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ORIGINAL ARTICLE

Distinct anabolic signalling responses to amino acids in C2C12 skeletal muscle cells

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Abstract The essential amino acids (EAA) activate anabolic signalling through mechanisms, which are unclear in detail but include increased signalling through the mammalian target of rapamycin complex 1 (mTORC1). Of all the EAA, the branched chain amino acid (BCAA) leucine has been suggested as the most potent in stimulating protein synthesis, although there have been no studies investigating the effects of each EAA on anabolic signalling pathways. We therefore undertook a systematic analysis of the effect of each EAA on mTORC1 signalling in C2C12 myotubes whereby cells were serum (4 h) and amino acid (1 h) starved before stimulation with 2 mM of each amino acid. Immunoblotting was used to detect phosphorylated forms of protein kinase B (Akt)/mTORC1 signalling enzymes. The phosphorylation of Akt was unchanged by incubation with EAA. Phosphorylation of mTOR and 4E binding protein-1 (4EBP1) were increased 1.67 ± 0.1-fold and 2.5 ± 0.1-fold, respectively, in response to leucine stimulation but not in response to any other EAA. The phosphorylation of ribosomal s6 kinase (p70S6K1) was increased by stimulation with all EAA with the exceptions of isoleucine and valine. However, the increase with leucine was significantly greater, 5.9 ± 0.3-fold compared to 1.6–2.0-fold for the non-BCAA EAA. This pattern of activation was identical in ribosomal protein s6 (RPS6) with the additional effect of leucine being 3.8 ± 0.3-fold versus 1.5–2.0-fold. Phosphorylation of eukaryotic initiation/elongation factors eIF2α and eEF2 were unaffected by EAA. We conclude that leucine is unique amongst the amino acids in its capacity to stimulate both mTOR and 4EBP1 phosphorylation and to enhance p70S6K1 signalling.

Keywords Amino acids · Anabolic signalling · Skeletal muscle · mTOR · Protein synthesis

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Introduction

Increasing intracellular amino acid availability in skeletal muscle cells serves two major functions: (1) providing substrate for polypeptide biosynthesis, and (2) directing activation (phosphorylation) of the regulatory mechanisms of mRNA translation. This dual function ensures that in postprandial periods, increased availability of amino acids acquired from protein stimulates a transient increase in muscle protein synthesis (MPS). The amino acids can be broadly classified into non-essential amino acids (NEAA) which are subject to de novo synthesis and the essential amino acids (EAA) which cannot be synthesized and are thus derived from dietary protein. Of the EAA, the branched-chained amino acids (BCAA) leucine isoleucine and valine are further distinguished by having aliphatic side-chains that are non-linear and make up approximately one-third of skeletal muscle protein.

Significantly, the capacity of amino acids to stimulate the regulatory mechanism of mRNA translation and thus MPS is not universal. For example, increasing the availability of the NEAA arginine, glycine, or serine and does not induce increases in protein synthesis (Smith et al. 1998) in humans whereas increasing plasma EAA in the absence of NEAA is sufficient to stimulate MPS (Tipton et al. 1999)

in humans. Intriguingly, additional studies have reported that of the EAA, the subset of BCAA may be more important in mediating this effect than the other EAA. In an early demonstration of this, Garlick and Grant (1988) showed that after infusion of various mixtures of amino acids to food-deprived rats, the branched-chain amino acids (BCAA) enhance MPS with similar efficacy, as does a complete amino acid mixture. Moreover, a number of additional studies have shown that a large fraction of the effect of the BCAA is directly attributable to leucine. For example, leucine stimulates MPS to the same extent as a complete protein or complete mixture of amino acids in rat muscle (Fulks et al. 1975) and these effects are not limited to rodents; Smith et al. (1992) reported that infusing large doses of a number of EAA, increased incorporation into skeletal muscle of stable isotopically labelled amino acids in human beings.

