10-1-2010

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Recommended Citation

Miaozong Wu, Hua Liu, Jacqueline Fannin, Anjaiah Katta, Yeling Wang, Ravi Kumar Arvapalli, Satyanarayana Paturi, Sunil K. Karkala, Kevin M. Rice, and Eric R. Blough. Acetaminophen Improves Protein Translational Signaling in Aged Skeletal Muscle. Rejuvenation Research. October 2010, 13(5): 571-579. doi:10.1089/rej.2009.1015.

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REJUVENATION RESEARCH
Volume 13, Number 00, 2010

DOI: 10.1089/rej.2009.1015

Acetaminophen Improves Protein Translational Signaling in Aged Skeletal Muscle

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Abstract

Background: Age-related muscle atrophy is characterized by increased oxidative stress, diminished Akt enzymatic function, and reduced phosphorylation of the mammalian target of rapamycin (mTOR), which can be attenuated by chronic acetaminophen ingestion. Here we hypothesize that age-related impairments in Akt/mTOR function are associated with reduced protein translational signaling, and that these changes, if present, can be attenuated by acetaminophen treatment.

Results: Compared to 6- and 27-month old animals, the expression of the mTOR-complex proteins raptor and GβL and the phosphorylation of tuberin/TSC2 (Thr1462) were reduced in the soleus muscles of very aged rats (33 months old). These changes in Akt/mTOR pathway signaling proteins were in turn associated with decreased phosphorylation of S6 kinase p85S6K (Thr412) and eukaryotic translation initiation factor-4E (eIF4E) binding protein-1 (4EBP1, Thr37/46), reduced phosphorylation of S6 ribosomal protein (Ser235/236), and increased inhibition of eIF4E by binding to 4EBP1. Age-associated alterations in the Akt/mTOR pathway signaling and in the phosphorylation of the stress-responsive eIF2α protein were attenuated by chronic acetaminophen treatment (30 mg/kg body weight per day). *Ex vivo* incubation of adult muscles with hydrogen peroxide mimicked the age-related decreases seen in eIF4E and 4EBP1 phosphorylation, whereas the inclusion of acetaminophen in the muscle bath attenuated this effect.

Conclusion: Aging is associated with impairments in the regulation of proteins thought to be important in controlling mRNA translation, and acetaminophen may be useful for the treatment of age-related muscle atrophy by reducing oxidative stress.

Introduction

The age-associated loss of skeletal muscle mass and muscle strength, or sarcopenia, has a profound effect on quality of life and is of significant socioeconomic interest.[1–3] The accumulation or loss of muscle protein is determined by the balance between muscle protein synthesis and degradation. The factors regulating the initiation and progression of sarcopenia are not fully understood; however, recent data have demonstrated that the rate of muscle protein synthesis is decreased in advanced age of human and different animal models[4–6] and that this effect may be mediated, at least in part by the reactive oxygen species (ROS) levels.[7–10] How increased age or ROS levels may affect the regulation of protein synthesis is not well understood.

The mammalian target of rapamycin (mTOR) pathway is a critical regulator of protein synthesis,[6,11,12] whose activity is regulated by phosphorylation at Ser2448 (via protein kinase B/Akt[13,14]) and by binding to its regulatory partners, the regulatory associated protein of mTOR (raptor) and GβL[15–17] (both positive regulators), or Tuberin/TSC2,[13,14] (negative regulator). Regulation of protein translation by mTOR is effected through the phosphorylation of S6 kinase, which functions to phosphorylate the S6 ribosomal protein[18,19] and eukaryotic translation initiation factor-4E (eIF4E) binding protein-1 (4EBP1), with the latter event leading to the release of eIF4E from 4EBP1, a step necessary for initiation of mRNA translation.[20–24] Like eIF4E, the eIF2 also plays a role in regulating translational initiation because it mediates the binding of initiator methionyl-tRNA and guanosine triphosphate

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(GTP) to the ribosome 40S subunit.[25,26] The ability of eIF2 to regulate initiation is inhibited, at least in part, under conditions of elevated stress, which can lead to the phosphorylation of the eIF2 α subunit (eIF2α) and the formation of a stable eIF2–GDP–eIF2B complex.[25,26] Whether aging or increased oxidative stress affects the amount or phosphorylation of molecules thought to participate in the regulation of protein synthesis rates has to our knowledge not been investigated.

Previous studies from our laboratory have demonstrated that age-related muscle atrophy is characterized by increased tissue ROS levels, impaired Akt enzymatic function, decreased mTOR phosphorylation/activation, diminished myocyte size, and decreased expression of the contractile proteins myosin and actin.[7,10] Other work from our laboratory has demonstrated that chronic acetaminophen ingestion can be used to attenuate these age-related changes in Akt/mTOR signaling and muscle structure.[10] Using the same animals and tissues that we examined previously,[10] here we hypothesize that impairments in Akt/mTOR signaling will be accompanied by a dysregulation of downstream protein translational signaling and that chronic acetaminophen ingestion can be used to ameliorate these age-associated changes. Although incapable of proving cause and effect, the data of the present study suggest that acetaminophen may be useful for improving age-related alterations in translational signaling.

