Oral Administration of Leucine Stimulates Phosphorylation of 4E-BP1 and S6K1 in Skeletal Muscle but Not in Liver of Diabetic Rats

Fumiaki YOSHIZAWA,* Sachiyo HIRAYAMA, Haruhito SEKIZAWA, Takashi NAGASAWA1 and Kunio SUGAHARA
Department of Animal Science, Faculty of Agriculture, Utsunomiya University, Utsunomiya, Tochigi 321-8505, Japan
1Department of Agro-Bioscience, Faculty of Agriculture, Iwate University, Morioka, Iwate 020-8550, Japan
(Received August 31, 2001)

Summary  Leucine performs a signaling role to enhance protein synthesis by phosphorylating eukaryotic initiation factor (eIF) 4E-binding protein 1 (4E-BP1) and 70-kDa ribosomal protein S6 kinase (S6K1), two key regulatory proteins involved in the initiation of mRNA translation. The purpose of the current study was to assess whether the phosphorylation of 4E-BP1 and S6K1 was increased in skeletal muscle and liver by an oral administration of leucine to diabetic rats and to determine the in vivo contribution of insulin to a leucine-dependent induction of 4E-BP1 and S6K1 phosphorylation. Food-deprived (18 h) normal and diabetic rats were orally administered 135 mg/100 g body weight L-leucine and sacrificed at 1 h after administration. Leucine administration resulted in enhanced phosphorylation of 4E-BP1 and S6K1 in skeletal muscle and in liver of nondiabetic rats. The stimulatory action of leucine on the phosphorylation of 4E-BP1 and S6K1 in skeletal muscle was not abolished in rats with streptozotocin-induced diabetes. In contrast, leucine administration did not stimulate the phosphorylation of 4E-BP1 and S6K1 in the liver of diabetic rats. These findings suggest that in skeletal muscle, leucine functions as a nutritional signaling molecule that independently regulates the phosphorylation states of 4E-BP1 and S6K1. In contrast to skeletal muscle, insulin is essential in mediating the leucine-dependent induction of 4E-BP1 and S6K1 phosphorylation in liver.

Key Words  leucine, 4E-BP1, S6K1, translation initiation, diabetes

Dietary amino acids stimulate protein synthesis in skeletal muscle and liver after food intake (1, 2). This anabolic effect may be attributed in part to an increase in amino acid supply to tissues, thereby augmenting substrate availability for peptide synthesis. Further, amino acids function as nutritional signaling molecules that regulate mRNA translation. Numerous reports have 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 (3–8).

A principal site in the regulation of translation initiation involves the binding of mRNA to 40S ribosome (9). This step requires a multisubunit complex, referred to as an eukaryotic initiation factor (eIF) 4F. The assembly of the elf4F complex is regulated by the phosphorylation state of the translational repressor, elf4E-binding protein 1 (4E-BP1). When hypophosphorylated, 4E-BP1 prevents the formation of the elf4F complex by sequestering the mRNA cap-binding protein, elf4E, into an inactive complex. The hyperphosphorylation of 4E-BP1 promotes the assembly of the elf4F complex and thus increases the translation of capped mRNAs.

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 (10). The activation of S6K1 is associated with its phosphorylation state (11, 12). 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 (13). It had been shown that the oral administration of leucine stimulates global rates of protein synthesis in skeletal muscle concomitant with increased elf4F assembly and S6K1 phosphorylation (6, 7). Recently, we demonstrated that an oral administration of leucine results in the enhanced phosphorylation of 4E-BP1 and S6K1 in skeletal muscle and liver (14). Insulin stimulates protein synthesis in rat skeletal muscle and other tissues in vivo (15), but the relative contribution of insulin to the leucine-dependent stimulation of muscle protein synthesis remains to be determined. More than 20 years ago, leucine was shown to stimulate protein synthesis in muscle isolated from diabetic rats (16). The mechanism through which insulin regulates protein synthesis initiation involves phosphorylation of 4E-BP1 and S6K1 (17, 18). Recently, Anthony et al. showed that leucine, administered orally
to fasted rats, restored rates of protein synthesis to levels observed in animals fed ad libitum (7). Such increases in protein synthesis were accompanied by an assembly of eIF4E and the activation of S6K1 and occurred independently of changes in plasma insulin levels (7). The purpose of the present study was to assess whether phosphorylation of 4E-BP1 and S6K1 was increased in the skeletal muscle and liver of diabetic rats by the oral administration of leucine and to determine the contribution of insulin to the leucine-dependent induction of 4E-BP1 and S6K1 phosphorylation in vivo.

