Aging influences cellular and molecular responses of apoptosis to skeletal muscle unloading

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Siu, Parco M., Emidio E. Pistilli, David C. Butler, and Stephen E. Alway. Aging influences cellular and molecular responses of apoptosis to skeletal muscle unloading. Am J Physiol Cell Physiol 288: C338–C349, 2005. First published October 13, 2004; doi:10.1152/ajpcell.00239.2004.—The influence of aging on skeletal myocyte apoptosis is not well understood. In this study we examined apoptosis and apoptotic regulatory factor responses to muscle atrophy induced via limb unloading following loading-induced hypertrophy. Muscle hypertrophy was induced by attaching a weight to one wing of young and aged Japanese quails for 14 days. Removing the weight for 7 or 14 days after the initial 14 days of loading induced muscle atrophy. The contralateral wing served as the intra-animal control. A time-released bromodeoxyuridine (BrdU) pellet was implanted subcutaneously with wing weighting to identify activated satellite cells/muscle precursor cells throughout the experimental period. Bcl-2 mRNA and protein levels decreased after 7 days of unloading, but they were unchanged after 14 days of unloading in young muscles. Bcl-2 protein level but not mRNA level decreased after 7 days of unloading in muscles of aged birds. Seven days of unloading increased the mRNA level of Bax in muscles from both young and aged birds. Fourteen days of unloading increased mRNA and protein levels of Bcl-2, decreased protein levels of Bax, and decreased nuclear apoptosis-inducing factor (AIF) protein level in muscles of aged birds. BrdU-positive nuclei were found in all unloaded muscles from both age groups, but the number of BrdU-positive nuclei relative to the total nuclei decreased after 14 days of unloading compared with 7 days of unloading. The TdT-mediated dUTP nick end labeling (TUNEL) index was higher after 7 days of unloading in both young and aged muscles and after 14 days of unloading in aged muscles. Immunofluorescent staining revealed that almost all of the TUNEL-positive nuclei were also BrdU immunopositive, suggesting that activated satellite cell nuclei (both fused and nonfused) underwent nuclear apoptosis during unloading. There were significant correlations among levels of Bcl-2, Bax, and AIF and TUNEL index. Our data are consistent with the hypothesis that apoptosis regulates, at least in part, unloading-induced muscle atrophy and loss of activated satellite cell nuclei in previously loaded muscles. Moreover, these data suggest that aging influences the apoptotic responses to prolonged unloading following hypertrophy in skeletal myocytes.

Aging is characterized by a significant loss of skeletal muscle mass (i.e., sarcopenia), which is a result of muscle fiber atrophy and a decrease in total fiber number (23). This loss contributes to the functional impairment and declined quality of life in elderly populations (31). Although the muscle’s hypertrophic response to functional overload is retained in aged skeletal muscle, it has been shown that the plasticity and function of muscles are diminished with aging (16, 20). Moreover, a greater detriment of muscle mass and function during muscle disuse was observed in aged muscles compared with young muscles (11, 21). Nonetheless, the mechanism accounting for the differences in muscle adaptations between aged and young muscles is unclear.

Aptosis is an internally encoded suicide program and has been widely accepted to be crucial in coordinating the balance between cell survival and death in various cell types. Although most apoptosis studies have been conducted in actively dividing or mitotic cells (e.g., lymphocytes), it was recently demonstrated that postmitotic cells (e.g., skeletal myocytes) also exhibit apoptosis under various conditions. For instance, apoptosis has been documented in skeletal myocytes under disuse including unloading and denervation (1, 8, 12, 27). Although the significance of apoptosis in muscle remodeling has yet to be determined, these findings suggest that apoptosis may have a physiological role in coordinating muscle remodeling in response to unloading. Moreover, several studies imply that normal physiological aging per se influences apoptosis in skeletal muscles and other postmitotic tissues (22, 30, 42–44). It is possible that the apoptotic response to unloading-induced muscle atrophy in aged muscles is different from that in young muscles. It is likely that aging-related differences in apoptotic responses to muscle unloading could be part of the mechanisms that regulate the differential adaptations to unloading in skeletal myocytes between aged and young individuals.

Although apoptosis has been demonstrated during unloading-induced muscle atrophy that reduces muscle mass below the control muscle mass level, only a few studies have been conducted to examine the apoptotic responses to unloading-induced muscle atrophy following hypertrophy, which returns muscle mass to the control muscle mass level. Moreover, the influence of aging in unloading-induced apoptosis in skeletal myocytes has not been well investigated. Therefore, the purpose of this study was to examine the responses of apoptosis and apoptotic regulatory factors to unloading-induced muscle atrophy following hypertrophy in young adult and aged quails. We hypothesized that 1) apoptosis is associated with the unloading-induced muscle atrophy following hypertrophy and is independent of aging, and 2) apoptotic gene responses to prolonged unloading following muscle hypertrophy are aging dependent.

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Animals. Japanese Coturnix quails were hatched and raised in pathogen-free conditions in the central animal care center at West Virginia University School of Medicine. The birds were housed at a room temperature of 22°C with a 12:12-h light-dark cycle and were provided with food and water ad libitum. Sixteen young adult birds (2 mo old) and sixteen aged birds (24 mo old) were examined in the present study. The average lifespan of Japanese quails is ~26–28 mo. They are both physically and sexually mature by 1.5 mo of age and do not grow thereafter (35, 41). All experimental procedures carried approval from the Institutional Animal Use and Care Committee of West Virginia University School of Medicine. The animal care standards were followed by adhering to the recommendations for the care of laboratory animals as advocated by the American Association for Accreditation of Laboratory Animal Care and fully conformed with the American Physiological Society’s “Guiding Principles for Research Involving Animals and Human Beings.”