Materials and Methods

Materials

Primary antibodies against tuberin/TSC2 (#3612), phospho-tuberin/TSC2 (Thr1462) (#3617), GβL (#3274), raptor (#2280), p70S6 kinase (#9202), phospho-p70S6 kinase (Thr389) (#9205), S6 ribosomal protein (#2217), phospho-S6 ribosomal protein (Ser235/236) (pS6, #4858), eIF4E (#2067), phospho-eIF4E (Ser209) (#9741), 4EBP1 (#9452), phospho-4EBP1 (Thr37/46) (#9459), eIF2α (#9722), phosphor-eIF2α (Ser51) (#3597), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, #2118), secondary antibodies conjugated with horseradish peroxidase (HRP) (anti-rabbit [#7074] or anti-mouse [#7076]) were purchased from Cell Signaling Technology (Beverly, MA). Protein A/G PLUS Agarose (sc-2003) were from Santa Cruz Biotechnology (Santa Cruz, CA). The Laemmli 4× sample buffer was from Sigma-Aldrich (St. Louis, MO). Pierce Tissue Protein Extraction Reagent (T-PER), Pierce 660nm protein assay reagent (#22660), GE Healthcare Amersham ECL™ Western Blotting Detection Reagents (RPN 2106), and Advance Western Blotting Detection Kit (RPN2135) were from Thermo Fisher Scientific Inc. (Rockford, IL). The PAGEr Gold Precast gels (10%) were from purchased from Lonza (Rockland, ME).

Animals

Animal care and procedures were conducted in accordance with the Animal Use Review Board of Marshall University using the criteria outlined by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) as proclaimed in the Animal Welfare Act (PL89-544, PL91-979, and PL94-279). The animals and tissues used in this study have been previously examined.[10] Six- and 27-month-old male Fischer 344/NNiaHSD X Brown Norway/BiNia F1 (F344BN) rats were procured from the National Institute on Aging (Bethesda, MD) and housed in an AAALAC approved vivarium with a 12:12 h light/dark cycle and at the temperature of $22 \pm 2^{\circ}$C. Two animals were kept in each cage with *ad libitum* access to water and food (LabDiet 5001, PMI Nutrition International, LLC, Brentwood, MO).

Acetaminophen treatment and tissue collection

The 27-month-old F344BN rats were daily given acetaminophen (30 mg/kg body weight per day) continuously for 6 months in their drinking water (denoted as 33T). Age-matched rats were maintained as controls (33C). Soleus muscles were removed from anesthetized animals under anesthesia using a ketamine–xylazine (4:1) cocktail (50 mg/kg, intraperitoneally), frozen in liquid nitrogen, and stored at -80°C.

Ex vivo *muscle incubation*

Two strips of muscle were isolated from each soleus muscle from 6-month-old rats and mounted to plastic plates to keep them at their *in situ* resting length. The *ex vivo* muscle incubation were performed at 25°C in Krebs Henseleit buffer (KHB, pH 7.4), that had been equilibrated with 95% O_2 and 5% CO_2 as described previously.[10] After preincubation in KHB for 15 min, muscles were incubated in KHB for another 40 min (control), or in KHB containing hydrogen peroxide (H_2O_2) (200 μM) and/or acetaminophen (1.65 mM) for 20 min each. The acetaminophen concentration (1.65 mM) was equivalent to that in drinking water provided for rats. Muscle samples were then washed with KHB, immediately frozen in liquid nitrogen, and stored at -80°C.

Tissue protein extraction

Soleus muscles were homogenized in a Pierce Tissue Protein Extraction Reagent (T-PER) (10 mL/g tissue) contained protease inhibitors (P8340; 1:100) and phosphatase inhibitors (P5726; 1:100), and then sonicated for 0.5 min three times at 4°C. The homogenate was collected by centrifuging at 12,000 $\times$ *g* for 5 min at 4°C. Protein concentration of homogenates was determined using a Pierce 660nm protein assay reagent (#22660). The homogenate were boiled in Laemmli 4 $\times$ sample buffer for 5 min for further immunoblotting analysis.

Determination of eIF4E/4EBP1 association

The binding of 4EBP1 to eIF4E were determined by co-immunoprecipitation. Briefly, 200 μg of homogenate (180 μL) was incubated with anti-eIF4E antibody (1:50 dilution) and 20 μL of protein A/G PLUS Agarose (sc-2003) overnight at 4°C. Agarose beads were collected by centrifuging at 8,000 $\times$ *g* for 30 sec at 4°C, and washed with 500 μL of T-PER buffer containing protease (P8340; 1:100) and phosphatase inhibitors (P5726; 1:100) three times. Forty microliters of Laemmli 2 $\times$ sample buffer was added to the pellet, and the samples were boiled for 5 min. Twenty microliters of each supernatant was used for immunoblotting analysis to detect the abundance of 4EBP1.

