Hypoxia-induced dysfunction of rat diaphragm: role of peroxynitrite

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Hypoxia, a common feature in several respiratory diseases, including chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS), and pneumonia, adversely affects respiratory muscle function. Diaphragm force generation is impaired and fatigability is increased under hypoxic conditions both in vitro and in vivo (4, 15, 25, 59). Impaired respiratory muscle function may result in ventilatory failure. The precise mechanisms involved in hypoxia-induced impairment in contractile performance are incompletely understood, but oxidative and nitrosative stress could be at play (20, 38).

Nitric oxide (NO), a highly reactive second messenger, plays an important role in skeletal muscle physiology, including contractility (3, 29). It is produced by NO synthases (NOS) (35). The activity of the constitutive NOS isoforms (neuronal and endothelial NOS, nNOS and eNOS, respectively) is primarily regulated by intracellular Ca2+ concentration (34, 40).

Hypoxia may impair intracellular Ca2+ homeostasis, which in turn could increase NO production by NOS (13, 17, 60). Little information is available regarding the role of NO in muscle dysfunction during hypoxia. Previously, we have shown that inhibition of NOS improves rat diaphragm contractility under hypoxic conditions (22, 57), the reaction product of NO and superoxide anions (O2−·) (7, 41). Several studies showed evidence of superoxide generation in the hyperoxic rat diaphragm (31, 43). Interestingly, hypoxia increases generation of reactive oxygen species in cardiac myocytes (14). We have shown that antioxidants improve diaphragm contractility under hypoxic conditions (20). It is unknown whether peroxynitrite is generated in skeletal muscle and plays a role in impaired force generation induced by hypoxia. This is an important question, since peroxynitrite is a potent nitrating and oxidizing agent (41). It can result in cellular injury and cell death by causing oxidation of sulfhydryls, lipid peroxidation, and nitration of tyrosine residues in protein to form nitrotyrosine (7, 41).

The first hypothesis of the present study was that hypoxia-induced impairment of rat diaphragm contractility under hypoxic conditions is associated with increased generation of peroxynitrite. To test this hypothesis we measured in vitro force generation of the rat diaphragm under hypoxic and hyperoxic conditions. After completion of contractile experiments, total nitrotyrosine formation was measured in these muscle bundles as a marker for peroxynitrite formation (7). In addition, the effects of exogenous peroxynitrite, the peroxynitrite scavenger ebselen, and the NOS inhibitor Nω-monomethyl-L-arginine acetate (L-NMMA) were assessed on in vitro contractility and nitrotyrosine formation of the rat diaphragm muscle strips to a predetermined level of contractile performance.

Nitrite oxide (NO), a highly reactive second messenger, plays an important role in skeletal muscle physiology, including contractility (3, 29). It is produced by NO synthases (NOS) (35). The activity of the constitutive NOS isoforms (neuronal and endothelial NOS, nNOS and eNOS, respectively) is primarily regulated by intracellular Ca2+ concentration (34, 40).
METHODS

Animal Preparation

This study was approved by the Animal Ethics Committee, Radboud University, Nijmegen, The Netherlands. Adult male outbred Wistar rats with a mean body weight of 351 ± 9 g were used. The rats were anesthetized with pentobarbital sodium (70 mg/kg body wt ip). A tracheotomy was performed, a polyethylene cannula was inserted into the trachea, and the animals were mechanically ventilated with 100% O2. The diaphragm strips were prepared as described previously (20). Briefly, the diaphragm and adherent lower ribs were quickly excised after a combined thoracotomy and laparotomy and were immediately submerged in cooled oxygenated (95% O2 and 5% CO2) Krebs solution at pH 7.4. This Krebs solution consisted of 137 mM NaCl, 4 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 1 mM KH2PO4, 24 mM NaHCO3, 7 mM glucose, and 25 μM α-tubocurarine (Sigma, Bornem, Belgium). From the central costal region of the hemidia- phragm a muscle strip was dissected along the parallel axis of the muscle fibers. Silk sutures were tied firmly to both ends of the muscle strip.

