Muscle-derived ROS and thiol regulation in muscle fatigue

Leonardo F. Ferreira and Michael B. Reid
Department of Physiology, University of Kentucky, Lexington, Kentucky

Ferreira LF, Reid MB. Muscle-derived ROS and thiol regulation in muscle fatigue. J Appl Physiol 104: 853–860, 2008. First published November 15, 2007; doi:10.1152/japplphysiol.00953.2007.—Muscles produce oxidants, including reactive oxygen species (ROS) and reactive nitrogen species (RNS), from a variety of intracellular sources. Oxidants are detectable in muscle at low levels during rest and at higher levels during contractions. RNS depress force production but do not appear to cause fatigue of healthy muscle. In contrast, muscle-derived ROS contribute to fatigue because loss of function can be delayed by ROS-specific antioxidants. Thiol regulation appears to be important in this biology. Fatigue causes oxidation of glutathione, a thiol antioxidant in muscle fibers, and is reversed by thiol-specific reducing agents. N-acetylcysteine (NAC), a drug that supports glutathione synthesis, has been shown to lessen oxidation of cellular constituents and delay muscle fatigue. In humans, NAC pretreatment improves performance of limb and respiratory muscles during fatigue protocols and extends time to task failure during volitional exercise. These findings highlight the importance of ROS and thiol chemistry in fatigue, show the feasibility of thiol-based countermeasures, and identify new directions for mechanistic and translational research.

exercise; skeletal muscle; oxidative stress; free radicals; antioxidants; glutathione; reactive oxygen species

A ROLE FOR RNS?

Fast-type skeletal muscle fibers constitutively express the type 1 or neuronal-type NO synthase (54). The type 2 or endothelial isoform of NO synthase is associated with muscle mitochondria (55). Both isoforms may contribute to RNS production by muscle under physiological conditions, which could influence fatigue during strenuous exercise.

Muscle function is sensitive to exogenously applied RNS. NO donors have complex effects on open probability of the sarcoplasmic reticulum (SR) ryanodine-sensitive calcium-release channel (1, 41, 76, 109). NO donors also depress SR calcium-dependent ATPase activity (46), inhibit actin-myosin cross-bridge cycling (92), lessen the activity of cytochrome-c oxidase (21), disrupt calcium regulation (93), depress force (54), diminish mitochondrial oxygen utilization (21), and accelerate fatigue (4, 14, 122). Similarly, high levels of RNS secondary to inducible NO synthase upregulation cause weakness and dysfunction in inflammatory states (15, 37, 102).

The role of endogenous RNS in healthy muscle is less clear. Data from intact muscle preparations show that blockade of RNS synthesis lessens fatigue (32, 36), promotes fatigue (4, 5, 16), or has no effect (8). In part, these divergent findings reflect RNS effects on vascular regulation (4, 5). These are eliminated...
by studying isolated muscle preparations in vitro. Such experiments show that NOS blockade slows the decline of force during repetitive, neurally stimulated contractions (33, 120) due to delayed rundown of the isolated motor nerve (120). RNS effects on muscle fiber function are most evident in directly-stimulated preparations. Under standard in vitro conditions, fatigue is unaffected by NO synthase inhibition or NO scavenging (120), whereas NOS blockade promotes fatigue under hypoxic conditions (120, 121).

Overall, the role that muscle-derived RNS play in fatigue is complex and the physiological importance remains controversial. It is not clear that RNS are robust mediators of fatigue in healthy muscle. Nor are RNS an obvious target for therapeutic interventions to inhibit fatigue. Accordingly, RNS biology is not incorporated into subsequent sections of this review.

**ROS AND FATIGUE**

ROS are produced at multiple sites within skeletal muscle fibers. Under resting conditions, the mitochondrial electron transport chain (114, 115), phospholipase A2 (35, 84), and metabolism of arachidonic acid by the lipoxygenase pathway (123) appear to participate in the production of ROS. In contracting muscles, technical challenges have precluded a clear definition of the main sites of ROS generation. Both NAD(P)H oxidase (29, 44, 49, 71, 91) and phospholipase A2 (35, 84, 85) contribute to increased superoxide anion production. Other sources, including the mitochondrial electron transport chain, have not been ruled out.