Muscle loading and unloading protocol. The patagial (PAT) muscle is flexed with the wing on the bird’s back at rest, but it is stretched when the wing is extended. In our experimental stretch-overloading model, we placed a tube containing 10–12% of the bird’s body weight over the left humeral-ulnar joint (13). This maintained the joint in extension throughout the period of stretch and induced stretch at the origin of the PAT muscle. Previous studies have shown that this stretch-loading protocol results in moderate hypertrophy of the PAT muscles (7, 12, 50). The unstretched right PAT muscle served as the intra-animal control muscle for each bird. Consistent with the fact that Japanese quails show no maturational changes in body weight and carcass composition beyond ~1.5–2 mo after hatching (33, 34, 56), it has been demonstrated that the body weights of Japanese quails do not change throughout stretch overloading and do not differ between adult and aged quails (6, 13–15, 19, 32). Therefore, the responses to the same absolute and relative loads could be compared in muscles from young adult and aged quails.

The left wing was loaded for 14 days, and then the weight was removed. Seven days after weight removal, eight young and eight aged quails were killed with an overdose of pentobarbital sodium. The remaining young and aged quails were killed 14 days after weight removal. Whole PAT muscles were dissected from surrounding connective tissue, removed, weighed, frozen in isopentane cooled to the temperature of liquid nitrogen, and then stored at ~80°C until used for analyses.

Bromodeoxyuridine administration. A subcutaneous time-released bromodeoxyuridine (BrdU) pellet (21-day release, 0.22 µg BrdU·g body mass⁻¹·day⁻¹; Innovative Research, Sarasota, FL) was implanted in each quail while anesthetized with 2% isoflurane, at the 1.5–2 mo after hatch. The BrdU pellet was removed with an aseptic protocol at 55.5°C. All PAT samples were confirmed by restriction digestion. Preliminary experiments were performed with each gene to assure that the number of cycles (36 cycles) represented a linear portion for the PCR OD curve for the muscle samples. The cDNAs from all muscle samples were amplified simultaneously by using aliquots from the same PCR mixture. After the PCR amplification, 20 µl of each reaction were electrophoresed on 1.5% agarose gels stained with ethidium bromide. Images were captured, and the signals were quantified in arbitrary units as OD × band area by using the Kodak one-dimensional (1-D) image analysis system (Eastman Kodak, Rochester, NY). The size (number of base pairs) of each of the bands corresponded to the size of the processed mRNA. All RT-PCR signals were normalized to the ribosomal 18S mRNA signal of the corresponding RT product. This eliminated the measurement error from uneven sample loading and provided a semiquantitative measure of the relative changes in gene expression.

Protein extraction and fractionation. The method described by Rothermel et al. (45) was used to obtain the cytoplasmic and nuclear protein extracts of the PAT muscles. Briefly, 50 mg of PAT muscles were homogenized on ice in 1 ml of ice-cold lysis buffer (10 mM NaCl, 1.5 mM MgCl₂, 20 mM HEPES, pH 7.4, 20% glycerol, 0.1% Triton X-100, and 1 mM dithiothreitol). The homogenates were centrifuged at 1,000 rpm for 1 min at 4°C. The supernatants contained the cytoplasmic protein fraction and were collected. The remaining nuclear pellet was washed and resuspended in 360 µl of lysis buffer, and 49.8 µl of 5 M NaCl were added to lyse the nuclei. The mixture was then rotated for 1 h at 4°C and centrifuged at 14,000 rpm for 15 min at 4°C. The supernatants contained the nuclear protein fraction and were collected. Purity of the extracted fractions was confirmed by performing immunoblotting on the extracted proteins, using an antihistone H2B (a nuclear protein) rabbit polyclonal antibody (1:2,000 dilution, 07371; Upstate, Lake Placid, NY) and an anti-Cu/Zn SOD (a cytoplasmic antioxidant enzyme) rabbit polyclonal antibody (1:500 dilution, sc-11407; Santa Cruz Biotechnology, Santa Cruz, CA) (Fig. 1). A protease inhibitor cocktail containing 104 mM ABEBSF, 0.08 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64 (P8340; Sigma) was added to the collected cytoplasmic and nuclear protein fractions. The total protein contents of the cytoplasmic and nuclear extracts were quantified in duplicate by using the bicinchoninic acid reagents (Pierce, Rockford, IL) and bovine serum albumin (BSA) standards. The cytoplasmic fraction protein was then used for immunoblotting of Bcl-2, Bax, and apoptotic protease-activating factor-1 (Apaf-1), whereas apoptosis-inducing factor (AIF) protein content was measured in both the cytoplasmic and nuclear fraction protein.

Western immunoblots. Protein expression of apoptotic regulatory factors including Bcl-2, Bax, Apaf-1, and AIF was determined in the PAT muscles of experimental (left) and intra-animal control (right)
were quantified as OD films (BioMax MS-1; Eastman Kodak), and digital records of the membranes were exposed to X-ray developed using West Pico chemiluminescent substrate (34080; Pierce).