Immunoblotting analysis

Immunoblotting analysis were performed to detect target proteins as described previously.[7,10] Briefly, protein samples was separated on a 10% PAGEr Gold Precast gel and then transferred to nitrocellulose membranes. After incubation with primary antibody overnight at 4°C and secondary antibody for 1 h at room temperature, target proteins were visualized following reaction with GE Healthcare Amersham ECL™ Western Blotting Detection Reagents (RPN 2106) or Advance Western Blotting Detection Kit (RPN2135). Target protein levels were quantified using AlphaEaseFC image analysis software (Alpha Innotech, San Leandro, CA) and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Data analysis

Results are presented as mean ± standard error (SE). The effects of age and acetaminophen treatment were analyzed using the general linear model (GLM) procedure (SAS 9.1 for Windows, SAS Institute Inc., Cary, NC). Means were calculated by the least-squares means (LSMEANS) procedure, and multiple comparisons were performed using the Tukey–Kramer test to determine differences between groups. Values of $p < 0.05$ were considered to be statistically significant.

Results

Acetaminophen restores tuberin/TSC2 phosphorylation in aged skeletal muscle

The phosphorylation of the mTOR negative regulator tuberin/TSC2 at Thr1462 was decreased in the soleus of 33-month-old aged control rats (−55.7% and −68.4% when compared to that in 6- and 27-month-old rats; $p < 0.05$; Fig. 1). Chronic acetaminophen treatment restored the tuberin/TSC2 phosphorylation level to that observed in the adult (6-month) rat ($p > 0.05$; Fig. 1).

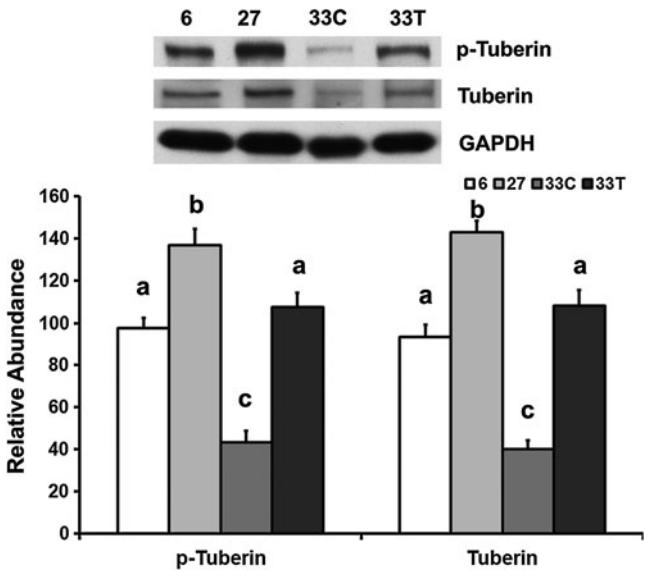

FIG. 1. Acetaminophen restores tuberin/TSC2 phosphorylation in aged skeletal muscle. Tuberin/TSC2 total protein and the phosphorylation of tuberin/TSC2 at Thr1462 in soleus muscle from 6-, 27-, 33-month-old control (33C) and acetaminophen-treated (33T) F344BN rats were determined by immunoblotting. Data are mean ± standard error. (abc) Groups without the same letter are significantly different ($p < 0.05$).

Acetaminophen attenuates aging-associated reductions in mTOR-complex regulators

Compared to that found in 27-month-old animals, the expression of the mTOR-complex proteins raptor and GβL were decreased in 33-month aged control rats (−26.8% and −15.0%, respectively; $p < 0.05$; Fig. 2A,B). Chronic acetaminophen treatment increased both raptor and GβL expression compared to that in 33-month control rats (13.9% and 11.4%, respectively; $p < 0.05$; Fig. 2A,B).

