Published in final edited form as:

Mech Ageing Dev. 2008 September ; 129(9): 542–549. doi:10.1016/j.mad.2008.05.005.

Age-related activation of mitochondrial caspase-independent apoptotic signaling in rat gastrocnemius muscle

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Abstract

Mitochondria-mediated apoptosis represents a central process driving age-related muscle loss. However, the temporal relation between mitochondrial apoptotic signaling and sarcopenia as well as the regulation of release of pro-apoptotic factors from the mitochondria has not been elucidated. In this study, we investigated mitochondrial apoptotic signaling in skeletal muscle of rats across a wide age range. We also investigated whether mitochondrial-driven apoptosis was accompanied by changes in the expression of Bcl-2 proteins and components of the mitochondrial permeability transition pore (mPTP). Analyses were performed on gastrocnemius muscle of 8-, 18-, 29- and 37-month-old male Fischer₃₄₄×Brown Norway rats (9 per group). Muscle weight declined progressively with advancing age, concomitant with increased apoptotic DNA fragmentation. Cytosolic and nuclear levels of apoptosis inducing factor (AIF) and endonuclease G (EndoG) increased in old and senescent animals. In contrast, cytosolic levels of cytochrome c were unchanged with age. Mitochondrial Bcl-2, Bax and Bid increased dramatically in 37-month-old rats, with no changes in the Bax/Bcl-2 ratio in any of the age groups. Finally, expression of cyclophilin D was enhanced at very old age. Our findings indicate that the mitochondrial caspase-independent apoptotic pathway may play a more prominent role in skeletal muscle loss than caspase-mediated apoptosis.

Keywords

sarcopenia; apoptosis; permeability transition pore; AIF; endonuclease G

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Introduction

The age-related loss of muscle mass, strength and quality, referred to as sarcopenia, is a common feature of aging and is characterized by a decline of both the number and size of muscle fibers, with a preferential loss of type II (fast-twitch) fibers (Larsson et al. 1978). Progressive demise of myonuclei via an apoptosis-like process (myonuclear apoptosis) is deemed as a fundamental mechanism driving sarcopenia (Dirks and Leeuwenburgh 2002; Marzetti and Leeuwenburgh 2006; Pistilli et al. 2006a; Marzetti et al. 2008a; Marzetti et al. 2008b). Mitochondria are centrally involved in this process, being at the same time the repository and target of several apoptotic mediators. Noticeably, mitochondria can induce apoptosis in both a caspase-dependent and independent manner (van Gurp et al. 2003). Upon apoptotic stimuli, mitochondrial outer membrane permeabilization (MOMP) can occur, followed by release of cytochrome *c*, which initiates the intrinsic pathway of apoptosis. Once in the cytosol, cytochrome *c* promotes oligomerization of apoptosis protease activating factor-1 (Apaf-1) in the presence of ATP/dATP. The resulting apoptosome activates caspase-9, which in turn engages caspase-3. In addition, other apoptogenic factors residing in the mitochondrial intermembrane space (IMS), such as apoptosis inducing factor (AIF) and endonuclease G (EndoG), can be released into the cytosol, translocate to the nucleus and cleave DNA independent of caspase activation.

Despite the central role of mitochondria in cell death, the exact mechanisms whereby mitochondrial apoptogenic factors are released have yet to be elucidated. However, substantial evidence indicates that the Bcl-2 family proteins and the so-called mitochondrial permeability transition pore (mPTP) are involved in this process. The Bcl-2 family members can either promote (e.g., Bax, Bak, Bid, Bad and Bim) or prevent (e.g., Bcl-2 and Bcl-x_L) MOMP (Chao and Korsmeyer 1998). In particular, levels of pro-apoptotic Bax and anti-apoptotic Bcl-2 (and their ratio) play a pivotal role in regulating mitochondrial membrane stability and hence cell fate. Following an apoptotic stimulus, Bax inserts into the mitochondrial outer membrane (OM) and oligomerizes, forming a pore that allows the efflux of apoptogenic factors. This process is promoted by Bid, which induces a conformational change of Bax allowing it to oligomerize and integrate in the OM (Eskes et al. 2000). Bcl-2, which is constitutively anchored to the OM, prevents oligomerization of membrane-bound Bax, thus inhibiting apoptosis.

