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# REACTIVE OXYGEN SPECIES: IMPACT ON SKELETAL MUSCLE

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## Abstract

It is well established that contracting muscles produce both reactive oxygen and nitrogen species. Although the sources of oxidant production during exercise continue to be debated, growing evidence suggests that mitochondria are not the dominant source. Regardless of the sources of oxidants in contracting muscles, intense and prolonged exercise can result in oxidative damage to both proteins and lipids in the contracting myocytes. Further, oxidants regulate numerous cell signaling pathways and modulate the expression of many genes. This oxidant-mediated change in gene expression involves changes at transcriptional, mRNA stability, and signal transduction levels. Furthermore, numerous products associated with oxidant-modulated genes have been identified and include antioxidant enzymes, stress proteins, and mitochondrial electron transport proteins. Interestingly, low and physiological levels of reactive oxygen species are required for normal force production in skeletal muscle, but high levels of reactive oxygen species result in contractile dysfunction and fatigue. Ongoing research continues to explore the redox-sensitive targets in muscle that are responsible for both redox-regulation of muscle adaptation and oxidant-mediated muscle fatigue.

### Keywords

oxidative stress; exercise; reactive oxygen species; reactive nitrogen species; skeletal muscle

## Introduction

It is clear that contracting skeletal muscles generate free radicals and that prolonged and intense exercise can promote oxidative damage to active myofibers (11, 96, 108, 115, 183, 329, 333). Although high levels of free radicals can damage cellular components, physiological levels of radicals and other oxidants play an important role in cells including the control of gene expression, regulation of cell signaling pathways, and modulation of skeletal muscle force production (111, 310, 325, 326, 375).

This article will discuss the role that free radicals play in skeletal muscle function and adaptation to exercise. Our approach will be to provide a synopsis of major principles rather than a detailed analysis of individual investigations. We will begin with an overview of radical species, the concept of oxidative stress and a discussion of cellular antioxidant systems. This will be followed with a historical synopsis of research in the field of exercise-induced oxidative stress and a discussion of cellular sources of oxidants during exercise. We will also discuss the redox modulation of muscle force production/fatigue and address redox

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sensitive targets within skeletal muscle. The article will end with a discussion of redox control and skeletal muscle adaptation to exercise training. Although we will discuss a broad range of issues related to exercise-induced oxidative stress, it is impossible for a brief review to address all aspects of this expansive field of study. For topics not covered in this article, the interested reader is referred to several reviews on specific aspects of exercise and oxidative stress (9, 30, 85, 101, 103, 112, 120, 182, 184, 191, 194, 223, 257, 305, 309, 311, 312, 314, 321, 362, 368, 399).

## 1. Free radicals and other reactive oxygen species

We begin of our discussion of free radicals with an introduction to the structure and properties of common free radicals. A free radical is defned as an atom/molecule that contains one or more unpaired electrons (149). This unpaired electron makes radicals unstable and reactive, although the chemical reactivity of radicals varies widely. The fact that biological material produces free radicals was first reported in 1954 and is now widely accepted (81).

Radicals can be formed in cells by losing a single electron or from gaining an electron. These radicals can be generated as products of homolytic, heterolytic or redox reactions, producing either charged or uncharged radical species. The name reactive oxygen species (ROS) is a general term that refers to not only oxygen centered radicals but also includes non-radical but reactive derivatives of oxygen (e.g., hydrogen peroxide)(149). Further, the term reactive nitrogen species (RNS) refers to both nitrogen radicals along with other reactive molecules where the reactive center is nitrogen. Finally, the term reactive oxygen and nitrogen species (RONS) is also used as a collective name for both ROS and RNS and includes both free radical and non-free radical species.

The main free radicals formed in cells are superoxide ($O_{2\cdot}^{-}$) and nitric oxide (NO). Superoxide is generated through either incomplete reduction of oxygen in electron transport systems or as a specific product of enzymatic systems, whereas NO is generated via enzymatic reactions. Both superoxide and NO are reactive and can readily react to form a series of other ROS and RNS. A discussion of major and secondary ROS and RNS follows.

**Superoxide—**Superoxide is commonly generated as an intermediate in several biochemical reactions (148). Superoxide is negatively charged and is relatively impermeable to cell membranes. Nonetheless, it has been argued that protonation of superoxide to form the hydroperoxyl radical ($HO_{2\cdot}$) occurs at physiological pH and that $HO_{2\cdot}$ radicals can cross cell membranes (352). Compared to many free radicals, superoxide has a relatively long half-life that permits diffusion within the cell and therefore, the number of the cellular targets is increased.

Many inflammatory cells can produce significant amounts of superoxide in an effort to protect against invading organisms (126). Although superoxide is considered to be relatively unreactive compared with other radicals, it can react quickly with both NO and iron-sulphur clusters in proteins (149). As a redox active species, superoxide can reduce some biological materials (e.g. cytochrome c) and oxidize others such as ascorbate. Dismutation of superoxide, both spontaneous and catalyzed by the superoxide dismutases, provides a major source of hydrogen peroxide in cells (reaction 1).

$$\mathbf{O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2} \quad \mathbf{(reaction\ 1)}$$

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**Hydrogen peroxide—**Hydrogen peroxide ($H_2O_2$) is a non-radical ROS that can produce free radicals such as the hydroxyl radical ($OH\cdot$). Hydrogen peroxide is stable, permeable to cellular membranes and has a relatively long half-life. Hydrogen peroxide is a relatively weak oxidizing agent but at high levels is cytotoxic. Several enzyme systems can generate $H_2O_2$ including the superoxide dismutases and amino acid oxidases. Hydrogen peroxide is unable to oxidize DNA or lipids directly, but can inactivate some enzymes (149). The cytotoxicity of $H_2O_2$ is primarily due to its ability to generate hydroxyl radical through metal-catalyzed reactions, such as the Fenton reaction (reaction 2).

$$\mathbf{H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + {\cdot}OH + {}^{-}OH \ (reaction\ 2)}$$

In cellular biology this reaction is important as part of the Haber-Weiss reaction where iron (or copper) are maintained in a reduced state by superoxide and therefore, catalyze the formation of the hydroxyl radical from hydrogen peroxide (178). The overall reaction is:

$$\mathbf{O_2^{\cdot -} + H_2O_2 \xrightarrow{Metal\ catalysis} {\cdot}OH + {}^{-}OH + O_2 \ (reaction\ 2a)}$$

Specifically, the cellular reactions involved are:

$$\mathbf{O_2^{\cdot -} + Fe^{3+} \rightarrow Fe^{2+} + O_2 \ (reaction\ 2b)}$$
$$\mathbf{H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + {\cdot}OH + {}^{-}OH \ (reaction\ 2 - as\ above)}$$

or:

$$\mathbf{O_2^{\cdot -} + Cu^{2+} \rightarrow Cu^{1+} + O_2 \ (reaction\ 2c)}$$
$$\mathbf{H_2O_2 + Cu^{1+} \rightarrow Cu^{2+} + {\cdot}OH + {}^{-}OH \ (reaction\ 2d)}$$

**Hydroxyl radicals—**Hydroxyl radicals ($OH\cdot$) possess a strong oxidizing potential and are highly reactive (148). Therefore, hydroxyl radicals typically damage molecules close to their site of production. They are potentially the most damaging ROS present in biological materials and their reactivity is such that it is virtually impossible to confirm that they exist in living organisms other than through demonstration of the presence of specific products of their reactions. It follows that, because of their high reactivity, these radicals are not membrane permeable.

**Singlet oxygen—**Singlet oxygen is another ROS that has a very short half-life, but is capable of diffusion and is permeable to membranes. Singlet oxygen is an electronically excited form of oxygen but is not a radical since it does not contain unpaired electrons. Singlet oxygen exists in one of two states: 1) the first excited state ($^1\Delta gO_2$); or 2) the more reactive second excited state ($^2\Sigma gO_2$) (148). This ROS has no spin restriction and therefore the oxidizing ability is relatively high (149). Dismutation of the superoxide anion in water can lead to the formation of singlet oxygen in biological systems. Currently, it is unknown if singlet oxygen is produced in contracting skeletal muscles during exercise.

**Nitric oxide—**Nitric oxide (NO) is synthesized in many cell types from the amino acid L-arginine. This synthesis occurs through three NO synthases (NOS): 1) neuronal NOS (NOS1); endothelial NOS (NOS3); and 3) inducible NOS (NOS2). Each of these NO synthases convert L-arginine into NO and L-citrulline utilizing NADPH.

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NO can bind to transition metals and its major actions in cells are linked to its ability to bind to the ferrous ion in guanyl cyclase; this activates guanyl cyclase and promotes the formation of cyclic GMP. This binding of NO to iron is an important mechanism of NO action and also plays a key role in its inactivation and removal through binding to the iron in hemoglobin. Nitric oxide is a weak reducing agent, reacts with oxygen to form nitric dioxide, and reacts very quickly with superoxide to produce peroxynitrite (147).

**Peroxynitrite—**The reaction of superoxide with NO to produce peroxynitrite (reaction 3) occurs three times faster than the dismutation of superoxide to produce hydrogen peroxide and even faster than the reaction of NO with heme proteins. It follows that this is the primary reaction when both superoxide and NO are present. Peroxynitrite (or its protonated form ONOOH) is a strong oxidizing agent that can lead to depletion of thiol groups, DNA damage, and nitration of proteins.

$$\mathbf{O_2^{\cdot-} + NO^{\cdot} \rightarrow ONOO^{-}}\ \mathbf{(reaction\ 3)}$$

**Hyperchlorite—**Hyperchlorite is formed by the action of myeloperoxidase utilizing hydrogen peroxide (reaction 4). Hyperchlorite is commonly formed by neutrophils and can damage biomolecules by oxidizing thiols, lipids, ascorbate, and NADPH with the generation of secondary products (149). Further, when in the acid form (i.e., hypochlorous acid), this oxidant can cross cell membranes and promote fragmentation and aggregation of proteins (149).

$$\mathbf{H_2O_2 + Cl^{-} \rightarrow HOCl + {}^{-}OH}\ \mathbf{(reaction\ 4)}$$

## 2. Enzymatic and non-enzymatic antioxidant defense systems

Given the importance of maintaining redox homeostasis in cells, it is not surprising that cells, including muscle fibers, contain a network of antioxidant defense mechanisms to reduce the potential for oxidative damage during periods of increased ROS production. The term “antioxidant” has been defined in many ways but in the context of our discussion, antioxidants will be broadly defined as any substance that delays or prevents the oxidation of a substrate (149).

To prevent oxidative damage to cells a well-organized system of antioxidants act in a synchronized fashion. In this section, we provide an overview of cellular antioxidants and summarize how antioxidants function to protect muscle fibers against oxidative injury. Moreover, we will sum up the literature related to chronic exercise-induced changes in both enzymatic and non-enzymatic antioxidants in skeletal muscle.

**Biological approaches to regulate ROS—**Cells contain both enzymatic and non-enzymatic antioxidants that work together to regulate ROS. These antioxidants are strategically located throughout cellular compartments (e.g., cytoplasm and organelles). Moreover, enzymatic and nonenzymatic antioxidants exist in both the extracellular and vascular space. Collectively, these antioxidants protect muscle fibers from oxidative injury during periods of increased oxidant production (e.g., intense or prolonged exercise).

Several antioxidant systems are employed to protect against ROS-mediated damage. For example, some antioxidant enzymes (i.e., catalase) convert ROS into less active molecules. An additional antioxidant strategy is to minimize the availability of pro-oxidants such as

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iron and copper ions via metal binding proteins. Further, several low molecular weight molecules are capable of scavenging ROS species. Examples of this antioxidant strategy include endogenously synthesized substances such as glutathione, uric acid, and bilirubin and other compounds contained in the diet (e.g., ascorbic acid and vitamin E).

**Antioxidant enzymes—**The primary antioxidant enzymes in cells include superoxide dismutase, glutathione peroxidase, and catalase (Fig. 1). Other antioxidant enzymes such as peroxiredoxins, glutaredoxins, and thioredoxin reductases also contribute to cellular protection against oxidation.

**Superoxide dismutase (SOD):** Superoxide dismutase (SOD) was discovered in 1969 (262) and provides protection against superoxide radicals. Specifically, SOD dismutates superoxide radicals to form hydrogen peroxide ($H_2O_2$) and oxygen ($O_2$). Three isoforms of SOD (SOD1, SOD2, SOD3) exist in mammalian cells and each incorporates a transition metal in the active site to accomplish the catalytic breakdown of the superoxide anion (91, 389). One of the SOD isoforms is located within the extracellular space whereas the remaining SOD isoforms are found within the cell (389). SOD1 is located in both the cytosol and the mitochondrial intermembrane space and requires copper-zinc as a cofactor. SOD2 uses manganese as a cofactor and is located in the mitochondrial matrix. whereas SOD3 incorporates copper-zinc as a cofactor and is found in the extracellular space. A summary of the properties of human SOD isoenzymes is contained in Table 1.

