Signaling Pathways and Molecular Mechanisms through which Branched-Chain Amino Acids Mediate Translational Control of Protein Synthesis

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ABSTRACT BCAAs stimulate protein synthesis in in vitro preparations of skeletal muscle. Likewise, the stimulation of protein synthesis in skeletal muscle produced by intake of a mixed meal is due largely to BCAAs. Of the three BCAAs, leucine is the one primarily responsible for the stimulation of protein synthesis under these circumstances. The stimulatory effect of leucine on protein synthesis is mediated through upregulation of the initiation of mRNA translation. A number of mechanisms, including phosphorylation of ribosomal protein S6 kinase, eukaryotic initiation factor (eIF)4E binding protein-1, and eIF4G, contribute to the effect of leucine on translation initiation. These mechanisms not only promote global translation of mRNA but also contribute to processes that mediate discrimination in the selection of mRNA for translation. A key component in a signaling pathway controlling these phosphorylation-induced mechanisms is the protein kinase, termed the mammalian target of rapamycin (mTOR). The activity of mTOR toward downstream targets is controlled in part through its interaction with the regulatory-associated protein of mTOR (known as raptor) and the G protein β-subunit-like protein. Signaling through mTOR is also controlled by upstream members of the pathway such as the Ras homolog enriched in brain (Rheb), a GTPase that activates mTOR, and tuberin (also known as TSC2), a GTPase-activating protein, which, with its binding partner hamartin (also known as TSC1), acts to repress mTOR. Candidates for mediating the action of leucine to stimulate signaling through the mTOR pathway include TSC2, Rheb, and raptor. The current state of our understanding of how leucine acts on these signaling pathways and molecular mechanisms to stimulate protein synthesis in skeletal muscle is summarized in this article. J. Nutr. 136: 227S–231S, 2006.

KEY WORDS: • leucine • mRNA translation • mammalian target of rapamycin • insulin-like growth factor-1

Orally administered leucine stimulates protein synthesis in skeletal muscle

In skeletal muscle, provision of the BCAAs mimics the effect of a complete mixture of amino acids in stimulating protein synthesis both in vitro and in vivo. For example, in isolated rat diaphragm, addition of BCAAs to the incubation medium stimulates protein synthesis to the same extent as does inclusion of a complete mixture of amino acids (1). Similarly, in perfused preparations of rat skeletal muscle, addition of BCAA at 5 times the concentrations present in plasma of fasted animals stimulates protein synthesis to an extent comparable to that observed in response to a complete mixture of amino acids (2). In contrast, perfusion with an amino acid mixture containing all the amino acids except the BCAAs has no effect on protein synthesis. A protein anabolic effect is also observed in skeletal muscle of food-deprived rats following intravenous administration of BCAAs or a complete mixture of amino acids (3).

Of the BCAAs, leucine appears to be the most important in stimulating protein synthesis in skeletal muscle. In both incubated diaphragm (1,4) and perfused gastrocnemius (2) preparations of leucine alone is nearly as effective in stimulating protein synthesis as supplying all three BCAAs. In addition, leucine stimulates protein synthesis in incubated preparations of soleus or extensor digitorum longus (5). However, despite the convincing evidence provided by in vitro studies, early attempts to show that leucine alone stimulates protein synthesis in skeletal muscle in vivo met with less success. Neither intravenous (6) nor intraperitoneal (7) administration of leucine to fasted rats stimulates protein synthesis. However, intraperitoneal administration of leucine in combination with...
Role of insulin in leucine-induced protein synthesis in muscle

Oral administration of leucine produces a slight transient rise in the serum insulin concentration. Whether the transient increase in insulin contributes to the leucine-mediated effect on protein synthesis in skeletal muscle has been addressed in studies in which somatostatin is administered intravenously by primed-constant infusion before the administration of leucine (12). These studies show that somatostatin maintains insulin concentrations at the fasting basal level throughout the time course. It also attenuates the effect of leucine on protein synthesis. Thus, although physiological increases in serum insulin induced by carbohydrate administration do not independently stimulate protein synthesis in skeletal muscle of food-deprived rats, a transient increase in the hormone appears to be permissive for the leucine-induced stimulation of protein synthesis. Additional studies on the relationship between insulin and leucine-induced stimulation of protein synthesis in skeletal muscle have been carried out in rats having experimentally induced diabetes (13). These studies show that protein synthesis in the skeletal muscle of diabetic rats is reduced to 35% of the rate observed in food-deprived, nondiabetic controls. Administration of leucine to the diabetic rats stimulates protein synthesis by ~50%, but the rate remains well below that of the nontreated, nondiabetic control. The stimulatory response to leucine is enhanced in diabetic rats treated acutely with insulin, however, the recovery of protein synthesis is incomplete with the rate being equivalent to food-deprived control values yet substantially less than values observed in control rats administered leucine. Overall, the studies demonstrate that a portion of the protein-synthetic response to leucine in skeletal muscle occurs through an insulin-independent mechanism because the rate is greater in diabetic rats administered leucine than that of the diabetic controls.

