

Emerging Roles of ROS/RNS in Muscle Function and Fatigue

Håkan Westerblad¹ and David G. Allen²

Abstract

Reactive oxygen and nitrogen species (ROS/RNS) are involved in numerous aspects of cellular signaling. Classically ROS/RNS have been associated with cellular dysfunction and disease, but it is now clear that they are also of integral importance under normal conditions. In this review, we discuss ROS/RNS effects in skeletal muscle, with special focus on changes in contractile function. The review deals with the tentative roles of ROS/RNS for acute changes that can occur during strenuous exercise resulting in muscle fatigue, for the recovery from fatigue, and for the effects of training/overtraining. We also discuss two groups of inherited diseases; muscle dystrophies, where recent data suggest that ROS/RNS may be of unexpectedly large importance, and mitochondrial myopathies, where the role of ROS seems more limited than originally thought. *Antioxid. Redox Signal.* 15, 2487–2499.

Introduction

CELLULAR FUNCTION IS CONTROLLED by a myriad of signaling events and reactive oxygen and nitrogen species (ROS/RNS) are involved in many aspects of this signaling. ROS/RNS have classically been associated with “oxidative/nitrosative stress” resulting in cellular dysfunction and disease. However, it is becoming increasingly clear that ROS/RNS are also important components in normal cellular signaling. For instance, ROS can affect muscle cell function via altering the activity of kinases and phosphatases and hence the cellular phosphorylation status (9, 113). ROS/RNS can induce both acute reversible effects and long-term effects, which are mediated via altered gene and protein expression or via partially irreversible changes in existing proteins and lipids. Positive acute effects of ROS/RNS in skeletal muscle include a role in contraction-mediated glucose uptake (29, 83, 87) and a long-term effect is the involvement in induction of beneficial adaptations in response to endurance training (82). On the other hand, an excessive ROS production can cause an acute decrease in force production during repeated contractions (67), and prolonged increases in ROS/RNS appear to have pivotal roles in the skeletal muscle dysfunction observed in diseases such as muscle dystrophies and generalized inflammatory diseases (99, 115). In this review, we will discuss ROS/RNS effects in skeletal muscle with special focus on results obtained in intact adult muscle fibers, which allow detailed studies of contractile function and cellular Ca^{2+} handling in an intact cellular environment.

Superoxide anion ($\text{O}_2^{\bullet-}$) is the primary oxygen free radical. In skeletal muscle, $\text{O}_2^{\bullet-}$ is considered to be mainly produced by mitochondria and by NADPH oxidases (76). In mitochondria $\text{O}_2^{\bullet-}$ is produced as a by-product of oxidative phosphorylation and hence the rate of production would be expected to increase during physical exercise and in other situations with increased mitochondrial respiration. In mitochondria, $\text{O}_2^{\bullet-}$ is mainly produced by complexes I and III, which release $\text{O}_2^{\bullet-}$ to the matrix, and complex III, which releases $\text{O}_2^{\bullet-}$ to the intermembrane space (68, 76). Early data indicated that the rate of mitochondrial $\text{O}_2^{\bullet-}$ production amounts to 1%–2% of the O_2 consumption (24). However, this cannot be the situation in exercising skeletal muscle, because prolonged physical exercise and endurance training would then result in dangerously high ROS levels, severe oxidative stress, and muscle damage. Accordingly, more recent measurements of mitochondrial $\text{O}_2^{\bullet-}$ production show that the $\text{O}_2^{\bullet-}$ production is only ~0.15% of the O_2 consumption (93). It should also be noted that there is no fixed relation between the rate of mitochondrial O_2 consumption and ROS production; for instance, uncoupling of oxidative phosphorylation with 2,4-dinitrophenol results in increased O_2 consumption accompanied by decreased ROS levels (71) and mitochondrial ROS production appears to be higher during state 4 (basal) as compared to state 3 (maximal ADP-stimulated) respiration, and the latter dominates during aerobic exercise (76).

It has been difficult to detect ROS increases in the mitochondrial matrix with fluorescent indicators during fatiguing stimulation, which increases mitochondrial respiration

¹Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden.

²Sydney Medical School and Bosch Institute (F13), University of Sydney, Sydney, Australia.

towards its maximum. For instance, fatigue induced by repeated tetanic stimulation of isolated fast-twitch mouse flexor digitorum brevis (FDB) fibers did not result in increased ROS production in the mitochondrial matrix as measured either by the dihydroethidium-derived fluorescent indicator MitoSOX Red (10, 45) or a novel reversible redox sensitive green fluorescent protein construct targeted to mitochondria (mito-roGFP) (65). On the other hand, increased mitochondrial ROS production during fatigue of FDB fibers is indicated by the fact that recovery after fatiguing stimulation depends on the expression level of superoxide dismutase 2 (SOD2), which is located in the mitochondria and catalyzes the conversion of $O_2^{\bullet-}$ to hydrogen peroxide (H_2O_2) (22). Taken together, it appears that mitochondrial ROS production increases during intense exercise and, although this increase is likely to be modest and hence difficult to detect, it can induce important changes in contractile function and cellular Ca^{2+} handling.

Several studies using fluorescent indicators to measure cytosolic ROS levels show moderate increases in muscles during repeated contractions. For instance, contraction-induced increases in ROS have been observed with the general cytosolic ROS indicator dichlorofluorescein in rat diaphragm (81), rat FDB fibers (22), and mouse FDB fibers (65), the latter in which an ROS increase in the mitochondrial matrix was not detected (see above). These results indicate that the increase in ROS production in muscle fibers during repeated contractions mainly involves sources outside of the mitochondrial matrix. One such source is $O_2^{\bullet-}$ generated in mitochondrial complex III and released to intermembrane space (68). Another source is NADPH oxidases; an increased ROS production that appeared, at least partly, to be mediated via NADPH oxidases was observed in experiments with cultured rat myotubes activated by electrical pulses or K^+ -induced depolarization (34). Thus, an increase in ROS during physical exercise may originate mainly from sources outside of the mitochondrial matrix, such as NADPH oxidases, rather than as a by-product of mitochondrial respiration. Though it must be noted that precise spatial and temporal analyses of ROS production are hampered by method limitations; future experiments with improved techniques are required to achieve a detailed understanding of contraction-mediated ROS production.

Nitric oxide (NO^{\bullet}) is the primary nitrogen free radical. NO^{\bullet} is synthesized from the amino acid L-arginine by nitric oxide synthases (NOS) (50). In addition, it was recently shown that NO^{\bullet} can be formed from the inorganic anions nitrate (NO_3^-) and nitrite (NO_2^-) (57). Adult skeletal muscle constitutively coexpresses two NOS isoforms: the neuronal (nNOS or type I) and the endothelial (eNOS or type III) isoform (95). A third isoform, inducible NOS (iNOS or type II), is expressed in skeletal muscle in some inflammatory conditions (95). The release of NO^{\bullet} from isolated rat fast-twitch extensor digitorum longus (EDL) muscles and mouse diaphragm and slow-twitch soleus muscles was increased by electrically stimulated contractions (14, 47). The NO^{\bullet} release both under resting conditions and during repeated contractions was similar in eNOS-deficient and wild-type mouse muscles, which indicates that nNOS is the dominating constitutively expressed isoform (47). It should be noted that, with the usage of intact whole muscles, it cannot be excluded that an increased NO^{\bullet} production occurred in other cell types than the skeletal muscle fibers. However, a more recent study showed an increased rate of intracellular NO^{\bullet} production with electrically

induced contractions in isolated adult mouse FDB fibers (77). Thus, although less studied than ROS, there is experimental support also for increased RNS production in muscle fibers during repeated contractions.

The contraction of skeletal muscle fibers is initiated by a series of events starting with an action potential propagating along the surface membrane and into the transverse tubules (t-tubules), where it activates specialized voltage sensors, the dihydropyridine receptors (DHPRs). The DHPRs activates the sarcoplasmic reticulum (SR) Ca^{2+} release channels (ryanodine receptors 1, RyR1) and Ca^{2+} is released into the cytoplasm where the free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) rapidly increases. Ca^{2+} subsequently binds to the actin filament regulatory protein troponin C, which leads to cross-bridge cycling and contraction. Ca^{2+} is actively pumped back into the SR by the SR Ca^{2+} -ATPase (SERCA). When action potentials are no longer generated and SR Ca^{2+} release ends, SERCA rapidly lowers $[Ca^{2+}]_i$ whereby cross-bridge cycling stops and the fiber relaxes. Any of these events can be affected by ROS/RNS, leading to either increased or decreased contractile performance.

In the present review, we will discuss some conditions where ROS/RNS can affect contractile function, with a focus on isometric contractions and studies where cellular mechanisms have been investigated in adult muscle (Fig. 1). We will first discuss acute changes that can occur during strenuous exercise resulting in muscle fatigue. The next section will discuss tentative long-lasting effects of ROS/RNS, which can be observed during the recovery from fatigue and which are likely to be important in relation to endurance training/overtraining. Finally, we will discuss two groups of inherited diseases; muscle dystrophies, where recent data suggest that prolonged effects of increased ROS/RNS may be of unantic-

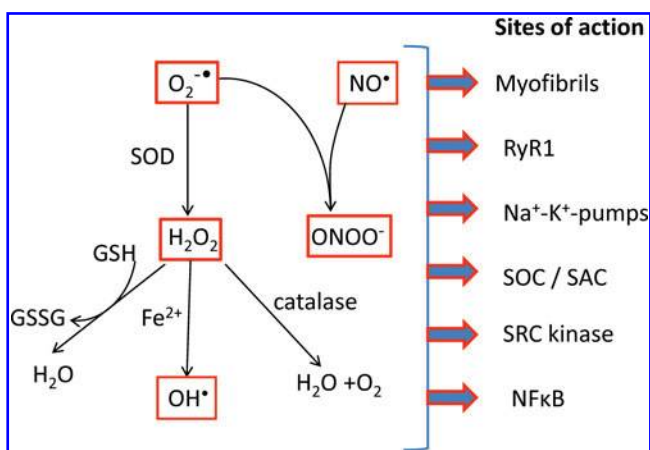


FIG. 1. Major reactive oxygen/nitrogen species (ROS/RNS) in muscle and tentative targets. The ROS/RNS discussed in this review are: H_2O_2 , hydrogen peroxide; NO^{\bullet} , nitric oxide; $O_2^{\bullet-}$, superoxide anion; OH^{\bullet} , hydroxyl radical; $ONOO^{\bullet}$, peroxynitrite. Targets discussed are: myofibrils, nuclear factor κ B (NF κ B), ryanodine receptor 1 (RyR1), Na^+-K^+ -pumps, store-operated channels (SOC), stretch-activated channels (SAC), and SRC kinase. GSH-GSSG, reduced and oxidized glutathione; SOD, superoxide dismutase. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars).

ipated importance, and mitochondrial myopathies, where the role of ROS seems more limited than originally thought.

