Reactive oxygen species formation in the transition to hypoxia in skeletal muscle

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Submitted 13 September 2004; accepted in final form 17 February 2005

Zuo, Li, and Thomas L. Clanton. Reactive oxygen species formation in the transition to hypoxia in skeletal muscle. Am J Physiol Cell Physiol 289: C207–C216, 2005. First published March 23, 2005; doi:10.1152/ajpcell.00449.2004.—Many tissues produce reactive oxygen species (ROS) during reoxygenation after hypoxia or ischemia; however, whether ROS are formed during hypoxia is controversial. We tested the hypothesis that ROS are generated in skeletal muscle during exposure to acute hypoxia before reoxygenation. Isolated rat diaphragm strips were loaded with dihydrofluorescein-DA (Hffluor-DA), a probe that is oxidized to fluorescein (Fluor) by intracellular ROS. Changes in fluorescence due to Fluor, NADH, and FAD were measured using a tissue fluorometer. The system had a detection limit of 1 μM H2O2 applied to the muscle superfusate. When the superfusion buffer was changed rapidly from 95% O2 to 0%, 5%, 21%, or 40% O2, transient elevations in Fluor were observed that were proportional to the rise in NADH fluorescence and inversely proportional to the level of O2 exposure. This signal could be inhibited completely with 40 μM ebselen, a glutathione peroxidase mimic. After brief hypoxia exposure (10 min) or exposure to brief periods of H2O2, the fluorescence signal returned to baseline. Furthermore, tissues loaded with the oxidized form of the probe (Fluor-DA) showed a similar pattern of response that could be inhibited with ebselen. These results suggest that Fluor exists in a partially reversible redox state within the tissue. When Hffluor-loaded tissues were contracted with low-frequency twitches, Fluor emission and NADH emission were elevated in a way that resembled the hypoxia-induced signal. We conclude that in the transition to low intracellular PO2, a burst of intracellular ROS is formed that may have functional implications regarding skeletal muscle O2-sensing systems and responses to acute metabolic stress.

dihydrofluorescein; tissue fluorometer; ebselen; N-acetylcysteine; rat

THE FORMATION OF REACTIVE OXYGEN SPECIES (ROS) in skeletal muscle has been described in response to muscle stimulation, heat stress, and ischemia-reperfusion injury (21, 23, 37). However, whether ROS are generated during hypoxia before reoxygenation is controversial. It is generally held that in low-O2 environments, it is difficult to form superoxide (O2−), the precursor of most forms of ROS (2). Furthermore, it is well known that conditions of high-O2 exposure promote the formation of ROS (34) and that vascular O2-sensing systems have been attributed to increases in ROS formation that are proportional to rising PO2 within the vasculature (14, 15).

ROS generation has been thought to be more likely during reoxygenation because hypoxia causes a significant accumulation of reducing equivalents in the mitochondria, such as NADH and FADH. Sudden exposure to O2 can promote the formation of O2− through intermediate electron carriers and one electron reduction of molecular O2 in the mitochondrial electron transport chain. However, there is also considerable evidence for ROS formation in hypoxia or ischemia in some tissues, such as the heart. For example, Vanden Hoek et al. (35) and Damerau et al. (4) observed increased ROS production during hypoxia in cardiac myocytes. These data are consistent with earlier observations by Park et al. (20), who used electron spin resonance (ESR) in intact hearts during ischemia, and Kevin et al. (9), who used redox-sensitive fluorescent probes in the intact heart. Liu et al. (13) also identified ROS formation in hypoxic distal pulmonary arteries using ESR. The possibility that both low and high O2 can cause ROS formation in specific circumstances stands as one of the more interesting paradoxes of free radical biology.

In previous studies, we observed that antioxidants administered during hypoxia exposure protected skeletal muscle contractile function (16, 36). On the basis of that previous work, we hypothesized that ROS are produced in skeletal muscle during hypoxia and that they produce a signal that has functional significance with regard to acute adaptations to hypoxia. In the present study, we used a tissue fluorescence system to monitor ROS production and simultaneously monitor changes in intracellular pyridine nucleotides, in real time, during hypoxia in intact, nonperfused diaphragm muscle. The pyridine nucleotides (NADH and FAD) were used as indirect indicators of muscle tissue oxygenation. We then applied this method to evaluate whether tissue hypoxia occurs during fatiguing muscle contractions in vitro superfused rat diaphragm and whether there are associated elevations in ROS production.

