Superoxide, hydroxyl radical, and hydrogen peroxide effects on single-diaphragm fiber contractile apparatus

L. A. CALLAHAN,1 Z. W. SHE,2 AND T. M. NOSEK3
1Division of Pulmonary and Critical Care Medicine, Department of Medicine, and 2Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, Ohio 44109; and 3Department of Medicine, Medical College of Georgia, Augusta, Georgia 30912

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Callahan, L. A., Z. W. She, and T. M. Nosek. Superoxide, hydroxyl radical, and hydrogen peroxide effects on single-diaphragm fiber contractile apparatus. J Appl Physiol 90: 45–54, 2001.—Reactive oxygen species contribute to diaphragm dysfunction in certain pathophysiological conditions (i.e., sepsis and fatigue). However, the precise alterations induced by reactive oxygen species or the specific species that are responsible for the derangements in skeletal muscle function are incompletely understood. In this study, we evaluated the effect of the superoxide anion radical ($O_2^-$), hydroxyl radical (•OH), and hydrogen peroxide ($H_2O_2$) on maximum calcium-activated force ($F_{max}$) and calcium sensitivity of the contractile apparatus in chemically skinned (Triton X-100) single rat diaphragm fibers. $O_2^-$ was generated using the xanthine/xanthine oxidase system; •OH was generated using 1 mM FeCl$_2$, 1 mM ascorbate, and 1 mM $H_2O_2$; and $H_2O_2$ was added directly to the bathing medium. Exposure to $O_2^-$ or •OH significantly decreased $F_{max}$ by 14.5% ($P < 0.05$) and 43.9% ($P < 0.005$), respectively. •OH had no effect on $Ca^{2+}$ sensitivity. Neither 10 nor 1,000 μM $H_2O_2$ significantly altered $F_{max}$ or $Ca^{2+}$ sensitivity. We conclude that the diaphragm is susceptible to alterations induced by a direct effect of •OH and $O_2^-$, but not $H_2O_2$, on the contractile proteins, which could, in part, be responsible for prolonged depression in contractility associated with respiratory muscle dysfunction in certain pathophysiological conditions.

free radicals; reactive oxygen species; skinned muscle fibers; respiratory muscle; skeletal muscle

The diaphragm generates oxygen-derived free radical species at rest and during contraction (12, 21, 37). Several of the most important of these reactive oxygen species (ROS) include hydrogen peroxide ($H_2O_2$), the superoxide anion radical ($O_2^-$), and the hydroxyl radical (•OH). Whereas low levels of these intermediates are thought to be necessary for optimal function of skeletal muscles under normal conditions (36), in pathological states, including sepsis (6, 16) and ischemia-reperfusion injury (46) and during fatigue (5, 21), it has been postulated that increased oxidative stress leads to tissue injury and, consequently, skeletal muscle dysfunction (4, 41). Measurements of increased levels of lipid peroxidation by-products (3, 44) and the observation that administration of selective or nonselective free radical scavengers partially ameliorates muscle dysfunction provide indirect evidence that enhanced ROS generation is an important factor in skeletal muscle dysfunction in a variety of conditions (11, 39, 40, 43). More direct evidence for the toxicity of ROS has been demonstrated by infusion of a free radical-generating solution into the diaphragm of dogs, which resulted in decreased force generation and a shift in the force-frequency relationship downward and to the right (31). The precise alterations induced by ROS or the specific species that are responsible for the derangements in skeletal muscle function are incompletely understood. Whereas some studies have evaluated the effects of free radicals on skeletal muscle sarcoplasmic reticulum (SR) and mitochondria (13, 35), only limited work has been performed to determine the effects of ROS on skeletal muscle contractile proteins. Moreover, most of the reports that have examined this issue in skeletal muscle have studied the effects produced by exposure of intact muscle fibers to exogenous free radical-generating solutions (2). These previous experiments have fundamental limitations, because indirect metabolic effects resulting from the actions of free radicals on intermediary metabolic pathways may well have been responsible for all of the phenomena observed. Methodologically, a more direct approach to this problem is to determine the effects of free radical-generating solutions on “skinned” muscle fibers (i.e., single fibers in which the sarcolemma, SR, and mitochondria have been removed, thus permitting direct functional assessment and access to the contractile proteins). Only a few reports have directly evaluated the effects of free radical species on contractile protein function in skeletal muscle using skinned fibers, and only the effects of nitric oxide, peroxynitrite, and $H_2O_2$ have been examined (8, 32, 45). Importantly, no study has explicitly examined the direct effects of •OH or $O_2^-$ on contractile protein function in any skeletal muscle using skinned fibers. It is known that different free radical species possess different propensities to chemically modify cel-
ular constituents. For example, peroxynitrite and nitric oxide produce nitrosylation of aromatic amino acid side chains, whereas superoxide and hydroxyl anions do not. Hydroxyl and peroxynitrite induce lipid peroxidation, whereas superoxide and nitric oxide do not (19). As a result, it is possible that individual free radical species may produce qualitatively and quantitatively different effects on contractile protein function, and it is necessary to study each free radical species individually to obtain a comprehensive understanding of the potential functional alterations of ROS on skeletal muscle.

