Published in final edited form as:

Apoptosis. 2006 December ; 11(12): 2115–2126. doi:10.1007/s10495-006-0194-6.

Death receptor-associated pro-apoptotic signaling in aged skeletal muscle

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Abstract

Tumor necrosis factor- α (TNF- α) is elevated in the serum as a result of aging and it promotes pro-apoptotic signaling upon binding to the type I TNF receptor. It is not known if activation of this apoptotic pathway contributes to the well-documented age-associated decline in muscle mass (i.e. sarcopenia). We tested the hypothesis that skeletal muscles from aged rodents would exhibit elevations in markers involved in the extrinsic apoptotic pathway when compared to muscles from young adult rodents, thereby contributing to an increased incidence of nuclear apoptosis in these muscles. The plantaris (fast) and soleus (slow) muscles were studied in young adult (5–7 mo, $n = 8$) and aged (33 mo, $n = 8$) Fischer₃₄₄ $\times$ Brown Norway rats. Muscles from aged rats were significantly smaller while exhibiting a greater incidence of apoptosis. Furthermore, muscles from aged rats had higher type I TNF receptor and Fas associated death domain protein (FADD) mRNA, protein contents for FADD, BCL-2 Interacting Domain (Bid), FLICE-inhibitory protein (FLIP), and enzymatic activities of caspase-8 and caspase-3 than muscles from young adult rats. Significant correlations were observed in the plantaris muscle between caspase activity and muscle weight and the apoptotic index, while similar relationships were not found in the soleus. These data demonstrate that pro-apoptotic signaling downstream of the TNF receptor is active in aged muscles. Furthermore, our data extend the previous demonstration that type II fibers are preferentially affected by aging and support the hypothesis that type II fiber containing skeletal muscles may be more susceptible to muscle mass losses via the extrinsic apoptotic pathway.

Keywords

Tumor necrosis factor- α ; Sarcopenia; Cytokine; Muscle atrophy

Introduction

The aging-associated loss of muscle mass and strength (i.e., sarcopenia), is an unavoidable consequence of advanced age. Although this phenomenon is well documented in animal and human studies, the underlying mechanisms leading to sarcopenia remain to be fully elucidated. The contributing mechanism(s) leading to sarcopenia are multi-factorial and may include: denervation and reinnervation of motor units, especially within skeletal muscles composed of a high degree of type II muscle fibers [1–3]; an alteration in the hormonal

milieu in which anabolic hormone concentrations progressively decline [4, 5]; elevated concentrations of inflammatory mediators that are not only associated with disease states, but also occur in healthy older adults [6–13]; and myonuclear loss through apoptotic mechanisms [14–19].

The process of nuclear apoptosis has been shown to contribute to skeletal muscle mass losses during disease states [20], periods of inactivity [15, 17, 19, 21], and as a result of aging [14]. The presence of apoptotic nuclei within skeletal myofibers has been well-documented [18, 19, 22, 23], although the precise signaling mechanisms that result in these nuclear losses are just beginning to be fully understood. Proapoptotic signaling has been shown to arise from mitochondria in aged skeletal muscles [14, 15, 17, 19] and this has been termed the intrinsic apoptotic pathway. Pro-apoptotic members of the BCL-2 family of proteins, such as Bax and Bak, can promote cytochrome-c release from the mitochondrial intermembrane space initiating a caspase cascade resulting in apoptotic myonuclear loss [24]. Although it appears that this intrinsic apoptotic program is well-conserved across muscles of differing fiber type and contractile activity, a pro-apoptotic environment appears to exist in aged skeletal muscles that can exacerbate myonuclear loss following apoptotic stimuli [15, 17, 19].

The contribution of the extrinsic apoptotic pathway to skeletal muscle mass losses, especially during aging, has been less studied [25]. The increase in circulating concentrations of the cytokine, tumor necrosis factor- α (TNF- α), may initiate pro-apoptotic signaling upon binding to the type I TNF receptor (TNFR). Upon binding, a death inducing signaling complex (DISC) is formed at the cytoplasmic portion of the TNFR, composed of adaptor proteins such as Fas associated death domain protein (FADD), TNFR associated death domain protein (TRADD) and procaspase-8 (reviewed in [26]). Formation of the DISC stimulates cleavage of procaspase-8 into the functional initiator caspase-8. Once cleaved, caspase-8 stimulates cleavage and activation of the executioner caspase-3, which is directly linked to pro-apoptotic changes. Thus, this pathway represents an extrinsic pathway of apoptosis activated by binding of a ligand (TNF- α) to a cell surface death receptor (type-I TNFR).