Rabbit polyclonal antibody (1:500 dilution, AB16503; Chemicon International), anti-Apaf-1 antibody (1:200 dilution, sc-6236; Santa Cruz Biotechnology), anti-Bcl-2 mouse monoclonal antibody (1:200 dilution, sc-7382; Santa Cruz Biotechnology) were blotted to nitrocellulose membranes (Bio-Rad, Hercules, CA) and separated by SDS-PAGE for 1.5 h at 20°C. The gels were boiled for 5 min at 95°C in Laemmli buffer, loaded on each lane of a 12% polyacrylamide gel, and separated by SDS-PAGE for 1.5 h at 20°C. The gels were blotted to nitrocellulose membranes (Bio-Rad, Hercules, CA) and stained with Ponceau S red (Sigma) to confirm equal loading and transferring of proteins to the membrane in each lane. As another approach to verify similar loading between the lanes, gels were loaded in duplicate with one gel stained with Coomassie blue for further confirmation. The membranes were then blocked in 5% nonfat milk in phosphate-buffered saline with 0.05% Tween 20 (PBS-T) and probed with anti-Bcl-2 mouse monoclonal antibody (1:200 dilution, sc-7382; Santa Cruz Biotechnology), anti-Bax rabbit polyclonal antibody (1:200 dilution, sc-6236; Santa Cruz Biotechnology), anti-Apaf-1 rabbit polyclonal antibody (1:500 dilution, AB16503; Chemicon International, Temecula, CA), or anti-AIF mouse monoclonal antibody (1:500 dilution, sc-1316HRP; Santa Cruz Biotechnology) diluted in PBS-T with 2% BSA. Secondary antibodies were conjugated to horseradish peroxidase (HRP; Chemicon), and the signals were developed using West Pico chemiluminescent substrate (34080; Pierce). The signals were visualized by exposing the membranes to X-ray films (BioMax MS-1; Eastman Kodak), and digital records of the films were captured with a Kodak 290 camera. The resulting bands were quantified as OD × band area by using a 1-D image analysis system (Eastman Kodak) and expressed in arbitrary units. The molecular sizes of the immunodetected proteins were verified using a prestained standard (LC5925; Invitrogen Life Technologies).

**Immunofluorescent staining.** Activated, proliferated satellite cells/muscle precursor cells were identified by double immunofluorescent staining with BrdU and laminin. Frozen 10-μm-thick muscle cross sections from the experimental and control PAT muscles were cut in a freezing cryostat at −20°C and placed on the same glass slide to control for processing differences (e.g., incubation time, temperature, etc.). The sections were air dried at room temperature, fixed in ice-cold methanol-acetone (1:1) for 10 min, permeabilized with 0.2% Triton X-100 in 0.1% sodium citrate at 4°C for 5 min, and blocked in 1.5% goat serum in PBS. All incubations were performed at room temperature and incubated with fluorescein-conjugated TUNEL reaction mixture on the tissue sections. The sections were then labeled with laminin or dystrophin to visualize the basal lamina or the sarcolemma, respectively. After TUNEL labeling, the muscle sections were incubated with an anti-chick laminin mouse monoclonal antibody (for visualizing the basal lamina, 20 μg/ml, clone 31–2) or an anti-dystrophin mouse monoclonal antibody (for visualizing the sarcolemma, 1:2 dilution, DS05; Vector Laboratories) followed by an anti-mouse IgG Cy3 conjugate F(ab′)2 fragment incubation (1:200 dilution, C2181) and mounted with DAPI Vectashield mounting medium. TUNEL- and DAPI-positive nuclei and laminin or dystrophin staining were examined under a fluorescence microscope, and the captured images were stacked using SPOT RT software as described in **Immunofluorescent staining.** The numbers of TUNEL- and DAPI-positive nuclei were counted, and only the labeled nuclei that were under the laminin staining were counted, to include solely the muscle-originated nuclei. Data were expressed as TUNEL index, which was calculated by counting the number of TUNEL-positive nuclei divided by the total number of nuclei (i.e., DAPI-positive nuclei) × 100. The TUNEL index for each muscle was calculated from six random, nonoverlapping fields at an objective magnification of ×40.

In a separate set of measurements, we performed the BrdU labeling on the muscle sections after the TUNEL labeling procedure to determine whether the TUNEL-positive nuclei under the laminin staining had undergone proliferation. After the tissue sections were labeled according to the TUNEL protocol, they were incubated with an anti-BrdU mouse monoclonal antibody (1:20 dilution, 555627; BD Pharmingen, San Diego, CA) followed by an anti-mouse IgG Cy3 conjugate F(ab′)2 fragment incubation (1:200 dilution, C2181; Sigma). Negative control experiments were done by omitting the BrdU antibody from the tissue sections. To visualize the basal lamina of the PAT muscles and therefore identify whether the BrdU-positive nuclei were muscle-originated nuclei (e.g., muscle satellite cell nuclei), the tissue sections were then incubated with an anti-chick laminin mouse monoclonal antibody (20 μg/ml, clone 31–2; D. M. Fambrough, The Johns Hopkins University, Baltimore, MD) followed by an anti-mouse IgG biotin-conjugated antibody (Vector Laboratories, Burlingame, CA) and then fluorescein-avidin DCS incubation (1:200 dilution, A2011; Vector Laboratories). The sections were finally mounted with 4’,6-diamidino-2-phenylindole (DAPI) mounting medium (Vectashield mounting medium; Vector Laboratories). BrdU- and DAPI-positive nuclei and laminin staining were examined under a fluorescence microscope with excitation wavelengths of 330–380 nm for DAPI blue fluorescence, 485–585 nm for Cy3 red fluorescence, and 450–490 nm for fluorescein green fluorescence (biological research microscope Eclipse E800; Nikon, Melville, NY). Images were obtained using a SPOT RT camera (Diagnostic Instruments, Sterling Heights, MI), and SPOT RT software (Universal Imaging, Downingtown, PA) was used to stack the images of BrdU- and DAPI-positive nuclei and laminin staining. The numbers of BrdU- and DAPI-positive nuclei were counted from six random, nonoverlapping fields at an objective magnification of ×40. Only the labeled nuclei that were under the laminin staining were counted, to exclude any nonmuscle nuclei in the sections. Data were expressed as BrdU index, which was calculated as the number of BrdU-positive nuclei divided by the total number of nuclei (i.e., DAPI-positive nuclei) × 100.