The proximal intracellular mechanisms by which EAA, and particularly leucine, stimulate MPS are not understood but likely involve the activation of "anabolic signalling pathways" such as the ste-20 related mitogen-activated protein kinase kinase kinase kinase 3 [MAP4K3 (Findlay et al. 2007)], the class III phosphatidylinositol 3′-kinase vacuolar protein sorting 34 [Vps34 (Nobukuni et al. 2005)], and/or recombination activating proteins [Rags1–4 (Sancak et al. 2008; Kim et al. 2008)] which converge on signalling through mammalian target of rapamycin complex 1 (mTORC1) (Anthony et al. 2000b). Consequently, mTORC1 has often been referred to as the cellular amino acid sensor and it is clear that signalling through mTORC1 has an essential role in EAA-signalling as administration of rapamycin—a potent inhibitor of mTORC1 activity—blunts EAA-induced mTORC1 signalling and protein synthesis (Vary et al. 2007). Moreover, leucine clearly signals through these pathways as activation of mTORC1 is dose-dependently modulated in the presence of leucine (Norton et al. 2009). Despite this surprisingly few direct substrates of mTORC1 are known, although two of the best characterised which are activated in response to EAA include 4E-binding protein 1 (4E-BP1), which regulates cap-dependent translation via promoting eukaryotic initiation factor 4F (eIF4F) assembly (Kimball et al. 1999), and ribosomal s6 kinase 1 (p70S6K1) (Balage et al. 2001) which in/directly regulates multiple substrates involved in the initiation (e.g. eukaryotic initiation factor 4B; eIF4B) (Raught et al. 2004) and elongation (e.g. eukaryotic elongation factor; eEF2) (Wang et al. 2001) phases of protein synthesis.

Nevertheless, the intracellular signalling mechanisms regulating the evidently greater anabolic properties of leucine over all other EAA are not known. Therefore, our aim was to investigate the distinct effects of each amino acid on anabolic signalling activity. We hypothesised that provision of EAA, but not NEAA would have a stimulatory effect on anabolic signalling with leucine demonstrating the greatest effects in terms of (1) quantity of target kinases, and (2) amplitude of signalling enzyme phosphorylation.

Methods

Cell culture

Murine C2C12 myocytes were cultured in DMEM containing 10% (v/v) foetal calf serum and antimycotic antibiotic solution (100 unit/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B) at 37°C in an atmosphere of 5% CO_2/95% air. At ~80% confluency, serum concentrations were reduced to 2% for 7 days to promote formation of multinucleated myotubes (Atherton et al. 2009). Cells were deprived of serum by incubation in serum-free α-MEM for 4 h followed by a 1-h amino acid-deprivation period in Hepes buffered saline (HBS, 20 mM Hepes/Na pH 7.4, 140 mM NaCl, 2.5 mM $MgSO_4$, 5 mM KCl and 1 mM $CaCl_2$). Cells (6-wells/condition) were inoculated with isoleucine, leucine, valine (BCAA) lysine, methionine, phenylalanine, threonine, tryptophan, valine (EAA) or alanine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, proline, serine, tyrosine (NEAA) at a concentration of 2 mM (Peyrollier et al. 2000) for 30 min.

Immunoblotting

Cell lysates were homogenised in ice-cold extraction buffer (10 μl/mg) containing 50 mM Tris–HCl (pH 7.4), 0.1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.1% 2-mercaptoethanol, 10 mM β-glycerophosphate, 50 mM NaF, 0.5 mM activated sodium orthovanadate (all Sigma-Aldrich, Poole, UK) and a complete protease inhibitor cocktail tablet (Roche, West Sussex, UK). Homogenates were rotated on a Vibramax for 10 min at 4°C, centrifuged at 10,000*g* for 10 min at 4°C, before recovery of supernatants representing sarcoplasmic fractions. Bradford assays were used to determine sarcoplasmic protein concentrations after which samples were standardised to 1 mg/ml by dilution with 3× Laemmli loading buffer in order to measure relative phosphorylated protein concentrations of Akt Ser473, mTOR Ser2448, p70S6K1 Thr389, 4EBP1 Thr37/46, RPS6 Ser235/236, eIF2α Ser51 and pan-actin (Sigma-Aldrich, Poole, UK). Samples were mixed and heated at 95°C for 7 min before 15 μg of protein/lane was loaded onto Criterion XT Bis–Tris 12% SDS-PAGE gels (Bio-Rad, Hemel Hempstead, UK) for electrophoresis at 200 V for ~60 min. Gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol) for 30 min before proteins were electroblotted onto 0.2 μm PVDF membranes (Bio-Rad) at 100 V for 30 min. After blocking with 5% low-fat

milk in TBS-T (Tris Buffered Saline and 0.1% Tween-20; both Sigma-Aldrich, Poole, UK) for 1 h, membranes were rotated overnight with primary antibody against the aforementioned targets at a concentration of 1:2,000 at 4°C. Membranes were washed (3 × 5 min) with TBS-T and incubated for 1 h at room temperature with HRP-conjugated anti-rabbit secondary antibody (New England Biolabs, UK), before further washing (3 × 5 min) with TBS-T and incubation for 5 min with ECL reagents (enhanced chemiluminescence kit, Immunstar; Bio-Rad). Blots were imaged and quantified by assessing peak density after ensuring bands were within the linear range of detection using the Chemidoc XRS system (Bio-Rad, Hemel Hempstead, UK).

