Redox modulation of maximum force production of fast- and slow-twitch skeletal muscles of rats and mice

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Plant, David R., Paul Gregorevic, David A. Williams, and Gordon S. Lynch. Redox modulation of maximum force production of fast- and slow-twitch skeletal muscles of rats and mice. J Appl Physiol 90: 832–838, 2001.—We used intact fast-twitch extensor digitorum longus (EDL) and slow-twitch soleus muscles from rats and mice to test the hypothesis that exogenous application of an oxidant would increase maximum isometric force production (P\textsubscript{0}) of slow-twitch muscles to a greater extent than fast-twitch skeletal muscles. Exposure to an oxidant, hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}; 100 μM to 5 mM, 30 min), affected P\textsubscript{0} of rat muscles in a time- and dose-dependent manner. P\textsubscript{0} of rat soleus muscles was increased by 8 ± 1 (SE) and 14 ± 1% (P < 0.01) after incubation with 1 and 5 mM H\textsubscript{2}O\textsubscript{2}, respectively, whereas in mouse soleus muscles P\textsubscript{0} was only increased after incubation with 500 μM H\textsubscript{2}O\textsubscript{2}. P\textsubscript{0} of rat EDL muscles was affected by H\textsubscript{2}O\textsubscript{2} biphasingly; initially there was a small increase (3 ± 1%), but then P\textsubscript{0} diminished significantly after 30 min of treatment. In contrast, all concentrations of H\textsubscript{2}O\textsubscript{2} tested decreased P\textsubscript{0} of mouse EDL muscles. A reductant, dithiothreitol (DTT; rat = 10 mM, mouse = 1 mM), was added to quench H\textsubscript{2}O\textsubscript{2}, and it reversed the potentiation in P\textsubscript{0} in rat soleus but not in rat EDL muscles or in any H\textsubscript{2}O\textsubscript{2}-treated mouse muscles. After prolonged equilibration (30 min) with 5 mM H\textsubscript{2}O\textsubscript{2} without prior activation, P\textsubscript{0} was potentiated in rat soleus but not EDL muscles, demonstrating that the effect of oxidation in the soleus muscles was also dependent on the activation history of the muscle. The results of these experiments demonstrate that P\textsubscript{0} of both slow- and fast-twitch muscles from rats and mice is modified by redox modulation, indicating that maximum P\textsubscript{0} of mammalian skeletal muscles is dependent on oxidation.

cellular redox balance; reactive oxygen species; hydrogen peroxide, muscle contraction; fatigue

**MATERIALS AND METHODS**

**Muscle Preparation**

All experiments were approved by the Animal Experimentation Ethics Committee of The University of Melbourne and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Adult male Sprague-Dawley rats (350–550 g) and male C57BL × CBA mice (28–35 g) were anesthetized deeply with intraperitoneal pentobarbital sodium (60 mg/kg body wt for rats, 10 mg/kg body wt for mice; Rhone Merieux, Pickenah, Queensland, Australia), with supplemental doses given to ensure that the animals remained unresponsive to tactile stimuli. The fast-twitch extensor digitorum longus (EDL) and the predominantly slow-twitch soleus muscles of the lower hindlimb were dants located in most mammalian cells (7). Antioxidant protection within muscle cells is also proportional to oxidative metabolism, with slow-twitch skeletal muscles containing a greater antioxidant protection than fast-twitch muscles (9).

**Recent evidence suggests that skeletal muscle contractility is sensitive to ROS. Application of low concentrations of the oxidant hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) to the muscle bathing solution enhanced the twitch force of muscle fiber bundles and intact single muscle fibers (1, 15, 20). In contrast, force production is compromised after exogenous application of an antioxidant (20). This proposed relationship between ROS and force production indicates that muscles at rest exist in a reduced state and that addition of an oxidant alters the redox balance of the muscle to optimize force production (18). To date, most experiments investigating the effects of oxidants on force production have focused on submaximal tetanic force or twitch contractions of isolated muscles. Assuming that there is a relationship between force production and oxidation, we tested the hypothesis that exogenous application of an oxidant would increase maximum isometric force production (P\textsubscript{0}) of slow-twitch muscles to a greater extent than fast-twitch skeletal muscles. The results from our experiments indicate that addition of an exogenous oxidant to intact skeletal muscles enhanced P\textsubscript{0} with a greater increase in force observed in slow- than fast-twitch skeletal muscles of rats and mice.

