Effects of hypoxia on diaphragm relaxation rate during fatigue

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Van Lunteren, Erik, Augusto Torres, and Michelle Moyer. Effects of hypoxia on diaphragm relaxation rate during fatigue. J. Appl. Physiol. 82(5): 1472–1478, 1997.—Skeletal muscle fatigue is associated with a slowing of relaxation rate. Hypoxia may increase the rate at which fatigue occurs, but, surprisingly, mild to moderate hypoxia has not been found to augment the degree of slowing of relaxation during fatigue. The present study tested the hypothesis that severe hypoxia interacts with fatigue in slowing the rate of muscle relaxation and that this can be modulated by altering membranous ionic conductances. Rat diaphragm muscle strips were studied in vitro while aerated with 95% O₂-5% CO₂ (normoxia) or 95% N₂-5% CO₂ (hypoxia). During continuous 0.1-Hz stimulation, relaxation rate and force remained stable over time, and relaxation rate was not slowed by hypoxia. Hypoxia accelerated force decline during continuous 5-Hz but not intermittent 20-Hz stimulation. During both 5- and 20-Hz stimulation, relaxation rate became slower over time as force declined, the extent of which was increased significantly by hypoxia. The extent of hypoxia-augmented slowing of relaxation rate during fatigue increased over time and was greater than expected for a given degree of force loss. 4-Aminopyridine did not attenuate or partially attenuate, whereas lowering extracellular Cl concentration fully attenuated, the hypoxia-induced prolongation of relaxation rate during repetitive stimulation. Thus hypoxia slows relaxation rate to a greater extent than expected for a given degree of force decline, an effect that increases over time, is at most partially attenuated by lowering K⁺ conductance, and is fully attenuated by lowering membranous Cl⁻ conductance.

Suggested by the finding of slowed rate of relaxation by severe hypoxia in cardiac muscle (13), the purpose of the present study was to test the overall hypothesis that severe hypoxia further slows the rate of diaphragm relaxation during fatigue. In addition, we tested the hypotheses that the hypoxia-induced slowing of relaxation during fatigue increases over time, is more prominent at higher rates of muscle stimulation, and is altered by changing membranous K⁺ and Cl⁻ conductances.

METHODS

Sprague-Dawley rats (male, weight 200–250 g) were anesthetized with urethan (1–1.5 g/kg administered ip). The costal diaphragm was removed surgically and placed in room temperature, oxygenated physiological solution. Small strips of muscle (diameter 1–1.5 mm) were dissected, with the bony and tendinous origins and insertions kept intact. The muscle strips were mounted vertically and bathed in physiological solution (temperature 37°C) of the following composition (in mM): 135 NaCl, 5 KCl, 2.5 CaCl₂, 1 MgSO₄, 1 NaH₂PO₄, 15 NaHCO₃, and 11 glucose, with the pH adjusted to 7.35–7.45 while the solution was being aerated with 95% O₂-5% CO₂. Muscle strips underwent field electrical stimulation (pulse width 1 ms, supramaximal voltage) via platinum electrodes, and their length was adjusted to that at which twitch force was maximal. With this stimulation paradigm, addition of curare (0.025 mM) to the bath does not alter twitch force, indicating that the muscles were activated directly (23, 24). To verify that neurotransmission failure did not contribute to fatigue, diaphragm force decline was compared in the absence and presence of curare during repetitive 20-Hz stimulation. If neurotransmission failure were present, the rate of fatigue should have been faster in the absence than in the presence of curare, but this was found not to be the case (Fig. 1). Somatic force was measured with a high-sensitivity force transducer (Kent Scientific/Radnoti Glass Technology, Monrovia, CA), and force records were digitized, collected on-line (Axotape software, Axon Instruments, Foster City, CA), and stored on the hard drive of a computer for later data analysis.

