

# Control of protein synthesis by amino acid availability

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Control of protein synthesis by amino acid availability is an active and centrally important area of research that has produced several recent advances in our understanding of how these substrates serve not only as precursors but also as signaling molecules. One particularly noteworthy advance is the identification of the unique specificity of leucine in signaling to stimulate protein synthesis in skeletal muscle. Leucine mediated signaling results in a stimulation of initiation of mRNA translation and involves increases in the phosphorylation status of the translational repressor 4E-BP1 and the ribosomal protein S6 kinase S6K1. It requires sustained activation of the mammalian target of rapamycin protein kinase. Leucine, however, also signals to stimulate protein synthesis in skeletal muscle by a mammalian target of rapamycin protein kinase independent (i.e. rapamycin insensitive) pathway, suggesting that the amino acid may signal through multiple pathways. Furthermore, leucine signaling in skeletal muscle differs from that in liver, suggesting that various responses may be tissue specific. Finally, there continues to be active research on the beneficial effects of glutamine as a unique supplement in catabolic circumstances. In this case, however, the signaling properties and mechanism of action of glutamine remain as an unsolved mystery. *Curr Opin Clin Nutr Metab Care* 5:63–67. © 2002 Lippincott Williams & Wilkins.

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**Current Opinion in Clinical Nutrition and Metabolic Care** 2002, 5:63–67

## Abbreviation

**mTOR** mammalian target of rapamycin protein kinase

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## Introduction

There is increasing recognition of the need to understand the role of amino acids as signaling molecules in the regulation of protein synthesis. Indeed, it is becoming abundantly clear that such an understanding is critically needed for effective therapeutic approaches to many pathophysiological circumstances that result in the loss of skeletal muscle tissue. It would appear that multiple amino acids act as signaling molecules and that their actions probably are mediated by multiple mechanisms. Two amino acids recently receiving a great deal of attention are leucine and glutamine. Thus, the purpose of this article is to review briefly some of the most recent findings on the control of protein synthesis by amino acid availability, with a particular focus on those reports related to leucine and glutamine.

## Stimulation of protein synthesis by increased availability of amino acids

Unquestionably, increased availability of amino acids stimulates protein synthesis in skeletal muscle in both humans and rodents. The stimulation of protein synthesis observed in response to increased availability of amino acids, however, is unlikely to be maintained indefinitely, and few studies have examined the duration of the effect. In this regard, a recent report reveals that a constant infusion of amino acids sufficient to raise their plasma concentrations approximately 1.7-fold above basal values stimulates protein synthesis in skeletal muscle 2.8-fold within 2 h of the onset of administration [1•]. During the subsequent 60 min of infusion, however, muscle protein synthesis returns to basal values even though plasma amino acid concentrations remain elevated. Moreover, both the initial stimulation and subsequent fall in synthesis are observed in all muscle fractions examined, including myofibrillar, sarcoplasmic, and mitochondrial, suggesting that a general control mechanism is involved in the observed response.

The composition of the diet by which increased availability of amino acids is provided to intact animals significantly affects the magnitude and duration of the stimulation of protein synthesis. In this regard, a recent study shows that whole body protein homeostasis in rats is better supported by dietary casein-bound amino acids than by crystalline free amino acids, and that protein-bound leucine is used more efficiently for liver protein synthesis than dietary free leucine [2]. These results in combination with those of an earlier study [3], suggest that slow absorption of amino acids from the gut, as occurs during digestion of protein, supports higher

postprandial rates of protein synthesis compared with the effect observed in response to rapid absorption of the same amount of dietary free amino acids.

In addition to fostering a general higher rate of protein synthesis, increased availability of amino acids also enhances the stimulation of muscle protein synthesis that occurs in response to exercise. The magnitude of the stimulation, however, depends on when the amino acids are administered relative to the period of exercise. For example, administration of a dietary supplement containing protein, carbohydrate, and fat immediately after exercise stimulates muscle protein synthesis, whereas administration of the same supplement 3 h after exercise does not [4]. Moreover, oral administration of an amino acid-carbohydrate supplement prior to exercise stimulates muscle protein synthesis to a significantly greater extent compared with administration immediately after exercise [5•]. Although the results of the two studies cannot be directly compared because of differences in composition of the dietary supplements as well as the exercise protocols employed, it is tempting to speculate that in order to optimally stimulate muscle protein synthesis, the best time to ingest amino acids is before, rather than after, exercise.