METHODS

Animal treatment and tissue preparation. Male Sprague-Dawley rats (350–450 g) were housed and treated according to approved protocols of The Ohio State University Institutional Laboratory Animal Care and Use Committee. The rats were anesthetized with ketamine (~76 mg/kg)/xylazine (~15 mg/kg), tracheotomized, and mechanically ventilated. Each diaphragm was removed and dissected into several muscle strips. For muscles mounted in fixed chambers for hypoxia studies, the tissues were stretched to ~120% slack length and attached to clear plastic frames using superglue gel (Scotch; 3M, Minneapolis, MN) on the tendon, rib, and lateral borders of the muscle (Fig. 1, A and B). For diaphragm strips used in fatigue studies, a portion of the central tendon was superglued to a plastic strip and attached to a force transducer, while the rib was tied to a glass holder with suture (Fig. 1C). Before the experiments, the strips were kept in

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Ringer solution (in meq/l: 21 NaHCO₃, 1.0 MgCl₂, 1.2 Na₂HPO₄, 0.9 Na₂SO₄, 2.0 CaCl₂, 5.9 KCl, and 121 NaCl, as well as 2.07g/l glucose and 10 μM d-tubocurarine) at room temperature and bubbled with 95% O₂-5% CO₂.

**Chemicals.** The following chemicals were used: dihydrofluorescein diacetate (Hfluor-DA; Molecular Probes, Eugene, OR), fluorescein diacetate (Fluor-DA; Molecular Probes), H₂O₂ (ACROS Organic), ebselen (Alexis Biochemicals), and N-acetyl-cysteine (NAC; Sigma, St. Louis, MO). Hfluor-DA, Fluor-DA, and ebselen were dissolved in DMSO stock solutions. The DMSO concentration in the tissue bath was kept at <0.8% (vol/vol) in all experiments to avoid possible artifact (24, 39). H₂O₂ stocks were made in distilled water.

For H₂O₂ detection, tissue strips were loaded with 50 μM Hfluor-DA at room temperature for 30 min. Hfluor was chosen over other similar redox-sensitive probes because it has considerable advantages with regard to loading and for its relative lack of sensitivity to nitric oxide, Fe²⁺, and peroxidase activity compared with more commonly used probes, such as dichlorofluorescein or dihydrorhodamine 123 (7, 38).

The tissues were washed in fresh buffer for 10 min after the loading period (38). In some cases, the loading time was doubled to increase the detection sensitivity, although this had little effect on the overall outcome of the experiments. For evaluation of the behavior of the oxidized probe within the muscle tissue, 0.05 μM Fluor-DA was loaded for 30 min at room temperature. Ebselen, a glutathione peroxidase mimic used primarily as a scavenger of ROS (30), was applied to the treated tissues at 40 M in the loading solution and throughout the rest of the experiment. Ebselen was found to be extremely sensitive to degradation in oxygenated buffer solutions, and therefore fresh ebselen stock was added directly to the buffers shortly before use.

**Tissue chamber and fluorometer system.** For the majority of hypoxia experiments, after fluorophore loading, tissue strips were transferred to a superfusion chamber. During initial development of this technique, and for some of the data collection, we used a commercially available chamber (model RC-27; Warner Instruments) that allowed ~1 mm of space under the tissue for continuous superfusion of both sides of the tissue. This system had a large head space region of both sides of the tissue. This system had a large head space region that was covered loosely with a plastic top, making changes in tissue O₂ less rapid and temperature difficult to control. The excitation and emission optical fibers were combined in one cable for epifluorescence measurements. We refer to this technique as “surface fluorometry.” For the majority of data collected, a custom-made enclosed chamber was used as shown in Fig. 1, A and B, which allowed for better control of temperature, chamber PO₂, more uniform flow, and near-elimination of air bubbles. An O₂ electrode (ISO₂; World Precision Instruments) was used in some experiments for monitoring chamber PO₂ levels, and a thermistor was used to measure and servocontrol the chamber temperature (Warner Instruments). For this configuration, the fluorescent signal was measured on the opposite side of the tissue with independent excitation and emission cables, a configuration that we refer to as “transmission fluorometry.” This technique has some advantages at some wavelengths. For example, on the basis of the optical properties of the rat diaphragm, we have modeled the light scattering, absorption, and escape of both the excitation and emission light and have determined that this configuration results in a relatively uniform estimate of fluorescence across the thickness of the tissue for wavelengths used in each Fluor measurement (3).