Contractile Measurements

Contractile properties were measured as described previously (20). Briefly, the strips were mounted vertically in tissue baths containing Krebs solution bubbled with 95% O2–5% CO2 with a pH of 7.4. Temperature of the solution was maintained at 26°C. The muscle was stimulated directly by using platinum plate electrodes placed in close apposition of the bundle. Stimuli were applied with a pulse duration of 0.2 ms and train duration of 400 ms. Muscle preload force was adjusted until optimal fiber length (L0) for maximal twitch force (Pt) was achieved. After 10 min of thermodilution, baseline measurements were determined: force was measured at 1, 15, 30, and 100 Hz (P1, P15, P30, and Pn, respectively) at 2-min intervals. Next, the gas mixture and Krebs solution were replaced by appropriate experimental conditions (see below). After 60 or 90 min (see specific protocols), P1, P15, P30, and Pn measurement were repeated. In a subgroup, isotonic fatigability was determined as published previously (20). Briefly, the load clamp level of the lever arm was set for maximum power output (assumed to be at 33.3% Pn in both groups), and the muscle was stimulated at 100 Hz (330-ms train duration) every 2 s. Stimulation continued until no muscle shortening could be observed, and this was defined as isotonic endurance time. After completion of contractile experiments, pH, PO2, and PCO2 of the Krebs solution was measured using a blood-gas analyzer.

Nitrotyrosine Measurement

Peroxy nitrite formation was evaluated by detection of nitrotyrosine residues using Western immunoblotting with a monoclonal antinitro- tyrosine antibody (clone 1A6; Upstate Biotechnology, Lake Placid, NY) (5, 8). Crude muscle homogenate proteins (22 μg) were heated for 5 min at 95°C in sample buffer, centrifuged for 5 min, and then separated by electrophoresis (100 V for 1 h) on 8% sodium dodecyl sulfate (SDS) polyacrylamide gels. High- and low-molecular-weight standards of nitrotyrosine (Upstate Biotechnology) were run in parallel as positive control. Proteins were transferred electrophoretically (100 V, 300 mA for 1.5 h) to nitrocellulose membranes. The nitrocellulose membranes were subsequently incubated for 16 h at 4°C with primary monoclonal antibodies raised against nitrotyrosine in 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) with 0.1% Tween 20 (PBST). After three 10-min washes with PBST on a rotating shaker, the nitrocellulose membranes were incubated with a secondary antibody (polyclonal anti-mouse IgG horse-radish peroxidase conjugated; Pierce, Rockford, IL) for 1 h at room temperature. The membranes were finally washed twice for 10 min with PBST. Afterwards, protein bands were visualized using a 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 with GeneTools software (Syngene). Total nitrotyrosine OD was calculated for each sample by adding 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 of nitrated BSA just before the membrane incubation for 1 h at room temperature (32). Nitrated BSA was prepared by solubilizing 15 mg of BSA in 10 ml of PBS and incubation for 30 min at room temperature with 1 mM peroxynitrite. The nitration of the tyrosine residues in BSA was determined by the absorbance at 430 nm (27).

Measurement of Lipid Peroxidation

Concomitant measurement of malondialdehyde (MDA) was made to determine whether the lipid membranes or the protein components are the plausible target of peroxynitrite. MDA levels were assessed by high-performance liquid chromatography, as previously reported (56). Muscle samples of 40 mg were homogenized and centrifuged, and the supernatant was collected and stored on ice. Samples were hydrolyzed by boiling in diluted phosphoric acid. MDA, one of low-molecular- weight end products formed via the decomposition of lipid peroxidation products, was reacted with thiobarbituric acid (TBA) to form MDA-TBA adducts (24). The adduct was eluted from the column with methanol-phosphate buffer and quantified spectrophotometrically at 532 nm. 4-Hydroxynonenal (HNE) was determined as a second, chemically distinct measure for lipid peroxidation. Crude muscle homogenate proteins (22 μM) were loaded on a 8% SDS-polyacryl- amide gel, separated by electrophoresis, and transferred to nitrocellu- lose membranes for 1 h at 100 V. Membranes were incubated with an HNE polyclonal antibody (Calbiochem, La Jolla, CA) for 1 h at room temperature. After three 10-min washes with PBST, membranes were incubated with peroxidase-conjugated goat anti-rabbit (Pierce) for 1 h at room temperature. After being washed with PBST, protein bands were visualized as described above in Nitrotyrosine Measurement.

