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SKELETAL MUSCLE

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Contributions of the ubiquitin–proteasome pathway and apoptosis to human skeletal muscle wasting with age

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Abstract The primary mechanism that contributes to decreasing skeletal muscle strength and size with healthy aging is not presently known. This study examined the contribution of the ubiquitin–proteasome pathway and apoptosis to skeletal muscle wasting in older adults (n = 21; mean age = 72.76 ± 8.31 years) and young controls (n = 21; mean age = 21.48 ± 2.93 years). Subjects underwent a percutaneous muscle biopsy of the vastus lateralis to determine: (1) ubiquitin ligase gene expression (MAFbx and MuRF1); (2) frequency of apoptosis; and (3) individual fiber type and cross-sectional area. In addition, a whole muscle strength test was also performed. A one-way ANOVA revealed significant increases in the number of positive TUNEL cells in older adults (87%; $p < 0.05$), although no significant increase in caspase-3/7 activity was detected. Additionally, ubiquitin ligase gene expression, individual muscle fiber type and CSA were not different between old and young subjects. Muscle strength was also significantly lower in old compared to young subjects ($p < 0.05$). In conclusion, this study indicates a preferential role for apoptosis contributing to decreases in muscle function with age.

Keywords Apoptosis · Protein metabolism · Skeletal muscle

Introduction

US census studies have predicted that by the year 2020, 52 million Americans will be over the age of 65. Skeletal muscle function has been shown to deteriorate with increasing age [21, 23]. These changes in whole muscle strength and size may cause impairments in locomotion and perhaps an increased risk for falls in older adults [8]. Reductions in skeletal muscle function related to sarcopenia (skeletal muscle wasting as a function of age), may have intense social and economic impacts for older adults and the health care system as it is estimated that as much as 26 billion dollars of annual health care costs are directly related to sarcopenia [17].

The mechanisms of skeletal muscle wasting may be attributable to an actual decrease in the total number of functioning skeletal myocytes (apoptosis), or an increase in myofibrillar protein breakdown via the ubiquitin–proteasome pathway (UPP). To date there have been studies that demonstrated an increase in skeletal muscle apoptosis under pathophysiological or other physiological conditions such as chronic heart failure [33], muscle denervation [5], muscular dystrophy [29], and muscle unweighting [1]. Similarly, previous research on the increased activity of the UPP is overwhelming in pathological states such as cancer, sepsis, and trauma [2, 6, 24, 31] as well as on the effects of denervation atrophy and simulated weightlessness [10, 26]. Despite the ample information of cellular mechanisms affecting muscle loss under disease and disuse conditions, there are currently no investigations explaining the cellular mechanisms of sarcopenia in a healthy, physically inactive adult population.

Determination of the relative contribution to, or regulation of, skeletal muscle wasting, whether it is from protein degradation or programmed cell death, will reveal possible areas for physical activity intervention in the aging process. The current investigation provides insight into the role of the UPP and apoptosis mechanisms in sarcopenia in a physically inactive, healthy adult population. The current investigation recruited physically inactive old volunteers as well as a gender and activity matched young control group to ensure that formal exercise training would not have an influence on the findings of the study.

To date previous research has only attempted to examine the mechanisms of skeletal muscle wasting in

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animal tissue [4, 5, 12] or specific pathological conditions [1, 33, 39], with no focus being given to a healthy aging population, which comprises a much larger percentage of the older adult population than those with a diagnosed pathological condition. This research examined, for the first time, possible mechanisms of skeletal wasting leading to a decrease in skeletal muscle strength, size, and function that accompanies the aging process. Although the dominating factor that is causing this loss of skeletal muscle function during the aging process remains a question, it is known that a decline in force generating capability is associated with a smaller cross-sectional area (CSA) of skeletal muscle [8, 20, 23]. However, it is unknown whether this decrease in CSA is due to a decreased number of muscle fibers from apoptosis, or an increase in protein degradation that causes subsequent atrophy of skeletal muscle fibers via the UPP. Thus, the purpose of this investigation was to investigate the relative contributions of apoptosis and the UPP to skeletal muscle wasting in a group of healthy aging volunteers. We tested the hypothesis that both pathways of apoptosis and the UPP would be up regulated in older adults when compared to young controls. Additionally, we examined the hypothesis that individual fiber size would be smaller in older adults due primarily to the influence of protein breakdown through the UPP.

Methods

To investigate the contribution of programmed cell death and protein degradation to skeletal muscle wasting in a physically inactive healthy aging population, a cross-sectional study design was employed. Subjects underwent a percutaneous needle biopsy of the vastus lateralis. This muscle sample was used to determine the relative activities of the UPP, and apoptosis in each subject. Volunteers also completed a Yale Physical Activity Survey [11], a medical history questionnaire, and two muscle function tests of the quadriceps muscle group to investigate the effects of skeletal muscle wasting on whole muscle strength.

