Aging Sustains the Hypertrophy-Associated Elevation of Apoptotic Suppressor X-Linked Inhibitor of Apoptosis Protein (XIAP) in Skeletal Muscle During Unloading

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This study tested the hypotheses that apoptotic suppressors: (a) increase during muscle overload, (b) decrease in response to unloading following hypertrophy, and (c) respond to unloading in an aging-dependent fashion. Following 14 days of stretch-induced overloading, the X-linked inhibitor of apoptosis protein (XIAP) was elevated by 140% and 116% in patagialis (PAT) muscles of young and old quail, respectively, when compared to the contralateral control side. XIAP messenger RNA (mRNA) or protein was not different in experimental and control muscles of young birds after 7 or 14 days of unloading. In old birds, PAT XIAP mRNA and protein were 47% and 67% greater in experimental than in control muscles, respectively, after 7 days of unloading. Furthermore, XIAP mRNA had returned to control level by 14 days of unloading, but XIAP protein content was 57% greater than control muscles after 14 days of unloading. Higher levels of XIAP during unloading in old than in young muscles may be an attempt to counterbalance apoptosis-induced muscle atrophy.

APOPTOSIS has been suggested to have a physiologic role in regulating muscle atrophy including muscle denervation, muscle unloading, hind-limb unweighting, neuromuscular disorders, muscle dystrophy, and aging-associated sarcopenia (1–14). It has been shown that aging reduces the ability of skeletal muscle to achieve hypertrophy in response to overload (15–17). Moreover, there is evidence suggesting that aging may exacerbate the extent of muscle loss during muscle disuse (18,19), although some studies (20,21) have shown a similar muscle loss between young and aged animals following hind-limb unloading. Nevertheless, it has been demonstrated that muscle atrophy in the models of unloading where muscle mass is reduced below control muscle level (e.g., hind-limb unloading) is associated with apoptosis (1,22). We have also found that apoptosis is evident in muscles from old and young quails that are first hypertrophied by loading, and then atrophied by unloading. However, regulation of apoptosis was aging dependent. For example, we have recently shown a reduced pro-apoptotic tendency including decreases in Bax and AIF, and increase in Bcl-2 in muscles of aged birds that were loaded to induce hypertrophy then unloaded for 14 days (12,13). These changes appear to be an attempt by aging muscles to offset the apoptosis-associated muscle losses occurring during unloading.

Apoptosis is controlled in a highly coordinated manner. The cellular decision for the execution of the apoptotic program is driven by simultaneous influences of both pro- and anti-apoptotic signaling which are orchestrated by a specific cluster of apoptotic proteins (e.g., BCL-2 family) (23,24). Among a number of apoptotic proteins that have been identified, X-linked inhibitor of apoptosis protein (XIAP), apoptosis repressor with caspase recruitment domain protein (ARC), and (Fas-associated death domain protein-like interleukin-1β-converting enzyme)-like inhibitory protein (FLIP) establish a group of endogenous apoptotic suppressors which primarily function in modulating the anti-apoptotic signaling (25–30). Although we have found that aging affects the response of apoptotic regulatory factors to unloading-induced atrophy in previously hypertrophied quail muscles (13), it is not known if apoptotic suppressor proteins participate in regulating apoptosis in this situation. Furthermore, it is unclear whether (a) muscle overloading has an effect on the expression of the apoptotic suppressors and (b) aging muscle influences the apoptotic suppressor proteins during unloading, which helps to reduce the rate of loss of muscle mass during unloading following a period of loading-induced hypertrophy as compared to muscles of young birds. Hence, this study examined the response of apoptotic suppressors XIAP, ARC, and FLIP to overloading and subsequent unloading in young adult and aged quail muscles. It is noted that the present study was designed to investigate the process of muscle loss from the “overloaded state” to the “normally loaded state” of the muscle. The term “unloading” used in this study refers to the removal of the load from the hypertrophied muscle, and this removal induces the atrophy of the hypertrophied muscle. We tested the hypotheses that (a) apoptotic suppressors are increased in response to muscle hypertrophy and are down-regulated during subsequent unloading, and (b) apoptotic suppressors respond differently to...
unloading following muscle hypertrophy in young adult and aged muscles.

