HIGHLIGHTED TOPIC | Free Radical Biology in Skeletal Muscle

Hypoxia-induced reactive oxygen species formation in skeletal muscle

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Clanton TL. Hypoxia-induced reactive oxygen species formation in skeletal muscle. J Appl Physiol 102: 2379–2388, 2007. First published February 8, 2007; doi:10.1152/japplphysiol.01298.2006.—The existence of hypoxia-induced reactive oxygen species (ROS) production remains controversial. However, numerous observations with a variety of methods and in many cells and tissue types are supportive of this idea. Skeletal muscle appears to behave much like heart in that in the early stages of hypoxia there is a transient elevation in ROS, whereas in chronic exposure to very severe hypoxia there is evidence of ongoing oxidative stress. Important remaining questions that are addressed in this review include the following. Are there levels of PO2 in skeletal muscle, typical of physiological or mildly pathophysiological conditions, that are low enough to induce significant ROS production? Does the ROS associated with muscle contractile activity reflect imbalances in oxygen uptake and demand that drive the cell to a more reduced state? What are the possible molecular mechanisms by which ROS may be elevated in hypoxic skeletal muscle? Is the production of ROS in hypoxia of physiological significance, both with respect to cell signaling pathways promoting cell function and with respect to damaging effects of long-term exposure? Discussion of these and other topics leads to general conclusions that hypoxia-induced ROS may be a normal physiological response to imbalance in oxygen supply and demand or environmental stress and may play a yet undefined role in normal response mechanisms to these stimuli. However, in chronic and extreme hypoxic exposure, muscles may fail to maintain a normal redox homeostasis, resulting in cell injury or dysfunction.

- reduced nicotinamide adenine dinucleotide; free radicals; ischemia; oxidase; mitochondria; exercise; altitude

The basic chemistry and biology of free radicals and oxidants is a very large and evolving topic and far beyond the scope of this review. For more general background information in this field, readers are urged to refer to a large number of excellent reviews and book chapters that are available (e.g., 8, 16, 43, 44, 73, 77).

HYPOXIA AND ITS RELATIONSHIP TO OXIDATIVE AND REDUCTIVE STRESS

Oxidative stress. ROS formation is usually associated with the term “oxidative stress,” although it is clear that all cells use low levels of ROS for normal function that cannot at all be linked to stress. Therefore, the term oxidative stress is largely descriptive and often misused. There have been numerous attempts at redefining oxidative stress, but perhaps the most useful was recently put forward by H. Sies and D. P. Jones as “an imbalance between oxidants and antioxidants in favor of oxidants, leading to disruption of redox signaling and control and/or molecular damage” (see Ref. 52, footnote, p. 1865). To make things confusing, to define something as an “oxidant” in chemical terms depends entirely on its role in a specific reaction with a “reductant.” What might be an oxidant in one reaction will be a reductant in another within the same environment, and redox reactions are not always in equilibrium.

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One can imagine that if you could sample all of the redox pairs in a cell you might be able to define a condition in which the majority have shifted to a more oxidized state and then be able to also define specific redox-sensitive proteins, lipids, or nucleic acids that have been oxidized and have diminished function. From this information you might be able to define a state of oxidative stress, but such well-defined conditions are rare. Jones has subsequently put forward yet another definition of oxidative stress as “a disruption of redox signaling and control” (52). This definition would presumably encompass both disorders of oxidation and reduction.

**Reductive stress.** Cellular hypoxia is a state that is generally characterized by being in a more cellular reductive state and in some cases has been described as a form of “reductive stress.” This reflects the elevations in reducing equivalents (mostly NADH and FADH₂) that build up, particularly in the mitochondria, when insufficient O₂ is available for reduction by the electron transport chain. This buildup of reducing equivalents also makes electrons more available for reduction reactions such as the reduction of O₂ to superoxide (O₂⁻; a moderate reductant but also a ROS). Reductive stress has also been used to describe the intracellular environment caused by “chemical hypoxia” from poisoning of the electron transport system (25). Both chemical hypoxia and tissue hypoxia lead to elevations in reducing equivalents, but in chemical hypoxia sufficient O₂ is presumably always available for reduction, whereas in tissue hypoxia, as one approaches anoxia, oxygen availability can become a critical substrate for production of ROS. As described by Dawson et al. (25), it may be that hypoxia- or ischemia-induced ROS production requires conditions of low-flow or intermittent ischemia where cycles of anoxia and reoxygenation are occurring almost concurrently. This may, in fact, be the most common form of hypoxia in living organisms (22), potentially producing oscillations in electron sources and sinks within the microenvironment that are out of equilibrium and out of phase: in other words, a state of redox disequilibrium. For conditions applicable to hypoxia, this term may be most descriptive and relevant.

