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# Translational regulation of protein synthesis in the liver and skeletal muscle of mice in response to refeeding

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*To identify the mechanism that modulates the rate of protein synthesis in different tissues responding to food intake, several parameters of translational activities were measured together with the rate of protein synthesis in the liver and skeletal muscle. In 18-hour fasted mice, protein synthesis in muscle and the liver was stimulated by refeeding a complete diet after 1 hour. Refeeding a protein-free diet increased the protein synthesis in the liver but not in muscle. Injection of anti-insulin serum suppressed the response to refeeding in both tissues except to a complete diet in the liver. The response of liver protein synthesis to food intake is not necessarily mediated by insulin, provided an abrupt, large increase in plasma amino acid concentration occurs. In contrast to the liver, an elevation of insulin level is essential for the stimulation of protein synthesis in muscle along with a high concentration of plasma amino acids. The stimulation of elongation activity in addition to stimulation of the initiation activity contributed to the enhancement of protein synthesis induced by refeeding in both the liver and muscle. In another set of experiments, we also observed a delayed rise of elongation coupled with an immediate rise of initiation in the liver after refeeding.* (J. Nutr. Biochem. 6:130–136, 1995.)

**Keywords:** protein synthesis; muscle; liver; insulin; polysome size; initiation and elongation

## Introduction

A rise of protein synthesis in skeletal muscle[1,2] and the liver[2] is observed immediately after the beginning of feeding a complete diet. With the onset of refeeding, the serum concentration of substrates such as glucose and amino acids increases, and changes occur in endocrine status, including a rise of insulin, and in the circulating $T_3$ level.[3] These factors and others have been implicated in the regulation of protein synthesis. However, many studies[4–9] have deduced that concentrations of insulin and amino acids strongly influence protein synthesis. Preedy and Garlick postulated that the response of muscle protein synthesis to food intake in vivo is mediated primarily by increases in plasma concentrations of insulin and amino acids acting cooperatively.[1] An acute rise of protein synthesis in diabetic mice was also observed in the liver, but not in muscle in response to refeeding a complete diet.[2] It was also observed that an injection of L-leucine to diabetic mice stimulated the rate of protein synthesis in the liver but not in muscle.[10] In addition, the work on perfused liver has shown that insulin can reproduce the effects of food intake by inhibiting proteolysis, along with minor stimulation of protein synthesis.[11] It was therefore suggested that the increase in liver protein synthesis by refeeding might be independent of insulin, involving amino acids and/or other factors.

The present studies had two objectives. The first was to ascertain extracellular mediators that signal the translational apparatus to drive in response to refeeding. We considered the three factors as possible extracellular mediators responding to refeeding; namely, insulin, amino acids, and glucose. Glucose has been reported to stimulate muscle protein synthesis.[7,12] We describe here the rate of protein syn-

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thesis in the liver and skeletal muscle of mice with or without anti-insulin serum 1 hour after refeeding a complete or a protein-free diet. The second objective was to identify the components of this translational apparatus and to assess their operating mechanisms that modulated the rate of protein synthesis in response to the signals. We found that an enlargement of polysome size resulted from coupling an immediate rise of initiation activity with a delayed rise of elongation activity in response to refeeding.

## Materials and methods

### *Animals*

Male mice (Crj:CD-1, Charles River Japan Inc., Atsugi, Japan, 4 wk of age) were individually housed in stainless steel wire cages and maintained at 23 °C on a 12-hr light-dark cycle. They were given a purified diet (see below) and water ad libitum for 4 days. To accustom the mice to eating within a short period, the diet was given at 1:00 p.m. and withdrawn at 1:00 a.m. for 4 days, and then at 1:00 p.m. and withdrawn at 7:00 p.m. for 6 days. The composition of the complete diet was 25% casein, 27.5% α-cornstarch, 8% corn oil, 27.5% sucrose, 5% mineral mixture (mineral mix 1, prepared by Oriental Yeast Co., Tokyo, Japan), 1% vitamin mixture (vitamin mix 1, prepared by Oriental Yeast Co., Tokyo, Japan), 4% cellulose powder, and 2% agar. At the end of the feeding period mice were fasted for 18 hours and were then divided into five groups. The first group was lightly anesthetized and, without refeeding, tissues and blood were sampled (18-hr fasted). The second group was fed a complete diet containing 25% casein for 1 hour (25C refed), and the third was fed a protein-free diet for 1 hour (0C refed). The protein-free diet was prepared by replacing casein with α-cornstarch and sucrose. One hour before refeeding, the other two groups were injected with anti-insulin serum (AIS) to determine the role of insulin and were refed a 25% casein diet (25C refed + AIS) or a protein-free diet (0C refed + AIS). Anti-insulin serum (anti pig-insulin serum from guinea pig) was given intraperitoneally as a single injection at a dosage (0.3 mL per head) which caused a large rise in serum concentration of glucose after 2 hours of anti-insulin serum injection. We also consulted a report by Preedy and Garlick.[1]

Another set of animal experiments was performed to examine the time courses of initiation and elongation activity and polysome size after a diet ingestion. Mice were handled in same fashion as for the first experiments. At the end of the feeding period mice were fasted for 18 hours and were refed a 25% casein diet for the specified times described in *Figure 3*.

