In vivo effect of L-leucine administration on protein synthesis in mice

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To clarify the in vivo *effect of leucine on protein synthesis within a short period, the fractional formation rate of peptidyl puromycin (FFR) was measured in the liver and skeletal muscle of mice. FFR was calculated as the ratio of specific activity of peptide-bound puromycin at 10 minutes after injection of [^{3}H]puromycin to the area under the specific activity-time curve of free puromycin. The value of FFR corresponds to the amount of tracer puromycin bound in unit time, expressed as a fraction of the total amount of puromycin potentially bound to the aminoacyl-site on the functional ribosome pool. In 12-hour fasted, streptozotocin-induced diabetic mice, L-leucine injection (360 μmol/100 g body weight) increased the FFR in the liver but not in muscle, while the injection of insulin (0.5 unit/100 g body weight) raised the rate in muscle but not in the liver. The leucine-induced rise of FFR in the liver was blocked by a cyclooxygenase inhibitor, indomethacin, although this inhibitor did not block the insulin-stimulated rise of FFR in muscle. No significant increase in FFR was detected in the liver or muscle of non-diabetic mice by leucine injection. L-leucine injection caused a slight decrease of ribosome aggregation in the liver but not in muscle, while insulin injection led to ribosome aggregation in muscle but not in the liver of diabetic mice. The enhancement of FFR in the liver of diabetic animals by leucine injection suggests that leucine may stimulate protein synthesis in part by increasing the rate of elongation.*

Keywords: leucine; insulin; streptozotocin-induced diabetes; protein synthesis; peptide chain elongation; peptidyl puromycin

Introduction

Leucine, a branched-chain amino acid, has been shown to stimulate protein synthesis in vitro in isolated muscles of the rat under various physiological and nutritional conditions.[1-6] Promotion of protein synthesis was found in a perfused hindquarters using leucine-enriched perfusate at 10 times the normal level in 80-g rats.[7]

Rates of muscle protein synthesis increased in post-absorptive young rats that were given intravenous infusion of insulin alone.[8] When a complete amino acid mixture was included in the infusion solution, the rate of stimulation was much greater.[8] The effect of a complete amino acid mixture could be reproduced by a mixture of essential amino acids or branched-chain amino acids, but not by a non-essential mixture.[8] The authors concluded that amino acids, particularly branched-chain ones, increased the sensitivity of muscle protein synthesis to insulin.[8] Diabetes depressed the acute rise of protein synthesis in response to food intake in all tissues of mice, but its magnitude was different among tissues.[9] Diet-induced enhancement of protein synthesis in skeletal muscle was severely affected by diabetes, while that in the liver and intestine was mildly affected by this condition.[9] It was suggested that the liver and intestine were less sensitive to insulin than was skeletal muscle.[9] Insulin dose stimulated in vivo protein synthesis in the cardiac, gastrocnemius, plantaris, and soleus muscle of young post-absorptive rats, but failed to stimulate it in the liver. The stimulation was blocked by a cyclooxygenase inhibitor, indomethacin, except in the soleus muscle.[10]

The present studies were designed to ascertain the potential of leucine to stimulate protein synthesis in

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vivo in skeletal muscle and in the liver without the concerted action of insulin, and also to find sites of stimulatory action of leucine on protein synthesis in the liver or muscle of mice, comparing the action of leucine with that of insulin.