**REACTIVE OXYGEN SPECIES (ROS)** are produced as a byproduct of normal metabolism in skeletal muscles (5, 8, 11, 17). The major site of production of endogenous oxidants within skeletal muscles is via ATP regeneration (5). As such, ROS production is associated with muscle contraction, and the elevated level of ROS observed during prolonged contractile activity has implicated ROS in muscle fatigue (2, 6, 10, 19, 22). ROS are highly reactive and have the ability to modify intracellular processes; however, these damaging effects of ROS are tightly regulated by the presence of antioxidant defense systems located in most mammalian cells (7). Antioxidant protection within muscle cells is also proportional to oxidative metabolism, with slow-twitch skeletal muscles containing a greater antioxidant protection than fast-twitch muscles (9).

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used in these experiments. Proximal and distal tendons of each muscle were exposed and tied with braided silk suture (3/0, Pearsalls Sutures, Somerset, UK), without impinging on muscle fibers. The arterial blood supply to the muscle was then severed, and the intact muscle was dissected free. When all of the muscles to be investigated had been dissected, the animals were killed by an overdose of anesthetic and opening of the thoracic cavity. Each muscle was blotted once on filter paper and placed immediately in a custom-built Plexiglas bath filled with oxygenated Krebs-Ringer solution [composition (mM): 118 NaCl, 4.75 KCl, 1.18 KH2PO4, 1.18 MgSO4, 7H2O, 24.8 NaHCO3, 2.5 CaCl2, 0.03 d-tubocurarine chloride, and 10 n-glucose, pH 7.4], perfused constantly with 95% O2-5% CO2 (BOC Gases, Preston, Victoria, Australia), and thermostatically maintained at 25°C, which is optimal for maintaining the patency of muscles in vitro for the duration of the experimental period (21).

Contractile Measurements

Each muscle was aligned horizontally and tied directly between an immovable arm and an isotonic force transducer (Research grade 60-2999, Harvard Apparatus, South Natick, MA). Muscles were stimulated directly by an electric field between two platinum plate electrodes either side of the muscle. Square-wave pulses (0.2-ms duration) were amplified (DC-300A, Crown Instruments, Elkhart, IN) to increase and sustain current intensity to a sufficient level to produce a maximal tetanic contraction. Stimulation train durations of 350 and 1,200 ms were used to produce tetanic contractions in the EDL and soleus muscles, respectively. Optimum muscle length (L0) was determined from the length at which the isometric twitch force was maximal. Optimum fiber length (Lf) was determined by multiplying L0 by previously measured Lf/L0 ratios of 0.44 for the EDL and 0.71 for the soleus muscles (3). The frequency-force relationship was established for each muscle, and Po was determined from the plateau of the frequency-force curve. Specific force (kN/m2) was determined for each muscle on the basis of an estimation of total muscle cross-sectional area (muscle mass divided by the product of L0 and 1.06 mg/mm2, the density of mammalian skeletal muscles) (14).

Experimental Procedure

Protocol 1: Dose response of H2O2 in intact muscles from the rat and mouse. After the determination of Po, rat muscles were allocated into one of four H2O2 treatment groups: 0 mM (control), 100 μM, 1 mM, or 5 mM H2O2 (n = 6 muscles/group). The range of H2O2 concentrations used in these experiments (100 μM to 5 mM) was based on those used in previous experiments that have demonstrated alteration in cellular redox state and modulation of muscle contraction (1, 15, 16, 20, 23). H2O2 (30% solution, Sigma Chemical, Castle Hill, NSW, Australia) was added directly to the bathing medium and the muscles incubated for 30 min and Po determined every 5 min. Each muscle was used as its own control and Po was expressed as a percentage of the initial untreated value. Recovery of Po was assessed after 10 min of treatment with 10 mM dithiothreitol (DTT). In these experiments, DTT in powdered form was added directly to the organ bath without washout of H2O2 and was mixed thoroughly with the oxygenated Ringer solution.

