+}-ATPase, the fluorescent probes FITC and N-cyclohexyl-N’-(dimethylamino-o-naphthyl)carboodimide (NCD-4) were used to measure changes in the regions of the ATP binding site and the Ca\textsuperscript{2+} binding sites of the Ca\textsuperscript{2+}-ATPase, respectively. We hypothesized that Ca\textsuperscript{2+}-ATPase activity would be lower in ischemic SR compared with control and that the lower Ca\textsuperscript{2+}-ATPase would be accompanied by a reduction in FITC binding, indicative of structural alterations to the ATP binding site.

METHODS

Animal Description and Care

Adult female Sprague-Dawley rats weighing 256 ± 6.7 g (mean ± 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. The hindlimbs were not reperfused before muscle sampling. The G and TA muscles from the CC animal were sampled and excised in the same manner immediately after anesthetization.

Experimental Protocol

To investigate the effects of complete ischemia on SR Ca\textsuperscript{2+}-ATPase structure and function, animals were randomly assigned to a control control (CC) group (n = 9) to be killed immediately after anesthetization or to experimental (E) (n = 27) groups. For each E animal, the experimental condition, 4 h of total ischemia, was randomly assigned to one hindlimb (I) while the contralateral limb served as a control limb (C). Due to tissue requirements for the isolation procedure used to obtain SR vesicles, experiments were conducted on one CC and three E animals each day. 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 (7). Total ischemia was confirmed on the basis of almost total depletion of muscle phosphocreatine and ATP after 4 h of ischemia (see Table 1).

Before the induction of ischemia, the rats were weighed and anesthetized. Anesthesia was initially accomplished by using an intraperitoneal injection of pentobarbital sodium (6 mg/100 g body wt) and then was maintained by using supplementary intraperitoneal injections as required. To prevent dehydration, experimental rats were administered 2 ml of saline by injection just under the skin before the induction of ischemia. Throughout the ischemic periods, body temperature was maintained between 37 and 39°C by having the rats lay in a prone position on a warm heating pad. At the end of each of the ischemic periods, a small piece of white gastrocnemius (G) muscle was rapidly sampled from each of the I and C limbs and frozen in liquid nitrogen for later analysis of muscle metabolites. The remainder of the G muscle (both red and white portions) and the entire tibialis anterior (TA) muscle were excised and placed in ice-cold buffer to be used for SR isolation by differential centrifugation. The hindlimbs were not reperfused before muscle sampling. The G and TA muscles from the CC animal were sampled and excised in the same manner immediately after anesthetization.

Analytic Procedures

Sample preparation for SR assessment in vitro. Ischemic and control muscles were prepared according to Heilmann et al. (11). Mixed G and TA muscles were diluted −1.5 (wt/vol) in ice-cold homogenizing buffer containing (in mM) 5 HEPES (pH 7.5), 250 sucrose, 0.2% sodium azide, and 0.2 phenylmethylsulfonyl fluoride (PMSF) and mechanically homogenized with a polytron homogenizer (PT 3,100) at 16,500 rpm, for two 30-s bursts. An aliquot of this sample was quick frozen in liquid nitrogen, stored at −70° to −80°C and used for homogenate determinations of Ca\textsuperscript{2+}-ATPase activity. To obtain an enriched SR membrane fraction, a combination of two SR isolation protocols was used (6, 11). 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 15 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 homogenizing buffer plus 600 mM KCl and allowed to incubate at 4°C for 30 min. This suspen-
sion was then centrifuged at 15,000 g for 10 min to pellet all
the mitochondria. The supernatant was centrifuged at 50,000 g for 52 min. The final pellet, enriched in SR membranes (no
succrose cushion), was resuspended in homogenizing buffer at
a protein concentration of 2–6 mg/ml. SR isolation was car-
ried out by differential centrifugation by use of a Beckmann
ultracentrifuge with a 70.1 Ti fixed-angle rotor. The SR
membrane fraction was used for measurements of Ca\(^{2+}\)-
ATPase activity and isoform composition and to examine
structural alterations to the enzyme. All homogenates and
SR membrane isolations were made with only PMSE as a
proteolytic inhibitor.

