Hypoxia-induced skeletal muscle fiber dysfunction: role for reactive nitrogen species

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Ottenheijm, Coen A. C., Leo M. A. Heunks, Maartje C. P. Geraedts, and P. N. Richard Dekhuijzen. Hypoxia-induced skeletal muscle fiber dysfunction: role for reactive nitrogen species. Am J Physiol Lung Cell Mol Physiol 290: L127–L135, 2006.—Hypoxia impairs skeletal muscle function, but the precise mechanisms are incompletely understood. In hypoxic rat diaphragm muscle, generation of peroxynitrite is elevated. Peroxynitrite and other reactive nitrogen species have been shown to impair contractility of skinned muscle fibers, reflecting contractile protein dysfunction. We hypothesized that hypoxia induces contractile protein dysfunction and that reactive nitrogen species are involved. In addition, we hypothesized that muscle reoxygenation reverses contractile protein dysfunction. In vitro contractility of rat soleus muscle bundles was studied after 30 min of hypoxia (PO2 ~90 kPa), hypoxia (PO2 ~5 kPa), hypoxia + 30 μM N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA, a nitric oxide synthase inhibitor), hypoxia + 30 μM L-NMMA, and hypoxia (30 min) + reoxygenation (15 min). One part of the muscle bundle was used for single fiber contractile measurements and the other part for nitrotyrosine detection. In skinned single fibers, maximal Ca\textsuperscript{2+}-activated specific force (F\textsubscript{max}), fraction of strongly attached cross bridges (α\textsubscript{sc}), rate constant of force redevelopment (k\textsubscript{f}), and myofibrillar Ca\textsuperscript{2+} sensitivity were determined. Thirty minutes of hypoxia reduced muscle bundle contractility. In the hypoxic group, single fiber F\textsubscript{max}, α\textsubscript{sc}, and k\textsubscript{f} were significantly reduced compared with hypoxic, 1-L-NMMA, and reoxygenation groups. Myofibrillar Ca\textsuperscript{2+} sensitivity was not different between groups. Nitrotyrosine levels were increased in hypoxia compared with all other groups. We concluded that acute hypoxia induces dysfunction of skinned muscle fibers, reflecting contractile protein dysfunction. In addition, our data indicate that reactive nitrogen species play a role in hypoxia-induced contractile protein dysfunction. Reoxygenation of the muscle bundle partially restores bundle contractility but completely reverses contractile protein dysfunction.

Recent studies have indicated that hypoxia enhances generation of reactive oxygen (ROS) and nitrogen species (RNS) in muscle (32, 47). For instance, hypoxia has been shown to enhance generation of superoxide anion in cardiac myocytes (13). Hypoxia-induced elevated free radical generation may impair contractile function. Indeed, our laboratory group and others have demonstrated that antioxidants improve rat diaphragm contractility under hypoxic conditions (20, 32, 44). Also, inhibition of nitric oxide (NO) synthases (NOS) has been shown to improve rat diaphragm contractility under hypoxic conditions, indicating that NO plays a role in hypoxia-induced contractile dysfunction of the diaphragm (45). Peroxynitrite, the reaction product of NO and superoxide anion, can induce cell injury and death by oxidation of sulfhydrys, induction of lipid peroxidation, and nitrination of tyrosine residues in protein to form nitrotyrosine (4, 37). On the other hand, depending on its concentration, peroxynitrite is able to act as a cytoprotective signaling agent, for instance, during reperfusion injury (29).

From our data as reported in laboratory (47) revealed that hypoxia-induced rat diaphragm dysfunction is associated with elevated diaphragm muscle nitrotyrosine levels.

In animal skinned single fibers, ROS and RNS have been shown to affect contractile properties, in a time- and concentration-dependent manner (10, 21, 30, 36), reflecting altered contractile protein function. As hypoxia enhances generation of ROS and RNS (32, 47), it is likely that hypoxia may affect contractile protein function. This was the first hypothesis of the present study. In addition, we evaluated the role of RNS in hypoxia-induced contractile protein dysfunction. In the present study we also hypothesized that inhibition of NOS attenuates hypoxia-induced contractile protein dysfunction and peroxynitrite generation in skeletal muscle. A previous study (9) suggested that hypoxia with subsequent reoxygenation does not affect contractile protein function. This also was tested in the present study.