Reductive stress has also been used to describe conditions in which there is an abnormally high reduction state of sulfhydryls (−SH) within the cell. This is usually chemically induced by exposure to reducing agents such as DTT (80). However, this form of reductive stress is not applicable to hypoxia since there is no evidence of shifts to greater reduced −SH content in hypoxic cells, and prolonged hypoxia can often result in general loss of −SH reducing equivalents in the form of glutathione (54). Furthermore, NADPH, required for normal reduction of oxidized glutathione through glutathione reductase, does not generally follow elevations in NADH (100), although the two are connected by several metabolic pathways. In fact, the rate of NADP-to-NADPH conversion is severely limited in hypoxic environments (100) and can represent another source of oxidative stress by not providing sufficient cytosolic reducing substrate (NADPH) to maintain the antioxidant status of NADPH-coupled reactions such as those necessary for reduction of oxidized glutathione or thioredoxin.

**Conditions necessary for ROS formation in hypoxia.** ROS are more likely to be produced in hypoxia when there is both a high reductive capacity (e.g., high NADH/NAD⁺ ) and sufficient O₂ available for reaction (Fig. 1). Therefore, one or both substrates can be predominantly important for a given ROS-generating system, depending on its relative Km for reaction with its electron donor and O₂. It is fortunate that safely contained electron carriers such as NADH, FADH₂, and NADPH do not react directly with O₂ at high enough rates to be significant, and therefore they must generally interact with reaction centers that can assist in moving electrons between donors and acceptors. These catalysts generally include enzymes such as xanthine oxidase, NAD(P)H oxidase, or cyclooxygenase; electron carriers such as ubiquinone; and sulfur clusters or other metal centers such as peroxidase enzymes. Therefore, the availability of these catalysts in their appropriate redox state is the third ingredient necessary for ROS to be formed.

In most conditions of “physiological” hypoxia, i.e., levels of O₂ that are still compatible with life, considerable O₂ is clearly available for reaction. On the basis of the solubility of O₂ in water and lipid (93), even at 1 mmHg Po₂, there is ≈1.3 and 6.5 μM of O₂ available for reactions, respectively. In pathological conditions of severe injury leading to eventual cell death, whether sufficient O₂ is available for ROS production is debatable but still highly probable. To give some relevant pathophysiological examples of the lowest Po₂ values seen in biological systems, in lower limb muscle made ischemic by complete obstruction of blood flow, mean tissue Po₂ drops to something less than 1.9 mmHg (66). By comparison, isolated spinotrapezius skeletal muscle cells demonstrate only the beginnings of respiratory inhibition (increasing NADH) when extracellular Po₂ drops below ≈1.3 mmHg (86). In the isolated ischemic heart, 10 min of global ischemia results in Po₂ of ≈0.44 mmHg (116), although much higher values have also been reported recently (5). In tissue bath studies common to many laboratories, it is difficult to reduce Po₂ by bubbling with pure N₂-CO₂ mixtures much below 6–10 mmHg, demonstrating the difficulty of studying hypoxic responses in tissue preparations that have wide diffusion gradients. The point of this discussion is that although it is likely that during severe hypoxic conditions, O₂ availability could limit ROS production by the mitochondria, it is as likely that in other cellular compartments, sufficient O₂ may be available for physiologi-
cally significant ROS production. Support of a “critical” range of hypoxia necessary for ROS formation were provided by Duranteau et al. (29), who showed in cardiac myocytes using the fluorescent probe dichlorofluorescein that the largest ROS signal occurred near 7 mmHg PO2 in the perfusate surrounding isolated cells.

**EVIDENCE FOR ROS FORMATION DURING HYPOXIA IN NONSKELETAL MUSCLE CELLS**

A number of reports have provided evidence for ROS formation in acute conditions of hypoxia or ischemia, but, as mentioned, there is no general consensus regarding the physiological significance of these observations (21). Importantly, the amounts of ROS measured during ischemia and hypoxia are considerably less and appear more transient than ROS seen in cardiac reperfusion (e.g., 97). Such relatively subtle signals are difficult to quantify and unequivocally verify with existing technology. They do not lend themselves, for example, to studies using spin traps or electron paramagnetic resonance approaches (11). Additionally, coexisting conditions such as acidosis, nutrient or energy depletion, shear stress, nitric oxide (NO) accumulation or depletion that are present during conditions of ischemia or hypoxia can influence ROS detection, either directly or through their effects on competing antioxidant defense reactions and/or the reductive state of the cells (11).

