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Myosin light chain 3f attenuates age-induced decline in contractile velocity in MHC type II single muscle fibers

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Summary

Aging is characterized by a progressive loss of muscle mass and impaired contractility (e.g., decline in force, velocity, and power). Although the slowing of contraction speed in aging muscle is well described, the underlying molecular mechanisms responsible for the decrement in speed are unknown. Myosin heavy chain (MHC) isoforms are the primary molecules determining contractile velocity; however, the contraction speed of single fibers within a given MHC isoform type is variable. Recent evidence proposes that the decline in shortening velocity (V_o) with aging is associated with a decrease in the relative content of essential myosin light chain 3f (MLC_{3f}) isoform. In the current study, we first evaluated the relative content of MLC_{3f} isoform and V_o in adult and old rats. We then used recombinant adenovirus (rAd) gene transfer technology to increase MLC_{3f} protein content in the MHC type II semimembranosus muscle (SM). We hypothesized that (i) aging would decrease the relative MLC_{3f} content and V_o in type II fibers, and (ii) increasing the MLC_{3f} content would restore the age-induced decline in V_o . We found that there was an age-related decrement in relative MLC_{3f} content and V_o in MHC type II fibers. Increasing MLC_{3f} content, as indicated by greater % MLC_{3f} and MLC_{3f}/MLC_{2f} ratio, provided significant protection against age-induced decline in V_o without influencing fiber diameter, force generation, MHC isoform distribution, or causing cellular damage. To the best of our knowledge, these are the first data to demonstrate positive effects of MLC_{3f} against slowing of contractile function in aged skeletal muscle.

Keywords

contraction velocity; MLC_{3f}; isometric muscle contraction; myofibrillar proteins; sarcopenia; gene delivery

Introduction

Aging is associated with a progressive loss of muscle mass and impairment of muscle contractile functions (i.e., strength, velocity, and power generation). The relevance of those phenomena with age is widely recognized as the life expectancy greatly increases. To date, the underlying mechanisms responsible for muscle weakness and slowing of contractile speed are not clearly identified. Single muscle fiber contractile studies and *in vitro* motility

experiments support the idea that the age-related decline in strength is, in part, because of modifications of myosin (Lowe *et al.*, 2001). Although several studies point to myosin as one of the key players, the reported age-induced slowing of velocity cannot be fully explained by myosin modification or myosin isoform expression. Myosin is composed of two myosin heavy chains (MHCs) and four myosin light chains (MLCs). In rodents, four different types (I, IIA, IIX, IIB) of MHC isoforms are identified. Each MHC consists of two types of MLCs: a pair of essential light chains (ELC) and a pair of regulatory light chains (RLC). In adult skeletal muscle, there are three isoforms of ELC (MLC_{1f}, MLC_{1s}, and MLC_{3f}) and two isoforms of RLC (MLC_{2f}, MLC_{2s}). MHC isoforms are primarily responsible for determining contractile velocity, whereas the ELC isoforms have a modulatory, or fine-tuning, role in the regulation of muscle speed. The contraction speed, as demonstrated by unloaded shortening velocity (V_o), in skeletal muscle increases three to ninefold in the order of MHC type I $\rightarrow$ IIA $\rightarrow$ IIX $\rightarrow$ IIB (Bottinelli *et al.*, 1991, 1994). However, the large variability of V_o within a given MHC fiber type supports the idea that other factors, in addition to MHC, contribute to V_o . Evidence suggests that the variability is, in part, related to the modulatory influence of the ELC isoforms (Sweeney *et al.*, 1988; Lowey *et al.*, 1993a,b; Bottinelli *et al.*, 1994). Using the *in vitro* motility assay (isolated proteins), the removal of the ELCs from myosin results in a 10-fold decrease in sliding velocity of actin filaments compared with native myosin (Lowey *et al.*, 1993a,b). Likewise, myosin containing only the MLC_{1f} isoform move actin at a significantly slower velocity than myosin containing the MLC_{3f} isoform. Using the permeabilized fiber preparation, MHC type II fibers with a relatively larger amount of MLC_{3f} shorten faster than fibers with a greater amount of MLC_{1f} and V_o is proportional to the relative content of MLC_{3f} (Sweeney *et al.*, 1988; Lowey *et al.*, 1993b; Bottinelli *et al.*, 1994). These results suggest that the relative content of the essential myosin light chains, MLC_{1f} and MLC_{3f}, is an important determinant in the regulation of V_o in MHC type II single fibers.

To date, the aging effect on the relative MLC_{1f} and MLC_{3f} isoform content in skeletal muscle fibers, mainly MHC type II fibers, is unknown. We predict that a decrease in relative MLC_{3f} (concomitant increase in MLC_{1f} content) content with age underlies the slowing of contraction in MHC type II fibers. Therefore, the primary purpose of this study was to use single, permeabilized fibers to determine whether aging decreases the relative MLC_{3f} content (increases the relative MLC_{1f} content) and V_o . The second goal was to restore the loss in V_o with age by increasing MLC_{3f} content via recombinant adenovirus (rAd) gene transfer technology in MHC type II fibers from the semimembranosus (SM) muscle (a muscle composed predominantly of MHC type II fibers). We hypothesize that (i) aging decreases the relative MLC_{3f} content (increases the relative MLC_{1f} content) and V_o in MHC type II SM fibers, and (ii) increasing the MLC_{3f} content by rAd-MLC_{3f} in the SM fibers would restore V_o of individual fibers. We further postulate that rAd-empty vector and MLC_{3f} injection in SM muscle will not result in cellular damage, protein damage, or changes in single fiber diameter and force generation in aged rats.