Fluorescence was detected using a ratiometric tissue fluorometer (Radnoti). The excitation beam from a 150-W Xenon lamp was focused on a 6-mm-diameter fiber optic cable, and the light passed through a filter wheel containing four specific band-pass filters. A second filter wheel with four emission filters was used to isolate emission light at specific wavelengths. This light was focused on the photomultiplier tube (PMT) (model HC 120-05MOD-6308; Hamamatsu PMT Assembly, Japan). Thus the tissue fluorometer could sequentially measure four channels of fluorescence. The three fluorescent channels used for this study were NADH autofluorescence (NADH, excitation 330-380 nm, emission 470-530 nm), fluorescein fluorescence (FAD/Fluor, excitation 490-500 nm, emission 535-545 nm), and FAD autofluorescence (FAD, excitation 455-460 nm, emission 630-650 nm). These three different excitation and emission ranges provided a relatively complete observation window for tissue autofluorescence for both NADH and FAD and for the fluorescence from the Fluor probe. There was no overlap of the Fluor fluorescence signal in the other two channels and no overlap of NADH in any other channel. There was overlap of the FAD autofluorescence signal in the Fluor channel as described herein. To minimize light interference, the tissue chamber was housed in a solid black metal box. The PMT output was collected on a personal computer using an analog-to-digital converter, and measurements from each filter set were signal averaged, recorded, and graphed using a program developed in a modified programming language (Workbench PC; Strawberry Tree). The light shutters were driven by an automated system in which the tissue was exposed for between 3 and 7 s every 30 s. The rotation of
the filter wheel exposed the tissue to each excitation wavelength for ~8.5 ms every 40 ms. The amount of light reaching the tissue was therefore reduced as much as possible to avoid photobleaching and photooxidation of the fluorophores. The tests of photooxidation and photobleaching that were performed were similar to those described by Murrant et al. (17), and we observed no measurable effects of this exposure time on the rate of Hfluor oxidation or Fluor disappearance (data not shown).

**Hypoxia experiments.** Most experiments were performed at 23°C because the oxidized probe (Fluor) apparently leaked at a faster rate from tissues maintained at 37°C. However, some entire experiments were performed at 37°C, where noted, and qualitatively similar results were observed at both temperatures. Experiments were generally conducted with groups of four tissues from each animal, with tissues maintained in oxygenated buffer at 23°C until use. Once mounted in the chamber, tissues were superfused with Ringer solution and then prebubbled with 95% O₂-5% CO₂ for 10–15 min or until the fluorescent emission was relatively stabilized. Drift of the Fluor signal was often observed during the experiment, but it was attributed to the balance of background ROS production and leakage or reduction of the oxidized probe. A slow drift upward in the Fluor signal was most evident at 23°C, whereas Fluor signals tended to drift downward at 37°C.

Hypoxia was induced by switching the superfusate to a separate buffer source, preequilibrated at the set temperature and O₂ concentration (0%, 5%, 21%, and 40%) and CO₂ concentration (5%). Approximately 3 min were required to achieve 90% of the final plateau in PO₂ within the chamber as measured using the O₂ electrode. After hypoxia, tissues were reoxygenated with 95% O₂. The level of O₂/CO₂/N₂ during hypoxia exposure was varied in the superfusate source using a gas mixing system (Reming Bioinstruments).

**H₂O₂ addition on tissues with antioxidant treatments.** To determine the sensitivity of the muscle and the detection system to ROS, tissues in the chamber were superfused with 1 or 10 μM H₂O₂ solution for 10 min. In parallel experiments, ebselen and NAC were administered with H₂O₂ as antioxidants to block the signal.

**Muscle contraction experiments.** Muscle strips were preloaded with 50 μM Hfluor-DA in oxygenated Ringer solution for 30 min at room temperature and then rinsed for 10 min in buffer. As shown in Fig. 1C, a specially designed tissue bath was used for these experiments. The emission and excitation cables were installed in the bath for transmission fluorometry apposing the tissue 1–1.5 mm from its surfaces. Once the strip was placed into a tissue bath at 37°C, it was maintained during an equilibration period with slow-twitch contractions at 0.05 Hz for 5–10 min and 95% O₂-5% CO₂. The tissue was then stimulated at 1 and 4 Hz for 5 min each, and it was allowed to rest for 10 min between stimulations. At the same time, fluorescence of NADH, Fluor, and FAD channels were monitored, collected, and analyzed.

