ORIGINAL ARTICLE

Increased desmin expression in hindlimb muscles of aging rats

David W. Russ · Jessica S. Grandy

Abstract

Background Aging skeletal muscle frequently exhibits a reduction in force produced per unit muscle tissue, variously termed muscle quality, specific tension or dynapenia. Muscles from animals in which desmin expression is reduced exhibit similar properties, raising the possibility that reduced desmin expression contributes to impaired force production in aging muscles.

Methods We examined expression of desmin and synemin, both intermediate filament proteins, in the plantarflexor muscles of adult (6–8 months) and older (24 months) rats. We have previously reported age-related reductions in muscle quality and sarcoplasmic reticulum function in these animals.

Results Significant effects of age and muscle were found for the expression of desmin ($P=0.040$ and <0.001 respectively), but not synemin. Desmin expression was increased in the aging muscles, with the greatest changes observed in the gastrocnemius muscles. Muscle quality, but not muscle mass, was reduced in the aging plantarflexor muscles.

Conclusions Loss of desmin does not account for reduced force production in aging muscles. The potential effects of the age-related increase in desmin on muscle function remain unclear, but may include dissipation of contractile force.

Keywords Sarcopenia · Cytoskeleton · Synemin · Dynapenia

Abbreviations

E–C	Excitation–contraction
EDTA	Ethylenediaminetetraacetic acid
PVDF	Polyvinylidene fluoride
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SR	Sarcoplasmic Reticulum
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with TWEEN20
Tris	Tris(hydroxymethyl)aminomethane

1 Introduction

Desmin is an intermediate filament protein of ~53-kD molecular weight and is a major component of the cytoskeleton of skeletal muscle [1]. Desmin is associated with z-discs and nebulin in the thin filaments of striated muscle sarcomeres and has connections to the sarcolemma, mitochondria, and nucleus [2, 3]. Functionally, desmin appears to be important in stabilizing sarcomeres and maintaining cell compliance [4], and is essential for maintaining the structural integrity of skeletal muscle [5]. Not surprisingly then, alterations in desmin have been linked to a number of specific myopathies in humans [6–8]. It has been suggested that desmin supports sarcomere alignment and transmission of both active and passive

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muscle force [5, 9]. Extensive research has been performed on transgenic mice that do not express desmin (i.e., desmin knockout (KO) mice). Several studies have shown that these animals produce less force when compared to wild-type control, even when force was normalized to muscle physiological cross-sectional area (i.e., specific force was reduced) [10–13], although this finding is not universal [9].

Increased age has also been associated with a reduction in muscle force production, even when changes in muscle size and mass have been taken into account [14–20]. This loss of force produced per unit muscle tissue is variously termed muscle quality, specific tension or dynapenia [21]. Several potential mechanisms have been put forth to account for this loss of force production, including: selective contractile protein loss [22], modification of contractile proteins that impairs crossbridge binding [19] and impairments of excitation–contraction (E–C) coupling [15, 17, 20, 23]. However, age-related changes in cytoskeletal force transmission could also play a role, particularly in light of the aforementioned reductions in specific force reported in desmin knockout mice. However, there are surprisingly few studies of changes in cytoskeletal proteins with aging, although there have been some suggestions that cytoskeletal protein expression might decrease with age [24].

A few studies have examined the effects of aging on desmin. Qualitative differences in desmin staining of cryosections from old and young muscles have been reported in both rats [25] and humans [26]. More recently, Piec and colleagues [27] assessed desmin in a more systematic and quantitative manner in the gastrocnemius muscles of old and young rats. Interestingly, these studies all reported either an increase or no change in desmin with increasing age. However, none of these studies reported any information regarding muscle force production. Furthermore, none of these studies systematically evaluated potential age-muscle interactions with regard to desmin expression, despite the fact that increasing evidence indicates that many age-related changes in muscle (e.g., oxidative enzyme activity, calcium release, myosin isoforms, and specific tension) are muscle specific [20, 28, 29].

We therefore chose to examine the effect of aging on desmin expression in the four major rat plantarflexor muscles in a subset of animals in which we had previously found age-related reductions in muscle quality and sarcoplasmic reticulum function. Of note, these changes occurred in the absence of major muscle atrophy. In addition, we determined the expression of a second intermediate filament protein, synemin, to get a sense of whether any age-related changes might be generalized to intermediate filaments, or if they were specific to desmin.

We hypothesized that desmin, but not synemin, expression would be reduced with aging, consistent with data from desmin knockout studies reporting reduced force generation.

