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Essential Amino Acid (EAA) Regulation of Skeletal Muscle Protein Turnover with Age

Mary Komp
University of Arkansas, Fayetteville

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_____
Jamie Baum, Ph.D.
Thesis Director

_____
Yan Huang, Ph.D.
Committee Member

_____
Elisabet Børsheim, Ph.D.
Committee Member

_____
Sami Dridi, Ph.D.
Committee Member

ABSTRACT

Skeletal muscle (SM) is vital for both long term health and quality of life. Recent research suggests an increase in catabolic signals with age triggers pathologic conditions, such as sarcopenia. Although results from *in vitro* studies model how EAA can regulate muscle protein synthesis (MPS), the relevance of these models to muscle protein breakdown (MPB) and the presence of physiological EAA concentrations remains to be established. Therefore, the objective of this study was to determine the effects of a low, normal, and supra physiological dose of EAA (0.2, 1.0, and 3.0 x EAA) in a young (passages 2-10) and aging (passages 16-24) C2C12 murine muscle cell model. We hypothesized that increased levels of EAA will increase MPS in aging cells and suppress MPB via mTORC1 when compared to young cells. Myoblasts were seeded ($1x10^5$) into 6-well plates and differentiated into myotubes when they reached 80% confluency. Myotubes were serum and AA starved for 24 hours before receiving one of the following treatments: control (CON), 0.2 x EAA, 1.0 x EAA, 3.0 x EAA with or without rapamycin (100 nm; rapamycin (RAP) for 1, 6, and 24 hours. All treatments were performed in triplicate and then each experiment was repeated three times, yielding nine wells per treatment. Phosphorylation for phospho and total protein of p70 S6 kinase 1 (p70S6K1), 4E binding protein 1 (4EBP1), mechanistic target of rapamycin (mTOR), general control nonderepressible 2 (GCN2), eukaryotic initiation factor 2- alpha (eIF2α) and Sestrin 2 (SESN2) was measured using Western Blot analysis. RAP inhibited phosphorylation of MPS markers p70S6K1 and 4EBP1 in all treatments. Whereas the activation of GCN2 in young and old muscle cells is independent of the availability of EAA and mTORC1 activation. The phosphorylation of 4EBP1 increased ($p<0.05$) following 0.2 x EAA in young cells and 3.0 x EAA in old cells compared

to CON. Phosphorylation of p70S6K1 increased ($p<0.05$) following 0.2, 1.0, and 3.0 x EAA in young cells compared to the CON. In conclusion, we demonstrated EAA can regulate pathways associated with sarcopenia and increase molecular markers related to MPS in young and old muscle cells. Therefore, it can be proposed increasing EAA may be effective for regulating the rate of MPS and MPB via the mTORC1 pathway in both young and aging muscle cells.

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INTRODUCTION

With advancing age, a decline in skeletal muscle mass and function (sarcopenia) is observed in both humans [1, 2] and animals [3, 4]. Sarcopenia is associated with a loss of muscle strength, which directly affects the loss of mobility and increases the risk of disability and mortality in aging adults [5-9]. The mechanisms leading to sarcopenia are not well-defined. Protein turnover is a constant metabolic process that regulates skeletal muscle mass and reflects the rates of muscle protein synthesis (MPS) and muscle protein breakdown (MPB). The preservation of skeletal muscle mass is achieved by a net protein balance between MPS and MPB. Skeletal muscle loss is increased when there is an imbalance between MPS and MPB over a prolonged period, as observed with aging and sarcopenia. In healthy muscle the consumption of protein stimulates an increase in MPS [10, 11]. Whereas, it is believed muscle loss with age and age-related diseases is due to a blunted response of MPS to the anabolic properties of amino acids (AA) [12].

Data originating from nitrogen balance studies in older adults indicate higher protein consumption (1.0-1.3 g/kg day) containing essential amino acids (EAA) could counteract the decrease in the availability of AA and preserve skeletal muscle mass. [13]. A subsequent study measuring MPS and MPB rates in muscle atrophying rats as compared to normal healthy rats identified the branched chain amino acid (BCAA), leucine, can stimulate MPS in normal rats, but EAA must be present to maintain the leucine stimulation of MPS [3]. In comparison to normal rats, leucine infusion in hindlimb-immobilized rats did not change muscle protein turnover rates, but EAA suppressed MPB [3]. Rates of MPS and MPB were also observed in healthy young (30 +/- 3 yr) and old adults (72 +/- 1 yr) after oral administration of 40 g of

crystalline AA in the same proportion as what is found in beef proteins [14]. The response of MPS to the AA mixture was found to be significantly reduced in older adults compared to the young [14]. To gain a better understanding of the intracellular mechanisms involved in the stimulation of MPS by AA and their role in the decrease of muscle sensitivity to AA during aging, the effects of a complete profile of AA and high leucine concentration (200 µmol/L) was assessed *in vitro* on muscle from young and aged rats [15]. By assessing intracellular targets of protein synthesis, it was demonstrated aged rats have a similar protein synthetic response to the young when high leucine concentrations are present with AA [15]. *In vivo* studies began showing increasing the availability of the EAA as a beneficial regulator of postprandial stimulation of MPS. For instance, bolus ingestion of EAA (15 g) was effective in stimulating MPS in both young (34 ± 4 yr) and old (67 ± 2 yr) subjects [16]. However, ingestion of a smaller bolus of EAA (6.7 g) was unable to stimulate MPS in older adults unless the mixture of EAA is further increased with leucine enrichment (41% leucine) [17]. Similar to the stimulation of MPS in response to an EAA and leucine mixture, MPS in older adults was further increased by increasing the dose of EAA [16, 18, 19].

The availability of dietary EAA regulate skeletal muscle protein metabolism and can counteract the blunted stimulation of MPS in older adults with provision of a sufficient dose of EAA or leucine [20-26]. However, the cellular mechanisms linking EAA to the regulatory mechanisms of MPS and MPB are currently not well understood. A better understanding could aid in the development of nutritional strategies to prevent or delay the onset of sarcopenia.

The mechanistic target of rapamycin complex 1 (mTORC1) is recognized as the key cellular mechanism for regulating protein synthesis in response to anabolic stimuli [27]. EAA act

as a signaling molecule via mTORC1, and an increase in the activity of mTORC1 phosphorylates two key downstream proteins: p70 S6 kinase 1 (p70S6K1) and 4E binding protein 1 (4EBP1), [28]. The activity of these two proteins contribute to the regulation of translation initiation and are dependent upon the activity of mTORC1 [29]. In L6 myoblasts and animals, an EAA or leucine supplementation was found to increase MPS through mTORC1 and its downstream proteins [30-32]. However, administration of rapamycin (RAP), an inhibitor of mTORC1, markedly reduced mTORC1 activation and blocked the phosphorylation of p70S6K1 and 4EBP1 [31, 32]. This data and others suggest that the increase in MPS is dependent on EAA- induced mTORC1 signaling to the phosphorylation of p70S6K1 and 4EBP1 [19, 33]. In human skeletal muscle, administration of chloroquine disrupted mTORC1's activation at the lysosome and impaired the EAA induced MPS response to EAA [34]. In aging muscle the activation of mTORC1 has also been shown to be impaired, observed through a decreased responsiveness of MPS [31]. Yet, according to some research the anabolic resistance in aging muscle may be overcome by EAA administration [31]; however, it is unknown whether this is related to the EAA availability at the muscle level.

In vitro experiments have also suggested mammalian cells possess a second amino acid-sensing mechanism, the general control nonderepressible 2 (GCN2) kinase, which senses AA deficiency and consequently phosphorylates eukaryotic initiation factor 2 alpha (eIF2α) , an inhibitory factor of translation initiation [35]. Important links between GCN2 and mTORC1 signaling in response to amino acid availability have been demonstrated following regulation of single amino acids, such as leucine [36, 37]. During AA deprivation a link between the two pathways was demonstrated by GCN2 directing mTORC1 suppression through activation of

Sestrin 2 (SESN2), the recently identified leucine sensor [37]. Current evidence suggests that the relative roles of the GCN2 and mTORC1-mediated responses to amino acid availability may differ depending on the specific amino acid(s) that is (are) deficient and the particular tissue of interest [36, 38, 39]. In C2C12 muscle cells, the modulation of signaling activity by EAA was found to be limited to the regulation of translation initiation via mTORC1 and p70S6K1 phosphorylation and not by regulation of elongation factors, such as eIF2α [40].

Although results from *in vitro* studies model how single EAA can regulate MPS [40-42], a complete profile of the EAA and the relevance of physiological doses to an aging cell model remains to be established. Thus, the aim of this research was to determine the regulatory effects of EAA on the signaling pathways associated with MPS and MPB using an aging C2C12 muscle cell model. We hypothesize increased levels of EAA will increase the phosphorylation of molecular markers associated with MPS through regulation of translation initiation via mTORC1. The specific aims of this thesis were to first identify the optimal starvation and refeeding time of EAA to generate a MPS response in both young and old C2C12 muscle cells. Followed by using Western blot analysis to determine the phosphorylation of markers of MPS and MPB: p70S6K1, 4EBP1, mTOR, GCN2, eIF2α and SESN2 in response to a low, normal, and supra physiological dose of EAA in young and old C2C12 muscle cells.

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LITERATURE REVIEW

The decline in muscle mass and function with advancing age is generally referred to as sarcopenia. Several groups have redefined sarcopenia with the primary outcomes for diagnosis being the loss of skeletal muscle mass and strength and physical performance, often referred to as dynapenia [5-9]. Secondary outcomes include the loss of mobility and increased risk of disability and mortality [5-9]. Sarcopenia affects 5–10% of people over 65 years of age and more than 50% of people over 80 years of age. Recent estimates suggest by the year 2050, 200 million people will be affected [43]. Due to the lack of consensus on a working definition and diagnosis criteria, sarcopenia is frequently underdiagnosed and undertreated [44]. Consequently, this disease places an economic and social burden on public healthcare systems and caregivers, especially in countries with a growing population of older adults. In the United States, the estimated cost of hospitalizations attributable to sarcopenia was estimated at $40.4 billion in 2019, with ~$19 billion attributed to individuals greater than 65 years of age [45]. Regarding caregivers, people with sarcopenia have a 13.8 percent increased risk of dependency in their activities of daily living, compared to those of similar age without the condition [46]. Currently, no pharmacological treatments exist for managing sarcopenia; therefore, the negative clinical outcomes and socio-economic consequences, such as poor quality of life and increased health care expenditure, indicate a significant need to better understand the changes contributing to this age-related condition [47].

The multifactorial nature of sarcopenia presents a challenge in identifying one single cause or mechanism to explain the disease. Sarcopenia is associated with impaired muscle structure and function. Structurally muscle fiber cross-sectional area is lost and functional changes in

slow and fast motor units occur [48]. These physiological changes that occur are well understood, yet an understanding of the underlying mechanisms driving muscle loss has not been unidentified. Age-related changes to the anabolic response to amino acid (AA) stimuli and a decline in protein consumption may be significant contributors to the pathogenesis of age-related sarcopenia by causing a deregulation of MPS [26, 49]. It has been reported synthesis rates of muscle protein are reduced by 30% in older adults compared to younger adulds [26]. With the multifactorial nature of sarcopenia it is unclear if the reductions in synthesis rates of muscle protein is in part to growth factors (e.g., insulin-like growth factor (IGF)), hormones (e.g., testosterone and leptin), nutrients (e.g., amino acids and glucose), or muscular activity (e.g., exercise) [41, 50-53].

Skeletal muscle is an important site for regulation of muscle protein synthesis (MPS) and muscle protein breakdown (MPB), defined by a constant turnover of skeletal muscle proteins to AA. A balance between MPS and MPB is essential for maintaining muscle integrity, growth, and functionality [41]. Aging reduces the response of myofibrillar protein synthesis and anabolic signaling to AA observed by a loss of skeletal muscle mass and decreased activation of the mechanistic target of rapamycin complex 1 (mTORC1), a key cellular mechanism for regulating protein synthesis [13, 18]. Therefore, it has been concluded the impaired anabolic response of aging skeletal muscle to AA may contribute to a decrease in MPS through the inhibition of mTORC1 resulting in sarcopenia [18].

In attempts to increase skeletal muscle mass, strength, and MPS in older adults, several studies have examined the effects of low and high dose boluses of orally ingested AA in both young (34 +/- 4 yr) and old 67 +/- 2 yr) adults. [16, 54, 55]. Controlled trails have found protein

intake of 30-40 g per meal can maximally stimulate MPS in older adults and could therefore minimize the risk for sarcopenic [13, 23, 56]. Evidence indicates an adequate dose of EAA (>7 g) and/or leucine must be available to elicit an increase in MPS rates in older adults comparable to that of young adults[17, 26, 57]. In young and older human skeletal muscle, the response to EAA enriched with leucine stimulated MPS and activated translation initiation through the activation of mTORC1 [58, 59]. One of the central roles of mTORC1 is the regulation of protein synthesis, which is coordinated by the phosphorylation of downstream substrates [29]. The two best characterized markers of mTORC1 activity, p70 S6 kinase 1 (p70S6K1) and 4E binding protein 1 (4EBP1), controls the activity of protein synthesis and translation initiation [28]. mTORC1 stimulation is largely responsible for muscle's anabolic response to EAAs, recognized by the regulation of MPS by the availability of EAA [19, 33]. *In vitro* research continues to focus on how EAA are sensed and regulated during fed and fasted conditions [60, 61]. Two major regulatory mechanisms exist in mammalian cells for regulating protein synthesis in response to EAA availability: mTORC1 and general control nonderepressible 2 (GCN2). These kinases have opposing effects on protein synthesis. mTORC1 promotes protein synthesis when activated by EAA, especially leucine, arginine, and methionine [62]. Recent discoveries have specified upstream leucine and arginine sensors of mTORC1, Sestrin2 (SESN2) and CASTOR1, serve as potential targets to promote mTORC1 signaling and protein synthesis to counteract muscle wasting conditions [27]. Conversely, depletion of even a single EAA or NEAA is sensed by GCN2 through binding of uncharged tRNA, which in turn inhibits protein synthesis and represses translation leading to muscle loss [63, 64]. Previous experimentation has proven when leucine is deficient GCN2 activation participates in a constant inhibition of mTORC1 activity [36, 37].

Although results from *in vitro* studies model how single EAA can regulate mechanisms of MPS and MPB [40-42], a complete profile of the EAA and the relevance of physiological doses to an aging cell model remains to be established. Further research is also needed to establish the activities of the GCN2 and mTORC1 pathways in response to the EAA with age.