The purpose of present study, therefore, was to examine the direct effects of O$_2^\cdot$ and -OH on the contractile apparatus in chemically skinned, single skeletal muscle fibers. Because it was necessary to utilize H$_2$O$_2$ as a component of our -OH generating solution, we also determined the effects of H$_2$O$_2$ per se in our experimental system. We chose to study fibers isolated from the diaphragm rather than limb skeletal muscle, both because of the physiological importance of the diaphragm (this is the only skeletal muscle whose function is critical to sustain life) and because a number of studies have identified the diaphragm as a target of free radical-mediated injury in several pathophysiological states. We specifically investigated the direct effects of the three free radical species studied on maximum calcium-activated force (F$_{\text{max}}$) of the contractile proteins and, when possible, also determined the effects of these species on the calcium (Ca$^{2+}$) sensitivity of the contractile apparatus. Because it is known that -OH are more chemically reactive with proteins, we hypothesized that -OH would produce greater alterations in contractile protein function than either O$_2^\cdot$ or H$_2$O$_2$ (33). Moreover, because one previous study has shown that calcium binding to isolated skeletal muscle troponin C is reduced by exposure to free radical-generating solutions, we postulated that -OH and O$_2^\cdot$ would reduce calcium sensitivity of the contractile proteins, shifting the force vs. pCa relationship to the right (18).

**METHODS**

**General Approach**

All experiments were conducted on single diaphragm fibers removed from adult Sprague-Dawley rats (all weighing $<200$ g), which were killed by cervical dislocation. This study was approved by the Medical College of Georgia Institutional Animal Care and Use Committee, and all procedures were performed in accordance with experimental guidelines for that institution and in accordance with American Physiological Society guidelines. After death, the diaphragm was carefully detached from its intercostal insertions and placed in a dissecting dish containing a relaxing solution with the following composition (in mM): 1.0 Mg$^{2+}$, 5.0 MgATP, 15 phosphocreatine, 140.0 potassium phosphatase, 50.0 imidazole, and 10.0 EGTA, with pCa >8.5 and pH 7.0. Ionic strength was adjusted to 200. This solution also contained the following protease inhibitors to protect the fibers from the damaging effects of proteolysis: 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, 1.0 mM benzamidine, 10 $\mu$M aprotinin, and 1 mM dithiothreitol (DTT).

The diaphragm was subsequently divided into small strips, and some bundles were stored at $-20^\circ$C in relaxing solution containing 50% glycerol and protease inhibitors. In this storage solution, CTP was used in place of ATP to prevent phosphorylation of myosin light chains. All single-fiber assessments were completed within 48–72 h of dissection.

On the day that fiber characteristics were assessed, diaphragm strips were removed from storage solution, placed in DTT-free relaxing solution (i.e., all components were the same as listed in the previous paragraph except that DTT was not included), and allowed to warm to room temperature. Small bundles of $\approx$10 fibers were then separated from the whole muscle by gently pulling on one end of the muscle with a pair of fine-tipped forceps while the other end of the muscle was held stationary with a second pair of forceps. Fiber bundles were immersed for 30 min in 0.1% Triton X-100, an ionic detergent that eliminates the membranes of the sarcolemma, SR, and mitochondria, leaving only the contractile proteins intact.

After incubation, bundles were removed from Triton X-100 and placed in relaxing solution, and individual fibers were teased from the muscle bundles. Single fibers were then mounted between an optoelectric force transducer (Scientific Instruments, Heidelberg, Germany) and a movable arm by wrapping the ends of each fiber around stainless steel wires. Fiber length was adjusted to achieve a resting sarcomere length of 2.6 $\mu$m as indicated by its helium-neon laser diffraction pattern. Length remained constant throughout the experiments. The cross-sectional area of each fiber was determined after sarcomere length was adjusted, by measuring the diameter of the fiber using a micrometer attached to the eyepiece of the microscope binocular. Area was calculated, assuming a cylindrical shape for the fiber.

Force vs. pCa curves were constructed for fibers under baseline conditions by immersing them in solutions of increasing calcium concentrations and recording tension on a strip recorder. Once peak tension was achieved in a given solution, fibers were rapidly switched to the next solution by means of a spring-loaded Plexiglas tray. The composition of all solutions used in this study was calculated by using a computer program (Borland International, Scotts Valley, CA) that takes into account stability constants and stock solutions to produce final solutions of the correct ionic strength and pCa (12). Specifically, the solutions used to examine the force vs. pCa relationship were of the following composition (in mM): 1.0 Mg$^{2+}$, 1.0 MgATP, 15 phosphocreatine, 110.0 potassium phosphatase, 20.0 imidazole, and 5.0 EGTA, pH 7.0. Ionic strength was 200. Ca$^{2+}$ was added to yield solutions of the desired pCa.

The general experimental protocol consisted of the following steps. 1) Fibers were submerged in a solution containing no added calcium (pCa 8.5), followed by sequential exposure to 11 different calcium solutions, namely pCa 6.0, 5.90, 5.80, 5.70, 5.60, 5.50, 5.40, 5.30, 5.20, 5.0, and 4.0, to establish the baseline force vs. pCa relationship. 2) Fibers were relaxed in pCa 8.5 solution and then transferred to another trough containing pCa 8.5, where they were exposed to the ROS-generating solution or individual components of the ROS-generating solutions. 3) After ROS exposure, a second force vs. pCa curve was generated. Modifications to the above protocol are outlined specifically below. All experiments were performed at room temperature, which averaged 22°C.
Experimental Procedures

We conducted three groups of experiments to determine the effect of each individual oxygen-derivative species being investigated on $F_{max}$ and the calcium sensitivity in single, chemically skinned diaphragm fibers from adult animals. Groups 1, 2, and 3 consisted of fibers exposed to $H_2O_2$, $O_2^-$, or $OH^-$, respectively. Details of the specific protocols for each group of experiments are described below.