MATERIALS AND METHODS

Animal and experimental design. The animal care protocol for this experiment was approved by the Utsunomiya University Animal Research Committee under the Guidelines for Animal Experiments of Utsunomiya 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 a 4:1 weight ratio. The rats in the other group, designated nondiabetic animals, received citrate buffer alone. Both the diabetic and nondiabetic animals were fed the 20% casein diet for another 2 d and were food-deprived for 18 h, from 16:00 to 10:00 the following morning. One half of the rats in the diabetic and nondiabetic groups were administered 135 mg/100 g body weight L-leucine by oral gavage (6, 7). The dose for L-leucine was 2.5 mL/100 g body weight (prepared as 54.0 g/L in distilled water). Rats not receiving leucine were gavaged with 2.5 mL of distilled water. The concentrations of glucose in the serum of streptozotocin-induced diabetic rats were significantly higher than the normal rats (Table 1). The serum insulin concentration in diabetic rats was lower than the normal rats treated with saline/100 g body weight (prepared as 54.0 g/L in distilled water).

Insulin ELISA, Mercodia AB, Uppsala, Sweden). Serum insulin was analyzed with a commercial enzyme immunoassay kit for rat insulin (Merckodia Rat Insulin ELISA, Merckodia AB, Uppsala, Sweden). Serum amino acid concentrations were measured by an automatic amino acid analyzer (JLC-300, JEOL, Tokyo, Japan) after sulfosalicylic acid treatment (final concentration 1.5%).

Statistical analyses. Data are means ± SE. Data were analyzed with 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, the differences among individual means were assessed by the Tukey-Kramer multiple comparisons test. The level of significance was set at p < 0.05 for all statistical tests.

RESULTS

The concentrations of glucose in the serum of streptozotocin-induced diabetic rats were significantly greater than in normal rats (Table 1). The serum insulin concentration in diabetic rats was lower than the detection limit of the ELISA kit used in this experiment.

Table 1. Serum glucose and insulin concentrations and body weight in normal and diabetic rats that were food-deprived or administered leucine orally.

| Treatment group | Glucose (mg/100 mL) | Insulin (pmol/L) | Body weight (g) |
|-----------------|---------------------|-----------------|-----------------|
| NS              | 75.5 ± 4.2* (8)     | 39 ± 4* (8)     | 88.5 ± 3.1* (8) |
| NL              | 57.1 ± 4.5* (8)     | 45 ± 7* (8)     | 80.0 ± 3.1* (8) |
| DS              | 226.4 ± 22.5* (7)   | <12*            | 70.1 ± 3.9* (7) |
| DL              | 275.2 ± 50.7* (7)   | <12*            | 71.4 ± 3.0* (7) |

1 Values are means ± SE (n).
2 Means not sharing a common superscript are significantly different (p < 0.05) by the Tukey-Kramer multiple comparison test.
3 NS, normal, saline treated; NL, normal, leucine treated; DS, diabetic, saline treated; DL, diabetic, leucine treated.
* The detection limit of the ELISA kit used in this experiment is 12 pmol/L.
Leucine Stimulates Phosphorylation of 4E-BP1 and S6K1 in Skeletal Muscle of Diabetic Rats

Leucine administration had no effect on serum glucose and insulin concentrations (Table 1). The oral administration of leucine elevated the serum concentration of leucine and reduced the circulating concentration of isoleucine and valine in diabetic and nondiabetic rats (Fig. 1). The changes in plasma concentrations of isoleucine and valine were essentially the reverse of those of leucine. Leucine infusion has been demonstrated to decrease the plasma concentrations of isoleucine and valine in rats (21). The mechanism of this effect is unknown, but it may involve the specific stimulation of branched-chain amino acid oxidation to consume excess leucine (21), an increase in uptake of those amino acids to support enhanced rates of protein synthesis (6) or a combination of these factors.

The phosphorylation state of 4E-BP1 can be conveniently examined by a resolution of the phosphorylated forms of 4E-BP1 during SDS-polyacrylamide gel electrophoresis. During SDS-polyacrylamide gel electrophoresis, 4E-BP1 is resolved into multiple electrophoretic forms, termed \( \alpha \), \( \beta \), and \( \gamma \), representing differentially phosphorylated forms of the protein. The most highly phosphorylated form, the \( \gamma \) form, exhibits the slowest electrophoretic mobility and is the only one of the three that does not bind to eIF4E. Therefore in this study, the phosphorylation of 4E-BP1 was expressed as the percent 4E-BP1 in the \( \gamma \) form.