Contractile activity alters the physiological milieu within skeletal muscle, predisposing muscle fibers to higher rates of oxidant production. Increased oxygen consumption lowers tissue oxygen tension during exercise (100), which favors ROS production (20, 124). Increased temperature, increased CO2 tension, and decreased pH are other exercise-associated changes that stimulate intracellular oxidant activity in muscle fibers (9).

**ANTIOXIDANTS IN MUSCLE**

Muscles are endowed with a system of antioxidant enzymes that degrade ROS. The sarcoplasm contains CuZn-superoxide dismutase (CuZn-SOD; SOD1), catalase, and glutathione peroxidase (62). The mitochondrial matrix contains MnSOD (SOD2) and glutathione peroxidase. Other thiol-based antioxidant enzyme systems, thioredoxin and thioredoxin reductase (83) and the peroxiredoxins (99), are also expressed by skeletal muscle (38, 67, 68, 101), but little is known about the localization and function of these proteins in muscle fibers.

The function of antioxidant enzymes is complimented by nonenzymatic antioxidants. Vitamin E, carotenoids, and ubiquinol are lipid soluble and localized to cell membranes. Ascorbate, lipoate, urate, and glutathione are water soluble and widely distributed within the myocyte. Glutathione is the most abundant nonprotein thiol, present at near millimolar concentrations, and is a primary determinant of the reducing environment within cells. The ratio of reduced-to-oxidized glutathione (GSH/GSSG) is an indicator of tissue redox status (103). In the context of muscle fatigue, glutathione is among the most important nonenzymatic antioxidants.

**INTRACELLULAR TARGETS OF ROS ACTION**

ROS are relatively unstable, short-lived molecules that have dose-dependent effects in biological systems. Cellular structures that are located nearest the sites of ROS production see the highest local concentrations and are most likely to be affected. Figure 1 illustrates possible sources and functional targets of ROS in skeletal muscles. Mitochondria resemble a reticulum that circumscribes the myofibrils, predominantly around the I band, and spans a large portion of the muscle cell (88). NADH oxidase is associated with the SR (118). The sarcolemma and T tubules contain NAD(P)H oxidase (44, 49), a multimeric enzyme complex that generates ROS in close proximity to ion channels of the t-tubular and the SR terminal cisternae and promotes SR calcium release (29, 44). Thus ROS sources are in close proximity to functional elements of the muscle fiber that may influence fatigue.

At the biochemical level, it is clear that ROS modify muscle components during strenuous exercise. Changes detected after exercise include lipid peroxidation (107), oxidation of mitochondrial and nuclear DNA (82), heme oxidation (116), tyrosine nitration (12, 53), protein carbonylation (12, 13), and thiol oxidation (63, 107). Among these, thiol oxidation may be the most sensitive marker of oxidative stress and is most strongly implicated in fatigue. The thiol moiety (–SH) of the amino acid cysteine can undergo reversible, covalent reactions with muscle-derived oxidants, e.g., to form disulfide bonds (28). Thiol oxidation can alter protein function by interfering with biochemical reactions or by altering protein structure and the availability of regulatory sites. Numerous proteins undergo reversible thiol-disulfide interactions. These include the ryanodine-receptor Ca2+ release channel (2, 64), SR Ca2+-ATPase (119), troponin (95), tropomyosin (117), myosin (3, 58), actin (19, 23), and Na+/K+-ATPase pump (17) among other ion transporters (59) (Fig. 1).

A subset of thiol-regulated proteins appears to mediate oxidant-induced fatigue. In a series of human studies, McKenna and associates (72–74) have shown that pretreatment with N-acetylcysteine (NAC; antioxidant and reduced thiol donor) enhances muscle cysteine and glutathione availability, preserves Na+/K+ pump activity, lessens changes in circulating potassium levels, and delays fatigue during prolonged cycling exercise. Moopanar and Allen (78) have shown that muscle fatigue involves a decrease in myofibrillar Ca2+ sensitivity that is mediated by oxidative stress and is temperature dependent. Subsequent studies by the same group (79) showed that loss of myofibrillar function was reversible by dithiothreitol (DTT), a thiol reducing agent that reverses fatigue of intact muscle fibers (26).