The purpose of this study was to determine if the extrinsic apoptotic pathway is an active process in aged skeletal muscle and would thus be a contributing mechanism of sarcopenia. Using an accepted rodent model of aging, the plantaris and soleus muscles from young adult (5–7 mo.) and aged (33 mo.) Fischer₃₄₄ × Brown Norway rats (FBN) were examined for the incidence of apoptosis as well as mRNA expression and protein content of markers downstream of the type I TNFR. We hypothesized that skeletal muscles from aged rodents would exhibit elevations in markers involved in the extrinsic apoptotic pathway when compared to muscles from young adult rodents, which would contribute to an increased incidence of apoptosis in these muscles. As such, these data demonstrate that not only is the extrinsic apoptotic pathway an active process within aged skeletal muscle, but our data support the hypothesis that cross-talk between this pathway and the mitochondrial intrinsic pathway may occur through the pro-apoptotic protein BCL-2 Interacting Domain (Bid).

Methods

Experimental animals

All procedures followed the guidelines of the National Institutes of Health, and were approved by the Institutional Animal Care and Use Committee of the West Virginia University School of Medicine. Eight young adult (5–7 mo.) and eight senescent (33 mo.) male Fischer₃₄₄ × Brown Norway (FBN) rats were obtained from the National Institute on Aging barrier-raised colony that is housed at Harlan Animal Colonies (Indianapolis, IN). The animals were housed at 20–22°C in barrier-controlled conditions under a 12:12-h light-dark cycle. They were provided rat chow and water ad libitum.

Experimental animals were sacrificed with an overdose of a xylazine (100 mg · ml⁻¹) and ketamine (50 mg ml⁻¹) 1:1 mixture diluted in sterile PBS. The plantaris and-soleus muscles were removed from each hindlimb and trimmed of visible connective tissue. Immediately upon dissection, muscles were flash frozen in isopentane cooled to the temperature of liquid nitrogen and subsequently stored at –80°C until biochemical analyses were performed.

Semi-quantitative estimates of mRNA

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis was conducted to examine transcriptional changes in markers of the extrinsic pathway. Briefly, frozen muscle samples (~50 mg) were homogenized in 1 ml of TriReagent (Molecular Research Center, Cincinnati, OH) with a mechanical homogenizer. Total RNA was isolated by centrifugation and washed in ethanol according to the manufacturer's instructions. RNA was solubilized in 20 µl of RNase-free H₂O. RNA was treated with Dnase I (Ambion, Austin, TX) and reverse transcribed (RT) with random primers (Invitrogen/Life Technologies, Bethesda MD) to make complimentary DNA (cDNA).

Primers were constructed from published sequences (Table 1). Primer pairs for the genes of interest were coamplified in the same reaction tube with 18S primer pairs and competitors to the 18S primers (Ambion, Austin, TX). Two different 18S primer pairs that produced either 324 base pair (bp) or 489 bp bands following PCR amplification were utilized, depending on the bp size of the gene of interest. The number of PCR cycles was determined for each gene to ensure analyses were done in the linear range of amplification. The signal from the gene of interest was expressed as a ratio to the 18S signal from the same PCR reaction in order to eliminate any loading or amplification errors. Previous studies from our laboratory have shown that ribosomal 18S RNA is suitable as an internal control for RT-PCR and does not change as a result of aging or following muscular atrophic stimuli [17, 19]. Following amplification, 20 µl of each reaction was electrophoresed on 1.5% agarose gels. Gels were stained with ethidium bromide. PCR signals were captured with a digital camera (Kodak 290) and the signals were quantified in arbitrary units as optical density × band area, using Kodak image analysis software (Eastman Kodak Company, Rochester, NY).

Quantitative real-time PCR verification of RT-PCR

Real-time quantitative PCR was utilized as another approach to examine transcript levels in muscles of aged rats. All experiments were performed using the Applied Biosystems 7500

Real-Time PCR system which utilizes TaqMan gene expression assays. Each well of a 96-well optical reaction plate contained: 12.5 μ l TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 1.8 μ l TaqMan Gene Expression Assays, 9.7 μ l sterile H₂O, and 1.0 μ l cDNA. In addition to test samples, each plate contained no template controls and no primer controls. All samples were examined in duplicate and 18S ribosomal RNA (Applied Biosystems, Foster City, CA) was used as an endogenous control. The data were analyzed using the CT method that has been shown to produce comparable results to an absolute quantification procedure [27] and has been previously used to examine cytokine mRNA levels in skeletal muscle [28, 29]. Two different fluorescent dyes were utilized to examine the genes of interest (FAM) and the 18S RNA control (VIC). The CT was calculated for each sample using the following equation: (CT_{FAM}–CT_{VIC}). The CT was then calculated using the following equation: (CT_{Calibrator}–CT_{Sample}). In this study, the young samples served as the calibrator to determine mRNA expression in aged samples relative to young. The fold-induction or relative quantification (RQ) was calculated using the formula: 2^{–(CT)}. Using this calculation, the young samples had an RQ value of 1.0 with all aged samples expressed relative to this value.

