Plasticity in Skeletal, Cardiac, and Smooth Muscle
Invited Review: Redox modulation of skeletal muscle contraction: what we know and what we don’t

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Reid, Michael B. Invited Review: Redox modulation of skeletal muscle contraction: what we know and what we don’t. J Appl Physiol 90: 724–731, 2001.—Over the past decade, reactive oxygen species (ROS) and nitric oxide (NO) derivatives have been established as physiological modulators of skeletal muscle function. This mini-review addresses the roles of these molecules as endogenous regulators of muscle contraction. The article is organized in two parts. First, established concepts are briefly outlined. This section provides an overview of ROS production by muscle, antioxidant buffers that oppose ROS effects, enzymatic synthesis of NO in muscle, the effects of endogenous ROS on contractile function, and NO as a contractile modulator. Second, a selected group of unresolved topics are highlighted. These more controversial issues include putative source(s) of regulatory ROS, the relative importance of the two NO synthase isoforms constitutively coexpressed by muscle fibers, molecular mechanisms of ROS and NO action, and the physiological relevance of redox regulation. By discussing current questions, as well as the established paradigm, this article is intended to further debate and stimulate research in this area.

WHAT WE KNOW

This section briefly outlines principles that are generally accepted regarding ROS and NO effects on muscle contraction. Subsequent sections consider selected observations or questions that remain unresolved, limiting our progress in the field. By focusing on what we do not understand, rather than what we do, I hope to highlight current bottlenecks in this field, to prod scientific discussion of these issues, and perhaps to stimulate new research to resolve these conundrums.

OVER THE PAST DECADE, WE HAVE made rapid strides in our understanding of the physiological roles that reactive oxygen species (ROS) and nitric oxide (NO) derivatives play in skeletal muscle. Ample evidence of this progress can be found in the literature; publications on ROS or NO effects on limb and respiratory skeletal muscle are burgeoning (Fig. 1). Existing data indicate that ROS and NO are continually generated in the muscles of healthy individuals and that these molecules modulate processes ranging from development to metabolism and from blood flow to contractile function.

The present mini-review addresses one aspect of this biology: redox regulation of muscle contraction. Given the explosion of information on this topic, a minireview cannot adequately summarize the field. Therefore, this article has a different slant. The robust concepts, those reproduced in multiple laboratories and relatively well

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Superoxide dismutase (SOD) catalyzes the dismutating the conversion reactions that occur spontaneously. In skeletal muscle. Three ROS-specific enzymes selective degrade individual molecular species, accelerating the conversion reactions that occur spontaneously. Superoxide dismutase (SOD) catalyzes the dismutating of superoxide anions to hydrogen peroxide. Cytosolic SOD has a copper/zinc center at the catalytic site (CuZnSOD), whereas the mitochondrial isoform has a manganese center (MnSOD). In turn, hydrogen peroxide is enzymatically dehydrated to water and molecular oxygen by catalase, which is widely distributed in the cell. Glutathione peroxidase (GPX) is less substrate specific. GPX catalyzes the reduction of hydrogen peroxide to water and also reduces organic hydroperoxides to alcohol. In both reactions, reduced glutathione (GSH) functions as the electron donor. Oxidized glutathione, the by-product of this activity, is enzymatically recycled to GSH via the enzyme glutathione reductase. All of these antioxidant enzymes are expressed constitutively by skeletal muscle. Distribution tends to vary according to metabolic properties such that SOD, catalase, and GPX levels are higher in aerobically adapted fibers. The thioredoxin and glutaredoxin enzyme systems may also contribute to antioxidant activity; however, these systems are more novel, and their roles in skeletal muscle have not been well defined.