AGE RELATED DETERIORATION OF SKELETAL MUSCLE

Body composition often changes with age [65, 66], and is characterized by a loss of muscle mass, strength, and physical performance [67-69]. Previous studies have shown no change in body weight and physical activity is necessary to account for increased fat and decreased lean mass often observed with age [66, 67, 70, 71]. Peak skeletal muscle mass and strength occurs during the third decade of life and begins to show early declines beginning at ages 30-50 years [72]. Muscle mass decreases approximately 3–8% per decade after the age of 30, and pronounced changes occur after the age of 50 [53, 73]. For instance, a leg lean body mass loss of 1–2% per year and a strength loss of 1.5–5% per year are reported for individuals older than 50 years [69, 74]. By the age of 70 years, muscle mass declines to nearly 25-30% of total body mass [1], and in parallel, the loss of strength accelerates 25% to 40% per decade after 70 years of age [69, 75]. The decline of muscle mass and strength with advancing age is known as, sarcopenia. Sarcopenia is associated with poor physical performance and functional decline [76]. For preventative measures, many studies have explored the impact of exercise and nutrition on the regulation of skeletal muscle mass in the aging population [77, 78].

SARCOPENIA: PREVALENCE, CAUSES, AND MANAGEMENT

The term sarcopenia was first introduced in 1989 by Rosenburg and has yet to hold a universal definition for diagnosis and assessment. Several groups have redefined sarcopenia with the primary outcomes for diagnosis being the loss of skeletal muscle mass, strength and performance, and the secondary outcomes including loss of mobility and increased risk of disability and mortality [5-9]. Sarcopenia affects 5–10% of people over 65 years of age and more than 50% of people over 80 years of age. Recent estimates suggest by the year 2050, 200 million people will be affected [43]. Due to the lack of consensus on a working definition and diagnosis criteria, sarcopenia is frequently underdiagnosed and undertreated [44]. Consequently, this disease places an economic and social burden on public healthcare systems and caregivers, especially in countries with a growing population of older adults. In the United States, the estimated cost of hospitalizations attributable to sarcopenia was estimated at $40.4 billion in 2019, with ~$19 billion attributed to individuals greater than 65 years of age [45]. Regarding caregivers, people with sarcopenia have a 13.8 percent increased risk of dependency in their activities of daily living, compared to those of similar age without the condition [46]. Currently, no pharmacological treatments exist for managing sarcopenia; therefore, the negative clinical outcomes and socio-economic consequences, such as poor quality of life and increased health care expenditure, compel a significant need to better understand the changes contributing to this age-related condition [47].

Factors contributing to the maintenance of skeletal muscle mass throughout the lifespan remains a challenging area of research because of the multifactorial contributions of age, nutrition, hormones, comorbidities, and activity level to changes in skeletal muscle over

time. Skeletal muscle acts as a key metabolic tissue for muscle and whole-body protein turnover [79]; therefore, it is an intriguing and relevant area of investigation. Recent studies have confirmed skeletal muscle tissue serves as the primary AA reservoir to maintain whole-body protein synthesis and breakdown [51, 80, 81]. The contribution of skeletal muscle protein to whole-body protein turnover varies significantly from 25-50% [25]. An imbalance of MPS and MPB can lead to the loss of muscle mass, and a perturbation of muscle protein turnover with aging has been proposed to play a role in the development of sarcopenia. The maintenance of skeletal muscle mass is achieved by a daily net protein balance between rates of MPS and MPB. A disruption to the equilibrium of protein turnover can lead to the loss of skeletal muscle mass, which is often observed during the aging process and in catabolic diseases, such as sarcopenia.

The balance between MPS and MPB is essential for maintaining muscle quality, growth, and functionality [41]. Sarcopenia defined as a gradual loss of lean mass over a prolonged period. The contribution of muscle protein to whole body protein metabolism has been found to be significantly reduced in older adults by review of the decreased rates of myofibrillar protein turnover [13]. However, the multiple factors contributing to the extent of protein turnover and the many different methodologic approaches for measuring protein turnover produce variability within the literature. When observing the muscle fractional synthetic rate of young and old adults following the incorporation of isotopically labeled AA a 25% difference amongst the fasting rates of MPS in older adults was observed; however, a decrease in the rate of MPB was not observed [82-84]. Other studies using intravenous infusion and muscle biopsy methodologies have observed minimal to no differences in the fasting rates of MPS between

young and old adults [10, 57, 85]. It is commonly recognized that the difference in fasted rates of MPS or MPB do not differ in healthy older adults prompting the hypothesis that the imbalance in protein turnover during the postprandial state must be associated with the etiology of sarcopenia [49]. Further exploration of how the postprandial state regulates protein turnover differently in young and old adults will be addressed later in this review.

METHODOLOGY FOR ASSESSING PROTEIN TURNOVER

Protein turnover is challenged by the consequences of nutrition, aging, and disease. Regarding nutrition, when food sources or supplements containing protein are ingested proteolysis occurs providing the body with amino acids (AA), which are metabolized by cells to build other proteins. There are 20 different amino acids (AA), 9 of which are essential (must be supplied by the diet), and 11 which are non-essential (they are made by the body). A complete protein profile of both the NEAA and EAA are necessary to increase muscle and whole-body protein synthesis. In recent years, numerous research studies have been initiated to investigate the regulatory roles of AA on protein synthesis [86-88].

Since the development of stable isotope tracer methodology alongside muscle biopsies, measuring rates of protein metabolism from the whole body (i.e., aggregated across all body proteins) down to a tissue-specific level (e.g., skeletal muscle only) and even an individual protein level has been made possible in both animals and humans [12, 89, 90]. The use of tracer methodologies drove the understanding of skeletal muscle tissue pools being in a continuous state of turnover with coordinated regulation during periods of feeding and fasting [88]. Utilization of the tracer-based methodology reflects how AA regulate the turnover rate of

skeletal muscle because multiple amino acid tracers can be used simultaneously, and multiple labels can be used with any individual amino acid allowing for its detection [91].

A dedicated effort has also been made to study how AA can influence muscle protein turnover in older adults. The interaction of AA in muscle protein turnover involves a complex interaction of signal transduction, gene transcription, translation, and protein degradation, among many other changes. A combination of rt-PCR to measure mRNA level expression and Western blot to detect protein level expression is commonly used to assess the proteins and genes that regulate protein turnover. Therefore, a great number of studies incorporate stable isotope methodology alongside rt-PCR and Western blot analysis to examine the regulation of protein metabolism and cell signaling by the EAA in young and old muscle.

AVAILABILITY OF EAA REGULATE SKELETAL MUSCLE PROTEIN TURNOVER

It is well recognized skeletal muscle protein turnover is a highly regulated process responsive to AA intake. Protein turnover is a relatively slow process that reflects the rates of a continuous exchange between muscle proteins and the free AA pool by anabolic and catabolic processes. In healthy adults at rest, skeletal muscle protein turns over at a rate of approximately 1–2% per day, and is largely dependent on AA stimuli during fasting and feeding [92].

Skeletal muscle protein shifts between states of negative and positive protein balance in response to both fasting (i.e., postabsorptive) and feeding (i.e., postprandial). In the fasted state, the rate of MPB exceeds that of MPS, resulting in a net loss of muscle protein. During

MPB, muscle protein becomes catabolized into free AAs and released into plasma to serve as a continuous supply of EAAs for other tissues and organs. Since there is a release of AAs from muscle in the post-absorptive state all EAA precursors to stimulate MPS are intracellular EAAs derived from MPB [19]. Furthermore, some EAAs released from MPB may also be oxidized through which they will be released into plasma and taken up by other tissues to serve as precursors for protein synthesis. Thus, the rate of MPS will always be lower than the rate of MPB in the post-absorptive state, by reason of intracellular EAAs utilization in protein synthesis, oxidative pathways, or efflux to plasma for other tissues [19, 81]. It has been consistently reported in humans at rest the rate of MPB exceeds the rate of MPS by approximately 30% in humans in the post-absorptive state [93]. Periods of negative protein balance become reversed during the fed state when MPS occurs to replace the protein lost during MPB.

During the postprandial phase, digestion and absorption of dietary protein derived AAs elevate plasma AA concentrations. Increased plasma AA concentrations are associated with an increase of the skeletal muscle amino acid pool and a rise in MPS rates, all of which reflect utilization for the synthesis of new muscle protein. For synthesis of new muscle protein, all the EAA, along with the NEAA, must be present in adequate amounts. When EAA precursors for new muscle protein are not available, endogenous stores from MPB will serve as derivatives to elicit an anabolic response. Although the EAA can be recycled, the body cannot produce EAA on its own, meaning the supply of EAA will eventually not be able to meet the global body demands. When a single EAA is present in a limited quantity, the amount of protein that can be synthesized is reduced. Therefore, as noted above, for skeletal muscle protein accretion, MPS must exceed MPB, and for this occurrence exogenous AAs must be provided through the diet.

Thus, dietary EAA are required to stimulate an increase in MPS and replenish the amino acid pool [94].

Initial work defined protein ingestion unaccompanied by other nutrients mimicked the postprandial increases in MPS and inhibition of MPB associated with consumption of a mixed-meal [95]. This work highlighted the regulation of protein metabolism in relation to the adequacy of protein intake. In further demonstration, Rennie and colleagues established that an increase of MPS is related to the blood concentration of AAs, especially of the EAA, and, in particular, leucine, in a dose-dependent way [96].

Among the EAAs, exists the branched chain amino acids (BCAA): leucine, valine, and isoleucine. The BCAA can bypass splanchnic extraction, thus making them readily available for muscle uptake. Given that the BCAAs are predominantly taken up by the muscle, much attention in the literature has been given to their role in skeletal muscle protein turnover. The hypothesis that one or more of the BCAAs may elicit a greater anabolic response through signaling to MPS drove multiple successive studies [97-99]. In support of the hypothesis, an initial study of protein metabolism incubated rat hemi-diaphragm muscle with individual BCAAs and ^{14}C-lysine tracer (indicative of MPS) [97]. The addition of all three BCAA increased MPS rates and the addition of leucine alone resulted in a protein synthetic response of a similar extent, but neither isoleucine, valine, nor any other AA tested produced the solitary effect as seen with leucine. Studies using inhibitory drugs suggest that leucine both decreases MPB and stimulates MPS, but did not do so through the stimulation of transcription or translation [97]. Later, additional studies *in vitro* and in perfused rat hindlimbs have suggested that leucine exerts anabolic effects via enhanced rates of mRNA translation [98, 99].

Since then, subsequent *in vivo* studies have strengthened the view that leucine is unique among the BCAAs in its ability to independently stimulates MPS during both the postabsorptive and postprandial state [100-102]. These studies also pioneered the findings that leucine-dependent stimulation of translation initiation and protein synthesis *in vivo* occurs via a rapamycin-sensitive pathway, known as mechanistic target of rapamycin complex 1 (mTORC1). Through studying mTORC1 signaling, it was observed leucine increases MPS in both normal and muscle-atrophying rats; however, the stimulatory response becomes limited by the availability of EAA [3]. In human muscle, the anabolic properties of leucine were confirmed following a large bolus infusion [103], and after oral intake of leucine (3.42 g Leu) [104]. In addition to leucine, several other EAA have been shown to stimulate MPS following large bolus infusions [105]. In most human studies, the ingestion of a high-quality protein or AA solution with extra leucine did not further increase MPS rates [57, 106]. Mitchhell et al. discovered fractional synthesis rates of muscle in older male adults (70 years of age or greater) received no additional benefit from a bolus of 3 grams of leucine following an intake of 15 g of EAAs during the postprandial state [107]. Thus, extra leucine does not further enhance the anabolic effect of an increased EAA intake, justifying the fact that the control of MPS is attributed to the availability of the EAA as opposed to the anabolic stimulatory factor of leucine [19]. However, studies have confirmed the added leucine in addition to the EAA may promote a greater overall anabolic response through a decrease in MPB [106].

EAA in combination with insulin have also been shown to enhance MPS and to reduce MPB [108, 109]. However, insulin does not contribute to the anabolic effects of EAA on MPS as demonstrated by large doses of EAA infusions stimulating MPS even when insulin is clamped at

postabsorptive concentrations [110]. Thus, a decline of MPB in contrast to MPS has been shown to be regulated through the release of insulin following the postprandial state rather than through a direct effect of EAA alone [111]. It follows that the change in MPS is far greater than that in MPB, thus EAA regulate MPS to a greater extent than MPB.

EFFECT OF AGE ON EAA REGULATING SKELETAL MUSCLE PROTEIN TURNOVER

Changes in protein turnover in response to anabolic stimuli are associated with the observed changes in skeletal muscle mass and strength that occur with age [26, 49]. Early studies addressing the role of protein turnover in age-related sarcopenia reported that muscle loss in older adults was due to a decline in basal rates of MPS, elevated basal rates of MPB, or a combination of the two processes. Studies have confirmed in the absence of anabolic stimuli (postabsorptive state) rates of MPB are similar amongst young and older adults, however there is still discussion regarding whether basal rates of MPS are further reduced with aging due to unnoticeable differences in post-absorptive MPS in young and old men [18, 112] and women [16, 113]. The inconclusive findings of basal MPS rates in young and older adults could be a factor may be explained by study differences in protein quality, subject digestibility and absorption rates, and protein timing.

An initial study demonstrated that basal rates of MPS were similar in old and young adults, but older adults demonstrated an increase in the splanchnic extraction of dietary AA [94]. Volpi and colleagues observed similar postprandial MPS rates for young and old adults following ingestion of 40 g of oral AA [16, 85].

Recently, similar conclusions were drawn with EAA. The understanding of MPS being a process dependent on the availability of EAAs was acknowledged by Cuthbertson et al. [29] when comparing the muscle synthetic response to bolus oral doses of crystalline (EAA) in young and older adults [18]. As hypothesized, Cuthbertson showed that basal fasted rates of MPS were indistinguishable between young and old, but MPS in older adults was less responsive to ingestion of crystalline EAA. Katsanos et al. later showed that a small bolus of EAA (~7 g) was unable to stimulate MPS in older adults comparable to young adults [57]. However, when EAA intake was doubled [16] or leucine was supplemented with EAA [17] optimal stimulation of MPS comparable to that of younger adults occurred. The resistance of older muscles to a physiological dose of AA was subsequently confirmed by others [17, 26, 57] , and it is now generally accepted older adults require a higher dose of EAAs to acutely stimulate MPS similar to young adults [20-22].

Dietary strategies to curtail anabolic resistance includes meal fortification with leucine and ensuring adequate high quality protein sources containing EAA are provided above the minimum threshold necessary for enhancing postprandial MPS in aging muscle. Excess leucine continues to be effective in stimulating MPS [104]; however, leucine can only stimulate MPS for a short time in absence of the other EAA [114]. Therefore, high quality protein containing a complete EAA profile also has an influence on the observed anabolic response of older adults following a meal. For example, animal and plant protein-containing foods differ in their AA content, absorption kinetics, and nutrient to food matrix interactions. Protein quality and digestibility are distinguishing features between animal and plant proteins. It has been shown

that (40 g) soy protein ingestion results in a lower MPS compared to whey protein, likely due to difference in digestibility and absorption kinetics and relative leucine content [115].

Furthermore, recent investigations have gone on to show dose-dependent effects of 10 g EAA [18] equivalent to ∼20 g of high quality protein [55] yielding more robust anabolic responses in older adults, and bolus doses of EAAs that include 3 g of leucine significantly increasing MPS rates in older adults [17]. Pre and post- feeding synthetic response effects have also shown 30 minutes post ingestion of EAA results in increased myofibrillar protein synthesis which later peaks 90-120 minutes post feed replacing protein loss from the post absorptive state [54]. Specifically, given the blunted response of aging muscle to low doses of AA, it is recommended older adults consume sufficient quantities of 20 g-30 g of high-quality protein per meal to provide the EAA required to elevate rates of MPS and elicit a positive net protein balance [26].

