Symposium: Leucine as a Nutritional Signal

Signaling Pathways Involved in Translational Control of Protein Synthesis in Skeletal Muscle by Leucine[1]

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ABSTRACT Numerous reports established that in skeletal muscle the indispensable branched-chain amino acid leucine is unique in its ability to initiate signal transduction pathways that modulate translation initiation. Oral administration of leucine stimulates protein synthesis in association with hyperphosphorylation of the translational repressor, eukaryotic initiation factor (eIF) 4E binding protein 1 (4E-BP1), resulting in enhanced availability of the mRNA cap-binding protein eIF4E, for binding eIF4G and forming the active eIF4F complex. In addition, leucine enhances phosphorylation of the 70-kDa ribosomal protein S6 kinase (S6K1). These results suggest that leucine upregulates protein synthesis in skeletal muscle by enhancing both the activity and synthesis of proteins involved in mRNA translation. The stimulatory effects of leucine on translation initiation are mediated in part through the protein kinase mammalian target of rapamycin (mTOR), where both insulin signaling and leucine signaling converge to promote a maximal response. J. Nutr. 131: 856S–860S, 2001.

KEY WORDS: • *leucine* • *insulin* • *translation initiation* • *protein synthesis* • *skeletal muscle*

Remarkable progress was made during the past few years in identifying insulin- and growth factor–induced modulators of translation initiation. However, investigations into the signaling events that culminate in amino acid–induced changes in mRNA translation were limited. Studies using cells in culture indicate that, although amino acids can directly initiate signal transduction pathways that modulate translation initiation, these signals are not propagated through upstream components of insulin or growth factor signaling pathways (Hara et al. 1998, Patti et al. 1998). Thus amino acids independently function as nutritional signaling molecules that regulate protein synthesis. Numerous reports established that in skeletal muscle the indispensable branched-chain amino acid (BCAA[3]) leucine is unique in this regard (Anthony et al. 2000a, Buse et al. 1979, Buse and Reid 1975, Li and Jefferson 1978). This review examines the role of leucine in mediating the stimulatory effects of dietary amino acids on signaling pathways that modulate translation initiation in skeletal muscle.

Leucine stimulates protein synthesis in skeletal muscle

Several early studies indicated that the BCAAs regulate protein synthesis in skeletal muscle. In isolated rat diaphragms, addition of the BCAAs to the incubation medium at five times fasting plasma concentrations stimulates protein synthesis compared to diaphragms incubated in the absence of the BCAAs (Fulks et al. 1975). A similar protein anabolic effect is observed in hindlimb preparations from fasted young rats perfused with a mixture of leucine, isoleucine and valine at five times plasma concentrations (Li and Jefferson 1978). Further, Garlick and Grant (1988) reported that a combination of the BCAAs and glucose stimulates skeletal muscle protein synthesis in postabsorptive rats. The authors infused food-deprived rats intravenously for 1 h with various mixtures of amino acids and glucose and found that infusion of the BCAAs and glucose enhances protein synthesis in skeletal muscle as efficiently as does a combination of a complete amino acid mixture and glucose. These data suggest that the protein anabolic effect of a complete amino acid mixture can be replicated by providing a mixture of only the BCAAs.

A number of studies previously indicated that most, and perhaps all, of the effect of a mixture of the BCAAs on skeletal muscle protein synthesis may be attributable to leucine alone. Buse and Reid (1975) demonstrated that incubating isolated rat hemidiaphragms in the presence of leucine stimulates the incorporation of labeled precursors into muscle proteins compared to diaphragms incubated in the absence of the amino acid. Further, the authors reported that leucine stimulates protein synthesis as effectively as does a mixture of all three

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[3] Abbreviations used: BCAA, branched-chain amino acid; eEF, eukaryotic elongation factor; eIF, eukaryotic initiation factor; met-tRNA$_i$, initiator methionyl-tRNA; mTOR, mammalian target of rapamycin; PDK1, 3-phosphoinositide–dependent protein kinase 1; PI 3-K, phosphoinositol 3-kinase; PKB, protein kinase B; PKC, protein kinase C; S6K1, 70-kDa ribosomal protein S6 kinase; TOP, terminal oligopyrimidine; 4E-BP, eukaryotic initiation factor 4E binding protein.

