Effects of buthionine sulfoximine treatment on diaphragm contractility and SR Ca\(^{2+}\) pump function in rats


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Tupling AR, Vigna C, Ford RJ, Tsuchiya SC, Graham DA, Denniss SG, Rush JW. Effects of buthionine sulfoximine treatment on diaphragm contractility and SR Ca\(^{2+}\) pump function in rats. J Appl Physiol 103: 1921–1928, 2007. First published August 23, 2007; doi:10.1152/japplphysiol.00529.2007.—The purpose of this study was to examine the effects of glutathione (GSH) depletion and cellular oxidation on rat diaphragm contractility and sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) function in vitro under basal conditions and following fatiguing stimulation. Buthionine sulfoximine (BSO) treatment (n = 10) for 10 days (20 mM in drinking water) reduced (P < 0.05) diaphragm GSH content (nmol/mg protein) and the ratio of GSH to glutathione disulfide (GSSG) by 91% and 71%, respectively, compared with controls (CTL) (n = 10). Western blotting showed that Hsp70 expression in diaphragm was not increased (P = 0.05) with BSO treatment. As hypothesized, basal peak twitch force (g/mm\(^2\)) was increased (P < 0.05), and fatigability in response to repetitive stimulation (350-ms trains at 100 Hz once every 1 s for 5 min) was also increased (P < 0.05) in BSO compared with CTL. Both Ca\(^{2+}\) uptake and maximal SERCA activity (μmol·g protein\(^{-1}\)·min\(^{-1}\)) measured in diaphragm homogenates that were prepared at rest were increased (P < 0.05) with BSO treatment, an effect that could be partly explained by a twofold increase (P < 0.05) in SERCA2α expression with BSO. In response to the 5-min stimulation protocol, both Ca\(^{2+}\) uptake and maximal SERCA activity were increased (P < 0.05) in CTL but not (P > 0.05) in BSO diaphragm. We conclude that 1) cellular redox state is more optimal for contractile function and fatigability is increased in rat diaphragm following BSO treatment, 2) SERCA2α expression is modulated by redox signaling, and 3) regulation of SERCA function in working diaphragm is altered following BSO treatment.

IN RESTING SKELETAL MUSCLE, reactive oxygen species (ROS) and reactive nitrogen species (RNS) exist at low but measurable concentrations and determine the basal intracellular thiol/disulfide redox state (6, 15, 34). Redox homeostasis in skeletal muscle is maintained by the balance of both ROS and/or RNS act as important regulatory signals for various physiological processes, whereas excessive accumulation of ROS and/or RNS causes oxidative stress and damage resulting in loss of muscle function (6, 24, 33, 34).

Redox regulation of skeletal muscle contractility has been examined extensively, and a model developed by Reid et al. (32) depicts the biphasic effects of cellular redox state on isometric force (for review, see Ref. 33). Modest ROS supplementation shifts intracellular redox from the basal state toward a slightly more oxidized state that is more optimal for force production, causing isometric force to increase (3, 28, 32). On the other hand, excessive ROS production that occurs with strenuous exercise (5, 31, 44, 49), or exposure to high concentrations of ROS causes oxidative stress and inhibits force production (i.e., causes muscle fatigue) (33).

Glutathione is an important nonenzymatic cellular antioxidant that functions as an electron donor for the glutathione peroxidase (GPX) reaction and has direct antioxidant properties (16, 41). GPX catalyzes the conversion of reduced glutathione (GSH) to its oxidized state (GSSG) in the presence of increased peroxides (i.e., H\(_2\)O\(_2\)); subsequently GSSG can be recycled back to GSH in the glutathione reductase (GR) reaction (41). Administration of the glutamate-cysteine ligase inhibitor buthionine sulfoximine (BSO) has been shown to deplete cellular glutathione (i.e., reduce antioxidant defense) and to reduce the GSH/GSSG ratio (i.e., shift basal redox to a more oxidized state) in rat diaphragm (26) and hindlimb skeletal muscle (39). As would be predicted by the model proposed by Reid et al. (32), BSO treatment (20 mM BSO in drinking water for 7 days) resulted in increased basal peak twitch force and exacerbated fatigue in rat diaphragm muscle subjected to inspiratory resistive loading (26). Therefore, BSO treatment appears to be a useful research model for investigating redox regulation of various physiological processes in skeletal muscle under basal conditions and in response to fatiguing muscle contractile activity.

Sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPases (SERCAs) are ~110-kDa integral membrane proteins that catalyze the ATP-dependent transport of Ca\(^{2+}\) from the cytosol to the lumen of the sarco(endo)plasmic reticulum (SR) (21). The two main SERCA isoforms that are expressed in skeletal muscle, namely SERCA1α and SERCA2α, contain 24 and 26 Cys residues, respectively, and therefore are highly susceptible to oxidative damage and loss of function when exposed to oxidative stress (40, 50). For example, SERCA activity is depressed following prolonged skeletal muscle ischemia (48), myocardial ischemia-reperfusion (27), fatiguing exercise (45), aging (42), and heat stress (4, 19, 37, 46). However, to what extent SERCA function is modulated by redox signaling in skeletal muscle under basal conditions is not well known.

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In this study, we treated rats with BSO for 10 days to examine the effects of GSH depletion and changes in basal redox state on diaphragm contractility and SERCA function measured in vitro under resting conditions and following fatiguing stimulation. It was hypothesized that BSO treatment would result in increased basal isometric force and increased fatigability of diaphragm muscle strips assessed in vitro. Second, we hypothesized that SERCA function measured in homogenates would not be altered under basal conditions but would be reduced to a greater extent in response to fatiguing stimulation following BSO treatment.

MATERIALS AND METHODS

Animal description and care. The animals used for this study were also used for another study examining the effects of GSH depletion in vivo on isolated blood vessel function. These results along with details of the animal model have been published elsewhere (10). In brief, a total of 20 adult male Sprague-Dawley rats were used for this study. The animals were housed two to three per cage in a climate-controlled facility on reverse 12-h light cycle. All rats were maintained on a normal diet of 22/5 rodent diet lab chow (Harlan), with the control (CTL) rats receiving normal tap water and the BSO rats water containing (in mM) 118 NaCl, 25 KCl, 1.15 NaHCO₃, 1.2 KH₂PO₄, 1.2 MgSO₄, pH 7.4, and maintained at 33°C between a Plexiglas clamp and a servomotor (Cambridge Technologies, model 300H Dual Mode Servo) connected to a National Instruments 16-bit analog-to-digital card and analyzed using the Dynamic Muscle Control and Data Acquisition (DMC) and Dynamic Muscle Analysis (DMA) Software (Aurora Scientific). Muscle length (L₀) was adjusted to obtain maximal isometric twitch force (g).

Fatigue protocol. To investigate the hypotheses that diaphragm fatigability would be greater and that SERCA function would be impaired to a greater extent in response to intense contractile activity following BSO treatment compared with CTL, a 5-min high-frequency stimulation protocol consisting of 350-ms contractions at 100 Hz, once per second, was employed. Fatigue was assessed by determining the number of contractions needed to induce a 50% loss in maximum force (50% rundown) and by the overall force loss after 5 min expressed as a percentage of initial force. After the contractile and fatigue properties were measured, the diaphragm muscle strips were trimmed of the remnants of the central tendon and the rib, blotted on filter paper, and weighed on an analytical balance. The total muscle fiber cross-sectional area of the muscles was determined by dividing the muscle mass (mg) by the product of L₀ (mm) and 1.06 mg/mm³, the density of mammalian skeletal muscle (25). Force data were normalized for total muscle fiber cross-sectional area.