Leucine stimulates the mRNA binding step in translation initiation

To investigate mechanisms involved in the stimulatory effect of leucine on protein synthesis in skeletal muscle, studies have assessed the modulation of 2 key regulatory steps in the initiation of mRNA translation, a process identified in earlier studies to be responsive to both BCAA and insulin. These studies demonstrate that leucine has no effect on the mRNA binding step in translation initiation, as assessed by the phosphorylation status of the eIF4E binding protein (4E-BP1) and by the association of eIF4E with 4E-BP1 and eIF4G. Leucine also has a stimulatory effect on the phosphorylation status of eIF4G as well as ribosomal protein S6 kinase (S6K1) and its downstream substrate S6. Because phosphorylation of 4E-BP1, eIF4G, and S6K1 is mediated in part by the mammalian target of rapamycin (mTOR), the results suggest that leucine stimulates a signaling pathway involving this serine/threonine protein kinase (Figure 1). A more detailed view of leucine signaling through mTOR to effector mechanisms involved in translation initiation is presented below.

In studies focused on the interaction between the stimulatory effects of leucine and insulin on protein synthesis in skeletal muscle, somatostatin attenuates the leucine-induced increases in 4E-BP1 and S6K1 phosphorylation and completely blocks the increase in S6 phosphorylation but has no effect on the association of eIF4E and eIF4G (12). Moreover, in studies of rats with experimentally induced diabetes (13), leucine administered alone has no effect on mTOR signaling to 4E-BP1 or S6K1 but nonetheless produces a stimulation of protein synthesis. Leucine administered in combination with insulin infusion sufficient to restore the serum concentration of the hormone to a level equivalent to that present in a food-deprived control rat, or approximately twice that level, rescues or even enhances mTOR signaling to 4E-BP1 and S6K1 (13). Overall, the results suggest that leucine stimulates protein synthesis in skeletal muscle through both insulin-dependent and independent mechanisms. The insulin-dependent mechanism is associated with signaling through mTOR to 4E-BP1 and S6K1, whereas the insulin-independent effect is mediated by other studies show that oral administration of leucine enhances polysome aggregation in skeletal muscle of fasted rats compared with administration of glucose and insulin alone (8). This suggests that insulin may be required for the leucine-induced in vivo stimulation of protein synthesis in skeletal muscle. More definitive evidence provided by other studies shows that oral administration of leucine enhances protein synthesis not only in skeletal muscle (9,10) but also in other tissues (11). In contrast, administration of either isoleucine or valine alone has no effect. Thus, leucine is unique among BCAAs with regard to its effectiveness as a nutrient regulator of protein synthesis in skeletal muscle. Overall, the data suggest that BCAA, and in particular leucine, replicates the effect of a complete mixture of amino acids in stimulating protein synthesis in skeletal muscle.

FIGURE 1 Diagrmatic representation of signaling from leucine and insulin/IGF-1 through mTOR. The mechanism through which leucine is sensed is unknown and is depicted as X. The dashed arrows labeled with a question mark denote possible links between leucine and mTOR regulatory proteins. Lines terminating in a perpendicular line represent inhibition, whereas arrows represent stimulation. The individual steps and proteins are described in more detail in the text.