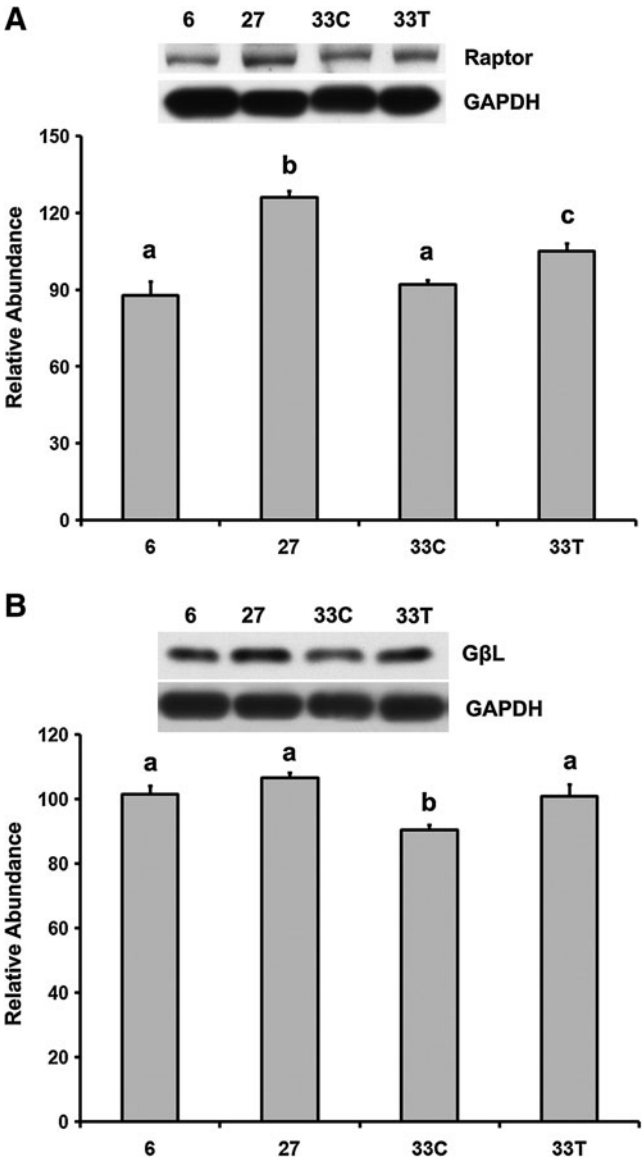

FIG. 2. Acetaminophen attenuates aging-associated reductions in the amount of raptor and GβL. Raptor (**A**) and GβL (**B**) protein levels in 6-, 27-, 33-month-old controls (33C) and acetaminophen-treated (33T) rats as determined by immunoblotting. Data are mean ± standard error. (abc) Groups without the same letter are significantly different ($p < 0.05$).

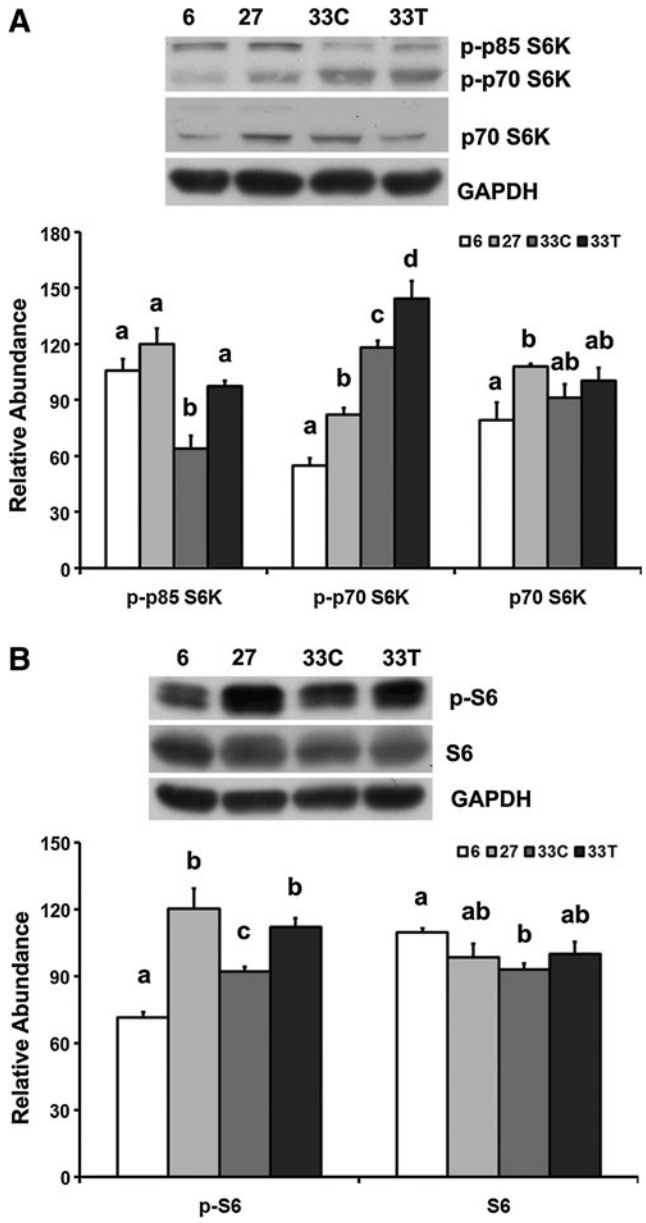

FIG. 3. Reductions in the amount of phosphorylation of S6 kinase and S6 ribosomal protein with aging are attenuated by chronic acetaminophen ingestion. (**A**) S6 kinase p85S6K (Thr412) and p70S6K (Thr389) protein levels in 6-, 27-, 33-month-old control (33C) and acetaminophen-treated (33T) rats. (**B**) The phosphorylation (Ser235/236) and total protein of S6 ribosomal protein as determined by immunoblotting. Data are mean ± standard error. (abcd) Groups without the same letter are significantly different ($p < 0.05$).

Age-associated reductions in the phosphorylation of S6 kinase and S6 ribosomal protein in muscle are attenuated by acetaminophen

The abundance of phosphorylated S6 kinase p85S6K (Thr412) in the aged soleus was lower than that in the 6- and 27-month animals (−39.6% and −46.8%, respectively; $p < 0.05$; Fig. 3A), while chronic acetaminophen treatment restored the amount of phosphorylated p85S6K to a level equivalent to that seen in 6- and 27-month old animals ($p > 0.05$; Fig. 3A). Phosphorylation of S6 kinase p70S6K (Thr389) was increased with aging ($p < 0.05$; Fig. 3A), and chronic acetaminophen treatment increased p70S6K phosphorylation compared to that in 33-month control rats (22.2%; $p < 0.05$; Fig. 3A). Compared to that observed in 27-month animals, the phosphorylation of S6 ribosomal protein at Ser235/236 was decreased in the soleus of 33-month aged control rats (−23.3%; $p < 0.05$; Fig. 3B), whereas chronic acetaminophen treatment restored the phosphorylated level of S6 ribosomal protein to that seen in 27-month rats ($P > 0.05$; Fig. 3B).

