Geniohyoid muscle properties and myosin heavy chain composition are altered after short-term intermittent hypoxic exposure

Eung-Kwon Pae, 1 Jennifer Wu, 1 Daniel Nguyen, 1 Ryan Monti, 1 and Ronald M. Harper 2

1Section of Orthodontics, University of California at Los Angeles School of Dentistry, and 2Department of Neurobiology, David Geffen School of Medicine at University of California at Los Angeles, Los Angeles, California

Submitted 7 September 2004; accepted in final form 12 November 2004

Pae, Eung-Kwon, Jennifer Wu, Daniel Nguyen, Ryan Monti, and Ronald M. Harper. Geniohyoid muscle properties and myosin heavy chain composition are altered after short-term intermittent hypoxic exposure. J Appl Physiol 98: 889–894, 2005. First published November 19, 2004; doi:10.1152/japplphysiol.00978.2004.—Patients with obstructive sleep apnea (OSA) often exhibit fatigued or inefficient upper airway dilator and constrictor muscles; an upper airway dilator, the geniohyoid (GH) muscle, is a particular example. Intermittent hypoxia (IH) is a frequent concomitant of OSA, and it may trigger muscle fiber composition changes that are characteristic of a fatigable nature. We examined effects of short-term IH on diaphragmatic and GH muscle fiber composition and fatigue properties by exposing 24 rats to alternating 10.3% O2-balance N2 and room air every 480 s (240 s duty cycle) for a total duration of 5, 10, 15, 20, or 30 h. Sternohyoid fiber composition was also examined. Control animals were exposed to room air on the same schedule. Single-fiber analyses showed that GH muscle fiber types changed completely from myosin heavy chain (MHC) type 2A to MHC type 2B after 10 h of exposure, and the conversion was maintained for at least 30 h. Sternohyoid muscle fibers showed a delayed transition from MHC type 2A/2B to MHC type 2B. In contrast, major fiber types of the diaphragm were not significantly altered. The GH muscles showed similar tension-frequency relationships in all groups, but an increased fatigability developed, proportional to the duration of IH treatment. We conclude that short-term IH exposure alters GH muscle composition and physical properties toward more fatigable, fast-twitch types and that it may account for the fatigable upper airway fiber types found in sleep-disturbed breathing.

METHODS

Mice and IH procedure. Twenty-four male Sprague-Dawley rats (290–430 g; Charles River, Wilmington, MA) were randomly assigned to one of six conditions. Animals were housed under 12:12-h light-dark conditions (light phase, 6:00 AM to 6:00 PM) with food and water available ad libitum. Animals were housed in a commercial

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
chamber 30 × 20 × 20 in. (Reming Bioinstruments, Redfield, NY) with a modified control system. Five subgroups of four animals each were exposed to IH during the light phase for 7.5 h per day for 5, 10, 15, 20, or 30 h; e.g., an animal in the 10-h group would be exposed to hypoxia during the first day and to an additional 2.5-h exposure the following day. The remaining four animals were housed outside of the chamber and breathed room air. Chamber temperature was maintained at 22–24°C. The experimental process was carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and it was approved by the institutional Animal Research Committee.

Tissue harvest and gel electrophoresis. Two animals from each group were used for single-fiber analysis. One hour before euthanasia, heparin (10 ml/kg) was injected intraperitoneally, and the rats were killed by intraperitoneal injection with an overdose of pentobarbital sodium (Nembutal) at 100 mg/kg. The GH, sternohyoid, and diaphragm muscles were removed and snap frozen in isopentane cooled by liquid N2.