The work of Lemasters and colleagues (38) demonstrated that a significant component of cell injury caused from reductive stress induced by chemical hypoxia appears to arise from ROS formation. Other early work by Park and Kehrer showed that hypoxic (95% N2-5% CO2) perfusion of rat (76) and rabbit (75) heart resulted in marked elevations in lipid peroxidation products, protein carbonyls, and glutathione disulfide that occurred within 10 min of exposure and continued to rise over 90 min of hypoxia, without reperfusion. Similar findings have been shown by Schumacker and colleagues in a variety of cell preparations using ROS-sensitive fluorescent probes (29, 101–103). Recently these results have been verified in several cell types using a novel and more specific fluorescence resonance energy transfer (FRET) probe (41). ROS produced in the intact, ischemic myocardium has also been measured using hydroethidium (a O2•–-sensitive probe) and derivatives of dihydrofluorescein, a nonspecific oxidant probe analogous to dichlorofluorescein (53, 97).

In the lung, increased ROS signaling occurs in the early stages of ischemia (1). This, however, may be a completely different phenomenon, since it is related directly to loss of shear stress at the time of flow reduction and not to tissue hypoxia (94). Changes in shear stress are always an important factor to keep in mind because in perfused systems local changes in blood flow associated with hypoxia or ischemia can induce a shear phenomenon. In isolated systemic vessels, hypoxia alone can result in rapid elevations in ROS signaling in local vascular beds (95). The phenomenon has also been observed in the pulmonary vasculature, but opposite results have been reported as well, a point of considerable controversy in vascular control literature, as recently reviewed (108).

**EVIDENCE FOR HYPOXIA-INDUCED ROS FORMATION IN SKELETAL MUSCLES**

Our laboratory has focused largely on an apparent ROS signal that is associated with transient hypoxia in isolated, nonperfused diaphragm muscle. Our first observation of this possibility occurred in experiments by Mohanraj et al. (70), who gave a series of antioxidants during exposure of isolated diaphragm bundles to severe hypoxia (95% N2-5% CO2). Surprisingly, contractile function was greatly preserved in antioxidant-treated tissues after a 30-min exposure. This work was largely substantiated for superoxide scavengers in subsequent studies by Wright et al. (110), where it was demonstrated that the effects were independent of the changes in high-energy phosphate content. Somewhat different results were reported by Heunks et al. (47) in a model of more modest hypoxic exposure at room temperature. In this study antioxidants decreased maximum force production and slowed maximum shortening velocity in hypoxia (47).

In later experiments, we used a method to measure intracellular ROS formation during hypoxia in isolated skeletal muscle using tissue fluorescence spectroscopy (114). Although the signal was observed using several redox-sensitive probes, including hydroethidium, the most reliable and sensitive indicator was dihydrofluorescein, which converts to fluorescein in the presence of H2O2 and other oxidants. A transient increase in fluorescence was observed that mimicked exposure of the muscle to low levels of exogenous H2O2. On the basis of estimates of sensitivity of the intracellular probe to exogenous H2O2 and further estimates regarding the reduction in H2O2 concentration between the inside and outside of the cell (96), we estimated the transient intracellular signal to be equivalent to ~100–500 nM H2O2. The signal was also shown to be proportional to the degree of hypoxia in the tissue bath and to the rise in autofluorescence of NADH/NAD+. In fact, only when a significant rise in NADH was observed was there a detectable change in ROS formation, but notably significant NADH elevations were observed in resting rat diaphragm in baths with PO2 values as high as 40% O2. Likewise, in isolated diaphragms stimulated to fatigue, only when NADH was concurrently elevated was there a rise in detectable ROS formation, suggesting that the ROS observed with muscle stimulation may require elevations in NADH or equivalent shifts to a more reducing intracellular environment. One critique of these kinds of studies is that all fluorescein-based probes have considerable complications with respect to their interpretation, and, in general, one must address these findings with educated skepticism. However, verification of the results by other methods and in other tissues, as described in the preceding paragraphs, and the ability to block the signal with appropriate antioxidants lends support to the credibility of the observations.

Further support of the hypothesis that physiologically relevant conditions of transient hypoxia in skeletal muscles may lead to ROS formation was provided by Bailey et al. (9) in exercising humans. Using an ex vivo phenylbutylnitrone (PBN) spin trapping method, the authors demonstrated increasing PBN adducts (a monitor of lipid derived alkoxyalkyl radicals) across the vascular bed of the exercising limb as exercise intensity increased. Interestingly, the authors did not attribute the rising levels of radical generation to the level of...
mitochondrial electron transport flux, as measured by the elevation in oxygen consumption, but rather the data tracked with known values of decreasing tissue PO2 levels occurring at these same levels of exercise, measured in previous studies by the same authors (85). These data, coupled with observations in isolated cells, strongly suggest that ROS associated with moderate to intense exercise may be associated with low levels of tissue PO2, coupled with changes in muscle redox state, as discussed later in this review.

