PKB signaling and atrogene expression in skeletal muscle of aged mice

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Gaugler M, Brown A, Merrell E, DiSanto-Rose M, Rathmacher JA, Reynolds TH 4th. PKB signaling and atrogene expression in skeletal muscle of aged mice. J Appl Physiol 111: 192–199, 2011. First published May 5, 2011; doi:10.1152/japplphysiol.00175.2011.—The purpose of this study was to determine if PKB signaling is decreased and contractile protein degradation is increased in extensor digitorum longus (EDL) and soleus (SOL) muscles from middle-aged (MA) and aged (AG) mice. We also examined the effect of age on atrogene expression in quadriceps muscle. PKB activity, as assessed by Thr308 and Ser473 phosphorylation, was significantly higher in EDL and SOL muscles from AG compared with MA mice. The age-related decrease in PKB activity appears to be due to an increase in expression of the kinase, as PKB-α and PKB-β levels were significantly higher in EDL and SOL muscles from AG than MA mice. The phosphorylation of forkhead box 3a (FOXO3a) on Thr124, a PKB target, was significantly higher in EDL muscles from AG than MA mice. The rate of contractile protein degradation was similar in EDL and SOL muscles from AG and MA mice. Atrogin-1 and muscle-specific RING finger protein 1 (MuRF-1) mRNA levels did not change in muscles from AG compared with MA mice, indicating that ubiquitin-proteasome proteolysis does not contribute to sarcopenia. A significant decrease in Bcl-2 and 19-kDa interacting protein 3 (Bnip3) and GABA receptor-associated protein 1 (Gabarap1) mRNA was observed in muscles from AG compared with MA mice, which may contribute to age-related contractile dysfunction. In conclusion, the mechanisms responsible for sarcopenia are distinct from experimental models of atrophy and do not involve atrogin-1 and MuRF-1 or enhanced proteolysis. Finally, a decline in autophagy-related gene expression may provide a novel mechanism for impaired contractile function and muscle metabolism with advancing age.

SARCOPENIA, THE AGE-RELATED LOSS of muscle mass, is present in ~45% of people ≥65 yr of age (14). Sarcopenia is a core component of a frailty syndrome that increases the risk of falling, reduces functional independence, and increases susceptibility to acute and chronic disease (9). In the United States, the direct healthcare costs associated with sarcopenia have been estimated to exceed $18 billion per year, an amount that is likely to increase dramatically because of the expansion of the aging population (14). The precise cause of sarcopenia is not established, but the condition is associated with physical inactivity, insulin resistance, nutritional deficiencies, elevated proinflammatory cytokines, and decreases in growth-promoting hormones.

Ultimately, muscle size is determined by the relative rates of protein synthesis and degradation. Since the average protein turnover rate is ~4–6 g protein·kg body wt−1·day−1, or 280 g protein/day for a 70-kg adult (19), small persistent changes in the rates of protein synthesis or protein degradation could lead to substantial changes in muscle mass. The role of protein synthesis in the development of sarcopenia has been studied extensively (3, 11, 21, 28, 31), but little information exists regarding protein degradation and age-related muscle atrophy. The lack of information regarding skeletal muscle proteolysis and sarcopenia is surprising, since experimental models of muscle atrophy have led to the discovery of several “atrogenes” that promote muscle atrophy by increasing skeletal muscle protein degradation (4, 16, 25, 34). Muscle atrophy induced by hindlimb suspension (4), fasting (17), or glucocorticoid treatment (24) is associated with increased expression of atrogenes that regulate ubiquitin-proteasome and autophagy/lysosomal protein degradation.

PKB is an insulin- and growth factor-responsive Ser/Thr kinase that controls metabolism, growth, apoptosis, and differentiation (13). The PKB signaling pathway plays an important role in the regulation of skeletal muscle mass, in part, by suppressing the expression of atrogenes. For example, several muscle atrophy model systems result in an increase in atrogin-1 [muscle atrophy F-box (MAFbx)] and muscle-specific RING finger protein-1 (MuRF-1), two E3 ubiquitin ligases whose expression is rate-limiting step in ubiquitin-proteasome proteolysis (4, 24). The ability of PKB to suppress atrogin-1 and MuRF-1 expression in skeletal muscle appears to be mediated by forkhead box (FOXO) 3a, a transcription factor that is phosphorylated by PKB and excluded from the nucleus. In addition to controlling atrogin-1 and MuRF-1 expression, FOXO3a activity appears to induce autophagy-related gene expression and promote muscle atrophy (17). Perhaps the loss of muscle mass with advancing age is due to a decline in the ability of insulin and growth factors to activate PKB signaling and suppress atrogene expression and protein degradation.