*Abbreviations used: 4E-BP1, eIF4E binding protein 1; eIF, eukaryotic initiation factor; GAP, GTPase-activating protein; mTOR, mammalian target of rapamycin; raptor, regulatory associated protein of mTOR; Rheb, ras homologue enriched in brain; S6K1, ribosomal protein S6 kinase; TSC, tuberous sclerosis complex; TOS, TOR signaling motif.
by an unknown mechanism that may involve phosphorylation of eIF4G and/or its association with eIF4E. Support for the latter suggestion has been provided by studies performed in a different experimental model system, that is, a perfused hind-limb preparation from postabsorptive rats (15). In this model, raising the leucine concentration in the perfusate from 1 time to 10 times in the presence of 1-time levels of all other amino acids results in a 60–70% increase in protein synthesis that is associated with no change in mTOR signaling to 4E-BP1 and S6K1. Instead, the higher leucine concentration causes increased phosphorylation of eIF4G on Ser1108 and increased association of eIF4G with eIF4E. Thus, the perfused muscle preparation may provide a good experimental model system in which to investigate the insulin-independent effect of leucine on protein synthesis.

The studies described above examined the responses of translation initiation and protein synthesis in skeletal muscle to the administration of rather large amounts of leucine. To assess the responses to lesser amounts of the amino acid, studies have been performed in which food-deprived rats are administered by oral gavage leucine in amounts ranging from 0.068 to 1.35 g/kg body weight, the latter amount is equivalent to that administered in the studies described above (14). The studies show that stimulation of protein synthesis by leucine reaches a maximal value of ~135% of the untreated control rate with 0.135 g/kg of the amino acid, i.e., 10% of the amount used in the previously described studies. The response in protein synthesis parallels that of eIF4G phosphorylation and eIF4G association with eIF4E, whereas signaling through mTOR to 4E-BP1 and S6K1 continues to increase in proportion to the increasing amounts of leucine administered and its serum concentrations. An increase in the serum insulin concentration is observed only at the highest amounts of leucine administered, that is, 50% and 100% of that used in the previous studies. Signaling through mTOR appears to be enhanced by the increase in insulin but is not associated with a further increase in protein synthesis. In summary, these studies show a closer correlation of protein synthesis to eIF4G phosphorylation and its association with eIF4E than with signaling through mTOR to 4E-BP1 and S6K1.

Orally administered leucine enhances signaling through mTOR, but does not stimulate global rates of protein synthesis in liver

In contrast to findings in skeletal muscle, oral administration of leucine to fasted rats does not stimulate global rates of protein synthesis in liver, as assessed by the incorporation of radioactive phenylalanine into protein (16,17). However, leucine administration promotes phosphorylation of both 4E-BP1 and S6K1 (16,17), suggesting that, as in muscle, leucine enhances signaling through mTOR in liver. The finding that administration of rapamycin prevents the leucine-induced increase in 4E-BP1 and S6K1 phosphorylation (17) provides further support for this suggestion. Importantly, although leucine does not stimulate global rates of protein synthesis, administration of the amino acid to fasted rats promotes an increase in the translation of mRNAs encoding specific proteins, as assessed by changes in the association of mRNAs with polysomes. For example, in fasted rats the major portion of the mRNAs encoding ribosomal proteins L26, S4, and S8 is not associated with polysomes, and therefore is not being actively translated (17). However, within 60 min of oral leucine administration, there is a dramatic shift of all three mRNAs into polysomes. This finding is in contrast to the mRNA encoding β-actin, which is predominantly polysomal in both fasted rats and fasted rats administered leucine. This result suggests that leucine enhances the translation of mRNAs encoding some, but not all, proteins.

Leucine-induced signaling through mTOR

As noted in the previous sections, administering leucine to fasted animals promotes phosphorylation of proteins that are regarded as direct substrates of mTOR, including 4E-BP1 and S6K1. This suggests that the amino acid activates mTOR in muscle and liver (Figure 1). Further support for this suggestion is provided in studies in which food-deprived rats are injected intravenously with rapamycin, a specific inhibitor of mTOR, before administering leucine (10,17). These studies demonstrate that rapamycin blocks entirely the effects of leucine on the downstream targets of mTOR signaling, and attenuates the stimulatory effect of the amino acid on protein synthesis in muscle. Moreover, the leucine-induced shift of ribosomal-protein mRNAs into polysomes in liver is blocked by administering rapamycin before leucine, which suggests that mTOR signaling is necessary for the effect.