To test our hypotheses, we determined the effect of hypoxia, NOS inhibition, and reoxygenation on contractility of soleus muscle bundles and, subsequently, skinned single fibers isolated from these bundles. After completion of contractile experiments, nitrotyrosine formation was determined in these bundles, as a marker of peroxynitrite generation.

METHODS

Animal Preparation

All experiments were approved by the local Animal Ethics Committee, Radboud University, Nijmegen. Adult male outbred Wistar rats (mean body weight 351 ± 7 g) were anesthetized with isoflurane.

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HYPOXIA, A COMMON FEATURE in several respiratory diseases, such as chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS), and severe pneumonia, impairs force generation and increases fatigability of respiratory and peripheral skeletal muscles (3, 14, 20, 24, 31, 45, 46). Impaired peripheral muscle function contributes to exercise intolerance in several chronic diseases, such as COPD (16). Hypoxia is known to reduce the excitability of the sarcoclemma (39) and decrease Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (9). However, little is known about the effects of hypoxia on contractile protein function.

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The right soleus muscle was excised quickly and immediately submerged in cooled, oxygenated (95% O2-5% CO2) Krebs solution at pH 7.40. The Krebs solution consisted of 137 mM NaCl, 4 mM KCl, 2 mM CaCl2, 1 mM KH2PO4, 24 mM NaHCO3, 7 mM glucose, and 25 μM d-tubocurarine (Sigma, Bornem, Belgium). A bundle was dissected from the central part of the muscle. Silk sutures were tied firmly to both ends of the bundle.

**Soleus Muscle Bundle Contractile Measurements**

Contractile experiments were performed as described previously (47). Briefly, muscle bundles were mounted vertically in a tissue bath containing Krebs solution bubbled with 95% O2-5% CO2, with a pH of 7.40. Temperature of the solution was maintained at 26°C. The muscle was stimulated directly by using platinum plate electrodes placed in close apposition to the bundle. Stroimi were applied with a pulse duration of 0.2 ms and a train duration of 400 ms. Muscle preload force was adjusted until optimal fiber length (L0) for maximal twitch force was achieved. After 10 min, thermoequalibration baseline measurements were determined: submaximal tetanic force was measured at 20 Hz, and maximal tetanic force at 100 Hz (P20 and P0, respectively) with a 1-min interval. Next, the gas mixture and Krebs solution were replaced with experimental conditions: in the hypoxia group (n = 5), the Krebs solution was constantly bubbled with a gas mixture of 95% O2-5% CO2, and in the hypoxia group (n = 5), with a gas mixture of 95% N2-5% CO2. The aspecific NOS inhibitor L-nomonomethyl-L-arginine acetate (L-NMMA) was used to inhibit NO production. For the L-NMMA-hyperoxia (n = 5) and L-NMMA-hypoxia groups (n = 5), the experimental conditions were identical to the hyperoxia and hypoxia groups, respectively, except that 30 μM L-NMMA was added to the Krebs solution [previous dose-response data from our laboratory (45) demonstrated that 30 μM l-NMMA exerted a maximal effect on muscle contractility under hypoxic conditions]. After 30 min, P20 and P0 measurements were repeated. To evaluate the effect of reoxygenation on soleus muscle bundle contractility, we determined the contractile responses to 30 min of hypoxia and subsequent reoxygenation in five bundles. The duration of reoxygenation was 15 min, because force recovery plateaued after 10–15 min (Fig. 1). To evaluate the time effect on in vitro force generation during these 15 additional minutes, we assessed the contractile responses to 45 min of hyperoxia in three bundles. These bundles were considered hyperoxic time controls for the reoxygenated bundles and were not used for further determinations. For clarity, contractile responses to L-NMMA and reoxygenation are presented separately (see RESULTS). After completion of the contractile measurements, length and weight of the soleus muscle bundles were determined. Cross-sectional area was calculated by dividing soleus bundle weight (g) by strip length (cm) multiplied by specific density (1.056). Force is expressed per cross-sectional area (in N/cm2). Muscle bundles were divided into two parts; one part was blotted dry and quickly frozen for nitrotyrosine analysis, and the other part was used for single fiber measurements.

**Single Fiber Dissection**

Immediately after completion of muscle bundle contractile measurements, the part destined for single fiber measurements was transferred to ice-cold (5°C) relaxing solution containing 1% Triton X-100 to permeabilize membranous structures. Single fiber segments (~2 mm) were isolated within 1–2 min after removal of muscle bundle from the tissue bath. The skinning solution rapidly disrupts the sarcolemma, sarcoplasmic reticulum, and mitochondria. Therefore, very shortly after completion of the muscle bundle experiments, single fibers were skinned. Subsequently, fiber ends were attached to aluminum foil clips. Three single fibers were dissected from each soleus muscle bundle for contractile experiments.