Although superoxide radicals are not highly reactive, they can extract electrons from biological membranes or other cellular components, resulting in a chain of radical reactions. Further, superoxide radicals can participate in the formation of more reactive species such as hydroxyl radicals and peroxynitrite. Therefore, it is essential for cells to keep superoxide radicals in check. Direct evidence to support this notion is illustrated by the fact that the mutations in SOD1 in humans promotes apoptosis of spinal neurons resulting in amyotrophic lateral sclerosis (125).

The relative allocation of the SOD1 and SOD2 isoenzymes varies across tissues. In skeletal muscle fibers, 15 to 35% of the total SOD activity is in the mitochondria with the remaining 65-85% located within the cytosol (195, 306). Superoxide dismutase activity is greatest in oxidative fibers (e.g. type I fibers) compared to muscles with low mitochondrial volumes (e.g., type IIx fibers) (89, 306).

Note that SOD activity in skeletal muscle can be greatly influenced by muscle activity history. Although some reports indicate that chronic (weeks to months) endurance exercise training does not increase SOD activity in muscle (10, 156, 229, 231), most studies conclude that regular bouts of endurance exercise increases the activities of both SOD1 and SOD2 in the exercised muscles (89, 165, 233, 237, 238, 288, 306, 307, 319, 404, 405). Methodological differences in the assay of SOD activity, variations in the intensity and duration of exercise training, and differences in fiber types between the muscles investigated could contribute to the differences reported in the literature. For example, a 10-fold difference exists in the relative sensitivity between common methods used to assay SOD activity (292). It follows that SOD assay techniques with low sensitivity would fail to detect small group differences in SOD activity and could explain the failure to observe exercise-induced increases in muscle SOD activity in some studies. Moreover, the magnitude of exercise-mediated changes in skeletal muscle SOD activity increases as a function of the intensity and duration of exercise; hence, studies exercising animals at higher intensities and/or longer daily durations of exercise commonly report a larger increase in SOD activity (306, 307). Lastly, the extent of the exercise-induced increase in SOD activity in muscle fibers is largest in skeletal muscles composed of highly oxidative fibers (e.g., type I and type

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IIa) (89, 306). The explanation for this finding is likely due to the recruitment patterns of muscle fibers during submaximal endurance execise whereby highly oxidative fibers are more highly recruited compared to faster fibers (i.e., type IIx) (350).

**Glutathione peroxidase (GPX):** Five different glutathione peroxidases have been reported in mammals (GPX1-GPX5; Table 2) (59, 110). Each of these GPX enzymes catalyze the reduction of $H_2O_2$ or organic hydroperoxide (ROOH) to water ($H_2O$) and alcohol (ROH), respectively, using reduced glutathione (GSH) (49, 50, 175). Specifically, a pair of GSH molecules donate hydrogen ions and are oxidized to glutathione disulfide (GSSG):

$$2\,\mathbf{GSH} + \mathbf{H_2O_2} \rightarrow \mathbf{GSSG} + \mathbf{2H_2O}$$
$$\text{or} \quad 2\,\mathbf{GSH} + \mathbf{ROOH} \rightarrow \mathbf{GSSG} + \mathbf{ROH} + \mathbf{H_2O}$$

Although the reaction catalyzed by all GPXs is similar, individual GPXs differ in substrate specificity (i.e., different types of hydroperoxides) and cellular localization (i.e., cytosol vs. mitochondria) (60). This varying substrate specificity and cellular location between GPX isoforms may be a cellular strategy to optimize GPX's role as a cellular antioxidant enzyme. Regardless, the fact that GPX isoenzymes can reduce a variety of different hydroperoxides makes GPX an important intracellular antioxidant to protect against ROS-mediated damage (196).

To function, GPX requires a supply of GSH to provide electrons and since GSH is oxidized by GPX to form GSSG, cells must possess a path capable of regenerating GSH. The reduction of GSSG back to GSH is achieved by glutathione reductase, a flavin containing enzyme whereby NADPH provides the reducing power (269). In this regard, most cells generate NADPH by glucose-6-phosphate dehydrogenase via the pentose pathway but skeletal muscle fibers produce NADPH primarily via isocitrate dehydrogenase (232, 234, 268).

Similar to SOD, the relative amounts of GPX present in skeletal muscle fibers differ across fiber types. Specifically, oxidative fibers (i.e., type I) possess the highest GPX activity whereas rodent muscle fibers with low oxidative capacity (i.e., type IIX) contain the lowest levels of GPX (235, 306). Glutathione peroxidase is also inducible in skeletal muscles and GPX increases +20% to +177% in skeletal muscles that are actively recruited during exercise training (89, 156, 195, 208, 229, 233, 237, 238, 306, 307, 315, 364, 402, 403). It is noteworthy that endurance exercise increases both cytosolic and mitochondrial GPX activity (195). Identical to SOD, the magnitude of the exercise-induced increase in GPX in skeletal muscle is a function of both the exercise intensity and exercise duration. Indeed, compared to low intensity exercise, high intensity exercise produces a greater increase in muscle GPX activity(306).

**Catalase (CAT):** Catalase is a ubiquitous antioxidant enzyme that catalyzes the breakdown of $H_2O_2$ into $H_2O$ and $O_2$:

$$2\mathbf{H_2O_2} \rightarrow \mathbf{2\,H_2O} + \mathbf{O_2}$$

Catalase is widely distributed within the cell (219) and iron is a required co-factor attached to the active site of the enzyme (73, 219, 421). Although CAT and GPX share common substrates, compared to GPX, CAT has been reported to have a lower affinity for $H_2O_2$ at low concentrations (i.e., GPX $K_m$ = 1μM vs. CAT $K_m$ = 1mM) (371).

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Similar to both SOD and GPX, CAT activity is the highest in highly oxidative muscle fibers and lowest in fibers with low oxidative capacity (231, 237, 299, 306). Whether or not CAT expression in skeletal muscle increases in response to chronic exercise is controversial with studies reporting an increase (319, 405), decrease (231, 237, 306), or no change (306) following exercise training. The ambiguity of these findings may be due to a variety of factors including issues associated with assaying CAT activity. For more details on this topic, see Powers and Jackson (313).

**Ancillary antioxidant enzymes:** Along with the previously discussed primary antioxidant enzymes, cells also contain several additional enzymes that participate in the maintenance of redox balance. In this regard, the thioredoxin (TRX), glutaredoxin (GRX), and peroxiredoxin (PRX) systems are important contributors. The TRX antioxidant system is comprised of both TRX and thioredoxin reductase (27, 48, 175, 418). Cells contain two TRX isoforms with one isoform located in the cytosol (TRX1) whereas the second isoform is located within the mitochondrial compartment (TRX2) (48). In reference to function, TRX participates in maintaining proteins in their reduced state (27). Once oxidized, TRX is then reduced by electrons from NADPH using the enzyme thioredoxin reductase (173). In addition to the prevention of protein oxidation, other physiological functions of TRX have been described including the reduction of transcription factors, protection against oxidative stress, and prevention of apoptosis (27). Furthermore, thioredoxin reductase also contributes as an antioxidant enzyme by reducing hydroperoxides and functioning as an NADPH-dependent dehydroascorbate reductase to recycle vitamin C (27).

GRX is a thiodisulfide oxidoreductase that participates in the protection and repair of protein and non-protein thiols during oxidative stress (48, 172). Specifically, glutaredoxin protects thiols by the transfer of electrons from NADPH to disulfide substrates and this cycle is connected with glutathione and glutathione reductase (48). Human cells contain three different GRX isoforms. GRX1 is located in cytosol while GRX2 and GRX5 are located within the mitochondria (133, 245, 411).

While both TRX and GRX control the redox state of thiol groups of cysteinyl side chains, their concurrent presence in cells suggests different functions for each protein (254). For example, although TRX and GRX have some overlapping functions, GRXs are exclusively reactive with glutathione-mixed disulfides (174).

PRX and is a peroxidase that can reduce both hydroperoxides and peroxynitrate using electrons provided by physiological thiols (215, 216, 335). In mammals, cells express six isoforms of PRX (PRX I-VI) that are located throughout the cell. Specifically, PRX I, II, and VI are found in the cytosol, PRX III is located in the mitochondrion, PRXIV is located in the extracellular space, and PRX V is located within both mitochondria and peroxisomes (335). In regard to function, the molar efficiencies of PRXs are generally smaller than GPX or CAT (123). Hence, while PRXs defend against cellular oxidative stress, the importance of their antioxidant role in mammalian cells remains uncertain (123). Finally, growing evidence suggests that in addition to antioxidant properties, these peroxidases may also play a role in the regulation of $H_2O_2$ as a second messenger in receptor-mediated signaling (205, 336, 337).

At present, the effects of regular exercise on the TRX, GRX, and PRX systems in skeletal muscles remain unclear. Nevertheless, it is feasible that exercise-induced changes in one or all of these antioxidant systems could participate in protecting muscle fibers against the ROS produced during contractions. However, additional research will be required to determine whether the TRX, GRX, and PRX systems adapt to exercise training.

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**Non-enzymatic antioxidants—**Many nonenzymatic antioxidants are found in cells (e.g., GSH, uric acid, bilirubin, etc.) and a detailed discussion of this topic is beyond the scope of this chapter. Nonetheless, it is appropriate to discuss and provide a short overview of selected nonenzymatic antioxidants located in cells. For additional details on nonenzymatic antioxidants, the reader is referred to other published reviews (107, 134, 189, 190, 196, 304, 312, 313, 412).

Perhaps the most important nonenzymatic antioxidant in muscle fibers is GSH. The tripeptide GSH is the most abundant nonprotein thiol in cells (269). Glutahione is primarily synthesized in the liver and transported to tissues via the blood. Glutathione concentration in cells is in the millimolar range for most tissues but GSH varies across organs depending upon their function (269). For instance, tissues with high exposure to oxidants (e.g., liver) contain high concentrations of GSH. Similarly, the levels of GSH in skeletal muscle fibers varies across fiber types with type I fibers in rats containing 4-5 fold higher GSH content (i.e., 2-3mM) compared to type IIb fibers (i.e., ~0.5mM) (238).

As an antioxidant, GSH serves a variety of roles. First, GSH can directly react with several ROS by donating a hydrogen atom (419). Further, as previously discussed, a key antioxidant action of GSH is to act as a substrate for GPX to eliminate $H_2O_2$ and organic hydroperoxides (269). Glutathione participates in reducing other antioxidants in the cell (e.g., vitamins E and C). For example, GSH can reduce vitamin E radicals that are formed in chain-breaking reactions with lipid peroxyl radicals. Furthermore, GSH can reduce the semidehydroascorbate radical (i.e., vitamin C radical) derived from the recycling of vitamin E. Together, these GSH-mediated reactions contribute to the maintance of vitamin E and C in the reduced state (196).

Numerous investigations demonstrate that skeletal muscle fibers adapt to high intensity endurance exercise by increasing the cellular levels of GSH (237, 238, 252, 289, 364). This exercise-induced increase in GSH within muscle fibers is likely due to increased activity of a key enzyme involved in GSH synthesis (190). This enzyme, γ-glutamylcysteine synthase, is the rate limiting enzyme for GSH biosynthesis and is increased in exercise trained muscles following endurance training (227, 252, 364).

α-lipoic acid is another important non-enzymatic antioxidant that has received much experimental attention. α-lipoic acid is a naturally occurring compound and is found in a variety of foods (201, 294, 296, 342). Functionally, α-lipoic acid serves as a cofactor for α-dehydrogenase complexes and also participates in other cellular reactions (201, 294). α-lipoic acid is normally found in small quantities in animal cells and is commonly bound to an enzyme complex which limits its function as an antioxidant (296). In contrast, unbound (reduced) α-lipoic acid (dihydrolipoic acid) and several of its metabolites are valuable antioxidants (201, 296). Moreover, many studies conclude that α-lipoic acid can provide antioxidant effects by recycling vitamin C (26, 51, 67, 82, 127, 201, 296, 416, 430). Although an acute bout of exercise may increase α-lipoic acid levels in skeletal muscle, chronic exercise training does not appear to change muscle levels of α-lipoic acid (212).