 Protocol 1: Exposure to $H_2O_2$. To achieve exposure to $H_2O_2$ in single, skinned diaphragm fibers, either 10 or 100 $\mu$M $H_2O_2$ were added directly to the pCa 8.5 solution using a diluted standard 30% stock solution (Sigma Chemical, St. Louis, MO) to achieve the final desired concentration. The concentrations of the diluted $H_2O_2$ solutions were directly measured by spectrophotometer at 250 nm, assuming a molar extinction coefficient of 0.071 $\times$ 10$^3$ M$^{-1}$cm$^{-1}$ (9). We chose to evaluate two concentrations of $H_2O_2$; both doses have been commonly used in previous studies that evaluated the effects of $H_2O_2$ (7, 8, 10, 25, 26).

The protocol for exposure of single fibers to $H_2O_2$ consisted of steps 1–3 as outlined above; in step 2, the fibers were exposed for 5 min in pCa 8.5 to either 10 or 1,000 $\mu$M $H_2O_2$. In a separate group of in-time control fibers, $H_2O_2$ was not added to the solution in step 2.

 Protocol 2: Exposure to superoxide. In the second series of experiments, the xanthine-xanthine oxidase system was used to generate $O_2^-$. Before initiation of the skinned fiber protocol, $O_2^-$-generation with 50 $\mu$M xanthine and 0.91 $\mu$M xanthine oxidase in the pCa 8.5 solution was confirmed spectrophotometrically by measuring the reduction of 0.05 mM cytochrome $c$ at 550 nm. To confirm that the reactive species was indeed $O_2^-$, superoxide dismutase (SOD; 15 $\mu$g/ml) was added to the above $O_2^-$-generating system. The amount of $O_2^-$, as determined by the amount of SOD-inhibitable cytochrome $c$ reduction, was calculated based on the changes in the spectrophotometric readings at 550 nm in the presence and absence of SOD, assuming a molar extinction coefficient of 18.5 $\times$ 10$^3$ M$^{-1}$cm$^{-1}$ and remained at this level for at least 10 min. The absence of $OH^-$ in this system was confirmed by using the salicylate trapping method (12). For these latter determinations, 50 $\mu$M xanthine and 0.91 $\mu$M xanthine oxidase in pCa 8.5 solution were incubated with 1 mM sodium salicylate for 10 min ($n = 3$ determinations). Samples from the reaction mixture were assayed using HPLC for determination of 2,3- or 2,5-dihydroxybenzoic acid peaks (12). We found no evidence of 2,3- or 2,5-dihydroxybenzoic acid formation in any sample using this approach. This concentration of $O_2^-$ is lower than the maximum reported rates of mitochondrial $O_2^-$-generation (1.2 nmol-min$^{-1}$mg protein$^{-1}$) (14) but is comparable to that which has previously been used to evaluate the effects of $O_2^-$ on the contractile properties of skinned cardiac fibers (24). Additionally, we have previously shown that, during a fatiguing stimulation, there is extracellular release of $O_2^-$ at a rate of 0.70 $\pm$ 0.17 nmol/min in whole diaphragm preparations (21).

In preliminary experiments, we observed that varying the concentrations of the components of the generating system produced higher $O_2^-$-generation. However, high concentrations of $O_2^-$ and an exposure time of 5 min made the fibers extremely fragile, such that we were unable to evaluate the entire force vs. pCa relationship for this series of experiments. Because the individual components of the $O_2^-$-generating system could be responsible for any observed effects on $F_{max}$, we controlled for this possibility.

In these experiments, the protocol consisted of the following steps: 1) baseline $F_{max}$ (pCa 5.3) was obtained in all fibers, followed by relaxation in pCa 8.5. 2) Fibers were then exposed for 2 min in pCa 8.5 to which one of the following was added: xanthine, xanthine oxidase, SOD, xanthine + xanthine oxidase, or xanthine + xanthine oxidase + SOD. 3) After exposure, a repeat measurement of $F_{max}$ in pCa 5.3 was performed in all fibers. In-time control fibers were carried through the same protocol but without exposure to $O_2^-$ or any component of the generating system.

 Protocol 3: Exposure to $OH^-$. In the final series of experiments, $OH^-$ was generated in pCa 8.5 using 1 mM FeCl$_2$, 1 mM ascorbic acid, and 1,000 $\mu$M $H_2O_2$. Because our normal pCa 8.5 solution contains a significant amount of EGTA (5 mM), the solution used in these experiments had to be modified by reducing the EGTA to 0.05 mM to prevent chelation of the iron necessary to generate $OH^-$. Production of $OH^-$ was confirmed by HPLC using the salicylate trapping method, as previously described (12). HPLC analysis showed that 31 $\mu$mol of 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid was produced in this system over 5–10 min.

The protocol used to assess the effects of $OH^-$ and each individual component of the $OH^-$ generating system on the force vs. pCa relationship was similar to protocol 2. In step 2, fibers were relaxed in pCa 8.5 (5 mM EGTA) and subsequently exposed for 5 min in pCa 8.5 (0.05 mM EGTA) to either 1 mM FeCl$_2$, 1 mM FeCl$_2$ + 1 mM ascorbic acid, or 1 mM FeCl$_3$ + 1 mM ascorbic acid + 1,000 $\mu$M $H_2O_2$. Because we had already performed experiments to examine the effects of exposure to 1,000 $\mu$M $H_2O_2$ as well as run-down in the control fibers after 5 min (see Protocol 1: Exposure to $H_2O_2$), we used the results from these experiments in the present protocol. Furthermore, because no significant changes in $F_{max}$ were seen with a 5-min exposure to ascorbic acid in a series of preliminary experiments, full force vs. pCa curves were not performed for this component of the $OH^-$ generating system.