In cells in culture, availability of amino acids, and in particular leucine (see below), enhances protein synthesis in the absence of other stimuli. The same, however, may not be true *in vivo*. In fasted rats, feeding a complete meal, but not a meal lacking amino acids, stimulates protein synthesis in skeletal muscle [6••]. In contrast, treatment with diazoxide to prevent the feeding-induced increase in plasma insulin concentration prevents completely the stimulation of protein synthesis. Thus, both amino acids and insulin appear to be required for maximal stimulation of muscle protein synthesis.

One mechanism through which amino acid availability and insulin stimulate muscle protein synthesis involves activation of the ribosomal protein S6 kinase, S6K1, as well as the mRNA binding step in translation initiation. The binding of mRNA to the 40S ribosomal subunit is mediated by a heterotrimeric complex consisting of three different translation initiation factors: eIF4A, an RNA helicase, eIF4E, the protein that binds to the m<sup>7</sup>GTP cap structure at the 5'-end of the mRNA, and eIF4G, a scaffolding protein that binds to both eIF4A and eIF4E as well as to the 40S ribosomal subunit [7]. Thus, association of mRNA with the 40S ribosomal subunit requires the binding of eIF4E to eIF4G. Formation of the eIF4E-eIF4G complex is regulated by the eIF4E binding protein 4E-BP1. 4E-BP1 and eIF4G have overlapping binding sites on eIF4E such that binding of the two proteins to eIF4E is mutually exclusive. This

means that when 4E-BP1 is bound to eIF4E, the eIF4E-mRNA complex cannot bind to the ribosome and translation initiation is impaired. Formation of the 4E-BP1-eIF4E complex is regulated by multisite phosphorylation of 4E-BP1, where hyperphosphorylated 4E-BP1 does not bind to eIF4E and hypophosphorylated and unphosphorylated forms do.

In skeletal muscle of fasted rats fed a complete meal, 4E-BP1 is hyperphosphorylated, eIF4E dissociates from the 4E-BP1-eIF4E complex, and formation of the eIF4G-eIF4E complex is enhanced compared with the same parameters in the unfed controls [6••]. Moreover, S6K1 is present in hyperphosphorylated forms in the fed compared with the unfed controls. As is the case for protein synthesis, however, diazoxide prevents completely the feeding-induced changes in initiation factor phosphorylation and association as well as S6K1 activation.

Glucocorticoid mediated signaling provides an alternative mechanism for decreasing the insulin-induced stimulation of protein synthesis skeletal muscle. In this regard, dexamethasone treatment of fasting rats prevents the stimulation of muscle protein synthesis that occurs in response to infusion of amino acids or insulin [8]. Dexamethasone also prevents the amino acid and insulin-induced hyperphosphorylation of 4E-BP1 and S6K1.

Another mechanism through which amino acid availability stimulates protein synthesis is by promoting the binding of initiator methionyl-tRNA<sub>i</sub> (met-tRNA<sub>i</sub>) to the 40S ribosomal subunit. Met-tRNA<sub>i</sub> binds to the ribosome as a ternary complex consisting of eIF2, GTP, and met-tRNA<sub>i</sub>. During a later step in initiation, the GTP bound to eIF2 is hydrolysed to GDP, and eIF2 is released from the 40S ribosomal subunit as an eIF2-GDP complex. Prior to binding met-tRNA<sub>i</sub>, the GDP bound to eIF2 must be exchanged for GTP, a process catalysed by the guanine nucleotide exchange factor, eIF2B. The activity of eIF2B is regulated by phosphorylation of its  $\epsilon$ -subunit as well as by phosphorylation of the  $\alpha$ -subunit of eIF2, which converts it from a substrate into a competitive inhibitor of eIF2B. This mechanism is involved when fasted rats are fed a complete meal lacking single essential amino acids, which results in a failure of the feeding-induced response of both the mRNA and the met-tRNA<sub>i</sub> binding steps [9••]. Thus, in response to feeding a complete diet lacking either tryptophan or leucine, 4E-BP1 and S6K1 do not become hyperphosphorylated, but rather remain in the relatively hypophosphorylated states that exist in the unfed controls. In addition, compared with what is observed in muscle of rats fed a complete, balanced diet, the amount of eIF2 $\alpha$  in the phosphorylated form is increased and eIF2B activity is decreased. The observed changes

in initiation factors are associated with a failure of the feeding-induced stimulation of global rates of protein synthesis as well as the translation of mRNAs encoding ribosomal proteins. Unlike the majority of mRNAs, ribosomal protein mRNAs contain an uninterrupted stretch of pyrimidine residues adjacent to the m<sup>7</sup>GTP cap structure, which is referred to as a terminal oligopyrimidine sequence. Messages containing a terminal oligopyrimidine sequence exhibit upregulated translation under conditions that foster activation of S6K1, suggesting that phosphorylation of ribosomal protein S6 leads to their preferential recruitment to ribosomes [10].