The experimental protocol was approved in accordance with the guide for the care and use of laboratory animals prepared by Tokyo Noko University.

### *Measurement of protein synthesis*

For measurement of protein synthesis in vivo, we used glycyl-L-tyrosine (Ajinomoto Co., Tokyo, Japan) in place of alanyl-L-tyrosine in a large dose,[13] because the solubility of glycyl-L-tyrosine was higher than that of alanyl-L-tyrosine. Mice were injected intraperitoneally with glycyl-L-tyrosine (170 μmol/100 g of body weight) combined with L-[U-$^{14}$C]tyrosine (435 kBq/100 g of body weight, 17.6 GBq/mmol, Amersham, U.K.) and killed 20 min later. Liver and skeletal muscle were then excised, frozen in liquid $N_2$, and stored at −80 °C until needed for analysis. The fractional synthesis rate ($k_s$) was determined as described previously.[13]

### *Polysome profiles*

Polysome profiles from the liver and hindleg muscle were measured as described by Kikuchi et al.[14] The postmitochondrial supernatant of liver homogenate was made in 1% sodium deoxycholate (Sigma Chemical Co., St. Louis, MO, USA) and 1% Triton X-100 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) to obtain the total ribosomes including membrane-bound ribosomes on which secretory protein is synthesized. The percentage of ribosomes in the form of polysomes was defined as: [polysome area/(polysome area + monomer − dimer area)] × 100.

### *Initiation activity assays*

Crude protein extract for the assays of translational inhibitor was prepared from the liver of 18-hour fasted or refed mice according to the method of Delaunay et al.[15] This liver inhibitor blocked peptide chain initiation in rabbit reticulocyte lysate. Briefly, livers were finely minced with scissors and then washed in a cold buffer containing 50 mM Tris-HCl (pH 7.6), 75 mM KCl, 5 mM magnesium acetate, 6 mM 2-mercaptoethanol, 250 mM sucrose. Extracts were prepared by gentle homogenization in 3 volumes of the same buffer with a Potter-Elvehjem-type homogenizer. A ribosome-free supernatant (S-150) was obtained by centrifugation at 150,000*g* for 3 hours. Solid ammonium sulfate was added to the S-150 fraction to bring it to 40% saturation. The precipitate was collected by centrifugation and dissolved in buffer containing 5 mM HEPES (pH 7.2), 200 mM KCl. After dialyzing against the same buffer, the preparation was used for translational inhibitor. Assay of the translational inhibitor was performed with a protein-synthesizing system derived from rabbit reticulocyte lysate.[15]

Skeletal muscle was extracted essentially as described by Foulkes et al.[16] The extracts inhibited peptide chain initiation in rabbit reticulocyte lysate. Briefly, muscle was homogenized with 1.5% (w/v) cold trichloroacetic acid (TCA), 4 mM EDTA and centrifuged at 6000*g* for 45 min. Supernatants were made at 15% (w/v) with TCA and, after 3 hours at 4 °C, were recentrifuged at 20,000*g* for 10 min. Precipitates were resuspended in 0.5 M Tris-HCl (pH 7.4), diluted with water, and dialyzed against four changes of 5 mM Tris-HCl (pH 7.0) at 4 °C. After 6 min in a boiling water bath, suspensions were cooled on ice and centrifuged at 20,000*g* for 20 min. Supernatants were lyophilized and stored at −70 °C until assay in the reticulocyte lysate system.

Protein synthesis was assayed by the incorporation of L-[U-$^{14}$C]leucine (11.6 GBq/mmol, NEN Research Products, Boston, MA, USA) into protein in reaction mixtures (50 μ L) containing 18.5 KBq L-[U-$^{14}$C]leucine and 20 μM hemin (Wako Pure Chemical Industries, Osaka, Japan). The $^{14}$C radioactivity was counted with a liquid scintillation spectrometer (Beckman, model LS5000TD).

### *Elongation activity assays*

Liver elongation activity was measured in cell-free protein-synthesizing systems employing high-speed supernatant (S-100) and salt-washed polysomes. S-100, which contains elongation factors and termination factors, was prepared from control or refed mice liver according to the method of Falvey and Staehelin.[17] The polysomes were prepared from another set of mice which had been fasted for 18 hours. To strip soluble protein synthesis factors such as elongation factors from the polysomes, the polysomes were treated with 0.5 M KCl. The postmitochondrial supernatant was layered over discontinuous sucrose gradients consisting of 2.5 mL each of 2.0 M sucrose and 0.5 M sucrose, both containing 200 mM Tris-acetate (pH 8.5), 0.5 M KCl, 10 mM magnesium acetate, and 6 mM 2-mercaptoethanol. The gradients were centrifuged for 24 hours at 105,000*g*, and polysomes were obtained as pellets. The

polysomes were suspended in 0.35 M sucrose containing buffer (200 mM Tris-acetate [pH 8.5], 50 mM KCl, 10 mM magnesium acetate, and 6 mM 2-mercaptoethanol). The cell-free protein synthesis assay system contained the following constituents in a final volume of 50 μL/50 mM Tris-HCl (pH 7.4), 80 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 0.2 mM GTP, 7 mM creatine phosphate, 0.5 μg of creatine phosphokinase, 1 mM dithiothreitol, 10 μL S-100, 2 $O.D._{260}$ units of polysomes, 10 μ M each of 19 different amino acids and 74 KBq [$^3$H]leucine (2.22 TBq/mmol, American Radiolabeled Chemicals Inc., St. Louis, MO USA). Reaction was initiated by the addition of polysomes. Incubation was carried out at 37 °C for 5 min. The $^3$H radioactivity was counted with a liquid scintillation spectrometer (Beckman, model LS5000TD).

