Single-Fiber Myosin Heavy-Chain Isoform Composition of Rodent Laryngeal Muscle

Modulation by Thyroid Hormone

Ya Zhen Wu, MD; Michael J. Baker, MS; Roger L. Crumley, MD; Vincent J. Caiozzo, PhD

Background: Studies have shown that canine laryngeal muscle contains a large number of muscle fibers that coexpress varying combinations of myosin heavy-chain (MyHC) isoforms. Currently, it is not clear whether this phenomenon is unique to canine laryngeal muscle or occurs in all mammals.

Objectives: To examine the single-fiber MyHC isoform composition of rodent laryngeal muscle and to examine the plasticity of single-fiber MyHC isoform composition via manipulation of thyroid state.

Results: (1) Findings of single-fiber electrophoretic analyses clearly demonstrate that most fibers in both the posterior cricoarytenoid and thyroarytenoid muscles exhibit MyHC polymorphism. However, the proportions and patterns of polymorphism appear to be muscle specific. (2) Although the fast type IIL isoform was observed in fibers from both muscles, it was always coexpressed in combination with other MyHC isoforms (ie, no pure type IIL fibers were found), and always represented a minor proportion of the total MyHC pool. (3) Altering the thyroid state proved a useful tool for exploring the scope of MyHC isoform expression in these muscles. While the posterior cricoarytenoid muscle seemed more sensitive to the thyroid state, transitions in both muscles were primarily confined to the fast type IIX and IIB MyHC isoforms.

Conclusion: The findings of this study support the concept that single-fiber MyHC polymorphism occurs commonly in mammalian laryngeal muscle.

Arch Otolaryngol Head Neck Surg. 2000;126:874-880

Little is known about the biochemical and mechanical properties of laryngeal muscle despite their central role in fundamental processes like swallowing, phonation, and breathing. Within this context, there has been a growing interest in the myosin heavy-chain (MyHC) isoform composition of laryngeal muscle. Myosin heavy chains are the molecular motors of muscle, and convert chemical energy into mechanical work and heat. By using electrophoretic and immunohistochemical techniques, 4 adult MyHC isoforms have been identified in the hind-limb muscles of a number of mammalian species. These have been classified as the slow type I, fast type IIA, fast type IIX, and fast type IIB MyHC isoforms. With respect to rate of adenosine triphosphate hydrolysis and maximum shortening velocity, MyHC isoforms can be ranked as type I slower than type IIA, type IIX slower than type IIB (slowest to fastest). On this basis, MyHC isoforms have been characterized as "physiological markers," providing insight into the mechanical properties of a fiber.

Recent studies have begun to characterize the MyHC isoform composition of laryngeal muscles. In addition to the slow and fast MyHC isoforms typically found in the rodent hind-limb musculature, DelGaudio et al reported that the rodent posterior cricoarytenoid (PCA) and thyroarytenoid (TA) muscles also expressed an “atypical” MyHC isoform, which these investigators classified as type IIL. A similar finding was also made in rabbit laryngeal muscle by Lucas et al. However, Lucas et al suggested that the additional laryngeal MyHC isoform was really identical to that found previously in the extraocular musculature (ie, the extraocular myosin [EOM]). More recently, Jung et al showed that the rodent type IIL and EOM MyHC isoforms are, in fact, the same. While the findings of these whole-muscle analyses have provided an important database, ultimately it is more important to understand how these MyHC isoforms are distributed at the single-
MATERIALS AND METHODS

ANIMAL MODEL AND CARE

Female Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing 250 to 275 g were randomly assigned to 1 of 3 experimental groups: (1) control, n=5; (2) −T3, n=7; or (3) +T3, n=5. All rats were individually housed in temperature- and light-controlled quarters, and received food and water ad libitum. All experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee at the University of California, Irvine.