Protein measures

The cytoplasmic protein fragment was isolated from frozen muscle samples according to procedures reported previously in our laboratory [16, 21]. Briefly, muscle samples (~50–75 mg), were homogenized in 1 ml of ice-cold lysis buffer (10 mM NaCl, 1.5 mM MgCl₂, 20 mM HEPES at pH 7.4, 20% glycerol, 0.1% Triton X-100, and 1 mM dithiothreitol) to obtain cytoplasmic protein extracts according to the methods of Rothermel et al. [30]. Muscle homogenates were centrifuged at 3000 rpm for 5-min at 4°C. The supernatants were collected that contained the cytoplasmic protein fraction. The protein concentration of the total muscle homogenate was assayed spectrophotometrically at 562 nm (BioRad, SmartSpec 3000) using a commercial bicinchoninic acid (BCA) method as recommended by the manufacturer (Pierce, Rockford, IL) with bovine serum albumin used as standards. Thirty micrograms of cytoplasmic protein was loaded into each lane of a pre-cast 4–12% NuPAGE Bis-Tris gel (Invitrogen, Carlsbad, CA) and separated by routine SDS-polyacrylamide gel electrophoresis (PAGE) for 1.5 h at 20°C. The proteins were transferred to nitrocellulose membranes and verification of equivalent protein loading and transfer was verified by Ponceau S red (Sigma). The membranes were blocked at room temperature for 1 h in 5% non-fat milk (NFM) in tris-buffered saline containing 0.05% Tween (TBS-T). Membranes were probed with the following primary antibodies overnight at 4°C: TNFR rabbit polyclonal antibody (1:1000 dilution, sc-7895, Santa Cruz Biotechnology, Santa Cruz, CA), Bid rabbit polyclonal antibody (1:750 dilution, sc-11423, Santa Cruz), FADD rabbit polyclonal antibody (1:1000 dilution, ab19891, AbCam Inc., Cambridge, MA), and FLICE-inhibitory protein (FLIP) polyclonal antibody (1:500 dilution, ab4042, AbCam Inc.) diluted in TBS-T with 5% NFM. A cytoskeletal β -tubulin antibody (1:500 dilution, ab6160, AbCam) was used as a loading control. Secondary antibodies were conjugated to horseradish peroxidase (HRP; Chemicon) and signals were developed using a chemiluminescent substrate (ECL Advanced, Amersham Biosciences/GE Healthcare Bio-Sciences Corp, Piscataway, NJ). Signals were visualized by exposing the membranes to X-ray films (BioMax MS-1; Eastman Kodak, Rochester, NY). Digital records were captured by

a Kodak 290 camera and protein bands were quantified using 1-D imager analysis software (Eastman Kodak). Bands were quantified as optical density (OD) X band area, normalized to β -tubulin bands, and expressed as arbitrary units.

Cell death ELISA and calculation of apoptotic index

Cytoplasmic protein extracts were used to quantify DNA fragmentation in all muscle samples using a commercially available ELISA kit (Cell Death Detection ELISA, Roche Diagnostics, Mannheim, Germany). Briefly, the wells of a 96-well plate were coated with a primary anti-histone mouse monoclonal antibody. Following the addition of 100 μ l of each sample, a secondary anti-DNA mouse monoclonal antibody coupled to peroxidase was added to each well. The substrate, 2,2'-azino-di-(3-ethylbenzthiazoline sulfonate) (ABST) was used to photometrically determine the amount of peroxidase retained in the immunocomplex. The color change of each well was determined at a wavelength of 405 nm using a Dynex MRX plate reader and computer software (Revelation, Dynatech Laboratories, CA). The resulting optical density (OD) was normalized to the protein concentration of each sample and recorded as the apoptotic index ($OD_{405} \text{ mg protein}^{-1}$).

