Hindlimb unloading increases muscle content of cytosolic but not nuclear Id2 and p53 proteins in young adult and aged rats

Parco M. Siu, Emidio E. Pistilli, Zsolt Murlasits, and Stephen E. Alway

Laboratory of Muscle Biology and Sarcopenia, Division of Exercise Physiology, West Virginia University School of Medicine, Morgantown, West Virginia

Submitted 22 August 2005; accepted in final form 4 November 2005

Siu, Parco M., Emidio E. Pistilli, Zsolt Murlasits, and Stephen E. Alway. Hindlimb unloading increases muscle content of cytosolic but not nuclear Id2 and p53 proteins in young adult and aged rats. J Appl Physiol 100: 907–916, 2006. First published November 10, 2005; doi:10.1152/japplphysiol.01012.2005.—This study tested the hypothesis that inhibitor of differentiation-2 (Id2), p53, and heat shock proteins (HSP) are responsive to suspension-induced muscle atrophy. Fourteen days of hindlimb suspension were used to unload the hindlimbs and induce atrophy in gastrocnemius muscles of young adult and aged rats. Following suspension, medial gastrocnemius muscle wet weight was reduced by ~30%, and the muscle wet weight normalized to the animal body weight decreased by 11 and 15% in young adult and aged animals, respectively. mRNA abundances of Id2, p53, HSP70–2, and HSP27 did not change with suspension, whereas HSP70–1 mRNA content was lower in the suspended muscle compared with the control muscle in both young adult and aged animals. Our immunoblot analyses indicated that protein expressions of HSP70 and HSP60 were not different between suspended and control muscles in both ages, whereas HSP27 protein content was increased in suspended muscle relative to control muscle only in young adult animals. Id2 and p53 protein contents were elevated in the cytosolic fraction of suspended muscle compared with the control muscle in both young and aged animals, but these changes were not found in the nuclear protein fraction. Furthermore, compared with young adult, aged muscles had a lower HSP70–1 mRNA content but higher HSP70–2 mRNA content and protein contents of Id2, p53, HSP70, and HSP27. These findings are consistent with the hypothesis that Id2 and p53 are responsive to unloading-induced muscle atrophy. Moreover, our data indicate that aging is accompanied with altered abundances of HSP70–1 and HSP70–2 mRNA, in addition to Id2, p53, HSP70, and HSP27 protein in rat gastrocnemius muscle.

sarcopenia; apoptosis; muscle atrophy; muscle disuse; hindlimb suspension

ACTIVATION OF APOPTOSIS has been consistently reported under different situations of skeletal muscle loss. Furthermore, it has been hypothesized that apoptosis may have a physiological role in regulating muscle wasting (1, 2, 5, 14, 15, 28, 29, 51, 52, 54–57, 60, 61, 64, 65, 70, 71). With an objective of unraveling the mediators of the apoptotic signaling pathway, it is possible that they may also be involved in the regulation of apoptosis-associated muscle wasting.

Id2 is one of four identified members (Id1–Id4) of the inhibitor of DNA binding/differentiation protein family. Id2 has been suggested to be essential in regulating both cell proliferation and apoptotic cell death under different circumstances (7, 9, 16, 45, 75). These seemingly two contradictory functions of Id2 are presumably related to the structure of DNA binding domain-negative helix-loop-helix motif, which promotes cell division, whereas the apoptotic capability of Id2 is mediated by the proapoptotic Bax protein, which is independent of helix-loop-helix dimerization (7, 9, 16, 45, 75). Id2 expression is typically high during cellular proliferation and before differentiation in keratinocytes and tumor cell lines, whereas the NH2-terminal region of Id2 has been implicated in mediating the proapoptotic cascade, which is probably related to mitochondria-associated apoptotic signaling. In agreement with the dual role of Id2 in cell growth and death, our laboratory has provided data demonstrating that Id2 is involved in both overload-induced muscle hypertrophy and apoptosis-related atrophy of hypertrophied muscle during unloading and sarcopenia with aging (2–5, 60). Although the exact mechanism(s) by which Id2 regulates muscle hypertrophic or atrophic process is largely unknown, we have previously demonstrated that cytosolic but not nuclear Id2 is associated with muscle atrophy during unloading following muscle hypertrophy, where the loss of muscle mass is still above the basal level (60). These observations suggest that the subcellular compartmentalization of protein expression (nuclear vs. cytosolic) may be of significance in coordinating the physiological role of Id2. Nonetheless, the role of subcellular Id2 in muscle atrophy has not been verified in muscle wasting, where the muscle loss occurs below the basal nonatrophied level (e.g., hindlimb suspension-induced muscle atrophy).

