Nuclear translocation of EndoG at the initiation of disuse muscle atrophy and apoptosis is specific to myonuclei

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SKELETAL MUSCLE ATROPHY is associated with numerous pathological and physiological conditions, such as congestive heart failure, cancer, AIDS, aging, and disuse, and carries a poor prognosis when present in disease states. Therefore, determining the mechanisms and molecules involved in the induction of atrophy is of pivotal importance for the development of therapeutic interventions. Decreased muscle protein synthesis, as well as increased muscle protein degradation have been shown to occur during skeletal muscle atrophy, and signaling pathways involved in these processes have been studied (reviewed in Refs. 30, 38, 41, 51, and 74). Additionally, myofiber nuclear number decreases during atrophy (3–5, 22, 23, 27, 62), and this could be a mechanism by which transcriptional potential decreases in skeletal muscle. However, it is not clear whether loss of nuclei initiates or is a consequence of the loss of muscle mass during atrophy.

The elimination of nuclei during atrophy appears to be through apoptosis because it involves chromatin condensation and DNA fragmentation (2, 49). The cellular and molecular mechanisms responsible for apoptosis in skeletal muscle are largely unknown. In other cell types, cytochrome c release and caspase activation have been regarded as hallmarks of apoptosis, but because of the multinucleated nature of skeletal muscle, apoptosis may follow different pathways. It was shown that the sarcopenia-related increase in apoptosis may be independent of cytochrome c (15) and the role of caspase-3 in apoptosis in skeletal muscle is not as well established. For example, caspase-3 in skeletal muscle has been suggested to be the initial protease responsible for the breakdown of myofibrillar protein, which subsequently can be processed by the proteasome resulting in muscle atrophy (17, 33), and it is also involved in the activation of phosphorylase b kinase (34). Moreover, it has recently been shown that caspase-3 contributes to muscle weakness induced by inflammation (70), indicating that the role of caspase-3 in muscle atrophy is not as clear as in other cell types. Indeed, caspase-3 activity was not changed after denervation in humans (72) or hindlimb suspension in rats (67) in contrast to its elevation after denervation in rats (66). Moreover, conflicting data exist about the activity of different caspases and different members of the Bcl-2 family of proteins during atrophy-induced apoptosis in skeletal muscle (for review, see Ref. 18). The fact that nuclear apoptosis in myofibers occurs without cellular death raises the question of whether the mechanisms by which nuclear apoptosis ensues in skeletal myofibers are distinct from those involved in apoptosis in mononucleated cells where nuclear, as well as cytoplasmic contents, are cleared. Thus as muscle atrophy involves both interstitial cell and myofiber nuclear loss (2, 49, 54), distinct mechanisms may regulate interstitial cell death compared with myofiber nuclear loss.

We previously investigated the involvement of a caspase-independent mechanism in muscle atrophy: endonuclease G (EndoG) localization in nuclei of atrophic muscles. EndoG is a mitochondrial protein that translocates to the nucleus on release from mitochondria and induces large-scale DNA fragmentation and apoptosis (10, 50, 76). We showed that EndoG localization to nuclei following unloading. The goal of this study was to determine whether the onset of apoptosis in response to disuse was consistent with the hypothesis that EndoG is involved in myofiber nuclear loss. Atrophy was induced by hindlimb suspension for 12 h or 1, 2, 4 and 7 days in 6-mo-old rats. Soleus myofiber cross-sectional area decreased significantly by 2 days, whereas muscle mass and muscle-to-body mass ratio decreased by 4 and 7 days, respectively. By contrast, a significant increase in apoptosis, evidenced by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive nuclei, occurred as early as 12 h after suspension, preceding the elevation in muscle atrophy F-box gene expression. The early increase in apoptosis appeared to be specific to myofiber nuclei, whereas TUNEL-positive interstitial cells did not become significantly elevated until 2 days after suspension. Furthermore, TUNEL-positive myofiber nuclei colocalized with EndoG as early as 12 h after suspension, and no such localization was observed in interstitial cells. Although no significant change in total activated caspase-3, -7, or -12 protein abundance was apparent, activated caspase-3 was expressed in positive myofiber nuclei colocalized with EndoG as early as 12 h after suspension. Furthermore, TUNEL-positive myofiber nuclei colocalized with EndoG as early as 12 h after suspension, and no such localization was observed in interstitial cells. Thus these data indicate that apoptosis is an early, and therefore consistent with the hypothesis that EndoG is involved in myofiber nuclear loss.

Skeletal muscle atrophy is associated with an increase in apoptosis, whereas interstitial cells may undergo apoptosis via a more classical, caspase-dependent pathway.

caspase; muscle atrophy F-box; hindlimb suspension; endothelial cells; endonuclease G

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localized to nuclei in muscles atrophied due to either disuse or aging and that the abundance of EndoG protein was increased in muscles from old animals undergoing disuse atrophy (49). Therefore, the translocation of EndoG to nuclei in skeletal muscle could be a specific mechanism by which myofiber nuclei are removed.