2 Methods

2.1 Animals and ethical approval

Animal use and all procedures were approved by the Ohio University Institutional Animal Care and Use Committee, and the *Principles of laboratory animal care* (NIH publication No. 86–23, revised 1985), were followed throughout the study. Data from total of six adult (6–8 months) and six older (24 months), male F344/BN hybrid rats, representing a subset of animals used in a previous study, are presented here. The aging animals used here were at an age of ~90% survival rate [30], but have been shown to exhibit marked declines in both muscle quality [20] and locomotor function [31]. Animals were housed in an environmentally controlled facility (12–12-h light–dark cycle, 22°C), and had *ad libitum* access to food and water. These animals represent a subset from a previously published study [20] that evaluated age-related changes in muscle quality and sarcoplasmic reticulum function. For purposes of this brief report, we focus on the immunoblotting experiments related to intermediate filament proteins; however, we also present the contractile data from the subset of previously studied animals

2.2 Muscle contractile testing

Animals were anesthetized with an intraperitoneal injection of a ketamine/xylazine (40:10 mg kg^{-1} body mass, respectively). The plantarflexor muscle group was dissected free and clamped via the Achilles tendon in series with a force transducer, as previously described [20]. Pulses (200 μs) from a Grass S48 stimulator (Astro-Med) were delivered via a hook electrode (Harvard Apparatus) to the sciatic nerve at supra-maximal (125% voltage needed to produce maximum twitch force) intensity. Muscles were potentiated with ten 100-ms, 100-Hz trains (one train per 10 s) [32], followed by administration of 500-ms trains of 1, 5, 10, 30, 50, 75, 100, and 125 Hz (also one train per 10 s) to determine the force frequency relationship. Two trains of each frequency were delivered in each trial, with the order randomly determined for each animal. Following testing, the plantarflexor muscles (soleus, plantaris, and medial and lateral gastrocnemii) were dissected free of surrounding tissues, blotted dry, and weighed. Animals were sacrificed following tissue collection by an intracar-

dial injection of anesthesia (euthasol, 100 mg kg^{-1}). Muscle quality was determined by dividing plantarflexor force by sum of the masses of the four plantarflexor muscles, as has been performed previously [33–35].

2.3 Tissue processing

After weighing, each of the four plantarflexor muscles was dissected and was divided into two portions. The first portion was frozen in liquid N_2 and stored at −80°C for later immunoblotting experiments. The second was homogenized (PRO 200, Oxford, CT, USA) fresh on ice for SR Ca^{2+} uptake and release assays presented elsewhere [20]. Frozen muscle samples were brought to 4°C, weighed, minced on cold glass, homogenized in buffer (10 mM sodium phosphate, pH 7.2, 2 mM EDTA, 10 mM sodium azide, 120 mM sodium chloride, 2% NP-40, plus HALT (Pierce) protease inhibitor), incubated on ice for 1 h, and centrifuged at 14,000×g for 30 min. Pellets were resuspended in 200 μL of buffer (150 mM postassium chloride, 20 mM Tris, pH 7.0). Both pellet and supernatant fractions were saved. Total protein concentrations were estimated using a Modified Bradford Assay (Pierce) for both fractions.

2.4 SDS-PAGE and Western blotting

Samples were diluted 1:1 with 2× Laemmli sample buffer and 30-μg protein were separated via sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% gels with 5% stacking gels, at 125 V. Preliminary testing found no desmin or synemin in the supernatant fraction. Therefore, only the results of the pellet analyses are presented here.

Following SDS-PAGE, proteins were transferred to PVDF for 2 h at 4°C in transfer buffer (15% methanol, 25 mM Tris, and 192 mM glycine). Once transfer was complete, membranes were blocked at room temperature for 1 h in blocking buffer (SuperBlock, Thermo Scientific, IL), then incubated with primary antibodies overnight at 4°C. Primary antibodies for both desmin (Cell Signaling Technologies) and synemin (Sigma) were diluted 1:2,000 in blocking buffer. After primary incubation, membranes were washed 5×5 min in Tris-buffered saline+Tween20 (TBS-T) and incubated for 1 h at room temperature in secondary antibody (Goat anti-Rabbit, LiCor) which was diluted in blocking buffer (1:10,000). Following secondary incubation, membranes were once again washed 5×5 min in TBS-T, then rinsed for 5 min with TBS. Membranes were dried in the dark overnight prior to scanning with the LiCor Odyssey system. The membranes were scanned via the LiCor Odyssey system for densitometric band analysis. After scanning, the membranes were stained with Coomassie Brilliant Blue R250 and within blot band intensities were normalized to total protein per lane determined from the stained, scanned membrane. The blots were all performed in duplicate. A known amount of rabbit IgG was run on each gel and used as a standard for normalization of bands from different blots.