Statistical analysis

Phosphorylation data were normalised to pan-actin to correct for loading anomalies. Differences in phosphorylation were detected by One-Way Anova using Tukey's Post Hoc test, with $P < 0.05$ considered as significant. Data are presented as mean ± SEM.

Results

Effects of amino acid classification (BCAA, EAA and NEAA) on anabolic signalling

NEAA had no effect upon phosphorylation of any signalling element (data not shown). Of the EAA, only leucine stimulated mTOR, 4EBP1, p70S6K1 and RPS6 signalling activity. All other EAA with the exception of valine and isoleucine (which had no effect), stimulated signalling through p70S6K1 and RPS6, albeit to a lesser extent than leucine.

Specific responses in phosphorylation of Akt, mTOR and 4EBP1

The phosphorylation of Akt was unchanged by incubation with EAA (Fig. 1a). Phosphorylation of mTOR on Ser2448 was increased 1.67 ± 0.1-fold in response to leucine stimulation (Fig. 1b) but not by any other EAA. This was comparable to phosphorylation of 4EBP1 Thr37/46 which increased 2.5 ± 0.1-fold with leucine (Fig. 1c), but not with any other EAA.

Specific responses in phosphorylation of p70S6K1 and RPS6

The phosphorylation of p70S6K1 was significantly increased by the presence of all EAA with the exception of isoleucine and valine (Fig. 2a). Stimulation with lysine, methionine, phenylalanine, tryptophan, and threonine

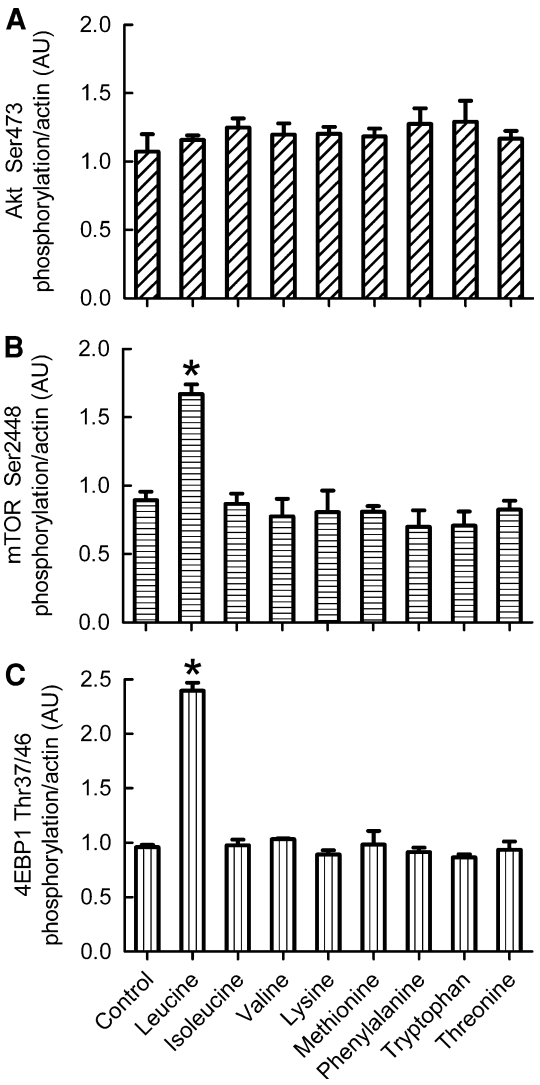

Fig. 1 Phosphorylation of Akt (**a**), mTOR (**b**) and 4EBP1 (**c**) in response to each EAA. *Asterisk* indicates increased phosphorylation versus control; $P < 0.05$. Data are presented as mean ± SEM

increased p70S6K1 phosphorylation by ~1.6–2.0-fold. Leucine increased phosphorylation of p70S6K1 by 5.9 ± 0.5-fold, a more significant rise than with any other EAA. Phosphorylation of RPS6 followed a similar pattern (Fig. 2b) with increases in lysine, methionine, phenylalanine, tryptophan, and threonine ranging from ~1.5 to 2.0-fold, but no increases with isoleucine or valine. The 3.8 ± 0.3-fold stimulation of RPS6 phosphorylation by leucine was also significantly greater than the other EAA.