The goal of the current study was to investigate the temporal sequence of events occurring during atrophy and apoptosis in skeletal muscle in response to disuse, and we hypothesized that EndoG is involved in myofiber nuclear apoptosis in skeletal muscle undergoing atrophy. Disuse atrophy was induced by hindlimb suspension in rats, and the appearance of apoptotic myofiber nuclei, as well as apoptotic interstitial cells, was studied at early time points after the onset of disuse.

METHODS

Animal Procedures and Tissue Collection

All procedures on animals were performed in accordance with institutional guidelines for the care and use of laboratory animals, and protocols were approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences (UAMS). Male, Sprague-Dawley rats were purchased from Harlan (Indianapolis, IN) at 6 mo of age, allowed to acclimate for 1 wk in the animal facilities of UAMS, fed standard laboratory rat chow, and maintained on a 12:12-h light-dark cycle before the start of the experiment. Rats were randomly assigned to the following six groups: control, hindlimb suspended for 12 h, 1 day, 2 days, 4 days, or 7 days (n = 8). Rats in the hindlimb-suspended groups were suspended for the indicated amount of time using a method previously described (25, 27, 49). Briefly, a tail device containing a hook was attached with gauze and cyanoacrylate glue while the animals were anesthetized with ketamine and xylazine (60 and 10 mg/kg body wt ip, respectively). 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 as above. The system was designed in such a way that the rats could not rest their hindlimbs against any side of the cage to ensure complete unloading of the hindlimbs. Control rats were also anesthetized, but no tail device was attached. At the designated time points, rats were euthanized by an overdose of pentobarbital sodium (100 mg/kg body wt), and 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 freezing medium at resting length, frozen in liquid nitrogen-cooled isopentane, and stored at −80°C for immunohistochemical and histological analyses.

Histology and Immunohistochemistry

For all antibodies and for the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay, negative control slides were included without primary and secondary antibodies or terminal deoxynucleotidyl transferase enzyme, respectively, to ensure specificity of the antibody binding, enzyme activity, and fluorescence (pictures not shown).

Cross-sectional area determination. 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 in PBS, and a standard hematoxylin and eosin stain was performed to measure the total myofiber cross-sectional area as described previously (20, 27, 49). Muscle sections were viewed and captured as digital images using a Nikon Eclipse E600 microscope, CoolSnap camera, and MetaView software. Cross-sectional area was determined on a total of 150 fibers from three different areas of the midbelly region of the soleus muscle, and the mean myofiber cross-sectional area was calculated.

Determination of nuclear apoptosis. Nuclei exhibiting apoptotic changes were identified by TUNEL as described below (22, 49) and according to the manufacturer’s recommendations (Roche Molecular Biochemicals, Pleasanton, CA). To determine whether TUNEL-positive nuclei were inside the muscle membrane or in interstitial cells, soleus muscle sections were first immunoreacted with an anti-dystrophin antibody (Vector Labs, Burlingame, CA). Sections were rehydrated in PBS, and anti-dystrophin was added at 1:4 dilution for 4 h at room temperature after which a Texas Red conjugated goat anti-mouse IgG secondary antibody (KPL, Gaithersburg, MD) was applied at 1:200 for 2 h at room temperature. Sections were then fixed in 4% paraformaldehyde at 4°C for 1 h, blocked in 3% H2O2 in 100% methanol at room temperature for 30 min, and permeabilized in 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice. TUNEL reaction mix was added in a 1:7.5 dilution and incubated at 37°C for 90 min in the dark. Sections were reacted with fluorescein antibody for 30 min at 37°C followed by incubation in tyramide signal amplification (TSA) buffer (Perkin Elmer, Boston, MA) for 10 min at room temperature. Sections were coverslipped using Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector). Positive nuclei were counted and at high power (∼400); it was determined whether the positive nuclei were inside or outside the myofiber membrane as visualized by dystrophin. Nuclei inside the muscle membrane are myofiber nuclei, and those outside the muscle membrane were called interstitial nuclei and could potentially include cell types such as fibroblasts, endothelial cells, leukocytes, and macrophages, as well as satellite cells. The number of positive nuclei was expressed per whole muscle section.