Results

Adenoviral vector transduction in SM muscle

We investigated the fluorescent properties of the SM muscles between nontreated controls and treated (rAd injection) experimental groups. As shown in Fig. 1, there was a clear difference in adenovirus DNA delivery between nontreated muscle, which was nonfluorescent (Fig. 1A,B) and treated muscle with red fluorescence because both empty vector (rAd-empty) and MLC_{3f} vector (rAd-MLC_{3f}) carry DsRed gene (Fig. 1C,D). Both empty vector and MLC_{3f} vector injections showed stripe and speckle fluorescent patterns. We also noticed that the fluorescence covered 20-30% of the total muscle area. The 20-30%

transduction levels of rAd in post-mitotic muscle may be partly due to inefficient binding of rAd particles to the cell surface (Nalbantoglu *et al.*, 2001).

No changes in fiber and protein damage with rAd injection

To determine whether the adenoviral vector injection causes muscle damage and subsequent repair, macrophage markers ED1⁺ and ED2⁺ were evaluated by immunohistochemistry. We observed an increase in % area of ED1⁺ with age and no further increase in ED1⁺ following injection with the vectors (Fig. 2A). In contrast to ED1⁺, the percentage of nonphagocytic ED2⁺ macrophages was greater with both rAd-empty vector and rAd-MLC_{3f} injection compared to adult and old groups (Fig. 2B). To determine whether the adenovirus injection causes an increase in protein damage, we determined protein carbonylation in both the cytosolic (Fig. 2C) and the myofibrillar fractions (Fig. 2D). Overall, the adenovirus injection did not appear to induce an increase in protein damage.

No changes in MHC type II fiber diameter, force, and composition with rAd injection

A total of 500 single fibers were evaluated for diameter and force generation. We observed a significant decrease in the diameter of MHC type II single fibers with age (Fig. 3A; $P = 0.015$). More importantly, the treatment of either rAd-empty vector or rAd-MLC_{3f} into the muscles of aged rats did not affect the diameter of the single fibers. Single fibers in the OC group showed lower absolute force (P_o) than those fibers from the AC group (Fig. 3B; $P < 0.001$). Specific force (P_o/CSA) of single fibers from the OC rats was significantly lower than fibers from the AC rats (Fig. 3C; $P = 0.015$). However, there was no difference in P_o and P_o/CSA between the OC and rAd-treated groups. These data provide evidence to support that contractile parameters, fiber size, P_o , and P_o/CSA are not affected following rAd injection.

We evaluated the MHC isoform composition of 587 single fibers. The percentage of type IIB fibers was significantly higher in the AC group compared with the OC group (Fig. 3D; $P < 0.001$). The expression of MHC type IIX/IIB fibers was significantly greater in the OC group compared with the AC group ($P = 0.03$). The proportion of MHC type IIX fibers was not different between the AC and OC groups. Noticeably, the rAd injection into the SM muscle did not affect the MHC isoform composition of type II fibers.

Single fiber MLC isoform identification

To identify the essential and regulatory myosin light chain proteins in our one-dimensional silver-stain gels, we used Mass spectrometry (MS) technology. As listed in Fig. 4, MS identified the 21 and 19 kD bands (Spots 1 and 2) as the fast myosin alkali light chain_{1f} and the phosphorylatable fast myosin light chain with 46% and 41% sequence coverage, respectively. These results indicate that the 21 and 19 kD bands in our silver-stain gels are the MLC_{1f} and MLC_{2f} proteins. The 17 kD band/Spot 3 was matched with a fast myosin alkali light chain_{1f} with 19% sequence coverage. Evidence suggests that MLC_{1f} (190aa) and MLC_{3f} (149aa) have complete sequence homology for the last 141aa at the C-terminus and are different in length and sequence (MLC_{1f} = 49aa, MLC_{3f} = 8aa) in N-terminus (Periasamy *et al.*, 1984). Therefore, the extension in amino termini of MLC_{1f} results in a greater molecular mass and, hence, lowers electrophoretic mobility (21 kD). The results of other papers identify the MLC_{3f} protein band almost at the bottom on the 12% SDS-PAGE silver-stain gels (below MLC_{2f} protein band), which also support the idea that 17 kD band is the MLC_{3f} protein (Bottinelli *et al.*, 1994; Larsson *et al.*, 1997).

The rAd-MLC_{3f} attenuates age-induced decline in MLC_{3f} content and Vo in MHC type II fibers

To determine whether there are age and adenovirus-associated differences in MLC isoform compositions, we examined the relative contents of fast MLCs in each MHC type II single fiber with/without physiology testing. There was a shift in fast MLC isoforms of type II fibers with age. The relative content of MLC_{1f} was significantly greater in the OC group than in the AC group (Fig. 5A; $P < 0.001$). In contrast, the relative content of MLC_{2f} (Fig. 5B; $P < 0.001$) and MLC_{3f} (Fig. 5C; $P < 0.001$) was significantly greater in the AC group than in the OC group. We also analyzed the ratio of MLC_{3f}/MLC_{2f} as an indicator for the fraction of MLC_{3f} per myosin head because the amount of regulatory MLC isoforms (MLC_{2f} or MLC_{2f} + MLC_{2s}) reflects the content or amount of myosin present (Lowey & Risby, 1971). The ratio of MLC_{3f}/MLC_{2f} was lower in the OC group compared to the AC group (Fig. 5D; $P < 0.001$). Noticeably, rAd-MLC_{3f} injection significantly increased the % MLC_{3f} content to 8% and the MLC_{3f}/MLC_{2f} ratio to 0.20 in type II fibers.

