Time Course of Leucine-induced 4E-BP 1 and S6K 1 Phosphorylation in the Liver and Skeletal Muscle of Rats

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Summary The purpose of the current study was to assess the involvement of the branched-chain amino acid leucine in the regulation of translation initiation in the liver and to compare the time course of leucine action on the translation initiation in the liver and skeletal muscle of rats. The phosphorylation of the eukaryotic initiation factor (eIF) 4E-binding protein 1 (4E-BP 1) frees eIF4E and stimulates protein synthesis by accelerating translation initiation. Phosphorylation of the 70-kDa ribosomal protein S6 kinase (S6K1) is thought to be involved in regulating the synthesis of certain ribosomal proteins and other selected proteins with polypyrimidine clusters near the transcription start site.

Food-deprived (18 h) male rats were orally administered 135 mg/100 g body weight of leucine and sacrificed at 0, 1, 3, or 6 h after administration. The oral administration of leucine resulted in an enhanced phosphorylation of 4E-BP 1 and S6K1 in both the liver and skeletal muscle. A time-dependent change in the phosphorylation state of 4E-BP 1 and S6K1 was more acute in the skeletal muscle than in the liver and closely paralleled the changes in plasma leucine concentration. Our results indicate that the primary mediator in 4E-BP 1 phosphorylation and S6K1 phosphorylation by the oral administration of leucine is an increase in the plasma concentration of leucine. Furthermore, our findings suggest differential sensitivity in the tissue response to oral administration of leucine.

Key Words leucine, 4E-BP 1, S6K1, translation initiation, rats

Feeding induces a rapid increase in the synthesis rate of total proteins in the skeletal muscle (1-4) and liver (1, 4) of growing postabsorptive animals. The signals responsible for stimulating protein synthesis remain unclear, though several studies have suggested a mediatory role for amino acids in this anabolic response (4-8). Dietary amino acids stimulate protein synthesis in skeletal muscle (4, 5, 8) and the liver (4, 5) by enhancing translation efficiency through stimulating peptide-chain initiation. Of all the amino acids, the branched-chain ones (leucine, isoleucine, and valine) play a unique role in this process. The stimulation of initiation caused by a complete mixture of amino acids can be reproduced by the provision of only the branched amino acids (9, 10). Of the branched-chain amino acids, leucine appears to be the specific effector on protein synthesis and translation initiation in muscle (9, 11-13).

A principal site in the regulation of translation initiation involves the binding of mRNA to 40S ribosome (14). This step requires a multi-subunit complex, referred to as eukaryotic initiation factor (eIF) 4F. The assembly of the eIF4F complex is regulated by the phosphorylation state of the translational repressor, eIF4E-binding protein 1 (4E-BP1). The formation of the eIF4F complex is prevented by 4E-BP1, which sequesters the mRNA cap-binding protein, eIF4E, into an inactive complex under the conditions of hypophosphorylation.

The hyperphosphorylation of 4E-BP 1 promotes assembly of the eIF4F complex and thus increases the translation of capped mRNAs.

The increased activity of the 70-kDa ribosomal protein S6 kinase (S6K1) has been implicated in stimulating protein synthesis under conditions that promote 4E-BP1 phosphorylation (15). The phosphorylation of S6K1 is associated with its activation (16, 17). The hyperphosphorylation of S6K1 appears to augment the translation of a specific class of genes characterized by the presence of an oligopyrimidine tract at the immediate 5’ end of the transcript (18). Recently it was shown that the oral administration of leucine stimulates global rates of protein synthesis in skeletal muscle concomitant with an increased eIF4F assembly and S6K1 phosphorylation (19, 20). It is unclear, however, whether the oral administration of leucine stimulates translation initiation in tissues other than skeletal muscle.

The purpose of the present study is to assess whether the phosphorylation of 4E-BP1 and S6K1 in the liver is increased by the oral administration of leucine and to compare the time course of leucine action on translation initiation in the liver and skeletal muscle.

MATERIALS AND METHODS

Animal and experimental design. The animal care protocol for this experiment was approved by the Iwate University Animal Research Committee under the
Guidelines for Animal Experiments of Iwate University. Three-week-old male Wistar rats purchased from CLEA Japan (Tokyo, Japan) were individually housed in stainless steel wire cages and maintained at 22°C and 55% relative humidity under a 12-h-light-dark cycle (06:00–18:00). They were allowed free access to water and a 20% casein diet according to AIN-93 (21) for 10 d. At the end of the feeding period, the rats were randomly assigned to one of four groups and deprived of food for 18 h, from 16:00 to 10:00 the following morning. One group of rats was anesthetized with diethylether and sacrificed at 10:00 (unfed for at least 18 h) just after the administration of 2.5 mL/100 g body weight saline (0.155 mol/L) by oral gavage (0 h after the administration of leucine). The rats in the remaining three groups were administered 135 mg/100 g body weight L-leucine by oral gavage (19, 20). The dose for L-leucine was 2.5 mL/100 g body weight (prepared as 54.0 g/L in distilled water). The rats were anesthetized with diethylether and sacrificed at 1, 3, or 6 h after administration.