BCAAs. Additionally, Hong and Layman (1984) demonstrated that incubating either soleus or extensor digitorum longus muscles isolated from food-deprived rats in Krebs–Ringer–bicarbonate buffer supplemented with leucine enhances protein synthesis compared to muscles similarly incubated in control buffer without leucine. In perfused rat hindlimb preparations, leucine at 10 times fasting plasma concentrations stimulates protein synthesis in muscle as effectively as does a mixture of all three BCAAs (Li and Jefferson 1978). These data indicate that leucine stimulates protein synthesis in skeletal muscle independent of the other BCAAs.

Initial attempts to demonstrate that leucine stimulates protein synthesis in skeletal muscle in vivo were not always successful. Buse et al. (1979) showed that intraperitoneal injection of leucine in combination with glucose and insulin to starved rats increases the proportion of ribosomes in polysomes in skeletal muscle compared to rats administered only glucose plus insulin. In contrast, McNurlan and colleagues (1982) reported no in vivo effects after intravenous injection of leucine in 2-d–starved or protein-deprived rats. Additionally, Funabiki and collaborators (1992) observed no alterations in muscle protein synthesis in either food-deprived or diabetic mice following intraperitoneal administration of leucine. The reason for the discrepancy between the in vitro and in vivo evidence remains unclear, but may be related to differences in study design. Alternatively, based on data from Buse et al. (1979), insulin may be required to facilitate the leucine-mediated stimulation of muscle protein synthesis in vivo.

Role of insulin

The relative contribution of insulin to the leucine-dependent stimulation of muscle protein synthesis remains to be determined. Several reports indicate that physiological increases in circulating insulin concentrations are not sufficient to stimulate rates of protein synthesis in postabsorptive rats (Anthony et al. 1999, 2000a, Garlick et al. 1983, Gautsch et al. 1998, Yoshizawa et al. 1995, 1998). When food-deprived rats are administered an oral bolus of carbohydrate alone, no change in protein synthesis rates is observed compared to that in starved rats (Anthony et al. 2000a, Gautsch et al. 1998). In contrast, when food-deprived rats receive an isocaloric combination of carbohydrate plus leucine, rates of protein synthesis are stimulated by nearly 40% and equivalent to freely fed controls (Anthony et al. 2000a). Plasma insulin concentrations of rats administered either the carbohydrate meal or the carbohydrate plus leucine meal were similar; hence, the enhanced rate of recovery cannot be attributed to a differential insulin response between the two groups. These results suggest that leucine has a unique role in facilitating recovery of protein synthesis in skeletal muscle of food-deprived rats. Additionally, oral administration of leucine stimulates protein synthesis in skeletal muscle following exercise (Anthony et al. 1999), food-deprivation (Anthony et al. 2000a) and glucocorticoid treatment (Shah et al. 2000). The stimulation of protein synthesis following an oral bolus of leucine alone is as great as when providing a combination of carbohydrate and leucine (Anthony et al. 1999, 2000). However, when leucine is administered alone there is no concomitant increase in circulating insulin concentrations 1 h after gavage.

These results are initially surprising, because leucine was previously shown to stimulate insulin secretion (Malaisse 1984). However, subsequent studies showed that provision of leucine to food-deprived rats promotes a transient rise in serum insulin from 15 to 45 min after administration (Anthony, J. C. et al., unpublished observations). Circulating insulin concentrations are maximal 30 min following leucine administration when values are approximately 75% of freely fed rats. Serum insulin returns to food-deprived control values sometime between 45 and 60 min. This transient spike in circulating insulin may facilitate the stimulatory effect of leucine on muscle protein anabolism. Garlick and Grant (1988) measured rates of protein synthesis in skeletal muscle of postabsorptive rats that had been given intravenous infusions of various combinations of insulin and amino acids. They reported that, although insulin independently stimulates rates of skeletal muscle protein synthesis, maximal effects are observed only at circulating concentrations of the hormone in excess of what is normally seen in freely fed rats. In contrast, when a complete mixture of amino acids is coinfused with insulin, maximal rates of protein synthesis are obtained at insulin concentrations of ~100 pmol/L, well within the physiological range for food-deprived rats. The results suggest that leucine independently stimulates protein synthesis in the presence of fasting or basal concentrations of circulating insulin. Although these studies indicate necessary contributions of both insulin and leucine to promote muscle protein anabolism following feeding, the mechanisms through which these mediators signal for increased rates of protein synthesis remain to be elucidated.