Effects of ROS/RNS During the Induction of Skeletal Muscle Fatigue

When repeatedly activated, the contractile performance of skeletal muscle fibers declines, and this is referred to as skeletal muscle fatigue (3). In skeletal muscle fibers, fatigue can be caused by impaired function of any of the events in the excitation-contraction pathway described above. A multitude of factors can contribute to fatigue and the importance of each of these depends on, for instance, the intensity and duration of the activity. Moreover, the susceptibility to develop fatigue, as well as underlying mechanisms, differs between fast-twitch easily fatigued (type IIb), intermediate fatiguable (type IIx), and fatigue resistant (type IIa) fibers (55), and slow-twitch fatigue resistant (type I) fibers (3).

Numerous studies have shown slower fatigue development in the presence of ROS scavengers, but there are also many studies showing no effect of ROS on fatigue development (76). The effects of RNS on fatigue are uncertain with data indicating that NO[•] can both accelerate and delay fatigue development (76). Adult skeletal muscle constitutively expresses nNOS and eNOS (95). If NO[•] generated by NOS affects fatigue, this would occur by increased nNOS activity because fatigue development was not affected in mouse muscles deficient of eNOS (47). Interestingly, recent human studies show that an increased dietary intake of nitrate results in decreased O₂ consumption during exercise (13, 53, 54) and increased time to exhaustion (12, 13). Dietary nitrate can be metabolized to NO[•] (57) and the positive effects of increased nitrate intake on exercise performance is considered to be mediated via increased NO[•], but the underlying mechanisms remain to be determined.

ROS/RNS effects on fatigue tend to be more marked with submaximal than with near-maximal contractions (76). At the muscle cell level, this suggests effects either on SR Ca²⁺ handling or myofibrillar Ca²⁺ sensitivity, because even minor changes in these have large effects on force production during submaximal contractions, which occur on the steep part of the force-[Ca²⁺]_i relationship (see Fig. 6 in Ref. (3)). We will therefore in greater detail discuss how acute fatigue-induced changes in ROS/RNS can affect events related to SR Ca²⁺ handling and myofibrillar function.

ROS/RNS and Ca²⁺ release in fatigue

Ca²⁺ release through RyR1 triggers contraction and there is good evidence that Ca²⁺ release decreases during fatigue of isolated muscle fibers (4, 58, 96, 109). Given that there are many mechanisms by which this Ca²⁺ decline can occur (2) and that ROS/RNS scavengers have been shown to prolong the time to fatigue under various experimental conditions (80), it seems possible that post-translational oxidative/nitrosative modification of RyR1 is one mechanism in fatigue. Of course many proteins contribute to the amount of Ca²⁺ available for release and other tentative targets include the channels contributing to the action potential, the plasma membrane Na⁺-K⁺-pumps, and SERCA.

The action potential. The action potential activates DHPR in the t-tubules that interact with RyR1 in the SR. Reductions

in the amplitude of the action potential may reduce SR Ca²⁺ release (27). Extracellular K⁺ rises in muscle during fatigue and this may reduce the amplitude of the action potential; hence, this is one possible cause of failing SR Ca²⁺ release during fatigue (89). Numerous studies on many different cell types have shown that the function of Na⁺-K⁺-pumps can be adversely affected by ROS (42, 63). Studies from McKenna's laboratory have established that the ROS scavenger *N*-acetyl cysteine (NAC) can prolong the period of submaximal cycling in trained-humans (64). In a subsequent study, muscle biopsies were taken and the activity of the Na⁺-K⁺-pumps was found to be reduced after the exercise period and this reduction was partly ameliorated by NAC (63). In addition, NAC reduced the rise in extracellular K⁺, presumably through its action on the Na⁺-K⁺-pumps. Thus, these studies suggest that increased ROS during fatigue may inhibit the Na⁺-K⁺-pumps and exacerbate a K⁺-induced reduction of the action potential amplitude and thereby contribute to fatigue. However, it appears that decreased membrane excitability is not a limiting factor during most types of intense exercise, which can be explained by a large number of factors counteracting the increase in extracellular K⁺ and/or its depolarizing effects (for review, see Ref. (3)).

A study on skinned rat EDL fibers electrically stimulated to produce twitches at 1 min intervals showed a markedly accelerated decrease in twitch force at 37°C (~80% in 7 min) as compared to at 25°C (103). This decrease in twitch force at high temperature was less marked in the presence of the antioxidant Tempol and was ascribed to a ROS-induced decrease in membrane excitability. However, intact single muscle fibers showed no evidence of action potential failure during tetanic contractions when exposed to ROS (5, 7) or elevated temperature (37°C for FDB and 43°C for soleus fibers) (73). In fact, force was well maintained and there were no signs of action potential failure, even when intact soleus fibers were fatigued at 43°C in the presence of 10 μM H₂O₂ or tert-butyl hydroperoxide (73). Thus, studies on intact muscle fibers indicate that ROS do not exert any acute inhibitory effects on membrane excitability.

The SR Ca²⁺ release channels, RyR1. Each RyR1 subunit has 100 cysteines, of which 12 appear capable of modification by S-glutathionylation, S-nitrosylation, or disulfide bridge formation (8). Of these, 9 show signs of endogenous modification while 3 sites appear to respond only to exogenous modification. Various oxidants increase the activity of isolated RyR1 while antioxidants (e.g., glutathione (GSH)) reduce RyR1 activity (8). These data seem to suggest that altered SR Ca²⁺ release due to ROS-induced changes in RyR1 function might be widespread, but intriguingly this appears not to be the case in the intact system. For instance, while H₂O₂ depressed depolarization-induced SR Ca²⁺ release in skinned fibers (20), this effect was not apparent when the release is triggered by action potentials in the t-tubules in either skinned fibers (74) or in intact single fibers (5, 7). Moreover, changes in tetanic [Ca²⁺]_i observed in intact fibers during fatigue induced by repeated tetanic stimulations were similar in control and in conditions with markedly increased ROS levels (66, 73). Furthermore, the number of fatiguing tetani required to decrease force to 40% of the original in single intact FDB fibers was not affected by the presence of the ROS scavenger NAC (22) and the fatigue-induced changes in [Ca²⁺]_i were also similar (Fig. 2).

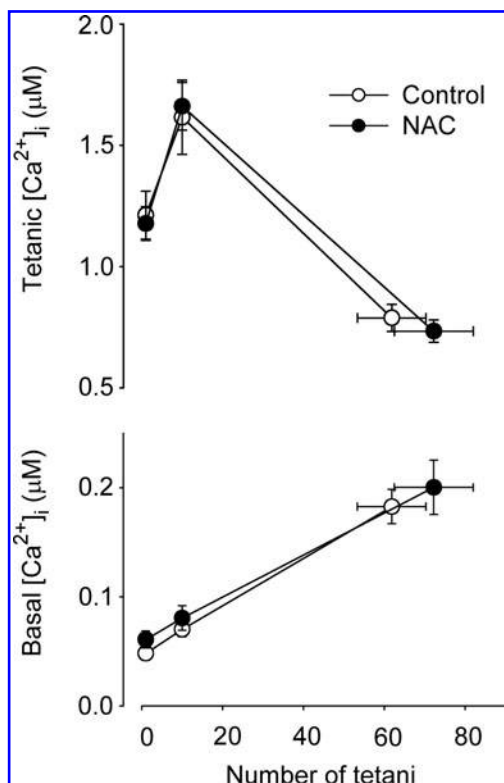


FIG. 2. The antioxidant NAC did not affect $[Ca^{2+}]_i$ during fatigue of single mouse FDB fibers. Mean (\pm s.e.m.) data of tetanic (upper part) and basal (lower part) $[Ca^{2+}]_i$ during fatigue induced by repeated tetanic stimulation continued until force was decreased to 40% of the initial in standard Tyrode solution (Control; $n=7$) or in the presence of 0.5 mM NAC ($n=4$). FH Andrade and H Westerblad, unpublished results.

Recent data suggest that NO^{\bullet} is capable of nitrosylating RyR1 (17), but it is uncertain whether NO^{\bullet} has any acute role in SR Ca^{2+} release during fatigue. A slight increase in $[Ca^{2+}]_i$ during submaximal tetani was observed when intact mouse FDB fibers were exposed to NO^{\bullet} donors (6). On the other hand, NOS inhibitors shifted the voltage dependence of SR Ca^{2+} release towards negative values in experiments on voltage-clamped mouse FDB fibers (75). Eu *et al.* suggested that NO^{\bullet} exerts a stimulatory effect on RyR1 Ca^{2+} fluxes at low O_2 pressure and that this effect is missed in many experiments on isolated whole muscles and muscle fibers because these were performed at unphysiologically high O_2 pressures (36, 37). Increased NO^{\bullet} during fatigue might then counteract other fatigue-induced effects, resulting in decreased SR Ca^{2+} release. However, the conclusions of Eu *et al.* are controversial and other investigators have not observed a critical role of O_2 pressure on NO^{\bullet} -induced effects on RyR1 (26). Furthermore, the physiological relevance of fatigue studies performed on isolated whole muscle preparations are likely to be limited by insufficient O_2 diffusion to deeper parts of the muscle and hence the development of an artificial hypoxia rather than hyperoxia (15, 117). For instance, force was only reduced by 20% after 120 fatiguing tetani in rat soleus muscles stimulated *in situ* (85), which is similar to the force decrease observed in single soleus fibers (59, 117) but markedly smaller than the 60% decrease after ~80 tetani

observed in isolated whole rat soleus muscles stimulated with a similar protocol *in vitro* (58). Thus, although there are no clear signs of altered function induced by hyperoxia in experiments on isolated intact muscle fibers, further studies are required to rule out the possibility that NO^{\bullet} effects on SR Ca^{2+} release are masked by high O_2 pressure in these experiments.

To sum up, both ROS and RNS have the potential to affect RyR1 function but their importance for the decrease in SR Ca^{2+} release during acute fatigue remains uncertain. A likely reason for this is that metabolic factors (*e.g.*, increased concentration of inorganic phosphate ions (P_i) due to breakdown of creatine phosphate) exert larger depressive effects on SR Ca^{2+} release during the induction of acute fatigue (3). On the other hand, ROS/RNS-induced modifications of RyR1 may affect the recovery from fatigue (when metabolic changes have been reversed) as well as how the muscle responds to prolonged stress, as discussed below.