The purpose of the present study was to determine the effect of aging on PKB signaling, atrogene expression, and protein degradation in skeletal muscle. We hypothesized that 1) advancing age would result in a decrease in insulin-stimulated PKB signaling and 2) atrogene expression and protein degradation rates would be elevated in senescent muscle. Our hypotheses were tested by assessing insulin-stimulated PKB signaling and contractile protein degradation rates in extensor digitorum longus (EDL) and soleus (SOL) muscles and atrogene expression in quadriceps muscles from middle-aged (MA) and aged (AG) mice.

METHODS

Animals. MA (11–13 mo old) and AG (25–27 mo old) male C57B6 mice were purchased from the Aged Rodent Colony of the National Institute on Aging. Upon arrival at the animal facility at Skidmore College, mice were housed individually with cage-enrichment nestlets...
and ad libitum chow and water. All animal care and surgery were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996). All experimental protocols were approved by the Institutional Animal Care and Use Committee.

**Surgical procedures for MA and AG mice.** At ~1 mo following their arrival, mice were fasted overnight and anesthetized with 1:1:1 promace-ketamine hydrochloride-xylazine (0.015 ml/10 g body wt ip). EDL and SOL muscles were isolated intact for in vitro incubation, and quadriceps muscles were rapidly dissected, frozen in liquid N₂, and stored at −80°C until analysis.

**Isolated intact muscle incubation.** EDL and SOL muscles were dissected out intact, blotted on gauze, and transferred to 25-ml Erlenmeyer flasks (1 muscle/flask) containing 2 ml of Krebs-Henseleit buffer (0.16 M NaCl, 0.0046 M KCl, 0.0012 M KH₃PO₄, 0.0025 M NaHCO₃, 0.0025 M CaCl₂, and 0.0012 M MgSO₄), 5 mM glucose, 0.75 mM L-glutamine, and 1 × MEM amino acids for 30 min at 37°C with shaking. Insulin and amino acids were included in the incubation medium to mimic in vivo physiology. After this 30-min incubation, muscles were transferred to a second set of 25-ml Erlenmeyer flasks (1 muscle/flask) containing fresh medium and incubated for a 2-h experimental period with shaking. To prevent the reutilization of amino acids released from muscles during incubations, 0.5 mM cycloheximide was added to the medium (20, 27). This concentration of cycloheximide inhibits 95% of protein synthesis without altering protein degradation in isolated rodent skeletal muscle (29). All flasks were pregressed with 95% O₂–5% CO₂ for 3 min immediately prior to both incubations as well as every 20 min (for ~15 s) during the 2-h incubation. After the 2-h incubation, the media were collected and stored at −20°C until further analysis. The muscles were frozen in liquid N₂ and stored at −80°C.

**Contractile protein degradation.** Contractile protein degradation was assessed by measurement of 3-methylhistidine (3-MH) release from EDL and SOL muscles incubated in vitro. The amount of 3-MH in the incubation medium of the isolated muscles was determined according to a modified GC-MS method (Metabolic Technologies, Ames, IA) previously described by Rathmacher et al. (22). Briefly, 1 ml of incubation medium and 10 nmol of 3-[methyl-2H₃]methylhistidine (d₃-3-MH; MassTrace, Woburn, MA) internal standard were transferred to a 12 × 75 mm tube and acidified with 2 ml of 1 N HCl. The samples were centrifuged at 2,300 × g for 10 min and the resultant supernatants were determined by the bicinchoninic acid method (Pierce). The remaining skeletal muscle extract was utilized for electrophoretic analysis and immunoblotting experiments.