**In situ TdT-mediated dUTP nick end labeling staining.** The nuclei with DNA strand breaks were assessed using a fluorometric TdT-mediated dUTP nick end labeling (TUNEL) detection kit according to the manufacturer’s instructions (1684795; Roche Applied Science, Indianapolis, IN). In brief, 10-μm-thick frozen muscle cross sections from muscles were cut in a freezing cryostat at −20°C. Tissue sections were air dried at room temperature, fixed in 4% paraformaldehyde in PBS, pH 7.4, at room temperature for 20 min, permeabilized with 0.2% Triton X-100 in 0.1% sodium citrate at 4°C for 2 min, and incubated with fluorescein-conjugated TUNEL reaction mixture in a humidified chamber at 37°C for 1 h in the dark. Negative control experiments were done by omitting the TdT enzyme in the TUNEL reaction mixture on the tissue sections. The sections were then labeled with laminin or dystrophin to visualize the basal lamina or the sarcolemma, respectively. After TUNEL labeling, the muscle sections were incubated with an anti-chick laminin mouse monoclonal antibody (for visualizing the basal lamina, 20 μg/ml, clone 31–2) or an anti-dystrophin mouse monoclonal antibody (for visualizing the sarcolemma, 1:2 dilution, DS05; Vector Laboratories) followed by an anti-mouse IgG Cy3 conjugate F(ab′)2 fragment incubation (1:200 dilution, C2181) and mounted with DAPI Vectashield mounting medium. TUNEL- and DAPI-positive nuclei and laminin or dystrophin staining were examined under a fluorescence microscope, and the captured images were stacked using SPOT RT software as described in **Immunofluorescent staining.** The numbers of TUNEL- and DAPI-positive nuclei were counted, and only the labeled nuclei that were under the laminin staining were counted, to include solely the muscle-originated nuclei. Data were expressed as TUNEL index, which was calculated by counting the number of TUNEL-positive nuclei divided by the total number of nuclei (i.e., DAPI-positive nuclei) × 100. The TUNEL index for each muscle was calculated from six random, nonoverlapping fields at an objective magnification of ×40.
adjacent fiber (i.e., fused nuclei) or nuclei that had proliferated but did not fuse to reside inside the adjacent muscle fiber (nonfused nuclei). BrdU-negative nuclei under the basal lamina were taken to be the postmitotic myocyte nuclei.

Statistical analyses. Statistical analyses were performed using the SPSS 10.0 software package. Student’s t-test for paired data was used to examine differences between the experimental and contralateral control muscles. Relationships between given variables were examined by computing the Pearson product-moment correlation coefficient (r). Multivariate analysis of variance (MANOVA) was performed on the percent changes in all measured variables, including muscle mass, BrdU index, and all apoptotic measurements, to examine the main effects of time (7 and 14 days of unloading), age (young and aged muscles), and interaction (time × age). Estimation of the effect size by computing \( \eta^2 \) was included in the MANOVA with the aim of indicating how much of the total variance in the present experiment was explained by the main effect or interaction. Statistical significance was accepted at \( P < 0.05 \). All data are given as means ± SE.

RESULTS

Change in muscle mass. The percent differences in muscle mass between the experimental and contralateral control muscles were estimated from the whole PAT muscle wet weight. Previous studies in which the same stretch-loading procedure adopted in the present study was used have consistently shown that the extent of muscle hypertrophy relative to the contralateral control muscle after 14 days of stretch overloading is ∼35 and ∼15% in young adult and aged quails, respectively (7, 13, 50). The degree of hypertrophy decreased to 15 and 12% after loading followed by 7 days of unloading. After 14 days of unloading, no difference in muscle mass was found between the experimental and control muscles in young adult quails (\( P > 0.05 \)), whereas the mass of the experimental muscle was still 6% greater than that of the control muscle in aged quails (Fig. 2). In agreement with the finding that edema did not contribute to the increased muscle wet weight of stretch-enlarged muscle (10), we did not observe any histological abnormality that indicated severe inflammation or edema in all the examined muscle sections in the present study.