Acetaminophen intervention reduces the inhibitory effect of 4EBP1 on eIF4E in aged muscle

Compared to that seen in the 6- and 27-month-old rats, the phosphorylation of translational initiation factor eIF4E at Ser209 was decreased in the soleus of 33-month aged control rats (−51.7% and −39.3%, respectively; $p < 0.05$; Fig. 4A). Chronic acetaminophen treatment restored eIF4E phosphorylation to levels observed in 6- and 27-month-old rats ($p > 0.05$; Fig. 4A). The abundance of phosphorylated 4EBP1 (Thr37/46) in the aged soleus was lower than that found in the 6- and 27-month-old animals (−17.2% and −12.3%, respectively; $p < 0.05$; Fig. 4B), while it was increased after 6 months of chronic acetaminophen treatment (+36.4%, $p < 0.05$; Fig. 4B). Co-immunoprecipitation experiments demonstrated that the abundance of 4EBP1 bound to eIF4E was increased in the soleus of 33-month-old aged control rats when compared to that in 27-month-old rats (+32.9%, $p < 0.05$; Fig. 4C) and that chronic acetaminophen treatment decreased this binding by 21.7% ($p < 0.05$; Fig. 4C).

To investigate whether oxidative stress affects the activation of eIF4E, muscle strips from control adult animals were incubated with H_2O_2 and/or acetaminophen for 20 min. The inclusion of H_2O_2 to the muscle bath decreased phosphorylation of eIF4E and 4EBP1 whereas this effect was reversed by the addition of acetaminophen to the muscle bath (Fig. 4D). Incubation with acetaminophen alone had no effect.

Acetaminophen reduces phosphorylation of eIF2α in aged skeletal muscle

Compared to 6-month-old animals, the phosphorylation of eIF2α at Ser51 was increased in the soleus of 33-month-old aged control rats (+19.5%; $p < 0.05$; Fig. 6). Chronic acetaminophen treatment restored the eIF2α phosphorylation to a level similar to that observed in the 6- and 27-month-old rats.

Discussion

Age-related decreases in muscle protein synthesis are thought to play a significant role in the loss of muscle mass that occurs during aging.[4,5] The main finding of this study is that aging in the F344BN rat model is characterized by alterations in the phosphorylation status of ribosomal protein S6, eIF2α, eIF4E, and 4EBP1. This latter modification is accompanied by increased binding of 4EBP1 to eIF4E, which would be predicted to impair the initiation step of protein synthesis. In addition, it appears that the inhibition of eIF4E by 4EBP1 may be mediated, at least partially, by increased