Determination of EndoG location. Soleus muscle cross sections were immunoreacted with the anti-dystrophin antibody as described above and with the anti-EndoG antibody (Abcam, Cambridge, UK) as described previously (49). Briefly, after dystrophin detection, sections were fixed in 4% paraformaldehyde for 30 min at 4°C and permeabilized in 1% IGEPA/PBS. Endogenous peroxidase activity was blocked using Peroxide Block (InnoGenex, South San Francisco, CA) twice for 2 h at room temperature. Sections were then incubated in anti-EndoG antibody (5 μg/ml; Abcam, Cambridge, MA) for 2 h at room temperature, followed by an overnight incubation at 4°C. A biotin-conjugated mouse anti-rabbit IgG secondary antibody (Pierce, Rockford, IL) was applied at 1:300 dilution and incubated for 30 min at room temperature followed by horseradish peroxidase (HRP)-conjugated streptavidin at 1:400 for 6 min at room temperature (Zymed, South San Francisco, CA). Sections were then incubated in TSA buffer (Perkin Elmer) for 10 min at room temperature and coverslipped using Vectashield mounting medium with DAPI (Vector). The number of myofibers and the number and location of EndoG-positive

Table 1. Soleus muscle size measurements at indicated times after hindlimb suspension

<table>
<thead>
<tr>
<th>Time</th>
<th>Muscle weight, mg</th>
<th>Muscle weight/body weight, mg/g</th>
<th>Muscle fiber cross sectional area, μm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>184.6±5.8</td>
<td>0.397±0.008</td>
<td>4136±188</td>
</tr>
<tr>
<td>12 h</td>
<td>172.0±8.6</td>
<td>0.414±0.011</td>
<td>3779±72</td>
</tr>
<tr>
<td>1 Day</td>
<td>175.4±10.8</td>
<td>0.415±0.020</td>
<td>4006±219</td>
</tr>
<tr>
<td>2 Days</td>
<td>167.8±5.1</td>
<td>0.384±0.015</td>
<td>3447±194*</td>
</tr>
<tr>
<td>4 Days</td>
<td>150.6±4.3*</td>
<td>0.387±0.008</td>
<td>3323±195*</td>
</tr>
<tr>
<td>7 Days</td>
<td>142.3±3.6*</td>
<td>0.355±0.009*</td>
<td>2804±138*</td>
</tr>
</tbody>
</table>

Values are means ± SE; *P < 0.05 significantly different from control.
nuclei were counted in four nonoverlapping areas per section. Nuclei were counted as EndoG positive only if clear colocalization of Hoechst dye and EndoG antibody was observed, as evidenced by a change in color from dark blue to light blue of the nucleus. Nuclei surrounded only by EndoG were not counted as positive if there was no clear colocalization inside the nucleus. A total of ~100 fibers per section were counted.

**Determination of colocalization of EndoG and DNA fragmentation.** DNA fragmentation was identified by TUNEL staining as described above. After the completion of the TUNEL staining, EndoG antibody (Abcam) was applied at 2.5 μg/ml in PBS with 2% normal goat serum and incubated overnight at 4°C. After incubation and washes, Texas Red conjugated goat anti-rabbit IgG secondary antibody was applied in 1:200 dilution in PBS and incubated for 1 h at room temperature (anti-rabbit Ig ImmPRESS reagent kit, Vector). Caspase-3 antibody (Cell Signaling) diluted 1:100 in ImmPRESS blocking solution was incubated overnight at 4°C after which ImmPRESS anti-rabbit Ig peroxidase-labeled secondary antibody was applied. TSA fluorescein and Hoechst staining was performed as described above, sections were visualized, and the localization of caspase-3 positive cells relative to the myofiber membrane was determined.

**Identification of TUNEL-positive endothelial cells.** Soleus muscle cross sections were assayed for TUNEL as described above. Following TUNEL assay, sections were fixed in acetone/methanol (90:10) for 5 min at 4°C, washed, and incubated overnight at 4°C with monoclonal mouse anti-αI-CD31 (PECAM-1) antibody (BD Biosciences Pharmingen, San Jose, CA) at 2.5 μg/ml in PBS. After being washed in PBS, sections were incubated with a biotinylated rat anti-mouse IgG1 secondary antibody (Zymed) at a dilution of 1:300 in PBS for 1 h at room temperature, following which Texas Red streptavidin was applied at a 1:250 dilution in PBS. Sections were then washed and incubated in Hoechst dye as described previously (22) and as suggested by the manufacturer (Invitrogen/Molecular Probes, Carlsbad, CA). Sections were viewed with Nikon Eclipse E600 fluorescent microscope and photographed using CoolSnap camera and MetaView imaging software.