Subjects

Twenty-one physically inactive (male $n = 11$, female $n = 10$) older adult volunteers (≥60 years); and a group of gender matched young physically inactive controls (male $n = 10$, female $n = 11$) ranging from 18 years to 30 years were recruited to participate in this study. The mean age, height, and weight of the old and young groups are listed in Table 1.

The exclusion criteria for all volunteers was obesity (body mass index ≥ 30 kg/m^2), inactive volunteers who performed any type of formal aerobic or resistance-training exercise program within the last 2 years, any form of heart disease, cigarette smokers, persons taking gluccocorticoids (as they may affect the UPP), diabetes mellitus, or any other musculoskeletal disorder limiting physical performance. The research protocol was designed and conducted in accordance with the Declarations of Helsinki and had been approved by the University of Kansas Institutional Review Board. Written consent was obtained from each subject prior to the commencement of any testing or data collection after being informed of all procedures and risks associated with this investigation.

Muscle biopsy

Percutaneous needle muscle biopsies (75–125 mg) of the vastus lateralis were obtained in each subject for muscle tissue analysis [3]. Muscle sections were placed in RNA *later* (Ambion; Austin, TX) and liquid nitrogen then stored for analysis.

Quantification of apoptosis

TUNEL staining

DNA fragmentation via the mechanism of apoptosis was quantified using a colorimetric TUNEL (terminal deoxyribonucleotidyl transferase (TdT)-mediated biotin-16-dUTP nick-end labeling) assay kit (Promega; Madison, WI). Longitudinal cross-sections were treated according to the manufacturer's instructions and developed using diaminobenzidine substrate buffer and chromogen solution with hydrogen peroxide. Samples were then analyzed by counting positively stained nuclei per cubic millimeter of sample using a digital stereo light microscope.

All TUNEL staining assays utilized approximately six subjects in conjunction with a negative and positive control. Cross-sections of all subjects were treated with

Table 1 The subject demographics for the present investigation are shown. Included in these demographics are age, height, weight, and daily caloric expenditure above resting metabolic rate for the old and young groups. All values are presented as the mean ± SD

Group	Age (years)	Height (cm)	Weight (kg)	Energy expenditure (kcals/day)
Old ($n = 21$)	72.76 ± 8.31	169.91 ± 10.49	78.15 ± 16.23	546.00 ± 289.66
Males ($n = 11$)	75.27 ± 8.95	177.76 ± 7.60	88.31 ± 15.72	458.77 ± 278.35
Females ($n = 10$)	70.00 ± 6.94	161.29 ± 4.78	66.96 ± 6.70	641.95 ± 284.30
Young ($n = 21$)	21.48 ± 2.93	173.67 ± 8.07	73.40 ± 11.71	449.54 ± 229.84
Males ($n = 10$)	22.00 ± 3.43	179.13 ± 6.16	79.85 ± 10.57	407.40 ± 238.34
Females ($n = 11$)	21.00 ± 2.45	168.70 ± 6.27	67.55 ± 9.73	487.84 ± 226.14

DNase I to induce DNA fragmentation as a positive control. Tissue sections treated as the negative control received deionized water in place of the TdT enzyme.

Caspase-3 detection

Caspase activity was detected according to manufacturer's instructions using the Apo-One Homogeneous Caspase-3/7 Assay (Promega), a fluorescent assay that measures caspase-3 and −7 activity specifically. Tissue was homogenized in an eight-fold volume of buffer adopted by Wagner et al. [35], comprised of 25 mM HEPES (pH 7.5), 0.1% Triton X-100, 5 mM $MgCl_2$, 2 mM DTT, 74 μg antipain, 0.15 μM aprotinin, 1.3 mM EDTA, 1 mM EGTA, 15 μM pepstatin, 20 μM leupeptin using a Polytron homogenizer. The assay was conducted in a 96-well plate with each homogenized sample being run in triplicate. Samples were then measured in a plate-reading fluorometer (Bio-Tek; Winooski, VT) at an excitation and emission wavelength of 485 and 520 nm, respectively.

The protocol for Apo-One Homogeneous Caspase-3/7 Assay was modified for the current investigation as it was originally designed for use in cultured cells. To validate the assay, inter- and intra-assay variability was assessed using tissue homogenate from two laboratory volunteers. Coefficient of variation (CV) was determined for freshly homogenized tissue vs flash freezing of the supernatant (CV = 1.97%) to ensure that flash freezing would not affect caspase activity. CV results for inter-assay and intra-assay variability was determined to be 1.85 and 0.41%, respectively.