Animals assigned to the −T3 group were thyroidectomized and given daily intraperitoneal injections of propoithouracil (12 mg/kg) for 6 weeks. This dose substantially reduces plasma T3 levels.28 Hyperthyroidism was induced through daily intraperitoneal injections of aqueous T3 (175 µg/kg) for 6 weeks. This dose induces significant hyperthyroidism, while not initiating any signs of thyrotoxicosis.25 Given that the thyroid state influences the mass of the heart (−T3 decreases; +T3 increases), heart weight was used as an index of thyroid state.

At the end of the experimental period, animals were weighed and then killed with an overdose of pentobarbital sodium (50 mg/kg). The hearts were then surgically removed and weighed. Next, a surgical laryngectomy was performed on each animal, and the larynx placed in a small glass dish filled with glycerol. Under a surgical microscope (Technival II; Jena, Germany), the PCA and TA muscles of each larynx were removed and weighed. These samples were then placed in vials containing a cooled glycerol-relaxing solution (2-mmol/L EDTA, 1-mmol/L magnesium chloride, 4-mmol/L adenosine triphosphate, 0.5-mmol/L dithiothreitol, 5% [volume per volume] β-mercaptoethanol), heated for 2 minutes at 95°C, and vortexed for 1 minute. The MyHC isoform composition of each individual fiber was then determined using gel electrophoresis.

ELECTROPHORETIC SEPARATION OF MYHC ISOFORMS

Myosin heavy-chain isoforms were separated using a discontinuous gel electrophoresis (SDS-polyacrylamide gel electrophoresis) technique that separated 5 MyHC isoforms in rodent laryngeal muscle (type I, type IIA, type IIX, type IIB, and type III).30 The separating gel contained 8% acrylamide, 0.5% bis-acrylamide, 29% glycerol, 0.4% SDS, 0.2-mol/L Tris (hydroxymethyl)-aminomethane (pH 8.8), and 0.1-mol/L glycine. After allowing the solution to degas for 30 minutes, polymerization was initiated by adding N, N, N, N′-tetramethylethylene diamine (TEMED, 0.05% final concentration) and ammonium persulfate (0.1% final concentration). The separating gel was layered with a 10% ethanol solution and allowed to polymerize for 30 minutes. After polymerization of the separating gel, a stacking gel was prepared containing 4% acrylamide, 0.2% bis-acrylamide, 30% glycerol, 70-mmol/L Tris (pH 6.8), 4-mmol/L EDTA, and 4% SDS. This stacking gel was allowed to degas for 15 minutes, with polymerization initiated by adding TEMED (0.05% final concentration) and ammonium persulfate (0.1% final concentration). This gel was then poured on top of the separating gel and allowed to polymerize for 60 minutes. For single-fiber analysis, 1 muscle fiber was loaded into each well. Gels used a running buffer containing 0.1-mol/L Tris, 0.15-mol/L glycine, and 0.1% SDS. Electrophoresis was performed using an 250-200 vertical slab gel system (C.B.S. Scientific, Del Mar, Calif) and gels were run at a constant voltage of 275 V for 24 hours (4°C). Gels were stained using a Silver Stain Plus Kit (Bio-Rad, Richmond, Calif) and scanned wet using a high resolution personal densitometer running Version 5.0 Imagequant software (Molecular Dynamics, Sunnyvale, Calif).

STATISTICAL ANALYSES

All statistical analyses were performed using a computer program (Systat, Evanston, Ill). Heart weights, body weights, and muscle weights were analyzed using a 1-way analysis of variance. The single-fiber distributions from the control, −T3, and +T3 groups were compared with one another using χ2 analyses. Only the comparisons that were significant for χ2 analyses are discussed. Comparisons were considered statistically significant when P was .05 or smaller.
limb muscles. Specifically, the thyroid hormone is thought to exhibit positive regulation on the fast MyHC isoform genes, and negative regulation on the slow MyHC isoform gene. If laryngeal muscle responds to thyroid state in a manner similar to that seen in hind-limb muscle, we would hypothesize that the faster MyHC isoforms would be up-regulated by hyperthyroidism (+T3), while hypothyroidism (−T3) would produce an opposite effect.