EVIDENCE FOR SKELETAL MUSCLE ROS FORMATION IN CHRONIC HYPOXIA

Climbers who have been exposed for prolonged periods to extreme altitude exhibit evidence of oxidative stress in skeletal muscles as demonstrated by the presence of increased lipofuscin in muscle biopsy samples (65) and muscle DNA oxidative damage (63). Lipofuscin is a product of lipid peroxidation and largely attributed to mitochondrial membrane oxidative breakdown products associated with aging and disease. Similar oxidative changes in muscle biopsies, including elevations in lipid peroxidation and protein oxidation products, have been observed in hypoxic chronic obstructive pulmonary disease (COPD) patients compared with similarly obstructed COPD patients who do not exhibit hypoxia (57). This suggests that the primary contributing factor in COPD accounting for the increased evidence of oxidative stress in skeletal muscle is hypoxia. Supporting evidence has been provided recently in rodents exposed to extreme altitude simulation (8,500 m) for 2 days. Muscle biopsies show striking evidence of muscle oxidative stress, including oxidized proteins, oxidized lipids, and sensitivity to vitamin E supplementation (64). Interestingly, throughout the animal kingdom the most dominant genomic response to sustained hypoxia is an elevation in a variety of antioxidant enzymes (46), indirectly suggesting that survival in prolonged hypoxia involves defense strategies against elevations in oxidant production. However, the influence of various levels and durations of hypoxia on the effectiveness of specific antioxidant defense mechanisms is poorly understood and an important area of research. In total, these observations are consistent with the hypothesis that chronic exposure to tissue hypoxia results in oxidative stress and that this may have significant impacts on normal responses of skeletal muscle to altitude or disease.

TISSUE HYPOXIA IN SKELETAL MUSCLE

What is skeletal muscle hypoxia? In establishing that hypoxia may be a physiologically relevant promoter of ROS formation in skeletal muscle, it is necessary to consider the meaning of muscle tissue hypoxia. Hypoxia cannot be easily defined in any in vivo system as a threshold PO2 below which cells are O2 deficient. There is a range, for example, over which cells fully adapt to lower PO2 levels, sometimes referred to as “adaptive cell hypoxia,” where compensatory mechanisms are in place to sustain metabolism and energy utilization in a changing environment (23). Mitochondrial ROS formation has, for example, been considered a potential effector of such a sensing system (19).

The PO2 that might be considered hypoxic is best defined as the level at which there is a more reduced state of the mitochondrial electron transport chain, as evidenced by increased mitochondrial NADH/NAD+ (97), which can be considered a sentinel for insufficient O2 availability at the level of mitochondrial electron flow (17, 114). The specific tissue PO2 that induces a rise in NADH/NAD+ depends on many variables, including diffusion distances, blood flow velocity, metabolic rate, myoglobin concentrations, the type of contraction, the fiber type, the Kn for O2 of cytochrome oxidase, and the dynamic influence of NO on mitochondrial electron transport (27). The fact that skeletal muscles use local reductions in cellular PO2 to their advantage during exercise to improve the overall flux of O2 from the capillary provides a window into the complexity of defining skeletal muscle tissue hypoxia, since muscles lower PO2 to a minimum value to sustain O2 delivery and prevent O2 deprivation during contraction.

Levels of tissue PO2 in exercising muscle. Based on recent improvements in resolution of NMR techniques for myoglobin saturation, the resting intracellular PO2 in human skeletal muscle has been estimated to be \( \approx \)34 mmHg, dropping to \( \approx \)23 mmHg when subjects breathe ambient 10% O2 (83). However, near maximal exercise, intracellular PO2 drops to values as low as 2–5 mmHg (84). Is this hypoxia? Is oxygen availability limiting exercise at cytochrome oxidase within these ranges? These questions, which are far beyond the scope of this review, exist in a zone of uncertainty and have been the subjects of ongoing debates for decades in the physiological literature. However, to evaluate the potential for ROS production, it is relevant to consider the probability that skeletal muscle experiences hypoxia in relatively normal physiological conditions. Furthermore, considering that the best estimates of intracellular PO2 are based on average values within a relatively large muscle mass, it is likely that local temporal (27) and spatial (61) PO2 inhomogeneities exist during dynamic exercise, resulting in areas of localized reductions in PO2.

