Size and Composition Changes in Diaphragmatic Fibers in Rats Exposed to Chronic Hypercapnia*

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Objective: To test the hypothesis that chronic hypercapnia changes the composition of the respiratory muscle by continuous augmentation of ventilation.

Materials and methods: Eighteen male Wistar rats were housed in 10% CO₂ in air for 19 weeks, and their minute ventilation (V˙E) was measured every 6 weeks. The diaphragm, excited at 19 weeks of exposure, was classified as fiber type I, IIA, or IIb. Cross-sectional areas of individual fibers were measured. Fibers with a target-like appearance on reduced nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR) stain also were counted. The data were compared with those of rats kept in room air.

Results: The mean (± SD) PaCO₂ after 19 weeks of sustained hypercapnia was 71.0 ± 4.7 mm Hg. The V˙E remained at a high level until 12 weeks of exposure, and then it significantly decreased at week 18. In a comparison with the control rats, a larger number of type I fibers and a smaller number of type IIb fibers were found in the diaphragm of the chronically hypercapnic rats. In addition, the latter group’s cross-sectional area revealed fibers of a significantly smaller diameter. Target-like fibers were observed in 5% of the NADH-TR-stained fibers in the chronically hypercapnic rats but were not seen in the control rats.

Conclusion: By increasing the ratio of fatigue-resistant fibers, the diaphragm was able to adapt to a sustained load induced by hypercapnia. However, this adaptive process was accompanied by a degenerative change in the tissue.

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Key words: adaptation; respiratory failure; respiratory load; target-like fiber

Abbreviations: ATPase = adenosine triphosphatase; GTR = Gomori’s trichrome; NADH-TR = reduced nicotinamide adenine dinucleotide-tetrazolium reductase; V˙E = minute ventilation

Insufficient function of the respiratory muscles is known to cause hypercapnic respiratory failure. However, to our knowledge, the role played by chronic hypercapnia in chronic respiratory failure has not been investigated. The diaphragm of patients with COPD, one of the major causes of chronic respiratory failure, has been described in the literature as atrophied1,2 or hypertrophied.3,4 Several possible mechanisms, including mechanical ineffectiveness due to thoracic wall deformation5 and nutritional depletion,6 have been suggested to explain the changes that occur in the respiratory muscles of patients with COPD. Several conditions associated with respiratory failure, such as congestive heart failure, also may cause the observed muscular changes.7 Reports have demonstrated that domiciliary mechanical ventilation decreases PaCO₂ during spontaneous breathing8 and provides a better prognosis for patients with chronic hypercapnic respiratory failure.9 These findings raise the question of whether or not chronic hypercapnia exerts deteriorative effects on the respiratory muscles. Chronic hypercapnia continuously stimulates the central respiratory system in healthy humans10 and in experimental animals.11,12 This augmented ventilation increases the amount of work involved in breathing. It also has been reported that a sustained respiratory load can alter the size and composition of respiratory muscle fibers.13–15 The present study tests the hypothesis that chronic hypercapnia can change the composition of the respiratory muscle by continuously augmenting centrally driven respiratory commands. We investigated chronological changes in minute ventilation (V˙E) in cases of experimentally...
induced chronic hypercapnia. We then conducted an analysis of changes in size and composition of the affected diaphragm fibers.

**Materials and Methods**

**Animals**

The experimental subjects were 25 awake, unrestrained, male Wistar rats between 4 months and 5 months of age, with a mean (± SD) body weight of 293 ± 83 g. Seven of the rats lived in room air at 25°C and served as controls (control rats) for the muscular changes. The remaining 18 rats were placed in a chamber ventilated with 10% CO$_2$ in room air for 19 weeks or 24 weeks (chronically hypercapnic rats). The rats were examined for changes in V&iota;E and muscular composition. The gas in the experimental chambers was sampled each week to confirm that the CO$_2$ level was maintained between 9.6% and 10.4%. The laboratory environment was maintained at 25°C throughout the study.