Materials and methods

Animal experiments

Sixty male mice (Crj:CD-1, Charles River Japan Inc., Atsugi, Japan, 4 weeks of age) were individually housed in stainless-steel wire cages and maintained at 23° C on a 12-hour light-dark cycle. They were fed a purified diet (see below) and water ad libitum for 2 days and divided randomly into 10 groups of six each. The composition of the diet was as follows: 10% amino acid mixture of whole-egg protein pattern that allows maximum growth in growing animals,[11] 35% α-cornstarch, 8% corn oil, 35% sucrose, 5% mineral mixture (mineral mix 1, prepared by Oriental Yeast Co., Tokyo, Japan), 1% vitamin mixture (vitamin mix 1, Oriental Yeast), 4% cellulose powder, and 2% agar. After being fasted for 12 hours, 42 animals from seven groups received an intraperitoneal injection of 200 μL of streptozotocin (Sigma Chemical Co., St. Louis, MO, USA, 25 mg/100 g body weight, dissolved in 100mmol/L citrate buffer; pH4.5) to produce diabetes. Eighteen animals from three groups received 200 μL of the citrate buffer alone and were designated as non-diabetic animals. Both the diabetic and non-diabetic animals were fed the purified diet for another 2 days and fasted for 12 hours before insulin or leucine injection. Insulin (Novo Actrapid MC, Novo Industry, Denmark, 40 IU/mL) was injected subcutaneously (0.5 IU/100 g body weight). L-Leucine (Ajinomoto Co., Tokyo, Japan, 360 μmol/100 g body weight) was given by intraperitoneal injection. The control group received a diluent injection (physiological saline) instead of insulin or leucine. Five minutes before the injection of insulin or leucine, each animal of one group was injected with 200 μL of indomethacin (Sigma Chemical Co.) in ethanol (2 mg/100 g body weight). Animals in the other group received 200 μL of ethanol alone. Fifty minutes following insulin injection, each mouse was intraperitoneally administered with [^{3}H]puromycin dichloride (Amersham International plc, Amersham, UK, 185GBq/mmol; 4 μmol and 740 KBq/100 g body weight). In the experiment on insulin action, the hormone was injected 20 minutes before the labeled puromycin and 30 minutes before sacrifice. Exactly 10 minutes after the injection of [^{3}H]puromycin, animals were decapitated and blood was collected, left to clot, and centrifuged to obtain serum. Tissue samples (the liver and hindleg muscles) were quickly removed, washed twice with cold 0.9% NaCl solution, blotted on filter paper, frozen in liquid nitrogen, and stored at −80° C until analyzed. A portion of each tissue was weighed and homogenized with 9 volumes of cold 5% (wt/vol) trichloroacetic acid (TCA). The homogenates were then filtered through a nitrocellulose filter (Toyo Roshi Co., Tokyo, Japan; pore size 40μm) to separate unchanged, free [^{3}H] puromycin as TCA-soluble material from the peptidyl puromycin formed as TCA precipitable material on the filter. After being washed with 8% (wt/vol) TCA, total radioactivity of the combined filtrate and each corresponding filter were measured with a liquid scintillation spectrometer (TRI-CARB 300C, Packard Instrument Co., Downers Grove, IL, USA). The specific radioactivity of free puromycin and peptidyl puromycin from each sample was expressed as d.p.m. per mg of fresh tissue.

Calculation of fractional formation rate

The fractional formation rate of peptidyl puromycin (abbreviated as :FFR, symbol:k_f, unit:day^{-1}) was calculated from the specific radioactivities of peptidyl puromycin (Sb) and free puromycin (Sa) in the tissue after the injection of [^{3}H]puromycin. The change in the specific radioactivity, dSb/dt, is given in Equation 1 in the steady-state, stating precursor-product relationship[12] as,

$$dSb/dt = k_f\,(Sa - Sb) \tag{1}$$

where Sa and Sb are the specific radioactivities of precursor and product, respectively. Assuming that the loss of the labeled peptidyl puromycin formed is negligible during the incorporation period, the omission of the term, $k_f \times Sb$ is permitted and Equation 1 becomes Equation 2.

$$dSb/dt = k_f \times Sa \tag{2}$$

Integration of Equation 2 yields Equation 3,

$$k_f = \frac{Sb}{\int_0^t \times Sa} \tag{3}$$

where the value of $\int_0^t \times Sa$ is the total area under the precursor-specific radioactivity-time curve from 0 to 10 minutes after the injection. Equation 3 can be rewritten by substituting $Sa \times t$ for $\int_0^t$ as Equation 4.

$$k_f = \frac{Sb}{Sa \times t} \tag{4}$$

Because the Sa has constant value within 15 minutes as shown in *Figure 1.* The specific radioactivities of Sb and Sa at 10 minutes (t) after [^{3}H]puromycin injection were used to calculate the FFR.

The value of FFR corresponds to the amount of tracer puromycin bound in unit time, expressed as a fraction of the total amount of puromycin, potentially attached at the aminoacyl-site of the functional ribosome pool. Implication of the value of FFR is similar to that of the fractional synthesis rate of protein (abbreviated as: FRS, symbol:ks, unit:day^{-1}). When radioactive tracers are used for measurements, FSR corresponds to the amount of tracer amino acid incorporated in unit time, expressed as a fraction of the total amount of the same amino acid in the protein pool.[13] The values of FFR and FSR are a function of three variables. The first is the concentration of ribosomes, the second is the proportion of ribosomes in polysomes, and the third is the average rate of peptide-chain elongation.[14] The proportion of ribosomes in polysomes depends on the relative rate of initiation and elongation, and does not necessarily reflect the overall rates of protein synthesis in vivo. The FFR of the liver indicates synthesis of two categories of proteins, fixed liver proteins and exported plasma proteins, so that measurement made within a short interval (10 minutes) could include the synthesis of exported plasma proteins.