The MHC type II single fibers had a significant decrement in Vo with age by 30% (Fig. 5E; $P < 0.001$). Importantly, the Vo of the rAd-MLC_{3f}-treated group was significantly faster than that of the OC group ($P = 0.013$) and no different than the individual fibers from the AC group. We also found that the rAd-MLC_{3f} injection showed a faster Vo compared to the empty vector treated counterpart ($P < 0.001$). These results are consistent with the hypothesis that rAd-MLC_{3f} injection may provide significant protection against age-induced decline in contraction speed in type II fibers.

The rAd-MLC_{3f} rescues age-associated decline in Vo in MHC type IIB and type IIX/IIB fibers

To investigate whether age and adenovirus injection affect Vo of single fibers within a specific combination of MHC isoform expression, we separated the MHC type II fibers based on MHC isoforms. Table 1 illustrates the change of Vo depending on the presence of the different MHC isoforms within the single fibers. The fibers with the same MHC isoform content from the AC group showed a significantly higher Vo than those from the OC group (3.3 ± 0.2 vs. 2.5 ± 0.2 FL s⁻¹, $P = 0.02$ in MHCIIB and 3.2 ± 0.5 vs. 2.3 ± 0.2 FL s⁻¹, $P = 0.04$ in MHCIIX/IIB). The Vo in MHCIIB and MHCIIB/IIX fibers was not different between the OC and OE groups. Noticeably, the Vo of MHCIIB fibers in the OM group was significantly higher than in the OC and OE groups. However, the Vo in MHCIIX/IIB fibers from the OM was not different than OC and OE groups.

The MLC isoform composition of the MHCIIB fibers in the AC group was 29.4% MLC_{1f}, 56.6% MLC_{2f}, and 14.1% MLC_{3f}, which was different than the OC group (51.6% MLC_{1f}, 44.5% MLC_{2f}, and 3.9% MLC_{3f}). Similarly, MHCIIX/IIB fibers showed a higher amount of MLC_{1f} and a lower amount of MLC_{2f} and MLC_{3f} with age. The MLC_{3f}/MLC_{2f} ratio overlapped considerably among the different fiber types, but the AC group showed a higher MLC_{3f}/MLC_{2f} ratio in MHCIIB and MHCIIX/IIB fibers compared with the OC group. There was a significant difference between the OC and OM groups in MLC_{3f}/MLC_{2f} ratio of MHCIIB fibers, but not between the OE and OM groups, whereas the MLC_{3f}/MLC_{2f} ratio in MHCIIX/IIB fibers was significantly higher in the OM group compared with the OC and OE groups.

Figure 6 illustrates the relationship between MLC_{3f}/MLC_{2f} ratio and Vo. We found that there was a significant correlation between MLC_{3f}/MLC_{2f} ratio and Vo in all type II fibers ($R^2 = 0.22$, $P < 0.001$) (Fig. 6A). We also found that there was a positive relationship between these two variables in each MHC isoform (Fig. 6B–D). Combined with MHC data, these results provide strong evidence that the relative contents of MLC_{3f} affect shortening velocity involved in age-induced dysfunction independent of fiber size and force generation.

Discussion

The primary purposes of this study were to examine (i) whether aging would decrease the relative MLC_{3f} content (increase the relative MLC_{1f} content) and Vo, and (ii) whether increasing MLC_{3f} content via rAd-MLC_{3f} is able to restore the age-related loss in Vo in MHC type II fibers. There were three major findings in the current study. First, the rAd injection procedure into the SM muscle did not appear to increase cellular inflammation or protein damage. Single fiber diameter and force generation capacity were not altered with the rAd injection. Second, there was a significant age-related decline in diameter, Po, Po/CSA, Vo, and shift in MHC and MLC isoforms in single fibers. More importantly, a decrease in relative MLC_{3f} content (concomitant increase in relative MLC_{1f} content) was highly associated with the age-induced decline in Vo of MHC type II fibers. Third and finally, we demonstrated that rAd-MLC_{3f} injection in SM muscle significantly increased the relative content of MLC_{3f} (i.e., % MLC_{3f} and MLC_{3f}/MLC_{2f} ratio) and attenuated the age-associated decrease in Vo in MHC type II fibers. To the best of our knowledge, these are the first data to indicate that increased MLC_{3f} content via rAd-MLC_{3f} gene transfer has positive effects against age-associated slowing of muscle contraction without changing fiber size, force, damage, and MHC isoforms in permeabilized single muscle fibers. Furthermore, our data offer evidence that gene delivery of MLC_{3f} would be beneficial for muscle aging by providing a selective advantage for enhancing contractile function.