The mPTP is a protein complex comprising the voltage-dependent anion channel (VDAC) in the OM, the adenine nucleotide translocator (ANT) in the inner membrane (IM), and cyclophilin D (CyPD) in the matrix (Crompton 1999). Under pathological conditions (e.g., oxidative stress), ANT modifies its function and interacts with VDAC, generating an unselective pore spanning the IM and OM (Tsujimoto and Shimizu 2007). This interaction is stabilized by CyPD. Opening of the mPTP causes a sudden increase in membrane permeability to solutes with molecular weight up to 1,500 Da. This may result in collapse of membrane potential, mitochondrial swelling and rupture of the OM (Tsujimoto and Shimizu 2007). This series of events has been associated with the execution of both apoptotic and necrotic cell death (Grimm and Brdiczka 2007). However, it is unclear whether mPTP opening is always required for MOMP to occur (Baines et al. 2005). Interestingly, cross-talk between mPTP and Bcl-2 proteins has been reported. In fact, Bid and Bax have been shown to promote mPTP opening (Marzo et al. 1998; Zamzami et al. 2000), whereas anti-apoptotic members of the Bcl-2 family (e.g., Bcl-2 and Bcl-x_L) exert an inhibitory effect (Zamzami et al. 1998). Opening of the mPTP is provoked by several factors, including elevated levels of oxidative (Rajesh et al. 2003) and nitrosative stress (Marriott et al. 2004; Zhang et al. 2006). Furthermore, it has been shown that enhanced production of reactive oxygen (ROS) and nitrogen species (RNS) may induce a pro-apoptotic shift of the pattern of expression of Bcl-2 proteins (e.g., increased Bax to Bcl-2 ratio) (Savory et al. 1999; Mishra et al. 2006; Qin et al. 2006). Notably, mitochondrial membrane polyunsaturated fatty acids are especially prone to ROS-mediated damage, and therefore are

highly susceptible to peroxidation (Esterbauer et al. 1991). Among the reactive aldehydes stemming from lipid peroxidation, 4-hydroxy-2-nonenal (HNE) has been shown to interfere with mitochondrial function (Chen et al. 2001) and to promote mPTP opening (Vieira et al. 2001). ROS can also elicit RNS production (Zhang, Li, Prabhakaran, Borowitz, and Isom 2006), via up-regulation of the inducible isoform of nitric oxide synthase (iNOS) (Zhen et al. 2008). Chronic overproduction of RNS can lead to irreversible nitration of proteins, with an apparent selectivity for those of mitochondrial origin (Aulak et al. 2001), and to enhanced production of ROS (Poderoso et al. 1996). The resulting vicious cycle may eventually cause mitochondrial dysfunction, imbalance of Bcl-2 proteins, mPTP opening and cell death.

Based on these premises, we investigated whether mitochondrial apoptotic signaling and expression levels of mPTP components increased over the course of the aging process in rat gastrocnemius muscle. We further investigated whether changes in the expression of Bcl-2 family proteins and enhanced oxidative and nitrosative stress were associated with mitochondria-driven apoptosis.

Methods

Animals

Thirty-six 8-, 18-, 29- and 37-month-old male Fischer₃₄₄ × Brown Norway (F344×BNF1) hybrid rats (9 per group) were purchased from the National Institute on Aging colony at Harlan Industries (Indianapolis, IN). This strain of rat was chosen because of its increased longevity (median life span: 33.3 months; maximum life span: 40 months) and decreased cumulative lesion incidence compared to other strains (Lipman et al. 1996). Furthermore, F344×BNF1 rats display an age-related muscle mass decline resembling that observed in humans (Rice et al. 2005). The ages were chosen from published growth and survival data (Turturro et al. 1999) to reflect a young age (8 months), adulthood (18 months), advanced age (29 months) and senescence (37 months). Rats had free access to NIH-31 average nutrient composition pellets and tap water. Animals were individually housed and maintained on a 12-hour light/dark cycle, at constant temperature and humidity, in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Health status, body weight and food intake were monitored daily. All procedures were in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and reviewed and approved by the University of Florida's animal care and use committee before commencement.

Preparation of subcellular fractions and whole cell homogenates

Rats were sacrificed by rapid decapitation and the gastrocnemius muscle removed, trimmed of adipose tissue and tendons, and weighed. Immediately afterward, muscles were snap-frozen in liquid nitrogen and subsequently stored at −80°C.