Overview of translation initiation

Rates of protein synthesis are determined by the number of ribosomes present in a cell as well as by the translational efficiency per ribosome. Acute changes in nutritional status, such as those occurring during the postprandial state, were not shown to alter tissue ribosomal content but instead to increase translational efficiency (Svanberg et al. 1997, Yoshizawa et al. 1995, 1997, 1998). Buse and Reid (1975) were the first to suggest that leucine exerts its effects at a posttranscriptional level and most likely during initiation. Their hypothesis was based on in vitro data demonstrating that pretreatment of rat diaphragms with actinomycin D does not inhibit the stimulatory effects of leucine on protein synthesis. This was further supported by Li and Jefferson (1978), who reported that the stimulatory effect of leucine on protein synthesis in rat hindlimb preparations is associated with a decrease in the level of free ribosomal subunits.

The initiation of mRNA translation is a complex multistep process requiring more than a dozen eukaryotic initiation factors (eIFs) (Pain 1996, Voorma et al. 1994). At least two steps in the initiation pathway are subject to regulation in vivo: *1*) binding of initiator methionyl-tRNA (met-tRNA$_i$) to the 40S ribosomal subunit and *2*) binding of mRNA to the 43S preinitiation complex. In the first step, met-tRNA$_i$ binds to the 40S ribosomal subunit as a ternary complex with eIF2 and GTP. Subsequently, the GTP bound to eIF2 is hydrolyzed to GDP, and eIF2 is released from the ribosomal subunit as a complex with GDP. For eIF2 to participate in another round of initiation, it must exchange GDP for GTP prior to formation of a new ternary complex. A second initiation factor, eIF2B, mediates guanine nucleotide exchange on eIF2. Inhibition of eIF2B activity results in a decrease in the amount of eIF2·GTP available to form the ternary complex, thereby restraining translation initiation. eIF2B activity is reciprocally regulated in part by phosphorylation of eIF2. Phosphorylation of the α-subunit of eIF2 converts eIF2 from a substrate to a competitive inhibitor of eIF2B (Kimball and Jefferson 1994).

Another potential rate-controlling step in the process of translation initiation involves the recognition and unwinding of the mRNA to allow binding to the 40S ribosome. This step requires a group of proteins referred to as eIF4F (**Fig. 1**). eIF4F

