

Apoptosis in skeletal muscle with aging

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Dirks, Amie, and Christiaan Leeuwenburgh. Apoptosis in skeletal muscle with aging. *Am J Physiol Regulatory Integrative Comp Physiol* 282: R519–R527, 2002. First published October 18, 2001; 10.1152/ajpregu.00458.2001.—Sarcopenia may be partly due to a loss in total fiber number by apoptosis. We have investigated age-related alterations in the mitochondria-mediated pathway leading to apoptosis in the gastrocnemius muscle from 6-mo-old and 24-mo-old male Fisher 344 rats. Apoptosis (mono- and oligonucleosome fragmentation) in the gastrocnemius muscle was increased by 50% in the old rats compared with the adult animals. Furthermore, there was a significant correlation between cytosolic cytochrome *c* and caspase-3 activity, although neither cytochrome *c* nor caspase-3 activity increased significantly with age. Furthermore, there was a significant correlation between caspase-3 activity and mono- and oligonucleosome fragmentation in the old rats only. Mitochondrial Bcl-2 and Bax were not altered with age. In vitro experiments demonstrated that activation of the caspase cascade in skeletal muscle might be limited by procaspase-9 activation. This is the first study to explore the role of apoptosis in sarcopenia and suggests that subtle changes in apoptosis are involved.

free radicals; oxidative stress; apoptotic protease activating factor-1; Bcl-2 family; cytochrome *c*; caspases; mitochondria

SKELETAL MUSCLE MASS decreases with age, via a decrease in fiber number and atrophy of the remaining muscle fibers, by largely unidentified mechanisms (26, 32). One potential mechanism of atrophy originates from proteolytic pathways (13, 18, 23). In addition, other mechanisms, including neurological mechanisms (loss in motor neurons) and hormonal changes with age, are likely to contribute to muscle loss (37). Alternatively, the loss of muscle mass due to apoptosis with normal aging may play an important role, but it has not been well investigated.

Accelerated apoptosis with normal aging has been reported in several mitotic tissues, such as liver and white blood cells, which serve to prevent age-associated tumorigenesis and to maintain overall control of immunocompetent cells, respectively (24, 25). However, it is still controversial whether apoptosis occurs in postmitotic tissues with normal aging, such as brain, heart, and skeletal muscle (24, 27, 34, 45). With pathophysiological and certain physiological conditions,

there is sufficient evidence to demonstrate that apoptosis plays a key role in skeletal muscle cell loss. For example, apoptosis has been documented to occur in muscular dystrophy (38), chronic heart failure (2), skeletal muscle denervation (8), muscle unweighting (3), and during acute exercise (38). With normal aging, there are several reports that indicate a loss in fiber number (4, 30), but only one report investigated apoptosis in skeletal muscle showing that the rhabdosphincter muscle cells of aged humans may be partly lost by apoptosis (43).

Although apoptosis may occur via several mechanisms, mitochondria have recently been implicated as major regulatory centers for apoptosis (11, 20, 21). It has been suggested that internal cellular stimuli, such as high levels of calcium or reactive oxygen intermediates, may trigger apoptosis by the cytochrome *c*-dependent pathway (15, 16, 35, 39). Other pathways require an alternate upstream activator(s) to initiate the caspase cascade (cysteine-dependent, aspartate-specific proteases, i.e., caspase-8 and caspase-10). For example, binding of tumor necrosis factor (TNF)- α to its receptor can induce apoptosis in an effector cell by the activation of procaspase-8, which cleaves and activates procaspase-3 and initiates the caspase cascade (44). In addition, endoplasmic reticulum stress could also partly contribute to apoptosis by releasing calcium into the cytosol and thereby activating procaspase-12 (7).

The mitochondria, which are extensively damaged by oxidants in aged skeletal muscle of humans and rodents (14, 29), could release cytochrome *c* into the cytosol, a first step for the initiation of apoptosis. After the release of cytochrome *c* from the mitochondria (Fig. 8), an apoptosis-initiating complex is formed with apoptotic protease activating factor-1 (Apaf-1), dATP, and procaspase-9, also called an apoptosome, which results in the self-cleavage and activation of procaspase-9. The active caspase-9 cleaves and activates procaspase-3 (20, 21), which in turn activates a cascade of caspases. Caspase activation leads to reorganization of the cytoskeleton, shuts down DNA replication and repair, destroys DNA, disrupts the nuclear structure, and disintegrates the cell into apoptotic bodies, eventually destroying the cell (20, 21).

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We were interested in investigating mitochondria-mediated signaling of apoptosis by cytochrome *c* since mitochondria of aged skeletal muscle produce more oxidants, accumulate calcium, and exhibit increased oxidative damage, all stimuli for apoptosis (6). In addition, we investigated possible age-related adaptations, such as alterations in the Bcl-2-to-Bax ratio, which may serve to protect against the apoptotic stimuli, thereby preventing loss of irreplaceable muscle fibers.

MATERIALS AND METHODS

Animals. Male 6-mo-old ($n = 8$) and 24-mo-old ($n = 8$) Fischer 344 rats (National Institute of Aging colony, Harlan Sprague Dawley, Indianapolis, IN) were used. The rats were housed two per cage in a temperature (18–22°C)- and light-controlled environment with a 12:12-h light-dark cycle and provided with food and water ad libitum. After 1 wk of acclimation, the animals were randomly killed on 4 consecutive days, with equal numbers of adult and old animals on each day.