METHODS

Animals

Japanese Coturnix quails were hatched and raised in pathogen-free conditions in the central animal care center at the West Virginia University School of Medicine. The birds were housed at a room temperature of 22°C with a 12-hour light/dark cycle and were provided with food and water ad libitum. Sixteen young adult (2-month-old) birds and 16 24-month-old birds were examined in the present study. The life span of Japanese quails is ~26–28 months, and they are both physically and sexually mature by 1.5 months of age (31,32).

Stretch-Induced Overloading and Unloading Protocol

The patagialis (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, a tube containing ~12% of the bird’s body weight was placed over the left humeral-ulnar joint (33). This weight maintains the joint in extension throughout the period of stretch and induces stretch at the origin of the PAT muscle. Previous studies (3,34,35) have shown that this stretch-overloading protocol results in moderate hypertrophy of the PAT muscles (i.e., 14-day stretch-loading induces ~35% and ~15% increases in muscle mass of young adult and aged birds, respectively). The left wing of animals was overloaded for 14 days, then the load was removed. Seven days after the removal of the weight, eight young and eight aged birds were killed by an overdose of pentobarbital sodium. The remaining young and aged animals were killed 14 days after the weight removal. The unstretched right PAT muscle served as the intra-animal control muscle for each bird. PAT muscles were dissected from the surrounding connective tissue, removed, weighed, frozen in isopentane cooled to the temperature of liquid nitrogen, then stored at −80°C until used for analyses.

All experimental procedures carried approval from the Institutional Animal Use and Care Committee from the 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 (AAALAC) and following the policies and procedures detailed in the Guide for the Care and Use of Laboratory Animals as published by the U.S. Dept. of Health and Human Services and proclaimed in the Animal Welfare Act.

Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was obtained from PAT muscles with TriReagent (Molecular Research Center, Cincinnati, OH), which is based on the guanidine thiocyanate method. Briefly, frozen muscle following mincing was mechanically homogenized on ice in 1 ml of ice-cold TriReagent. Total RNA was solubilized in RNase-free H2O and quantified in duplicate by measuring the optical density (OD) at 260 nm. Purity of RNA was determined by examining the OD260/OD280 ratio. Two micrograms of RNA was reverse transcribed with decamer primers and Superscript II reverse transcriptase (RT) in a total volume of 20 µl according to standard methods (Invitrogen Life Technologies, Bethesda, MD). Control RT reaction was done in which the RT enzyme was omitted. The control RT reaction was polymerase chain reaction (PCR)-amplified to ensure that DNA did not contaminate the RNA. One microliter of complementary DNA (cDNA) was then amplified by PCR using 100 ng of forward and reverse primers, ribosomal 18S primer pairs (Ambion, Austin, TX), 250 µM deoxyribonucleotide triphosphates (dNTPs), 1 × PCR buffer, and 2.5 U Taq DNA polymerase (USB Corp., Cleveland, OH) in a final volume of 50 µl. PCR was performed using a programmed thermocycler (Biometra, Göttingen, Germany). The primer pairs were designed from sequences published in GenBank (Table 1), and PCR products were verified by restriction digestions. Preliminary experiments were conducted with each gene to assure that the number of cycles represented a linear portion for the PCR OD curve for the muscle samples. It is noted that the mRNA expression of ARC was not examined in this study because the sequence of the ARC gene of quail or chicken has not been reported previously. The cDNA from all muscle samples were amplified simultaneously using aliquots from the same PCR mixture. After the PCR amplification, 30 µl of each reaction was subjected to electrophoresis on 1.5% agarose gels and stained with ethidium bromide. Images were captured, and the signals were quantified in arbitrary units as OD × band area using a Kodak 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. Ribosomal 18S primers (Ambion) were used as internal controls, and all RT-PCR signals were normalized to the 18S signal of the corresponding RT product to eliminate measurement error from uneven sample loading.

