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Differential Effects of Leucine Supplementation in Young and Aged Mice at the Onset of Skeletal Muscle Regeneration

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Abstract

Aging decreases the ability of skeletal muscle to respond to injury. Leucine has been demonstrated to target protein synthetic pathways in skeletal muscle thereby enhancing this response. However, the effect of aging on leucine-induced alterations in protein synthesis at the onset of skeletal muscle regeneration has not been fully elucidated. The purpose of this study was to determine if aging alters skeletal muscle regeneration and leucine-induced alterations in markers of protein synthesis. The tibialis anterior of young (3 months) and aged (24 months) female C57BL/6J mice were injected with either bupivacaine or PBS, and the mice were given *ad libitum* access to leucine-supplemented or normal drinking water. Protein and gene expression of markers of protein synthesis and degradation, respectively, were analyzed at three days post-injection. Following injury in young mice, leucine supplementation was observed to elevate only p-p70S6K. In aged mice, leucine was shown to elicit higher p-mTOR content with and without injury, and p-4EBP-1 content post-injury. Additionally in aged mice, leucine was shown to elicit higher content of relative p70S6K post-injury. Our study shows that leucine supplementation affects markers of protein synthesis at the onset of skeletal muscle regeneration differentially in young and aged mice.

Keywords

Injury; aging; BCAAs; protein synthesis; mTOR

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1. Introduction

According to the United States Census Bureau, adults 65 years of age and older accounted for 13% of the population in 2010 and this population is expected to grow to 20% of the total population by 2050 (Howden & Meyer, 2010). As this population increases in size, so too will the cost associated with age-related loss of skeletal muscle mass and force, or sarcopenia (Ryall, Schertzer, & Lynch, 2008). The development of sarcopenia is often associated with increased motor neuron denervation, impaired protein synthetic response, and impaired skeletal muscle regeneration (Edström et al., 2007). This can lead to frailty, disability and loss of independence (Fried & Guralnik, 1997; Karlsson et al., 2004). In order to counteract the effects of sarcopenia, elderly individuals are recommended to be physically active and increase consumption of whey protein (Rieu et al., 2007), a food source containing a high concentration of branched-chain amino acids (BCAAs). Dietary supplementation of BCAA, particularly leucine, has been well documented to aid in protein synthesis in the young and aged populations alike (Anthony, Anthony, & Layman, 1999; Koopman et al., 2006; Pereira et al., 2014). However, little is known about the effects of leucine supplementation during muscle regeneration, especially in the aged population.

Regeneration of skeletal muscle is a multi-step process characterized by degeneration, inflammation, regeneration, remodeling, and maturation/functional repair (Musarò, 2014). An additional component of the regenerative phase in skeletal muscle is insulin-like growth factor-1 (IGF-1)-stimulated protein synthesis which leads to skeletal muscle growth (Charge & Rudnicki, 2004). IGF-1 activates the PI3K-Akt-mTOR pathway which, in turn, phosphorylates p70S6K and 4E-BP-1 leading to increases in mRNA translation initiation and subsequent protein synthesis (Anthony, Anthony, Kimball, & Jefferson, 2001; Greiwe, Kwon, McDaniel, & Semenkovich, 2001; Stipanuk, 2007). Clavel et al. (2006) reported a marked decrease in IGF-1/Akt signaling corresponding with an increase in the atrophy markers, MuRF-1 and Atrogin-1, in the tibialis anterior of aged rats (Clavel et al., 2006). In contrast, Sandri et al. (2013) reported no significant differences in markers of either the protein synthesis or protein degradation pathways of aged mice or human subjects (Sandri et al., 2013). Taken together, it is clear that the cellular mechanisms involved in sarcopenia have yet to be fully elucidated.