![Fig. 2. Muscle mass change. The loss of muscle mass during unloading following stretch-induced hypertrophy was monitored by examining the decrease in the extent of muscle hypertrophy. The extent of muscle hypertrophy was simply estimated by examining the percent difference between the experimental and contralateral control whole patagial (PAT) muscle wet weights after 7 days of unloading following 14 days of stretch overloading (7d unloading) or after 14 days of unloading following 14 days of stretch overloading (14d unloading). Data are presented as means ± SE. *\( P < 0.05 \), **\( P < 0.01 \), compared with control muscles.](http://ajpcell.physiology.org/)

Bcl-2 mRNA and protein levels. As estimated using RT-PCR, we found that the Bcl-2 mRNA level of the experimental muscle was 15 ± 5% (\( P < 0.05 \)) lower than that of the control muscle after 7 days of unloading, whereas we found no difference after 14 days of unloading in young adult quails (Fig. 3A). Furthermore, in aged quails, the Bcl-2 mRNA level of the experimental muscle was similar to that of the control muscle after 14 days of unloading (Fig. 3A).

In our Western blot analyses, we identified an immunoreactive band of ∼25 kDa corresponding to the predicted molecular mass of Bcl-2 protein. The Bcl-2 protein level of the experimental muscle was 15 ± 5% (\( P < 0.05 \)) lower than that of the control muscle after 7 days of unloading, whereas no difference was found in Bcl-2 protein level between the experimental and control muscles after 14 days of unloading (Fig. 3A).

![Fig. 3.](http://ajpcell.physiology.org/)
control muscles after 14 days of unloading in young adult quails (Fig. 3B). In aged quails, the Bcl-2 protein level of the experimental muscle was $37 \pm 6\%$ ($P < 0.01$) lower and $29 \pm 11\%$ ($P < 0.05$) higher than that of the control muscle after 7 and 14 days of unloading, respectively (Fig. 3B).

**Bax mRNA and protein levels.** The Bax mRNA level of the experimental muscle was $23 \pm 7\%$ ($P < 0.01$) greater than that of the control muscle after 7 days of unloading, whereas the Bax mRNA levels were similar in the experimental and control muscles after 14 days of unloading in young adult quails (Fig. 4A). In aged quails, the Bax mRNA level of the experimental muscle was $12 \pm 4\%$ ($P < 0.01$) greater than that of the control muscle after 7 days of unloading, but the Bax mRNA levels were similar in the experimental and control muscles after 14 days of unloading (Fig. 4A).

We detected an $\sim 21$-kDa immunoreactive band corresponding to the predicted molecular mass of Bax protein in the immunoblots. There was no difference in the Bax protein level between the experimental and control muscles after both 7 and 14 days of unloading in young adult quails (Fig. 4B). In aged quails, although the Bax protein level of the experimental muscle appeared to increase $34 \pm 25\%$ compared with that of the control muscle after 7 days of unloading, the difference did not reach a statistically significant level ($P = 0.312$). However, the Bax protein level of the experimental muscle was $25 \pm 7\%$ lower than that of the control muscle after 14 days of unloading (Fig. 4B).

**Apaf-1 and AIF protein levels.** In our Western blot analyses, we detected an $\sim 130$-kDa immunoreactive band corresponding to Apaf-1 protein. However, there was no difference in Apaf-1 protein level between the experimental and control muscles in all groups of quails (Fig. 5). An immunoreactive band of $\sim 67$ kDa corresponding to the predicted molecular mass of AIF protein was detected in both the cytoplasmic and nuclear protein fraction of all muscle samples. No difference in cytoplasmic AIF protein level was found between the experimental and control muscles after both 7 and 14 days of unloading in both young adult and aged quails (Fig. 6A). The nuclear AIF protein level was similar in the experimental and control muscles after 7 days of unloading in young adult and aged quails as well as after 7 days of unloading in aged quails. However, we found that the AIF protein level of the experimental muscle was $16 \pm 5\%$ ($P < 0.01$) lower than that of the control muscle after 14 days of unloading in aged quails (Fig. 6B).

**TUNEL index.** Nuclear DNA breaks in skeletal muscles were detected using TUNEL staining and were expressed using a TUNEL index to estimate the extent of apoptosis. Figure 7A shows an example of a TUNEL-positive nucleus that was located under the basal lamina in an experimental PAT muscle from an aged quail after 7 days of unloading. Although TUNEL-positive nuclei were rarely detected in the young control muscles, we found that the TUNEL index of the experimental muscle was $246 \pm 157\%$ ($P < 0.01$) greater than that of the control muscle after 7 days of unloading but that no difference was found in the TUNEL index between the experimental and control muscles after 14 days of unloading in young quails (Fig. 7B). In aged quails, the TUNEL index of the
preliminary muscle was 91\% and 44\% greater than that of the control muscle after 7 and 14 days of unloading, respectively (Fig. 7B).

Our double immunofluorescent analyses of TUNEL and BrdU labeling from a separate set of measurements showed that almost all of the TUNEL-positive nuclei were also labeled with BrdU (Fig. 8). This finding indicates that the TUNEL-positive nuclei under the basal lamina staining, as illustrated in Fig. 7A, were proliferated satellite cell/muscle precursor cell nuclei. In addition, the double immunocytochemical staining performed with TUNEL and dystrophin revealed that the TUNEL-positive nuclei were present both under (Fig. 9A) and on (Fig. 9B) the dystrophin staining. This finding suggests that the TUNEL-positive nuclei included both the fused (under dystrophin staining) and nonfused (on dystrophin-stained sarcolemma) proliferated satellite cell/muscle precursor cell nuclei.