**Single Fiber Contractile Measurements**

Activating and relaxing solutions used for contractile measurements were previously described (11). Relaxing solution had a pCa of 9.0, whereas activating solutions had a pCa of either 6.0 (for submaximal activation) or 4.5 (for maximal activation). The composition of the solution for maximum rigor activation was the same as that of the pCa 4.5 solution, except that Na2ATP was omitted.

Single fiber contractile measurements were performed as published previously (21), with minor modifications. Fibers were mounted in a temperature-controlled flow-through acrylic chamber (120-μl volume), with a glass coverslip bottom, on the stage of an inverted microscope (model IX-70; Olympus, Amsterdam, The Netherlands). Two stainless steel hooks were used to mount the fiber horizontally in the chamber. One end of the fiber was attached to a force transducer (model AE-801; SensoNor, Horten, Norway) with a resonance frequency of 10 kHz, whereas the other end was attached to a servomotor (model 308B; Aurora Scientific, Aurora, ON, Canada) with a step time of 250 μs. Sarcomere length was set at 2.4 μm. During experiments, sarcomere length was stabilized with the Brenner cycling method (7) as modified by Sweeney et al. (42). MIDAC software (Radboud University, Nijmegen, The Netherlands) and a data-acquisition board were used to record signals. Muscle fiber length (~1–1.5 mm) was measured using a reticle in the microscope eyepiece (×10 Olympus Plan 10, 0.30 numerical aperture (NA)]. The XY fiber diameter (width) was measured with a ×40 objective [×40 Olympus Plan 40, 0.60 NA]. The ×40 objective also was used to measure the XZ fiber diameter (depth) by noting the displacement of the microscope’s objective while focusing on the top and bottom surfaces of the fiber. The fiber cross-sectional area (CSA) was calculated from the average of three width and depth measurements made along the length of the fiber while it was mounted in relaxing solution at a sarcomere length of 2.4 μm, assuming that the fiber was ellipsoid in shape.

Maximal and submaximal specific force were determined by dividing the isometric force generated at pCa 6.0 and 4.5, respectively, by CSA. Force at pCa 4.5 is referred to as Fmax.

The rate constant for tension redevelopment (k0) was measured as described by Brenner and Eisenberg (8) during activation at pCa 6.0 and 4.5. Shortly thereafter, fibers were rapidly released by ~15% and then ~50 ms later, restretched to their original length. During the rapid release and restretch, cross bridges detach and force drops to zero. The cross bridges then reattach, and force redevelops. The k0 value was determined using a computer algorithm for least-squares fit of a first-order exponential.

Fiber stiffness was determined from sinusoidal length oscillations of 0.4% at 1 kHz, and delta force was normalized to CSA (21).
Stiffness provides an estimation of the number of strongly bound cross bridges (26). Stiffness was determined when fibers were in relaxing solution and during activation at pCa 6.0 and 4.5 in the presence and absence (rigor solution) of ATP. Assuming that, during rigor activation, all cross bridges are in the strongly bound state, the ratio of stiffness during Ca$^{2+}$ activation to that during rigor activation provides an estimate of the fraction of cross bridges in the strongly attached state ($\alpha_{tr}$).

**Single Fiber Experimental Protocol**

Maximal specific force was determined by perfusing the experimental chamber at, successively, pCa 9 and 4.5. During the plateau phase of maximal activation at pCa 4.5, $k_e$ was determined. pCa 9 solution was then perfused through the chamber to relax the fiber, and fiber stiffness was determined. Subsequently, the fiber was perfused at pCa 6.0, and after a plateau was reached, again at pCa 4.5. During activation at pCa 6.0, $k_e$ and fiber stiffness were determined. Stiffness was determined again during activation at pCa 4.5. Subsequently, the fiber was perfused with rigor solution of pCa 4.5 to determine stiffness during rigor activation. Finally, the fiber was perfused at pCa 9 to verify baseline force.

Structural and functional instability of skinned single fibers, reflected by an increased heterogeneity of sarcomere spacing during activation and reduced force generation over time, is a well-recognized problem (6). To improve functional stability of muscle fibers, we applied Brenner cycling (7) throughout the experimental protocol, except during determination of $k_e$ and stiffness, and accordingly, no functional decline over time was observed.

