Regulation of myogenin protein expression in denervated muscles from young and old rats

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Kostrominova, Tatiana Y., Peter C. D. Macpherson, Bruce M. Carlson, and Daniel Goldman. Regulation of myogenin protein expression in denervated muscles from young and old rats. Am J Physiol Regulatory Integrative Comp Physiol 279: R179–R188, 2000.—Myogenin is a muscle-specific transcription factor participating in denervation-induced increases in nicotinic ACh receptor (nAChR) gene expression. Although myogenin RNA expression in denervated muscle is well documented, surprisingly little is known about myogenin protein expression. Therefore, we assayed myogenin protein and RNA in innervated and denervated muscles from young (4 mo) and old (24–32 mo) rats and compared this expression to that of the nAChR α-subunit RNA. These assays revealed increased myogenin protein expression within 1 day of denervation, preceding detectable increases in nAChR RNA. By 3 days of denervation, myogenin and nAChR α-subunit RNA were increased 500- and 130-fold, respectively, whereas myogenin protein increased 14-fold. Interestingly, old rats (32 mo) had 6-fold higher myogenin protein and ~80-fold higher mRNA levels than young rats. However, after denervation, expression levels were similar for young and old animals. The increased myogenin expression during aging, which tends to localize to small fibers, likely reflects spontaneous denervation and/or regeneration. Our results show that increased myogenin protein in denervated muscles correlates with the upregulation of its mRNA.

nicotinic acetylcholine receptor; aging; gene expression

SKELETAL MUSCLE IS A DYNAMIC system that responds quickly and adapts to changes in intrinsic and extrinsic stimuli. A whole repertoire of genes can be activated in response to these stimuli. The family of myogenic basic helix-loop-helix (bHLH) transcription factors (MyoD, myogenin, MRF4, Myf-5, Id) plays a major role in coordinating the muscle developmental program, as well as the process of adaptation (reviewed in Ref. 27). The mechanism by which myogenic bHLH family members regulate gene expression involves homodimerization or heterodimerization with ubiquitously expressed bHLH proteins (E12, E47) and binding to an E-box sequence in the promoter region of the genes (18, 23). Formation of heterodimers with Id, however, prevents binding to the E-box and leads to downregulation of gene expression (2). Expression levels of different myogenic bHLH proteins vary in different muscle types and might regulate and reflect phenotypic and physiological differences among muscles (16, 35).

One of the most studied members of the family of myogenic bHLH factors is myogenin. Myogenin knockout mice have a severe deficiency of skeletal muscle and neonatal lethality (14, 25). In contrast, mice lacking MyoD (31), Myf-5 (3), or MRF4 (37) are viable and do not have skeletal muscle defects. Myogenin participates in the muscle response to a wide variety of conditions. Expression of myogenin mRNA changes in muscle during development (9), passive stretch (20), regeneration (11), denervation (1, 4, 9, 35), disease (29), and aging (10, 22, 24), etc.

One of the hallmarks of skeletal muscle development is innervation, which is ultimately required for cell survival. When mature innervated muscle is denervated, an apparent recapitulation of at least portions of the developmental process takes place (9). After denervation, an increase in expression of many of the myogenic transcription factors occurs, but the increase in myogenin mRNA appears to be the most robust (1, 35). Myogenin message gradually increases during the first few days of muscle denervation and is followed by an increase in transcription of activity-dependent genes such as those encoding the nicotinic ACh receptor (nAChR) subunits (9). This finding is complemented by the observation that overexpression of myogenin in adult muscles of transgenic mice leads to an increase in expression of nAChR transcripts (12). Furthermore, direct injection of a myogenin antisense expression vector into denervated muscle causes a significant decrease in the transcriptional activation of the nAChR α-subunit promoter (26). Finally, muscle denervation extending beyond 1 mo results in diminished myogenin and nAChR subunit RNA levels over the ensuing months of denervation (1). These observations suggest

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that myogenin plays a key role in regulating the expression of the nAChR subunits after denervation.