Muscle damage and function with rAd

We used rAd DNA gene transfer technology to overexpress MLC_{3f} in the SM muscle of aged rats because rAd-mediated gene transfer is being widely investigated as a therapeutic vector for a host of muscle-related dysfunction. Although rAd-mediated gene transfer is a suggested therapy, efficient delivery of the specific gene to the target tissue without causing damage and pathogenic or immunogenic responses is a major issue. Macrophages, ED1⁺ and ED2⁺, play an important role in the process of inflammation (St Pierre & Tidball, 1994; McLennan, 1996). ED1⁺ macrophages, which are initially derived from blood monocytes, are the first subpopulation to respond when damage occurs. ED1⁺ macrophages remove damaged cells via phagocytosis. ED2⁺ macrophages contribute to satellite cell activation and myogenic proliferation required for repair (Tidball *et al.*, 1999). The adenovirus injection does not appear to induce muscle damage because there was no significant difference in the percentage of ED1⁺ cells between adenovirus injected groups and age-matched control groups. Interestingly, the degree of ED2⁺ macrophages without infiltration was higher in the adenovirus-treated groups than in the age-matched control groups. Although it is not possible to identify the mechanism underlying the increase in ED2⁺ macrophages, one possibility is the injection by itself induces muscle regeneration because both the empty vector and MLC_{3f} DNA injections increase ED2⁺. On the other hand, it is possible the adenovirus itself might increase ED2⁺ macrophages through the activation of myogenic regulatory factors such as MyoD, Myf-5, and myogenin (Tsivitsse *et al.*, 2003).

To confirm minimal cellular inflammation/damage following injection, we evaluated overall protein damage with the universal marker, protein carbonylation. Consistent with our ED1⁺ data, rAd injection into the SM muscle did not affect cytosolic and myofibrillar protein carbonylation. Lastly, the adenovirus injection into the SM muscle did not alter individual fiber function. While there was the predicted age-related decline in Po and Po/CSA and changes in MHC distribution, neither rAd-empty vector nor rAd-MLC_{3f} injection caused further reductions in those contractile parameters or alterations in MHC distribution. Most important to our study design is the fact that the rAd-empty vector injection did not change Vo, MHC distribution, or MLC isoform composition compared with age-matched control.

Taken together, the findings described previously support that 7 days postinjection of the rAd does not significantly influence contractile function nor cause cellular damage or protein damage in the SM muscle. These findings are consistent with several studies, demonstrating no detrimental effect of rAd injections into skeletal muscle (Kimura *et al.*, 2001; Lefesvre *et al.*, 2002).

Vo and MLC isoforms with age

An age-induced decline in Vo is documented in humans (Larsson *et al.*, 1997; D'Antona *et al.*, 2003) and rodents (Li & Larsson, 1996; Degens *et al.*, 1998; Thompson & Brown, 1999) using permeabilized single muscle fibers. For example, in elderly humans, MHC type I and MHC type IIA fibers have slower speeds of shortening compared to those fibers from young subjects. Meanwhile, in aged rats, the Vo of MHC type I fibers from the soleus (Li & Larsson, 1996; Degens *et al.*, 1998) and MHC type IIB fibers from the SM decreased by 20–50% relative to those fibers from younger rats.

In the present study, single fibers expressing MHC type II isoforms show a significant age-related slowing of contraction speed consistent with our previous report. Notably, the age-induced decline in contraction speed persists when we compare single fibers containing the same MHC type II isoform. The persistent reduction in contraction speed, within single fibers with the same MHC isoform, suggests the involvement of proteins that regulate contraction speed. The relative content of the MLC isoforms may be one of the molecular mechanisms responsible for the age-related slower velocities in single fibers with the same MHC isoform because it is well documented that a decrease in the relative content of essential MLC_{3f} isoform (parallel with an increase in relative content of essential MLC_{1f} isoform) is associated with slower velocities (Sweeney *et al.*, 1988; Bottinelli *et al.*, 1994; Zhong & Thompson, 2007). Currently, investigations focused on the role of the essential MLCs as the major determinant for age-related slowing of contraction are few. In two rodent studies, the results report a relationship between the relative MLC_{3f} content in MHC type IIB fibers and Vo with age. Our results in the current study are consistent with these studies and document a significant decline in MLC_{3f} isoform content and a concomitant increase in MLC_{1f} isoform content with age.

Vo and MLC isoforms with rAd-MLC_{3f}

The primary goal of the current study was to rescue the age-induced slowing of muscle contraction by increasing the relative content of MLC_{3f} in the SM muscles of old rats. Foremost, the rAd-MLC_{3f} gene transfer into the SM muscle does not alter the MHC isoform distribution; however, increasing MLC_{3f} relative content via rAd-MLC_{3f} injection (i.e., % MLC_{3f} and MLC_{3f}/MLC_{2f} ratio) attenuates the age-related decline in Vo in MHC type II single muscle fibers. Specifically, there is an overall increase in relative MLC_{3f} protein content in MHC type II fibers by 7 days after rAd-MLC_{3f} injection. Moreover, increased % MLC_{3f} content to 7.7% from 4.2–4.6% and MLC_{3f}/MLC_{2f} ratio to 0.19 from 0.10 in MHC type II fibers via rAd gene transfer to SM muscle significantly offset age-induced decline in Vo by 24–61%.

It is also interesting to note that rAd-MLC_{3f} gene transfer improves Vo within the given MHC type II fibers differentially. The observed difference in the slope of the regression line between the ratio of MLC_{3f} to MLC_{2f} and Vo suggests that the influence of MLC_{3f} on Vo is more prominent in MHC type IIB fibers than in MHC type IIX/IIB and MHC type IIX fibers. The strong influence of MLC_{3f} on contraction speed in single fibers with MHC type IIB isoform has been demonstrated before (Bottinelli *et al.*, 1994). As noted previously, the percent increase in relative MLC_{3f} content does not have to be large to have an influence on contraction speed. The rAd-MLC_{3f} gene transfer in aging muscle, which results in increased

% MLC_{3f} content to 8.6% from 3.9–6.0% and MLC_{3f}/MLC_{2f} ratio to 0.21 from 0.09, enhances Vo by 44–80% in MHC type IIB fibers.