Nonenzymatic antioxidants also convey protection against ROS. GSH has direct antioxidant properties, independent of GPX, and may be the most important antioxidant in mammalian cells. In addition, GSH functions to recycle other antioxidants, e.g., vitamins E and C. GSH levels in skeletal muscle range from 0.5 to 3 mM. Antioxidant nutrients important to muscle include vitamins E, which comprises a family of lipid-soluble antioxidants, including \( \alpha \)-tocopherol. These molecules are hydrophobic and primarily function to protect muscle membranes against oxidation. In contrast, vitamin C (ascorbic acid) distributes in the aqueous phase where it directly scavenges ROS and facilitates redox cycling of vitamin E. \( \beta \)-Carotene is another lipid-soluble antioxidant that can scavenge ROS and acts to limit lipid peroxidation. Other antioxidants such as \( \alpha \)-lipoic acid and uric acid may also be important, but their actions in muscle have not been studied systematically.

**NO in muscle.** NO is generated continuously by skeletal muscle. Like ROS, NO output is low in resting muscle and is exaggerated during periods of contractile activity. NO gives rise to a second cascade of redox-active derivatives. These low-molecular-weight molecules catalyze a variety of signaling events that are thought to regulate intracellular processes and cell-cell interaction. At low levels, NO derivatives may exert antioxidant actions that oppose ROS effects. NO also interacts with superoxide anions to generate peroxynitrite, a highly toxic radical species that is second only to the hydroxyl radical in reactivity. Peroxynitrite formation promotes oxidative or nitrosative injury. NO metabolism culminates in the accumulation of nitrite and nitrate, two relatively stable derivatives that can be measured in biological systems.

NO is synthesized by NO synthase (NOS), a family of enzymes expressed by skeletal muscle fibers. The constitutive NOS isoforms are calcium dependent. They metabolize L-arginine and oxygen to generate NO; citrulline is a by-product. NOS activity varies among...
muscles. Generally, higher activities are measured in fast-twitch muscles than in slow. This reflects distribution of the neuronal-type NOS isoform (nNOS), which localizes to the sarcolemma of fast-type muscle fibers and is associated with the dystrophin complex. The endothelial-type NOS (eNOS) is associated with mitochondria; eNOS levels do not correlate with myosin heavy chain content. Individual muscle fibers may constitutively express nNOS, eNOS, or both. Inflammatory disease may stimulate expression of a third isoform, the inducible-type NOS (iNOS). NO production by iNOS is not regulated by calcium and appears to progress at a rate that is substrate limited. Accordingly, iNOS produces NO at much higher rates than the two constitutive isoforms. This exaggerates NO action on affected cells and can result in oxidative or nitrosative stress.

**ROS effects on muscle contraction.** ROS have biphasic effects on the contractile function of unfatigued skeletal muscle. The low ROS levels present under basal conditions are essential for normal force production. Selective depletion of ROS from unfatigued muscle by use of SOD or catalase causes force to fall. Conversely, modest ROS supplementation causes force to increase. This positive effect is reversed at higher ROS concentrations; force production falls in a time- and dose-dependent manner. These negative effects can be inhibited by pretreating muscles with antioxidants or can be reversed by post hoc administration of reducing agents.

The rise in ROS production that occurs during strenuous exercise contributes to the development of acute muscle fatigue. Muscle-derived ROS are generated faster than they can be buffered by endogenous antioxidants. As ROS accumulate in the working muscle, they inhibit force production. This is analogous to the drop in force that occurs when unfatigued muscle is exposed to high levels of exogenous ROS. As in unfatigued muscle, ROS effects in fatiguing muscle can be blunted by pretreatment with selected antioxidants and can be partially reversed by post hoc exposure to reducing agents.

Other factors may also increase ROS activity in muscle. Aging appears to increase the oxidant load to which muscles are exposed. Muscle injury, e.g., due to reperfusion or stretch, also results in oxidative stress that is linked to loss of function. Finally, muscles may experience oxidative stress in inflammatory disease processes, including hyperthyroid myopathy, sepsis, malignant hyperthermia, and heart failure.