Specific responses in phosphorylation of eIF2α, eIF2Bε and eEF2

Phosphorylation of eIF2α (Fig. 3a), eIF2Bε (Fig. 3b) and eEF2 (Fig. 3c) were unaffected by EAA stimulation at any time-point.

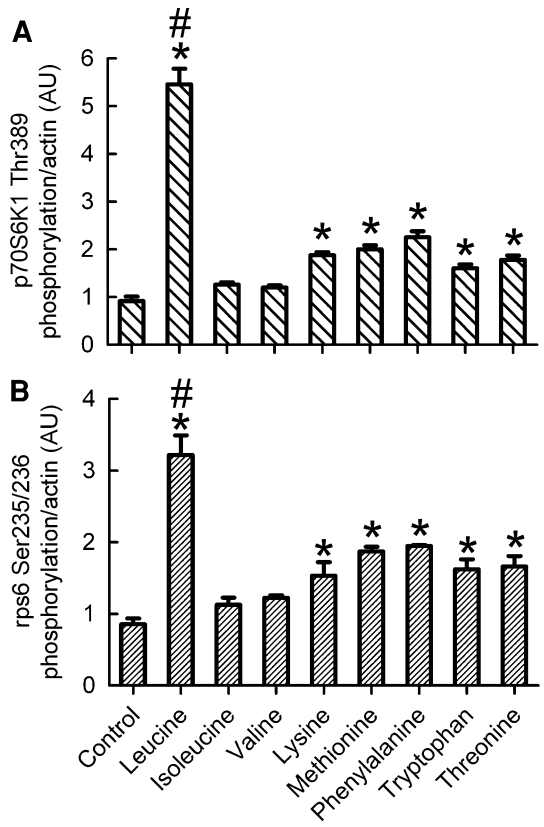

Fig. 2 Phosphorylation of p70S6K1 (**a**) and RPS6 (**b**) in response to 30 min stimulation with each EAA. *Asterisk* indicates increase phosphorylation versus control, #indicates a greater response than all other EAA; $P < 0.05$. Data are presented as mean ± SEM

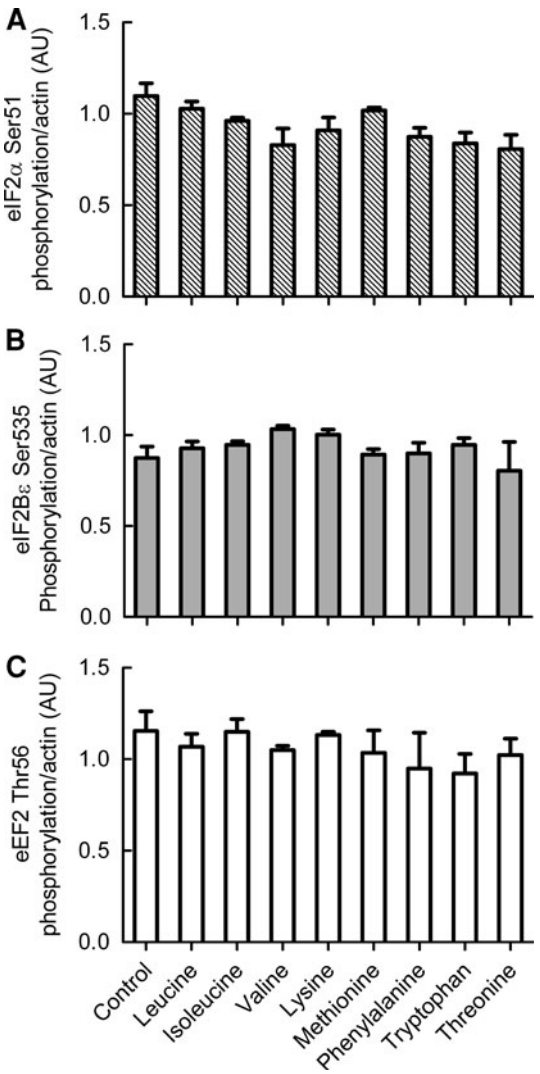

Fig. 3 Phosphorylation of eIF2α (**a**) eIF2Bε (**b**) and eEF2 (**c**) in response to 30 min stimulation with each EAA. Data are presented as mean ± SEM