Leucine administration was found to stimulate the phosphorylation of 4E-BP1 in skeletal muscle of nondiabetic rats (Fig. 2). The increase in the amount of 4E-BP1 in the \( \gamma \) form observed in the skeletal muscles of nondiabetic rats in response to leucine administration was also observed in the skeletal muscles of diabetic rats (Fig. 2). In the liver of nondiabetic rats, leucine administration caused a significant increase in the amount of 4E-BP1 in the \( \gamma \) form (Fig. 3). In contrast, no change in the amount of 4E-BP1 in the \( \gamma \) form was observed in the liver of diabetic rats in response to leucine administration (Fig. 3).

Upon activation, S6K1 is typically resolved into multiple electrophoretic forms after separation by electrophoresis on SDS-polyacrylamide gel, with increased phosphorylation being associated with decreased electrophoretic mobility (22). Therefore in the present study, the effect of leucine administration on the phosphorylation of S6K1 was investigated in skeletal muscle and liver by a protein immunoblot analysis. For S6K1, we quantified the ratio of the more heavily phosphorylated (more slowly migrating) forms to the total immune reactivity because it is the phosphorylated forms that possess kinase activity.

Leucine administration had an obvious stimulatory effect on the phosphorylation of S6K1 in the skeletal muscles of nondiabetic rats (Fig. 4). The hyperphosphorylation of S6K1 in response to leucine administration...
Fig. 4. Effect of leucine administration on the phosphorylation state of S6K1 in the skeletal muscles of food-deprived normal and diabetic rats. S6K1 is resolved into multiple electrophoretic forms on SDS-polyacrylamide gels. The rapidly migrating band is arbitrarily designated α and the more slowly migrating bands β and γ. A bar graph displays the amount of S6K1 in the β and γ forms, expressed as a proportion of the total S6K1. The inset shows a representative immunoblot with the positions of α-, β-, and γ-forms of S6K1 noted to the left. NS, normal, saline treated; NL, normal, leucine treated; DS, diabetic, saline treated; DL, diabetic, leucine treated. Each value is the mean±SE for 8 (NS and NL) or 7 (DS and DL) rats. Means not sharing a common superscript are significantly different (p<0.05) by the Tukey-Kramer multiple-comparison test.

Fig. 5. Effect of leucine administration on the phosphorylation state of S6K1 in livers of food-deprived normal and diabetic rats.

was also observed in the skeletal muscles of diabetic rats (Fig. 4). In contrast, in the liver of diabetic rats the phosphorylation state of S6K1 was unaffected by leucine administration (Fig. 5). The phosphorylation of S6K1 in the liver of diabetic rats (DS and DL) was higher than that in food-deprived controls (NS) (Fig. 5). The basis for this increase in S6K1 phosphorylation is unknown. We cannot discount the possibility that insufficient endogenous insulin secretion disrupts phosphate turnover on the protein.

DISCUSSION

Insulin stimulates protein synthesis in several types of cells in culture (23, 24) as well as in rat skeletal muscle and other tissues in vivo (15). In skeletal muscle, stimulation occurs in part through the enhanced initiation of mRNA translation (25). Several reports indicate that physiological increases in circulating insulin concentrations are not sufficient to stimulate rates of protein synthesis in postabsorptive rats (1, 2, 7, 26, 27). One of us [Yoshizawa et al. (1)] previously demonstrated that refeeding a protein-containing diet to rats fasted for 18 h stimulated protein synthesis in skeletal muscle and liver concomitant with eIF4F assembly and S6K1 phosphorylation. However, even though a protein-free diet was as effective at raising plasma insulin concentration as a protein-containing diet, it was unable to stimulate protein synthesis in skeletal muscle or liver (1). Moreover, a recent study of diabetic mice revealed that changes in protein synthesis, 4E-BP1 phosphorylation, and 4E-BP1 and eIF4G binding to eIF4E in response to feeding are the same in the skeletal muscles of diabetic animals as in controls, though plasma insulin concentrations are only increased in control animals (28). Taken together, the findings of these studies strongly implicate amino acid supply, perhaps in combination with a permissive concentration of insulin, as a mediator of the response of protein synthesis to feeding and an eIF4F regulatory step in skeletal muscle and liver.

