Age-related apoptotic responses to stretch-induced hypertrophy in quail slow-tonic skeletal muscle

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Siu, Parco M., and Stephen E. Alway. Age-related apoptotic responses to stretch-induced hypertrophy in quail slow-tonic skeletal muscle. Am J Physiol Cell Physiol 289:C1105–C1113, 2005. First published June 22, 2005; doi:10.1152/ajpcell.00154.2005.—In the present study, we examined the responses of apoptosis and apoptotic regulatory factors to muscle hypertrophy induced by stretch overload in quail slow-tonic muscles. The wings from one side of young and aged Japanese quails were loaded by attaching a tube weight corresponding to 12% of the bird’s body weight for 7 or 21 days. Muscle from the contralateral side served as the intraanimal control. Relative to the intraanimal control side, the muscle wet weight and muscle weight significantly increased by 179% and 102% in young and aged birds, respectively. Heat shock protein (HSP)72 and HSP27 protein contents in the loaded sides were higher than on the control sides exclusively in young birds after 7 days of loading. Compared with the contralateral control muscle, the extent of apoptotic DNA fragmentation and the total cytosolic protein content of second mitochon-drial activator of caspases/direct inhibitor of apoptosis-binding protein with low isoelectric point were elevated compared with the intraanimal control muscle from young birds. Furthermore, after 7 days of loading the muscles of aged birds, H2O2 content and the total cytosolic protein content of second mitochondrial activator of caspases/direct inhibitor of apoptosis-binding protein with low isoelectric point were elevated compared with the intraanimal control side. These data suggest that stretch overload-induced muscle hypertrophy is associated with changes in apoptosis in slow-tonic skeletal muscle. Moreover, discrepant apoptotic responses to muscle overload in young and aged muscles may account in part for the age-related decline in the capability for muscle hypertrophy.

aging; sarcopenia; Bcl-2; Bax; heat shock proteins; apoptosis-inducing factor

APOPTOSIS IS A CELLULAR process that has been revealed to be essential in regulating a range of biological events (28, 60). In addition to the etiologic role of apoptosis in the pathogenesis of several severe diseases (e.g., cancer) (60), it has been demonstrated that apoptosis may play a role in regulating muscle wasting under different physiological and pathophysiological conditions, including muscle denervation, muscular dystrophy, neumuscular disorders, hindlimb unweighting, muscle unloading, and strenuous physical exercise (1, 6, 8, 31, 48–50, 52, 55, 56, 59). In company with the hypothesized regulatory role of apoptosis in muscle atrophy, there has been evidence showing the relationship between apoptosis and aging muscle, including accelerated apoptotic signaling in skeletal muscle with aging (6, 7, 19, 20, 30, 31, 44, 45, 51, 58) and the influence of aging in the apoptotic response to muscle disuse (31, 51, 55, 56).

Sarcopenia is an unavoidable age-related loss of muscle mass that leads to significant muscle weakness (21). It has been suggested that sarcopenia is related to declined quality of life, increased morbidity, and reduced life expectancy of the elderly population. Sarcopenia is attributed to both reductions in muscle fiber size and total fiber number (18, 32). Although the mechanisms responsible for sarcopenia have not been elucidated fully, metabolic, hormonal, nutritional, neuropathic, and immunological factors may contribute to the development of sarcopenia (47). In addition to the diminishing of muscle quality with aging (4, 5, 7, 12, 17, 39), it has also been demonstrated that the hypertrophic response to either functional or stretch overload is decreased in aged muscle relative to muscle from young adults (3, 4, 7, 12, 14–17, 29). However, the exact mechanisms of the age-related attenuation in hypertrophic responses are largely unclear.