**SR Ca\(^{2+}\)-ATPase activity measurements.** Spectrophotometric (Schimadzu UV 160U) measurement of SR Ca\(^{2+}\)-
ATPase activity was performed on homogenates by using
procedures developed by Simonides and van Hardeveld (34)
and SR samples analyzed according to methods of Leberer
et al. (18) with minor modifications. Total (Mg\(^{2+}\)-activated)
ATPase activity was measured in the presence of the Ca\(^{2+}\)
ionophore A-23187, across a range of CaC\(_2\) concentrations.
Basal activity was measured in the presence of 40 \(\mu\)M cyco-
piazonic acid, which completely inhibits SR Ca\(^{2+}\)-ATPase
activity (32). The difference between total and basal activi-
ty represents the Ca\(^{2+}\)-activated ATPase activity. Maximal
activity and the Ca\(^{2+}\) dependency of Ca\(^{2+}\)-ATPase activity
were assessed by adding 1–11 \(\mu\)l of 100 mM CaCl\(_2\) in 0.5-\(\mu\)l
additions. Ca\(^{2+}\)-ATPase activity increases with [Ca\(^{2+}\)]\(_t\) until
a plateau occurs, once maximal activity is reached. The
[Ca\(^{2+}\)]\(_t\) corresponding to each CaCl\(_2\) addition was assessed
separately, on a different SR aliquot, by using dual-wave-
length spectrofluorometry and the Ca\(^{2+}\)-fluorescent dye indo
1. Ca\(^{2+}\)-ATPase activity was then plotted against the nega-
tive logarithm of [Ca\(^{2+}\)]\(_t\) (pCa), and the Hill coefficient and
the [Ca\(^{2+}\)]\(_t\) that gives half-maximal activity (Ca\(_{50}\)) were de-
termined. These properties were measured through nonlin-
ear regression with computer software (Graph Pad Software)
using the following sigmoidal dose-response equation

\[
Y = \frac{Y_{\text{bot}} + Y_{\text{top}}}{1 + 10^{\left(\log C - \log C_{50}\right) \times H}}
\]

where \(Y_{\text{bot}}\) is the value at the bottom of the plateau, \(Y_{\text{top}}\) is
the value at the top of the plateau, \(\log C\) is the concen-
tration that gives a response halfway between \(Y_{\text{bot}}\) and \(Y_{\text{top}}\),
and \(H\) is the Hill coefficient. For calculation of these properties,
only a portion of the curve that corresponded to between 20
and 80% of maximal activity was used. Total protein was
determined by the method of Lowry as modified by Schacterle
and Pollock (31). All Ca\(^{2+}\)-ATPase activities are expressed
relative to total protein content. On a given analytical day,
samples from all conditions were analyzed in duplicate.

**SDS-PAGE and Western blotting.** A suspension of 0.5
mg/ml SR protein in either 40 \(\mu\)l reducing buffer (1.25 M
succrose, 0.1 M dithiothreitol (DTT), 0.25 M Tris-HCl, pH 6.8,
5% SDS, 0.01% bromophenol) or nonreducing buffer (1.25 M
succrose, 0.25 M Tris-HCl, pH 6.8, 5% SDS, 0.01% bromophen-
ol) brought to 200 \(\mu\)l by distilled water was heated for 10
min at 100°C, and solid DTT was added to the sample in
reducing buffer to raise the final concentration of DTT to 100
mM. All samples were then sonicated for 10 s in a probe
sonicator, and 5 \(\mu\)g of each sample was analyzed in duplicate
on separate 7% polyacrylamide SDS gels (BIO-RAD Mini-
PROTEAN II) with a 3.75% stacking gel.

After SDS-PAGE and a 15 min equilibration in cold trans-
fer buffer (25 mM Tris, 192 mM glycine, and 20% vol/vol
methanol), the proteins were transferred to a polyvinylidene
difluoride membrane (Bio-Rad) by placing the gel in trans-
fer buffer and applying a high voltage (100 V) for 45 min
(Trans-Blot Cell, Bio-Rad). Nonspecific binding sites were blocked
with 10% skim milk powder in Tris-buffered saline (pH 7.5),
and applied overnight at room temperature. Immunoblotting was
performed with use of the primary monoclonal antibody
IIH11 specific for rat (Affinity Bioreagents) for determination
of SERCA1 protein and the aggregation state of SERCA1.
Incubation with the primary antibodies was performed for 60
min at room temperature. After washing, a secondary anti-
body (anti-mouse IgG\(_1\) conjugated to horseradish peroxidase)
was applied for 60 min at room temperature. Protein quanti-
tication was performed by using densitometry and an en-
hanced chemiluminescence immunodetection procedure
(Amersham-ECL-RPN2106P1). After exposure to photo-
graphic film (Kodak Hyperfilm-ECL), the blot was developed
for 90 s in Kodak GBX developing solution and fixed in Kodak
GBX fixer. Relative SERCA1 protein levels were determined
by scanning densitometry, and values were expressed as a
percentage of the CC value.