ROS/RNS and myofibrillar function in fatigue

Experiments in which intact mouse FDB fibers were exposed to the oxidant H_2O_2 showed large changes in myofibrillar Ca^{2+} sensitivity, whereas maximum myofibrillar force production was little affected (5, 7). Unexpectedly, the initial response to H_2O_2 exposure was an increased Ca^{2+} sensitivity, which was followed by decreased sensitivity with prolonged exposure. Experiments on skinned fibers provide an explanation for this bi-phasic response. Skinned fibers are rather insensitive to application of H_2O_2 by itself, but a marked decrease in myofibrillar Ca^{2+} sensitivity was observed when H_2O_2 was applied together with myoglobin (69); this effect was probably caused by the hydroxyl radical, OH^{\bullet} , produced in the Fenton reaction when Fe^{2+} in myoglobin reacted with H_2O_2 (76). Conversely, an increased sensitivity was observed when H_2O_2 and myoglobin was added in the presence of glutathione (69). Since both myoglobin and glutathione are normal constituents in intact muscle fibers, H_2O_2 can increase or decrease Ca^{2+} sensitivity depending on the cellular redox state, for example, reflected by the proportion of reduced to oxidized glutathione (GSH/GSSG).

A marked reduction of the myofibrillar Ca^{2+} sensitivity is a general feature in fatigued fast-twitch muscle fibers (3, 109). Fatiguing stimulation of fast-twitch muscle is also accompanied by major changes in energy metabolites and an increased concentration of P_i derived from breakdown of creatine phosphate appears to be the major factor underlying the decrease in myofibrillar Ca^{2+} sensitivity (3). Recently, a very fast force decrease was observed when isolated mouse FDB fibers were stimulated by repeated tetani at increased temperature (37°C). This rapid force decline was due to decreased myofibrillar Ca^{2+} sensitivity and could be prevented by ROS scavengers (66). Subsequently it was shown that the premature fatigue development was caused by Fe^{2+} leakage from a stainless steel heat exchanger, and fatigue development was not accelerated at increased temperature when an aluminum heat exchanger was used (79). Moreover, muscles of mice injected with iron showed impaired contractile function and signs of increased ROS production (78). Thus, the combination of increased temperature and Fe^{2+} had a major force depressing effect via inducing a decrease in myofibrillar Ca^{2+} sensitivity, most likely mediated by OH^{\bullet} produced in the Fenton reaction (Fig. 3). On the other hand, increased tem-

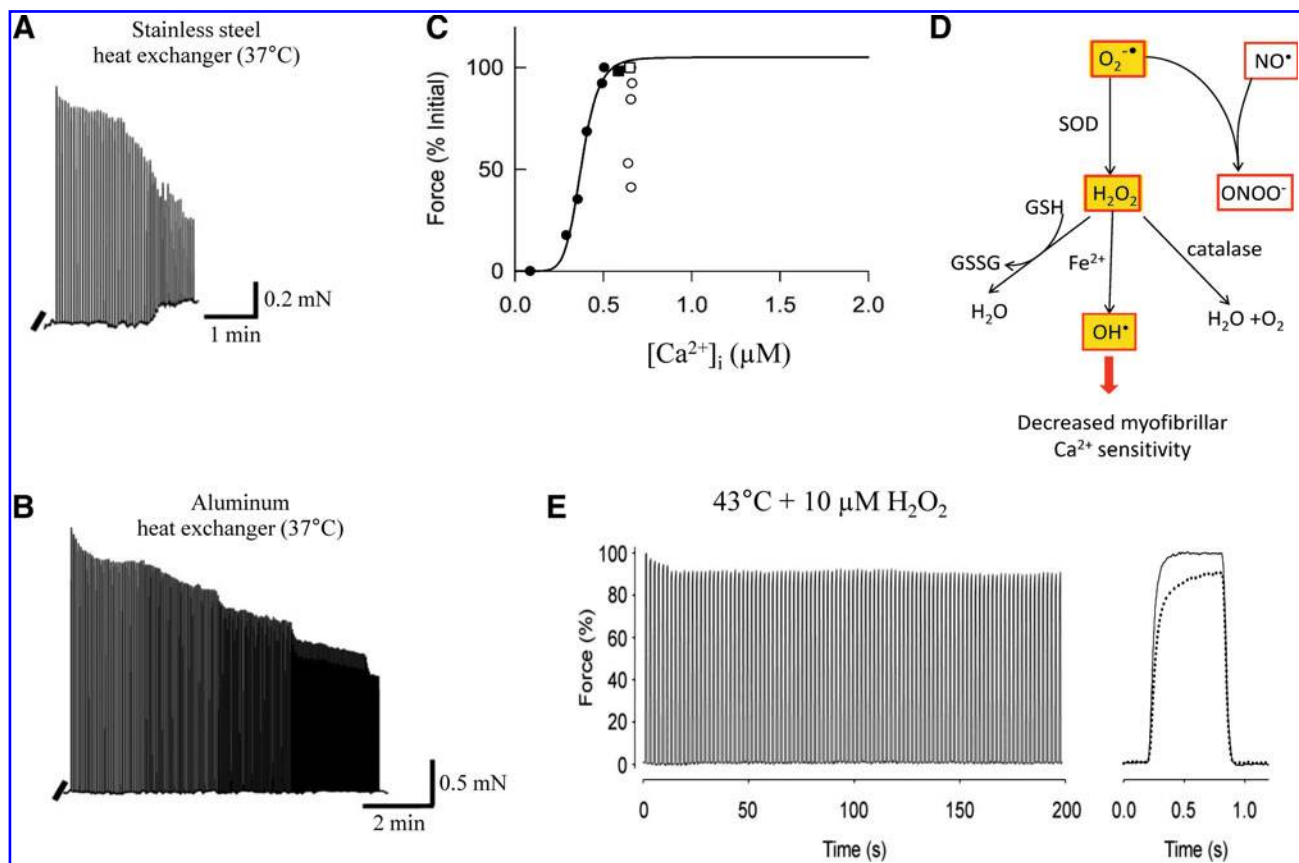


FIG. 3. Fe^{2+} causes rapid force decrease during repeated tetanic stimulation. Continuous force records showing a markedly faster force decrease in mouse FDB fiber bundles fatigued by repeated tetani (100 Hz, 400 ms) at 37°C when using a stainless steel (A) as compared to an aluminum (B) heat exchanger (adapted from (79)). (C) Simultaneous force and $[\text{Ca}^{2+}]_i$ measurements during fatigue at 37°C using a stainless steel heat exchanger showed that the rapid force decrease was due to decreased myofibrillar Ca^{2+} sensitivity (adapted from (66)). (D) The rapidly developing reduction in myofibrillar Ca^{2+} sensitivity is likely to be mediated by increased OH^\bullet generated via the highlighted pathway. (E) Fatigue development was not accelerated when a single soleus fiber was fatigued at 43°C (glass heat exchanger) while being exposed to 10 μM H_2O_2 ; first and last fatiguing tetani shown to the right (adapted from (73)). (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars).

perature itself does not accelerate fatigue development in isolated muscle fibers (73,79). To conclude, increased ROS has the potential to decrease myofibrillar Ca^{2+} sensitivity during induction of acute muscle fatigue but factors related to increased energy metabolism (e.g., increased P_i) are more important under normal conditions.

The rate of NO^\bullet production has been shown to increase with electrically stimulated contractions in mouse FDB fibers (77). Intact mouse FDB fibers exposed to NO^\bullet donors showed decreased myofibrillar Ca^{2+} sensitivity (6). Decrease myofibrillar Ca^{2+} sensitivity was also observed in fast-twitch skinned fibers exposed to NO^\bullet donors (92). Thus, a fatigue-induced increase in NO^\bullet might contribute to the decrease in myofibrillar Ca^{2+} sensitivity generally observed during acute fatigue in fast-twitch muscle fibers (109), but, as discussed above, changes in energy metabolites are probably more important (3). Interestingly, NO^\bullet donors did not affect the myofibrillar Ca^{2+} sensitivity in skinned slow-twitch fibers (92), which might contribute to slow-twitch fibers generally being more fatigue resistance than fast-twitch fibers, although other factors such as higher aerobic capacity and lower rate of ATP consumption are likely to be more important (3).

Changes in myofibrillar Ca^{2+} sensitivity can be caused by altered Ca^{2+} interaction with the actin filament regulatory troponin-tropomyosin protein complex and/or altered cross-bridge function changing the interaction between cross-bridge attachment and actin filament activation (44). Acute exposure of single mouse FDB fibers to increased ROS or RNS had no significant effect on maximum force production (5–7), which reflects the ability of cross-bridges to generate force. Moreover, increased NO^\bullet had no acute effect on maximum shortening velocity or the rate of force redevelopment after a shortening step in FDB fibers (6). These findings speak against major effects on cross-bridge cycling as the mechanism underlying acute ROS/RNS-induced changes in myofibrillar Ca^{2+} sensitivity. However, the actual mechanisms by which ROS/RNS alter the Ca^{2+} activation of the actin filament remain to be established.

Effects of ROS/RNS on the Recovery from Fatigue

The recovery of muscle force production after fatiguing exercise can be very slow, sometimes requiring days for full recovery (33). The force deficit is most marked at low stimulation