While apoptosis has been implicated in various muscle atrophic conditions (1, 6, 8, 31, 48–52, 54–56, 59), including age-associated sarcopenic muscle loss (6, 7, 19, 20, 30, 31, 44, 45, 58), it is unknown whether muscle hypertrophy is accompanied by alterations in apoptotic signaling. However, our laboratory recently provided preliminary evidence suggesting that regulation of apoptotic signaling may be important during muscle hypertrophy on the basis of a finding of upregulation of the apoptotic suppressor X-linked inhibitor of apoptosis (XIAP) after stretch-induced overload (56). It seemed likely that if this antiapoptotic protein were increased with hypertrophy, other apoptotic proteins might also be involved in apoptotic signaling during loading-induced hypertrophy. Furthermore, because apoptosis has been shown to increase during proliferation of some cell types (40), it is not inconceivable that apoptosis might be involved in regulating nuclear number during hypertrophic adaptations to loading, in which satellite cell proliferation is extensive (8, 16, 63). We speculated that antiapoptotic proteins would be upregulated to support the survival of satellite cells activated during overload to permit hypertrophic adaptations. Furthermore, it is unclear whether...
apoptosis of muscle nuclei is related to the attenuated ability of aged skeletal muscle to achieve hypertrophic levels that are similar to those found in young animals in response to the same loading stimulus. Thus the objective of this study was to examine the responses of apoptosis and apoptotic regulatory factors to muscle hypertrophy induced by stretch overload in slow-tonic skeletal muscles from young adult and aged quails. We hypothesized that the abundance of proapoptotic regulatory factors would be reduced, which would lead to an antiapoptotic cellular milieu during muscle hypertrophy. We further tested the hypothesis that the apoptotic changes that occur during loading in young adult muscle would be absent during loading in muscles from aged birds.

In the present study, apoptotic DNA fragmentation, Bax, Bcl-2, cytochrome c, second mitochondrial activator of caspases (Smac)/direct inhibitor of apoptosis-binding protein with low isoelectric point (DIABLO), XIAP, apoptosis repressor with caspases recruitment domain (ARC), apoptosis-inducing factor (AIF), H\textsubscript{2}O\textsubscript{2} content, superoxide dismutases (SOD; Mn-SOD and Cu,Zn-SOD), heat shock protein (HSP)70, HSP27, and HSP60 were examined in the muscle samples. On the basis of the previous findings in our laboratory (HSP)70, HSP27, and HSP60 were determined in the total cytosolic protein fraction. The left wings of birds were loaded for 7 or 21 days (eight young and eight aged birds were included in each experimental setting), and then the birds were killed by administering an overdose of pentobarbital sodium. ALD muscles were dissected from the surrounding connective tissue, removed, weighed, and frozen in isopentane cooled to the temperature of liquid nitrogen and then stored at $-80^\circ\text{C}$ until used for analyses.

**Protein preparation.** The fractionation method described by Rothermel et al. (46) was adopted with minor modification to extract the nuclei-free cytosolic protein fraction from the ALD muscle. We have previously obtained the fractionated cytosolic and nuclear proteins from skeletal or heart muscles using this modified protocol (51–53, 55). Briefly, after removal of connective tissues, muscle was homogenized on ice in lysis buffer (10 mM NaCl, 1.5 mM MgCl\textsubscript{2}, 20 mM HEPES, pH 7.4, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol) supplemented with a protease inhibitor cocktail containing (in mM) 104-4-(2-aminooethyl)benzenesulfonyl fluoride, 0.08 aprotinin, 2 leupeptin, 4 bestatin, 1.5 pepstatin A, and 1.4 E-64 (Sigma-Aldrich, St. Louis, MO). After we performed centrifugation at 5,000 rpm for 5 min at 4°C to pellet the nuclei and cell debris, the supernatants were collected and centrifuged three times at 6,000 rpm for 5 min at 4°C to remove residual nuclei. The final collected supernatants were stored as nuclei-free total cytosolic protein fraction. This cytosolic protein fraction was used for cell death ELISA, H\textsubscript{2}O\textsubscript{2} assay, and Western immunoblots. The above-described protein fractionation procedures have been used routinely in our laboratory to obtain high-purity protein fractions as assessed by immunoblotting the fractions with an anti-histone H2B (a nuclear protein) and an anti-Cu,Zn-SOD (a cytosolic isoform of superoxide dismutase) antibody (52, 55).