NADH redox status in muscle. As mentioned, one way to assess intracellular oxygen availability is to monitor ongoing NADH/NAD+ redox status. Unfortunately, there is little agreement as to what happens to NADH/NAD+ in intact exercising muscle (87), and results depend entirely on exercise intensity. The most convincing data, however, come from chemical measurements in muscle biopsies at various levels of exercise (87). After 10 min of 40% of maximal steady work load, NADH content drops significantly, but at 75% or 100% of maximal exercise, NADH content increases from resting levels. Similar findings of a drop in NADH/NAD+ were found in isolated Xenopus fibers at elevated PO2, but these responses reversed at low PO2 during exercise (48). The elevation in NADH during heavy exercise may not represent local hypoxia, as such. For example, Wilson and colleagues (107) have reported that the electron transport chain becomes more reduced in conditions in which electron flux does not change and PO2 is orders of magnitude higher than the Kn for the reaction of O2 with cytochrome oxidase. This increase in the reduction state of the electron transport chain has been described as one of several normal mechanisms used by mitochondria to maximize the rate of ATP formation in high metabolic states such as exercise (87). Regardless of whether it represents hypoxia, as such, it still represents a necessary precondition for promotion of ROS formation from some intracellular generator.

Steady-state levels of NADH/NAD+ do not provide information regarding changes in redox state that can occur in transitions of exercise. Techniques employing NADH autofluorescence
spectroscopy are the only tools currently available for these measurements. There are questions regarding the accuracy of this method in blood-perfused muscle, but considerable efforts have been made to overcome these limitations (67). In essentially all tissues studied, over 90% of the signal arising from surface fluorescence comes from mitochondrial NADH (67). This not only may be due to the overall compartmental concentrations of NADH but could also relate to the fluorescence lifetime of NADH in mitochondria being ~5 times longer than in the cytosolic compartment (106). In perfused dog limb muscle studies using repeated contractions, surface NADH autofluorescence (using tissue fluorometry) is shown to go down (i.e., becomes more oxidized) with exercise, believed in part to be due to increasing ADP available with increased work load (51). In contrast, during sustained contractions in humans, in which blood flow is restricted by elevations in tissue pressure, rapid elevations in NADH are observed at forces of ~50% of maximum, exceeding two times the resting values over a few seconds (39). Although real-time measurements of autofluorescence NADH in intact muscles have some limitations with respect to interpretation, it appears that in certain kinds of contractions or conditions of limited blood flow, anemia, or hypoxia due to heart or lung disease, NADH levels are likely to increase with physiological exercise. Whether this can strictly be defined as tissue hypoxia remains an open question, but we speculate that these conditions are likely to underlie ROS formation.

**Intermittent hypoxia in muscle.** Perhaps the most common form of hypoxia in nature may be “intermittent” hypoxia where transient falls in oxygen delivery occur over time in a variety of disease states or during conditions of exercise in hypoxic environments (22). Our experiments have demonstrated that ROS formation in skeletal muscle appears to peak over a short time during the transition to hypoxia, where NADH is rapidly rising and PO2 is falling (114), making conditions of intermittent hypoxia a likely facilitator of increased ROS formation. This may explain the oxidative stress, now relatively well established, that is associated with clinical and experimental conditions of intermittent hypoxia (78). Transient dips into tissue hypoxia may also occur in some subjects in the transition to steady-state levels of exercise, as observed from skeletal muscle microvascular measurements in older animals (10).

### Table 1. K<sub>m</sub> values for reaction with O₂ in ROS-generating or O₂-consuming reactions

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; for O₂, mmHg</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome &lt;i&gt;c&lt;/i&gt; oxidase&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.03–0.3</td>
<td>26, 104</td>
</tr>
<tr>
<td>Mito NADH oxidase (complex I)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>See below</td>
<td>59</td>
</tr>
<tr>
<td>NAD[P]H oxidase&lt;sup&gt;e&lt;/sup&gt;</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>NADH oxidase&lt;sup&gt;a,d&lt;/sup&gt;</td>
<td>11.4</td>
<td>20</td>
</tr>
<tr>
<td>Xanthine oxidase&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29–139</td>
<td>104</td>
</tr>
<tr>
<td>nNOS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>158</td>
<td>30</td>
</tr>
<tr>
<td>nNOS&lt;sup&gt;b&lt;/sup&gt;</td>
<td>202</td>
<td>98</td>
</tr>
<tr>
<td>eNOS&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.3</td>
<td>98</td>
</tr>
</tbody>
</table>

**Notes:**
- ROS, reactive oxygen species; Mito, mitochondrial; nNOS, neuronal nitric oxide synthase (NOS); eNOS, endothelial NOS. *Used for comparison. <sup>b</sup>25°C. <sup>c</sup>37°C. <sup>d</sup>From kidney. <sup>e</sup>Complex I, as a superoxide generator, shows a linear response to PO2 up to 1 atm.
derived NO would be shut down, removing its potential governing effects on local oxidation reactions and on mitochondrial respiration (56). Finally, in hypoxia the inhibitory influence of local NO on cytochrome oxidase becomes more dominant. This could also influence ROS formation by producing more upstream electronegativity within the electron transport chain.

