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Severe atrophy of slow myofibers in aging muscle is concealed by myosin heavy chain co-expression

Fennigje M. Purves-Smith [a], Nathan M. Solbak [b], Sharon L. Rowan [b], Russell T. Hepple [a,*]

[a] Muscle Aging Diagnostics Laboratory, Department of Medicine, McGill University, Montreal, QC, Canada
[b] Muscle and Aging Laboratory, Faculty of Kinesiology, University of Calgary, Calgary, AB, Canada

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ABSTRACT

Although slow myofibers are considered less susceptible to atrophy with aging, slow fiber atrophy may have been underestimated previously. First, the marked atrophy of the aging rat soleus (Sol) muscle cannot be explained by the atrophy of only the fast fibers, due to their low abundance. Second, the increase in small fibers co-expressing both fast and slow myosin heavy chains (MHC) in the aging rat Sol is proportional to a decline in pure MHC slow fibers (Snow et al., 2005), suggesting that these MHC co-expressing fibers represent formerly pure slow fibers. Thus, we examined the size and proportion of MHC slow, MHC fast, and MHC fast-slow co-expressing fibers in the Sol and mixed region of the gastrocnemius (Gas) muscle in young adult (YA) and senescent (SEN) rats. Our results suggest that formerly pure MHC slow fibers are the source of MHC co-expressing fibers with aging in both muscle regions. Accounting for the atrophy of these fibers in calculating MHC slow fiber atrophy with aging revealed that MHC slow fibers atrophy on average by 40% in the Sol and by 38% in the mixed Gas, values which are similar to the 60% and 31% atrophy of pure MHC fast fibers in the Sol and mixed Gas, respectively. Probing for the atrophy-dependent ubiquitin ligase, MAFbx (atrogin 1), it was suggested that former slow fibers acquire atrophy potential via the up-regulation of MAFbx coincident with the co-expression of fast MHC. These results redefine the impact of aging on slow fiber atrophy, and emphasize the necessity of addressing the atrophy of fast and slow fibers in seeking treatments for aging muscle atrophy.

1. Introduction

Sarcopenia is the age-related loss of skeletal muscle mass and function (Hepple, 2003). It is characterized by atrophy and loss of individual muscle fibers (Lexell et al., 1988; Lushaj et al., 2008), as well as shifts in myosin heavy chain (MHC) expression (Andersen, 2003; Caccia et al., 1979; Monemi et al., 1999). One of the most widely accepted tenets of muscle aging is that there is preferential atrophy of fast twitch myofibers, with rather convincing evidence in the literature (Caccia et al., 1979; Lexell et al., 1988; Lushaj et al., 2008). As a result, the majority of intervention strategies for sarcopenia, from strength training (Rolland et al., 2011) to the use of anabolic pharmacological agents (Glass and Roubenoff, 2010), are based upon this belief. Despite the acceptance of this view, more recent evidence raises the possibility that the atrophy of slow twitch fibers with aging may have been dramatically under-estimated heretofore.

Specifically, the rat soleus muscle (Sol; predominantly slow) exhibits a strikingly similar rate of atrophy between late middle age (28–30 months) and senescence (SEN; 35–36 months) as the gastrocnemius muscle (Gas; predominantly fast) (Rowan et al., 2011). The substantial atrophy observed in the SEN Sol muscle cannot be explained by the atrophy of only fast twitch fibers, due to their very small proportion in this muscle. In addition, serial sections immunolabeled for fast and slow MHC show the shift toward an increase in fast MHC expression in the aging rat Sol muscle (Carter et al., 2010) is due to a marked increase in fibers that co-express both fast and slow MHC isoforms, and a proportional decline in fibers expressing slow MHC only (Snow et al., 2005). Although the presence of MHC fast-slow co-expressing fibers in aging muscle has been reported previously in several studies (Andersen et al., 1999; Ansved and Larsson, 1989; Edstrom and Larsson, 1987; Rowan et al., 2011; Snow et al., 2005), no prior study has accounted for the possibility that a fraction of these co-expressing fibers are derived from formerly pure slow fibers when ascribing the degree of atrophy in slow versus fast myofibers with aging.