Tumor suppressor protein p53 has been demonstrated to be one of the central regulators in cell growth and death by inducing cell growth arrest via p21Cip1/Waf1 mediation or apoptosis through the activation of Bax in various mitotic cell lineages (12). Only a few studies have attempted to investigate the response of p53 in postmitotic skeletal muscle, and these have been under conditions of disuse, laser irradiation, and ischemia (19, 24, 41, 47, 59, 60). Although a common agreement on the role of p53 in muscle wasting has not been established, there has been at least some evidence indicating that p53 is responsive to certain muscle atrophic conditions...

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
(i.e., rat muscle during spaceflight and quail muscle during unloading following hypertextrophy) (47, 60). Additionally, HSPs have been implicated as an ant apoptotic component in determining apoptotic cell death in response to a variety of cellular stresses (8, 67, 68). In particular, HSP70 and HSP27 have been shown to have an inhibitory role on apoptosis by interfering with key effectors of mitochondria-associated apoptosis, including formation of the apoptosome and, therefore, precluding the activation of subsequent caspases (e.g., caspase-9) (17). Furthermore, HSP70 has been demonstrated to suppress the apoptogenic properties of apoptosis-inducing factor, a mitochondrial-released apoptotic protein, which has been advocated to be a caspase-independent factor (18). Nevertheless, the possible role of HSPs in apoptosis-associated muscle wasting remains to be fully identified. Collectively, understanding the possible roles of Id2, p53, and HSP in mediating the apoptosis-associated muscle atrophy will provide insight in revealing the mechanisms leading to significant muscle loss during the mechanisms disuse as well as sarcopenia.

Although the activation of apoptosis has been demonstrated in hindlimb-suspended skeletal muscle (1, 29), it is unclear whether the apoptosis-associated factors, including Id2, p53, and HSPs, may be involved. These proteins have been previously shown to be involved in apoptosis-related atrophy of hypertrophied muscle during unloading, where muscle mass is reduced from a hypertrophy to control levels or in muscle damage (34, 60, 64). Nevertheless, it is unknown whether they also contribute to muscle wasting as induced by hindlimb suspension, where the loss of muscle mass is well below the basal level. Therefore, this study investigated the gene and subcellular protein expressions of these apoptosis-associated factors following 14 days of hindlimb suspension-mediated muscle unloading. We tested the hypothesis that Id2, p53, and HSPs are responsive to suspension-induced muscle atrophy. The medial gastrocnemius muscle was examined because it is composed of both type I and type II fibers and undergoes considerable atrophy during unloading. Furthermore, apoptotic signaling is increased in this muscle group in response to 14 days of hindlimb unloading (63).

MATERIALS AND METHODS

Animals. Experiments were conducted on 6-mo-old young adult and 30-mo-old aged Fischer 344 × Brown Norway rats (Harlan, Indianapolis, IN). The rats were housed in pathogen-free conditions at 20°C and were exposed to a reverse light condition of 12:12-h of light/darkness each day. They were fed rat chow and water ad libitum throughout the study period.