The decreased regenerative potential of muscle in aged individuals and limited treatment options necessitates a clinically relevant solution that will attenuate sarcopenia and increase skeletal muscle regeneration in the aged population. Leucine supplementation has been demonstrated to aid in muscle recovery following various forms of injury (Anthony et al., 1999; Koopman et al., 2006). Leucine (*2-Amino-4-methylpentanoic acid*), is an essential, anabolic, branched-chain amino acid. It is well established that leucine can promote protein synthesis independent of the PI3K-Akt pathway by increasing mammalian target of rapamycin (mTOR) activation. Leucine-induced protein synthesis in skeletal muscle has been repeatedly observed in young subjects and is also common with older adults (Katsanos, Kobayashi, Sheffield-Moore, Aarsland, & Wolfe, 2006). Additionally, leucine supplementation also affects proteolysis by inhibiting relevant transcription factors such as FOXO3a (Pereira et al., 2014). However, the effects of leucine supplementation in older subjects is more variable than young subjects, and this variability is based on concentration

of leucine, acute or chronic supplementation and physical activity and disease state of the older subjects (Casperson, Sheffield-Moore, Hewlings, & Paddon-Jones, 2012; Rieu et al., 2007; Verhoeven et al., 2009).

Many studies reporting the effects of leucine supplementation on protein synthesis and markers of protein synthesis conduct their analyses of these factors acutely following ingestion of leucine (within 36 hours) or after chronic supplementation (2 weeks to 6 months) (Anthony et al., 1999; Caspersen et al., 2012). Leucine supplementation studies that look at skeletal muscle regeneration in the acute phase (2-4 days post-injury), commonly prime the body by administering leucine a few days prior to the induction of injury (Nicastro et al., 2012; Pereira et al., 2014). Pereira et al. (2015) studied the effects of leucine supplementation on muscle regeneration in aged rats and show an improved regeneration potential. Though they report changes in downstream markers of the Akt/mTOR pathway, it is still not known whether this improved regeneration from leucine supplementation is due to an overall increase in the total content of these markers or from an increase in the activation of these markers. This is the first study to observe markers of protein synthesis and degradation during the onset of skeletal muscle regeneration when leucine supplementation and injury, via bupivacaine injection, are administered simultaneously. The purpose of this study is to determine if aging alters skeletal muscle regeneration and if a single, environmental change, a modest increase in leucine intake, induces alterations in markers of protein synthesis and degradation. We hypothesized that age blunts protein synthesis at the onset of skeletal muscle regeneration but leucine supplementation would attenuate this effect.

2. Methods

2.1 Animals and Housing

Female C57BL/6 mice were purchased from Jackson Laboratories and were housed in the University of Arkansas Central Laboratory Animal Facility as previously described (Washington et al., 2013). The mice were kept on a 12:12-h light-dark cycle with *ad libitum* access to normal rodent chow and water. Young (3 months) and aged (24 months) mice were randomly assigned to one of eight treatment groups: 1) young/no leucine/uninjured (n = 6); 2) young/no leucine/injured (n = 6); 3) young/leucine/uninjured (n = 6); 4) young/leucine/injured (n = 6); 5) aged/no leucine/uninjured (n = 6); 6) aged/no leucine/injured (n = 6); 7) aged/leucine/uninjured (n = 15); 8) aged/leucine/injured (n = 6). All procedures were approved by the University of Arkansas Institutional animal Care and Use Committee (IACUC).

2.2 Leucine Administration

Animals in the leucine supplementation groups were provided leucine in their water at a dose of 1.5g/100mL (Costa Junior et al., 2015; Li, Xu, Lee, He, & Xie, 2012) following either saline or bupivacaine injection into the TA. Leucine was dissolved in the drinking water at 70°C for 40 minutes. We measured water consumption in the leucine treated groups only, and their water consumption was within reported values for C57BL/6J mice (Bachmanov, Reed, Beauchamp, & Tordoff, 2002). In addition, multiple studies show that

leucine-treated water does not alter consumption (Costa Junior et al., 2015; Guo, Yu, Hou, & Zhang, 2010; Li et al., 2012; Nairizi, She, Vary, & Lynch, 2009).