**NADPH oxidases in skeletal muscle.** A third category of important enzyme systems that could be involved in local \( O_2^- \) production are the NAD(P)H oxidases, which have been shown to be expressed in skeletal muscle cells (50). Their functional role, how they are regulated, and the expression of various isoforms under differing conditions and in different muscle phenotypes have not been well characterized. Their relatively high \( K_m \) for \( O_2 \) (Table 1 comes from studies on kidney tissue) makes them appear an unlikely candidate for significant ROS formation in severe hypoxia. Being localized on the sarclemma (50), they would be exposed to a somewhat higher \( P_{O_2} \) than enzyme systems deeper in the muscle cell, particularly compared with the mitochondria. Although there is some evidence that NADPH oxidases are stimulated to produce ROS under conditions of hypoxia, in most systems, particularly in the pulmonary vasculature, ROS production from NADH(P)H oxidase has been shown to decrease with hypoxia (for review, see Refs. 60, 108). Other points of NAD(P)H oxidase control include activation and assembly of the protein complex at the membrane and the availability of reducing equivalents in the form of NADPH or NADH to drive the reaction, both of which can act as substrates. Cytosolic NADH rises, either transiently or steadily during hypoxia, and could influence NAD(P)H oxidase activity if sufficient \( P_{O_2} \) were available. Exposure of skeletal muscle to extracellular NADH and NADPH has been shown to cause apparent NAD(P)H-dependent ROS formation to increase (50), although it has never been understood how these substrates can access the NAD(P)H oxidase, since they diffuse poorly across membranes. An intriguing finding that exposure of vascular tissue to lactate can increase ROS formation, presumably through elevations in NADH produced by the lactate dehydrogenase reaction (109), raises the possibility of an additional mechanism for control of ROS formation in vivo hypoxic or exercising skeletal muscle.

**Myoglobin oxidation.** Another potential source of ROS in hypoxia or ischemia could come from the autoxidation of myoglobin. Recently, it has been shown that like hemoglobin, autoxidation of myoglobin to metmyoglobin is increased markedly by elevations in cytosolic NADH. Decreases in \( P_{O_2} \) and acidification further facilitate the autoxidation (71). Autoxidation of myoglobin can be a significant source of ROS and has been proposed to contribute to myocardial injury in ischemia-reperfusion (40). Whether such a mechanism is important in hypoxic skeletal muscle is not known at this time.

**Mitochondrial sources of ROS.** The strongest candidate for the source of hypoxia-induced ROS formation may be the mitochondria. Recent evidence has demonstrated considerable ROS production in skeletal muscle mitochondria, particularly from type II fibers compared with type I fibers (2). For a very lucid discussion of the potential for mitochondria to produce ROS during hypoxia in nonmuscle cells, refer to the recent review by Guzy and Schumacker (42). In short, evidence is based on studies in which blocking complex I and II in cardiomyocytes attenuates the severity of hypoxia-induced ROS when measured with fluorescent probes (29), and myxothiazol, a specific inhibitor of complex III at the binding site of ubiquinol to Qo, also inhibits hypoxia-induced ROS formation (102). These results suggest that the primary source of ROS formation in hypoxia in most cells studied thus far is the Q cycle, where the ubisemiquinone radical can share an electron with \( O_2 \). Exactly why this would happen in hypoxic environments is not clear, but Gilie and Nohl (37) have been proponents of the idea that leakage of electrons at the Q cycle requires changes (increases or decreases) in the fluidity of the inner mitochondrial membrane, which makes the ubisemiquinone more accessible for longer periods necessary for oxygen reduction. In some preparations, hypoxia or ischemia has been shown to alter mitochondrial membrane fluidity as measured by fluorescence anisotropy (49); therefore, this mechanism remains a viable contender.