Hindlimb suspension. The animals were randomly assigned to a suspension group (Young n = 10, Aged n = 10) or a control group (Young n = 10, Aged n = 8). The procedure of hindlimb suspension described by Morey-Holton and Globus (38) was adopted in the present study. Briefly, an adhesive (tincture of benzoin) was applied to the tail and air dried, and an orthopedic tape was put along the proximal one-third of the tail. This practice distributed the load evenly and avoided excessive tension on a small area of the tail. The tape was then placed through a wire harness that was attached to a fish line swivel at the top of a specially designed National Aeronautics and Space Administration-approved hindlimb-suspension cage. This provided the rats with 360° of movement around the cage, and the forelimbs maintained contact with a grid floor, allowing the animals to move and access food and water freely. Sterile gauze was wrapped around the orthopedic tape and was subsequently covered with a thermoplastic material, which formed a hardened cast (Vet-Lite, Veterinary Specialty Products, Boca Raton, FL). The distal tip of the tail was examined to verify that the procedure did not occlude the blood flow to the tail (i.e., tail remained pink). The suspension height was adjusted to prevent the animal’s hindlimb from touching any supportive surface, with care taken to maintain a suspension angle of ~30° (22). The suspension height and animal behavior were monitored daily. Control animals were allowed to move unconstrained around the cages. Following 14 days of suspension, animals were killed with an overdose of pentobarbital. Medial gastrocnemius muscles from the hindlimbs were excised, weighed, and frozen in isopentane cooled to the temperature of liquid nitrogen and stored at −80°C until used for analyses. All experimental procedures carried approval from the Institutional Animal Use and Care Committee from 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 following the policies in the Animal Welfare Act. RT-PCR analyses. Total RNA was extracted from the medial gastrocnemius muscle of both suspended and control animals with TRI Reagent (Molecular Research Center, Cincinnati, OH), which is based on the guanidine thiocyanate method. Frozen muscle was mechanically homogenized on ice in 1 mL of ice-cold TRI Reagent. Total RNA were solubilized in RNaase-free H2O and quantified in duplicate by measuring the optical density (OD) at 260 nm. Purity of RNA was assessed by examining 260-nm OD-to-280-nm OD ratio. Ten micrograms of RNA were reverse transcribed with decamer primers and Superscript II reverse transcriptase (RT) in a total volume of 30 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 PCR amplified to ensure that DNA did not contaminate the RNA. One microliter of cDNA was then amplified by PCR by using 100 ng of forward and reverse primers, ribosomal 18S primer pairs (Ambion, TX), 250 µM deoxyribonucleotide triphosphates, 1 × PCR buffer, and 2.5 units of Taq DNA polymerase (USB, Cleveland, OH) in a final volume of 50 µL. PCR was performed by 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 ensure that the number of cycles represented a linear portion for the PCR OD curve for the muscle samples. 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 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 using 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 were used as internal controls, while all RT-PCR signals were normalized to the 18S signal of the corresponding RT product to eliminate the measurement error from uneven sample loading and provide a semiquantitative measure of the relative changes in gene expression. Subcellular protein fractionation. The fractionation method described by Rothermel et al. (53) was adopted with minor modification to extract the cytosolic and nuclear protein fractions from the gastrocnemius muscles. Our laboratory has previously obtained the fractionated cytosolic and nuclear proteins from skeletal and heart muscles using this modified protocol (61, 62, 64). In brief, after removal of connective tissues, muscle was homogenized on ice in 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) supplemented with a protease inhibitor cocktail containing 104 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.08 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM peptatin A, and 1.4 mM E-64 (Sigma-Aldrich, St. Louis, Missouri).
MO). Following centrifuging at 5,000 rpm for 5 min at 4°C to pellet the nuclei and cell debris, the supernatants were collected, and the remaining cytosolic proteins were verified by using prestained standard (LC5925, Invitrogen) when loading the immunoblot. Young, young adult muscle; Aged, aged muscle were stored as nuclei-free total cytosolic protein fraction. The remaining cytosolic protein fractions (C) probed with anti-histone H2B and anti-CuZn-SOD antibodies. The nuclear and cytosolic protein fractions were obtained from a young or aged rat gastrocnemius muscle, and equal amount of protein was loaded in the immunoblot. Young, young adult muscle; Aged, aged muscle. The data show that the nuclear or cytosolic fractions were not contaminated by contents of the other protein fraction.