Sample preparation for assessment of SERCA function in vitro. To investigate the effects of BSO treatment and fatiguing contractile activity on SERCA function, Ca⁺⁺-ATPase activity and Ca⁺⁺ uptake were measured in diaphragm homogenates. Basal (i.e., resting) SERCA function was determined in homogenates that were prepared from costal diaphragm samples taken immediately following removal of the whole diaphragm from both CTL and BSO-treated animals. The effects of fatiguing contractile activity on SERCA function were determined in homogenates that were prepared from diaphragm muscle strips immediately following the fatigue protocol. Homogenates were prepared with 30–40 mg of tissue diluted 11:1 (vol/wt) in an ice-cold buffer containing (in mM) 250 sucrose, 5 HEPES, 10 NaF, 0.2 PMSF, and 10 Na+, and 0.2 PMSE (pH 7.5) by using a handheld glass homogenizer (Daul 20, Kontes). The homogenates were separated into multiple aliquots and frozen immediately in liquid nitrogen for later analysis. On a given analytical day, aliquots were thawed and processed for the duplicate measurements of Ca⁺⁺-ATPase activity in diaphragm and Ca⁺⁺ uptake. Total protein concentration of the homogenates was measured by the method of Lowry, as modified by Schacterle and Pollock (35).

Ca⁺⁺-dependent Ca⁺⁺-ATPase activity. Measurements of Ca⁺⁺-induced Ca⁺⁺-ATPase activity were made at 37°C using a spectrophotometric assay as described previously (47). Briefly, the assay buffer contained (in mM) 20 HEPES, 200 KCl, 1.5 MgCl₂, 1 EGTA, 10 NaF, 5 ATP, and 10 phosphonopyruvate, pH 7.0. Immediately before initiating the reaction with CaCl₂ additions, 18 μM lactate dehydrogenase enzyme, 18 μM pyruvate kinase enzyme, 0.3 mM NADH, 1 μM Ca⁺⁺ ionophore A23187 (Sigma, C-7522), and the tissue (50 μg of total protein) were added to 1 ml of assay buffer. Ca⁺⁺-dependent Ca⁺⁺-ATPase activity was assessed by adding 4–11 μl of 100 mM CaCl₂ in 0.5-μl additions until Vmax was reached. The final concentration of each CaCl₂ addition was assessed separately, on a different SR aliquot, by use of dual-wavelength spectrophotometry and the Ca⁺⁺-fluorescent dye indo-1. Basal activity was measured in the presence of 40 μM cyclopiazonic acid, which is a specific SERCA inhibitor. The difference between total and basal activities represents the Ca⁺⁺-activated SR Ca⁺⁺-ATPase activity. The data were analyzed by nonlinear regression with computer software (GraphPad Software) and the Kₘ values were calculated using an equation for a general cooperative model for substrate activation. The values for maximal Ca⁺⁺-ATPase activity that occurred at pCa 5.6 to pCa 5.2 were taken directly from the experimental data and normalized for total protein concentration.
SR Ca\(^{2+}\) uptake. Oxalate-supported Ca\(^{2+}\) uptake was measured using the Ca\(^{2+}\) fluorescent dye indo-1 as described previously (47). Fluorescence measurements were collected on a dual-emission wavelength spectrofluorometer (Ratiomaster System, Photon Technology International). The excitation wavelength was set to 355 nm, and 405 and 485 nm correspond to the emission wavelength for bound (F) and free (G) indo-1, respectively. Photon counts were simultaneously collected for each wavelength. Before each analytical session, the background fluorescence was determined in the absence of indo-1 and subtracted before starting each assay. The reaction buffer contained 200 mM KCl, 20 mM HEPES, 10 mM NaN\(_3\), 0.005 \(\mu\)M N,N',N",N"-tetrakis(2-pyridylmethyl)ethylendiamine (TPEN), 5 mM oxalate, and 15 mM MgCl\(_2\), pH 7.0. Before initiating data collection, the assay buffer was heated to 37°C, and 1.5 \(\mu\)M CaCl\(_2\), and 50 \(\mu\)M of diaphragm homogenate were added to a cuvette containing 2.0 ml of buffer. Once these additions were made, data collection was started. After a brief period, 40 \(\mu\)l of 250 mM ATP was added to initiate Ca\(^{2+}\) uptake. Initial [Ca\(^{2+}\)]\(_i\) was \(~2.5\) \(\mu\)M.