Initially it was proposed that impaired AA absorption might contribute to reduced AA delivery to muscle [116, 117]. Meanwhile, it was later demonstrated older adults experience similar hyperaminoacideamia to younger adults following large doses of AA, suggesting that impaired digestion/absorption of AA is not a limiting factor in MPS [118]. Further studies at the cellular level have attributed anabolic resistance in older adults to the inability for muscle tissue to incorporate intracellular AA into muscle protein leading to an increase in skeletal MPB to meet physiological demands [26, 53, 119].

The cellular mechanisms responsible for the resistance of aging muscle to AA continue to be revealed. The next section will provide an overview of the mechanistic signaling pathways regulating translation and protein synthesis in response to AA at the cellular level.

The sensing of AA will be addressed with further explanation of plausible factors contributing to the dysregulation of aging muscle in response to AA. Lastly, studies exploring how specific AA, especially the EAAs regulate translation and protein synthesis will be discussed.

REGULATORY MECHANISMS FOR AMINO ACID SENSING

It is well established that the GCN2 and mTORC1 signaling pathways are regulated by AA availability and have opposing effects on protein synthesis. When cytosolic and lysosomal amino acid levels are sufficient mTORC1 is activated, which is fundamental for protein synthesis [120]. Leucine has been the focus for many studies, as it alone is one of the most potent regulators of protein turnover, through stimulating mTORC1 signaling [121-123]. Conversely, when AA are not available mTORC1 is in an inactive state resulting in an increase in uncharged tRNA [124]. In answer to accumulating levels of uncharged tRNA, a specific stress response is generated through the GCN2/ATF4 pathway of the integrated stress response[63, 64]. Previous experimentation has proven when leucine is deficient GCN2 activation participates in a constant inhibition of mTORC1 activity [36, 37]. In contrast, despite recent progress in the understanding of the regulation of mTORC1 and GCN2 by AA availability, key aspects of this process in response to EAA remains unknown.

AMINO ACIDS REGULATE TRANSLATION VIA MTORC1

Protein synthesis, including the translation of specific messenger RNA (mRNA), is a dynamic process that is under strict regulation in response to external stimuli and nutrient levels within cells [20]. It is fundamental that cells balance the rate of protein synthesis to the availability of

nutrients. Therefore, complex regulatory mechanisms exist to control protein synthesis in response to intracellular stimuli (EAA). The mTORC1 kinase, often referred as the cellular amino acid sensor, is the primary signaling pathway that controls protein synthesis by both enhancing mRNA translation and upregulating ribosomal protein levels [87, 125]. mTORC1 stimulation is largely responsible for muscle's anabolic response to EAAs, and it is recognized increased MPS only results when sufficient EAAs are available to serve as precursors for protein synthesis and translation [19, 33]. EAAs activate the mTORC1 pathway and modulate downstream targets which promote protein synthesis and translation initiation [28, 29]. It is established administration of rapamycin, an inhibitor of the mTORC1 pathway, blunts EAA-induced mTORC1 signaling of key downstream translation components and protein synthesis [126]. It is accepted for models both *in vitro* and *in vivo* that mTORC1 is fundamental in EAA signaling [34, 126]. The mechanistic understanding of EAAs ability to induce protein synthesis and translation is limited; however, there is continued research focusing on how EAAs are sensed and regulate protein synthesis during fed and fasted conditions [60, 61].

UPSTREAM OF MTORC1- THE ROLE OF AA TRANSPORTERS IN MTORC1 ACTIVATION

After absorption of AA following protein digestion, circulating plasma AA correspond to an increase in the intracellular AA pool for uptake by muscle tissue. AA cannot readily cross the cellular membrane and thus require AA transporters to facilitate entrance into the cell. AA transporters have a functional role in AA sensing to further facilitate downstream signaling to the mTORC1 pathway. Beugnet *et al.* showed that increases in the intracellular amino acid pool up-regulates mTORC1 signaling, resulting in an increased rate of protein synthesis [127]. To

further explore AA induced signaling, it was observed leucine is transported into the cell by specific AA transporters that activate mTORC1 [128]. For instance, intracellular glutamine accumulation was needed to facilitate leucine transport by the system L amino transporter [129]. Over 60 AA transporters have been discovered in mammalian cells, however only a handful are involved in the regulation of mTORC1 activation leading to translation [130]. Thus, it is beyond the scope of this review to discuss all AA transporters. As described in a review by Broer et al. SNAT1 (SLC38A1), SNAT2 (SLC38A2), LAT1 (SLC7A5), ASCT1 (SLC1A4), ASCT2 (SLC1A5), and PAT1 are all transporters that mediate the transport of AA into the cell based upon AA availability [60].

Sodium-coupled neural amino acid transporter 1 and 2 (SNAT1/2) act as transceptors, which can be defined as transporters that exhibit functions as transporters and sensors [131]. SNAT 1/2 import AA such as glutamine, alanine, serine, asparagine, and cysteine. Once these specific AA are inside the cell they serve as exchange substrates to import other AA that cannot be actively transported without assistance; Broer et al., stated these are many of the EAA, such as the BCAA. For example, SNAT1/2 imports glutamine which acts as an exchange substrate for the BCAA, leucine [131]. The presence of intracellular glutamine transported by SNAT 2 is crucial for cellular uptake of leucine through another transporter LAT1(L-type amino acid transporter 1) [61].LAT1 transports large AA such as phenylalanine, tyrosine, leucine, and tryptophan. Also PAT1, proton assisted AA transporter, not only facilitates the transport of alanine, glycine, and proline, but is also is thought to act as a receptor through a signaling mechanism that regulates mTORC1 activation [132]. SNAT1/2, Lat1, and PAT1 are all expressed in various tissues including skeletal muscle where they act as key modulators of mTORC1

signaling [133, 134]. *In vitro* studies were amongst the first evidence to show the crucial role of AA transporters in the AA-induced activation of mTORC1. In L6 muscle cells, silencing SNAT2 expression inhibited AA transport causing a marked reduction in protein synthesis due to reduced activation of translation initiation factors downstream of mTORC1 [135]. Furthermore, SNAT2 inhibition caused the depletion of cellular glutamine, resulting in depletion of leucine [128].

It is known that amino acid deprivation profoundly affects overall protein synthesis by impairing mTORC1 activation and increasing the rate of uncharged tRNAs which activate the GCN2 pathway. Additionally, mTORC1 signaling has been proposed to be a regulator of ATF4 and AA transporter expression, as demonstrated by cell culture experiments utilizing the inhibitor drug rapamycin [136-138]. Under specific conditions involving the transport of the non-metabolizable SNAT2 substrate MeAIB (α-methylaminoisobutyrate), it was demonstrated this compound could activate mTORC1 signaling via SNAT2 during AA starvation and increase cell growth in MCF7 breast cancer and L6 myotubes [139]. A recent cell culture study in human MCF-7 breast cancer and HEK-293 cell lines also indicated PAT1's primary location on the lysosome regulates mTORC1 by inducing translocation, which is required for the kinase's activation [140, 141]. Follow up *in vivo* research confirmed PAT1's complexes with the Rag GTPases and activates mTORC1 at the lysosomal membrane in mammalian cell types [142]. Though, It is worth noting that in contrast to other findings, overexpression of PAT1 has also been shown to reduce mTORC1 signaling [143]. Presumably, amino acid binding and/or translocation could lead to conformational changes in the transporter and/or altered

interaction with an associated protein complex, which then alters protein synthesis via mTORC1 signaling.

It is cautioned *in vitro* work often does not translate to *in vivo* conditions, particularly in complex tissues such as skeletal muscle; however previous work in biopsies of human skeletal muscle has shown a responsiveness of some AA transporter genes to EAA feeding [59, 144]. EAA feeding increased blood leucine concentrations and was followed by increases in expression of SNAT2, LAT1, and PAT1 in human skeletal muscle 1–3 hours after ingestion of EAA [144]. In this same study ATF4 protein expression of the GCN2 pathway reached significant increases by 2 h post-EAA bring forward the conclusion that an increase in EAA availability upregulates human skeletal muscle AA transporter expression, in an mTORC1-dependent manner and mTORC1 plays a part in driving ATF4 expression.

In terms of the differences of AA transport in young vs old skeletal muscle, it was established postexercise EAA ingestion enhances AA transporter, SNAT2, only in younger adults indicating that age may influence the function of specific AA transporters [145] However, older adults have exhibited a constant higher LAT1 expression compared to younger adults after ingesting leucine [145]. In skeletal muscle of neonatal pigs, SNAT2 protein abundance is consistent with the aging decline in the AA induced activation of mTORC1 that was present in the human data [146]. Yet, contradicting the results in human skeletal muscle LAT1and PAT1 expression in skeletal muscle of older pigs was significantly less than during the neonatal period and is positively correlated with the activation of mTORC1 [146]. These observations on the role of transporters in mTORC1 activation in cell cultures and skeletal muscle of mammalian species have shed new light regarding the role of AA in the mTORC1 pathway.

UPSTREAM OF MTORC1- THE ROLE OF REGULATORY COMPLEXES AND AA SENSORS IN MTORC1 ACTIVATION

Recent discoveries have identified several mechanisms of the AA sensing complex, including cytosolic AA sensors: leucyl-tRNA synthetase, TSC/Rheb, the castors, and the sestrins [27, 147]. These sensors act as regulatory mechanisms of the mTORC1 pathway and have greatly expanded our understanding of the regulation of mTORC1 activity by AA, which will be discussed in further detail after explanation of regulatory complexes controlling mTORC1's activation.

The first step in AA-dependent activation of mTORC1 consists of the translocation of mTORC1 to the lysosomal surface. The discovery of the Ras-related GTP binding (Rag) GTPases at the lysosomal membrane through their association with the Ragulator Complex initiated the understanding of AA signaling to mTORC1 [29, 148]. The presence of AAs modifies the Rag GTPases to an active state that prompts their binding to Raptor allowing for the translocation of mTORC1 to the lysosomal surface where Rheb, a direct activator of mTORC1 resides [29, 149]. Rheb has been shown to directly bind to mTORC1 *in vitro* and provides a key link to extracellular stimuli through the phosphatidylinositide 3-kinase (PI3K) or MAP kinase (extracellular ligand regulated kinase (ERK)) signaling pathways [29]. Furthermore, Rheb is regulated by the tuberous sclerosis complex 1 and 2 (TSC1/2). TSC2 acts as a GTPase activator protein for Rheb and has been shown to inhibit mTORC1 signaling by prompting conversion to an inactive GDP-bound state [150]. There is contradictory evidence whether TSC2 is involved in regulating mTORC1 in response to AA availability [151, 152]. Several lines of evidence indicate that the sensors for AA are located downstream of TSC1/2 [153]. Wolfson and Sabatini further

explained unlike the regulation of mTORC1 by other growth factors via TSC1/2 and Rheb, AA induced activation of mTORC1 appears to involve a separate set of small GTPases of the Rag family (Rag A,B,C,D) [29]. Studies have revealed the lysosomal surface is comprised of a large protein complex including, H+-ATPase (v-ATPase), Lamtor/Ragulator complex, and Rag small GTPases, all of which mediate the AA-sensing of mTORC1 [154]. Within the AA sensing complex, The Rag GTPases form hetero-dimeric complexes consisting of RagA or RagB bound to RagC or RagD. The Rag complexes are attached to the lysosomal membranes via the Lamtor/Ragulator complex [149]. When RagA or RagB are bound to GTP and RagC or Rag D are bound to GDP the Rag GTPases exist in an active state. In the presence of AA, v-ATPase interacts with the Lamtor/Ragulator complex,and initiates the active forms for the Rag small GTPases. The active Rag small GTPases recruit inactive mTORC1 to the lysosomal surface, where it becomes activated by Rheb [148, 149].

In the absence of AA, mTORC1 exists in an inactive state, because the Rag small GTPases are unable to initiate the binding of mTORC1 and Rheb on the lysosomal surface. A recently identified complex, GATOR1/2 are responsible for the regulation of the activity of Rag A/B [155]. GATOR1 functions as a GTPase activating protein (GAP) of Rag A/B and an inhibitor of the AA-sensing pathway, whereas GATOR2 functions as an inhibitor of GATOR1 [156]. The newly discovered Sestrin proteins have been found to interact with GATOR 2 in an AA induced fashion by regulating the subcellular localization of mTORC1 [156]. Sestrin 2, identified as an intracellular sensor for leucine, contains binding pocket that enables leucine to bind causing dissociation of Sestrin 2 from GATOR2 [27, 157]. The negative interaction of Sestrin 2 and GATOR2 negatively regulates GATOR 1, which allows the Ragulator complex to position

mTORC1 on the lysosomal surface to become activated by Rheb [156, 158]. *In vivo* studies using treatments of mTORC1 inhibitors, rapamycin and Tornin1, did not prevent an AA induced decrease in the GATOR2-Sestrin2 interaction, indicating that mTORC1 activity does not control the interaction and the AA sensing takes place upstream of mTORC1 [156].

Additionally, two arginine sensors upstream of mTORC1 have been discovered. One is a solute carrier family 38 member 9 (SLC38A9), a lysosomal transmembrane protein that interacts with both Rag small GTPases and Ragulator [159]. Following SLC38A9, cellular arginine sensor for mTORC1 (CASTOR1), has been identified as an inhibitor of GATOR 2 in the presence of arginine. The binding of arginine and CASTOR1 causes dissociation of CASTOR1 from GATOR 2 [159]. Lastly, Leucyl tRNA synthetase, an enzyme that attaches leucine to its cognate tRNA, also binds to the GTPases, however it's exact influence on mTORC1 is undefined at this time [160].

Transporters and sensors remain as necessary links between AA availability and mTORC1 signaling. In human skeletal muscle, it appears that mTORC1 activity increases the expression of several amino acid transporters and an increased phosphorylation occurs after ingestion of an AA supplementation [24]. Thus, targeting the transporters and sensors associated with AA induced mTORC1 activation may be an important adaptive response to sensitize muscle to a subsequent increase in AA availability. It is worth noting that the consensus model of the amino acid-sensitive mechanism of mTORC1 activation has been described in many excellent reviews [29, 143].

DOWNSTREAM OF MTORC1- MTORC1 SIGNALING IN RESPONSE TO EAA

One of the central roles of mTORC1 is the regulation of protein synthesis through the activation and phosphorylation of downstream substrates that stimulate translational machinery [29]. In response to AA, downstream signaling proteins associated with the mTORC1 pathway have consistently been shown to be hypersensitive to the availability of intracellular AA [161]. In the presence of AA, activated mTORC1 phosphorylates downstream targets including Eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4EBP1) and the 70-kDa ribosomal protein s6 kinase (p70s6K), and key markers of mTORC1 activity and intermediates involved in the regulation of translation initiation and protein synthesis [18, 28]. Phosphorylation of 4EBP1 by mTORC1 reduces the interaction of 4EBP1 and eIF4E, enabling eIF4E to participate in the formation of the eIF4F complex that is required for translation initiation [162]. The phosphorylation of mTORC1 also activates p70s6K, which increases the phosphorylation of ribosomal protein S6 and facilitates the synthesis of some ribosomal proteins, initiation factors, and elongation factors that play important roles in protein synthesis [15].