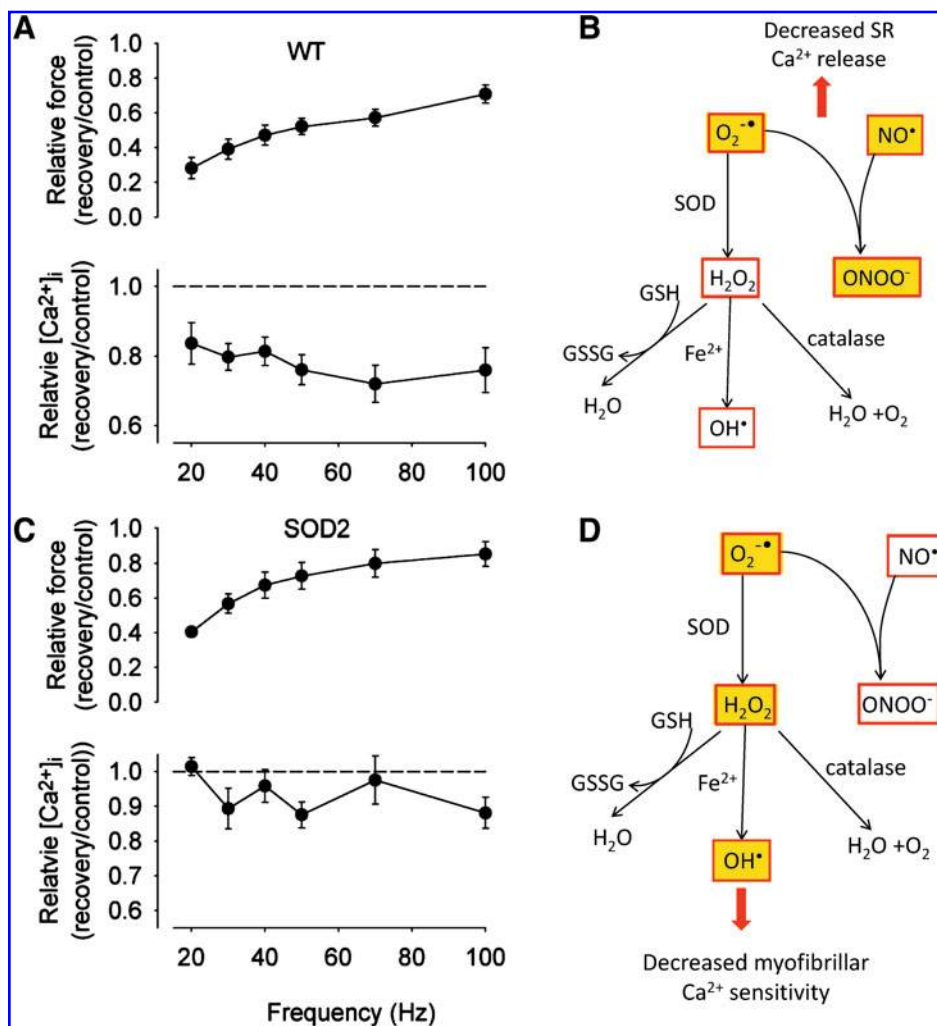


FIG. 4. Both wild-type and SOD2 overexpressing fibers display marked PLFFD, but the underlying mechanism differs. Mean data (\pm s.e.m.) of the relative change in tetanic force (*top*) and $[Ca^{2+}]_i$ (*bottom*) obtained in mouse wild-type (A) and SOD2 overexpressing (C) fibers ($n=4$) (adapted from (22)). Relative changes were calculated as ratio 30 min after (recovery) to before (control) fatiguing stimulation. Note that while the pattern of force depression during recovery was similar in the two groups, the force decrease was due to decreased SR Ca^{2+} release in wild types and decreased myofibrillar Ca^{2+} sensitivity in SOD2 overexpressing mice. The likely ROS/RNS involved are highlighted in B and D, respectively. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars).

frequencies and it was therefore initially named "low frequency fatigue" (LFF) (33). The term LFF is now unfortunately being used to describe many different fatigue-related properties, and we recently proposed an alternative term "prolonged low-frequency force depression" (PLFFD) to specifically describe the delayed force recovery (3). In humans, PLFFD has been reported after many different types of muscle activity, such as isometric contractions (33), concentric/eccentric contractions (32), and global exercise such as running (28, 33, 61). PLFFD has important consequences on physical performance and perception of effort because most voluntary contractions are performed with relatively low motoneuron discharge frequencies where the relationship between force production and frequency is steep (19).

At the muscle fiber level, a more marked force decrease preferentially at low stimulation frequencies can be explained by either decreased SR Ca^{2+} release or reduced myofibrillar Ca^{2+} sensitivity (see Fig. 6 of Ref. (3)). Although the exact cellular mechanisms underlying PLFFD remain uncertain, there are data supporting a role of ROS-induced changes underlying the slow force recovery after fatigue; for instance, application of the disulfide reducing agent dithiothreitol resulted in increased force production in isolated rat diaphragm strips recovering from fatigue induced by repeated tetani (30). Recent data also indicate a central role for ROS-induced

modifications. In a study by Bruton and colleagues (22), the recovery of force after fatigue induced by repeated tetani was followed in FDB fibers of wild-type and transgenic mice overexpressing SOD2. During fatigue, SOD2 overexpressing muscle would be expected to show reduced $O_2^{\bullet-}$ and increased H_2O_2 levels as compared to wild-types (91). Interestingly, FDB fibers of both groups of mice displayed PLFFD but the defect differed; decreased myofibrillar Ca^{2+} sensitivity in SOD2 overexpressing fibers and impaired SR Ca^{2+} release in wild-type fibers (Fig. 4). Furthermore, PLFFD was due to decreased myofibrillar Ca^{2+} sensitivity in rat FDB fibers; compared to mouse FDB fibers these show higher SOD activity and an increase in cytosolic ROS during fatiguing stimulation (22). These results indicate that increased H_2O_2 , which would dominate in SOD2 overexpressing and in rat FDB fibers, preferentially induces decreased myofibrillar Ca^{2+} sensitivity, consistent with experiments with exogenous application of H_2O_2 (5, 7). As discussed above, the ROS that actually mediate the depressant effect on myofibrillar Ca^{2+} sensitivity is likely to be OH^{\bullet} , which is generated from H_2O_2 via the Fenton reaction.

Comparatively higher $O_2^{\bullet-}$ levels, as would be expected during fatigue in wild-type mouse FDB fibers (although not detected with fluorescent indicators; see above), were accompanied by a prolonged decrease in SR Ca^{2+} release. The

fact that different mechanisms underlying PLFFD depend on SOD2, which is located in the mitochondrial matrix, suggests that the $O_2^{\bullet-}$ was of mitochondrial origin. For membrane impermeable $O_2^{\bullet-}$ produced in the mitochondrial matrix to affect SR Ca^{2+} release, it has to pass through anion channels (101). Alternatively it might react with NO^{\bullet} to form $ONOO^{\bullet-}$, which then directly or indirectly mediates the effect. Thus, while it seems clear that development of PLFFD can be ROS dependent, the exact mechanisms and species involved remain to be established.

Effects of Prolonged ROS/RNS Challenges and Their Relation to Training and Overtraining

Prolonged increases of ROS/RNS are likely to induce post-translational changes in various proteins, which may directly affect contractile function and fatigue resistance. Alternatively, a prolonged elevation of ROS/RNS levels may function indirectly by mediating changes in gene transcription and hence the cellular protein composition. Recently, Bellinger *et al.* subjected mice to an intense exercise regimen: two 90 min bouts of swimming daily for 3 weeks (18). This, which can be seen as a model for overtraining, resulted in marked changes in RyR1 with serine-2844 phosphorylation, increased nitrosylation of free cysteines, and depletion of the associated proteins 12 kDa FK506 binding protein (FKBP12, also called calstabin-1) and phosphodiesterase 4D3 (PDE4D3). Similar changes in the RyR1 protein complex were seen after a 3-day period of intense exercise in trained cyclists (3 h of 70% V_{O2max}). The period of intense exercise increased endothelial NOS in mouse EDL muscles (18), which then provided a potential source of NO^{\bullet} for the RyR1 nitrosylation. At the end of the training period, mice showed reduced exercise capacity and force production was decreased in isolated EDL muscles. Mice with muscle-specific KO of calstabin-1 or PDE4D3 showed reduced exercise capacity similar to that of the exercised mice. A causal relation between the RyR1 changes and the reduced exercise capacity after training was suggested by the use of a drug, S107, which stabilizes the binding of calstabin-1 and PDE4D to RyR1. This drug had no direct effect on muscle performance in control mice but prevented the exercise-induced depletion of calstabin-1 and exercise deficiency. Thus, the study by Bellinger *et al.* provides a model of 'overtraining' in which intense exercise precipitates a decline in performance due to NO^{\bullet} -associated modifications of the RyR1 protein complex. This model shows similarities to PLFFD observed in wild-type mouse fibers and, taken together, these findings indicate that exercise-induced ROS/RNS-mediated changes in RyR1 can cause long-lasting decreases in SR Ca^{2+} release.

Another example of tentatively ROS/RNS-dependent changes in the RyR1 protein complex was recently described in experiments on muscles of cold-acclimated mice (11). Mammals initially shiver to maintain body temperature when placed in a cold environment (46). With prolonged cold exposure, shivering is normally replaced by the recruitment of uncoupling protein (UCP) 1-dependent heat production in brown adipose tissue (23), whereas UCP1 KO mice continue to depend on heat generated by shivering (43). Soleus muscle fibers of cold-acclimated UCP1 KO mice displayed marked RyR1 phosphorylation and calstabin-1 depletion, accompanied by decreased SR Ca^{2+} release and impaired contractility

(11). Conversely, FDB fibers of cold-acclimated UCP1 KO as well as wild-type mice showed only minor RyR1 phosphorylation and calstabin-1 depletion and these changes were associated with increased SR Ca^{2+} release (Fig. 5). An important difference between the two muscles is that soleus, but not FDB, muscles participate in the shivering response. Thus, soleus muscles of cold-acclimated UCP1 KO mice were exposed to both prolonged shivering and the stress induced by cold exposure, which includes chronically increased sympathetic activity in skeletal muscle (31). These stressors resulted in a state of muscle dysfunction, which might be equivalent to severe overtraining and similar to that described by Bellinger *et al.* (18). On the other hand, FDB muscles were only exposed to the general stress induced by a cold environment and this resulted in increased resting and tetanic $[Ca^{2+}]_i$, which were accompanied by an increased mitochondrial biogenesis and increased fatigue resistance (21), (*i.e.*, beneficial changes similar to those obtained with endurance training). As discussed above for intense exercise, the induction of changes in the RyR1 channel complex caused by cold exposure is likely to involve ROS/RNS and provides yet another example of a fine

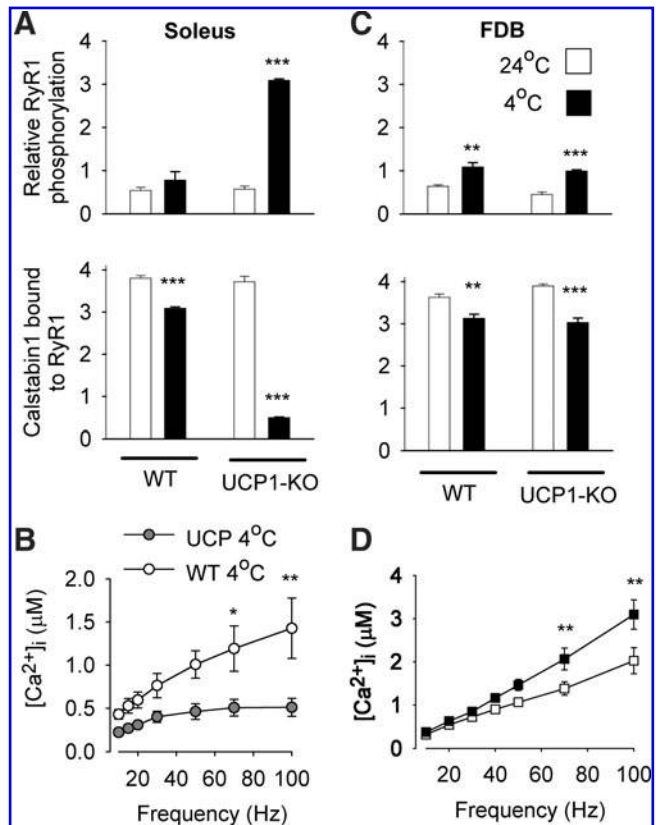


FIG. 5. Severe changes in the RyR1 channel complex are accompanied by decreased tetanic $[Ca^{2+}]_i$, whereas minor changes have the opposite effect. Mean (\pm s.e.m) data of relative RyR1 phosphorylation and calstabin-1 binding of soleus (A) and FDB (C) muscles of wild-type and UCP1-KO mice. The severe changes in the RyR1 channel complex in soleus muscles of cold-acclimated UCP1-KO mice were accompanied by decreased tetanic $[Ca^{2+}]_i$ (B), whereas the minor changes in cold-acclimated FDB muscles led to increased tetanic $[Ca^{2+}]_i$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. A–C adapted from (11), and D from (21).

balance between beneficial and deleterious effects of these species.