Isolation of mitochondria. Animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt). The gastrocnemius muscle was excised and weighed. One section of the gastrocnemius was cut from the lateral head, frozen in liquid nitrogen, and stored at -80°C for further analysis, and another section was cut from the medial head and used for mitochondrial isolation. The medial muscle section (300–500 mg) was homogenized in isolation buffer (0.225 M mannitol, 0.075 M sucrose, 0.2% BSA) (1:25 wt/vol) using a Potter-Elvehjem glass homogenizer. Homogenate was centrifuged at 1,000 g for 10 min. The supernatant was decanted into a centrifuge tube and centrifuged at 14,000 g for 10 min. The 14,000 g supernatant was stored at -80°C for other biochemical analysis. The mitochondrial pellet was resuspended in 5 ml of wash buffer (0.225 M mannitol, 0.075 M sucrose, 1 mM EGTA, pH 7.4) and centrifuged at 14,000 g for 10 min. The final mitochondrial pellet was resuspended in 0.5 ml storage buffer (0.25 M sucrose, 2 mM EDTA, pH 7.4), and mitochondrial membrane integrity was immediately determined. The remaining mitochondria were stored at -80°C for further analysis.

Determination of mitochondrial membrane integrity. We used two assays to evaluate if there were differences between the 6-mo-old and 24-mo-old rats in mitochondrial membrane integrity. Cytochrome *c* reduction in isolated intact mitochondria was determined immediately after the isolation procedure. Differences in membrane damage would result in higher levels of cytochrome *c* reduction by superoxide produced by the inner membrane. The incubation buffer consisted of 6 mM succinate, 70 mM sucrose, 220 mM mannitol, 2 mM HEPES, 25 mM KH_2PO_4 , 2.5 mM MgCl_2 , 0.5 mM EDTA, 5 $\mu\text{g/ml}$ catalase, pH 7.4, and 40 μM acetylated cytochrome *c*. The change in absorbance was measured at 550 nm at 37°C using a spectrophotometric plate reader from Molecular Devices (Sunnyvale, CA). Furthermore, to determine if there were differences in mitochondrial membrane damage between the adult and old animals, we measured citrate synthase activity in the cytosolic and mitochondrial fractions using a previously described method (41).

Determination of the levels of Bcl-2 and Bax by ELISAs. To quantify the amount of mitochondrial Bcl-2 and Bax proteins, ELISAs were performed. Plates were coated with 1 μg of mitochondrial protein in a physiological buffer solution (PBS) and sealed overnight at 4°C . Bcl-2 and Bax peptide

standards (Oncogene, Boston, MA) were included as positive controls. The plates were washed with buffer containing PBS with 0.02% sodium azide and 0.05% Tween 20. The wells were blocked with 300 μl of 1% BSA in PBS with 0.02% sodium azide and incubated at room temperature for 60 min. After samples were washed four more times, 50 μl of the primary antibody (Oncogene) at a concentration of 5 $\mu\text{g/ml}$ diluted in 1% BSA in PBS/azide was incubated for 60 min at room temperature. Each well was washed four times. Next, 50 μl of the secondary antibody [goat anti-rabbit IgG ALK-PHOS conjugate (Sigma A 8025) at a 1:2,000 dilution into a solution of 1% BSA in PBS/azide] was added to each well, and the plate was incubated for 60 min at room temperature. The washing procedure was then repeated, and 100 μl of freshly made substrate containing *p*-nitrophenyl phosphate (Sigma N-2765) at a concentration of 1 mg/ml in substrate buffer (carbonate-bicarbonate, pH 9.6) was added. The plate was then incubated at room temperature for 60 min, and the absorbance at 405 nm was read.

Determination of the levels of Apaf-1 by Western blot analysis. The level of Apaf-1, an adaptor molecule essential for caspase-9 activation, was determined in the gastrocnemius muscle. Muscle (300–500 mg) was homogenized in phosphate buffer (1:10 wt/vol) and centrifuged at 1,000 g for 10 min. Proteins were separated on a precast 4–12% polyacrylamide gel (BMA) using 60 μg of protein per well and then transferred onto a nitrocellulose membrane. Nitrocellulose membranes were blocked overnight using a blocking solution containing 0.05% Tween 20 and 5.0% milk. Membranes were incubated with the polyclonal antibody for Apaf-1 (dilution 1:500; Biovision, Palo Alto, CA) for 90 min. Membranes were then incubated for 90 min in anti-rabbit Ig horseradish peroxidase (Amersham Life Science) diluted 1:1,000. Blots were analyzed using the Apple-J program downloaded from the NIH website. Values are expressed as arbitrary optical density (OD) units calculated by multiplying the area of each band by its OD.

Other biochemical analyses. Specific apoptotic DNA fragmentation was quantified by measuring the amount of cytosolic mono- and oligonucleosomes using a Cell Death ELISA kit (Roche Molecular Biochemicals) according to instructions from the manufacturer. Endogenous endonucleases that cleave double-stranded DNA in the linker region between nucleosomes generate mono- and oligonucleosomes of 180 bp or multiples (12). This is a very sensitive technique in quantifying apoptosis compared with terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) or DNA laddering, both of which are qualitative assessments of DNA fragmentation. Results were reported as arbitrary OD units normalized to milligram of protein. Cytosolic cytochrome *c* was quantified using an ELISA kit (R&D Systems, Minneapolis, MN) that employs the sandwich enzyme immunoassay technique. Caspase activity was measured using the synthetic peptide n-Ac-DEVD-AMC (BD PharMingen, San Diego, CA). This assay detects activated caspase-3 and to a lesser extent caspases -6, -7, and -8. Active caspases will cleave the AMC from the peptide, and the free AMC will fluoresce. Briefly, 1 ml of assay buffer (20 mM HEPES, 10% glycerol, 1 M dithiothreitol, and 14 μl of n-Ac-DEVD-AMC/ml of buffer) and 50 μl of sample were added to a microcentrifuge tube and protected from the light. Samples were incubated at 37°C for 60 min, after which fluorescence was measured on a spectrofluorometer with an excitation wavelength of 380 nm and an emission wavelength of 440 nm. Protein concentration of mitochondria and cytosol was measured using the Bradford method (9).