**Nitrotyrosine Measurement**

 Peroxynitrite formation was evaluated by detection of nitrotyrosine residues by performing Western immunoblotting with a monoclonal anti-nitrotyrosine antibody of high specificity (clone 1A6; Upstate Biotechnology, Lake Placid, NY), as discussed previously (47). Crude muscle homogenate proteins (10 μg) were heated for 5 min at 95°C in sample buffer and then separated by electrophoresis (200 V for 1 h) on 10% sodium dodecylsulfate-polyacrylamide gels. High- and low-molecular-weight standards of nitrotyrosine (Upstate Biotechnology) were run in parallel as positive controls. Proteins were transferred electrophoretically (100 V, 300 mA for 1 h) to methanol-presoaked polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were subsequently incubated with primary monoclonal antibodies raised against nitrotyrosine in 0.1% bovine serum albumin (BSA) in phosphate-buffered saline with 1% Tween 20 (PBST). After three 10-min washes with PBST on a rotating shaker, the PVDF membranes were incubated with a secondary antibody (polyclonal anti-mouse IgG horseradish peroxidase conjugated) for 1 h at room temperature. The membranes were finally washed twice for 10 min with PBST. Afterward, protein bands were visualized using an enhanced chemiluminescence detection kit (Amersham Biosciences Europe). The blots were scanned with an imaging densitometer, and optical densities (OD) of positive nitrotyrosine protein bands were quantified using GeneTools software (Syngene, Cambridge, UK). Total nitrotyrosine OD was calculated for each samples by adding the OD of individual positive protein bands. To confirm the specificity of antibody for nitrotyrosine, we incubated the antibody with 10 mM nitrotyrosine, or 1 mg/ml nitrated BSA, just before the membrane incubation, for 1 h at room temperature (28). Nitrated BSA was prepared by solubilizing 15 mg of BSA in 10 ml of phosphate-buffered saline and incubating for 30 min at room temperature with 1 mM peroxynitrite. The nitration of the tyrosine residues in BSA was determined by absorbance at 430 nm (25).

We made an effort, with no success, to determine nitrotyrosine formation in single fibers to directly correlate those data to single fiber contractile function. Because we preferred to use the single fibers for nitrotyrosine detection, it was not possible to determine myosin heavy chain (MHC) isoform composition of the single fibers. However, MHC isoform composition of rat soleus muscle consists almost exclusively of the slow isoform (12, 33–35). Therefore, it is very unlikely that nonuniform distribution of MHC isoform composition between fibers of different experimental groups has influenced the robust changes in single fiber contractility between groups, as observed in the present study.

**Statistical Methods**

Data are presented as means ± SE. Differences between experimental groups were analyzed with one-way ANOVA and Tukey’s post hoc test, if appropriate. Muscle bundle contractile response to hypoxia and subsequent reoxygenation was analyzed with the paired $t$-test. Statistical analysis was performed with the SPSS package version 11.0 (SPSS, Chicago, IL). Differences were considered significant at $P < 0.05$.

**RESULTS**

**Gas Tension Analysis and Muscle Strip Dimensions**

Perfusion of the bundle tissue bath with the hypoxic gas mixture reduced $P_{O_2}$ in the Krebs solution to 5.0 ± 0.7 kPa during hypoxic conditions, compared with 89.5 ± 1.3 kPa during hyperoxic conditions ($P < 0.001$). The $pH$ and $P_{O_2}$ of the tissue baths were not significantly different between groups ($P > 0.05$). Average $pH$ was 7.42 ± 0.02, and average $P_{O_2}$ was 3.8 ± 0.2 kPa. Soleus muscle bundle dimensions were not significantly different between experimental groups. Average muscle bundle weight was 58.0 ± 3 mg, and muscle bundle length at $L_o$ was 17.8 ± 0.4 mm.

**Soleus Muscle Bundle Contractile Properties**

Baseline $P_o$ and $P_{20}$ (thus before exposure to hypoxic conditions and/or addition of L-NMMA) were not different among the experimental groups ($P > 0.05$). Values for $P_o$ for the hyperoxia, hypoxia, L-NMMA-hypoxia, L-NMMA-hyperoxia, and reoxygenation groups were 9.3 ± 0.6, 5.2 ± 0.6, 9.5 ± 0.9, 10.7 ± 0.3, 11.3 ± 0.6, and 11.0 ± 0.6 N/cm$^2$, respectively, and values for $P_{20}$ were 5.2 ± 0.6, 5.2 ± 0.6, 6.1 ± 1.2, 6.6 ± 0.6, and 6.7 ± 0.4 N/cm$^2$, respectively.