In most experimental conditions, rapamycin is used as an inhibitor of the mTORC1 pathway to observe the response of AA-induced signaling. Under most conditions tested rapamycin potently suppresses p70S6K1phosphorylation, whereas it only has a marginal effect on 4ebp1 [163]. However, the treatment of L6 myoblasts with rapamycin, has been shown to block the increase in p70S6K1 and 4EBP1 phosphorylation in response to elevated leucine levels [30]. Likewise, rapamycin administration prior to leucine supplementation has been shown to block the increase in p70S6K1 phosphorylation and prevent the normal increase in MPS [31, 32]

. This data suggests that the increase in protein synthesis in response to AA availability is reliant on mTORC1 activation. According to previous research, the phosphorylation of mTORC1 and the subsequent phosphorylation of proteins that regulate translation initiation; p70S6K and 4EBP1, are reduced in aging muscle in the presence of AA as compared with the young [18, 34, 164]. Differences in the availability of such key regulatory proteins may contribute to the reduced capacity of the muscle protein synthetic machinery to aging muscle [34]. In addition, the response to a combined AA and leucine supplement induced S6K1, 4EBP1, and mTOR, in older adults [165]. Thus, phosphorylation of p70S6K1 and 4EBP1 have routinely been used to assess the activity of the mTORC1 pathway [34, 52] and the muscle protein synthetic response to AA supplementation in older skeletal muscle compared to young [4, 166].

A complete analysis of EAA dependent activity regarding regulation of mTORC1 and protein synthesis has yet to be fully defined and remains undefined in aging muscle. *It is established for both in vitro* and *in vivo* models that single EAA can stimulate protein synthesis thru mTORC1 by enhancing mRNA translation and upregulating ribosomal protein levels [21, 34, 40, 167]. In response to a complete profile of the EAA the mechanisms responsible for sensing, transport, and mTORC1 regulation continue to be revealed [34, 126]. It is also recognized deprivation of EAA impairs mRNA translation and potentially inhibits protein synthesis via the GCN2 kinase and phosphorylation of eIF2α [21]. Current evidence suggests that the relative roles of the GCN2 and mTORC1-mediated responses to AA availability may differ depending on the specific AA(s) that is (are) deficient and the particular tissue [36, 38, 39]. *I*mportant links between GCN2 and mTORC1 signaling in response to AA availability have been demonstrated following regulation of single AA, such as leucine [36, 37]. Further exploration of the effects of

EAA supplementation and deprivation on the phosphorylation status of the GCN2 and mTORC1 pathways and protein synthesis are necessary to further the understanding of the EAAs regulatory roles. Although results from *in vitro* studies model how single EAAs can regulate MPS [40-42], a complete profile of the EAA and the relevance of physiological doses to an aging cell model remains to be established. Supplementary research is also needed to determine the regulatory effects of EAA on the signaling pathways associated with MPS and MPB.

EAA MEDIATE AA-SENSING KINASES ASSOCIATED WITH PROTEIN SYNTHESIS

Recent *in vitro* studies in Hela cells, mammary cells, and C2C12 muscle cells have revealed EAA act to regulate mTORC1, observed by an increased phosphorylation status of 4EBP1 [168-170]. Among the EAA, leucine is a principal anabolic stimulus that regulates protein synthesis via mTORC1 [121, 171]. The effects of single AA, especially leucine, on the mechanisms regulating protein synthesis have been studied in greater detail than the effects of a complete profile of the AA, including the EAA and NEAA. Atherton et al., completed an in-depth assessment of each AA, EAA and NEAA, using C2C12 skeletal muscle cells [40]. He and colleagues observed that the NEAA had no effect on activating the mTORC1 signaling mechanism or further phosphorylation of downstream protein 4EBP1, but of the EAAs, leucine stimulated mTORC1, 4EBP1, and p70S6K signaling activity. Other EAAs including lysine, methionine, phenylalanine, tryptophan, and threonine induced increases in the phosphorylation of p70S6K; however, the stimulation of p70s6K by leucine was a more significant rise than with any other AA. Interestingly, isoleucine and valine did not exhibit a stimulatory effect on p70S6K phosphorylation. Additionally, Atherton and colleagues

recognized the phosphorylation of eIF2α and eEF2 was unaffected by EAA stimulation. As follows it was concluded the modulation of anabolic signaling activity by EAA is limited to translation initiation via mTORC1 and p70S6K phosphorylation and not via modulation of the ternary complex or by regulation of elongation. Furthermore, leucine was the most unique amongst the EAA in stimulating anabolic signaling in skeletal muscle cells via mTORC1. Leucine's anabolic properties and the role of mTORC1 signaling was explored further by Talvas et al [42]. This study explored the regulation of protein synthesis by starving C2C12 myoblasts and myotubes of leucine for 1 h and 5 h, respectively. Regarding the mechanistic effects observed during leucine starvation, repressed mTORC1 signaling was recognized by a decreased phosphorylation of S6K1 in myotubes. However, in the presence of rapamycin a decreased phosphorylation of S6K2 and S6 were detected. Talvas and colleagues suggested the regulation of S6K1 phosphorylation may be mTORC1 independent and regulated through other signaling mechanisms [42]. In Talvas's study leucine starved myoblasts responded differently than myotubes. In myoblasts 4EBP1 and eIF4E association increased, subsequently increasing the phosphorylation of eIF2α and contributing to a decrease in protein synthesis. Since similar results were not observed in leucine starved C2C12 myotubes, researchers concluded the activity of 4EBP1 is not a rate limiting step in protein synthesis.

As discussed previously, starvation induces the loss of muscle by decreasing the rate of protein synthesis and increasing protein degradation. The use of *in vitro* analysis of AA deprivation reveals that protein degradation is differently induced than protein synthesis. In terms of protein degradation, the ubiquitin proteasome pathway is the predominant pathway for myofibrillar proteolysis [172]. Sadiq et al., provided evidence that depletion of a mixture of

AA in the media of C2C12 myotubes increases proteolysis and the expression of components of the ubiquitin-proteasome pathway [168]. Down regulation of the ubiquitin proteasome pathway was observed with increasing AA concentrations and leucine supplementation. Conversely, rapamycin was effective at inhibiting the increasing AA concentrations on limiting protein degradation, and it was ineffective at blocking the inhibitory effects of leucine. This study further demonstrated leucine's unique ability to suppress proteolysis in an mTOC1 dependent manner; thus, suggesting that the mTORC1 pathway is influenced by AA availability [168]. A complementary study also in C2C12 cells explored the effects of leucine supplementation on the signaling pathways related to protein degradation when mTORC1 is inhibited. Leucine's unique characteristic once again presented a new understanding, showing leucine's ability to inhibit the expression of protein degradation pathways directly linked to the ubiquitin-proteasome pathway and regulate protein turnover in C2C12 cells [173]. Taken together these studies reason that AA, specifically leucine, may signal through the mTOC1 and ubiquitin-proteasome pathways to regulate protein turnover.

While leucine is known to be a stimulatory factor for protein synthesis several studies have demonstrated important links between the GCN2 and mTORC1 signaling pathways in response its availability. It is suggested that GCN2 activation can contribute to mTORC1 inhibition following leucine depletion [174]. The molecular mechanism and the dynamics of the crosstalk between GCN2 and mTORC1 have taken some time to identify. In multiple mouse embryonic fibroblasts (MEFs) and human cancer cell lines, the mTORC1-GCN2 pathway has stimulated expression of genes involved in amino acid uptake, protein synthesis, and tRNA charging [175].

A study using wild-type mouse embryonic fibroblasts (MEFs) cultured in leucine-free medium, demonstrated within 30 minutes of leucine starvation a decrease in mTORC1 activity can be observed through the decreased phosphorylation of S6K [176]. Using wild-type and *Gcn2*$^{-/-}$ MEFs starved in leucine free medium it was further shown that long term mTORC1 suppression is dependent on GCN2 activation by inducing Sestrin2 [37]. Since ATF4 is a major downstream effector of GCN2 that regulates AA homeostasis [177], it was next tested whether ATF4 was required for mTORC1 suppression upon leucine deprivation. Averous et al., reevaluated this conclusion and observed the activation of GCN2 to be present when leucine or arginine is deficient in AA-growth media [176]. It was concluded the activation of GCN2 is necessary but not sufficient to inhibit mTORC1 signaling and the phosphorylation of eIF2-alpha is necessary for the regulation of mTORC1 by leucine but is independent of GCN2's downstream transcription factor ATF4 [176].

In further detail researchers have evaluated GCN2's activation during leucine deprivation plays a necessary role in inhibiting mTORC1 kinase activity through inducing expression of Sestrin 2, which blocks the localization of mTORC1 to the lysosome for activation by GTPase Rheb [37, 158]. Additionally, Sestrin2 stimulation has been shown to be necessary for cell survival during glutamine deprivation, indicating that Sestrin2 is a critical effector of GCN2 signaling that regulates AA homeostasis through mTORC1 suppression [178].

The effects of the medium lacking specific single EAAs, particularly leucine, arginine, histidine, and methionine, on eIF2 and 4EBP1 phosphorylation and measures of mRNA translation have been studied in HEK293T cells [38]. Methionine starvation caused the most drastic decrease in translation as assessed by polysome formation, ribosome profiling, and a

measure of protein synthesis, but had no significant effect on eIF2 phosphorylation, 4EBP1 hyperphosphorylation or 4EBP1 binding to eIF4E [38]. This finding is significant to compelling past research in rat hepatocytes, that concluded the deprivation of methionine having a greater inhibitory effect on protein synthesis than the deprivation of other EAA [179].

Regarding the effects of the addition of a complete profile of EAA after a starvation period, studies using bovine mammary tissue slices and MAC-T cells demonstrated a significant effect of EAA on eIF2 phosphorylation and mTORC1 activity [169, 180]. Increasing the intracellular EAA concentrations in bovine mammary tissue slices and MAC-T cells resulted in increased mTORC1 and 4EBP1 phosphorylation but was found to be negatively associated with eEF2 phosphorylation. The increased phosphorylation of 4EBP1 observed in both studies after EAA supplementation signifies EAA mediated stimulation of mRNA translation; thus, researchers indicated an mTORC1 independent mediation of 4EBP1 by EAA. A recent discovery by Zhang and Zheng, confirms the mTORC1-independent control of 4EBP1 phosphorylation corresponds to the linking of the EAA involvement in this control [181]. Supplementary experimentation of the effects of EAA starvation and refeeding on the GCN2/eIF2 signaling pathway and milk protein synthesis were analyzed using bovine mammary epithelial cells (MECs) [182]. In response to a 24h EAA starvation, an increased expression of the GCN2 pathway was observed. EAA refeeding to starved MECs resulted in similar GCN2 levels that were observed for the control treatment that did not experience a 24 h starvation period. The phosphorylation of eIF2α was also greater in cells starved of EAA compared with cells supplemented with EAA after the starvation period. Results from this study highlight the ability of mammalian cells to sense EAA availability and respond through the GCN2 mechanism [182]. Furthermore, a lack of

change in the levels of protein synthesis were observed introducing the possibility an insufficiency of EAA may attenuate the protein metabolic response to maintain homeostasis.

Although, the *in vitro* work presented in review provided compelling evidence for EAA to regulate protein synthesis through the mTORC1 pathway. One must note the concentration of AA that have been used in some *in vitro* experiments presented are greater than or nonrelevant to physiological levels. A recent review from Wolfson and Sabatini states, "it is not known how concentrations of amino acids in the media correlate with intracellular amino acid concentrations" therefore, these imitations create debate against the physiological relevance of these studies [27]. Currently gaps in the literature still remain, which provides justification for my thesis.

GAPS IN THE LITERATURE

In aging skeletal muscle, the precise role of mTORC1 regulation of protein synthesis and protein breakdown following an increase in EAA levels remains less defined due to the lack of mechanistic studies. Despite important advances in this field, it remains unclear if the increase of MPS following an elevation of EAA availability is mTORC1 dependent and if the regulatory interactions between GCN2 and mTORC1 influence MPS and MPS when EAA are available. Furthermore, although the anabolic sensitivity to EAA is recognized using *in vivo* studies the regulation of protein synthesis machinery in response to increasing EAA availability in aging skeletal muscle is not defined.

Therefore, the **overall objective** of this thesis was to determine the regulatory effects of EAA on the signaling pathways associated with MPS and MPB using an aging C2C12 muscle cell model.

The **specific aims** of the study were:

Aim 1: Identify the optimal starvation and refeeding time of EAA to generate a MPS response in both young and old C2C12 muscle cells.

Aim 2: Determine the role of physiological EAA doses on markers of MPS and MPB in young and old C2C12 muscle cells using western blot analysis.

We **hypothesized:**

Increasing levels of EAA will increase the phosphorylation of molecular markers associated with MPS and decrease MPB in aging muscle cells through regulation of translation initiation via mTORC1.

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MATERIALS AND METHODS

ANTIBODIES

The following primary antibodies were used at a dilution of 1:1,000 in EveryBlot Blocking Buffer: [The following primary antibodies were purchased from Cell Signaling Technologies, Danvers, MA, USA: p70S6 kinase (#2708) and phospho-p70S6 kinase$^{(Thr389)}$ (#9234), 4EBP1(#9644) and phospho 4EBP1$^{(Ser\ 65)}$ (#9451), mTOR (#2983) and phospho-mTOR$^{(S2448)}$ (#55536), GCN2 (#3302), eIF2α (#3398), and GAPDH (#2118). The following primary antibodies were purchased from Abcam, Cambridge, UK: Anti-EIF2S1$^{(phospho\ S51)}$ (#32157), Anti-GCN2 $^{(phospho\ T899)}$ (#75836), Anti-SESN2/Sestrin-2 (#178518).] The HRP-linked secondary antibody, anti-rabbit IgG, (#7074, Cell Signaling Technologies, Danvers, MA, USA) was diluted at 1:2000 in TBST for primary antibody detection. Precision Protein Strep Tactin conjugate (#1610380, BioRad, Hercules, CA, USA) was diluted at 1:5,000 in the same TBST as the conjugated secondary antibody and used to detect the standard.

CELL CULTURE AND TREATMENTS

The mouse myoblast cell line C2C12 (ATCC® CRL-1772™, Lot #70024392) was purchased from the American Type Culture Collection (Manassas, VA, USA) and used between passage numbers 2-24. Cells were cultured in high glucose 2 mM glutamine, 1 mM sodium pyruvate Dulbecco's modified Eagle's growth medium (DMEM, Gibco™ #11995040; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Gibco™ #16000044, Thermo Fisher Scientific, Waltham, MA, USA), and 1% (v/v) penicillin-streptomycin (Gibco™ #15140122, Thermo Fisher Scientific, Waltham, MA). Cells were grown in filter-top

flasks in a humidified environment of 5% CO_2 at 37°C. Myoblasts were passed when they reached >70% confluency until passage numbers for both a young (passages 2-10) and aging (passages 16-24) cell model was achieved.