To this end, we examined the size and proportion of fibers expressing only slow MHC, only fast MHC, and both slow and fast MHC (co-expressing fibers) by immunofluorescence in two muscle types; the Sol muscle and the Gas muscle of YA and SEN rats. We

Abbreviations: MHC, myosin heavy chain; Gas, gastrocnemius muscle; Sol, soleus muscle; SEN, senescence; YA, young adult; MAFbx, muscle atrophy F-box.

* Corresponding author at: Critical Care Division, Royal Victoria Hospital, 687 Pine Ave W, Room L3.01, Montreal, Qc, Canada H3A 1A1. Tel.: +1 514 398 4184.
E-mail address: russell.hepple@mcgill.ca (R.T. Hepple).

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hypothesized that accounting for the origins of co-expressing fibers in aging muscle would reveal a greater severity of slow fiber atrophy with aging than has been previously indicated.

2. Methods

2.1. Ethics

All experimental procedures on animals were made with prior approval of the University of Calgary Animal Care Committee, protocol ID BI09R-11.

2.2. Experimental animals and tissue collection

5 young adult (YA; 8–10 months of age) and 6 senescent (SEN; 36 months of age) male Fisher 344×Brown Norway F1 (F344BN) rats were obtained from the colony maintained by the National Institute on Aging (NIA; Baltimore, MD). They were housed in the Biological Sciences vivarium at the University of Calgary in single cages (12/12 h light/dark cycle, 21 °C) and provided food and water ad libitum. On tissue harvest day, animals were anesthetized with sodium pentobarbital (55–65 mg kg^{-1}) and the hind limb muscles were removed. The muscles were dissected free of fat, weighed and an approximately 8 mm thick slice through the entire midbelly was mounted on cork in an optimal cutting temperature compound. The muscle tissue was then frozen in liquid isopentane (cooled in liquid nitrogen) and stored at −80 °C until sectioning. For the purposes of this study, 10-μm thick cross-sections of the soleus muscle (Sol) and gastrocnemius muscle (Gas) were cut using a crytostat (−18 °C) and were mounted on lysine-coated slides (Superfrost). They were air dried for one hour and then stored at −80 °C until used for immunolabeling.

2.3. Immunolabeling

Serial sections of Sol and Gas muscles from each age group were immunolabeled for MHC slow and MHC fast isoform expression, in keeping with the following procedures:

After reaching room temperature, stored tissue slides (frozen at −80 °C) were rehydrated in phosphate buffered saline (PBS; pH 7.4—5 min) and incubated in permeabilization solution (0.1% Triton X-100 in PBS—15 min). Next, slides were washed in PBS (3×5 min) and incubated in blocking solution (10% goat serum, 1% bovine serum albumin in PBS—30 min), and then in primary antibody solution (mouse anti-MHCs or mouse anti-MHCf, Novocastra 1:10 dilution; mouse anti-dystrophin, Sigma 1:200 dilution in blocking solution—overnight at 4 °C). Slides were again washed in PBS (3×5 min) and incubated in blocking solution (30 min), followed by a 60 min incubation at room temperature with a secondary antibody solution (goat anti-mouse AlexaFluor 633, Invitrogen 1:200 dilution in blocking solution). Finally, slides were washed in PBS (3×5 min), rinsed in distilled water, and then cover-slipped with Prolong Gold (Invitrogen) and stored at 4 °C until they were imaged the following day. For the Sol muscle only, one serial section per muscle was first fixed in 4% paraformaldehyde (in PBS) after being brought to room temperature and then labeled for slow MHC, dystrophin and rabbit anti-Muscle Atrophy F-box (MAFbx; ECM Biosciences 1:100 dilution) according to the above labeling protocol, using a secondary antibody cocktail containing goat anti-mouse AlexaFluor 633 (Invitrogen 1:200 dilution) and goat anti-rabbit AlexaFluor 546 (Invitrogen 1:200 dilution). Slides were stored at 4 °C until they were imaged the following day. Note that within a given tissue cross-section, the MHC (slow or fast) and dystrophin primary antibodies were labeled with the same secondary antibody such that both the MHC (slow or fast) and dystrophin were excited at the same frequency during fluorescence imaging.