### 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>T_A, °C</th>
<th>Cycles</th>
<th>Product Length, bp</th>
<th>Restriction Enzyme</th>
<th>Restriction Products, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Id2</td>
<td>NM_013060</td>
<td>F: 5’-GCCGGCAGCACTCTACCCG-3’</td>
<td>3–20</td>
<td>58.4</td>
<td>36</td>
<td>534</td>
<td>EcoRI</td>
<td>416, 118</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5’-TGAGAAGAAAAAGAAGAGAACTGAAATTTAA-3’</td>
<td>508–536</td>
<td></td>
<td></td>
<td></td>
<td>PstI</td>
<td>258, 246, 30</td>
</tr>
<tr>
<td>p53</td>
<td>NM_040989</td>
<td>F: 5’-GCCGGCCATCTTACCTGAACGTG-3’</td>
<td>920–940</td>
<td>59.6</td>
<td>33</td>
<td>328</td>
<td>AliI</td>
<td>91, 88, 64, 44, 41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5’-TGGGACGAGCCGTCTCTCATC-3’</td>
<td>1229–1247</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSP70-1</td>
<td>NM_031971</td>
<td>F: 5’-GGGCGAAGAGGCGAGCCTGTTT-3’</td>
<td>983–1000</td>
<td>60.4</td>
<td>33</td>
<td>267</td>
<td>AliI</td>
<td>247, 20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5’-GGCCGCTTGAAGGAATTGACG-3’</td>
<td>1232–1249</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSP70-2</td>
<td>X77208</td>
<td>F: 5’-CTCTCTCTATGTGTGTGCTAACAGG-3’</td>
<td>1604–1625</td>
<td>59.0</td>
<td>33</td>
<td>109</td>
<td>N/A</td>
<td>sequneced</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5’-GGCTCTCGTCTGTTGAATTCAG-3’</td>
<td>1691–1712</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSP27</td>
<td>NM_031970</td>
<td>F: 5’-GCCGGCGGCTCCCTCTTC-3’</td>
<td>74–90</td>
<td>62.4</td>
<td>33</td>
<td>349</td>
<td>HindIII</td>
<td>263, 86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5’-GCCCTCTTCGTGCTTCTCCTG-3’</td>
<td>400–422</td>
<td></td>
<td></td>
<td></td>
<td>Rsal</td>
<td>291, 58</td>
</tr>
</tbody>
</table>

Accession No., GenBank accession number; T_A, annealing temperature; F, forward primer; R, reverse primer; Id2, inhibitor of differentiation-2; HSP, heat shock protein; N/A, not applicable.

Muscle weight. Sarcopepic muscle loss was observed by comparing the whole medial gastrocnemius muscle wet weight between young adult and aged control animals. Muscle wet weight has been previously reported in these animals (63). The wet weight of the medial gastrocnemius muscle of aged control rats was 23% lower than that of young control adults (young adult vs. aged: 993 vs. 761 mg, Fig. 2A). The muscle weight was...
normalized to the animal body weight in aged rats was 48% lower than that of the young adult rats (young adult vs. aged: 2.7 vs. 1.4 mg/g, Fig. 2B). Fourteen days of unloading reduced the medial gastrocnemius muscle wet weight by ~30% in both young adult and aged rats (young control vs. suspended: 993 vs. 699 mg; aged control vs. suspended: 761 vs. 520 mg) (Fig. 2A), while the muscle weight normalized to the animal body weight decreased by 11% and 19% in young adult and aged rats, respectively (young control vs. suspended: 2.7 vs. 2.4 mg/g; aged control vs. suspended: 1.4 vs. 1.1 mg/g) (Fig. 2B).

*Id2 mRNA, nuclear and cytosolic protein contents.* As indicated by the RT-PCR analyses, no difference was found in the mRNA content of Id2 between the suspended and control muscles (Fig. 3A). According to our immunoblot analysis, nuclear Id2 protein content of the suspended muscle was not different from the control muscle.

Fig. 2. A: muscle wet weight. The extent of muscle loss following hindlimb suspension was estimated by examining the muscle mass loss between the suspended and the control whole gastrocnemius muscle wet weight. B: muscle wet weight normalized to body weight. The extent of muscle loss following hindlimb suspension was further determined by examining the decline in gastrocnemius muscle wet weight normalized to the animal's body weight between the suspended and control groups. Values are means ± SE. *P < 0.05, suspended group was significantly different from the control group under the same age group. **P < 0.05, aged animals were significantly different from the young animals under the same experimental condition. The main effects of age and suspension were analyzed by a 2 × 2 ANOVA.