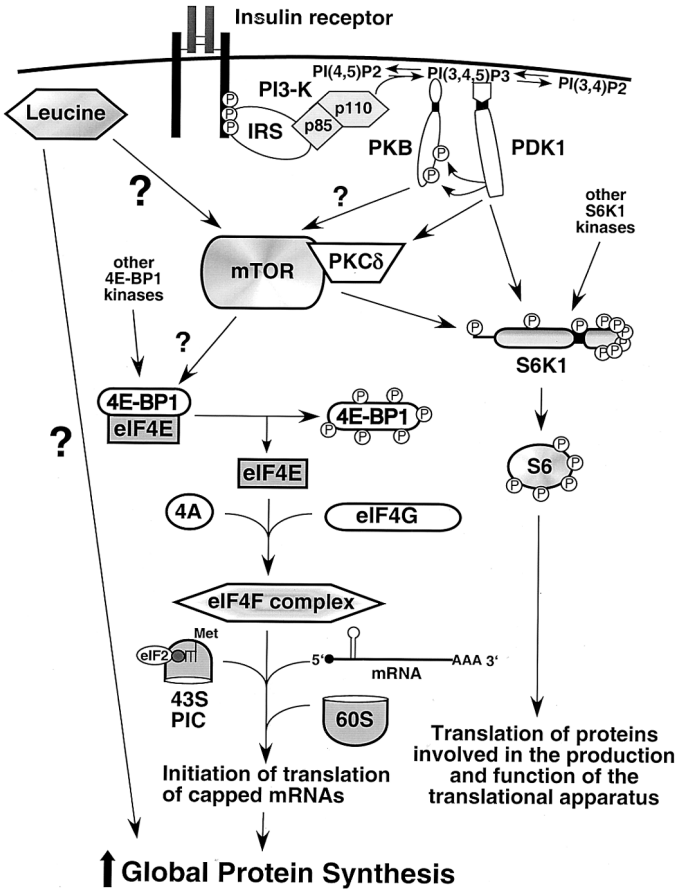

FIGURE 1 Signaling events in the stimulation of translation initiation by leucine.

is a multisubunit complex consisting of *1*) eIF4A, a RNA helicase that functions in conjunction with another protein, eIF4B (not shown), to unwind secondary structure in the 5′-untranslated region of the mRNA; *2*) eIF4E, a protein that binds the m^7GTP cap present at the 5′-end of the mRNA; and *3*) eIF4G, a large, 220-kDa polypeptide that functions as a scaffold for eIF4E, eIF4A, the mRNA (via association with eIF4E) and the ribosome (via association with eIF3; not shown). The eIF4F complex collectively serves to recognize, unfold and guide the mRNA to the 43S preinitiation complex (Pain 1996). One mechanism through which changes in eIF4F function alter translation initiation involves modulation of eIF4E availability for binding eIF4G and forming the active eIF4F complex. The availability of eIF4E for eIF4F complex formation appears to be regulated by a family of translational repressors, the eIF4E-binding proteins (4E-BPs) (Pause et al. 1994). 4E-BP1 competes with eIF4G for binding eIF4E and is able to sequester eIF4E into an inactive complex. The binding of 4E-BP1 to eIF4E is regulated by phosphorylation of 4E-BP1, with increased phosphorylation of the protein causing a decrease in the affinity of 4E-BP1 for eIF4E.

Leucine stimulates translation initiation in skeletal muscle

Leucine appears to regulate protein synthesis in skeletal muscle independent of changes in the formation of the ternary initiation complex (Anthony et al. 2000a). Significant alterations in either the activity of eIF2B or in the phosphorylation state of eIF2α are not observed following leucine administration (Anthony et al. 2000a). Rather, leucine enhances the association of eIF4E with eIF4G (Anthony et al. 2000a). Following administration of small meals containing either leucine or a combination of carbohydrate plus leucine to growing rats, rates of protein synthesis in skeletal muscle are increased in association with hyperphosphorylation of 4E-BP1 compared to that of food-deprived controls. Consequently, formation of 4E-BP1·eIF4E is inhibited and eIF4G·eIF4E is enhanced. In contrast, carbohydrate alone has no significant effect on 4E-BP1 phosphorylation or eIF4E availability, even though serum insulin values are equivalent to those in rats fed carbohydrate plus leucine and significantly greater than those in rats fed leucine alone. Therefore, physiological increases in serum insulin are not sufficient to enhance rates of translation initiation. These results suggest that the stimulatory effects of a protein containing meal on translation initiation may be modulated in part by dietary leucine enhancing the formation of the active eIF4F complex.

In addition to promoting the hyperphosphorylation of 4E-BP1, oral administration of leucine increases the phosphorylation state of the 70-kDa ribosomal protein S6 kinase (S6K1), particularly at Thr^{389}, a residue whose phosphorylation is associated with increased activation of the protein (Burnett et al. 1998). Increased activity of S6K1 was previously shown to regulate the translation of specific mRNAs by modulating the phosphorylation of ribosomal protein S6. S6 phosphorylation plays an important role in regulating the synthesis of ribosomal proteins and elongation factors (eEF), which are encoded by mRNAs containing terminal oligopyrimidine tracts at the 5′-end of the message (TOP mRNAs) (reviewed by Jefferies and Thomas 1996). In L6 myoblasts in culture, leucine enhances phosphorylation of S6K1 and ribosomal protein S6 in association with increases in the synthesis of eEF1A (Kimball et al. 1999). The mechanism involved in the translation of TOP mRNAs in association with ribosomal protein S6 phosphorylation is not known. However, in vitro studies employing chemical cross-linking to investigate the structural arrangement within the initiation complex reveal that S6 present in ribosomes is in close proximity with both the eIFs and mRNA (Nygard and Nilsson 1990). Therefore, phosphorylation of S6 could alter the interaction of the protein with either of these components and promote the translation of TOP mRNAs. The ability of leucine to promote the hyperphosphorylation of both 4E-BP1 and S6K1 suggests a common signaling pathway through which leucine upregulates protein synthesis by enhancing both the activity and the synthesis of proteins involved in mRNA translation.

