Are Hybrid Fibers a Common Motif of Canine Laryngeal Muscles?

Single-Fiber Analyses of Myosin Heavy-Chain Isoform Composition

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

Background: The canine lateral cricoarytenoid muscle contains a large proportion of muscle fibers that coexpress various combinations of myosin heavy-chain isoforms (ie, so-called hybrid fibers).

Objective: To test the hypothesis that hybrid fibers are a common motif throughout laryngeal muscles.

Design: The posterior cricoarytenoid, canine cricothyroid, and thyroarytenoid muscles were removed from 5 beagle dogs. The posterior cricoarytenoid and canine cricothyroid muscles were each dissected into horizontal, oblique, and rectus regions. The thyroarytenoid was separated into medial and lateral regions. Approximately 40 single fibers were microdissected from each region (=1800 total fibers were sampled) and placed into a denaturing sample buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was then used to separate the individual myosin heavy-chain isoforms.

Results: Each laryngeal muscle contained hybrid fibers; however, the types and proportions of hybrid fibers were clearly muscle specific. Within a given muscle, there were relatively minor regional differences in the types and proportions of hybrid fibers.

Conclusion: If the myosin heavy-chain isoform composition of a single fiber can be used as a “physiological marker,” then the extent of hybridism may reveal the diversity of activity required of a given laryngeal muscle.

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Early observations of the mechanical properties of skeletal muscle demonstrated the existence of so-called slow and fast twitch muscles.1 Results of subsequent histochemical studies2-4 demonstrated that these differences in mechanical properties were related to so-called fiber types that differed in a variety of properties (eg, oxidative, glycolytic, and myofibrillar adenosine triphosphatase [ATPase] activities). Fibers that were classified on the basis of myofibrillar ATPase histochemical properties were grouped as slow type I (slowest contractile properties), fast type IIA (intermediate contractile properties), or fast type IIB (fastest contractile properties). During the past 20 to 30 years, the concept of “muscle fiber type” has undergone continuous refinement as more sophisticated and sensitive techniques have evolved. For instance, electrophoretic and immunohistochemical techniques have been used to identify 4 types of fibers on the basis of myosin heavy-chain (MyHC) isoform composition.4-7

Until recently, it was believed that most skeletal muscle fibers, under steady state conditions, expressed only one MyHC isoform, hence the identification of discrete fiber types. During the past 5 to 10 years, highly sensitive electrophoretic techniques have been developed for analyzing the MyHC composition of single fibers.8-11 These analyses have provided important evidence to suggest that muscle fibers, under steady state conditions, can coexpress multiple MyHC isoforms. Such fibers have been described as “polymorphic” or “hybrid” fibers, and their presence suggests that muscle fiber composition is much more complex than previously thought.

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It was recently reported12 that approximately 40% of the fibers in the lateral cricoarytenoid (LCA) muscle of the canine larynx coexpressed multiple MyHC isoforms. The presence of these so-called hybrid fibers in the LCA raised the possibility that such fibers might not be “novel” but might represent a common motif across a spectrum of canine laryngeal muscles. Results of previous studies13 have shown that the muscle fiber composition of laryngeal muscles is also regionalized. On this basis, it seems reasonable to sug-
gest that the types and proportions of hybrid fibers found within a given laryngeal muscle might also depend on the region of the muscle examined. Given these considerations, we examined the MyHC isoform composition of single fibers in canine cricothyroid (CT), posterior cricoarytenoid (PCA), and thyroarytenoid (TA) muscles. The findings of this study clearly demonstrate that each of these muscles contains hybrid fibers and that the types and proportions of these fibers are primarily muscle specific. Although there seem to be regionalized differences in the types of hybrid fibers within a given laryngeal muscle, such differences are relatively minor.

RESULTS

CT MUSCLE

Regional distributions of MyHC isoforms in the CT muscle are shown in Figure 1. Each region primarily expressed only slow type I and fast type IIA MyHC isoforms. The horizontal region had a relatively even distribution of these 2 isoforms, whereas the rectus and oblique regions had greater proportions of the fast type IIA vs the slow type I MyHC isoform (≈60% vs ≈35%-40% of the total MyHC isoform pool).