Experimental Protocols

Effects of hypoxia. In the first series of experiments, the effect of hypoxia on contractility, nitrotyrosine formation, and tissue MDA level was determined. After an initial measurement of force-frequency characteristics, the Krebs solution was replaced by fresh standard Krebs solution, and the gas mixture was maintained at 95% O2 and 5% CO2 (hyperoxia, n = 10) or changed to 95% N2 and 5% CO2 (hypoxia, n = 10). After 60 min, the force-frequency relationship was remeasured, and subsequently bundles were blotted dry, quickly frozen in liquid nitrogen, and stored at −80°C. From each group, five bundles were used for measurement of nitrotyrosine and five bundles for MDA measurement. Experimental conditions may affect lipid peroxidation of the diaphragm muscle bundle. Therefore, in a subgroup, diaphragm muscle bundles were immediately after excision from the rat transferred into liquid nitrogen for later MDA measurement (control, n = 5).

Effects of fatigue. In the second series of experiments, the effects of fatiguing contractions on task endurance and nitrotyrosine formation were determined under hypoxic and hyperoxic conditions. After initial measurement of the force-frequency relationship, Krebs solution was replaced by fresh standard Krebs solution, and the gas mixture was either maintained at 95% O2 and 5% CO2 (hyperoxia-fatigue, n = 5) or changed to 95% N2 and 5% CO2 (hypoxia-fatigue, n = 5) for 60 min. After remeasurement of the force-frequency relationship, iso- tonic fatigue was determined as described above. After completion of contractile experiments, bundles were blotted dry, quickly frozen in liquid nitrogen, and stored at −80°C for later nitrotyrosine analysis.

Effects of peroxynitrite and ebselen. The effects of peroxynitrite (Upstate Biotechnology, Venendaal, the Netherlands) and ebselen [2-phenyl-1,2-benzisothiazol-3-(h)-one; Calbiochem] were deter-
mained on contractile properties and nitrotyrosine formation under hypoxic conditions. In pilot experiments we studied dose-response relation of peroxynitrite on P0 during hyperoxia. Diaphragm muscle bundles were treated with increasing concentrations of peroxynitrite: 10, 100, 250, 500, and 1,000 μM. Peroxynitrite was prepared according to the manufacturer’s instructions. Because 0.3 M NaOH serves as a diluent of peroxynitrite, pH in tissue bath was measured to detect any alkaline shift, which even at a high peroxynitrite dose was <0.1 unit. The half-life of peroxynitrite is <1.0 s at neutral to low pH (6) but is ~3.3 h in diluent 0.3 M NaOH (37). Although peroxynitrite has a short half-life time at physiological pH, the changes in skeletal and smooth muscle function in vitro appeared to evolve long after 1 h of exposure (28, 48). A single pulse of peroxynitrite was added in the bottom of the tissue bath. After 60 min, P1 and P0 were remeasured. The effects of peroxynitrite on P1 and P0 were compared with that in standard Krebs solution during hyperoxia (n = 3 per group). From these experiments it appeared that 250 μM of peroxynitrite reduced P1 by ~35% from baseline (data not shown). Increasing peroxynitrite concentration further reduced P1. Therefore, in subsequent experiments final peroxynitrite concentration was 250 μM. Final concentration of ebselen was 50 μM (1, 33). Ebselen was dissolved in the lowest volume of DMSO, and consequently a final amount of DMSO in the tissue bath was 0.0009 vol/vol. In pilot studies we tested the effect of the solvents for peroxynitrite (0.3 M NaOH, equal volume, n = 4) and for ebselen (0.0009 vol/vol DMSO, n = 3) and also the effect of degraded peroxynitrite (n = 4, Upstate Biotechnology) on in vitro force generation under hypoxic conditions. An identical protocol was used as in the final studies. These data were compared with a control hyperoxic group. No differences in force generation were observed among these groups (data not shown).

To study the effect of peroxynitrite and ebselen on contractility under hypoxic conditions, baseline measurements were determined as described above. Subsequently, Krebs solution was replaced by experimental Krebs solutions: standard, 250 μM peroxynitrite, or 50 μM ebselen. After 60 min, the force-frequency measurement was repeated, and muscle bundles were quickly frozen as described above. Similar experiments were conducted under hyperoxic conditions; accordingly, in this series of experiments six groups of rats were studied: hyperoxia (n = 10), hyperoxia-peroxynitrite (n = 10), hyperoxia-ebselen (n = 10), hyperoxia-peroxynitrite (n = 10), hyperoxia-ebselen (n = 10), and hyperoxia-ebselen (n = 10). From each group, five bundles were used for measurement of nitrotyrosine and five bundles for MDA concentration. HNE was determined as a second measure for lipid peroxidation in three groups of bundles: hyperoxia, (n = 10), hyperoxia, and hyperoxia-peroxynitrite (n = 10).