**Measurements**

In the chronically hypercapnic rat group, V&iota;E was determined every 6 weeks. Rats were examined while unrestrained and awake by a body plethysmograph system, as described by Bartlett and Tenney. The temperature in the plethysmograph ranged from 25°C to 27°C. The humidity in the plethysmograph was maintained at a near constant level (ie, >90%) by allowing gases to bubble through water and into the chamber. The mean tidal volume and respiratory frequency were determined by a thermal array that recorded no fewer than 10 consecutive breaths. The mean V&iota;E was calculated as the mean of the tidal volume times the respiratory frequency, expressed as milliliters per minute per gram.

**Protocols**

Thirteen of the 18 chronically hypercapnic rats were exposed to 10% CO$_2$ in air for 19 weeks. V&iota;E, blood gas levels, and muscular changes were measured and analyzed. Five rats were exposed to 10% CO$_2$-mixed air for 24 weeks for the V&iota;E analysis. An animal’s baseline response to inspiring 10% CO$_2$ for 20 min was determined at week 0 immediately prior to placing the animal in the 10% CO$_2$ environment. Each chronically hypercapnic rat was moved from the CO$_2$ chamber to the plethysmograph every 6 weeks to determine V&iota;E while breathing 10% CO$_2$ in air. At week 19, the 13 chronically hypercapnic rats were anesthetized with halothane, and an arterial catheter (polyethylene No. 5) filled with heparinized saline solution was inserted in the rat’s carotid artery. Blood gas samples were taken while the animals breathed 10% CO$_2$ in air and were analyzed (model IL1304 analyzer; Instrumentation Laboratory; Milan, Italy). The rats were killed with an overdose of pentobarbital and then were used for the muscle analysis. Seven control rats were kept in room air for 19 weeks. They were used for the muscular analysis at week 19, as described above.

Muscle strips obtained from the costal part of the diaphragm and the quadriceps femoralis were fixed to a cork holder with the muscle perpendicular to the surface of the cork. Specimens were frozen immediately in isopentane cooled to its melting point by liquid nitrogen. Cross-sections of the muscle were sliced in 10-μm thicknesses using a cryostat maintained at ~20°C. Serial sections were stained according to the following histochemical techniques: alkaline-stable and acid-stable adenosine triphosphatase (ATPase) at pH 10.5 and pH 4.2; reduced nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR) stain; cytochrome c oxidase stain; and Gomori’s trichrome (GTR) stain. With these techniques, muscle fibers were classified as type I, type IIa, or type IIb.

We determined also the development of target-like fibers described by Ciesielki et al and Campbell et al. Target-like fibers have been observed in diaphragms subjected to long-term high-frequency stimulation of the phrenic nerve or in patients with COPD. If there was a poorly stained region in the central part of the type I fiber stained with NADH-TR and if fine reddish networks disappeared in a GTR stain, the fiber was regarded to be a target-like fiber. If necessary, a specimen stained with cytochrome C oxidase also was referred to for comparison. The target-like change suggests the disruption of myofibrils and the absence of activity of mitochondrial enzymes.

**Microscopic Images**

Microscopic images of the muscular sections were acquired by a video camera (CS220; Tokyo Electronic Industry; Tokyo, Japan) and were used for the computer analyses. Outlines of the muscle fibers were determined by computer software (Photoshop; Adobe Systems; Mountain View, CA). Target-like fibers were counted on specimens stained by NADH-TR and GTR. Cross-sectional areas of each muscle fiber were determined within the outlined boundary of the fiber (NIH-image; National Institutes of Health; Bethesda, MD [ftp://zippy.nimh.gov/pub/nih-image/]). The protocols were approved by the animal ethics committee of Tokai University School of Medicine.

**Statistical Analysis**

All data regarding individual rats were expressed as the mean ± SD. Statistical analyses were performed by an analysis of variance with repeated measures. Differences with p values of < 0.05 were accepted as significant.