Single fibers were mechanically isolated (1). MHC protein isoforms were separated by using the SDS-PAGE protocol (45). To isolate single fibers, muscle samples were placed in relaxing solution (in g: 1.0 EGTA, 0.16 MgCl, 1.0 KCl, 1.1 imidazole, 0.44 ATP, 0.74 creatine phosphate, and 1.5 × 10⁻⁵ creatine phosphate) overnight. The muscle sample was removed from the relaxing solution and pinned in a 6-cm Sylgard-coated petri dish. By using a dissection scope and microtweezers, fibers were mechanically isolated from the nonendomysial end of the sample and placed directly into a sample buffer [100 mM Tris base, 100 mM Tris (pH 6.8), 5% glycerol, 4% SDS, 0.05% bromophenol blue, 5% β-mercaptoethanol]. The single fibers were denatured in the sample buffer and boiled at 100°C for 6 min. Protein analyses were performed on a vertical slab gel unit (CBS Scientific, Solana Beach, CA). Large 22 × 16.5-cm plates with 0.75-mm spacers were used. The separating gel [30% glycerol, 8% total acrylamide (2%) bis, 0.2 M Tris base (pH 8.8), 0.1 M glycine, 0.4% SDS, 0.1% ammonium persulfate, 0.05% N,N',N',N''-tetramethylethylenediamine] was injected between the two plates. The preparation was subjected to 245 V for 1 h, followed by 375 V for 24 h. The gel was stained with Coomassie blue G250 for 1–2 h under observation, destained in 10% acetic acid and 25% methanol, and mounted on a drying frame for 24 h. Each gel was scanned and captured by using Image Quant 5.0 software (Molecular Dynamics, Sunnyvale, CA). Expression of each band was compared with bands containing muscle fibers that have all four MHC isoforms (5, 45). Molecular masses estimated using the rat sequence are 223,095 Da for MHC type 2B (GenBank locus X.M. 213345), and 225,156 Da for MHC type 2B (GenBank locus X.M. 340817).

Fatigue test. Six animals were used for muscle fatigue tests. Two animals each from control, 15- and 30-h-treated subgroups were maintained under deep anesthesia by intraperitoneal injection of pentobarbital sodium (80 mg/kg). Because of the narrow anesthetic safety margin, extreme caution was used to maintain a pain-free preparation. The digastric muscle, followed by the mylohyoid muscle, were reflected to reveal the medial and lateral branches of the hypoglossal nerve. After the mylohyoid muscle was detached and severed from the midline, the GH muscle was exposed and isolated, together with the mandible symphysis and the hyoid bone. All infrhayoid and stylohyoid muscles were severed. The lateral and medial branches of the hypoglossal nerve were transected, leaving a collateral of the medial branch intact. This collateral branch solely innervates the GH muscle in rats (Fig. 1B). To confirm this relationship, we successively transected each branch as we stimulated the main trunk of nerve XII and ablated other muscles supplied by that nerve. The mandible symphysis was secured to the fixture of a custom-designed in situ system by using orthodontic wire ligatures, and the midsagittal part of the hyoid bone was tied to a force transducer (model FT03, Astro-Med, West Warwick, RI) by using 3-0 silk suture. A skin pouch, filled with mineral oil, was made to provide baths for isolation of the nerve trunks; the trunks were stimulated with a pair of wire electrodes.

For in situ data collection, the GH muscles were stimulated by using a Grass S48 stimulator (Astro-Med), and forces were assessed using a force transducer and 15LT Bipolar Amplifier System (Astro-
Med), together with a desktop computer. The muscle length was adjusted until a peak twitch tension was observed; the passive tension at this length provided a baseline. After the baseline voltage was determined (6–9 V), a tension-frequency study was performed, with the frequency varying from 10 to 100 Hz. After 5 min of rest, fatigue was induced by stimulation at 30 Hz with 300-ms trains for 120 s. Peak-force and force-output declines were measured. In addition, the time taken to reach 70% of the initial force was measured and used as a separate index of fatigability (3, 7). Data acquisition and analysis were performed by a Polyview system (Astro-Med) and LabView (National Instruments, Austin, TX).