**Fluorescence calculation and statistics.** Except where mentioned, the following approach was used to calculate the fluorescence signal. The Fluor signal during hypoxia and H₂O₂ exposure was characterized by a positive deflection, which returned approximately to baseline after exposures. In each curve, a baseline was established by selecting a stable point before and after the hypoxia or H₂O₂ administration. The vertical deflection between the extrapolated baseline and the peak point of the signal was divided by the extrapolated baseline signal at the same time point as a way of normalizing the overall fluorescence. For some Fluor and NADH signals, there was no real peak, and the “peak” selected was approximately where the NADH signal reached the maximum. For some FAD determinations, which have a negative deflection during hypoxia exposure, similar vertical distances between the baseline and the minimum were used in calculation. These data were then grouped and analyzed using statistics described in the figure legends.

**RESULTS**

**Tissue autofluorescence in hypoxia.** Typical autofluorescence with exposure to varying degrees of hypoxia at room temperature is shown in Fig. 2A. As expected, fluorescence in the range of NADH (channel 1) increased in hypoxia and decreased in the range of FAD (channels 2 and 3). For clarity,

![Fig. 2](http://ajpcell.physiology.org/)
the fluorescence intensity in Fig. 2 and all subsequent graphs was normalized as a fraction of initial background fluorescence during baseline measurements. Exposure to 0% O₂-5% CO₂-95% N₂ resulted in a 2–8% O₂ level in the chamber and was highly dependent on the location of the electrode within the chamber and its position relative to the muscle. The grouped data for autofluorescence in response to 10 min of 0% O₂ are shown in Fig. 2B. All measurements in this experiment were performed at 23°C (n = 6).

Effects of hypoxia on ROS production in Hfluor-loaded tissues. In Hfluor-loaded tissues (Fig. 3A), exposure to hypoxia resulted in elevations of the fluorescence intensity in the Fluor channel in the reverse direction from the autofluorescence measurements observed in unloaded tissue (i.e., Fig. 2A). Note that the signal returns to near-baseline at reoxygenation. This response could largely be eliminated by treatment with ebselen (Fig. 3B). Ebselen is a cell-permeable glutathione (GSH) peroxidase mimic and works with GSH to scavenge H₂O₂ and other ROS (8, 20). The grouped data for the responses, with and without ebselen, are shown in Fig. 3C (n = 5, P < 0.05, for post hoc contrasts at individual time points). Identical experiments were conducted with other antioxidants, namely, 10–20 mM NAC (n = 5) and 500 μM deferoxamine (n = 4). However, these had no significant effect on the elevation in the Fluor signal with hypoxia (data not shown). To determine whether the influence of ebselen was reversible, the tissues in some experiments were rinsed for 30 min in the absence of ebselen and the hypoxia exposure was repeated. This procedure resulted in a return of the hypoxia-induced increases in the Fluor signal (data not shown). Finally, experiments were performed with other redox-sensitive probes, 5-(and 6-)carboxy-2',7'-dichlorodihydrofluorescein diacetate (chemically similar to Hfluor), and hydroethidine, a probe sensitive to O₂•⁻ (37, 38). Both showed similar responses to acute hypoxia (data not shown).

Fig. 3. Reactive oxygen species (ROS) detection in tissues loaded with Hfluor. A: typical data in response to 0% O₂ exposure at 23°C. Note the transient rise in channel 2 with hypoxia. B: identical experiments in tissues preincubated in 40 μM ebselen. C: grouped data for Hfluor-loaded tissues in the presence and absence of ebselen during hypoxia. *P < 0.05 between hypoxia + ebselen and hypoxia alone at each time point by post hoc contrast analysis after two-way ANOVA, with time and treatment being the factors of interest (n = 5).
shown), suggesting that the oxidation of Hfluor and its apparent reversibility observed during hypoxia is not specific to the probe.

**Hypoxia-induced increases in ROS at various O2 levels.** Figure 4 (left) shows the influence of various levels of hypoxia (0%, 10%, 21%, and 40% O2) on the Fluor signal in Hfluor-loaded tissues at 37°C. The hypoxia-induced increase in the Fluor signal was also proportional to the degree of hypoxia. In Fig. 4 (middle and right), the autofluorescence signals for NADH and FAD were proportional to the degree of hypoxia. Qualitatively similar signals were observed for 0% O2 studies at 37°C compared with those observed at 23°C (Fig. 2). Interestingly, even at acute exposure to 40% O2 at 37°C, there is a significant elevation in NADH and reduction in FAD, suggesting the possibility that tissue hypoxia occurs in the core of diaphragm samples at this level of O2 in the superfusate.