Muscle elongation activity was measured in a cell-free protein-synthesizing system employing a cytoplasmic enzyme fraction (S-339) that contains elongation and termination factors in addition to aminoacyl-tRNA synthases and salt-washed polysomes as described by Bjercke et al.[18] The cell-free protein synthesis assay contained the following constituents in a final volume of 50 μL/50 mM Tris-HCl (pH 7.5), 40 mM KCl, 4 mM $MgCl_2$, 1 mM ATP, 2 mM GTP, 20 mM creatine phosphate, 1 μg creatine phosphokinase, 6 mM 2-mercaptoethanol, 400 μg (approximately 10 μL) S-339, 2 $O.D._{260}$ units of polysomes, 10 μM each of 19 different amino acids, and 74 KBq [$^3$H]leucine (2.22 TBq/mmol, American Radiolabeled Chemicals Inc., St. Louis, MO, USA). Incubation was carried out under the same conditions as described for the liver.

### *Insulin, glucose, and amino acid assays*

Serum insulin concentration was determined by radioimmunoassay using an insulin assay kit (Pharmacia Biosystems, Tokyo, Japan). Serum glucose was measured by the glucose oxidation method using a glucose B test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). A portion of serum was deproteinized and defatted, and the free amino acid concentrations of serum were determined with a Hitachi 835 amino acid analyzer (Hitachi, Ltd., Tokyo, Japan) equipped with ion-exchange resion.

### *Determination of RNA and DNA*

RNA and DNA were measured by the ethidium bromide method.[19]

### *Statistical analyses*

The data are expressed as means ± SE. The effect of refeeding and anti-insulin serum (*Table 4*) were analyzed by two-way analysis of variance (ANOVA) and Duncan's multiple range test.[20,21] All other data were evaluated by one-way ANOVA and Duncan's multiple range test. Differences at $P < 0.05$ were considered significant.

## Results

Refeeding a 25% casein diet (25C) and a protein-free diet (0C) for 1 hour caused increases in the serum concentration of glucose and insulin (*Table 1*). Serum concentrations of alanine, methionine, isoleucine, leucine, and tyrosine rose 3-fold and that of valine rose 4.5-fold by refeeding a 25C diet for 1 hour. Most free amino acid concentrations in serum decreased by refeeding a 0C diet for 1 hour, and those showing the most remarkable decrease were the branched-chain amino acids: leucine, valine and isoleucine, tyrosine and arginine (*Table 2*). We did not measure serum concentration of tryptophan, which was a key amino acid leading to ribosomal aggregation.[22] When rats were fed a complete diet containing 20% casein for 1.5 hours, the serum concentration of tryptophan rose 2-fold (Kajikawa et al., personal communication); the serum concentration of tryptophan seems to rise to the same extent with refeeding a 25C diet.

**Table 1** Serum glucose and insulin level in postabsorptive mice refed for 1 hour

| | Glucose (mg/100 mL) | Insulin (μ unit/mL) |
|---|---|---|
| 18-hr fasted | 96.7 ± 5.8$^a$ (7) | 5.5 ± 0.4$^a$ (7) |
| 25C refed | 189.5 ± 8.8$^b$ (6) | 28.4 ± 1.8$^b$ (6) |
| 0C refed | 302.4 ± 20.3$^c$ (6) | 30.8 ± 1.7$^b$ (6) |

In the three experimental groups, the values not sharing the same superscript letter are significantly different ($P < 0.05$) by Duncan's multiple range test. Values are means ± SEM for the number of mice indicated in parentheses.

In response to the refeeding for 1 hour, there was a decrease in liver RNA concentration expressed in RNA/tissue weight ratio, but the RNA/DNA ratio in the liver did not change (*Table 3*). In muscle, the RNA concentration expressed in both RNA/tissue weight ratio and RNA/DNA ratio remained constant with the refeeding (*Table 3*).

Protein synthesis of the liver was stimulated by refeeding a 25C diet for 1 hour, and the injection of anti-insulin serum (AIS) did not suppress the response. In contrast, the increase in protein synthesis by refeeding a 0C diet for 1 hour was blocked by the anti-insulin serum, and thus interaction was found between casein levels (0C, 25C) and the presence or absence of AIS on the effect of liver protein synthesis by refeeding (*Table 4*). In muscle, the increase in protein synthesis by refeeding a 25C diet for 1 hour was completely blocked by the antiserum. When the mice were refed a 0C diet for 1 hour, there was no increase in protein synthesis irrespective of AIS (*Table 4*).