2.5 Statistical analyses

Animal and muscle masses were compared using unpaired t tests. Muscle quality was analyzed using two-way (age×frequency) repeated measures analysis of variance (ANOVA), with frequency as the repeated factor. In the event of a significant age×frequency interaction, post hoc tests were performed using unpaired t tests. Desmin and synemin expression were analyzed with mixed-models two-way (age×muscle) ANOVAs with muscle as a repeated factor. Because we were primarily interested in age differences within a given muscle, we planned *a priori* to compare old and young animals within using unpaired t tests, in the event a significant effect of age was found. All data are presented as means±SE, and all statistic analyses were performed using SPSS, with the threshold for significance set at $P \leq 0.05$.

3 Results

3.1 Immunoblotting

Desmin expression was greatest in the soleus muscle, regardless of age ($P=0.001$) and age was associated with an overall increase in desmin expression ($P=0.040$), but there was no age×muscle interaction ($P=0.461$). Within-muscle, age-related differences were significant in both the medial and lateral gastrocnemius muscles ($P=0.048$ and 0.018, respectively), but not the soleus or plantaris (Fig. 1a). In contrast, no significant main effects or interactions for muscle and age were observed for synemin expression (Fig. 1b).

3.2 Muscle quality

Muscle quality, determined by dividing plantarflexor group force by total plantarflexor muscle mass, was similar in adult and aging muscles at low stimulation frequencies. At higher frequencies (≥ 75 Hz) however, the adult muscles exhibited higher muscle quality than the older muscles (Table 1). Consistent with these findings, there was a significant age×frequency interaction ($P=0.021$), though the main effect of age did not reach significance ($P=0.074$).

Fig. 1 a *Top*, mean (+SE) desmin expression in hindlimb muscles of adult (*A*) and older (*O*) rats (*N*=6/group). *Bottom*, representative immunoblot for desmin. b *Top*, mean (+SE) synemin expression in hindlimb muscles of *A* and *O* rats (*N*=6/group). *Bottom*, representative immunoblot for synemin. *Asterisk*, significant within-muscle difference between adult and aging rats
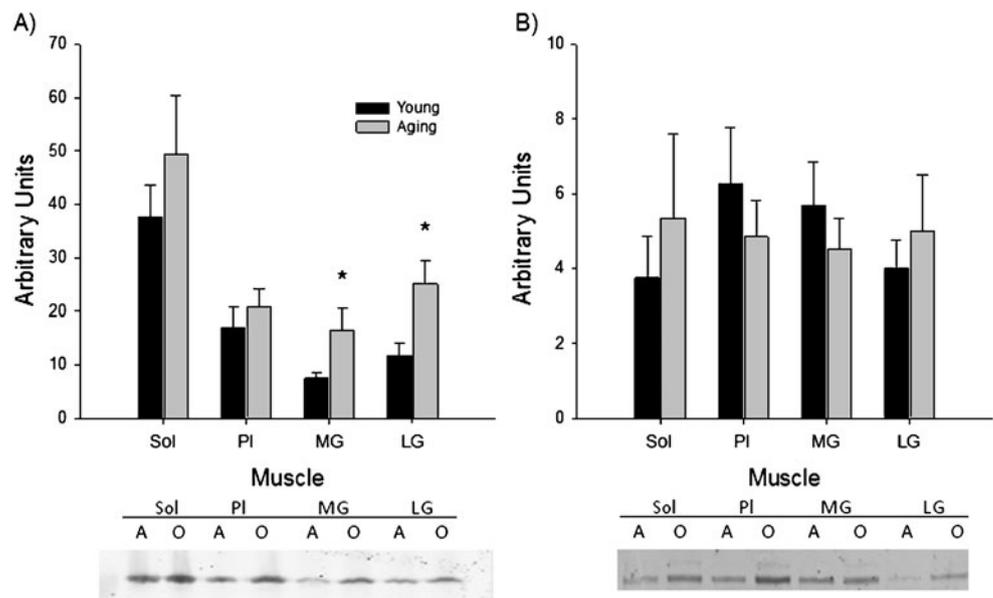
3.3 Animal and muscle mass

The older rats were significantly heavier than the adult ones. Overall, there was no significant difference in total plantarflexor muscle mass. Among the individual muscles, only the medial gastrocnemius exhibited any age-related decline in muscle mass, and the soleus muscles of the older animals were actually heavier than those of the adult animals (Table 1).