Heart failure

Humans with heart failure have greatly increased fatigability of their skeletal muscles (111). Much of this presumably derives from impaired blood flow responses but there is some evidence that intrinsic changes in skeletal muscle also contribute. An early study by Perreault *et al.* showed that isolated muscle bundles from rats with heart failure exerted less force, fatigued more rapidly, and had reduced $[Ca^{2+}]_i$ transients (72). Ward *et al.* confirmed the reduction in $[Ca^{2+}]_i$ transients in muscles of rats with heart failure and they also showed hyperphosphorylation of RyR1 combined with dissociation of calstabin-1 (106). A later study from the same laboratory showed that treating heart failure mice with JTV519, a benzodiazepine that inhibits the dissociation of calstabin-1 from RyR1, improved fatigue performance of isolated soleus muscles (108). Thus, this result provides a link between RyR1 hyperphosphorylation and calstabin-1-depletion to the increased fatigability of skeletal muscle observed in heart failure. The hyperphosphorylation of RyR1 in these experiments was attributed to increased sympathetic activity associated with heart failure (106, 108). Nevertheless, these changes in the RyR1 protein complex show great similarities with those observed after intense exercise (discussed above) and may then also be related to the increased ROS/RNS frequently observed in heart failure (102, 114). However, it should be noted that Lunde *et al.* failed to observe changes in the tetanic $[Ca^{2+}]_i$ or the fatigability of fast-twitch skeletal muscle fibers of heart failure rats (58). A later article from this group focused on slow muscles, which were found to be more fatigable in the presence of heart failure, but the underlying mechanism appeared to be myofibrillar rather than changes in SR Ca^{2+} handling (59). This myofibrillar impairment appeared to involve a marked reduction in the Ca^{2+} sensitivity which, as discussed above, can be observed also after intense exercise and which is very sensitive to ROS/RNS. Thus, heart failure is associated with impairments in skeletal muscle related to SR Ca^{2+} handling and/or myofibrillar function and both these may involve ROS/RNS-induced modifications.

ROS/RNS in Muscle Disease

Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD) is a disease in which mutations in the dystrophin gene lead to a loss of dystrophin protein. While the chronic effects are clear and include muscle wasting and weakness coupled with inflammation and fibrosis, the early stages of the pathology are still uncertain. It is generally accepted that increased Ca^{2+} entry into muscle cells, either through damaged membrane or activation of channels that allow Ca^{2+} entry, contributes to the pathology, notably by activating calpains (41, 70). Increased production of ROS in dystrophic muscle has been recognised for many years (99) and a number of recent studies suggest that ROS-induced modification of RyR1 may play a role in the disease process, possibly by increasing the propensity to SR Ca^{2+} sparks and leakage.

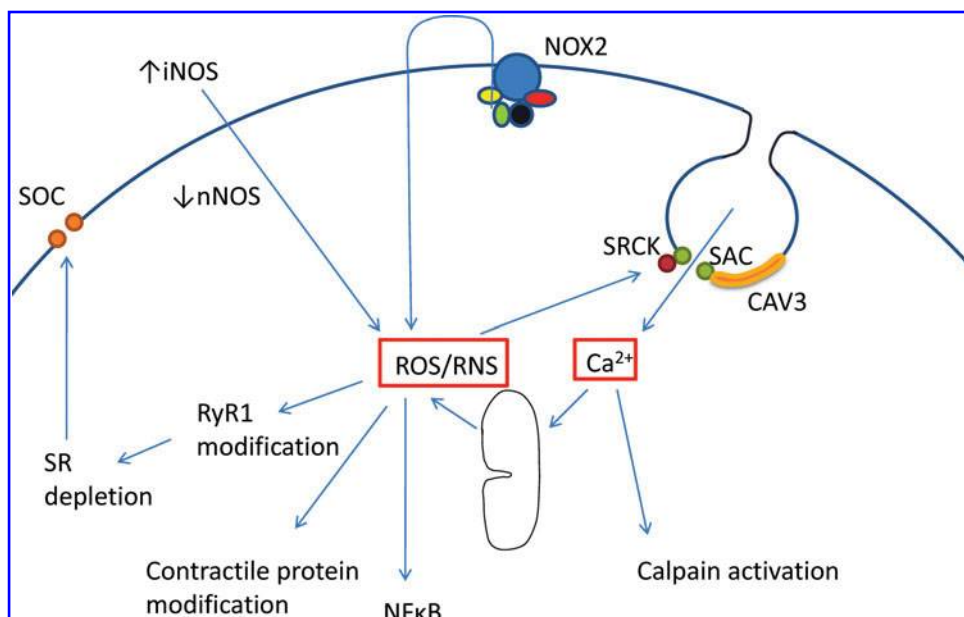
Ca^{2+} sparks are temporally and spatially restricted increases of $[Ca^{2+}]_i$ induced by the simultaneous activation of

clusters of RyRs (25, 49). Wang *et al.* showed that Ca^{2+} sparks, which are normally hard to detect in mammalian skeletal muscle, can be elicited transiently by osmotic shock (105). These Ca^{2+} sparks did not require extracellular Ca^{2+} and are blocked by ryanodine, suggesting that RyR1 is the source. Furthermore, when studied in a mouse model of DMD, the *mdx* mouse, these sparks were enhanced, became irreversible and occurred spontaneously in fibers obtained from mice which were exercised before isolation (105). Wang *et al.* suggested that the uncontrolled Ca^{2+} sparks in *mdx* fibers may result in SR Ca^{2+} depletion and activation of store-operated Ca^{2+} entry, which is one of the Ca^{2+} entry pathways that has frequently been implicated in muscular dystrophy (104). Martins *et al.* showed that Ca^{2+} sparks induced by osmotic shock could be largely eliminated by pretreatment with a variety of ROS scavengers or by the NADPH oxidase inhibitors DPI and apocynin (62). A subsequent study from the same group confirmed these findings in *mdx* fibers and also showed upregulation of gp91phox, the membrane-bound catalytic subunit of NADPH oxidase (90). They also showed increased mitochondrial uptake of Ca^{2+} and increased ROS generation in mitochondria of *mdx* fibers indicating a cycle of damaging events.

Another pathway in DMD was recently proposed by Bellinger *et al.*, who showed RyR1 modifications with increased nitrosylation and calstabin-1 dissociation in skeletal muscle of *mdx* mice (17). Neuronal NOS (nNOS) is normally bound to the dystrophin-associated protein complex, which is lost in dystrophic muscles, and therefore levels of nNOS are reduced (95). In contrast, inducible NOS (iNOS) was greatly increased in *mdx* muscle fibers and co-immunoprecipitated with RyR1, an association not found in wild-type muscles (17). Bellinger *et al.* confirmed, as previously shown (8), that exogenous NO donors also cause RyR1 nitrosylation and calstabin-1 depletion. They also showed that the RyR1-stabilizing drug S107 reversed the calstabin-1 depletion and decreased the SR Ca^{2+} leak without affecting RyR1 nitrosylation. Administration of S107 to *mdx* mice from 1 month age led to an improvement in muscle strength, reduced serum creatine kinase (a marker of membrane damage), and reduced calpain activity. Thus, Bellinger *et al.* proposed that Ca^{2+} leak from the SR elevates $[Ca^{2+}]_i$ causing calpain activation and the downstream consequences of muscular dystrophy. However, simply redistribution of Ca^{2+} from the SR to the myoplasm cannot be the whole explanation because it is widely accepted that total muscle Ca^{2+} is elevated in muscular dystrophies (41).

Several groups have found evidence that stretch-activated channels are more active and have changed properties in *mdx* muscle (38, 97). Furthermore, blocking these channels minimizes some of the features of muscle damage, suggesting that this Ca^{2+} entry pathway is important in early stages of the disease (116). Although stretch-activated channels were discovered by mechanical stretch imposed in patch-clamp experiments, this is not necessarily the mechanism of activation under physiological or pathological circumstances. In fact, several stretch-activated channels seem to be activated by pathways that include ROS (107) and in *mdx* muscle there is evidence that ROS can activate the same entry pathway (40). Recent work shows that both expression and activity of NADPH oxidase are increased in *mdx* muscle and confirms that blockers of NADPH oxidase inhibit the Ca^{2+} entry through stretch-activated channels (110).

FIG. 6. Pathways involved in changed ROS/RNS activity in muscular dystrophy. NADPH oxidase (NOX2) and several of its subunits are up-regulated in muscular dystrophy, leading to increased ROS production. Neuronal NOS (nNOS) shows reduced activity, while inducible NOS (iNOS) shows increased activity. The stretch-activated channel (SAC) may bind to caveolin 3 (CAV3) locating it in caveolae. Src kinase (SRCK) can bind to both CAV3 and SAC and is activated by ROS. ROS/RNS modify RyR1, which may result in raised $[Ca^{2+}]_i$ and calpain activation and/or cause store depletion which activates store operated channels (SOC), resulting in increased cellular $[Ca^{2+}]_i$. Contractile protein modification and NF κ B activation by ROS are other downstream consequences in dystrophic muscle. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars).



To sum up, there is substantial evidence for an increased ROS production by NADPH oxidase and probably also by mitochondria in dystrophic muscle. Increased NO production by iNOS and decreased production by membrane-bound nNOS are also well established (17, 39, 95). The interaction between ROS/RNS and its potential targets in dystrophic muscle is illustrated in Figure 6. The targets include SR, contractile proteins, and activation of nuclear factor κ B (NF κ B), which contributes to the inflammation, fibrosis, and atrophy (52). Note that in Figure 6 NADPH oxidase (NOX2) is shown as the membrane-bound, catalytic subunit gp91^{phox} together with its activating subunits (p67^{phox}, p22^{phox}, p47^{phox}, rac 1). Its production of superoxide is initially to the extracellular space (16). Superoxide itself is charged and membrane-impermeant and it is thought to enter the intracellular space either through anion-permeable channels (60) or as the uncharged hydroperoxy radical, HO₂[•] (86) or as H₂O₂ after dismutation by extracellular SOD3 (16, 76).

