Reactive oxygen species formation during tetanic contractions in single isolated Xenopus myofibers

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Zuo L, Nogueira L, Hogan MC. Reactive oxygen species formation during tetanic contractions in single isolated Xenopus myofibers. J Appl Physiol 111: 898–904, 2011. First published June 23, 2011; doi:10.1152/japplphysiol.00398.2011.—Contracting skeletal muscle produces reactive oxygen species (ROS) that have been shown to affect muscle function and adaptation. However, real-time measurement of ROS in contracting myofibers has proven to be difficult. We used amphibian (Xenopus laevis) muscle to test the hypothesis that ROS are formed during contractile activity in isolated single skeletal muscle fibers and that this contraction-induced ROS formation affects fatigue development. Single myofibers were loaded with 5 μM dihydrofluorescein-diazodium (HFluor-DA), a fluorescent probe that reacts with ROS and results in the formation of fluorescein (Fluor) to precisely monitor ROS generation within single myofibers in real time using confocal microscopy. Three identical periods of maximal tetanic contractions (1 contraction/3 s for 2 min, separated by 60 min of rest) were conducted by each myofiber (n = 6) at 20°C. Ebselen (an antioxidant) was present in the perfusate (10 μM) during the second contractile period. Force was reduced by ~30% during each of the three contraction periods, with no significant difference in fatigue development among the three periods. The Fluor signal, indicative of ROS generation, increased significantly above baseline in both the first (42 ± 14%) and third periods (39 ± 10%), with no significant difference in the increase in fluorescence between the first and third periods. There was no increase of Fluor in the presence of ebselen during the second contractile period. These results demonstrated that, in isolated intact Xenopus myofibers, 1) ROS can be measured in real time during tetanic contractions, 2) contractile activity induced a significant increase above resting levels of ROS production, and 3) ebselen treatment reduced ROS generation to baseline levels but had no effect on myofiber contractility and fatigue development.

Address for reprint requests and other correspondence: L. Zuo, Dept. of Medicine-0623A, Univ. of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92093-0623 (e-mail: lzuos@ucsd.edu).
in real time from intact skeletal muscle single fibers with confocal microscopy.

METHODS

All procedures were approved by University of California, San Diego, institutional animal care and use committee (IACUC). Adult female Xenopus laevis were quickly euthanized, lumbrical muscles (II–IV) were removed from the foot, and intact single fibers were microdissected. Primarily fast-twitch, glycolytic skeletal muscle fibers were selected for use in the present study, as described previously (46). After isolation, the tendons of each fiber were attached with platinum clips and mounted in a glass-bottomed chamber to a force transducer (1500A Small Intact Muscle Test System and force transducer model 400A, Aurora Scientific). The chamber was perfused with Ringer’s solution (in mM: 116.5 NaCl, 2 KCl, 1.9 CaCl2, 2 NaH2PO4, and 0.1 EGTA, pH 7.0) at room temperature (20°C).

Each fiber was loaded with 5 μM Hfluor-DA (Sigma), a probe that is sensitive to intracellular ROS, for 30 min, and then the excess dye was washed out using Ringer’s solution. When ebselen (Alexis Biochemicals) was used, it was kept in the bath for 30 min before and during the contractile period, followed by a thorough washout procedure before the next contractile period. A laser scan confocal microscope system (McBain Systems with a Nikon inverted microscope) was used to record fluorescent signals from optical sections of the interior of the fibers. Fibers were stretched to produce maximal tetanic force and electrically stimulated (S48 stimulator, Grass Technologies, West Warwick, RI; 250-ms trains, 2-ms pulse duration, 70 Hz, 8 V) every 3 s for 2 min at room temperature. Three identical contractile periods, separated by 60 min of rest, were applied to each fiber. Fibers were perfused during the first period with Ringer’s solution, followed in the second contractile period by Ringer’s containing 10 μM ebselen, and the last contractile period was conducted after the washout of ebselen. The setup parameters for confocal imaging detection of ROS were illustrated as the following: laser, argon; excitation, 488 nm; emission, 525 ± 15 nm. The emitted signal captured by a PMT was an image of 512 × 512 pixels on a computer monitor. The background fluorescence was kept minimal and could be easily corrected when the initial baseline was normalized in data analysis. An image was captured every 5 s (to prevent photobleaching) with a 300-ms scanning time, and the mean fluorescence of each image was used for the calculation of ROS production.