Sample collection. Immediately after a blood sample was taken, both gastrocnemius muscles and the liver were excised in that order and rinsed in ice-cold saline. Tissues were excised and immediately weighed and homogenized in 7 volumes of buffer A [20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) at pH 7.4, 100 mM KCl, 0.2 mM EDTA, 2 mM ethylene glycol-bis (β-aminoethyl ether)-N,N',N'-tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 50 mM NaF, 50 mM β-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 0.5 mM sodium vanadate] using either a Dounce homogenizer (for the liver) or a Polytron homogenizer (for the muscle). Each homogenate was centrifuged at 10,000 × g for 10 min at 4°C (4).

Examination of 4E-BP1 phosphorylation state. An aliquot of the 10,000 × g supernatant was boiled for 10 min, then centrifuged at 10,000 × g for 30 min at 4°C. The resulting supernatant was mixed with an equal volume of 2X SDS sample buffer, and the diluted sample was subjected to electrophoresis on 15% polyacrylamide gel. The samples were then subjected to a protein immunoblot analysis by using rabbit anti-4E-BP1 polyclonal antibodies (purchased from Santa Cruz Biotechnology, Inc., CA, USA), as described previously (22).

Phosphorylation of S6K1. An aliquot of the 10,000 × g supernatant was combined with an equal volume of 2X SDS sample buffer, and the diluted sample was subjected to electrophoresis on 7.5% polyacrylamide gel. The samples were then subjected to a protein immunoblot analysis by using rabbit S6K1 polyclonal antibodies (purchased from Santa Cruz Biotechnology, Inc., CA, USA), as described previously (23).

Measurement of plasma amino acid concentrations. Plasma amino acid concentrations were measured by an automatic amino acid analyzer (AminoTac, JL-500/V, JEOL, Tokyo, Japan) after sulfosalicylic acid treatment (final 1.5%).

Statistical analyses. Data are means ± SE. The data were analyzed by using a one-way analysis of variance to assess the main effects with the treatment group as the independent variable. When a significant overall effect was detected, differences among individual means were assessed by using the Tukey-Kramer Multiple Comparisons test. The level of significance was set at p < 0.05 for all statistical tests.

RESULTS

An oral administration of leucine elevated its plasma concentration (Fig. 1), which peaked within 1 h and returned to the basal level by 6 h postadministration. Leucine administration reduced the circulating concentration of isoleucine and valine in a time-dependent manner (Fig. 1). The changes in plasma concentrations of isoleucine and valine were essentially the reverse of those of leucine. Leucine infusion decreases the plasma
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Fig. 2. Changes in the phosphorylation of 4E-BP1 in the liver of rats after leucine administration. 4E-BP1 is resolved into three bands on SDS-polyacrylamide gels, with the top band (γ-band) corresponding to the most highly phosphorylated species. A line graph displays the amount of 4E-BP1 in the γ-phosphorylated form, expressed as a proportion of the total 4E-BP1. The inset shows a representative immunoblot with the position of α-, β-, and γ-forms of 4E-BP1 noted to the left. Each value is the mean ± SE for 5 rats. Means not sharing a common superscript are significantly different (p<0.05) by the Tukey-Kramer multiple-comparison test.

concentrations of isoleucine and valine in rats (24). The mechanism of this effect is unknown, but it could involve specific stimulation of branched-chain amino acid oxidation by the consumption of excess leucine (24).

The phosphorylation state of 4E-BP1 can conveniently be examined by resolution of the phosphorylated forms of the protein during SDS-polyacrylamide gel electrophoresis. During SDS-polyacrylamide gel electrophoresis, 4E-BP1 is resolved into multiple electrophoretic forms, termed α, β, and γ, representing differentially phosphorylated forms of the protein. The most highly phosphorylated form, the γ form, exhibits the slowest electrophoretic mobility and is the only one of the three that does not bind to eIF4E. In this study, therefore, the phosphorylation of 4E-BP1 was expressed as the percentage of the protein in the γ form. Leucine administration stimulated phosphorylation of 4E-BP1 in both the liver (Fig. 2) and skeletal muscle (Fig. 3). In the liver (Fig. 2), maximal stimulation was observed between 1 and 3 h with a decrease in the phosphorylation of 4E-BP1 at 6 h. In the skeletal muscle (Fig. 3), the amount of 4E-BP1 in the γ form reached a maximal level within 1 h, then decreased at 3 h, but the values at 3 and 6 h remained elevated.