**FUNCTIONAL SIGNIFICANCE OF HYPOXIA-INDUCED ROS FORMATION**

**ROS signaling in hypoxia and other stressful environments.** Skeletal muscles have the incredible ability to withstand high levels of stress. They are extremely resistant to heat exposure, osmotic stress, deformation, and hypoxia. For example, compared with the kinds of ischemia-reperfusion injury seen in cardiac or brain tissue, it requires \( \sim 3 \) h of complete ischemia to cause the beginnings of significant permanent injury to limb muscle on reperfusion (for review, see Ref. 12). The working hypothesis of our laboratory has been centered on the idea that ROS produced in hypoxia, or other stress environments common to skeletal muscle such as heat stress (115) or osmotic stress (72), have an underlying role in protecting muscle cells. In the studies of Mohanraj et al. (70) and Wright et al. (110) antioxidant treatments during hypoxia resulted in improvements in contractile force, suggesting that hypoxia-induced ROS inhibits contractile function in a way that is not related to loss of energy status of the tissue (110). This points to a potential functional role for ROS in protecting hypoxic muscle cells by inhibiting contractile function and preserving energy stores during low energy states. This idea is analogous to the “protective” role of fatigue in overstimulated muscle and may reflect the effects of ROS on calcium signaling as discussed below. It is also analogous to downregulation of ATP utilization observed in hypoxic cells as described by Chandel et al. (18) and Budinger et al. (15). In hypoxic environments, cells enter a kind of hibernation state, which has been linked to a mitochondrial ROS signaling event (42).

Another important pathway by which ROS could contribute to cell signaling responses to hypoxia involves its potential influence on the hypoxia-inducible factor (HIF) family of proteins (91). Although this is an area of active research and ongoing debate as discussed in recent reviews (42, 92), considerable evidence exists for a role of ROS in stabilization of HIF-1\( \alpha \), particularly at low \( P_{O_2} \) environments. HIF-1\( \alpha \) is constantly degraded in normoxic conditions but is stabilized in hypoxia, leading to activation of a variety of hypoxia-sensitive genes. Continuous degradation is ensured by the hydroxylation of a critical prolyl group on the HIF-1\( \alpha \) protein by proline hydroxylases (31). In turn, the activity of proline hydroxylases has been shown to be exquisitely sensitive to inhibition by ROS, with >50% inhibition following exposure of cells to 10
μM peroxide (74). Therefore, ROS formation could play an important primary or complementary role with other mediators in signaling adaptive responses to hypoxia.

**ROS and calcium regulation.** The potential influence of ROS on calcium regulation is not completely understood. Several studies have suggested that ryanodine receptors, skeletal muscle Ca\(^{2+}\) release channels (RyR1), are activated by exposure to oxidants (36, 36), by the activity of local NADH oxidases on the sarcoplasmic reticulum (SR) (111), or by the influence of reactive nitrogen species (33, 34). However, in intact muscle, mild exposure to oxidants inhibits calcium release and elevates Ca\(^{2+}\) sensitivity (4), whereas exposure to higher levels of oxidants for longer periods increases Ca\(^{2+}\) release but reduces force production (3). All of these studies have evaluated exogenously administered oxidants. The impact of endogenous oxidants of various species within the microenvironment of the SR, as might occur in hypoxia, remains unknown.

Alternatively, ROS produced in hypoxia could have important influences on ion channels at the sarcolemmal membrane. In many cells, voltage-dependent potassium channels (Kv) decrease their conductance in hypoxia (for review, see Ref. 62). The loss of Kv conductance leads to membrane depolarization, Ca\(^{2+}\) mobilization via voltage-dependent Ca\(^{2+}\) channels, and activation of an effector response (e.g., contraction, secretion, etc.). We know that skeletal muscle membranes depolarize when exposed to even mild hypoxia (32), and in severe hypoxia, significant calcium begins to accumulate in the cell within a few minutes, causing contraction (110). Contraction in hypoxia is due to elevations in cytosolic Ca\(^{2+}\) because it can be eliminated by lowering extracellular calcium to zero. The underlying mechanism that causes contraction in severe hypoxia is not known, but treatment with antioxidants, specifically O\(^{2-}\) scavengers, prevents its occurrence, suggesting that it is linked in some way to ROS formation during hypoxia and not to the energy state of the muscle (110). When subjected to levels of exogenous H\(_2\)O\(_2\) of >50 μM, muscles demonstrate a similar contracture (unpublished observation). These observations suggest that ROS play a role during hypoxia by promoting movement of calcium from either the extracellular fluid or the SR, or by preventing its removal from the cytosol, possibly through inhibition of sarcoplasmic Ca\(^{2+}\)-ATPase. It is difficult to imagine that the latter has a protective effect on the cells but may be a more general programmed response to hypoxia that a variety of cells use in their respective roles as oxygen sensors and effectors of response to hypoxia (62). Regardless, the net effect of high intracellular Ca\(^{2+}\) over long periods of time would be to promote protease activity, membrane degradation, and ultimately injury to the cell.