The observed effects of ROS have been integrated in the homeostatic model depicted in Fig. 2. This model assumes that cytosolic redox state is a physiologically regulated variable that muscle fibers balance oxidant production against antioxidant buffering capacity. The model predicts that an intracellular redox state exists that is optimal for force generation. All else being equal, deviations from this optimum lead to loss of force. Such relationships are predicted for other homeostatically regulated variables, for example, pH, temperature, or osmolarity, and are consistent with what we know about ROS effects on contraction. Under basal conditions, unfatigued muscle appears to maintain lower oxidant levels than are optimal for contraction. Antioxidants further deplete oxidant levels, depressing force. ROS supplementation has biphasic effects; as oxidant levels are progressively increased, force first increases slightly and then falls. The oxidative stress caused by fatiguing exercise, muscle injury, or disease acts to shift the muscle rightward along this relationship, thereby depressing force. Under such conditions, antioxidants tend to increase force by returning cellular redox state toward optimal.

**NO effects on contraction.** NO depresses force production in unfatigued muscle. This action is similar to NO effects on the myocardium and on smooth muscle of the vasculature, airways, and uterus. The effect of endogenous NO can be demonstrated by pharmacological blockade of NOS activity; the force of twitch and submaximal tetanic contractions increases. Exogenous NO donors have the opposite effect, decreasing twitch and submaximal tetanic forces. Approximately one-half of the contractile inhibition induced by NO is mediated via the second messenger cyclic guanosine monophosphate (cGMP). The other half is cGMP independent and appears to reflect direct effects of NO on redox-sensitive regulatory proteins.

NO effects in fatiguing muscle are less clear. Studies in vivo show that muscle fatigue is accelerated by systemic administration of either NO donors or NOS inhibitors. These effects are attributed to loss of vascular control. In contrast, NOS inhibitors have little effect on fatigue of isolated muscles in vitro. It is therefore unlikely that endogenous NO is a primary...
mediator of the intracellular events that depress force. NO effects on fatigue, if any, are likely to be indirect.

The story is different in sepsis, peritonitis, and other inflammatory processes that stimulate iNOS expression in skeletal muscle. NO production by iNOS is limited only by substrate availability, and l-arginine is abundant in muscle. As a result, iNOS generates a profusion of NO that inhibits contractile function, contributing to muscle weakness. Such weakness is likely to reflect nonspecific nitration of regulatory proteins, which inhibits function.

WHAT WE DON'T

This section deals with unresolved issues. Given a lack of consensus, the text necessarily reflects my individual perspective and may differ from the position of other investigators in this field. Primary references and recent review articles are noted throughout. Readers are encouraged to evaluate the underlying data and draw their own conclusions.

Whither ROS? Our understanding of the factors that regulate ROS production has been quite sketchy. Over the past year, however, Supinski and colleagues have published a series of innovative papers (50–52, 68, 70) that markedly advances our knowledge in this area. The rise in intracellular ROS during repetitive muscle contraction appears to be mediated by the 14-kDa isoform of phospholipase A2, i.e., PLA2 (52), and is dependent on influx of extracellular calcium via L-type channels (70). Superoxide anion release into the vascular compartment is blunted by a decrease in stimulation frequency, a decrease in muscle length, or a rise in carbon dioxide partial pressure but is not altered by the cyclooxygenase inhibitor indomethacin (68). Mitochondria isolated from muscles of normal animals release hydrogen peroxide; this signal is stimulated by calcium and adenosine diphosphate, diminished by PLA2 inhibitors, and exaggerated in mitochondria from septic animals (50). These observations implicate mitochondria as the major source of ROS in skeletal muscle and identify PLA2 as a regulator of ROS production. This model is consistent with previous reports that mitochondria are the source of ROS in tumor necrosis factor-α-stimulated muscle (44).

A conceptual problem lies in the observations that superoxide anions produced by exercising muscle can be detected in the extracellular space (62, 80) and vascular compartment (41, 53, 68). It is unappealing to suppose that superoxide anions generated within mitochondria can be measured outside the cell. This scenario requires that electrically charged and relatively reactive radicals escape antioxidant buffering in the mitochondrial matrix and diffuse through the inner and outer mitochondrial membranes, cytosol, and sarcolemma without undergoing chemical reaction. Further diffusion across the capillary endothelium and into the vascular compartment seems even less likely.