**Hypoxia and L-NMMA.** Figure 2 shows contractile response in soleus muscle bundles in the hyperoxia, hypoxia, and L-NMMA groups. L-NMMA partially prevented hypoxia-induced decrease of $P_o$ ($P < 0.05$ vs. hypoxia and hyperoxia) but had no effect on $P_{20}$ ($P > 0.05$ vs. hypoxia).

**Hypoxia and reoxygenation.** Figure 3 shows contractile response in soleus muscle bundles in the reoxygenation group and in the hyperoxic time controls. Bundle reoxygenation partially restored force generation ($P < 0.05$ vs. hyperoxia and hypoxia).

**Soleus Single Fiber Contractile Properties**

**Specific force.** HYPOXIA AND L-NMMA. Figure 4A shows that hypoxia significantly decreased $F_{max}$ compared with other groups ($P < 0.05$). Incubation with L-NMMA prevented hypoxia-induced depression of $F_{max}$ ($P > 0.05$ vs. hyperoxia). Hypoxia did not affect $Ca^{2+}$ sensitivity of force generation, because the ratio of force generated at pCa 6.0 to $F_{max}$ was not different between groups ($P > 0.05$; average 0.61 ± 0.02), except between the L-NMMA-hyperoxia and hypoxia groups (0.72 ± 0.03 and 0.59 ± 0.02, respectively; $P < 0.05$).
Hypoxia did not affect $\alpha_{fs}$ at submaximal activation, and therefore, not surprisingly, $\alpha_{fs}$ also was not different among reoxygenation, hypoxia, and hyperoxia groups at pCa 6.0 ($P > 0.05$) and averaged 0.56 ± 0.03.

**Cross-bridge cycling kinetics. Hypoxia and L-NMMA.** Figure 6A shows that hypoxia significantly decreased $k_{tr}$ at pCa 4.5 compared with other groups ($P < 0.05$). Incubation with L-NMMA prevented hypoxia-induced depression of $k_{tr}$ ($P > 0.05$ vs. hyperoxia). In contrast, $k_{tr}$ at submaximal activation was not affected by hypoxia; the ratio of $k_{tr}$ at pCa 6.0 to $k_{tr}$ at pCa 4.5 was not different between hypoxia and other groups ($P > 0.05$) and averaged 0.47 ± 0.03.

**Reoxygenation.** As shown in Fig. 6B, reoxygenation completely reversed $k_{tr}$ at pCa 4.5 to hyperoxic values ($P > 0.05$). Again, not surprisingly, at submaximal activation, reoxygenation did not affect $k_{tr}$; the ratio of $k_{tr}$ at pCa 6.0 to $k_{tr}$ at pCa 4.5 was not different among the three groups ($P > 0.05$) and averaged 0.47 ± 0.04.

**Nitrotyrosine formation in rat soleus muscle bundles.** Monoclonal anti-nitrotyrosine antibody detected roughly two ni-
treated protein bands in hyperoxic soleus muscle bundles, with molecular masses of ~30 and 50 kDa (see Fig. 7B). Nitrotyrosine level in hyperoxic bundles was similar to nitrotyrosine level in soleus muscle bundles immediately removed from the animal (data not shown), which is in line with previous data from our laboratory (47).

HYPOXIA AND L-NMMA. Hypoxia significantly increased total nitrotyrosine level compared with the other groups ($P < 0.05$; Fig. 7A). Roughly, four additional protein bands were detected in the hypoxic group, with molecular masses between ~36 and 45 kDa (Fig. 7B). Also, nitrotyrosine OD of protein bands of ~30 and 50 kDa was increased in the hypoxic group compared with the other groups. L-NMMA completely prevented hypoxia-induced nitrotyrosine formation (Fig. 7, A and B). To exclude nitrite as the nitrating agent (5), we quenched nitrite with 1% sulfanilamide in muscle homogenates from hypoxic muscles. Quenching of nitrite did not affect nitrotyrosine levels (data not shown), thereby confirming peroxynitrite as the nitrating agent.