Five separate experiments were performed, the sample sizes of which are indicated in Table 1. Muscle strips were randomized across arms of a given experiment but not across experiments. After a 15-min equilibration period, muscles underwent twitch stimulation at 0.1 Hz for a 3-min baseline period. Strips in which force changed by >5% during the baseline period were discarded from further analysis. After the baseline period, addition of 0.3 mM 4-aminopyridine to block K⁺ channels (6, 17) (or placebo) was performed in experiments C and E, and lowering bath Cl⁻ concentration from 135 to 67.5 mM by substituting Na-gluconate for NaCl to lower Cl⁻ conductance (12) (or no change in bath Cl⁻ concentration) was performed in experiment D. A second equilibration period ensued, 4 min for the 4-aminopyridine studies (23) and 5-min for the low-Cl⁻ studies (based on Ref. 12). Subsequently, the gas with which the solution was aerated was either switched to 95% N₂-5% CO₂ (hypoxia) or maintained at
95% O2-5% CO2 (normoxia control). Bath oxygen was monitored in a subgroup of studies with a dissolved oxygen meter (model ISO-2, World Precision Instruments, Sarasota, FL). Muscles underwent continued 0.1-Hz stimulation during all of the above experiments to monitor twitch force. Finally, strips underwent one of three stimulation protocols (Table 1): continued 0.1-Hz stimulation (experiment A), continuous 5-Hz stimulation (experiments B–D), or intermittent 20-Hz stimulation (train duration 0.33 s, with 1 train delivered every second; experiment E). Drugs and reagents were obtained from Sigma Chemical (St. Louis, MO).

Data analysis was performed off-line with use of manually controlled cursors and included measurements of peak force and twitch half relaxation time (time for force to decay by 50%) performed at 10-s intervals after the onset of stimulation. Analysis was restricted to the first 60 s of repetitive stimulation, based on the following considerations: a desire to avoid long periods of stimulation associated with large degrees of muscle fatigue and a need to examine data for the 4-aminopyridine studies when the drug effect was at a stable plateau. (For Fig. 6 only, half relaxation time was analyzed as a function of specific degrees of force decline. Measurements of half relaxation time were performed at the points where force had declined by 10, 20, and 30% of initial force. This range of force declines was chosen because it corresponded roughly to the extent of force decline over 60 s in normoxic muscle.) During the 20-Hz stimulation paradigm, half relaxation times were derived from the last twitch of each train.

Force values are presented in absolute terms and after normalization relative to twitch force preceding onset of intermittent 20-Hz stimulation (train duration 0.33 s, with 1 train delivered every second). n, No. of muscle strips. There were no significant effects of curare on force or half relaxation time. Normalization of force values was performed to factor out the effects of variability in muscle strip size and hence baseline force; statistical analysis of force data was, therefore, performed only for normalized values. Data reported are means ± SE. Statistical comparisons were made with two-way analysis of variance (ANOVA) or two-way repeated-measures ANOVA followed by the Newman-Keuls test when the ANOVA indicated statistical significance. A P value of < 0.05 (2 tailed) was considered to indicate statistical significance.

### RESULTS

Changing the gas with which the physiological solution was bubbled from one containing 95% O2 to one containing 0% O2 produced a rapid fall in bath oxygen saturation, which reached a nadir at <5% (Fig. 2). Hypoxia did not significantly reduce force or slow the

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<th>Table 1. Stimulation frequencies, experimental conditions, and sample sizes of the five experiments</th>
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<td>Experiment</td>
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<td>Stimulation frequency, Hz</td>
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<td>Experimental conditions</td>
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Nos. in parentheses are sample size. N, normoxia; H, hypoxia; ND, no drug; AP, 4-aminopyridine; NC, normal chloride; LC, low chloride.

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**Fig. 1.** Comparison of diaphragm muscle force decline in presence and absence of curare (0.025 mM) in muscle bath. Force is presented in absolute values and after normalization to twitch force preceding onset of intermittent 20-Hz stimulation (train duration 0.33 s, with 1 train delivered every second). n, No. of muscle strips. There were no significant effects of curare on force or half relaxation time.

**Fig. 2.** Changes in oxygen saturation of bathing solution over time. Values are means ± SE. Arrows indicate time at which aerating gas was changed from 95% O2-5% CO2 to 95% N2-5% CO2 for hypoxia studies and time at which 5- or 20-Hz intermittent stimulation commenced for all studies.
relaxation rate of muscle strips undergoing twitch contractions (i.e., those stimulated at 0.1 Hz; Fig. 3).