Xanthine oxidase represents an alternative molecular source of ROS for which there is some experimental support. In skeletal muscle tissue, xanthine oxidase primarily localizes to vascular endothelial cells (45). Inhibitors of the enzyme depress superoxide anion release into the vasculature by contracting muscle (68) and partially inhibit muscle fatigue in vivo (7). However, xanthine oxidase inhibitors do not inhibit the entire superoxide anion signal during exercise (68) nor do they uniformly inhibit oxidative stress in fatiguing muscle (71). In contrast to the basal state, xanthine oxidase-derived ROS may be more important in the inflammatory responses to eccentric exercise (24) or reperfusion injury (34).

Divergences in the regulation and distribution of ROS have led to the speculation that a single source may not be responsible for all the ROS generated by exercising muscle (68). The robust observation that superoxide anions are detectable outside muscle fibers suggests that these radicals may be generated at the cell surface. It is conceivable that the sarcolemma contains one or more oxidoreductases that synthesize superoxide anions in an activity-dependent manner. However, such enzymes remain theoretical; none has yet been described.

Roles of NOS isoforms. Skeletal muscle fibers were the first cell type in which constitutive coexpression of two NOS isoforms was observed (28, 39). Subsequently, questions have persisted about the functional importance of these two enzymes in muscle cells. Recent studies of eNOS-deficient mice (26) have shed a little light on this issue. In muscles of eNOS-deficient animals, NO production and contractile function were indistinguishable from data obtained in control muscles. Such findings demonstrate that eNOS is not essential for normal contractile regulation and suggest that contraction may be regulated by the other isoform, nNOS. The association of nNOS with cytoskeletal proteins may also confer a role in mechanical signal transduction (72); nNOS-derived NO appears to mediate changes in muscle gene expression in response to mechanical stimuli. Other possible roles attributed to the nNOS isoform include modulation of glucose transport and neuromuscular transmission (5, 21). What is the role of eNOS in muscle cells? This isoform is associated with muscle mitochondria and has been suggested to modulate mitochondrial oxygen consumption (39, 78). However, experimental support for NO as a regulator of oxygen consumption in skeletal muscle (37) is largely outweighed by evidence opposing its importance (10, 27, 76). Thus the role of eNOS in skeletal muscle fibers remains enigmatic.

Mechanism(s) of contractile modulation. How is it that force production by muscle can either be increased or decreased via redox perturbations? What are the molecular targets that respond to ROS and NO signaling? These fundamental questions continue to occupy investigators in this field despite an array of putative answers, detailed below.

Calcium regulation is a prime candidate. It has long been recognized that proteins in the sarcoplasmic reticulum (SR) are sensitive to redox modulation. Among SR proteins, the ryanodine-sensitive calcium release channel has received the greatest attention as detailed
in a number of recent reviews (4, 16, 18, 22, 55, 64). Briefly, ROS and other oxidants increase the probability of channel opening, thereby promoting calcium release from SR stores. Antioxidants and reducing agents oppose or reverse ROS action on the channel. NO effects appear to be concentration dependent. At low levels, NO derivatives inhibit oxidative activation of the channel without altering the open probability. At higher levels, NO mimics the action of ROS and stimulates channel opening directly. Considerable progress has recently been made in our understanding of the protein chemistry underlying redox sensitivity of the channel. Each 565-kDa subunit of this huge tetrameric protein contains a small number of regulatory cysteines. ROS and NO oxidize thiol residues on neighboring cysteines to form disulfide bonds that induce channel opening. Disulfide formation is reversed by reducing agents, providing a mechanism for direct redox modulation of channel activity. Activity may also be modulated indirectly via redox effects that alter sensitivity of the channel to other regulatory proteins, e.g., calmodulin.