Table 1. Mitochondrial membrane integrity in the gastrocnemius muscle of 6- and 24-mo-old male Fischer 344 rats determined by levels of cytochrome *c* reduction as well as mitochondrial and cytosolic citrate synthase activity

	6-Mo	24-Mo
Cytochrome <i>c</i> reduction	0.08 ± 0.01	0.08 ± 0.04
Citrate synthase (mitochondrial)	2.0 ± 0.15	2.1 ± 0.30
Citrate synthase (cytosolic)	0.13 ± 0.02	0.15 ± 0.01

Results are expressed as means ± SE. Units of cytochrome *c* reduction are in arbitrary absorbance units at 550 nm/mg protein and citrate synthase units are in $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$.

Statistical analysis. Analysis was performed in triplicate, and the mean was used for statistical analysis. For statistical analysis, we used an independent *t*-test. Pearson correlation coefficients were determined using a Graph-pad Prism statistical analysis program (San Diego, CA). A *P* value of <0.05 was considered significant.

RESULTS

Body weight and muscle mass of animals. Body weight increased ~20% in the 24-mo-old rats (400 ± 8.3 g; mean ± SE) compared with the 6-mo-old animals (319.0 ± 9.0 g; mean ± SE). The gastrocnemius muscle mass showed a decrease between 6 mo (1.3 ± 0.02 g; mean ± SE) and 24 mo (1.2 ± 0.02 g; mean ± SE) of age. The loss in muscle mass is consistent with that of other studies (17, 31). In addition, expressed as a percentage of body weight, the gastrocnemius wet weight decreased significantly by 25% in the 24-mo-old animals compared with 6-mo-old animals (0.4 ± 0.006 vs. 0.3 ± 0.006 g; mean ± SE; *P* < 0.001).

Membrane integrity was not different between the two groups because of the mitochondrial isolation procedure. To ensure that mitochondria isolated from adult and old animals showed no differences in damage during the isolation procedure, we used two assays to determine membrane integrity (Table 1). One assay, to assess outer membrane integrity, involved measuring cytochrome *c* reduction by superoxide in intact isolated mitochondria. Differences in outer mitochondrial membrane damage would allow different amounts of cytochrome *c* to enter the mitochondria and become reduced by superoxide produced by the inner membrane. In addition, minimal damage to the inner (as well as the outer) mitochondrial membrane during the isolation procedure would result in a very low cytosolic citrate synthase activity compared with mitochondrial citrate synthase activity. We found that the cytosolic citrate synthase activity was approximately 5–7% of the total mitochondrial citrate synthase activity in both the 6- and 24-mo-old animals (Table 1). Moreover, there were no significant differences in cytosolic or mitochondrial citrate synthase activity between the 6- and 24-mo-old rats.

Mono- and oligonucleosome content in muscle of 6-mo-old and 24-mo-old rats. Apoptosis results in the activation of endonucleases that cleave double-stranded DNA between nucleosomes into 180-bp mononucleosome

or multiple oligonucleosome fragments. We quantified the amount of DNA fragmentation in gastrocnemius muscle in the 6- and 24-mo-old animals. We found a 50% increase in cytosolic mono- and oligonucleosomes in the 24-mo-old animals compared with the 6-mo-old animals (*P* = 0.0017), strongly suggesting an increase in cell death by apoptosis (Fig. 1).

Cytosolic cytochrome *c* and caspase-3 activity in skeletal muscle of 6- and 24-mo-old rats. Cytochrome *c* is a cofactor for procaspase-9 activation, which cleaves and activates procaspase-3 in the presence of Apaf-1 and initiates the caspase cascade. We did not detect a significant change in cytosolic cytochrome *c* levels between the 6- and 24-mo-old animals (8.53 ± 2.44 vs. 9.91 ± 2.43 ng/mg protein, respectively; mean ± SE; Fig. 2A). Caspase-3, a pivotal protease involved in the destruction of the cell, did not increase significantly in the 24-mo-old rats compared with the 6-mo-old animals (576 ± 60.3 vs. 508 ± 43.4 arbitrary OD units/mg protein, respectively; mean ± SE; Fig. 2B). It is likely that we could not detect significant changes in the cytosol by our techniques, since most likely only very few fibers were undergoing apoptosis.

Significant correlation between cytosolic cytochrome *c* levels and the activity of caspase-3. We correlated cytosolic cytochrome *c* levels and caspase-3 activity to determine if the levels of cytochrome *c* present in the cytosol could directly affect the activity of caspase-3 (Fig. 3). Indeed, we found a significant correlation (*r* = 0.68; *P* = 0.0035) between cytochrome *c* concentration and caspase-3 activity. Furthermore, when comparing within each age group, we also found high correlations in the 6-mo-old rats (*r* = 0.79; *P* = 0.019), but the correlation did not reach significance in the 24-mo-old rats (*r* = 0.62; *P* = 0.102). These findings strongly suggest that cytosolic levels of cytochrome *c* correlate well with caspase-3 activities in vivo.