Upon activation, S6K1 is typically resolved into multiple electrophoretic forms after its separation by electrophoresis on SDS-polyacrylamide gel, with increased phosphorylation being associated with decreased electrophoretic mobility (25). In the present study, therefore, the effect of leucine administration on the phosphorylation of S6K1 was investigated in skeletal muscle and the liver by a protein immunoblot analysis. For S6K1, we quantified the ratio of the more heavily phos-
phosphorylated (more slowly migrating) forms to the total immune reactivity, because it is the phosphorylated forms that have kinase activity. Leucine administration had an obvious stimulatory effect on the phosphorylation of S6K1. In both tissues, maximal leucine-stimulated S6K1 phosphorylation occurred 1 h after leucine administration (Figs. 4, 5). S6K1 phosphorylation in the liver (Fig. 4) remained elevated at 3 h after leucine administration, whereas that in skeletal muscle (Fig. 5) returned to near the baseline level by 3 h.

**DISCUSSION**

Studies in cells and in vivo have demonstrated that amino acids stimulate protein synthesis congruous with the hyperphosphorylation of 4E-BP1 and S6K1, two proteins important in the selective control of mRNA translation (4, 19, 20, 26). Beyond their use as substrates, the branched-chain amino acids, and especially leucine, function as signaling molecules to promote the initiation of mRNA translation (19, 20, 26). Recently, Anthony et al. reported that the oral administration of leucine stimulates protein synthesis in skeletal muscle in association with the enhanced assembly of the mRNA cap recognition complex, eIF4F (20). These effects were singular to leucine because the administration of isoleucine and valine were ineffective in stimulating protein synthesis in skeletal muscle (19). However, little is known about the role of leucine in the regulation of hepatic protein biosynthesis. Studies of the perfused rat liver demonstrate that leucine enhances eIF4F assembly without altering the global rates of protein synthesis (27). We show for the first time that leucine administration promotes the hyperphosphorylation of 4E-BP1, implying enhanced rates of eIF4F assembly and translation initiation in the liver.

Insulin stimulates protein synthesis in several types of cells in culture (28, 29) and in rat skeletal muscle and other tissues in vivo (30). In skeletal muscle, the stimulation occurs in part through an enhanced initiation of mRNA translation (31). In overnight-fasted rats, an infusion of insulin at a rate that increments plasma insulin to levels that match those of fed animals while maintaining euglycemia reproduced the phosphorylation of S6K1 seen with feeding. This insulin infusion, however, did not affect the phosphorylation state of 4E-BP1 when compared with fasted saline-infused rats (32). Along with arginine and methionine, leucine is one of the insulin secretagogue amino acids (33). In the present study, the serum insulin concentration was essentially constant after leucine administration (data not shown). These findings provide evidence that the primary mediator in 4E-BP1 phosphorylation and S6K1 phosphorylation by an oral administration of leucine is not an increase in plasma insulin concentration.

In skeletal muscle, the time course of 4E-BP1 phosphorylation (Fig. 3) and S6K1 phosphorylation (Fig. 5) was remarkably similar and notably resembled the changes in leucine concentration (Fig. 1). In contrast, a dissociation between the time course of the phosphorylation state of two proteins (Figs. 2 and 4) and the plasma leucine concentration (Fig. 1) was observed in the liver. The plasma leucine concentration was immediately increased after oral administration before decreasing to 30% of the maximum level at 3 h after treatment (Fig. 1). The phosphorylation of 4E-BP1 (Fig. 2) and S6K1 (Fig. 4) was still stimulated at this time point. McNurlan et al. (34) failed to detect any effect of 100 µmol of leucine per 100 g body weight administration on the rate of protein synthesis in the liver and skeletal muscle. Funabiki et al. (35) reported that leucine injection (360 µmol/100 g body weight) stimulated protein synthesis in the liver, but not in skeletal muscle. Anthony et al. (20) showed that the oral administration of leucine (1,030 µmol/100 g body weight) stimulates protein synthesis in skeletal muscle. These findings suggest that leucine stimulation of protein synthesis in both the liver and skeletal muscle is dose-dependent. Similarly, Xu et al. (36) showed that leucine stimulates 4E-BP1 phosphorylation in a concentration-dependent manner in pancreatic RINm5F cells in cultures. Moreover, liver protein synthesis could be more sensitive to leucine than muscle protein synthesis could. At 3 h after administration, plasma leucine concentration declined by 70%, but it was still five times the food-deprived control level. The plasma leucine concentration at 3 h may be sufficient to stimulate the phosphorylation of 4E-BP1 and S6K1 in the liver, but not in skeletal muscle. Supporting this notion, a perfusion of rat liver with leucine alone at a concentration 4 times that reported in plasma of fasted rats resulted in increases in the phosphorylation of 4E-BP1 and S6K1 compared with livers administered a mixture of amino acid at 1X (27).

In summary, we showed in the present study that an oral administration of leucine results in enhanced phosphorylation of 4E-BP1 and S6K1 in both the liver and skeletal muscle. A time-dependent change in the phosphorylation state of 4E-BP1 and S6K1 was more acute in the skeletal muscle than in the liver and closely paralleled the changes in the plasma leucine concentration. Our findings suggest differential sensitivity in tissue response to the oral administration of leucine.

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