The interactive effects of repetitive stimulation and hypoxia on mean values for force and half relaxation time during 5- and 20-Hz stimulation are depicted in Figs. 4 and 5. Neither force nor rate of relaxation was affected by hypoxia at the beginning of repetitive stimulation. During 5-Hz stimulation, normalized force was significantly lower in hypoxic muscle than normoxic muscle 30–60 s after the onset of stimulation, whereas hypoxia did not affect normalized force during 20-Hz stimulation (Fig. 4). During both 5- and 20-Hz stimulation protocols, hypoxia significantly slowed the rate of relaxation during the second half of the stimulation period (Fig. 5). During 20-Hz stimulation, normalized force was comparable for hypoxic and normoxic muscle, yet half relaxation time was significantly longer for hypoxic than normoxic muscle (see 40- to 60-s data in Figs. 4 and 5), indicating that the degree of hypoxia-augmented slowing of relaxation was greater than expected for a given degree of force decline. To examine whether this was also the case for 5-Hz stimulation, we quantified changes in half relaxation time as a function of degree of force decline (Fig. 6). Hypoxia prolonged half relaxation time significantly when force had declined by 30% of initial force, and similar trends were noted for smaller degrees of force decline. The degree to which relaxation time was prolonged at the end of the 60-s stimulation period was greater during 20- than 5-Hz stimulation under both normoxic and hypoxic conditions (Fig. 7).

In both normoxic and hypoxic muscle, 4-aminopyridine (0.3 mM) had either no effect (5-Hz stimulation; Fig. 8) or a small effect (20-Hz stimulation; Fig. 9) on relaxation rate at the onset of repetitive stimulation. After 60 s of stimulation, however, relaxation rate was prolonged substantially by 4-aminopyridine during both 5- and 20-Hz stimulation, as was reported previously for normoxic muscle (23). In the absence of 4-aminopyridine, hypoxia prolonged relaxation time significantly after 60 s of both 5- and 20-Hz stimulation. In the presence of 4-aminopyridine, hypoxia still prolonged relaxation time significantly after 60 s of 5-Hz contraction. However, the hypoxia-induced prolongation of relaxation time in the presence of 4-aminopyridine was small and not statistically significant during 20-Hz stimulation.

In normoxic muscle during 5-Hz stimulation, lowering extracellular Cl\(^{-}\) did not change relaxation time at the onset of repetitive contraction. However, the change
in half relaxation time over 60 s of repetitive contractions was greater in low-Cl\(^-\) than normal-Cl\(^-\) conditions (Fig. 10). In contrast, lowering extracellular Cl\(^-\) had no effect on the change in relaxation time with repetitive stimulation of hypoxic muscle. With normal extracellular Cl\(^-\), there was a greater change in relaxation time after 60 s of repetitive stimulation during hypoxia than normoxia, whereas with low extracellular Cl\(^-\) there was no apparent effect of hypoxia on the change in relaxation time after repetitive stimulation.

**DISCUSSION**

**Effects of hypoxia on fatigue.** Previous studies conflict on whether hypoxia accelerates muscle fatigue, with some data indicating a lack of effect (2, 9, 20) and other data indicating a detrimental effect (2, 14, 15, 20). Part of the discrepancy among studies may be due to use of different models (in vivo, in situ, in vitro), different muscles (diaphragm, gastrocnemius), and different species (human, dog, cat, hamster). However, studies using the same model and species indicate that a detrimental effect of hypoxia on fatigue resistance is more likely with greater degrees of hypoxia (2, 20) and possibly with a higher duty cycle of contraction (Ref. 2; but also see Ref. 15). Bark and Supinski (2) varied the intensity of contraction by increasing the duration of electrical stimulation but keeping stimulation frequency constant and found greater effects of hypoxia on fatigue during high-intensity than low-intensity contraction. In the present study, both duty cycle and stimulation frequency were varied. As a result, slowing of relaxation with fatigue and hypoxia increased the degree of contractile fusion and led to better maintenance of peak force during the 20-Hz stimulation.

