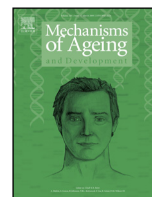
Mechanisms of Ageing and Development 157 (2016) 7–16

Contents lists available at ScienceDirect

Mechanisms of Ageing and Development

journal homepage: www.elsevier.com/locate/mechagedev



Differential effects of leucine supplementation in young and aged mice at the onset of skeletal muscle regeneration

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ARTICLE INFO

Article history:
Received 14 March 2016
Received in revised form 24 May 2016
Accepted 27 May 2016
Available online 18 June 2016

Keywords:
Injury
Aging
BCAAs
Protein synthesis
mTOR

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.
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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 and 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 et al., 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 and 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 et al., 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 (Tidball, 2011). 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 and 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 et al., 2001; Greiwe et al., 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 tib-

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http://dx.doi.org/10.1016/j.mad.2016.05.007
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ialis 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 et al., 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 are more variable than young subjects, and this variability is based on concentration of leucine, acute or chronic supplementation, physical activity and disease state of the older subjects (Casperson et al., 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 h) or after chronic supplementation (2 weeks to 6 months) (Anthony et al., 1999; Casperson 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

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 1.5 g/100 mL (Costa et al., 2015; Li et al., 2012) following either saline or bupivacaine injection into the TA. Leucine was dissolved in the drinking water at 70 °C for 40 min. We measured water consumption in the leucine treated groups only, and their water consumption was within reported values for C57BL/6 J mice (Bachmanov et al., 2002). In addition, multiple studies show that leucine-treated water does not alter consumption (Costa et al., 2015; Guo et al., 2010; Li et al., 2012; Nairizi et al., 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 (3 mg/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 (0.5×16 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 1× Tris-buffered saline (TBS) with 0.1% Tween-20 (TBST) for 2 h. 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 1:2000 in 5% BSA or non-fat milk, in TBST, and incubated at room temperature for 1 h or 4 °C overnight. Anti-rabbit (7074S) and anti-mouse (7076S) secondary antibodies (Cell Signaling, Danvers, MA) were diluted 1:1000 to 1:2000 in 5% BSA or non-fat milk, in TBST, and incubated at room temperature for 1 h. Enhanced Chemiluminescence (ECL) was performed using Fluorochem M Imager (Protein Simple, Santa Clara, California) to visualize antibody-antigen interaction.

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