Despite evidence suggesting that after denervation, an increased expression of myogenin mRNA leads to activation of other denervation responsive genes, very little is known about the actual myogenin protein expression profile after denervation. Expression of myogenin protein is not only regulated at the level of transcription, but also by mRNA stability, translation efficiency, protein stability, and protein phosphorylation (reviewed in Ref. 27). For example, 8.5 days postcoitus (dpc) mouse embryos accumulate a significant pool of unprocessed myogenin RNA in their developing somites, but do not have detectable levels of myogenin protein until 10 dpc (6, 32, 33). In addition, immunostaining has shown that myogenin protein accumulates in rat muscle fiber myonuclei 3 days after denervation (36). Unfortunately, immunostaining does not allow for a quantitative estimation of myogenin protein expression. Therefore, it is difficult to tell if accumulation of myogenin protein after denervation is a result of increased protein expression or a posttranslational modification that leads to the accumulation of the protein in the nuclei.

To determine if myogenin RNA levels reflect protein levels, we assayed myogenin RNA and protein expression in innervated and denervated young (4 mo) and old (24–32 mo) rats. We related these changes to nAChR α-subunit mRNA expression, which is regulated by myogenin (12, 26). In the present study, gastrocnemius muscles from young rats were denervated over a period of 4 mo and myogenin and nAChR α-subunit RNAs and myogenin protein expression were characterized. Experiments on old animals were also included in this study on the basis of the observation that the levels of myogenin and nAChR γ- and ε-subunit mRNAs were significantly higher in muscles from 31-mo-old rats than from either 3- or 18-mo-old rats (10, 22). Our data showed that after experimental denervation of muscles from young and old rats and during the normal aging process, an increase in myogenin mRNA expression correlated with an increase in myogenin protein that was localized to muscle nuclei. Compared with myogenin mRNA expression, accumulation of nAChR α-subunit mRNA after experimental denervation was delayed for ~1 day but, in general, followed the pattern of myogenin expression both after denervation and during aging. In innervated muscles from old rats, increased basal levels of myogenin expression were consistent with the presence of a population of denervated and/or regenerating muscle fibers.

The proximal stump of the sciatic nerve was ligated and then implanted and sutured into the hip musculature. The distal stump was implanted as far from the proximal stump as possible. This procedure results in permanent denervation of the lower hindleg (34). Denervation experiments were carried out over a period of 1 to 120 days. Gastrocnemius and tibialis anterior muscles were then excised for analysis. Usually muscles from the contralateral legs served as a control. In agreement with previously published observations (15), we could not detect differences between muscles from unoperated animals and from contralateral legs of operated animals (data not shown). All operations and subsequent animal care were carried out in accordance with the guidelines of the Unit for Laboratory Animal Medicine at the University of Michigan and National Institute of Health Guide for the Care and Use of Laboratory Animals.

RNA isolation and RNase protection assay. Total RNA was isolated by homogenizing the muscles in Trizol (GIBCO BRL, Grand Island, NY) followed by the single-step purification method as described by the manufacture protocol. Antisense probes used to detect muscle creatine kinase (MCK), myogenin, and the nAChR α-subunit were the same as those described by Adams et al. (1). RNase protection assays were carried out as previously described (1). The probe for MCK was included in each experiment and served to normalize for differences in the amount of RNA in each of the samples as has been reported previously (1). The RNA for MCK was not regulated by any of the conditions employed in this report. The myogenin probe was made from mouse DNA and consistently protects two bands when hybridized to rat RNA. RNase resistant hybrids were analyzed on 6% polyacrylamide–8 M urea gels. After electrophoresis, gels were dried and exposed to the X-ray film. Probe signals were quantitated by scanning densitometry, and values were normalized to the RNA signal obtained for MCK. The specificity of the protected bands was confirmed by hybridizing probes to tRNA, which resulted in no protected fragments on the gel. Probe integrity was monitored for each experiment by running an aliquot of nonhybridized probe on each gel.