**Table 2** Serum amino acid concentration

| | 18-hr fasted | 25C refed | 0C refed |
|---|---|---|---|
| Asp | 31.5 ± 1.7$^a$ | 26.1 ± 2.0$^a$ | 19.5 ± 1.8$^b$ |
| Thr | 384.2 ± 52.0$^a$ | 496.7 ± 36.0$^b$ | 328.5 ± 12.6$^a$ |
| Ser | 250.1 ± 8.5$^a$ | 405.8 ± 29.2$^b$ | 135.6 ± 2.9$^c$ |
| Glu | 275.2 ± 24.4$^a$ | 255.0 ± 28.3$^a$ | 267.0 ± 8.0$^a$ |
| Gly | 270.9 ± 11.0$^a$ | 258.1 ± 16.7$^a$ | 131.2 ± 5.7$^b$ |
| Ala | 336.9 ± 15.6$^a$ | 1088.7 ± 68.4$^a$ | 308.5 ± 10.2$^a$ |
| Cys | 58.0 ± 5.9$^a$ | 61.9 ± 4.4$^a$ | 32.7 ± 1.9$^b$ |
| Val | 290.9 ± 19.9$^a$ | 1311.3 ± 50.5$^b$ | 112.3 ± 4.4$^c$ |
| Met | 62.2 ± 7.9$^a$ | 211.8 ± 14.7$^b$ | 27.5 ± 2.6$^c$ |
| Ile | 142.9 ± 13.6$^a$ | 453.2 ± 19.8$^b$ | 42.5 ± 2.9$^c$ |
| Leu | 237.4 ± 19.4$^a$ | 770.3 ± 32.9$^b$ | 73.2 ± 3.9$^c$ |
| Tyr | 88.5 ± 5.9$^a$ | 269.4 ± 11.1$^b$ | 21.7 ± 1.4$^c$ |
| Phe | 172.0 ± 20.2$^a$ | 292.4 ± 8.0$^b$ | 86.0 ± 1.9$^c$ |
| Lys | 298.2 ± 15.0$^a$ | 851.4 ± 32.2$^b$ | 154.8 ± 5.1$^c$ |
| His | 95.1 ± 17.9$^a$ | 153.7 ± 6.9$^b$ | 48.7 ± 2.1$^c$ |
| Arg | 145.3 ± 9.8$^a$ | 249.4 ± 19.2$^b$ | 45.7 ± 7.0$^c$ |

Values are means ± SEM for seven mice; units are nmol/mL. In the three experimental groups, the values not sharing the same superscript letter are significantly different ($P < 0.05$) by Duncan's multiple range test.

**Table 3** DNA and RNA concentrations of liver and muscle in mice

| Treatment | Liver | | | Muscle | | |
|---|---|---|---|---|---|---|
| | RNA/tissue (μg/mg) | DNA/tissue (μg/mg) | RNA/DNA | RNA/tissue (μg/mg) | DNA/tissue (μg/mg) | RNA/DNA |
| 18-hr fasted | 8.88 ± 0.55[a] | 5.60 ± 0.18[a] | 1.59 ± 0.10[a] | 0.96 ± 0.04[a] | 0.66 ± 0.05[a] | 1.51 ± 0.16[a] |
| 25C refed | 6.14 ± 0.64[b] | 4.29 ± 0.17[b] | 1.43 ± 0.12[a] | 1.04 ± 0.06[a] | 0.71 ± 0.05[a] | 1.49 ± 0.10[a] |
| 0C refed | 7.68 ± 0.55[a,b] | 4.85 ± 0.13[c] | 1.59 ± 0.12[a] | 1.08 ± 0.06[a] | 0.76 ± 0.02[a] | 1.42 ± 0.05[a] |

In the three experimental groups, the values not sharing the same superscript letter are significantly different ($P < 0.05$) by Duncan's multiple range test. Values are means ± SEM for six mice.

Refeeding a 25C diet for 1 hour increased the proportion of heavier polysomes in both liver and muscle (*Figures 1* and *2*). With a 0C diet, no increase in polysome aggregations occurred in muscle (*Figure 2*), whereas aggregations were observed in the liver (*Figure 1*). The initiation activities in both tissues rose by refeeding for 1 hour except with a 0C diet in muscle (*Figures 1* and *2*). The elongation activities increased in both tissues by refeeding for 1 hour except with a 0C in the liver (*Figures 1* and *2*).

In another set of experiments, the significant rise of liver ribosome aggregation was observed at 20 minutes after refeeding a 25C diet. Also, the initiation of liver activity began to rise immediately and increased significantly for 30 minutes. In contrast, the elongation activity did not change within the first 30 minutes but then rose significantly at 1 hour after refeeding had commenced (*Figure 3*).