**Fluorescence measurements.** Fluorescence measurements
were made on an SLM-4800S spectrophotometer (SLM
Instruments, Urbana, IL) according to Lalone et al. (17). FITC
(Sigma Chemical) and NCD-4 (Molecular Probes) were stored
at a concentration of 5 mM in ethanol at –20°C. FITC
emission spectra (500–550 nm) were collected by exciting
samples at 490 nm (see Fig. 3A). FITC labeling was done by
washing the SR samples once in wash buffer (homogenizing
buffer without sucrose) with no DTT, then resuspending the
samples in FITC labeling buffer (wash buffer plus 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 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 incre-
ments. NCD-4 labeling was done by washing the SR samples
once in wash buffer with no DTT, then resuspending in
NCD-4 labeling buffer (wash buffer plus 150 \(\mu\)M NCD-4, pH
6.2) and incubating in darkness for 5 h at 25°C. As before, the
sample was washed to remove unbound label.

**Muscle metabolites.** Muscle metabolites were measured
on samples of stored tissues (–80°C) that were freeze dried and
cleared of visible connective tissue and blood. After extrac-
tion of the metabolites by perchloric acid (0.5 M) and neu-
tralization, the contents of phosphocreatine (PCr), creatine,
and lactate were assessed by using fluorometric procedures
according to techniques previously published by our labora-
tory (10). A portion of the extract was also used for the
determination of ATP, ADP, AMP, IMP, inosine, and hypo-
xanthine by using ion-pair, reversed-phase HPLC techniques
(13) as described earlier (10).

**Statistical Analysis.**

For all measurements, a one-way ANOVA was used to test
for differences between means. When 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 ±
SE.

**RESULTS**

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

Maximal Ca\(^{2+}\)-ATPase activity (\(\mu\)mol·g protein\(^{-1}\)·
min\(^{-1}\)) measured in homogenates between the ische-
mic muscle (970 ± 54) and contralateral control (907 ±
70) was not different. Similarly, there were no differ-
ences between these muscles and the muscles from
nonischemic animals (826 ± 46). In enriched SR vesicles, maximal Ca\(^{2+}\)-ATPase activity, which occurred at a \([\text{Ca}^{2+}]_\text{f}\) of 6–10 μM in all groups (data not shown), was 73% (P < 0.05) and 90% (P < 0.05) of CC compared with I and C, respectively (Table 1). There was no difference between CC and C. However, although maximal activity was depressed after ischemia, there was no effect on enzyme kinetics (Table 1; Fig. 1). 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.

**Electrophoresis and Western Blot Analysis**

Total SR Ca\(^{2+}\)-ATPase protein contents were compared between groups by Western blot analysis. Total protein was only analyzed on SR samples subjected to reducing conditions (with DTT) SDS-PAGE, because all of the Ca\(^{2+}\)-ATPase protein should be in the monomer form under these conditions and easily detected by the monoclonal antibody IIH11 (22). Six discrete bands were present under these conditions, corresponding to the monomer form of SERCA1 and smaller molecular weight SERCA1 products (Fig. 2B). No discrete bands corresponding to dimers or higher molecular weight aggregates were observed. Relative total protein content was taken as the sum intensity of all six bands and was expressed relative to CC. There was no difference between groups in total SERCA1 protein content. Compared with CC, the values for C and I groups were 94.8 ± 8.9 and 104 ± 10.3%, respectively.