**Response to hypoxia after loading with the oxidized form of the probe (Fluor-DA).** Because of the reversibility of the hypoxia-induced signals after reoxygenation as shown in Fig. 3, we wished to observe the behavior of the oxidized form of the Hfluor probe when loaded into the tissue. Tissues were loaded with 0.05 μM Fluo-DA, which, by trial and error, resulted in baseline Fluor fluorescence roughly equivalent to that observed after 50 μM Hfluor loading. Figure 5A demonstrates typical results for the hypoxia signal after Fluor loading. Curiously, the signal during hypoxia was qualitatively similar to that observed with the reduced form of the probe. Furthermore, Fig. 5B demonstrates that 40 μM ebselen inhibits the response as observed with Hfluor loading. Compared with autofluorescence, the rise in Hfluor with hypoxia was highly significant as shown in Fig. 5C (n = 5; P < 0.001). These data...
suggest the possibility that Fluor can be reduced to Hfluor by the tissue.

Additional control experiments were performed to rule out the possibility that O2 quenching of the fluorophore was responsible for the reversibility of the hypoxia-induced signal. In vitro cuvette experiments appeared to rule out a significant O2-quenching effect, but confirmation of this result was demonstrated by testing the influence of hypoxia and hyperoxia exposure on diaphragm that was no longer living (40-h refrigeration after removal from rat) but was loaded with Fluor at baseline fluorescence levels equivalent to that of living tissue. No hypoxia-induced signal could be reproduced in dead tissue (data not shown).

Sensitivity of the ROS detection system to H2O2. Figure 6 shows the results of H2O2 superfusion onto Hfluor-DA-loaded tissues. Figure 6A shows a typical response to 1 and 10 μM H2O2. Note the rapid reversibility of the H2O2-induced rise in fluorescence that resembles the nature of the hypoxia-induced signal. Changes of both NADH and FAD autofluorescence were essentially no different from noise in response to 1 and 10 μM H2O2. In Fig. 6B, both 1 and 10 μM H2O2 resulted in significant, transient increases in the Fluor fluorescence compared with baseline autofluorescence (n = 5; P < 0.05). Compared with control (H2O2), these signals were greatly attenuated in tissues treated with the antioxidants ebselen (n = 5; P < 0.01), NAC (n = 5; P < 0.05), and ebselen + NAC (n = 4; P < 0.05). The change of Fluor signal induced by 10 μM H2O2 was significantly larger than 1 μM H2O2 (n = 5; P < 0.05), suggesting that the tissue was optimally loaded with probe for ROS detection within this range of ROS exposure. Additional experiments were performed on oxidized probe (Fluor-DA-loaded tissue), which also showed sensitivity to H2O2 compared with autofluorescence (n = 6; P < 0.05). This sensitivity could be blocked significantly by ebselen or NAC treatment (P < 0.05).

ROS production during twitch stimulation. Typical fluorescent data for studies of ROS production in response to twitch (TW) stimulation are shown in Fig. 7. For purposes of illustration and for comparison with previously described data, brief exposure to hypoxia was performed, which resulted in an immediate increase of both the NADH and Fluor signals and a decrease of FAD autofluorescence. In response to 1- and 4-Hz twitch stimulations, the change of fluorescence in these three channels showed a pattern similar to that of previously described hypoxia data, suggesting the generation of ROS during low-frequency stimulation and apparent tissue hypoxia in this setting. Figure 7, top, shows the raw force data obtained during the stimulation, and it is clear that at these stimulation frequencies and in this testing chamber, the muscles were slowly fatiguing. The grouped data are summarized in Fig. 8. We found that 1-Hz TW significantly increased Fluor signal compared with autofluorescence (n = 6 for 1-Hz TW autofluorescence, n = 9 for 1-Hz TW loaded; P < 0.05), as did the 4-Hz stimulation frequency (n = 6 for 4-Hz TW autofluorescence, n = 8 for 4-Hz TW loaded; P < 0.05). However, there was no significant difference between 1- and 4-Hz-treated tissues.