Uric acid is a by-product of purine metabolism and is potentially an important low-molecular-mass antioxidant in human biological fluids (15, 159). Under normal conditions, almost all uric acid is converted to urate (374). Evidence indicates that uric acid is a useful scavenger of peroxyl radicals, hydroxyl radicals, and singlet oxygen (15, 97, 177, 367). In this regard, urate can protect against oxidative damage by acting as an electron donor (149). Also, urate can chelate iron and copper ions and prevent them from producing hydroxyl radicals via the Fenton reaction (149). The influence of exercise training on muscle urate

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levels is unknown, but it is feasible that urate could function as an antioxidant scavenger in muscle fibers during exercise (158, 159).

Another potentially important non-enzymatic cellular antioxidant is bilirubin. Bilirubin is the end product of hemoprotein breakdown as heme oxygenase cleaves the heme ring to form biliverdin. Biliverdin is then reduced by biliverdin reductase to produce bilirubin (382). Although both biliverdin and bilirubin are reductants, bilirubin is considered to be the better physiological antioxidant (41). In this regard, bilirubin possesses antioxidant potential against peroxyl radicals and can also protect cells from hydrogen peroxide (41, 383, 384). It has been proposed that the antioxidant actions of bilirubin are a result of an amplification cycle whereby bilirubin acting as an antioxidant, is itself oxidized back to biliverdin and then recycled back to bilirubin via biliverdin reductase (41). It is established that prolonged and intense exercise increases blood levels of bilirubin (119, 264). However, it is unclear if exercise training increases bilirubin content in human skeletal muscle.

The final non-enzymatic antioxidant worthy of discussion is coenzyme Q10. Coenzyme Q10 (ubiquinone) is synthesized in cells and is essential in mitochondrial electron transport (149). *In vitro* studies suggest that coenzyme Q10 can function as a non-enzymatic antioxidant by scavenging $RO_2\cdot$ radicals and inhibiting lipid peroxidation. However, the contribution of coenzyme Q10 to antioxidant defense *in vivo* remains unspecified. Although several studies have investigated the effects of coenzyme Q10 supplementation on skeletal muscle function during exercise (157, 202, 271, 341, 369, 390), the impact of endurance exercise training on coenzyme Q10 levels in muscle remains unclear (93).

**Dietary antioxidants—**In theory, several dietary antioxidants can contribute to myofiber protection against radicals and other ROS. Widely studied dietary antioxidants include vitamin E, vitamin C, and carotenoids. Vitamin E is a widely distributed in nature and it is the primary chain breaking antioxidant in cell membranes (186, 295). The generic term vitamin E refers to at least eight structural isomers of tocopherols or tocotrienols (186, 355). Among these, α-tocopherol possesses the greatest antioxidant activity (186). In addition to its direct antioxidant properties, evidence indicates that the beneficial effects of vitamin E in cells also comes from its ability to control gene expression of several proteins (34, 35, 151, 358).

Numerous reports have investigated the effects of acute and chronic exercise on vitamin E levels in skeletal muscles of rodents. Unfortunately, the findings are not consistent with some studies reporting an exercise-induced decrease in muscle vitamin E concentration (55, 136) whereas others conclude that both acute and chronic muscular activity does not change muscle vitamin E levels (84, 351, 381). Also, studies investigating the impact of chronic exercise on vitamin E levels in human skeletal muscle indicate that exercise training does not alter vitamin E levels (394, 395).

Carotenoids (e.g., β-carotene) are lipid soluble antioxidants located primarily in cell membranes. The antioxidant properties of carotenoids comes from their structural arrangement consisting of long chains of conjugated double bonds; this arrangement affords the scavenging of numerous ROS species including superoxide and peroxyl radicals (116, 228, 379). Because of their cellular location and their radical scavenging capacity, carotenoids are efficient biological antioxidants against lipid peroxidation (228). At present, the effects of chronic exercise on muscle levels of carotenoids remains unknown.

Vitamin C (ascorbic acid) is hydrophilic and therefore, can function as an antioxidant in an aqueous environment. At physiological pH, the ascorbate anion is the predominant form of vitamin C (419). Ascorbate is widely distributed in mammalian tissues and its role as an

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antioxidant is widely appreciated. In regard to its antioxidant functions, vitamin C can directly scavenge superoxide, hydroxyl, and lipid hydroperoxide radicals (68). Also, vitamin C plays an important role in the recycling of vitamin E, a process that results in the formation of a vitamin C (semiascorbyl) radical (293). Note, however, that the semiascorbyl radical can be reduced to vitamin C by NADH semiascorbyl reductase, or via cellular thiols such as glutathione or dihydrolipoic acid (295).

## 3. Definition of oxidative stress

Historically, the term oxidative stress has been defined as “a disturbance in the pro-oxidant-antioxidant balance in favor of the former” (371, 372). Although this definition has been employed for many years, the definition of oxidative stress has recently been modified to account for two different mechanistic outcomes of oxidative conditions. Indeed, because of the complexity associated with the assessment of cellular redox balance, the term “oxidative stress” defies a simple pro-oxidant versus antioxidant definition and that the description of an “oxidant stress” is only useful if the molecular details of the imbalance are known (33, 199). In an effort to refine the meaning of oxidative stress, it has been proposed that this term should be redefined as “macromolecular oxidative damage along with a disruption of redox signaling and control” (198, 199, 373). Independent of whether this new definition achieves widespread acceptance, it can be predicted that the definition of oxidative stress will undergo future modifications as the field of redox biology advances.

Regardless of how oxidative stress is defined, a persistent pro-oxidant environment in cells can damage cellular structures and modify redox-sensitive molecules. Historically, the most common approach to appraise oxidative stress in cells has been to measure the increase or decrease in a redox-sensitive molecule that responds to oxidative stress. In general, dependable biomarkers of oxidative stress contain the following qualities: 1) chemically unique and detectable; 2) increased or decreased during periods of oxidative stress; 3) possess relatively long half-lives; and 4) not impacted by other cellular processes (e.g., cell cycle, energy metabolism, etc.) (149, 316).

Several molecules that meet one or several of these criteria have been identified and procedures to detect these biomarkers have been described (98, 109, 150, 179, 199, 236, 339, 346). In this regard, oxidative stress in biological systems is often characterized by the following biomarkers: 1) increase in the formation of radicals and other oxidants; 2) decrease in small molecular weight and/or lipid soluble antioxidants; 3) disturbance in cellular redox balance; and 4) oxidative damage to cellular components (i.e., lipids, proteins, and/or DNA). Hence, biomarkers of oxidative stress typically fall into one of four categories (Fig. 2). The first category of biomarkers involves the detection of oxidants. Unfortunately, direct measurement of radical production in living cells is difficult because radicals are highly reactive and have a short half-life. Therefore, exogenous molecules such as fluorescent probes or spin traps are commonly used to measure oxidant production in cells. When added to a biological system, the probe or spin trap is converted into a unique modified radical product with a relatively long half-life that can be quantified as a measurement of oxidant production (150, 179). Nonetheless, because an increase in oxidant production does not necessarily define a pro-oxidant condition, measures of increased oxidant production alone are not definitive markers of oxidative stress. Further, a disadvantage of using probes or spin traps is that these molecules may disturb the biological system being investigated (149). Further, many spin traps and fluorescent probes are also toxic to cells (149).

A second category of oxidative stress biomarkers involves the measurement of antioxidants in tissues. In theory, a decrease in cellular antioxidants (e.g., glutathione, ascorbate, α-tocopherol, etc.) should occur during oxidative insults, and therefore, measurement of a

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decrease in a specific antioxidant has been used as a biomarker of oxidative stress. Although assessment of tissue antioxidant levels has value as a biomarker of oxidative stress, this approach does contain several weaknesses. For instance, other factors such as changes in cellular metabolism and diet can influence antioxidant levels in cells. Another concern associated with the measurement of tissue antioxidants is the possibility of auto-oxidation during sample handling resulting in antioxidant depletion in the tissue (149).

A third class of biomarkers of oxidative stress is the quantification of oxidatively modified molecules. For example, ROS attack of lipids, protein, or DNA generates uniquely oxidized molecules that can be used as biomarkers to detect oxidative stress in cells. Common measures of bio-oxidation include the measurement of protein carbonyls as an indicator of protein oxidation, assessment of isoprostanes, malondialdehyde, and 4-hydroxyl-2-nonenol as signs of lipid peroxidation, and evaluation of DNA oxidation by assaying the levels of the oxidized base, 8-hydroxy-2′-deoxyguanosine (8-OH-dG) (98, 179, 209, 236). Although it can be argued that oxidized molecules are the most important biomarker of oxidative stress, their measurement in biological systems is often difficult because oxidized molecules exist in limited amounts in cells even during periods of oxidative stress. Moreover, identical to other biomarker methodologies, measurement of oxidation products is subject to assay artifacts if tissue samples are handled improperly. A final limitation associated with the use of oxidized proteins as biomarkers of oxidative stress is as follows. Oxidized proteins can be rapidly degraded by the 20S proteasome so an increase in proteasome activity in cells would accelerate the removal of these damaged products (144, 308). It follows that comparing levels of oxidized proteins in tissues obtained from two groups of animals with different rates of protein turnover would lead to spurious conclusions about the magnitude of oxidative damage in tissues from two experimental groups.

The final class of oxidative stress biomarkers involves the measurement of cellular redox balance. One of the most frequently reported biomarkers of cellular redox balance is the ratio of GSH to GSSG. This assay is valuable because increased oxidant production results in a diminished GSH/GSSG ratio. This indicates a lower level of reduced GSH along with an increase in the levels oxidized GSH (i.e., GSSG). While this assay is conceptually simple, experimental artifacts are widespread and can occur during tissue removal and sample processing due to improper tissue handling resulting auto-oxidation of GSH and therefore decreasing the GSH/GSSG ratio (149).

To summarize, many tactics to evaluate oxidative stress in biological systems have been reported in the literature. Regrettably, each class of oxidative stress biomarkers has limitations. Consequently, while there are several measures that have been used to quantify oxidative stress, the development of a single and ideal biomarker has yet to emerge. Therefore, it appears that no one biomarker best assesses oxidative stress and that in most cases, the measurement of several biomarkers is required to confirm the presence of oxidative stress in tissues (149).

## 4. Sources of radical production during exercise

The first report of exercise-induced oxidant production in humans appeared in 1978 (108). This study used a whole body indicator of radical production (i.e., release of expired pentane) and therefore, the tissue source of ROS production during exercise was undetected. Davies et al. (96) were the first group to demonstrate that muscular exercise promotes radical production in both liver tissue and skeletal muscles. Further, these experiments were the first document that intense exercise results in lipid peroxidation in both liver and skeletal muscles. Since this early work, many studies have also demonstrated that whole body exercise results in increased lipid oxidation (e.g. (29)), DNA oxidation (e.g. (410)) and protein oxidation in the blood (e.g. (366)). However, in addition to liver and skeletal

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muscles, there are many other potential sources for ROS and RNS production during exercise and surprisingly, few studies have investigated the predominant tissues responsible for exercise-induced oxidant production. This is probably due to both the restricted access to most tissues in humans and the complex nature of exercise that involves many organ systems that are linked via the increased metabolic requirement of contracting skeletal muscles. Nonetheless, it is feasible that in some conditions other tissues such the heart, lungs or white blood cells may contribute significantly to the total body generation of ROS (313). Indeed, some authors have suggested that common metabolic changes that occur during most exercise protocols such as the increased release of catecholamines may play a role in the increased ROS generation (85), but the general consensus has been that ROS generation occurs predominantly by contracting skeletal and heart muscle. An exception to this rule could be experimental protocols that result in significant muscle damage, and in this situation, inflammatory processes may play an important role in radical production. In the following three sections, we will discuss potential sites of exercise-induced production of superoxide radicals and NO in muscle along with the sources of ROS production in muscle following damage.

**Endogenous sites for superoxide generation in skeletal muscle—**Skeletal muscle fibers can generate superoxide at several cellular locations. Interestingly, both increased muscle contractile activity and prolonged periods of muscle disuse can promote increased superoxide production within skeletal muscle fibers(313). Figure 3 illustrates some of the key locations in skeletal muscles where superoxide production occurs. A brief overview of each of these sites follows.