The mechanism through which leucine regulates mTOR signaling is incompletely defined. However, the effect likely involves two proteins that directly associate with kinase, the regulatory-associated protein of mTOR (raptor) and ras-homolog enriched in the brain (Rheb). Raptor is identified by its ability to bind to mTOR and form a nutrient-sensitive complex (18,19). In addition, raptor binds to both 4E-BP1 and S6K1 (18). Subsequent studies (20,21) showed that 4E-BP1 and S6K1 share a domain that contains a structure referred to as a TOR signaling motif (TOS), that mediates the association of the proteins with raptor. A single point mutation in the 4E-BP1 TOS motif reduces cell size (21) and impairs mTOR-mediated phosphorylation of the protein (20). Moreover, repressing raptor expression using RNAi decreases signaling through mTOR (19). However, it should be noted that in that study, downregulation of raptor expression also results in decreased expression of mTOR, making the interpretation of the results more difficult. Overall, the available evidence is consistent with a model wherein raptor acts as a molecular adaptor that recruits substrates, such as 4E-BP1 and S6K1, to mTOR for phosphorylation.

The interaction between mTOR and raptor is regulated in part by changes in amino acid availability. For example, in HEK293T cells deprived of all amino acids or deprived of leucine alone, the amount of raptor found in mTOR immunoprecipitates is increased relative to cells maintained in amino acid-containing medium (19). In contrast, the readdition of leucine to leucine-deprived cells restores raptor association with mTOR to control values. However, when cells are lysed in the presence of the crosslinking reagent, dithiobis(succinimidyl)propionate, leucine deprivation and readdition no longer modulate the amount of raptor present in mTOR immunoprecipitates (19). This finding suggests that leucine does not enhance dissociation of the raptor-mTOR complex per se, but instead may promote a conformational change in the complex whereby it adapts a less stable conformation in the presence compared to the absence of the amino acid. In this regard, it has been proposed that amino acids promote a shift in the raptor-mTOR complex from a stable, inactive complex to an unstable, active complex (19). Although the molecular details of such a conformational change have not been elucidated, a possible explanation for the observations above is that, in leucine-deprived cells, the TOS-binding domain on raptor is occluded by mTOR. With such a model, the provision of leucine through an undefined mechanism would induce a conformational change...
in raptor and/or mTOR that results in exposure of the TOS-binding domain on raptor, permitting it to bind to 4E-BP1 and S6K1 and thereby recruiting them to the complex to be phosphorylated. Importantly, the leucine-induced shift from an inactive to active raptor-mTOR complex is not simply a result of a destabilization of raptor binding to mTOR, because rapamycin also weakens the association of raptor with mTOR (19,22), but instead of enhancing signaling through mTOR, rapamycin represses it.

A second mTOR-binding protein that has been implicated in the regulation of mTOR signaling by amino acids is Rheb. Similar to many other ras homologs, Rheb is a small guanine nucleotide-binding protein and GTPase (23). Overexpression of the protein results in increased phosphorylation of both 4E-BP1 and S6K1 (24,25), suggesting that Rheb enhances signaling through mTOR. This suggestion is supported by the observation that rapamycin, but not the PI-3 kinase inhibitor wortmannin, prevents the Rheb-induced phosphorylation of 4E-BP1 (26). However, until recently, the mechanism through which Rheb might modulate signaling through mTOR had not been defined. Two recent studies (27,28) report the novel finding that Rheb binds directly to mTOR and to the mTOR-interacting protein referred to as G protein β-subunit-like protein (GβL, also known as mLST8). The binding of Rheb to mTOR does not depend on GDP or GTP, as a Rheb variant that does not bind guanine nucleotides is able to form the Rheb-mTOR complex (27,28). However, expression of a Rheb variant lacking guanine nucleotide-binding activity acts to repress mTOR function in cells in culture and in in vitro kinase assays, suggesting that association with guanine nucleotides is necessary for Rheb function in vivo. Importantly, a Rheb variant that exhibits constitutively high binding to GTP results in substantially greater mTOR protein kinase activity compared to wildtype Rheb (27), suggesting that when Rheb is associated with GTP it enhances mTOR activity to a greater extent than when it is associated with GDP.

The GDP/GTP binding status of RhoB is controlled in part by a GTPase-activating protein (GAP) referred to as tuberin (also known as TSC2) (24,25,29). However, overexpression of tuberin alone has little or no effect on signaling through mTOR (30). Instead, co-expression of tuberin and hamartin (also known as TSC1) results in decreased mTOR function, suggesting that it is a tuberin-hamartin complex that regulates mTOR signaling. Tuberin function is regulated through phosphorylation on multiple residues by a number of protein kinases. For example, PKB/Akt (31–34), the extracellular-signal regulated protein kinase ERK (36), and the AMP-activated protein kinase (37) all phosphorylate tuberin. Phosphorylation by PKB/Akt, p90Rsk (35), the extracellular-signal regulated protein kinase ERK (36), and the AMP-activated protein kinase (37) all phosphorylate tuberin. Phosphorylation by PKB/Akt, p90Rsk, or ERK inhibits the GAP activity of tuberin, resulting in increased GTP association with Rheb, and enhanced signaling through mTOR. In contrast, phosphorylation by AMPK enhances tuberin function and represses mTOR signaling.