**Western immunoblot analyses.** Protein expression of B-cell leukemia/lymphoma-2 (Bcl-2), Bcl-2-associated X protein (Bax), AIF, second mitochondria-derived activator of caspase (Smac/DIABLO), X-linked inhibitor of apoptosis (XIAP), ARC, superoxide dismutases (Mn-SOD and Cu,Zn-SOD), and heat shock proteins HSP72, HSP27, and HSP60 were determined in the total cytosolic protein fraction.

**Apoptotic cell death ELISA.** A cell death detection ELISA kit (Roche Applied Science, Indianapolis, IN) was used to determine apoptotic DNA fragmentation quantitatively by measuring the cytosolic histone-associated mono- and oligonucleosomes. In brief, the nuclei-free cytosolic fraction of ALD muscle was used as an antigen source in a sandwich ELISA with a primary anti-histone mouse monoclonal antibody coated onto the microtiter plate and a second anti-DNA mouse monoclonal antibody coupled to peroxidase. The amount of peroxidase retained in the immunocomplex was determined photometrically by incubating it with 2,2'-azino-di-[3-ethylbenzthiazoline-sulfonate] as a substrate for 30 min at 20°C. The change in color was measured at a wavelength of 405 nm using a Dynex MRX plate reader controlled with personal computer software (Revelation; Dynatech Laboratories, Chantilly, VA). Measurements were performed with loaded and contralateral control samples analyzed on the same microtiter plate at the same setting. The optical density (OD)\textsubscript{405} reading was then normalized to the milligrams of protein used in the assay and presented as an apoptotic index.
Louis, MO) to verify equal loading and transferring of proteins to the membrane in each lane. Another approach used to validate similar loading between the lanes was to load gels in duplicate, with one gel being stained with Coomassie blue. The membranes were then blocked in 5% nonfat milk in phosphate-buffered saline (PBS) with 0.05% Tween 20 (PBST) at room temperature for 1 h and probed with the following primary antibodies diluted in PBST with 2% BSA: anti-Bcl-2 mouse monoclonal antibody (1:200 dilution; sc-7382), anti-Bax rabbit polyclonal antibody (1:200 dilution; sc-6236), anti-human inhibitor of apoptosis-like protein/XIAP mouse monoclonal antibody (1:250 dilution, 610762; BD Biosciences, San Jose, CA), anti-ARC rabbit polyclonal antibody (1:200 dilution; sc-11435), anti-AIF mouse monoclonal antibody (1:500 dilution; sc-13116HRP), anti-Smac/DIABLO mouse monoclonal antibody (1:500 dilution, 612244; BD Biosciences), anti-Mn-SOD goat antibody (1:2,000 dilution, A30049A; Bethyl Lab, Montgomery, TX), anti-Cu,Zn-SOD rabbit polyclonal antibody (1:500 dilution; sc-11407), anti-HSP72 rabbit polyclonal antibody (1,200 dilution; SPA812), anti-HSP27 rabbit polyclonal antibody (1,200 dilution; SPA801), or anti-HSP60 rabbit polyclonal antibody (1,2000 dilution; SPA804). Bcl-2, Bax, ARC, AIF, and Cu,Zn-SOD antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), while HSP72, HSP27, and HSP60 antibodies were purchased from StressGen (Victoria, BC, Canada). All primary antibody incubations were performed overnight at 4°C. Secondary antibodies were conjugated to horseradish peroxidase (Chemicon International, Temecula, CA), and signals were developed using an ECL detection kit (Amersham Biosciences, Piscataway, NJ). The signals were then visualized by exposing the membranes to X-ray films (BioMax MS-1; Eastman Kodak, Rochester, NY), and digital records of the films were captured using a Kodak 290 camera. The resulting bands were quantified as OD × band area using a one-dimensional image analysis system (Eastman Kodak, Rochester, NY) and recorded in arbitrary units. The molecular sizes of the immunodetected proteins were verified using prestained standard (LC5925; Invitrogen Life Technologies, Bethesda, MD).