C2C12 myoblasts reached 70-80% confluency before being seeded ($1x10^5$cells/cm^2) in 6 well plates and grown for two days in DMEM, high glucose, supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin until >80% confluency was achieved. To initiate differentiation of myoblasts into myotubes, FBS in the DMEM medium was replaced with 2% (v/v) horse serum (HS, Gibco™ #26050088, Thermo Fisher Scientific, Waltham, MA, USA). Myotube progression was monitored for 5-7 days, during which the medium was changed every 24 hours (h) until 100% differentiation was achieved. Differentiated cells were serum and AA starved for 24 h in DMEM without AA (#0500274, Athena Enzyme Systems, Baltimore, MD, USA) and classified as either young or old based on the respective cell passage number.

Within each young and old cell model the following treatments were assigned: control (CON, no treatment), 0.2 x EAA, 1.0 x EAA and 3.0 x EAA treated with or without rapamycin (100 nM; RAP, #553210, Calbiochem, San Diego, CA, USA). All treatments were performed in triplicate and then each experiment was repeated three times, yielding nine wells per treatment. The physiological concentration of AA in each EAA culture media can be found in **Table 1.**

The EAA treatment medium used in this study were purchased through Athena Enzyme Systems (Baltimore, MD, USA). EAA treatment medium was supplemented with a nonlabelled lysine tracer (0.35mM; #0417, Athena Enzyme Systems, Baltimore, MD, USA). At the last hour

of the 24 h starvation period, cells were exposed to RAP for 1 h. Prior to the addition of EAA medium at this stage being 0 h, cells were washed twice with cold 1 x phosphate buffer saline (PBS, Gibco™ #10010023, Thermo Fisher Scientific, Waltham, MA, USA) and afterwards incubated for 1, 6, and 24 h in the respective EAA culture media. The CON treatments preceded to be harvested at 0 h described below. An overview of the study design containing the treatments and harvesting procedure can be found in **Figure 1.**

HARVESTING AND PREPARATION OF CELLULAR PROTEIN LYSATES

After the incubation period, cells were harvested at 0, 1, 6, and 24 h. Cellular protein lysates were prepared on ice as follows. Media was removed and each well was rinsed twice with cold PBS. Cells were dislodged by scraping with a cell scraper and harvested in 200 µl of Laemmli sample buffer 2x (4% SDS; 10% 2-mercaptoethanol; 20% glycerol; 0.004% bromophenol blue; 0.125 M Tris-HCl). Lysed cells were collected in a chilled microcentrifuge tube and incubated on ice for 15 minutes. Samples were then centrifuged at 12,000 x rpm for 5 minutes at 4℃ to pellet the cellular debris and stored at -80 °C until analysis. When ready for analysis, samples were thawed on ice and boiled for 10 minutes at 90°C to achieve denaturation for SDS/PAGE (sodium dodecyl polyacrylamide gel electrophoresis).

WESTERN BLOTTING AND IMMUNOBLOTTING

Proteins were separated by SDS-Page, where equal amounts of total protein of cell lysate (12 µL) along with the Precision Plus Protein Western C Standard (#1610376, BioRad, Hercules, CA, USA) were loaded in each lane of a 7.5% or 12% (depending on protein size) Mini-

PROTEAN TGX Precast Protein Gel (Bio-Rad, Hercules, CA, USA) and subsequently transferred to a polyvinylidene difluoride membrane (PVDF, BioRad, Hercules, CA, USA) using the Mini-PROTEAN Tetra Cell System (BioRad, Hercules, CA, USA). Tris Glycine SDS running buffer (#1610732, BioRad, Hercules, CA, USA) and Tris/Glycine transfer buffer (#1610734, BioRad, Hercules, CA, USA) were used for protein separation and transfer. Gels ran for 1 h at a constant voltage of 150 V. For complete transfer to be achieved, low molecular weight proteins (<150 kDa) were transferred for 1 h at a constant voltage of 100 V, whereas large molecular weight proteins (>150 kDa) were transferred at 70 V for 3 H. Membranes were blocked in EveryBlot Blocking Buffer (#12010020, BioRad, Hercules, CA, USA) for 5 minutes at room temperature and were then incubated in primary antibody at 4 °C overnight.

The next day, membranes were washed 3 times for 5 minutes with Tris-buffered saline with Tween 20 (TBST, 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% v/v Tween 20) and exposed to the HRP- conjugated secondary antibody for 1 h at room temperature. Following another series of three washes in TBST, blots were developed in Clarity Western ECL Substrate (#170-5060, BioRad, Hercules, CA, USA) for protein detection during enhanced chemiluminescence (ECL) imaging. Images were captured using FluorChem M (ProteinSimple San Jose, California, USA). Membranes probed with phosphorylated proteins were stripped and restained for total proteins. Efficiency of stripping was checked by chemiluminescent imaging. Once stripping was satisfactory, the membranes were washed with TBST 3 times for 5 minutes each, then blocked before proceeding to the antibody incubation.

AlphaView software (ProteinSimple San Jose, California, USA) multiplex band analysis tool with background subtraction was used for band densitometry measurements.

To calculate, relative phosphorylation index for proteins, phospho-protein band intensity was divided by total protein band intensity and normalized to the control. This normalized phosphorylation index was first calculated for each sample and replicate blot and then averaged between blots from replicate experiments. GAPDH was used as a loading control and was used to normalize SESN2. Phosphorylated proteins were normalized to the total protein content of p70S6K, 4EBP1, mTOR, GCN2, eIF2α, and SESN2. Finally, all treatments were expressed as the fold change compared with control.

Regarding study I, cellular protein lysates for both young and old muscle cells from the 6 h incubation period not treated with RAP were used to determine p70S6K and 4EBP1 phosphorylation in response to the physiological EAA dose. Study II used cellular protein lysates from 1, 6, and 24 h with and without the RAP treatment to determine the phosphorylation of p70S6K, 4EBP1, mTOR, GCN2, eIF2α, and SESN2 in response to the physiological EAA dose.

STATISTICAL ANALYSIS

Statistical significance was determined using GraphPad Prism 9.3.1 Software (La Jolla, CA) by a one-way analysis of variance (ANOVA) followed by Tukey's test. Results are presented as means ± SEM and are representative of at least three independent experiments. Statistical significance is defined as P values < 0.05.

RESULTS

In study I, the effects of EAA on downstream signaling proteins of the mTORC1 pathway were investigated in both young and old C2C12 murine muscle cells. It was found young muscle cells treated with 0.2, 1.0, and 3.0 x EAA increased phosphorylation of p70S6K at the mTORC1 specific site Thr^{389} compared to the CON ($p<0.05$) (**Figure 2B**). Phosphorylation of 4EBP1 on serine 65, a major downstream target of mTORC1, increased following 0.2 x EAA in young cells and 3.0 x EAA in old cells compared to CON ($p<0.05$) (**Figure 2C**).

In study II, the effects of EAA and RAP on molecular signaling pathways and markers of protein synthesis were examined in young and old murine skeletal muscle cells. At 1,6, and 24 hours of EAA treatment RAP inhibited the effect of EAA on stimulating phosphorylation of p70S6K in both young and old muscle cells (**Figure 3**). In young cells, 1 h supplementation of 0.2, 1.0, and 3.0 x EAA increased phosphorylation of p70S6K at Thr^{389} compared to the CON ($p<0.05$) (**Figure 3B**). No significant differences amongst treatments were observed in old muscle cells after 1 H of EAA supplementation (**Figure 3C**). When 0.2, 1.0, and 3.0 x EAA were supplied for 6 h, p70S6K phosphorylation increased in both young and old muscle cells compared to the CON ($p<0.05$) (**Figure 3D, 3E**).

Additionally, in young cells phosphorylation of p70S6K at 6 h increased to a larger extend in response to all EAA treatments compared to treatments with EAA+ RAP ($p<0.01$) (**Figure 3D**). Compared to treatments only receiving EAA ($p<0.05$), RAP inhibited the phosphorylation of p70S6K at 6 h in old muscle cells treated with 1.0 and 3.0 x EAA+RAP (**Figure 3E**). The phosphorylation of p70S6K in young and old muscle cells was unaffected by EAA and RAP supplementation at 24 h (**Figure 3F, 3G**).

Phosphorylation of 4EBP1 in response to RAP and EAA showed no differences amongst treatments in both young and old muscle cells at 1 and 24 h (**Figure 4B, 4C, 4F, 4G**). However, at 6 h phosphorylation of 4EBP1 in young muscle cells increased following 0.2 x EAA compared to CON ($p<0.01$) (**Figure 4D**). In comparison to 0.2 x EAA ($p<0.05$), RAP + 1.0 and 3.0 x EAA blunted phosphorylation of 4EBP1 at 6 h in young muscle cells (**Figure 4D**). Although not significant, hyperphosphorylation signified by darker band intensity in the Western Blot images shows phosphorylation of 4EBP1 is more predominant when RAP is not present with the EAA (**Figure 4A**).

mTORC1, the upstream regulator of p70S6K and 4EBP1, responded differently in young and old cells during RAP and EAA supplementation. mTORC1 phosphorylation activity was not significantly altered by EAA or RAP at 1 h and 6 h in young and old muscle cells (**Figure 5B, 5C, 5D, 5E**). While in young cells, a 24 h treatment of 0.2 and 1.0 x EAA increased the phosphorylation of mTORC1 compared to cells treated with EAA + RAP ($p<0.05$) (**Figure 5F**). Additionally, old cells treated with 3.0 x EAA for 24 h exhibited a greater phosphorylation of mTORC1 compared to CON+ RAP and EAA+RAP treatments ($p<0.01$) (**Figure 5G**).

Concerning the key pathway that senses AA deficiency through binding to uncharged tRNAs, regulation of GCN2 by EAA and RAP, revealed no key differences in young and old muscle cells (**Figure 6 B-G)**. During AA deficiency, activated GCN2 phosphorylates eIF2α. Regarding this study, eIF2α phosphorylation in old muscle cells decreased with 24 h treatments of RAP+EAA compared to the CON and EAA treated cells ($p<0.01$, $p< 0.0001$, respectively) (**Figure 7G**). Like the effects of EAA and RAP on GCN2 in young and old muscle cells, no

differences were observed for the phosphorylation of Sestrin2, a critical effector of GCN2 signaling (**Figure 8 B-G**).

All representative western blot analyses (A) were performed to determine the phosphorylation of phosphorylated and total protein and controlled to the housekeeping protein, GAPDH (**Figure 9A**).

DISCUSSION

The mTORC1 pathway has received considerable attention as a key regulator of protein synthesis following an increase in amino acid availability. Data obtained from *in vitro* and *in vivo* studies suggest that the EAA are generally responsible for the stimulation of MPS [1-3]. It is also recognized the AA stimulation of muscle protein anabolism in aging muscle is less responsive compared to young muscle [4-7]. The activation of mTORC1 and phosphorylation of its downstream proteins, p70S6K1 and 4EBP1, by the anabolic properties of EAAs are well defined [8, 9]. For example, treatment of cells with rapamycin, an inhibitor of the mTORC1 pathway has been shown to block the increase in p70S6K1 and 4EBP1 phosphorylation in response to elevated leucine levels in L6 myoblasts [10]. Likewise, rapamycin administration prior to leucine supplementation has been shown to block the increase in S6K1 phosphorylation and prevent the normal increase in MPS [11, 12] . This data suggests that the increase in protein synthesis in response to AA availability is reliant on mTORC1 activation. Thus, phosphorylation of p70S6K1 and 4EBP1 have routinely been used to assess the activity of the mTORC1 pathway [13, 14] and the muscle protein synthetic response to EAA supplementation in older skeletal muscle compared to young [15, 16]. According to previous research,

differences in the availability of such key regulatory proteins may contribute to the reduced capacity of the muscle protein synthetic machinery to sense EAA in aging muscle [13]. In addition, the response to AA and leucine induced S6K1, 4EBP1, and mTOR, phosphorylation has been reduced in older adults [17].

In vitro experiments have also suggested mammalian cells possess a second amino acid-sensing kinase aside from mTORC1. The GCN2 kinase senses AA deficiency and consequently phosphorylates eIF2α , an inhibitory factor of translation initiation [18]. Important links between GCN2 and mTORC1 signaling in response to AA availability has been demonstrated following regulation of single amino acids, such as leucine [19]. The molecular mechanism and the dynamics of the GCN2 and mTORC1 pathways have taken some time to identify. Therefore, this thesis provides insight to the activation of both the GCN2 and mTORC1 pathways in response to EAA in an aging model.

Although results from *in vitro* studies model how single EAAs can regulate MPS [20-22], a complete profile of the EAA and the relevance of physiological doses to an aging cell model remains to be established. Supplementary research is also needed to enhance the establishment of EAA signaling via the mTORC1 to control MPS and MPB.

Study I, aimed to demonstrate the effects of EAA on downstream signaling proteins of the mTORC1 pathway in both young and old C2C12 murine muscle cells. In line with previous results, this study found that the most potent increase in p70S6K phosphorylation occurred when young muscle cells compared to old muscle cells were treated with EAA. Therefore, the decreased phosphorylation of p70S6K in old muscle cells was reflective of the impaired response of older muscle to EAA. Considering the human data suggesting aging muscle is less

responsive to lower doses of EAA and the phosphorylation of 4EBP1 is dampened in older muscle, our data demonstrates an increase in 4EBP1 phosphorylation following 0.2 x EAA in young cells and 3.0 x EAA in old cells compared to CON ($p<0.05$). In support of other research, we conclude a larger dose of EAA is necessary to generate a comparable response of 4EBP1 phosphorylation in old muscle cells to that of the young. The increased phosphorylation of p70S6K and 4EBP1 with the presence of EAA in the media signified EAA supplementation mediated stimulatory signals on mRNA translation and MPS in C2C12 cells.

Study II was designed to evaluate if the EAA positively regulate translation via a mTORC1- dependent mechanism in a rapamycin sensitive manner. Rapamycin is a potent inhibitor of mTORC1 activity [23] and the EAA are potent stimulators of the mTORC1 pathway [13]. Therefore, to understand the mechanistic role of mTORC1 signaling the activity of key signaling proteins downstream of mTORC1 were examined at time points known to be influenced *in vitro* by EAA supplementation [24, 25]. Also, since studies have reported that aged skeletal muscle exhibits a blunted protein anabolic response to AA that is more evident at lower doses [26] a small, normal, and supra physiological EAA treatment was administered. These physiological doses were similarly used in other vitro studies [25]. Altogether, the effects of EAA dose, refeeding time, and RAP on the phosphorylation of the mTORC1 and GCN2 molecular pathways and their regulatory proteins were examined in both young and old murine skeletal muscle cells.