## Discussion

In addition to the rise of serum insulin level that follows a 25C diet refeeding, there were also increases in the concentration of glucose and almost every free amino acid in serum compared with a fasted group (*Tables 1* and *2*). With refeeding a 0C diet, the serum concentration of insulin was the same as that in the 25C diet-fed mice; however, the concentrations of glucose were higher than the 25C diet-fed mice (*Table 1*), and the concentrations of amino acids were lower than the 18-hr fasted group (*Table 2*). The rise of liver protein synthesis induced by a 0C diet was blocked by the anti-insulin serum injection (*Table 4*). In contrast, the rise of liver protein synthesis with refeeding a 25C diet was not blocked by anti-insulin serum (*Table 4*). The stimulatory action of leucine injection on liver protein synthesis was also demonstrated in 12-hr fasted diabetic mice. A 6-fold increase of leucine concentration in serum was observed by leucine injection.[10] In the experiment reported here, the concentration of branched-chain amino acids, methionine and alanine, in mice that were fed a 25C diet was three to four times higher than in the 18-hr fasted and the 0C diet-fed mice (*Table 2*). When mice were fed the 25C diet, the changes in total liver protein synthesis paralleled the changes in serum amino acid concentration (*Tables 2* and *4*). This relationship suggests that certain amino acids in serum play a role as an extracellular signal molecule rather than as a mere substrate in the liver cell when the abrupt increase of the amino acid concentration could be brought about by refeeding a complete diet. The detailed mechanism by which liver protein synthesis is induced is not known, and participation of many endocrine factors other than insulin is beyond the scope of this discussion. Blocking of the stimulation of liver protein synthesis in the 0C diet-fed mice treated with anti-insulin serum suggests that the involvement of insulin together with glucose is necessary. Both

**Table 4** The effects of refeeding a 25C diet and a 0C diet and of anti-insulin serum on liver and muscle protein synthesis in postabsorptive mice

| | Fractional rates of protein synthesis (%/day) | |
|---|---|---|
| | Liver | Muscle |
| 18-hr fasted | 98.3 ± 11.4[a] (6) | 6.9 ± 0.6[a] (7) |
| 25C refed | 131.5 ± 6.8[b,d] (5) | 10.9 ± 1.4[b] (6) |
| 25C refed + AIS | 139.8 ± 4.7[c,d] (7) | 8.2 ± 0.6[a] (7) |
| 0C refed | 158.8 ± 4.8[c] (6) | 8.3 ± 0.4[a] (6) |
| 0C refed + AIS | 113.0 ± 8.6[a,b] (7) | 6.8 ± 0.6[a] (7) |

In the five experimental groups, the values not sharing the same superscript letters are significantly different ($P < 0.05$) by Duncan's multiple range test. Values are means ± SEM for the number of mice indicated in parentheses.

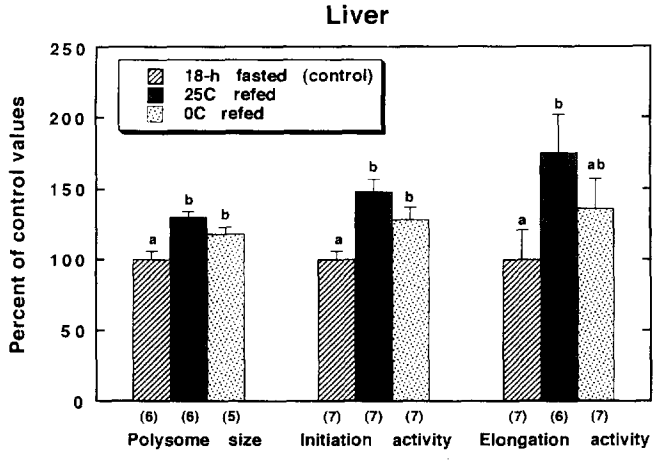


**Figure 1** Comparison of polysome size, initiation activity, and elongation activity in livers of mice fasted for 18 hours (18 h fasted), refed a 25C diet for 1 hour (25C refed), and refed a 0C diet for 1 hour (0C refed). Each value represents the mean for the number of mice indicated in parentheses, and each vertical bar indicates one SE. The values not sharing the same superscript letter are significantly different ($P < 0.05$) by Duncan's multiple range test.

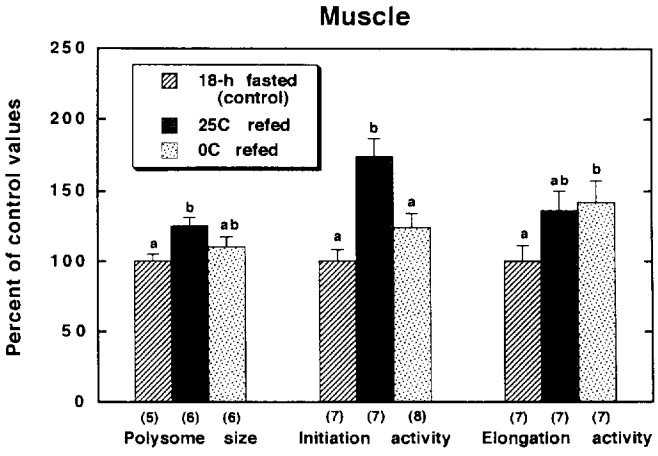


**Figure 2** Comparison of polysome size, initiation activity, and elongation activity in muscles of mice fasted for 18 hours (18h fasted), refed a 25C diet for 1 hour (25C refed), and refed a 0C diet for 1 hour (0C refed). Each value represents the mean for the number of mice indicated in parenthesis, and each vertical bar indicates one SE. The values not sharing the same superscript letter are significantly different ($P < 0.05$) by Duncan's multiple range test.