We evaluated group differences in fatigability at each data point by using one-way ANOVA (SPSS version 12.0), followed by a post hoc multiple comparison test with Bonferroni correction. The times required to reach 70% of the initial force output were assessed using the nonparametric signed-rank test, with the level of statistical significance set at $P < 0.05$.

RESULTS

**MHC protein expression after IH challenge.** MHC isoform expression results are shown in Fig. 2. Gels for single fibers of the GH muscle showed no coexpression in control animals; i.e., all were type 2A (Fig. 2A). In contrast, fibers from the diaphragm demonstrated coexpression of MHC isoforms, such as MHC type 2A and MHC type 1 in a single fiber, i.e., two bands in a lane. Polymorphism of MHC composition of the diaphragm was maintained with IH. However, fiber types of the GH muscle began to exhibit changes from MHC type 2A to MHC type 2B after 5 h of IH exposure, and this conversion was readily evident at 10 h. Five-hour-treated muscle showed transition forms; some single fibers were MHC type 2A, some were type 2B, and some showed a polymorphism of type 2A and 2B in a single fiber.

Sternohyoid muscles harvested from the control animals showed a coexpression of MHC type 2A and MHC type 2B protein in single fibers. The sternohyoid muscles maintained a similar degree of MHC composition at 5 h; however, its principal fibers began to change at 10 h and completed the transition from type 2A/2B to type 2B by 20 h (Fig. 2B).

**Fatigability changes after IH challenge.** To evaluate whether the fiber-type transitions were commensurate with muscle functional alterations, isometric muscle tension-frequency relationships and fatigability were measured and compared between controls and 15- and 30-h-treated animals (Fig. 2A).
3, A and B). Weights of the animals were 339 ± 57 g (mean ± SD), and no significant weight change was noted after IH stimulation. Wet weights of the GH muscle were 0.18 ± 0.04 g. No significant differences in animal weights or in wet weights of the GH muscle emerged between groups. All tension-frequency curves showed the same trend. However, the fatigability test showed 67.2% of the initial force for control, 56.5% for 15 h, and 49.1% for 30-h-treated muscle after 120 s of stimulation of the tetanus train with 300-ms pulses at 30 Hz. Evaluation of force development by ANOVA with Bonferroni post hoc multiple comparisons revealed that the control group differed significantly (P < 0.05) from the 30-h group at 120 s. In addition, the times required to reach 70% of the initial force output were 115 s for control, 95 s for 15 h, and 78 s for 30-h exposure. The times required to reach 70% of the initial force outputs significantly differed (P < 0.05).

An increase of force production over baseline values was measured from the control animals at 20-, 40-, and 60-s points, as shown in Fig. 3B.

**DISCUSSION**

The findings indicate that IH results in a dose-dependent fiber-type change in the GH muscle and that the switch in fiber composition is accompanied by significant alterations in fatigability. The findings may have implications for airway patency control in humans with obstructed breathing during sleep.

In the human, the GH muscle opens the pharynx by drawing the hyoid bone forward, but it depresses the lower jaw when the length of supra- and infrahyoid muscles are fixed. We stimulated a small collateral branch of the medial branch of the hypoglossal nerve, which exclusively innervates the rat GH muscle, and confirmed the innervation by successively severing each branch as we stimulated the main trunk of nerve XII, and ablating other muscles supplied by that nerve. Type 2 fibers are abundant in the GH muscle (10); type 1 fibers have been reported as well (29, 30). In our single-fiber study, the GH muscle in control rats consisted of type 2A fibers but contained no type-1 fibers. The disparity in muscle type found by others may result from strain differences [Wistar used by McGuire et al. (29, 30); Sprague-Dawley in the present study].