As Ca\(^{2+}\) decreases because of negative SR Ca\(^{2+}\) uptake, R decreases. Using Felix software (Photon Technology International), the [Ca\(^{2+}\)]\(_i\) was calculated from the following equation:

\[
[Ca^{2+}]_i = K_d \times \frac{(G_{\text{max}}/G_{\text{min}}) (R - R_{\text{min}})/(R_{\text{max}} - R)}
\]

where \(K_d\) represents the equilibrium constant for the interaction between Ca\(^{2+}\) and indo-1. A \(K_d\) value of 250 nm was used (12). \(R_{\text{min}}\) is the minimum value of R at addition of 250 \(\mu\)M EGTA, \(G_{\text{max}}\) is the maximum value of G at addition of 250 \(\mu\)M EGTA, \(G_{\text{min}}\) is the minimum value of G at addition of 1 \(\mu\)M CaCl\(_2\), and \(R_{\text{max}}\) is the maximum value of R at addition of 1 \(\mu\)M CaCl\(_2\). For all Ca\(^{2+}\) uptake trials, \(R_{\text{min}}\) and \(R_{\text{max}}\) were not determined until Ca\(^{2+}\) uptake had plateaued, which occurred at \(~75\) nM [Ca\(^{2+}\)]. The generated curve from Eq. 1, [Ca\(^{2+}\)]\(_i\) vs. time, was smoothed over 21 points using the Savitsky-Golay algorithm. Linear regression was performed on values of [Ca\(^{2+}\)]\(_i\) between 1.4 and 1.6 \(\mu\)M (i.e., \(p_Ca\) 5.82). Differentiating the linear fit curve allowed the determination of Ca\(^{2+}\)-uptake rates, which were normalized for total protein concentration.

**Western blot analysis.** Western blotting was performed to determine the relative expression levels of Hsp70, SERCA1a, and SERCA2a in diaphragm muscle from CTL and BSO-treated rats. After ensuring linearity of band density, 20 \(\mu\)l of the homogenate (Hsp70, 5 \(\mu\)g total protein; SERCA1a, 1 \(\mu\)g total protein; SERCA2a, 20 \(\mu\)g total protein) were applied to 10\% (Hsp70) or 7\% (SERCA1a and SERCA2a) polyacrylamide gels, and proteins were separated using standard SDS-PAGE protocols (17) and then transferred to polyvinylidene difluoride (PVDF) membranes (Roche Diagnostics, Mannheim, Germany). A biotinylated ladder was used as a molecular weight standard (Cell Signaling Technology, Beverly, MA). The membranes were cut horizontally to separate proteins of molecular weight 60 kDa before applying the primary antibodies. After blocking with 10\% skim milk suspension, the membranes containing the lower molecular weight proteins (i.e., above \(~60\) kDa) were incubated for either 16 h with anti-Hsp70 monoclonal antibody SPA-810 (Stressgen Biotechnologies) or 1 h with either anti-SERCA1a monoclonal antibody A52 (54) or anti-SERCA2a antibody 2A7-A1 (Affinity Bioreagents), and the membranes containing the higher molecular weight proteins (i.e., below \(~60\) kDa) were incubated for 1 h with anti-\(\alpha\)-sarcomeric actin antibody 5C5 (Sigma) to control for protein loading. Then, after washing in Tris-HCl, pH 7.5, 150 mM NaCl, 0.1\% Tween 20 (Tris-buffered saline-0.1\% Tween), the membranes were treated with horseradish peroxidase-conjugated anti-mouse secondary antibody (Santa Cruz Biotechnology). Membranes were washed in Tris-buffered saline-0.1\% Tween, and the signals were detected with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech) using a bioimaging system, and densitometric analysis was performed using the GeneSnap software (Syngene). All samples were run in duplicate on separate gels, and the Hsp70, SERCA1a and SERCA2a expression levels were expressed relative to \(\alpha\)-actin content and normalized to the CTL rest sample.