Quantification of ubiquitin–proteasome pathway

RNA isolation

Eighteen unknown samples were processed for total RNA isolation from a portion of the skeletal muscle biopsies sampled from the vastus lateralis. Twenty-four samples had mRNA extracted due to a change in the Applied Physiology Laboratory protocol for RNA isolation. The total RNA isolation was performed according to the manufacturer's specifications for the RiboPure Kit (Ambion; Austin, TX). mRNA was isolated from skeletal muscle biopsies utilizing the μMACS mRNA Isolation Kit (Miltenyi Biotec; Auburn, CA). Both total and mRNA samples were DNase treated and stored at −80°C until analysis.

Real-time PCR

Skeletal muscle protein degradation from the UPP was determined by measuring the expression of two ubiquitin ligase genes, human muscle atrophy F-box (MAFbx) and muscle specific ring finger 1 (MuRF1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene (HKG). Expression of all genes was determined using standard real-time quantitative RT-PCR. Primers and Taqman probes for MAFbx and GAPDH were obtained from previous publications authored by Jones et al. [18] and Imai et al. [16], respectively. The human mRNA sequence for MuRF1 was acquired from GenBank and primers and Taqman probe were designed using Beacon Designer 3 software (Premier Biosoft International; Palo Alto, CA). Probes and primers were manufactured by Sigma Genosys (The Woodlands, TX); probes were labeled with a reporter dye, FAM at the 5′ end, and a dark quencher, Black-Hole Quencher (BHQ) at the 3′ end. Primers for all genes were designed to be intron spanning to ensure amplification of cDNA and the exclusion of gDNA. Sequence information for the gene specific primer and probe design can be found in Table 2.

Real-time PCR assays were completed using the Rotor-Gene 3000 (Corbett Research; Mortlake, Australia) with a 72-well rotor following a standard procedure of a reverse-transcription step at 55°C for 15 min, denaturation at 95°C for 3 min, followed by 45 repeats of the cycling protocol, 95°C for 15 s and a combined annealing/extension at 60°C for 1 min, after which fluorescence is recorded. Optimal probe and primer concentrations were determined to be 100 nM probe and 200 nM sense and anti-sense primer for GAPDH and MuRF1. Probe and primer concentrations for MAFbx followed published guidelines [18] at 125 and 300 nM, respectively. A master mix was created using components from the SuperScript III Platinum One-Step qRT-PCR Kit (Invitrogen; Carlsbad, CA) for all three genes containing (volumes provided are per reaction) 5 μl 2× reaction buffer, 0.2 μl Superscript III/Platinum Taq polymerase, 0.2 μl sense primer, 0.2 μl anti-sense primer, 0.1 μl probe, 0.4 μl 50 mM Mg^{2+}, and 1.9 μl water. Samples were run in triplicate 10 μl reactions including 8 μl Master Mix and 2 μl RNA template.

Table 2 Real-time qPCR primer and probe sequences

	Sense primer (5′–3′)	Anti-sense primer (5′–3′)	Probe (5′-FAM–BHQ1–3′)
GAPDH	GAAGGTGAAGG TCGGAGTC	GAAGATGGTG ATGGGATTTC	CAAGATTCCCGTTC TCAGCC
MAFbx	CAGCTCTGCAAAC ACTGTCACA	GAGCAGCTCTCTG GGTTATTGG	AAGGGCACTGACCA TCCGTGCAC
MuRF1	CAAGGAGAACAGT CACCAGGTAA	GGGCCTCGATG AAGCTAAGC	TTCCTCCTGCTCCTGCG TGATCCG

To validate the HKG, the GAPDH primer/probe set was used with young ($n = 9$) and old ($n = 11$) mRNA samples (in duplicate) in the same real time run. Cycle threshold (C_T) was measured for comparison of changes in gene expression of GAPDH with age. Relative changes in gene expression of MuRF1 and MAFbx as compared to GAPDH was analyzed using the $\Delta\,\Delta C_T$ method. Primers and probe sets were validated for the $\Delta\,\Delta C_T$ method by generating a standard curve of ten-fold dilutions of RNA template for each gene. Efficiencies of all three reactions were within 5% (GAPDH = 97%, MAFbx = 95%, and MuRF1 = 100%) indicating that the comparative $\Delta\,\Delta C_T$ method could be used. Products of the standard curve experiments were separated on a 2% agarose gel and stained with ethidium bromide to confirm expected product size of each genes amplicon (Fig. 1).