**Mitochondria:** Mitochondria have often been cited as the predominant source of ROS in muscle fibers (e.g. (95, 224)) and early reports suggested that 2-5% of the total oxygen consumed by mitochondria may undergo one electron reduction to produce superoxide (54, 244). More recent research has identified the major site(s) of superoxide generation within mitochondria and it is generally agreed that complexes I and III of the electron transport chain are the primary locations of mitochondrial superoxide production (44, 277). In complex I, the main site of electron leakage to oxygen appear to be the iron-sulphur clusters and in complex III it appears to be the $Q_{1o}$ semiquinone (277). Note that complex III releases superoxide to both the inner membrane space and the mitochondrial matrix (277). It is unclear if this superoxide crosses the outer mitochondrial membrane or is dismutated by SOD1 located in the mitochondrial intermembrane space. During exercise, a number of researchers have assumed that the increased ROS generation that occurs during contractile activity is directly related to the elevated oxygen consumption that occurs with increased mitochondrial activity (e.g. see (206, 397)). Nonetheless, a recent finding suggests that mitochondria may not be dominant source of ROS during exercise (185). Moreover, mitochondria produce more ROS during basal (state 4) respiration than during active state 3 respiration(16). Collectively, these findings question the notion that mitochondria are the primary source of ROS production in contracting skeletal muscle and future studies will be required to fully define the role that mitochondria play in exercise-induced production of ROS in muscle.

In regard to the rate of mitochondrial ROS production, recent evidence suggests that the upper estimate of the total fraction of mitochondrial oxygen utilized that forms superoxide is ~0.15% (377). Clearly, this value is markedly less than the original estimate of 2-5%. This low rate of superoxide production may include a role for uncoupling proteins (specifically UCP3 in skeletal muscle) as regulators of mitochondrial production of ROS (57, 58) acting to protect mitochondria against oxidative damage. Again, growing evidence indicates that mitochondria produce more ROS during state 4 (basal) respiration compared to state 3 (maximal ADP stimulated respiration) (6, 16, 104, 160, 225). This is significant because

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during aerobic contractile activity, skeletal muscle mitochondria are predominantly in state 3 and this limits their generation of ROS during contractions (104, 160, 225). Collectively, these recent findings reveal that mitochondria are not the primary source of ROS production in skeletal muscle during exercise.

Finally, recent evidence suggests that compared to type I fibers, type II skeletal muscle fibers possess unique properties that promote mitochondrial ROS production. Specifically, using an *in situ* approach to measure $H_2O_2$ release from mitochondria in permeabilized rat muscle fiber bundles, research reveals that compared to type I fibers, mitochondrial ROS emission (i.e., $H_2O_2$ release/$O_2$ consumed) was two to three fold greater in type II fibers (16). The mechanism responsible for this observation remains unclear and remains an interesting area for future work.

**Sarcoplasmic reticulum (SR):** Studies have identified NAD(P)H oxidase enzymes associated with the SR of both cardiac (75) and skeletal muscle (413). The superoxide generated by these enzymes can affect calcium release by the SR through oxidation of the ryanodine receptor (75). The skeletal muscle NAD(P)H oxidase preferentially uses NADH as substrate (413). Although it has been suggested that NAD(P)H oxidases are responsible for the superoxide release from contracting muscle (297), the muscle specific NAD(P)H oxidase described by Xia *et al* (413) is localized to the SR and therefore, this NAD(P)H oxidase seems unlikely to contribute to the extracellular release.

**Transverse tubules:** Studies also suggest that the transverse tubules of skeletal muscle contain a NAD(P)H oxidase whose activity is increased by depolarization (117, 164). Note that this enzyme contains some of the sub-units found in the NAD(P)H oxidase of phagocytic cells and appears to release superoxide to the cytosol of skeletal muscle cells.

**Sarcolemma:** Many studies indicate that skeletal muscle cells release superoxide into the extracellular space (e.g. see (258, 297, 329, 333, 434)). In this regard, all cells contain plasma membrane redox systems capable of producing electron transfer across the plasma membrane. Although the sources of this electron transfer continues to be investigated, evidence indicates that an NAD(P)H oxidase complex is constitutively expressed in diaphragm and limb muscles of the rat and is localized in the sarcolemma (187). The enzyme contains four of the subunits that are found in the enzyme in phagocytic cells ($gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$), all of which were associated with the cell membranes (239). It is unknown if this enzyme complex predominantly releases superoxide to the inside or the outside of the plasma membrane (187) and whether or not this enzyme is similar to the NAD(P)H oxidase located in the transverse tubules (117, 164) is currently unknown.

In addition to NAD(P)H oxidases, there are other plasma membrane redox systems that are capable of transferring electrons from intracellular reductants to appropriate extracellular electron acceptors (353). Nonetheless, to date, no such system has been described in skeletal muscle. However, in other cell types, Morré (276) has described external NADH oxidase (ECTO-NOX) proteins that exhibit a hydroquinone (NADH) oxidase activity and a protein disulphide-thiol exchange activity. These systems appear to accept electrons from the hydroquinones of the plasma membrane and can reduce a number of non-physiological (e.g. ferricyanide and WST-1) and physiological (e.g. protein thiols or oxygen) electron acceptors outside the cell although oxygen is likely to be a major acceptor *in vivo* (100). Transfer of electrons from cytosolic NAD(P)H to the plasma membranes has been proposed to occur through either NADH-cytochrome $b_5$ oxidoreductase or NAD(P)H quinone oxidoreductase (NQO1) (100). Thus, through a series of linked steps, intracellular NAD(P)H can act as substrate for superoxide generation on the cell surface. The relevance of these processes to

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skeletal muscle contractions is unclear, but it is possible that these systems are activated during contractile activity because the characteristics of the release of superoxide from skeletal muscle are compatible with this type of system.

**$PLA_2$-dependent processes:** Phospholipase $A_2$ ($PLA_2$) is an enzyme that cleaves membrane phospholipids to release arachidonic acid which is a substrate for ROS-generating enzyme systems such as the lipoxygenases (433). Also, activation of $PLA_2$ can stimulate NAD(P)H oxidases (427) and increased $PLA_2$ activity has been reported to stimulate ROS generation in muscle mitochondria (281), cytosol (140) and release ROS into the extracellular space (433). Both calcium-dependent and calcium-independent forms of $PLA_2$ are involved in muscle ROS generation. Specifically, it has been suggested that the calcium-independent enzymes ($iPLA_2$) contribute to cytosolic oxidant production in skeletal muscle cells (140), while a 14kDa calcium-dependent isoform ($sPLA_2$) located within mitochondria has been reported to stimulate intracellular ROS generation during contractile activity (282). In non-muscle cells, activity of another calcium-dependent $PLA_2$ isoform, $PLA_2$ cytosolic ($cPLA_2$) has been linked to ROS generation (278). Reid and colleagues (140) hypothesized that the calcium-independent $PLA_2$ is a major contributor to ROS production during resting (i.e., noncontractile) conditions whereas during contractions the calcium-dependent $PLA_2$ is activated and stimulates ROS production.

**Xanthine oxidase (XO):** Numerous studies suggest XO can promote superoxide generation in skeletal muscle (138, 409). At present, the evidence to support this supposition is primarily based on the effects of the XO inhibitors, allopurinol or oxypurinol (e.g. see (139, 161)). Although rat skeletal muscles contain significant levels of XO (200), human skeletal muscle cells *per se* appear to possess low amounts of xanthine dehydrogenase or oxidase (156). Clearly, additional research is required to determine the role that XO plays in exercise-induced ROS production.

**Endogenous sites for NO production—**NO is generated continuously by skeletal muscle and this production is increased by contractions (40, 221). Skeletal muscle normally expresses the neuronal (nNOS) and the endothelial (eNOS) isoforms of NO synthase (NOS). nNOS is strongly expressed in fast-twitch muscle fibers and localized to the muscle sarcolemma where it is associated with the dystrophin-glycoprotein complex (DGC). In contrast, eNOS is localized to the muscle mitochondria (222). iNOS is also expressed in skeletal muscle in some inflammatory conditions, but it does not play a significant role in normal muscle (380). Analysis of myotubes in culture has confirmed that skeletal muscle cells release increased amounts of NO during contractile activity (297). In this regard, nNOS appears to be the prime source of the NO released from skeletal muscle (166). Further, passive stretching of muscle has also been shown to increase NO release from rat skeletal muscle *in vitro* (393) and to increase nNOS expression.

**Generation of ROS in muscle following damage—**Non-muscle sources of ROS (e.g., phagocytic white cells) can play an important role in disturbing muscle redox state following events that lead to tissue damage. For example, significant damage to muscle fibres is accompanied by invasion of macrophages and other phagocytic cells (e.g. see (259)) and although this process appears to be essential for effective fiber regeneration to occur, it also involves the release of ROS from the phagocytic cells (249). Unfortunately, the magnitude of this ROS release can damage previously undamaged muscle cells and therefore, additional fiber injury occurs (422).

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## 5. Radicals and muscular fatigue

As discussed earlier, numerous sources of ROS and RNS production exist in skeletal muscle and it is now clear that muscle fibers continuously produce ROS and RNS. This section will discuss the impact of reactive species on skeletal muscle force production. Because of the large volume of literature on this topic, it is vital to compress the material presented in this segment. For additional details about the impact of reactive species on muscle force production readers should consult the following reports (12, 76, 251, 260, 313, 324-326, 328, 375, 380, 386, 387).

**Influence of NO on muscle force production—**As presented earlier, NO is produced in mammalian cells via the enzymatic action of a family of NO synthases that are expressed in all muscle fiber types (221, 222, 343). It is established that isolated skeletal muscle fibers produce low levels of NO at rest and that NO production increases during muscle contractions (221, 318, 327). Further, evidence indicates that endogenous production of NO can modulate skeletal muscle force production (324, 326, 327, 331). Indeed, numerous investigations reveal that force production during submaximal tetanic contractions is depressed by NO donors (8, 20, 222, 300, 338) whereas muscle force production is increased by NOS inhibitors and NO scavengers (20, 221). However, NO does not influence maximal tetanic force production. Therefore, endogenous NO production shifts the force-frequency curve to the right without decreasing maximal tetanic force production. Further, because NOS3 deficiency does not alter muscle function, it appears that the influence of NO on skeletal muscle contractile properties is mediated by NOS1 (166).

**ROS modulation of muscle force production—**Similar to NO and muscle force production, ROS also influence muscle force production. For example, the low levels of ROS present in skeletal muscle during resting conditions (i.e., unfatigued) are essential for normal force production (325, 326, 387). Indeed, antioxidant-mediated scavenging of ROS from skeletal muscle fibers results in a depression of muscle force generation (83, 330-332). In contrast, a modest increase in ROS in skeletal muscle fibers results in an increase in force production (330). Note, however, that the positive impact of ROS on muscle force production is reversed at higher ROS concentrations as force production decreases in both a time and dose-dependent manner (330).

Although numerous investigators have contributed to our understanding of the influence of ROS on muscle force production, Reid and colleagues (330) were the first to develop a theoretical model to explain the relationship between redox balance and muscle force production. This model is illustrated in Fig. 4. This model assumes that the muscle redox state in muscle fibers is a physiologically regulated variable that is balanced by matching the rates of ROS production with cellular antioxidant buffering capacity. The Reid et al. (325, 330) model predicts that an optimal redox state exists whereby conditions are ideal for muscle force production. This model also predicts that a deviation from the optimal redox balance leads to a loss of muscle force production.

**Radicals contribute to exercise-induced muscular fatigue—**The observation that redox disturbances in skeletal muscle can significantly decrease force production has stimulated interest in the notion that ROS contribute to muscular fatigue during prolonged exercise. In this regard, the contribution of oxidants to muscle fatigue has been studied using a variety of animal models via *in vitro* and *in situ* muscle preparations and during exercise in intact animals. Further, studies have determined the role of antioxidant supplementation in delaying muscle fatigue during exercise in humans. In the forthcoming sections, we review the data indicating that ROS contribute to muscular fatigue. For the purpose of this

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discussion, we have defined muscle fatigue as “an exercise-induced decrease in muscle force generation” (130, 408).

**Redox disturbances and skeletal muscle fatigue: *in vitro* and *in situ* animal studies—**As discussed throughout this chapter, it is clear that production of ROS increases in contracting skeletal muscle and animal studies provide convincing evidence that ROS contribute to muscle fatigue induced by prolonged muscular contractions (313, 324). In this regard, an early study demonstrated that the ROS scavenger N-acetylcysteine delayed muscle fatigue in an *in situ* diaphragm muscle preparation (370). Since this initial report, many investigations using both *in vitro* and *in situ* preparations have explored the role that redox disturbances play in the development of muscular fatigue and a majority of studies have concluded that scavenging ROS via enzymatic and nonenzymatic antioxidants delays muscle fatigue during submaximal contractions (24, 42, 214, 275, 329, 330, 388). On the contrary, antioxidants do not appear to be effective in delaying fatigue when muscle contractions are near maximum (255, 329). Finally, studies that probe the impact of antioxidants on muscle function during recovery from fatiguing contractions are inconsistent with one report indicating a faster recovery of force production (106) whereas others fail to observe a faster recovery time (214, 329, 333).