Cytochrome c ELISA. It would have been ideal to measure the mitochondrial release of the mitochondria-housed apoptotic factors, including cytochrome c, AIF, and Smac/ DIABLO. However, the ALD muscles were too small to obtain both the total cytosolic and mitochondria-free cytosolic fractions (which would have been required to estimate mitochondrial release) with a reasonable concentration of protein. Thus we decided to examine cytochrome c accumulation in the total cytosolic protein fraction. According to the manufacturer’s protocol, 60 μl of the total cytosolic protein fraction was used as an antigen source in a sandwich ELISA with a horseradish peroxidase-conjugated anti-cytochrome c polyclonal antibody in microwell strips coated with an anti-cytochrome c antibody. After being washed, the horseradish peroxidase retained in the immunocomplex was detected by incubation with a chromogenic substrate, tetramethylbenzidine/H2O2, followed by addition of an acid solution to terminate the enzyme reaction and to stabilize the developed color. The change in color was monitored at a wavelength of 450 nm using a Dynex MRX plate reader. Measurements were performed with the loaded and contralateral control samples analyzed on the same microplate, and the cytochrome c content was expressed as OD450/mg of protein.

H2O2 fluorometric assay. The content of H2O2 in the ALD muscle homogenate was measured in the total cytosolic fraction using a fluorometric H2O2 detection kit (FLOH 100-3; Cell Technology, Mountain View, CA). In accordance with the manufacturer’s instructions, 50 μl of the total cytosolic fraction protein of the muscles were incubated in 50 μl of reaction cocktail containing horseradish peroxidase and 10-acetyl-3,7-dihydroxyphenoxazine (a nonfluorescent substrate that turns fluorescent after being oxidized by H2O2) in sodium phosphate buffer at room temperature in the dark for 10 min. The fluorescence was measured on a spectrofluorometer with an excitation wavelength of 530/25 nm and an emission wavelength of 590/35 nm (CytoFluor; Applied Biosystems, Foster City, CA) after the incubation. H2O2 content was estimated in arbitrary fluorescence units normalized to milligrams of protein used in the assay. Measurements were performed with the loaded and contralateral control samples run on the same microplate in the same setting.

Statistical analyses. Statistical analyses were performed using the SPSS 10.0 software package (SPSS, Chicago, IL). ANOVA followed by Tukey’s honestly significant difference post hoc analysis was performed to examine differences between groups. Statistical significance was accepted at P < 0.05. All data are means ± SE.

RESULTS

Change in muscle mass after stretch overload. The percentage difference in the whole ALD muscle wet weight between the loaded and contralateral control wings was examined to estimate the degree of muscle hypertrophy. After 7 days of loading, muscles of young adult birds showed 96% hypertrophy, whereas no significant extent of hypertrophy was found in the muscles of aged birds (Fig. 1A). After 21 days of loading, we observed 179% and 102% hypertrophy in the muscles of young and aged birds, respectively (Fig. 1A). The percentages of hypertrophy in young muscles after 7 and 21 days of loading were both significantly greater than those observed in the aged muscles (Fig. 1B). Our results confirm that the extent of hypertrophy as estimated on the basis of the increase in whole ALD muscle wet weight in response to stretch overload was attenuated in aged muscles compared with the muscles from young adult birds.

HSP72, HSP27, and HSP60 protein content. In all groups of birds, we did not find any differences in the protein contents of HSP72 and HSP27 between the loaded and the contralateral control sides, except for the young birds after 7 days of loading (Fig. 2). In the young adult muscle after 7 days of loading, the protein contents of HSP72 and HSP27 were 33% and 34% higher, respectively, than in the control muscle (Fig. 2, A and B). No significant difference was found in the protein content of HSP60 between the loaded and control muscles in all birds examined in the present study (data not shown).

