Age-related differences in apoptosis with disuse atrophy in soleus muscle

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Leeuwenburgh, Christiaan, Cathy M. Gurley, Beau A. Strotman, and Esther E. Dupont-Versteegden. Age-related differences in apoptosis with disuse atrophy in soleus muscle. Am J Physiol Regul Integr Comp Physiol 288: R000–R000, 2005. First published January 13, 2005; doi:10.1152/ajpregu.00576.2004.—Muscle atrophy is associated with a loss of muscle fiber nuclei, most likely through apoptosis. We investigated age-related differences in the extent of apoptosis in soleus muscle of young (6 mo) and old (32 mo) male Fischer 344 × Brown Norway rats subjected to acute disuse atrophy induced by 14 days of hindlimb suspension (HS). HS-induced atrophy (reduction in muscle weight and cross-sectional area) was associated with loss of myofiber nuclei in soleus muscle of young, but not old, rats. This resulted in a significant decrease in the myonuclear domain (cross-sectional area per nucleus) in young and old rats, with changes being more pronounced in old animals. Levels of apoptosis (TdT-mediated dUTP nick end labeling and DNA fragmentation) were higher in soleus muscles of old control rats than young animals. Levels were significantly increased with HS in young and old rats, with the greatest changes in old animals. Caspase-3 activity in soleus muscle tended to be increased with age, but changes were not statistically significant (P = 0.052). However, with HS, caspase-3 activity significantly increased in young, but not old, rats. Immuno-histochemistry showed that the proapoptotic endonuclease G (EndoG, a mitochondrion-specific nuclease) was localized in the subsarcomellar mitochondria in control muscles, and translocation to the nucleus occurred in old, but not young, control animals. There was no difference between EndoG total protein content in young and old control rats, but EndoG increased almost fivefold in soleus muscle of old, but not young, rats after HS. These results show that deregulation of myonuclear number occurs in old skeletal muscle and that the pathways involved in apoptosis are distinct in young and old muscles. Apoptosis in skeletal muscle is partly mediated by the subsarcomellar mitochondria through EndoG translocation to the nucleus in response to HS.

hindlimb suspension; sarcopenia; nuclear apoptosis; caspase-3; endonuclease G; oxidative stress; aging

Changes in skeletal muscle size also include alterations in the number of myofiber nuclei in an attempt to maintain a constant myonuclear domain (amount of cytoplasm per nucleus) (4). Skeletal muscle hypertrophy is correlated with an increase in myofiber nuclei (3, 14, 42), whereas skeletal muscle atrophy, such as that following spinal cord injury (25), hindlimb suspension (HS) (2, 30), immobilization (56), denervation (10), or chronic heart failure (1), is associated with a loss of nuclei from the existing fibers, most likely by apoptosis-like processes (2, 25, 56). Aged muscle also exhibits an increased incidence of apoptosis in the rhabdosphincter, as well as skeletal muscle (5, 6, 20, 21, 58). Because there is a loss of muscle fibers in addition to a loss of CSA per fiber (7, 35), apoptosis in aged muscle could be the mechanism for the cellular, as well as nuclear loss that occurs during muscle atrophy.

Extrinsic or intrinsic stimuli are responsible for apoptosis, with some cross talk between signaling pathways (41, 50). For example, extrinsic or ligand-induced apoptosis through the tumor necrosis factor receptor superfamily causes the activation of cysteine-dependent, aspartate-specific protease (caspase)-8 and, consequently, caspase-3, a central cell death protease. Caspase-3 can activate caspase-activated DNase, leading to DNA fragmentation and, in mononucleated cells, cell death (8). The mitochondrial pathway of apoptosis is an example of an intrinsic pathway and is usually initiated by cell-internal stimuli such as reactive oxygen species (41, 49). On proapoptotic stimulation, mitochondria release cytochrome c, which combines with Apaf-1, caspase-9, and dATP in the cytoplasm, forming an apoptosome, which activates the central caspase-3 (41, 49). Caspase-independent mechanisms also exist, such as the release of apoptosis-inducing factor (AIF) and endonuclease G (EndoG) from mitochondria, inducing large-scale DNA fragmentation and apoptosis after translocation to the nucleus (15, 16, 38, 63). Few in vivo studies in skeletal muscle have been conducted to investigate intrinsic and extrinsic pathways of apoptosis. Dirks and Leeuwenburgh (20) showed that caspase-3 activity, Bcl-2-to-Bax ratio, and cytochrome c release were not increased in the gastrocnemius muscle of old rats, even though apoptosis was increased. In a recent study, the same group showed that levels of procaspase-3, caspase-3, and XIAP (an inhibitor of caspase-3 activity) were elevated and that caspase-3 protease activity was not altered with age (21). Other proapoptotic proteins such as caspase-12 and AIF increased with age. These studies suggest an elevated potential for apoptosis activation with age as well as adaptive responses to prevent apoptosis. Which of these mechanisms may be responsible for the age-associated increase in apoptosis in