**Effects of hypoxia and relaxation time and interactions with time and stimulation intensity.** Previous studies have not demonstrated an effect of hypoxia on slowing of relaxation rate during fatigue (2, 9, 20). This contrasts with the marked effect of hypoxia on relaxation rate during fatigue noted in the present study. This may very well be due to milder degrees of hypoxia and/or use of an in vivo rather than an in vitro preparation used in previous studies. Esau (9) used a gas mixture containing 15% O\(_2\) in vitro; although the bath PO\(_2\) was not specified, it is very likely less hypoxic than that of the present study in which the bath was bubbled with a gas mixture containing 0% O\(_2\). Previous in vivo studies achieved a mean arterial PO\(_2\) of ~50–57 Torr for “mild hypoxia” and ~29–34 Torr for “severe hypoxia” (2, 20). Although the above value for severe hypoxia is similar to that obtained in the present study, it is likely that the degree of tissue hypoxia is more...
severe in vitro than in vivo because of larger diffusion distances for oxygen under these conditions.

Esau (9) found that combined hypoxia and hypercapnic acidosis in vitro accelerated fatigue and augmented the degree to which relaxation rate slowed during fatigue, whereas either stimulus by itself had little effect. Edwards et al. (8) found that the rate of human quadriceps muscle relaxation was slowed during fatigue but only when forces were large enough to occlude the vascular supply (which most likely also produces combined hypoxia and acidosis). In the present study, we found that the hypoxia-augmented slowing of relaxation was greater during 20- than 5-Hz stimulation and, furthermore, became more prominent over time during continued stimulation. The previous and present studies are consistent with the concept that a sufficiently severe stimulus is required to slow relaxation.

Diaphragm motor units typically fire at frequencies ranging from 10 to 30 Hz during resting breathing and from 20 to 40 Hz when breathing is stimulated by hypoxia (19, 22). The interactive effects of fatigue and hypoxia in slowing the rate of muscle relaxation will increase the degree of contractile fusion during these intermediate firing frequencies, thereby leading to better maintenance of peak force despite the adverse effects of hypoxia on twitch force.

Potential mechanisms, including roles of K⁺ and Cl⁻ conductances. Muscle relaxation results from reuptake of Ca²⁺ into the sarcoplasmic reticulum, a process that requires energy (3, 4, 7). Studies of fatigue under normoxic conditions have attributed slowing rate of relaxation to metabolic factors. Specifically, the degree of relaxation slowing has been found to be related to intracellular phosphocreatine, creatine, inorganic phosphate, ATP, and H⁺ concentrations as well as to rate of ATP hydrolysis (7). Thus possible explanations for the greater effects of severe than mild to moderate hypoxia on rate of relaxation during fatigue include an accelerated rate of intracellular acidosis or an accelerated rate of depletion of high-energy phosphates. However, in the present study during 20-Hz stimulation, the relaxation rate was slowed to a greater extent during hypoxia than normoxia despite comparable changes in force (see especially 40 s after onset of stimulation, Figs. 4 and 5), arguing against metabolic factors being the sole determinant of the augmented slowing of relaxation.

Relaxation may also be prolonged by increased sarcoplasmic Ca²⁺ and by delayed repolarization (5). Repetitive contractions and hypoxia both lead to resting

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<th>Initial</th>
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<td>4 Hz, normoxia (n=16)</td>
<td>4 Hz, hypoxia (n=18)</td>
<td>20 Hz, normoxia (n=5)</td>
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<td>20 Hz, hypoxia (n=18)</td>
<td>20 Hz, hypoxia (n=5)</td>
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*Statistically significant difference compared with normoxia, 5 Hz, P < 0.05. **Statistically significant difference compared with hypoxia, 5 Hz, P < 0.05. *Statistically significant difference compared with normoxia, 20 Hz, P < 0.05.

Fig. 7. Combined effects of hypoxia and stimulation frequency (5-Hz continuous vs. 20-Hz intermittent stimulation) on half relaxation time. Values are means ± SE and are shown for relaxation time at onset of and after 60 s of repetitive contraction as well as for changes in relaxation time over course of 60 s; n, no. of muscle strips. Data are from experiment C. *Statistically significant difference compared with normoxia, no drug, P < 0.05. **Statistically significant difference compared with hypoxia, no drug, P < 0.05. *Statistically significant difference compared with normoxia, 4-AP, P < 0.05.