Table 1. Primers Used for PCR Amplification of cDNA

<table>
<thead>
<tr>
<th>Product</th>
<th>Accession No.</th>
<th>Sequence</th>
<th>Position</th>
<th>Tm, °C</th>
<th>Cycles</th>
<th>Product Length, bp</th>
<th>Restriction Enzyme</th>
<th>Restriction Products, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>XIAP</td>
<td>AF451854</td>
<td>F: 5′-CCCCCCTATGGCCCTATTGAC-3′</td>
<td>584–603</td>
<td>54.9</td>
<td>32</td>
<td>288</td>
<td>NcoI</td>
<td>266, 22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5′-CGGCCCTCATATTCGCACT-3′</td>
<td>851–871</td>
<td></td>
<td></td>
<td></td>
<td>AluI</td>
<td>93, 92, 58, 34, 11</td>
</tr>
<tr>
<td>FLIP</td>
<td>XM_421935</td>
<td>F: 5′-GCCAGAAGATGAGACAGAATATCTCACTA-3′</td>
<td>1161–1186</td>
<td>52.7</td>
<td>36</td>
<td>297</td>
<td>AluI</td>
<td>196, 101</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5′-TGGCTTGGTCGTTGTCTATATCC-3′</td>
<td>4134–1457</td>
<td></td>
<td></td>
<td></td>
<td>EcoRV</td>
<td>129, 109, 59</td>
</tr>
</tbody>
</table>

Note: PCR = polymerase chain reaction; cDNA = complementary DNA; Tm = annealing temperature; Accession No. = GenBank accession number; F = forward primer; R = reverse primer; XIAP = X-linked inhibitor of apoptosis protein; FLIP = (Fas-associated death domain protein-like interleukin-1β-converting enzyme)-like inhibitory protein.
and to provide a semiquantitative measure of the relative changes in gene expression.

**Protein Extraction and Immunoblot Analysis**

Total cytosolic protein was extracted from PAT muscles according to the procedure originally described by Rothemel and colleagues (36) with modifications as previously reported (12,13,37). In brief, muscles were homogenized in ice-cold lysis buffer [10 mM NaCl, 1.5 mM MgCl₂, 20 mM HEPES (pH 7.4), 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol]. The cytoplasmatic protein fraction-containing supernatant was obtained after centrifugation. A protease inhibitor cocktail (P8340; Sigma-Aldrich, St. Louis, MO) was added to the protein extracts. The protein concentration of the protein extract was quantified in duplicate by the BCA Protein Assay (Pierce, Rockford, IL) based on the Biuret reaction and the bicinchoninic acid detection of cuprous cation. As a further means to confirm the protein contents, all the protein samples were measured in duplicate on a different occasion with a DC Protein Assay (Bio-Rad, Hercules, CA) based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent, which was similar to the Lowry assay.

Forty micrograms of protein was boiled in Laemmli buffer (161-0737; Bio-Rad) in the presence of 2-mercaptoethanol and was loaded on each lane of a 12% polyacrylamide gel and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) at room temperature. The gels were blotted to nitrocellulose membranes (VWR, West Chester, PA) and stained with Ponceau S red (Sigma-Aldrich, St. Louis, MO) based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent, which was similar to the Lowry assay.

Protein Extraction and Immunoblot Analysis

**RESULTS**

The present 14-day stretch-loading procedure has been consistently shown to result in a moderate extent of muscle hypertrophy relative to the contralateral control muscle (35% and 15% in young adult and aged birds, respectively) (33–35). We have previously reported a reduced degree of muscle hypertrophy in the experimental muscle that was unloaded following loading-induced hypertrophy relative to the contralateral control muscle (13). Following 7 days of unloading, the muscle mass of the experimental muscles was 15% and 12% greater than that of the contralateral control muscle in the young adult and aged birds, respectively (Table 2). After 14 days of unloading, the young adult birds the muscle mass of the experimental side had returned to the contralateral control level, whereas in the aged birds there was still a 6% hypertrophy in the experimental muscle when compared to the contralateral control muscle (Table 2).