Of all the amino acids, leucine appears to be most effective at recapitulating the responses in protein synthesis and eIF4F regulation produced by feeding a protein-containing meal (7). In the skeletal muscles of rats fasted for 18 h, the oral administration of leucine stimulates protein synthesis in association with hyperphosphorylation of 4E-BP1 and S6K1. When leucine is administered alone, there is no concomitant increase in circulating insulin concentration 1 h after gavage (6, 7). The results suggest that leucine independently stimulates muscle protein synthesis in the presence of fasting or basal concentrations of circulating insulin. In the present study, increases in phosphorylation of 4E-BP1 and S6K1 were observed in the skeletal muscles of non-diabetic and diabetic rats in response to leucine administration. However, phosphorylation levels of 4E-BP1 and S6K1 in diabetic rats administered leucine were significantly lower than those in nondiabetic rats administered leucine. The serum insulin concentration in streptozotocin-induced diabetic rats was undetectable by an enzyme immunoassay (Table 1). These results suggest that insulin is playing a permissive role; i.e., the hormone facilitates, but is not required for, the stimulation of 4E-BP1 and S6K1 phosphorylation in response to leucine administration. In skeletal muscle, the amino-acid-specific input may initiate an unidentified insulin-independent signaling pathway and thereby alter the phosphorylation states of 4E-BP1 and S6K1.

In contrast to skeletal muscle, leucine administration has a stimulatory effect on the phosphorylation of 4E-BP1 and S6K1 in the livers of nondiabetic rats, but not in the livers of diabetic rats. This result indicates that insulin is essential in mediating the leucine-dependent induction of 4E-BP1 and S6K1 phosphorylation in the liver. Although it appears that in the hepatocyte the an-
abolic effects of insulin are secondary to those of amino acids, insulin and amino acids synergistically promote the assembly of eIF4F and the activation of S6K1 (29, 30). Our findings in the liver corroborate these reports. We observed that in combination with insulin, leucine promotes hyperphosphorylation of 4E-BP1 and S6K1 in the liver. The simplest interpretation of the present data is that phosphorylation of 4E-BP1 and S6K1 in the liver requires input from two independent signal transduction pathways, one stimulated by leucine and the other by insulin.

In summary, we have shown in the present study that the oral administration of leucine enhances the phosphorylation of 4E-BP1 and S6K1 in skeletal muscle and livers of nondiabetic rats. The stimulatory action of leucine on the phosphorylation of 4E-BP1 and S6K1 in skeletal muscle was not abolished in rats with streptozotocin-induced diabetes. In contrast, leucine administration had no stimulatory effect on the phosphorylation of 4E-BP1 and S6K1 in the liver of diabetic rats. These findings suggest that leucine independently functions as a nutritional signaling molecule, regulating the phosphorylation states of 4E-BP1 and S6K1 in the liver of diabetic rats. The simplest interpretation of the present data is that phosphorylation of 4E-BP1 and S6K1 in the liver. The simplest interpretation of the present data is that phosphorylation of 4E-BP1 and S6K1 in the liver.

Acknowledgments
This work was supported in part by Grants-in-Aid for scientific research (No. 12760098 and No. 12460054) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The authors thank Dr. Hideyuki Tanaka (Utsunomiya University) for the analysis of serum amino acids concentrations.