Compared to the large volume of literature regarding the impact of ROS on muscle fatigue, evidence that NO production directly contributes to the development of muscle fatigue is sparse. In theory, because of the effects of NO on muscle proteins involved in force production (i.e., contractile and calcium handling proteins), it could be predicted that NO should contribute to muscle fatigue (375). However, the current literature does not provide strong support for the concept that NO production contributes to muscular fatigue. For example, although one report suggests that NOS inhibition does delay fatigue in electrically stimulated diaphragm muscle (128), other studies conclude that NO does not directly influence skeletal muscle fatigue resulting from prolonged submaximal contractions (74, 221). Further, in perfused *in situ* muscle preparations, NOS blockade appears to accelerate muscle fatigue due a dysregulation of blood flow (8, 14). Together, these reports do not support an important role for NO in mediating contraction-induced muscle fatigue.

**Redox disturbances and skeletal muscle fatigue: *in vivo* studies—**Many studies have reported that supplementation with common dietary antioxidant vitamins does not improve endurance exercise tolerance in humans (32, 64, 129, 309, 340, 376). These results suggest that dietary antioxidants such as vitamin E and C do not improve human exercise performance (77, 207, 309). Nonetheless, numerous animal studies using a variety of pharmacological antioxidants have clearly demonstrated that exercise-induced production of ROS in skeletal muscle contributes to muscle fatigue. For example, numerous animal studies using both spin traps and other ROS scavengers have reported that scavenging ROS in muscle during exercise delays the onset of muscular fatigue (63, 99, 121, 285). Similarly, an increasing number of studies suggest that administration of the antioxidant N-acetylcysteine (NAC) delays muscle fatigue during submaximal exercise in humans (255, 263, 266, 267, 334, 396). As an antioxidant, NAC acts as a reduced thiol donor that supports glutathione resynthesis and may exhibit other antioxidant properties (102, 211). Table 3 highlights some of the studies indicating that NAC administration delays muscular fatigue during submaximal exercise tasks including electrically stimulated human limb muscle (334), breathing against an inspiratory load (396), cycle exercise (263, 266, 267), and repetitive handgrip contractions (255). The reported improvements in exercise performance ranged from 15 to 62 percent (Table 3). Similar to these positive findings in humans, NAC administration has also been shown to delay fatigue in animal models using both *in vitro* and *in situ* muscle preparations (105, 214, 370, 388). Note, however, that NAC does not appear

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to retard muscle fatigue during high intensity exercise (i.e. near or above $VO_2$ max) (105, 255, 265).

## 6. Redox sensitive sites in skeletal muscle

It is clear that many redox-sensitive sites exist within skeletal muscle. This section will describe these sites beginning with those that are important in the actions of ROS and NO in regulating force production in skeletal muscle. This will be followed by a discussion of how ROS and NO modify cellular signaling pathways and coordinate gene expression of muscle proteins.

**Redox sensitive targets in skeletal muscle that impact force production—** There are numerous mechanisms by which NO and ROS can influence skeletal muscle force production. Fig. 5 illustrates putative molecular targets and processes involved in skeletal muscle contraction that are influenced by NO and/or ROS. In addition to affecting calcium regulation, and/or myofilament function, NO can theoretically influence muscle force production by lowering ATP production. Specifically, the NOS3 isoform is closely associated with skeletal muscle mitochondria and NO production from this site could limit mitochondrial ATP production via inhibition of cytochrome oxidase (61, 62) or other mitochondrial enzymes (52). NO derivatives also inhibit the activity of both glyceraldehyde-3-phosphate dehydrogenase (272, 273) and creatine kinase (143) that could also limit ATP production. Nonetheless, whether or not NO production negatively impacts ATP production in muscle during repetitive contractions remains an open question.

Several studies have determined whether NO influences force production via its second messenger, cGMP, or through non-cGMP dependent processes. Both muscle contractions and NO donors increase cGMP concentration in skeletal muscle and inhibition of NOS depresses cGMP levels (230). Immunohistochemical studies indicate that cGMP levels is not fiber type specific and that cGMP is found near the subsarcolemma and in close proximity to NOS1 (291). Although it is not universally accepted (43, 56), several authors argue that interventions that increase cGMP signaling (e.g., NO donors, cGMP analogs, etc.) reduce skeletal muscle force production (1, 46, 221, 250, 251, 327). Similarly, contractile force can be increased by treatments that decrease cGMP levels (e.g. NOS inhibitors and guanylate cyclase inhibitors) (221). Nonetheless, the magnitude of the cGMP-dependent changes in muscle contraction is consistently smaller than the cumulative effect of NO on muscle force production (326). In this regard, it has been suggested that approximately 50% of the NO-induced depression in muscle force production is mediated via cGMP action (325). This observation leads to the conclusion that NO-induced depression of muscle force production occurs via both a cGMP-dependent and cGMP-independent mechanism (327).

**Redox regulation of sarcoplasmic reticulum calcium—**The redox influence on sarcoplasmic reticulum (SR) function has been investigated in both cardiac and skeletal muscle (2, 3, 23, 253, 348, 413, 420, 431). The ryanodine receptor (RyR) calcium release channel in the SR is a large homotetramer composed of four 565-kDa subunits. Each of these subunits contain sulfhydryl groups that are sensitive to redox modulation (287, 385, 423, 432) and is activated by direct exposure to ROS and by changes in the cellular redox status of key thiols such as glutathione (431). In situ, the RyR appears to be in close association with the NADP(H) oxidase(s) situated within the SR and locally produced superoxide appears to be the major ROS that influences this channel (413).

Exposure of skeletal muscle fibers and isolated SR proteins to exogenous ROS indicates that regulatory proteins within the sarcoplasmic reticulum calcium release channels are oxidatively modified (2, 23, 301). In isolated *in vitro* preparations, high levels of ROS

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augments the opening the ryanodine-sensitive calcium release channel resulting in increased calcium release from the SR (23, 118). In contrast, reducing agents and antioxidants have the opposite effect and prevent calcium release from the SR (330). Although ROS exposure can influence the release of calcium from the SR in isolated systems, research suggests that the redox potential across the SR does not influence excitation-contraction coupling in skinned rat muscle fibers (303). Further, oxidation of the calcium release channels in the SR and does not influence cytosolic calcium levels in skeletal muscle during twitch contractions (303). More specifically, Andrade et al. have investigated this issue using intact single muscle fibers and their data indicate that exposure of fibers to ROS results in an increase in cytoplasmic calcium levels in resting (i.e., non-contracting) muscle (18, 21). This increase in resting calcium levels in fibers appears to be due to a reduced rate of SR calcium uptake rather than an increase in calcium release form the SR. Further, two independent groups have reported that exposure of muscle fibers to ROS has little impact on SR calcium release during submaximal tetanic contractions (18, 21). Together, these results suggest that exposure of muscle fibers to ROS does not have a major impact on cytoplasmic calcium transients during both twitch and tetanic muscular contractions.

Interestingly, ROS also inhibit SR calcium ATPase (SERCA) activity (146, 414), and paradoxically, reductive stress also inhibits SERCA function by reducing sulfhydryl groups on SERCA that are required for ATP hydrolysis (92). Hence, optimal SERCA activity is critically dependent upon an optimal redox balance. In this regard, SERCA1 and SERCA2 proteins contain critical thiol residues that are influenced by the cellular redox status. ROS can inhibit SERCA function by both interfering with the ATP binding site and by uncoupling calcium uptake from ATP hydrolysis (356, 414). It is noteworthy that some variability exists in the responses of different SERCA isoforms to inhibition by ROS (45).

NO also has a significant effect on SR calcium handling although the specific target of NO/cGMP signaling remains unknown. Inhibition of soluble cGMP in isolated single muscle fibers results in increased cytosolic calcium transients during a tetanic contraction (20). A potential mechanism for the influence of NO on both skeletal and cardiac muscle is through the inhibition of phospholamban, a protein that retards SERCA activity (345, 424, 426). Nonetheless, phospholamban is expressed only in cardiac and type I skeletal muscle fibers and therefore this mechanism cannot explain the NO/cGMP inhibition of muscle force production in fast type II fibers (280). As discussed previously, SERCA contain a small number of sulfhydryl groups that control SERCA activity (406, 407) and exposure to high levels of NO inhibits SERCA activity via both thiol oxidation (407) and nitration of tyrosine residues (406). Overall, NO-mediated changes in SERCA sulfhydryl groups results in reduced calcium uptake into the SR and increased cytosolic calcium concentrations.

NO donors have a complex effect of the RyR (163) which is manifested by a biphasic response to NO (349, 380). At low levels, NO prevents oxidative activation of the RyR by acting as an antioxidant (i.e., scavenging superoxide) without directly altering channel function (152). In contrast, prolonged exposure to high levels of NO inhibits ryanodine activity and prevents calcium release (152, 162).

**ROS and NO impact on myofibrillar proteins—**Although it is established that both ROS and NO can modify SR calcium handling, growing evidence also indicates that ROS can also influence myofilaments structure and function. In this regard, Reid et al. have studied the pathways that are involved in control of skeletal muscle force production and predict that the most likely explanation resides in ROS influencing the calcium sensitivity of myofilaments (for a review see (375). They based this prediction on observations indicating that: 1) high levels of ROS exposure can alter myofilament structure (65, 66, 90, 154); 2) that exogenous ROS diminish calcium sensitivity of myofilaments (19, 21); 3) ROS

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exposure influences cross-bridge kinetics (21); and 4) that muscle-derived ROS can potentiate the calcium insensitivity of muscle during fatiguing contractions (275).

Several myofilament proteins can be oxidized during ROS exposure and myofilament function is impaired by prolonged exposure to high levels of oxidants (80, 415). For example, myosin heavy chain molecules are targets of ROS and oxidation of myosin heavy chain proteins results in impaired in myosin function (80, 146, 415). Troponin C is also sensitive to ROS-mediated oxidation and oxidation can impact the normal function of this protein (203, 302).

**Levels of ROS required to activate redox sensitive targets—**You will note that throughout this chapter we have used terms such as “low” or “high” levels of ROS in our discussions about the influence of ROS on muscle force production and other cellular signaling processes. These qualitative descriptions of cellular ROS levels lack scientific rigor and are unsatisfying to the investigator designing experiments to examine redox signaling. However, we currently lack reliable data to quantify ROS within cells, even where specific ROS are relatively stable (e.g., hydrogen peroxide). Few studies of the hydrogen peroxide content of body fluids have been performed, but microdialysis studies reveal that the skeletal muscle interstitial hydrogen peroxide concentration ranges from 10-15μM (400). Non-muscle cells have been shown to respond to concentrations of hydrogen peroxide of this order (15μM) with adaptive changes in redox-sensitive gene expression (213). Muscle fibers have been reported to activate adaptive changes in gene expression when exposed to 25-100μM extracellular hydrogen peroxide and muscle fibers lose viability when exposed to >1mM hydrogen peroxide (256, 259, 261). In regard to experiments that expose cells to exogenous hydrogen peroxide, Antunes and Cadenas (22) have examined the concentration difference across membranes when hydrogen peroxide is used in this experimental context and calculated that an ~7 fold drop occurs across biological membranes. In this regard, concentrations of hydrogen peroxide of 25-100 μM have been used to mimic the effects of the rise in ROS levels observed during contractions and/or during periods of prolonged muscle inactivity (261, 317). The fact that this range of hydrogen peroxide concentrations has been shown increase gene expression implies that an intracellular concentration of 2-15μM hydrogen peroxide may be sufficient to induce adaptive changes in gene expression (261).

## 7. ROS control of cellular signalling pathways and gene expression

**Physiological significance—**Maintenance of oxidative –antioxidant homeostasis is critical for the normal function and survival of all aerobic organisms (149). Oxygen consumption in resting muscle is relatively low but increases by several fold during rigorous contractions. This metabolic demand not only increases energy needs but also elicits a disturbance to intracellular oxidative –antioxidant homeostasis. During the past 25 years, increasing evidence indicates that skeletal muscle is remarkably plastic in terms of upregulating antioxidant defense in response to oxidative stress (192). This plasticity is due largely to the evolutionally conserved pathways in muscle cells that are redox-sensitive (13). It is now clear that in healthy skeletal muscle, contraction-induced generation of ROS can stimulate a host of events that inhibit ROS production and/or facilitate ROS removal. The majority requires *de novo* protein synthesis through transcription, translation and transport. These cellular events have been abstractly termed “signal transduction” or simply “signaling”. It is noteworthy that signaling pathways do not operate separately but often interact with each other to process and transfer signals, termed “crosstalk”. Whereas most of the antioxidants are encoded by nuclear genes, their post-translational modification, assembly and cross-membrane transportation involve multiple organelles and cellular compartments. Finally, muscle inflammation, mitochondrial biogenesis and expression of

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uncoupling proteins, though being unique biological events controlled by specific mechanisms, are highly relevant and regulated by redox signaling (347, 378, 391). Due to the importance of the topic, the definition of oxidative stress has recently been postulated to be redefined as a *disruption of redox signaling and control* (199).