For subcellular fractionation, ~250 mg of the left medial gastrocnemius muscle were minced in 1:5 w/v ice-cold lysis buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EDTA, and 0.2% fatty acid-free bovine serum albumin, 0.01% protease inhibitor cocktail, pH 7.4), homogenized on ice in a glass-glass Dull homogenizer and centrifuged for 10 min at 1,000 × g at 4°C to pellet cellular debris and nuclei. The supernatant was collected and further centrifuged for 15 min at 14,000 × g at 4°C. The resulting supernatant, representing the mitochondria-free cytosolic fraction was collected, aliquoted and stored at −80°C for later biochemical analyses. The mitochondrial pellet was resuspended in 1.5 mL ice-cold wash buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EDTA, pH 7.4) and centrifuged for 15 min at 14,000 × g at 4°C. The supernatant was decanted and the mitochondrial pellet resuspended in 50 µL storage buffer (250 mM sucrose, 1 mM EDTA, pH

7.4) and stored at -80°C . For the preparation of nuclear extracts, nuclear pellets were resuspended in 400 μL nuclear extraction buffer (20 mM HEPES, 25% glycerol, 500 mM, NaCl, 10 mM MgCl_2 , 0.1% Triton X-100, 0.2 mM EDTA, 0.05 mM dithiothreitol, 0.2 mM phenylmethanesulfonyl fluoride, 0.01% protease inhibitor cocktail, pH 8.0) and centrifuged for 5 min at $3,000 \times g$ at 4°C . The resulting supernatant was transferred into a 10 KDa nominal molecular weight limit Amicon Ultra-4 Centrifugal Filter Unit (Millipore, Bedford, MA, USA) and centrifuged for 30 min at $4,500 \times g$ at 4°C . The concentrated nuclear extracts were then aliquoted and stored at -80°C .

Whole cell homogenates were prepared as previously described (Marzetti, Groban, Wohlgemuth, Lees, Lin, Jobe, Giovannini, Leeuwenburgh, and Carter 2008a), with minor modifications. Briefly, ~ 100 mg of the left medial gastrocnemius muscle were pulverized under liquid nitrogen with a porcelain mortar and pestle. The powder was suspended in 400 μL ice-cold lysis buffer (250 mM sucrose, 10 mM Tris-HCl, 1 mM EDTA, 2% SDS, 0.01% protease inhibitor cocktail, pH 6.5), vortexed for 30 seconds and centrifuged at $12,000 \times g$ at 4°C for 10 minutes to pellet residual cellular debris. The supernatant was collected, aliquoted and stored at -80°C .

Protein concentration in the cytosolic and mitochondrial fractions was determined by the method developed by Bradford (Bradford 1976), whereas the detergent-compatible D_C assay (BioRad, Hercules, Ca) was employed for nuclear and tissue extracts.

Western blot analysis

Prior to loading, samples were boiled at 95°C for 5 minutes in Laemmli buffer (62.5 mM Tris.HCl, 2% SDS, 25% glycerol, 0.01% Bromophenol Blue, pH 6.8; BioRad) with 5% β -mercaptoethanol. Proteins were separated by using 7.5% and 15% pre-cast Tris-HCl gels (BioRad). Quantification of mitochondrial VDAC, ANT, CyPD, Bcl-2, Bax and Bid was achieved by loading 20 μg of protein, whereas 120 μg were used to assess cytosolic cytochrome *c*, active caspase-9, and cytosolic and nuclear AIF and EndoG. Separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon P, 0.45 μm , Millipore, Billerica, MA) using a semidry blotter (BioRad). Transfer efficiency was verified by staining the gels with GelCode Blue Stain Reagent (Pierce Biotechnology, Rockford, IL) and membranes with Ponceau S (Sigma-Aldrich, St. Louis, MO). Ponceau S staining of total protein was also used as a loading control. For the analysis of cytosolic cytochrome *c* and cytosolic and nuclear AIF and EndoG, the Vectastain ABC-AmP immunodetection kit (Vector Laboratories, Burlingame, CA) was used, according to the manufacturer's instructions. For all other immunoblots, membranes were blocked in 2% casein with 0.05% Tween 20 for 1 h at room temperature, washed in Tris-buffered saline (TBS), and incubated overnight in the corresponding primary antibody at 4°C . Subsequently, membranes were washed in TBS with 0.05% Tween 20 (TBS-t) and incubated with alkaline phosphatase-conjugated secondary antibody (Sigma-Aldrich), 1:30,000, at room temperature for 1 h. Membranes were then washed in TBS-t, rinsed in TBS, and washed in Tris-HCl (100 mM, pH 9.5). Finally, the DuoLux chemiluminescent/fluorescent substrate for alkaline phosphatase (Vector Laboratories) was applied, and the chemiluminescent signal captured with an Alpha Innotech Fluorchem SP imager (Alpha Innotech, San Leandro, CA). The digital images were analyzed using AlphaEase FC software (Alpha Innotech). Spot density of the target band was normalized to that of the most prominent band on the corresponding Ponceau S-stained membrane and expressed as arbitrary optical density (OD) units. The following primary antibodies and relative dilutions were used: mouse monoclonal anti-VDAC (Mitosciences, Eugene, OR), 1,000; mouse monoclonal anti-ANT (Mitosciences), 1,000; mouse monoclonal anti-CyPD (Mitosciences), 1,000; rabbit polyclonal anti-Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA) 1:200; rabbit polyclonal anti-Bax (Santa Cruz Biotechnology), 1:200; rabbit polyclonal