**Electrophoretic analysis and immunoblotting.** Skeletal muscle extracts and molecular weight standards (Bio-Rad, Hercules, CA; Magic Mark, Invitrogen) were subjected to SDS-PAGE. The proteins were electrophoretically transferred to Immobilon membranes and immunoblotted with phospho-specific PKB (pThr308 and pSer473) antibodies. The pThr308 and pSer473 PKB antibodies recognize PKB when phosphorylated on Thr308 or Ser473, two sites at which phosphorylation is necessary for PKB activity (1). The phosphorylation of FOXO3α was assessed by a phospho-specific antibody that recognizes FOXO3α only when phosphorylated on Thr32. After the membranes were washed, the light generated by the alkaline phosphatase-conjugated secondary antibody and CDP-Star reagent was detected using a digital imaging system (UVP, Upland, CA). Phospho-specific immunoblots were stripped and reprobed with the PKB-α or PKB-β antibody. To account for gel loading differences, all immunoblots were stripped and reprobed with a GAPDH antibody. Relative signal intensities of the PKB-α, PKB-β, pThr308 PKB, pSer473 PKB, and GAPDH bands were determined using Total Lab software (Nonlinear, Durham, NC). All data were normalized to GAPDH and expressed as a percentage of MA. The GAPDH antibody was obtained from Abcam (Cambridge, MA), the phospho-specific PKB and FOXO3α antibodies were obtained from Cell Signaling Technology (Beverly, MA), and the PKB-α and PKB-β antibodies were a generous gift from Dr. Morris J. Birnbaum (University of Pennsylvania, Philadelphia, PA).

**Skeletal muscle RNA extraction and real-time quantitative PCR.** Frozen quadriceps muscles from mice were manually ground with a porcelain mortar and pestle chilled in liquid N₂. Total RNA was extracted from powdered muscle using an RNA extraction kit (RNeasy Fibrous Tissue Kit, Qiagen) and quantified by measurement of absorbance at 260 nm using a spectrophotometer (Beckman Coulter, Brea, CA). A 1-μg aliquot of total RNA was reverse transcripted using the RETROscript kit (Ambion, Austin, TX). The resultant cDNA (20 ng cDNA/sample in triplicate) was subjected to quantitative PCR using target-specific TaqMan gene expression assays and a real-time PCR system (StepOne Plus Real-Time PCR System, Applied Biosystems, Foster City, CA). Relative quantitation of amplified cDNA targets was determined by the cycle threshold (ΔΔCT) method using StepOne version 2.1 software (Applied Biosystems). The specific expression of mRNAs for atrogin-1, MuRF-1, and Atg12 was assessed. The resultant cDNA was quantified using a TaqMan gene expression assay and a real-time PCR system (Applied Biosystems). The specific expression of mRNAs for atrogin-1, MuRF-1, and Atg12 was assessed.

**Statistical analysis.** To detect statistical significance for all dependent variables, a one-way ANOVA was utilized. Values are means ± SE. The level of statistical significance was set at P ≤ 0.05.

**RESULTS**

**Body weight and muscle mass.** To demonstrate the presence of age-related muscle atrophy, we assessed the wet weight of intact EDL muscles. As shown in Table 1, the EDL muscle mass was significantly lower (~21%) in AG than MA mice. To gain insight into the changes in muscle mass relative to body weight, we calculated the EDL muscle mass-to-body weight ratio. Although there was no difference in body weight between AG and MA mice, Table 1 shows a substantial decline in the EDL muscle mass-to-body weight ratio in AG compared with MA mice. This finding indicates that the age-related decline in EDL muscle size is greater than changes in total...
body size. No changes in SOL muscle mass or SOL muscle mass-to-body weight ratio were observed in AG compared with MA mice (Table 1).