BrdU index. Previously, investigators in our laboratory demonstrated that the number of activated satellite cells/muscle precursor cells after 14 days of stretch overloading in young adult PAT muscle is \(~7\%\), determined as the number of muscle-related BrdU-labeled nuclei relative to the total nuclei population (12). BrdU-immunopositive nuclei were rarely found in the control muscles of all groups. Figure 10A shows an example of a BrdU-immunopositive nucleus under the laminin staining in the experimental PAT muscle from a young quail after 14 days of unloading. As an estimate of the number of activated satellite cells/muscle precursor cells, the BrdU index was 2.7 and 1.3\% of the total nuclei population in the experimental muscles of young quails after 7 and 14 days of unloading, respectively. In the aged quails, the BrdU index was 2.6 and 1.8\% in the experimental muscles after 7 and 14 days of unloading, respectively (Fig. 10B). These BrdU indexes were generally lower than our previously reported BrdU indexes after 7 and 14 days of unloading (12) because only the
BrdU-immunopositive nuclei under the laminin staining were counted in the present study.

Relationships among apoptotic regulatory factors and apoptosis. The relationships among Bcl-2, Bax, AIF, and TUNEL index were analyzed by examining the corresponding Pearson’s correlation coefficient (r). When the data for experimental and control PAT muscles of all groups were collapsed and treated as a single group, Bax protein content was positively correlated with the apoptotic index (r = 0.393, P < 0.001, n = 64; Fig. 11A), whereas Bcl-2 protein content was negatively correlated with the apoptotic index (r = −0.577, P < 0.0001, n = 64; Fig. 11B).

When the data from muscle samples were collapsed and analyzed as different subgroups (e.g., all control muscle or all aged muscles), we found that, generally, Bax protein and mRNA levels were positively correlated with TUNEL index and cytoplasmic AIF, respectively, whereas Bcl-2 protein and mRNA levels were negatively correlated with TUNEL index and nuclear AIF, respectively (P < 0.05; Table 1).

Main effects of age, time, and age × time. The outcomes of the multivariate tests in MANOVA indicated that there were significant main effects of age [F(10, 19) = 2.393, P < 0.05, η² = 0.557] and time [F(10, 19) = 4.808, P < 0.01, η² = 0.717]. Moreover, we found that a significant main effect of interaction (time × age) existed in explaining the variances of the present findings [F(10, 19) = 2.872, P < 0.05, η² = 0.602].

DISCUSSION

The present study delineates the responses of apoptosis as estimated by TUNEL and apoptotic regulatory factors including Bcl-2, Bax, AIF, and Apaf-1 to unloading-induced muscle atrophy following stretch-induced muscle hypertrophy in...
young adult and aged quails. Previous findings have shown a role for apoptosis in muscle atrophy below the control muscle mass level induced by hindlimb suspension/unweighting, muscle denervation, and limb immobilization (1, 8, 17, 27, 28, 37, 48, 54). In this study, we have demonstrated that apoptosis is also involved in unloading-induced muscle atrophy, in which muscle mass is reduced from a hypertrophied state to control levels. Our data indicate that elimination of the activated satellite cell nuclei (both fused and nonfused activated nuclei) via apoptotic mechanisms occurs during unloading-induced muscle atrophy following muscle hypertrophy. Moreover, we provide evidence suggesting that differential apoptotic responses exist to prolonged unloading-induced muscle atrophy following muscle hypertrophy between young adult and aged animals. Conclusively, our findings support the hypotheses that 1) apoptosis plays a physiological role in unloading-induced muscle atrophy following hypertrophy and 2) aging suppresses the apoptotic responses to muscle atrophy induced by muscle unloading (i.e., 14 days of unloading in the present study) following hypertrophy.

Apoptosis during unloading-induced muscle atrophy following hypertrophy. In the present study, the decreased degree of muscle hypertrophy due to unloading following stretch-induced hypertrophy in both young adult and aged quails denoted muscle atrophy with unloading. It has been commonly shown that muscle mass loss during disuse is associated with a decreased number of muscle nuclei (3–5, 25, 38); therefore, it is reasonable to suggest that muscle mass loss during muscle disuse is mediated by decreasing the nuclei number and thereby maintaining a homeostatic balance in the myonuclear domain (i.e., the cytoplasmic volume of myonuclei) in the multinucleated skeletal myocytes (3–5, 25, 38–40). On the basis of the fact that apoptosis is consistently demonstrated during hindlimb suspension/unweighting, muscle denervation, and limb immobilization (1, 8, 17, 27, 28, 37, 48, 54), it has been proposed that the loss in nuclei number could possibly be achieved through the activation of pathways resulting in apoptosis in the skeletal myocytes. Indeed, Allen et al. (1) demonstrated that apoptosis is associated with the loss of muscle mass and myonuclei during hindlimb unweighting, because they found that TUNEL-positive nuclei increased in muscles after 14 days of unweighting. Their findings suggested that the decreased myonuclei number during hindlimb unweighting-induced muscle atrophy is accounted for by elimination of the existing myonuclei by apoptotic mechanisms.

Recently, investigators in our laboratory demonstrated that unloading-induced muscle atrophy following hypertrophy in young adult quails is also related to the activation of apoptosis as indicated by increased caspase protease activities and poly-(ADP-ribose) polymerase-positive nuclei (12). Consistent with these findings, in the present study, we have demonstrated proapoptotic changes including increased TUNEL index, increased Bax content, and decreased Bcl-2 content in the experimental muscle after 7 days of unloading following muscle hypertrophy in both the young adult and aged quails. Furthermore, we have shown that significant relationships exist among TUNEL index, Bax, Bcl-2, and AIF in our quail muscles. We have provided evidence confirming that apoptosis occurs during muscle remodeling induced by atrophy following hypertrophy (i.e., muscle loss after hypertrophy that returns to control muscle mass level), and we have extended our findings by showing that apoptosis is associated with unloading-induced muscle atrophy following hypertrophy not only in young adult quails but also in aged quails.