**Isolation of Protein and Western Blot Analysis.** Western blot analysis of proteins was performed as described previously with minor modifications (21). Briefly, soleus muscles were homogenized in a buffer containing 10 mM MgCl2, 10 mM K2HPO4, 1 mM EDTA, 5 mM EGTA, 1% IGEPAL, 50 mM βGPO4, 1 mM PMSF, 1 mM Na3VO4, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 10 μg/ml benzamidine, 1 μg/ml apro tin, 1 μg/ml chymostatin, and 1 μg/ml pepstatin. After homogenization, samples were centrifuged for 10 min at 1,000 g at 4°C. Protein concentration of the supernatants was determined according to Bradford (6) using the Bio-Rad (Hercules, CA) protein assay reagent. For determination of activated caspase-12 and EndoG protein content, 100 μg total protein was loaded and separated on a 10% polyacrylamide gel (Bio-Rad), and for activated caspase-3 and caspase-7 protein abundance, 100 μg total protein was separated on a 15% polyacrylamide gel (Bio-Rad). After electrophoretic separation, proteins for activated caspase-12 and EndoG analysis were transferred to nitrocellulose membranes (Bio-Rad) and for activated caspase-3 and activated caspase-7 to PVDF membranes with 0.22 μm pore size. Subsequently, the membranes were incubated in Ponceau S solution (Sigma, St. Louis, MO) for 5 min for visualization of the protein and assurance of equal loading in all the lanes and pictures were saved for normalization of total protein after Western blot analysis. Membranes were incubated in blocking solution [5% blocking grade nonfat dry milk (Bio-Rad) in Tris-

*Fig. 1. Abundance of muscle atrophy F-box (MAFbx) mRNA is significantly increased 2 days after hindlimb suspension. MAFbx mRNA abundance normalized to β2-microglobulin mRNA in soleus muscle from control rats and at 12 h, 1 day, 2 days, 4 days, and 7 days after hindlimb suspension was quantified by real time RT-PCR. *Significant difference from control, P < 0.05.*

*Fig. 2. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive nuclei are located inside and outside myofibers. Soleus muscle cross sections (A and B) were immunoreacted with dystrophin antibody to identify the sarcolemma (red), and the TUNEL assay was performed to identify apoptotic nuclei (green). 4',6-diamidino-2-phenylindole (DAPI; blue) identified all nuclei. Nuclei inside the dystrophin stain (arrow in A and inset magnified below) were identified as myofiber nuclei, whereas nuclei outside (arrow in B and inset magnified below) were counted as interstitial cell nuclei. Bar indicates 10 μm.*
buffered saline + 0.1% Tween] for 1 h at room temperature followed by incubation with activated caspase-12 (1:750; Cell Signaling, Beverly, MA), activated caspase-3 (1:550; Cell Signaling), activated-caspase-7 (1:700; Cell Signaling), or EndoG (1:800; Abcam) antibodies. All membranes were incubated overnight at 4°C, washed, and further incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:1,000; Pierce). Antibody binding was detected by incubating membranes for 5 min in SuperSignal (Pierce) at room temperature. Membranes were exposed to X-ray film or ChemiImager 5500 (AlphaInnotech, San Leandro, CA), and density of the bands was determined by ChemiImager software (AlphaInnotech) and normalized to Ponceau S.

Isolation and Analysis of RNA

RNA isolation was performed using Totally RNA isolation kit (Ambion, Austin, TX) according to the manufacturer’s instructions. Total RNA was treated with DNase (Ambion) before measurement of mRNA abundance by RT-PCR, as previously described (19). Quantitative real-time RT-PCR was performed using the protocols, chemistries, and the amplification and detection systems of Applied Biosystems (Foster City, CA). For each sample, cDNA was synthesized from 1 μg of DNase-treated total RNA using components from Taqman Reverse Transcription Reagents (Applied Biosystems). The reaction contained 1× RT buffer, 5.5 mM MgCl₂, 0.5 mM dNTPs, 2.5 mM random hexamers, 40 units of RNase inhibitor, and 375 units of multiscribe reverse transcriptase. The primers were allowed to anneal for 10 min at 25°C before the reaction proceeded for 1 h at 37°C followed by 5 min at 95°C. The resulting cDNA samples were aliquoted and stored at −80°C. Primer sequences were selected from the accession numbers in NCBI database using the Taqman Probe and Primer Design function of the Primer Express v1.5 software (Applied Biosystems) and were as follows: β-2 microglobulin (NM_012512) forward, 5’-cgtgcttgccattcagaaaa-3’; reverse, 5’-gaagttgggcttcccattctc-3’; muscle atrophy F-box (MAFbx), forward, 5’-gacctgcatgtgctcagtgaag-3’; reverse, 5’-ggatctgccgctctgagaagt-3’. PCR reactions were assembled using the SYBR Green PCR Master Mix that required only the addition of cDNA template and primers. Control reactions were run lacking template to check for reagent contamination and to determine the melting temperature of any primer dimer. To optimize assay efficiency, PCR standard curves were produced using a pool containing each sample cDNA. Data points were generated using fourfold serial dilutions of cDNA. Gene expression was compared in individual samples by using 16 ng RNA equivalents of cDNA. The reactions were performed using the ABI Prism™ 7700 Sequence Detection System (Applied Biosystems) and the instrument’s universal cycling conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s, and then 60°C for 1 min. An additional cycle was added in which the ramp time to 95°C was increased to 19 min and 59 s during which time data were collected for melting curve analysis. RNA abundance for each gene of interest is expressed as a ratio normalized to RNA abundance of β-2 microglobulin in the same sample.

