Stimulative Effect of Dietary Protein on the Phosphorylation of p70 S6 Kinase in the Skeletal Muscle and Liver of Food-deprived Rats

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The effect of dietary protein on p70S6k phosphorylation was examined in rats starved for 18 h and then fed either a 20% casein diet (20C) or a protein-free diet (0C). Refeeding the 20C diet, but not the 0C diet, increased p70S6k phosphorylation in both the skeletal muscle and liver. The plasma insulin concentrations were the same after refeeding the 20C or 0C diet, suggesting that a combination of dietary protein and insulin may be required to stimulate p70S6k phosphorylation.

Key words: protein synthesis; p70S6k; food intake; dietary protein; rat

The rate of protein synthesis in growing rodents is highly sensitive to the immediate intake of food.1-4 The acute increase in protein synthesis in response to refeeding occurs through stimulation of the translation of mRNA. Furthermore, the stimulation of protein synthesis caused by refeeding is mediated by an increase in the initiation of mRNA translation.3-5 The process of initiation comprises numerous steps and is mediated by 12 proteins that are referred to as eukaryotic initiation factors (eIFs). Of the many steps, two appear to be particularly important in the physiological regulation of translation, i.e., the binding of initiator methionyl-tRNA, (met-tRNA), to the 40S ribosomal subunit, a reaction mediated by eukaryotic initiation factor (eIF) 2, and the initial binding of 40S ribosome to the 5'-end of mRNA, a reaction mediated by eIF4F. In a previous study,6 the increase in translation initiation in both skeletal muscle and liver in response to refeeding a complete diet was not associated with any detectable change in the activity of eIF2B nor in the phosphorylation state of eIF2a, both regulating binding of initiator methionyl-tRNA, to the 40S ribosomal subunit. Instead, stimulation was associated with the enhanced formation of the active eIF4F complex. One of the eIF4F family members, eIF4E, plays an important role in binding of mRNA to the 40S ribosomal subunit, because it is the initiation factor that binds the m`GTP cap present at the 5'-end of eukaryotic mRNAs. The availability of eIF4E for eIF4F complex formation can be modulated by changes in the association of eIF4E with an eIF4E-binding protein termed 4E-BP1.7 4E-BP1 sequesters eIF4E into an inactive complex. The binding of eIF4E to 4E-BP1 is regulated by phosphorylation of the binding protein, which results in a decrease in the affinity of eIF4E for 4E-BP1. The signal(s) that causes the stimulation of protein synthesis in response to refeeding has been the subject of investigation for decades, with the majority of studies reporting that an increase in either insulin or amino acids is responsible for the stimulation. The regulation of 4E-BP1 phosphorylation by insulin occurs through the activation of a signal transduction pathway that also activates p70 S6 kinase (p70S6k).8 It has also been observed that amino acids regulate the phosphorylation of 4E-BP1 by activating the p70S6k signalling pathway.9,10 Therefore, the p70S6k signalling pathway is thought to be important in regulating the rate of translation in response to refeeding.

Activation of p70S6k is associated with its phosphorylation on multiple Ser and Thr residues. In the present study, the effect of refeeding on the phosphorylation of p70S6k was investigated in rat skeletal muscle and liver by a protein immunoblot analysis, and we also examined the role of the protein component of the diets in mediating the immediate response of the phosphorylation of p70S6k to food intake.

Male Wistar rats 3 wk of age were purchased from SLC (Shizuoka, Japan). They were individually housed in stainless steel wire cages and maintained at 22°C and 55% relative humidity with a 12 h-light-dark cycle (6:00-18:00). They were allowed free access to water and a 20% casein diet (20C) according to AIN7611,12 for 3d. To acclimatize them to feeding within a short period, the daily feeding schedule was changed as follows: the diet was given from 10:00 to 22:00 for 3d, then from 10:00 to 19:00 for 3d, and finally from 10:00 to 16:00 for 6d. At the end of the feeding period, the rats were starved for 18 h, from 16:00 to 10:00 the following morning, and refed a 20C or an isocaloric protein-free diet (0C) for 1 h. The protein-free diet was prepared by replacing casein with α-cornstarch and sucrose. The animals were anesthetized with diethylether and killed -1 (before feeding), 0, 3, 6, 9, or 12 h after refeeding had been completed. Immediately after removing a blood sample, 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