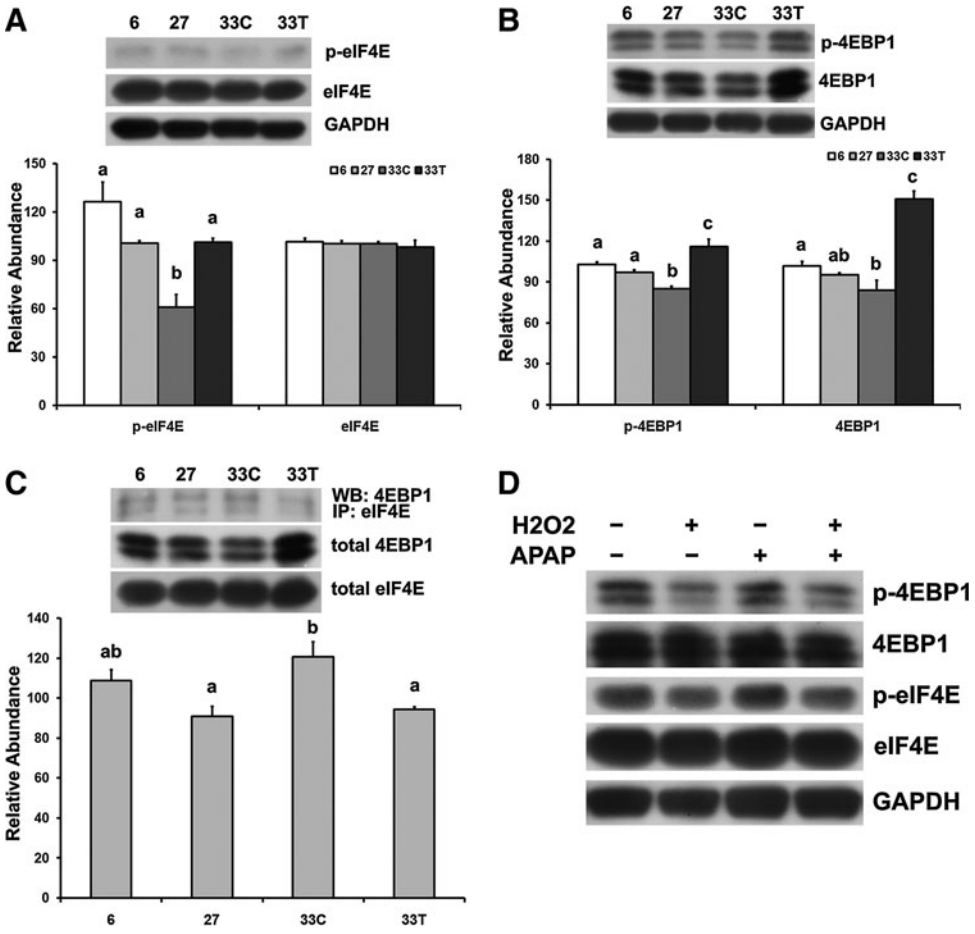

FIG. 4. Acetaminophen reduces the inhibitory effect of eukaryotic initiation factor 4E binding protein (4EBP1) on eukaryotic initiation factor 4E (eIF4E) in aged muscle. eIF4E total protein and phosphorylation of eIF4E at Ser209 in 6-, 27-, 33-month-old controls (33C) and acetaminophen-treated (33T) rats (**A**). Phosphorylation (Thr37/46) and total protein of eIF4E binding protein-1 (4EBP1 or PHAS-1) (**B**). Binding of 4EBP1 to eIF4E as determined by co-immunoprecipitation and immunoblotting (**C**). Soleus muscle strips from 6-month control animals were *ex vivo* incubated with hydrogen peroxide (H_2O_2) and/or acetaminophen as described in Materials and Methods. Phosphorylation of eIF4E and 4EBP1 after *ex vivo* incubation were detected by immunoblotting (**D**). Muscle incubated with Krebs Henseleit buffer (−) was used as control of H_2O_2 and acetaminophen response. Data are mean ± standard error. (abc) Groups without the same letter are significantly different ($p < 0.05$). H2O2, hydrogen peroxide; APAP, Acetaminophen.

oxidative stress, as *ex vivo* muscle incubation with H_2O_2 acutely reduced eIF4E and 4EBP1 phosphorylation. Importantly, these age-associated alterations were attenuated by chronic acetaminophen intervention using a dosage (30 mg/ kg body weight per day) that has previously been found to be safe and well tolerated.[7]

mTOR plays an important role in the stimulation of myocyte growth and prevention of muscle atrophy.[6,11,12] It is thought that the mTOR activity is stimulated by phosphorylation of Ser2448 by Akt[13,14] and by the binding of mTOR to raptor and GβL.[15–17] Conversely, mTOR activity has been shown to be inhibited following binding with Tuberin/TSC2 in a process that is negatively regulated through the phosphorylation of Tuberin/TSC at Thr1462 by Akt/PKB.[13,14]

Previous data from our laboratory have demonstrated that age-associated impairments in Akt/mTOR signaling are characterized by a mismatch between the degree of Akt phosphorylation and the activation of its downstream substrates mTOR and GSK-3β.[10] Similar instances of Akt "uncoupling" have been reported in human studies following the infusion of amino acids and insulin, and with exercise.[27,28] Further studies have indicated that although higher Akt phosphorylation (Ser473 and Thr308) is observed, Akt kinase enzymatic activity is impaired in aged atrophic skeletal muscle and that this impairment contributes to the reduced phosphorylation of mTOR because reversal of Akt dysfunction by acetaminophen is associated with increased mTOR phosphorylation.[10] Supporting our previous findings of reduced Akt activity,[10] we observed a reduction of tuberin/TSC2 (Thr1462) phosphorylation with aging (Fig. 1). This effect was reversed by chronic acetaminophen ingestion, a finding that is consistent with the notion that alterations in tuberin/TSC2 phosphorylation may be involved in reducing mTOR activation in aged skeletal muscle.

To further investigate how mTOR function may be altered in aged skeletal muscle, we next determined the expression of the mTOR-binding proteins, raptor and GβL, and the

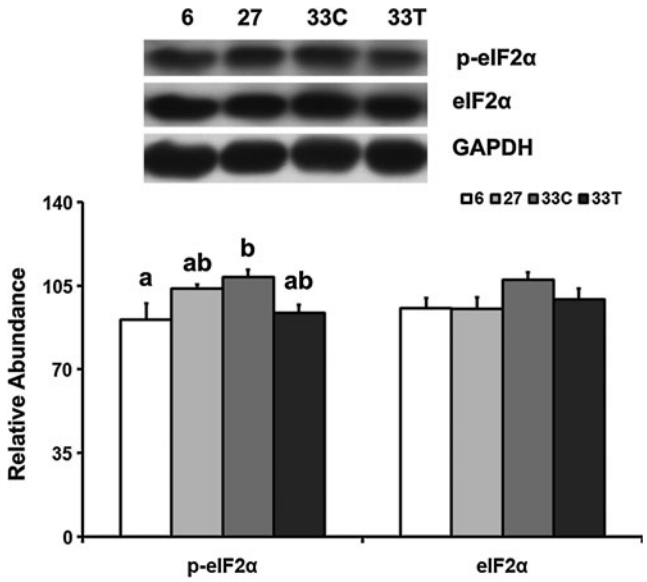

FIG. 5. Acetaminophen reduces the phosphorylation of eukaryotic initiation factor 2a-subunit (eIF2α) in aged skeletal muscle. eIF2α total protein and phosphorylation of eIF2α at Ser51 in 6-, 27-, 33-month-old control (33C) and acetaminophen-treated (33T) rats. Data are mean ± standard error. (ab) Groups without the same letter are significantly different ($p < 0.05$).