substances were needed to obtain a maximum rate of albumin synthesis in the perfused liver.[23] Increase of serum glucose level by refeeding a 0C diet did not affect liver protein synthesis without the cooperation of insulin, whereas the elevation of serum amino acid level did enhance total liver protein synthesis without insulin collaboration. We did not determine whether or not increment of the serum glucose level could be a requirement for the stimulation of liver protein synthesis in the 25C diet-fed mice injected with anti-insulin serum.

Refeeding a 25C diet to postabsorptive mice for 1 hour caused an increase in the rate of muscle protein synthesis (*Table 4*), whereas refeeding a 0C diet did not stimulate muscle protein synthesis. However, the serum insulin level was the same as when a 25C diet was fed (*Table 4*). Garlick et al.[8] reported that rates of muscle protein synthesis in postabsorptive rats were not as high as those in fed rats,

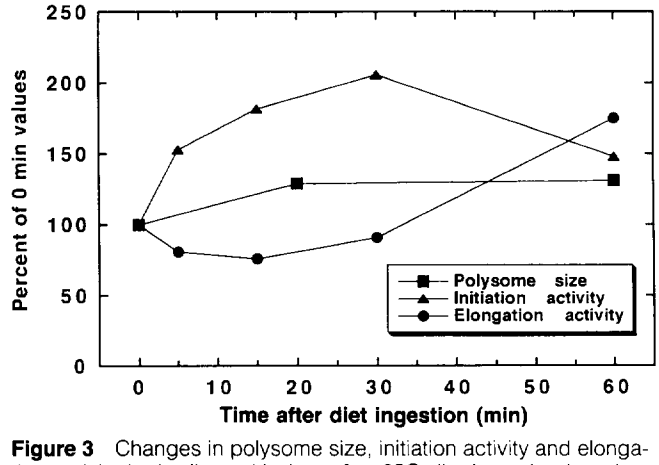


**Figure 3** Changes in polysome size, initiation activity and elongation activity in the liver with time after 25C diet ingestion by mice. Values are means for four (polysome size) or six (initiation activity and elongation activity) mice.

even when stimulated maximally by insulin, or in postabsorptive rats refed a complete diet for 60 minutes. Studies with incubated and perfused muscle have shown that amino acids can increase the rate of protein synthesis and that this response can be attributed entirely to the branched-chain amino acids, particularly leucine.[7,12] It is therefore likely that free amino acids act cooperatively with insulin. When insulin was removed by injection of the anti-insulin serum before feeding commenced, no increase in protein synthesis was observed.[1] This clearly demonstrated that insulin was essential in mediating the acute stimulation of muscle protein synthesis on food intake and that insulin was not the sole responsible factor in feeding response.

Increase of tissue protein synthesis in an early phase after refeeding must be achieved at the translational level. The rate of protein synthesis in a tissue depends on the number of ribosomes per cell and the activity of these ribosomes. The RNA/DNA ratio both in the liver and skeletal muscle did not change by refeeding (*Table 3*). Because 80% of measured total RNA was considered to be the ribosomal RNA,[24] it seems that the number of ribosomes per cell did not change with the 1-hr refeeding. Liver and muscle ribosomes from refed mice contained much heavier polysomes (*Figures 1* and *2*). It is generally accepted that the extent of polysomal aggregation depends on both the rate of initiation and the elongation rate of peptide synthesis. The shifting of heavy polysomes induced by refeeding suggested that the rate of initiation increased relative to elongation, or that the rate of elongation fell relative to initiation. In many situations it is said that the initiation limits the overall translation rate as judged by analysis of polysome size distributions under various conditions,[25] but it is not clear what effects changes in elongation rates might have. Nevertheless, under some circumstances, elongation is thought to be important. A novel action of insulin that could affect the rate of elongation was reported using NIH 3T3 cells.[26] Activation of translational activity by refeeding seems to be achieved mainly in the initiation steps, but stimulation of elongation also contributes to the enhancement of protein synthesis.

Translational initiation activity in the liver was increased by refeeding not only a 25C diet but also a 0C diet (*Figure 1*). Refeeding a 25% casein diet to 18-hr fasted mice caused considerable stimulation of elongation activity in the liver. We reported previously that leucine stimulates protein synthesis in part by increasing the rate of elongation in the liver,[10] and it was also reported that the administration of alanine increased the rate of elongation in this organ.[27] Thus, these amino acids could be playing an important regulatory role in hepatic polypeptide chain elongation.