**XIAP and FLIP mRNA Level**

As estimated by RT-PCR with 18S RNA as an internal control, following 14 days of overloading, the XIAP and...

<table>
<thead>
<tr>
<th>Quail</th>
<th>14d Stretch-Overloading</th>
<th>7d Unloading</th>
<th>14d Unloading</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young adult</td>
<td>-35%</td>
<td>15 ± 3%*</td>
<td>2 ± 1%</td>
</tr>
<tr>
<td>Old</td>
<td>-15%</td>
<td>12 ± 2%*</td>
<td>6 ± 2%*</td>
</tr>
</tbody>
</table>

**Note:** Muscle mass loss during unloading following stretch-induced hypertrophy was monitored by examining the decrease in the extent of muscle hypertrophy. The extent of muscle hypertrophy was determined by examining the percent difference in the muscle wet weight between the experimental and the contralateral control whole patagial muscles. Data are presented as means ± standard error. 7d unloading = 7 days of unloading following 14 days of stretch-overloading; 14d unloading = 14 days of unloading following 14 days of stretch-overloading. *p < .05, significant percent difference in muscle wet weight relative to control muscle.
FLIP mRNA content was not different between the experimental and control muscles in both the young adult and aged birds (Figure 1). During the subsequent unloading, in young adult birds, both the XIAP and FLIP mRNA contents of the experimental muscles were not different ($p > .05$) when compared to the contralateral control after either 7 or 14 days of unloading (Figure 1). However, in the aged birds, the XIAP mRNA content was 47% greater ($p < .05$) in the experimental muscle relative to the contralateral control side after 7 days of unloading, but by 14 days of unloading, the XIAP mRNA content was not different between the experimental and control muscles (Figure 1A). In contrast, we did not find any significant difference in the mRNA content of FLIP between the experimental and control muscles from the aged birds that were unloaded for 7 or 14 days (Figure 1B).

**XIAP and ARC Protein Level**

In our Western blot analyses, we detected an immunoreactive band of $\sim 57$ kDa and $\sim 25$ kDa, corresponding to the predicted molecular mass of XIAP and ARC protein, respectively. Following 14 days of overloading, the protein content of ARC was not different between the experimental and control muscles in both the young adult and aged birds, whereas the XIAP protein content in the experimental muscle was 140% and 116% higher than the contralateral side in young adult and aged birds, respectively (Figure 2). After 7 or 14 days of the removal of overload, in the young adult birds, we did not find any differences in the XIAP and ARC protein content between the experimental and contralateral control muscles ($p > .05$, Figure 2). However, in the aged birds, XIAP protein content in the experimental muscle following 7 days of unloading was 67% higher ($p < .05$) relative to the contralateral control side, whereas it was still 57% higher than the contralateral control level ($p < .05$) after 14 days of unloading (Figure 2A). The outcomes of the 2 $\times$ 2 ANOVA indicated main effects of age [F(1, 60) = 48.96, $p = .0001$] on the XIAP protein content in the unloaded animals (Figure 2A). For the protein content of ARC in the aged muscles, no significant differences were found between the experimental and contralateral control sides following 7 or 14 days of unloading ($p > .05$, Figure 2B).

**Relationship Between XIAP Protein Content and Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling Index**

The relationship of XIAP protein content and terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) index was analyzed by examining the corresponding Pearson’s correlation coefficient ($r$). We have previously shown that apoptosis was evident as indicated by the increase in TUNEL-positive nuclei (expressed as apoptotic index) and the changes in apoptosis regulatory factors including Bcl-2, Bax, and AIF which were consistent with elevated apoptosis (13). In the current study, when the unloaded and control PAT muscles of all groups were collapsed and treated as a single group, a positive relationship was found between XIAP protein content and apoptotic index ($r = 0.506$, $p = .001$, $N = 64$) (Figure 3).