AGING IS ASSOCIATED with a loss of skeletal muscle mass known as sarcopenia (53). By 60–70 yr of age, muscle mass in humans decreases by 25–30% as a result of a loss of fibers as well as a decrease in the total cross-sectional area (CSA) of the remaining fibers (12, 29, 37). Mechanisms responsible for sarcopenia are being actively investigated and include changes in neuroendocrine and hormonal function, deficiencies in satellite cell activation, proliferation, and differentiation, increases in free radicals and oxidative stress, and accumulation of mitochondrial abnormalities (for a review, see Refs. 22, 36, 65).
skeletal muscle is largely unknown and should also be investigated in young and old animals exposed to atrophy-inducing conditions.

It is well established that recovery from losses in muscle mass is impaired for old animals after an atrophy-inducing event such as immobilization (17, 66) or starvation (44). In addition, we recently showed that muscles from old animals are also impaired in their ability to maintain muscle mass with increased activity in the face of an atrophy-inducing event (30). Satellite cells are required to replenish damaged and lost nuclei after events such as muscle injury and atrophy, and it is known that satellite cell function is decreased with age (19, 48, 52, 64). Taken together, nuclear losses through apoptotic mechanisms during atrophy may determine the extent of muscle mass recovery, but this has not been experimentally addressed. Hence, the primary objective of this study was to investigate whether there are age-related differences in the extent of apoptotic nuclear losses after acute disuse atrophy. Moreover, we were interested to discover whether specific mechanisms such as caspase-3 activity or the release of the proapoptotic mitochondrial EndoG protein are involved in nuclear apoptotic loss. We utilized HS as a means to induce acute muscle atrophy and to investigate apoptosis signaling. HS is an excellent model to mimic disuse and, consequently, to better understand the mechanisms of acute disuse atrophy during aging (13, 28, 45, 60).

**METHODS**

**Animals and experimental procedures.** All procedures were performed in accordance with institutional guidelines for the care and use of laboratory animals following a protocol approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences. Male Fischer 344 X Brown Norway rats (6 and 32 mo) were purchased from the National Institute on Aging. This strain of rat was chosen because it has increased longevity and decreased cumulative lesion incidence compared with other strains; therefore, aging aspects can be studied in the relative absence of disease (39). The different ages were chosen to reflect a mature rat (6 mo) and an old rat at ~50% mortality (32 mo). Rats of both ages were divided into two groups (n = 6 per group): nonsuspended control and HS for 14 days. Rats were allowed free access to food and water. Animals were housed in a 12:12-h light-dark cycle. HS was performed as previously described (24). Briefly, a tail device containing a hook was attached with gauze and cyanoacrylate glue, while the animals were anesthetized with pentobarbital sodium (50 mg/kg body wt). After the animal regained consciousness, the tail device was connected via a thin cable to a pulley sliding on a vertically adjustable stainless steel bar running longitudinally above a high-sided cage with standard floor dimensions. The system was designed in such a way that the rats could not rest their hindlimbs against any side of the cage. After 14 days of control housing or HS, rats were killed with an overdose of pentobarbital sodium. The soleus muscles were dissected, weighed, and frozen. Muscles from one leg were frozen in liquid nitrogen and stored at ~80°C for biochemical analyses, and muscles from the other leg were embedded in a freezing medium, frozen in liquid nitrogen-cooled isopentane, and stored at ~80°C for immunohistochemical and histological analyses.

**Immunohistochemistry and histology.** Cross sections of soleus muscles were cut on a cryostat (6 μm), air dried, and stored at ~20°C until further analysis. Sections were rehydrated and then stained with standard hematoxylin and eosin for measurement of total muscle fiber CSA, as described previously (23). Muscle sections were viewed and captured as digital images using a Nikon Eclipse E600 microscope, CoolSnap camera, and MetaView software. CSA was determined on a total of 150 fibers from three different areas of the midbelly region of the soleus muscle, and the mean CSA was calculated.