2.3 Bupivacaine Injection

Bupivacaine was administered as previously described (Brown et al., 2015; Washington et al., 2013). Mice were anesthetized with a subcutaneous injection of a cocktail containing ketamine hydrochloride (45 mg/kg body weight), xylazine (3mg/kg body weight), and acepromazine (1 mg/kg body weight). Muscle damage was induced by injecting 0.03 mL of 0.75% bupivacaine (Marcaine) in the left and right tibialis anterior (TA). A 25-gauge, 5/8 (0.5 X 16 mm) needle was inserted along the longitudinal axis of the muscle, and the bupivacaine was injected slowly as the needle was withdrawn. Bupivacaine was delivered in an isotonic solution of NaCl. Uninjured groups were injected with 0.03 mL of phosphate buffered saline (PBS).

2.4 Muscle and Tibia Extraction

Three days post-injection, the TA and tibias were extracted as previously described (Washington et al., 2013). Mice were anesthetized with a subcutaneous injection of a cocktail containing ketamine hydrochloride (90 mg/kg body weight), xylazine (3 mg/kg body weight), and acepromazine (1 mg/kg body weight). The TA was snap frozen in liquid nitrogen and stored at -80°C for protein and gene expression analysis.

2.5 Western Blotting

Western blot analysis was performed as previously described (Washington et al., 2013). Tissue was homogenized in Mueller Buffer and protein concentration was determined using the Qubit 2.0® fluorimeter (Invitrogen). Muscle homogenate (30 µg) was fractionated in 8%-12% SDS-polyacrylamide gels. Gels were transferred overnight to polyvinylidene difluoride (PVDF) membranes. Membranes were Ponceau stained before blotting to verify equal loading of the gels. Membranes were blocked in either 5% bovine serum albumin (BSA) or 5% non-fat dry milk in 1x Tris-buffered saline (TBS) with 0.1% Tween-20 (TBST) for 2 hours. Primary antibodies p-Akt (Ser473), Akt, p-mTOR (Ser2448), mTOR, p-p70S6K (Thr389), p70S6K, p-4E-BP1 (Thr37/46), and 4E-BP1 were obtained from Cell Signaling. Primary antibodies were diluted 1:500 to 1:2,000 in 5% BSA or non-fat milk, in TBST, and incubated at room temperature for 1 hour or 4°C overnight. Anti-rabbit (7074S) and anti-mouse (7076S) secondary antibodies (Cell Signaling, Danvers, MA) were diluted 1:1,000 to 1:2,000 in 5% BSA or non-fat milk, in TBST, and incubated at room temperature for 1 hour. Enhanced Chemiluminescence (ECL) was performed using Fluorochem M Imager (Protein Simple, Santa Clara, California) to visualize antibody-antigen interaction. Blotting images were quantified by densitometry using AlphaView software (Protein Simple). The Ponceau-stained membranes were digitally scanned. The 45-kDa actin bands were quantified by densitometry and used as a protein loading correction factor for each lane as previously described (Brown et al., 2015; Greene et al., 2014; Washington et al., 2011).

2.6 RNA Isolation, cDNA Synthesis, and Quantitative RT-PCR

RNA was extracted with Trizol reagent (Life Technologies, Grand Island, NY, USA) as previously described (Washington et al., 2011; Washington et al., 2013). Briefly, TA muscles were homogenized in Trizol. Total RNA was isolated using an RNA isolation (Abcam kit) and DNase treated with DNase. RNA concentration and purity was determined by UV spectrophotometry. cDNA was reverse transcribed from 1 µg of total RNA using the Superscript Vilo cDNA synthesis kit (Life Technologies, Carlsbad, CA, USA). Real-time PCR was performed and results were analyzed using the ABI 7300 thermocycler Real-Time detection system (Applied Biosystems, Foster City, CA, USA). cDNA was amplified in a 25 µL reaction containing appropriate primer pairs and ABI SYBR Green or TaqMan Universal Mastermix (Applied Biosystems, Grand Island, NY). Samples were incubated at 95°C for 4 minutes, followed by 40 cycles of denaturation, annealing, and elongation at 95°C, 55°C, and 72°C, respectively. TaqMan fluorescence was measured at the end of the extension step each cycle. Commercially available Taqman probes were used for the following gene targets: FoxO1 (FAM), FoxO3 (FAM), MuRF-1 (FAM), Atrogin-1 (FAM), GAPDH (FAM) MyoD (FAM), Myogenin (FAM). Fluorescent probes were purchased from Applied Biosystems and quantified with Taqman Universal Mastermix (cat# 4304437). Cycle threshold (Ct) was determined and the Ct value was calculated as the difference between the Ct value and the GAPDH value. Final quantification of gene expression was calculated using the Ct method $Ct = [Ct(\text{calibrator}) - Ct(\text{sample})]$. Relative quantification was then calculated as 2^{-Ct} .