phosphorylation of two critical mTOR-substrates, S6 kinase and 4EBP1. It has been postulated that the interaction of raptor with mTOR is required for the phosphorylation of S6 kinase and 4EBP1 by mTOR[15,16] and that this event is reliant on GβL, which has been shown to stabilize the raptor–mTOR interaction.[17] In agreement with our findings of decreased mTOR expression in aged skeletal muscle,[10] here we demonstrate that the expression of both raptor and GβL is diminished with aging (Fig. 2A,B). This finding could be predicted to diminish mTOR function, a notion that is supported by our observation that aging is associated with diminished phosphorylation of the two critical mTOR-substrates, S6 kinase and 4EBP1 (Figs. 3A and 4B). Similar to our findings with tuberin/TSC2, these age-related changes in S6 kinase and 4EBP1 phosphorylation were reversed with chronic acetaminophen ingestion (Figs. 3A and 4B). Taken together, these data suggest that mTOR function is impaired in aged skeletal muscle and that chronic acetaminophen ingestion may be useful for the prevention of age-associated mTOR dysfunction in skeletal muscle.

The phosphorylation of the S6 ribosomal protein by S6 kinase is critical for the translation of ribosomal proteins and the elongation factors required to increase the synthetic potential of the cell.[18,19] In the present study, we found that the phosphorylation of S6 ribosomal protein at Ser235/236 was lower in very aged muscle (33-month) compared to that observed in the muscles obtained from 27-month-old rats, and that this decrease was associated with reduced phosphorylation of S6 kinase, p85S6K (Fig. 3A,B). Consistent with the data for S6 kinase and 4EBP1, we also demonstrate that the phosphorylation of p85S6K and S6 ribosomal protein can be preserved with chronic acetaminophen ingestion, suggesting once again that acetaminophen may be helpful for the treatment of age-associated muscle atrophy.

The eukaryotic translation initiation factor eIF4E is thought to bind with the mRNA 5′-cap structure and mediate the initiation of translation, a rate-limiting step for mRNA translation.[23,24] The binding of eIF4E to the mRNA 5′-cap structure is inhibited if eIF4E is sequestered by 4EBP1. This process is regulated by phosphorylation state of 4EBP1, because the phosphorylation of 4EBP1 by mTOR prevents its binding to eIF4E.[20–22] In agreement with our previous finding of the ability of mTOR to phosphorylate S6 kinase, we found that the phosphorylation of 4EBP1 is reduced in aged skeletal muscle (Fig. 4B). To further examine the potential functional ramifications of these data we next performed co-immunoprecipitation experiments, using an antibody to pull down eIF4E and then probing the immunocomplex for the presence of 4EBP1. As expected from our 4EBP1 phosphorylation data, we found that the binding of 4EBP1 to eIF4E is increased in aged skeletal muscle (Fig. 4C). These results, along with our finding of a decreased phosphorylation of eIF4E with aging, may suggest that aging in the skeletal muscles of the F344BN rat may be associated with an impairment of the processes regulating the initiation of translation and that alterations in Akt/mTOR signaling may contribute to this condition. Interestingly, we also observed that this age-associated increase in 4EBP1 and eIF4E interaction was diminished following chronic acetaminophen ingestion, a finding that is supportive of the possibility that acetaminophen ingestion may also increase mTOR activity (Fig. 5).

To better understand if changes in oxidative stress may participate in regulating the association between eIF4E and 4EBP1, we performed a series of *ex vivo* incubation experiments where we added H_2O_2 to the muscle bath. We found that the inclusion of H_2O_2 to the bath decreased the phosphorylation of eIF4E and 4EBP1 and that these decreases in phosphorylation could be attenuated by the presence of acetaminophen (Fig. 4D). These data are in agreement with the possibility that age-associated increase in oxidative stress could play a role in affecting the ability of the aged muscle to initiate protein synthesis by its ability to increase the phosphorylation of eIF4E and 4EBP1. This postulate is consistent with previous data from our laboratory that have demonstrated that chronic acetaminophen ingestion in the aged rat is associated with diminished age-related increases in muscle ROS, oxidatively modified proteins, and decreased hyperactivation of stress-responsive kinase p38–mitogen-activated protein kinase (MAPK).[7]

In an effort to further examine the potential adverse effects of oxidative stress on processes associated with the initiation step of protein synthesis, we measured the phosphorylation of eukaryotic initiation factor eIF2α. eIF2 mediates the binding of initiator methionyl-tRNA and GTP to the ribosome 40S subunit and participates in forming the 43S pre-initiation complex.[26] It is thought that this process is inhibited by eIF2α phosphorylation,[25,26] with the latter also occurring under conditions of increased oxidative stress.[29,30] Here we observed that phosphorylation of eIF2α at Ser51 is increased with aging and that chronic acetaminophen treatment was associated with a return of phosphorylated eIF2α to a level observed in the 6- and 27-month-old animals (Fig. 5). These findings are consistent with recent data