To count myofiber nuclei, muscle sections were reacted with dystrophin antibody and Hoechst dye, as described previously (25, 26). Briefly, sections were rehydrated, and dystrophin antibody (Vector Laboratories, Burlingame, CA) was added at 1:4 dilution. An alkaline phosphatase-conjugated secondary antibody (Zymed, San Francisco, CA) was added, and sections were incubated with alkaline phosphatase substrate (Vector Laboratories) for color development. Subsequently, sections were fixed in 2% paraformaldehyde, and Hoechst 33258 nuclear dye (Molecular Probes, Eugene, OR) was applied at 1.2 ng/ml. Sections were viewed through a fluorescent microscope (Nikon) with an ultraviolet filter package, and digital pictures were saved and analyzed. Nuclei within the dystrophin-positive sarcolemma were counted in 120–150 fibers of three different areas of a section; the number of nuclei was expressed per 100 fibers. The CSA per nucleus was calculated by dividing the mean CSA by the number of nuclei per fiber.

EndoG staining was performed to determine the localization and quantitation of this protein. Sections were fixed in 2% paraformaldehyde, and endogenous peroxidase activity was blocked by immersion of sections in 3% H2O2 in 100% methanol for 30 min. Sections were then permeabilized in 1% Igepal (Sigma, St. Louis, MO) in PBS, and all further incubations and washes were performed in the presence of 0.1% Igepal. EndoG antibody (Abcam, Cambridge, UK) was applied at 3 μg/ml, and sections were incubated for 1–2 h at room temperature. Subsequently, a biotinylated mouse anti-rabbit IgG secondary antibody (Pierce, Rockford, IL) was applied for 1 h at room temperature. Sections were then incubated with streptavidin-peroxidase (Zymed), and dianisobenzidine substrate was applied. Sections were viewed, and the cellular location of EndoG was determined visually. Digital pictures of EndoG staining were analyzed using MetaView software. To determine the area occupied by EndoG on a section, digital pictures were taken in gray-scale format and thresholded. A random sample from an image of a control soleus muscle was taken, and the range of threshold was set on this image. This threshold value was then used on all images. A total area of 80,000 μm2 was analyzed for each soleus muscle. The number of fibers within each field was counted to normalize the fiber number as well as the area measured. Values are reported as a percentage of the threshold area per unit area or as a percentage of the threshold area per fiber. No difference between the samples was observed in the intensity of the staining; therefore, the area occupied by EndoG is an indication of the amount of EndoG within the tissue. To determine whether EndoG was colocalized with nuclei in the different experimental groups, additional staining of EndoG was performed using the Renaissance tyramide signal amplification (TSA) fluorescence system (Perkin Elmer, Boston, MA) as follows. Sections were fixed and blocked for endogenous peroxidase activity as described above; EndoG antibody (Abcam) was applied at 5 μg/ml, and sections were incubated overnight at 4°C. Sections were washed, and TSA blocking buffer was applied. Sections were further incubated in biotinylated mouse anti-rabbit secondary antibody (Pierce) and then in 1:400 dilution of ZhMax streptavidin-horseradish peroxidase (Zymed). After three washes, sections were incubated in TSA-fluorescein at 1:150 dilution in amplification buffer. To stain the nuclei, sections were covasellipped using Vectashield mounting medium with 4’,6-diamidino-2-phenylindole (DAPI, Vector Laboratories). Pictures were taken with a digital camera (Nikon), as described above.

**Detection of apoptotic nuclei.** Nuclei exhibiting apoptotic changes were identified by TdT-mediated dUTP nick end labeling (TUNEL), as described elsewhere (25) and according to the manufacturer’s recommendations (Roche Molecular Biochemicals, Pleasanton, CA). Briefly, soleus muscle cross sections were cut on a cryostat (6 μm) and fixed in 4% paraformaldehyde at room temperature, blocked in 3% H2O2 in 100% methanol at room temperature, and permeabilized in 0.1% Triton X and 0.1% sodium citrate. TUNEL reaction mix was applied to the slides, and the slides were then incubated at 37°C in a humidified atmosphere for 1 h. After washing, the slides were incubated in a blocking solution for 10 min and then incubated with anti-fluorescein antibody (1:150) for 1 h. Finally, the slides were incubated in FITC-labeled streptavidin antibody (1:150) for 1 h. The slides were then washed and mounted with Vectashield. Pictures of the sections were taken with a digital camera (Nikon). The percentage of TUNEL-positive nuclei was counted in 120–150 fibers of three different areas of a section; the number of TUNEL-positive nuclei was expressed per 100 fibers.