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Abbreviations: p70S6, p70 S6 kinase; eIF, eukaryotic initiation factor; SDS, sodium dodecyl sulfate
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',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(1) 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, an aliquot of the resulting supernatant was combined with an equal volume of an SDS sample buffer, and the diluted sample was subjected to electrophoresis on 7.5% polyacrylamide gel.(13) The samples were then subjected to an protein immunoblot analysis, using rabbit anti-p70S6K polyclonal antibodies (purchased from StressGen Biotechnologies Corp., BC, Canada), as described previously. The plasma insulin concentration was determined by an enzyme immunoasay with an insulin assay kit (Grazyme Insulin EIA Test, Wako Pure Chemicals, Osaka, Japan).

Upon activation, p70S6K 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. Therefore, in the present study, the effect of food intake on the phosphorylation of p70S6K was investigated in skeletal muscle and liver by a protein immunoblot analysis. As shown in Fig. 1, refeeding the 20C diet for 1 h caused a shift in the distribution of p70S6K to the slower-migrating species, indicative of phosphorylation of the protein, in both the skeletal muscle and liver. The distribution of the kinase among the electrophoretic forms returned to the pattern observed in the 18 h-starved rats 12 h after refeeding. In contrast, refeeding the isocaloric diet lacking protein had no effect on p70S6K phosphorylation.

One hour after 18 h-fasted mice had been refed a complete diet containing 25% casein, the size spectrum of polysomes, which is a useful indicator of translational activities, shifted to the heavier polysomes in the skeletal muscle and liver. When the rate of translation initiation increases relative to that of elongation, ribosomes are aggregated and display a heavier polysome profile. The proportion of heavier polysomes in skeletal muscle(15) was increased by refeeding a complete diet for 1 h and was reduced to the basal level 6 h after refeeding. The size spectrum of polysomes in the liver changed in a similar fashion. Refeeding the complete diet for 1 h increased the proportion of heavier polysomes in the liver by up to 134% of the 18 h-starved (control) value, and subsequent fasting reduced it to the control level in 6 h (Yoshizawa, F., unpublished observations). The change in the activity of p70S6K (Fig. 1) was consistent with the change in the size spectrum of polysomes in the skeletal muscle(15) and liver (Yoshizawa, F., unpublished observations), suggesting that feeding the 20C diet stimulated the translational activities in part through utilization of the p70S6K signal transduction pathway.

A number of studies(7,8,16,17) have shown that insulin activated the p70S6K signal transduction pathway. To determine whether or not the plasma insulin concentration was proportional to p70S6K phosphorylation, the concentration of insulin in the plasma from animals fed on either the 20C or 0C diet was measured. The plasma insulin concentration was increased by about 2.5-fold by refeeding with either the 20C or 0C diet, this being followed by a rapid decrease after the feeding period (Fig. 2). Thus, the rise in plasma insulin appears to have been dependent on the caloric intake, but independent of protein ingestion. Furthermore, an increase in plasma insulin was not sufficient to activate the p70S6K in either the skeletal muscle or liver of rats fed on the 0C diet. However, this result does not exclude the possibility that an increase in plasma insulin may be required for the activation of p70S6K by the 20C diet. Recent studies(9,10,18,19) have shown that amino acids increased p70S6K phos-
phorylation. Thus, it is possible that the abrupt, large increase in serum amino acids that occurred after feeding the 20C diet may have led to the activation of p70<sup>65k</sup>. Overall, the results suggest that the activation of the p70<sup>65k</sup> signal transduction pathway in response to food intake is not necessarily mediated by insulin alone, but instead is regulated by the increase in plasma amino acid concentration that occurs after feeding a 20C diet in combination with the increased plasma insulin concentration.

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