Single-fiber distributions of MyHC isoforms for the CT muscle are shown in Figure 2 and Figure 3. In all 3 regions, most fibers expressed either slow type I or fast type IIA MyHC isoforms (ie, most fibers expressed just one MyHC isoform). The horizontal region contained approximately equal proportions of slow type I and fast type IIA fibers. In the rectus and oblique regions, fast type IIA fibers represented the largest pool of fibers (≈50%-55% of the total population). Although each region contained hybrid fibers, these represented a small proportion of the total pool of fibers (≈<10% of the total population).

MATERIALS AND METHODS

ANIMAL CARE

Five healthy adult beagle dogs weighing 20 to 30 kg were used in this study. All aspects of the study conformed to the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee of the University of California, Irvine, approved this study.

DISSECTION OF LARYNGEAL MUSCLES AND ISOLATION OF SINGLE FIBER SEGMENTS

After the dogs were humanely killed with barbiturate overdose, the larynx was exposed and then removed. The TA muscle was isolated and divided into the TA vocalis (referred to as the medial TA) and the lateral TA. The PCA muscle was isolated by removing the overlying cricopharyngeus muscle and esophageal mucosa and was then divided into horizontal, oblique, and vertical regions according to the anatomical description by Sanders et al. The CT muscle originates along the anterior and lateral aspects of the cricoid cartilage and inserts along the inferior border of the thyroid cartilage. Based on the anatomical descriptions of Arnold, Tschiassny, and Zaretsky and Sanders, the CT muscle was divided into the pars recta (rectus), pars obliqua (oblique), and pars interna (internal/horizontal).

On dissection, each muscle sample was put immediately into a cooled glycerol relaxing solution containing 2-mmol/L EDTA, 1-mmol/L magnesium chloride, 4-mmol/L adenosine triphosphate, 10-mmol/L imidazole, and 100-mmol/L potassium chloride (pH 7.0). Muscle samples were stored in a ~20°C freezer until isolation of single-fiber segments.

Approximately 40 single muscle fibers were microdissected from each region using a dissection microscope (Technival II; Jena, Germany) (magnification ×25-×50) and fine Dumont tweezers. Each fiber was approximately 2 to 4 mm long. These methods are similar to those described previously.

Figure 1. Regional distribution of myosin heavy-chain (MyHC) isoforms in the canine cricothyroid muscle. Regions are shown in order of their MyHC isoform profile, with the slowest MyHC isoform profile in the top panel and the fastest in the bottom panel. Values are mean ± SE. Asterisk indicates P<.05 compared with the horizontal region.
Regional distributions of MyHC isoforms in the PCA muscle are shown in Figure 4. Each region of the PCA muscle expressed 4 MyHC isoforms; however, there were distinctive regional differences. For instance, the horizontal region had the slowest profile, with the slow type I MyHC isoform representing the largest proportion (45%-50%) of the total MyHC pool. At the opposite end of the isoform spectrum, the vertical region had the fastest MyHC isoform profile. The vertical region had relatively equal proportions of slow type I, fast type IIA, and fast type IIB MyHC isoforms. However, the putative canine fast type IIX MyHC isoform migrated slightly ahead of that from rodent skeletal muscle.

Figure 2. Single-fiber gels taken from the various regions of the canine cricothyroid muscle. Reference lanes for the horizontal (lanes 1 and 2), rectus (lanes 1 and 10), and oblique (lanes 1 and 9) regions are included. Most fibers in the canine cricothyroid muscle express slow type I or fast type IIA myosin heavy-chain isoforms. There are few hybrid fibers in the canine cricothyroid muscle.

SODIUM DODECYL SULFATE–POLYACRYLAMIDE GEL ELECTROPHORESIS FOR MyHC ANALYSIS

As described previously,9,10,12 isolated single muscle fibers were placed into 30 µL of sample buffer containing 9% β-mercaptoethanol, 100-mmol/L Tris base, 5% glycerol, 4% sodium dodecyl sulfate (SDS), and 1% bromophenol blue. Each single muscle fiber was then heated to 95°C for 2 minutes and sonicated for 30 seconds. The separating gel contained 8% acrylamide, 0.6% bis-acrylamide, 29% glycerol, 0.4% SDS, 0.2-mol/L Tris, and 0.1-mol/L glycine. The stacking gel contained 4% acrylamide, 0.2% bis-acrylamide, 30% glycerol, 70-mmol/L Tris (pH 6.7), 4-mmol/L EDTA, and 0.4% SDS. N,N,N′,N′-tetramethylethylenediamine (0.05%) and ammonium persulfate (10%) were used as cross-linkers during polymerization. Gels were immersed in a running buffer (0.1-mol/L Tris, 0.15-mol/L glycine, and 0.1% SDS), and a total of 15 µL from each sample was then loaded into each well. Electrophoresis was performed at a constant of 275 V for 24 hours at 4°C. Gels were then silver stained (Silver Stain Plus Kit; Bio-Rad, Hercules, Calif) and scanned for MyHC isoforms using an automated densitometer (Molecular Dynamics, Sunnyvale, Calif).