At 1,6, and 24 hours of EAA treatment RAP completely blocked an increase in the phosphorylation of p70S6K in both young and old muscle cells. Findings of other studies using various cell lines have shown the administration of leucine after AA starvation stimulates

protein synthesis and promotes phosphorylation and activation of S6K1 via mTORC1 [10, 27-29]. Our data supports the findings and indicates that mTORC1 is required for EAA-dependent activation of p70S6k. However, previous studies in human embryonic kidney 293 cells have reported that regulation of translation by AA is only partially sensitive to rapamycin and does not require S6K1 activity [30]. A recent study observed supplementation of leucine in the presence of rapamycin is unable to restore S6K1 phosphorylation in C2C12 myotubes [22]. Thus, the idea was established that S6K1 phosphorylation may be regulated by leucine availability through an mTOR-independent pathway [22]. In our study, to restore p70S6K phosphorylation in old muscle cells a 6-hour incubation with EAA was needed to increase p70S6K activation greater than the CON ($p<0.05$). There is no definitive data for a time analysis of EAA refeeding in young and old C2C12 cells. Under the conditions of our study, we are limited because we are not able to make direct comparisons amongst each time trial or the young and old model. However, our results correspond to Talvas's study who observed the activation of S6K1 after leucine starvation and later leucine incorporation was greatest between 3-5 hours compared to 1 hour [22].

Many experiments have shown that leucine can modulate mTOC1 and 4EBP1 phosphorylation but does not necessarily lead to enhanced protein synthesis [31, 32]. In our results in C2C12 myotubes, the addition of EAA at three different concentrations induced differential responses in mTOC1 and 4EBP1 phosphorylation throughout all timepoints. Our data indicates that in young cells even the lowest EAA concentration (0.2) could elicit a maximal phosphorylation response of mTORC1 and 4EBP1 phosphorylation greater than the control at 24h ($p<0.05$) and 6h ($p<0.01$), respectively. Similar phosphorylation responses of mTORC1 and

4EBP1 followed the addition of the lowest concentration (1.5 mM) of leucine in C2C12 myotubes; however, in this study the lowest concentration was only increased above the control and no differences amongst treatments were observed [32].

While rapamycin potently inhibited p70S6K phosphorylation when myotubes were treated with rapamycin and EAA, incomplete blocking was observed for 4EBP1 and mTORC1. The incomplete inhibition of mTORC1 signaling has also been observed following AA feeding studies in rodents [33]. Furthermore, studies in various cell types have observed that rapamycin treatment does not completely inhibit the phosphorylation of 4EBP1 at levels that do inhibit S6K1 phosphorylation [34-36]. Choo and colleagues precisely examined rapamycin's potent inhibition of S6K activity throughout a 24 h treatment with 4ebp1 phosphorylation having an initial inhibition of 1-3 h and a reemerged activity within 6 h. In addition to finding 4EBP1 phosphorylation to be rapamycin resistant, this study also concluded the phosphorylation of 4EBP1 does requires mTORC1 activity for cap dependent translation to be initiated [36].

It has also been noted the phosphorylation of mTOR on Ser2448 is not an absolute requirement for mTORC1 kinase activity since all EAA other than isoleucine and valine have been shown to stimulate the phosphorylation of p70S6K in the presence of rapamycin [20]. Therefore, these results explain how rapamycin may not provide complete inhibition of mTORC1 dependent phosphorylation of 4EBP1 or additional pathways must exist to elicit an increase in 4EBP1 phosphorylation following amino acid availability. Also, the signaling of EAA through p70S6K may be initiated through other pathways.

Despite these findings, the data from our current study in C2C12 cells suggests that the dose of rapamycin administered likely did not inhibit mTORC1 activity and was not overcome by an increase EAA availability. However, it was observed the availability of EAA for 24 h was able to increase mTORC1 phosphorylation to overcome the effects of rapamycin. It also appears that in our *in vitro* muscle cell model, a critical threshold may exist at 6 h for the EAA-inducible phosphorylation of mTORC1-mediated signaling proteins p70S6K and 4EBP1. It seems once 6h is surpassed, the anabolic signaling of EAA to these two proteins plateaus, but further work is required to substantiate this theory.

Collectively, the data indicating a relationship between GCN2 and mTORC1 in specific AA conditions are inconsistent. It has been reported that aa starvation does not lead to an accumulation of uncharged tRNA in HeK-293 cells [37], however more recent studies challenge this statement [38, 39]. Jousse and Ron reported that GCN2 is not required for the regulation of the mTORC1 pathway by AA. In addition GCN2 contributes to mTORC1 inhibition by leucine deprivation through an ATF4 independent mechanism [40]. The GCN2 mechanism sustains mTORC1 suppression upon AA starvation by inducing Sestrin2 [41]. Other data established that GCN2 is involved in the inhibition of mTORC1 upon leucine or arginine deprivation [39]. However, the activation of GCN2 alone is not sufficient to inhibit mTORC1 activity, indicating that leucine and arginine exert regulation via additional mechanisms. While the mechanism by which GCN2 contributes to the initial step of mTORC1 inhibition involves the phosphorylation of eIF2α, we show that it is independent of the mTORC1 pathway.

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CONCLUSION

To our knowledge the present study is the first to demonstrate that in C2C12 muscle cells (1) EAA stimulate molecular signaling pathways associated with sarcopenia, (2) that the EAA activate molecular markers of MPS in young and old muscle cells, (3) that EAA stimulate the phosphorylation of p70S6K to a greater extend in young muscle cells compared to old muscle cells, whereas phosphorylation of 4EBP1 is stimulated to a similar degree, and (4) modulation of anabolic signaling activity by EAA is limited to the regulation of MPS via mTORC1 and p70S6K and not via modulation of the GCN2/eIF2α pathway. The primary findings from the present study suggest that the increase in EAA increases the phosphorylation of key molecular proteins associated with MPS and RAP administration may block the activation of key downstream components of the mTORC1 signaling pathway. Taken together, this data supports the fundamental role for mTORC1 activation in the stimulation of MPS in response to EAA.

TABLES

TABLE 1

Amino Acids Concentration in the 0.2 x EAA, 1.0 X EAA, and 3.0 x EAA culture media.

	0.2 x EAA		**1.0 x EAA**		**3.0 x EAA**
Amino Acid	Concentration (mM)	Amino Acid	Concentration (mM)	Amino Acid	Concentration (mM)
Alanine	0.41	Alanine	0.41	Alanine	0.41
Arginine	0.18	Arginine	0.18	Arginine	0.18
Asparagine	0.07	Asparagine	0.07	Asparagine	0.07
Aspartate	0.04	Aspartate	0.04	Aspartate	0.04
Cysteine	0.06	Cysteine	0.06	Cysteine	0.06
Glutamate	0.11	Glutamate	0.11	Glutamate	0.11
Glutamine	0.74	Glutamine	0.74	Glutamine	0.74
Glycine	0.28	Glycine	0.28	Glycine	0.28
Histidine	0.014	Histidine	0.07	Histidine	0.21
Isoleucine	0.018	Isoleucine	0.09	Isoleucine	0.27
Leucine	0.03	Leucine	0.15	Leucine	0.45
Lysine	0.35	Lysine	0.35	Lysine	0.35
Methionine	0.01	Methionine	0.05	Methionine	0.15
Phenylalanine	0.016	Phenylalanine	0.08	Phenylalanine	0.24
Proline	0.33	Proline	0.33	Proline	0.33
Serine	0.53	Serine	0.53	Serine	0.53
Threonine	0.05	Threonine	0.25	Threonine	0.75
Tryptophan	0.014	Tryptophan	0.07	Tryptophan	0.21
Tyrosine	0.07	Tyrosine	0.07	Tyrosine	0.07
Valine	0.038	Valine	0.19	Valine	0.57

FIGURES

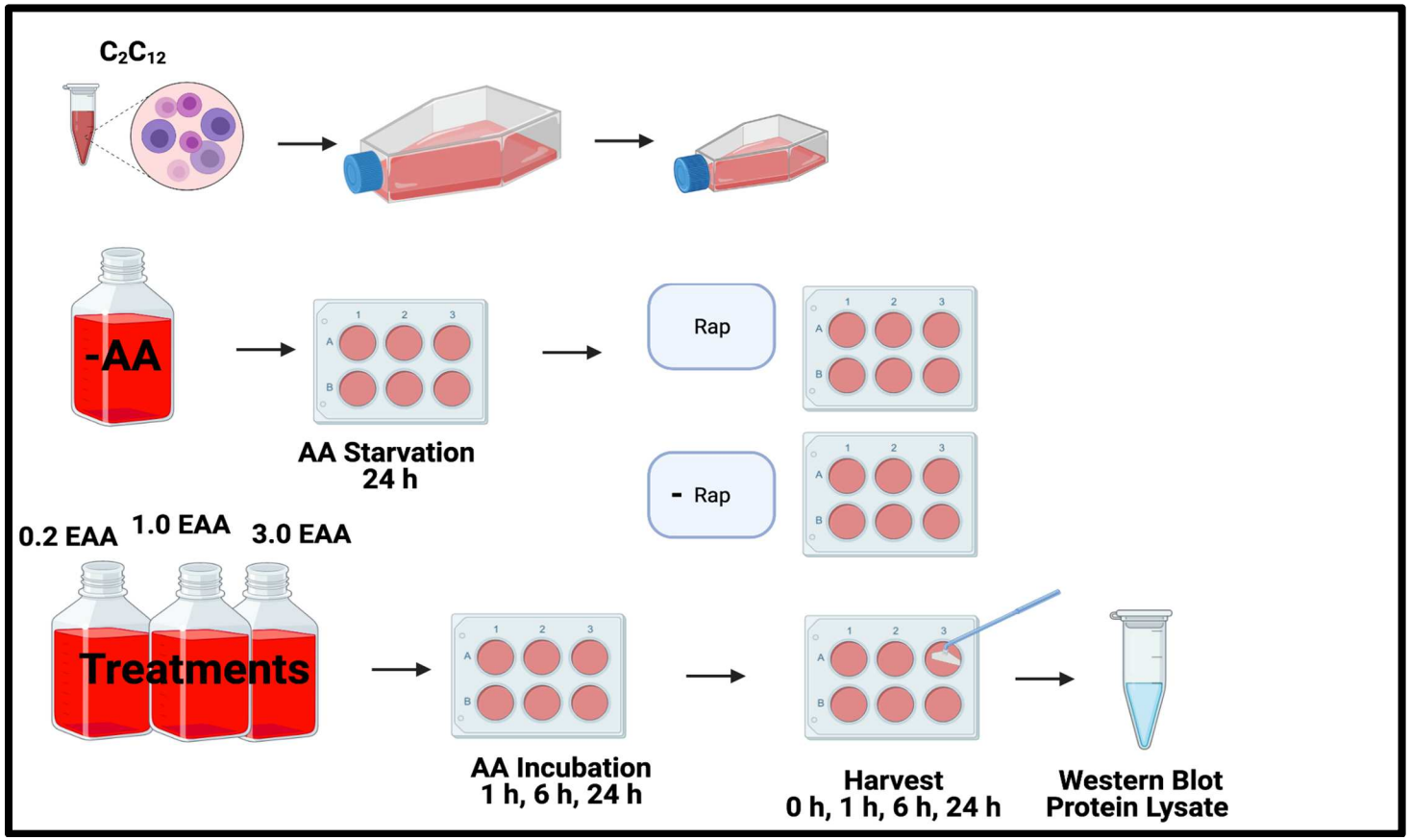

FIGURE 1. Flowchart for the study design of culturing and passing C2C12 cells from flasks to 6 well plates prior to treatment with amino acid (AA) free media, rapamycin (RAP), and essential amino acid (EAA) treatments. An AA starvation period of 24 h was followed by the EAA treatment incubation period of 1,6, and 24 h. The protein lysate was collected at 0, 1, 6, and 24 h and used for Western Blot analysis. All treatments were performed in triplicate and then each experiment was repeated three times, yielding nine wells per treatment.

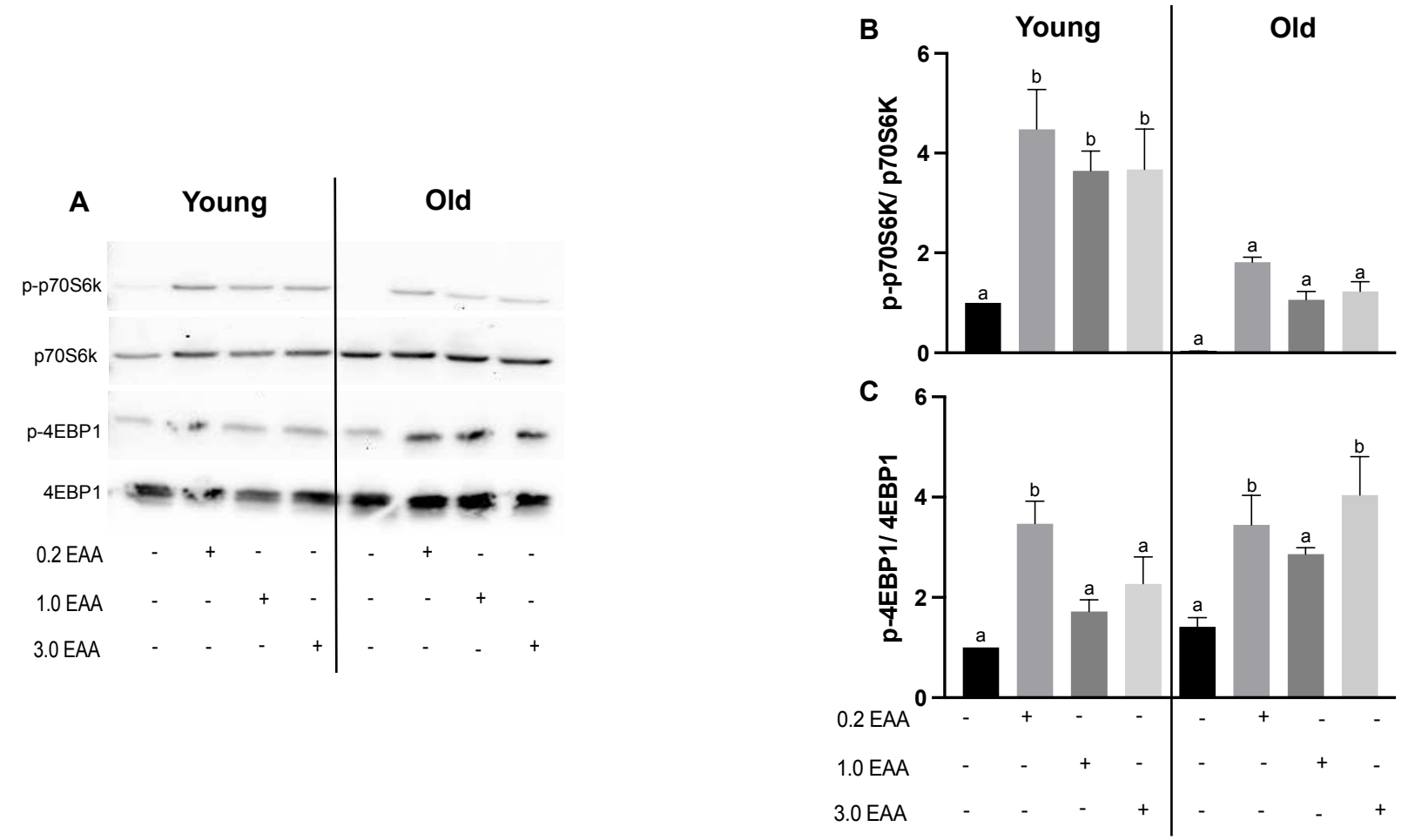

FIGURE 2. Essential amino acids (EAA) activate downstream proteins of the mechanistic target of rapamycin complex 1 (mTORC1) in young and old C2C12 myotubes treated with 0.2, 1.0, and 3.0 x EAA for 6 h. Representative Western Blot Analysis (A) was performed to determine the phosphorylation of phosphorylated and total p70S6K1 and 4EBP1 (A). The relative phosphorylation level of p70S6K1 (B) and 4EBP1 (C) were expressed as the fold-change to control group as a ratio of phosphorylated protein to total protein. Values not sharing the same letter are significantly different; $P<0.05$. Values represent mean ± SE (n=9).