**Discussion**

By using the present hypertrophied-muscle unloading model, we have previously demonstrated that apoptosis is involved in unloading-induced muscle atrophy where muscle mass is reduced from a hypertrophied state toward basal level (13). We described the pro-apoptotic changes including decreased Bcl-2 and increased Bax, p53, cytosolic Id2, and TUNEL-positive nuclei in the experimental muscles after unloading in young adult or aged quails (12,13). These
previous findings also indicated that apoptosis is responsible for eliminating the excessive activated/proliferated muscle precursor cell nuclei (e.g., satellite cell nuclei) during unloading in hypertrophied muscles. Moreover, our previous data showed that aging influences the apoptotic regulation during muscle unloading by increasing Bcl-2, decreasing Bax and nuclear AIF, but concomitant with increased TUNEL-positive nuclei in hypertrophied muscle from aged birds after 14 days of unloading following hypertrophy (13). Here, we extend our previous findings by demonstrating that XIAP protein expression is increased in response to muscle overload in both young adult and aged muscles while this elevated XIAP protein level returns to the basal level after 7 or 14 days of subsequent unloading exclusively in the young adult muscle. On the contrary, this hypertrophy-associated elevation of XIAP is partially sustained in the muscle from aged birds, even though the TUNEL-indicated apoptosis was evident in the muscles of old birds after unloading (13). This distinct aging-associated response of apoptotic suppressor XIAP further suggests that (a) aging complicates the regulation of apoptotic machinery and (b) a more complex mechanism may be involved in mediating the apoptotic signaling in the aged skeletal muscle.

**XIAP Expression Is Elevated in Response to Overload and Is Declined During Subsequent Unloading in Young Adult Muscle**

A considerable amount of research in mitotic cells/tissues has demonstrated that apoptosis is a conserved cellular process as significant as the vital proliferating events, such as mitosis (24,38–40). This notion was fundamentally established by the exhibited importance of apoptosis in maintaining the cell survival/death homeostasis and by the fact that a number of severe diseases (e.g., Alzheimer’s disease and cancers) are attributed, at least in part, to aberrant regulation of apoptosis (24,38–40). Lately, there have been novel findings showing that apoptosis is consistently evident under various muscle atrophic situations (e.g., muscle unloading), and thereby implicating that, in postmitotic skeletal muscle, apoptosis may further have a role in the regulation of muscle loss (1–12,14,41). Recently, the apoptotic events have been investigated in our laboratory by using a quail hypertrophied-muscle unloading model which permits us to examine apoptosis and the regulatory mechanisms during muscle atrophy where the reducing muscle mass was still above the basal level (3,12,13). Data from our laboratory have shown apoptotic changes in unloaded muscle that was first hypertrophied including increases in caspase protease activities, cleaved...
poly(ADP-ribose) polymerase (PARP)-positive nuclei, pro-apoptotic Bax, p53, cytosolic Id2, and TUNEL-positive nuclei as well as decreases in anti-apoptotic Bcl-2 (3,12,13).

We have also demonstrated that apoptosis functions to eliminate excessive activated/proliferated muscle precursor cell nuclei (e.g., satellite cell nuclei) during muscle regression from a state of hypertrophy toward control levels (13). In the current study, we further demonstrated that the protein expression of apoptotic suppressor XIAP is elevated during muscle overloading in both young adult and aged muscles, and that this hypertrophy-associated elevation of XIAP is then down-regulated during subsequent removal of the overload in the young adult muscle. It is noted that the findings of XIAP are in accordance with our previous observations of increased apoptotic markers (e.g., TUNEL nuclei) in these unloaded muscles (12,13) and the identified anti-apoptotic properties of the apoptotic suppressors (25–27). These findings indicate that down-regulation of the elevated XIAP expression during previous hypertrophy is required for mediating the activation of apoptosis during unloading in young adult muscle.