Mitochondrial myopathies

Inherited disorders of mitochondrial oxidative phosphorylation are the most common group of inborn errors of metabolism (48). These mitochondrial diseases are highly variable with respect to age of disease onset and affected organs or tissues (88). Nevertheless, symptoms are most often observed from tissues with high-energy demand, such as skeletal muscle (56, 84), and muscle weakness and exercise intolerance are frequent symptoms in patients with mitochondrial diseases (98). Defective mitochondrial function has been suggested to increase ROS production and cause oxidative stress and these have been suggested to have pivotal roles in the disease process (35, 94, 98). Muscle function has been studied in mice with specific disruption of the nuclear gene for mitochondrial transcription factor A (*Tfam* KO mice) in fast-twitch muscle fibers. Fast-twitch muscles of these mice

display important hallmarks of mitochondrial myopathy, including muscle weakness (112). However, mitochondrial O₂[•] (measured with the fluorescent indicator MitoSOX Red) did not increase during fatiguing stimulation of *Tfam* KO FDB fibers (10). Moreover, increased ROS production has been associated with decomposition of polyunsaturated fatty acids, resulting in formation of reactive carbonyl species that can bind to protein (1), but *Tfam* KO muscles showed no increase in carbonyl protein adducts (10). These results are in accordance with other recent studies, which also failed to show an increased ROS production or ROS-induced cellular damage in mouse mitochondrial disease models (51, 100). Thus, data from recently developed mouse models do not support an essential role of increased ROS production and oxidative stress in the pathology of mitochondrial diseases.

Concluding Remarks

In this review we have described acute and prolonged effects of ROS/RNS on muscle contractile function. It is clear that the understanding of normal and pathological roles of ROS/RNS in muscle has accelerated dramatically in the past decade. However, there are still areas where knowledge is relatively limited and where rapid progress can be expected in the near future; for example, effects of ROS/RNS on cellular signaling in adult muscle that occur via modulating the activity of kinases and phosphatases (113). There are also major impediments to further progress and these include inadequacy of the tools for detecting, quantifying, and localizing ROS/RNS in cells. In addition, tools for detecting the protein and lipid modifications that are secondary to ROS/RNS are at an early stage of development. Technical developments of measurement tools leading to improved quantification would provide the impetus for a fuller understanding of the regulatory roles of ROS/RNS in normal muscle function and disease.

Acknowledgments

DGA acknowledges support from the Australian National Health and Medical Research Council and the Australian Research Council. HW is supported by the Swedish Research Council, the Swedish National Center for Sports Research, and Association Française contre les Myopathies (AFM).

References

1. Aldini G, Dalle-Donne I, Facino RM, Milzani A, and Carini M. Intervention strategies to inhibit protein carbonylation by lipoxidation-derived reactive carbonyls. *Med Res Rev* 27: 817–868, 2007.
2. Allen DG, Lamb GD, and Westerblad H. Impaired calcium release during fatigue. *J Appl Physiol* 104: 296–305, 2008.
3. Allen DG, Lamb GD, and Westerblad H. Skeletal muscle fatigue: Cellular mechanisms. *Physiol Rev* 88: 287–332, 2008.
4. Allen DG, Lee JA, and Westerblad H. Intracellular calcium and tension during fatigue in isolated single muscle fibres from *Xenopus laevis*. *J Physiol (Lond)* 415: 433–458, 1989.
5. Andrade FH, Reid MB, Allen DG, and Westerblad H. Effect of hydrogen peroxide and dithiothreitol on contractile function of single skeletal muscle fibres from the mouse. *J Physiol (Lond)* 509: 565–575, 1998.
6. Andrade FH, Reid MB, Allen DG, and Westerblad H. Effect of nitric oxide on single skeletal muscle fibres from the mouse. *J Physiol (Lond)* 509: 577–586, 1998.
7. Andrade FH, Reid MB, and Westerblad H. Contractile response of skeletal muscle to low peroxide concentrations: Myofibrillar calcium sensitivity as a likely target for redox-modulation. *FASEB J* 15: 309–311, 2001.
8. Aracena-Parks P, Goonasekera SA, Gilman CP, Dirksen RT, Hidalgo C, and Hamilton SL. Identification of cysteines involved in S-nitrosylation, S-glutathionylation, and oxidation to disulfides in ryanodine receptor type 1. *J Biol Chem* 281: 40354–40368, 2006.
9. Avner BS, Hinken AC, Yuan C, and Solaro RJ. H₂O₂ alters rat cardiac sarcomere function and protein phosphorylation through redox signaling. *Am J Physiol Heart Circ Physiol* 299: H723–H730, 2010.
10. Aydin J, Andersson DC, Hänninen SL, Wredenberg A, Tavi P, Park CB, Larsson NG, Bruton JD, and Westerblad H. Increased mitochondrial Ca²⁺ and decreased sarcoplasmic reticulum Ca²⁺ in mitochondrial myopathy. *Human Mol Genet* 18: 278–288, 2009.
11. Aydin J, Shabalina IG, Place N, Reiken S, Zhang SJ, Bellinger AM, Nedergaard J, Cannon B, Marks AR, Bruton JD, and Westerblad H. Nonshivering thermogenesis protects against defective calcium handling in muscle. *FASEB J* 22: 3919–3924, 2008.
12. Bailey SJ, Fulford J, Vanhatalo A, Winyard PG, Blackwell JR, DiMenna FJ, Wilkerson DP, Benjamin N, and Jones AM. Dietary nitrate supplementation enhances muscle contractile efficiency during knee-extensor exercise in humans. *J Appl Physiol* 109: 135–148, 2010.
13. Bailey SJ, Winyard P, Vanhatalo A, Blackwell JR, DiMenna FJ, Wilkerson DP, Tarr J, Benjamin N, and Jones AM. Dietary nitrate supplementation reduces the O₂ cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. *J Appl Physiol* 107: 1144–1155, 2009.
14. Balon TW and Nadler JL. Nitric oxide release is present from incubated skeletal muscle preparations. *J Appl Physiol* 77: 2519–2521, 1994.
15. Barclay CJ. Modelling diffusive O₂ supply of isolated preparations of mammalian skeletal and cardiac muscle. *J Muscle Res Cell Motil* 26: 225–235, 2005.
16. Bedard K and Krause KH. The NOX family of ROS-generating NADPH oxidases: Physiology and pathophysiology. *Physiol Rev* 87: 245–313, 2007.
17. Bellinger AM, Reiken S, Carlson C, Mongillo M, Liu X, Rothman L, Matecki S, Lacampagne A, and Marks AR. Hypernitrosylated ryanodine receptor calcium release channels are leaky in dystrophic muscle. *Nat Med* 15: 325–330, 2009.
18. Bellinger AM, Reiken S, Dura M, Murphy PW, Deng SX, Landry DW, Nieman D, Lehnart SE, Samaru M, Lacampagne A, and Marks AR. Remodeling of ryanodine receptor complex causes "leaky" channels: a molecular mechanism for decreased exercise capacity. *Proc Natl Acad Sci USA* 105: 2198–2202, 2008.
19. Bigland-Ritchie B, Johansson R, Lippold OC, Smith S, and Woods JJ. Changes in motoneurone firing rates during sustained maximal voluntary contractions. *J Physiol (Lond)* 340: 335–346, 1983.
20. Brotto MA, and Nosek TM. Hydrogen peroxide disrupts Ca²⁺ release from the sarcoplasmic reticulum of rat skeletal muscle fibers. *J Appl Physiol* 81: 731–737, 1996.
21. Bruton JD, Aydin J, Yamada T, Shabalina IG, Ivarsson N, Zhang SJ, Wada M, Tavi P, Nedergaard J, Katz A, and Westerblad H. Increased fatigue resistance linked to Ca²⁺-stimulated mitochondrial biogenesis in muscle fibres of cold-acclimated mice. *J Physiol (Lond)* 588: 4275–4288, 2010.
22. Bruton JD, Place N, Yamada T, Silva JP, Andrade FH, Dahlstedt AJ, Zhang SJ, Katz A, Larsson NG, and Westerblad H. Reactive oxygen species and fatigue-induced prolonged low-frequency force depression in skeletal muscle fibres of rats, mice and SOD2 overexpressing mice. *J Physiol (Lond)* 586: 175–184, 2008.
23. Cannon B and Nedergaard J. Brown adipose tissue: Function and physiological significance. *Physiol Rev* 84: 277–359, 2004.
24. Chance B, Sies H, and Boveris A. Hydroperoxide metabolism in mammalian organs. *Physiol Rev* 59: 527–605, 1979.
25. Cheng H, Lederer WJ, and Cannell MB. Calcium sparks: Elementary events underlying excitation-contraction coupling in heart muscle. *Science* 262: 740–744, 1993.
26. Cheong E, Tumbe V, Stoyanovsky D, and Salama G. Effects of pO₂ on the activation of skeletal muscle ryanodine receptors by NO: A cautionary note. *Cell Calcium* 38: 481–488, 2005.
27. Costantin LL. Contractile activation in skeletal muscle. *Prog Biophys Mol Biol* 29: 197–224, 1975.
28. Davies CT and White MJ. Muscle weakness following dynamic exercise in humans. *J Appl Physiol* 53: 236–241, 1982.
29. Deshmukh AS, Long YC, de Castro Barbosa T, Karlsson HK, Glund S, Zavadski WJ, Gibbs EM, Koistinen HA, Wallberg-Henriksson H, and Zierath JR. Nitric oxide increases cyclic GMP levels, AMP-activated protein kinase (AMPK) α 1-specific activity and glucose transport in human skeletal muscle. *Diabetologia* 53: 1142–1150, 2010.
30. Diaz PT, Costanza MJ, Wright VP, Julian MW, Diaz JA, and Clanton TL. Dithiothreitol improves recovery from *in vitro* diaphragm fatigue. *Med Sci Sports Exerc* 30: 421–426, 1996.
31. Dulloo AG, Young JB, and Landsberg L. Sympathetic nervous system responses to cold exposure and diet in rat skeletal muscle. *Am J Physiol* 255: E180–E188, 1988.