To determine whether prolonged ischemia results in SERCA1 protein oxidation and protein aggregation, SR samples from all groups were subjected to nonreducing (no DTT) and reducing (DTT) SDS-PAGE. Under nonreducing conditions, only two main bands were detected, corresponding to the monomer form of SERCA1 (110 kDa) and a smaller molecular weight SERCA1 product (Fig. 2A). No discrete bands corresponding to dimers or higher molecular weight aggregates were observed. Relative total protein content was taken as the sum intensity of all six bands and was expressed relative to CC. There was no difference between groups in total SERCA1 protein content. Compared with CC, the values for C and I groups were 94.8 ± 8.9 and 104 ± 10.3%, respectively.

**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 domain of the Ca\(^{2+}\)-ATPase after prolonged ischemia, respectively. FITC covalently labels Lys515, which is close to the ATP binding site and competitively inhibits ATP binding (26). Similarly, the label NCD-4 binds near the two Ca\(^{2+}\) binding sites in addition to some nonspecific labeling, inhibiting binding of Ca\(^{2+}\) without inhibiting the binding of ATP (3).
A sample emission spectrum of FITC from each group is shown in Fig. 4A. The average maximum intensity, which occurs at 520 nm, was 26% lower in I (P < 0.05) compared with both CC and C (Fig. 4B). There was no difference between CC and C. The maximum emission intensity of NCD-4 was 0.36 ± 0.2, 0.36 ± 0.2, and 0.34 ± 0.2 for the CC, C, and I groups, respectively. No differences were found between any of the groups.

Ischemia resulted in extensive changes in the metabolic status of the muscle (Table 2). For the metabolites measured, ischemia induced near-complete depletion of ATP and PCr. These changes were accompanied by large relative increases in IMP, inosine, and hypoxanthine. In contrast, ADP, but not AMP, was reduced with ischemia. Total creatine was not different in any of the groups. In addition, lactate content was elevated ~12-fold over control muscles. No differences were found for any of the metabolites between the CC and C groups.

DISCUSSION

In summary, we have found that 4 h of ischemia resulted in a reduction in maximal Ca\(^{2+}\)-ATPase activity in SR vesicles that was accompanied by a reduction in FITC but not NCD-4 binding. The reduction in maximal Ca\(^{2+}\)-ATPase activity was not accompanied by changes in either the Hill coefficient or pCa\(_{50}\). Interestingly, no changes in maximal Ca\(^{2+}\)-ATPase activity were observed with ischemia in homogenates.

**Table 2. Muscle metabolite concentrations for ischemic and control groups**

<table>
<thead>
<tr>
<th></th>
<th>CC</th>
<th>C</th>
<th>I</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>30.6 ± 0.50</td>
<td>29.7 ± 0.54</td>
<td>1.2 ± 0.35*</td>
</tr>
<tr>
<td>ADP</td>
<td>6.0 ± 0.14</td>
<td>5.5 ± 0.19</td>
<td>2.7 ± 0.21*</td>
</tr>
<tr>
<td>AMP</td>
<td>0.15 ± 0.01</td>
<td>0.15 ± 0.01</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>IMP</td>
<td>0.42 ± 0.18</td>
<td>0.20 ± 0.02</td>
<td>28.4 ± 0.64*</td>
</tr>
<tr>
<td>Inosine</td>
<td>0.07 ± 0.02</td>
<td>0.05 ± 0.01</td>
<td>5.69 ± 0.70*</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>0</td>
<td>0</td>
<td>1.10 ± 0.20</td>
</tr>
<tr>
<td>PCr</td>
<td>77.1 ± 2.1</td>
<td>77.2 ± 3.0</td>
<td>1.3 ± 0.4*</td>
</tr>
<tr>
<td>Creatine</td>
<td>84.9 ± 2.0</td>
<td>78.5 ± 2.5</td>
<td>150 ± 2.3*</td>
</tr>
<tr>
<td>Lactate</td>
<td>22.4 ± 1.0</td>
<td>24.5 ± 2.7</td>
<td>249.0 ± 5.6*</td>
</tr>
</tbody>
</table>

Values are means ± SE in \(\mu\)mol/g dry wt. *Significantly different (P < 0.05) from CC and C.
Differences Between Homogenates and SR Fractions