Effects of NOS inhibition. Because peroxynitrite is generated from superoxide and NO, inhibition of NO synthesis is expected to reduce peroxynitrite formation. Recently, we have shown that the NOS inhibitor l-NMMA (30 μM for 90 min) increases force generation of the rat diaphragm under hypoxic conditions. Accordingly, in the fourth series of experiments the effects of l-NMMA (AcoH; Calbiochem, Breda, The Netherlands) on in vitro force generation and diaphragm nitrotyrosine formation were assessed. After initial force-frequency measurements (P1, P10, P30, P50), the gas mixture was changed to hypoxia (to 95% N2 and 5% CO2), and Krebs solution was replaced by fresh standard Krebs solution (hypoxia, n = 6) or Krebs solution containing 30 μM l-NMMA (hypoxia-l-NMMA, n = 6). After 90 min, force-frequency measurements were repeated. Subsequently, bundles were blotted dry and quickly frozen for nitrotyrosine analysis.

Data Treatment and Statistics

After completion of contractile experiments, the length and dry weight of the diaphragm muscle bundle were measured. Cross-sectional area (CSA) was calculated by dividing diaphragm strip weight (g) by strip length (cm) times specific density (1.056). Force is expressed per CSA in N/cm2. Muscle shortening velocity in fatigue experiments was calculated as the change in muscle length during a 30-ms period and expressed as muscle lengths per s (L/s). To eliminate the effect of muscle compliance, the time window for shortening velocity measurements was set to begin 10 ms following the first detectable change in length. Data are presented as means ± SE. Differences in single baseline contractile properties, bundles dimensions, gas pressure, and nitrotyrosine levels among the experimental groups were analyzed with either Student’s t-test or one-way ANOVA and, if appropriate, Student-Newman-Keuls (SNK) post hoc testing. Parameters requiring repeated measures over time (force frequency, fatigue) were estimated using repeated-measurement models and, if appropriate, SNK post hoc testing. Statistical analysis was performed with the SPSS package version 12.0 (SPSS, Chicago, IL). Comparisons were considered significant at P < 0.05.

RESULTS

Gas Tension Analysis, Muscle Strip Dimensions

Perfusion of the tissue baths with the hypoxic gas mixture reduced PO2 of the Krebs solution to ~6.5 ± 0.2 kPa compared with 88.2 ± 1.6 kPa in the hyperoxic groups (P < 0.001). The pH and PCO2 of the tissue baths were not significantly different between hypoxic and hyperoxic groups (pH, 7.40 ± 0.01 vs. 7.38 ± 0.02; PCO2, 4.6 ± 0.1 vs. 4.8 ± 0.4 kPa in hypoxic and hyperoxic groups, respectively; P > 0.05).

No difference was observed in muscle strip dimensions among all experimental groups. Average muscle strip weight was 44.2 ± 1.0 mg, and strip length at L0 was 19.0 ± 0.3 mm.

Contractile Studies

Hypoxia. Initial force-frequency relationship was not different between hypoxia and hyperoxia (P > 0.05) and averaged 7.4 ± 0.2, 11.8 ± 0.5, 15.1 ± 0.6, and 16.3 ± 0.6 N/cm2 for P1, P10, P30, and P50, respectively. Hypoxia resulted in a significant downward shift of the force-frequency curve compared with hyperoxia (Fig. 1, P < 0.001).

Fatigue. Repetitive contractions resulted in progressive loss of power, which was more rapid during hypoxia than during

![Fig. 1. Force-frequency relationship in the rat diaphragm after 60 min in either hypoxia (hypox) or hyperoxia (hyperox). Hypox shifted the force-frequency curve downward. *P < 0.001 compared with hyperox.](http://ajplung.physiology.org/doi/10.1152/ajplung.00324.2017)
hyperoxia (Fig. 2A, \( P < 0.05 \)). Time to task failure was significantly reduced by hypoxia (Fig. 2B, \( P < 0.005 \)).