32. Dundon JM, Cirillo J, and Semmler JG. Low-frequency fatigue and neuromuscular performance after exercise-induced damage to elbow flexor muscles. *J Appl Physiol* 105: 1146–1155, 2008.
33. Edwards RH, Hill DK, Jones DA, and Merton PA. Fatigue of long duration in human skeletal muscle after exercise. *J Physiol (Lond)* 272: 769–778, 1977.
34. Espinosa A, Leiva A, Peña M, Müller M, Debandi A, Hidalgo C, Carrasco MA, and Jaimovich E. Myotube depolarization generates reactive oxygen species through NAD(P)H oxidase; ROS-elicited Ca^{2+} stimulates ERK, CREB, early genes. *J Cell Physiol* 209: 379–388, 2006.
35. Esposito LA, Melov S, Panov A, Cottrell BA, and Wallace DC. Mitochondrial disease in mouse results in increased oxidative stress. *Proc Natl Acad Sci USA* 96: 4820–4825, 1999.
36. Eu JP, Hare JM, Hess DT, Skaf M, Sun J, Cardenas-Navina I, Sun QA, Dewhirst M, Meissner G, and Stamler JS. Concerted regulation of skeletal muscle contractility by oxygen tension and endogenous nitric oxide. *Proc Natl Acad Sci USA* 100: 15229–15234, 2003.
37. Eu JP, Sun J, Xu L, Stamler JS, and Meissner G. The skeletal muscle calcium release channel: Coupled O_2 sensor and NO signaling functions. *Cell* 102: 499–509, 2000.
38. Franco-Obregon A and Lansman JB. Changes in mechanosensitive channel gating following mechanical stimulation in skeletal muscle myotubes from the mdx mouse. *J Physiol (Lond)* 539: 391–407, 2002.
39. Froehner SC. Just say NO to muscle degeneration? *Trends Mol Med* 8: 51–53, 2002.
40. Gervasio OL, Whitehead NP, Yeung EW, Phillips WD, and Allen DG. TRPC1 binds to caveolin-3 and is regulated by Src kinase. Role in Duchenne muscular dystrophy. *J Cell Sci* 121: 2246–2255, 2008.
41. Gillis JM. Understanding dystrophinopathies: An inventory of the structural and functional consequences of the absence of dystrophin in muscles of the mdx mouse. *J Muscle Res Cell Motil* 20: 605–625, 1999.
42. Glynn IM. Transport adenosinetriphosphatase' in electric organ. The relation between ion transport and oxidative phosphorylation. *J Physiol* 169: 452–465, 1963.
43. Golozoubova V, Cannon B, and Nedergaard J. UCP1 is essential for adaptive adrenergic nonshivering thermogenesis. *Am J Physiol Endocrinol Metab* 291: E350–E357, 2006.
44. Gordon AM, Homsher E, and Regnier M. Regulation of contraction in striated muscle. *Physiol Rev* 80: 853–924, 2000.
45. Grundtman C, Bruton J, Yamada T, Östberg T, Pisetsky DS, Harris HE, Andersson U, Lundberg IE, and Westerblad H. Effects of HMGB1 on *in vitro* responses of isolated muscle fibers and functional aspects in skeletal muscles of idiopathic inflammatory myopathies. *FASEB J* 24: 570–578, 2010.
46. Hemingway A. Shivering. *Physiol Rev* 43: 397–422, 1963.
47. Hirschfield W, Moody MR, O'Brien WE, Gregg AR, Bryan RM, Jr., and Reid MB. Nitric oxide release and contractile properties of skeletal muscles from mice deficient in type III NOS. *Am J Physiol Regul Integr Comp Physiol* 278: R95–R100, 2000.
48. Kirby DM and Thorburn DR. Approaches to finding the molecular basis of mitochondrial oxidative phosphorylation disorders. *Twin Res Hum Genet* 11: 395–411, 2008.
49. Klein MG and Schneider MF. Ca^{2+} sparks in skeletal muscle. *Prog Biophys Mol Biol* 92: 308–332, 2006.
50. Kobzik L, Stringer B, Balligand JL, Reid MB, and Stamler JS. Endothelial type nitric oxide synthase in skeletal muscle fibers: Mitochondrial relationships. *Biochem Biophys Res Commun* 211: 375–381, 1995.
51. Kujoth GC, Hiona A, Pugh TD, Someya S, Panzer K, Wohlgemuth SE, Hofer T, Seo AY, Sullivan R, Jobling WA, Morrow JD, Van Remmen H, Sedivy JM, Yamasoba T, Tanokura M, Weindruch R, Leeuwenburgh C, and Prolla TA. Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. *Science* 309: 481–484, 2005.
52. Kumar A, Takada Y, Boriek AM, and Aggarwal BB. Nuclear factor-kappaB: Its role in health and disease. *J Mol Med* 82: 434–448, 2004.
53. Larsen FJ, Weitzberg E, Lundberg JO, and Eklom B. Effects of dietary nitrate on oxygen cost during exercise. *Acta Physiol (Oxf)* 191: 59–66, 2007.
54. Larsen FJ, Weitzberg E, Lundberg JO, and Eklom B. Dietary nitrate reduces maximal oxygen consumption while maintaining work performance in maximal exercise. *Free Radic Biol Med* 48: 342–347, 2010.
55. Larsson L, Ansved T, Edström L, Gorza L, and Schiaffino S. Effects of age on physiological, immunohistochemical and biochemical properties of fast-twitch single motor units in the rat. *J Physiol (Lond)* 443: 257–275, 1991.
56. Larsson NG and Oldfors A. Mitochondrial myopathies. *Acta Physiol Scand* 171: 385–393, 2001.
57. Lundberg JO and Weitzberg E. NO-synthase independent NO generation in mammals. *Biochem Biophys Res Commun* 396: 39–45, 2010.
58. Lunde PK, Dahlstedt AJ, Bruton JD, Lännergren J, Thorén P, Sejersted OM, and Westerblad H. Contraction and intracellular Ca^{2+} handling in isolated skeletal muscle of rats with congestive heart failure. *Circ Res* 88: 1299–1305, 2001.
59. Lunde PK, Sejersted OM, Thorud HM, Tonnessen T, Henriksen UL, Christensen G, Westerblad H, and Bruton J. Effects of congestive heart failure on Ca^{2+} handling in skeletal muscle during fatigue. *Circ Res* 98: 1514–1519, 2006.
60. Lynch RE and Fridovich I. Permeation of the erythrocyte stroma by superoxide radical. *J Biol Chem* 253: 4697–4699, 1978.
61. Martin V, Millet GY, Martin A, Deley G, and Lattier G. Assessment of low-frequency fatigue with two methods of electrical stimulation. *J Appl Physiol* 97: 1923–1929, 2004.
62. Martins AS, Shkryl VM, Nowycky MC, and Shirokova N. Reactive oxygen species contribute to Ca^{2+} signals produced by osmotic stress in mouse skeletal muscle fibres. *J Physiol (Lond)* 586: 197–210, 2008.
63. McKenna MJ, Medved I, Goodman CA, Brown MJ, Bjorksten AR, Murphy KT, Petersen AC, Sostaric S, and Gong X. N-Acetylcysteine attenuates the decline in muscle Na^+ , K^+ -pump activity and delays fatigue during prolonged exercise in humans. *J Physiol (Lond)* 576: 279–288, 2006.
64. Medved I, Brown MJ, Bjorksten AR, Murphy KT, Petersen AC, Sostaric S, Gong X, and McKenna MJ. N-Acetylcysteine enhances muscle cysteine and glutathione availability and attenuates fatigue during prolonged exercise in endurance-trained individuals. *J Appl Physiol* 97: 1477–1485, 2004.
65. Michaelson LP, Shi G, Ward CW, and Rodney GG. Mitochondrial redox potential during contraction in single intact muscle fibers. *Muscle Nerve* 42: 522–529, 2010.
66. Moopanar TR and Allen DG. Reactive oxygen species reduce myofibrillar Ca^{2+} sensitivity in fatiguing mouse skeletal muscle at 37 degrees C. *J Physiol (Lond)* 564: 189–199, 2005.
67. Moopanar TR and Allen DG. The activity-induced reduction of myofibrillar Ca^{2+} sensitivity in mouse skeletal