A key concern is why the effects of ischemia on Ca^{2+}-ATPase activity were observed only in the SR vesicles and not in the homogenates. Interestingly, we have previously observed increases in maximal activity in homogenates with shorter periods of ischemia (8). It is possible that ischemia is without effect on homogenates and that the reductions in Ca^{2+}-ATPase activity in SR vesicles simply reflect a selective discard of undamaged SR during the isolation procedure. This has been observed previously in ischemic hearts (27). On the other hand, the failure to find reductions in Ca^{2+}-ATPase activity in homogenates could be due to activation of other cellular ATPases, masking the effects of the inhibiting agents or recruitment of additional enzyme by covalent modification, leading to phosphorylation or production of an activator substance during ischemia, which binds to the Ca^{2+}-ATPase and activates it (8). It is possible that these changes become lost during the vesicle isolation process. Regardless of the mechanism, the overall effect would be to minimize the inactivation of the pool of enzymes that occurs during ischemia. This finding is potentially very important because isolation artifact could conceivably explain much of the existing controversy in the literature regarding ischemic effects on SR function (24).

Structural Changes in Ca^{2+}-ATPase

In this study, we attempted to elucidate the structural alterations that occur to the SR Ca^{2+}-ATPase in vivo with prolonged ischemia in rat skeletal muscle, which could explain the predicted reduction in Ca^{2+}-ATPase activity that occurs with ischemia. Our results for the SR vesicles are consistent with our hypothesis, namely that the reductions in Ca^{2+}-ATPase activity are accompanied by alterations in the region of the nucleotide binding site on the enzyme.

The reductions in maximal Ca^{2+}-ATPase activity that we observed in this study in ischemic SR compared with CC and C paralleled the reductions in FITC binding that occurred with ischemia. On the other hand, kinetic analysis of Ca^{2+}-ATPase activity shows that both the Hill coefficient and sensitivity of the enzyme to Ca^{2+} (Ca_{50}) were unaffected. These results are consistent with a problem in binding ATP, but only in a selected population of Ca^{2+} pumps. A similar effect has been postulated to occur in rabbit muscle after chronic muscle activity (18). The reduction in FITC binding is not due to reductions in Ca^{2+}-ATPase protein as determined by Western blot analysis. Rather, it appears that a reduction in the number of FITC binding sites per Ca^{2+}-ATPase protein occurred in response to the prolonged ischemia.

We did probe for structural alterations in the region of the Ca^{2+} binding domain of the Ca^{2+}-ATPase, by using the fluorescent probe NCD-4. We found that ischemia had no effect on the maximum binding capacity of NCD-4, suggesting that the structure of the Ca^{2+} binding domain was unaltered after prolonged ischemia. This is in close agreement with our kinetic analysis of ATPase activity because the Hill coefficient, which theoretically represents the number of Ca^{2+} binding sites, was not different between groups. However, it must be emphasized that the NCD-4 probe not only binds near to Ca^{2+} binding sites but displays some nonspecific binding as well (3).

Potential Role of Free Radicals

Although several mechanisms may be involved, our metabolic data indicate a strong possibility that free radical production, mediated during the 4 h of ischemia, is involved. The ischemic period resulted in massive reductions in the high-energy phosphates ATP and PCR in ischemic muscle but not in control muscles. As a consequence, large elevations in hypoxanthine and xanthine, which are substrates for the enzyme xanthine oxidase, occurred, as would be expected (29). The enzyme catalyzes the reduction of O_2, leading to the formation of superoxide and H_2O_2, and it has been proposed as a central mechanism of oxidative injury (21). Therefore, although we did not directly measure free radical production in this study, it can be assumed that there was significant free radical production in the ischemic muscle compared with control.

Our results indicate that the structural changes in Ca^{2+}-ATPase with ischemia are similar to those reported after exposure of the Ca^{2+}-ATPase to oxidizing conditions in vitro. In the in vitro study, it was found that exposure of the Ca^{2+}-ATPase to levels of hydroxyl radicals similar to that measured during postischemic reperfusion denatures the Ca^{2+}-ATPase and inhibits ATPase activity by directly attacking the ATP binding site without damaging the primary structure of the enzyme (41). This would suggest that prolonged ischemia also leads to structural reorganization of the region of the nucleotide binding domain, which would likely impair ATP binding and ATPase activity.