**Muscle cross-sectional area measurements.** CSA was determined on a total of 150 fibers from three different areas of the midbelly region of the soleus muscle, and the mean CSA was calculated. After 14 days of control housing or HS, rats were killed with an overdose of pentobarbital sodium. The soleus muscles were dissected, weighed, and frozen. Muscles from one leg were frozen in liquid nitrogen and stored at ~80°C for biochemical analyses, and muscles from the other leg were embedded in a freezing medium, frozen in liquid nitrogen-cooled isopentane, and stored at ~80°C for immunohistochemical and histological analyses.

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added in a 1:7.5 dilution, and the sections were incubated at 37°C for 1 h. Sections were reacted with fluorescein antibody for 30 min at 37°C, and substrate was added for color development. To determine whether positive TUNEL staining was observed inside the muscle membrane, sections were stained first with dystrophin. Sections were rehydrated, dystrophin antibody (Vector Laboratories) was added at 1:4 dilution, and a Texas red-conjugated goat anti-mouse IgG secondary antibody (KPL, Gaithersburg, MD) was applied (5 μg/ml). TUNEL staining was performed using a fluorescein TUNEL kit at 1:7.5 dilution as recommended by the manufacturer (Roche Molecular Biochemicals). Positive nuclei were counted, and at high power (∼400), it was determined whether they were associated with the myofiber or with the interstitial space. The number of positive nuclei was expressed per whole muscle section.

Cytosolic mono- and oligonucleosomes. Apoptotic DNA fragmentation was quantified by measuring the amount of cytosolic mono- and oligonucleosomes in the muscle using a Cell Death ELISA kit (Roche Molecular Biochemicals) as previously described (20, 21). Muscles were homogenized using a Polytron in isolation buffer: 220 mM D-mannitol, 75 mM sucrose, 0.1% fatty acid-free bovine serum albumin, 0.5 mM EGTA, and 2 mM HEPES, pH 7.4 (1:10 wt/vol). The homogenate was centrifuged at 700 g at 4°C for 10 min, and the supernatant was centrifuged again at 8,000 g at 4°C for 10 min. The supernatant was carefully collected, and protein concentration was determined according to the Bradford method (11). Mono- and oligonucleosomes in the muscle using a Cell Death ELISA kit (Roche Molecular Biochemicals) as described previously (21). The substrate Ac-DEVD-aminomethylcoumarin is cleaved proteolytically by caspase-3, and the fluorescence of free aminomethylcoumarin is measured. Fluorescence was determined at 460 nm using a Spectra Max fluorescent microplate reader (Molecular Devices, Sunnyvale, CA). Values are expressed as arbitrary optical density units normalized to milligram of protein.

Caspase-3 activity determination. Caspase-3 activity was measured in the cytosolic fraction of the muscle homogenate using a fluorometric activity assay kit (Roche Molecular Biochemicals), as described previously (21). The substrate Ac-DEVD-aminomethylcoumarin is cleaved proteolytically by caspase-3, and the fluorescence of free aminomethylcoumarin is measured. Fluorescence was determined with an excitation wavelength of 380 nm and an emission wavelength of 460 nm using a Spectra Max fluorescent microplate reader (Molecular Devices, Sunnyvale, CA). Values are expressed as arbitrary optical density units per milligram of protein.

Statistics. To test for statistically significant differences, two-way ANOVA was used; in case of significant differences, Tukey’s multiple comparisons test was applied. Statistical significance was assessed at P < 0.05.

RESULTS

Muscle size and nuclear number. Soleus muscle weight and CSA were 24% and 26% lower, respectively, in the 32-mo-old than in the 6-mo-old rats. Moreover, the soleus muscle weight-to-body weight ratio was 35% lower in the older animals (Table 1), indicating that significant sarcopenia had occurred by 32 mo of age. HS in 6-mo-old rats was associated with a 49% and 38% decrease in soleus muscle weight and muscle weight-to-body weight ratio, respectively, and a 72% decrease in CSA, whereas in 32-mo-old rats muscle weight and muscle weight-to-body weight ratio decreased by 33% and 22%, respectively, with HS, and CSA was 58% lower (Table 1). Therefore, HS was associated with a decrease in muscle size in young and old animals, but the response was somewhat attenuated in the older animals.