Fig. 3. A: inhibitor of differentiation-2 (Id2) mRNA content. The mRNA content of Id2 was determined by RT-PCR with 18S rRNA as an internal control. The data are expressed as arbitrary ratio of Id2/18S. Insets: representative results for the Id2 mRNA. B: nuclear Id2 protein content. The protein content of Id2 was assessed in nuclear fraction by immunoblot analysis. The data are expressed as optical density (OD) × resulting band area and expressed in arbitrary units. Insets: representative blots for Id2 in control and suspended muscles isolated from young adult and aged animals. C: cytosolic Id2 protein content. The protein content of Id2 was measured in cytosolic fraction. The data are expressed as OD × resulting band area and expressed in arbitrary units. Insets: representative blots for Id2 in control and suspended muscles isolated from young adult and aged animals. Values are means ± SE. *P < 0.05, suspended group was significantly different from the control group under the same age group. **P < 0.05, aged animals were significantly different from the young animals under the same experimental condition. The main effects of age and suspension were analyzed by a 2 × 2 ANOVA.
different from that of the control muscle (Fig. 3B), whereas the cytosolic Id2 protein content in the suspended muscle was 1.7- and 1.4-fold higher than that of the control muscle in young adult and aged animals, respectively (Fig. 3C). The $2 \times 2$ (suspension $\times$ age) ANOVA indicated that there is a greater Id2 protein content in the aged muscle compared with the young adults (Fig. 3, B and C).

$p53$ mRNA, nuclear and cytosolic protein contents. There was no significant difference in the $p53$ mRNA content between the suspended and control muscles (Fig. 4A). The nuclear $p53$ protein content of the suspended muscle was not different from that of the control muscle (Fig. 4B). In contrast, the cytosolic $p53$ protein content was 44 and 52% greater in the suspended muscle compared with the control muscle in young adult and aged animals, respectively (Fig. 4C). The $2 \times 2$ (suspension $\times$ age) ANOVA indicated that aged muscle has higher levels of both nuclear and cytosolic $p53$ protein relative to the young adult muscle (Fig. 4, B and C).

**HSP70 mRNA and protein contents.** Transcriptional expressions of both HSP70–1 and HSP70–2 genes that individually encode HSP70 were examined by RT-PCR analyses. We found that the mRNA content of HSP70–1 was 44 and 74% reduced in the suspended muscle relative to the control muscle in young adult and aged animals, respectively (Fig. 5A). Although no difference was found in the mRNA content of HSP70–2 between the suspended and control muscles of both ages, HSP70–2 mRNA content was greater in the aged muscle compared with young muscle (Fig. 5B). The protein content of HSP70 was not different between the suspended and control muscle in both young and aged muscles, whereas aged muscle exhibited a higher level of HSP70 protein relative to the young muscle (Fig. 5C). The $2 \times 2$ (suspension $\times$ age) ANOVA suggested that aged muscle has a lower level of HSP70–1 mRNA but greater level of HSP70–2 mRNA and HSP70 protein relative to the young adult muscle (Fig. 5, A–C, respectively).

**HSP27 mRNA and protein contents.** No difference was found in the HSP27 mRNA content between the suspended and control muscles in both young and aged animals (Fig. 6A). Although the protein content of HSP27 did not change with suspension in aged muscle, we found that the HSP27 protein content was increased by 89% in the young suspended muscle relative to the young control muscle (Fig. 6B). The $2 \times 2$ (suspension $\times$ age) ANOVA suggested that aged muscle has a higher levels of HSP27 protein compared with the young adult muscle (Fig. 6B).