Western blotting. Rat muscles were dissected, frozen in liquid nitrogen, pulverized, and homogenized in solution containing 20 mM Tris·HCl (pH 6.8), 4% (wt/vol) SDS, 1 mM of phenylmethylsulfonyl fluoride (PMSF), and 1 μM each of leupeptin and pepstatin A. Protein concentrations were determined using the Bio-Rad detergent compatible protein assay (Hercules, CA). Protein samples were mixed with loading buffer, subjected to SDS-PAGE (10%), and transferred electrophotically to Immobilon-P membranes (Millipore, Bedford, MA). Gels with identical samples were stained with Coomassie brilliant blue and used as an additional control of equilibration of protein loading. After transfer Immobilon-P membranes were blocked in Blotto buffer containing 5% dry milk in PBS-0.05% Tween 20 (PBST) and then incubated overnight at 4°C with mouse monoclonal antibody against myogenin (clone F5D), obtained from the Developmental Studies Hybridoma Bank, The University of Iowa, Iowa City, IA). Immundetection was done using peroxidase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Lab, West Grove, PA) with subsequent chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). Band intensity was quantitated by scanning densitometry.

Immunohistochemistry. Dissected muscles were fixed in 2% (wt/vol) paraformaldehyde, washed in PBS, embedded in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC), and sectioned on a cryostat (10 μm). Sections were incubated in blocking buffer (20% goat serum in PBST) for 1 h and then in the solution of anti-myogenin antibody

MATERIALS AND METHODS

Animals and muscle denervation. Experiments were performed on 4- to 32-mo-old Wistar rats of the WI/HicksCar strain maintained at the University of Michigan. Two to five animals were used for each time point (n = 54). Rats were anesthetized with ether during the induction of experimental denervation and before euthanasia. For denervation experiments, the right legs of 4-mo-old rats were denervated by a high sciatic nerve section in the hip region of the hindlimb.
mRNA was increased; P 300-fold (in young (4 mo old) rats, myogenin mRNA increased nAChR mRNA levels (1). After 10 days of denervation Muscle denervation results in increased myogenin and nAChR α-subunit mRNA levels (1). From 1 to 120 days of denervation, the overall pattern of expression of the myogenin protein paralleled that of the myogenin RNA (Fig. 2). Myogenin and nAChR α-subunit RNA levels increased after muscle denervation, reaching their maximum level by 30 days (Fig. 2A). However, these levels of myogenin and nAChR RNAs were not maintained and decreased significantly (P < 0.05) in muscles that remained denervated for >30 days (Fig. 2A). Therefore, it appears that the level of transcription of myogenin-responsive genes, such as the nAChR α-subunit, follows the level of myogenin RNA expressed in the muscle. These results suggest that myogenin RNA will reflect myogenin protein levels.

To address this latter issue, we assayed for myogenin protein in these same denervated muscle samples (Fig. 2B) and compared it to myogenin RNA expression. Western blotting with antibody against myogenin detected one major band that was increased in denervated muscle samples. To confirm the specificity of the antibody, denervated muscles and cultured rat myoblasts and myotubes (both primary cultures and L6 cell line) were assayed in the same Western blot. The major band in denervated muscle samples corresponded to the major band that accumulated in myotubes (13) but was not present in myoblasts (data not shown). In general, after muscle denervation, the pattern of myogenin protein paralleled that of myogenin RNA expression. During the first 3 days after denervation, nAChR α-subunit and myogenin RNAs increased as myogenin protein increased (Fig. 2). After 10 days of denervation there was a modest, but discernible, decrease in the expression of both myogenin mRNA and protein (P < 0.05). In contrast, after 10 days of denervation, the level of expression of the nAChR α-subunit was still increasing (Fig. 2A). Around 1 mo of denervation, myogenin RNA and protein and the nAChR α-subunit RNA had already reached their highest level of expression (~600-, 14-, and 600-fold, respectively) and then their mRNAs began to decline (Fig. 2). By 120 days postdenervation, the levels of myogenin and nAChR RNA declined to ~20 and 10% (P < 0.05), respectively, of the levels observed at 30 days postdenervation. Myogenin protein level at 120 days postdenervation was ~70%

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Fig. 1. Changes in the level of expression of myogenin and the nicotinic ACh receptor α-subunit (alpha) mRNA after denervation and during aging. RNase protection assays were used to evaluate changes in myogenin (solid bars) and α-subunit (open bars) mRNA in gastrocnemius muscles of rats. Presented are a representative RNase protection assay and the averaged levels of myogenin and α-mRNA expression in innervated muscles from 4-, 24-, and 32-mo-old rats and 10-day denervated muscles from 4-mo-old rats (4m/Den). For each protection assay, the levels of mRNA for myogenin and nAChR α-subunit were normalized to the level of expression of muscle creatine kinase (MCK). Bars represent means ± SE.