**Results**

After 18 weeks in the CO$_2$ environment, animals weighed 364 ± 41 g. The mean weight of the chronically hypercapnic rats was approximately 10% less than that of the control rats. The mean arterial blood gas levels in the chronically hypercapnic rats were as follows: pH, 7.355 ± 0.033; P$_{CO_2}$, 71.0 ± 4.7 mm Hg; and P$_{O_2}$, 118.9 ± 8.8 mm Hg.

**V&iota;E of Chronically Hypercapnic Rats**

Although V&iota;E was determined every 6 weeks in the 18 chronically hypercapnic rats, it was not possible to obtain a measurement in two rats, and one rat died at week 18 before V&iota;E could be measured. Therefore, V&iota;E was completely measured in 15 of the chronically hypercapnic rats. Figure 1 shows the mean V&iota;E of these rats. At week 0, after the rats had been living in room air, the V&iota;E was 0.65 ± 0.10 mL/min/g. With their placement in the 10% CO$_2$ environment (ie, acute hypercapnia), the V&iota;E increased to 1.70 ± 0.41 mL/min/g (ie, 256% of the value obtained in room air). After sustained CO$_2$ exposure for 6 or 12 weeks, the V&iota;E was not different from that observed during

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the acute initial exposure to 10% CO₂ at week 0. However, at week 18, the mean V̇e had significantly decreased from that measured at both weeks 6 and 12.

In five rats, sustained CO₂ exposure was continued until week 24. The mean of their V̇e also is shown in Figure 1. At week 24, the V̇e was still lower than that observed at weeks 0 through 12. However, statistical significance was not obtained because of the small sample size.

**Gross Changes in Fiber Composition**

Figure 2 shows the changes in muscular composition wrought by sustained hypercapnia. There was a relatively large number of darkly acid ATPase-stained fibers (ie, type I fibers) in the diaphragm of the chronically hypercapnic rat (Fig 2, top right, B) compared with those of the control rat (Fig 2, top left, A). The sizes of type I fibers in Figure 2, top left, A, and top right, B, were not different, but the weakly acid ATPase-stained fibers (ie, type II fibers) in Figure 2, top right, B, were larger than those in Figure 2, top left, A. In contrast, no differences, either in the number or size of the type I and type II fibers, were found in the quadriceps femoralis between the chronically hypercapnic rat (Fig 2, bottom right, D) and the control rat (Fig 2, bottom left, C). We quantitatively assessed these changes in the following analyses.

**Fiber Composition**

Muscular composition was analyzed in 12 chronically hypercapnic rats and in 5 control rats. Figure 3 depicts the ratio of each type of fiber to the total number of fibers in the control rat group and the
chronically hypercapnic rat group. The muscle groups examined included the diaphragm (Fig 3, left, A) and the quadriceps femoralis (Fig 3, right, B). In the diaphragms of the chronically hypercapnic rats, the relative number of type I fibers was significantly larger and the number of type IIa and IIb fibers was significantly smaller than those in the control rats (Fig 3, left, A). In contrast, there were no significant differences in the number of any type of muscle fiber in the quadriceps femoralis between the chronically hypercapnic rats and the control rats (Fig 3, right, B).

Cross-Sectional Area

Figure 4 shows a mean cross-sectional area of each type of fiber in the diaphragm of rats in the control rat group and those in the chronically hypercapnic rat group (Fig 4, left, A). Corresponding images are given for the quadriceps femoralis (Fig 4, right, B). The cross-sectional area of type IIb fibers was significantly decreased in the chronically hypercapnic rats. The difference in cross-sectional area among type I fibers did not reach statistical significance (p = 0.053), and no significant differences were found among type IIa fibers (Fig 4, left, A). In the quadriceps femoralis, no significant differences were seen in any type of fiber values in either control rats or chronically hypercapnic rats (Fig 4, right, B).