Aging Alters the Response of XIAP to Unloading in Previously Hypertrophied Muscle

The response of apoptotic suppressor XIAP to unloading following loading-induced hypertrophy differed between young adult and aged muscles. We have recently demonstrated that the number of muscle-related BrdU-positive nuclei decreases back toward the basal level after reperfusion injury or with aging (45,46). Taken together, it is not unreasonable that our observed sustained increase in XIAP during unloading following hypertrophy might have a compensatory regulatory role in the aged muscle. Nonetheless, we are aware that the present data did not allow us to elucidate the precise mechanisms in explaining the physiologic function of XIAP in the present experimental situation. Further investigation is needed to fully understand this distinct aging-associated response of XIAP to unloading in hypertrophied muscle.

According to the existing evidence, it has been proposed that the regulatory process of skeletal muscle hypertrophy and atrophy is related to the alteration of the myonuclear number. Although this idea originates from the “myonuclear domain hypothesis,” which suggests that the cytoplasmic volume per myonucleus is strictly maintained in a homeostatic manner in the multinucleated skeletal muscle fibers (47–49), there also have been data suggesting that aging may influence the homeostatic balance of myonuclear domain under atrophic conditions in a rodent hind-limb unweighting model (50). Nonetheless, by using the technique of BrdU incorporation, previous investigations have demonstrated that the number of muscle-related BrdU-positive nuclei increases with stretch-overload in young and aged quails (3,51). Moreover, BrdU-positive nuclei are eliminated during unloading because the number of BrdU-positive nuclei decreases back toward the basal level after subsequent removal of the load in the hypertrophied muscle.
muscles. The decline of these muscle-related BrdU-positive nuclei is associated with apoptosis as demonstrated by the concomitant labeling of TUNEL and BrdU (3,13). Taken together, these data show that myogenic precursor cell populations (e.g., muscle satellite cells) are activated and proliferate, and they contribute to the increase in myonuclear number in response to stretch-overload. The newly incorporated (i.e., BrdU positive) myonuclei gained during overload are eliminated possibly through apoptosis when the load is subsequently removed in the quail hypertrophied-muscle unloading model.

Overall, our findings are in accordance with the hypothesis that elimination of the myonuclei gained during previous hypertrophy (possibly through apoptotic machinery) may be an important factor in mediating the process of muscle loss from hypertrophic state to normally loaded state in skeletal muscle of young adult birds (12,13). However, as indicated by the present XIAP data and our previous findings, the apoptotic events that occur in the aged hypertrophied muscle during unloading are evidently different from the muscle of young adult animals. It is not completely clear why the aged quail muscle exhibits up-regulation of the anti-apoptotic machinery during unloading following hypertrophy, as the losses of muscle mass and BrdU-positive muscle-related nuclei are still apparent with unloading (13). Based on the fact that aged skeletal muscle shows evidence of accelerated apoptosis (5,7,22), it is noted that our interpretations are limited by speculating that this age-related decreased pro-apoptotic tendency maybe a compensatory response to unloading following hypertrophy. We speculate that the increase in anti-apoptotic proteins may be an adaptive event, which is designed to partially offset the increase in apoptosis. This increase could have a functional effect by preserving muscle mass and therefore muscle force to a greater extent in old animals. Although muscle force increases with loading (52,53), we have not evaluated the functional components of the unloaded quail muscles.

Conclusion
We have demonstrated that XIAP protein expression is elevated in response to muscle overload in both young adult and aged muscles, although this elevated XIAP protein level returns to the basal level after 7 or 14 days of subsequent unloading in the muscles from young adult birds. We have provided evidence demonstrating that the response of apoptotic suppressor XIAP to unloading following hypertrophy is different between young adult and aged muscles. We have shown that the mRNA and protein contents of XIAP in the 7- or 14-day unloaded muscles were higher than those in the contralateral control muscles in the aged birds, but these changes were not found in young adult birds. Also, the XIAP protein content was positively correlated with the TUNEL index in all the muscle samples. We speculated that the sustained increase in XIAP in aged hypertrophied muscle maybe a compensatory response to unloading. Although the exact reason for this aging-related distinct response is unknown, these findings agree that the apoptotic regulation is more complicated in aged muscle than in young muscle. More research is warranted in investigating the influence of aging in the apoptotic regulation with the aim of fully understanding the complexity of the apoptotic mechanisms in the aged skeletal muscle.

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