Replicates of the C_T for all genes were averaged and evaluated by subtracting the value for the HKG GAPDH from the C_T value of each gene of interest (GOI) yielding the first ΔC_T value. Averages of the ΔC_T for both GOI (MAFbx and MuRF1) were taken for all groupings of subjects, by age (old vs young), by gender (male vs female), and the combination of age and gender (old males (OM), old females (OF), young males (YM) and young females (YF)). The average ΔC_T value for each group was then compared to that of its respective group (i.e., ΔC_T old$-\Delta C_T$ Young $= \Delta\,\Delta C_T$) to give an indication of relative expression. Triplicate values were used where possible; however, there were some outliers that had to be removed leaving some subjects with only duplicate data points. These subjects were in the minority as there was a relative proportion of only 13/123 of outliers/all values. Outlying values were determined as those that did not amplify above the chosen threshold and subsequently were not assigned a C_T value.

Histochemistry

A bundle of the skeletal muscle sample was mounted longitudinally, frozen in isopentane, and stored in liquid nitrogen for analysis. Several transverse sections (~10 μm) of the mounted muscle sample were cut for histochemical analysis at −20°C in a cryostat microtome (American Optical; Buffalo, NY). Fiber type analysis was then performed using the ATPase method, preincubated at pH 4.3 [7]. After fiber type was determined for the "pure" fibers (I, IIa, and IIb) the fibers were analyzed for individual fiber CSA. CSA of the individual fibers was determined using computerized planimetry (NIH Scion Image Program (Version 4.0.2)).

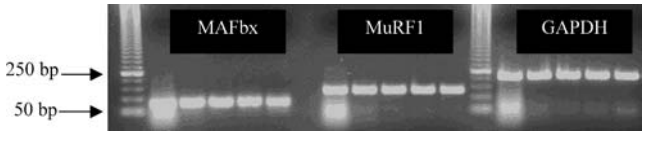

Fig. 1 Illustrates the qRT-PCR products from the ten-fold serial dilutions of the standard curves generated for each gene. *Lane 1*, DNA ladder (50 bp); *lanes 2–6*, MAFbx dilutions from 1000 to 0.1 ng total RNA; *lanes 8–12*, MuRF1 dilutions from 1000 to 0.1 ng total RNA; *lane13*, DNA ladder (50 bp); *lanes 14–18*, GAPDH dilutions from 1000 to 0.1 ng total RNA. Expected product size for MAFbx = 79 bp, MuRF1 = 139 bp, and GAPDH = 226 bp

Muscular strength

Muscle function evaluations were conducted on each subject to evaluate functional capacity of the knee extensors (quadriceps). The quadriceps muscle strength evaluation consisted of two parts: (1) maximal isometric strength at a knee angle of 60° (with neutral representing a 90° knee position); and (2) isokinetic measurements at velocities of 60, 180, and 300°/s. Subjects performed two trial repetitions for isometric strength at the 60° joint angle followed by one maximal effort lasting for 5 s. At each isokinetic velocity, subjects completed two warm-up repetitions (~50% effort) to familiarize them with the velocity and range of motion. Subsequent to the warm-up, each volunteer executed four maximal repetitions with 90 s rest between each testing velocity. All measurements were conducted using a Cybex II dynamometer (Lumex Corp., Ronkonkoma, NY). CV for all subjects from familiarization to test trial was 8.07, 7.95, 7.36, and 12.62% for isometric, 60, 180, and 300°/s, respectively.

Statistical analysis

All statistical analysis was carried out using the Statistical Package for the Social Sciences software program version 12.0 (SPSS, Chicago, IL). The data were subjected to a one-way ANOVA. A Bonferroni follow-up test was utilized when comparing all four groups separated by gender and age. Finally, variables were analyzed for existing correlations using a Pearson product–moment correlation. Descriptive statistics (mean ± SD) were calculated for all variables (TUNEL staining, caspase-3/7 activity, ubiquitin ligase gene expression, individual muscle fiber CSA and fiber type composition, and whole muscle strength). The overall significance level was set at 5% ($\alpha = 0.05$).

Results

The data for all subjects were separated and compared in three ways for statistical differences, by age only (old vs young, results posted in Table 3), by gender only (males vs females, results posted in Table 4), and by separating age and gender into four distinct groups (old males, old females, young males, and young females; results posted in Table 5).