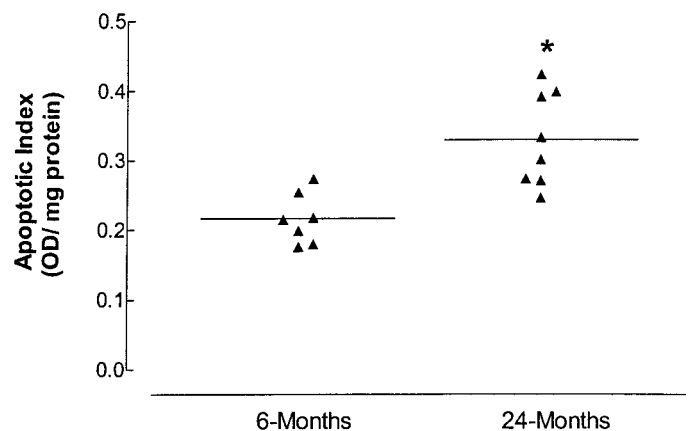


Fig. 1. Apoptosis determined by the quantification of mono- and oligonucleosomes (apoptotic index) in gastrocnemius muscle of 6- and 24-mo-old rats using an ELISA. The 24-mo-old rats had significantly greater levels (+50%) of cytosolic mono- and oligonucleosomes compared with the 6-mo-old rats (0.33 ± 0.02 vs. 0.22 ± 0.01, means ± SE; **P* = 0.0017). Results are reported as arbitrary optical density (OD) units/mg protein.

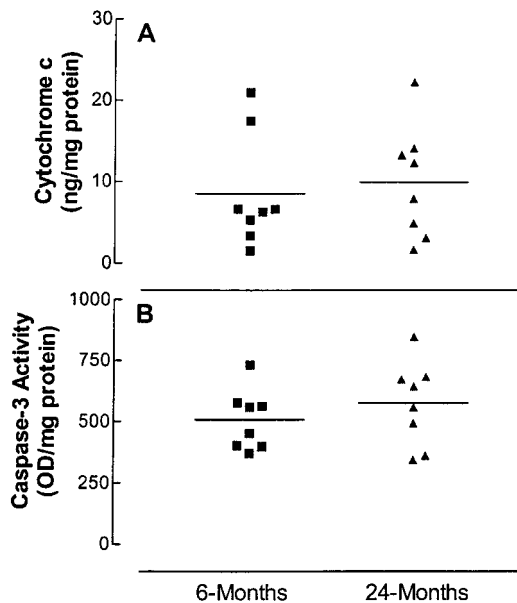


Fig. 2. Cytosolic cytochrome *c* levels (A) and caspase-3 activity (B) in the gastrocnemius muscle in 6- and 24-mo-old rats. There was no significant difference in cytosolic cytochrome *c* between the 6- and 24-mo-old animals (8.53 ± 2.44 vs. 9.91 ± 2.43 , respectively; means \pm SE). Results are reported as ng/mg protein. Cytosolic caspase-3 activity was not significantly different between the 6- and 24-mo-old animals (508 ± 43.4 vs. 576 ± 60.3 , respectively; means \pm SE; $n = 8$ in each group). Caspase-3 activity is reported as arbitrary OD units/mg protein.

Significant correlation between caspase-3 activity and mono- and oligonucleosome content. Because the activation of caspases is partly responsible for the formation of mono- and oligonucleosomes in that they cleave inhibitors of endonucleases, such as caspase-activated DNase (CAD), we correlated caspase-3 activity with the amounts of mono- and oligonucleosome content in both age groups (Fig. 4). We found no correlation ($r = -0.007$; not significant) in the 6-mo-old animals (Fig. 4A) but a highly significant positive

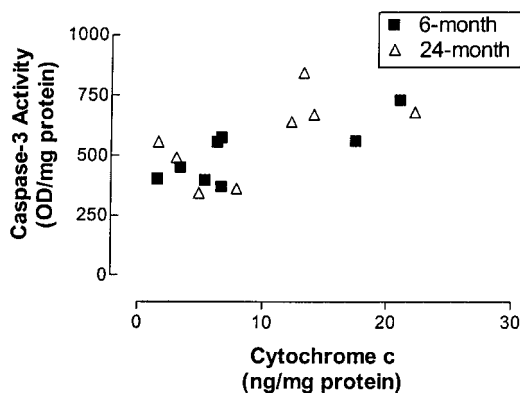


Fig. 3. Correlation between cytosolic cytochrome *c* and caspase-3 activity of the 6- and 24-mo-old animals. The overall correlation for both 6- and 24-mo-old animals was $r = 0.68$ ($P = 0.0035$). We found a significant positive correlation ($r = 0.79$; $P = 0.019$) between cytochrome *c* and caspase-3 activity in the 6-mo-old rats, but the correlation did not reach significance in the 24-mo-old rats ($r = 0.62$; $P = 0.102$).