Discussion

We have shown (1) that neither the NEAA or surprisingly the BCAA valine and isoleucine stimulate anabolic signalling protein phosphorylation, (2) that all other EAA stimulate phosphorylation of p70S6K1 and RPS6 to a similar degree, with the exception of leucine which is ~3-fold more potent, (3) that leucine is unique in its capacity to stimulate mTOR and 4EBP1 phosphorylation, and (4) modulation of anabolic signalling activity by EAA is limited to regulation of translational initiation via mTORC1, p70S6K1 and RPS6 phosphorylation and not via modulation of the ternary complex or by regulation of elongation; as eIF2α and eEF2 are unaffected.

Our demonstration of a lack of stimulation of anabolic signalling by increasing availability of the NEAA is in agreement with past studies demonstrating no effect of NEAA (arginine, glycine or serine) infusions on MPS (Smith et al. 1998) in humans. Moreover, our data show that the lack of response in MPS to increasing NEAA availability is related to a failure of NEAA to stimulate anabolic signalling activity. Such a sensory mechanism makes evolutionary sense, such that increases in MPS are restricted to times in which sufficient dietary-derived EAA are available.

Incubation of C2C12 muscle cells with EAA did not affect eIF2α or eIF2Bε phosphorylation, which is in line with a previous report (Anthony et al. 2000a). Evidently then, modulation of anabolic signalling activity by EAA is principally independent of assembly of the ternary initiation complex, at least as demonstrated by a lack of dephosphorylation of eIF2Bε or eIF2α. Furthermore, incubation with EAA did not affect the phosphorylation status of Akt, which is in line with other studies showing that suppressing PI3K activity does not inhibit EAA-induced mTORC1 signalling (Bolster et al. 2004). Therefore, although Akt is a potential upstream regulator of mTORC1 via tuberous sclerosis complex 2 (TSC2) and proline rich Akt substrate (PRAS40) phosphorylation (Sancak et al. 2007), EAA apparently

stimulate mTORC1 independently of Akt and to date there is a growing list of putative upstream regulators. For example, MAP4K3, a kinase that is involved in the activation of mTORC1 is inactivated when amino acids are removed and rapidly reactivated when amino acids are replaced (Findlay et al. 2007). In addition, Vps34 can signal amino acid and glucose levels to mTORC1 through undefined mechanisms (Nobukuni et al. 2005; Byfield et al. 2005). Furthermore, GTP charging of the rag A/B partner, probably dependent on amino acid sufficiency, promotes association of the rag heterodimer with mTORC1 and its translocation to a membrane compartment enriched in Rheb, thereby enabling mTORC1 activation when Rheb is GTP charged (Sancak et al. 2008). Though it is not known whether these elements are part of the same or different pathways, we speculate that as EAA stimulate distinct anabolic signalling responses it is likely that this occurs via discrete modulation of upstream elements.