REOXYGENATION. Figure 8 shows that bundle reoxygenation reversed hypoxia-induced nitrotyrosine formation to a level comparable with that of the hyperoxia group ($P > 0.05$ vs. hyperoxia, Fig. 8A). To exclude destruction of nitrated proteins during boiling of the homogenates of reoxygenated bundles, we evaluated nitrotyrosine levels in nonboiled homogenates of reoxygenated muscle bundles. Our results revealed that avoiding boiling did not affect nitrotyrosine levels in the homogenates of reoxygenated muscle bundles (data not shown).

**DISCUSSION**

The present study is the first to demonstrate that acute hypoxia impairs skinned single fiber contractility in skeletal muscle, indicating hypoxia-induced contractile protein dysfunction. The fact that inhibition of NOS prevented hypoxia-induced reduction of single fiber contractility indicates that NO, or products downstream of NO synthesis, plays a role in hypoxia-induced contractile protein dysfunction. Indeed, hyp-

**Fig. 4.** A: effect of hypoxia and L-NMMA on force generation of skinned single fibers at maximal activation ($F_{\text{max}}$; force generated at $pCa$ 4.5, see METHODS). B: effect of hypoxia and reoxygenation on $F_{\text{max}}$. Data for B were obtained from single fibers dissected from reoxygenated muscle bundles (bundle contractile data presented in Fig. 3) and 30-min hypoxic and hyperoxic bundles (bundle contractile data presented in Fig. 2). Force generation was normalized to fiber cross-sectional area. Data are presented as means ± SE. *$P < 0.05$, different from other groups.

**Fig. 5.** A: effect of hypoxia and L-NMMA on the fraction of strongly attached cross bridges ($\alpha_{\text{fs}}$, derived from single fiber stiffness measurements) at maximal activation ($pCa$ 4.5, see METHODS). B: effect of hypoxia and reoxygenation on $\alpha_{\text{fs}}$ at maximal activation. Data for B were obtained from single fibers dissected from reoxygenated muscle bundles (bundle contractile data presented in Fig. 3) and 30-min hypoxic and hyperoxic bundles (bundle contractile data presented in Fig. 2). Data are presented as means ± SE *$P < 0.05$, different from other groups.
oxia was associated with elevated nitrotyrosine formation, which could be prevented by NOS inhibition. Reoxygenation of the muscle bundle partially restored bundle contractility but completely reversed the effects of hypoxia on skinned fiber contractile function. Concomitantly with restored single fiber contractility, reoxygenation reversed hypoxia-induced nitrotyrosine formation, also suggesting that NO products affect single fiber contractility.

**Muscle Bundle Contractility During Hypoxia and Reoxygenation**

Acute hypoxia-induced impairment of muscle bundle force generation, as found in the present study, is in line with previous studies from our group and others (14, 20, 43, 45). Several mechanisms may play a role in impaired force generation during hypoxia, such as increased production of free radicals, downregulation of mitochondrial enzymes, and reduced excitability of the sarcolemma (39). In the present study, l-NMMA attenuated hypoxia-induced decrease of P0. NO is known to affect force generation through redox effects on regulatory proteins (38). Eu et al. (15) have shown that oxygen tension regulates S-nitrosylation of thiols on the Ca2+ release ryanodine receptor (RyR1) channels of the sarcoplasmic reticulum. Also, previous work from our laboratory demonstrated that NO affects sarcoplasmic calcium release in myotubes, probably by modification of thiols on RyR1 (22). In addition, thiols on myosin are also sensitive to NO modulation (36). In line with previous studies in murine diaphragm bundles (9), the present study shows that muscle bundle reoxygenation partially recovers force generation. Subsequent exposure of reoxygenated muscle bundles to 10 mM caffeine has been shown to fully restore force generation, which suggests that hypoxia has an irreversible inhibitory effect on Ca2+ release from the sarcoplasmic reticulum (9). This is in line with the present study, because we found that muscle bundle reoxygenation completely restored contractile protein function.

**Hypoxia and Single Fiber Function**

The present study is the first to demonstrate that hypoxia impairs skinned single fiber contractility, indicating hypoxia-
attached cross bridges and thereby decreased \( F_{\text{max}} \). Our observation that reoxygenation completely restored \( F_{\text{max}} \) suggests that 30 min of hypoxia did not decrease the total number of cross bridges in parallel per half sarcomere or, in other words, myosin content, as could be expected. However, because the reduction of \( \alpha_{fs} \) is not of sufficient magnitude to fully account for the \(-32\%\) decline of \( F_{\text{max}} \), reduction of available cross bridges and/or decreased \( \bar{f}_m \) might play a role as well.