Target-Like Fibers

Figure 5 shows the target-like fibers in the diaphragm of a chronically hypercapnic rat. As shown in Fig 5, left, A, there were regions in the central part of the type I muscle fibers that were poorly stained with NADH-TR (Fig 5, left, A). One can observe the disappearance of internyofibrillar networks in these fibers. We confirmed the disappearance of these networks by performing a GTR stain on the same specimen (Fig 5, right, B). The target-like fibers were visualized in the diaphragms of all of the chronically hypercapnic rats. The mean incidence of target-like fibers was 6.4 ± 4.4% (range, 1.2 to 16.5%) of the fibers that were darkly stained with NADH-TR (ie, type I and IIa fibers). Target-like fibers were not seen in the diaphragms of the control rats. In the quadriceps femoralis muscle, target-like fibers were not seen in any of the specimens in either of the groups.

Discussion

In this study, we demonstrated the effects of chronic hypercapnia on V\textsuperscript{E} and diaphragmatic fiber composition. Initially, the rats exposed to sustained hypercapnia had elevated V\textsubscript{E} levels that remained at a high and nearly constant level for 12 weeks, which
significantly decreased after week 18. A second finding was that the number of type I fibers increased, whereas the number of type IIa and IIb fibers decreased in the diaphragm. Third, the cross-sectional area of type IIb fibers in the diaphragm in the chronically hypercapnic rats was significantly smaller than that of the control rats. Finally, target-like fibers appeared in the diaphragms of the chronically hypercapnic rats but not in those of the control rats. These differences in fiber size and composition were not observed in the quadriceps femoralis muscles of rats in either group.

**Chronological Changes in V˙E**

As has been reported previously,12 long-term exposure to 10% CO2 caused a sustained and constant elevation of V˙E until week 12; however, V˙E significantly decreased at week 18. In the present study, the decreased level of V˙E was observed again at week 24. We previously analyzed the pattern of breathing in chronically hypercapnic rats every 6 weeks for 18 weeks. It became deep and slow at week 12, and thereafter the pattern did not change until end of the study.12 Blood gases attained a steady state after a few days of long-term CO2 exposure.22 These reports suggest that the central respiratory system may not be responsible for the decrease in V˙E that occurred after week 18. In this study, we analyzed changes in the composition of the diaphragm as a possible mechanism of the decrease in V˙E.

**Changes in Diaphragmatic Fibers**

In the diaphragm of the chronically hypercapnic rats, type I fibers increased relative to type IIa and IIb fibers; the cross-sectional area of type IIb fibers significantly decreased. Thus, in rats that underwent long-term CO2 exposure, the diaphragm became load-resistant and lost fast-twitch capability. Similar changes in the fiber composition of the rat diaphragm have been reported by Keens et al13 and by Prezant et al.14 Keens and coworkers13 applied 5 weeks of extrathoracic banding and found an increase in the number of type I fibers (ie, slow-twitch and high-oxidative) and a decreased number of type IIb fibers (ie, fast-twitch and low-oxidative). Cross-sectional areas of individual fibers had not changed significantly. Prezant and associates14 applied continuous respiratory resistive loading for 24 to 28 weeks. They reported increases in both the proportion and cross-sectional area of type I fibers. The load in our study was different from that of previous studies, but the results as regards fiber composition were qualitatively identical. Therefore, it may be said that the diaphragmatic fiber composition shifted to a
fatigue-resistant state due to the long-term application of a respiratory load. Furthermore, changes were similar whether the load was that of a sustained augmentation or a restriction of the diaphragmatic motility.

Despite the load-resistant changes in the diaphragmatic fiber composition, the $V_{\text{E}}$ of our chronically hypercapnic rats significantly decreased at week 18. This finding suggested that diaphragmatic adaptation is incomplete in chronic severe hypercapnia.

**Comparison With Other Studies**

Table 1 shows the results from previous studies of changes in diaphragmatic fibers caused by the long-term application of several types of load. As mentioned above, our results most closely agreed with those of Keens et al\textsuperscript{13} and Prezant et al.\textsuperscript{14} In contrast, two treadmill studies, those of Green et al\textsuperscript{23} and Powers et al,\textsuperscript{24} did not show an increased ratio of type I fibers. Therefore, it may be concluded that the effects of continuous respiratory load and treadmill exercise on fiber composition are different. Although fiber composition seems to be affected by the type of loading, fiber size is affected only to a small degree by long-term loading.