Mouse muscles were treated identically to rat muscles but were incubated with different concentrations of H2O2: 0 mM (control), 500 μM, 1 mM, or 5 mM H2O2 (n = 6 muscles/group), DTT (1 mM). H2O2 and DTT were added directly from stock solutions to the organ bath and mixed thoroughly to ensure uniform exposure of each muscle to the redox agent.

Protocol 2: Effects of prolonged H2O2 exposure on Po in rat muscles. In another group of rat muscles, optimum length and voltage were established, and maximum "control" Po was determined. Each muscle was then incubated with 5 mM H2O2 for 30 min, and Po was determined only at the end of the 30-min incubation. The muscle was not stimulated during the 30-min treatment period with H2O2.

Protocol 3: Effects of brief DTT exposure on Po in rat muscles. The direct effects of DTT exposure on muscle function were assessed in rat muscles after determination of Po, without washout of H2O2 and was mixed thoroughly with the Ringer solution. The DTT-Ringer solution was then removed and replaced with fresh Ringer solution to determine the response after a 10-min washout period. An oxidant (5 mM H2O2) was added after the washout period to assess reversibility of the effects of the reductant. During each treatment, Po was determined every 5 min. Each muscle was used as its own control, with Po expressed as a percentage of the initial Po before treatment.

Statistical Analysis

Values in the text are presented as means ± SE. Treated and control groups were compared by using either repeated-measures analysis of variance with Newman-Keuls post hoc analysis or Student’s paired t-test where appropriate. Results were considered significant when P < 0.05.

RESULTS

Dose Response of H2O2 in Intact Skeletal Muscles from the Rat

In muscles from the rat, Po was not different in any group before treatment (rat EDL, 306 ± 9 kN/m2; rat soleus, 232 ± 5 kN/m2). The effects of H2O2 on Po were dose dependent, with the highest concentration (5 mM) having the most effect on Po. In the soleus muscles, 100 μM H2O2 had no effect on Po, whereas 1 and 5 mM H2O2 potentiated Po throughout the 30-min treatment period. Peak potentiation of Po during 1 mM H2O2 treatment occurred at 25 min (108 ± 1% of initial Po) and during 5 mM H2O2 treatment at 15 min (114 ± 1% initial Po; Fig. 1A). Po of the rat soleus muscles in the 5 mM H2O2-treated group was greater (P < 0.01) than that of muscles in the 1 mM H2O2-treated group throughout the treatment period. After the 30-min H2O2 incubation, addition of DTT returned Po of rat soleus muscles to control values in all H2O2-treated groups.

In EDL muscles from the rat, H2O2 affected Po bi-phasically over the course of incubation. Initially (5–10 min), 1 and 5 mM H2O2 potentiated Po (103 ± 1% of initial Po, for both 1 and 5 mM H2O2; P < 0.05; Fig. 1B). However, incubation with 5 mM H2O2 depressed Po after 25 min (92 ± 1% of initial Po) and 30 min of exposure (87 ± 1% of initial Po; P < 0.05). Treatment of rat EDL muscles with the lowest concentration (100 μM) of H2O2 did not affect Po. Incubation with DTT (10 mM) for 10 min decreased Po in all H2O2-treated and untreated EDL muscles, such that Po of muscles of the
5 mM H$_2$O$_2$-treated group remained depressed compared with that of control rat EDL muscles (Fig. 1B).

**Dose Response of H$_2$O$_2$ in Intact Skeletal Muscles From the Mouse**

$P_o$ was not different in any group before H$_2$O$_2$ treatment in the mouse muscles (mouse EDL, 244 ± 14 kN/m$^2$; mouse soleus, 229 ± 13 kN/m$^2$). Effects of H$_2$O$_2$ on $P_o$ of mouse muscles were also dose dependent, with the highest concentration (5 mM) causing the greatest change in muscle contractility. H$_2$O$_2$ (500 μM) potentiated $P_o$ of mouse soleus muscles, with peak potentiation occurring after 10-min incubation (106 ± 2% of initial $P_o$, $P < 0.01$, Fig. 2A). Treatment with 1 mM H$_2$O$_2$ also caused a biphasic response with a small potentiation followed by a diminution of $P_o$ after 30 min of incubation. Treatment with 5 mM H$_2$O$_2$ caused strong inhibition of $P_o$ in mouse soleus muscles throughout the 30-min treatment period. In mouse EDL muscles, all concentrations of H$_2$O$_2$ progressively diminished $P_o$ in a dose-dependent manner (Fig. 2B).