In a previous study,12 4 MyHC isoforms were identified in the canine LCA muscle based on immunoblots, immunohistochemical analysis, and electrophoretic migration distance relative to that of rodent skeletal muscle. There was good agreement between the electrophoretic mobility of the rodent and canine slow type I, fast type IIA, and fast type IIB MyHC isoforms. However, the putative canine fast type IIX MyHC isoform migrated slightly ahead of that from rodent skeletal muscle.

STATISTICAL ANALYSES

Whole-muscle analyses were performed using 1-way analysis of variance. If a significant F ratio was obtained, then supplemental analyses were used to determine which groups were different from one another. Differences between single-fiber population distributions (ie, either regional or muscle dependent) were determined using χ² analysis. Only those χ² tests that were statistically significant are reported in the “Results” section. All statistical analyses were performed using a computer program (Systat, Evanston, Ill). All statistical analyses were considered significant at P≤.05.
There are 3 key findings. First, in each region, slow type I fibers represented the largest pool of fibers; however, note the regional difference. Second, each region contained a sizable proportion of fast type IIA/IIX hybrid fibers. Finally, the largest proportion of fast type IIX fibers was found in the vertical region.

**TA MUSCLE**

Distributions of MyHC isoforms in the medial and lateral TA regions are shown in **Figure 7**. There were clear differences between the 2 regions. The medial TA region contained a large population of single fibers that expressed only the fast type IIX MyHC isoform (<15% of the total population) and IIX/IIB (~18% of the total population) populations of hybrid fibers. The lateral TA region contained approximately 7 different types of hybrid fibers, with the IIX/IIB fibers representing the largest pool of hybrid fibers (~20% of the total population).

**OVERALL MyHC ISOFORM COMPOSITION OF CANINE LARYNGEAL MUSCLES**

For comparative purposes, the overall MyHC isoform composition of the CT, LCA, PCA, and TA muscles (in order of speed) is shown in **Figure 11**.

Consistent with these differences, the medial TA region contained a large population of single fibers that expressed only the fast type IIX MyHC isoform (**Figure 8** and **Figure 9**). The lateral TA region had a large population of single fibers that expressed only the fast type IIB MyHC isoform (**Figure 9** and **Figure 10**). Both regions also contained a significant proportion of hybrid fibers (~30%-40% of the total population).
For many years, the dominant approach for determining muscle fiber type involved histochemical identification of myofibrillar ATPase activity. The pH lability of myosin causes fast muscle fibers to stain dark under alkaline (pH 9.2) and light under acidic (pH 4.2) conditions. Opposite staining patterns are produced by slow fibers. At a pH of 4.5, three different staining intensities are observed, with slow type I fibers staining dark, fast type IIA fibers staining light, and fast type IIB fibers staining intermediate. Occasionally, investigators noted that staining at a pH of 4.2 produced 3 staining intensities. Fibers with an intermediate intensity were labeled as fast type IIC fibers and were thought to either contain developmental myosin isoforms or coexpress slow and fast MyHC isoforms. Type IIC fibers were thought to be fibers undergoing a transformation from one type of fiber to another.

During the past 5 to 10 years, it has become clear that type IIC fibers are hybrid fibers (ie, fibers expressing more than one MyHC isoform) and that hybrid fibers exist even under so-called steady state conditions. During the past 5 to 10 years, it has become clear that type IIC fibers are hybrid fibers (ie, fibers expressing more than one MyHC isoform) and that hybrid fibers exist even under so-called steady state conditions.