The mechanisms underlying age-related decline in Vo with regard to MLC regulation are not known. However, the modulatory role of MLCs in Vo with age may be associated with the difference in sequence of MLC isoforms. Evidence suggests that the 45 amino acid N-terminal extension of MLC_{1f} compared with MLC_{3f} which has relatively shorter amino acid sequence can cause unfavorable interactions with actin's negatively charged C-terminus. The interactions between MLC_{1f} and actin are linked with slower speed of filament sliding on the assumption that they increase the lifetime of the attached state of the myosin heads and delay their dissociation during a cross-bridge cycle (Lowey *et al.*, 2007). The results of the present study are congruent with this theory whereby there was an age-related decline in Vo together with an increase in relative MLC_{1f} content (concomitant decrease in MLC_{3f} content). Moreover, we found that an increase in MLC_{3f} content by rAd gene transfer could ameliorate the age-induced decline in Vo of MHC type II single muscle fibers. The positive relationships between relative MLC_{3f} content and Vo in MHC type II fibers suggest that MLC_{3f} may have therapeutic and clinical value in preserving contractile speed in aging skeletal muscle.

One caveat requiring a notation is the species-dependent variation in MHC gene expression. This species-to-species variation in MHC isoform expression is, perhaps, most notable for MHCIIB, which is highly expressed in rodent muscle, yet effectively not expressed in healthy human skeletal muscle. In contrast to healthy muscle, expression of MHCIIB in human skeletal muscles appears to increase with muscle fiber degeneration/regeneration (Harrison *et al.*, 2011). Because there is evidence of increased degeneration/regeneration in aging muscle and MLC_{3f} is found in both fast and slow human skeletal muscle fibers, it is possible to speculate that the results of the current study in rats have potential to translate into humans (Canepari *et al.*, 2000). However, a more extensive analysis of human skeletal muscle may help to determine whether, in fact, MLC_{3f} has any functional consequences.

In summary, contractile velocity in SM single-MHC type II fibers was slowed with age and the decline of Vo was significant in both MHC type IIB and MHC type IIX/IIB fibers. Our data also indicate that rAd-MLC_{3f} transduction in SM muscle did not increase cellular inflammation nor contractile protein damage. Importantly, this is the first study to demonstrate that the increased relative MLC_{3f} content (i.e., % MLC_{3f} and MLC_{3f}/MLC_{2f} ratio) via rAd-MLC_{3f} increases contraction speed of individual fibers independent of the age-related shift in MHC isoforms. Our data offer evidence that gene delivery of MLC_{3f} would be beneficial for muscle aging by providing a selective advantage for enhancing contractile function.

Experimental procedures

Animals

Adult (10–12 months) and old (24–26 months) male F344 rats were purchased from the NIA colony (Harlan). The experiments were approved by the IACUC (U of Minnesota) and were in accordance with the NIH and APS standards. After acclimatization for 1 week, all rats were randomly assigned to one of the following experimental groups: adult control (AC; *n* = 8), old control (OC; *n* = 8), old empty vector treated (OE; *n* = 14), and old MLC_{3f} vector treated (OM; *n* = 14).

rAd construction and injection in SM muscle

To construct a rAd vector for rat MLC_{3f} gene, total RNA was isolated from the SM muscle of adult rats using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was

synthesized using the Transcriptor first strand cDNA synthesis kit (Roche Applied Science, Indianapolis, IN, USA). The amplified MLC_{3f} cDNA (forward primer, 5'-TCT CCA GTC CCG CTG CTG TTT TGC-3'; reverse primer, 5'-ATT TGT GGG ATT GGT GCC CAG AGC-3') by RT-PCR with Pfx Polymerase (Invitrogen) was first cloned into the pCRII-TOPO vector by TOPO TA cloning (Invitrogen). Plasmid DNAs were purified using QIAprep Miniprep kit (QIAGEN, Valencia, CA, USA) and verified by DNA sequence analysis (BioMedical Genomics Center, University of Minnesota). MLC_{3f} cDNA was excised from pCRII-MLC_{3f} with BamHI and XhoI and cloned into BamHI/SalI site of pDNR-CMV. To confirm MLC_{3f} expression, the expression vector for MLC_{3f} was transfected into primary myoblasts with Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. The expression of MLC_{3f} was verified by anti-MLC_{1f/3f} antibody (F310, Developmental Study Hybridoma Bank, Iowa). To create pLP-Adeno-X-MLC_{3f}-DsRed (rAd-MLC_{3f}) and pLP-Adeno-X-DsRed (rAd-empty) adenoviral vector with the Adeno-X™ ViraTrak™ Expression System 2, we followed the manufacturer's protocol (Clontech, Mountain View, CA, USA). Titration of the adenoviral vectors was determined by the methods described in the manufacturer's protocol (Clontech). Briefly, HEK 293 cells were infected with adenoviral vectors. By 48 h after infection, DsRed positive cells were counted under the fluorescent microscope. The ifu (infectious unit) was calculated for the resulting average number of DsRed positive cells/unit dilution. Adenoviral vector solution (250 µL of 1×10^{11} to 1×10^{12} ifu mL⁻¹) was carefully injected into five areas of the SM muscle *in vivo* via a Kendall syringe equipped with a 28-gauge needle because the SM muscle is very large, 0.25 × 1.5 inches. The SM of the right hindlimb was injected with rAd-MLC_{3f} vector solution (OM), and the SM of the left hindlimb was injected with a same volume of rAd-empty vector solution (OE). Following injection, the rats were allowed to recover from anesthesia and monitored to ensure adequate recovery (i.e., eating, drinking, and cage behavior).