Fluorometric caspase-activity assay

The activity of caspase-8 and caspase-3 was examined using commercially available fluorogenic substrates (caspase-8/10 AC-IETD-AMC, caspase-3 AC-DEVD-AFC, Alexis Biochemical, San Diego, CA). Previous research has demonstrated that embryonic, but not adult skeletal muscle, contains detectable levels of caspase-10, thus allowing the assumption that the caspase-8/10 substrate is specific to the activity of caspase-8 in this study [31]. To each well of a 96-well fluorescent microplate (Nalgene Nunc Int., Rochester, NY) was added 50 μ l of caspase activity buffer, 50 μ l of cytoplasmic protein without protease inhibitor from each muscle, and 10 μ l of substrate (1 mM). Samples were incubated at 37°C for 2-h with caspase activity accessed using a fluorescent microplate reader at the following wavelengths: caspase-8 excitation 380 nm and emission 460 nm; caspase-3 excitation 400 nm and emission 505 nm. Caspase activity was quantified by subtracting OD readings at time 2-h from the initial reading at time 0-h. The resulting optical density was normalized to the protein concentration of each muscle sample to provide a caspase activity index ($OD \cdot \text{mg protein}^{-1}$).

Statistics

Statistical analyses were performed using the SPSS software package, version 10.0. Data are presented as MEANS $\pm$ SE. Mean values from young adult and aged muscles were compared using independent *t*-tests with significance set a $p < 0.05$. Relationships between given variables were examined by computing the Pearson correlation coefficient.

Results

Bodyweight and muscle characteristics

Bodyweight was 47% greater in aged rats compared to young adult rats (Fig. 1(A)). Absolute plantaris and soleus muscle wet weights from aged animals were 21% and 15% less, respectively, than muscles from young adult animals (Fig. 1(B)). When expressed

relative to bodyweight, plantaris and soleus muscle weights from aged animals were 47% and 41% less, respectively, than muscles from young adult animals (Fig. 1(C)).

Apoptotic index

A cell death ELISA kit was used to quantify the incidence of DNA fragmentation in plantaris and soleus muscle homogenates. The apoptotic index was 600% greater in aged plantaris muscles and 300% greater in aged soleus muscles, when compared to muscles from young adult animals (Fig. 1(D)).

mRNA alterations in components of the death receptor pathway

The mRNA changes for TNF- α and the type I TNFR, as well as components known to be involved in the apoptotic signaling downstream of the TNFR, were analyzed in hindlimb muscles from young adult and aged rats to determine whether they were regulated at a transcriptional level. No differences in the mRNA concentration of TNF- α were observed when comparing plantaris and soleus muscles from young adult and aged rodents (Fig. 2(A)). However, mRNA for the type I TNFR was 43% and 60% greater in aged plantaris and soleus muscles, respectively, when compared to young adult muscles (Fig. 2(B)). The mRNA signal for TRADD was unchanged in young adult and aged plantaris and soleus muscles (Fig. 2(C)). In contrast, the mRNA for FADD was 50% and 94% greater in aged plantaris and soleus muscles, respectively, when compared to young adult muscles (Fig. 2(D)).

As a second approach, mRNA was measured by real-time quantitative PCR. Generally, there was greater variability in the genes of interest when assessed by the real-time method than in the semi-quantitative approach. In all cases, the changes in mRNA expression as assessed by 2^{-CT} did not reach significance. Specifically, the RQ values for each gene of interest were: TNF- α (young PL = 1.0 ± 0.19 ; aged PL = 0.23 ± 0.27 ; young SOL = 1.0 ± 0.17 ; aged SOL = 0.69 ± 0.34), TNFR (young PL = 1.0 ± 0.13 ; aged PL = 0.35 ± 0.32 ; young SOL = 1.0 ± 0.13 ; aged SOL = 1.0 ± 0.24), FADD (young PL = 1.0 ± 0.19 ; aged PL = 0.24 ± 0.23 ; young SOL = 1.0 ± 0.11 ; aged SOL = 0.47 ± 0.30), TRADD (young PL = 1.0 ± 0.11 ; aged PL = 0.39 ± 0.24 ; young SOL = 1.0 ± 0.15 ; aged SOL = 0.67 ± 0.27).

Protein alterations in components of the death receptor pathway

Based on the observed alterations in mRNA concentrations, western immunoblot analyses were performed for the TNFR and FADD as well as pro-apoptotic Bid and anti-apoptotic FLIP proteins. No differences were observed in the protein content for the soluble TNFR when comparing young adult and aged plantaris and soleus muscles (Fig. 3(A)). Consistent with mRNA changes as assessed by the semi-quantitative method, the protein content of FADD was greater in aged plantaris and soleus muscles when compared to young adult muscles (Fig. 3(B)). In a similar fashion, plantaris and soleus muscles from aged rodents had a higher protein content full-length Bid, when compared to young adult muscles (Fig. 3(C)). The anti-apoptotic protein, FLIP, was also greater in aged plantaris and soleus muscles when compared to young adult muscles (Fig. 3(D)).