Given this background, there were 2 primary objectives of our study. The first was to determine the MyHC isoform composition of individual muscle fibers found in the rodent PCA and TA muscles. Currently, it is not clear whether the single-fiber MyHC polymorphism in canine laryngeal muscle occurs among all mammalian species or represents a phenomenon unique to canines. The second objective was to use −T3 and +T3 as tools to gain insight into the malleability of MyHC isoform expression within rodent laryngeal muscles.

To our knowledge, the current study is the first to directly examine the single-fiber MyHC isoform composition of rodent laryngeal muscle. This approach yielded 3 unique findings. First, results of the single-fiber analyses clearly demonstrate that most fibers in both the PCA and TA muscles exhibit MyHC polymorphism. However, the proportions and patterns of polymorphism seem to be muscle specific. Second, although the type IIL isoform occurred in fibers from both muscles, it was always coexpressed in combination with other MyHC isoforms (ie, no pure type IIL fibers were found), and always represented a minor proportion of the total MyHC pool in a given fiber. Third, altering the thyroid state proved to be a useful tool for exploring the scope of MyHC isoform expression in these muscles. While the PCA muscle seemed to be more sensitive to thyroid state, transitions in both muscles were primarily confined to the fast type IIIX and IIB MyHC isoforms.

### Description of Animal, Heart, and Muscle Weights

<table>
<thead>
<tr>
<th>Weights</th>
<th>Control</th>
<th>−T3</th>
<th>+T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body, g</td>
<td>218 ± 4.6</td>
<td>206 ± 14.1</td>
<td>227 ± 9.7</td>
</tr>
<tr>
<td>Heart, mg</td>
<td>886 ± 174</td>
<td>572 ± 57</td>
<td>1035 ± 108</td>
</tr>
<tr>
<td>TA, mg</td>
<td>1.8 ± 0.8</td>
<td>1.9 ± 1.1</td>
<td>2.8 ± 0.8</td>
</tr>
<tr>
<td>PCA, mg</td>
<td>2.1 ± 0.9</td>
<td>1.9 ± 0.6</td>
<td>2.4 ± 0.5</td>
</tr>
</tbody>
</table>

*All data are mean ± SE. −T3 indicates hypothyroid; +T3 hyperthyroid; TA, thyroarytenoid muscle; and PCA, posterior cricoarytenoid muscle. For each weight category, statistical significance was determined for the 3 experimental groups (analysis of variance). Tukey tests were performed to make multiple comparisons.

†Significant difference from the −T3 group. No P values are available.
‡Significant difference from the control group. No P values are available.

### Results

#### Physical Description of Animals and Muscles

The effects of −T3 and +T3 on animal, heart, and muscle weights are given in the Table. The mean heart weight of the −T3 group was 35% lower than that of the control group (P<.001). In contrast, the mean heart weight of the +T3 group was approximately 50% greater than that of the control group (P<.001).

As shown in Figure 1, the control PCA muscles expressed 5 MyHC isoforms (ie, slow type I, fast type IIA, fast type IIIX, fast type IIB, and fast type IIL MyHC). Of these 5, however, the fast type IIIX and IIB MyHC isoforms represented approximately 85% of the total MyHC pool (≈35% and 50%, respectively).

Relative to the control group, −T3 decreased the proportion of fast type IIB MyHC isoform (P<.05) and increased the relative amounts of the fast type IIA and IIIX MyHC isoforms (P<.05) (Figure 1). In contrast, +T3 increased the proportion of the fast type IIB MyHC isoform (P<.05) and concomitantly reduced the fast type IIIX MyHC isoform (P<.05) relative to the control group.