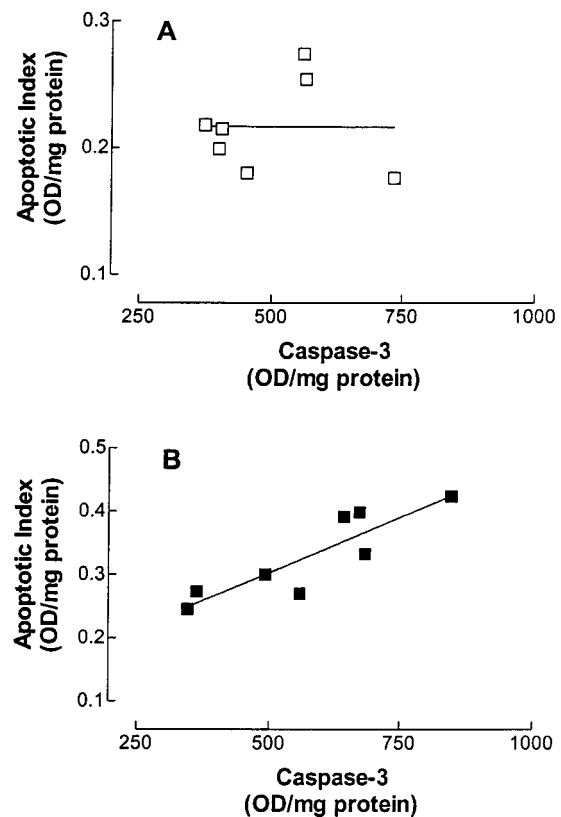


Fig. 4. Correlation between caspase-3 activity and the apoptotic index (mono- and oligonucleosomes) in the 6-mo-old (A) and 24-mo-old rats (B). We found no correlation ($r = -0.007$) between caspase-3 activity and the apoptotic index in the 6-mo-old rats but a strong positive correlation in the 24-mo-old rats ($r = 0.88$; $P = 0.0037$).

correlation in the 24-mo-old rats ($r = 0.88$; $P = 0.001$; Fig. 4B).

Thus the overall caspase-3 activity did not increase, but in those samples with a higher activity, the mono- and oligonucleosome levels were elevated compared with other samples, suggesting a correlation.

Detectable levels of Apaf-1 in rat skeletal muscle. Burgess et al. (10) have shown that Apaf-1, a required apoptotic cofactor protein for procaspase-9 activation and therefore caspase-3 activation, was lacking in human skeletal muscle. Because this is a debated finding, we determined if Apaf-1 was present in skeletal muscle of rats. We found that Apaf-1 was detectable in muscle samples from adult and old rats ($37,550 \pm 2,291$ vs. $26,440 \pm 3,580$, respectively; $n = 5$; mean \pm SE of arbitrary OD units/mg protein) as determined by Western blot analysis.

Mitochondrial Bcl-2 and Bax protein content in muscle of adult and old rats. We determined if there were any changes in the antiapoptotic (Bcl-2) and proapoptotic (Bax) proteins in the mitochondria with age and determined the Bcl-2-to-Bax ratio (Fig. 5). These Bcl-2 family proteins and others partly control the release of cytochrome *c* from the mitochondria. We found no significant alteration in Bcl-2 (Fig. 5A) and Bax (Fig. 5B) with age. In addition, the Bcl-2-to-Bax ratio in the mitochondria did not increase significantly (Fig. 5C).

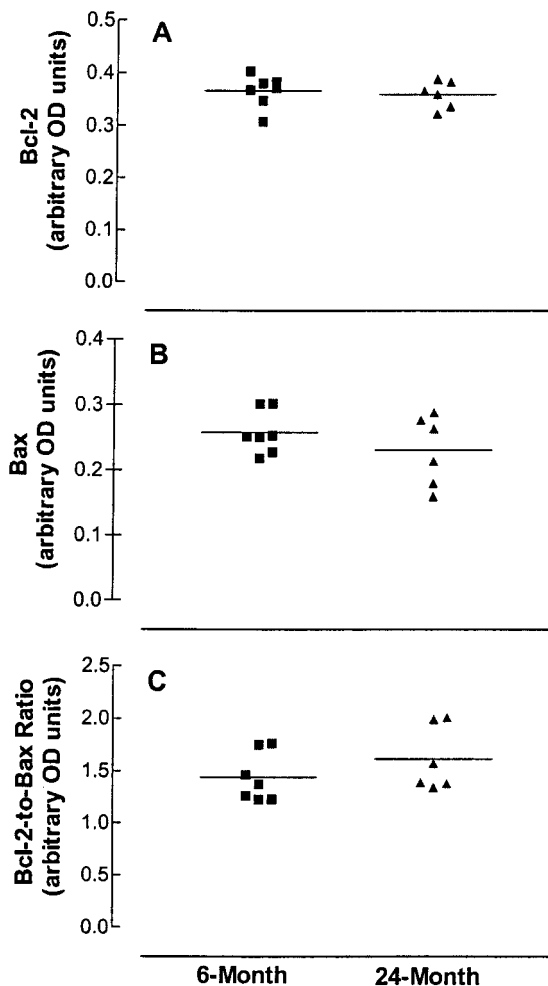


Fig. 5. Mitochondrial Bcl-2 (A), Bax (B), and the Bcl-2-to-Bax ratio (C) in gastrocnemius muscle of 6- and 24-mo-old rats. Levels of the Bcl-2 family proteins were analyzed using an ELISA method (see MATERIALS AND METHODS). A: the mitochondrial Bcl-2 protein levels in the adult compared with the old rats were 0.37 ± 0.01 vs. 0.36 ± 0.01 , respectively (means \pm SE; arbitrary OD units/mg protein). B: mitochondrial Bax protein levels in the adult compared with the old rats were 0.258 ± 0.01 vs. 0.231 ± 0.02 (means \pm SE; arbitrary OD units/mg protein). C: gastrocnemius mitochondrial Bcl-2-to-Bax ratio in the adult compared with the old rats was 1.44 ± 0.09 vs. 1.62 ± 0.13 .

Activation requirements of caspase-3 in skeletal muscle in vitro. We determined if we could activate caspase-3 activity in vitro with the addition of cytochrome *c*. We incubated gastrocnemius muscle homogenates with 5 μ g cytochrome *c* (from rat heart; Sigma) and ATP (1 mM; Sigma) or buffer (0.225 M mannitol, 0.075 M sucrose, 0.2% BSA, 1.5 mM MgCl₂, 1 mM EDTA) for 1 h and measured caspase-3 activity (Fig. 6). This concentration of cytochrome *c* has been previously used to activate caspase-3 activity in rabbit reticulocyte lysates (36). We found no significant differences in caspase-3 activity in muscle homogenates of adult and old rats after incubation with exogenous cytochrome *c*. In contrast, liver homogenate from 6-mo-old animals, used as a positive control, did show a \sim 50% increase in caspase-3 activity after cytochrome *c* incubation.