Caspase enzymatic activity

Caspase-8 and caspase-3 specific fluorogenic substrates were utilized to quantify the enzymatic activity in plantaris and soleus muscle homogenates. Caspase-8 activity was 72% and 33% greater in aged plantaris and soleus muscles, respectively, when compared to young adult muscles (Fig. 4(A)). Similarly, caspase-3 activity was 27% and 20% greater in aged plantaris and soleus muscles, respectively, when compared to young adult muscles (Fig. 4(B)).

Correlation analyses

The changes in measures of apoptosis were examined for their correlation with the degree of plantaris and soleus muscle mass losses by calculating the Pearson correlation coefficient. The plantaris muscle weight was highly negatively correlated with the apoptotic index (Fig. 5(A)), especially when muscle weight was normalized to the rodent bodyweight (Fig. 5(B)). In contrast, only the normalized soleus weight was negatively correlated with the apoptotic index (Fig. 5(F)). The changes in caspase activity of the plantaris muscle were also highly correlated with the apoptotic index (Figs. 5(C) and (D)), especially the changes in caspase-8 activity. In contrast, there were no significant correlations between the changes in caspase activity of the soleus muscle and the apoptotic index (Figs. 5(G) and (H)).

Discussion

Apoptotic signaling downstream of the type I TNFR (i.e., extrinsic apoptotic pathway) has been shown in a variety of tissues, but it has not been studied in detail in skeletal muscle. Based on the well-documented increase in circulating TNF- α levels with aging [6–13] and increases in apoptosis of myonuclei in aged skeletal muscles, [18, 19, 22], we examined whether apoptotic signaling via the extrinsic pathway contributed to sarcopenia. Our data show that pro- and anti-apoptotic proteins in the extrinsic apoptotic pathway are affected by aging in fast (plantaris) and slow (soleus) skeletal muscles of rats.

Muscles from aged rats were significantly smaller and exhibited a larger incidence in fragmented DNA, which would indicate a higher level of nuclear apoptosis in these muscles. In addition, muscles from aged rodents had higher TNFR and FADD mRNA content (measured by semi-quantitative RT-PCR) and protein contents for FADD, Bid, and FLIP, and enzymatic activities of caspase-8 and caspase-3, when compared to muscles from young adult rodents. Although we observed an increase in mRNA expression for the TNFR as measured by the semi-quantitative approach, the protein content for the TNFR was unchanged in this study. This may be explained by the fact that the TNFR antibody utilized in western immunoblots recognizes the soluble form of the receptor. Thus, the changes in the membrane bound form of the receptor, measured by PCR, and the amount of the soluble TNFR may not be equivalent.

It was surprising that the data obtained using semi-quantitative RT-PCR to measure apoptotic transcripts were not completely consistent with those obtained by the real-time method we utilized in this study. It is possible that the changes in mRNA expression may have been below the detectable limit of the real-time PCR method, based on the fact that the

percent changes in mRNA obtained by semi-quantitative PCR were all less than 1-fold (40–95%). Nevertheless, the changes in protein content as determined from western blots and changes in enzyme activity clearly show that extrinsic apoptotic signaling was activated in muscles from old rats.

The pro-apoptotic changes observed in this study occurred in a similar fashion in both plantaris and soleus muscles, although strong relationships were observed between markers of apoptosis and muscle loss in the fast plantaris muscle that were not observed in the soleus. These data extend the previous demonstration that type II fibers are preferentially affected by aging and suggest that type II fiber containing skeletal muscles may be more susceptible to muscle mass losses via the extrinsic apoptotic pathway.

Effects of TNF- α in skeletal muscle

One potential mechanism contributing to the onset of sarcopenia may be the increase in circulating cytokines which activates the extrinsic apoptotic pathway. The circulating concentrations of specific cytokines have been shown to be elevated in the serum as a result of aging. In humans, serum levels of catabolic cytokines, such as TNF- α [7, 9, 12] and IL-6 [6, 11, 32], are increased in healthy elderly compared to young adults. Serum concentrations of TNF- α have been proposed as a prognostic marker of all cause-mortality in centenarians [7] and with age-associated pathology and mortality in 80-year old adults [9]. Several studies have also drawn associations between the increases in circulating cytokines and the sarcopenic process [11, 13]. Specifically, elevated circulating levels of TNF- α are associated with lower appendicular skeletal muscle mass [11] and reduced knee extensor and grip strength [13]. Because we have found activation of the extrinsic apoptotic signaling pathway in muscles of old rats, we speculate that circulating TNF- α may be the initiator of this pathway in skeletal muscle. Nevertheless, we cannot rule out the possibility that other pathways that we did not examine may have been activated by circulating TNF- α in aging muscles. For example, TNF- α has been shown to directly promote protein degradation [33–36] and apoptosis [37] within skeletal muscle. Furthermore, intravenous injection of recombinant TNF- α increases protein degradation in rat skeletal muscles and this is associated with the increased activity of the ubiquitin-dependent proteolytic pathway [33, 34, 36, 38]. In addition, elevated TNF- α concentrations in cell culture for 24–48 h increases apoptosis in skeletal myoblasts as determined by DNA fragmentation [39, 40]. A reduction of procaspase-8 occurs within 6 h of incubating myoblasts *in vitro* with recombinant TNF- α , suggesting a TNF- α mediated cleavage and activation of this initiator caspase in myoblast cultures [41].