Brenner and Eisenberg (8) proposed a model in which the transition between the weakly and strongly bound cross bridges was described by two apparent rate constants, one for cross-bridge attachment (\( f_{\text{app}} \)) and one for cross-bridge detachment (\( g_{\text{app}} \)). In this model, the fraction of cross bridges in the force-generating state is given by Eq. 2:

\[
\alpha_{fs} = \frac{f_{\text{app}}}{f_{\text{app}} + g_{\text{app}}}
\]

and the relationship among \( f_{\text{app}}, g_{\text{app}}, \) and the rate constant for force redevelopment, \( k_{tr} \), is defined by Eq. 3:

\[
k_{tr} = \frac{f_{\text{app}}}{g_{\text{app}}}
\]

At maximal activation (pCa 4.5), hypoxic fibers had a slower \( k_{tr} \) compared with hyperoxic fibers, reflecting slower cross-bridge cycling kinetics. \( k_{tr} \) is initially dominated by cross-bridge reattachment and thus provides an approximation of \( f_{\text{app}} \) (40). Therefore, our results indicate that hypoxia specifically slows \( f_{\text{app}} \) at maximal activation. A slower cross-bridge attachment rate decreases the fraction of strongly attached cross bridges (as reflected by Eq. 2) and consequently could have attributed to decreased \( F_{\text{max}} \) in hypoxic fibers. During submaximal activation (pCa 6.0), hypoxic fibers showed no altered force generation, \( \alpha_{fs} \) and \( k_{tr} \), indicating no hypoxia-induced changes in \( Ca^{2+} \) sensitivity of cross-bridge recruitment.

**Role of RNS in Hypoxia-Induced Single Fiber Dysfunction**

Because inhibition of NOS during hypoxia resulted in higher values for \( F_{\text{max}}, \alpha_{fs}, \) and \( k_{tr} \) compared with hypoxic fibers, the present study clearly demonstrates that NO plays a prominent role in hypoxia-induced contractile protein dysfunction. The effects of exogenous NO on single fiber contractility during maximal activation have been shown to be modest, in contrast to greater effects of NO during submaximal activation (1, 17, 21). Therefore, the relatively large reduction of \( F_{\text{max}} \) and unaltered contractility during submaximal activation in hypoxic fibers suggest additional mechanisms, in addition to direct effects of NO, to be at play in hypoxia-induced contractile protein dysfunction. Hypoxia has been shown to increase superoxide production in cardiomyocytes (13). The reaction of superoxide with NO, with an extremely high reaction rate, generates the highly reactive free radical peroxynitrite. Exogenous peroxynitrite has shown to decrease skinned single fiber \( F_{\text{max}} \) of rat diaphragm single fibers (41) without alterations in submaximal force generation (i.e., pCa3.5). These observations reflect the hypoxia-induced changes in skinned single fiber contractility as presented in this study. Therefore, increased levels of peroxynitrite might act as a mediator in hypoxia-induced contractile protein dysfunction. Peroxynitrite generation in hypoxic soleus muscle bundles was assessed by determining nitrotyrosine formation in the soleus muscle bundles. Although nitrotyrosine can be generated through mechanisms

\[
\text{Eq. 1: } F_{\text{max}} = n \times f_m \times \alpha_{fs}
\]

where \( n \) is the number of available cross bridges in parallel per half sarcomere, \( f_m \) is the mean force per attached cross bridge in the force-generating state, and \( \alpha_{fs} \) is the fraction of cross bridges in the force-generating state.

The reduced \( F_{\text{max}} \) of hypoxic fibers should be reflected by a reduction of \( n, f_m, \) or \( \alpha_{fs} \) or by a combination of these determinants of force generation. In the present study, \( \alpha_{fs} \) was reduced from \(-0.90 \) in hyperoxic fibers to \(-0.76 \) in hypoxic fibers. Apparently, hypoxia reduced the fraction of strongly

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**Fig. 8. Nitrotyrosine residues in soleus muscle homogenates: effect of hypoxia and reoxygenation.** Nitrotyrosine data were obtained from muscle bundles that also were used for single fiber measurements, and not from bundles from which contractile data are presented in Fig. 3. A: total OD values for nitrotyrosine bands of rat soleus muscle after hyperoxia, after hypoxia, and after 30-min hypoxia and subsequent 15-min reoxygenation (reoxygenation). Nitrotyrosine level in hypoxia group was significantly higher compared with other groups. *P < 0.05 compared with other groups. B: representative immunoblots of rat soleus muscle homogenates from hyperoxia, hypoxia, and reoxygenation groups. Molecular mass markers are shown in kDa. After reoxygenation, the 4 additional protein bands present in the hypoxia group disappeared. Also, nitrotyrosine OD of protein bands of \(-30 \) and \(-50 \) kDa was less after reoxygenation compared with the hypoxia group.