Polysome profiles

Another set of animal experiments was performed. To obtain information on the relative rates of peptide-chain initiation and elongation, polysome profiles from the liver and hindleg muscles were measured as described by Kikuchi et al.[15] The postmitochondrial supernatant of liver homogenate was made in 1% sodium deoxycholate (Sigma Chemical Co.) and 1% Triton X-100 (Wako Pure Chemical Industries, Ltd., Osaka,

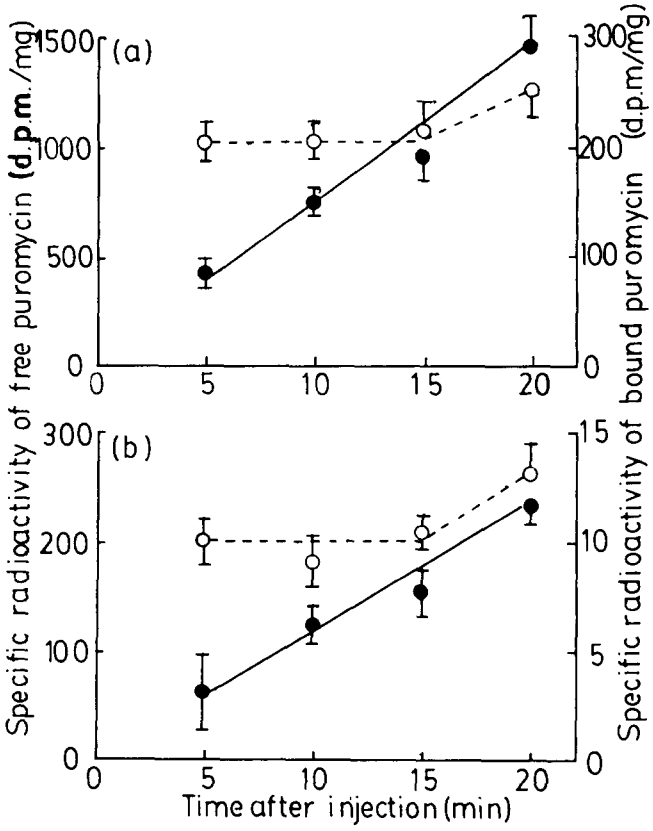

Figure 1 Time course of specific radioactivity of free puromycin (○) and bound puromycin (●) in the liver (a) or in muscle (b) of mice after i.p. injection of [^{3}H]puromycin. Each point represents the mean of three mice and each bar represents SE.

Japan) to obtain the total liver ribosomes including membrane-bound ribosomes on which secretory protein is synthesized. Animals, diets, and experimental design of the polysome profile study were almost the same as in the puromycin study.

The 12-hour fasting prior to streptozotocin injection was not done in animals in the polysome study but 26 mg of streptozotocin per 100 g body weight was injected to produce diabetes. Animals were killed by decapitation 60 minutes after leucine injection or 30 minutes after insulin injection and tissue samples were quickly removed and frozen in liquid nitrogen until preparation of polysomes. The percentage of ribosomes in the form of polysomes was defined as: [polysome area/(polysome area + monomer-dimer area)] × 100.

Insulin, glucose, and amino acid assays

Serum insulin concentration was determined by radioimmunoassay using an insulin assay kit (Daiichi Radioisotope Research Co., Tokyo, Japan). Serum glucose was measured by the glucose oxidation method adapted to the assay system of dextrostix and dextrometer by Miles-Sankyo Co. (Tokyo, Japan). The free leucine concentration of serum and the liver was determined with a Hitachi 835 amino acid analyzer (Hitachi Ltd., Tokyo, Japan).

Statistical method

Results are expressed as mean ± SE. Statistical analysis was done using the Student t test with $P < 0.05$ as the level of significance.

Results

The radioactivity of free puromycin per mg of tissue was maintained at a constant level for at least 10 minutes while the radioactivity of bound puromycin (peptidyl puromycin) per mg of tissue was increased linearly from 5 to 20 minutes in the liver *(Figure 1a)* and muscles *(Figure 1b)* of mice following an injection of [^{3}H]puromycin. Extrapolation of bound puromycin value(Sb) to zero time goes through the origin *(Figure 1 a and b)*. This is essential to calculate the FFR using the Sa value at 10 minutes. The area under the curve of the specific radioactivity of free puromycin(Sa) from zero to 10 minutes was calculated on the assumption that the Sa may reach the plateau value promptly after the labeled puromycin injection.

In non-diabetic mice, no significant rise of FFR in the liver or muscle was observed by leucine injection. The concurrent administration of indomethacin and leucine also exerted no significant effect on FFR in either tissue of non-diabetic animals *(Figure 2)*.

