Physiological and Genomic Consequences of Intermittent Hypoxia
Selected Contribution: Improved anoxic tolerance in rat diaphragm following intermittent hypoxia

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Clanton, Thomas L., Valerie P. Wright, Peter J. Reiser, Paul F. Klawitter, and Nanduri R. Prabhakar. Selected Contribution: Improved anoxic tolerance in rat diaphragm following intermittent hypoxia. J Appl Physiol 90: 2508–2513, 2001.—Intermittent hypoxia (IH), associated with obstructive sleep apnea, initiates adaptive physiological responses in a variety of organs. Little is known about its influence on diaphragm. IH was simulated by exposing rats to alternating 15-s cycles of 5% O2 and 21% O2 for 5 min, 9 sets/h, 8 h/day, for 10 days. Controls did not experience IH. Diaphragms were excised 20–36 h after IH. Diaphragm bundles were studied in vitro or analyzed for myosin heavy chain isoform composition. No differences in maximum tetanic stress were observed between groups. However, peak twitch stress (P < 0.005), twitch half-relaxation time (P < 0.02), and tetanic stress at 20 or 30 Hz (P < 0.05) were elevated in IH. No differences in expression of myosin heavy chain isoforms or susceptibility to fatigue were seen. Contractile function after 30 min of anoxia (95% N2-5% CO2) was markedly preserved at all stimulation frequencies during IH and at low frequencies after 15 min of reoxygenation. Anoxia-induced increases in passive muscle force were eliminated in the IH animals (P < 0.01). These results demonstrate that IH induces adaptive responses in the diaphragm that preserve its function in anoxia.

skeletal muscle; chronic obstructive pulmonary disease; obstructive sleep apnea; myosin heavy chain; anoxia

OBSTRUCTIVE SLEEP APNEA (OSA) is associated with a constellation of clinical conditions, including elevations in arterial blood pressure (25), increases in tonic sympathetic activity (13), potentiation of ventilatory and chemoreflex responses to hypoxia (14), changes in cardiac function (24), and numerous alterations in hormonal and paracrine signaling (9, 15, 18). The underlying physiological or pathological stimuli responsible for these changes are not well understood. However, models of intermittent hypoxia (IH), which mimic the oscillations in arterial saturation occurring in OSA, suggest that IH may be different from chronic hypoxia and may be an important trigger for specific adaptations (23).

Little is known about the influence of OSA on the diaphragm or other skeletal muscles. Metabolic responses to exercise have been shown to be altered (27), and the exercise responses in OSA patients are improved after treatment with continuous positive airway pressure (26). This suggests a causal relationship between OSA and changes in muscle function or metabolism. The present study was designed to evaluate the potential for short-cycle IH, a model of OSA, to alter the function of diaphragm muscle. The results demonstrate baseline alterations in contractile function and twitch kinetics and marked improvements in the contractile responses during and after an anoxic challenge.

METHODS

General Procedures

All procedures were done on male Sprague-Dawley rats (245–325 g), in accordance with The Ohio State University (OSU) and Case Western Reserve University (CWRU) lab animal use guidelines. Experimental rats (n = 5) housed at CWRU were exposed to 10 days of IH as follows. Animals were unrestrained and freely mobile in standard rat cages with food and water available ad libitum. The cages were placed in a custom-built chamber and flushed with 15-s
alternating cycles of 5% O\textsubscript{2} and 21% O\textsubscript{2} for 5 min (9 sets/h). Timed solenoid valves controlled the durations of the gas flows. Ambient O\textsubscript{2} levels were continuously monitored using an oxygen analyzer (LB-2 analyzer). Animals were exposed to IH 8 h/day during the hours from 9:00 AM to 5:00 PM, a time when rodents normally sleep. Each day, after exposure to IH, the rats were returned to the Animal Resource Center at CWRU where they were maintained on a 12:12-h light-dark cycle. After 10 days of IH, they were transported to OSU in a heated automobile and allowed to recover overnight with food and water ad libitum. Muscle testing was performed 20–36 h after leaving CWRU. Control rats, shipped directly to OSU (n = 6), were not exposed to IH but were otherwise handled identically.