Fiber Typing

After each experiment, fibers were stored in SDS buffer solution containing 0.125 M Tris, 4% SDS, 20% glycerol, and 20 mM DTT at $-40^\circ$C. Fibers were analyzed for slow myosin heavy chain (MHC) using Western Blot analysis. Samples were denatured at 95°C, and SDS-PAGE was conducted under reducing conditions on a 6% separation gel with a 4% stacking gel. Proteins were transferred to a nylon membrane by electroblotting (BioRad Immuno-Lite Assay kit). The blot was blocked overnight in a 5% nonfat milk powder dissolved in Tris-buffered saline (500 mM NaCl, 20 mM Tris $\cdot$ HCl, pH 7.5) solution and washed for 1 h in Tris-buffered saline + 0.05% Tween 20 (TTBS). The blot was incubated with a 1:50 diluted monoclonal anti-MHC slow antibody (Accurate Chemical & Scientific, Westbury, NY) in TTBS containing 1% nonfat milk powder and 0.1% sodium azide for 4 h at room temperature. After immunoreaction, the membrane was stored at 4°C overnight in the same solution. The next day, the membrane was washed for 1 h in TTBS. Immunodetection of the primary antibody against the anti-MHC slow antibody was carried out with a 1:3,000 diluted anti-mouse-horseradish peroxidase secondary antibody solution (TTBS with 1% nonfat milk powder and 0.1% sodium azide) for 2 h at room temperature. The membrane was washed again for 1 h, incubated with chemiluminescent detection reagent for 5 min, and exposed to an X-OAMAT AR X-ray film for 5–10 min. Rabbit soleus muscle (10 $\mu$g total protein) was used as a control. The presence of the slow MHC classified the fiber
type as slow. Of the 86 fibers used in this study, only two were identified as slow fibers, and these were excluded from the data analysis.

**Data Analysis and Statistics**

SigmaPlot software (version 2.0, Jandel Scientific) was used to determine the constant \( N \) related to the steepness of the force vs. \( p\text{Ca} \) relationship (\( N \) is a measure of the extent of cooperativity among the thin filaments) and the calcium concentration required for half-maximal activation (\( \text{Ca}_{50} \)) values for the force-\( p\text{Ca} \) relationships from a best fit of the data to the modified Hill equation.

\[
\%\text{Maximum force} = 100\left(\frac{[\text{Ca}^{2+}]^N}{[\text{Ca}^{2+}]^{N} + [\text{Ca}^{2+}]^{3}}\right)
\]

Averages and standard errors of the mean for \( N \) values, \( \text{Ca}_{50} \), cross-sectional area, percentage of \( F_{\text{max}} \), and absolute force (normalized for cross-sectional area) for individual diaphragm fibers were calculated for baseline conditions and after exposure to \( \text{H}_2\text{O}_2 \), \( \text{O}_2 \text{-}, \) and \( \text{-OH} \), or one of the components of the generating system. The significance of the effects of exposure to \( \text{H}_2\text{O}_2 \), \( \text{O}_2 \text{-}, \) and \( \text{-OH} \), or to any component of one of the generating systems in each individual fiber, was calculated using the paired \( t \)-test (SigmaStat, version 2.0, Jandel Scientific). The effects of each condition on groups of fibers utilized in protocols 1, 2, and 3 were compared with time-controlled fibers using one-way ANOVA, followed by post hoc comparisons using the Student-Newman-Keuls all-pairwise multiple comparison procedure. Because the individual components of the ROS generating systems induced alterations in the contractile proteins, the differences due to the specific ROS being studied in protocols 1, 2, and 3 were assessed by comparing the results of ROS-exposed fibers to their appropriate control fibers. All data were corrected for any direct effect of the individual components of the generating systems on the contractile apparatus. A \( P \) value < 0.05 was considered to be statistically significant. All fast fibers studied were included in the data analysis.

**RESULTS**

A total of 84 fast fibers was used in this study. Absolute \( F_{\text{max}} \) under baseline conditions (i.e., before exposure to any of the three specific ROS being examined) averaged 126.2 \( \pm \) 8.1 kPa. Cross-sectional area of the fibers averaged 4.29 \( \pm \) 0.22 \( \times \) 10\(^{-9}\) m\(^2\). There were no significant differences in absolute force or cross-sectional area in any of the different experimental groups under baseline conditions.