Recent studies using cells in culture indicate that the hyperphosphorylation of 4E-BP1 and S6K1 in the presence of amino acids, particularly leucine, involves a signaling pathway that includes the protein kinase referred to as mammalian target of rapamycin (mTOR) (Kimball et al. 1999, Patti et al. 1998, Xu et al. 1998). To investigate the role of mTOR signaling in the stimulation of translation initiation and protein synthesis in vivo, food-deprived rats were injected intravenously with the immunosuppressant drug rapamycin, a specific inhibitor of mTOR, 2 h prior to leucine administration (Anthony et al. 2000b). Rapamycin completely prevents the leucine-dependent hyperphosphorylation of both 4E-BP1 and S6K1 (**Table 1**). These results suggest that mTOR signaling is essential for the leucine-dependent stimulation of translation initiation. However, available evidence indicates that the stimulation of skeletal muscle protein synthesis following leucine administration may involve additional intracellular signaling pathways. Although rapamycin attenuates rates of protein synthesis in rats administered leucine, the drug also inhibits protein synthesis in food-deprived animals (Table 1).

TABLE 1

Phosphorylation of eukaryotic initiation factor (eIF) 4E-binding protein (4E-BP1) and of the 70-kDa ribosomal protein S6 kinase (S6K1) and protein synthesis in skeletal muscle of food-deprived rats treated with rapamycin and/or orally administered leucine[1,2]

Treatment group[3]	4E-BP1 phosphorylation, % control	S6K1 phosphorylation, % control[4]	Protein synthesis, % control
Control	100 ± 25[b]	100 ± 81[b]	100 ± 10[bc]
Rapamycin	19 ± 13[c]	15 ± 15[b]	76 ± 7[c]
Control + Leucine	600 ± 103[a]	1547 ± 543[a]	142 ± 11[a]
Rapamycin + Leucine	40 ± 17[c]	20 ± 20[b]	103 ± 5[b]

[1] Data adapted from Anthony et al. (2000b).
[2] Values are means ± SE, $n = 4–8$. Means in a column not sharing the same superscript are different ($P < 0.05$).
[3] Food-deprived rats were administered 0.75 mg/kg rapamycin (Rapamycin) or an equal volume of excipient (Control) via the tail vein. After 2 h one half of the rats in the Rapamycin and Control groups were orally administered 1.35 g/kg body weight leucine (Rapamycin + Leucine and Control + Leucine, respectively). All measurements were made 1 h after leucine administration.
[4] Phosphorylation of S6K1 is expressed as the amount of phosphorylation at Thr^{389}, a residue whose phosphorylation is associated with increased activity of the protein.

Thus, leucine promotes a significant stimulation of protein synthesis in rats fed rapamycin. These results suggest that signaling through mTOR alone is not sufficient to explain the leucine-dependent stimulation of muscle protein synthesis. This conclusion is supported by additional studies demonstrating that oral administration of isoleucine can also promote the hyperphosphorylation of both 4E-BP1 and S6K1 without a concomitant increase in rates of protein synthesis in skeletal muscle (Anthony et al. 2000b). Alternate pathways activated by leucine that could result in the stimulation of muscle protein synthesis are currently under investigation.

Upstream signaling events in the leucine-dependent stimulation of translation initiation

It is clear from the available evidence that, although amino acids can directly activate components of signal transduction pathways, these signals are not propagated through upstream components of insulin or growth factor signaling pathways. Addition of amino acids to Fao cells at a fourfold concentration present in cell culture medium has no effect on insulin receptor tyrosine kinase autophosphorylation, or on the phosphorylation of the insulin receptor substrate (IRS) proteins (Patti et al. 1998). The addition of amino acids to cells in culture at concentrations that maximally stimulate S6K1 phosphorylation are also without effect with respect to phosphoinositol 3-kinase (PI 3-K) phosphorylation or activation of a serine/threonine kinase downstream of PI 3-K called protein kinase B (PKB, also referred to as Akt) (Patti et al. 1998).