Tissue preparations and determination of protein concentration

After 1 week of adenovirus injection, rats were anesthetized with sodium pentobarbital (50 mg mL⁻¹ i.p.). The SM muscles were rapidly removed and trimmed clean of visible fat and connective tissue. Because the adenovirus vectors were not fully infected in the SM, we identified the fluorescent regions and isolated these regions using fluorescence microscopy. Protein concentration, required for protein carbonylation quantification, was determined using the bicinchoninic acid assay kit (Thermo Scientific, Rockford, IL, USA), with bovine serum albumin as the standard. Cytosolic and myofibrillar fractions were isolated (McDonough *et al.*, 2002; Thompson *et al.*, 2006). The cytosolic and myofibrillar protein content was measured in triplicate and expressed as µg of cytosolic or myofibrillar protein per mg wet weight.

Determination of muscle damage and repair

To determine whether the rAd injection caused muscle damage and repair, we used immunohistochemistry to quantify changes in ED1⁺ macrophages and ED2⁺ macrophages, two markers of muscle damage and repair, respectively. Serial cross-sections from one of the isolated fluorescent bundles were cut (-20 °C) and dried (30'). Sections were fixed in ice-cold acetone (4 °C, 10'), air-dried (30'), and then rinsed with PBS. After quenching the tissues in 0.3% hydrogen peroxide (30'), the sections were blocked (10% horse serum in Tris-Tween 20 buffer for 30'). The desired monoclonal primary antibody (ED1⁺ 1:200 and ED2⁺ 1:200, AbD Serotec) was then applied in blocking buffer and placed on the section for 60' at room temperature. After rinsing in PBS, biotinylated secondary antibody (Horse anti-mouse IgG, PK-6102, Vector) was applied (1:200) for 30'. The sections were amplified with Vectastain ABC reagent for 30' and then incubated in DAB development reagent (10'; Vector, SK-4100). Images were captured on a Nikon Eclipse E400 microscope (20×). NIH

image J program was used for quantification (<http://rsb.info.nih.gov/ij/>). After opening the image, the image was separated into the red/green/blue (RGB) windows. Critical components of the analysis include: an automatic threshold function [(average background + average object)/2] and the fractional area of positive stain for ED1⁺ and ED2⁺. Additionally, a negative control was used to evaluate nonspecific background staining.

Protein carbonylation

To determine whether protein damage occurred following the adenovirus injection, we measured levels of carbonylation using Oxyblot™ detection kits (Millipore, MA) according to the manufacturer's instructions with slight modifications. Briefly, cytosolic and myofibrillar fractions (110 ng) were denatured with 12% SDS and derivatized with DNPH. Samples were incubated at room temperature (15') with agitation and treated with neutralization solution. Then, the samples were loaded on a slot-blot apparatus and transferred to a PVDF membrane (12 h). Membranes were blocked in 5% FISH (20 mM Tris, 0.5 M NaCl, 0.1% Tween 20, 5% Fish gelatin) for 1 h and incubated with a 1:150 dilution of anti-DNP polyclonal rabbit antibody for 12 h. Following washing with 1% FISH buffer, membranes were incubated with a HRP-conjugated anti-rabbit IgG secondary antibody diluted in 1:300 ratios (1 h). Each sample was evaluated in triplicate and the intensity of chemiluminescence was quantified by arbitrary optical density. Protein carbonylation was expressed as percentage of the AC group.

Permeabilized single fiber preparations

After dissection, the SM bundles were quickly immersed in ice-cold relaxing solution (pH = 7.0, 200 mM imidazole, 100 mM EGTA, 1 M KCl, 100 mM ATP, 100 mM creatine phosphate, 100 mM CaCl₂, 1 M MgCl₂, pCa = 9). To maintain optimal length, each end of the bundles was tied with surgical silk (4-0) to glass capillary tubes and stored in skinning solution (pH = 7.0, 50% glycerol: 50% relaxing solution composed of 1 M propionate, 200 mM imidazole, 100 mM EGTA, 1 M MgCl₂, 4.4 mM ATP) up to 4–5 weeks (–20 °C).

Single muscle fiber physiology experiments

To determine whether single fiber contractile function, including force and velocity, is influenced by aging and adenovirus injection, we performed single muscle fiber physiology experiments. A fiber segment (1–2 mm) was isolated from a bundle and transferred to an experimental bath filled with relaxing solution (Permeabilized Fiber Apparatus Model 802B; Aurora Scientific, Aurora, Ontario, CAN). The fiber was securely mounted to a force transducer and a high speed length controller by two aluminum T-clips carefully folded over each end of the fiber. Sarcomere length was set to 2.4 μm, and the fiber length (Lo) was measured. The fiber diameter was determined from three places along the length of the fiber. The force transducer and length controller signals were analyzed by custom software program (ASI Model 600A, ver 2.0, Aurora Scientific, Aurora, Ontario, CAN).