**Group 1: Response to 10 or 1,000 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) Exposure**

Exposure to 10 or 1,000 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) caused no significant decrease in \( F_{\text{max}} \) compared with in-time control fibers (5 min in \( p\text{Ca} \) 8.5 without \( \text{H}_2\text{O}_2 \)). Specifically, \( F_{\text{max}} \) decreased to 91.9 \( \pm \) 10.0 and 90.3 \( \pm \) 1.7% of initial baseline force in fibers treated with 10 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) and in the control fibers, respectively (Fig. 1A). Similar results were obtained in fibers exposed to 1,000 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) (94.4 \( \pm \) 1.2% of initial baseline force). When we examined the effects of \( \text{H}_2\text{O}_2 \) exposure on the calcium sensitivity of the contractile apparatus, there were no significant changes in the \( \text{Ca}_{50} \) in fibers treated with 10 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) (see Fig. 1B) or 1,000 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) or in the in-time control fibers. The difference in the \( \text{Ca}_{50} \) after 5 min averaged 0.11 \( \pm \) 0.05 \( \mu \text{M} \) \( \text{Ca}^{2+} \) in fibers treated with 10 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \), 0.13 \( \pm \) 0.09 \( \mu \text{M} \) \( \text{Ca}^{2+} \) in fibers treated with 1,000 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \), and 0.22 \( \pm \) 0.06 \( \mu \text{M} \) \( \text{Ca}^{2+} \) in the in-time control fibers. Furthermore, analysis of the Hill coefficient also showed no significant differ-
Fig. 2. Effects of a 2-min exposure of single skinned diaphragm fibers to superoxide anion radical (O$_2^-$) and the individual components of the O$_2^-$ generating system on F$_{\text{max}}$: control fibers (Cont; n = 8); xanthine-treated fibers (X; n = 6); xanthine oxidase-treated fibers (XO; n = 6); superoxide dismutase-treated fibers (SOD; n = 7); fibers treated with X + XO + SOD (n = 6); and O$_2^-$ treated fibers (n = 6). All exposures were performed in pCa 8.5. ∗Significant difference compared with the in-time control fibers, P < 0.05. †Significant difference compared with fibers treated with any component of the generating system, with in-time control fibers, and with the appropriate control conditions (X + XO + SOD), P < 0.05.

ences among groups. After 5 min, N values averaged 6.00 ± 0.40 in fibers treated with 10 μM H$_2$O$_2$, 4.56 ± 0.25 in fibers treated with 1,000 μM H$_2$O$_2$, and 5.04 ± 0.40 in the in-time control fibers. These results demonstrate that exposures to 10 or 1,000 μM H$_2$O$_2$ had no significant effect on F$_{\text{max}}$ or the calcium sensitivity (Ca$_{50}$) of the contractile apparatus of single diaphragm fibers.

**Group 2: Response to O$_2^-$ Exposure**

In this group of experiments, F$_{\text{max}}$ was obtained in pCa 5.3, followed by a 2-min exposure in pCa 8.5 to either 50 μM xanthine, 0.91 mU xanthine oxidase, 15 μg/ml SOD, 50 μM xanthine with 0.91 mU xanthine oxidase to produce O$_2^-$, or 50 μM xanthine with 0.91 mU xanthine oxidase and 15 μg/ml SOD. After exposure, a second measurement of F$_{\text{max}}$ in pCa 5.3 was performed in all fibers. These results are shown in Fig. 2. Exposure to O$_2^-$ reduced force to 64.7 ± 2.3% of initial baseline force (P < 0.001) in the same fiber. Compared with the appropriate controls (i.e., xanthine + xanthine oxidase + SOD), the reduction in force after a 2-min exposure to O$_2^-$ represents a 14.5% decline, which is significant (P < 0.05). This indicates that O$_2^-$ significantly alters the contractile properties of single diaphragm fibers by inhibiting F$_{\text{max}}$. After 2 min, F$_{\text{max}}$ for the in-time control fibers was 94.1 ± 1.4% of initial baseline force. In fibers exposed to either xanthine or SOD for 2 min, F$_{\text{max}}$ was 96.8 ± 1.9 and 98.1 ± 1.7%, respectively, of the initial baseline force, which is not significant. However, after exposure to xanthine oxidase alone, force was 75.8 ± 3.1% of initial baseline force (P < 0.001). In fibers treated with xanthine + xanthine oxidase + SOD, force diminished to 75.7 ± 3.1% of initial baseline force (P < 0.001). These reductions in F$_{\text{max}}$ are almost identical and suggest that xanthine oxidase has a nonspecific inhibitory effect on force generation.

**Group 3: Response to -OH Exposure**

In these experiments, an initial force vs. pCa relationship was determined for fibers under baseline conditions, and fibers were then exposed to either 1 mM FeCl$_2$, 1 mM FeCl$_2$ with 1 mM ascorbic acid, or 1 mM FeCl$_2$ with 1 mM ascorbic acid and 1,000 μM H$_2$O$_2$ (to generate -OH) for 5 min in pCa 8.5. After the 5-min exposure, a second force vs. pCa relationship was determined. In preliminary experiments, a 5-min exposure to 1 mM ascorbic acid in pCa 8.5 produced no significant decrease in F$_{\text{max}}$ compared with control fibers (93.3 ± 2.7% vs. 90.3 ± 1.7% in controls). Furthermore, as demonstrated above in Group 1: Response to 10 or 1,000 μM H$_2$O$_2$ Exposure, 1,000 μM H$_2$O$_2$ has no significant effect on either F$_{\text{max}}$ or Ca$_{50}$.

When we examined the effects of the other components of the -OH generating system and of -OH per se, we found significant changes in F$_{\text{max}}$ (Fig. 3). Specifically, exposure to FeCl$_2$ reduced force to 83.8 ± 7.9% of the initial force, and exposure to FeCl$_2$ with ascorbic acid reduced force further to 68.6 ± 6.7% of initial.
baseline force. However, when FeCl₂, ascorbic acid, and H₂O₂ were used to generate \( \cdot \text{OH} \), F\(_{\text{max}}\) declined to 38.5 ± 5.0% of initial baseline values \( (P < 0.0001) \). Compared with the appropriate controls (FeCl₂ with ascorbic acid), exposure to \( \cdot \text{OH} \) resulted in a decline in force of 43.9% \( (P < 0.005) \). The effects of FeCl₂ and FeCl₂ with ascorbic acid on the force vs. pCa relationship are shown in Fig. 4, and those of \( \cdot \text{OH} \) are shown in Fig. 5.