Statistical Analysis

To test for statistically significant differences between the groups, one-way ANOVA was used; when significant F-ratios were observed, a Student-Newman-Keuls multiple comparisons test was applied to test individual means. Statistical significance was assumed at P < 0.05.

RESULTS

Progression of Muscle Atrophy with Hindlimb Suspension

Soleus muscle weight decreased progressively with hindlimb suspension, reaching significance at 4 days of hindlimb suspension (19%) and declined further to 23% less than control at 7 days (Table 1). Since the rats also lost body weight suspended, the muscle weight-to-body weight ratio showed a significant 11% decline only at 7 days after the onset of suspension (Table 1). Soleus myofiber cross-sectional area, on the other hand, decreased more rapidly, showing a significant
17% decline at 2 days and that progressed further to a 32% decrease compared with control at 7 days after hindlimb suspension (Table 1). These data indicate that the earliest measurable decrease in muscle size was detected at 2 days after the onset of hindlimb suspension. MAFbx has been suggested as an early marker of skeletal muscle atrophy (29), and therefore its gene expression was measured. No change was observed compared with control after 12 h of suspension, and the 2.1-fold increase at 1 day failed to reach significance (Fig. 1). However, MAFbx mRNA abundance showed a significant 2.8-fold increase compared with control after 2 days of suspension and remained elevated at 4 and 7 days (Fig. 1), suggesting that MAFbx-dependent ubiquitin ligation is not elevated until after 1–2 days of hindlimb suspension.

Quantification of TUNEL-Positive Nuclei

To investigate the onset and progression of apoptosis and to determine which nuclei were undergoing apoptotic changes during the disuse-induced atrophy, soleus muscle cross sections were immunoreacted with an antibody against dystrophin, outlining the muscle membrane, and the TUNEL assay was performed to identify nuclei undergoing DNA fragmentation (Fig. 2). TUNEL-positive nuclei inside the dystrophin stain were counted as myofiber nuclei (Fig. 2A), and those outside were marked as interstitial nuclei (Fig. 2B). The total number of TUNEL-positive nuclei was elevated compared with control as early as 12 h after the onset of hindlimb suspension, reaching a maximum threefold difference compared with control at 2 days after suspension (Fig. 3A). The number of myofiber TUNEL-positive nuclei was elevated more than twofold as early as 12 h after the onset of suspension and remained higher until 4 days after suspension (Fig. 3B). This indicates that apoptotic nuclear changes occurred rapidly in myofiber nuclei and were observed before markers of muscle loss, such as an increase in MAFbx gene expression or a decrease in cross-sectional area, were detected. On the other hand, TUNEL-positive interstitial cells were not elevated until 2 days after the onset of hindlimb suspension after which they slowly declined (Fig. 3C). Therefore, the increase in apoptosis observed early after the onset of an atrophy-inducing stimulus can be attributed to apoptotic changes in myofiber nuclei, whereas the interstitial loss of cells accounts for the observed increase in total apoptosis at later time points. Also, this indicates that apoptosis is a very early event in the process of atrophy and may play a causative role in the development of muscle atrophy.

Caspase Analysis

We further investigated whether activated caspase-3, -7, or -12 played a role in the increase in apoptosis. These caspases were chosen for the following reasons. Caspase-3 is the main executioner caspase that, once activated, cleaves the inhibitor of caspase-activated DNase (iCAD), thereby releasing CAD from its inhibition. CAD is then free to translocate to the nucleus and induce DNA fragmentation (63). Caspase-7 was investigated because it can cleave iCAD and activate CAD, independent of caspase-3 (35, 65), and its activation therefore

### Table 2. Protein abundance

<table>
<thead>
<tr>
<th></th>
<th>Activated Caspase-3</th>
<th>Activated Caspase-12</th>
<th>Activated Caspase-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.577 ± 0.075</td>
<td>0.073 ± 0.009</td>
<td>1.313 ± 0.339</td>
</tr>
<tr>
<td>12 h</td>
<td>0.657 ± 0.075</td>
<td>0.076 ± 0.015</td>
<td>1.324 ± 0.278</td>
</tr>
<tr>
<td>1 Day</td>
<td>0.681 ± 0.079</td>
<td>0.086 ± 0.019</td>
<td>1.555 ± 0.305</td>
</tr>
<tr>
<td>2 Days</td>
<td>0.661 ± 0.112</td>
<td>0.060 ± 0.009</td>
<td>1.467 ± 0.277</td>
</tr>
<tr>
<td>4 Days</td>
<td>0.713 ± 0.153</td>
<td>0.069 ± 0.015</td>
<td>1.343 ± 0.339</td>
</tr>
<tr>
<td>7 Days</td>
<td>0.845 ± 0.101</td>
<td>0.070 ± 0.009</td>
<td>1.221 ± 0.342</td>
</tr>
</tbody>
</table>

Values are means ± SE in normalized arbitrary units.