anti-Bid (Santa Cruz Biotechnology), 1:200; rabbit polyclonal anti-cytochrome *c* (Santa Cruz Biotechnology), 1:200; rabbit polyclonal anti-active caspase-9 (Santa Cruz Biotechnology), 1:200; rabbit polyclonal anti-EndoG (Abcam, Cambridge, MA), 1:1,000; rabbit polyclonal anti-AIF (BD PharMingen, San Diego, CA), 1:500.

Dot blot analysis for nitrotyrosine and HNE

For the determination of nitrotyrosine and HNE-adducts, 3 and 5 μ L of gastrocnemius homogenate, respectively, were loaded onto a nitrocellulose membrane (BioRad). Membranes were air-dried for 20 min and blocked in 2% casein with 0.05% Tween 20 for 1 h at room temperature. Membranes were subsequently incubated in primary antibody for 30 min at room temperature and washed three times in TBS-t. The following primary antibodies and relative dilutions were used: mouse monoclonal anti-3-nitrotyrosine (Santa Cruz Biotechnology), 1:200; goat polyclonal anti-HNE (Alpha Diagnostic, San Antonio, TX), 1:500. Secondary antibody incubation was carried out for 30 min at room temperature, using anti-mouse (Sigma-Aldrich, 1:30,000) and anti-goat (Santa Cruz, 1:5,000) alkaline phosphatase-conjugated secondary antibodies. Membranes were washed in TBS-t, rinsed in TBS, and washed in Tris-HCl (100 mM, pH 9.5). Generation of the chemiluminescent signal, digital acquisition and densitometry analysis were performed as described above. Density of the target spot was normalized to total protein concentration as determined by the D_C assay and expressed as arbitrary OD units % of total protein.

Cell death ELISA for determination of the apoptotic index

The extent of apoptotic DNA fragmentation (apoptotic index) was quantified by measuring the amount of cytosolic mono- and oligonucleosomes (180 base pair nucleotides or multiples) using an enzyme-linked immunosorbent assay (ELISA) kit (Cell Death Detection ELISA; Roche Diagnostics, Mannheim, Germany), following the manufacturer's instructions. Briefly, cytosolic extracts were incubated in a microtitre plate coated with primary mouse monoclonal anti-histone, followed by the addition of peroxidase-conjugated anti-DNA antibody. The amount of peroxidase retained in the immunocomplex was determined spectrophotometrically with ABTS (2,2'-azino-di-[3-ethylbenzthiazoline sulfonate]) as a substrate. Absorbance was read at 405 nm using a Synergy HT Multi-Detection microplate reader (BioTek, Winooski, VT) and reported as arbitrary OD units/mg protein.

Statistical analysis

Statistical analysis was performed using GraphPrism 4.0.3 software (GraphPad Software, Inc., San Diego, CA). Differences among experimental groups for normally distributed variables were determined by one-way ANOVA followed by Tukey's post hoc test when applicable. Kruskal-Wallis H (with Dunns' post test, when applicable) was used to assess differences for not normally distributed data. Pearson's test (or Spearman's, when appropriate) was used to explore correlations between variables. All tests were two-sided, with significance set at $p < 0.05$. All data are reported as mean $\pm$ SEM.

Results

Morphological characteristics

Body weight increased from 8 to 29 months of age and declined thereafter (Table 1). Gastrocnemius muscle wet weight displayed a slight, yet significant increase from 8 to 18 months and declined thereafter, with an abrupt loss from 29 to 37 months ($>170\%$, Table 1), indicating the development of substantial sarcopenia. Interestingly, the decline of gastrocnemius weight relative to body weight (MW/BW) was already evident at 18 months of

age and progressed until 37 months. Similar to the absolute muscle weight, MW/BW declined dramatically between 29 and 37 months of age (>120%, Table 1).