**Redox-sensitive signaling pathways—**A redox-sensitive signaling pathway uses ROS to influence cell signaling pathways to stimulate growth, differentiation, proliferation, apoptosis, and other cellular processes (270). Most antioxidant enzymes contain redox-sensitive gene regulatory sequences in their promoter and/or intron regions that can interact with transcription factors to trigger upregulation of gene expression (13). Intracellular redox status, controlled mainly by GSH and thioredoxin (Trx), also plays an important role in the various stages of signal transduction (365). Among the main pathways are nuclear factor (NF) κB, the phosphoinositide 3-kinase ($PI_3K$)/Akt pathway, p53, heat shock proteins and mitogen activated protein kinase (MAPK) cascades, among which NFκB and MAPK are often considered the most critical for the cells to cope with oxidative stress (38). Fig. 6 illustrates the various redox-sensitive signaling pathways and their role in antioxidant gene expression in response to exercise, inflammation, growth factors, infection, stress and ischemia-reperfusion (I-R). These pathways will be discussed in detail in the following segments.

**NFκB:** The NFκB/Rel transcription factors are present in the cytoplasm in an inactive state, conjugated with the inhibitory IκB proteins (270). NFκB is activated by a variety of external stimulants, such as $H_2O_2$, pro-inflammatory cytokines (TNFα, IL-1, IL-6), lipopolysaccharide (LPS) and phorbol esters. These signals are believed to activate IκB kinase (IKK), due to activation of either double strand-RNA activated protein kinase (PKR), protein kinase C (PKC) (122, 240), or NFκB-inducing kinase (NIK), a family member of MAPK kinase kinase (MEK) (132, 241). Phosphorylation of ser-32 and ser-36 of IκBα (or ser-19 and -23 of IκBβ) leads to IκB ubiquitination and proteolytic degradation by the 26S proteasome. IκB dissociation releases the p50/p65 subunits of NFκB to translocate into the nucleus and bind the DNA sequence of the gene targets. Proteins and enzymes that require consensus binding of NFκB in the promoter include SOD2, GCS, inducible NOS (iNOS), cyclooxygenase-2 (COX-2), vascular cell adhesion molecule-1 (VCAM-1), and several cytokines. These genes are involved in a wide variety of biological functions such as antioxidant defense, inflammation, immunity, and anti-apoptosis (69, 131).

**MAPK:** MAPK has a complicated hierarchy including extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK) and $p38^{MAPK}$, which are regulated by their respective upstream kinases (MEK/MKK) (13). The primary stimulators of MAPK pathway include growth factors (GF), inflammatory cytokines (e.g., TNFα, IL-1), LPS phorbol esters, and ROS. In the better known ERK and JNK pathways, Ras plays an important role in the initial phase of MAPK activation. After receptor binding by GFs, membrane-associated proteins Sos, Grb-2 and Shc undergo conformational changes leading to the activation of Ras. TNFα and IL-1 bypass the Ras pathway by increasing cytosolic concentration of $H_2O_2$, which activates several isoforms of PKC (122). PKC appears to serve as a pivot enzyme in activating MAPK pathways by stimulating multiple MEK/MKKs (197).

NFκB and MAPK are distinct signaling pathways in the cell. NFκB is primarily responsive to stress, toxins and cytokines leading to inflammation, apoptosis and adaptation, whereas the primary consequence of MAPK activation is growth, development, transcription, translation, and remodeling. However, there are considerable functional overlap and crosstalk between the two pathways. MAPK participates in the regulation of gene expression controlled primarily by NFκB signaling. For example, ERK and p38 have recently been

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shown to play an important role in the temporal regulation of NFκB activation by IL-1β and $H_2O_2$ (70, 210). These results, however, were derived from smooth muscle or cultured muscle fibers. Their relevance to contracting skeletal muscle remains to be established.

**AP-1:** AP-1 is another important transcription factor that regulates expression of numerous genes in a redox-sensitive manner. AP-1 is not a single protein but a dimer composed of activating (c-Fos and c-Jun) and inhibitory (Fos-related antigen [Fra]-1 and 2) subunits (47). Depending on the cellular redox milieu and cell type, Fos and Jun can dimerize or interact with other transcription factors such as ATF, C/EBP and Maf leading to activation or inhibition of gene transcription of antioxidant and immunoactive proteins (169, 360). It is well known that TNFα and IL-1 can induce c-Fos expression. Recent research suggests that AP-1 function is largely dependent on MAPK and NFκB signaling pathways (47). IL-1 has been shown to induce c-Fos and Fra-1 thereby stimulating IL-8 (also known as cytokine-induced neutrophil chemoattractant, CINC) expression, whereas pharmacological blockade of MEK1 by PD98059 suppresses the expression of almost all AP-1 subunits, indicating that ERK activation was required (169). Furthermore, the IL-8 promoter also has a p65 NFκB binding site occupancy of which plays an important role in the synergistic action of c-Fos. Zhou et al (428) reported that $H_2O_2$ and paraquat treatment induced AP-1 binding in nuclear extracts of C2C12 muscle cells and that the major components of the AP-1 complexes were identified as *c-jun/fos.*

**PGC-1α:** PGC-1α plays a pivot role in a wide range of mitochondrial adaptations such as energy metabolism, thermogenesis, biosynthesis, and UCP upregulation, which may have direct or indirect impact on intracellular oxidant-antioxidant homeostasis. PGC-1α integrates signals from several important upstream enzymes such as cAMP (through PKA and CREB phosphorylation), cGMP (through NO), AMPK, $Ca^{2+}$ (through CaMK), MEF-2 (through Calcineurin A) and p38 MAPK, and exerts direct control over transcription of the nucleus-encoded respiratory subunits and indirect control of the mitochondrial subunits. When transcriptionally activated and phosphorylated, PGC-1α regulates a number of down-stream transcription factors such as Tfam, MEF-2, NRF-1, NRF-2, ERRα and PPARα, which are involved in the expression of selective enzymes in ETC, fatty acid oxidation, UCP3, and MtDNA replication (354). Recent research indicates that PGC-1α is also required for the induction of antioxidant enzymes GPX1, catalase and SOD2, as well as UCP2 (122, 378).

The PGC-1α promoter has a potent cAMP response element (CRE) that serves as a target of CREB-mediated transcriptional activation. Thus, physiological conditions that increase catecholamines and glucagon concentrations can activate the PGC-1α pathway via elevated intracellular cAMP levels. NO can also induce PGC-1α through cGMP-dependent signaling. For example, it has been shown that mice with a genetic eNOS defect had lower mitochondrial density and energy expenditure along with decreased mRNA levels of PGC-1α, Tfam and NRF-1 (283). Intracellular $Ca^{2+}$ concentration may affect PGC-1α expression either by activating CaMK and phosphorylating CREB, or via Calcineurin A and MEF-2. Finally, PGC-1α has been shown to be stimulated by activation of p38 MAPK, a well-known redox-sensitive signaling pathway (7). Transgenic over-expression of its upstream kinase MKK3E was found to boost PGC-1α and mitochondrial enzyme COXIV in rat skeletal muscle. Since p38 MAPK could be activated by an acute bout of exercise in a redox-sensitive manner (137, 141), a link can be made between elevated ROS level and mitochondrial biogenesis via MAPK and PGC-1α controlled signaling process.

**Exercise activates numerous muscle redox signaling pathways—**Activation of JNK, ERK1/2 and $p38^{MAPK}$ was first reported after treadmill running in rat (141) and after bicycle exercise in human skeletal muscle along with activation of MEK1 and Raf-1

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(MEKK) (28). Numerous studies have confirmed that MAPK signal transduction pathways can be activated by contractile activity in skeletal muscle (36, 79, 279, 344, 398). The signals triggering the MAPK activation have been attributed to a variety of physiological stimuli associated with exercise including ROS, hormones, calcium ion, neural activity, and mechanical force. Interestingly, training tended to attenuate the activation intensity of several MAPK enzymes by acute exercise (53, 79, 180). Biological implications of MAPK activation are wide-spread including such important functions as mitochondrial biogenesis, glucose transport, muscle and heart hypertrophy, angiogenesis, and vascular adaptation (7, 153, 176, 243, 347).

NFκB activation was first observed in L6 muscle cells in response to $H_2O_2$ treatment and was controlled by intracellular GSH:GSSG status (363). Hollander et al. (171) first reported that NFκB and AP-1 binding was significantly elevated in rat skeletal muscle after an acute bout of prolonged exercise in a fiber-specific manner. The increased NFκB and AP-1 binding was accompanied by increased SOD2 mRNA abundance and protein content in exercised muscle. Further, work by Ji et al. (193) show higher levels of NFκB binding after exercise, along with increased IKK activity, IκBα phosphorylation and degradation, and nuclear P50 accumulation in rat DVL muscle. Ho et al. (167) reported that NFκB activation was elevated two-fold in the soleus and red gastrocnemius muscles during 60 minutes of treadmill exercise in rats, accompanied by IKKα/β phosphoralation. Importantly, application of p38 and ERK inhibitors reduced IKKα/β activation, suggesting MAPK and NFκB may work synergistically during exercise. Available data in both rodents and humans indicate that NFκB content and activation pattern are muscle fiber type dependent (31, 114, 170). In adult mice, basal NFκB activity was two-fold higher in the soleus and diaphragm muscles than in gastrocnemius and EDL; in rats, soleus muscle showed 3-fold higher NFκB protein content than EDL muscle. Unloading dramatically decreases NFκB activity suggesting contractile activity and/or nerve stimulation are required for the basal activity of this signaling pathway (113, 114, 178).

PGC-1α expression and signal transduction have displayed profound changes in response to physical exercise in skeletal muscle. An acute bout of endurance exercise and stimulated muscle contraction can upregulate PGC-1α and activate mitochondrial protein synthesis and proliferation (37, 181, 284, 392). Furthermore, training can result in elevated PGC-1α, NRF-1, and Tfam protein levels (37, 142, 181). Kang et al. (204) recently demonstrated that PGC-1α protein content was increased by >5 fold in rats after an acute bout of sprinting exercise along with a two-fold increase in NRF-1 and Tfam contents. Exercised rats had doubled content of the phospho-CREB and tripled phosphorlated-p38, whereas these effects were reduced when ROS generation was inhibited. The data indicate that contraction activated PGC-1α signaling pathways in skeletal muscle are redox-sensitive and that non-mitochondrial ROS play an important role in stimulating mitochondrial biogenesis.

**Molecular mechanism of redox signaling—**A variety of chemical and physical agents are capable of serving as the signaling molecules in response to oxidative stress, the most well-known of which are $H_2O_2$, NO, $Ca^{2+}$, and cytokines. When cells are exposed to UV irradiation, phorboesters, toxins (such as LPS), redox-disturbing agents (such as paraquat, Trx, menadione, DTT, NAC, growth factors, and anoxia/hypoxia/hyeroxia), intracellular levels of the abovementioned chemical messengers are increased. This may be caused by increased metabolism (such as mitochondrial respiration), inflammation, ion-channel opening, activation of enzymes, or simply chemical reactions. These messengers transfer signals from the cell surface to the nucleus to stimulate gene expression. A glance at the list of potential signaling molecules provided by Allen and Tresini (13) indicates that $H_2O_2$ appears to be the most common ROS messenger. The reason for $H_2O_2$ to serve this role is several fold: (1) $H_2O_2$ is constantly produced in the mitochondria during normal

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metabolism; (2) $H_2O_2$ is a relatively stable molecule; (3) it is a strong oxidant capable of oxidizing a variety of moieties (such as sulfhydryl, hydroxyl, sulfoxide, etc), yet not highly destructive; and (4) $H_2O_2$ is a small enough molecule to defuse across most, but not all, biomembrane barriers (72). It is worthy to note that intracellular level of $H_2O_2$ is often elevated in response to other signaling molecules such as cytokines (270).

The molecular mechanism for ROS signaling lies mainly on the interactions between gene regulatory sequences, usually, but not always in the promoter region, and the transcription factors (TF). ROS can influence the activity of TF binding through (a) activation of kinases resulting in sequential phosphorylation cascade; (b) modulation of phosphatase activity, often due to the oxidation of cysteine on active sites (274). For example, NIK appears to be activated by $H_2O_2$-induced inhibition of its phosphatase (241). Synthesis and degradation of TFs thereby determine their steady-state concentration and/or their ability to form homodimer or heterodimer (such as AP-1) (169).