Table 3 The data for apoptosis and individual muscle fiber CSA and fiber type distribution as it was analyzed by age only. TUNEL positive is expressed as the number of positive cells/mm^3 of tissue sample. Caspase-3/7 activity is expressed in arbitrary units (AU) of fluorescence. Ubiquitin gene expression is expressed as fold difference relative to the young group with ↑ and ↓ denoting increase or decrease in expression, respectively

*Significantly different from old ($p < 0.05$)

	Old	Young
Apoptosis		
TUNEL Positive (mm^3)	270.76 ± 105.58	144.72 ± 79.05*
Caspase (AU)	5685.26 ± 1415.99	5838.42 ± 1534.85
Ubiquitin ligase relative gene expression		
MAFbx	↑ 1.20	–
MuRF1	↓ 1.17	–
Cross-sectional area (μm^2)		
Type I	4723.61 ± 1259.96	4776.07 ± 1129.88
Type IIa	5032.90 ± 1669.61	5700.22 ± 1572.50
Type IIb	4552.87 ± 1511.62	4704.26 ± 1170.99
Fiber type distribution (%)		
Type I	53.68 ± 9.95	50.29 ± 15.57
Type IIa	22.94 ± 6.82	25.85 ± 11.80
Type IIb	23.38 ± 7.80	23.86 ± 6.98

Table 4 The data for apoptosis and individual muscle fiber CSA and fiber type distribution as it was analyzed by gender only. TUNEL positive is expressed as the number of positive cells/mm^3 of tissue sample. Caspase-3/7 activity is expressed in arbitrary units (AU) of fluorescence. Ubiquitin gene expression is expressed as fold difference relative to the male group with ↑ and ↓ denoting increase or decrease in expression, respectively

	Males	Females
Apoptosis		
TUNEL positive (mm^3)	195.55 ± 100.12	218.77 ± 123.20
Caspase (AU)	5440.70 ± 1343.18	6071.34 ± 1534.64
Ubiquitin ligase relative gene expression		
MAFbx	–	↑ 1.18
MuRF1	–	↑ 1.58
Cross-sectional area (μm^2)		
Type I	5139.77 ± 1381.82	4399.54 ± 845.57*
Type IIa	6159.57 ± 1754.30	4680.85 ± 1159.03*
Type IIb	5352.68 ± 1429.18	3980.63 ± 813.60*
Fiber type distribution (%)		
Type I	49.09 ± 15.22	54.44 ± 10.70
Type IIa	25.21 ± 11.32	23.80 ± 8.31
Type IIb	25.70 ± 6.87	21.76 ± 7.29

*Significantly different from males ($p < 0.05$)

Table 5 The data for apoptosis and individual muscle fiber CSA and fiber type distribution for each of the four groups as separated by a combination of age and gender. TUNEL positive is expressed as the number of positive cells/mm^3 of tissue sample. Caspase-3/7 activity is expressed in arbitrary units (AU) of fluorescence

	Old males	Old females	Young males	Young females
Apoptosis				
TUNEL positive (mm^3)	245.82 ± 96.64	295.70 ± 133.19	139.69 ± 73.34[a]	148.84 ± 86.77[a]
Caspase (AU)	5355.29 ± 1727.91	6015.23 ± 1002.62	5526.10 ± 898.05	6122.35 ± 1949.23
Cross-sectional area (μm^2)				
Type I	5464.55 ± 1406.56	4056.76 ± 611.01	4847.47 ± 1363.76	4711.16 ± 932.55
Type IIa	6186.58 ± 1778.63	3994.59 ± 484.08[b] *	6135.26 ± 1828.03	5304.73 ± 1255.66
Type IIb	5576.19 ± 1589.95	3631.89 ± 577.32[b] *	5151.51 ± 1320.28	4297.67 ± 889.80
Fiber type distribution (%)				
Type I	51.74 ± 10.00	55.43 ± 10.10	46.71 ± 19.00	53.54 ± 11.63
Type IIa	21.58 ± 6.63	24.17 ± 7.10	28.48 ± 13.86	23.46 ± 9.61
Type IIb	26.69 ± 8.25	20.40 ± 6.37	24.82 ± 5.67	23.00 ± 8.17

*Significant from old males
[a]Significant from old females
[b]Significant from young males ($p < 0.05$)

Apoptosis

The outcome of the TUNEL staining demonstrates a significantly higher number (87%) of muscle fibers stained positively for DNA fragmentation in the old subjects as compared to the young (270.76 ± 105.58 positive fibers/mm^3 and 144.72 ± 79.05 positive fibers/mm^3, respectively ($p < 0.05$)). When the data are

separated further into four groups by gender and age, a significant difference is found more specifically between both young males (YM) and young females (YF), and the old female (OF) group, (139.69 ± 73.34, 148.84 ± 86.77, and 295.70 ± 133.19 positive fibers/mm^3, respectively). No significant differences were found when the subjects were divided by gender.