- muscle is reversed by dithiothreitol. *J Physiol (Lond)* 571: 191–200, 2006.
68. Murphy MP. How mitochondria produce reactive oxygen species. *Biochem J* 417: 1–13, 2009.
 69. Murphy RM, Dutka TL, and Lamb GD. Hydroxyl radical and glutathione interactions alter calcium sensitivity and maximum force of the contractile apparatus in rat skeletal muscle fibres. *J Physiol (Lond)* 586: 2203–2216, 2008.
 70. Murphy RM, Verburg E, and Lamb GD. Ca^{2+} activation of diffusible and bound pools of μ -calpain in rat skeletal muscle. *J Physiol (Lond)* 576: 595–612, 2006.
 71. Okuda M, Lee HC, Kumar C, and Chance B. Comparison of the effect of a mitochondrial uncoupler, 2,4-dinitrophenol and adrenaline on oxygen radical production in the isolated perfused rat liver. *Acta Physiol Scand* 145: 159–168, 1992.
 72. Perreault CL, Gonzalez-Serratos H, Litwin SE, Sun X, Franzini-Armstrong C, and Morgan JP. Alterations in contractility and intracellular Ca^{2+} transients in isolated bundles of skeletal muscle fibers from rats with chronic heart failure. *Circ Res* 73: 405–412, 1993.
 73. Place N, Yamada T, Zhang SJ, Westerblad H, and Bruton JD. High temperature does not alter fatigability in intact mouse skeletal muscle fibres. *J Physiol (Lond)* 587: 4717–4724, 2009.
 74. Posterino GS, Cellini MA, and Lamb GD. Effects of oxidation and cytosolic redox conditions on excitation-contraction coupling in rat skeletal muscle. *J Physiol (Lond)* 547: 807–823, 2003.
 75. Pouvreau S and Jacquemond V. Nitric oxide synthase inhibition affects sarcoplasmic reticulum Ca^{2+} release in skeletal muscle fibres from mouse. *J Physiol (Lond)* 567: 815–828, 2005.
 76. Powers SK and Jackson MJ. Exercise-induced oxidative stress: Cellular mechanisms and impact on muscle force production. *Physiol Rev* 88: 1243–1276, 2008.
 77. Pye D, Palomero J, Kabayo T, and Jackson MJ. Real-time measurement of nitric oxide in single mature mouse skeletal muscle fibres during contractions. *J Physiol (Lond)* 581: 309–318, 2007.
 78. Reardon TF and Allen DG. Iron injections in mice increase skeletal muscle iron content, induce oxidative stress and reduce exercise performance. *Exp Physiol* 94: 720–730, 2009.
 79. Reardon TF and Allen DG. Time to fatigue is increased in mouse muscle at 37 degrees C; The role of iron and reactive oxygen species. *J Physiol (Lond)* 587: 4705–4716, 2009.
 80. Reid MB. Free radicals and muscle fatigue: Of ROS, canaries, and the IOC. *Free Radic Biol Med* 44: 169–179, 2008.
 81. Reid MB, Haack KE, Franchek KM, Valberg PA, Kobzik L, and West MS. Reactive oxygen in skeletal muscle. I. Intracellular oxidant kinetics and fatigue *in vitro*. *J Appl Physiol* 73: 1797–1804, 1992.
 82. Ristow M, Zarse K, Oberbach A, Klötting N, Birringer M, Kiehnopf M, Stumvoll M, Kahn CR, and Blüher M. Antioxidants prevent health-promoting effects of physical exercise in humans. *Proc Natl Acad Sci USA* 106: 8665–8670, 2009.
 83. Roberts CK, Barnard RJ, Scheck SH, and Balon TW. Exercise-stimulated glucose transport in skeletal muscle is nitric oxide dependent. *Am J Physiol* 273: E220–E225, 1997.
 84. Rossignol R, Letellier T, Malgat M, Rocher C, and Mazat JP. Tissue variation in the control of oxidative phosphorylation: Implication for mitochondrial diseases. *Biochem J* 347: 45–53, 2000.
 85. Roy RR, Zhong H, Monti RJ, Vallance KA, and Edgerton VR. Mechanical properties of the electrically silent adult rat soleus muscle. *Muscle Nerve* 26: 404–412, 2002.
 86. Salvador A, Sousa J, and Pinto RE. Hydroperoxyl, superoxide and pH gradients in the mitochondrial matrix: A theoretical assessment. *Free Radic Biol Med* 31: 1208–1215, 2001.
 87. Sandström ME, Zhang SJ, Bruton J, Silva JP, Reid MB, Westerblad H, and Katz A. Role of reactive oxygen species in contraction-mediated glucose transport in mouse skeletal muscle. *J Physiol (Lond)* 575: 251–262, 2006.
 88. Schapira AH. Mitochondrial disease. *Lancet* 368: 70–82, 2006.
 89. Sejersted OM and Sjøgaard G. Dynamics and consequences of potassium shifts in skeletal muscle and heart during exercise. *Physiol Rev* 80: 1411–1481, 2000.
 90. Shkryl VM, Martins AS, Ullrich ND, Nowycky MC, Niggli E, and Shirokova N. Reciprocal amplification of ROS and Ca^{2+} signals in stressed mdx dystrophic skeletal muscle fibers. *Pflügers Arch* 458: 915–928, 2009.
 91. Silva JP, Shabalina IG, Dufour E, Petrovic N, Backlund EC, Hultenby K, Wibom R, Nedergaard J, Cannon B, and Larsson NG. SOD2 overexpression: Enhanced mitochondrial tolerance but absence of effect on UCP activity. *EMBO J* 24: 4061–4070, 2005.
 92. Spencer T and Posterino GS. Sequential effects of GSNO and H_2O_2 on the Ca^{2+} sensitivity of the contractile apparatus of fast- and slow-twitch skeletal muscle fibers from the rat. *Am J Physiol Cell Physiol* 296: C1015–C1023, 2009.
 93. St-Pierre J, Buckingham JA, Roebuck SJ, and Brand MD. Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J Biol Chem* 277: 44784–44790, 2002.
 94. St Pierre J, Drori S, Uldry M, Silvaggi JM, Rhee J, Jager S, Handschin C, Zheng K, Lin J, Yang W, Simon DK, Bachoo R, and Spiegelman BM. Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. *Cell* 127: 397–408, 2006.
 95. Stamler JS and Meissner G. Physiology of nitric oxide in skeletal muscle. *Physiol Rev* 81: 209–237, 2001.
 96. Stary CM and Hogan MC. Impairment of Ca^{2+} release in single *Xenopus* muscle fibers fatigued at varied extracellular PO_2 . *J Appl Physiol* 88: 1743–1748, 2000.
 97. Suchyna TM and Sachs F. Mechanosensitive channel properties and membrane mechanics in mouse dystrophic myotubes. *J Physiol (Lond)* 581: 369–387, 2007.
 98. Tarnopolsky MA and Raha S. Mitochondrial myopathies: Diagnosis, exercise intolerance, and treatment options. *Med Sci Sports Exerc* 37: 2086–2093, 2005.
 99. Tidball JG and Wehling-Henricks M. The role of free radicals in the pathophysiology of muscular dystrophy. *J Appl Physiol* 102: 1677–1686, 2007.
 100. Trifunovic A, Hansson A, Wredenberg A, Rovio AT, Dufour E, Khvorostov I, Spelbrink JN, Wibom R, Jacobs HT, and Larsson NG. Somatic mtDNA mutations cause aging phenotypes without affecting reactive oxygen species production. *Proc Natl Acad Sci USA* 102: 17993–17998, 2005.
 101. Turrens JF. Mitochondrial formation of reactive oxygen species. *J Physiol (Lond)* 552: 335–344, 2003.
 102. Umar S and van der Laarse A. Nitric oxide and nitric oxide synthase isoforms in the normal, hypertrophic, and failing heart. *Mol Cell Biochem* 333: 191–201, 2010.

103. van der Poel C, Edwards JN, Macdonald WA, and Stephenson DG. Mitochondrial superoxide production in skeletal muscle fibers of the rat and decreased fiber excitability. *Am J Physiol Cell Physiol* 292: C1353–C1360, 2007.
104. Vandebrout C, Martin D, Colson-Van Schoor M, Debaix H, and Gailly P. Involvement of TRPC in the abnormal calcium influx observed in dystrophic (mdx) mouse skeletal muscle fibers. *J Cell Biol* 158: 1089–1096, 2002.
105. Wang X, Weisleder N, Collet C, Zhou J, Chu Y, Hirata Y, Zhao X, Pan Z, Brotto M, Cheng H, and Ma J. Uncontrolled calcium sparks act as a dystrophic signal for mammalian skeletal muscle. *Nat Cell Biol* 7: 525–530, 2005.
106. Ward CW, Reiken S, Marks AR, Marty I, Vassort G, and Lacampagne A. Defects in ryanodine receptor calcium release in skeletal muscle from post-myocardial infarct rats. *FASEB J* 17: 1517–1519, 2003.
107. Ward ML and Allen DG. Stretch-activated channels in the heart: Contribution to cardiac performance. In: *Mechanosensitivity of the Heart, Mechanosensitivity in Cells and Tissues*, edited by Kamkin A, Kisleva I. Springer Science; 2009. pp. 141–167.
108. Wehrens XH, Lehnart SE, Reiken S, van der Nagel R, Morales R, Sun J, Cheng Z, Deng SX, de Windt LJ, Landry DW, and Marks AR. Enhancing calstabin binding to ryanodine receptors improves cardiac and skeletal muscle function in heart failure. *Proc Natl Acad Sci USA* 102: 9607–9612, 2005.
109. Westerblad H and Allen DG. Changes of myoplasmic calcium concentration during fatigue in single mouse muscle fibers. *J Gen Physiol* 98: 615–635, 1991.
110. Whitehead NP, Yeung EW, Froehner SC, and Allen DG. NADPH oxidase is a primary source of oxidative stress and triggers stretch-induced muscle damage in the mdx mouse. *PLoS ONE* 5: e15354, 2010.
111. Wilson JR. Exercise intolerance in heart failure. Importance of skeletal muscle. *Circulation* 91: 559–561, 1995.
112. Wredenberg A, Wibom R, Wilhelmsson H, Graff C, Wiener HH, Burden SJ, Oldfors A, Westerblad H, and Larsson NG. Increased mitochondrial mass in mitochondrial myopathy mice. *Proc Natl Acad Sci USA* 99: 15066–15071, 2002.
113. Wright VP, Reiser PJ, and Clanton TL. Redox modulation of global phosphatase activity and protein phosphorylation in intact skeletal muscle. *J Physiol* 587: 5767–5781, 2009.
114. Xu Q, Dalic A, Fang L, Kiriazis H, Ritchie R, Sim K, Gao XM, Drummond G, Sarwar M, Zhang YY, Dart A, and Du XJ. Myocardial oxidative stress contributes to transgenic β_2 -adrenoceptor activation-induced cardiomyopathy and heart failure. *Br J Pharmacol* 162: 1012–1028, 2011.
115. Yamada T, Place N, Kosterina N, Östberg T, Zhang SJ, Grundtman C, Erlandsson-Harris H, Lundberg IE, Glenmark B, Bruton JD, and Westerblad H. Impaired myofibrillar function in the soleus muscle of mice with collagen-induced arthritis. *Arthritis Rheum* 60: 3280–3289, 2009.
116. Yeung EW, Whitehead NP, Suchyna TM, Gottlieb PA, Sachs F, and Allen DG. Effects of stretch-activated channel blockers on $[Ca^{2+}]_i$ and muscle damage in the mdx mouse. *J Physiol (Lond)* 562: 367–380, 2005.
117. Zhang SJ, Bruton JD, Katz A, and Westerblad H. Limited oxygen diffusion accelerates fatigue development in mouse skeletal muscle. *J Physiol (Lond)* 572: 551–559, 2006.

Address correspondence to:

Prof. Håkan Westerblad
Department of Physiology and Pharmacology
Karolinska Institutet
171 77 Stockholm
Sweden

E-mail: hakan.westerblad@ki.se

Date of first submission to ARS Central, January 31, 2011; date of acceptance, March 6, 2011.

Abbreviations Used

$[Ca^{2+}]_i$	= cytoplasmic free Ca^{2+} concentration
CAV	= caveoline
DHPR	= dihydropyridine receptor
DMD	= Duchenne muscular dystrophy
EDL	= extensor digitorum longus
eNOS	= endothelial NOS
FDB	= flexor digitorum brevis
FKBP12	= 12kDa FK506 binding protein
GSH	= reduced glutathione
GSSG	= oxidized glutathione
iNOS	= inducible NOS
LFF	= low frequency fatigue
NAC	= N-acetylcysteine
NF κ B	= nuclear factor κ B;
nNOS	= neuronal NOS
NOS	= nitric oxide synthase
NOX	= NADPH oxidase
PDE4D3	= phosphodiesterase 4D3
P_i	= inorganic phosphate ions
PLFFD	= prolonged low-frequency force depression
RNS	= reactive nitrogen species
ROS	= reactive oxygen species
RyR	= ryanodine receptor
SAC	= stretch-activated channels
SERCA	= SR Ca^{2+} ATPase
SOC	= store-operated channels
SOD	= superoxide dismutase
SR	= sarcoplasmic reticulum
SRCK	= SRC kinase
Tfam	= mitochondrial transcription factor A
UCP	= uncoupling protein

This article has been cited by:

1. Antonios Kyparos, Michalis G. Nikolaidis, Konstantina Dipla, Andreas Zafeiridis, Vassilis Paschalis, Gerasimos V. Grivas, Anastasios A. Theodorou, Maria Albani, Chrysoula Matziari, Ioannis S. Vrabas. 2012. Low-Frequency Fatigue as an Indicator of Eccentric Exercise-Induced Muscle Injury: The Role of Vitamin E. *Oxidative Medicine and Cellular Longevity* **2012**, 1-9. [[CrossRef](#)]