**Hypoxia-Induced Single Fiber Dysfunction**

Although nitrotyrosine can be generated through mechanisms
independent from peroxynitrite, this is unlikely in our preparation as discussed previously (47).

In accordance with our hypothesis, hypoxia enhanced nitrotyrosine formation, indicating hypoxia-induced increased peroxynitrite generation in soleus muscle bundles. In addition, L-NMMA completely prevented hypoxia-induced elevated nitrotyrosine formation. These observations are in line with previous work from our laboratory in rat diaphragm muscle bundles (47). In the present study, hypoxia enhanced nitrotyrosine OD of protein bands already present under hyperoxic conditions but also resulted in the appearance of additional protein bands between ~36 and 45 kDa. The nature of these proteins is unknown. However, whereas L-NMMA only partially prevented reduction of bundle contractility under hypoxic conditions, hypoxia-induced nitrotyrosine formation was completely prevented by L-NMMA. This suggests that no causal relationship exists between nitrotyrosine formation and contractility but that other pathways are at play as well. Previously, we have shown that L-NMMA decreases diaphragm nitrotyrosine formation under hypoxic conditions (47). However, L-NMMA did not affect diaphragm bundle contractility under these conditions. This is an apparent discrepancy. However, differences in the duration of L-NMMA-incubation and phenotype of the muscle under investigation could provide an explanation.

Effect of Bundle Reoxygenation on Single Fiber Contractility and Nitrotyrosine Formation

In the present study, reoxygenating the muscle bundle partially restored bundle contractility but completely reversed the effects of hypoxia on skinned fiber contractile function. This discrepancy shows that reoxygenation restored contractile protein function, whereas some steps upstream, the excitation-contraction coupling process remained impaired.

Our findings extend data by Brotto et al. (9), who reported unaltered force generation of single fibers isolated from reoxygenated murine diaphragm bundles. The present study also showed that cross-bridge cycling kinetics and the fraction of strongly attached cross bridges are unaltered in single fibers from reoxygenated muscle bundles.

As discussed previously, inhibiting NO production prevented hypoxia-induced contractile protein dysfunction. The fact that reoxygenation reversed hypoxia-induced skinned single fiber dysfunction therefore strongly suggests that reoxygenation counteracts the negative effects of hypoxia-induced generation of NO and NO products on contractile protein function. Interestingly, after bundle reoxygenation, nitrotyrosine levels were comparable to those in hyperoxic bundles. Apparently, hypoxia-induced nitrotyrosine formation was completely reversed by reoxygenating the muscle bundle. To our knowledge, the present study is the first to report denitrification of tyrosine in muscle. This is an important finding, because nitrotyrosine modifications of proteins have long been thought to be irreversible and just a “footprint of peroxynitrite.” Only recently, studies revealed denitrification of tyrosine residues in mitochondria (2, 27) and enzymatic activity in tissue capable of denitrating nitrotyrosine (23). The reversibility of nitrotyrosine formation after muscle bundle reoxygenating adds to the accumulating evidence that nitrotyrosine formation is involved in posttranslational signaling by altering protein function (19).

The exact mechanism responsible for reoxygenation-induced reversibility of protein nitration is unknown. However, reoxygenation-induced nitrotyrosine denitration should be interpreted with caution in relation to force generation, because reoxygenation completely reversed hypoxia-induced nitrotyrosine formation, whereas muscle bundle force generation was only partially recovered. The fact that L-NMMA had similar effects on nitrotyrosine formation and bundle contractility (as discussed previously) suggests that other mechanisms are at play causing reduced hypoxia-induced muscle contractility.

Clinical Relevance

Hypoxemia is a common feature in pulmonary diseases such as COPD, severe pneumonia, and ARDS. Hypoxia impairs skeletal muscle function and could thereby contribute to exercise intolerance and ventilatory failure in these patients. However, the cellular mechanisms of hypoxia-induced impairment of contractile performance are incompletely understood. Hypoxia is known to increase production of RNS in skeletal muscle. Investigation of the role of RNS in the pathophysiology of hypoxia-induced contractile protein dysfunction is needed to develop successful strategies for improving muscle function under hypoxic conditions.

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