Western blot analysis of mPTP components and Bcl-2 family proteins

Opening of the mPTP and imbalance between pro- and anti-apoptotic Bcl-2 family proteins (e.g., increased Bax to Bcl-2 ratio) have been identified as fundamental mechanisms leading to MOMP and subsequent release of apoptogenic factors (Chao and Korsmeyer 1998; Tsujimoto and Shimizu 2007). Therefore, we investigated whether advancing age was associated with changes in the composition of the mPTP and in the mitochondrial pattern of expression of Bcl-2 proteins. Protein levels of VDAC and ANT were not affected by age (Table 2). However, we detected an upward trend for CyPD with advancing age ($p=0.057$, 8-mo vs. 37-mo) (Table 2). Furthermore, advanced age was associated with increased expression of CyPD relative to ANT and VDAC (Fig. 1a–b). Mitochondrial content of Bcl-2, Bax and Bid remained unchanged between the ages of 8 and 29 months, after which it increased significantly (Fig. 2a–c). Because mitochondrial content of Bcl-2 and Bax increased to a similar extent, Bax to Bcl-2 ratio remained constant over the course of aging (Fig. 2d).

Western blot analysis of mitochondrial apoptotic signaling proteins

Release of apoptogenic proteins from the mitochondrial IMS represents a critical step in the execution of the intrinsic pathway of apoptosis. Once in the cytosol, cytochrome *c* initiates the caspase-dependent apoptotic pathway, whereas AIF and EndoG induce cell death independent of caspase activation (Marzetti, Lawler, Hiona, Manini, Seo, and Leeuwenburgh 2008b). Cytosolic cytochrome *c* content, as determined by Western blot analysis, was unchanged among the experimental groups (Fig. 3a). However, we detected an increased expression of active caspase-9 at 29 months of age, which was no longer evident in 37-month-old rats (Fig. 3b). In contrast, cytosolic levels of AIF increased significantly at 29 months of age compared to the young controls. A further increase was evident at the age of 37 months (Fig. 4a). Similarly, nuclear levels of AIF were increased at 29 months of age compared to the young rats and remained elevated in senescent animals (Fig. 4b). Cytosolic content of EndoG was increased in 29-month-old rats compared to both 8- and 18-month-old animals and remained elevated in senescent rats (Fig. 4c). A similar pattern was evident for the nuclear levels of EndoG (Fig. 4d).

Dot blot analysis for the quantification of lipid peroxidation and nitrosative stress

Elevated levels of oxidative and nitrosative stress have been shown to promote mPTP opening (Rajesh, Sasaguri, Suzuki, and Maeda 2003; Marriott, Ali, Read, Mitchell, Whyte, and Dockrell 2004; Zhang, Li, Prabhakaran, Borowitz, and Isom 2006) and to induce a pro-apoptotic shift of the mitochondrial Bcl-2 family pattern (Savory, Rao, Huang, Letada, and Herman 1999; Mishra, Randis, Ashraf, and Iorio-Papadopoulos 2006; Qin, Yan, Patel, Liu, and Dong 2006). Therefore, we determined tissue levels of lipid peroxidation and nitrosative stress by assaying for HNE-modified proteins and 3-nitrotyrosine, respectively. Both these markers were unchanged between 8 and 29 months of age, but increased significantly in 37-month-old rats (Fig. 5a–b).

Apoptotic index

Internucleosomal DNA fragmentation represents a hallmark of apoptotic cell death. A sandwich ELISA was employed to quantify the amount of cytosolic mono- and oligonucleosomes (180 base pair nucleotides or multiples). Apoptotic DNA fragmentation increased at 29 months of age compared to 8- and 18-month-old rats (Fig. 6). A further increase was observed in the 37-month-old group.

Correlation analyses

Pearson's tests (or Spearman's, when necessary) were run to explore correlations between experimental variables. Gastrocnemius muscle weight either absolute or normalized to body weight was negatively correlated with the extent of apoptotic DNA fragmentation (Fig 7a–b). Additionally, positive correlations were found between nuclear content of AIF and EndoG and the extent of DNA fragmentation (Fig 7c–d). On the contrary, no significant correlation was detected between levels of active caspase-9 and the apoptotic index (Pearson's r : 0.13, $p=0.48$; data not shown). Finally, MW/BW was negatively correlated with nuclear levels of AIF and EndoG (Fig 7e–f).