Higher concentrations of cytochrome *c* (10 μ g) gave the same results. Our results suggest that under these in vitro conditions, procaspase-3 cannot be activated by addition of cytochrome *c* to skeletal muscle homogenate of 6- and 24-mo-old animals.

We further investigated why caspase-3 activity did not increase with the addition of cytochrome *c*. We incubated muscle homogenate with recombinant active caspase-9 and found that caspase-3 activity increased in both the 6- and 24-mo-old animals (Fig. 7). These results indicate that procaspase-9 may be the limiting step in the activation of the caspase cascade. Furthermore, these data suggest that activation of procaspase-9 may require in vivo conditions not present in our in vitro experimental environment, such as the release of other apoptotic proteins from the mitochondria that may suppress inhibitors of apoptosis that may be present. These possibilities remain unexplored in skeletal muscle.

DISCUSSION

An increased rate of apoptosis in skeletal muscle has been documented to occur under several pathophysiological conditions (1–3, 8, 38). However, there is little evidence and investigation as to whether apoptosis occurs in postmitotic tissues during normal physiological aging. We were able to detect a significant increase in DNA fragmentation in skeletal muscle from aged animals. This supports the recent findings of Strasser et al. (43), who found an increased incidence of apoptosis by the TUNEL technique in human rhabdosphincter skeletal muscle with age. Our results provide the first report to show a significant increase in DNA fragmentation in aged locomotor skeletal muscle of rodents, reflective of the levels of apoptosis.

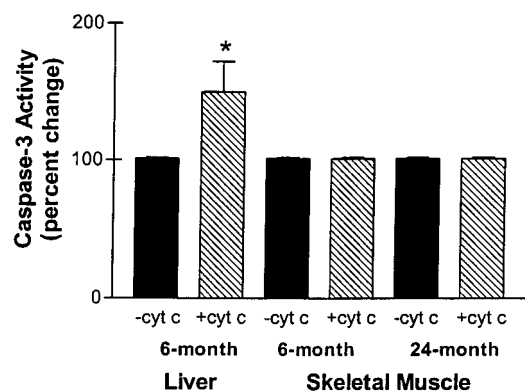


Fig. 6. Cytochrome *c* addition (+cyt *c*) in vitro was unable to activate procaspase-3 in gastrocnemius muscle of 6- and 24-mo-old rats. Muscle and liver were homogenized 1:6 (wt/vol) in buffer (0.225 M mannitol, 0.075 M sucrose, 0.2% BSA, 1.5 mM MgCl₂, 1 mM EDTA, and 1 mM ATP). Homogenates were centrifuged at 1,000 *g* for 10 min. Five micrograms of cytochrome *c* (Sigma) or isolation buffer was added to 100 μ l of cytosolic supernatant and incubated for 1 h at 37°C followed by determination of caspase-3 activity (see MATERIALS AND METHODS). Incubation with cytochrome *c* did not increase caspase-3 activity in muscle. However, caspase-3 activity was increased by \sim 50% in liver homogenate (**P* < 0.01). The same results were obtained in 2 other independent experiments performed in triplicates.

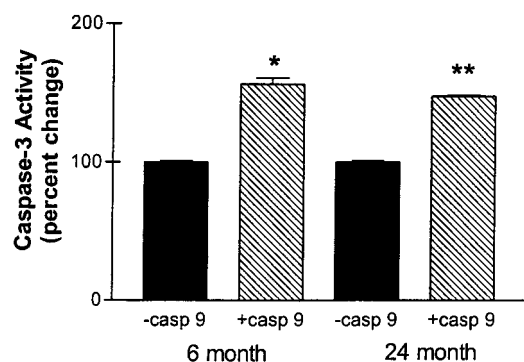


Fig. 7. Recombinant active caspase-9 (US Biological) activates pro-caspase-3 in vitro in skeletal muscle of 6- and 24-mo-old rats. Muscle was homogenized 1:6 (wt/vol) in buffer (0.225 M mannitol, 0.075 M sucrose, 0.2% BSA, 1.5 mM MgCl₂, 1 mM EDTA, and 1 mM ATP). Homogenates were centrifuged at 1,000 g for 10 min. Samples were incubated for 90 min at 37°C with (+casp9) or without (-casp9) 0.5 U of active human recombinant caspase-9 (1 U reflects the enzyme activity that cleaves 1 nmol of caspase substrate per hour at 37°C). Caspase-3 activity was determined using spectrophotometric techniques (see MATERIALS AND METHODS). Incubation with active caspase-9 did increase caspase-3 activity in gastrocnemius of 6- and 24-mo-old rats (* $P < 0.01$, ** $P < 0.001$). To ensure that caspase-9 was not directly cleaving the synthetic peptide, we incubated recombinant caspase-9 with the peptide in incubation buffer without tissue sample and found no significant cleaving of the synthetic peptide. The same results were obtained in 2 other independent trials performed in triplicate.