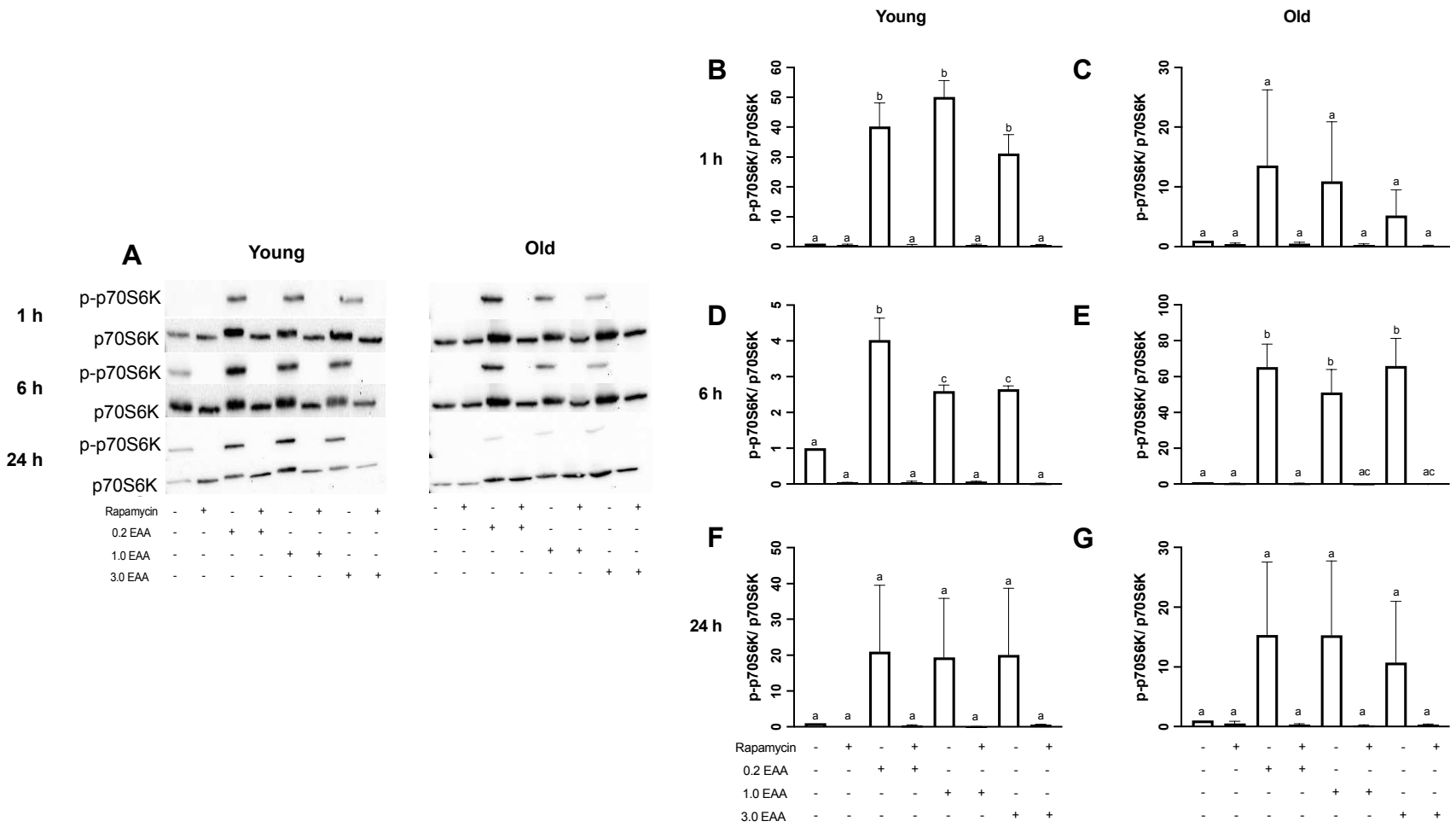

FIGURE 3. Phosphorylation of p70S6K1 in young and old C2C12 myotubes receiving the following treatments: 0.2, 1.0, and 3.0 x EAA, with or without at rapamycin (RAP) for 1, 6, and 24 h. Representative Western Blot Analysis (A) was performed to determine the phosphorylation of phosphorylated and total p70S6K1. The relative phosphorylation level of p70S6K1 in young cells (B) and old cells (C) at 1 h, young cells (D) and old cells (E) at 6 h, and young cells (F) and old cells (G) at 24 h was expressed as the fold-change to control group as a ratio of phosphorylated protein to total protein. Values not sharing the same letter are significantly different; $P<0.05$. Values represent mean ± SE (n=9).

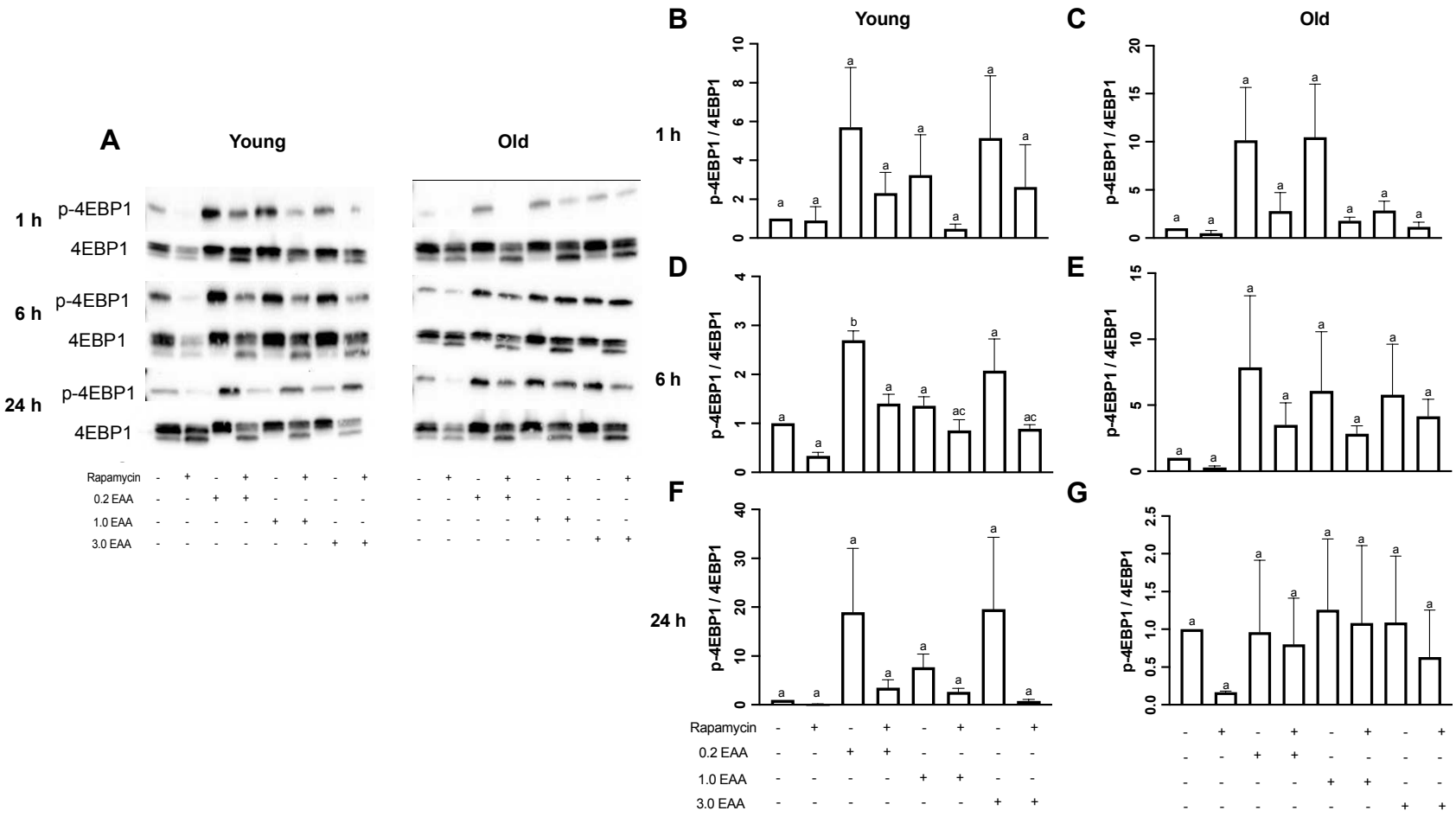

FIGURE 4. Phosphorylation of 4EBP1 in young and old C2C12 myotubes receiving the following treatments: 0.2, 1.0, and 3.0 x EAA, with or without at rapamycin (RAP) for 1, 6, and 24 h. Representative Western Blot Analysis (A) was performed to determine the phosphorylation of phosphorylated and total 4EBP1. The relative phosphorylation level of 4EBP1 in young cells (B) and old cells (C) at 1 h, young cells (D) and old cells (E) at 6 h, and young cells (F) and old cells (G) at 24 h was expressed as the fold-change to control group as a ratio of phosphorylated protein to total protein. Values not sharing the same letter are significantly different; $P<0.05$. Values represent mean ± SE (n=9).

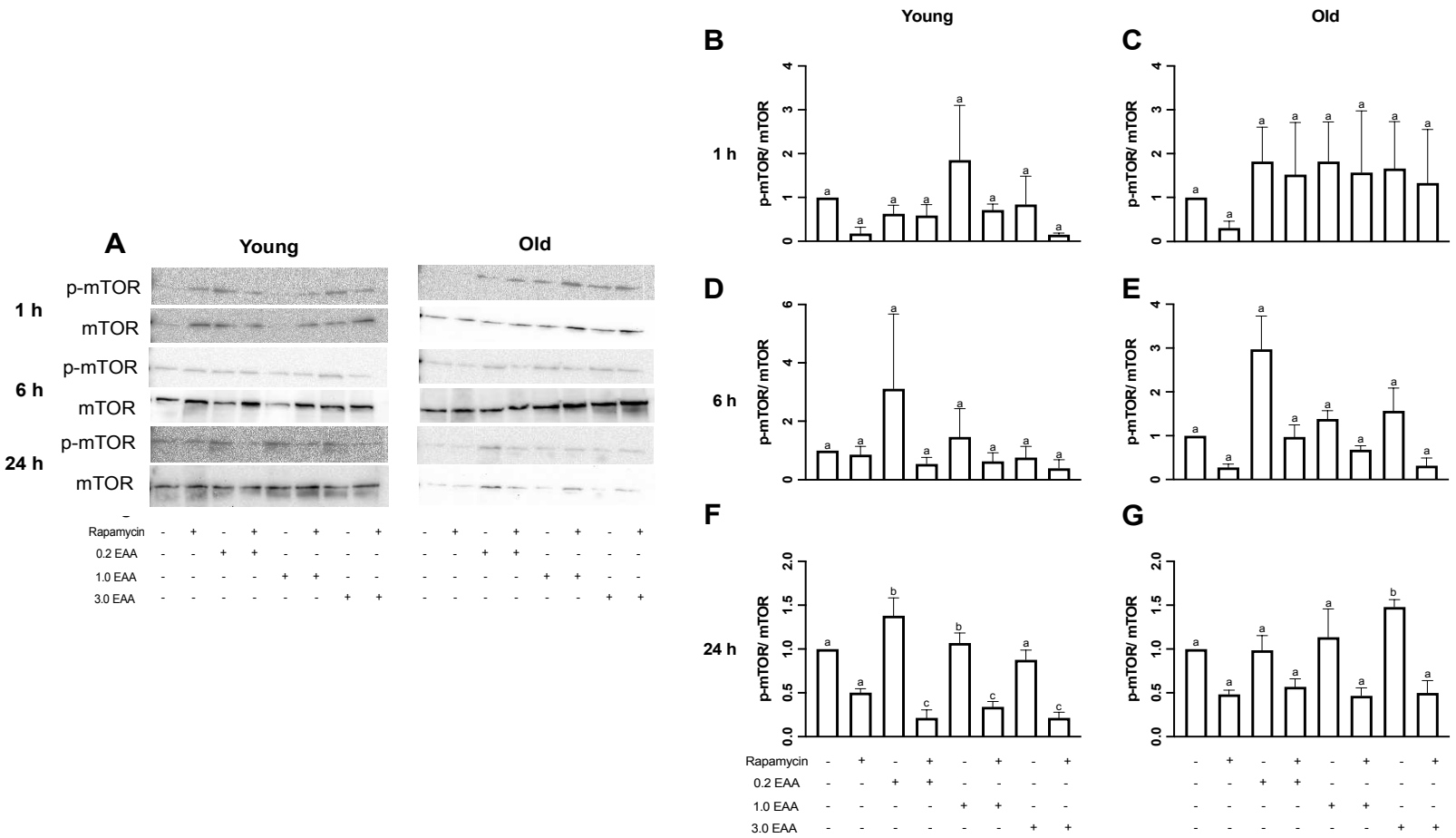

FIGURE 5. Phosphorylation of mTOR in young and old C2C12 myotubes receiving the following treatments: 0.2, 1.0, and 3.0 x EAA, with or without at rapamycin (RAP) for 1, 6, and 24 h. Representative Western Blot Analysis (A) was performed to determine the phosphorylation of phosphorylated and total mTOR. The relative phosphorylation level of mTOR in young cells (B) and old cells (C) at 1 h, young cells (D) and old cells (E) at 6 h, and young cells (F) and old cells (G) at 24 h was expressed as the fold-change to control group as a ratio of phosphorylated protein to total protein. Values not sharing the same letter are significantly different; $P<0.05$. Values represent mean ± SE (n=9).

