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Proteomics and immunohistochemistry identify the expression of α-cardiac myosin heavy chain in the jaw-closing muscles of sooty mangabeys (order Primates)

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ABSTRACT

Objective: The jaw-closing muscles of humans and nonprimate mammals express alpha-cardiac fibers but MyHC α-cardiac has not been identified in the jaw adductors of nonhuman primates. We determined whether MyHC α-cardiac is expressed in the superficial masseter and temporalis muscles of the sooty mangabey (*Cercocebus atys*), an African Old World monkey that specializes on hard seeds.

Design: LC–MS/MS based proteomics was used to identify the presence of MyHC Iα.

Immunohistochemistry was used to analyze the composition and distribution of fiber types in the superficial masseter and temporalis muscles of eight *C. atys*. Serial sections were stained against MyHC α-cardiac (MYH6), as well as MyHC-1 (NOQ7.5.4D), MyHC-2 (MY-32), and MyHC-M (2F4).

Results: Proteomics analysis identified the presence of Myosin-6 (MyHC α-cardiac) in both heart atrium and superficial masseter. MyHC α-cardiac was expressed in abundance in the superficial masseter and temporalis muscles of all eight individuals and hybrid fibers were common.

Conclusions: The identification of MyHC α-cardiac in the jaw adductors of sooty mangabeys is a novel finding for nonhuman primates. The abundance of MyHC α-cardiac indicates a fatigue-resistant fiber population characterized by intermediate speed of contraction between pure MyHC-1 and MyHC-2 isoforms. We suggest that α-cardiac fibers may be advantageous to sooty mangabeys, whose feeding behavior includes frequent crushing of relatively large, hard seeds during the power stroke of ingestion. Additional studies comparing jaw-adductor fiber phenotype of hard-object feeding primates and other mammals are needed to explore this relationship further.

1. Introduction

Fiber type composition of the chewing muscles is well documented in mammals (Bredman et al., 1991; English, Eason, Schwartz, Shirley, & Carrasco, 1999; Hoh, 2002; Hoh, Kim, Sieber, Zhong, & Lucas, 2000; Kang, Hughes, Pettigrew, & Hoh, 1994; Mascarello, Aureli, & Veggetti, 1979; Schiaffino & Reggiani, 1996; Sciote, Horton, Rowlerson, & Link, 2003; Sciote & Rowlerson, 1998; Sciote, Rowlerson, Hopper, & Hunt, 1994; Sfondrini, Reggiani, Gandini, Bovenzi, & Pellegrino, 1996; Van Wessel et al., 2005). In addition to the myosin heavy chain (MyHC) isoforms expressed in limb muscles, including type 1, and faster isoforms such as type 2B, 2X and 2A, the jaw-closing muscles of a variety of mammals express α-cardiac (Bredman et al., 1991; Sciote & Rowlerson, 1998; Sciote et al., 1994; Widmer, Morris-Wilman, & Nekula, 2002), a MyHC that is also expressed in heart muscle (Hoh, McGrath, & Hale, 1978). Alpha-cardiac fibers are capable of a higher speed of contraction compared to type 1 fibers while maintaining roughly equivalent fatigue resistance (Hoh, Kang, Sieber, Lim, & Zhong, 2006). Thus, the presence of α-cardiac fibers broadens the functional range of fiber types in mammalian jaw muscles as compared with the limb muscles.

Sooty mangabeys (*Cercocebus atys*) are an African Old World monkey that specializes on seeds (McGraw, Vick, & Daegling, 2011). It has been hypothesized that a significant percentage of their feeding behavior involves crushing the seed casings to access the enclosed nut (McGraw et al., 2011). Nonprimate mammals that spend a significant portion of their day chewing, such as grazers, have been shown to exhibit a slow, fatigue-resistant (MyHC-1or MyHC α-cardiac) fiber type

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(Hoh et al., 2000; Kang et al., 1994; Mascarello et al., 1979). We thus hypothesized that the jaw adductors of sooty mangabeys would exhibit a similar fiber type, expressing both MyHC-1 and MyHC α-cardiac. Previous work has demonstrated the presence of slow twitch (fatigue resistant, type 1), fast twitch (fast fatigue resistant and fast fatigable, type 2) and the jaw-specific MyHC-M isoforms in the jaw-closing muscles of macaques (Maxwell, Carlson, & Faulkner, 1979; Rowlerson, Mascarello, Veggetti, & Carpene, 1983; Miller & Farias, 1988) and baboons (Wall, Briggs, Huq, Hylander, & Schachat, 2013), which are closely related to the sooty mangabey (Harris & Disotell, 1998). In the order Primates, however, the presence of α-cardiac MyHC has been confirmed only for humans (Sciote et al., 1994).

In this study, we used proteomics to identify the presence of the α-cardiac myosin heavy chain protein in the sooty mangabey superficial masseter muscle. We then used immunohistochemistry (IHC) to determine the major MyHC isoforms present in the superficial masseter and temporalis muscles of the sooty mangabey. We show that sooty mangabey masseter and temporalis express an abundance of MyHC α-cardiac as well as varying amounts of the major myosin heavy chain isoforms.