**HSP60 protein content.** No difference was found in HSP60 protein content between the suspended and control muscles in both young and aged animals (Fig. 7).
DISCUSSION

Although it has been consistently shown that accelerated apoptosis is evident during unloading-induced muscle atrophy (1, 5, 29, 64), the underlying cellular and molecular mechanisms that contribute to activation of the apoptotic signaling pathway leading to unloading-induced apoptosis remain to be fully identified. In this study, we have provided novel evidence showing that Id2 and p53 may be involved in the regulation of hindlimb suspension-induced muscle loss. We demonstrated that the subcellular cytosolic fractions of Id2 and p53 proteins are responsive to hindlimb suspension-mediated muscle unloading. With hindlimb unloading, we observed that cytosolic but not nuclear Id2 and p53 protein contents were elevated in unloaded muscles from both young adult and aged rats. These findings are consistent with the hypothesis that subcellular compartmentalization of accumulated proteins is one factor determining the physiological role of Id2 and p53 in muscle atrophy. Furthermore, we found that the HSP70–1 mRNA content is lower, whereas the mRNA content of HSP70–2 and protein contents of Id2, p53, HSP70, and HSP27 are greater in the control gastrocnemius muscle of aged rats relative to the control muscle in young adult rats. These results indicate that sarcopenic muscle loss is accompanied by the changes of Id2, p53, and HSPs expression in rat gastrocnemius muscle.

In addition to the demonstrated differentiation-inhibitory and proliferating functions of Id2 (27, 44, 75), it has also been shown that Id2 can initiate apoptosis through the apoptogenic properties resident in the amino-terminal region of Id2 (16). Consistent with this dual role of Id2, previous studies from our laboratory have demonstrated the possible role of Id2 in mediating cell growth/proliferation and apoptotic cell death in mature skeletal muscle in response to muscle overload and unload, respectively (3–5, 60). By using an avian model of unloading following a period of loading, we have previously shown that the atrophic role of Id2 during muscle unloading may be mediated by the subcellular localization of its protein accumulation. Specifically, our data showed that cytosolic but not nuclear Id2 protein abundance is responsive to unloading in birds (60). However, it is noted that this regulatory role of subcellular Id2 is inferred from the observations during unloading of muscle that was first hypertrophied, and muscle loss returned muscle mass back to the resting control mass level (60). In the present study, we extend our previous findings in quail muscles, in that we show that the subcellular location (i.e., cytosolic protein fraction) of Id2 protein expression also responds to hindlimb suspension-mediated unloading, where muscle mass is reduced well below control mass levels, in a similar subcellular compartmentalized manner in both young adult and aged gastrocnemius muscles (60). Collectively, the
hypothesis of the physiological role of cytosolic Id2 in muscle loss is supported by the findings in these two muscle atrophy experimental models, where the loss of muscle is at or just above control mass levels, or below the basal muscle mass level.

Tumor suppressor p53 has been demonstrated to be essential in the regulation of cell survival and programmed cell death in mitotic cell lineages (58, 72). Although the expression of p53 is well conserved in mature muscle (10, 24, 41, 47, 60, 61), the role of p53 in postmitotic skeletal myocytes is relatively unknown. Based on the proapoptotic properties of p53, it has been hypothesized that p53 may be related to the activation of apoptosis during muscle disuse-induced muscle loss (19, 24, 41, 47, 59, 60). In addition, p53 has been demonstrated to be able to mediate the apoptotic cascades by both transcriptional-dependent mechanism through the upregulation of the transcriptional expressions of certain proapoptotic genes (e.g., Bax, Noxa, and PUMA) (23, 46, 58) and/or a transcriptional-independent mechanism via translocating to the mitochondria, thereby activating the cytosolic proapoptotic factors (e.g., Bax) (11, 35, 50, 58). Hence, it is required to test whether the role of p53 in muscle loss may be linked in a nuclear/cytosolic compartment-specific fashion. Previously, results from our laboratory have suggested that p53 may be involved in mediating the apoptosis-associated muscle regression in hypertrophied muscle during unloading. We have reported that both the nuclear and cytosolic p53 protein abundances are increased concomitant with the elevation of proapoptotic markers during unloading in formerly hypertrophied quail muscle (60, 64). Consistent with these findings, here we demonstrated that the protein expression of p53 is also elevated in both young and aged rat muscles after hindlimb suspension, but these changes are only found in the cytosolic fraction. The current results are in support of the findings that the total p53 protein content was increased in atrophied rat skeletal muscle following unloading that was induced by 14 days of spaceflight (47). It is noted that the increase in nuclear p53 protein that we have observed in the quail hypertrophied muscle following unloading is not evident in the present rat hindlimb-suspended muscle, albeit the change in cytosolic p53 is found in both atrophic situations (60). However, these discrepancies in the change of nuclear p53 protein content may be reasonably attributed to the different nature of muscle loss in these atrophic conditions (i.e., muscle loss that stops above the basal control muscle level in the quail model vs. muscle loss below the basal level in the rodent hindlimb suspension model of the present study) and between species (i.e., birds vs. rodents). Overall, our findings in both models of quail unloading and rat hindlimb suspension generally agreed with the hypotheses that p53 is associated with