### Leucine as a regulator of protein synthesis

Earlier studies established an important role for the branched-chain amino acids in regulating protein synthesis in skeletal muscle [11–13]. More recent studies have extended the earlier investigations to show that leucine is the most potent of the branched-chain amino acids in enhancing mRNA translation. One recent example demonstrates that oral administration of leucine to fasted rats invokes the same stimulation of protein synthesis in skeletal muscle as is observed following consumption of a complete meal [14]. Moreover, another recent example shows that leucine, but not arginine or histidine, stimulates protein synthesis in incubated preparations of epitrochlearis muscle [15•]. Finally, another example shows that ingestion of a mixture of the three branched-chain amino acids prior to 1 h of aerobic exercise exerts a protein-sparing effect during the subsequent 2 h recovery period [16]. Overall, the results suggest that branched-chain amino acids, and in particular leucine alone, can stimulate muscle protein synthesis after an overnight fast.

In skeletal muscle of fasted rats, 4E-BP1 is present in hypophosphorylated forms, 4E-BP1 binding to eIF4E is elevated, and eIF4G binding to eIF4E is repressed [14]. Feeding fasted rats either a complete meal or leucine alone results in hyperphosphorylation of 4E-BP1, release of 4E-BP1 from the 4E-BP1–eIF4E complex, and enhanced binding of eIF4G to eIF4E. In addition, provision of a complete meal or oral administration of leucine alone promotes hyperphosphorylation, and thus activation, of S6K1. Similarly, in fasted humans, infusion of leucine sufficient to raise the plasma concentration of the amino acid to the fed level results in increased phosphorylation of S6K1 in skeletal muscle [17•]. Finally, in in-vitro studies, leucine causes phosphorylation of S6K1 in both pancreatic  $\beta$ -cells [18•] and incubated preparations of epitrochlearis muscle [15•] as well as promoting hyperphosphorylation of 4E-BP1 in primary cultures of adipocytes [19••]. Of interest is the finding that the concentration of leucine required to maximally stimulate protein synthesis and activate S6K1

in incubated epitrochlearis muscle from young (4–5 week) or adult (6–8 month) rats is equivalent to the concentration observed in plasma of fed rats, that is approximately 200  $\mu$ M [15•]. In contrast, the concentration of leucine required for maximal stimulation in old (20 month) rats is approximately 400  $\mu$ M. At 400  $\mu$ M, however, leucine was able to stimulate both protein synthesis and S6K1 activity in old rats to the same extent as that observed for 200  $\mu$ M leucine in young and adult animals. Thus, muscle sensitivity to the stimulatory effect of leucine on protein synthesis declines with age. Overall, the available evidence suggests that leucine stimulates translation initiation by enhancing mRNA binding to the 40S ribosomal subunit as well as by activating S6K1.

The signal transduction pathway through which leucine promotes hyperphosphorylation of 4E-BP1 and S6K1 is not completely defined. It is clear, however, that the mammalian target of rapamycin protein kinase (mTOR) must be active in order for leucine to be effective. For example, in either rat muscle [20•] or pig muscle or liver [21], pretreatment of fasted animals with the mTOR inhibitor rapamycin completely prevents the leucine or feeding-induced hyperphosphorylation of 4E-BP1 and S6K1. Similarly, in in-vitro systems, rapamycin has been shown to prevent the amino acid-induced hyperphosphorylation of 4E-BP1 and S6K1 [18•,19•,22]. There is no direct evidence, however, that amino acids activate the protein kinase activity of mTOR. Furthermore, amino acids do not activate protein kinases upstream of mTOR as do growth promoting hormones such as insulin and insulin-like growth factor I [22]. Another unanswered question is whether leucine itself or a leucine metabolite is the activator. In this regard, a recent report using RINm5F cells claims that leucine must be metabolized in the mitochondria in order to promote S6K1 hyperphosphorylation [18•]. The conclusions drawn in that study are in direct contrast to an earlier report using primary cultures of rat adipocytes, where evidence was presented demonstrating that leucine metabolism is not required for its stimulatory effects on S6K1 phosphorylation [23].