**ARE HYBRID FIBERS A COMMON MOTIF OF CANINE LARYNGEAL MUSCLES?**

In a previous study, Wu et al examined the MyHC isoform composition of single fibers from the canine LCA muscle and found that approximately 40% of the fibers in this muscle coexpressed various combinations of MyHC isoforms. This finding raised the possibility that hybrid fibers might be a common theme among canine laryngeal muscles. Within this context, each muscle (ie, CT, PCA, and TA) examined in the present study contained hybrid fibers. However, the types and proportions of hybrid fibers differed significantly from one muscle to another and, in some cases, from one region to another. With respect to differences between muscles, the CT had the smallest proportion of hybrid fibers (≈5%-10% of the total population of fibers), whereas the TA had the greatest proportion of hybrid fibers (≈30%-40% of the total population). In the slower CT muscle, the primary pool of hybrid fiber was the I/IIA fiber type. In contrast, the primary pool of hybrid fiber in the faster TA muscle was the IIX/IIB fiber type. For the CT and PCA muscles, the types and proportions of hybrid fibers were similar from one region to another. Although the medial and lateral regions of the TA also had similar types of hybrid fibers, there was a significant difference in the proportions of IIA/IIX fibers between the 2 regions.

As reported in 2 companion articles, investigators also examined the MyHC composition of single fibers taken from human and rodent PCA and TA laryngeal muscles. The findings of the companion studies demonstrate that there is also a significant degree of hybridism in human and rodent laryngeal muscle. However, the types of hybrid fibers differ significantly across species. For instance, as shown in Figure 4, the canine PCA muscle contains significant proportions of slow type I, fast type IIA, and fast type IIX MyHC isoforms. At the single-fiber level, the largest population of hybrid fibers (≈10%-20% of the total population of fibers) coexpressed the IIA/IIX MyHC isoforms. The rodent PCA muscle, in contrast, is a fast muscle that primarily expresses fast type IIX (≈30%) and IIB (≈45%) MyHC isoforms and contains many fibers that coexpress the IIX/IIB MyHC isoforms. Relative to the canine and rodent...
PCA muscle, the human PCA muscle is slower and the types of hybrid fibers are biased toward slower combinations of MyHC isoforms (e.g., I/IIA). With respect to the TA muscle, the MyHC isoform profile is predominantly fast in canines, rodents, and humans. However, the MyHC profile becomes progressively slower (IIB → IIX → IIA) in accordance with the mass of the animal. For instance, the predominant MyHC isoforms in the rodent TA muscle were fast type IIX and IIB, whereas in the human TA muscle the fast type IIA MyHC isoform accounted for the largest proportion of the myosin pool. The most common hybrid fibers found among human, canine, and rodent laryngeal muscles involve various combinations of fast MyHC isoforms. For some reason, laryngeal muscles that contain a high proportion of the slow type I MyHC isoform have a paucity of hybrid fibers involving this isoform.

From a global perspective, the findings of the present study further support the concept that hybrid fibers seem to be a common motif not only within laryngeal muscles but also across a broad spectrum of muscles. For instance, Staron and Pette observed hybrid fibers in rabbit and rodent skeletal muscle. Subsequent to these studies it was shown that hybrid fibers also exist within human skeletal muscle. More recently, we examined the single-fiber MyHC isoform composition of fibers taken from approximately 15 different rodent muscles and found hybrid fibers in each of the different muscles studied. The common occurrence of hybrid fibers raises several interesting issues related to muscle fiber type and the physiological role of such fibers.

**WHAT IS THE PHYSIOLOGICAL SIGNIFICANCE OF HYBRID FIBERS?**

The classic studies by Szent-Gyorgi and Barany demonstrated the central role of myosin in the contractile process and the mechanistic linkage between myofibrillar ATPase activity and maximal shortening velocity. More recently, both whole-muscle and single-fiber studies demonstrated a high correlation between myosin isoforms and maximal shortening velocity. Collectively, these findings support the concept that myosin isoforms can be used, in a sense, as physiological markers, hence the potential importance of studying the MyHC isoform composition of laryngeal muscle. If the MyHC isoform composition of single fibers can be used as a physiological marker, then the single-fiber distribution of MyHC isoforms should provide indirect evidence about the activity patterns of laryngeal muscle. Within this context, the single-fiber MyHC isoform distributions of the PCA and TA muscles are more diverse than that of the CT muscle. On this basis, it seems reasonable to hypothesize that the activity patterns of the PCA and TA muscles are more varied than that of the CT muscle.
To date, the significance of hybrid fibers relative to the concept of fiber types is still unclear. From a simplistic perspective, hybridism (polymorphism) might represent a strategy whereby 4 different types of molecular motors (ie, slow type I, fast type IIA, fast type IIX, and fast type IIB MyHC isoforms) can be mixed in various combinations to yield a population of muscle fibers that has a more complete continuum of contractile properties. With respect to the genotype of skeletal muscle, this phenomenon might represent a more parsimonious strategy compared with the evolution of new MyHC genes.