**Peroxynitrite and ebselen.** Under hypoxic conditions, neither peroxynitrite nor ebselen affected the force-frequency relationship (Fig. 3A, \( P > 0.05 \)). However, under hyperoxic conditions, peroxynitrite resulted in a significant downward shift of the force-frequency curve (Fig. 3B, \( P < 0.01 \)). If force generation after exposure to peroxynitrite is normalized for its respective control, the effect under hyperoxic conditions is still much more prominent: relative force generation in hypoxia-peroxynitrite vs. hypoxia was 78, 80, 77, and 83% for \( F_r, P_{15}, P_{30}, \) and \( P_o \), respectively. Relative force generation in hyperoxia-peroxynitrite vs. hyperoxia was 66, 68, 65, and 60% for \( F_r, P_{15}, P_{30}, \) and \( P_o \), respectively. Ebselen did not affect force-frequency under hyperoxic conditions (Fig. 3, A and B; both \( P \geq 0.05 \)).

\( L^- \text{NMMA}. \) In both hypoxia and hypoxia-\( L^- \text{NMMA}, \) a significant depression in the force-frequency relationship was observed after 90 min of incubation (Fig. 4A, \( P \leq 0.001 \)). When expressed as percentage of initial force, \( L^- \text{NMMA} \) tended to improve the force-frequency relationship under hypoxic conditions (Fig. 4B, \( P = 0.095 \)).

**Nitrotyrosine Formation in the Rat Diaphragm**

**Hypoxia.** Monoclonal antinitrotyrosine antibody detected roughly eight nitrated protein bands in diaphragms of hyperoxic rats, with molecular masses ranging from \( \sim 230 \) to 28 kDa (Fig. 5B). Nitrotyrosine protein band OD in control diaphragm was similar to hypoxia (\( P > 0.05 \), Fig. 5A). Hypoxia significantly increased diaphragm nitrotyrosine level (\( P < 0.001 \), Fig. 5A). Roughly, additional nitrotyrosine proteins bands were detected at 295 and 243 kDa in the hypoxia group. Nitrotyrosine OD at 150 kDa was increased in hypoxia (\( P < 0.01 \) vs. hyperoxia).

**Fatigue.** At the time of task failure, nitrotyrosine OD was not significantly different between hypoxia-fatigue and hyper-
oxia-fatigue ($P > 0.05$, Fig. 6A), nor between hypoxia and hypoxia-fatigue. However, under hyperoxic conditions, fatiguing contractions significantly increased nitrotyrosine OD ($P < 0.05$, hypoxia vs. hypoxia-fatigue). Interestingly, after fatiguing contractions under hyperoxic conditions, nitrotyrosine protein expression at 295 and 243 kDa resembled the hypoxic pattern.

**Peroxy nitrite and ebselen.** Peroxynitrite exposure under hypoxic conditions did not affect total nitrotyrosine OD ($P > 0.05$, hypoxia vs. hypoxia-peroxynitrite, Fig. 7A). Ebselen reduced total nitrotyrosine OD under hypoxic conditions ($P < 0.01$, hypoxia vs. hypoxia-ebselen), although not completely to the OD of the hyperoxia group. Peroxynitrite significantly increased nitrotyrosine OD under hyperoxic conditions ($P < 0.05$, hyperoxia vs. hyperoxia-peroxynitrite, Fig. 7B). With peroxynitrite an additional protein band was detected at 325 kDa (Fig. 7C). Also, nitrotyrosine OD at 150 kDa was higher than that in diaphragm of hyperoxic group ($P < 0.05$). Ebselen

![Fig. 4. Effect of nitric oxide synthase (NOS) inhibitor l-NMMA on the force-frequency relationship under hypox conditions. A: solid symbols represent initial force generation, open symbols represent force-frequency relationship after 90 min in experimental conditions; squares represent hypox, circles represent hypox-l-NMMA. Initial measurement of force generation was not different between hypox and hypox-l-NMMA. Also, 90-min incubation with l-NMMA did not significantly affect decline in force under hypox conditions. B: when normalized for initial force, l-NMMA tended to increase force generation ($P = 0.095$ hypox vs. hypox-l-NMMA, $n = 6$ per group). $*P < 0.001$ compared with initial force.](image1)