**PKB phosphorylation and expression.** PKB is an important signaling molecule as its activity is necessary for many actions of insulin, including the stimulation of glucose transport and the suppression of protein degradation (13). Therefore, a reduction in PKB activity or expression could play a role in the development of insulin resistance and sarcopenia. In the present study, we assessed the ability of insulin to promote the phosphorylation of PKB on Thr308 and Ser473, two sites at which phosphorylation is associated with increased kinase activity. Figure 1A shows representative immunoblots of EDL muscles prepared with phospho-specific PKB antibodies (pThr308 and pSer473), or the loading control antibody GAPDH. Both pThr308 and pSer473 immunoreactivity was significantly greater in EDL muscles from AG than MA mice (Fig. 1, B and C). The pThr308 and pSer473 immunoblots were then stripped and reprobed with a PKB-α or PKB-β antibody, as shown by the representative immunoblots in Fig. 2A. Figure 2, B and C, demonstrates that PKB-α and PKB-β immunoreactivity is higher for EDL muscles from AG than MA mice. Figure 3A shows representative immunoblots of SOL muscles prepared with phospho-specific PKB antibodies (pThr308 and pSer473) or the loading control antibody GAPDH. Immunoreactivity of pThr308 and pSer473 was similar in SOL muscles from AG than MA mice (Fig. 3, B and C). The pThr308 and pSer473 immunoblots were then stripped and reprobed with a PKB-α or PKB-β antibody, as shown by the representative immunoblots in Fig. 4A. Figure 4, B and C, demonstrates that PKB-α and PKB-β immunoreactivity is higher for SOL muscles from AG than MA mice. These findings indicate that, in EDL and SOL muscles, the increase in PKB phosphorylation on Thr308 and Ser473 is likely due to increased expression of PKB-α and PKB-β.

**FOXO3a phosphorylation.** PKB regulates protein degradation by promoting the phosphorylation and nuclear exclusion of the transcription factor FOXO, thereby decreasing expression of the E3 ubiquitin ligases atrogin-1 and MuRF-1. In the present study, we assessed the phosphorylation of FOXO3a on Thr32, a site phosphorylated by PKB (30). Figure 5A shows a representative immunoblot of EDL muscles prepared with phospho-specific FOXO3a antibody (pThr32) or the loading control antibody GAPDH. pThr32 immunoreactivity was significantly greater in EDL muscles from AG than MA mice (Fig. 5D). Therefore, it appears that the increase in PKB activity leads to an increase in FOXO3a phosphorylation on Thr32 in EDL, but not SOL, muscles.

**Atrogin-1 protein levels.** Experimental models of muscle atrophy have shown an increase atrogin-1, a ubiquitin E3 ligase that controls ubiquitin-proteasome-dependent proteolysis. Therefore, we have assessed atrogin-1 levels in EDL and SOL muscles from MA and AG mice by immunoblotting with a commercially available antibody (EMC Biosciences, Versailles, KY). In Fig. 6, a peptide competition experiment reveals a ~60-kDa immunoreactive band that is abolished by

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**Table 1. Body weight and muscle mass of MA and AG mice**

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<tr>
<th>Group</th>
<th>MA</th>
<th>AG</th>
<th>P Value</th>
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<tr>
<td></td>
<td>Body wt, g</td>
<td>30.8 ± 0.8</td>
<td>29.5 ± 0.7</td>
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<td></td>
<td>SOL muscle mass, mg</td>
<td>19.2 ± 0.8</td>
<td>16.8 ± 1.3</td>
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<tr>
<td></td>
<td>EDL muscle mass, mg</td>
<td>18.9 ± 1.2</td>
<td>15.6 ± 0.9*</td>
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<tr>
<td></td>
<td>SOL muscle mass/body wt</td>
<td>0.627 ± 0.031</td>
<td>0.555 ± 0.036</td>
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<tr>
<td></td>
<td>EDL muscle mass/body wt</td>
<td>0.610 ± 0.026</td>
<td>0.529 ± 0.028</td>
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Values are means ± SE; n = 9, except for aged (AG) group soleus (SOL) muscle mass and SOL muscle mass/body wt, where n = 8. MA, middle-aged; EDL, extensor digitorum longus. *Significantly different from MA, P < 0.05.

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**Figure 1.** Effects of aging on phosphorylation of PKB on Ser473 and Thr308 in extensor digitorum longus muscles (EDL). Muscles from middle-aged (MA) and aged (AG) mice were incubated in vitro. Muscle extracts were prepared, and PKB activity was assessed by immunoblotting with phospho-specific [Thr308 and Ser473 (p308 and p473, respectively)] PKB antibodies (A). Phosphorylated (Thr308 and Ser473) PKB immunoreactive bands were quantified, and data were normalized to total amounts of GAPDH (B and C). Values are means ± SE; n = 9. *Significantly different from MA (P < 0.05, by 1-way ANOVA).
a synthetic peptide corresponding to amino acids 23–35 of atrogin-1, the site in the protein recognized by our atrogin-1 antibody. The peptide-sensitive 60-kDa immunoreactive band was used to assess the effect of aging on atrogin-1 protein levels in EDL and SOL muscles. Representative atrogin-1 immunoblots containing EDL and SOL muscle extracts are shown in Fig. 7, A and C, respectively. After normalization to GAPDH immunoreactivity, quantification of atrogin-1 immunoreactivity revealed that atrogin-1 protein levels are similar in EDL and SOL muscles of MA and AG mice (Fig. 7, B and D).