(50% F5D hybridoma supernatant-50% PBST) overnight at 4°C. Cy3-conjugated goat anti-mouse secondary antibody was used for visualization. Nuclei were stained with a bis-benzimide solution (Sigma, St. Louis, MO) in PBST.

Statistics. Means and SE were determined for samples from different animals. To determine differences in mean values of expression of myogenin and nAChR α-subunit RNA and myogenin protein, one-way analyses of variance were performed. If the F statistic of the analysis of variance showed significance, differences among means were detected by using the Tukey-Kramer multiple comparisons post hoc test. The level of significance was set a priori at P < 0.05. Values are expressed as means ± SE.

RESULTS

Myogenin and nAChR mRNA expression in aged rodent muscle suggest the existence of denervated fibers. Muscle denervation results in increased myogenin and nAChR mRNA levels (1). After 10 days of denervation in young (4 mo old) rats, myogenin mRNA increased 300-fold (P < 0.05; Fig. 1). Similarly, nAChR α-subunit mRNA was increased ~100-fold (P < 0.05) after 10 days of denervation (Fig. 1). Thus myogenin and nAChR RNA levels appeared to reflect whether the muscle was innervated or denervated.

Consistent with the observation that the amount of myogenin mRNA increased in old rats (10), we observed an 80-fold (P < 0.05) increase in myogenin mRNA as rats aged from 4 to 32 mo (Fig. 1). These increases in myogenin expression were paralleled by increases in nAChR α-subunit mRNA (Fig. 1). On the basis of the pattern of gene expression during short-term denervation, the 80- to 90-fold increase in the expression of myogenin and nAChR α-subunit in muscles from 32-mo-old rats suggested that a significant number of muscle fibers in old animals were denervated.

Expression of myogenin in denervated young muscle. RNase protection and Western blot assays were used to determine if myogenin protein reflected myogenin RNA levels after denervation of skeletal muscle in young rats. In addition, because myogenin appears to mediate denervation-induced increases in nAChR gene transcription (12, 27, 28), we also assayed nAChR α-subunit RNA levels after muscle denervation. From 1 to 120 days of denervation, the overall pattern of expression of the myogenin protein paralleled that of the myogenin RNA (Fig. 2). Myogenin and nAChR α-subunit RNA levels increased after muscle denervation, reaching their maximum level by 30 days (Fig. 2A). However, these levels of myogenin and nAChR RNAs were not maintained and decreased significantly (P < 0.05) in muscles that remained denervated for >30 days (Fig. 2A). Therefore, it appears that the level of transcription of myogenin-responsive genes, such as the nAChR α-subunit, follows the level of myogenin RNA expressed in the muscle. These results suggest that myogenin RNA will reflect myogenin protein levels.

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(P < 0.05) of the 30-day postdenervation value. The smaller decrease in myogenin protein between 30 and 120 days of denervation may be a consequence of muscle atrophy, resulting in a substantial shift in the nuclear protein to total protein ratio (see DISCUSSION).

Fig. 2. Time course of the changes in the expression of myogenin and nicotinic ACh receptor α-subunit mRNA and myogenin protein after denervation in 4-mo-old rats. The levels of expression of the respective mRNAs and protein were evaluated at 0, 1, 2, 3, 10, 30, 60, and 120 days after denervation. A: representative RNase protection assay and averaged data for the levels of expression of myogenin (solid bars) and α-subunit (open bars) mRNA. B: representative Western blot and averaged data normalizing myogenin levels to the control innervated level. Coomassie blue-stained polyacrylamide gel is shown as an additional control of protein loading. Bars represent means ± SE.