Of all the EAA, much early work demonstrated that the BCAA are key regulators of MPS. For example, Buse and Reid (1975) demonstrated that a mixture of the BCAA stimulated MPS in the absence of other amino acids. Moreover, they reported that leucine increased MPS as effectively as a mixture of all three BCAA suggesting that leucine stimulated MPS independently of inputs from isoleucine and valine. Indeed, since BCAA account for a substantial portion ($\sim$1/3rd) of muscle protein, more recent studies have suggested that the role of the other BCAA may be to ensure that their corresponding aminoacyl-tRNA do not become rate limiting in the stimulation of MPS by leucine (Escobar et al. 2007). In agreement with this, we report that leucine exclusively stimulates mTORC1 signalling (using 4EBP1 and p70S6K1 phosphorylation as surrogate measures) which is indicative of its unique role amongst the BCAA in stimulating protein synthesis (Anthony et al. 2000b). Furthermore, we surprisingly found that despite structural similarity to leucine, neither valine nor isoleucine modulated phosphorylation of any measured kinases in C2C12 cells. The reason for the exclusivity of leucine over other BCAA as a signal transducer is unclear, but may relate to specific moieties on leucine or to some covalently liganded or metabolically transformed product of leucine metabolism such as α-ketoisocaproate, rather than leucine itself. Indeed, there is support for these suppositions; for example, modification of the α-amino group (i.e. acetylation, methylation) partially eliminates the ability of leucine to promote p70S6K1 phosphorylation in H4-EII hepatoma cells (Shigemitsu et al. 1999). In addition, α-ketoisocaproate is as effective as leucine in stimulating p70S6K1 and 4EBP1 hyperphosphorylation, although the rapid reversible transamination to leucine could also mediate this effect (Yoshizawa et al. 2004). Another equivocal aspect relating to the anabolic effects of leucine is whether stimulation of anabolic signalling occurs at an extracellular—suggesting a "leucine receptor"—or intracellular site. Some workers have reported that the effects of leucine on mTORC1 is probably initiated subsequent to leucine entry as expression of a recombinant system L transporter confers p70S6K1 phosphorylation responsiveness to extracellular leucine in Xenopus oocytes (Christie et al. 2002), as does intracytoplasmic injection of leucine. However, there has been identification of an amino acid receptor in *S. cerevisiae* of non-transporting members of the amino acid permease family (Eckert-Boulet et al. 2006), though not yet in mammals. In addition, in humans, it was proposed that extracellular leucine was the signal increasing MPS following the observation of a curvilinear positive relationship between increase in MPS and extracellular but not intracellular EAAs (Bohe et al. 2003; Beugnet et al. 2003). Thus, more work is required to understand the precise mechanisms by which leucine induces anabolic responses.

The regulation of mTORC1 signalling is complex, involving multiple inputs which regulate phosphorylation, cellular localisation (Sancak et al. 2008) and also associations with regulatory cellular binding partners (Escobar et al. 2005). Activity of one of the best-characterised mTORC1 substrates, p70S6K1 was initially thought to increase cellular capacity for protein synthesis by specifically upregulating mRNA containing 5′ oligopyrimidine tracts which encode components of the translational apparatus (Jefferies et al. 1994). However, translation of these mRNA subsets is unaffected in p70S6K1$^{-/-}$ cells suggesting that under these conditions, alternative kinases such as the ribosomal s6 kinases (RSKs) (Roux et al. 2007) could instead phosphorylate p70S6K1 substrates such as RPS6 which has been shown as important in regulating cell size (Ruvinsky et al. 2005), or perhaps that p70S6K1 could have little role at all. Indeed, our data support the notion that signalling through RPS6 is a direct result of p70S6K1 activity since Ser235/236 on RPS6 is modulated in a similar manner to p70S6K1 on Thr389. More importantly, signalling responses of p70S6K1 and RPS6 to leucine were $\sim$5 and $\sim$3-fold increased, respectively, over the other EAA suggesting that the anabolic potency of leucine is at least partly mediated via enhancing the amplitude of p70S6K1 signalling. What could be causing this increased amplitude over other EAA? Our data support the notion that phosphorylation of mTOR on Ser2448 is not an absolute requirement for mTORC1 kinase activity since all EAA other than isoleucine and valine stimulated rapamycin sensitive Thr389 phosphorylation on p70S6K1. Moreover, mutation of Ser2448 to alanine does not affect mTORC1 kinase activity (Copp et al. 2009) suggesting a restricted role for phosphorylation of this site in overall mTORC1 regulation. However, since leucine exclusively

stimulated mTOR Ser2448 and 4E-BP1 phosphorylation as well as potentiating p70S6K1 phosphorylation, it is tempting to speculate that Ser2448 may either regulate mTORC1 substrate specificity (which may explain leucine exclusivity in affecting 4EBP1) and/or mTORC1 signalling amplitude. In addition, recent work has demonstrated that Ser2448 is a direct target of p70S6K1 and thus should act as a readout of p70S6K1 activity (Holz and Blenis 2005); however, if this were the case, then it is unclear why Ser2448 phosphorylation was unaffected when other amino acids (i.e. methionine, phenylalanine) significantly stimulated Thr389 phosphorylation.

We conclude that leucine is unique amongst the BCAA/EAA in stimulating anabolic signalling in skeletal muscle cells through the unique modulation of mTOR and 4EBP1 phosphorylation, in addition to potentiating the amplitude of p70S6K1 signalling over other EAA.

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