---

©2000 American Medical Association. All rights reserved.
Whole-muscle MyHC isoform analyses for the TA muscles from the control, −T3, and +T3 groups are shown in Figure 2.

Unlike the control PCA muscles that expressed all 5 MyHC isoforms, the control TA muscles expressed only the fast type IIX, IIB, and IIL isoforms. Of these 3 MyHC isoforms, the type IIB represented approximately 65% of the total MyHC pool. Hypothyroidism, compared with the control group, produced relatively small but significant changes in the relative amounts of the fast type IIX, IIB, and IIL. As seen in the PCA muscle, +T3 produced effects that were opposite to those of −T3, increasing the proportion of the fast type IIB MyHC isoform and concomitantly decreasing the relative amount of the fast type IIX MyHC isoform (Figure 2).

PCA single-fiber MyHC isoform composition

The PCA single-fiber MyHC isoform compositions for each of the 3 experimental groups are shown in Figure 3 and Figure 4. There were approximately 12 or 13 different fiber types in the PCA muscle. Most of the fibers from the control group (=80% of the total population of fibers) fell into 3 distinct pools: fast type IIX fibers (=15%), fast type IIB fibers (30%), and polymorphic IIX/IIB/IIL fibers (=35%).

Relative to the control group, −T3 produced a significant reduction in the proportion of fast type IIB fibers and an increase in the proportion of polymorphic IIX/IIB/IIL fibers (Figures 3 and 4). In contrast, +T3 produced a large increase in the proportion of fast type IIB fibers and a concomitant decrease in the proportion of IIX/IIB fibers (Figures 3 and 4).

TA single-fiber MyHC isoform analyses

The TA single-fiber MyHC isoform compositions of the 3 groups are shown in Figure 5 and Figure 6. The control TA muscles were composed of 5 fiber types (Figure 5). However, approximately 85% of the total population of fibers were represented by just the IIB/IIL (=65%) and IIX/IIB/IIL (=25%) fibers.

Relative to the control group, −T3 produced a substantial reduction in the proportion of IIB/IIL fibers that was accompanied by an increase in the proportion of IIX/IIB/IIL fibers (Figure 5). In contrast, +T3 produced an opposite effect, increasing the proportion of IIB/IIL fibers and reducing the proportion of IIX/IIB/IIL fibers.

Comment

No studies have directly examined the MyHC isoform composition of single fibers from rodent laryngeal muscle.
Few studies have examined the MyHC isoform composition of laryngeal muscle. DelGaudio et al used a combination of adenosine triphosphatase histochemical and immunohistochemical analysis to determine the MyHC isoform composition of muscle fibers in rodent laryngeal muscle. These investigators found, however, that it was difficult to categorize fibers in the TA and PCA muscles using this approach. Based on the findings of the
In 1995, 2 independent research groups reported the existence of a MyHC isoform in laryngeal muscle different from that typically occurring in the hind limb musculature of mammals. In contrast, DelGaudio et al studied rodent laryngeal muscle and classified this additional isoform as the fast type III MyHC. More recently, Jung et al reported that the DNA sequence of the additional isoform found in rodent laryngeal muscle matched that of the EOM MyHC isoform. Based on this most recent evidence, it would seem that the so-called fast type III MyHC isoform found in rodent laryngeal muscle is really the EOM MyHC isoform.

As shown in Figures 1 and 2, the fast type III MyHC isoform represents a relatively small proportion of the total MyHC isoform pool in both the PCA and TA muscles of the rodent. In this regard, however, the TA muscle clearly has a larger proportion of fast type III MyHC isoform than the PCA muscle. These observations are consistent with those made previously. As shown in Figures 4 and 6, none of the fibers in either the PCA or TA muscles expressed exclusively the fast type III MyHC isoform. Within both the PCA and TA muscles, the fast type III MyHC isoform was found primarily in IIB/IIL and IIX/IIB/IIL fibers. Note that the fast type III MyHC isoform was never the dominant isoform within any given fiber type (Figures 4 and 6). On this basis, it can be concluded that the rodent PCA and TA muscles do not contain so-called type III fibers (ie, fibers that only express this isoform).