Translational initiation activity of muscle was stimulated by refeeding a 25C diet, but not by refeeding a 0C diet (*Figure 2*). In contrast, the elongation activity was stimulated by refeeding a 0C diet (*Figure 2*). The evidence in the present paper (*Figure 2*) suggests that insulin exerts its stimulatory action cooperatively with amino acids at the initiation step and that glucose stimulates peptide chain elongation in collaboration with insulin. The serum concentration of glucose in the 0C diet group was significantly higher than in the 25C group (*Table 1*). In muscle, insulin has been reported to increase protein synthesis exclusively by enhancing initiation.[28] In isolated soleus muscle of mice, the

rate of peptide chain elongation increased in the presence of insulin and glucose (unpublished observation). The elucidation of a molecular mechanism that controls the rate of initiation or elongation is beyond the scope of this study; nevertheless, the conclusion seems to be supported by the observation that the administration of glucose was effective in increasing liver protein synthesis and that alanine, an amino acid, increased the rate of peptide elongation in the liver of starved rats in the meaning of their potential role as metabolic effectors rather than fuels or building blocks.[27]

The increased ribosome aggregation by refeeding for 1 hour is thought to be due to the enhancement of peptide chain initiation relative to that of elongation, or due to the lag in increase between the initiation and elongation rates. *Figure 3* shows the time course of the activity of initiation and elongation, and of ribosome aggregation during feeding. A delayed rise in elongation coupled with an immediate increase in initiation is effective for an acute enlargement of polysome size, and enhancement of peptide chain elongation would serve to enhance the translation rate as a whole.

The regulation of translational activities for a long term could be due to the changes in the tissue content of the individual components of the translational machinery. For example, the amount of eukaryotic initiation factor 2 (eIF-2) relative to total protein tended to decrease with increasing age. Furthermore, eIF-2 content was directly proportional to the rate of protein synthesis in various tissues.[29] In contrast, the present study suggests that the transient changes in the activities of the translational apparatus account for the regulation of translational activities for a short term. It seems that the stimulation of translational initiation and elongation activity after refeeding is brought about by the increase in the proportion of active form of initiation and elongation factors to inactive form. Several protein factors of the translational machinery are subject to phosphorylation.[30] Regulation of the activity of translational apparatus by phosphorylation seems to be a general mechanism for the control of rates of protein synthesis. The protein kinase(s) and phosphoprotein phosphatase(s) are responsible for establishing the extent of phosphorylation. We measured the activity of liver translational inhibitor which displays properties similar to those of hemin-controlled inhibitor (HCI). HCI is a protein kinase that phosphorylates $\alpha$ subunit of eIF-2, and phosphorylation of eIF-2 leads to inhibition of translational initiation. In skeletal muscle, we measured the activity of heat- and acid-stable, TCA-precipitable and non-dialyzable factor(s) that inhibit peptide chain initiation. These extracts include phosphoprotein phosphatase inhibitors I and II and possibly also peptides that promote the phosphorylation of eIF-2$\alpha$ and are thought to have eIF-2 kinase activity.[31]

In conclusion, the primary mediators in the acute rise of protein synthesis by refeeding seem to be increases in the serum concentrations of amino acids, glucose, and insulin. The diet-induced enhancement of liver protein synthesis is not necessarily mediated by insulin provided an abrupt, large increase in serum amino acids occurs with refeeding. However, stimulation of liver protein synthesis by a protein-free diet is mediated by both a large increase in serum glucose and a surge of insulin. In contrast to the liver, insulin is essential for the increase in muscle protein synthesis, but is not the sole mediator in the acute rise of protein synthesis; a high concentration of serum amino acids is necessary in addition to the insulin action. The mechanisms responsible for the modulation of protein synthesis by refeeding could operate to alter the rate of elongation and the polysome size and involve the time-scheduled changes of activity in both initiation and elongation. Further study will elucidate the signaling pathways that mediate the extracellular signals to the translational apparatus in response to refeeding.

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## References

1 Preedy, V.R., and Garlick, P.J. (1986). The response of muscle protein synthesis to nutrient intake in postabsorptive rats: the role of insulin and amino acids. *Biosci. Rep.* **6,** 177–183

2 Funabiki, R., Nyumura, N., Hara, H., and Yagasaki, K. (1986). Protein synthesis rate in brain, liver, skeletal muscle and intestines by starved or diabetic mice. *Rep. Res. Commun. Essen. Amino Acids* **112,** 17–19

3 Jepson, M.M., Bates, P.C., and Millward, D.J. (1988). The role of insulin and thyroid hormones in the regulation of muscle growth and protein turnover in response to dietary protein in the rat. *Br. J. Nutr.* **59,** 397–416

4 Garlick, P.J., and Grant, I. (1988). Amino acid infusion increases the sensitivity of muscle protein synthesis in vivo to insulin. *Biochem. J.* **254,** 579–584

5 Stirewalt, W.S., and Low, R.B. (1983). Effects of insulin in vitro on protein turnover in rat epitrochlearis muscle. *Biochem. J.* **210,** 323–330

6 Frayn, K.N., and Maycock, P.F. (1979). Regulation of protein metabolism by a physiological concentration of insulin in mouse soleus and extensor digitorum longus muscles. *Biochem. J.* **184,** 323–330

7 Fulks, R.M., Li, J.B., and Goldberg, A.L. (1975). Effects of insulin, glucose and amino acids on protein turnover in rat diaphragm. *J. Biol. Chem.* **250,** 290–298