In contrast to what is observed in skeletal muscle, oral administration of leucine to fasted rats has no effect on global rates of protein synthesis in liver [24•], suggesting that the regulatory role of leucine may be specific for muscle. Similarly, oral administration of either valine or isoleucine is without effect in rat liver. Although neither meal feeding nor oral administration of leucine has an apparent effect on synthesis of most hepatic proteins, both conditions result in enhanced incorporation of mRNAs encoding ribosomal proteins into polysomes [9•,24•], indicating that ribosomal proteins are being preferentially synthesized. Thus, the increased phos-

phorylation of both S6K1 and ribosomal protein S6 observed in liver in response to feeding [9<sup>•</sup>,24<sup>•</sup>] provides a likely explanation for the increased translation of ribosomal protein mRNAs.

### Glutamine as a regulator of protein synthesis

Evidence continues to accumulate to support the view that glutamine should be an essential part of total parenteral nutrition in various disease states. Indeed, much of the evidence was reviewed recently by a number of investigators who made presentations at the International Symposium on Glutamine, which was held in October 2000 in Bermuda. The proceedings of the Symposium are published as a supplement to *The Journal of Nutrition* (vol. 131, September 2001).

During the past year, two reports have appeared of studies on the effects of glutamine in normal, healthy subjects. Svanberg *et al.* [25] performed protein balance studies before and during infusions of a standard and a glutamine/tyrosine enriched amino acid solution. They concluded that provisions of glutamine neither stimulated protein synthesis nor attenuated breakdown of either globular or myofibrillar proteins in skeletal muscle of healthy volunteers. Mittendorfer *et al.* [26] measured whole body and skeletal muscle glutamine kinetics in the postabsorptive state and during the ingestion of an amino acid mixture containing either glutamine or glutamine plus glucose. They found that ingestion of an amino acid mixture that includes glutamine increases glutamine availability and uptake by skeletal muscle without causing an increase in the intracellular free glutamine pool. Simultaneous ingestion of glucose diminishes the intracellular concentration of glutamine in skeletal muscle despite increased availability in the blood due to decreased glutamine production. Both protocols result in increased utilization of muscle glutamine for protein synthesis despite the diminished intramuscular glutamine concentration induced by simultaneous glucose ingestion.

Protein catabolism in skeletal muscle, reflected by a decrease in protein synthesis and a negative nitrogen balance, can be reduced by administration of either glutamine or growth hormone. A recent study compares the effects of total parenteral nutrition containing either glutamine alone or glutamine together with growth hormone during the three postoperative days in patients undergoing an abdominal operation [27]. Growth hormone has an additive effect given together with glutamine on muscle amino acid metabolism, preventing the decrease in glutamine concentration in skeletal muscle and diminishing loss of whole body nitrogen. These improvements, however, are not associated with differences between the two treatment groups in muscle

protein synthesis postoperatively. Another study compares the efficacy of four diets differing in glutamine content and form in which glutamine is provided on tissue protein synthesis in rats in which a catabolic state is induced by glucocorticoid treatment [28]. Glutamine supplementation, either as free amino acid or in protein-bound form, is equally effective in stimulating protein synthesis in the jejunum. Only the free amino acid supplementation, however, causes an increase in protein synthesis in skeletal muscle.

### Conclusion

The accumulating evidence strongly supports a key role for amino acids as signaling molecules in the regulation of protein synthesis. One signaling pathway that is becoming better defined involves the protein kinase mTOR, the activity of which needs to be maintained in order for amino acids to mediate changes in the phosphorylation status of proteins such as 4E-BP1 and S6K1. mTOR appears to be a site of integration of signals generated by amino acids and those generated by growth factors such as insulin. Both types of signaling are required to maximally stimulate protein synthesis. Although the picture is becoming better defined for amino acid induced signaling involving mTOR, we continue to know very little about other pathways activated by amino acids. For example, there is evidence for signaling by the amino acid leucine to stimulate protein synthesis by a mTOR-independent (i.e. rapamycin insensitive) mechanism. Yet we have no knowledge of the signaling pathway involved in this effect. Likewise, we continue to understand little about how amino acid availability is sensed by the cell. Finally, a critical question requiring further understanding is why the stimulatory effect of amino acids on protein synthesis wanes despite a sustained increase in their availability.

### Acknowledgements

The studies described in this article that were performed in the laboratories of the authors were supported by research grants DK13499 and DK15658 from the National Institutes of Health.

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