REFERENCES
1) Yoshizawa F, Kimball SR, Vary TC, Jefferson LS. 1998. Effect of dietary protein on translation initiation in rat skeletal muscle and liver. Am J Physiol 275: E814–E820.
2) Yoshizawa F, Endo M, Ide H, Yagasaki K, Funabiki R. 1995. Translational regulation of protein synthesis in the liver and skeletal muscle of mice in response to refeeding. J Nutr Biochem 6: 130–136.
3) Buse MG, Reid SS. 1975. Leucine. A possible regulator of protein turnover in muscle. J Clin Invest 56: 1250–1261.
4) Li JB, Jefferson LS. 1978. Influence of amino acid availability on protein turnover in perfused skeletal muscle. Biochim Biophys Acta 544: 351–359.
5) Buse MG, Atwell R, Mancusi V. 1979. In vitro effect of branched chain amino acids on the ribosomal cycle in muscles of fasted rats. Horm Metab Res 11: 289–292.
6) Anthony JC, Yoshizawa F, Anthony TG, Vary TC, Jefferson LS, Kimball SR. 2000. Leucine stimulates translation initiation in skeletal muscle of postabsorptive rats via a rapamycin-sensitive pathway. J Nutr 130: 2413–2419.
7) Anthony JC, Anthony TG, Kimball SR, Vary TC, Jefferson LS. 2000. Orally administered leucine stimulates protein synthesis in skeletal muscle of postabsorptive rats in association with increased eIF4F formation. J Nutr 130: 139–145.
8) Anthony JC, Anthony TG, Kimball SR, Jefferson LS. 2001. Signaling pathways involved in translational control of protein synthesis in skeletal muscle by leucine. J Nutr 131: 856S–860S.
9) Pain VM. 1996. Initiation of protein synthesis in eukaryotic cells. Eur J Biochem 236: 747–771.
10) Sonenberg N. 1996. mRNA 5′ cap-binding protein eIF4E and control of cell growth. In: Translational Control (Hershey JBW, Mathews MB, Sonenberg N, eds), p 245–289. Cold Spring Harbor Laboratory Press, Plainview, New York.
11) Cheatham B, Vlahos CJ, Cheatham L, Wang L, Blenis J, Kahn CR. 1994. Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation. Mol Cell Biol 14: 4902–4911.
12) Chung J, Grammer TC, Lemon KP, Kazlauskas A, Blenis J. 1994. PDGF- and insulin-dependent pp70S6k activation mediated by phosphatidylinositol-3-OH kinase. Nature 370: 71–75.
13) Amaldi F, Pierandrei-Amaldi P. 1997. TOP genes: a translationally controlled class of genes including those coding for ribosomal proteins. Prot Mol Subcell Biol 18: 1–17.
14) Yoshizawa F, Sekizawa H, Hirayama S, Hatakeyama A, Nagasawa T, Sugahara K. 2001. Time course of leucine-induced 4E-BP1 and S6K1 phosphorylation in the liver and skeletal muscle of rats. J Nutr Sci Vitaminol 47: 311–315.
15) Kimball SR, Flaim KE, Peavy DE, Jefferson LS. 1990. Protein metabolism. In: Ellenberg and Rifkin’s Diabetes Mellitus. Theory and Practice (Rifkin H, Porte DJ, eds), p 41–50. Elsevier Science, New York.
16) Buse MG, Weigand DA. 1977. Studies concerning the specificity of the effect of leucine on the turnover of proteins in muscles of control and diabetic rats. Biochim Biophys Acta 475: 81–89.
17) Kimball SR, Horetsky RL, Jefferson LS. 1998. Signal transduction pathways involved in the regulation of protein synthesis by insulin in L6 myoblasts. Am J Physiol 274: C221–C228.
18) Flynn A, Proud CG. 1996. The role of eIF4 in cell proliferation. Cancer Surv 27: 293–310.
19) Reeves PG, Nielsen FH, Fahey GC Jr. 1993. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 123: 1939–1951.
20) Kimball SR, Jurasinski CV, Lawrence JC, Jefferson LS. 1997. Insulin stimulates protein synthesis in skeletal muscle by enhancing the association of eIF-4E and eIF-4G. Am J Physiol 272: C754–C759.
21) Harper AE, Miller RH, Block KP. 1984. Branched-chain amino acid metabolism. Annu Rev Nutr 4: 409–454.
22) Weng QP, Andrabi K, Kozlowski MT, Grove JR, Avruch J. 1997. Phosphorylation of 4E-BP1 and S6K1 in skeletal muscle. Cell 88: 2333–2340.
23) Kimball SR, Horetsky RL, Jefferson LS. 1995. Hormonal regulation of albumin gene expression in primary cultures of rat hepatocytes. Am J Physiol 268: E6–E14.
24) Palmer RM, Bain PA. 1989. Indomethacin inhibits the insulin-induced increases in RNA and protein synthesis.
in L6 skeletal muscle myoblasts. Prostaglandins 37: 193–203.

25) Kimball SR, Vary TC, Jefferson LS. 1994. Regulation of protein synthesis by insulin. Annu Rev Physiol 56: 321–348.

26) Garlick PJ, Fern M, Preedy VR. 1983. The effect of insulin infusion and food intake on muscle protein synthesis in postabsorptive rats. Biochem J 210: 669–676.

27) Gautsch TA, Anthony JC, Kimball SR, Paul GL, Layman DK, Jefferson LS. 1998. Availability of eIF4E regulates skeletal muscle protein synthesis during recovery from exercise. Am J Physiol 274: C406–C414.

28) Svanberg E, Jefferson LS, Lundholm K, Kimball SR. 1997. Postprandial stimulation of muscle protein synthesis is independent of changes in insulin. Am J Physiol 272: E841–E847.

29) Hara K, Yonezawa K, Weng QP, Kozlowski MT, Belham C, Avruch J. 1998. Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. J Biol Chem 273: 14484–14494.

30) Patti ME, Brambilla E, Luzi L, Landeker EJ, Kahn CR. 1998. Bidirectional modulation of insulin action by amino acids. J Clin Invest 101: 1519–1529.