Notably, our double immunofluorescent staining revealed that almost all the TUNEL-positive nuclei in the unloading muscles were BrdU immunopositive, indicating that the TUNEL-positive nuclei originated from the proliferating cell populations. Given that we had already excluded the non-muscle cells (e.g., fibroblasts) and included only the TUNEL-positive nuclei that were under the basal lamina according to the immunolabeling of laminin, it is most likely that the TUNEL-positive nuclei under the laminin staining were nuclei originating from activated satellite cells or other myogenic precursor cell populations. Moreover, our double staining with TUNEL and dystrophin demonstrated that the TUNEL-positive nuclei were found both under and on the dystrophin staining. Together, these data suggest that unloading-induced...
muscle atrophy following stretch-induced hypertrophy eliminates both the fused and nonfused activated satellite cell nuclei via apoptotic pathways in young adult and aged quails, and this is further supported by the finding of a decreased BrdU index along with unloading following hypertrophy. Our findings are in agreement with the suggestion that muscle remodeling is mediated by regulating the number of muscle nuclei so as to maintain the homeostatic myonuclear domain in multinucleated skeletal myocytes (2–5, 25, 36–40, 46). Because satellite cells have been shown to be more susceptible to apoptosis during chronic denervation (26, 27), it is not surprising that the activated satellite cell nuclei, rather than the existing myonuclei, were eliminated during unloading following hypertrophy to attain muscle atrophy. In addition, although “nuclear” apoptosis has been reported in skeletal myocytes during muscle atrophy below the control muscle mass level (e.g., by hindlimb unweighting), it is expected that those observed apoptotic nuclei belonged to the existing myonuclei population because it has been shown that the mitotic activity of satellite cells is significantly suppressed during the degenerative muscle states, including hindlimb unweighting and muscle denervation (24, 47).

Aging influences apoptotic responses to prolonged unloading following hypertrophy. Another notable finding of this study is that the rate of muscle mass change and the responses of apoptotic regulatory factors after 14 days of unloading in the experimental muscles of aged quails were different from those of young adult quails. After 14 days of unloading following hypertrophy, we found that the muscle mass of the experimental muscles was not different from the control muscles in young quails (i.e., the experimental muscles had returned to the control muscle mass level from the hypertrophic level), but the muscle mass of the experimental muscles was still greater than that of the control muscles in aged quails. Intriguingly, although the TUNEL index after 14 days of unloading was higher, we found some antiapoptotic changes, including

Table 1. Relationships among apoptotic regulatory factors in various muscle groups

<table>
<thead>
<tr>
<th>Correlated Factors</th>
<th>Muscle Samples Examined</th>
<th>N</th>
<th>r</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax protein and TUNEL index</td>
<td>All control muscles</td>
<td>32</td>
<td>0.476</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>All unloaded muscles</td>
<td>32</td>
<td>0.393</td>
<td>0.026</td>
</tr>
<tr>
<td></td>
<td>Young 14-day control and unloaded muscles</td>
<td>16</td>
<td>0.521</td>
<td>0.039</td>
</tr>
<tr>
<td>Bax mRNA and TUNEL index</td>
<td>All young muscles</td>
<td>32</td>
<td>0.377</td>
<td>0.033</td>
</tr>
<tr>
<td></td>
<td>Young 7-day control and unloaded muscles</td>
<td>16</td>
<td>0.581</td>
<td>0.018</td>
</tr>
<tr>
<td>Bax mRNA and cytoplasmic AIF</td>
<td>Young 14-day control and unloaded muscles</td>
<td>8</td>
<td>0.741</td>
<td>0.035</td>
</tr>
<tr>
<td>Bcl-2 protein and TUNEL index</td>
<td>All control muscles</td>
<td>32</td>
<td>−0.693</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>All unloaded muscles</td>
<td>32</td>
<td>−0.529</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>Young 7-day and 14-day unloaded muscles</td>
<td>32</td>
<td>−0.521</td>
<td>0.038</td>
</tr>
<tr>
<td></td>
<td>Young 14-day unloaded muscles</td>
<td>8</td>
<td>−0.723</td>
<td>0.043</td>
</tr>
</tbody>
</table>

Relationships among Bcl-2, Bax, apoptosis-inducing factor (AIF), and TdT-mediated dUTP nick end labeling (TUNEL) index were analyzed using Pearson’s correlation coefficient (r); N = sample size.

Fig. 11. Relationships between the TUNEL index and the protein levels of Bax and Bcl-2 were investigated by examining the Pearson product-moment correlation coefficient (r). Data collected from control and experimental muscles from both young adult and aged quails of all groups were collapsed and treated as a single pooled group (n = 64). Y7, young adult quails after 7 days of unloading; Y14, young adult quails after 14 days of unloading; A7, aged quails after 7 days of unloading; A14, aged quails after 14 days of unloading.
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generally increased Bcl-2 and decreased Bax levels in the experimental muscles of aged quails even with the existence of a few inconsistent alterations in protein vs. mRNA levels, possibly because the measurements were limited to the selected time points. Moreover, given that translocation of AIF from mitochondria to nuclei has been suggested to be an apoptotic event (18, 29), we found a decreased level of nuclear AIF in the 14-day unloaded muscles of aged quails. These data suggest that the apoptosis- unfavorable changes in the apoptotic regulatory factors may provide an “apoptosis-braking” machinery to decelerate the apoptotic elimination of the muscle nuclei and therefore slow down the muscle mass loss during unloading in the aged quails. However, the reason for the decelerating loss in muscle mass and the apoptosis- unfavorable changes after 14 days of unloading following stretch-induced hypertrophy in the aged quails is unknown. Nonetheless, we interpret this observation as an antiapoptotic adaptation to prolonged unloading (i.e., 14 days of unloading in this study) following muscle hypertrophy in aged quails, although this antiapoptotic response did not appear to be successful (on the basis of TUNEL data).