Expression of myogenin in aging muscle. The accumulation of myogenin protein in muscle as animals age is shown in Figs. 4 and 5. From 4 to 17 mo of age, myogenin protein levels did not change significantly (P > 0.05). However, by 24–32 mo of age, myogenin levels increased (Fig. 4). All myonuclei were negative for myogenin immunostaining in muscles from 4-mo-old rats (Fig. 5A). In muscles from 17-mo-old rats, a very small number of normal-sized fibers exhibited myonuclear staining for myogenin (Fig. 5E and F). The number of myogenin-positive fibers increased with age. There was a significant population of small fibers denervation, muscle fiber cross-sectional area was decreased significantly, but the size of the nuclei appeared unchanged (Fig. 3E), and the majority of the myonuclei was positive for myogenin (Fig. 3, E and F).
whose nuclei showed detectable myogenin accumulation in muscles from 26-mo-old rats (Fig. 5E). Within the 26-mo-old muscle there were also some small regenerating fibers, recognized by centrally located nuclei, that were myogenin positive (data not shown). Some larger fibers had centrally located nuclei and were myogenin negative (Fig. 5, E and F), suggesting that these fibers represent regenerated and innervated fibers. However, the majority of the fibers from 26-mo-old muscles was normal in size and had myogenin-negative nuclei (Fig. 5E).

Comparison of young and old denervated muscle. In muscles from 24- to 32-mo-old rats, myogenin and nAChR α- and γ-subunits mRNA increased >10-fold compared with 4-mo-old controls (Refs. 10, 22, and 24 and Fig. 1). These changes in gene expression during aging are presumed to reflect the presence of a population of denervated muscle fibers. To assess the ability

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**Fig. 3.** Accumulation of myogenin in the myonuclei after denervation. Cross sections of innervated (A, B) and 3 (C, D) and 120 (E, F)-days denervated tibialis anterior muscles from 4-mo-old rats were stained with antibody for myogenin (A, C, E) or with nuclear stain bis-benzimide (B, D, F). Arrowheads show nuclei localization. Bar 40 μm.

**Fig. 4.** Increased expression of myogenin protein during muscle aging. The level of expression of myogenin protein was evaluated in muscles from rats of different ages by Western blot analysis. Values were normalized to the level of expression in 4-mo-old innervated rats. Coomassie blue-stained polyacrylamide gel is shown as an additional control of protein loading. Bars represent means ± SE.
of old animals to respond to denervation, we compared levels of myogenin and nAChR α-subunit mRNA expression in muscles from young (4 mo) and old (24 mo) rats after denervation. After 10 days of denervation, the magnitude of the increase in myogenin and nAChR α-subunit mRNA was greater for young than for old animals, but the absolute level of expression in denervated muscles was similar (Fig. 6A). Western blotting experiments suggested a similar pattern of expression for the myogenin protein (Fig. 6B). Finally, immunostaining of innervated muscles showed little to no detectable myogenin-positive myonuclei in fibers from young rats and the majority of fibers from old rats (Fig. 7, A and B and E and F). After 30 days of denervation, a significant accumulation of myogenin was observed in practically all myonuclei in muscles from both young and old rats (Fig. 7, C and D and G and H).

DISCUSSION

Several studies have implicated myogenin as an important regulator of gene expression during muscle development, denervation, and aging (4, 9, 22, 24, 35). Myogenin is expressed at high levels during the late stages of myoblast differentiation and during myotube formation. After myotube innervation and maturation, myogenin returns to a low level of expression. In contrast to the innervated state, after denervation of mature muscle, myogenin expression increases dramatically and appears to induce the expression of activity-dependent genes such as those encoding the nAChR subunits (9). Although changes in myogenin mRNA after denervation and during aging have been reported previously (1, 9, 22, 24), we are unaware of any data that compare the level of myogenin protein expression with its mRNA profile. Our experiments showed that after denervation, the pattern of myogenin protein expression was similar to that of the myogenin mRNA. However, despite this similarity, there were considerable differences in the magnitude of the changes observed for these two molecules. For example, at 3 days postdenervation of young rats, myogenin RNA increased by 500-fold, whereas myogenin protein only increased 14-fold above control. This difference in the magnitude of the response to denervation may simply reflect differences in transcriptional and translational controls that ultimately influence myogenin protein levels. Indeed, it was previously reported that protein synthesis is slower in unweighted muscles than in control (17). In addition, RNA and protein stability also likely contribute to the steady-state levels reported here. Regardless of the reason for these differences, it is clear that the modest increase in myogenin protein, relative to its RNA, is sufficient for mediating the muscle’s response to denervation as demonstrated by the increase in nAChR α-subunit mRNA.