Fatigability of the upper airway dilators or constrictors has been suggested as one etiologic factor in OSA (19, 40), partially stemming from the finding of an abnormal ratio of fatigue-resistant to fatigue-sensitive fibers in the genioglossus of OSA patients (43) and resolution of this abnormal ratio to fatigue-resistant types after long-term use of nasal CPAP (8). Although the mechanisms underlying the increased fatigability or fiber-type changes have not been addressed, high sympathetic outflow occurs in sleep-disordered breathing (20) and alters muscle sympathetic nerve tone in humans (46, 48). Increased sympathetic outflow can lead both to fatigue (41) and to fiber-type changes (4, 16). IH recruits central sympathetic nervous system structures, and results in increased sympathetic tone, and in turn, an acute elevation of blood pressure (2). Irrespective of blood flow changes (27), muscles under fatigue have reduced proprioceptive capacity, which is associated with sympathtic activation (41). An increase in sympathetic outflow depresses the feedback control of muscle length in jaw muscles via disturbed muscle spindle afferent sensitivity (38). Sympathetic outflow is enhanced by steady-state hypoxia (11, 41); the patterns of sympathetic outflow to IH are not clear, but they presumably result in significant variability in outflow (17). We speculate that the combination of exaggerated motor outflow with enhanced, intermittent sympathetic tone may lead to the fiber changes noted here, although the precise mechanisms of interactions remain unclear.

Considering the concurrent and immediate changes in muscle structure and physiology emerging in the GH, we speculate that IH may initially influence the central nervous system by altering sympathetic and somatic motor outflow from neural structures known to be sensitive to hypoxia. Several autonomic nervous system and motor control neural structures are damaged in OSA, presumably in the same fashion as hippocampal and frontal cortex areas are injured from IH exposure (15). The damaged structures in OSA patients include sympathetic control areas of the cortex (insula) and cerebellar cortex and deep autonomic (fastigial) nuclei (21). Damage to cerebellar Purkinje neurons and deep nuclei emerges in rats after only 5 h to IH exposure (35). The injured neural areas reported in OSA patients presumably represent consequences of long-term exposure to hypoxic and other sequelae of apnea; the relationships of neural responses to short-term hypoxic exposure remain unknown, but acute exposure likely elicits pronounced...
excitement in certain areas and, in some areas, is sufficient recruitment to invite excitotoxicity (35).

The earlier described change in upper airway contractile properties and fiber types to more fatigable types result from episodic, not chronic hypoxia, although with accompanying hypercapnia (29). Repetitive, rather than sustained hypoxia, elicits a genioglossal response that mimics that which occurs during OSA (28). Compensatory reflexes to increased airflow resistance trigger increased GH tonic activity to overcome pharyngeal narrowing or even induce a lengthening contraction of the GH muscle during phasic activity (50). Increased overall activity of the genioglossus and mylohyoid muscles also develop to such increased airflow resistance in rabbits (44a). After severe hypoxic stimulation, fatigability of the GH muscle increases in cats (39).

Because the sternohyoid muscle maintains the same degree of MHC composition at 5 h (Fig. 2B), and begins to change its fiber composition by 10 h, the GH may succumb to IH more quickly than the sternohyoid muscle. Muscle-specific responses, such as different timing of fiber type change in each muscle, may depend on structural relationships. For example, the sternohyoid muscle exerts more force than the GH; when GH and sternohyoid muscles contract concomitantly, the GH must overwork or be lengthened (50), because each muscle attaches to either side of the hyoid bone. Thus the GH would become quickly fatigued. This early fatigue mechanism could be one process by which fiber-type changes in the GH began earlier than in the sternohyoid muscle.

Earlier studies (29, 30) that examined GH muscle properties under episodic hypoxia differ from our study in hypoxia exposure cycle duration (15- vs. 240-s duty cycle), extent of hypoxia (0% vs. 10.3% O2), and, as noted, nature of preparation (in vitro, rather than in vivo), with the last procedural difference likely accounting for many of the disparities in findings found here. Despite the procedural differences, several common findings emerged, including increased fatigue in GH muscles, although without conversion of muscle fiber types (29). However, an increase of MHC fast 2B types was reported under hypercapnic conditions in addition to functional property changes (30). None of these changes is as dramatic as with the present data in which we maintained CO2 levels at 0-0.1%. However, this difference can also be confounded by the difference in rat strain, as previously indicated.