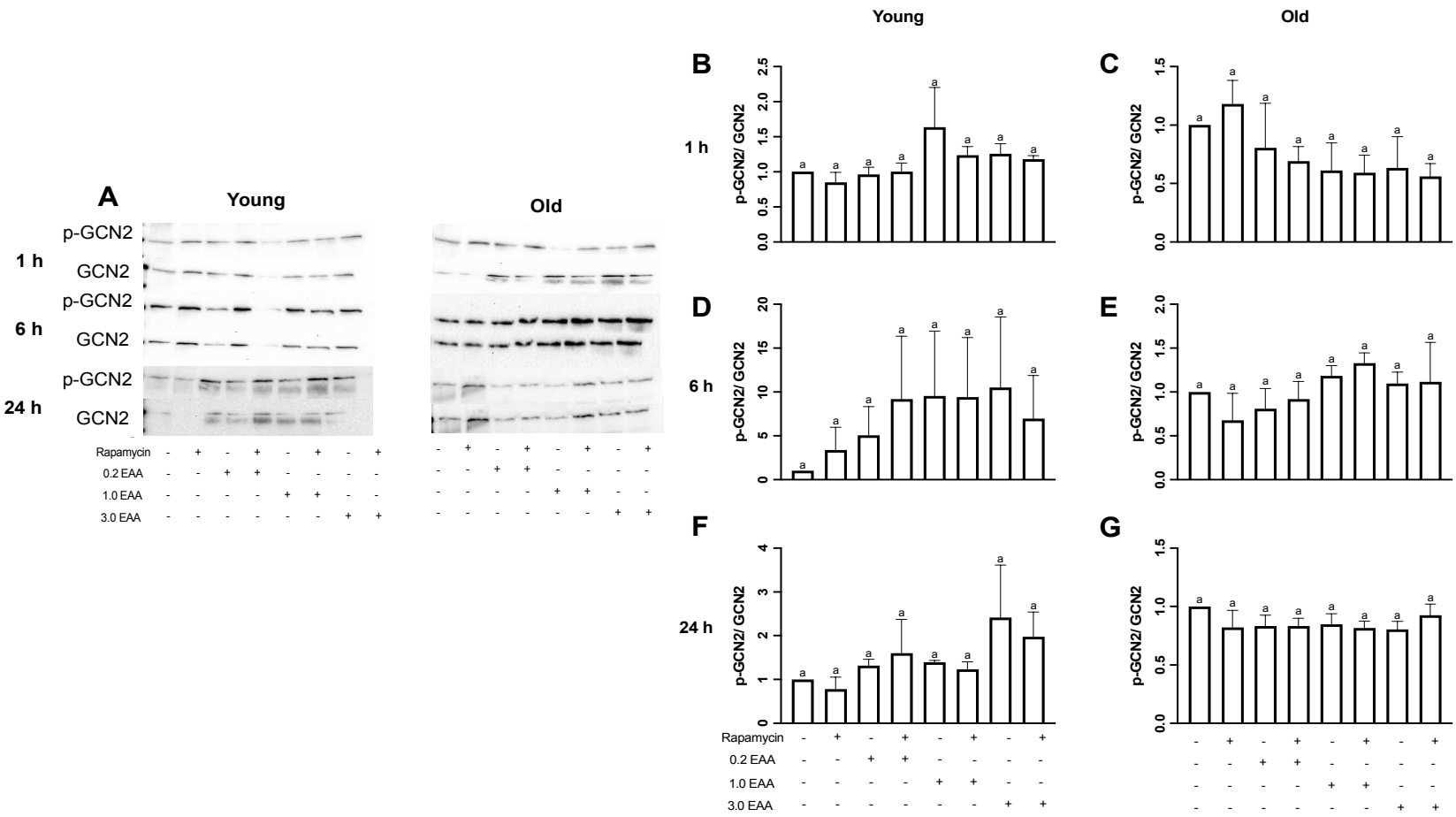

FIGURE 6. Phosphorylation of GCN2 in young and old C2C12 myotubes receiving the following treatments: 0.2, 1.0, and 3.0 x EAA, with or without at rapamycin (RAP) for 1, 6, and 24 h. Representative Western Blot Analysis (A) was performed to determine the phosphorylation of phosphorylated and total GCN2. The relative phosphorylation level of GCN2 in young cells (B) and old cells (C) at 1 h, young cells (D) and old cells (E) at 6 h, and young cells (F) and old cells (G) at 24 h was expressed as the fold-change to control group as a ratio of phosphorylated protein to total protein. Values not sharing the same letter are significantly different; $P<0.05$. Values represent mean ± SE (n=9).

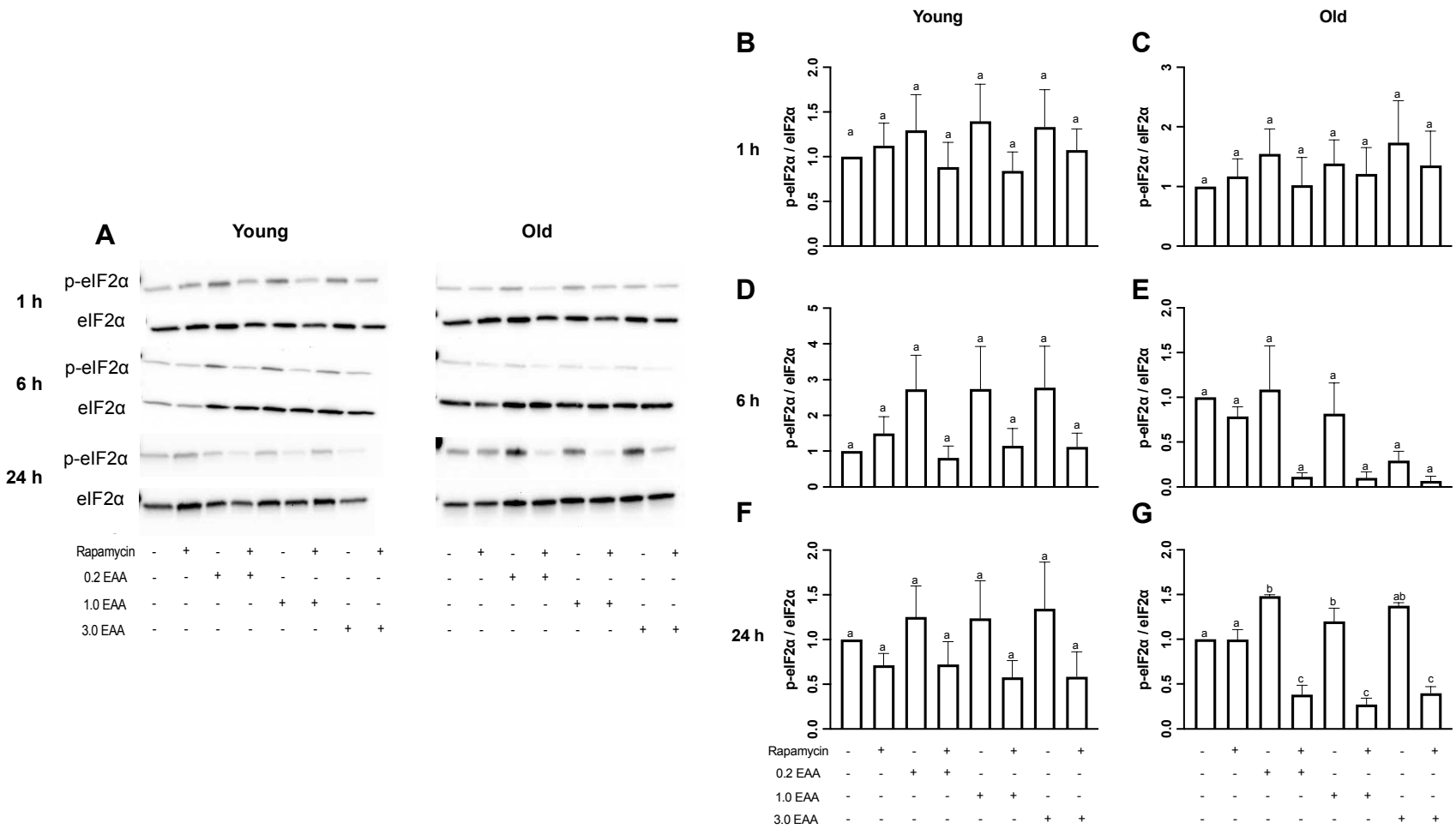

FIGURE 7. Phosphorylation of eIF2α in young and old C2C12 myotubes receiving the following treatments: 0.2, 1.0, and 3.0 x EAA, with or without at rapamycin (RAP) for 1, 6, and 24 h. Representative Western Blot Analysis (A) was performed to determine the phosphorylation of phosphorylated and total eIF2α. The relative phosphorylation level of eIF2α in young cells (B) and old cells (C) at 1 h, young cells (D) and old cells (E) at 6 h, and young cells (F) and old cells (G) at 24 h was expressed as the fold-change to control group as a ratio of phosphorylated protein to total protein. Values not sharing the same letter are significantly different; $P<0.05$. Values represent mean ± SE (n=9).

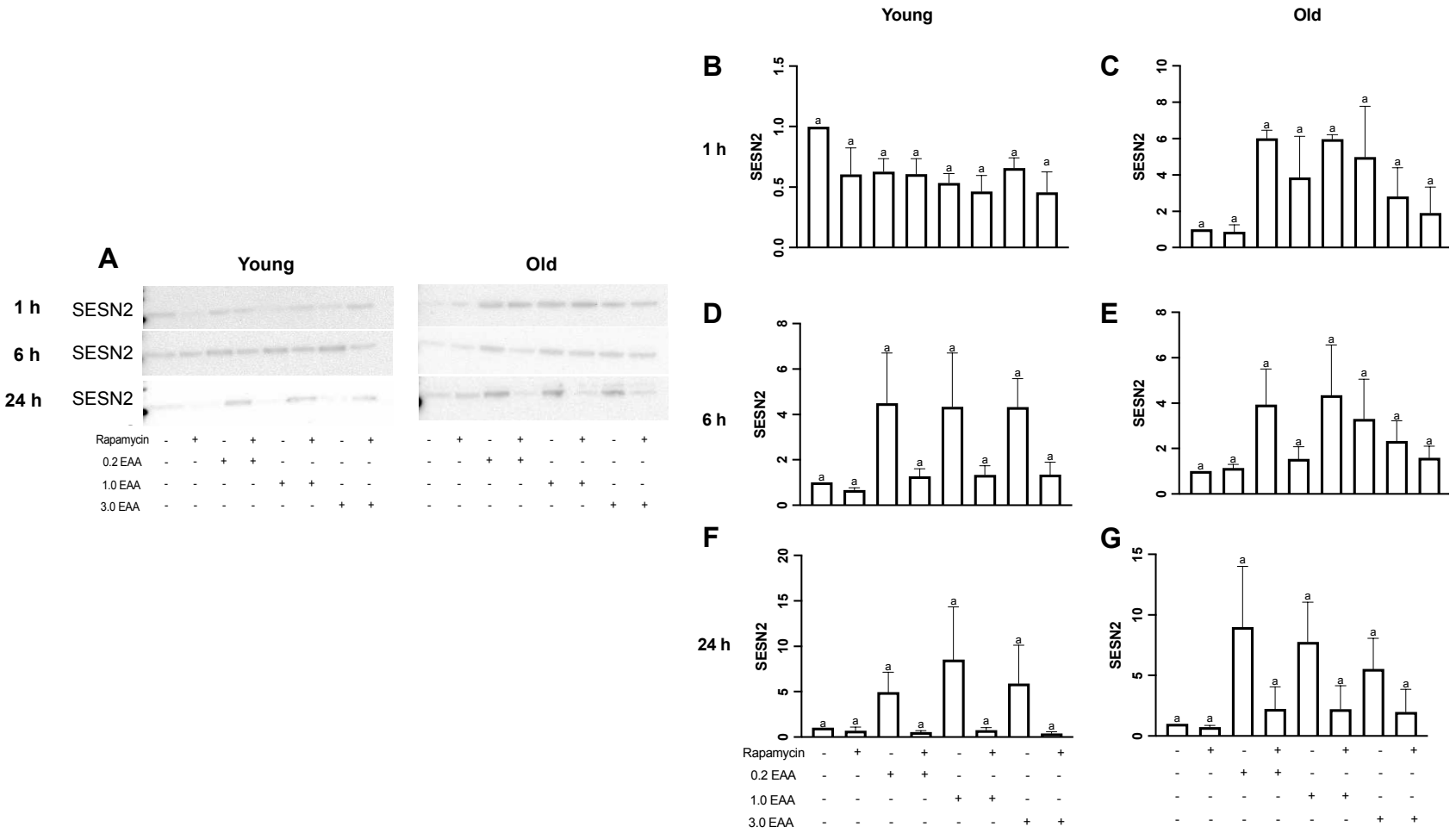

FIGURE 8. Phosphorylation of SESN2 in young and old C2C12 myotubes receiving the following treatments: 0.2, 1.0, and 3.0 x EAA, with or without at rapamycin (RAP) for 1, 6, and 24 h. Representative Western Blot Analysis (A) was performed to determine the phosphorylation of phosphorylated and total SESN2. The relative phosphorylation level of SESN2 in young cells (B) and old cells (C) at 1 h, young cells (D) and old cells (E) at 6 h, and young cells (F) and old cells (G) at 24 h was expressed as the fold-change to control group as a ratio of phosphorylated protein to total protein. Values not sharing the same letter are significantly different; $P<0.05$. Values represent mean ± SE (n=9).

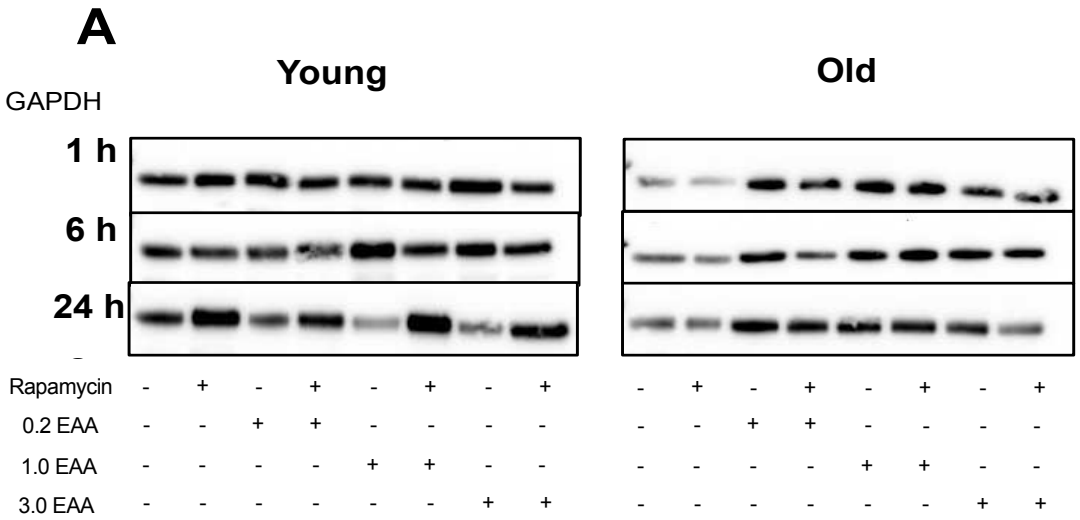

FIGURE 9. Phosphorylation of GAPDH in young and old C2C12 myotubes receiving the following treatments: 0.2, 1.0, and 3.0 x EAA, with or without at rapamycin (RAP) for 1, 6, and 24 h. Representative Western Blot Analysis (A) was performed as a control to the phosphorylated and total proteins measured