2.7 Statistical Procedures

Results are reported as mean ± SE. Pre-planned comparison between 3 month and 24 month uninjured, untreated controls (no injury, no leucine) were conducted by Student's t-tests. Two-way ANOVAs (leucine supplementation × injury) were conducted at each level of age to determine significant main effects and interactions (CA, SPSS 23). Post hoc analysis on significant interactions was done with a Student–Newman–Keuls test. Significance was established at an alpha level of 0.05.

3. Results

3.1 Body Weight and Muscle Mass

All body and muscle mass data can be found in Table 1. Body weight of aged mice was 27% higher compared to young mice ($p < 0.05$). In young mice, there was a significant interaction ($p < 0.05$) of leucine supplementation and injury on body weight. In the no leucine group, injured mice were 11% ($p < 0.05$) heavier compared to uninjured mice. However, body weight in the no leucine, injured mice was already significantly greater pre-injection (data not shown). Body mass did not differ between uninjured and injured mice receiving leucine. In the aged mice, there was a main effect of injury ($p < 0.05$) to increase body mass by 13% in the no leucine groups but only 3% in the leucine groups. However, body weight in the injured mice was already significantly greater than uninjured mice pre-injection (data not shown).

Age did not affect TA muscle mass relative to tibia length. In young mice there was an interaction ($p < 0.05$) between leucine supplementation and injury on TA muscle mass relative to tibia length. In young mice without leucine, there was no difference between the uninjured and injured groups. However, with leucine, there was a 16% decrease in TA muscle mass relative to tibia length in the injured group compared to the uninjured group. In aged mice, there was a main effect of leucine supplementation ($p < 0.05$) to lower TA mass relative to tibia length regardless of injury.

3.2 Markers of Skeletal Muscle Regeneration

It is well established that muscle regeneration requires proliferation and differentiation of satellite cells which can be monitored through expression of Myogenin and MyoD (Brown et al., 2015; Marsh, Criswell, Carson, & Booth, 1997; Washington et al., 2011). There was a main effect for injury to increase Myogenin and MyoD mRNA abundance regardless of age (Figures 1A, 1B). In the young, Myogenin and MyoD gene content were ~7 and ~14-fold greater ($p < 0.05$), respectively, in injured vs control mice. Aged mice showed a ~2.5 and ~2-fold increase ($p < 0.05$) in Myogenin and MyoD, respectively, 3 days post-injury.

3.3 IGF-1/Akt/mTOR Protein Synthesis Pathway

Age Effect—Age had no effect on expression levels of relative, phosphorylated or total Akt [see Figure 1A, 1E in (Perry et al., 2016)]. Expression of relative mTOR did not change with age, however, content of phosphorylated and total mTOR levels were 79% and 76% lower ($p < 0.05$), respectively, in the aged mice [see Figure 1B, 1E in (Perry et al., 2016)]. Aged mice had a 52% decreased expression of relative p70S6K [$p < 0.05$; see Figure 1C in (Perry et al., 2016)] compared to young mice. Content level of p-p70S6K did not change with age, but total p70S6K content was 48% higher in the aged animals compared to the young mice [$p < 0.05$; see Figure in 1E in (Perry et al., 2016)]. In aged mice, expression of relative 4EBP-1 was 16% lower [$p < 0.05$; see Figure 1D in (Perry et al., 2016)] compared to young mice. However, levels of p-4EBP and total 4EBP were 140% and 184% higher ($p < 0.05$), respectively, in aged mice compared to young mice [see Figure 1E in (Perry et al., 2016)].