Incubation of H$_2$O$_2$-treated mouse muscles with DTT (1 mM) for 10 min decreased $P_o$ (Fig. 2, A and B). DTT treatment was unable to reverse this H$_2$O$_2$-induced depression of $P_o$.

**Prolonged H$_2$O$_2$ Exposure in Intact Skeletal Muscles From the Rat**

The $P_o$ of rat EDL muscles was not altered by 30-min incubation with H$_2$O$_2$. However, in rat soleus muscles, $P_o$ was potentiated 10 ± 1% ($P < 0.05$) after prolonged exposure to 5 mM H$_2$O$_2$ in the absence of prior contractions (Fig. 3).

**DTT Exposure Affects $P_o$ of Skeletal Muscles From the Rat**

After 10 min of incubation in the Ringer-DTT solution (DTT, 10 mM), $P_o$ was 94 ± 6.2 and 97 ± 0.2% of initial $P_o$ in rat soleus and EDL muscles, respectively. The inhibition of $P_o$ was reversed by washout with fresh Ringer solution, and $P_o$ of soleus muscles returned to initial values but remained depressed for EDL muscles. Subsequent exposure to H$_2$O$_2$ (5 mM, 5 min) returned $P_o$ of EDL muscles to control levels and potentiated $P_o$ of the soleus muscles by 6 ± 1% above initial ($P < 0.05$). Figure 4 illustrates the return of $P_o$.
to initial values in rat EDL muscles and potentiation above initial in rat soleus muscles.

DISCUSSION

The findings of this study demonstrate clearly that the maximum force-producing capacity of intact skeletal muscles is modulated dynamically by redox manipulation, providing support for a relationship between redox perturbation and P_o. These experiments are novel in that they compare the effects of oxidation and reduction in slow- and fast-twitch mammalian skeletal muscles. A greater degree of modulation of P_o was...
observed in fast-than slow-twitch muscles, indicating a fiber specific susceptibility to redox manipulation. This fiber-type redox susceptibility is likely explained by the difference in the antioxidant levels of fast- and slow-twitch muscles (9). Antioxidant protection of rat EDL muscles is much less than that of soleus muscles, and, although it might be predicted that lower antioxidant protection would render EDL muscles more susceptible to exogenous oxidant exposure, our results argue against this proposal. The greater antioxidant protection of soleus muscles appears to delay the effects of the exogenous oxidant.

Muscles from the mouse were used to examine the effects of redox manipulation in smaller mammalian muscles to confirm that the effect of exogenous oxidant in rat muscles was not due to providing O₂ (a product of H₂O₂ breakdown) to the hypoxic core of the muscle. The effects of oxidation on force production in the smaller muscles from mice were similar to those observed in the larger rat muscles but occurred at different concentrations of exogenous H₂O₂, indicating that the effects of H₂O₂ in the rat muscles were not due to the liberated O₂.

Brief Redox Manipulation

The effects of H₂O₂ on both rat and mouse muscles were concentration dependent, with the highest concentrations of H₂O₂ causing the greatest change in P₀ in both fast- and slow-twitch muscles. At a given concentration of H₂O₂, the effect on P₀ of mouse muscles was different from that evident in rat muscles, indicating that muscle size, overall total antioxidant protection (4), and redox-sensitivity of regulatory proteins within the muscle are important determinants of the response of P₀ to redox modulation. These findings, however, do not cast light on the mechanisms responsible for mediating the changes in muscle contraction. The effects of H₂O₂ may not have been directly due to H₂O₂ exposure but possibly to an effect of other radicals. The absence of a transitional metal chelator in the Ringer solution allowed possible hydroxyl radical formation via a reaction between H₂O₂ and transitional metals. Another limitation of this investigation is the fact that the effects of oxidation of proteins cannot be distinguished from the effects of oxidation on membrane lipids. Given that application of an exogenous oxidant would initially target the sarcoplemmal membrane, we cannot exclude the possibility that the effects of oxidation on contractile function are mediated by the modulation of membrane lipids.