The effects of TNF- α on apoptosis are not limited to *in vitro* conditions, because a systemic elevation of TNF- α *in vivo* increases DNA fragmentation within rodent skeletal muscle [37]. Based on the observation in the current study that TNF- α mRNA was not different between muscles from young adult and aged rats, it is reasonable to assume that muscle-derived TNF- α does not act in an autocrine manner to stimulate the pro-apoptotic signaling observed in this study. Our data are consistent with the hypothesis that the well-documented systemic elevation of TNF- α with age, may increase the likelihood of ligand binding to the TNFR and stimulate apoptotic signaling of the extrinsic pathway downstream of the TNFR and

contribute to sarcopenia in skeletal muscle of old rats. Although this hypothesis was not directly examined in this study our data show that pro-apoptotic proteins associated with the extrinsic apoptotic pathway were increased in muscles of old animals as compared to muscles in young adult animals.

Apoptotic changes in aged skeletal muscle

Pro-apoptotic signaling is an active process in skeletal muscle during periods of reduced muscle activity (hindlimb suspension, denervation). Apoptosis is an early occurring event that can contribute to muscle atrophy at later time points [22]. Furthermore, nuclear apoptosis is a part of the normal aging process (reviewed in [42–44]) and is a contributing mechanism of muscle mass losses with aging. Dirks and Leeuwenburgh [14] measured mono- and oligonucleosome fragmentation and reported that apoptosis was elevated 50% in aged muscles compared to young adult. Hindlimb suspension (HS) in rodents has also been used to examine the effect of superimposing a muscle atrophic stimulus (i.e. – disuse) in aged skeletal muscle [15, 17, 19]. A common finding is that apoptotic signaling differs when comparing muscles from aged rodents to muscles from young adult rodents following periods of HS. Increases in apoptotic nuclei, as well as in molecular markers of the intrinsic apoptotic pathway (Bax, Bcl-2, Apaf-1, AIF, caspase-9, and caspase-3) were evident in aged muscles, supporting the hypothesis that an environment exists in aged skeletal muscle that favors apoptosis. The current study extends these results by showing that apoptotic signaling via the extrinsic pathway is active in aged skeletal muscles but not in muscles from young adult animals.

Muscles from aged animals also up-regulate anti-apoptotic proteins. Data from our lab [17, 19] and others [45] have consistently shown an increase in the protein content for Bcl-2 following disuse and in aged muscles, with further increases when disuse is superimposed with aging. This led to our hypothesis that aged muscles attempt to counteract pro-apoptotic stimuli by up-regulating anti-apoptotic proteins. The data from the current study is consistent with this hypothesis by showing that muscles from aged rodents up-regulate the anti-apoptotic protein, FLIP. FLIP protein can attenuate apoptotic signaling downstream of death receptors by inhibiting the cleavage of procaspase-8 [46–49]. However, despite these aging-related adaptations, the up-regulation of FLIP and Bcl-2 proteins appears to be ineffective in attenuating myonuclear loss in aged muscles.

Type II muscle fibers and aging

Analyses from human muscle biopsy samples have consistently demonstrated that type II fibers are more susceptible to aging-related atrophy when compared to type I fibers [3, 50]. Doherty [51] reported that the loss of type II fiber area was 20–50%, whereas type I area loss was much less (1–25%). Part of the loss of type II fiber size and number has been attributed to denervation/reinnervation which is a contributing factor of sarcopenia [51]. An alternative mechanism as suggested by the current data is that type II fibers (i.e., the plantaris muscle) may be more susceptible to age associated atrophy and nuclear loss as a result of pro-apoptotic signaling via the extrinsic pathway. Nevertheless, we cannot rule out the possibility that apoptotic signaling may be muscle and not fiber-type specific.