![Fig. 5. A: total optical densities (ODs) for nitrotyrosine bands of rat diaphragm immediately removed from animal (control), after 60 min under hyperox conditions or after 60 min under hypox conditions. Hypox significantly increased rat diaphragm nitrotyrosine level. $*P < 0.001$ compared with control; $+P < 0.001$ compared with hyperox. B: representative immunoblots of rat diaphragm homogenates from control (1), hyperox (2), and hypox (3) groups. Note the increased OD under hypox conditions but also the appearance of high molecular bands (>200 kDa) not observed in hyperox. Nitrotyrosine level was detected with monoclonal antinitrotyrosine antibodies (see METHODS).](image2)
**DISCUSSION**

The main findings of the present study are that 1) severe hypoxia enhances nitrotyrosine formation in rat diaphragm, 2) hypoxia-induced elevation in diaphragm nitrotyrosine levels can be (partially) prevented by peroxynitrite scavenging or NOS inhibition, 3) under hypoxic conditions NOS inhibition tends to improve in vitro force generation, 4) exogenous peroxynitrite increases nitrotyrosine levels and impairs force generation of the rat diaphragm under hypoxic conditions, and 5) fatiguing contractions enhance nitrotyrosine levels in the hyperoxic diaphragm.

**Methodological Considerations**

Ebselen, a seleno-organic compound (45), was used as a peroxynitrite scavenger. It exhibits a marked thiol-dependent glutathione peroxidase-like activity and especially displays antioxidant activity in the micromolar range (39, 45). It acts as a singlet oxygen quencher (39, 45) and peroxynitrite scavenger (11, 12, 33). The reaction of ebselen with peroxynitrite is a simple oxygen atom transfer and depends on pH and temperature (36). As a lipid-soluble compound, it is likely to exert its effect intracellularly (45).

On the basis of dose-response studies, peroxynitrite at 250 μM was used (see METHODS). It is unknown whether this concentration is within limits of intracellular concentration during (patho-) physiological conditions. Due to the extreme short half-life of peroxynitrite, actual concentration with muscle fibers is unknown. Although NO release has been measured from intact skeletal muscle tissue in vitro (3), no data are available on NO concentrations within the subcellular compartments. This is relevant because within muscle fibers NO expression is not uniform (29, 30), suggesting that localized areas of higher NO concentration may exist. Also, localized areas of high superoxide generation may exist within muscle fibers. Therefore, measuring the average concentration of NO, superoxide, or peroxynitrite does not provide accurate information regarding maximal concentration within muscle fibers.

Nitration of protein tyrosine residues results in the formation of 3-nitrotyrosine (see i.e., Refs. 7, 16). In the present study nitrotyrosine formation was detected using a monoclonal antibody that is known to have high specificity (7). Although other nitrogen-centered oxidants such as nitrogen dioxide and acidified nitrite may result in nitrotyrosine formation, this is unlikely to happen in vivo, since the concentrations of nitrogen dioxide and nitrite present in vivo are far lower than necessary to cause significant nitration (7). It has been proposed that nitrogen dioxide radical production by myeloperoxidase may be an important source for nitrotyrosine formation in vivo (21). However, the current studies were performed in isolated muscle bundles that do not contain myeloperoxidase, which rules out a contribution of this pathway for nitrotyrosine formation.

The effect of hypoxia on nitrotyrosine formation of the diaphragm muscle was investigated in vitro. Clearly, this might not completely represent the effects of hypoxia on nitrotyrosine formation in vivo. However, a limitation of in vivo studies would be that hypobaric hypoxic environment increases work of breathing, which will enhance generation of oxidants in the diaphragm (31). The model used in the present study permits us to investigate the effects of hypoxia alone on muscle nitrotyrosine level.

**Hypoxia and Skeletal Muscle Function**

Previously, we have discussed the severity of hypoxia as used in the present study (PO2 ~6.5 kPa) (50). This should be considered as severe hypoxia, especially because under present experimental conditions oxygenation of the muscle solely depends on diffusion of O2 into the central region of the muscle...
bundle. Therefore, the average PO2 of the muscle will be well below 6.5 kPa. Several studies have shown that hypoxia impairs diaphragm muscle function (15, 20, 50, 51). Recently, we have shown that under resting conditions hypoxia mainly affects isometric contractile properties with no effect on shortening velocity (20). For this reason we focused on isometric contractile properties in nonfatiguing experiments. The precise mechanisms of hypoxia-induced impairment in muscle contractility are unknown. Several recent studies indicate that free radicals may be at play (20). We have recently summarized current knowledge on the role of hypoxia in muscle dysfunction (20).

