The Effects of Chronic Episodic Hypercapnic Hypoxia on Rat Upper Airway Muscle Contractile Properties and Fiber-Type Distribution*

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Objective: Obstructive sleep apnea (OSA) is caused by episodes of upper airway (UA) obstruction due to an inability of UA muscles such as the geniohyoids and sternohyoids to maintain airway patency. This results in chronic episodic hypercapnic hypoxia. Chronic continuous hypoxia and episodic hypocapnic hypoxia affect skeletal muscle structure and function, but the effects of chronic episodic hypercapnic hypoxia on UA muscle structure and function are unknown.

Design: Rats breathed air and hypercapnic hypoxic gas twice per minute for 8 h/d for 5 weeks in order to mimic the intermittent hypercapnic hypoxia of OSA in humans. Isometric contractile properties were determined using strips of isolated geniohyoid and sternohyoid muscles in physiologic saline solution at 30°C. Fiber-type distribution was determined by adenosine triphosphatase staining.

Results: For both muscles, chronic episodic hypercapnic hypoxia had no significant effect on twitch or tetanic tension, twitch/tetanic tension ratio, and tension-frequency relationship. There was a significant (p < 0.05) increase in geniohyoid fatigue (50.5 ± 6.6% vs 43.6 ± 5.8% of initial tension), but sternohyoid fatigue was reduced (31.5 ± 5.2% vs 37.8 ± 6.0% of initial tension). Geniohyoid type 1 fibers were reduced and type 2B fibers increased, whereas sternohyoid muscle had an increase in type 1 and 2A fibers and a decrease in type 2B fibers.

Conclusions: Chronic episodic hypercapnic hypoxia alters UA muscle structure and function, changes that may affect the regulation of UA patency.

Key words: hypercapnia; hypoxia; geniohyoid; sternohyoid

Abbreviations: ATPase = adenosine triphosphatase; FIO2 = fraction of inspired oxygen; FICO2 = fraction of inspired carbon dioxide; OSA = obstructive sleep apnea; UA = upper airway
previously shown that chronic episodic hypocapnic hypoxia alters UA muscle contractile properties, and we have proposed that the chronic episodic blood gas changes associated with sleep-disordered breathing cause changes in UA muscle function that may contribute to the pathophysiology of the condition. However, since hypopnea/apnea is accompanied by hypocapnic hypoxia rather than hypocapnic hypoxia, and since hypocapnia has been shown to interact with hypoxia in its effects on other tissues, the present investigation was undertaken to examine the effects of chronic episodic hypocapnic hypoxia on UA muscle structure and contractile properties.

**Materials and Methods**

All of the procedures used were in accordance with the Cruelty to Animals Act, 1876 and European Union Directive 86/609/EC. Wistar rats were housed four to a cage under a 12 h/12 h (light/dark) photoperiod, and were given free access to food and water. Animals were randomly assigned to two groups of 16 rats each. During treatment periods, the rats were placed in restrainers with their heads surrounded by hoods. For the hypocapnic hypoxia group, a mixture of nitrogen and carbon dioxide was distributed into the hoods for 15 s to reduce the ambient fraction of inspired oxygen \( F_{\text{io}} \) to 6 to 8%, and to increase the ambient fraction of inspired carbon dioxide \( F_{\text{co}} \) to 10 to 14%. This was followed by an infusion of air for 15 s so that the \( F_{\text{io}} \) and the \( F_{\text{co}} \) returned to normal. This cycle was repeated twice per minute, 8 h/d for 5 weeks. The \( F_{\text{io}} \) and \( F_{\text{co}} \) in the hoods was measured daily, and arterial blood \( P_{\text{io}} \) and end-tidal \( P_{\text{co}} \) were measured in a sample of control animals as described previously. The nadir arterial \( P_{\text{co}} \) values were 55 to 65 mm Hg, and the peak end-tidal \( P_{\text{co}} \) values were 47 to 74 mm Hg. For the control group, air was distributed to the hoods for 15 s followed by air for 15 s at the same flow rates as the hypocapnic hypoxia rats. This cycle was repeated twice per minute, 8 h/d, 5 d/wk for 5 weeks.