Young Animals—In young mice, there was a main effect of IGF-1 mRNA content to be higher in the injured groups regardless of leucine treatment ($p < 0.05$) (Figure 2A). There was an interaction of leucine and injury on relative Akt protein content. In young mice not receiving leucine, relative Akt was 3-fold higher ($p < 0.05$; Figure 2C) in injured mice contrasted with uninjured mice. In young mice receiving leucine, relative Akt content did not differ between the uninjured and injured groups. Additionally, there was a main effect of injury to increase p-Akt content regardless of leucine supplementation ($p < 0.05$; Figure 2B, 2J). Lastly, there was a main effect of leucine to increase total Akt content [$p < 0.05$; see Figure 3A in (Perry et al., 2016)].

In young mice, relative and phosphorylated mTOR were higher in the injured group compared to the uninjured group ($p < 0.05$; Figures 2E and 2D respectively) in both leucine and non-leucine treated mice. Compared to the uninjured mice, p-mTOR content was ~2.5 fold higher in the injured mice regardless of leucine supplementation. Compared to the uninjured mice, relative mTOR content was 50% higher in the leucine, injured mice and was

15% higher in the non-leucine, injured mice. Total mTOR content was higher in the no leucine, injured mice than all other groups by approximately 2-fold [$p < 0.05$; see Figure 3B in (Perry et al., 2016)].

In young mice, an interaction existed in relative p70S6K. With mice not receiving leucine, differences were not observed between uninjured and injured mice. However, in the leucine-treated mice, relative p70S6K was 6-fold higher ($p < 0.05$; Figure 2G) in injured mice compared to uninjured mice. There was an interaction of leucine and injury on p-p70S6K. In mice receiving leucine, p-p70S6K was 7-fold higher ($p < 0.05$; Figure 2F, 2J) in the injured group compared to the uninjured group. In the groups not receiving leucine, there was a trend for p-p70S6K to be higher in the injured group compared to the uninjured group ($p = 0.07$). There was a main effect of injury to increase total p70S6K content [$p < 0.05$; see Figure 3C in (Perry et al., 2016)].

In young mice, there was a main effect of injury to decrease relative content of 4EBP-1 ($p < 0.05$; Figure 2I). Relative 4EBP-1 was 25% lower in the non-leucine group and 10% lower in the leucine group. An effect of injury raised the content of phosphorylated 4EBP-1 ($p < 0.05$; Figure 2H, 2J). Compared to the uninjured groups, p-4EBP-1 was ~70% higher in the injured mice regardless of leucine supplementation. There was a main effect of injury to increase total 4E-BP-1 content [$p < 0.05$; see Figure 3D in (Perry et al., 2016)].

Aged Animals—In aged mice, there was a main effect of IGF-1 mRNA content to be higher in the injured groups regardless of leucine treatment ($p < 0.05$) (Figure 2A). In aged mice, there was a trend for an interaction of leucine and injury on relative Akt content ($p = 0.087$; Figure 2C); however, an effect of injury to increase relative Akt ($p < 0.05$) was observed. An effect of injury was also observed to increase p-Akt ($p < 0.05$; Figure 2B, 2K). No changes were detected in total Akt content [see Figure 4A in (Perry et al., 2016)].

In aged mice, an effect of leucine was observed to increase relative and phosphorylated content of mTOR ($p < 0.05$; Figure 2E and 2D, respectively). Compared to the non-leucine group, relative mTOR was 90% higher in the leucine group regardless of whether or not the mice were injured. Compared to the non-leucine group, p-mTOR content was ~9-fold higher in the leucine group regardless of whether or not the mice were injured. Similarly, total mTOR content was increased by ~5 fold in the leucine group regardless of whether or not the mice were injured [$p < 0.05$; see Figure 4B in (Perry et al., 2016)].

For relative p70S6K, an interaction of leucine and injury was observed in aged mice. Whereas differences were not observed between uninjured and injured mice not receiving leucine, mice in the leucine group were observed to have 2.4-fold higher content of relative p70S6K in the injured group compared to the uninjured group ($p < 0.05$; Figure 2G). Additionally, content of relative p70S6K in the uninjured mice not receiving leucine was 2.7-fold higher compared to the uninjured mice receiving leucine ($p < 0.05$; Figure 2G). There was a main effect of injury to increase p-p70S6K ($p < 0.05$; Figure 2F, 2K). There was a main effect of leucine to increase total p70S6K content [$p < 0.05$; see Figure 4C in (Perry et al., 2016)].