The rats were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg), tracheotomized, and mechanically ventilated. The whole diaphragm was then excised and cut into strips with portions of the central tendon and ribs attached. Strips were then mounted vertically in tissue baths maintained at 37°C and oxygenated with 95% O\textsubscript{2}-5% CO\textsubscript{2} in Ringer salt solution (in meq/l: 21 NaHCO\textsubscript{3}, 0.9 NaSO\textsubscript{4}, 1.2 Na\textsubscript{2}HPO\textsubscript{4}, 1.0 MgCl\textsubscript{2}, 2.0 CaCl\textsubscript{2}, 5.9 KCl, and 121 NaCl with 2.07 g/l glucose and 10 \( \text{M D-tubocurarine} \). One strip was used for fatigue measurements. The remaining two strips duplicated an anoxia challenge and reoxygenation protocol. Strips in each experimental group, for both IH and controls, were taken from the same region of the diaphragm. An additional strip was cut for myosin heavy chain isoform analysis.

Once the strips were mounted in the baths, optimal length and maximum current were determined for each using standard methods (11). Strips were allowed to equilibrate for 15 min while they were stimulated with one twitch every 20 s. At the end of this time, force-frequency relationships were determined using twitches (20-, 30-, 60-, and 150-Hz tetanic simulations) maintained for 400 ms, with a 20-s interval between each stimulation. Baseline preloads were reset to the tension measured at optimal length. The strips were rested for 10 min, at which time individual protocols were started.

At the end of each protocol, strip length and wet weight were measured. Tissues were then dried, and dry weight was recorded. Forces were normalized to cross-sectional area using the technique of Close (3). Wet weight was corrected based on dry weight. This avoided errors due to loss of water during the weighing process.

Fatigue Protocol

Strips were stimulated at 20 Hz, 330-ms duration, one stimulation per second. At the end of 4 min, strips were given a single stimulus of 200 Hz to test for fatigue-induced changes in maximal tetanic stress.

Anoxia Challenge and Reoxygenation

This protocol was repeated on two tissue strips for each animal. Bathes were quickly changed to 95% N\textsubscript{2}-5% CO\textsubscript{2} by first changing the source gas to the bath, then draining the bath, and refilling with prenitrogenated Ringer solution at 37°C. They were then continually bubbled with 95% N\textsubscript{2}-5% CO\textsubscript{2} with the top of the tissue bath loosely covered. This resulted in a rapid step change to anoxia. Tissues were equilibrated in anoxia for 30 min. Measurements of passive force (preload) were recorded throughout the anoxic exposure. This was followed by a new force-frequency maneuver. Oxygenated Ringer was then reintroduced and continually bubbled with 95% O\textsubscript{2}-5% CO\textsubscript{2}. At the end of 15 min of reoxygenation, a final force-frequency maneuver was performed.

Myosin Heavy Chain Isoform Expression

Diaphragm samples were prepared for gel electrophoresis to analyze myosin heavy chain isoform expression. Samples were taken from the same region of the right costal diaphragm, ~2–3 mm dorsal from the phrenic nerve. The separating gels consisted of 7% acrylamide and 30% glycerol and were run for 24 h at a constant 275 V at 8°C. All other details of the methods for sample and gel preparation, the composition of the gels, the gel running conditions, and gel staining and scanning were as described in Blough et al. (1) and Reiser and Kline (19). The basis for the identification of the myosin heavy chain bands on the stained gels was as stated in Reiser and Kline.

Data Analysis

Absolute force development per cross-sectional area is expressed as stress, whereas all other normalized force measurements are referred to as force. For analysis of baseline contractile function, before hypoxia or fatigue, data from three strips (i.e., from one rat) were tested using repeated measures, one-way ANOVA, with treatment (IH vs. control) as the factor of interest and the repeated measure being samples from different regions of the diaphragm. Data points were expressed graphically as the average value of the means from the three samples and the average standard error (SE) of the three measured groups tested (representing strips from three anatomic origins on the diaphragm). Therefore, each SE represents an estimate of the SE from groups representing one diaphragm per animal. For the anoxia challenge, two strips were tested, but the data analysis was handled identically with repeated measures. For fatigue measurements (one strip), a two-sample t-test was used to test for differences between groups.

RESULTS

Baseline Contractile Characteristics

Diaphragms from IH animals demonstrated marked changes in baseline contractile properties (Table 1). A 28% average increase in peak twitch stress \((P < 0.005)\), a 17% increase in twitch half-relaxation time \((P < 0.02)\), and a 24% increase in tetanic stress at 20-Hz stimulation \((P < 0.04)\) were observed. Time to peak tension and maximum stress at 150 Hz remained unchanged. A shift was also observed in the force-frequency relationship, as illustrated by the ratio of 20 to 150 Hz (Table 1), which increased 28% in the IH diaphragms compared with controls \((P < 0.01)\).