Although, in general, myogenin protein levels reflected their RNA level in short-term denervated muscle, this was not the case for long-term denervated...
muscle (Fig. 2). The level of myogenin mRNA expression between 30 and 120 days of denervation decreased ~80%, whereas myogenin protein expression decreased only ~30% during this same time. This is an intriguing observation because nAChR α-subunit RNA levels seem to follow myogenin RNA, but not protein (Fig. 2). There are several possible explanations for the apparent discrepancy between the maintained level of myogenin protein expression after long-term denervation and the significant decrease in nAChR α-subunit mRNA expression. First, myogenin activity can be suppressed by phosphorylation (reviewed in Ref. 27). In our assays we only measured total myogenin protein levels, and the amount of active (nonphosphorylated) myogenin in muscle might be significantly smaller. Second, other regulatory factors, such as Id (1, 2) or the recently described MyoR (21) and Mist 1 (19), could be inhibiting nAChR α-subunit mRNA expression after prolonged denervation by forming inactive complexes with myogenin. Consistent with this idea, we have previously shown an increase in Id expression after 1 mo of muscle denervation (1). Third, one must consider...
muscle atrophy and decreased muscle volume after long-term denervation. After 2–4 mo of denervation, muscle mass decreases by >70% (5) and muscle fiber volume decreases by ~90% compared with innervated control muscle (34). In contrast, the relative volume of the nuclei per fiber increases (34). In our experiments, we compared the level of myogenin expression to the total amount of protein in muscle. Consequently, relatively large decreases in cytoplasmic proteins may have obscured decreases in the level of myogenin protein after long-term denervation.

Our results showing that 2–3 days after denervation practically all myonuclei stain for myogenin are consistent with the observation that after short-term denervation most of the increase in myogenin expression is localized to the existing myofibers (35, 36). In innervated and 2- and 3-day denervated myofibers, the number of activated satellite cells is very small, representing ~4% of total number of nuclei (30, 34). Between 1 and 2 mo of denervation, the number of satellite cell nuclei increases to 8–12% and then decreases gradually to ~2% after 7 mo of denervation (30, 34). Consequently, the positive myogenin staining that we observed after 120 days of denervation likely reflects a mix between existing and newly formed fibers.

The pattern of myogenin and nAChR α-subunit mRNA expression reported in the present study is similar to previously reported observations (1, 4, 9, 35). Using Northern blotting experiments, Eftimie et al. (9) reported a 40-fold increase in myogenin mRNA in muscles from mice 2 days after denervation. The accumulation of nAChR α-subunit mRNA was delayed and reached a maximal level (70-fold) 7 days after denervation. Buonanno et al. (4) reported that the maximal level of myogenin transcripts was found in gastrocnemius muscle 4 days after denervation. In the experiments of Voytik et al. (35), myogenin mRNA reached a maximal level of 150- to 200-fold above the contralateral innervated muscle within the first 10 days after denervation and then gradually decreased to ~50 times higher than control at 28 days of denervation. Adams et al. (1) reported an increase in both myogenin and nAChR subunit mRNA expression in short-term denervated muscle and a decline in these RNAs as denervation continued beyond 1 mo. The minor differences between studies in the temporal and quantitative increases of myogenin and nAChR subunit RNAs after denervation undoubtedly reflect variability between species, muscles evaluated, and the methods used to evaluate and quantify changes in gene expression. Nonetheless, after denervation, the rapid and dramatic increase in myogenin expression followed by nAChR subunit mRNA expression and then the subsequent decline in expression of both molecules remain a consistent observation across studies.