When we examined the effects of \( \cdot \text{OH} \) exposure or the individual components of the generating system (FeCl₂ or FeCl₂ with ascorbic acid) on the calcium sensitivity of the contractile apparatus, there were no significant differences observed among individual fibers or among groups. The Ca\(_{50}\) averaged 1.74 ± 0.05, 1.91 ± 0.10, and 1.90 ± 0.10 μM Ca\(^{2+}\) in fibers after exposure to FeCl₂, FeCl₂ with ascorbic acid, and \( \cdot \text{OH} \), respectively. Furthermore, analysis of the Hill coefficient also showed no significant differences among groups. These data indicate that \( \cdot \text{OH} \) exposure significantly decreases

Fig. 4. Effects of FeCl₂ (A) and FeCl₂ + AA (B) on the force vs. pCa relationship are compared with baseline conditions (solid symbols) and after exposure in pCa 8.5 (open symbols). Force is normalized to %maximum force obtained under baseline conditions. F\(_{\text{max}}\) is significantly reduced with the above conditions. Data points are means ± SE.

Fig. 5. Effect of \( \cdot \text{OH} \) on the force vs. pCa relationship after a 5-min exposure in pCa 8.5 (●) for 11 fibers. ○ Baseline force vs. pCa relationship. A: force is normalized to %maximum force obtained under baseline conditions. B: force is normalized to %maximum force obtained for each condition to demonstrate any alterations in Ca\(_{50}\). As shown, \( \cdot \text{OH} \) exposure has a profound effect on F\(_{\text{max}}\) but no significant effect on calcium sensitivity of the contractile proteins. Although there appears to be a trend toward alterations in the Hill coefficient, these changes were not statistically significant. Data points are means ± SE.
F\textsubscript{max} but does not alter calcium sensitivity of the contractile proteins in single diaphragm fibers (Fig. 5).

**DISCUSSION**

To our knowledge, this is the first study to examine the direct effects of O\textsubscript{2}\textsuperscript{•} and -OH on the contractile apparatus in single Triton-skinned skeletal muscle fibers. Exposure to either O\textsubscript{2}\textsuperscript{•} or -OH resulted in significant reductions in F\textsubscript{max}. We also found that the calcium sensitivity of the contractile proteins was not altered with exposure to -OH. In contrast, H\textsubscript{2}O\textsubscript{2} had no significant effect on F\textsubscript{max} or the calcium sensitivity of the contractile apparatus.

In keeping with our initial hypothesis, -OH had a more pronounced effect on depressing F\textsubscript{max} than did either O\textsubscript{2}\textsuperscript{•} or H\textsubscript{2}O\textsubscript{2}. Our hypothesis that calcium sensitivity would be altered is not supported by our findings, because neither -OH nor H\textsubscript{2}O\textsubscript{2} altered the C\textsubscript{a}\textsubscript{50}. These data provide a potential mechanism by which O\textsubscript{2}\textsuperscript{•} and -OH could contribute to respiratory muscle dysfunction via direct effects on the contractile apparatus.

**Methodological Considerations**

We employed a large number of control groups to assess the individual effects of components of the free radical-generating solutions used in this study. By doing so, we were able to separate the nonspecific effects produced by these components from the specific effects of O\textsubscript{2}\textsuperscript{•} and -OH on force generation. Surprisingly, we found that the nonspecific effects produced by several components of the generating systems were quite pronounced (e.g., xanthine oxidase produced a 24.2% reduction in F\textsubscript{max}). This raises the question as to whether or not these nonspecific effects may have altered the contractile proteins in such a way as to artifically heighten the depressant effects of O\textsubscript{2}\textsuperscript{•} and -OH. For example, it is conceivable that one of these chemical components might induce alterations in protein conformation and expose an oxidant-sensitive site that might not normally be susceptible to redox-mediated modification. There are, however, no studies in the literature that suggest that this might occur. Moreover, some data indicate that oxidant-sensitive sites are easily accessible to ROS when contractile proteins are in their native configuration. For example, Perkins et al. (32) have suggested that sulfhydryl groups within the actomyosin ATPase are readily accessible and exquisitely sensitive to redox modification. Therefore, whereas we cannot entirely exclude the possibility that some artifact may have magnified the depressant effects of O\textsubscript{2}\textsuperscript{•} and -OH on the contractile proteins, we think such a phenomenon is unlikely.