DISCUSSION

This study provides evidence for intracellular ROS production in skeletal muscle in the transition to acute hypoxia and in contracting superfused muscle. Because the diffusion gradient for O2 varies across the thickness of a nonperfused tissue such as rat diaphragm, it is not possible with the use of this technique to discern the level of cellular hypoxia at which significant ROS are produced. However, it seems likely that the...
signal occurs in physiological or mildly pathological states of muscle hypoxia, because 1) the magnitude of the Fluor signal was inversely proportional to the level of O2 exposure, 2) the signal was present even when only small changes in NADH or FAD could be detected, and 3) the Fluor signal emerged very early during the hypoxia exposure. We attribute our ability to see this hypoxia-induced signal in intact muscle to the sensitivity of the measurement technique.

At this low range of change in ROS activity, it appears that the fluorescent probe, Hfluor, and related compounds exist in a reversible redox state. This observation is based on the evidence that brief hypoxia, exposure to low concentrations of H2O2, and muscle contractions resulted in only transient increases in fluorescence, which were immediately reversed upon reoxygenation, perfusion with fresh buffer, or discontinuation of stimulation, respectively. The hypothesis is also supported by the observation that when the oxidized probe Fluor-DA is loaded into the tissue, similar responses to both hypoxia and H2O2 can be observed and can be inhibited by cotreatment with ebselen. The apparent ability of tissue to reduce Fluor to Hfluor has not generally been considered in the literature. However, similar findings of the reversibility of the fluorescent signal for several redox-sensitive fluorescent probes have been shown in response to ischemia-reperfusion injury in cardiomyocytes (29, 35) and in intact hearts (9).

Critique of the ROS detection method. Compared with most other fluorescein derivatives, Hfluor is insensitive to nitric oxide, has low sensitivity to Fe2+, and has relatively low sensitivity to changes in peroxidase activity; yet, it maintains excellent loading characteristics and good sensitivity to H2O2 (7). We speculate that the predominant ROS signal measured during hypoxia in this study was H2O2, in part because the signal was inhibited by ebselen. Although ebselen may scavenge other ROS such as peroxynitrite (31), its primary targets are thought to be peroxides. Experiments in which nitric oxide synthase was inhibited have shown no inhibition of the hypoxia-induced fluorescence signal (data not shown), thus making it likely that peroxynitrite is not a predominant species in this setting. The effectiveness of ebselen as a scavenger of peroxides arises from its ability to use glutathione as an
systems and could identify no such effect in both Hfluor- and Fluor-loaded dead tissues. The H$_2$O$_2$ addition experiments provided an estimate of the range of sensitivity of our system. Although we were able to detect a clear signal with as low as 1 µM H$_2$O$_2$ in the superfusion medium, and because the intracellular H$_2$O$_2$ concentration has been estimated to be 7- to 10-fold lower than the extracellular H$_2$O$_2$ in such experiments (33), we estimate that our system has a threshold sensitivity to intracellular H$_2$O$_2$ of $\sim$100–200 nM.

There are a number of ways to measure fluorescence in intact tissues. Previously, we used laser-scanning confocal microscopy to detect intracellular ROS formation (37), and many other investigators have used this technique or other, more conventional types of epifluorescence microscopy. Tissue fluorometry has three major advantages over microscopy for some applications. 1) It obtains fluorescent signals from hundreds of cells simultaneously because of the large diameter of its detection platform. 2) Because of the relatively large observation area, most artifacts due to tissue or cell movements can be greatly minimized; movement can result in substantial problems in microscope experiments. 3) As mentioned earlier, the light source has only minimal effects of photobleaching or photodamage, whereas this phenomenon is common in microscope experiments. Therefore, the tissue fluorometer can be used as an effective spectroscopy tool for investigations in which details at the cellular level can be sacrificed.

The intracellular levels of NADH and FAD greatly affect autofluorescence in their respective emission ranges. Our results illustrate the critical importance of measuring their autofluorescence when studying redox behavior during hypoxia using fluorescent probes, because these signals are large and extend through much of the visible spectrum. Of particular importance is the fact that Fluor has excitation and emission ranges that lie within those of FAD, and when loading is poor, the emission signal of Fluor does not provide reliable information about ROS formation, owing to the FAD interference. However, we were able to overcome this problem by measuring and documenting autofluorescence in unloaded muscles and by measuring FAD autofluorescence in an accompanying channel that did not overlap the one used for Fluor measurements. The other great advantage of measuring FAD and NADH in these experiments is that they provide insights into the time course and extent of tissue hypoxia during the experiment. Because O$_2$ availability becomes limiting for cytochrome c oxidase, reducing equivalents in the form of cytosolic or mitochondrial NADH and mitochondrial FADH buildup and can be considered fluorescent sentinels of local hypoxia.