Fig. 6. A: HSP27 mRNA content. The mRNA content of HSP27 was determined by RT-PCR with 18S rRNA as an internal control. The data are expressed as arbitrary ratio of HSP27/18S. Insets: representative results for the HSP27 mRNA. B: HSP27 protein content. The protein content of HSP27 was determined by immunoblot. The data are expressed as OD × resulting band area and expressed in arbitrary units. Insets: representative blots for HSP27 in control and suspended muscles isolated from young adult and aged animals. Values are means ± SE. *P < 0.05, suspended group was significantly different from the control group under the same age group. **P < 0.05, aged animals were significantly different from the young animals under the same experimental condition. The main effects of age, suspension, and interaction (age × suspension) were analyzed by a 2 × 2 ANOVA.

Fig. 7. HSP60 protein content. The data are expressed as OD × resulting band area and expressed in arbitrary units. Insets: representative blots for HSP60 in control and suspended muscles isolated from young adult and aged animals. Values are means ± SE.
muscle atrophy induced by muscle disuse, and the atrophic role of p53 may be related to the subcellular location of the protein accumulation. Nonetheless, future investigation is required to fully identify the biological significance and the underlying mechanism(s) of p53 in mediating muscle atrophy.

HSPs are commonly believed to function as a protective mechanism in response to various stressful events, including elevated temperature, oxidative stress, hypoxia, and other cellular damages (31). Previously, the response of HSP70 to hindlimb unloading has been examined, but the results are rather contradictory. By examining the soleus muscle, it has been shown that HSP70 protein content decreases after 18 h, 8 days, or 9 wk of hindlimb suspension in adult rats (26, 40, 49). However, it has also been reported that HSP70 protein content in adult soleus muscle is not altered with 5 days of hindlimb unloading (48). In addition, Desplanches and colleagues (13) demonstrated that the protein expression of HSP70 remains unaffected in response to 14 days of hindlimb suspension in both slow-fiber predominated muscles (soleus and adductor longus) and fast-twitch muscles (plantaris and extensor digitorum longus) of adult rats. In light of these previous findings, we investigated the gene and protein expressions of HSP70 in gastrocnemius muscle, which is a muscle that has not been well examined. Furthermore, in addition to young adult rats, aged rats were also investigated in the present study, as the data showing that the response of HSP70 to hindlimb unloading in aged muscle are lacking. We report that HSP70–1 mRNA content decreases concomitant with unchanged abundances of HSP70–2 mRNA and HSP70 protein in both young adult and aged gastrocnemius muscles following 14 days of hindlimb unloading. Although the protein abundance of HSP70 remains unchanged with hindlimb unloading, our novel mRNA data suggest that the expressions of HSP70–1 and HSP70–2 gene may respond differently, at least in gastrocnemius muscle, to hindlimb suspension-mediated muscle unloading, even though these HSP70 genes encode the same peptide sequence for HSP70 protein (36, 37, 73). It is interesting to note that this differential alteration of HSP70–1 and HSP70–2 mRNA abundance occurred in an age-related fashion, because we found an increase in mRNA level of HSP70–2 but decrease HSP70–1 mRNA abundance in the aged muscles. The underlying mechanism for this response with aging is not known. However, we speculate that the lack of increase in HSP70–1 might be due to already adequate or high basal levels of HSP70 protein in muscles of old animals, as is the case in the myocardium (69). Furthermore, Liu and colleagues (30) have previously shown that mRNA levels and protein expression of HSP70–2 are not tightly regulated in response to increased exercise, and this may also be the case for unloading.