Fatiguing exercise or ROS exposure of sufficient intensity will disrupt calcium regulation in skeletal muscle. However, SR calcium regulation is less sensitive to oxidative stress than myofibrillar proteins. In the studies of Moopanar and Allen (78), neither the fatigue protocol nor the concentration of DTT was sufficient to alter tetanic calcium concentration; redox changes occurred at the myofibrillar level. Mishima et al. (77) found that NAC inhibits the loss of contractile function in fatigue without affecting SR function. These findings are consistent with previous reports by Andrade and coworkers (6, 7) that exogenous hydrogen peroxide depletes force at concentrations too low to alter calcium regulation.
Note that existing data on cellular mechanism, although elegant and informative, are limited in scope. The available data come from in vitro studies of fast-twitch rodent muscle subjected to narrowly defined fatigue protocols. Mechanisms observed thus far may not be generalizable to slow-twitch muscle or muscles from nonrodent species and may not apply to fatigue induced using different patterns of muscle activation. A greater diversity of research is needed to determine the physiological robustness of these initial findings.

**ANTIOXIDANTS AND FATIGUE**

The logical question is whether antioxidant treatment can delay fatigue in healthy individuals? The short answer is yes but efficacy depends on biochemistry of the antioxidant. Most antioxidants tested thus far do not enhance performance. Nutritional antioxidants such as vitamin C, vitamin E, and β-carotene diminish biochemical markers of oxidative stress during exercise but do not inhibit fatigue, especially in humans (34, 94, 106). Antioxidant enzymes that selectively buffer ROS activity are more effective. Both SOD (96, 110) and catalase (96) lessen fatigue during repetitive, electrically stimulated muscle contractions. Note that these studies were conducted using experimental animals. The relevance to humans cannot be tested because SOD, catalase, and other ROS-specific probes are not approved for human use.

Other categories of antioxidants have not been tested systematically. The nutrition and phytochemical literature contains a huge number of antioxidant compounds such as resveratrol, ellagic acid, eicosapentanoic acid, and omega-3 fatty acids that may be beneficial in fatigue but have not been evaluated experimentally. Similarly, compounds that selectively inhibit ROS sources, e.g., NAD(P)H oxidase, xanthine oxidase, and lipooxygenase, are largely unstudied in this context. Such research is warranted. The clinical literature suggests antioxidants and cyclooxygenase inhibitors can inhibit the perception of fatigue, e.g., in cancer patients (66). It would be informative to test the effects of such compounds on fatigue of muscle per se.

**THIOL REGULATION AS A TARGET**

Muscle performance is most consistently improved by antioxidants that oppose thiol oxidation (Fig. 2). Novelli and associates (86) directly administered reduced glutathione to mice and observed an increase in swimming endurance. In a human study, Lands et al. (61) fed healthy volunteers a dietary supplement that contained a whey-based cysteine donor. After 3 mo, peak power and 30-s endurance time measured during isokinetic cycle exercise were increased by 13% each. These experiments suggested that glutathione redox state influences fatigue. Consistent with this postulate, animal studies have shown that pharmacological inhibitors of glutathione metabolism accelerate muscle fatigue (80, 81).

The most robust tool for thiol-related research has been NAC, a nonspecific antioxidant and reduced thiol donor that...
supports glutathione synthesis (104, 105). NAC was the first antioxidant shown to inhibit muscle fatigue. Shindoh et al. (108) made this landmark observation during studies of rabbit diaphragm in situ. Their report provided the first compelling evidence that oxidative stress plays a causal role in fatigue and is not simply a by-product. Subsequent animal studies confirmed the robustness of this finding. NAC has been shown to inhibit fatigue by 17–40% in studies of muscles in situ (108, 112). Experiments on isolated muscle preparations yielded similar results in vitro (25, 52, 77). The latter findings established that oxidants cause fatigue via direct effects on muscle fibers and not via indirect neural or vascular effects.

NAC is approved for clinical use, which has enabled breakthrough studies in humans. NAC improved indices of fatigue by 15–60% during exercise of individual muscles or muscle groups: tibialis anterior (98), quadriceps (56), forearm (69), and diaphragm (113). Electrical-stimulation studies confirmed that NAC effects in humans were peripheral and were not related to effects on the central nervous system (98). The next step was to demonstrate whether NAC could delay fatigue during whole body exercise, e.g., cycling or running. Experiments by McKenna and associates (72–74) have answered this question. In endurance-trained individuals, NAC increases time to fatigue by ~25% during near-maximal cycling exercise (72, 74).