Discussion

Several studies have indicated that mitochondrial-driven apoptosis may play a central role in the pathogenesis of age-related sarcopenia (Dirks and Leeuwenburgh 2004; Leeuwenburgh et al. 2005; Baker and Hepple 2006; Pistilli et al. 2006b). However, the temporal relation between mitochondria-mediated apoptosis and sarcopenia has been sparsely studied (Rice and Blough 2006; Baker and Hepple 2006). Similarly, age-related changes in mPTP composition have only recently been investigated (Chabi et al. 2008). Here we show that apoptotic DNA fragmentation increases progressively over the course of aging in rat gastrocnemius muscle, paralleling the loss of muscle mass. Furthermore, we found that cytosolic and nuclear levels of the caspase-independent apoptotic mediators AIF and EndoG increased with age. A positive correlation was evident between nuclear AIF and EndoG and DNA fragmentation. Furthermore, nuclear levels of both these markers were negatively correlated to muscle weight. On the contrary, cytosolic content of cytochrome *c* remained unchanged over the course of aging. Levels of active caspase-9 were elevated in the old, but not in senescent rats, and were not correlated with the apoptotic index. We therefore suggest that mitochondrial caspase-independent apoptotic signaling may play a more prominent role in age-related muscle wasting compared to the caspase-dependent pathway. Moreover, we demonstrate that the ratio between CyPD and ANT and between CyPD and VDAC is increased in senescent rats, consistent with a previous report on denervated skeletal muscle (Csukly et al. 2006). Our finding underlines the major role postulated for CyPD in the formation of the mPTP, and might suggest a higher susceptibility to mPTP opening at older age. Recent studies demonstrated that formation of the mPTP and apoptotic cell death could still occur in the absence of either ANT (Kokoszka et al. 2004) or VDAC (Baines et al. 2007). However, overexpression of CyPD induced mitochondrial swelling and spontaneous cardiac apoptosis, whereas mice lacking cardiac CyPD were protected from ischemia/reperfusion-induced cell death (Baines, Kaiser, Purcell, Blair, Osinska, Hambleton, Brunskill, Sayen, Gottlieb, Dorn, Robbins, and Molkentin 2005). Interestingly, Baines et al. (Baines, Kaiser, Purcell, Blair, Osinska, Hambleton, Brunskill, Sayen, Gottlieb, Dorn, Robbins, and Molkentin 2005) reported that addition of Bax and the truncated form of Bid (tBid) to purified mitochondria induced a similar degree of cytochrome *c* release from both wild-type and CyPD-deficient mitochondria.

In our study, mitochondrial levels of both anti-apoptotic Bcl-2 and pro-apoptotic Bax and Bid were significantly increased in gastrocnemius muscle from very old rats. This pattern was analogous to that observed for tissue levels of HNE and 3-nitrotyrosine, suggesting that the enhanced oxidative and nitrosative stress might have influenced the mitochondrial content of those proteins (Savory, Rao, Huang, Letada, and Herman 1999; Mishra, Randis, Ashraf, and Iovoria-Papadopoulos 2006; Qin, Yan, Patel, Liu, and Dong 2006). However, in contrast to previous studies (Alway et al. 2002; Alway et al. 2003; Song et al. 2006; Chung and Ng 2006), we did not detect an age-related increase in the Bax to Bcl-2 ratio. While this finding may be interpreted as a compensatory action aimed at preventing myonuclei loss, we found a concomitant increase of total mitochondrial content of Bid. Recent experimental evidence

indicates that full length Bid and tBid are sufficient to elicit mitochondrial cell death pathways (Kim et al. 2000;Konig et al. 2007). In an elegant experiment, Scorrano and coworkers (Scorrano et al. 2002), demonstrated that tBid induced a dose-dependent release of cytochrome *c* from isolated mitochondria. In our study, however, cytosolic levels of cytochrome *c* were not elevated with age, although a transient increase of active caspase-9 expression was evident in old rats. Similar to our findings, Chung and Ng (Chung and Ng 2006) also reported increased levels of active caspase-9 in the gastrocnemius muscle of old rats, in spite of unchanged cytosolic content of cytochrome *c*. In addition, the lack of an age-related increase of cytosolic levels of cytochrome *c* was previously shown by our group (Dirks and Leeuwenburgh 2002;Dirks and Leeuwenburgh 2004). Based on these reports, it could be argued that a limited fraction of cytochrome *c* had been released and that the resulting subtle increase of its cytosolic levels was not detected by our Western blot analysis. Furthermore, the absence of an age-dependent larger release of cytochrome *c* would explain the lack of sustained caspase-9 activation in our model. Taken together, our findings indicate that the mitochondrial caspase-dependent apoptotic pathway may be only marginally involved in age-related muscle atrophy.