Cellular reductant levels and redox status play an important role in the regulation of antioxidant signaling determined mostly by GSH and TRX status (122). When intracellular GSH/GSSG ratio is decreased, critical thiols located on the active site of key enzymes can be oxidized forming intra- or inter-protein disulfide, affecting signaling capacity. Predicted from its kinetics, GPX maintains a relatively stable cellular $H_2O_2$ concentration as long as GSH level is above 100 μM. TRX is involved in the reduction of cysteine residues in the oxidatively-modified proteins and is known to modulate p50/p65 binding to κB sequence in the target gene promoter (122). Interestingly, TRX plays different roles in different cell compartments: in the cytosol TRX keeps redox-sensitive proteins in the reduced state and thus attenuates ROS-induced signaling intensity; in the nucleus TRX facilitates p50 binding with its reducing power. Interestingly, TRX itself is a target gene of NFκB upon stimulation of ROS, lipopolysaccharide (LPS), phorbol ester PMA and inflammatory cytokine TNFα and IL-1 (357), presumably preventing over-expression of potentially deleterious gene products.

In addition to the transcriptional activation as mentioned above, ROS may directly influence activities of enzymes involved in the metabolic pathways, such as those in the Krebs cycle and the electron transport chain (ETC) (286). This may be viewed as an alternative means to modulate overall cell oxygen consumption.

**Gene Targets of Redox Signaling—**It appears that most, if not all of antioxidant enzymes rely on redox signaling to control their gene expression. Redox signaling also controls other gene products of such important biological functions as UCP, mitochondrial biogenesis, and muscle inflammation.

**SOD2:** SOD2 is a well-known target gene for NFκB activation and its expression has been shown to be upregulated by an acute bout of exercise. The SOD2 promoter contains NFκB and AP-1 binding sites and its upregulation by TNFα and IL-1 is mediated in part by NFκB activation (94, 168). SOD2 can also be activated by platelet-derived growth factor (PDGF) due to early growth-responsive-1 (egr-1) protein binding to a putative GC-rich region of SOD2 gene, which may be controlled by MEK1 and ERK1/2 signaling (248). In addition, TNFα, IL-1 and PMA have been shown to upregulate SOD2 gene through a complex intronic enhancer sequence binding involving NFκB, CCAAT-enhancer binding protein (C/EBP), Sp-1 and nuclear factor (NF)-1 (218, 247). Whereas physical interaction between C/EBP and NFκB proteins was evident and Sp-1 binding was is necessary for basal SOD2 expression, p65 translocation and binding was found to be essential for SOD2 transactivation (145). Gomez –Cabrera et al. (137) studied the role of ERK1/ERK2 and p38 $^{MAPK}$, which were shown to control egr-1 and ATF-2 binding to SOD2 gene, during an

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acute bout of prolonged exercise in rats. ERK1/2 and p38 were activated in the gastrocnemius muscle of exercised rats, accompanied with a two-fold increase in SOD2 mRNA level. Injection of allopurinol, a xanthine oxidase (XO) inhibitor, abolished exercise-induced ERK and p38 activation and SOD2 upregulation. As mentioned in the previous section, $H_2O_2$ may simultaneously activate enzymes in MAPK and NFκB pathway required for transactivation via DNA binding by various TFs shown in Fig. 6.

**SOD1:** SOD1 is a cytosolic enzyme with relatively uniform activity across different muscle fibers (189, 286). An acute bout of exercise has been shown to activate CuZnSOD activity, but most studies reported no change in its mRNA and enzyme protein levels suggesting the increased activity is due to post-translational control, probably the increased $O_2^{-\bullet}$ concentration which is known to activate the enzyme (72). However, since SOD1 has a quick turn-over rate and a short $t_{1/2}$ in the range of minutes, *de novo* synthesis of new enzyme protein cannot be ruled out. For example, Radak et al. (320) showed that enzyme activity and protein content of both SOD1 and SOD2 in rat soleus and tibialis muscles were elevated after a single bout of treadmill running lasting 60-70 min. The promoter of SOD1 contains AP-2 binding site between the CCAAT box and Sp-1 binding site, which plays a role in the upreguation of SOD1 by phytopanaxadiol $Rb_2$ (a ginsenoside fraction) (217). Yoo et al (417) showed that the $H_2O_2$-responsive element (HRE) on the promoter (−576) was responsible for the activation of SOD1 gene expression by $H_2O_2$, paraquat and heat shock in human Hep2 cells.

**GPX:** GPX is a homotetramer with each 22-kDa subunit bound to a selenium atom existing as a selenocysteine (149). Two oxygen response elements (ORE) located at −1232 to −1213 and −282 to −275 in the 5′-flanking region of human GPX gene have been identified (87). The expression of the GPX gene, *hgpx1*, occurs in a wide range of tissues controlled by development, hormones, and oxygen tension. In the myocardium, GPX activity induced by oxygen tension was found to be proportional to the mRNA levels, suggesting a transcriptional mechanism (88). Franco et al. (124) reported a 4-5 fold increase in GPX mRNA level in cultured myotubes in response to paraquat, $H_2O_2$ and menadione treatment, supporting the above notion. However, Clerch et al. (78) showed that a common protein in rat lung might bind to both GPX and SOD2 mRNA thereby increasing their stability, suggesting post-transcriptional regulation may play a role. In muscle cells, GPX promoter reportedly contains both NFκB and AP-1 binding sites (425, 428). It has been shown that $H_2O_2$ and paraquat-induced GPX mRNA expression in C2C12 cell culture was dependent on functional NFκB signaling. Introduction of IκB mutant abolished GPX mRNA expression. Relatively sparse data is available regarding its gene regulation during exercise.

**Glutamylcysteine synthetase (GCS):** GSH plays a critical role in muscle antioxidant defense during exercise by providing substrate for GPX, maintaining proper redox status and scavenging $^{\bullet}OH$ and $O_2^{\bullet-}$. Several studies have demonstrated that as the rate-limiting enzyme for GSH synthesis, GCS, can be induced by endurance training in rat skeletal muscle and liver (323, 361). Training also increases muscle GSH content, though the adaptation is fiber specific (188, 361). Surprisingly, little is known about the gene regulation of GCS in skeletal muscle. In mammalian cells, GCS is a heterodimer consisting of the catalytic heavy-chain subunit (GCS-HS) and regulatory light-chain subunit (GCS-LC) (322). GCS-HS expression is known to be regulated by redox-sensitive mechanism via a variety of oxidants, phenolic antioxidants and pro-inflammatory cytokines (TNFα and IL-1ß). Both GCS-HC and GCS-LC promoters contain antioxidant response element (ARE) and NRF-2 binding seems to play a critical role in oxidative stress-induced GCS upregulation. GCS-HC also has NFκB binding sites that are essential for GCS expression in some, but not all cell

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types (71). If these signaling pathways are also operational in muscle cells, they could be potential mechanisms for training-induced upregulation of GCS and GSH biosynthesis.

**iNOS:** NO at low concentration exerts an antioxidant function by neutralizing $O_2^{-\bullet}$ (327). Unlike the other forms of NOS, iNOS is not regulated by calcium ion and has been shown to be induced primarily by ROS and inflammatory cytokines through activation of NFκB and MAPK (4). In rat skeletal muscle myoblasts, the IL-1β-mediated iNOS induction was reduced by blocking ERK1/2 activation and completely abolished by the inhibition of NFκB. Moreover, a linear correlation was observed between NFκB activation and iNOS expression in human skeletal muscle (5). iNOS mRNA level has been reported to be elevated after an acute bout of exercise in rat skeletal muscles (39, 137). However, Vassilakopoulos et al. (401) reported that while chronic exercise training successfully increased nNOS and eNOS activity and protein expression, it failed to induce iNOS in rat gastrocnemius and diaphragm muscles. The role of iNOS is largely viewed as being catabolic and often co-expressed with pro-inflammatory cytokines and adhesion molecules during muscle injury and wasting (359). High levels of NO production also leads to the formation of peroxynitrite, a highly reactive species contributing to muscle oxidative damage.

**Pro-inflammatory cytokines and adhesion molecules—**During the early phase of muscle injury, inflammatory cytokines promote the gene expression of adhesion molecules such as VCAM-1, CINC-1 and MCP-1, and NOS expression. In addition, some cytokines can bind with membrane receptors and activate specific ROS-generating enzymes, such as COX-2, NADPH oxidase, and XO. Endothelial cells from injured muscle are known to secrete TNF-α, IL-1, IL-6 and IL-8, providing a positive feed-forward cycle (290, 298). While this process is largely viewed as prooxidative, selective expression of antioxidants is an important to prevent chronic inflammation.

During heavy exercise, especially work involving lengthening contractions, progressive increases in TNFα, IL-1 and IL-6 have been observed in the plasma and muscle cells. In cultured C2C12 cells, IL-6 production is regulated by IL-1β, and the p38 inhibitor SB-208350 or the ERK inhibitor PD-98059 reduces IL-6 production, suggesting that these two MAPK pathways regulate IL-6 production (246). ERK has also been shown to play a role in regulating IL-1-induced gene expression of iNOS and COX-2, but no VCAM-1 or SOD2 (197). Aoi et al (25) showed that in myotube L6 cells, $H_2O_2$ stimulated p65 nuclear translocation and expression of CINC-1 and MCP-1, whereas preincubation with α-tocopherol limited the increases. An acute bout of exercise increased CINC-1 and MCP-1 levels and nuclear p65 content in rat gastrocnemius muscle, but these changes were less in rats fed a high vitamin E diet. These results indicate that exercise-induced inflammation was caused by phagocyte infiltration and regulated in a redox-sensitive manner.

**Uncoupling proteins (UCP)—**Located in the mitochondrial inner membrane, uncoupling proteins (UCP) are a heterogeneous family of proteins that play an important role in partially dissipating the proton electrochemical gradient across the membrane (220). The best characterized UCP1 is expressed exclusively in the brown adipose tissue of rodents with a key function of adaptive thermogenesis, whereas UCP2 is expressed ubiquitously (226). UCP3, expressed primarily in the skeletal muscle, shares 59% homology to that of UCP1 and is regarded a plausible regulator of trans-membrane proton potential and hence efficiency of oxidative phosphorylation (242). UCP3 expression is increased in response to muscle contractile activity in mammalian skeletal muscle (86, 242, 429). Recent studies suggest that its upregulation reduces superoxide generation in the mitochondria (135). Jiang et al (197) reported that UCP3 mRNA and protein expression elevated progressively after prolonged exercise, whereas ROS production showed an initial increase but was

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dramatically decreased at 150 min when UCP3 protein reached the highest. Meanwhile state 3 respiration and respiratory control index (RCI) were deceased. These findings suggest that UCP3 may be induced in part to shunt protons back to the matrix and maintain a modest cross-inner membrane potential ($\Delta\psi$), thus reducing superoxide radical production at the expense of ATP production. Anderson et al (17) demonstrated that exercise-induced UCP3 gene expression was dependent on mitochondrial $H_2O$ production. In the $UCP3^{-/-}$ 2 mice, however, exercise failed to increase mitochondrial uncoupling respiration, whereas $H_2O_2$ production was significantly greater compared to that in the wild type mice.

How $H_2O_2$ induces UCP gene expression is currently unknown. St. Pierre et al. (378) demonstrated that PGC-1α plays an important role in the concerted expression of mitochondrial proteins including UCP3 and UCP2 transactivated by $H_2O_2$ challenge. In the PGC-1α knockout cell line, $H_2O_2$ did not elicit these effects. Thus, PGC-1α could be an important mediator in the upregulation of UCP3.

## Conclusion

Since the first report that muscular exercise promotes oxidative damage in human tissues, the field of redox biology has grown significantly and our understanding of the sources and consequences of exercise-induced free radical production continues to advance. Current evidence suggests that contracting muscles produce oxidants from a variety of cellular locations. Further, although mitochondria are a potential source of ROS in cells, growing evidence suggests that these organelles may play a less prominent role in oxidant production in contracting skeletal muscles than was previously thought.

Many early investigations of exercise and free radical production focused upon the damaging effects of oxidants in muscle (e.g., lipid peroxidation). However, a new age in redox biology currently exists with an ever growing number of reports detailing the advantageous biological effects of free radicals on cell signaling. Indeed, it is now clear that ROS and RNS are involved in regulation of cell signaling pathways and the control of numerous redox-sensitive transcription factors. Furthermore, physiological levels of reactive oxygen species are essential for optimal force generation in skeletal muscle. In contrast, high levels of ROS promote skeletal muscle contractile dysfunction promote muscle fatigue. Needless to say, there is much more to be learned about this exciting topic.