Optimal amino acid-induced signaling through mTOR requires both Rheb and TSC1-TSC2. For example, in cells overexpressing Rheb, S6K1 phosphorylation is maintained during starvation for amino acids (38,39), which suggests that Rheb is involved in transducing signals from amino acids through mTOR. Also, in Drosophila, downregulated expression of either tuberin or hamartin causes cells to become resistant to amino acid deprivation (40). Thus, S6K1 phosphorylation is largely maintained during amino acid starvation in cells with reduced expression of either tuberin or hamartin. Similarly, in mammalian cells lacking either tuberin or hamartin, S6K1 phosphorylation is resistant to amino acid deprivation (32). Moreover, in mammalian cells in culture, co-overexpression of tuberin and hamartin prevents amino acid-dependent activation of S6K1 (30). Superficially, these studies might be interpreted as evidence that amino acids regulate signaling through mTOR by repressing the function of the tuberin-hamartin complex. However, an alternative interpretation is that the increase in Rheb GAP activity caused by increasing tuberin and/or hamartin expression is dominant to positive input from amino acids to mTOR. In this model, amino acids might activate a Rheb guanine nucleotide exchange factor (GEF), but overexpression of tuberin and/or hamartin overcomes the endogenous GEF activity resulting in an increase in the amount of Rheb associated with GDP, even in the presence of elevated amino acid concentrations.

Evidence suggesting that amino acids do not modulate mTOR signaling by regulating tuberin-hamartin GAP activity toward Rheb is provided by studies showing that amino acid deprivation does not alter the proportion of Rheb in GTP bound form (41,42). Instead, amino acids may regulate Rheb function by modulating the binding of Rheb to mTOR. In this regard, a recent study reports that deprivation of all amino acids or just leucine results in dissociation of the Rheb-mTOR complex (41). Readdition of the deprived amino acid(s) reverses the effect. The effect of amino acid deprivation and readdition is also observed in cells expressing the Rheb variant that does not bind guanine nucleotides (41), providing further evidence that amino acid-induced dissociation of Rheb from mTOR is not mediated by changes in guanine nucleotide charging. However, it should be noted that a second study (28) reports that amino acids do not alter the association of Rheb with mTOR. A caveat to the results of the second study is that binding of Rheb to mTOR was measured in cells exogenously expressing Rheb at high levels, rather than examining the binding of the endogenous protein to mTOR. Previous studies have shown that when Rheb is expressed at high levels, the proportion of the protein associated with GTP is elevated (27,29,43). Whether exogenous expression of Rheb at high levels might alter the kinetics of Rheb-mTOR association is unknown.

FUTURE DIRECTIONS

Although recent studies have significantly enhanced our understanding of the mechanisms involved in leucine-induced signaling through mTOR, much remains to be discovered. In particular, a topic that was not discussed herein, but of considerable interest, is the nature of the signal that initiates activation of the pathway. Is the signal induced by the binding of leucine to a receptor? If so, is the receptor intracellular or is it a transmembrane protein that links extracellular leucine? Does leucine need to be metabolized to generate the signal, and if so, what is the metabolite that activates signaling through mTOR? More detailed information is also needed to define the upstream components of the mTOR signaling pathway regulated by amino acids. For example, is tuberin and hamartin function regulated by amino acids? If it is, then do amino acids promote changes in tuberin and/or hamartin phosphorylation? Which kinase(s) is involved in such changes? Recent studies imply an important role for Rheb in amino acid-induced signaling through mTOR. How does Rheb modulate signaling through mTOR, and what role does it play in amino acid signaling? What role do other mTOR-interacting proteins, such as raptor, GβL, and rictor play in amino acid signaling through mTOR? Are there unidentified mTOR-interacting proteins involved in the effect? Finally, what role do protein phosphatases have in amino acid signaling in mammalian cells? These, and many other questions remain to be answered before an understanding of how amino acids, and leucine in particular, regulate protein synthesis in mammals.
LITERATURE CITED