Caspase activity was not found to be significantly different in any of the three subject groupings by age, gender, or the combination of both (Tables 3, 4, and 5). However, a negative correlation was detected between caspase-3/7 activity and two measures of whole muscle functional capacity in the old subjects regardless of gender ($r = -0.52$; $p = 0.016$, and $r = -0.47$; $p = 0.031$ for 180 and 300°/s, respectively). The negative correlation signifies that whole muscle strength at fast velocities decreases with increasing activity of caspase-3/7. When caspase-3/7 was examined in the four groups separated by age and gender, the old males displayed a similar negative correlation with the 180°/s strength measure ($r = -0.62$, $p = 0.042$).

Ubiquitin–proteasome pathway

In order to ensure no significant changes in expression of the HKG with age, GAPDH expression was analyzed in Young ($n = 9$) and Old ($n = 11$) samples. The average C_T value for Young was 15.21 ± 1.41 and for Old 15.16 ± 1.36. Analysis of gene expression for the two ubiquitin ligase genes MAFbx and MuRF1 demonstrated no significant alterations in the relative expression in any of the subject groupings. In all instances of comparing age, gender, or the four groupings of age and gender combinations, MAFbx and MuRF1 gene expression was less than a two-fold difference (see Tables 3 and 4 for relative expression values for the age and gender groups). Gene expression is articulated in relative terms to the young group with respect to age, and the male group with respect to gender. In the instance of subjects being organized into four groups separated by the combination of their age and gender (i.e., OM, OF, YM, and YF), each group was treated as the control group (setting their relative expression to 1.00) to elucidate any possible increases or decreases in ubiquitin ligase gene expression across all combinations of the four groups. There were no evident alterations in gene expression in any groups regardless of which group the relative expression was set.

Histochemistry

When individual muscle fiber CSA was examined in the old and young grouping, there were no significant differences found in any of the three fiber types (I, IIa, or IIb). However, when the subjects were divided by gender the CSA values for all three fiber types were significantly lower in females as compared to males regardless of age (Table 2). Again, taking a closer look at the data by separating subjects into four groups of age and gender, the CSA measures in fiber types IIa and IIb were significantly lower ($p < 0.05$) in the OF group as compared the YM and OM groups (Table 5).

Analysis of fiber type distribution (as represented by a percentage of the three fiber types I, IIa, and IIb) revealed no significant differences regardless of the grouping of subjects (Tables 3, 4, and 5).

Muscular strength

Evaluation of the data for whole muscle strength established significant differences in several of the strength measurements within all of the possible grouping of subjects. Comparison of the old to the young group illustrates a significantly lower strength value in the old group (128.38 ± 46.18, 109.24 ± 41.71, 69.48 ± 32.85, and 50.33 ± 25.71 Nm for isometric, 60, 180, and 300°/s, respectively) for every measurement as compared to the young subject group (204.67 ± 63.23, 166.95 ± 47.28, 115.71 ± 41.05, and 88.14 ± 35.10 Nm for isometric, 60, 180, and 300°/s, respectively).

Results for strength measurements when evaluated by gender were also found to show a significantly lower value in females (141.86 ± 58.24, 115.86 ± 44.08, 73.29 ± 32.90, 53.81 ± 25.24 Nm for isometric, 60, 180, and 300°/s, respectively), when compared to their male counterparts (191.19 ± 67.20, 160.33 ± 52.36, 111.91 ± 44.96, and 84.67 ± 38.82 Nm for isometric, 60, 180, and 300°/s, respectively).

Further exploration of the data across the four groups of differing age and gender demonstrates significant differences at every strength measurement for the OF group ($p < 0.05$). Results of the strength measurements in all four groups are represented in Fig. 2.

Discussion

The purpose of this investigation was to examine the influences of the UPP and apoptosis on skeletal muscle wasting in healthy Old and Young adults. The direct effects of ubiquitin ligase gene expression and DNA damage and caspase activity on skeletal muscle size and function of the knee extensors were studied to determine the potential contributions of a specific protein degradative pathway (UPP) and programmed cell death (apoptosis). The primary findings of this study suggest a contributing role of apoptosis rather than the UPP to sarcopenia in healthy older adults.