Fig. 7. Effects peroxynitrite or ebselen exposure (60 min) on rat diaphragm total nitrotyrosine OD. A: peroxynitrite did not affect nitrotyrosine OD of the diaphragm under hypox conditions. However, after 60 min of exposure to ebselen, nitrotyrosine level of the diaphragm was significantly reduced. *P < 0.01 vs. hypox. B: under hyperox conditions, 60-min incubation with peroxynitrite significantly increased nitrotyrosine OD. Instead, ebselen did not affect nitrotyrosine level under hyperox conditions. †P < 0.05 vs. hyperox. C: representative immunoblots of rat diaphragm homogenates under different experimental conditions, for details see METHODS. Hyperox (1), hyperox-peroxynitrite (2), hyperox-ebselen (3), hypox (4), hypox-peroxynitrite (5), and hypox-ebselen (6). D: selectivity of the monoclonal antibody. Preincubation of the antinitrotyrosine antibody with nitrated BSA resulted in near disappearance of the positive nitrotyrosine bands in hyperox diaphragm homogenates (1) and homogenates of diaphragm bundles exposed to peroxynitrite (2).
Nitrotyrosine in Skeletal Muscle

Peroxy-nitrite is generated by reaction of superoxide with NO (7). This reaction rate is approximately six times faster than the scavenging of superoxide with superoxide dismutase (7), one of the physiological scavengers of superoxide. In line with previous observations (5, 52), the present study shows nitrotyrosine in the rat diaphragm muscle under baseline conditions. The molecular weight of the molecules nitrated in the present study is in line with observations by Vassilakopoulos et al. (52), but higher than that reported by Barreiro et al. (5). The reason for the discrepancy between these latter two studies (5, 52) is unclear.

Nitrotyrosine level can be increased under certain (patho-) physiological conditions. Barreiro et al. (5) show that LPS-induced sepsis increased nitrotyrosine formation in rat diaphragm, especially in the cytosolic fraction. In addition, exercise training increases gastrocnemius nitrotyrosine level (52). Our data show that acute hypoxia increases diaphragm muscle nitrotyrosine level. Elevated generation of NO and/or superoxide may enhance peroxynitrite formation. It has been shown in cardiomyocytes that generation of oxidants is increased, depending on the degree of hypoxia: the lower the PO2, the higher the oxidant generation (14). In addition, antioxidants reduce fatigability of the rat diaphragm under hypoxic conditions, indicating that reactive oxygen species affect force generation under hypoxic conditions (20). Little is known about the effects of acute hypoxia on NOS regulation or the rate of NO generation in skeletal muscle. Sixty days of hypobaric hypoxia increases eNOS and nNOS expression and activity in rat diaphragm muscle, but prolonged (9 mo) exposure decreased NOS activity and expression (26).

Hypoxia increased nitrotyrosine OD of protein bands already present under hyperoxic conditions but also resulted in the appearance of additional protein bands, for instance at ~295 and ~243 kDa (Fig. 5B). These same bands appeared in muscle bundles that were subjected to fatiguing contractions under hypoxic conditions (Fig. 6B). The nature of these proteins is unknown, but potential targets for peroxynitrite include myosin, actin, and troponin I, which have tyrosine groups that can be nitrated (7, 23). Peroxynitrite can also affect calcium homeostasis by inactivating Ca2+-ATPase (53–55), although data are conflicting (47). Studies with cultured cardiac myocytes revealed that plasma membrane proteins and myofibrillar creatine kinase are potential targets for peroxynitrite as well (23, 37). In addition, mitochondria potentially provide an abundant source of superoxide, and high concentrations of NOS are localized to mitochondria in skeletal muscle. Consequently, peroxynitrite may reach high concentrations in mitochondria, affecting nitration of mitochondrial proteins, and thereby impair mitochondrial function (10).