Surprisingly, the age-related decrease in muscle size was not associated with a decrease in myonuclear number (Fig. 1A), resulting in a decreased CSA per nucleus in 32-mo-old soleus muscle (Fig. 1B). This suggests that nuclei in old muscles may not function as efficiently as nuclei in young muscles and that more nuclei are needed for optimal function, hence, the smaller nuclear domain. As expected, in 6-mo-old rats the decrease in

Table 1. Soleus muscle weight, muscle weight-to-body-weight-ratio, and CSA in young and old male Fischer 344 × Brown Norway rats

<table>
<thead>
<tr>
<th></th>
<th>6 Months</th>
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<th>32 Months</th>
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<tr>
<td></td>
<td>Control</td>
<td>HS</td>
<td>Control</td>
<td>HS</td>
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<tr>
<td>Muscle wt, mg</td>
<td>167.9±4.9</td>
<td>86.2±2.3*</td>
<td>127.6±3.1†</td>
<td>85.3±3.6*</td>
</tr>
<tr>
<td>Muscle wt-to-body wt, ratio, mg/g</td>
<td>0.430±0.008*</td>
<td>0.266±0.003*</td>
<td>0.279±0.008†</td>
<td>0.219±0.014*</td>
</tr>
<tr>
<td>CSA, μm²</td>
<td>2,976.8±137.9</td>
<td>1,135.3±64.6*</td>
<td>2,213.9±128.9†</td>
<td>926.2±94.8*</td>
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</tbody>
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Values are means ± SE. CSA, cross-sectional area; HS, hindlimb suspension. *Significantly different from control (P < 0.05). †Significantly different from 6 mo (P < 0.05).
Table 2. TUNEL-positive nuclei per section in soleus muscle from young and old male Fischer 344 × Brown Norway rats

<table>
<thead>
<tr>
<th></th>
<th>6 Months</th>
<th></th>
<th>32 Months</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>HS</td>
<td>Control</td>
<td>HS</td>
</tr>
<tr>
<td>Total, no./section</td>
<td>1.0±0.9</td>
<td>11.3±1.6*</td>
<td>10.5±2.3†</td>
<td>21.2±1.6*†</td>
</tr>
<tr>
<td>Myofibrillar, no./section</td>
<td>0.7±0.2</td>
<td>7.5±1.1*</td>
<td>8.0±2.0†</td>
<td>15.2±1.7*†</td>
</tr>
<tr>
<td>Interstitial, no./section</td>
<td>1.0±0.4</td>
<td>3.8±0.8*</td>
<td>2.5±0.6</td>
<td>6.0±0.9*</td>
</tr>
<tr>
<td>Myofibrillar, % of total</td>
<td>45.0±15.1</td>
<td>66.4±4.2</td>
<td>73.7±14.0</td>
<td>71.0±4.4</td>
</tr>
</tbody>
</table>