**Statistical analysis.** To determine the effects of BSO treatment (CTL vs. BSO) and fatiguing stimulation (rest vs. fatigue) for both Ca\(^{2+}\)-ATPase activity and Ca\(^{2+}\)-uptake measures, a two-way ANOVA was employed. For all other measures, statistical analysis was performed by Student’s paired \(t\)-test between CTL and BSO-treated diaphragm. The significance level was set at \(P < 0.05\), and when appropriate a Newman-Keuls post hoc test was used to compare specific means. All data are presented as means ± SE.

**RESULTS**

**Diaphragm glutathione content.** The effects of BSO treatment on diaphragm GSH, GSSG, and the ratio GSH/GSSG are illustrated in Fig. 1. Compared with CTL animals, diaphragm GSH levels were reduced (\(P < 0.05\)) by \(~91\%\) (Fig. 1A), and GSSG levels were reduced by \(~62\%\) (Fig. 1B) in animals treated with BSO, which indicates that cellular antioxidant buffering capacity was reduced in BSO-treated diaphragm. The calculated ratio GSH/GSSG was also reduced by \(~71\%\) with BSO treatment (Fig. 1C), indicating a shift toward a more oxidized cellular redox state in diaphragm muscle from BSO-treated animals compared with CTL.

**Diaphragm Hsp70 Content.** It was of interest to further characterize the effects of BSO treatment on cellular redox state to determine whether the shift toward a more oxidized environment that occurred in BSO-treated diaphragm was sufficient to induce a cellular stress response. Western blot analysis was carried out on homogenates prepared from diaphragm muscles from CTL and BSO-treated animals to determine the relative expression levels of Hsp70, which is the most highly induced protein of the cellular stress response and is rapidly upregulated under conditions of oxidative stress (51). Overall, there were no differences (\(P > 0.05\)) in Hsp70 expression levels between CTL and BSO-treated diaphragm muscles (Fig. 2).

**In vitro basal isometric force in rat diaphragm.** For diaphragm muscle strips in vitro, “basal state” is defined as the unfatigued muscle (33). The effects of BSO treatment on in vitro basal peak twitch force and peak tetanic force (100 Hz) are shown in Fig. 3. Note that the first contraction during the fatigue protocol was taken to represent basal peak tetanic force. Basal peak twitch force was \(~31\%\) higher (\(P < 0.05\)) in diaphragm strips from BSO-treated animals compared with CTL (Fig. 3A). Basal peak tetanic force was also higher in diaphragm strips from BSO-treated animals compared with CTL; however, the difference between BSO and CTL was not significant (\(P = 0.11\)) (Fig. 3B).

**Effects of BSO treatment on diaphragm fatigability.** Diaphragm susceptibility to fatigue was assessed by repetitive stimulation of the muscle strips using the protocol outlined in MATERIALS AND METHODS. Compared with CTL, it took \(~16\) fewer contractions to induce a 50\% loss in maximum tetanic force, and the overall reduction in force by the end of the fatigue protocol was greater (\(P < 0.05\)) in diaphragm strips from BSO-treated animals (Table 1).

**SERCA function.** The effects of BSO treatment and fatiguing contractile activity on SERCA function are illustrated in Fig. 4. Compared with CTL, BSO treatment resulted in an increase in...
maximal Ca\textsuperscript{2+}-ATPase activity of \(~28\%\) (Fig. 4A, open bars) and an increase in Ca\textsuperscript{2+} uptake of \(~25\%\) (Fig. 4B, open bars) in diaphragm muscle under basal conditions (i.e., rest). Following fatiguing stimulation, both maximal Ca\textsuperscript{2+}-ATPase activity and Ca\textsuperscript{2+} uptake were increased \((P < 0.05)\) in CTL diaphragm by \(~31\%\) and \(~21\%\), respectively, whereas in BSO-treated animals, maximal Ca\textsuperscript{2+}-ATPase activity and Ca\textsuperscript{2+} uptake in diaphragm were not different \((P > 0.05)\) between rest and fatigue. Kinetic analysis of the Ca\textsuperscript{2+}-ATPase-
pCa curves showed that \(K_{Ca}\) (pCa units) was unaltered \((P > 0.05)\) by either BSO treatment (CTL 5.72 ± 0.04 vs. BSO 5.72 ± 0.05) or fatiguing stimulation (CTL 5.81 ± 0.04 vs. BSO 5.71 ± 0.05). Similarly, \(n_H\) was unaffected by either BSO treatment (CTL 1.72 ± 0.15 vs. BSO 1.59 ± 0.06) or fatiguing stimulation (CTL 1.55 ± 0.04 vs. BSO 1.51 ± 0.05).