The classic cross-innervation study by Buller et al\(^{26}\) gave rise to the concept that the phenotype of muscle fiber is determined by neural factors (eg, the pattern of impulses, the quantity of impulses, or neurotrophic factors). A variety of subsequent approaches have provided data that both support and reject this concept.\(^{27-30}\) If this is the case in laryngeal muscles, then the existence of a given hybrid fiber type suggests that its innervation pattern must be different from that of other fiber types, thereby raising important issues related to motor control. For instance, are there only 3 different types of motor units as widely reported? Are the phenotypic properties of muscle fibers within a given motor unit homogeneous? With respect to the first issue, if there are only 3 different types of motor neurons innervating laryngeal muscle fibers, then some motor units must be composed of monomorphic and polymorphic fibers. If this is the case, then the innervation pattern cannot be the only factor determining the phenotype of muscle fibers within a given motor unit. If the muscle fibers of a given motor unit are, in fact, ho-

**Figure 8.** Single-fiber gels for the medial and lateral regions of the thyroarytenoid muscle. Reference lanes for the medial (lanes 8 and 17) and lateral (lanes 9 and 18) regions are included. Most fibers express the fast type IIX MyHC isoform. In some fibers, there is coexpression of fast type IIA and IIX MyHC isoforms. In contrast, most fibers in the lateral region express the fast type IIB MyHC isoform.

**Figure 9.** Single-fiber myosin heavy-chain isoform distribution of each region of the thyroarytenoid muscle. The number of fibers sampled in each region is shown in each panel. Note the predominance of the fast type IIA fibers in the medial region, whereas in the lateral region there was a large proportion of fast type IIX fibers. In addition, in each region there were substantial pools of hybrid fibers.

**Figure 10.** Cross section of the lateral region of the thyroarytenoid muscle probed with a monoclonal antibody specific for the fast type IIB myosin heavy-chain isoform (ie, BF-F3). Note the large percentage of fibers that stain positive (dark) for the presence of the fast type IIB myosin heavy-chain isoform.
muscles. Hence, it seems highly unlikely that the presence of hybrid fibers is due to a single motor neuron (i.e., a given motor neuron can have multiple neuromuscular junctions on a given fiber). In addition, as noted previously, hybrid fibers occur in a broad spectrum of different muscles. Hence, it seems highly unlikely that the presence of hybrid fibers in laryngeal muscles occurs because of the unique form of innervation found in these muscles.

Results of several studies have shown that the MyHC isoform expression of skeletal muscle is affected not only by innervation pattern but also by hormonal milieu and mechanical factors. Within this context, little is known about the role of these 3 factors (i.e., innervation, hormonal milieu, and mechanical factors) in controlling the MyHC isoform expression of laryngeal muscles. Hence, new approaches are needed to clearly delineate the various extrinsic factors that affect the function of laryngeal muscles. In addition, a comprehensive biomechanical model is needed that incorporates important architectural properties with the mechanical properties of various fiber types found in laryngeal muscles.

In summary, each of the human, canine, and roentgen laryngeal muscles studied, to date, has been shown to contain so-called hybrid fibers (i.e., fibers that co-express various combinations of MyHC isoforms). From an anatomical perspective, the findings of the present study demonstrate that the types and proportions of the hybrid fibers differ significantly between the laryngeal muscles. In contrast, the regional differences in hybrid fibers are relatively minor. The findings of this study highlight the complexity of muscle fiber types within laryngeal muscles and emphasize the need to resolve basic issues about genotypic and phenotypic expression patterns and the consequent physiological role of laryngeal muscles.

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Reprints: Vincent J. Caiozzo, PhD, Department of Orthopaedics, College of Medicine, University of California, Medical Science I B-152, Irvine, CA 92717 (e-mail: vjcaiozz@uci.edu).

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