Myosin Heavy Chain Isoforms

As shown in Table 2 and Fig. 1, all four of the myosin heavy chain isoforms that are characteristic of adult rat skeletal muscle were expressed in the diaphragms of hypoxic and control rats, with the largest fraction being the IID isoform in both groups of rats. There were no significant differences between the two groups of animals in the relative proportions of any of the isoforms.
ANOVA, with repeated measures, intermittent hypoxia vs. control. Groups (right). There was also no significant difference between the fatigue protocol (single data point in Fig. 4, force at 200 Hz was measured at the last contraction of not significantly different (P 0.19, t-test). Maximum force at 200 Hz was measured at the last contraction of the fatigue protocol (single data point in Fig. 4, top right). There was also no significant difference between groups (P 0.21, t-test).

Fig. 1. Myosin heavy chain (MHC) region of a gel on which samples from intermittent hypoxia and control rats were loaded. Each lane represents a diaphragm strip from 1 rat.

Table 1. Effects of intermittent hypoxia on baseline stress and twitch kinetics

<table>
<thead>
<tr>
<th></th>
<th>Pt, N/cm²</th>
<th>TPT, ms</th>
<th>T½, ms</th>
<th>Stress, N/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.52 ± 0.27</td>
<td>287 ± 9</td>
<td>259 ± 13</td>
<td>20 Hz</td>
</tr>
<tr>
<td></td>
<td>(P &lt; 0.005)</td>
<td>(P = 0.91)</td>
<td>(P &lt; 0.02)</td>
<td>150 Hz</td>
</tr>
<tr>
<td>Intermittent hypoxia</td>
<td>5.83 ± 0.36</td>
<td>290 ± 14</td>
<td>302 ± 15</td>
<td>20/150 Hz Stress Ratio</td>
</tr>
<tr>
<td>(n = 5)</td>
<td></td>
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</tbody>
</table>

Values are means ± SE (i.e., grand means of 3 samples from each diaphragm; SE is the average of the standard error of means of the 3 samples for each animal). Pt, twitch stress; TPT, time to peak tension during twitch; T½, time to half relaxation. P values from 1-way ANOVA, with repeated measures, intermittent hypoxia vs. control.

Responses to Anoxic Challenge

As shown in Fig. 2A, contractile function of IH diaphragms in anoxia was preserved at all frequencies of stimulation compared with controls. After 15 min of reoxygenation (Fig. 2B), the responses to stimulation frequencies between 20 and 60 Hz were also significantly preserved in the IH diaphragms. At 150-Hz stimulation, there were no differences between the two groups.

Over the 30-min anoxia exposure, passive diaphragm force increased with time, ~1–2 g (i.e., 1–2% of maximum tetanic stress) in each control diaphragm strip (Fig. 3). However, in the IH group, there was no increase in passive stress during anoxia (P 0.01).

Responses to Fatiguing Stimulations

Figure 4 illustrates relative force production during the fatigue protocol. Note, that when displayed as a percentage of absolute maximum tetanic force at baseline, the IH group generated greater force at the initiation of the fatigue protocol. This could have been predicted by the results of Table 1. However, the forces quickly superimposed and were not significantly different beyond 45 s at any time point. When the data were expressed as a percentage of 20-Hz force at baseline (data not shown), these force tracings were nearly superimposable at all times during the fatigue test. Taken together, their expression of fatigue does not suggest marked improvements in endurance characteristics of the muscles exposed to IH. Endurance times, calculated as the time required to achieve 50% of the 20-Hz tetanic force measured at baseline, were 116 ± 6 s for control and 101 ± 8 s for IH rats and were not significantly different (P = 0.19, t-test). Maximum force at 200 Hz was measured at the last contraction of the fatigue protocol (single data point in Fig. 4, top right). There was also no significant difference between groups (P = 0.21, t-test).