To elucidate possible pathways of apoptosis, which may have lead to the cleavage of DNA to form mono- and oligonucleosomes in aged muscle, we evaluated the levels of cytochrome *c* and caspase activity in the cytosol of young and old rats. Scientists have shown, using a model of acute skeletal muscle burn injury, increases in cytosolic cytochrome *c* and caspase-3 activity as well as apoptosis (46). Furthermore, we have shown, using an acute rat model of oxidative stress induced by doxorubicin, a drug that generates oxidants in the mitochondria, significant increases in caspase-3 activity (19). However, with aging skeletal muscle, a slow chronic process, we did not find significant increases in cytosolic cytochrome *c* or caspase-3 activity, although further analysis revealed a significant positive correlation between the levels of cytosolic cytochrome *c* and caspase-3 activity. These findings may suggest that very few fibers are undergoing apoptosis at this given time point, and therefore we did not detect significant differences with our techniques using the cytosol of whole muscle homogenate. Small changes in specific proteins with age were very difficult to detect. However, we were able to pick up the more subtle correlation between cytosolic cytochrome *c* and caspase-3 activity. Therefore, it cannot be entirely ruled out that mitochondria-mediated apoptosis does not occur with aging in muscle.

Another important finding was the positive correlation between caspase-3 activity and mono- and oligonucleosomes in the 24-mo-old animals but not in the 6-mo-old animals. It is well established that caspase-3 is able to cleave endonuclease inhibitors and therefore activate CAD, which is responsible for the formation of

mono- and oligonucleosomes (40). Therefore, this strongly suggests that caspase-3 activity in the old rats may be responsible for the activation of CAD and the increase in DNA fragmentation seen in the old rats. In addition, caspase-independent DNA fragmentation has been shown to occur via release of apoptosis inducing factor (AIF) from the mitochondria and translocation to the nucleus (16) and may also play a role in DNA fragmentation of young and old animals, a possibility that requires further investigation.

The Bcl-2 family of proteins partly regulates the release of cytochrome *c* from the mitochondria. The mechanism by which this occurs is not yet clear; however, it is thought that the ratio of Bcl-2 to Bax may be one determining factor influencing cytochrome *c* release (21). The Bcl-2-to-Bax ratio was slightly increased, but this change was not significant and therefore cannot explain why there was little release of cytochrome *c* from the mitochondria in skeletal muscle with aging. We should point out that our isolation technique only isolates subsarcolemmal mitochondria from skeletal muscle. It may be possible that age-related alterations in mitochondrial Bcl-2 and Bax may occur in the interfibrillar fraction of mitochondria rather than the subsarcolemmal fraction. Future investigations remain to be done to clarify the role of these mitochondrial subpopulations in apoptosis. Many other Bcl-2 family proteins, such as bak, bid, bad, and mcl-1, can influence the release of cytochrome *c* and may also play a role in skeletal muscle. Moreover, once released, inhibitors of caspases, or even repressors of these inhibitors, could play a role in regulating the activation of caspases by cytochrome *c*. Specifically, inhibitors of apoptosis (IAPs) and apoptosis repressor with caspase recruitment domain (ARC) can inhibit caspases, whereas a second mitochondrial activator of caspases (Smac) can repress some of the inhibitors of caspases (28, 42).

We have attempted to elucidate the regulation of the mitochondria-mediated signaling pathway in skeletal muscle. In cell types, such as liver, the addition of cytochrome *c* to cytosol activates caspase-3 in vitro (33, 35). We demonstrated that the caspase cascade (as detected by procaspase-3 activation) in skeletal muscle was not activated in response to addition of cytosolic cytochrome *c*. Our findings are in agreement with Burgess et al. (10), who reported that the addition of cytochrome *c* to human skeletal muscle cytosol did not increase caspase-3 activity. However, they suggested that this was due to a lack of Apaf-1, a protein required for the activation of procaspase-9. However, we found significant levels of Apaf-1 in rat skeletal muscle and thus suggest that the inability of cytosolic cytochrome *c* to activate procaspase-9 in rat skeletal muscle was not due to the lack of this cofactor.

Additional experiments were performed to determine why increased cytosolic cytochrome *c* in vitro did not result in the increased activity of caspase-3. We demonstrated that procaspase-3 was indeed capable of being activated by active caspase-9, and therefore it appears that the activation of procaspase-9 may be the

limiting step in the activation of the caspase cascade via mitochondrial cytochrome *c* release.

Perspectives

This study is the first to explore the role of apoptosis in sarcopenia and suggests that subtle changes in apoptosis are involved, which may be critical over a longer period of time. Our results suggest that aged skeletal muscle is characterized by an increased rate of cell death; however, it is not clear if mitochondria-mediated pathways are prevalent in causing apoptosis in skeletal muscle, but they cannot be entirely excluded. Alternative techniques to ours may be required to elucidate the involvement of this apoptotic pathway in the death of very few muscle fibers occurring at an acute time point. Other pathways (see Fig. 8) may also significantly contribute to cell death, such as the receptor-mediated pathway via TNF- α . A recent study showed a significant increase in TNF- α protein expres-

sion with age in human skeletal muscle (22). Further investigation is required to elucidate the involvement of this pathway contributing to skeletal muscle fiber loss via apoptosis.

Furthermore, because whole muscle tissue was used to quantify apoptosis, the possibility remains that other cell types, such as connective tissue, endothelial tissue, and nervous tissue, contributed to the apoptosis observed. However, we removed connective tissues and nervous tissue during the dissection of muscle tissues, and therefore the majority of cells were myocytes.