In the in vitro study, it was also found that presaturation of the active site with ATP completely protected both cardiac and skeletal muscle SR Ca^{2+}-ATPase function from hydroxyl radical-induced inhibition. This suggests that depletion of cellular ATP, in the region of the enzyme, induces the structural alteration to the nucleotide binding domain of the Ca^{2+}-ATPase. In our study, depletion of muscle ATP stores, as observed with prolonged periods of skeletal muscle ischemia, not only suggests formation of free radicals in skeletal muscle but may also indicate the susceptibility of SR Ca^{2+}-ATPase to free radical-induced damage, specifically to the nucleotide binding domain.

The precise nature of the structural modification to the nucleotide binding domain in ischemic SR, which resulted in a reduced FITC binding capacity, cannot be directly ascertained from this study. However, several in vitro studies have helped to characterize the free radical-induced molecular modification of the SR Ca^{2+}-ATPase that is correlated with its functional properties (33, 39). Viner et al. (39) employed a peroxyl radical-generating system by using the free radical initiator...
2,2'-azobis(2-aminopropane) dihydrochloride to examine and identify oxidation-sensitive peptides within the SR Ca\textsuperscript{2+}-ATPase of fast-twitch rabbit skeletal muscle. They identified six oxidatively sensitive peptides on the cytoplasmic side of the SR membrane. One of these peptide segments (Glu\textsubscript{551}-Arg\textsubscript{604}) is located in the nucleotide binding domain and was found to participate in the formation of intermolecular bitryosine cross-links with the identical Glu\textsubscript{551}-Arg\textsubscript{604} peptide from a neighboring Ca\textsuperscript{2+}-ATPase polypeptide chain. Although this peptide does not contain the FITC binding site Lys\textsubscript{515} (4) or the actual ATP binding site around Arg\textsubscript{604} (36), cross-linking and aggregation in this region may interfere with both FITC and ATP binding. Whether cysteine residues are affected by prolonged ischemia, as might be expected if aggregation occurred, was not assessed in this study.

As reported, we detected smaller molecular weight SERCA\textsubscript{1} fragments by using reducing SDS-PAGE and Western blot analysis. However, the relative amount of each fragment was not different between groups (data not shown). Likely, the appearance of these products was due to proteolytic activity and/or oxidative fragmentation that took place during homogenization and preparation of SR vesicles, and this should be the same for all groups. Our homogenization buffer only included one proteolytic inhibitor (PMSF) and did not include the sulphydryl reducing agent, DTT. The decision not to use DTT was purposeful because we have reported that even 5 mM DTT in the homogenization buffer can alter the effects of 4 h ischemia on SR Ca\textsuperscript{2+}-ATPase activity (38).

Although aggregation of Ca\textsuperscript{2+}-ATPase in ischemic SR compared with control is suggested, as indicated by the lower relative monomer form of the protein in I, the gels were not supportive. The difference in monomer levels was corrected in reducing gels. This suggests that aggregation did occur with ischemia and was due to disulfide cross-links. A similar finding was reported by Senisterra et al. (33), with exposure of the SR Ca\textsuperscript{2+}-ATPase to the thiol-specific reagents diamide and arsenite.

In the present study, we used an ischemia model in rat skeletal muscle known to lead to elevations in oxygen free radical production and reductions in SR Ca\textsuperscript{2+}-ATPase activity to assess the structural alterations associated with in vivo oxidation of the SR Ca\textsuperscript{2+}-ATPase. We have shown that prolonged ischemia leads to reductions in FITC binding capacity in isolated SR preparations, which is associated with reductions in maximal Ca\textsuperscript{2+}-ATPase activity. We suggest that the molecular mechanism is likely oxidation of one or more of the cysteine residues within the nucleotide binding domain of the Ca\textsuperscript{2+}-ATPase by xanthine oxidase-produced superoxide and/or H\textsubscript{2}O\textsubscript{2}. Whether these structural alterations are manifested in vivo remains to be demonstrated.

This research was supported by grants from the Medical Research Council (Canada) and the Natural Sciences and Engineering Research Council (Canada).

REFERENCES

3. Chadwick CC and Thomas EW. Inactivation of sarcoplasmic reticulum (Ca\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPase by N-cyclohexyl-N-(4-dimethylamino-o-naphthyl) carbodiimide. Biochim Biophys Acta 730: 200–206, 1983.