**Measurement of Contractile Properties**

After the 5-week treatment period, animals were anesthetized (pentobarbitone sodium, 60 mg/kg intraperitoneal), tracheostomized, and placed on mechanical ventilation. Body temperature was maintained at 37°C using a thermostatically controlled heating blanket and radiant heat. A midline incision was made in the neck, and the digastric muscles were separated to reveal the omohyoid muscle, which was cut to expose the underlying geniohyoid muscles. The sternohyoid muscles running from the sternum to the hyoid bone were also exposed. The muscles were removed rapidly and prepared for adenosine triphosphatase (ATPase) staining or for contractile studies. For the latter, longitudinal strips of 1 to 2 mm in diameter were suspended vertically in a water-jacketed bath in warmed (30°C), oxygenated (95% oxygen, 5% carbon dioxide) physiologic saline solution (pH 7.4) containing NaCl, 120 mmol; KCl, 5 mmol; Ca gluconate, 2.5 mmol; MgSO4, 1.2 mmol; NaH2PO4, 1.2 mmol; NaHCO3, 25 mmol; and glucose, 11.5 mmol. The muscle strip was fixed at one end and attached at the other end to an isometric force transducer. Isometric twitch tension, tetanic tension, twitch/tetanic tension ratio, contraction time, half-relaxation time, the tension-frequency relationship, fatigue, and recovery from fatigue were measured using field stimulation (supramaximal voltage, 1 ms in duration) with platinum electrodes and recorded using an analog-to-digital converter and microcomputer.

**Protocol**

Muscles were allowed to equilibrate in the bath for 30 min. Optimal length (ie, the length producing maximal twitch tension) was determined, and the muscle was held for the remainder of the experiment at this length. The tension/frequency relationship was determined by stimulation at 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 Hz for 300 ms at each frequency, and allowing 2 min between each stimulus. A 10-min recovery period was allowed before the fatigue protocol. Fatigue was induced by stimulation at 30 Hz with 300-ms trains of 0.5 Hz for 5 min. Recovery from fatigue was determined by measuring twitch tension every 5 min for 30 min after the end of the fatigue protocol.

**ATPase Staining**

In order to distinguish between type 1 and type 2 fibers and to further subdivide the type 2 fibers into 2A and 2B, ATPase in incubating solutions of pH 4.2, pH 4.6, and pH 9.4 was used. Following excision of the muscles, excess blood and tissue fluid was removed using blotting paper. A section from the center of the muscle measuring approximately 5 × 5 mm was placed in talcum powder and then into liquid N2 (~180°C) for 1 min and then placed into a cryogenic vial and stored at ~70°C. Frozen sections of 12 μm in thickness were prepared using a cryostat and allowed to air-dry for 5 h. Sections were preincubated at 4°C in 0.1 mol/L sodium acetate/acetic acid buffer at either pH 4.2 (36.8 mL of 0.1 mol/L acetic acid, 13.2 mL of 0.1 mol/L sodium acetate, and 50 mL of H2O), pH 4.2 (47 to 74 mmol/L H2O, pH to 4.2 using 0.1 mol/L HCl) or pH 4.6 (25.5 mL of 0.1 mol/L acetic acid, 24.5 mL of 0.1 mol/L sodium acetate, and 50 mL of H2O, pH to 4.6 using either 0.1 mol/L HCl or 0.1 mol/L NaOH) for 10 min. For pH 9.4 incubation, sections were incubated in 0.1 mol/L glycine buffer with 0.75 mol/L CaCl2 and adenosine triphosphate (1 mg of adenosine triphosphate to 2 mL of glycine/CaCl2 solution, pH 9.4 using 0.1 mol/L NaOH) at 37°C for 45 min. The rest of the staining procedure was the same for all three pH values. The sections were rinsed in distilled water and immersed in a 2% cobalt chloride solution for 5 min. The sections were then rinsed in three changes of distilled water and immersed in a diluted (1:10) ammonium sulfate solution for 30 s. The slides were rinsed in running tap water for 5 min. Sections incubated at pH 4.2 were lightly stained in Harris’ hematoxylin. All sections were then dehydrated in 95%, 99%, and 99% alcohol and cleared in xylene.