Isometric force and shortening velocity

To determine maximal isometric force or Vo, the fiber was transferred into activating solution (pCa 4.5; 200 mM imidazole, 100 mM EGTA, 1 M KCl, 100 mM ATP, 100 mM creatine phosphate, 100 mM CaCl₂, 1 M MgCl₂). Isometric maximal force (Po) was determined in μN. Specific isometric force (Po/CSA) was calculated in kN m^{–2}. To determine maximal unloaded shortening velocity (Vo), the slack-test technique was utilized (Zhong & Thompson, 2007). Briefly, once the fiber reached Po, the fiber was instantly shortened (slacked) by a specific % of Lo (10%-20%) at which time the force output was zero. The time interval between the slack and when the individual fiber starts to generate force was measured. The fiber was then transferred back into relaxing solution. This procedure was

repeated at five to six different length steps. The linear regression line between length steps and time interval was calculated ($r^2 = 0.98$). V_o , represented by the slope of the line divided by L_o , was expressed as fiber length per second (FL s^{-1}). Throughout all single fiber contractility tests, the temperature of experimental solutions was set at 15°C .

MHC and MLC isoform determination of single muscle fibers

MHC and MLC isoform compositions of the single fibers were determined by SDS-PAGE (Zhong & Thompson, 2007). Ten μL of sample was loaded on an electrophoresis system with 4% stacking gel and either a 5% separating gel for MHC or 12% separating gel for the MLC. The gels were run at 250 V and at constant current (76 Amps) (24 h for MHC and 4 h for MLC). The gels were washed, silver-stained, and scanned using molecular multi-imaging system (GS-800; Bio-Rad, Hercules, CA, USA). To confirm reliability of our densitometry measurements, we performed protein concentration curves (12% SDS-PAGE) in duplicate followed by silver staining using the same myofibrillar SM samples. Briefly, the protein samples ranged from 0.03 to $4.0\text{ }\mu\text{g}$. The arbitrary density of 96 MLC_{1f} , MLC_{2f} , and MLC_{3f} protein bands was used as a measure of test-retest reliability ($R^2 > 0.99$).

MLC isoform identification using in-gel digestion and mass spectrometry

To identify the essential and regulatory myosin light chain proteins, we used powerful 'in-gel' digestion and MS techniques. Single fibers (10-15) were isolated and solubilized in 50 μL of sample buffer. One-dimensional SDS-PAGE was performed (Bio-Rad manufacturer's protocol). Then, the 17, 19, and 21 kD bands were excised from the gels, transferred into a microcentrifuge, and spun down. The enzymatic digestion protocol and MS were previously described (Shevchenko *et al.*, 2006). Briefly, gel pieces were dehydrated by acetonitrile (ACN) for 10'. The proteins were reduced with DTT (0.1 M) in ammonium bicarbonate (NH_4HCO_3 , 0.1 M) at 56°C for 30' followed by alkylation with iodoacetamide (0.055 M) in NH_4HCO_3 (0.1 M) for 20' at room temperature in the dark. Then, the gel pieces were dehydrated and shrunk with ACN for 10'. For the 'in-gel' digestion with trypsin, gel pieces were kept on ice-cold trypsin buffer containing $0.1\text{ }\mu\text{g }\mu\text{L}^{-1}$ trypsin, 0.1 M NH_4HCO_3 , and 1.9 M ACN for 30' and further incubated overnight at 37°C . Next, samples were desalted using the STAGE tip method (Rappsilber *et al.*, 2003). A LTQ-Orbitrap XL mass spectrometer (Thermo Scientific) was used for protein identification, and the data were analyzed with Scaffold 3 (http://www.proteomesoftware.com/Proteome_software_prod_Scaffold.html).

Statistical analysis

All data from SPSS software program (ver 18.0, IBM, Armonk, NY, USA) were expressed as mean $\pm$ SEM. A one-way ANOVA was used to evaluate statistical difference in diameter, P_o , P_o/CSA , V_o , MHC and MLC composition in MHC type II single fibers, damage, and protein contents among the four groups. A Tukey's HSD post hoc test was used to estimate the difference among means. Correlation between $\text{MLC}_{3f}/\text{MLC}_{2f}$ ratio and V_o in MHC type II fibers (MHCIIB, IIX/IIB, IIX) was examined by linear regression analysis. Because of the disparity in sample size of each MHC type II isoform, the nonparametric one-way ANOVA using Kruskal-Wallis test was applied to analyze the mean difference of V_o , MLC_{1f} , MLC_{2f} , MLC_{3f} , and $\text{MLC}_{3f}/\text{MLC}_{2f}$ ratio in each MHC isoform. The Mann-Whitney test was used to determine the existence of mean differences in each group.

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Fig. 1. Representative examples of recombinant adenovirus (rAd) transduction in SM muscle. The transduction in tissues was screened using a fluorescent microscope. (A) Nontreated adult control (AC), (B) nontreated old control (OC), (C) old rAd-empty vector (OE), and (D) old rAd-MLC_{3f}-treated (OM) groups. Bar = 1 mm.

Fig. 2.

Fiber damage and protein damage of SM muscle. (A) Average % area of phagocytic macrophages (ED1⁺) and (B) nonphagocytic macrophages (ED2⁺) per total area in SM muscle was determined using NIH image J program. Protein carbonylation, a marker of protein damage, in (C) cytosolic and (D) myofibrillar fractions was compared among the three experimental groups within the left and right hindlimbs. 'a' indicates significantly different than AC. '†' indicates significantly different than OM. Values represent mean $\pm$ SEM. Significance was set at $P < 0.05$.