Of particular interest in the present study was to correlate muscle contractile function and performance with ROS formation, both measured simultaneously in an intact single fiber using laser scan confocal microscopy. Perhaps the biggest challenge of this technique is the motion artifact caused by contracting fibers. To overcome this, we followed the rigorous criteria used with all of the fibers. When the fiber movement exceeded 10% of the size in the field in any direction based on visual pattern recognition, the data were discarded. The two tendon ends of the fiber were firmly secured in the perfusing chamber. To overcome the photobleaching and photooxidation problems caused by laser illumination, we used Hfluor because it is more resistant to photodamage compared with DCFH (11, 53). We did not use a continuous mode of measurement but used a shutter program that opened the laser once for 300 ms every 5 s to capture each image. Moreover, the laser power was also adjusted to a relatively low level without any significant death of sensitivity or resolution. The final fluorescence data were averaged and grouped. These precautionary measures eliminated any changes of fluorescence that may have been due to horizontal and vertical motion artifacts, or any photobleaching effects.

Furthermore, we tested all the chemicals used in the present study including ebselen to determine whether they have any overlapped fluorescence with Fluor. We did not detect any fluorescence with the current confocal setup parameters used for Fluor. There was also no chemical interactions between ebselen and oxidized Hfluor (53).

Although DCFH does react with NO under certain conditions, its analog Hfluor has very little reaction with NO (51).

Data were analyzed using a multi-way ANOVA and expressed as means ± SE (JMP, SAS Institute, Cary, NC). The differences between treatment and control were determined by post-ANOVA contrast methods available on the SAS JMP software. P < 0.05 was considered to be significant.

RESULTS

Typical representative images from Hfluor loaded fibers are shown in Fig. 1. In Fig. 1, A and B, under confocal microscopy, Fluor fluorescence increased markedly after 1 mM H2O2 addition. After one set of 2-min contractile work, the myofiber also demonstrated a marked increase in fluorescence from the resting state (Fig. 1C) to the end of the 2-min contractile period (Fig. 1D). However, ebselen treatment completely abolished the Fluor increase during a 2-min contraction period (see Fig. 1, E and F).

Figure 2A illustrates contractile force during the three 2-min contractile work bouts in a representative Hfluor-loaded single fiber. From this chart record of a set of raw data, it was clear that there was no difference between ebselen-treatment and control regarding the muscle contractile performance. However, from the fluorescent measurement of the same fiber, as shown in Fig. 2B, ebselen treatment ablated the Fluor signal,
and after ebselen washout the Fluor signal was restored during the last 2-min contraction period.

The grouped data of ebselen’s effect on fatigue are illustrated in Fig. 3. The fall in force (fatigue) during the ebselen treatment was not significantly different from the other contractile periods. There was a significant drop in the developed force from the initial maximal force to the developed force at the end of each contractile period (\(n = 6; P < 0.05\), significant from the start force in each contractile period, respectively). Furthermore, there was no significant difference among the three contractile periods in initial maximal force and subsequent fatigue development.

As illustrated in Fig. 4A, mean data for intracellular ROS generation during the three contractile periods are shown by changes in Fluor fluorescence in the isolated single myofibers. Significant levels (\(-40\%\) of baseline) of intracellular ROS were generated during tetanic contractions in the single myofibers during the first and third contractile periods. Ebselen treatment during the second contractile period blunted ROS generation but had no significant effect on myofiber fatigue development (\(n = 6; P < 0.05\), significant from baseline; each point represents a mean signal value every 30 s). Figure 4B shows the mean data of the first 30 s of intracellular ROS formation during the first and third contractile periods. ROS formation became significantly elevated above baseline 15 s after the initiation of contractions and then rose at the same rate and to the same maximal level in contractile periods 1 and 3.

DISCUSSION

Measurement of ROS in skeletal muscle. The results from the present study demonstrate that intracellular ROS formation can be accurately measured in real time during tetanic contractions in Xenopus single intact myofibers. As shown in Fig. 1, A and B, addition of \(\text{H}_2\text{O}_2\) caused a marked increase of Fluor signals and thereby provided a positive control for these experiments. In Fig. 1, C and D, we detected a similar increase of Fluor signal after fatigue induced by 2 min of contractions. As shown in Figs. 2 and 4, the ROS signal from Fluor fluorescence was significantly increased with repetitive contractions, and this signal was completely abolished by the presence of the antioxidant ebselen. These results confirm that the fluorescence signal detected in this study was a result of ROS generation. These data are consistent with some of the earlier work that suggested that low levels of ROS generation occur in resting skeletal muscle and that this production significantly increases during muscle stimulation (2, 7, 21, 38, 39). The present study, using real-time confocal imaging, extends these findings to intact single myofibers and thus suggests that intracellular ROS formation is a generalized response to moderate repetitive muscle contractions that can occur during a relatively short period. The increase in ROS signal during contractions (\(-40\%\) after 2 min of contractions) was similar to that reported in mouse myofibers in culture (\(-25\%\) increase after 15 min of contractions (30)) and in diaphragm muscle bundles (\(-40\%\) increase after 1h contractions (38)).