The reductant diminished P₀ in both muscle fiber types, indicating that reduction and oxidation have opposing effects on P₀. Rat soleus muscles demonstrated a greater increase in P₀ after incubation with H₂O₂ than rat EDL muscles, indicating a different susceptibility of slow- than fast-twitch muscles to oxidation.

In some muscles, the inability of DTT to reverse the H₂O₂-induced changes in P₀ suggests that these muscles were altered irreversibly by oxidation. The DTT...
incubation time (10 min) may not have been sufficient to reverse the effects of exposure to \( \text{H}_2\text{O}_2 \), or prolonged oxidation may have resulted in permanent damage to redox-sensitive proteins within the muscle. It is possible that reversal of the effects of \( \text{H}_2\text{O}_2 \) are time dependent, as is observed in the Na\(^+-\)K\(^+\)-ATPase activity of cardiac myocytes exposed to \( \text{H}_2\text{O}_2 \) \( \text{H}_2\text{O}_2 \). Such damage may cause, or be caused by, alterations in intracellular \( \text{Ca}^{2+} \) concentration \( ([\text{Ca}^{2+}]_i) \). Elevated \( [\text{Ca}^{2+}]_i \) has been observed after prolonged oxidation of single muscle fibers from the mouse (1). Lännergren and colleagues (12) suggested that the slowed \( P_e \) recovery after long duration, low-frequency fatiguing stimulation of mouse single muscle fibers was due to elevated \( [\text{Ca}^{2+}]_i \) disrupting cellular processes and may be similar to the lack of recovery from prolonged oxidation of muscles in vitro.

DTT may also have its own direct effects on excitation-contraction (E-C) coupling (24). Antioxidants and thiol donors have been demonstrated to suppress contractility and, when DTT was added to quench the effects of the oxidant \( \text{H}_2\text{O}_2 \), may also have had deleterious effects on the muscle, similar to the decreased \( P_e \) observed after exposure of rat muscles to DTT alone (Fig. 4). The magnitude of the decrease in \( P_e \) due to DTT was far less than the increase in \( P_e \) caused by \( \text{H}_2\text{O}_2 \).

**Prolonged \( \text{H}_2\text{O}_2 \) Exposure**

Previous investigations have suggested that the response of skeletal muscles to oxidation is time dependent (1, 16). After prolonged exposure (30 min) to \( \text{H}_2\text{O}_2 \) (5 mM) without stimulation (protocol 2), \( P_e \) was potentiated in rat soleus but not EDL muscles to a level similar to that after repeated activation (protocol 1; Fig. 1A). This indicates that the activation history of EDL muscles is also important in determining its response to \( \text{H}_2\text{O}_2 \). Muscle contraction accelerates the effect of exogenous oxidants due to the additive effect of oxidants produced from endogenous sources.

**Redox Modulation of Contractile Function**

The net effect of oxidation and reduction on susceptible components of the E-C coupling process contributes to the complex response of intact muscles to redox manipulation. Recently, it was proposed that force production is a function of cellular redox balance and that the point of baseline redox balance is a slightly reduced state (18). It was theorized that \( P_e \) of skeletal muscles occurs only after exposure to an oxidizing agent capable of shifting redox balance toward a more oxidized state. Clearly, a reductant would cause a shift in cellular redox balance to a more reduced state and therefore impair force production (18). Such a model for cellular redox balance is consistent with the observations of the present experiments in both fast- and slow-twitch muscles of the rat and mouse. Figure 5 represents the model for the relationship between cellular redox balance and \( P_e \) as proposed by Reid (18).

This model is also representative of mouse muscles, but due to differences in total antioxidant protection to that of rat muscles, mouse muscles at rest are closer to the apogee of the force-redox relationship.

In conclusion, these experiments demonstrate that \( \text{H}_2\text{O}_2 \) and DTT modify the contractility of both slow- and fast-twitch mammalian skeletal muscles, providing support for a relationship between redox manipulation and \( P_e \). We have also demonstrated that different responses are observed in slow- and fast-twitch muscles during redox modulation.

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