In aged mice, content of relative 4EBP-1 did not differ between uninjured and injured groups of either the non-leucine or leucine-treated ($p = 0.077$) groups. However, in injured mice, relative 4EBP-1 was 126% higher in the leucine group compared to the non-leucine group ($p < 0.05$; Figure 2I). An interaction of leucine and injury was observed to affect p-4EBP-1. In the no leucine group, p-4EBP-1 content was 64% lower in the injured group compared to the uninjured group ($p < 0.05$; Figure 2H, 2K). In aged mice, in the leucine-treated group, p-4EBP-1 content was 71% higher at the onset of muscle regeneration compared to uninjured mice ($p < 0.05$). Furthermore, in injured mice, p-4EBP-1 content was 2.6-fold greater in the leucine group compared to the non-leucine group ($p < 0.05$). There was a main effect of injury to decrease total 4E-BP-1 content [$p < 0.05$; see Figure 4D in (Perry et al., 2016)].

3.4 FoxO/MuRF-1/Atrogin-1 Protein Degradation Pathway

Aging did not affect gene expression of FoxO1, FoxO3, MuRF-1 or Atrogin-1 [See Figure 2B in (Perry et al., 2016)]. In young mice, FoxO1 (Figure 3A) and Atrogin-1 (Figure 3D) were not affected by leucine or injury. However, an effect of injury to increase FoxO3 and MuRF-1 was observed ($p < 0.05$; Figure 3B, 3C). In aged mice, Atrogin-1 was not affected by leucine or injury (Figure 3D); however, an effect of injury was observed to increase FoxO1, FoxO3 and MuRF-1 ($p < 0.05$; Figure 3A-C).

4. Discussion

Overall, this study shows markers of protein synthesis respond differently to leucine supplementation at the onset of muscle regeneration in young and aged mice. Specifically, leucine supplementation appears to elicit more profound alterations in the Akt/mTOR pathway in aged mice than in young mice. To our knowledge, only one other study has reported the effects of leucine supplementation at the onset of muscle regeneration in young and aged mice (Pereira et al., 2015). Pereira et al. reported changes in content of downstream proteins in the PI3K/Akt/mTOR pathway; however, this study did not report changes in the relative ratio of phosphorylated to total content of these downstream proteins. Our study provides more insight on the effect of leucine supplementation in aged mice by examining the relative ratio of phosphorylated to total content of both p70 and 4EBP1 as well as upstream modulators in the Akt/mTOR pathway at the onset of muscle regeneration.

4.1 Muscle Mass Alterations with Leucine Supplementation

Aging is associated with chronic, low-grade, systemic inflammation. Additionally, bupivacaine-induced muscle regeneration is associated with muscle necrosis, inflammation, and edema (Duguez, Feasson, Denis, & Freyssen, 2002; Musarò, 2014)). Because of this muscle mass changes at the onset of skeletal muscle regeneration are varied with studies showing increases, decreases, or no changes (Duguez et al., 2002; Plant, Colarossi, & Lynch, 2006; White, Baltgalvis, Sato, Wilson, & Carson, 2009). Aging muscle has a prolonged inflammatory time course during regeneration which could explain the impaired regenerative response in aging animals (van der Poel et al., 2011). Leucine supplementation has been observed to attenuate injury-induced inflammation/swelling in muscle of both young and older subjects (Nosaka, Sacco, & Mawatari, 2006; Pereira et al., 2015; Van Someren,

Edwards, & Howatson, 2005). Our data corroborates this finding in that we demonstrate a reduced TA mass relative to tibia length at the onset of muscle regeneration in young mice receiving leucine. Furthermore, a recent finding shows that leucine supplementation attenuates damage-induced inflammation in aged mice (Pereira et al., 2015) which might help explain why TA mass relative to tibia length was decreased in the aged group receiving leucine.