Our observations of potential muscle and/or fiber type specific differences in TNF- α associated apoptotic signaling differs from data reported by Phillips and Leeuwenburgh [25], who did not find differences in the expression of apoptotic markers between the soleus (primarily a type I fibered muscle) and the superficial vastus lateralis muscles (primarily a type II fibered muscle) of aged rats. These disparate findings may be due to the different strains and ages of rodents utilized in our study and the previous study [25]. Despite these differences, the data from our study and that of Phillips and Leeuwenburgh [25] suggest that the extrinsic apoptotic pathway is active in skeletal muscle of aged rodents and this may contribute to age-associated muscle loss via increases in apoptotic signaling.

Skeletal muscle function is reduced and the extent of hypertrophy to loading is reduced with aging. However we do not know the extent to which nuclear apoptosis initiated through the extrinsic pathway, might contribute to a reduction in muscle force or an attenuation of hypertrophic adaptations in aging. Our laboratory has previously reported the reduced force production and prolonged contractile properties in muscles of aged animals [52–54]. Maximal tetanic muscle force is reduced by more than 30% between the ages of 9 and 26 months of age in Fischer₃₄₄ $\times$ Brown Norway rats as used in the present study [54]. Aging also attenuates the hypertrophic response of the plantaris muscle in these rats [54, 55]. Recent evidence suggests that muscle function is impaired when circulating TNF- α levels are elevated. When extensor digitorum longus (EDL) muscles from guinea pigs were exposed to recombinant TNF- α (5 and 10 ng $\cdot$ ml⁻¹), the force-frequency relationship was shifted down and to the right indicating a reduced force output at specific frequencies of activation [56]. Furthermore, when coronary heart failure is induced in experimental animals, circulating TNF- α is elevated above control levels, and this is associated with reduced absolute force output as well as an increase in the incidence of apoptosis in the tibialis anterior muscle. These data demonstrate that not only is muscle function impaired with aging, but that TNF- α may also impair muscle function when circulating levels are elevated [20].

Pro-apoptotic signaling cross-talk

Cross-talk between extrinsic and intrinsic apoptotic pathways was recently reviewed [26]. Cross-talk between these pathways is the result of the cleavage of the pro-apoptotic BCL-2 family member Bid. Cleaved and activated caspase-8 cannot only serve to activate caspase 3, which is the executioner caspase, but also cleave full-length Bid into a truncated version (tBid) [57]. tBid then interacts with pro-apoptotic Bax, to stimulate apoptotic signaling from the mitochondria [58]. As has been previously shown, apoptotic signaling from the mitochondria stimulates cleavage of procaspase-9, which then serves to activate caspase-3 [59]. Thus, both the extrinsic and intrinsic apoptotic pathways converge on caspase-3, which then fully engages pro-apoptotic signaling.

Certain cell types seem to be more susceptible to proapoptotic signaling via the extrinsic pathway without involving the mitochondria. These cell types, termed type I cells, differ from type II cells based on the accumulation of DISC following ligand binding [60]. Upon ligand binding, type I cells accumulate a larger amount of DISC on the cytoplasmic side of the death receptor, and do not require additional pro-apoptotic signaling arising from the

mitochondria, to fully activate apoptosis. In contrast, type II cells accumulate considerably less DISC and require mitochondrial signaling to fully activate an apoptotic program. The current study as well as data from Nagaraju et al. [49] suggest that skeletal muscle cells may act as type II cells, in that apoptotic signaling arising from the death receptor can include subsequent mitochondrial apoptotic signaling through Bid activation. Future studies should directly address the ability of truncated Bid to mediate messages from the death receptor to the mitochondria. Nevertheless, in the current study, skeletal muscles from aged rodents contained a greater protein expression of full-length Bid, which raises the possibility that cross talk between the extrinsic pathway and the intrinsic pathway may occur in aged skeletal muscles (Fig. 6).

Conclusions

The results of the current study demonstrate that proapoptotic signaling downstream of the type I TNFR resulting in caspase-8 and caspase-3 activation, is active in aged skeletal muscles and is a contributing mechanism of sarcopenia. Secondly, although no differences were observed in the pattern of expression of apoptotic markers between the plantaris and soleus muscles, the changes in these markers and muscle loss were highly correlated in the plantaris muscle only. These data suggest that type II fiber containing muscles may be more susceptible to apoptotic signaling via the extrinsic pathway. Third, aged skeletal muscles undergo an adaptation in which anti-apoptotic proteins such as FLIP and Bcl-2 are increased, possibly in an effort to counteract pro-apoptotic signaling. Lastly, because skeletal muscles from aged rodents contained a greater protein content of full-length Bid than muscles of young adult rats, it is possible that that cross talk between the extrinsic pathway and the intrinsic pathway may occur in aged skeletal muscles (Fig. 6). Collectively, these data indicate that a pro-apoptotic environment exists in aged skeletal muscles that can contribute to sarcopenia.