Fig. 4. Caspase-3 localized in interstitial cells. Soleus muscle cross sections were immunoreacted with cleaved caspase-3 (green in A), and dystrophin antibody (red in B), reacted with Hoechst dye (blue in C), and a composite is depicted in D. Activated caspase-3 positive interstitial cells were observed in soleus muscles 2 days after hindlimb suspension (arrow), while immunoreactivity in myofibers was very weak. Bar in D is 10 μm for A–D.
could lead to apoptosis. Caspase-12 is localized at the sarcoplasmic reticulum, and its activation leads to apoptosis independent of the mitochondrial pathway of apoptosis (18). Caspase-12 was elevated in atrophic old muscles (16) and therefore is possibly involved in skeletal muscle atrophy. Western blot analysis showed no difference for activated caspase-12 or activated caspase-7 at these early time points after disuse, and a trend for increased activated caspase-3 with increased time after hindlimb suspension, but this did not reach significance (Table 2). The latter may reflect caspase-mediated apoptosis in interstitial cells, but due to the relative low abundance of interstitial cell number compared with myofiber number and mass, this effect may have been masked. This idea is supported by the observation that activated caspase-3-positive interstitial cells were found within atrophying muscles, whereas myofibers themselves reacted only weakly with the antibody (Fig. 4). These data suggest that caspases do not play a prominent role in myonuclear apoptosis at these early time points after disuse, but that interstitial cell apoptosis may be mediated by caspase-3.

Role of EndoG in Muscle Apoptosis

In our previous work we suggested that EndoG, which is caspase independent, plays a role in apoptosis in muscle during atrophy (49). Here we investigated whether EndoG is specific for myofiber nuclei or is also involved in apoptosis of interstitial cells. No differences in total EndoG protein abundance were observed at different time points after hindlimb suspension (Fig. 5). Since EndoG translocates to the nucleus on an apoptotic stimulus (50, 76), the location of EndoG protein was determined. Soleus muscle cross sections were immunoreacted with EndoG antibody (green) and with dystrophin (red) to identify the myofiber membrane (Fig. 6). DAPI staining enabled colocalization of EndoG with nuclei. EndoG was detected colocalized with nuclei as early as 12 h following hindlimb suspension (filled arrow in Fig. 6A and Fig. 7), reaching statistical significance at 1 day and a maximum at 2 days (Fig. 7). EndoG colocalization with nuclei was observed in myofibers in which there were also nuclei that had not taken up the nuclease (open arrow, Fig. 6A). Moreover, EndoG was quite often found surrounding, but not inside, nuclei, particularly at early time points (arrow, Fig. 6B), and these nuclei were often in close proximity to nuclei free of EndoG (Fig. 6C). The fact that distinct nuclei within the same cytoplasm can undergo distinct changes (Fig. 6A) and that EndoG surrounds nuclei without immediately entering them, suggests that...
there may be some nuclear autonomy for the entrance of small molecules into a myofiber nucleus. Importantly, EndoG-positive myofiber nuclei were often also TUNEL positive, consistent with the hypothesis that EndoG may in fact mediate myofiber nuclear apoptosis (Fig. 8). By contrast, EndoG-positive interstitial nuclei were very rare and the frequency did not differ between the groups (Fig. 7), suggesting that EndoG-mediated apoptosis during muscle disuse is likely specific for myofiber nuclei.

Identification of Apoptosis in Interstitial Cells

A variety of cell types is present in the interstitium surrounding myofibers including fibroblasts, endothelial cells, leukocytes, and macrophages, as well as satellite cells. To begin to determine which cell type is undergoing apoptosis in response to disuse, soleus muscle cross sections from rats 2 days after hindlimb suspension were immunoreacted with an antibody against CD31 that recognizes cell surface proteins on endothelial cells (14). TUNEL-positive nuclei were quite often, but not always, associated with CD31-positive cells (arrow Fig. 9), indicating that endothelial cell apoptosis occurs during disuse-induced muscle atrophy.