The load used in the present study can be categorized as continuous respiratory loading. Different results are observed when continuous loading and intermittent loading are compared. The number of type I fibers tended to increase and the number of type IIb fibers decreased in experiments with continuous loading, whereas the number of type I fibers did not increase in cases of intermittent respiratory or nonrespiratory loading.

![Figure 5](image-url) Target-like fibers in a chronically hypercapnic rat (arrows). **Left**, A: NADH-TR stain. **Right**, B: GTR stain (original ×400).

**Table 1—Studies of the Effect of Chronic Respiratory Load on the Diaphragm**

<table>
<thead>
<tr>
<th>Study/yr</th>
<th>Severity Type</th>
<th>Type</th>
<th>Term, wk</th>
<th>Fiber Size</th>
<th>Fiber Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keens et al\textsuperscript{13}/1978</td>
<td>Moderate respir</td>
<td>Continu</td>
<td>5</td>
<td>→</td>
<td>→</td>
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<tr>
<td>Green et al\textsuperscript{23}/1989</td>
<td>Heavy tread</td>
<td>Intermit</td>
<td>14</td>
<td>→</td>
<td>↓</td>
</tr>
<tr>
<td>Powers et al\textsuperscript{24}/1992</td>
<td>Heavy tread</td>
<td>Intermit</td>
<td>10</td>
<td>→</td>
<td>→</td>
</tr>
<tr>
<td>Prezant et al\textsuperscript{14}/1993</td>
<td>Moderate respir</td>
<td>Continu</td>
<td>24</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td>Rollier et al\textsuperscript{15}/1995</td>
<td>Low inspir</td>
<td>Intermit</td>
<td>8</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td>Present study</td>
<td>Heavy CO\textsubscript{2}</td>
<td>Continu</td>
<td>12</td>
<td>→</td>
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</tr>
</tbody>
</table>

*respir = respiratory load; tread = treadmill; inspir = inspiratory load; continu = continuous application; intermit = intermittent application; ↑ = increase; → = no change; ↓ = decrease.*
Target-Like Fibers

On NADH-TR staining, target-like fibers indicate enzyme-poor regions and focal disruption of myofilaments. These pathologic changes in diaphragmatic fibers have been observed in the dogs with relatively high-frequency continuous stimulation (27 to 33 Hz) of the phrenic nerve for 6 weeks. Target-like fibers also have been found in the intercostal muscles of patients with COPD, or chronic respiratory failure, when cases persisted for >3 months. Continuous overexertion of the respiratory muscles and a decrease in regional blood flow have been assumed as possible mechanisms of target-like fiber development; however, the mechanism of the development of target-like fibers remains unclear. We found similar pathologic changes in diaphragmatic fibers in chronically hypercapnic rats. Therefore, this study suggests that augmented diaphragmatic motion, either by stimulation of the peripheral (ie, phrenic) nerve or the central respiratory system, produced target-like degeneration. Similar to the results of previous studies, this observed degeneration was associated with the atrophy of type I fibers. We have reported that approximately 5% of the NADH-TR-stained fibers developed a target-like appearance in the diaphragms of the rats exposed for 12 weeks to 5% CO2. The ratio obtained elsewhere is not significantly different from that of the present study. Therefore, the development of target-like fibers may not be strongly dependent on the intensity or duration of sustained stimulation. Target-like fibers may be considered as an indication of degeneration of the muscular fibers. Although VE remained at high level throughout the 24 weeks of CO2 exposure, it significantly decreased after 18 weeks. Further analysis should be done to clarify the relationship between the target-like change and the decrease in VE.

In conclusion, this animal study revealed that in rats with chronic hypercapnia, the diaphragm adapted to a sustained load (ie, an increase in the number of type I fibers was observed). In addition, the development of target-like fibers and a decrease in the size of type IIb fibers was observed. These latter changes are assumed to be degenerative. Such changes were associated with persistently augmented ventilation; however, ventilation did slightly decrease after week 18 of the present study.

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