Values are means ± SE. TUNEL, Tdt-mediated dUTP nick and labeling. *Significantly different from control (P < 0.05). †Significantly different from 6 mo (P < 0.05).
soleus muscles of old and HS rats. Caspase-3 activity tended to increase with age (+47%), but these changes were not statistically significant (P = 0.052; Fig. 5). This finding is similar to what has been shown previously for gastrocnemius muscles (20, 21). HS-induced soleus muscle atrophy in 6-mo-old rats was associated with an 84% elevation of caspase-3 activity (Fig. 5). By contrast, in soleus muscles from 32-mo-old rats, the 22% elevation of caspase-3 activity in response to HS was not statistically significant (P = 0.052), likely because basal levels in control rats are already elevated. This suggests that additional mechanisms likely are involved in the execution of apoptosis in old muscles or that pathways other than caspase-3 activation are being recruited. We investigated whether a caspase-independent mechanism was involved in the elevation of apoptosis as a result of HS and aging. Muscle cross sections were immunoreacted with EndoG antibody to investigate whether there was a translocation of EndoG to the nucleus in muscles undergoing apoptotic nuclear loss. EndoG staining was performed alone (Fig. 6, A–D) or in combination with DAPI (Fig. 6, E–H) to determine whether EndoG colocalizes with the nuclei. In the double-stained sections, EndoG is colored green and DAPI stains the nuclei blue; when double staining occurs, the nuclei turn light blue. In soleus muscles from control 6-mo-old rats, EndoG staining was mainly observed under the sarcolemma, coinciding with the location of subsarcolemmal mitochondria (Fig. 6, A and E). In soleus muscles from 32-mo-old control rats, staining was observed in the same region as in the young animals, but staining also coincided with nuclear localization (Fig. 6, B and F, arrow), indicating that nuclear translocation of EndoG had taken place in soleus muscles from old animals. With HS in young rats, EndoG staining was more punctate (Fig. 6, C), and EndoG was
colocalized with nuclei, as indicated by the light blue nuclei in Fig. 6G (arrows). Similarly, in old rats after HS, EndoG staining was colocalized with nuclei at a very high frequency (Fig. 6, D and H, arrows). To quantify the amount of staining, we investigated the density of the staining and the area stained. Because the density of staining was not different between the groups, the area stained represented the amount of EndoG in the muscles. The percentage of stained area in soleus muscles from 6-mo-old rats was unchanged after HS, whether it was normalized to the number of fibers in the counted area (Fig. 7B) or not (Fig. 7A). By contrast, in soleus muscles from 32-mo-old rats, HS was associated with a 4.8-fold increase in the percent area stained by EndoG when not normalized to fiber number and a 2.7-fold increase after normalization to fiber number. Therefore, EndoG translocation occurs with HS in young and old soleus muscles, but in old rats, HS is also associated with an increase in the protein abundance of EndoG in soleus muscles.

**DISCUSSION**

Atrophy of skeletal muscle tissue is associated with an increase in apoptosis (2, 6, 25, 30, 56), which is also elevated in skeletal muscles from old animals (20, 21). In this study, we investigated whether age-related differences existed in the extent of nuclear loss and apoptosis and in pathways utilized for apoptosis after acute disuse. We found that apoptosis increased with sarcopenia in the soleus muscle, as has been shown previously for the gastrocnemius (20, 21) and rhaddosphincter (58) muscles. In addition, we showed that the...
difference in apoptosis is mainly due to an increase in apoptotic nuclei associated with myofibers and not interstitial cells in soleus muscles from the old rats. More importantly, we demonstrated that soleus muscles from aged rats exhibited an exaggerated increase in apoptosis in myofibrillar and interstitial nuclei in response to acute disuse induced by HS. This finding suggests that there is a loss of nuclei and/or cells in muscles from old rats that exceeds that in young animals and that the proportion of myofibrillar apoptotic nuclei is unchanged. However, the number of myofiber nuclei in soleus muscles did not decrease with disuse in muscles from old animals, in contrast to that in young animals, indicating that there may be deregulation in myonuclear number in muscles from old rats. In addition, the increased apoptosis observed in the face of a stable myonuclear pool implies that muscle stem cells may have fused to muscle fibers of old animals. We previously showed an increase in centrally located and bromodeoxyuridine-labeled myofibrillar nuclei in old control muscles (30), likely the result of fused satellite cells.

It is unknown why the nuclear domain is decreased in muscles from old animals and why there is a further decrease with disuse induced by HS. It is known that slow-twitch fibers, which are most prevalent in rat soleus muscle, contain a smaller myonuclear domain than fast-twitch fibers (3, 62) and that HS induces a shift from slow- to fast-twitch fibers (59, 61). Therefore, the smaller nuclear domain after HS cannot be explained by a possible shift in fiber type in soleus muscle. We hypothesize that nuclei in muscles from old animals are less efficient, possibly because of extensive DNA damage as occurs with aging; therefore, more nuclei per area of cytoplasm are needed to maintain normal function of the muscle fibers. It has indeed been shown that there is an increase in DNA damage in muscle nuclei from older animals (32, 51) because of an increase in oxidative stress (9). Whether the same explanation applies for the decrease in myonuclear domain after disuse is unknown. It has been shown that oxidative stress and gene expression of oxidative stress-related proteins are increased after an atrophy-inducing event (33, 34, 46, 57), but whether DNA damage is also increased remains to be determined.