Finally, our data suggest that skeletal muscle is not a tissue that initiates apoptotic signaling cascades in response to cytosolic cytochrome *c* alone. It appears that the activation of the caspase cascade mediated by mitochondrial cytochrome *c* release may be limited by the activation of procaspase-9, suggesting that this caspase may be associated with an inhibitor of some type. Alternatively, other proteins, such as heat shock

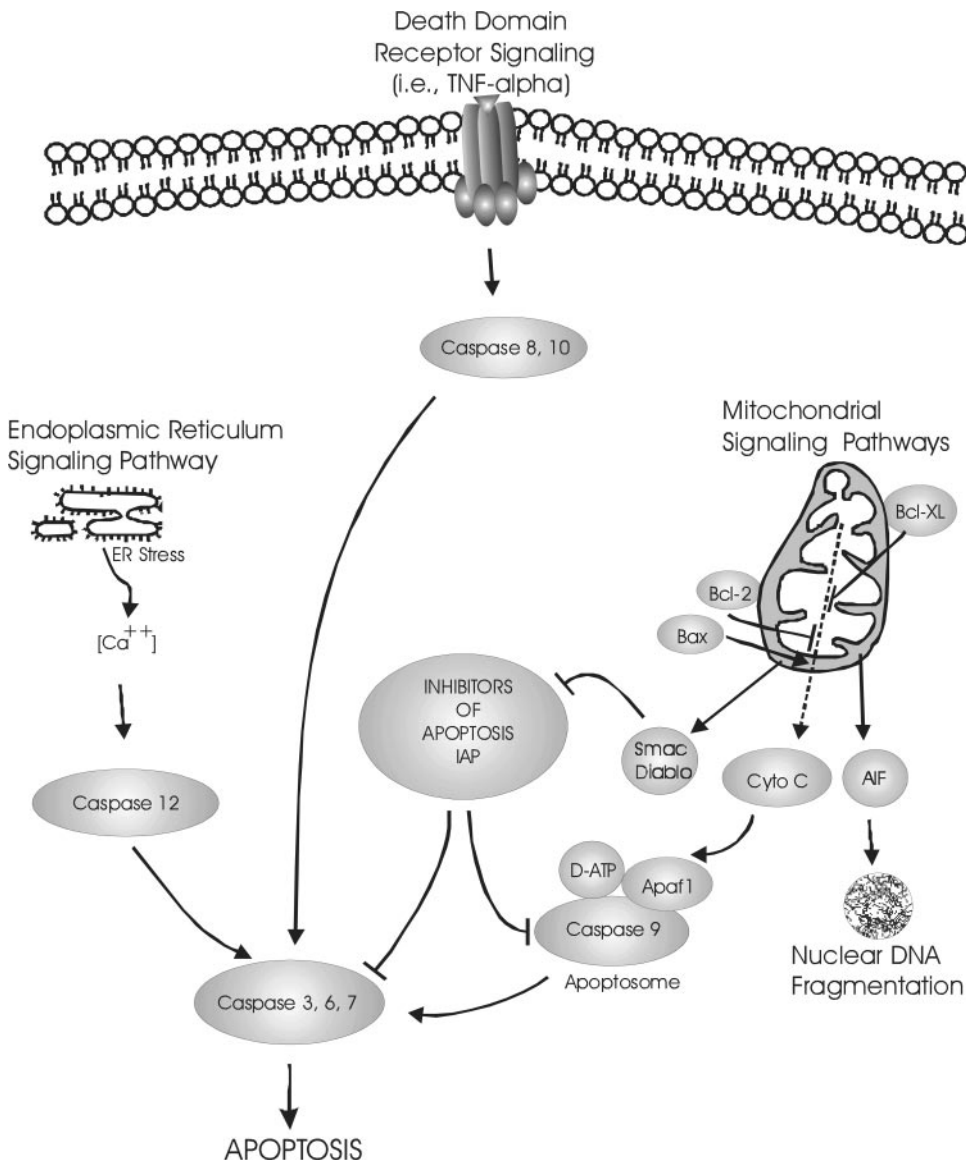


Fig. 8. Simplified cell signaling pathways implicated in inducing cell death. Receptor-mediated pathways may be initiated by ligand binding of tumor necrosis factor (TNF)- α , resulting in caspase activation (caspase-8, caspase-10) and further activation of procaspase-3. The endoplasmic reticulum (ER)-mediated pathway may be activated by "ER stress," resulting in increased intracellular Ca^{2+} content, leading to the activation of procaspase-12. The mitochondria-mediated pathway may be stimulated by oxidants or increases in Ca^{2+} levels, resulting in loss of mitochondrial membrane potential and mitochondrial dysfunction. The release of cytochrome *c* (Cyto *c*) can activate caspases if Smac/DIABLO is simultaneously released and represses the inhibitors of apoptosis (IAPs). Cytochrome *c* release could lead to the formation of the apoptosome [apoptotic protease activating factor-1 (Apaf-1), procaspase-9, and dATP], resulting in procaspase-9 activation followed by procaspase-3 activation. Mitochondrial proteins, such as Bcl-2, Bcl-X_L, and Bax, and their specific ratios can influence mitochondrial outer membrane channel permeability and may therefore be responsible for the release of cytochrome *c* from the mitochondria. Finally, mitochondria can also release apoptosis inducing factor (AIF), which translocates to the nucleus where it induces large-scale DNA fragmentation in a caspase-independent manner. Besides apoptotic and necrotic cell loss, sarcopenia has many other potential causes, including neurological causes (loss in motor neurons), physical inactivity, and hormonal alterations with age, that are likely to contribute to cell loss.

protein 70, could bind to Apaf-1 and inhibit the recruitment of procaspase-9 to the apoptosome, thereby inhibiting apoptosis (5), and may function as a protective mechanism against muscle fiber loss. These and other possible adaptations, such as posttranslational protein modification (nitrosylation) to procaspase-9 or Apaf-1, remain to be investigated.

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