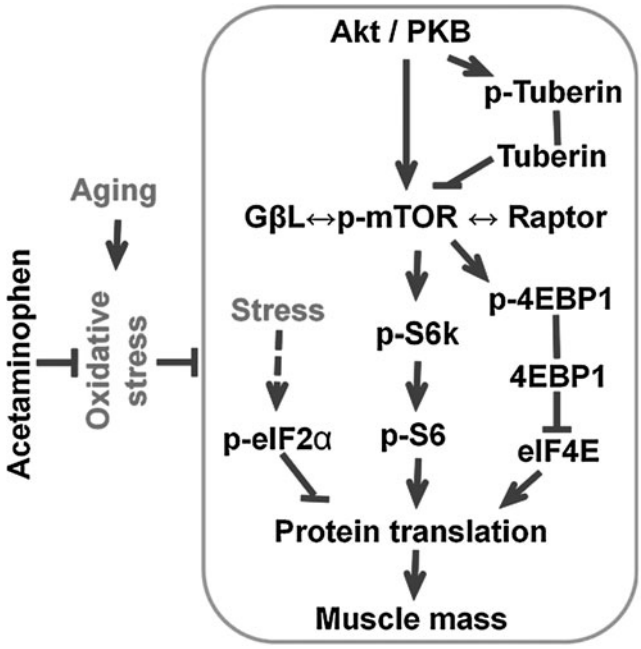

FIG. 6. Proposed mechanism(s) of how acetaminophen may function to improve protein translational signaling in aged skeletal muscle. Age-associated increases in oxidative stress impair the regulation of protein translational regulators thought to reside downstream of Akt/mTOR and the regulation of stress-mediated translation initiation in aged atrophic skeletal muscle (see the Discussion for details). Chronic acetaminophen intervention is thought to reduce reactive oxygen species (ROS) and oxidative stress, thereby improving translational signaling and the prevention of muscle mass loss in aged muscle. 4EBP1, Initiation factor 4E binding protein; eIF4E, eukaryotic initiation factor 4E; mTOR, mammalian target of rapamycin.

demonstrating that high glucose (hyperglycemia) increases the phosphorylation of eIF2α.[31] In addition, this finding is in agreement with previous work from our laboratory showing that aging is associated with increased blood glucose and that chronic acetaminophen ingestion can be used to reverse age-related hyperglycemia.[7] Taken together, these data support the possibility that age-associated hyperglycemia and/or increases in oxidative stress may decrease translational initiation by altering the phosphorylation status of eIF2α.

Although the effects of aging on tissue ROS levels have been investigated extensively, much less is known about how aging may affect the expression of molecules thought to be important in counteracting ROS. It is thought that the amount and activity of antioxidant enzymes are increased during the initial phases of aging before undergoing a gradual, but steady decline as the aging process progresses.[32,33] Recent work using Cu^{2+}/Zn^{2+} superoxide dismutase (SOD) (SOD1) transgenic mice have suggested a link between the expression of SOD1 and eIF2α phosphorylation.[34] Previous studies have demonstrated that acetaminophen exhibits potent antioxidant activity,[35–39] and that acetaminophen can decrease age-associated increases in muscle ROS and iron-induced cardiac damage.[7,40] Although not investigated here, given these data, it is possible that acetaminophen could have functioned to improve translational signaling via either its antioxidant activity or perhaps by altering the expression of molecules involved in scavenging cellular ROS. Future experiments designed to directly test this possibility will no doubt be useful in furthering our understanding of how oxidative stress may be associated with changes in protein synthesis and the regulation of muscle mass during aging.

In summary, we found that aging in the F344BN rat skeletal muscle is associated with alterations in the amount or phosphorylation of tuberin/TSC2, raptor, Gβ*L*, p85S6K, S6 ribosomal protein, eIF4E, 4EBP1, and eIF2α. In addition, we also noted evidence of increased interaction between eIF4E and 4EBP1. These changes, considered together, would be predicted to decrease the ability of the aged muscle to initiate and undergo protein synthesis. This finding is supportive of previous work demonstrating that age-associated increases in Akt *S*-nitrosylation lead to impaired Akt function and mTOR activation in the muscles of the aged F344BN.[10] In this aforementioned study, we found that chronic acetaminophen treatment was associated with a reversal of Akt function and mTOR phosphorylation.[10] We find a similar effect here given that acetaminophen ingestion was associated with a reversal in either the expression or phosphorylation of tuberin/TSC2, raptor, Gβ*L*, p85S6K, S6 ribosomal protein, eIF4E, 4EBP1, and eIF2α to levels similar to that found in their younger counterparts. In addition, we also observed that acetaminophen treatment diminished the possible inhibitory effects of tuberin/TSC2 and 4EBP1 on protein synthesis by increasing phosphorylation of these molecules. These improvements are in agreement with our previous findings that chronic acetaminophen ingestion is capable of attenuating age-associated changes in muscle cross-sectional area and the incidence of myocyte apoptosis.[10] The data of the present study, considered together with our previous work demonstrating that acetaminophen diminishes muscle ROS levels and improves Akt function, lead to further credence of the possibility that chronic acetaminophen ingestion may be useful for the treatment of age-related muscle atrophy (Fig. 6).

Acknowledgments

This work was supported by grants provided by McNeil Consumer Healthcare to E.R.B. We thank Mr. Madhukar Kolli, Siva Nalabotu, and Brent Kidd for their technical assistance.

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Received: December 28, 2009
Accepted: April 16, 2010