4.2 Aged muscle demonstrates a more sensitive response to leucine supplementation at the onset of muscle regeneration

Leucine supplementation increases muscle protein synthesis, and researchers have taken a great interest in its effect on aged tissue. Leucine supplementation has been shown to work most effectively in aged subjects when used in conjunction with another anabolic-inducing stimulus such as exercise (Breen & Phillips, 2011). It has been previously demonstrated that IGF-1 mRNA abundance is upregulated at the onset of skeletal muscle regeneration in aged muscle (Marsh, Criswell, Carson, & Booth, 1997). Our data is consistent with this finding. At the onset of muscle regeneration, we demonstrated an increase in IGF-1 mRNA abundance regardless of age or leucine supplementation. In young mice, we observed a greater abundance of phosphorylated Akt, mTOR, and 4EBP-1 at the onset of skeletal muscle regeneration. Leucine supplementation only affected activated p70S6K; therefore, this study shows that activated markers of protein synthesis, except p-p70S6K, respond in a similar manner at the onset of muscle regeneration in young mice regardless of leucine supplementation. This finding supports the results of Pereira et al. which also show no differential responses of protein synthesis markers in leucine-supplemented young rats three days after injury (Pereira et al., 2014). Both exercise and leucine supplementation provoke anabolic effects, but it is possible that the anabolic effect from the injury supersedes that of leucine during the onset of muscle regeneration in young mice (Nakai, Kawano, & Ohira, 2012). Conversely, the anabolic effect of exercise in aged subjects is not as high as it is in the young (Drummond et al., 2008). In our study, aged mice expressed accentuated changes in markers of protein synthesis with leucine supplementation (relative mTOR, p70S6K and 4EBP-1). Thus, the effects of leucine on these markers lead the authors to believe that leucine supplementation may increase protein synthesis in both young and aged mice at the onset of muscle regeneration, but the response to leucine varies between young and aged mice. We cannot discount the fact that the effects observed may be due to events immediately downstream of leucine that activates the Akt/mTOR pathway. Leucine has been demonstrated to induce other downstream events such as insulin signaling which could contribute to the observations observed in this study (Macotela et al., 2011).

4.3 Leucine does not alter mRNA abundance of FoxO, MuRF-1 or Atrogin-1

MuRF-1 and Atrogin-1 are two muscle-specific E3 ligases that are important for regulating protein degradation of poly-ubiquitinated proteins (Gumucio & Mendias, 2013). FoxO3 is a transcription factor that initiates transcription for genes of MuRF-1 and Atrogin-1. FoxO3 increases at the onset of muscle regeneration, and leucine supplementation blunts this response (Pereira et al., 2014). Our study shows mRNA abundance of FoxO3 to be higher three days after injury in both young and aged mice. However, we do not see attenuation of this response in the leucine supplemented group in the young. However, there is a trend ($p =$

0.065) for leucine supplementation to blunt the increase in FoxO3 after injury in the aged mice. Discrepancies between our findings and the findings of Pereira et al. (2014) in young mice can be attributed to several variables in the experimental designs such as mode of damage, time point of leucine supplementation, and concentration and administration of leucine. Concomitant with increased FoxO3 following injury, MuRF-1 was also increased in both young and aged mice at the onset of muscle regeneration regardless of leucine supplementation. However, no changes in Atrogin-1 were observed in either young or aged mice. During the first 24 hours of muscle regeneration, MuRF-1 increases whereas Atrogin-1 initially decrease (Louis, Raue, Yang, Jemiolo, & Trappe, 2007). These responses of MuRF-1 and Atrogin-1 may help explain why only MuRF-1 mRNA abundance was elevated at the onset of muscle regeneration in this study.

While fiber-type distribution was not measured in this study, the TA muscle is a fast-twitch muscle with its composition being ~92% Type II fibers in C57BL/6J mice (Augusto, Padovani, & Campos, 2004). However, aging is characterized by a switch from fast- to slow-twitch muscle fiber types (Ohlndieck, 2011), and leucine has been shown to have a greater effect on CSA of Type I fibers (Baptista et al., 2010). Therefore, differential effect of leucine supplementation in aged mice compared to young mice may be due to aged-induced alterations in fiber type.