DISCUSSION

The results demonstrate that rats exposed to 10 days of short-cycle IH during sleep exhibit functional adaptations that protect contractile function during and after an anoxic challenge. These adaptations resulted in improvements in tetanic force at all stimulation frequencies during anoxia (Fig. 2). Strikingly, anoxia-induced elevations in passive force were eliminated in the IH animals. Before anoxia, diaphragms exposed to IH also showed significant elevations in twitch stress, slowed relaxation kinetics (Table 1), and an upward shift of the force-frequency relationship at low frequencies of stimulation (Fig. 2A). These suggest inherent, chronic alterations in some component of excitation-contraction coupling, which are not evident in measures of maximum tetanic stress. Furthermore, the changes in contractile characteristics are not likely to reflect alterations in muscle fiber phenotype, as myosin heavy chain isoforms were unchanged between IH and control (Table 2, Fig. 1).

Comparisons Between Short-Cycle IH and OSA

Patients with OSA exhibit periodic airway occlusions during sleep, resulting in simultaneous reductions in blood oxygen saturation and activation of inspiratory muscles. These are characteristics in common with this model of IH. However, the model also differs from OSA in that hyperventilation and diaphragm shortening contractions would presumably accompany hypoxia, whereas hypercapnia and contractions against an occluded airway would characterize OSA. Whether these factors are important contributors to the response needs to be evaluated.

Another relevant question is whether resistance to anoxia is a functionally important adaptation in OSA patients. The diaphragm, in OSA, appears to be considerably taxed during periods of occlusion. For exam-

Table 2. Comparison of myosin heavy chain isoforms composition

<table>
<thead>
<tr>
<th></th>
<th>%MHC IIA</th>
<th>%MHC IID</th>
<th>%MHC IIB</th>
<th>%MHC I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 5)</td>
<td>24.8 ± 2</td>
<td>31.8 ± 1.8</td>
<td>20.9 ± 4.7</td>
<td>22.6 ± 1.8</td>
</tr>
<tr>
<td>Intermittent hypoxia</td>
<td>25.7 ± 1.7</td>
<td>33.7 ± 1.6</td>
<td>20.3 ± 3.9</td>
<td>20.3 ± 2.6</td>
</tr>
<tr>
<td>(n = 5)</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Values are means ± SE. MHC, myosin heavy chain.
ple, the pressures generated can be quite large, reaching $>150$ cmH$_2$O in some patients and averaging $>25$ cmH$_2$O in most by the end of the occlusion period (30). Furthermore, the duty cycle (contraction time/total time) nearly doubles by the end of the apneic phase, resulting in a relatively large pressure-time index for brief periods of time (30). These facts have led some investigators to suggest that the diaphragm is susceptible to fatigue during apneic episodes in OSA (28). However, this remains controversial (2, 30). Regardless of whether fatigue develops, heavy and prolonged contractions during hypoxia could result in tissue hypoxia and markedly disordered energy balance for brief periods. This could damage the muscle or make it susceptible to other forms of stress. In addition, although IH exposure did not appreciably affect susceptibility to fatigue in this model, it is possible that, when stimulation is concurrent with hypoxia, there may be an improved resistance to fatigue.

Possible Mechanisms of Adaptation

**Metabolism.** One possible source for improved anoxic tolerance could be alterations in metabolic pathways that favor energy availability in hypoxia. No studies have directly addressed the metabolic responses of skeletal muscle to short-cycle IH. However, it is clear that the adaptive responses of muscle to long-cycle or continuous hypoxia differ depending on the duration of exposure (6), the degree of hypoxia (6, 16), and the activity of the muscle during exposure (10). These differences are likely to also influence the responses to short-cycle IH but have not been studied extensively. Models of long-cycle IH (12-h cycles) for extended periods (4 wk) show reductions in citrate synthase activity and elevations in both hexokinase and lactate dehydrogenase activity (17). These results have been interpreted as a shift away from mitochondrial respiration and toward increases in glycolysis (7, 16, 17). However, in patients with OSA, maximal lactate production during exercise is reduced, a response similar to the “lactate paradox” of altitude acclimatization (7), suggesting a more complex response. Paradoxically, it is possible that either increased or decreased anaerobiosis could improve hypoxic tolerance in this model. For example, increased glycolytic capacity during a hypoxic challenge could result in more energy available in the form of creatine phosphate and ATP. On the other hand, the additional lactate and H$^+$ formed from glycolysis could inhibit contraction and Ca$^{2+}$ cycling, causing inhibition of contractile force during hypoxia (20).