2. Materials and methods

2.1. Muscle samples

All *Cercocebus atys* (sooty mangabey) tissues were from animals that had been assigned to Emory University's IACUC Protocol YER-2002775-ELMNTS-A, Comparative AIDS Core Program, Yerkes National Primate Research Center (Yerkes), which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The sooty mangabey is classified as an endangered species and Yerkes maintains a US Fish and Wildlife Service permit that governs the use of mangabeys in research. In accordance with their policies and strict guidelines regarding this endangered species, all tissues provided to us were collected following the clinically necessary sacrifice of these animals. The number of animals in the Yerkes sooty mangabey colony was not increased in order to provide the specimens for our work. All sooty mangabeys were fed the same IACUC-approved diet of monkey chow supplemented with fruit. The jaw muscles are from adult females (6.63–12.0 kg) ranging in age between 11.2–23.9 years and adult males (9.05–13.13 kg) ranging in age between 15–22.1 years, were fully dentate and without evidence of craniofacial or dental pathology or muscle atrophy.

For the IHC jaw-muscle sample, the heads were immediately formalin-fixed and stored in a conventional freezer. The heads were shipped frozen on dry ice, thawed in 10% buffered formalin and stored in 10% buffered formalin until use. Following fixation, the masseter and temporalis muscles were dissected free from their attachments. For the proteomics sample, the head and heart of one *C. atys* were frozen immediately postmortem, shipped frozen on dry ice and stored at −80 until use.

2.2. LC–MS/MS based proteomics validation of MyHC Iα

A liquid chromatography tandem mass spectrometry (LC–MS/MS) analysis was performed on fresh anterior superficial masseter and heart atrium tissue for one *C. atys* to determine whether MyHC α-cardiac protein was expressed in superficial masseter. Heart atrium is a positive test that confirms the presence of MyHC α-cardiac (also referred to as MYH6 and Myosin-6; the gene is identified as *MYH6*) in superficial masseter, and the ability of the α-cardiac antibody to recognize MyHC α-cardiac fibers in primate jaw-muscle tissue.

The MyHCs are highly conserved genes (Schiaffino & Reggiani, 2011). Thus, in the absence of a proteome for *C. atys*, we relied on protein homology (Gevaert & Vandekerckhove, 2000). Following data acquisition, all spectra (the identified peptide sequences) for *C. atys* were matched to the NCBI proteomic database across the annotated proteome for *Papio,* both genera belonging to the Papionini tribe of Old World monkeys based on nuclear (Harris & Disotell, 1998) and mitochondrial (Harris, 2000) gene sequences. A total of 1350 proteins was identified at a 1% false discovery rate. The Proteomics and Metabolomics Shared Resource at Core Facility, Duke Center for Genomic and Computational Biology at Duke University prepared and performed this analysis. Detailed analytical procedures can be found in Supplementary data.

2.3. IHC protocol

Small tissue blocks (approximately ~3 mm thick × ~2 mm wide in each perpendicular direction) were cut perpendicular to the long axis of the masseter and temporalis muscles. We sampled from the anterior superficial masseter and the anterior and posterior temporalis, averaging the two temporalis regions for data analysis. Prior to embedding, the muscle tissues were preserved in 70% ethanol solution for a period of 48–72 h. Tissue blocks then were paraffin-embedded and sectioned at 5 μm using a cryostat. Sections were pre-treated with 1% bovine serum albumin (BSA, Sigma) and dissolved in Tris-buffered saline, 0.1% with Tween 20 (TBST) for 20 min. Contiguous sections from each muscle block were stained against MyHC-1 (1:400 dilution; NOQ7.5.4D, Sigma), MyHC α-cardiac (1:400 dilution; MYH6, Sigma), MyHC-2 (1:400 dilution; MY-32, No. 4276, Sigma) and MyHC-M (1:200 dilution, 2F4, Developmental Studies Hybridoma Bank, University of Iowa). The MY-32 antibody varies in its ability to react with MyHC-M in mammalian jaw muscles (Kang et al., 1994; Sciote & Rowlerson, 1998). It is reported to react with 2M fibers in macaques (Stedman et al., 2004) and baboons (Wall et al., 2013), both closely related to *C. atys*, but not in several other nonhuman primate species (Kang et al., 1994). Here we refer to MyHC expressing a positive reaction to MY-32 as MyHC-2 and MyHC expressing a positive reaction to 2F4 as MyHC-M. IHC sample preparation was performed by the Research Immunohistology Lab, Department of Pathology, Duke University School of Medicine.

The muscle sections were photographed and saved as digital images at 20× magnification using either a Zeiss axiocam digital camera attached to an Axioplan microscope and Axiovision 2.05 software or a Nikon DS-Fi3 high-definition camera attached to a Nikon 50i microscope and NIS Elements 4.5 software. Microscope fields in serial and contiguous sections were matched by identifying specific muscle cells and fascicles based on their size and shape and using fixed markers such as fascial planes, nerves and vessels. Fibers were counted and scored for intensity as strong, intermediate, weak, or unstained (Wall et al., 2013).[1]

3. Results

3.1. Proteomics confirmation of MyHC α-cardiac

The top identified protein in the heart sample was Myosin-6 (MyHC α-cardiac) with 170 unique peptides to match. The presence of MyHC α-cardiac (Myosin-6 in Fig. 1) and MyHC-1 (Myosin-7 in Fig. 1) in both heart atrium and superficial masseter, and the absence of fast isoforms of MyHC in heart atrium (Myosin 2 in Fig. 1), together confirm the expression of MyHC α-cardiac in *C. atys* superficial masseter.

3.2. Immunohistochemistry

The anterior superficial masseter and temporalis muscles of all eight

[1] Variation in staining intensity is well documented in paraffin-embedded, formalin-fixed tissue sections (Werner et al., 2000) and staining intensity may be reduced relative to quantitative estimates using gel electrophoresis (Wall et al., 2013). Weakly stained fibers were reliably distinguished from unstained fibers. Thus, we report counts for all staining intensities.