A second potential target is the SR calcium-dependent ATPase (SERCA), an enzymatic pump that scavenges calcium from the cytosol. A small number of critical sulfhydryls near the SERCA active site have been shown to modulate enzyme activity (14, 66, 79). Redox extremes inhibit pump activity. Reductive stress inhibits SERCA function via effects on regulatory sulfhydryls that must be oxidized for ATP hydrolysis to proceed (14). At the other extreme, ROS-induced oxidative stress slows the reuptake of calcium into the SR (20, 30, 43, 49, 79). Exposure to elevated NO levels also inhibits SERCA activity via thiol oxidation (74) and nitration of tyrosine residues (75). Overall, oxidation of SR proteins tends to increase cytosolic calcium levels. This effect may contribute to loss of calcium homeostasis in conditions that produce oxidative stress, e.g., muscle fatigue (8).

Muscle myofilaments are also sensitive to direct redox modification (23). Studies of isolated muscle fibers indicate that myofilament function is altered by exposure to either ROS (2) or NO donors (3, 54). Several regulatory proteins could regulate such responses. Myosin heavy chains contain multiple sulfhydryl residues that provide useful sites for protein labeling; however, thiol modification generally does not alter myosin function dramatically (13). Troponin also exhibits redox sensitivity. Oxidation of critical sulfhydryls causes cardiac troponin C conformation to resemble the calcium-bound form, activating the protein (9). Actin and tropomyosin appear less sensitive to redox modulation (46, 77), whereas the redox sensitivity of myosin light chains remains poorly defined.

ROS and NO also may act on regulatory proteins indirectly via redox-sensitive second messenger systems. The paradigm for such regulation is NO signaling through cGMP. NO activates guanylate cyclase, increasing cGMP synthesis by skeletal muscle. In part, this pathway is responsible for NO depression of skeletal muscle force (1, 38), although the target of cGMP action remains enigmatic. It is possible that ROS also exert indirect effects via reversible modulation of kinase and phosphatase activities. Such targets might enable ROS to alter the phosphorylation state of regulatory proteins, thereby altering function.

The contractile changes mediated by ROS and NO are likely to involve more than one molecular target. The diversity of redox-sensitive proteins that participate in contractile regulation argues against a single site of action. Even within an individual protein, multiple residues can be sensitive to redox perturbations and these can interact in their effects on protein function. Consider the biphasic effects of redox state on isometric force. As illustrated in Fig. 3, the factors defining this performance envelope can be modeled using as few as two hypothetical proteins. This example assumes that both proteins are essential for force production and that each is inhibited by either oxidation or reduction. Redox sensitivity of the two hypothetical proteins would therefore define the upper limits of force across the entire range of attainable redox states. Force is maximal at the cellular redox state that optimizes function of both. Under physiological conditions, oxidative or reductive limitations to force may involve functional changes in more than one protein. Sequential inhibition of individual proteins could limit force progressively, with specific proteins defining the boundary at different redox states.

What units were those? Critical readers may have noted that the abscissas of Figs. 2 and 3 have no units. This reflects a major gap in understanding. We have no units for quantifying “redox state.” It is akin to studying acid-base physiology without pH units or studying...
water balance with no measure of osmolarity. Lacking a universal scale, data on the redox sensitivity of individual proteins obtained under different laboratory conditions cannot be integrated. This makes it impossible to identify the factor that limits contraction (or other biological process) in intact cells.

**Physiological relevance.** Previous sections illustrate the diversity of intracellular mechanisms by which ROS and NO might regulate muscle contraction. The question is, Which predominates under physiological conditions? Studies of intact muscle or excised tissue provide limited information about intracellular events. In addition, the relevance of data obtained in noncellular systems is restricted by our inability to reproduce the redox environment of the cell. The predominant sources and intracellular concentrations of ROS and NO are not known. Also, we cannot duplicate the molecular localizations that appear to influence signaling events. NOS isoforms associate with specific structures in the muscle fiber, including the dystrophin complex, costomeres, the motor endplate, and mitochondria. ROS also are produced at specific intracellular sites within the cell. Finally, ROS and NO signaling may be strongly influenced by second messenger systems and antioxidant buffers that have not yet been fully quantified in skeletal muscle.