Although the tension-stimulation frequency relationship did not differentiate IH-altered muscles from normal control values, fatigability increased in the IH-treated muscles, confirming that muscle fiber-type changes are commensurate with muscle functional properties. We view the increase of force production over baseline values in control animals at 20-, 40-, and 60-s points as an initial force potentiation. This phenomenon has been reported by others for the sternohyoid muscle (29). With submaximal stimulation during fatigue tests, it is uncommon to observe an initial force potentiation. This phenomenon has been reported in the present data in which we maintained CO2 levels at 0-0.1%.

The earlier described change in upper airway contractile properties and fiber types to more fatigable types result from episodic, not chronic hypoxia, although with accompanying hypercapnia (29). Repetitive, rather than sustained hypoxia, elicits a genioglossal response that mimics that which occurs during OSA (28). Compensatory reflexes to increased airflow resistance trigger increased GH tonic activity to overcome pharyngeal narrowing or even induce a lengthening contraction of the GH muscle during phasic activity (50). Increased overall activity of the genioglossus and mylohyoid muscles also develop to such increased airflow resistance in rabbits (44a). After severe hypoxic stimulation, fatigability of the GH muscle increases in cats (39).

Because the sternohyoid muscle maintains the same degree of MHC composition at 5 h (Fig. 2B), and begins to change its fiber composition by 10 h, the GH may succumb to IH more quickly than the sternohyoid muscle. Muscle-specific responses, such as different timing of fiber type change in each muscle, may depend on structural relationships. For example, the sternohyoid muscle exerts more force than the GH; when GH and sternohyoid muscles contract concomitantly, the GH must overwork or be lengthened (50), because each muscle attaches to either side of the hyoid bone. Thus the GH would become quickly fatigued. This early fatigue mechanism could be one process by which fiber-type changes in the GH began earlier than in the sternohyoid muscle.

Earlier studies (29, 30) that examined GH muscle properties under episodic hypoxia differ from our study in hypoxia exposure cycle duration (15- vs. 240-s duty cycle), extent of hypoxia (0% vs. 10.3% O2), and, as noted, nature of preparation (in vitro, rather than in vivo), with the last procedural difference likely accounting for many of the disparities in findings found here. Despite the procedural differences, several common findings emerged, including increased fatigue in GH muscles, although without conversion of muscle fiber types (29). However, an increase of MHC fast 2B types was reported under hypercapnic conditions in addition to functional property changes (30). None of these changes is as dramatic as with the present data in which we maintained CO2 levels at 0-0.1%. However, this difference can also be confounded by the difference in rat strain, as previously indicated.

Although the tension-stimulation frequency relationship did not differentiate IH-altered muscles from normal control values, fatigability increased in the IH-treated muscles, confirming that muscle fiber-type changes are commensurate with muscle functional properties. We view the increase of force production over baseline values in control animals at 20-, 40-, and 60-s points as an initial force potentiation. This phenomenon has been reported by others for the sternohyoid muscle (29). With submaximal stimulation during fatigue tests, it is uncommon to observe an initial force potentiation. This phenomenon has been reported in the present data in which we maintained CO2 levels at 0-0.1%.

Gupta MP, Gupta M, Dizon E, and Zak R. 894 GENIOHYOID CHANGES AFTER INTERMITTENT HYPOXIA

Gupta MP, Gupta M, Dizon E, and Zak R. 894 GENIOHYOID CHANGES AFTER INTERMITTENT HYPOXIA

Gupta MP, Gupta M, Dizon E, and Zak R. 894 GENIOHYOID CHANGES AFTER INTERMITTENT HYPOXIA

Gupta MP, Gupta M, Dizon E, and Zak R. 894 GENIOHYOID CHANGES AFTER INTERMITTENT HYPOXIA