Apoptosis

The current investigation found that involvement of apoptosis appears to be a contributing pathway to skeletal muscle wasting in healthy older adults compared

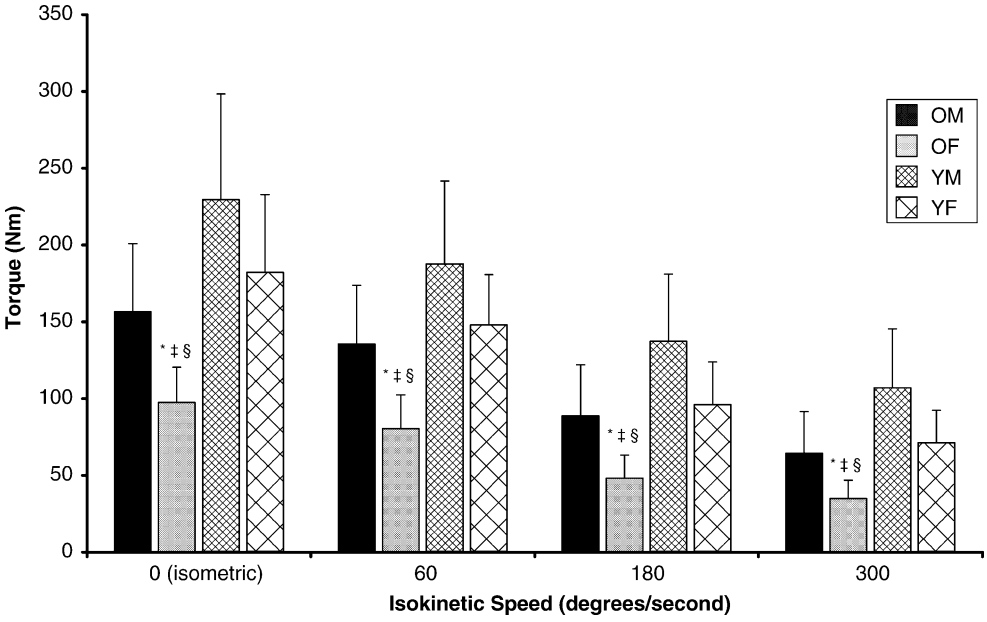

Fig. 2 Illustrates strength differences between old and young subjects. Data is conveyed as mean ± SD; units are in Nm of torque. *Denotes significant difference from old males. ‡ Denotes significant difference from young males. § Denotes significant difference from young females ($p < 0.05$)

to healthy young adults. This was marked by significant increases in TUNEL positive cells stained for DNA fragmentation (older adults showing an increase of 87% over young adults). The results from the current study are similar to those of Vescovo et al. [33, 34] in a heart failure population in regards to TUNEL staining (18.8 ± 19.6 in heart failure vs. 0 ± 0 positive myocytes in controls). However, this investigation failed to see the supportive evidence of elevated caspase-3 activity that was found in skeletal muscle of heart failure patients [33].

The idea of a caspase-independent mechanism of apoptosis is not novel, as many models of stress-induced apoptosis have already proven inhibition of caspase activity does not prevent cell death [9, 19, 25]. The absence of a significant increase in the effector caspase-3 activity in the current investigation could indicate that DNA fragmentation is occurring through a caspase-independent pathway. The authors do acknowledge there are a myriad of other caspases that contribute to apoptosis, however, caspase-3 is traditionally accepted as the major effector caspase at the end of the caspase cascade. One such caspase-independent pathway is Apoptosis Inducing Factor (AIF). Joza et al. [19] demonstrated the presence of a caspase-independent mechanism of cell death while displaying the same morphological features of apoptosis. This genetic evidence for a caspase-independent pathway of programmed cell death opens the door for new areas of gene and protein expression research in human subjects.

Ubiquitin–proteasome pathway

The current investigation did not find any significant differences in MAFbx or MuRF1 gene expression between Old and Young subjects. Though Welle et al. [36] previously documented no change in a leucine-rich Fbox gene, the mRNA sequence in Welle's investigation is not homologous to that used by Bodine et al. [4] or in the current investigation.

The lack of change in expression of MAFbx and MuRF1 with age was probably not due to change in the reference gene since GAPDH expression was not altered between young and old subjects in this study. It is speculated that the lack of physical activity in both Young and Old subjects may be responsible for the similarities in gene expression between the young and old groups. Perhaps both populations are experiencing an increased expression due to their physical inactivity. This hypothesis opens the door for future studies involving physical training of similar healthy Young and Old subjects.

One limitation to the current investigation is that protein expression of the UPP was not evaluated. Although there were no apparent changes in transcriptional regulation of the ubiquitin ligases, there could potentially be translational differences that would only be evidenced in examining protein expression. Such an increase in MAFbx protein was discovered in several muscle wasting models in rats [14, 28].