2.4. Imaging

Using an Olympus Fluoview confocal microscope, two to four serial images were obtained from each of the soleus muscle (Sol) and the mixed region of the gastrocnemius muscle (mixed Gas) at both 100× and 200× magnification. The mixed Gas region is composed of primarily MHC fast fibers and a relatively small abundance of MHC slow expressing fibers (Armstrong and Phelps, 1984). It is located between the deep red region of the Gas muscle (which has many MHC slow expressing fibers) and the white region of the Gas muscle (which is composed of only MHC fast expressing fibers). The mixed Gas region was determined by working from the deep red portion of the Gas muscle to the border of the white region of the muscle. Two images were then taken along this border. All images were then analyzed offline using Image-J software (NIH, Bethesda, MD, USA), using an internal reference frame within the image to prevent biasing fiber size (Hepple and Mathieu-Costello, 2001). For each image we determined the individual fiber cross sectional area and MHC labeling status. Fibers that were negative for MHC slow in the MHC slow labeled sections and positive for MHC fast in the MHC fast labeled sections were classified as pure MHC fast fibers; those negative for MHC fast in the MHC fast labeled section and positive for slow in the MHC slow labeled sections were classified as pure MHC slow fibers, and all others were classified as MHC fast-slow co-expressing fibers. A minimum of 450 fibers per muscle per animal was sampled for fiber type proportion analysis. A minimum of 100 fibers per muscle per animal was sampled for fiber size analysis.

2.5. Statistics

Comparisons between YA and SEN whole muscle mass and fiber type proportions were made using student t-tests or Mann–Whitney rank sum tests when there were conditions of unequal variance. Comparisons within and between YA and SEN groups for fiber type CSA were made using two-way ANOVAs for age, fiber type and CSA, followed by a Holm–Sidak post-hoc test. For all tests, $\alpha = 0.05$. All values are expressed as means ± standard deviation (SD).

3. Results

3.1. Muscle mass

In the soleus muscle, there was 15% lower muscle mass in SEN versus YA samples (YA = 155 ± 6 mg, SEN = 132 ± 6 mg, $p<0.05$). In the gastrocnemius muscle, there was 35% lower muscle mass in the SEN versus YA samples (YA = 2004 ± 78 mg, SEN = 1309 ± 50 mg, $p<0.05$).

3.2. Fiber type expression and size

Note that the fiber size in YA animals documented here is similar to that seen in another cohort of YA animals, as we have reported in the supplemental data of a prior report (Rowan et al., 2011). Similarly, our current results on fiber type alterations in the aging Sol muscle are consistent with the prior results of Snow et al. (2005) in the same rodent model and the same ages as studied here. Specifically, our results show that in the Sol, whereas the abundance of pure MHC fast fibers is unchanged between YA (1.3 ± 0.2%) and SEN (2.9 ± 1.3%), there was a marked increase in MHC co-expressing fibers from 3.9 ± 0.9% in YA to 46.8 ± 5.3%, and this was accompanied by a proportional decline in pure MHC slow fibers between YA (94.7 ± 0.9%) and SEN (50.3 ± 4.9%) (Fig. 1A and B). Similar changes were observed in the mixed region of the Gas muscle where the decline in abundance of pure MHC slow fibers was accompanied by an increase in MHC fast-slow co-expressing fibers and no change in the abundance of pure MHC fast fibers (Fig. 1D and E).