**ROS and preconditioning.** Another intriguing idea is that hypoxia-induced ROS play a role as a preconditioning stimulus, initiating cell signaling mechanisms that protect the muscle from subsequent stress exposure. Kohin et al. (38) have demonstrated preconditioning in isolated skeletal muscle fibers by exposure to brief hypoxic episodes. These cells were subsequently protected from severe hypoxia exposure. Myotubes preconditioned with H\(_2\)O\(_2\) exposure are also protected from subsequent stress (68), resembling known findings of preconditioning stimuli in cardiac tissue, believed to be dependent, in part, on ROS signaling (88).

**ROS and paracrine signaling.** ROS formation during hypoxia could act as a signaling agent, coordinating function between intracellular compartments (24), or in a paracrine role, signaling between adjoining cells (7). Inside the cell, recent experiments have demonstrated that creating a localized region of light-induced oxidation near mitochondria in isolated cardiac myocytes sets up a synchronized pattern of ROS production, NADH redox waves, and membrane potential oscillations in the mitochondria, across the cell (6). The physiological significance of these oscillations is not known, but they are believed to be important coordinated responses to ischemia-reperfusion and to influence Ca\(^{2+}\) currents and excitability across the entire cell volume (24). With respect to potential paracrine responses, unlike many other ROS species, H\(_2\)O\(_2\) diffuses well across lipid membranes and has relatively long diffusion distances. In the vasculature, it is known to be a vasoconstrictor under basal conditions and a vasodilator in agonist-constricted vessels (7) and has been hypothesized as an important vasodilator in skeletal muscle vasculature (79). Recently, Richardson et al. (82) have demonstrated that administration of an antioxidant cocktail to exercising humans causes vasoconstriction in the exercising limb. The location of intrafibrillar mitochondria adjacent to the t tubules and the high density of subsarcolemmal mitochondria at the cell membrane make the extracellular space highly accessible for diffusion. It is interesting to speculate that hypoxia-induced ROS formation could function in a feedback loop to promote vasodilatation of local vessels, a mechanism not unlike the role of NO, but could operate more effectively in conditions of hypoxia rather than ischemia.

**ROS and metabolism.** Another potential role of hypoxia-induced ROS is in activation of energy-utilization pathways. For example, GLUT-4 mobilization and glucose uptake across the cell membrane are regulated by many factors related to both insulin and contraction, but recently it has been shown that contraction-stimulated glucose uptake is augmented by endogenous and exogenous oxidants in skeletal muscle (89). This appears to operate via the AMP-activated protein kinase (AMPK) system, which other investigators have previously shown is ROS sensitive (45, 99, 113). However, these observations remain controversial (35). As little as 50 μM exogenous H\(_2\)O\(_2\) exposure appears to be all that is necessary to activate the AMPK pathway in isolated myoblasts (45). Since there is believed to be an eightfold decrease of H\(_2\)O\(_2\) signal in the intracellular compartment compared with the extracellular compartment (96), this may be in the range of normal physiology.

**SUMMARY AND FUTURE DIRECTIONS**

It appears that a small but significant ROS signal is produced during exposure to hypoxia that may be within the range of normal physiological or mildly pathophysiological signaling mechanisms. Similar signals have been observed in the heart and other tissues. More work needs to be done in all tissues to verify that the signal is actually ROS and not related to chemical or biophysical effects unrelated to ROS. Techniques such as spin trapping would be ideal improvements, but because of the fast reducing power of the intracellular environment in tissues, particularly in hypoxia, these methods have not proven useful to date for this purpose. The molecular and intracellular origins of the signal are simply not known, although mitochondria appear to be involved, on the basis of
studies done in the heart and in isolated cells (42). The physiological significance of the ROS produced under such conditions is also not known, but there are extremely intriguing possibilities discussed here that would suggest that ROS could play a variety of important roles in the responses to hypoxia, disorders of oxygen uptake-delivery, and other forms of metabolic stress. Finally, the importance of this signal may lie in pathological responses to sustained hypoxia or possibly repeated hypoxia-reoxygenation exposures that would be typical of chronic disease or conditions of exercise during long-term altitude acclimatization. The link between observations in normal physiological states and those seen in more chronic or pathological exposures is far from being resolved, but understanding this relationship could have important implications to health care and preservation of quality of life in patients with disorders of oxygen supply.

ACKNOWLEDGMENTS

The authors thank Valerie Wright and John Merola for helpful editing, discussions, and suggestions.

GRANTS

This study was supported by the National Heart, Lung, and Blood Institute Grant HL-53333.

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