Several lines of evidence suggest that old muscles have a population of denervated muscle fibers due to loss of motoneurons (reviewed in Ref. 7). Consistent with this observation, 31-mo-old rats express ~10-fold higher levels of myogenin and nAChR α-subunit mRNA than 2-mo-old rats (10, 22). Complementing these data, Musaro et al. (24) reported a significant increase in mRNA expression for myogenin, MyoD, and AChR α-subunit message in 2-yr-old mice. In agreement with previously published results (10, 22, 24), our data showed a gradual increase in myogenin and nAChR α-subunit mRNA expression in aging animals. The present study, for the first time, showed that the increase in myogenin mRNA expression in old rats correlated with an increase in myogenin protein expression. Muscles from 32-mo-old rats had ~80 times more myogenin mRNA and 6 times more myogenin protein than muscles from 4-mo-old rats. Furthermore, myogenin immunostaining showed that the majority of the myogenin-positive fibers in 24- to 32-mo-old rats is small-diameter atrophic fibers that are located in close proximity. This proximity likely results from spontaneous denervation during aging accompanied by collateral sprouting from nearby axons that leads to fiber type grouping (reviewed in Ref. 8). On denervation of these fibers, one would then observe clustering of myogenin-positive fibers as we described. Indeed, myogenin-positive fibers likely reflect the diminished capacity of motoneurons in old animals to sprout and/or...
maintain collateral sprouting. Some myogenin-positive fibers from old rats are also positive for the embryonic isoform of myosin and for neural cell adhesion molecule (A. Borisov and E. Dedkov, personal communication), which suggests the presence of a regenerative process. Whether these fibers are refractory to reinnervation is not known.

The results described above indicate that muscles from old animals have a population of presumably denervated fibers that express elevated levels of myogenin and nAChRs. To determine if muscles in old animals maintain their ability to respond to experimental denervation, we compared the response of muscles from young and old animals to short-term denervation. Despite initial differences in myogenin mRNA and protein and nAChR \( \alpha \)-subunit mRNA in innervated muscles from young and old rats, the level of expression was similar in young and old animals after denervation. Marsh et al. (22) reported similar results for young and old rats in response to injections of the myotoxin bupivacaine. In their experiments, old rats had much higher initial levels of myogenin than young rats. After bupivacaine injection, myogenin increased in young and old muscles to similar levels, but muscles from old animals were characterized by incomplete recovery of mass and a prolonged elevation of myogenin mRNA expression. These data suggest that innervated muscle fibers in old animals maintain their ability to activate the process of adaptation to experimental denervation but, once denervated, are less likely to become reinnervated.

In summary, our data indicate that myogenin protein levels reflect RNA levels and either can be used as an indicator of a muscle’s response to denervation. These results suggest that the increased myogenin expression detected in old animals is a result of spontaneous muscle denervation. In addition, myogenin levels correlate with the activation of target genes such as those encoding nAChR subunits. Finally, young and old muscle exhibit similar changes in myogenin and nAChR RNAs and myogenin protein after denervation, suggesting that aged muscle maintains signaling cascades involved in activation of these genes and presumably participating in adaptation to muscle denervation.

**Perspectives**

Aging is accompanied by muscle fiber denervation. In addition, muscles that become denervated for extended periods of time (beyond a couple of months) are poorly reinnervated. The limiting factors for these phenomena are not known. Therefore, an important issue for aging research is to identify mechanisms mediating muscle denervation and reinnervation and to identify ways to facilitate reinnervation after partial denervation. We show here that young and old muscle respond to denervation by upregulating nAChR and myogenin gene expression in a similar fashion. Because myogenin is an important regulator of muscle-specific gene expression, it appears that fibers in muscles of old animals are capable of responding appropriately to a denervation event. These results suggest that factors extrinsic to muscle are playing an important role in reinnervation of denervated fibers in old animals. However, there may be subpopulations of fibers in muscles of old animals that do not respond to denervation adequately. For example, the population of small myogenin-positive fibers we identified in the old animals may differ in their ability or capacity to attract axonal sprouts and express factors necessary for reinnervation. Therefore, future experiments must focus on this population of denervated fibers to determine if they are intrinsically different from their young counterparts.

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