DISCUSSION

Apoptosis has been implicated as a mediator of skeletal muscle atrophy, but its precise role and underlying mechanisms are just beginning to be investigated. In this study, we used the hindlimb suspension model to investigate early events in the onset of atrophy. The following conclusions can be drawn from the results. First, apoptosis is a very early event in disuse muscle atrophy. The question has been posed whether apoptosis causes inactivity-induced atrophy or is a consequence of the loss of muscle protein (18, 51). We show here that apoptosis is already increased at a time when no measurable loss of muscle mass or cross-sectional area occurred. Most studies have shown that the loss of soleus muscle mass becomes significant between days 3 and 7 after the induction of disuse (2, 19, 44), even though protein synthesis of selected proteins is suppressed as early as 6 h (55, 73, 77). It is generally thought that the decrease in muscle protein synthesis after disuse precedes the increase in protein degradation and the loss of total muscle protein (74), although recent studies have indicated that both synthesis and degradation may be coordinately regulated by the phosphatidylinositol 3-kinase (PI3-kinase)-Akt-forkhead box subgroup O signaling pathway (24, 45, 64, 69). Akt, which is crucial in regulating muscle protein synthesis through the phosphorylation of mammalian target of rapamycin (29), was also found to decrease gene expression of MAFbx and Murf-1, ubiquitin E3 ligases suggested as key markers for muscle atrophy, through phosphorylation of FOXO (45, 64, 69). In addition, muscle protein

Fig. 7. EndoG selectively enters nuclei in myofibers and not interstitial nuclei during disuse atrophy. Quantitation of EndoG positive nuclei of soleus muscle cross sections from control rats and 12 h, 1 day, 2 days, 4 days, and 7 days after hindlimb suspension. *Significant difference from control $P < 0.05$; $P = 0.07$ difference from control.

Fig. 8. EndoG-positive nuclei undergo DNA fragmentation. Soleus muscle cross sections were assayed for TUNEL (A, green), immunoreacted with EndoG antibody (B, red), and stained with Hoechst dye (C, blue). A composite picture (D) was created to show that nuclei colocalized with EndoG were undergoing DNA fragmentation (arrow in D). Bar in D is 10 μm for A–D.
Degradation through the ubiquitin proteasome pathway and apoptosis in muscle have been shown to be activated coordinately by PI3-kinase suppression (48). Indeed, Akt has been shown to be an important inhibitor of apoptosis through the phosphorylation of FOXO transcription factors (7, 8, 28, 31). Our data suggest that apoptosis of myofiber nuclei precedes muscle loss and the increase in markers for protein degradation, since apoptosis started at least 12 h earlier than the elevation in MAFbx mRNA, which has been suggested as an early marker for atrophy (29). The increases in muscle loss and MAFbx mRNA are temporally better correlated with the increase in interstitial cell apoptosis, and the significance of this observation needs further investigation.

The second conclusion from the current study is that translocation of EndoG to nuclei during apoptosis is specific for myofiber nuclei. We found that nuclear colocalization of EndoG in myofiber nuclei, but not in interstitial cell nuclei, increased with time after disuse up to 2 days and then declined even though apoptosis in interstitial cell nuclei was elevated after 2 days of disuse, and it has been shown previously that atrophy induces interstitial cell loss (49, 54). Since we did not investigate mitochondrial-free cytosolic fractions, the actual release of EndoG from mitochondria into the cytosol in relation to time after suspension could not be determined, but the timing of nuclear colocalization of EndoG was measured. EndoG is a mitochondrial nuclease that is thought to be involved in mitochondrial DNA replication (13), is released from mitochondria in response to proapoptotic signals, and localizes to nuclei, inducing DNA fragmentation (50, 76). In neurons, nuclear translocation of EndoG and subsequent DNA fragmentation in response to an ischemic insult occurred early and without an increase in total EndoG protein (47), consistent with results reported here in muscle. More importantly, we show here that EndoG colocalization with nuclei is coincident with DNA fragmentation, strongly suggesting that myofiber nuclear apoptosis is mediated through EndoG. Studies in other cell types have indicated that EndoG-induced DNA fragmentation and subsequent apoptosis are independent of caspases (32, 40, 60). Indeed, we show that caspase-3, -7 and -12 are not activated early after hindlimb suspension, indicating that myofiber nuclear apoptosis likely occurs independent of these caspases. Caspase-3 has previously been shown to be elevated during muscle atrophy after 14 days of denervation (66); however, no change after 14 days of hindlimb suspension was noted (67), indicating that the atrophy-inducing stimulus, the time point after onset of disuse, or the different cell types undergoing apoptosis may be important in the activation of distinct apoptotic pathways. Therefore, we investigated whether activated caspase-3 was associated with interstitial cell apoptosis. Indeed, activated caspase-3 was strongly observed in interstitial cells, while very weak activated caspase-3 was seen in myofibers, indicating that apoptosis in mononucleated cells in muscle may follow the more classical caspase-dependent pathway. Results from the current study corroborate our previous observation that EndoG nuclear colocalization was elevated after hindlimb suspension (49) and support our hypothesis that EndoG is involved in myofiber nuclear apoptosis in skeletal muscle.