Fig. 3. Fiber diameter, absolute force, specific force, and MHC isoform composition in type II single fibers. (A) Diameter, (B) absolute force (P_o), (C) specific force (P_o/CSA), and (D) MHC isoform distribution (%) of type II fibers were compared among experimental groups. 'a' indicates significantly different than AC. '†' indicates significantly different than OM. 'NS' indicates no significant difference. Values represent mean $\pm$ SEM. Significance was set at $P < 0.05$.

Fig. 4. Myosin light chain (MLC) isoform identification in SM muscle by Mass Spectrometry (MS). The specified protein’s accession number was used from the National Center for Biotechnology Information database. The amino acid peptides with identified sequence were highlighted in yellow, and modified amino acid peptides were highlighted in green.

Fig. 5. Myosin light chain (MLC) isoform composition and maximal unloaded shortening velocity (V_0) in MHC type II single fibers. (A-D) MLC isoform composition and (E) V_0 in MHC type II single fibers were compared among experimental groups. The relative content of the individual MLC isoforms (MLC_{1f}, MLC_{2f}, and MLC_{3f}) was determined densitometrically from 12% SDS-PAGE and silver staining. Single fiber V_0 was determined by the slack test. 'a' indicates significantly different than AC. '†' indicates significantly different than OM. Values represent mean $\pm$ SEM. Significance was set at $P < 0.05$.

Fig. 6. Relationship between V_o and the MLC_{3f}/MLC_{2f} ratio in MHC type II single fibers. The linear regression line for (A) all MHC type II fibers ($5.31x + 1.76$, $R^2 = 0.22$, $r = 0.46$, $P < 0.001$), (B) MHC IIB fibers ($5.73x + 2.01$, $R^2 = 0.25$, $r = 0.50$, $P < 0.001$), (C) MHC IIX/IIB fibers ($3.26x + 1.87$, $R^2 = 0.09$, $r = 0.30$, $P = 0.020$), (D) MHC IIX fibers ($5.42x + 1.38$, $R^2 = 0.23$, $r = 0.48$, $P = 0.014$) was expressed. Significance was set at $P < 0.05$.

Table 1

Vo and MLC isoforms of SM MHC type II fibers (MHC type IIB, IIX/IIB, IIX, IIAx)

Group	AC		OC		OE		OM						
	MHCs												
V ₀ MLCs	IIB	IIX/IIB	IIX	IIAX	IIB	IIAX	IIX/IIB	IIX	IIAX	IIB	IIX/IIB	IIX	IIAX
V ₀ (FL s ⁻¹)	3.3 ± 0.2 (40)	3.2 ± 0.5 (6)	2.7 ± 0.0 (1)	/	2.5 ± 0.2 ^{a†} (14)	2.4 ± 0.3 (6)	1.9 ± 0.2 ^{a†} (22)	1.7 ± 0.3 [†] (11)	0.7 ± 0.3 (2)	3.6 ± 0.4 (14)	2.5 ± 0.3 (17)	2.9 ± 0.4 (9)	2.4 ± 0.0 (1)
MLC1f (%)	29.4 ± 1.5 [†] (48)	26.4 ± 4.2 [†] (4)	26.0 ± 0.0 (1)	/	51.6 ± 2.7 ^a (28)	46.1 ± 5.1 (13)	50.4 ± 3.1 ^a (22)	42.3 ± 6.7 (11)	45.9 ± 10.8 (2)	47.7 ± 2.1 ^a (14)	50.9 ± 2.4 ^a (17)	46.1 ± 1.8 (9)	56.8 ± 0.0 (1)
MLC2f (%)	56.6 ± 1.7 [†] (48)	62.0 ± 3.4 [†] (4)	64.9 ± 0.0 (1)	/	44.5 ± 2.6 ^a (28)	48.6 ± 4.6 (13)	45.5 ± 2.8 ^a (22)	52.1 ± 6.1 (11)	53.7 ± 10.8 (2)	43.7 ± 2.2 ^a (14)	41.1 ± 2.4 ^a (17)	46.8 ± 1.3 (9)	42.3 ± 0.0 (1)
MLC3f (%)	14.1 ± 0.9 [†] (48)	11.6 ± 1.3 (4)	9.2 ± 0.0 (1)	/	3.9 ± 0.6 ^{a†} (28)	5.4 ± 0.9 (13)	4.1 ± 0.5 ^{a†} (22)	5.6 ± 0.9 (11)	0.4 ± 0.1 (2)	8.6 ± 1.1 ^a (14)	8.0 ± 1.0 (17)	7.1 ± 1.6 (9)	1.0 ± 0.0 (1)
MLC3F2f (%)	0.28 ± 0.03 (48)	0.19 ± 0.02 (4)	0.14 ± 0.00 (1)	/	0.09 ± 0.02 ^{a†} (28)	0.12 ± 0.02 (13)	0.09 ± 0.0 ^{a†} (22)	0.14 ± 0.03 (11)	0.01 ± 0.00 (2)	0.21 ± 0.03 (14)	0.21 ± 0.03 (17)	0.15 ± 0.04 (9)	0.02 ± 0.00 (1)

Vo and MLC isoforms in semimembranosus muscle (SM) myosin heavy chain (MHC) type II fibers were compared among groups: adult control (AC), old control (OC), old empty vector (OE), and old MLC3f (OM). The % MLC1f, % MLC2f, % MLC3f, and MLC3f/MLC2f ratio were determined from densitometric analysis of individual fibers.

^a indicates significantly different than AC.

[†] indicates significantly different than OM. Values represent mean ± SEM. Significance was set at *P* < 0.05.