Even though our experiments were designed in such a way that we attempted to examine the effects of O\textsubscript{2}\textsuperscript{•}, -OH, and H\textsubscript{2}O\textsubscript{2} individually on the contractile proteins, it must be understood that these chemical species are interrelated and solutions of any one of these may have been contaminated with the other radical species. Our findings, however, would argue that such potential contamination is of trivial importance. For example, H\textsubscript{2}O\textsubscript{2} had no effect on F\textsubscript{max}, O\textsubscript{2}\textsuperscript{•} had small effects on F\textsubscript{max}, and -OH had large effects on F\textsubscript{max}. It is not possible that significant amounts of O\textsubscript{2}\textsuperscript{•} or -OH were present in the H\textsubscript{2}O\textsubscript{2} generating solution, because such an eventuality should have resulted in a significant effect of H\textsubscript{2}O\textsubscript{2} on force generation, which was not seen. Conversely, H\textsubscript{2}O\textsubscript{2} alone had no effect on force generation; therefore, the presence of H\textsubscript{2}O\textsubscript{2} could not have affected the alterations seen with the O\textsubscript{2}\textsuperscript{•} or -OH. Furthermore, we excluded the presence of -OH in the O\textsubscript{2}\textsuperscript{•} generating solution (see METHODS). Nevertheless, there are no waterproof barriers between free radical species, and it is even possible that a variety of uncharacterized low-molecular-weight radical species may have been generated in the complex, biological milieu studied in the present experiments. However, in vivo responses to ROS are likely to involve the participation of such daughter reaction species. As a result, we would still argue that the physiological responses demonstrated in the present study represent the overall effects produced when the contractile proteins are exposed to O\textsubscript{2}\textsuperscript{•}, -OH, or H\textsubscript{2}O\textsubscript{2}.

DTT, a reducing agent, was selectively used in some of the solutions employed in these experiments. The only solutions containing DTT were those used to store or to dissect fibers. This chemical was not included in any of the solutions actually used to construct force vs. pCa relationships; i.e., this agent was not included in any of the activating solutions used in this study. DTT was also not included in the pCa 8.5 solution, nor was it included in any of the free radical-generating solutions. It is, therefore, extremely unlikely that the presence of DTT had any significant effects on the results obtained in our studies.

In the present study, we only present data for fast fibers in the diaphragm. Moreover, the technique we used for fiber typing did not distinguish between subtypes of fast fibers. However, we observed an extremely uniform response to the various ROS being tested. It seems unlikely, therefore, that there is significant subtype-dependent variation in the contractile protein response to O\textsubscript{2}\textsuperscript{•} or -OH.

**Comparison to Previous Studies**

Our findings in skinned diaphragm fibers exposed to H\textsubscript{2}O\textsubscript{2} confirm those previously reported in the literature. Broto and Nosek (8) examined the effects of a 5-min exposure to 1,000 \(\mu\)M H\textsubscript{2}O\textsubscript{2} on the contractile apparatus in single skinned fibers from the extensor digitorum longus of rats and found no changes in F\textsubscript{max} or calcium sensitivity. Similar studies in Triton-skinned cardiac myocytes using 10 mM H\textsubscript{2}O\textsubscript{2}, and exposure times of up to 60 min (25) showed that there were no significant changes in myofibrillar force generation or calcium sensitivity, leading the authors to conclude that H\textsubscript{2}O\textsubscript{2} was not an important modulator of the contractile proteins per se, even under pathophysiological conditions (25).
Andrade et al. (1) examined the effects of exogenously administered H₂O₂ on force and myoplasmic calcium concentrations ([Ca²⁺]ᵢ, where i is intracellular) in single, intact fibers from the flexor brevis muscle of mice and found that, at more physiological concentrations (150–300 μM H₂O₂), H₂O₂ effects were biphasic. With short exposure times, submaximal tetanic [Ca²⁺]ᵢ was unchanged, but force generation actually increased. With longer exposures, force declined and was independent of changes in submaximal tetanic [Ca²⁺]ᵢ. The authors suggest that contractile protein function in intact, single skeletal muscle fibers is sensitive to changes in cellular redox status, with the potential for biphasic responses (1). It is important to note, however, that, in the intact single-fiber preparation, the observed effects from exogenously administered H₂O₂ could be due to alterations at a myriad of sites within the myocyte (i.e., SR, pH, metabolism, etc.) or to effects of H₂O₂ derivatives. It is also possible that the observed effects are due to more than one modification in the cellular machinery, producing changes where physiological consequences are functionally opposing. In the present study, we have eliminated many of these confounding factors by studying single Triton-skinned fibers. In our experiments, potential alterations in the sarclemma, the SR, the mitochondria, the membranes of other subcellular organelles, or cytoplasmic enzymes by ROS can be ignored, because this preparation contains only the contractile proteins. Therefore, based on our results, we conclude that the contractile proteins per se are not directly modified by exposure to H₂O₂.

To our knowledge, our studies are the first to examine the direct effects of O₂⁻ exposure on contractile function in single skinned skeletal muscle fibers and, specifically, in the diaphragm. MacFarlane and Miller (24) previously examined the direct effects of O₂⁻ on skinned cardiac myocytes and found that the contractile apparatus was extremely sensitive to this particular reactive oxygen intermediate. In those studies, exposure to as little as 2 μM of xanthine oxidase for only 2 min markedly diminished absolute force generation to levels <20% of the initial preexposure force. These effects on force were both concentration and time dependent. Furthermore, despite changes in force, there were no changes in calcium sensitivity (24). Although the amount of O₂⁻ generated in our system was considerably lower than that used by MacFarlane and Miller, our findings in the diaphragm parallel these findings in cardiac muscle with regard to decrements in maximal force generation. We were not able to examine the force vs. pCa relationship in the O₂⁻ experiments and, therefore, are unable to make any conclusions with regard to the effects of O₂⁻ exposure on the calcium sensitivity of the contractile apparatus.