**Arrogene mRNA expression.** Using real-time quantitative PCR, we assessed several mRNA transcripts to genes that have been shown to be increased following experimentally induced muscle atrophy (atrogens) (4, 16, 25, 34). As shown in Table 2, mRNA levels for the E3 ubiquitin ligases atrogin-1 and MuRF-1 mRNA are similar in quadriceps muscles from AG and MA mice. These results indicate that ubiquitin-proteasome-dependent proteolysis is not altered in skeletal muscles of AG mice. Table 2 also demonstrates that mRNA levels for Bnip3 and Gabarap1 are significantly lower in muscles from AG than MA mice. The levels of LC3 and Atg12 were not significantly different in muscles of MA compared with AG mice. Overall, our results indicate that the effect of advancing age on atrogene expression in skeletal muscle is quite different...
from that observed in experimental models of muscle atrophy. Perhaps the decline in Bnip3 and Gabarap1 expression leads to the accumulation of damaged proteins/organelles, leading to contractile dysfunction.

**In vitro skeletal muscle protein degradation rates.** Experimental models of muscle atrophy have shown that skeletal muscle proteolysis is elevated (4, 17, 20, 24, 25, 27, 34). Therefore, we have assessed contractile protein degradation rates by measuring the release of 3-MH from isolated intact EDL and SOL muscles from MA and AG mice. As shown in Fig. 8, contractile protein degradation rates were not significantly different in EDL and SOL muscles of AG compared with MA mice (P = 0.406), respectively. These findings, unlike experimental models of muscle atrophy, indicate that age-related muscle atrophy is likely due to factors other than accelerated rates of contractile protein breakdown.

**DISCUSSION**

Sarcopenia is a major public health problem that is associated with poor nutrition, physical inactivity, increased proinflammatory cytokines, and resistance to growth-promoting hormonal action. Although it is established that PKB signaling regulates muscle mass by enhancing protein synthesis, only recently, studies have indicated that PKB signaling regulates muscle mass by controlling protein degradation (4, 17, 20, 24, 25, 27, 34). Therefore, the present study investigated the effects of advancing age on insulin-stimulated PKB signaling and protein degradation rates, as well as atrogate expression. Our results indicate that insulin-stimulated PKB signaling is increased and the expression of several atrogens is decreased or unchanged with advancing age. It is important to note that the present PKB signaling results are limited to the insulin-stimulated state, as we did not study basal PKB signaling.

PKB signaling plays an important role in regulating skeletal muscle mass by stimulating protein synthesis and suppressing protein degradation. However, little is known about PKB signaling and protein degradation in aging skeletal muscle. Because aging is associated with insulin resistance (7, 21, 32) and PKB is an insulin-responsive signaling molecule that inhibits protein degradation (10), it is a reasonable hypothesis that sarcopenia would be due, in part, to a decline in insulin-stimulated PKB activity and an increase in skeletal muscle proteolysis. The results of this study suggest otherwise, as we observed an increase in the expression of PKB-α and PKB-β in EDL and SOL muscles from AG compared with MA mice. The increased expression of PKB was associated with an increase in the phosphorylation of PKB on Thr308 and Ser473, two sites at which phosphorylation is necessary for kinase activity (1). Perhaps the increase in PKB expression and phosphorylation is a futile attempt to preserve muscle mass during aging, at least in the atrophying EDL muscle. This idea was suggested by Kimball et al. (15), who demonstrated that basal mammalian target of rapamycin signaling and protein synthesis increased with advancing age. Because the mammalian target of rapamycin binding partner rictor is the kinase that phosphorylates PKB on Ser473 (26), our data suggest that rictor activity is increased with advancing age.