In contrast, we found that advancing age was associated with increased cytosolic and nuclear levels of both AIF and EndoG, indicative of activation of caspase-independent cell death. The relevance of caspase-independent apoptosis to age-related muscle atrophy was supported by a positive correlation between nuclear content of both AIF and EndoG and the extent of apoptotic DNA fragmentation. Furthermore, nuclear levels of the two caspase-independent factors were negatively correlated with muscle mass. Our data confirm and extend previous findings from Leeuwenburgh et al. (Leeuwenburgh, Gurley, Strotman, and Dupont-Versteegden 2005), in which enhanced nuclear translocation of EndoG was observed in soleus muscle of old rats. However, to the best of our knowledge, this is the first study reporting an increased nuclear content of AIF in skeletal muscle of aged rats. In fact, previous investigations from our group (Dirks and Leeuwenburgh 2004) as well as from others (Siu et al. 2005) did not detect enhanced nuclear levels of AIF, in spite of increased cytosolic content of this mediator. This discrepancy may be due to the different experimental models investigated as well as to methodological differences between the studies. Interestingly, the increased cytosolic content of AIF and EndoG observed at 29 months of age was not accompanied by detectable changes in the mitochondrial levels of either Bax and Bid or CyPD. Furthermore, tissue levels of HNE and 3-nitrotyrosine were not elevated until 37 months. It is possible that other pro-apoptotic members of the Bcl-2 family (e.g., Bak, Bad, Bim) might have contributed to MOMP in old and senescent animals. However, those mediators were not investigated in the present study. In addition, it is conceivable that a small increase in oxidative and nitrosative damage might have occurred at 29 months of age in discrete cellular compartments (e.g., mitochondria), leading to activation of the apoptotic signaling.

Some aspects of our study deserve further discussion. First of all, we did not assess mPTP opening susceptibility. Therefore, it cannot be established whether the observed increased expression of mitochondrial CyPD translated into an actual enhanced propensity toward mPTP opening. However, we have preliminary data for quadriceps muscle of F344×BNF1 rats indicating that mitochondrial susceptibility to permeability transition is indeed elevated in old and senescent animals. Furthermore, due to the unavailability of commercial antibodies, we could not distinguish between full length and truncated Bid. However, as previously mentioned, both forms possess apoptogenic properties (Kim, Zhao, Barber, Kuharsky, and Yin 2000;Konig, Rehm, Gudorf, Krajewski, Gross, Ward, and Prehn 2007). Therefore, an increased mitochondrial content of Bid, regardless of its cleavage, may still be considered as part of the pro-apoptotic environment taking place in aged skeletal muscle. Finally, previous studies have reported that spontaneous physical activity decreases in rats with advancing age (Andrade et al. 2003;Behnke et al. 2006). Since physical exercise has been shown to reverse age-related myonuclear apoptosis (Song, Kwak, and Lawler 2006;Marzetti, Groban, Wohlgemuth, Lees,

Lin, Jobe, Giovannini, Leeuwenburgh, and Carter 2008a), it is conceivable that the increased myocyte apoptosis observed in old and senescent rats was at least partly attributable to inactivity. However, spontaneous levels of physical activity were not quantified in the present study.

Conclusions

Results from our study indicate that mitochondrial apoptotic signaling is elevated in rat gastrocnemius muscle at advanced age, likely contributing to the age-related muscle loss. Furthermore, our findings suggest that the mitochondrial caspase-dependent and independent apoptotic program may be sequentially and selectively activated in skeletal muscle over the course of aging. However, our data strongly suggest that the mitochondrial caspase-independent apoptotic pathway may play a more prominent role in skeletal muscle loss than caspase-9-mediated apoptosis. Activation of mitochondrial apoptotic signaling may be due to modification of the Bcl-2 proteins pattern of expression, possibly supported by enhanced levels of oxidative and nitrosative stress. In addition, changes in the composition of the mPTP (i.e., increased CyPD/ANT and CyPD/VDAC ratio) may contribute to mitochondrial-mediated apoptosis observed in aged muscle.

Acknowledgements

This research was supported by grants to CL (NIA R01-AG17994 and AG21042). EM, SEW and HS are supported by the University of Florida Institute on Aging and Claude D. Pepper Older Americans Independence Center (1 P30 AG028740). SG is supported by the Department of Gerontology, Geriatrics and Physiatrics, Catholic University of the Sacred Heart, Rome, Italy. The authors would also like to thank Drs. Tim Hofer and Stephane Servais for their critical input in the conduction of the study.