Recently, apoptosis was suggested to play an important role in the development of aging-associated sarcopenia, because aged skeletal muscles have been characterized by an increased level of apoptosis (9, 22, 30, 43, 44, 49). Furthermore, the detrimental effects of muscle disuse on muscle mass and function have been shown to be greater in aged muscles (11, 21). From the point of view of survival, it is not known whether the aged skeletal muscles could have developed an adaptive antiapoptotic response to prolonged disuse following muscle hypertrophy aimed to preserve the hypertrophied muscle status in the frail aged muscles. We speculate that this age-related adaptive response may function as an attempt to favor the preservation of the enlarged muscle masses of old animals during prolonged muscle disuse following hypertrophy. Nevertheless, this antiapoptotic adaptation is assumed to decelerate the hypertrophied muscle mass loss during prolonged unloading, but this is partially in contradiction to the suggestion that aged skeletal muscles are subjected to a greater muscle mass loss during muscle disuse. Further research is required to verify that this antiapoptotic adaptation also occurs during prolonged disuse in other experimental models (e.g., muscle denervation) following muscle hypertrophy.

In the present study, the apoptotic responses to unloading following hypertrophy have been discussed in terms of the findings obtained from a unilateral wing weighting/unloading model with the contralateral wing acting as the intra-animal control in these quails. Because we could not rule out the possibility that loading of one wing might have a slight effect on the mechanical environment or the central environmental milieu (circulating stress hormones, etc.) that would affect the contralateral control muscle, we conducted subsequent studies to address this issue. First, we closely monitored the daily living activities and biomechanics of the animals (e.g., eating habits, body center of gravity, body balancing ability, and movement and locomotion patterns) throughout the study period, and we did not observe any apparent abnormality in the studied animals or differences when comparing the experimental animals (with one wing weighted and one wing unweighted, or one control wing and one wing that was unloaded following stretch loading) with the normal, untreated animals that did not receive a stretch load/unload. Moreover, using immunoblotting, we found that Bcl-2, Bax, Apatf-1, and AIF protein contents of the contralateral control muscles from the experimental animals were not different from those of muscles of normal (nonexperimental) animals that had not experienced any stretch/unload (data not shown). These observations suggest that the influence of wing weighting/unloading of the experimental limb did not alter the normal activities of the animal or the muscle environment sufficient to alter markers for apoptosis in the contralateral control muscles. Thus we conclude that the contralateral control muscles were not different from the normal, nonexperimental control muscles in the present study.

The exact identity of the mitotic myogenic contributors that have been involved in the loading-unloading process was not comprehensively evaluated in this study. Thus we cannot rule out the possibility that, in addition to the muscle satellite cell population, some other mitotic cell populations (e.g., invading bone marrow, blood mesenchymal cells, and/or resident interstitial myogenic cells) may also have contributed nuclei to the overloaded quail muscles during muscle hypertrophy, as is the case in mammalian muscles (51–53).

In summary, we have demonstrated proapoptotic changes including decreased Bcl-2 content, increased Bax content, and increased numbers of nuclei with DNA breaks as estimated by TUNEL in the experimental muscles after 7 days of unloading following hypertrophy in both young adult and aged quails. We also have shown that moderate correlational relationships exist among TUNEL index and Bcl-2, Bax, and AIF contents in the quail muscles. These findings support the hypothesis that apoptosis may have a physiological role in mediating muscle remodeling during unloading-induced muscle atrophy following muscle hypertrophy. Our findings indicate that one of these possible physiological roles could be the elimination of excessive activated satellite cells (both fused and nonfused) to maintain the myonuclear domain. In addition, although the TUNEL index of the experimental muscles was still higher than that of the control muscles after 14 days of unloading in aged quails, we have found some antiapoptotic changes, including increased mRNA and protein levels of Bcl-2, decreased protein levels of Bax, and decreased nuclear AIF protein level in the 14-day unloaded aged muscles. These findings supports the hypothesis that aged muscles may respond differently from young muscles during unloading-induced muscle atrophy following muscle hypertrophy. Additional research is needed to further confirm this antiapoptotic adaptation and its physiological role during prolonged unloading in aged muscles.

Although we and others have provided evidence suggesting the physiological role of apoptosis during muscle remodeling and normal aging (1, 8, 9, 12, 22, 27, 28, 30, 43, 44), further research is required to clarify the cellular and molecular upstream-regulatory mechanism(s) contributing to the activation of the apoptotic signaling pathway that results in subsequent apoptosis. Understanding the upstream-regulatory pathways leading to apoptosis during muscle remodeling and aging will provide insight into developing novel preventive or therapeutic regimens to alleviate or delay the loss of postmitotic myocytes with muscle disuse (e.g., bed rest, space flight) and aging.
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