The transcription factor FOXO is a target of PKB that controls expression of atrogin-1 and MuRF-1 genes (4, 25, 34), as well as the autophagy-related genes (16, 17). PKB phosphorylates FOXO on Thr32, a process that results in nuclear exclusion and a decrease in the transcription of FOXO-dependent genes (30). The effect of advancing age on FOXO is not well established, as studies suggest an increase (23) and a decrease (8) in FOXO activity. Consistent with the present increase in PKB expression and activity (Thr308 and Ser473 phosphorylation), we observed an increase in FOXO3a phosphorylation on Thr32 in EDL muscles from AG compared with MA mice. This observation would be expected to protect EDL muscles from age-related atrophy, but that is not the case, as EDL muscles from AG mice weighed significantly less than EDL muscles from MA mice. Perhaps PKB-independent...
FOXO phosphorylation (30) has a more profound effect on skeletal muscle mass with advancing age. However, despite elevated PKB expression and phosphorylation, FOXO3a phosphorylation on Thr32 was similar in SOL muscles from AG and MA mice. This finding suggests that, in slow-twitch oxidative, but not fast-twitch glycolytic, muscle fibers, advancing age results in a resistance to the ability of PKB to phosphorylate FOXO3a. Perhaps in the slow-twitch oxidative muscle fibers, advancing age alters the subcellular location of PKB in a manner that does not facilitate efficient FOXO phosphorylation.

Because atrogin-1 expression is controlled by PKB/FOXO signaling (24, 25), it would not be surprising to observe a decrease in atrogin-1 levels, since we observed an increase in insulin-stimulated FOXO3a phosphorylation in EDL muscles of AG mice. Furthermore, the expression of atrogin-1 and MuRF-1 mRNA levels was similar in quadriceps muscles of AG and MA mice. This finding is different from the results of other studies reporting increases (2, 5, 23) or decreases (8) in atrogin-1 and MuRF-1 expression. Consistent with our atrogin-1 and MuRF-1 data in quadriceps muscles, contractile protein degradation rates and atrogin-1 protein levels were similar in EDL muscles from AG and MA mice, indicating that ubiquitin-dependent proteolysis may not play a major role in the development of sarcopenia. However, our results are limited to 25- to 27-mo-old mice. Although initial age-related muscle atrophy typically occurs by 25 mo of age, we cannot rule out the possibility that elevated protein degradation might be observed in slightly older mice that are experiencing greater deficits in muscle mass. Studies involving the oldest of old mice are problematic because of complicating factors such as disease and mortality.

In addition to the ubiquitin-proteasome proteolytic pathway, autophagy/lysosomal protein degradation plays a role in regulating muscle mass (6, 12, 34). Recently, it has been shown that the expression of the autophagy-related genes Bnip3, LCN, and Gabarap1, are increased by fasting and denervation (16, 17), two interventions that induce muscle wasting. However, other autophagic genes are necessary to maintain muscle mass, as Atg7 knockout mice experience severe muscle wasting (18). The role of autophagy in age-related muscle atrophy is not established, as only one study has examined autophagy-related genes in skeletal muscle of aged rats (33). A novel finding of the present study is the observation that Bnip3 and Gabarap1 expression is significantly lower in muscles of AG than MA mice. Since PKB/FOXO signaling controls autophagy-related gene expression in skeletal muscle (16, 17), the present results suggest that the observed increase in FOXO3a phosphorylation may have contributed to the decline in Bnip3 and Gabarap1 expression. However, this interpretation is not without limitations, as we assessed FOXO3a phosphorylation in EDL and SOL muscles and gene expression in quadriceps muscles. Nonetheless, the present decline in Bnip3 and Gabarap1 may contribute to the accumulation of damaged proteins/organelles and lead to age-related contractile dysfunction and/or impaired muscle metabolism. It is also important to note that the present decline in Bnip3 and Gabarap1 with advancing age is quite

**Fig. 5.** Effects of aging on phosphorylation of forkhead box 3a (FOXO3a) on Thr32 in EDL and SOL muscles. Muscles from MA and AG mice were incubated in vitro. Muscle extracts were prepared, and FOXO3a phosphorylation was assessed by immunoblotting with a phospho-specific (Thr32) FOXO3a antibody (A and C). Phosphorylated (Thr32) FOXO3a immunoreactive bands were quantified, and data were normalized to total amounts of GAPDH (B and D). Values are means ± SE; n = 9, except for AG group SOL muscle data, where n = 8. *Significantly different from MA (P < 0.05, by 1-way ANOVA).