Under hypoxic conditions, incubation with peroxynitrite did not increase diaphragm nitrotyrosine formation. We are not aware of studies showing that half-life or reactivity of exogenous peroxynitrite depends on O2 tension. Theoretically, peroxynitrite reactivity toward tyrosine could depend on O2 tension. Indeed, recently, it has been shown that the effects of NO on muscle contractility depend on muscle PO2 (18). The PO2 dependence for peroxynitrite in skeletal muscle is not known. A more likely explanation for the inability of peroxynitrite to enhance nitrotyrosine levels under hypoxic conditions is that all highly reactive tyrosine groups were already nitrated. Indeed, it has been suggested that not all tyrosine groups in biological environment are equally susceptible to nitration (16, 46). Protein characteristics, such as the location of tyrosine on a loop structure and its association with a neighboring negative charge, determine susceptibility to nitration by peroxynitrite. This could also explain the absence of elevated protein nitration after strenuous contractions under hypoxic conditions. In hypoxia, strenuous contractions significantly increase protein nitration but to a level approximately equal to protein nitration under baseline hypoxic conditions (Fig. 6).

Functional Consequences of Nitrotyrosine Formation in Skeletal Muscle

Nitrotyrosine formation has been suggested as just “footprints” of peroxynitrite formation. However, evidence is accumulating that nitrotyrosine formation is a posttranslational mechanism for altering protein function (16) and thereby muscle contractility. The present study is the first to describe the relationship between nitrotyrosine levels and in vitro contractility. Under hypoxic conditions, the reduction in contractility was accompanied by enhanced nitrotyrosine levels. A similar pattern was observed after peroxynitrite exposure under hyperoxic conditions. The peroxynitrite-induced decrease in force generation is in line with previous observations (48). Interestingly, Supinski et al. (48) found that peroxynitrite...
impaired force generation in skinned single skeletal muscle fibers, indicating a direct effect on contractile proteins. Reducing nitrotyrosine levels with the peroxynitrite scavenger ebselen did not improve contractility in the present study. However, it should be noted that although ebselen significantly reduced nitrotyrosine level under hypoxic conditions, levels were still higher compared with hyperoxia (Fig. 7, A and B). Thus the inhibition of nitrotyrosation may have been too modest to exert an effect on force generation. Alternatively, it should be kept in mind that ebselen is not solely a peroxynitrite scavenger but also scavenges superoxide (see Methodological Considerations), which may modulate force generation via different pathways. Also, L-NMMA significantly reduced hypoxia-induced nitrotyrosine levels, but the effects on contractility were modest, if present at all (Fig. 4B, P = 0.095, n = 6). Together, no causal relationship seems to exist between total nitrotyrosine level in diaphragm muscle and contractility. However, this is not surprising, since the effect of protein nitration on contractility depends on the final effect of all proteins nitrated.

The mechanisms of increased peroxynitrite formation under hypoxic conditions have not been investigated. It can be speculated that elevated generation of peroxynitrite is the result of hypoxia-induced inflammatory response. Evidence is emerging that hypoxia induces expression of cytokines such as tumor necrosis factor (TNF)-α both in vitro (9, 18) and in vivo (49). In turn, TNF-α has been shown to increase oxidant levels in diaphragm muscle fibers (44) and also to increase the generation of NO in peripheral skeletal muscle (2). However, whether the hypoxia-induced elevation in nitrotyrosine formation in skeletal muscle is related to the activation of proinflammatory cytokines remains to be investigated.

Clinical Relevance

Hypoxia is a common feature in various pulmonary diseases including COPD, pneumonia, and ARDS. The adverse effects of hypoxia on respiratory muscle function are commonly recognized, but relatively little is known about the underlying pathophysiology. Recent studies have shown that oxidants play a prominent role in muscle physiology at different steps in excitation-contraction coupling (for review see, e.g., Refs. 19, 42). Recent studies indicate that oxygen free radical scavengers improve muscle endurance under hypoxic conditions. Whereas
hypoaxia may directly increase oxidant generation by the respiratory muscles, the hypoaxia-induced elevated work of breathing will further enhance oxidant generation by the respiratory muscles. Further understanding of the role of oxidants in respiratory muscle function is needed to develop successful strategies for preventing hypoaxia-induced respiratory muscle failure.

GRANTS

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REFERENCES