**Data Analysis**

For the fatigue protocol, values were normalized by expressing the force generated by the first pulse of the stimulus train at 1, 2, 3, 4, and 5 min as a percentage of the value of the first pulse of the first train. Recovery from fatigue was determined by expressing the ratio (recovery tension – fatigue tension/
initial tension (fatigue tension) as a percentage where recovery tension is the twitch tension measured during recovery, fatigue tension is the twitch tension measured immediately after the 5-min fatigue protocol, and initial tension is the twitch tension measured immediately before the fatigue protocol. For the tension-frequency relationship, values were normalized by expressing them at different frequencies as a percentage of the maximal tetanic value. Specific tension was expressed in newtons per square centimeter of strip cross-sectional area. Cross-sectional area was approximated by weighing the muscle strip after removal from the bath and blotting dry, and dividing this by the product of the optimal length and muscle density, assumed to be 1.06 mg/mm³. Values were expressed as means ± SD. Different fiber types were determined by the different pHs in which the sections were incubated. The different fiber types were counted and expressed as a percentage ± SD of the total number of fibers. These values and contractile values were used to compare statistically the control and hypercapnic hypoxic groups using analysis of variance and the Fisher least significant difference test, with p < 0.05 taken as significant.

Results

Chronic episodic hypercapnic hypoxia had no significant effect on the weight of the geniohyoid muscle (98 ± 2 mg vs 96 ± 3 mg, control vs hypercapnic hypoxia) or the sternohyoid muscle (144 ± 23 mg vs 139 ± 31 mg, control vs hypercapnic hypoxia). For both muscles, chronic episodic hypercapnic hypoxia had no significant effect on twitch or tetanic tension, twitch/tetanic tension ratio (Table 1), or tension-frequency relationship (Figs 1, 2), but caused a significant reduction in geniohyoid contraction and half-relaxation time (Table 1). Chronic episodic hypercapnic hypoxia significantly increased geniohyoid fatigue and decreased the recovery from fatigue (Fig 3), but reduced fatigue and increased recovery from fatigue in the sternohyoid muscle (Fig 4).

The fiber-type distribution of both the geniohyoid and sternohyoid in control and chronic episodic hypercapnic hypoxia-treated animals is shown in Figure 5. In the geniohyoid muscle, the hypercapnic hypoxia group had significantly reduced type 1 fibers and significantly increased type 2B fibers. The sternohyoid muscle had a significant increase in type 1 and 2A fibers and a significant decrease in type 2B fibers. In general, there was a good correlation between fiber type and fatigue. For example, in the chronic episodic hypercapnic hypoxia-treated animals, there was a positive correlation between percentage of type 1 fibers and percentage of initial tension for both muscles. Thus, the Pearson product-moment correlation coefficient was 0.963 for the geniohyoid and 0.808 for the sternohyoid.

Discussion

OSA is caused by collapse of the UA during sleep, as a result of an inability of the UA muscles to maintain UA patency. The causes of this inability are poorly understood, but there is...
evidence for abnormalities of UA muscle contraction. In the English bulldog, an animal model of human OSA,8 there are abnormalities of sternohyoid and geniohyoid structure, and abnormalities in the structure of a number of other UA muscles have been reported in humans with OSA.9–11 These structural changes are characterized by an increase in fast-twitch fibers. Although little is known about the functional consequences of this, these changes could result in UA muscle dysfunction and a vicious cycle of further UA collapse.16

In OSA, UA muscle activity is chronically elevated, and activity becomes greatly elevated during obstructive events,16 and it has been proposed that this leads to an adaptive increase in the number of fast fibers. However, we have previously proposed an alternative mechanism due to the effect of chronic hypoxia. Although controversial, there is some evidence that chronic continuous hypoxia causes an increase in fast fibers.17 We have previously shown that chronic episodic hypoxia causes increased fatigability in rat geniohyoid and sternohyoid muscle. However, the hypoxia in these experiments was accompanied by hypocapnia due to the hypoxic ventilatory drive, whereas in OSA, hypoxia is accompanied by hypercapnia. Chronic continuous hypercapnia is known to reduce the right ventricular hypertrophy and pulmonary hypertension caused by chronic continuous hypoxia.13 However, the effects of chronic episodic hypercapnic hypoxia on skeletal muscle structure or function have not been previously investigated. The purpose of the present investigation was to determine the effects of chronic episodic hypercapnic hypoxia on UA muscle structure and function. We used an exposure period of 5 weeks because we consider this to be a reasonable period to induce chronic changes. This is based on the observations that 5 weeks of episodic blood gas changes induce arterial14,18 and pulmonary hypertension,14 increased hematocrit14,19 and changes in UA muscle function.12