**Fig. 6.** Peptide competition assay for atrogin-1 immunoreactivity in skeletal muscle extracts. Muscle extracts were prepared, and atrogin-1 immunoreactivity was assessed by immunoblotting with an atrogin-1 antibody (left) or an atrogin-1 antibody and a synthetic peptide corresponding to the amino acids recognized by the atrogin-1 antibody (right). MW, molecular weight marker.
different from the rapid increase in these genes due to experimentally induced muscle atrophy (16, 17).

In summary, the present study demonstrates that advancing age does not appear to result in an increase in skeletal muscle proteolysis or an increase in the expression of atrogenes. Although several experimental models of muscle atrophy describe a program of gene expression that leads to increases in ubiquitin-proteasome-dependent and autophagy/lysosomal protein degradation, age-related muscle atrophy seems to occur via different mechanisms. This idea is supported by the observation that sarcopenia is a slow gradual process, whereas muscle atrophy induced by denervation, fasting, or glucocorticoid treatment occurs rapidly (2). Finally, the present decline in the expression of the autophagy-related genes Bnip3 and Gabarap1 may contribute to the accumulation of damaged proteins/organelles in senescent skeletal muscle and lead to age-related dysfunction in skeletal muscle contractile properties or muscle metabolism.

ACKNOWLEDGMENTS

The authors are grateful for the technical support of John A. Tine (Center for Functional Genomics, University at Albany, SUNY, Rensselaer, NY) and Patricia J. Hilleren (Department of Biology, Skidmore College).

Table 2. Real-time quantitative PCR analysis of atrogenes in skeletal muscles of MA and AG C57B6 male mice

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<th>Gene</th>
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<th>AG</th>
<th>P Value</th>
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<td>Atrogin-1</td>
<td>0.268 ± 0.044</td>
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<tr>
<td>MuRF-1</td>
<td>1.136 ± 0.207</td>
<td>0.734 ± 0.172</td>
<td>0.162</td>
</tr>
<tr>
<td>Bnip3</td>
<td>0.787 ± 0.055</td>
<td>0.547 ± 0.033*</td>
<td>0.002</td>
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<tr>
<td>LC3</td>
<td>0.953 ± 0.118</td>
<td>0.953 ± 0.180</td>
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<tr>
<td>Gabarap1</td>
<td>0.907 ± 0.064</td>
<td>0.613 ± 0.057*</td>
<td>0.004</td>
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<tr>
<td>Atg12</td>
<td>0.852 ± 0.143</td>
<td>0.709 ± 0.081</td>
<td>0.399</td>
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Values (means ± SE) are relative units; n = 8 mice in each group. Total RNA was extracted from quadriceps muscles, reverse transcribed, and subjected to real-time quantitative PCR. MuRF, muscle-specific RING finger protein 1; Bnip3, Bel-2 and 19-kDa interacting protein 3; LC3, light chain 3; Gabarap1, GABA receptor-associated protein 1; Atg12, autophagy-related protein 12. *Significantly different from MA, P < 0.05.

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Table 2. Real-time quantitative PCR analysis of atrogenes in skeletal muscles of MA and AG C57B6 male mice

<table>
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<tr>
<th>Group</th>
<th>3-Methylhistidine Release (nM)</th>
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<td>A</td>
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<tr>
<td>MA</td>
<td>120</td>
</tr>
<tr>
<td>AG</td>
<td>100</td>
</tr>
<tr>
<td>B</td>
<td>Soleus Muscle</td>
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<tr>
<td>Middle Aged</td>
<td>120</td>
</tr>
<tr>
<td>Aged</td>
<td>100</td>
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Fig. 7. Effects of aging on atrogin-1 levels in EDL and SOL muscles. Muscles from MA and AG mice were incubated in vitro. Muscle extracts were prepared, and atrogin-1 was assessed by immunoblotting with atrogin-1 antibody (A and C). Atrogin-1 immunoreactive bands were quantified, and data were normalized to total amounts of GAPDH (B and D). Values are means ± SE; n = 9, except for AG group SOL muscle data, where n = 8.

Fig. 8. Effects of aging on contractile protein degradation in EDL and SOL muscles. Muscles from MA and AG mice were incubated in vitro, and 3-methylhistidine release was assessed. Values are means ± SE; n = 9, except for AG group SOL muscle data, where n = 8.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.
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