Fig. 8. Combined effects of hypoxia and 4-aminopyridine (4-AP; 0.3 mM) on half relaxation time during 5-Hz stimulation. Values are means ± SE and are shown for relaxation time at onset of and after 60 s of repetitive contraction as well as for changes in relaxation time over course of 60 s; n, no. of muscle strips. Data are from experiment C. *Statistically significant difference compared with normoxia, no drug, P < 0.05. **Statistically significant difference compared with hypoxia, no drug, P < 0.05. *Statistically significant difference compared with normoxia, 4-AP, P < 0.05.
membrane depolarization, the former as a direct consequence of enhanced K\textsuperscript{+} efflux (21, 25) and the latter possibly as a result of an adenosine-mediated reduction in Na-K pump activity (10). Repetitive muscle contraction also is associated with decreased membrane Cl\textsuperscript{-} conductance and slowing of action potential repolarization (12, 16, 18). Lowering Cl\textsuperscript{-} conductance (by reducing extracellular Cl\textsuperscript{-}) and blocking K\textsuperscript{+} channels (with 4-aminopyridine) each slows the rate of relaxation to a greater extent in fatigued than nonfatigued muscle (Refs. 12, 23; and confirmed by the present data), suggesting that reduced Cl\textsuperscript{-} and K\textsuperscript{+} conductances with consequential delayed action potential repolarization may play roles in the prolongation of relaxation during fatigue (12, 23).

4-Aminopyridine and low extracellular Cl\textsuperscript{-} had a similar impact on relaxation rate of resting hypoxic compared with normoxic muscle, which is not unexpected given that hypoxia itself also did not alter relaxation rate in resting muscle. The two interventions did affect relaxation of hypoxic, fatigued muscle, although in different manners: K\textsuperscript{+} channel blockade did not attenuate (during 5-Hz stimulation) or partially attenuated (during 20-Hz stimulation) the hypoxia-induced slowing of relaxation during fatigue, whereas lowering Cl\textsuperscript{-} conductance fully attenuated the hypoxia-induced slowing of relaxation during fatigue. In normoxic muscle, 4-aminopyridine had a considerably greater effect than did low extracellular Cl\textsuperscript{-} in slowing relaxation during fatigue. Although both interventions are known to delay cellular repolarization (1, 12, 17), 4-aminopyridine but not low extracellular Cl\textsuperscript{-} also prolongs contraction time. As a result, the former but not the latter will increase Ca\textsuperscript{2+} influx during the depolarization phase of the action potential, the reuptake of which will be prolonged, leading to a slower rate of relaxation. This additional mechanism may account for the greater effects of 4-aminopyridine than low-extracellular Cl\textsuperscript{-} concentration on slowing the rate of relaxation during fatigue. That hypoxia further slows rate of relaxation during fatigue even in the presence of K\textsuperscript{+} channel blockade suggests that hypoxia and fatigue may act similarly in altering K\textsuperscript{+} channel conductance and thereby relaxation rate. During 5-Hz stimulation, hypoxia and 4-aminopyridine interacted additively or possibly even multiplicatively to prolong relaxation rate during fatigue, whereas during 20-Hz stimulation the effects of hypoxia and 4-aminopyridine singly were greater than their combined effects. This is consistent with mecha-
nisms in addition to altered K\(^+\) conductance (e.g., metabolic factors) playing a greater role in determining rate of relaxation during high- than during low-intensity contraction. In contrast, the finding that lowering Cl\(^-\) conductance exaggerates the slowing of relaxation during fatigue but attenuates the hypoxia-induced slowing of relaxation during fatigue argues that fatigue and hypoxia have divergent effects on the manner in which Cl\(^-\) conductance regulates muscle relaxation rate.

Conclusions. In summary, the present study demonstrated that hypoxia produces a slowing of relaxation in actively contracting, but not in relatively quiescent, diaphragm muscle. The augmented slowing of relaxation by hypoxia during fatigue becomes more prominent over time and appears to be more pronounced during high- compared with low-frequency stimulation. Finally, the extent of slowing of relaxation is influenced by 4-aminopyridine and lowered extracellular Cl\(^-\) concentration, suggesting a role for altered membranous ionic conductances in the hypoxia-augmented slowing of relaxation.

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