H2O2 content in muscle homogenate and protein content of Mn-SOD and Cu,Zn-SOD. As estimated by performing a fluorometric assay, no difference was found regarding the H2O2 content between the loaded and control muscles in all groups except for the aged birds after 7 days of loading (Fig. 3). After 7 days of loading, the H2O2 content in the aged loaded muscle was 40% higher than that in the contralateral control muscle (Fig. 3). There was no difference in the protein contents of Mn-SOD and Cu,Zn-SOD between the loaded and control muscles in all groups of birds (data not shown).

Apoptotic DNA fragmentation and total cytosolic cytochrome c content. The cell death ELISA analysis showed that the level of the apoptotic DNA fragmentation was decreased with loading in all animals except the aged birds after 7 days of loading (Fig. 4A). The level of DNA fragmentation was 33% lower than that of the control side in muscles from young birds after 7 days of loading (Fig. 4A). After 21 days of loading, the level of DNA fragmentation in the loaded muscle was ~34% lower than that of the control muscle in young and aged birds (Fig. 4A). The cytochrome c ELISA analysis indicated that the total cytosolic cytochrome c protein content was reduced with loading in the young but not the aged birds (Fig. 4B). For the young adult birds, we found that the cytochrome c protein content in the loaded side was 34% and 31% lower than that in...
the contralateral control muscle after 7 and 21 days of loading, respectively (Fig. 4B).

Bax and Bcl-2 protein content. In the present study, the protein abundances of Bax and Bcl-2 were measured as markers for pro- and antiapoptotic proteins, respectively. Other pro- and antiapoptotic members of Bcl-2 family have not been investigated. According to our immunoblot data, the Bax protein content was decreased in the loaded side relative to the intraanimal control side in all groups of animals (Fig. 5A). After 7 days of loading, the total cytosolic AIF protein content in the loaded muscle was decreased by 21% compared with the control muscle in young birds, while the protein content of AIF in the loaded side was 36% and 28% lower than that of the control side after 21 days of loading in young and aged birds, respectively (Fig. 5A). We did not find any difference in the total cytosolic protein content of Smac/DIABLO between the loaded and control muscles in all birds, except for the aged

the contralateral control muscle after 7 and 21 days of loading, respectively (Fig. 4B).

Bax and Bcl-2 protein content. In the present study, the protein abundances of Bax and Bcl-2 were measured as markers for pro- and antiapoptotic proteins, respectively. Other pro- and antiapoptotic members of Bcl-2 family have not been investigated. According to our immunoblot data, the Bax protein content was decreased in the loaded side relative to the intraanimal control side in all groups of animals (Fig. 5A). The Bcl-2 protein content did not change with 7 days of loading in both young and aged muscle, but it was 41% and 53% lower in the loaded muscle compared with the control muscle in young and aged birds, respectively, after 21 days of loading (Fig. 5B).

Total cytosolic AIF, Smac/DIABLO, XIAP, and ARC protein content. Relative to the intraanimal contralateral control side, the AIF protein content in the total cytosolic fraction was reduced in the loaded muscle from all groups of animals except the aged birds after 7 days of loading (Fig. 6A). After 7 days of loading, the total cytosolic AIF protein content in the loaded muscle was decreased by 21% compared with the control muscle in young birds, while the protein content of AIF in the loaded side was 36% and 28% lower than that of the control side after 21 days of loading in young and aged birds, respectively (Fig. 6A). We did not find any difference in the total cytosolic protein content of Smac/DIABLO between the loaded and control muscles in all birds, except for the aged
birds after 7 days of loading. In the 7-day-loaded aged muscle, the Smac/DIABLO protein content was 29% lower than that in the contralateral control muscle (Fig. 6B).