5. Conclusions

In summary, in young and aged mice receiving leucine supplementation, we observed differential responses of proteins in the PI3K/Akt/mTOR pathway at the onset of muscle regeneration. The finding of this study suggests that leucine affects these proteins in an mTOR-independent fashion in young mice whereas leucine exerts its effects in an mTOR dependent fashion in aged mice. While the current study only implies this finding, it gives direction for future research studies aimed at definitively demonstrating an mTOR-dependent or mTOR-independent effect of leucine supplementation at the onset of skeletal muscle regeneration in young and aged mice.

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Highlights

- In young mice, leucine had a no effect on markers of protein synthesis post-injury
- In aged mice, leucine had an effect on markers of protein synthesis post-injury
- In young mice, leucine acts independently of mTOR post-injury
- In aged mice, leucine acts in an mTOR-dependent manner to increase protein synthesis

Figure 1. mRNA abundance of myogenic regulatory factors (MRFs), Myogenin and MyoD, in young and aged mice at the onset of skeletal muscle regeneration. A) Myogenin:GAPDH in young and aged mice B) MyoD:GAPDH in young and aged mice. Main effect of injury is indicated by “ME Injury”. $P < 0.05$.

Figure 2.

mRNA abundance and protein content of targets in the Akt/mTOR protein synthesis pathway in young and aged mice at the onset of skeletal muscle regeneration. A) IGF-1:GAPDH mRNA B) p-Akt:Ponceau C) p-Akt:Act D) p-mTOR:Ponceau E) p-mTOR:mTOR F) p-p70S6K:Ponceau G) p-p70S6K:p70S6K H) p-4EBP-1:Ponceau I) p-4EBP-1:4EBP-1 J) Representative blots of protein targets in young mice K) Representative blots of protein targets in aged mice. Main effect of injury is indicated by “ME Injury”, main effect of leucine is indicated by “ME Leucine”, and significant differences between two groups is indicated by bars. $P < 0.05$.

Figure 3.

mRNA abundance of atrophy targets in young and aged mice at the onset of skeletal muscle regeneration. A) FoxO1:GAPDH B) FoxO3:GAPDH C) MuRF-1:GAPDH D) Atrogin-1:GAPDH. Main effect of injury is indicated by “ME Injury”, main effect of leucine is indicated by “ME Leucine”, and significant differences between two groups is indicated by bars. $P < 0.05$.

Table 1

Body weight, tibialis anterior muscle weight, tibia length, tibialis anterior muscle weight normalized by tibia length three days after PBS or bupivacaine injection.

		Without Leucine Supplementation		With Leucine Supplementation	
		Uninjured	Injured	Uninjured	Injured
Body Weight (g)					
	3 months	19.2 ± 0.6	21.4 ± 0.4 [#]	19.3 ± 0.4	19.4 ± 0.3
	24 months	24.4 ± 1.7	27.5 ± 1.2 [‡]	25.5 ± 0.4	26.2 ± 0.8 [‡]
Tibialis Anterior (mg)					
	3 months	34.47 ± 1.15	34.61 ± 1.88	34.13 ± 0.91	29.09 ± 0.88 [#]
	24 months	34.42 ± 1.21	37.97 ± 1.50	34.86 ± 1.15	34.56 ± 2.44
Tibia Length (mm)					
	3 months	16.41 ± 0.16	16.82 ± 0.16 [‡]	16.47 ± 0.10	16.69 ± 0.12 [‡]
	24 months	17.32 ± 0.41	16.80 ± 0.28 [‡]	17.84 ± 0.18	17.04 ± 0.25 [‡]
TA mass/Tibia Length (mg/mm)					
	3 months	2.10 ± 0.06	2.06 ± 0.10	2.07 ± 0.05	1.74 ± 0.05 [#]
	24 months	2.04 ± 0.10	2.26 ± 0.09	1.90 ± 0.07 [‡]	1.97 ± 0.16 [‡]

Values are means ± SEM. Main effects of leucine supplementation distinguished by † and main effects of injury by ‡.

[#] Indicates the group's mean is statistically different from the other three groups, $P < 0.05$.