Expression of SERCA1a and SERCA2a. The relative expression of both SERCA1a (Fig. 5A) and SERCA2a (Fig. 5B) were compared between diaphragm muscles from BSO-treated animals and controls. No differences \((P > 0.05)\) in SERCA1a expression levels were noted between CTL and BSO-treated animals, whereas SERCA2a content was increased \(~2.2\) fold in diaphragm muscle from BSO-treated animals compared with CTL (Fig. 5C).

DISCUSSION

To assess the effects of a reduced antioxidant capacity and a more oxidized cellular redox state on diaphragm contractility and SERCA function, we treated rats with BSO to deplete cellular GSH and reduce the GSH/GSSG ratio, both of which did occur in this study, confirming treatment efficacy. Our results are in agreement with previous findings (26) that BSO treatment increases basal peak isometric force and increases diaphragm fatigability. We also show several novel effects of
BSO treatment on SERCA protein expression and function. First, we measured SR Ca\(^{2+}\)/H11001 uptake and maximal SERCA activity in homogenates prepared from diaphragm under basal conditions, and both were increased with BSO treatment. Second, SERCA2a protein expression in diaphragm was increased with BSO treatment, suggesting that the expression of SERCA2a can be regulated by redox signaling. Finally, intense contractile activity results in an increased Ca\(^{2+}\)/H11001 uptake and maximal SERCA activity in diaphragm strips from control animals but not from BSO animals.

It is difficult to quantify changes in intracellular redox state without measuring changes in ROS or RNS concentrations. We did not measure ROS or RNS in diaphragm tissue, but we have reported previously that liver homogenate H2O2 levels were increased by 42% in the BSO-treated rats that were used for this study (10). That the GSH/GSSG ratio was not altered in liver but was reduced in diaphragm in BSO-treated animals would suggest that ROS and/or RNS levels may have been even higher in diaphragm under basal conditions. Nevertheless, the fact that diaphragm Hsp70 expression was not different between CTL and BSO suggests that the increased ROS and/or RNS levels may have been below the threshold required to induce a stress response. In contrast to our findings, Hsp70 levels were increased by 63% in mouse soleus 24 h after a single 4 mmol/kg dose of BSO injected intraperitoneally (22). BSO treatment, species, and/or muscle fiber type differences may explain the different Hsp70 responses between the studies.

### Table 1. Effects of BSO treatment on diaphragm fatigability

<table>
<thead>
<tr>
<th>Group</th>
<th>50% Rundown</th>
<th>Final Force, %Rest</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>54.6±7.0</td>
<td>18.9±1.8</td>
</tr>
<tr>
<td>BSO</td>
<td>38.8±2.6*</td>
<td>11.3±1.6*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 10/group. BSO, buthionine sulfoximine. 50% Rundown is defined as the number of contractions needed to induce a 50% loss in tetanic force. \*Significantly different from control (CTL). (P < 0.05).

### Fig. 3. Basal isometric twitch and tetanic force of diaphragm strips from CTL rats and BSO-treated rats. Diaphragm muscle strips were prepared from CTL and BSO-treated animals, and basal peak isometric force amplitude was determined during a twitch and a maximal tetanic contraction (100 Hz) as described in MATERIALS AND METHODS. Force data were normalized for total muscle cross-sectional area (g/mm\(^2\)).