With unloading, we found that HSP27 protein content increases exclusively in suspended muscle from young animals, although HSP27 mRNA and HSP60 protein contents are unchanged with hindlimb suspension in muscles of both ages. Notably, our present findings in both mRNA and protein responses of HSP27 in gastrocnemius muscle are consistent with the findings of Kato and coworkers (25), who have demonstrated that, following 2–10 days of hindlimb suspension, the protein accumulations as well as the phosphorylation of two α-crystallin small HSPs, including αB-crystallin and HSP27, are increased together with elevated αB-crystallin mRNA content but unchanged HSP27 mRNA content, as estimated by Northern blot analysis in the soleus muscle. Based on the protective role of HSPs, we speculate that the increase in HSP27 protein content in suspended gastrocnemius muscle might be part of a compensatory adaptation in young adult rats, and this potentially protective increase in HSP27 during hindlimb unloading is not present in the aged suspended muscle. It is also possible that gastrocnemius muscles in old animals failed to increase HSP27 protein levels with unloading, because they had already reached the ceiling of a high resting level of this protein.

It is interesting to note that HSP70 and HSP27 responded differently to unloading in muscles of young animals, because the protein abundance of HSP70 did not change with 14 days of unloading, whereas HSP27 protein content increased. Both HSP70 and HSP27 are known to interfere with apoptotic signaling, and, as a result, they have been implicated to act as antiapoptotic factors (17, 18). Therefore, we would have expected both HSP70 and HSP27 to increase in the gastrocnemius during conditions known to induce unloading-induced apoptosis (63). We cannot rule out the possibility that the fiber-type composition of the muscle may have affected the different responses to unloading. The gastrocnemius muscle is a mixed-fibered muscle but predominantly contains type II fibers (6, 20). HSP70 is proportional to the type I myosin heavy chain content of the muscle and would be expected to be less abundant in the gastrocnemius than as primarily type I-fibered muscle like the soleus (32), and type I fibers in the gastrocnemius would be expected to have higher levels of HSP70 than type II fibers. Furthermore, HSP70 responses increased neural activity occur in a fiber-type-specific manner (31, 43). Although HSP27 is also preferentially expressed in type I fibers and in subpopulations of type II fibers in control muscles, increased activity can result in expression of this protein in type II fibers in an activity-dependent manner (42). Thus it is possible that the regulation of HSP70 and HSP70 may have, in part, different regulatory pathways within each fiber type. Nonetheless, additional research is required to further investigate the physiological significance of the elevation of HSP70 protein but in the absence of HSP70 protein alteration in gastrocnemius muscle during hindlimb unloading.

In conclusion, we have provided evidence indicating that the transcriptional repressor protein Id2 and the tumor suppressor protein p53 are associated with muscle wasting induced by hindlimb suspension in young adult and aged gastrocnemius muscles. Although the extent of apoptosis-associated myocyte loss has not been assessed, we have shown that protein expression of Id2 and p53 increased exclusively in the cytosolic fraction of the suspended muscle relative to the control muscle in both young and aged rats, whereas these changes were not found in the nuclear fraction. These observations suggest that the hypothesized role of Id2 and p53 in mediating apoptosis-related muscle loss during hindlimb suspension may be attributed to the compartmental localization of the subcellular protein accumulation. Nevertheless, additional research is needed to fully understand the regulatory mechanisms of Id2 and p53 in muscle disuse through other possible machineries (e.g., by phosphorylation) (21, 39, 74). Furthermore, we report that the sarcopenic gastrocnemius muscle mass decline is accompanied by decreased HSP70–1 mRNA but increased HSP70–2 mRNA and Id2, p53, HSP70, and HSP27 protein abundances in aged compared with gastrocnemius muscle in young adult
rats. Further investigation is warranted to evaluate the role of these alterations of apoptosis-associated factors in sarcopenia.

ACKNOWLEDGMENTS

This study was supported by National Institute on Aging Grant R01 AG-021530.

REFERENCES