One approach to this problem is to study intact muscle fibers. ROS and NO production are preserved in such a preparation, and related signaling pathways and antioxidant buffers remain relatively unperturbed. In individual fibers, cytosolic calcium levels and force can be simultaneously measured to obtain mechanistic information. Such studies indicate that myofilaments are more sensitive than the SR to redox changes. Exogenous ROS (2) and NO donors (3) evoke large perturbations in force with little or no change in resting calcium levels, tetanic calcium transients, or calcium reuptake by the SR.

Calcium regulation can be disrupted by exposing muscle fibers to high ROS concentrations. However, such events generally result in fiber damage. The data suggest that loss of calcium homeostasis is a late-stage event, more likely to occur under pathological than physiological conditions.

**Antioxidant interventions.** A topic of long-standing interest in this field is the potential use of antioxidant supplementation to offset ROS effects on human muscles. This is most evident in studies of acute muscle fatigue. Antioxidants, including SOD, catalase, vitamin E, dimethyl sulfoxide, deferoxamine, and spintrap compounds, have been used to inhibit fatigue of excised muscles in vitro or intact muscles in situ. Such approaches established the causal role of oxidative stress in mammalian muscle fatigue. However, it was difficult to demonstrate this principle in humans. Antioxidant nutrients such as vitamin C, vitamin E, and β-carotene have been evaluated extensively as ergogenic aids and generally proved ineffective (12, 19, 33, 35, 56). This is likely due to the fact that muscle cells regulate the uptake and distribution of nutrients. Such regulation may not be easily overridden for experimental purposes. More recently, the reduced thiol donor N-acetylcysteine was used with greater success, confirming the capacity of antioxidants to inhibit human muscle fatigue (63, 73). Scientific progress in this area has been slowed by the small number of antioxidant interventions available for use in humans. This bottleneck should improve as pharmacological agents now under development eventually reach the market. An increase in the availability of antioxidant therapies will be of considerable interest to clinicians, as well as exercise physiologists, since oxidative stress is thought to limit muscle function in a variety of pathological processes.

**Adaptation to exercise.** ROS and NO production are linked to muscle activity and are known to influence gene expression (48, 67). Accordingly, there is considerable interest in the potential of these mediators to regulate muscle adaptation to exercise (17, 31). This is one of the oldest postulates in the field, dating back to the suggestion by Davies and co-workers (15) that free radicals produced in exercising muscle might stimulate mitochondrial biogenesis. A growing body of evidence supports the prospect that muscle gene expression is redox sensitive. Muscles adapt to exercise by upregulating the expression of genes for antioxidant enzymes, including SOD, catalase, and GPX (57). Heat shock proteins are also upregulated in response to exercise (65). Oxidative signaling may mediate the latter response; preliminary reports indicate it can be blocked by antioxidant supplementation (32, 36). NO derivatives also appear to influence muscle adaptation. Recent data show that NO mediates the expression of cytoskeletal proteins (vinculin, talin) in response to mechanical stimuli (72) and is essential for the addition of sarcomeres when working length is chronically increased (40).

**CONCLUSION**

This field is at an exciting stage. Research over the past decade has established the physiological importance of ROS and NO as contractile modulators in skeletal muscle. This knowledge provides a firm foundation for future investigation into the cellular and molecular mechanisms of ROS and NO action. Research in this area will be informed by the rapid progress of redox biology as a whole. ROS and NO are proving to be ubiquitous effectors of physiological function in most cell types and organ systems. New tools are rapidly evolving to measure ROS and NO in cellular systems, to assess redox effects on regulatory proteins, and to modulate ROS and NO activities experimentally. Muscle biologists will contribute to these advances and profit from them. Research into redox mechanisms will provide a clearer picture of contractile regulation and will broaden our understanding of skeletal muscle function.

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