CAVEAT LECTOR

Thiol donors are not always effective against fatigue. For example, α-lipoic acid is an antioxidant that supports GSH synthesis (90) and diminishes exercise-induced oxidative stress (51) but does not affect muscle fatigue (22) for reasons that are not clear. In a second example, NAC failed to delay respiratory muscle failure in decerebrate rats subjected to a large inspiratory load (111). Follow-up studies showed that NAC effects were obscured by hypoxemia in the original protocol; experiments in normoxic animals confirmed that NAC improves respiratory muscle function and delayed ventilatory failure as predicted (112). Finally, NAC did not improve performance of nontrained individuals during near-maximal cycling exercise (73). The subjects in this study were unfamiliar with high-intensity activities and may have stopped exercise for reasons other than muscle fatigue (69).

BIOCHEMISTRY OF GLUTATHIONE SUPPORT

The glutathione cycle regulates thiol redox status in muscle fibers and is the biochemical gateway by which thiol donors inhibit fatigue (Fig. 3). Several strategies have been used in an attempt to support the glutathione cycle. Direct glutathione administration, either orally or by intraperitoneal injection, may not provide glutathione to muscles at levels sufficient to affect exercise performance (75, 104). An alternative option is

Fig. 2. Summary of studies showing significant beneficial effects of treatment with compounds that promote glutathione synthesis on muscle fatigue. Data show the average and range of increase in performance in different experimental paradigms. Black bar, minimum published value; gray bar, maximum published value. Absence of black bar (in situ and electrical stimulation) indicate only one study; in vitro, Refs. 25, 52, 77, and 111; in situ, Ref. 108; electrical stimulation, Ref. 98; small-muscle mass, Refs. 56, 61, 69, and 113; large-muscle mass, Refs. 72 and 74. All studies, except for Lands et al. (61), used the antioxidant N-acetylcysteine (NAC).

Fig. 3. Schematic representation of potential biochemical processes involved in the fatigue-sparing effects of compounds that increase the muscle glutathione pool. Proteins containing thiol groups (-SH) undergo oxidation creating, for example, a disulfide bond (S=O-S), which alters protein function. GSSG, oxidized glutathione; GSH, glutathione; CysH, cysteine; Cys2, cystine (oxidized form of cysteine); α-LP, α-lipoate; DHLP, dihydrolipoate. Numbered structures refer to enzymes (1, GSSG reductase; 2, γ-glutamylcysteine synthetase and GSH synthetase; 3, lipoamide dehydrogenase, GSSG reductase and thioredoxin reductase). NAC crosses the cell membrane, whereas hydrolysis of NAC results in CysH. CysH and Cys2 are transported by the alanine-serine-cysteine (ASC) and cystine/glutamate (Xc) systems, respectively. Modified from Refs. (104, 105). NAC appears to delay fatigue by increasing the intracellular pool of GSH, which helps maintain thiol groups of myofibrillar proteins in a reduced state. Note that formation of disulfide bond by interaction of thiol groups with ROS is one example out of many possible mediators and products of thiol oxidation (28).
to stimulate glutathione synthesis by administering substrates. Glutathione is composed of three amino acids: glutamate, glycine, and cysteine. Cysteine availability is the rate-limiting step in glutathione synthesis. Two compounds commonly used to increase cysteine availability and glutathione synthesis are α-lipoic acid and NAC (22, 39, 51, 104, 105). Fatigue is not affected by α-lipoic acid supplementation (22). Thus NAC has become the standard probe for experimental support of the glutathione cycle in fatiguing muscle.

NAC is the acetylated derivative of cysteine. The sulfhydryl residue of this amino acid confers antioxidant properties. NAC can react directly with a variety of biological oxidants, including ROS and RNS. NAC also supports glutathione biosynthesis by functioning as a cysteine donor. Similar to NAC, glutathione has direct antioxidant properties. Glutathione also serves as substrate for glutathione peroxidase in the enzymatic breakdown of peroxides, e.g., hydrogen peroxide and lipid hydroperoxides. Note that thiol compounds are oxidized by an array of biological mediators, including RNS and ROS (28), and that the antioxidant properties of NAC and glutathione are nonspecific. The supposition that NAC delays fatigue by a ROS-related mechanism stems from complimentary experiments using more selective interventions; ROS depletion inhibits fatigue (96, 110) whereas NO depletion generally does not (see above).