Our immunoblot analysis indicated that the XIAP protein content was higher on the loaded side compared with the contralateral control side after loading in the young but not in the aged birds (Fig. 6C). The XIAP protein content in the loaded muscle was ~40% greater than that in the control muscle in the young birds after 7 or 21 days of loading (Fig. 6C). It is noted that members other than XIAP (e.g., cIAP) in the inhibitor of apoptosis family have not been examined in the current study. No difference was found in the protein content of ARC between the loaded and control sides in all groups of animals (data not shown).

**DISCUSSION**

An expanding body of published data from several laboratories have shown that apoptosis may play a role in regulating the process of muscle loss under various conditions, including aging (1, 6, 8, 19, 20, 30, 31, 45, 48–53, 55, 56, 58, 59). However, it is relatively unknown whether apoptosis is involved in the remodeling of skeletal muscle during hypertrophy. By using an avian model of stretch-induced muscle hypertrophy, our laboratory has preliminarily shown that the elevation of apoptotic suppressor XIAP is evident in loaded patagialis muscle, which is a muscle composed predominantly of fast $\alpha$-fibers (56). In the present study, we have extended our previous findings by demonstrating that muscle hypertrophy as induced by stretch overload is accompanied by changes in apoptosis and apoptotic regulatory factors in slow $\beta$-fiber-predominant ALD muscle. Our findings indicate that in the young adult ALD muscle after stretch-induced overload, apoptotic DNA fragmentation, Bax, Bcl-2, total cytosolic cytochrome $c$, and AIF content are reduced, whereas XIAP, HSP72, and HSP27 are elevated with hypertrophy. Furthermore, we observed age-related differences in the apoptotic responses to muscle overload as shown by the increases in total cytosolic Smac/DIABLO and H$_2$O$_2$ content and unchanged total cytosolic cytochrome $c$, XIAP, HSP72, and HSP27 in the loaded muscle exclusively from aged animals.

**Apoptotic responses to stretch-induced muscle overload.** In accordance with the destructive or removal nature of apoptosis, it is generally accepted that the apoptotic program is initiated by internal and/or external stimuli linking to cellular stress or damage. The activation of apoptosis has been demonstrated consistently in atrophying skeletal muscle (1, 6, 8, 19, 20, 30, 31, 45, 48–53, 55, 56, 58, 59). This apoptotic activation may be accompanied by other cellular signaling involving NF-kB, ubiquitin ligases muscle-specific ring finger 1/muscle atrophy F-box, and/or myostatin, which are thought to be triggered by the largely unidentified atrophy-related stimuli during muscle disuse or with aging (23, 25). All of these atrophy-associated cellular changes presumably lead to decreased protein synthesis/accumulation and/or increased protein breakdown/degradation so that the muscle approaches a catabolic state (23, 25, 27). In contrast to muscle atrophy, overload causes muscle hypertrophy by increasing protein synthesis and accumulation of proteins as a result of adjusting the rate of anabolism over...
catabolism in muscle cells. Noticeably, these anabolic responses necessitate increases in transcription and/or translation of muscle-related genes (13, 33), and various signaling pathways are apparently required to connect the hypertrophic stimuli to the upregulation of transcriptional and/or translational processes (23, 25). Indeed, several signaling components [e.g., insulin-like growth factor-1 (IGF-1), phosphatidylinositol 3-kinase (PI3K), PKB/Akt, mammalian target of rapamycin (mTOR), p70 ribosomal protein S6 kinase (p70S6K), RhoA, serum response factor, focal adhesion kinase, and/or mechano-growth factor (MGF)] have been demonstrated to be potentially involved in mediating the anabolic process of muscle hypertrophy (22–26, 38, 43). In conjunction with the anabolic or survival nature of the demonstrated signaling proteins, it appears that a cellular milieu favoring survival/growth is anticipated for the overloaded muscle to achieve a hypertrophic state. In line with this idea, our results have shown that muscle hypertrophy induced by stretch overload is accompanied by antiapoptotic changes, including modulation of XIAP, AIF, Bax, cytochrome c, and DNA fragmentation in quail slow-twitch skeletal muscle. Although our data did not allow us to identify the exact physiological role of the observed antiapop-