Potential sources of ROS formation in skeletal muscle. It has been reported that there are a number of potential cellular sources of ROS in contracting skeletal muscle models. One potential source of elevated ROS production in contracting fibers is from mitochondria, which are believed to generate ROS via single-electron leakage from the respiratory chain to oxygen (4, 54). During respiration, most oxygen utilized during oxidative phosphorylation is reduced to water, whereas an
Therefore, it is likely that intracellular mitochondria may be the strongest potential candidate responsible for ROS generation, although the molecular source of ROS is still poorly understood in contracting muscles.

Finally, it is important to note that *Xenopus* skeletal muscle fibers used in this work do not express myoglobin (14). Myoglobin has been implicated as either an important scavenger of free radicals (e.g., ROS and nitric oxide) at physiological conditions (12) or as a stimulant for free-radical under non-physiological conditions (i.e., excess production of ROS) and can thereby increase skeletal muscle oxidative stress and dysfunction (27).

**Kinetics of ROS production.** Figure 4B shows that Fluor fluorescence was significantly elevated above baseline in both the first and the third contractile periods at 15 s ($P < 0.05$). After this 15-s time delay, the maximal rate of increase in fluorescence was not significantly different in these two contractile periods (0.49 ± 0.13 vs. 0.52 ± 0.11 RU/min; $n = 6$). There was no difference in the peak fluorescence from baseline achieved in the end of both contractile periods as shown in Fig. 4A. These results suggest that there was no preconditioning effect observed in the present study.

Furthermore, it has been shown in intact single muscle fibers that, during the transition from rest to work (repetitive contractions), it takes 12–15 s for intracellular oxygen concentration (14, 16) and NADH (16) to fall at the initiation of contractions, thereby showing a slight time delay for mitochondrial activation. The period of time between the beginning of contractions and the rise in intracellular ROS in the present investigation (~15 s; see Fig. 4B) was quite similar to both intracellular PO$_2$ and NADH kinetics from the investigations cited above, which demonstrates that the kinetics of ROS produced during contractions match the kinetics of mitochondrial activation in *Xenopus* single myofibers. Thus this suggests the mitochondria involvement in ROS generation during contractile activities.

**ROS production and oxygen tension.** It has been demonstrated that transitions from either extracellular normoxia to hypoxia or normoxia to hyperoxia increases cellular ROS production in skeletal muscle (5, 52). In the present investigation, the extracellular, and likely the intracellular, oxygen tensions (PO$_2$) were ~150 Torr (ambient PO$_2$) at rest. During contractions in *Xenopus* single fibers, the intracellular PO$_2$ decreases significantly (14), but even though extracellular PO$_2$ remained at ~150 Torr, the intracellular PO$_2$ would only be ~30–40 Torr lower (19). This intracellular PO$_2$ (~100 Torr) is much higher than that measured in skeletal muscle during exercise [2–3 Torr (41)]. Therefore, the ROS production measured in these single fibers occurred at relatively high levels of extracellular and intracellular PO$_2$ (19), suggesting that in these contracting myofibers a hypoxic environment was not necessary for ROS generation.

**Skeletal muscle fatigue and ROS.** The role of ROS in the development of muscle fatigue has become a well studied area of research (36). It has become clear that, under some contractile conditions, ROS generation and antioxidant activity can influence force production. In the present study, modest fatigue development occurred in all three contractile periods, but this was not altered when ROS generation was blunted by antioxidant administration (Fig. 2). The native antioxidants (AOXs) in cells include superoxide dismutase, catalase, glutathione, and thioredoxin redox system (7). By scavenging excess ROS, AOXs modify the cellular redox tone and redox-sensitive

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**Fig. 3.** Data showing the fall in force (fatigue) during the three contractile periods in *Xenopus* single fibers ($n = 6$). *Left:* force at the start of the first contractile period compared with the force at the end of contractile period. *Middle:* after ebelen treatment, force at the start of the second contractile period compared with the force at the end of contractile period. *Right:* force at the start of the third contractile period compared with the force at the end of contractile period. *Significantly different from the start force in each contractile period ($P < 0.05$). There was no significant difference among the three contractile periods in initial force development and subsequent fatigue development.

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estimated small portion (~0.15%) of oxygen could be reduced to form superoxide and hydrogen peroxide ($3$, $44$). As shown previously (19), mitochondria are activated shortly after the beginning of repetitive tetanic contractions, particularly after ~10 s of contractions. In the present investigation, the ROS fluorescence was significantly increased after approximately the similar period of time (15 s after the start of contractions; see Fig. 4B), which suggests potential mitochondrial involvement.