4 Discussion

The major finding of this study was that, contrary to our hypothesis, desmin expression increased with increasing

Table 1 Animal and Muscle Masses

	Adult mean (SE)	Old mean (SE)	P value
Body mass (g)	409 (21)	586 (6)	<0.001
Muscle mass (mg)			
Soleus	171 (10)	197 (12)	0.049
Plantaris	423 (22)	443 (15)	0.474
M. gastrocnemius	1094 (54)	919 (29)	0.017
L. gastrocnemius	1,049 (98)	1,088 (28)	0.717
Muscle quality (N/g)			
1 Hz	2.033 (0.152)	1.829 (0.168)	0.389
5 Hz	2.140 (0.175)	1.861 (0.164)	0.271
10 Hz	2.225 (0.185)	2.088 (0.134)	0.562
25 Hz	4.408 (0.555)	3.598 (0.241)	0.210
50 Hz	6.506 (0.522)	5.490 (0.574)	0.220
75 Hz	7.819 (0.379)	6.332 (0.502)	0.039
100 Hz	8.169 (0.413)	6.444 (0.555)	0.032
125 Hz	8.098 (0.404)	6.423 (0.634)	0.050

age. Synemin expression was unchanged with age, suggesting that an overall increase in intermediate filament proteins was not occurring with age. Despite the increase in desmin, plantarflexor muscle quality was lower in the older animals, in contrast to what we expected based on the results of previous studies of desmin knockout mice.

The increased desmin expression in the gastrocnemius muscles is similar to the data of Piec and colleagues [27], and extends these findings to the other major plantarflexors. Though less robust (i.e., not statistically significant), a trend for increased desmin expression with aging was also observed in the soleus and plantaris muscles, suggesting that this increase may not exhibit the muscle specificity associated with other age-related changes [28, 29, 36]. Consistent with others, we found that desmin expression was greater in the soleus muscle than the other hindlimb muscles (plantaris and gastrocnemii), which are known to contain greater amounts of type II myosin heavy chain isoforms [10, 37]. As aging is often associated with an increase in the muscular type I myosin heavy chain profile, it is possible that the age-related increase we observed was a function of fiber type transitions. However, our previously published data on these animals indicate that only minor changes in overall myosin isoform composition occurred [20], making it unlikely to account for the increased desmin expression.

The increase in desmin was not accompanied by an increase in synemin, another intermediate filament protein that has been shown to co-localize with desmin in intermediate filaments. While our methods were not sufficiently quantitative to determine exact molar ratios of synemin to desmin, as has been done elsewhere [38], our results suggest that the synemin:desmin ratio should decrease with age. It has been shown that increases in the

synemin:desmin ratio are associated with problems in correct assembly of intermediate filaments. However, the effects of a decrease in the ratio have not been reported, to our knowledge. It has been reported that synemin persists in the myotendinous and neuromuscular junctions, but is absent from the z-discs of desmin KO mice [39]. Though highly speculative, this may indicate that the increased desmin we observed in the older muscles here is not associated with the z-disc. Such a finding would be consistent with the report of Ansved who found that the pattern of desmin, but not spectrin, staining was altered with age [25]. These investigators suggested that the altered pattern of desmin staining in old muscles is associated with increased deposition of desmin in intermyofibrillar spaces that were increased with age, due to loss of myofibrils [25]. Increased cytoplasmic desmin expression was observed in muscles subjected to a denervation–reinnervation protocol [40]. It is widely accepted that aging is associated with a denervation–reinnervation process, and this may account for our observation of increased desmin. One possible method of addressing the issue of the localization of the increased desmin with aging in future studies would be to label longitudinal sections from old and young animals, fixed at various sarcomere lengths as described by Bowman and colleagues to study obscurin [41].

As reported previously [20], rats of this strain and age differential exhibited reduced plantarflexor force production, but minimal age-related muscle atrophy, resulting in reduced muscle quality. Clearly, reduced desmin expression was not the cause. However, one previous study [9] has reported increased specific force in muscles from desmin KO mice. These investigators posit that desmin acts a "viscous element that dissipates mechanical energy." If this is the case, then an age-associated increase in desmin could indeed have contributed to impaired muscle quality we observed in the aging rats. Given the number of other studies reporting reduced specific force with aging however, this remains an unsettled issue.

Another group has suggested that desmin is necessary for optimal E–C coupling [13]. Our previous study found that aging was associated with impaired sarcoplasmic reticulum calcium release (a key step in E–C coupling). Thus, it is tempting to speculate that the increase in desmin represents a failed, or only partially successful, compensation in the aging muscles. However, Wieneke and colleagues suggested that desmin's role in the excitation–contraction coupling process occurred upstream of the calcium release channel, and was possibly related to maintaining t-tubule integrity and function. In contrast, we observed impaired calcium release from sarcoplasmic reticulum vesicles, a process that was independent of t-tubule function and action potential propagation, thus it seems unlikely that the two processes are related.

The potential role of changes in desmin expression in age-related alterations in muscle function remains unclear. Our results confirm and extend previous findings of an age-related increase in expression of desmin in skeletal muscle. One study suggests that increased desmin could directly contribute to impaired muscle quality through mechanical blunting of force production. This is consistent with our results, but clearly the issue remains equivocal. It is difficult to study normal aging in desmin knockout animals, due to their shortened lifespan [42]. However future studies may be able to address this issue in aging animals by using advanced molecular biology techniques (e.g., RNAi knockdown).

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