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Fig. 5. A: Bcl-2-associated X protein (Bax) protein content. Data are expressed as OD resulting band area and are expressed in arbitrary units. The insets show representative blots for Bax in control and loaded muscles isolated from young adult and aged animals. Data are presented as means ± SE. *P < 0.05, significantly different from corresponding intraanimal control muscles. B: B-cell leukemia/lymphoma-2 (Bcl-2) protein content. Data are expressed as OD resulting band area and are expressed in arbitrary units. The insets show representative blots for Bcl-2 in control and loaded muscles isolated from young adult and aged animals. Data are presented as means ± SE. *P < 0.05, significantly different from corresponding intraanimal control muscles.

Fig. 6. A: total cytosolic apoptosis-inducing factor (AIF). The AIF protein content was estimated by performing immunoblot analysis on the total cytosolic fraction. Data are expressed as OD resulting band area and are expressed in arbitrary units. The insets show representative blots for AIF in control and loaded muscles isolated from young adult and aged animals. Data are presented as means ± SE. *P < 0.05, significantly different from the corresponding intraanimal control muscles. B: total cytosolic second mitochondrial activator of caspases (Smac/DIABLO) protein content. The Smac/DIABLO protein content was estimated by immunoblotting analysis on the total cytosolic fraction. The data are expressed as OD resulting band area, and expressed in arbitrary units. The insets show representative blots for Smac/DIABLO in control and loaded muscles isolated from young adult and aged animals. Data are presented as means ± SE. *P < 0.05, significantly different from the corresponding intraanimal control muscles. C: X-linked inhibitor of apoptosis (XIAP). The XIAP protein content was estimated by performing immunoblot analysis on the total cytosolic fraction. Data are expressed as OD resulting band area and are expressed in arbitrary units. The insets show representative blots for XIAP in control and loaded muscles isolated from young adult and aged animals. Data are presented as means ± SE. *P < 0.05, significantly different from corresponding intraanimal control muscles.
Apoptotic responses to muscle overload, the present findings are the first to demonstrate that apoptotic components are responsive to stretch-induced muscle hypertrophy.

**Aging influences the apoptotic responses to muscle overload.** Consistent with the previous reports showing that the ability of aged skeletal muscle to achieve muscle hypertrophy is diminished (3, 4, 7, 12, 14–17, 29, 38, 43, 62), the data from muscles overloaded for 7 or 21 days in the current study demonstrate the impairment of the ability for the aged muscle to gain muscle mass with overload. Although the exact underlying mechanisms contributing to the attenuation of muscle enlargement in the aged muscle are not completely understood, potential molecular and cellular factors [e.g., MRF/Id myogenic repressor, mTOR/p70(S6K)/extracellular signal-regulated kinase 1/2 (ERK1/2) signaling, AMP kinase, and IGF-1/MGF/IGF-1 receptor] have been suggested as candidates to explain, at least in part, the age-related limitation for muscle hypertrophy (7, 26, 33, 42, 43, 61).