However, there are other sources of enzyme-based ROS generation that are likely activated during muscular activity. Particularly, xanthine oxidase and NADPH oxidase (NOX) have been shown to play a role in ROS generation during skeletal muscle repetitive contractions and exhaustive exercise (16, 47). However, ROS production from xanthine oxidase has been mostly associated with more severe conditions of stress, such as exhaustive exercise (8, 47), and NOX is mostly located in endothelial cells (26, 28, 50). It was unlikely in the present study that either xanthine oxidase or NOX was the main source of the detected intracellular ROS production, since in the intact single myofiber model there was no significant amount of vascular tissue present on the muscle fiber after the dissection. Therefore, it is likely that intracellular mitochondria may be the strongest potential candidate responsible for ROS generation.
metabolism, reduce muscle injury, and preserve maximal energy supply during metabolic imbalance conditions, since those could occur during fatigue (6). In the present study, we used a powerful antioxidant, ebselen, since it performs as a cell-permeable glutathione peroxidase mimetic to scavenge \( \text{H}_2\text{O}_2 \) (10, 42, 53). It has been demonstrated that activities of major AOX enzymes including superoxide dismutase and glutathione (GSH) peroxidase, were significantly increased in response to various fatigue paradigms (6, 22, 35, 37). It should be noted, however, that other studies have demonstrated no benefit of AOX on the development of muscle fatigue development (34). Interestingly, McClung et al. reported that overexpression of AOX in skeletal muscle does not reduce fatigue development (24), but another group found that, in a series of more heavy exercise, AOX significantly reduced muscle fatigue (17). Since the efficacy of antioxidants on muscle performance depends on the biochemistry of the antioxidant and the conditions of contractile activity (9), this could partially explain why, during our mild fatigue protocols, AOX did not affect the fatigue development (Fig. 3). In particular, it should be noted that most studies in this field have used mammalian muscle models, and it is possible that AOX may act differently on fatigue development in our amphibian model.

It has been suggested that excessive ROS decrease muscle functional capacity, whereas a moderate or even low level of ROS is critically required for redox signaling (25, 28). The protocol of the present study was designed for moderate fatigue because a complete recovery from each contractile period was needed every 60 min (Fig. 2). Since oxidative stress results from an imbalance between ROS and AOX levels, particularly when ROS formation is excessive and not well buffered by AOX, it is possible that a decreased contractile function by a more intensive stimulation or a severe non-recovery fatigue protocol may have resulted in a significant effect of the AOX on fatigue development due to a more pronounced ROS production (43). It has been reported that massive ROS formation in response to muscle fatigue development can lead to extensive oxidative modifications of proteins, lipids, and other cellular compounds (6, 48), and thus negatively affect contractility and the development of fatigue (40). However, it should be noted that not all studies of muscle fatigue implicate ROS as causative agents of the fatigue process (33).

It should be pointed out that Hfluor is very insensitive to another major oxidant, superoxide \( (\text{O}_2^-) \) (11), and thus there is a possibility that \( \text{O}_2^- \) could have mediated the fatigue development seen in the present study. This could be the reason why we did not see the ebselen’s effect on muscle contractions. However, intracellular \( \text{O}_2^- \) is highly unstable and is quickly converted to another more stable product, \( \text{H}_2\text{O}_2 \), catalyzed by intracellular superoxide dismutase (SOD) or glutathione peroxidase (6, 51). Since Hfluor is mainly sensitive to \( \text{H}_2\text{O}_2 \), the fluorescent signals that we detected with confocal microscopy represent mostly the intracellular \( \text{H}_2\text{O}_2 \) formation. Particularly, ebselen, a cell-permeable glutathione peroxidase mimetic, has been...
broadly used as a powerful intracellular AOX that cooperates with GSH to scavenge H₂O₂ (10, 42, 53). Thus the scavenging of H₂O₂ by ebselen could potentially drive the dismutation reaction from O₂⁻ to H₂O₂ (53). Ebselen is also a scavenger for peroxynitrite and NO under certain conditions (20). Since peroxynitrite is a reaction product of O₂⁻ and NO, ebselen could be regarded as an indirect scavenger for O₂⁻. Overall, the present results suggest that ROS, particularly H₂O₂, does not seem to play a critical role in moderate fatigue development in intact Xenopus myofibers.

Summary and future directions. The results from the present study demonstrate that intracellular ROS formation can be accurately measured in real time during tetanic contractions in Xenopus single intact myofibers. Although the ROS generation was shown to be blocked by ebselen treatment, ebselen administration had no significant effect on the myofiber fatigue development. This suggests that, under the moderate fatigue conditions induced in these Xenopus single myofibers, fatigue development was independent of ROS formation. It is possible that the contraction-induced ROS generation may not have been sufficient under these conditions to affect fatigue development, particularly in this amphibian muscle model.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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