The present findings demonstrate that, when skeletal muscle contractile proteins are exposed to the ·OH, Fₘₐₓ decreases significantly without producing changes in calcium sensitivity. We are not aware of any other studies in skeletal muscle that have examined the direct effect of ·OH on the force-generating capacity of the contractile proteins. However, studies that employed spin traps in Triton skinned rabbit psoas muscle exposed to a ·OH generating system showed that ·OH can specifically modify the Cys-707 residue of the SH-1 group of myosin (22). In other studies, using in vivo iron overload as a model of oxidative stress, skeletal muscle actin and myosin have been shown to be oxidatively modified (30). Although these studies shed light on potential sites of protein modification by ·OH, the functional consequences of these modifications in skeletal muscle were not examined in these previous studies. Our results confirm that ·OH is a potent reactive oxygen intermediate that is able to alter the contractile proteins significantly.

Potential Sites of Free Radical-Induced Alterations of the Contractile Proteins

The specific site or sites of alteration in the contractile proteins induced by exposure to the different free radical-generating solutions used in the present study are not known. Potential targets include oxidation of critical thiol residues on myosin, actin, troponin, or tropomyosin. Other possible mechanisms include free radical-mediated biochemical modifications that induce changes in the tertiary structures of these proteins. Free radical-induced alterations in tertiary structure of proteins are well recognized as the major mechanism underlying protein degradation by the proteosome complex. It is quite possible that similar alterations in the contractile proteins could modify protein-protein contact, disrupting interactions among myosin, actin, tropomin, and tropomyosin.

One of the potential sites of alteration by the ROS examined in the present study is myosin. In skinned cardiac myocytes, MacFarlane and Miller (24) found that the contractile apparatus was extremely sensitive to O₂⁻ and that these effects were both concentration and time dependent. Despite significant alterations in force, there were no changes in Ca²⁺ sensitivity. However, during rigor, where there is no active cross-bridge cycling, exposure to O₂⁻ had no effect on force generation (24). These findings suggest that the alteration produced by O₂⁻ exposure is not a generalized or non-specific effect on the contractile apparatus, leading the authors to conclude that O₂⁻ does not promote cross-bridge detachment or weaken the cross bridges but most likely alters a part of the cross bridge that is not accessible in the attached state, that is, either subsequent attachment, cross-bridge kinetics, or ATPase activity (24). Recent studies by Perkins et al. (32) have shown that specific modification of the SH-1 subunit of myosin alters actomyosin ATPase activity and reduces Ca²⁺ sensitivity in skinned psoas fibers. Additionally, specific sulphydryl modifying agents have been used to modify myosin (20, 29, 48), and it has been suggested that cross-linking of the SH-1 and SH-2 of myosin can induce decrements in Fₘₐₓ without altering Ca²⁺ sensitivity (48), a finding that could explain our results.

Specific cysteine residues on actin have also been identified as potential sites of thiol modification by...
oxidants. However, studies show that, when actin is in its filamentous form, as in the skinned fiber preparation, the susceptibility of these residues to modification is reduced (23). Furthermore, although it has been suggested that troponin C is particularly sensitive to oxidative modification, Metzger and Moss (27) have shown that partial extraction of troponin C in skinned skeletal muscle fibers produces changes in both Ca\(^{2+}\) sensitivity and cooperativity of the contractile proteins. The absence of changes in Ca\(^{2+}\) sensitivity of the contractile proteins in the present study suggests that this is an unlikely mechanism. However, it is possible that biochemical modification of troponin C with resultant changes in the tertiary structure of the protein, and perhaps alteration in the Ca\(^{2+}\) sensitivity of troponin C per se, could be an explanation by which free radical-induced changes could alter the force generation of the contractile proteins (34). Whether or not other conformational changes in the other components of the troponin complex (i.e., troponin I or troponin T) could account for these findings is presently unknown.

Although it would be attractive to account for the findings in the present study by a single mechanism, it seems more likely that multiple sites on the proteins that comprise the contractile machinery are susceptible to redox modification. In fact, it is entirely possible that our findings may be due to several alterations that produce opposing effects on some aspects of the functional characteristics of the contractile proteins, while producing other effects that are synergistic.

**Implications in Pathophysiological States**

We and others have demonstrated that production of free radical species in muscle is increased in pathophysiological conditions, such as ischemia-reperfusion and sepsis (39, 46). In fact, we have recently established that, after endotoxin administration, force generation by intact diaphragm muscles, as well as the absolute force generated by the contractile proteins in Triton-skinned diaphragm fibers, is markedly reduced (42). Specifically, \(F_{\text{max}}\) is significantly reduced without changes in calcium sensitivity. This relationship was shown to be true for all fiber types in the diaphragm, as well as in the soleus and extensor digitorum longus, indicating that the muscle dysfunction produced in sepsis is widespread and, in part, due to a direct effect on the contractile proteins. The present findings provide a potential mechanism for these previous observations.

In conclusion, we have demonstrated that the contractile proteins are susceptible to modification by \(O_2^-\) and \(-OH\). Furthermore, exposure to these specific oxygen-derived free radicals leads to alterations in physiological function, specifically, decreases in the absolute force-generating capacity of the muscle without alterations in calcium sensitivity. It is, therefore, possible that one or more of these free radical species is responsible for producing specific alterations in the contractile proteins, which leads to muscle dysfunction in a variety of pathophysiological conditions. Albeit potentially complex, we believe that further investigation is warranted to elucidate the specific protein alteration or alterations and the mechanisms by which these changes produce derangements in the contractile apparatus.

**REFERENCES**