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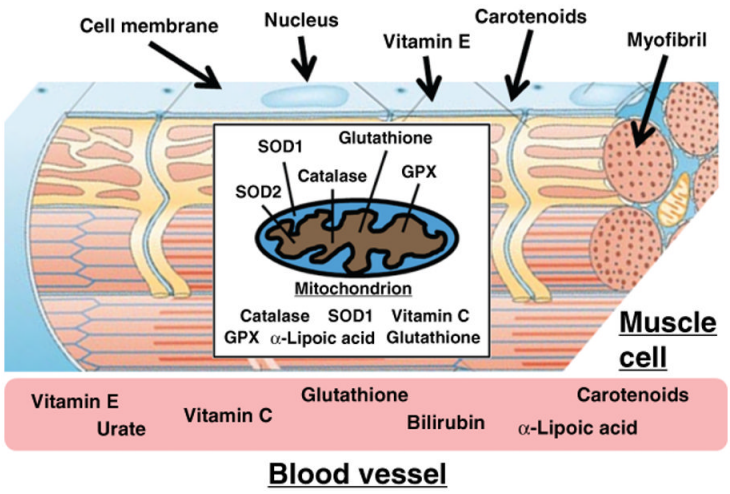


**Figure 1.**
Locations of the principal enzymatic and non-enzymatic antioxidants found in cells. Key to abbreviations: GPX = glutathione peroxidase; SOD1 = superoxide dismutase 1; SOD2 = superoxide dismutase 2.

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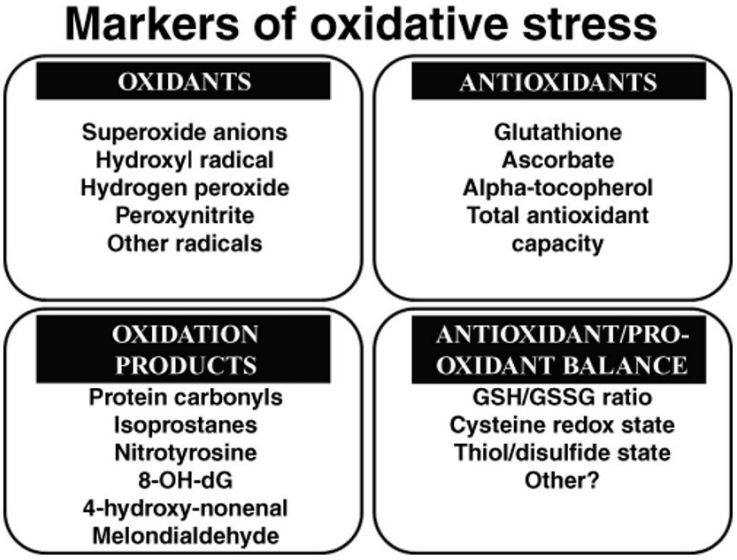


**Figure 2.**

Four broad classes of biomarkers are commonly used to assess the presence of oxidative stress in cells or tissues. These categories include the measurement of oxidants, cellular levels of antioxidants, oxidation products, and the antioxidant/prooxidant balance. Key to abbreviations: 8-OH-dG = 8-hydroxydeoxyguanosine; GSH/GSSG = ratio of reduced glutathione to oxidized glutathione.

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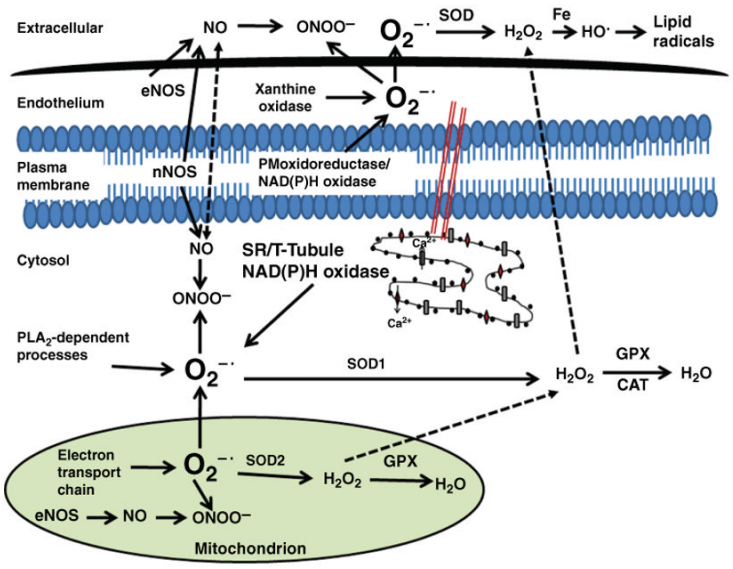


**Figure 3.**

Potential sites for the production of superoxide and nitric oxide (NO) in skeletal muscle. Key to abbreviations: CAT = catalase; SOD1 = superoxide dismutase 1; SOD2 = superoxide dismutase 2; GPX = glutathione peroxidase.

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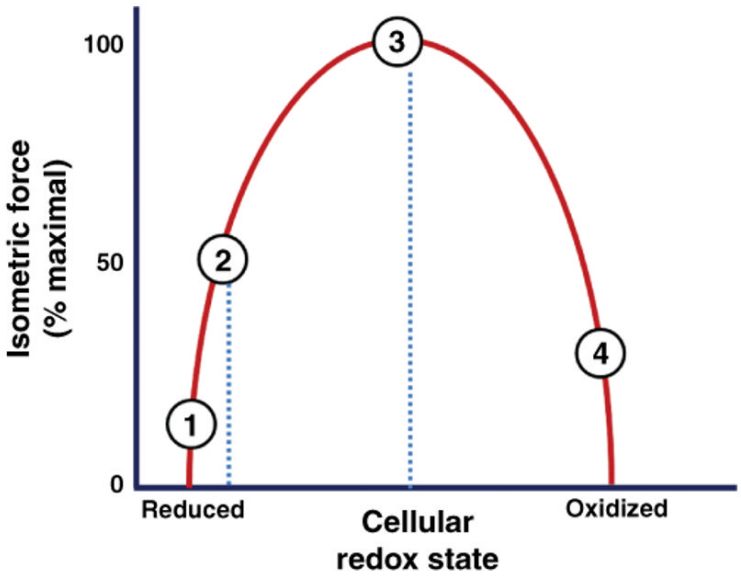


**Figure 4.**

A theoretical model proposed by Reid and colleagues (330) that describes the biphasic effect of ROS on skeletal muscle force production. Point 1 represents the force production by unfatigued muscle exposed to antioxidants or a reducing agent. Point 2 illustrates the force generated by muscle in its basal state (i.e., no antioxidants or oxidants added). Point 3 illustrates the force produced by unfatigued skeletal muscle exposed to low levels of oxidants; this represents the optimal redox state for force production. Point 4 illustrates the deleterious effects of excessive ROS on skeletal muscle force. Figure is redrawn from work by Mike Reid (325).

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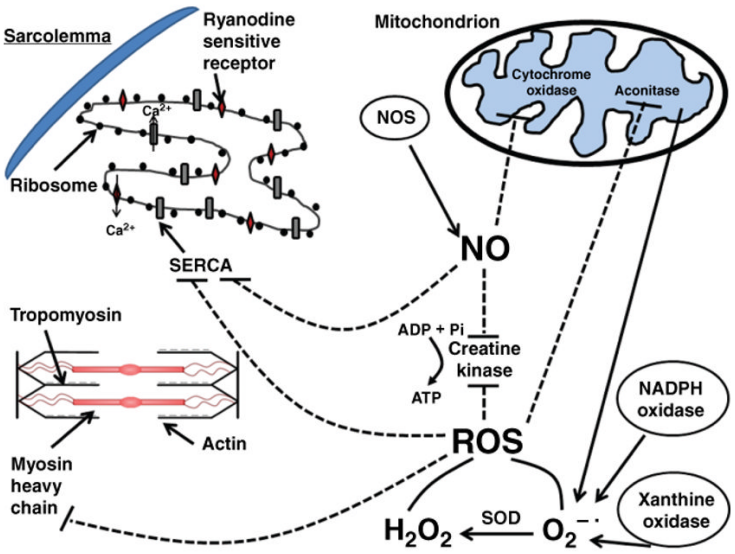


**Figure 5.**

Diagram of reputed redox sensitive targets in skeletal muscle that can impact muscle force production. Redrawn from Smith and Reid (375). Key to abbreviations: SOD = superoxide dismutase; NOS = nitric oxide synthase; NO = nitric oxide; SERCA = sarcoplasmic reticulum calcium ATPase.

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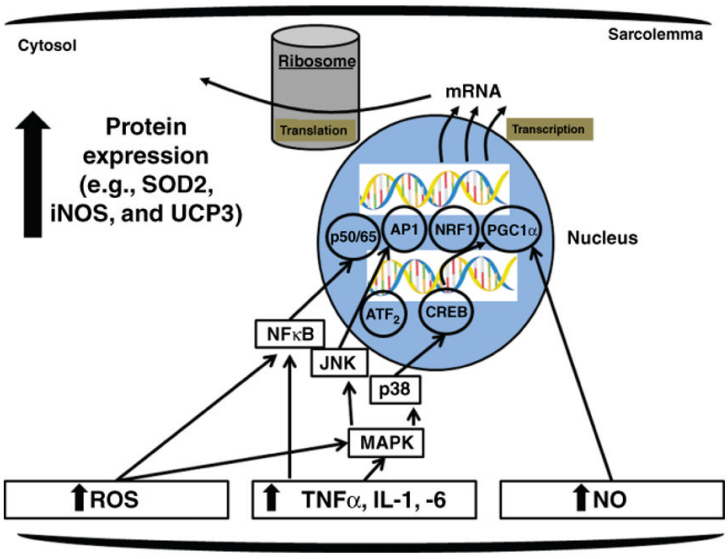


**Figure 6.**

Hypothetical illustration of function and expression of antioxidants in the skeletal muscle. Abbreviations: ROS = reactive oxygen species, NO = nitric oxide; CREB = cAMP-response element binding protein; NRF-1 = nuclear respiratory factor-1; SOD2 = superoxide dismutase 2; UCP3 = uncoupling protein 3; JNK = c-Jun amino-terminal kinase; MAPK = mitogen activated protein kinase; NFκB = nuclear factor (NF) κB; inducible nitric oxide synthase (iNOS), IL = interlurekin.

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## Table 1

Properties of human SOD isoenzymes. Data are from references (155, 389).

| Property | SOD1 | SOD2 | SOD3 |
|---|---|---|---|
| Cellular location | Cytosol and mitochondrial intermembrane space | Mitochondrial matrix | Extracellular |
| Metal/monomer | 1 Cu, 1 Zn | 1 Mn | 1 Cu, 1 Zn |
| Molecular weight (kDa) | 32.5 | 24.7 | 30 |
| Subunit | Dimer | Tetramer | Tetramer |
| Inhibition by $CN^-$ | yes | no | yes |
| Inhibition by $H_2O_2$ | yes | yes | yes |
| Rate constant for reaction with $O_2^-$ | $0.62 \times 10^9$ | $1.2 \times 10^9$ | $0.72 \times 10^9$ |

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## Table 2

Physical characteristics and tissue locations of the multiple GPX proteins in humans. Data are from references (59, 110).

| Property | GPX1 | GPX2 | GPX3 | GPX4 | GPX5 |
|---|---|---|---|---|---|
| Cellular location | Cytosol and mitochondria | cytosol | Extracellular space and cytosol | Membrane bound-nuclei and mitochondria | Extracellular and membrane bound |
| Subunit | tetrameric | tetrameric | tetrameric | monomeric | dimeric |
| Molecular weight (kDa) | 21 | 22 | 22.5 | 19 | 24 |
| Tissue location | All tissues | Stomach, intestine | All tissues | Testes, spermatozoa, heart, brain | Epididymis, spermatozoa, liver, kidney |

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## Table 3

A list of selected human studies indicating that N-acetylcysteine delays muscular fatigue during prolonged submaximal exercise.

| Mode of exercise | Subject pool | NAC treatment | Exercise dependent measure | Performance Improvement | Reference |
|---|---|---|---|---|---|
| Cycling to fatigue | Adult male endurance athletes | Multiple i.v. doses pre- and during exercise | Time to fatigue | +24% | McKenna et al. (263) |
| Breathing against inspiratory load | Adult men | Single i.v. dose pre-exercise | Time to task failure | +65% | Travaline et al. (396) |
| Repetitive handgrip exercise | Adult men and women | Single i.v. dose pre-exercise | Time to task failure | +15% | Matuszczak et al. (255) |
| Repeated electrical stimulation of limb muscle | Adult men | Single i.v. dose pre-exercise | Force decline during 30 minutes of contractions | +15% | Reid et al. (334) |