Individual muscle fiber size and type

Individual fiber CSA was not found to be significantly different between young and old volunteers in this study. These findings are different than previous research that has documented significantly smaller CSA values for both fast and slow fiber types with age [23]. Previous research does, however, support the theory that significant alterations in whole muscle CSA can exist between Young and Old individuals [13, 23]. Evidence from this investigation suggests that apoptosis is contributing to

declines in muscle function; indicating alterations in muscle size are most likely due to a loss of total myocyte number rather than individual fiber atrophy, indicating that a whole muscle evaluation may be more accurate in depicting skeletal muscle wasting in an older adult population.

There was a gender effect on muscle CSA, with females having significantly smaller CSA measures than their male counterparts regardless of age. Yet when subjects were examined in their four groups, the old females showed a smaller CSA in type IIa (3994.59 ± 484.08 μm^3 ($p < 0.05$)) and IIb (3631.89 ± 577.32 μm^3 ($p < 0.05$)) fibers than both the old and young males, indicating that it is primarily the old females that contribute to the gender differences in CSA, especially of the fast fiber types.

This investigation showed that there were no significant differences in fiber type composition between the old and young subjects. These findings do not agree with previous research that has displayed significant changes in fiber type composition with age resulting in a shift toward the slow type I fiber from the fast type II ($r = -0.47$, $p < 0.001$) [21]. It is important to note that the methodology for determining fiber type expression (ATPase staining [7]) in this investigation might not be sensitive to alterations in MHC expression that may be taking place within the skeletal muscle. Williamson et al. [38] found that after 12-weeks of progressive resistance training of older adults, changes in the MHC composition were significant, yet there was nothing detected in the ATPase method of fiber typing. Several other investigators have also documented shifts in MHC expression with age [22], bed rest [32], and spaceflight [37].

The lack of significant differences between old and young subjects in this investigation for both fiber type alterations and individual muscle fiber size again may be attributed to the similarities in the level of physical inactivity between the groups. The cross-sectional design of this study is a limitation given the potential for varied mode of physical activity. In addition, there is a possibility that individuals were physically active prior to the last 2 years and may have retained benefits and strength gains through this study. Smith et al. discovered that subjects after 3 years of detraining still recorded strength measurements above baseline prior to their training [30]. All subjects reported roughly the same amount of physical activity, however as shown in Table 1 the old subjects had slightly higher energy expenditure than the young. Therefore, given the healthy status of the old and young subjects, similarity in physical activity levels may account for the lack of differential findings in changes in muscle CSA.

Muscular strength

Whole muscle strength measurements in this investigation were consistent with other findings of decreased strength in aging muscle. Larsson et al. [21] reported consistent declines in isometric and isokinetic strength after the fifth decade of life. After conducting a 12-year longitudinal study, Frontera et al. [13] state that significant strength loss (20–30%) was seen accompanying decreases in muscle CSA (roughly 12.5% loss in total thigh CSA). There have been multiple other reports of decreases in muscle strength of knee and elbow flexors and extensors in aging skeletal muscle [15, 27].

This investigation did find strength differences between male and female volunteers in accordance with other results from Newman et al. [27] who studied 2,623 men and women between the ages of 70–79 years. Gender differences in strength were seen in both measurements of isokinetic knee extensor strength (specific torque = 12.36 and 14.35 Nm/kg for women and men, respectively) and isometric grip strength (24.48 and 39.8 kg for women and men, respectively) with the women having significantly lower absolute and relative values. The gender differences in strength in the current investigation could be partially explained by the gender differences in individual fiber CSA in all fiber types that were found in this investigation. Previous findings have demonstrated a correlation between decreases in single fiber CSA, specifically of the type II fibers in aging muscle, and decreases in strength [21].

Summary

This investigation found evidence that apoptosis is an active mechanism contributing to sarcopenia in healthy older adults. Results were not conclusive to state that apoptosis is the preferential mechanism responsible for skeletal muscle wasting, as there was no evidence of caspase-3 being up regulated in the old subjects. One of the hypotheses was supported in that the old subjects did exhibit a higher number of TUNEL positive myocytes over the young subjects. However, this finding alone is not enough to substantiate that apoptosis is the primary cause of the decreased muscle strength that was also seen in the old group. The results from other variables such as the UPP mRNA expression levels and individual fiber CSA contradict the hypotheses that the ubiquitin pathway would be up regulated in old subjects as well as show evidence as being the preferential pathway of sarcopenia over apoptosis.

Understanding the pathways and mechanisms that contribute to skeletal muscle wasting in a healthy aging population is essential in the battle to maintain independent living status. Previous research has reported several models, both acute (bed rest, spaceflight, limb suspension, etc.) and chronic (heart failure, renal disease, spinal cord injury, etc.) in nature, all citing the potential place for apoptosis and ubiquitin–proteasome activity. Perhaps the up regulation and involvement of these controlling pathways do not have as large of a role in healthy muscle loss.

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