**Potential physiological significance of ROS production in hypoxia.** Normal intracellular Po$_2$ in perfused skeletal muscle is thought to be $\sim$10 Torr at rest, dropping to a mean value of 3–5 Torr during intense exercise (25) and not reaching a critical level until cellular Po$_2$ drops to $\sim$1.5 Torr (26). Whether this deoxygenation of skeletal muscle can be defined as hypoxia depends on one’s definition of hypoxia. The transient deoxygenation associated with acute intense exercise would be a likely example of tissue hypoxia in skeletal muscle, where mismatches between local O$_2$ consumption and perfusion have been shown to occur over a time frame of 2–3 min at the onset of moderate-intensity exercise (5). This rapid deoxygenation may be sufficient to induce a transient reactive
oxygen signal as described in this study. Furthermore, it is reasonable to predict that if the signal exists in intact organisms, it might be amplified in hypoxic patients with chronic heart or lung disease or during exercise at high altitude.

The muscle stimulation experiments shown in Figs. 7 and 8 were performed to demonstrate a direct and practical application of this technology and to investigate whether tissue hypoxia and ROS formation are likely to occur in isolated non-perfused muscles of this thickness. On the basis of the changes in autofluorescence within the NADH and FAD ranges, it is clear that isolated rat diaphragm does become hypoxic at this level of stimulation and in this in vitro setting. In normally perfused, steady-state, moderately exercising muscles, NADH generally decreases, indicating that in steady-state exercise, skeletal muscles do not generally become critically hypoxic at the tissue level, except under conditions of compromised O2 delivery (19) or during transient states of high exercise intensity (5) as discussed earlier. It is possible that the experimental configuration used in this study, which required the electrodes to be relatively close to the muscle surfaces (~1 mm), compromised O2 availability to the tissue underneath the fiberoptic probes, thus amplifying the hypoxia. However, it is just as likely to be due to the thickness of the rat diaphragm and the long diffusion gradients necessary to deliver O2 to the core of the tissue (~0.5–0.6 mm in diameter). Although reactive oxygen signals associated with contraction have been described extensively in the literature (6, 11, 18, 22, 23, 32), it is interesting to speculate that at least part of the ROS signal observed in previous studies may be secondary to local hypoxia induced by inadequate O2 supply during high O2 demand in isolated or compromised preparations.

What possible function could a transient ROS signal provide to exercising muscle? In heart muscle, ROS is thought to play an important role in the phenomenon of preconditioning (see, e.g., Ref. 9), resulting in the protection of heart muscle and vasculature during subsequent exposure to ischemia. The exact cell signaling pathway that is stimulated by ROS has been studied extensively, but it is complex and still poorly understood. Kohin et al. (10) demonstrated a similar phenomenon in single frog skeletal muscles in which brief exposures to hypoxia appear to precondition the fibers, protecting them from subsequent hypoxia. At this time, we can only speculate that the ROS signal that we have observed could have a functional role in preconditioning.

Paradoxically, our laboratory previously observed that antioxidants have a protective influence on skeletal muscle function during 30-min exposure to near anoxia (16, 36), suggesting that oxidants have a negative influence on contractile function. However, the role of oxidants at very low metabolic states may also be thought of as protective of the muscle in the same way that muscle fatigue is thought of as an inherently protective mechanism to prevent injury as a result of overstimulation. Therefore, oxidants in severe or prolonged hypoxia might provide a different kind of functional role from those observed in brief exposures.

Summary. This report provides evidence for hypoxia-induced ROS generation in skeletal muscle using tissue fluorometry. The possibility of internal conversion of Hfluor and Fluor was also noted in this research. We speculate that ROS formation during these transient hypoxic exposures may have an important role as a mediator of O2 sensing and cell adaptation. Such ROS signals may also work in synchrony with those produced during exercise-induced temperature elevation in muscle (37). Future studies to confirm these experiments in intact muscle using other complementary methods are needed to address the molecular source of hypoxia-induced ROS, to evaluate its potential role as an effenter arm of an O2-sensing system, and to determine its functional role in adaptation to exercise and exposure to stressful environments.

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

REFERENCES