In rabbit laryngeal muscle, there seems to be a large variation in the expression of the fast type III MyHC isoform. Immunohistochemical analyses have shown that the rabbit TA muscle contains a large proportion of fibers that stain positive for the fast type III MyHC isoform. In contrast, rabbit cricothyroid muscle seems to be void of fibers expressing this MyHC isoform. Clearly, a broader survey of laryngeal muscle is needed to fully appreciate the significance of this isoform.

**LARYNGEAL MUSCLES ARE INFLUENCED BY –T3 AND +T3 IN A MyHC ISOFORM–SPECIFIC MANNER**

In a simplistic view, one might assume that the effect of thyroid hormone on all skeletal muscle would be similar. However, studies have shown that the thyroid hormone acts in a muscle-specific manner. Our study is the first to determine the effect of thyroid hormone on rodent laryngeal muscle MyHC isoform composition. Clearly, the whole-muscle and single-fiber shifts in MyHC isoform expression produced by –T3 and +T3 were not equivalent in the PCA and TA muscles, indicating that thyroid hormone also acts in a muscle-specific manner within rodent laryngeal muscle.

In the PCA muscle, thyroid state affected the relative amounts of the fast type IIA, IIX, and IIB MyHC isoforms. Of these 3 MyHC isoforms, the fast type IIB MyHC isoform seemed the most sensitive (Figure 1) to thyroid state. At the single-fiber level, most of the fast type IIB MyHC isoform in the control group was found in the IIB and IIX/IIB fibers (Figure 4). Hypothyroidism down-regulated the fast type IIB MyHC isoform primarily by reducing the proportion of fast type IIB fibers. In contrast, +T3 produced an increase in the relative amount of the fast type IIB MyHC isoform by producing a dramatic increase in the proportion of IIB fibers and reducing the proportion of IIX/IIB fibers. In the TA muscle,
-T3 reduced the proportion of IIB/IIL fibers and increased the proportion of IIX/IIB/IIL fibers. Hyperthyroidism produced an opposite effect.

With respect to MyHC isoform plasticity, Pette and Staron, Aigner et al., Staron and Pette, and Termin et al. proposed a simple sequential scheme of MyHC that can be summarized as follows: IIA/IIB. Currently, it is not clear whether such a model pertains to rodent laryngeal muscle. If such a model is applicable, then a modification of this scheme is required to account for the presence of the type IIL isoform. While the whole-muscle data in the current study create the impression that transitions occurred between the fast type IIX and IIB MyHC isoforms, the manifestation of such transitions at the single-fiber level was clearly more complex than this. Also, recent studies have shown that the IIA/IIB scheme may not apply to all rodent skeletal muscles or conditions. For instance, rodent muscles such as the diaphragm and plantaris contain fibers inconsistent with this scheme (V.J.C., unpublished data, 1999). Additionally, altered thyroid and mechanical states have been shown to produce patterns of single-fiber MyHC polymorphism that cannot be explained by this model (V.J.C., unpublished data, 1999).

**SUMMARY**

In summary, the findings of this study demonstrate that the mechanisms regulating the expression of MyHC isoforms in laryngeal muscle are highly complex. This is exemplified by the finding that a large proportion of fibers in both the PCA and TA muscles exhibit polymorphic expression of MyHC isoforms. Within this context, manipulation of thyroid state proved to be a useful tool in exploring the plasticity of single-fiber MyHC isoform composition.

Accepted for publication December 12, 1999.

Reprints: Vincent J. Caiozzo, PhD, Medical Sciences I, B-152, Department of Orthopedics, University of California, Irvine, CA 92697 (e-mail: vjcaiozzo@uci.edu).

**REFERENCES**


©2000 American Medical Association. All rights reserved.