Because aging is associated with a decrease in muscle fiber number (37, 43), some of the observed apoptosis may be associated with a loss of actual fibers and not just a loss of myofiber nuclei or interstitial cells. If fibers were completely lost due to apoptosis, it would be expected that apoptotic changes occurred in clusters. We did not observe these clusters of apoptotic nuclei; rather, the apoptotic nuclei appeared to be randomly dispersed throughout the muscle tissue, as we and others reported previously in response to disuse atrophy (2, 25). Also, the conclusions about the myonuclear domain size do not change if fiber loss were to be considered, because nuclear number would not have been counted in the lost fibers. Therefore, we suggest that fiber loss due to apoptosis is not a major mechanism involved in acute muscle disuse but may play a role in age-associated muscle loss, which occurs over a more extended period of time.

Caspase-3 is a central mediator of cell death, because many apoptotic signaling (e.g., receptor- and mitochondria-mediated) pathways converge at this point (41, 49). Previous studies have shown that caspase-3 activity was not elevated in muscles from old rats, even though the protein abundance of pro-caspase-3 and cleaved caspase-3 was elevated in aged gastrocnemius muscles (20, 21). Data from the present study examining the soleus muscle confirmed this finding, even though there was a strong trend for caspase-3 activity to be elevated in soleus muscle from old rats (P = 0.052). Whether caspase-3 is involved in disuse-induced muscle atrophy at different ages was previously unknown. We found that caspase-3 activity was increased in soleus muscles undergoing atrophy induced by HS in young rats, but the increase in old rats was not significant. Thus age-related differences exist in the apoptotic pathways utilized by muscles in young and old rats, and the potential to undergo apoptosis in skeletal muscle is greater in old than in young animals. Indeed, two recent studies have also indicated that aging influences the apoptotic responses of muscles during disuse atrophy (54, 55).

In multinucleated cells, such as skeletal muscle myocytes, apoptosis may not proceed by the classical pathway, because the goal of apoptosis during acute skeletal muscle atrophy is to eliminate nuclei and, to a lesser extent, cytoplasmic proteins or the entire cell. Caspases have cytosolic substrates such as actin (27), which is needed by the myocytes for maintenance of normal cell function; therefore, activation of caspases may not be desirable. Thus caspase-independent mechanisms are likely more prevalent, because such pathways act directly on the nucleus to induce DNA fragmentation (40), bypassing caspase activation. EndoG is a mitochondrion-specific nuclease that is released from the mitochondria in response to truncated Bid and translocates to the nucleus during apoptosis (38, 63) without the involvement of caspases. Because calpain can
activate Bid by cleavage to truncated Bid (18) and because calpain is elevated with aging (47), we investigated the role of EndoG in the age-associated and disuse-induced muscle atrophy of the soleus muscle. We found that EndoG was colocalized in the nucleus in the muscles of old animals; therefore, EndoG is likely involved in apoptosis observed in aged soleus muscles. Also, in young muscle, EndoG was colocalized in the nucleus in muscle fibers after HS; therefore, this caspase-independent endonuclease may be involved in disuse-induced, as well as age-associated, muscle atrophy. More importantly, in soleus muscles from old rats after HS, EndoG was not only localized in a nuclear position in most fibers, but the amount of EndoG in the muscle was also increased. It has been shown that the levels of EndoG in cardiac myocytes were correlated with oxidative damage in this tissue (31), but apoptosis was not studied in that report. In our study, the elevated levels of EndoG in soleus muscle from old HS rats could indicate elevated oxidative stress leading to elevated apoptosis. These results suggest that disuse-induced apoptosis in young muscles is likely caspase dependent, because caspase-3 activity was elevated. In contrast, caspase-3-independent pathways may play a more important role in muscles from old animals. Moreover, other proapoptotic proteins located in the mitochondria, such as AIF (21), could have facilitated the extent of nuclear fragmentation, and this warrants further investigation.

In summary, we found that the level of apoptosis was elevated in soleus muscles from aged rats, even though myonuclear number was not changed, suggesting a deregulation of the myonuclear domain. This becomes more apparent when atrophy is induced in the soleus muscle: the loss of myofiber nuclei, as observed in the soleus muscle from young rats, does not occur in old animals, even though apoptosis is increased dramatically. The importance of the increased apoptosis without a change in myonuclear number remains to be investigated. Pathways leading to apoptosis are distinct between young and old soleus muscles, such that in soleus muscles from young rats, caspase-dependent mechanisms are activated during atrophy, whereas in soleus muscles from old rats, additional pathways, such as the caspase-independent translocation of EndoG, may be more prominent. Future studies should further investigate signals leading to apoptosis in muscles undergoing atrophy and determine the differences that occur with age.

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