By examining hypertrophy of plantaris muscle induced by denervating its agonist plantarflexor muscles (i.e., gastrocnemius and soleus), Alway et al. (7) demonstrated that myogenic regulatory factor (MRF) myogenin, MyoD, and myogenic repressor Id2 are elevated in overloaded muscle from young adult rats, but these changes are not present in the aged overloaded muscle. Similar observations for the attenuated increase in MRFs in the aged muscle have also been reported at the mRNA level in a quail stretch-induced muscle overload model (33). By examining 6- and 30-mo-old Fischer 344 × Brown Norway rats, Parkington et al. (43) showed that the increases in the phosphorylation of mTOR, p70(S6K), and ERK1/2 after a single bout of in situ muscle contractile activity as elicited by high-frequency electrical stimulation of the sciatic nerve are attenuated in the aged muscle compared with the muscle from adult animals. In addition, impairment of the elevation of MGF or IGF-1 receptor and hyperphosphorylation of AMP kinase during muscle overload have also been found in the aged skeletal muscle with muscle overload (26, 42, 61). In the present study, we observed age-related differences in the apoptotic changes in the muscles after stretch-induced overload. We found that total cytosolic Smac/DIABLO protein content and H2O2 content are increased exclusively in the 7-day-loaded muscles from aged birds; however, these aged muscles did not show any sign of hypertrophy (i.e., muscle weight gain) in response to stretch overload. Moreover, the changes in total cytosolic cytochrome c, XIAP, HSP72, and HSP27 that we observed in the loaded young muscle are not found in identically loaded muscles obtained from aged birds. These proteins/genes designate the reduced proapoptotic or increased antiapoptotic tendencies that were observed in the young muscle with overloading were not present in the aged loaded muscle. We speculate that the aged muscle might have defects in handling the anticipated apoptotic changes during overload and unsuccessfully assemble a survival- and/or growth-favoring cellular environment and therefore fails to achieve the same muscle enlargement as young adult muscle. This speculation is supported by findings showing that muscles from old animals have increased proapoptotic tendencies compared with muscles from young adult animals (6, 7, 19, 20, 30, 31, 44, 45, 58) and that aging influences the apoptotic responses to muscle disuse (31, 51, 55, 56). Notably, similar responses of apoptotic DNA fragmentation, Bax, Bcl-2, total cytosolic AIF, and Smac/DIABLO were found in young adult and aged muscles after 21 days of stretch overload. Although significant muscle weight gain was evident in these loaded aged muscles, the extent of hypertrophy was attenuated in the muscles loaded for 21 days in aged compared with young adult birds. These data indicate that the age-related attenuation of muscle hypertrophy may not be explained completely by the apoptotic events. Nonetheless, discrepant responses of cytochrome c, XIAP, HSP72, HSP27, and Smac/DIABLO in the loaded muscles from young adult and aged birds were in agreement with the hypothesis that apoptosis may partly account for the age-related diminishment of the capability for muscle hypertrophy. More research is needed to fully explore the possible contribution of apoptosis to the impairment of muscle-hypertrophied capacity in aged muscle.

In summary, we report antiapoptotic changes, including decreases in apoptotic DNA fragmentation, Bax, total cytosolic cytochrome c, and AIF content and increases in the protein content of XIAP, HSP72, and HSP27, in young adult slow-tonic skeletal muscles after 7 or 21 days of stretch-induced overloading. These findings indicate that muscle overload is associated with alteration of apoptosis and apoptotic regulatory factors. While the exact physiological function of these antiapoptotic changes during muscle overload remains to be identified, we speculate that these antiapoptotic changes may have provided a cellular milieu favoring survival and/or growth of activated satellite cells, which facilitates the cellular processes (e.g., anabolism) that result in muscle enlargement with overload. Nevertheless, more investigation is needed to fully comprehend the role of these apoptotic changes in muscle hypertrophy. Furthermore, we observed differences in the apoptotic responses to overload between young adult and aged muscles. There are increases in total cytosolic Smac/DIABLO and H2O2 content, whereas the total cytosolic cytochrome c, XIAP, HSP72, and HSP27 were unchanged exclusively in the aged loaded muscle. These findings are consistent with the hypothesis that the age-related apoptotic responses explain, at least in part, the attenuated ability of aged muscle to achieve a considerable level of hypertrophy during overload. However, further research is required to fully explore the contribution of apoptosis to the age-related impairment in muscle hypertrophy. Additional investigations that stimulate or inhibit apoptosis may provide further information to delineate the relationship between apoptosis and muscle plasticity.

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**REFERENCES**