An accumulating amount of evidence points to mTOR as a convergence point for both amino acid– and insulin-mediated effects on translation initiation. Experiments in HEK-293 cells demonstrate insulin treatment to induce phosphorylation of mTOR at Ser 2448, a site that is considered crucial in the activation of the kinase (Nave et al. 1999). On the other hand, amino acid starvation reduces the phosphorylation of mTOR at the Ser 2448 residue and makes the phosphorylation of this site refractory to insulin. Further, in an in vitro kinase assay, activated PKB does not phosphorylate mTOR at Ser 2448 when mTOR is immunoprecipitated from amino acid–deprived cells (Nave et al. 1999). Interpretation of these results leads to two possibilities: either amino acid deprivation activates a phosphatase specific for mTOR at Ser 2448, or amino acids regulate a kinase that can activate a "priming" phosphorylation site on mTOR whose phosphorylation is a prerequisite for phosphorylation by PKB. Currently, there is no evidence to directly support activation of a phosphatase by amino acid withdrawal or data to show the existence of a priming kinase.

A possible role for the protein kinase C (PKC) family of proteins in regulating translation initiation by amino acids is beginning to emerge. The PKC family is a group of at least 11 proteins that are involved in the regulation of various cellular processes (Dekker et al. 1995). Recent evidence has identified PKCδ, classified as a novel PKC, to be involved in the control of mTOR activation and cap-dependent translation (Kumar et al. 2000, Parekh et al. 1999). Studies in amino acid–deprived HEK293 cells show that addition of amino acids to the culture medium results in phosphorylation of the kinase at Ser 662, a site that is associated with its maximal activation. This result can be reproduced by adding back leucine alone (Parekh et al. 1999).

Even though PKCδ may be involved in leucine signaling to mTOR, it is unlikely that PKCδ is the initial "leucine signal." Several lines of evidence support this conclusion (Kumar et al. 2000). First, whereas PKCδ is found to be constitutively associated with mTOR, PKCδ does not directly phosphorylate mTOR. Second, activation of mTOR is required for PKCδ-mediated phosphorylation of 4E-BP1. Finally, PKCδ-mediated phosphorylation of 4E-BP1 is rapamycin sensitive, and therefore not independent of mTOR. These lines of evidence suggest that the activities of both mTOR and PKCδ are required for leucine-induced maximal phosphorylation of 4E-BP1.

Taking into account what is currently known from both in vivo and in vitro studies, the following model can be proposed (Fig. 1). Administration of leucine in vivo results in a transient release of insulin and/or enhanced sensitivity of muscle cells to insulin. Presentation of insulin at the muscle cell surface results in its binding to the insulin receptor, which not only causes autophosphorylation of the receptor and activation of the IRS proteins but also thereby initiates the PI 3-K signaling cascade. Further, leucine initiates a novel signaling cascade through some unidentified mechanism that is independent of PI 3-K, PKB (Akt) or 3-phosphoinositide dependent protein kinase 1 (PDK1) activation. The insulin signal and the leucine signal appear to converge at mTOR, where both signals are required for maximal activation of the translation initiation. This convergence may also require the activation of PKCδ. Further, other unidentified signaling molecules and/or pathways also likely contribute, providing either a

degree of redundancy or an increased capability to fine-tune the response.

Several studies indicate that leucine functions as a nutritional signaling molecule that regulates protein synthesis in skeletal muscle following food intake. The stimulatory effect of leucine on protein synthesis occurs at the level of translation initiation and involves signaling through mTOR. However, signaling through mTOR alone is not sufficient to explain the increased rates of protein synthesis following leucine administration. Further studies are required to determine additional signaling pathways activated by leucine that regulate protein synthesis. It is likely that maximal rates of protein synthesis following food intake require both leucine and insulin signaling.

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