EXPERIMENTAL CONSIDERATIONS

NAC inhibits fatigue in a time- and dose-dependent manner. Plasma NAC and cysteine concentration peak within 60–120 min after ingestion (18). Although the bioavailability of orally administered NAC is low (89), Matuszczak et al. (69) observed that oral administration of 150 mg/kg NAC solution increased plasma NAC concentration by 20- to 30-fold, and plasma cysteine concentration by 10- to 15-fold. A similar dose administered via intravenous infusion yielded plasma NAC concentrations an order of magnitude higher (73, 74). Both routes of administration inhibited oxidation of circulating glutathione during exercise to a similar degree. This “ceiling effect” of NAC may be a consequence of glutathione regulation of glutathione synthesis through a negative feedback mechanism (75, 104).

Side-effects of NAC can limit experimental use. These include anaphylactoid reactions, hypotension, light-headedness, nausea, diarrhea, sleepiness, and dysphoria, among others (30, 50, 65, 69, 98). The severity and frequency of side effects depend on the dose and route of administration (50) and can be minimized by treatment with diphenhydramine (98). In healthy subjects, intravenous administration of 125 mg/kg NAC over 15 min followed by a maintenance dose of 25 mg·kg⁻¹·h⁻¹ resulted in no side effects (73) and oral administration of 150 mg/kg NAC solution caused only mild side effects (69). At much lower doses, patients with chronic obstructive pulmonary disease reported no side effects from ingesting 1,800 mg/day NAC (capsules) for 4 days (56). Finally, note that NAC bath concentrations that exceed 30 mM can depress force of unfatigued muscle in vitro (25, 52). This side effect is not seen after oral or intravenous administration of 150 mg/kg (98, 108), the maximal clinical dose, and therefore is not an issue in humans.

TOPICS FOR FUTURE RESEARCH

Fundamental questions persist about the mechanism by which muscle-derived oxidants promote fatigue. The site(s) of activity-related ROS production and the factors that regulate this signal need further study. The process by which ROS depletion contracture is also undefined. Recent studies (77–79) have shifted the attention from the SR to myofibrillar proteins and calcium sensitivity. However, the regulatory protein(s) affected by activity-related oxidants are not known. Nor is the biochemistry known. Does the function of myofibrillar proteins undergo direct redox modulation, e.g., via vicinal thiols? Or is the redox sensor located upstream, i.e., a redox-sensitive kinase or phosphatase that alters phosphorylation state of a myofibrillar protein? Answers to these questions will help guide the development of compounds to blunt ROS production and protect muscles from fatigue.

Translational studies also are needed to optimize the use of glutathione support in humans. We lack basic pharmacological information on the optimal preparation, dose, and route of NAC administration to inhibit fatigue. The capacity of NAC to lessen fatigue has not been tested outside the laboratory and this raises interesting issues. For example, the temperature dependence of muscle ROS production (9, 70, 123) suggests that NAC may have greater fatigue-sparing effects in individuals exercising in a hot environment. It is intriguing to speculate that a thiol donor might benefit patients who experience premature fatigue due to cancer, heart failure, fibromyalgia, or other diseases. However, we cannot assume that NAC is the ideal tool for such research. Novel nutritional or pharmacological interventions that support glutathione cycling may inhibit fatigue more effectively than NAC. This issue deserves systematic testing.

Commercial realities raise broader issues. NAC is sold in retail stores and over the internet as a nutritional supplement. Based on its observed benefits in acute fatigue, individuals might feel compelled to take NAC on a regular basis. This would chronically stimulate the glutathione cycle, causing long-term shifts in redox homeostasis of muscle and other tissues. ROS and RNS modulate transcription and translation (27, 31) and may be required for exercise adaptation, e.g., hypertrophy, mitochondrial biogenesis, and angiogenesis (24, 27, 31). Regular NAC consumption would oppose ROS and RNS signaling and might interfere with exercise training in young, healthy individuals. In contrast, aging and chronic inflammatory disease are associated with persistent states of oxidative stress (40). Long-term support of glutathione regulation could be beneficial under such conditions. For example, Hauer and associates (43) found that exercise training was more effective in improving strength in old individuals (>65 yr) that received NAC (1,800 mg/day) for 6 wk. Overall, long-term glutathione support is a vexed concept. We have few data on the effects of prolonged NAC supplementation, and the physiological responses cannot be predicted.

GRANTS

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