**Contractile responses.** The changes observed in baseline twitch contractions directly translate to the ob-

![Fig. 2. Force responses (relative to baseline maximum tetanic force at 150 Hz) at varying frequencies of stimulation (means ± SE). A: contractile responses during the last minutes of the 30-min anoxic challenge. B: contractile responses after 15 min of reoxygenation. *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$, one-way, repeated-measures ANOVA.](http://jap.physiology.org/)

**Fig. 3.** Changes in passive stress from the beginning to the end of the anoxic challenge, normalized to strip cross-sectional area (means ± SE).

**Fig. 4.** Relative force production during fatiguing stimulation in control diaphragms and diaphragms from animals exposed to intermittent hypoxia (means ± SE). There were no significant differences between points beyond 45 s.
erved shifts in the force-frequency relationship. Higher peak twitch forces with slower relaxation times would result in tetanic fusion at lower frequencies of stimulation. Functionally, this would mean that during submaximal stimulations a greater force reserve would be available.

The changes in twitch characteristics also suggest inherent alterations in excitation-contraction coupling. It is unlikely that they are due to inherent changes in contractile machinery, as myosin isoform composition and peak tetanic stress were unchanged. Therefore, the changes in baseline excitation-contraction coupling must arise from 1) altered membrane ion regulation, 2) changes in Ca\textsuperscript{2+} release and reuptake, or 3) alterations in Ca\textsuperscript{2+} buffering and/or myofilament Ca\textsuperscript{2+} sensitivity. We have no data to distinguish between these mechanisms. However, there is evidence that changes in several ATPases occur in response to altered oxygen availability. For example, in rats acclimated to high-altitude hypoxia, myocardial sarcosommal Mg\textsuperscript{2+}, Ca\textsuperscript{2+}, and Na\textsuperscript{+}-K\textsuperscript{+}-ATPases show a significant decrease in \( K_m \) (31). This would allow the ATPases to better maintain their activity, compared with nonacclimatized conditions, in the face of the lowered ATP concentration seen during an acute anoxic insult. If similar changes occurred in the diaphragm, it would also allow an improvement in contractility during hypoxia. Furthermore, it has been shown that exposure to hyperoxia upregulates Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (29) and that exercise training at altitude downregulates Na\textsuperscript{+}-K\textsuperscript{+}-ATPase channel expression (5). One mechanism that has been proposed for the upregulation in hyperoxia is the formation of reactive oxygen species (29). Reactive oxygen species formation would also be expected to increase in response to cycling between hypoxia and reoxygenation (4, 22).

We cannot rule out that changes in contractile properties of these muscles reflect the influence of increased exercise conditioning in IH. No doubt, for the 8-h periods of IH cycling, respiration and diaphragm activity were transiently increased. The fact that these conditioning stimuli did not translate to an improvement in endurance properties of the muscle is interesting (Fig. 4) and suggests a divergence of adaptive responses to hypoxia vs. exercise conditioning.

**Passive force during hypoxia.** After exposure to very low levels of \( O_2 \), the diaphragm muscle shortens against its series elastic component, resulting in elevations in passive tension and presumably in stiffness (Fig. 3). The mechanism for this phenomenon in skeletal muscle is not completely understood but could resemble similar responses in cardiac myocytes. In cardiac myocytes, it is referred to as “ATP-depletion rigor contracture” and may involve Ca\textsuperscript{2+} accumulation during ischemia or anoxia (21). The complete inhibition of this response in diaphragm after IH exposure, regardless of the mechanism, leads to an intriguing possibility that IH may represent a “preconditioned state.” That is, as in the heart, where ischemic preconditioning results in resistance to subsequent ischemia and inhibition of elevations in end-diastolic pressure and contracture (8, 21), IH may precondition skeletal muscle to resist a similar contracture response.

In summary, the results have demonstrated that the diaphragm is capable of remarkable adaptive responses to short-cycle IH over the short period of 10 days. These clearly function to protect its activity during anoxia and reoxygenation. There may be clinical significance to these observations in that the diaphragm under conditions of sleep apnea would be predicted to be susceptible to dysfunction (28, 30), but the evidence suggests that it is not (12). Perhaps the orchestration of responses potentiating chemoreceptor-stimulated respiration (14), augmentation of blood pressure (25), increased sympathetic activity (13), and improved hypoxic tolerance of muscles is initiated by a common stimulus associated with IH. These responses could conceivably be detrimental to the organism if unchecked, but they may also represent a sustained, primordial survival program that allows the animal to adapt to periods of apnea and have sufficient reserves to prevent system failure during prolonged apneic episodes.

We thank the technical staff at Case Western Reserve University for maintaining the animals on intermittent hypoxia.

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