A: peak twitch force. B: peak tetanic force measured at 100 Hz. Values are means ± SE; n = 10/group. \*Significantly different from CTL (P < 0.05).

### Fig. 4. Effects of BSO treatment and fatigue on sarcoplasmic reticulum Ca\(^{2+}\)-ATPase function. Ca\(^{2+}\)-ATPase function was assessed in crude homogenates that were prepared from diaphragm strips from both CTL and BSO-treated animals either at rest (open bars) or following a fatiguing stimulation protocol (filled bars). See MATERIALS AND METHODS for details about the fatigue protocol.

A: maximal Ca\(^{2+}\)-ATPase activity, which occurred at pCa 5.6–5.2, was measured using a spectrophotometric assay as described in MATERIALS AND METHODS. B: oxalate-supported Ca\(^{2+}\) uptake was measured at pCa 5.82 using Indo-1 as described in MATERIALS AND METHODS. Values are means ± SE; n = 8/group. \*Significantly different from Rest (P < 0.05). #Significantly different from CTL (P < 0.05).
One of the major findings of this study was that the stimulation-induced increase in both SERCA activity and $\text{Ca}^{2+}$ uptake that was observed in CTL diaphragm strips was completely blunted in BSO-treated animals. Since diaphragm ROS levels increase with repeated contractions (31) and since it is well established that ROS can inhibit SERCA activity (8, 14, 53), the fact that both SERCA activity and $\text{Ca}^{2+}$ uptake were increased following fatiguing stimulation in controls suggests that other signaling pathways that are activated with muscle contraction cause SERCA activity to increase, thus masking the negative effects of oxidative stress. If this interpretation is
correct, then a simple explanation for the results obtained from BSO animals is that contraction-induced oxidative stress would be higher in glutathione-depleted BSO diaphragm compared with controls and would completely offset any positive regulatory signals associated with muscle contraction, resulting in no change in either Ca\textsuperscript{2+} uptake or SERCA activity in response to fatiguing stimulation.

Our study is not the first to report an increase in Ca\textsuperscript{2+} uptake or maximal SERCA activity in response to repetitive muscle contractions. For example, both Ca\textsuperscript{2+} uptake and maximal Ca\textsuperscript{2+-ATPase} activity measured in rat hindlimb muscle are increased during recovery following a single bout of prolonged treadmill exercise (9, 38). In another study, Holloway et al. (13) found that fatiguing stimulation of rat hindlimb muscle caused an increase in both Ca\textsuperscript{2+} uptake and maximal SERCA activity in white gastrocnemius muscle, whereas, in contrast, both Ca\textsuperscript{2+} uptake and maximal Ca\textsuperscript{2+-ATPase} activity were reduced in red gastrocnemius and soleus. The different muscle-specific responses are related to the oxidative potential of the muscle (13) and may reflect differences in second messenger regulation of SERCAs and/or redox homeostasis. Similarly, in response to different types of exercise, SR Ca\textsuperscript{2+} uptake measured in rat diaphragm homogenates is either increased (43) or decreased (23), which may reflect differences in ROS production and/or second messenger activation, depending on the exercise protocol employed.

In summary, we have found that BSO treatment in rats for 10 days depletes cellular GSH and reduces the GSH/GSSG ratio in diaphragm. BSO treatment increased diaphragm basal peak twitch force and increased diaphragm fatigability. SERCA activity, assessed in homogenates prepared from diaphragm under basal conditions, was upregulated with BSO treatment, which corresponded with an increase in SERCA2a protein expression. Fatiguing stimulation resulted in an increased Ca\textsuperscript{2+} uptake and maximal SERCA activity in controls but not in BSO animals suggesting that regulation of SERCA activity in diaphragm likely involves a complex interaction between redox signaling and other signaling pathways that are activated with muscle contraction. This study contributes to a growing body of work supporting the view that free radicals play important biological roles in skeletal muscle (34).

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REFERENCES