Acknowledgments

The study was supported by the National Institutes of Health, National Institute on Aging Grant RO1 AGO21530 and the American College of Sports Medicine Doctoral Student Research Grant FRG-14. The authors would like to thank Dr. Richard Dey, Dr. Tom Batchelor, and Dr. Dawn Hunter for their assistance with the real-time PCR experiments and data analysis.

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Fig. 1.

Rodent and skeletal muscle characteristics. Rodent bodyweight (A), as well as absolute (B) and normalized (C) muscles weights and incidence of apoptosis (D) was determined for plantaris and soleus muscles. Data presented as means $\pm$ SE. * Significant effect of age ($p < 0.05$). PL-plantaris, SOL-soleus

Fig. 2.

RT-PCR analysis of muscle mRNA. The mRNA content for TNF- α (A), TNFR (B), TRADD (C), and FADD (D) was determined by RT-PCR, with PCR products normalized to the 18S gene. Representative agarose gel images following electrophoresis are displayed for each group. Data are presented as means $\pm$ SE. * Significant effect of age ($p < 0.05$)

Fig. 3.

Western-immunoblot analyses. The protein content for the soluble TNFR (A), FLIP (B), Bid (C), and FADD (D) was determined by western immunoblot. Representative immunoblots for TNFR, FADD, BID, and FLIP are displayed for each graph. * Significant effect of age ($p < 0.05$). PL-plantaris, SOL-soleus

Fig. 4.

Caspase enzymatic activities. The caspase activity of caspase-8 (A) and caspase-3 (B) was measured using specific fluoremetric substrates. Data are presented as means $\pm$ SE. * Significant effect of age ($p < 0.05$)

Fig. 5.

Correlational analyses in plantaris and soleus muscles, plantaris weight and the apoptotic index. The relationships between muscle phenotypes and apoptotic indicators were determined by calculating the Pearson correlation coefficient. The following relationships were analysed: absolute plantaris weight and the apoptotic index (A), normalized plantaris weight and the apoptotic index (B), plantaris caspase-8 activity and the apoptotic index (C), plantaris caspase-3 activity and the apoptotic index (D), absolute soleus weight and the apoptotic index (E), normalized soleus weight and the apoptotic index (F), soleus caspase-8

activity and the apoptotic index (G), soleus caspase-3 activity and the apoptotic index (sh=squ). = young adult samples, [$\diamond$] = aged samples

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Cross-Talk Apoptotic Pathways

Fig. 6.

Cross-talk between apoptotic pathways. The extrinsic apoptotic pathway, downstream of the type I TNFR, is active in aged skeletal muscle and consists of FADD, caspase-8, and caspase-3. Cleaved caspase-8 can activate BID, which can interact with Bax and promote further pro-apoptotic signaling arising from the mitochondria

Table 1

Primers used for semi-quantitative RT-PCR amplification of cDNA

Product	Accession no.	Sequence	Position	T_A (°C)	PCR Length (bp)	Restriction enzyme	Restriction products (bp)
TNF- α	NML012675	F: 5'-CGGGGGCCACCACGCTCTTCTGT-3'	125-147			<i>XhoI</i>	222, 134
		R: 5'-TCGGCTGACGGTGTGGGTGAGGA-3'	458-480	62.0	356	<i>BglII</i>	222, 134
TNFR	M63122	F: 5'-CCACCTGGTCCGATCGTCTTAC-3'	138-159			<i>DraIU</i>	282, 190
		R: 5'-CACGGTGTCCATGTCAGCTTTG-3'	588-609	58.5	472		
FADD	NM-152937	F: 5'-TGGCCTGGACCTGTCTCTCG-3'	186-204			<i>BglIII</i>	350, 163
		R: 5'-CGGGCTTGTCAAGGTTGTTTC-3'	679-698	59.5	513	<i>PstI</i>	405, 108
TRADD	AF517017	F: 5'-TGAGCTCTGCAAACTGACGTGT-3'	12-33			<i>PstI</i>	237, 66
		R: 5'-CCATCACGGCTCATACTCATAGG-3'	292-314	59.1	303		

T_A , annealing temperature; bp, base pair; TNF- α , tumor necrosis factor- α ; TNFR, tumor necrosis factor receptor; FADD, Fas-associated death domain protein; TRADD, TNF receptor associated death domain protein.