The identity of the interstitial cells undergoing apoptosis during atrophy has not been investigated in detail, but it is known that cell number decreases (53). Hindlimb unloading is associated with changes in the extracellular matrix (11, 12), and specifically in collagen expression (52). Since fibroblasts are responsible for the synthesis of collagen in skeletal muscle (43) and collagen type 1 decreased with atrophy due to hindlimb suspension (52), it is plausible that muscle fibroblasts undergo apoptosis. Moreover, disuse-induced atrophy is associated with decreased capillary-to-fiber density (36, 75) and Fujino et al. (26) reported increased apoptosis in vascular endothelial cells, mainly in anastomoses, after 14 days of hindlimb suspension, possibly accounting for some of the cell loss in capillaries with disuse. Indeed, we also found that...
interstitial cells undergoing apoptosis were quite often identified as endothelial cells by immunostaining with CD31 antibody, and apoptosis in this cell type may be responsible for the increase in apoptosis starting at the time that muscle atrophy is measurable. In addition, satellite cells, located outside the muscle membrane, have been shown to be more sensitive to apoptosis after disuse induced by denervation (39) and to undergo apoptosis in quail muscle during atrophy after pre-loading (68). However, we did not observe any satellite cells (Pax-7 positive) that were also TUNEL positive (data not shown), possibly because Pax-7 has been shown to be anti-apoptotic (61) and therefore satellite cells may be somewhat protected from the proapoptotic stimuli during hindlimb suspension. Most other studies have employed biochemical methods to investigate pathways and molecules involved in the apoptotic response to disuse-induced atrophy, and, therefore, in those studies, it is uncertain which cell types are affected. This study is the first to show that the different cell types present in skeletal muscle may use distinct apoptotic pathways.

Interestingly, colocalization of EndoG with nuclei in myofibers is not determined by the cytoplasmic environment, since we frequently observed myofibers in which some nuclei were colocalized with EndoG while other nuclei in close proximity were not. This finding is consistent with observations that apoptotic nuclei are found in the same myofibers as normal nuclei (2, 22, 68). The fact that EndoG is colocalized to only a subset of nuclei could indicate that the release of EndoG from mitochondria is spatially controlled and only mitochondria in a certain area of the myofiber are releasing proteins from their intermembranous space, which do not diffuse along long distances in the myofiber. This is similar to the observation that protein products stay within the vicinity of the nucleus in which they were produced, implying discretely controlled areas within myofibers (58). It has been shown recently that intermyofibrillar mitochondria release a greater amount of proapoptotic proteins in response to oxidative stress compared with subsarcolemmal mitochondria (1), indicating that not all mitochondria within a muscle fiber behave similarly. It is plausible that the abundance of EndoG or the susceptibility to EndoG release is different between subsets of mitochondria, and this possibility warrants further investigation. Alternatively, it could indicate that only certain nuclei are susceptible to EndoG permeating their membrane. This would imply that there is a certain amount of nuclear autonomy and that the release of EndoG from the mitochondria is a separate event from the entrance of EndoG into the nucleus. The latter suggestion would explain the observation that, particularly at early time points after the onset of disuse, numerous, seemingly random, nuclei were found surrounded by EndoG without it entering the nuclei. Similarly, Siu and Alway (66) showed that apoptosis-inducing factor, a caspase-independent mitochondrial protein translocating from mitochondria to nuclei to induce apoptosis (9, 71), was elevated in the cytosolic, but not nuclear fraction upon denervation, suggesting that it was being released from mitochondria, but did not enter nuclei. It is possible that in skeletal muscles undergoing atrophy, two separately controlled events occur to induce selective nuclear apoptosis in myofibers: first, proteins are released from the mitochondrial intermembranous space into the cytoplasm, and second, the nuclear membrane becomes permeable to selected proteins. We suggest that this second event could be caused by oxidative stress to the nuclear membrane protein and/or lipids, because oxidative stress is increased under atrophy-inducing stimuli (37, 42, 46, 57, 59). Alternatively, nuclear entrance by EndoG could be determined by a stochastic mechanism, such as shown for independent transcriptional regulation in myonuclei (56). Of interest, however, is the fact that we observed EndoG-positive nuclei undergoing DNA fragmentation, as measured by TUNEL staining, indicating that EndoG indeed may play a mediating role in the apoptotic nuclear loss of atrophying skeletal muscle. Future studies should be directed toward understanding the regulation of EndoG release from mitochondria and the differential colocalization to myofiber nuclei.

In summary, results from this study indicate that apoptosis during disuse-induced muscle atrophy is an early event and could therefore be causative to the loss of muscle mass, particularly since myofiber nuclei undergo apoptosis before interstitial cells. In addition, apoptotic nuclear loss in myofibers, but not interstitial cells, may be mediated by EndoG translocation to nuclei to induce DNA fragmentation. Therefore, therapeutic interventions targeted at EndoG may counteract or prevent disuse-induced skeletal muscle atrophy.

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