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Figure 1. Mitochondrial content of CyPD, as determined by Western blot analysis, was increased relative to ANT (a) and VDAC (b) in senescent rats. Identical indices represent significant difference between two groups (^a $p < 0.01$; ^b $p < 0.05$; ^c $p < 0.05$). Values are mean $\pm$ SEM, $n = 7-9$ per group.

Figure 2.

Mitochondrial content of Bcl-2 (a), Bax (b) and Bid (c), as determined by Western blot analysis, did not change between 8 and 29 months of age. However, a highly significant increase was detected for all the three proteins at 37 months. Bax to Bcl-2 ratio remained unchanged over the course of aging (D). Identical indices represent significant difference between two groups ($a-i$ $p < 0.001$). Values are mean $\pm$ SEM, $n=7-9$ per group.

Figure 3.

Western blot analysis of cytosolic cytochrome *c* (a) and active caspase-9 (b). Levels of cytosolic cytochrome *c* did not change across the age groups. An increase of active caspase-9 was detected in 29- compared to 18-month-old rats. Identical indices represent significant difference between two groups (^a $p < 0.05$). Values are mean $\pm$ SEM, $n = 7-9$ per group.

Figure 4.

Cytosolic and nuclear content of AIF (a–b) and EndoG (c–d) were assessed by Western blot analysis. For both these markers, an age-dependent increase was observed in the two cellular compartments. Identical indices represent significant difference between two groups (^{a,c,d} $p < 0.01$; ^b $p < 0.05$; ^{e–f} $p < 0.05$; ^{g–j} $p < 0.001$; ^k $p < 0.01$; ^{l–m} $p < 0.05$). Values are mean $\pm$ SEM, $n = 7–9$ per group.

Figure 5.

Tissue levels of HNE-modified proteins (a) and 3-nitrotyrosine (b) were measured by dot blot analysis. For both these markers, no changes were apparent between 8 and 29 months of age. A sharp increase was observed in 37-month-old rats. Identical indices represent significant difference between two groups (^{a-f} $p < 0.001$). Values are mean $\pm$ SEM, $n = 7-9$ per group.

Figure 6. Apoptotic index as determined by quantification of cytosolic mono- and oligonucleosomes. An age-related increase of the extent of DNA fragmentation was detected. Identical indices represent significant difference between two groups (^{a,c,e} $p < 0.01$; ^{b,d} $p < 0.001$). Values are mean $\pm$ SEM, $n = 7-9$ per group.

Figure 7.

Correlation analyses between muscle weight (MW) and the apoptotic index (a) and between muscle weight to body weight (MW/BW) and the apoptotic index (b). Additional correlation analyses were run between the apoptotic index and the content of nuclear AIF (c), and nuclear EndoG (d). Finally, correlation tests were performed between MW/BW and nuclear levels of AIF (e) and EndoG (f).

Table 1

Morphologic characteristics of 8-, 18-, 29- and 37-month-old F344.BNF1 rats.

	8-mo	18-mo	29-mo	37-mo
Body weight (g)	376.9±14.8 ^{a,b,c}	478.8±6.0 ^{a,d}	537.7±7.4 ^{b,d,e}	486.0±10.3 ^{c,e}
Gastrocnemius muscle weight (g)	1.89±0.07 ^{f,g}	2.11±0.03 ^{f,h,i}	1.79±0.05 ^{h,j}	0.73±0.04 ^{g,i,j}
Muscle weight to body weight	0.50±0.01 ^{k,l,m}	0.44±0.01 ^{k,n,o}	0.33±0.01 ^{l,n,p}	0.15±0.01 ^{m,o,p}

Identical indices represent significant difference between two groups.

^{a-c,g-o} p<0.001;^{d,e} p<0.01;^f p<0.05.

Values are mean ± SEM, n=9 per group.

Table 2

Mitochondrial content of VDAC, ANT and CyPD as determined by Western blot analysis.

	8-mo	18-mo	29-mo	37-mo
VDAC	3.43±0.37	3.45±0.56	3.13±0.38	2.52±0.52
ANT	0.36±0.03	0.39±0.06	0.37±0.05	0.28±0.05
CyPD	0.12±0.01	0.16±0.03	0.18±0.02	0.19±0.02

Expression of VDAC and ANT was not affected by age. However, an upward trend for CyPD with advancing age was detected ($p=0.057$, 8-mo vs. 37-mo). Values are expressed as arbitrary optical density units/mg protein, and are reported as mean $\pm$ SEM, $n=7-9$ /group.