We found that for both muscles, chronic episodic hypercapnic hypoxia had no significant effect on force production. However, there was a significant increase in geniohyoid fatigue, whereas sternohyoid fatigue was significantly less. These effects were consistent with our findings for fiber-type distribution. For the geniohyoid muscle, the hypercapnic hypoxia group had significantly reduced type 1 fibers and significantly increased
type 2B fibers. This reduction in fatigue-resistant type 1 fibers and increase in fatigable type 2B fibers is likely to be responsible for the increase in geniohyoid fatigability. For the sternohyoid muscle, there was a significant increase in type 1 and 2A fibers and a significant decrease in type 2B fibers. Again, this increase in fatigue-resistant type 1 and 2A fibers and decrease in fatigable type 2B fibers is likely to be responsible for the reduced fatigability of the sternohyoid muscle. These results are generally in agreement with our previous results with chronic episodic hypocapnic hypoxia, in which there was no effect on force production but fatigue was affected. Both geniohyoid and sternohyoid fatigue were greater following episodic hypocapnic hypoxia treatment. Therefore, hypocapnic hypoxia increased sternohyoid fatigue, whereas hypercapnic hypoxia reduced fatigue. Since we do not know the mechanism whereby these chronic blood gas changes cause fiber type transitions, it is difficult to speculate on why the geniohyoid and sternohyoid were affected differently by hypercapnic hypoxia or why hypocapnic and hypercapnic hypoxia affected the sternohyoid differently. Perhaps it is somehow related to the initial fiber distribution of the muscles since the sternohyoid has more type 2B fibers and less type 1 fibers than the geniohyoid. It is possible that the effects on one muscle might be secondary to those on the other. The geniohyoid and sternohyoid muscles are suprahyoid and infrahyoid muscles, respectively. They influence UA patency by determining the position of the hyoid bone. When the two muscles contract together, they pull on the hyoid arch and dilate the UA. There is also an inverse relationship between the length of these
muscles and the volume of the UA, with geniohyoid length showing greater coupling to UA volume than the sternohyoid. The mechanical interaction between the two muscles means that an effect on one muscle, such as a direct effect of systemic asphyxia, might affect the other muscle secondarily. For example, greater fatigability in the geniohyoid might result in greater loads being placed on the sternohyoid, which might result in reduced fatigability of the sternohyoid. Such a mechanism might contribute to the difference in the effect of hypocapnic and hypercapnic hypoxia on sternohyoid fatigu. However, the present experiments were not designed to investigate these possibilities.

The present results show that chronic episodic hypercapnic hypoxia significantly affected geniohyoid and sternohyoid muscle fatigue. These effects might have implications for the pathogenesis of OSA. We have previously shown that chronic episodic hypoxia increases both geniohyoid and sternohyoid fatigue, and we have suggested that the chronic episodic hypoxia of OSA might lead to increased vulnerability of the UA muscles to fatigue, further hypoxia, and a vicious cycle exacerbating the condition. The UA muscles may be especially vulnerable to fatigue since they have a relatively high content of fast-twitch fibers, and the activity in these muscles is high in patients with OSA. This vulnerability may be exacerbated by acute hypoxia when the UA collapses, since UA muscle activity becomes greatly increased, and since acute hypoxia has been shown to reduce geniohyoid endurance. The present results show that chronic hypercapnic hypoxia increases geniohyoid muscle fatigue. However, sternohyoid fatigue was reduced, suggesting that hypercapnia may offset to some extent the deleterious effects on fatigue on this muscle.

In conclusion, these results show that chronic intermittent hypercapnic hypoxia in rats alters the fiber type distribution of UA muscles, increases fatigue in geniohyoid muscle, and reduces fatigue in the sternohyoid muscle. These effects may contribute to the pathogenesis of OSA.

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