8 Garlick, P.J., Fern, M., and Preedy, V.R. (1983). The effect of insulin infusion and food intake on muscle protein synthesis in postabsorptive rats. *Biochem. J.* **210,** 669–676

9 Reeds, P.J., Hay, S.M., Glennie, R.T., Mackie, W.S., and Garlick, P.J. (1985). The effect of indomethacin on the stimulation of protein synthesis by insulin in young postabsorptive rats. *Biochem. J.* **227,** 255–261

10 Funabiki, R., Yagasaki, K., Hara, H., Nyumura, N., Yoshizawa, F., and Saito, K. (1992). In vivo effect of L-leucine administration on protein synthesis in mice. *J. Nutr. Biochem.* **3,** 401–407

11 Mortimore, G.E., and Mondon, C.E. (1970). Inhibition by insulin of valine turnover in the liver. *J. Biol. Chem.* **245,** 2375–2383

12 Li, J.B., and Jefferson, L.S. (1978). Influence of amino acid availability on protein turnover in perfused skeletal muscle. *Biochim. Biophys. Acta.* **544,** 351–359

13 Funabiki, R., Yagasaki, K., Hara, H., Nyumura, N., and Takeda, A. (1990). Measurement of the rate of whole body protein synthesis by intraperitoneal injection of a large dose of alanyltyrosine with [$^{14}$C]tyrosine. *Agric. Biol. Chem.* **54,** 113–119

14 Kikuchi, T., Okamoto, H., Chiku, K., and Natori, Y. (1986). Effects of glucose and amino acid depletions on protein synthetic parameters in liver and skeletal muscle of rats during parenteral nutrition. *J. Nutr. Sci. Vitaminol.* **32,** 601–612

15 Delaunay, J., Ranu, R.S., Levin, D.H., Ernst, V., and London, I.M. (1977). Characterization of a rat liver factor that inhibits initiation of protein synthesis in rabbit reticulocyte lysates. *Proc. Natl. Acad. Sci. USA* **74,** 2264–2268

16 Foulkes, J.G., Jefferson, L.S., and Cohen, P. (1980). The hormonal control of glycogen metabolism: dephosphorylation of protein phos-

phatase inhibitor in vivo in response to insulin. *FEBS Lett.* **112,** 21–24

17 Falvey, A.K., and Staehelin, T. (1970). Structure and function of mammalian ribosomes. *J. Mol. Biol.* **53,** 1–19

18 Bjercke, R.J., Goll, D.E., and Robson, R.M. (1984). Development of methods to measure activity of polysomes and cytoplasmic enzymes from bovine skeletal muscle in in vitro protein-synthesis assays. *J. Anim. Sci.* **59,** 666–683

19 Karsten, U., and Wollenberger, A. (1972). Determination of DNA and RNA in homogenized cells and tissues by surface fluorometry. *Anal. Biochem.* **46,** 135–148

20 Snedecor, G.W., and Cochlan, W.G. (1967). *Statistical Methods,* 6th ed., Iowa State University Press, Ames, IA

21 Duncan, D.B. (1955). Multiple range and multiple F tests. *Biometrics* **11,** 1–42

22 Munro, H.N. (1968). Role of amino acid supply in regulating ribosome function. *Fed. Proc.* **27,** 1231–1237

23 John, D.W., and Miller, L.L. (1969). Regulation of net biosynthesis of serum albumin and acute phase plasma proteins. *J. Biol. Chem.* **244,** 6134–6142

24 Scornik, O.A. (1974). *In vivo* rate of translation by ribosomes of normal and regenerating liver. *J. Biol. Chem.* **249,** 3876–3883

25 Pain, V.M. (1978). Protein synthesis and its regulation. In *Protein turnover in Mammaliam Tissues and in the Whole Body* (J.C. Waterlow, P.J. Garlick, and D.J. Millward, eds.), pp. 15–54, North-Holland Publishing Co., Amsterdam

26 Levenson, R.M., Mairn, A.C., and Blackshear, P.J. (1989). Insulin rapidly induces the biosynthesis of elongation factor 2. *J. Biol. Chem.* **264,** 11904–11911

27 Pérez-Sara, D., Parrilla, R., and Ayuso, M.S. (1987). Key role of L-alanine in the control of hepatic protein synthesis. *Biochem. J.* **241,** 491–498

28 Monier, S., and Le Marchand-Brustel, Y. (1982). Insulin affects only initiation and not elongation in protein synthesis in soleus muscles of lean and obese mice. *FEBS Lett.* **147,** 211–214

29 Kimball, S.R., Vary, T.C., and Jefferson, L.S. (1992). Age-dependent decrease in the amount of eukaryotic initiation factor 2 in various rat tissues. *Biochem. J.* **286,** 263–268

30 Hershey, J.W.B. (1990). Overview: phosphorylation and translation control. *Enzyme* **44,** 17–27

31 De Haro, C., Manne, V., De Herreros, A.G., and Ochoa, S. (1982). Heat-stable inhibitor of translation in reticulocyte lysates. *Proc. Natl. Acad. Sci. USA* **79,** 3134–3137