In diabetic mice, the injection of leucine stimulated FFR in the liver, while no significant change was observed in muscle. The leucine-induced rise of FFR in

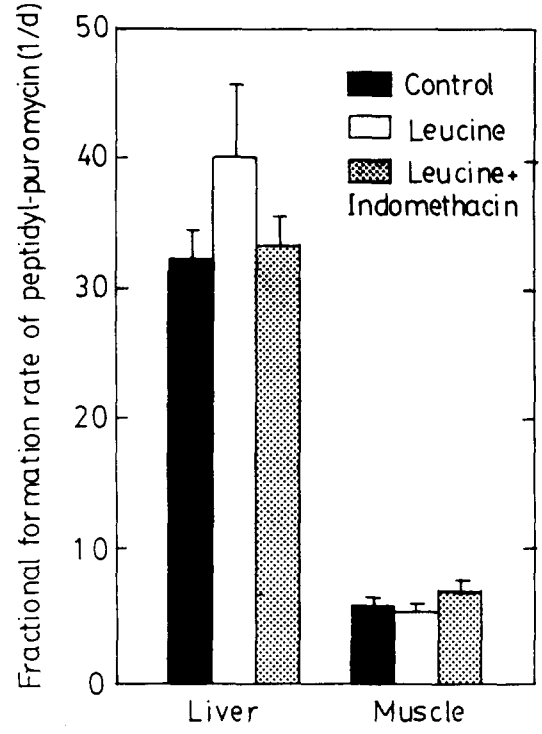

Figure 2 Effect of L-leucine or L-leucine + indomethacin on peptidyl puromycin formation in non-diabetic mice. Each value represents the mean of six mice and each vertical bar indicates one SE.

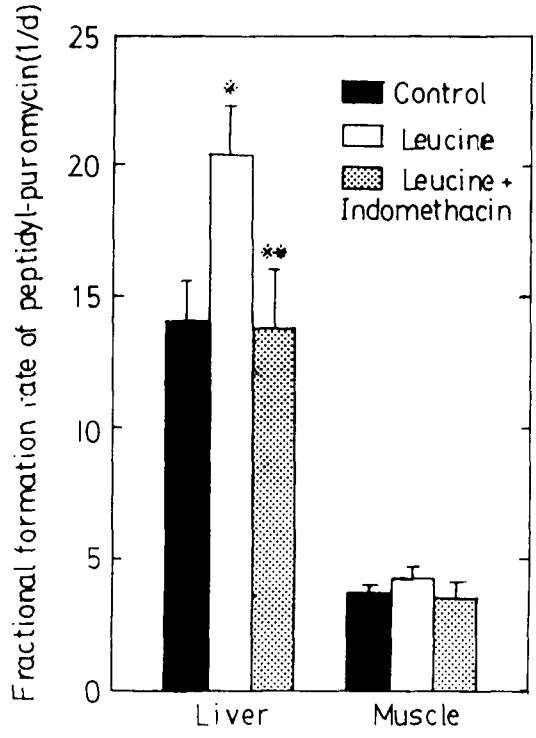

Figure 3 Effect of L-leucine or L-leucine + indomethacin on peptidyl puromycin formation in diabetic mice. Each value represents the mean of six mice and each vertical bar indicates one SE.
*Significantly different from control ($P < 0.05$)
**Significantly different from the L-leucine administered groups ($P < 0.05$).

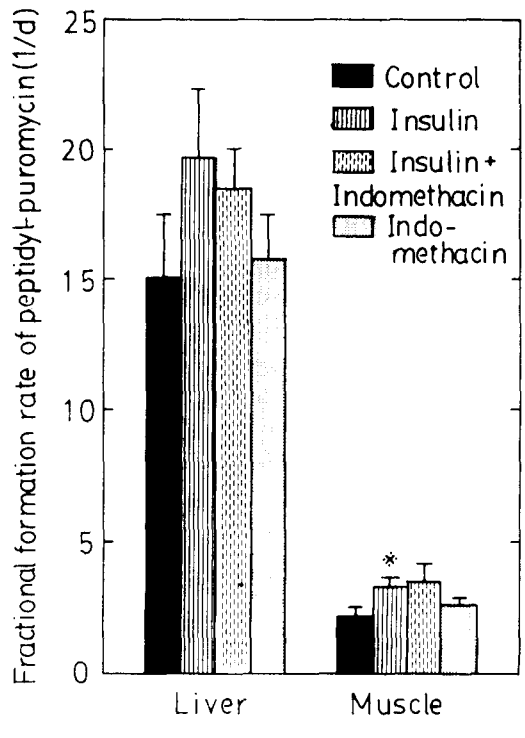

Figure 4 Effect of insulin or insulin + indomethacin on peptidyl puromycin formation in diabetic mice. Each value represents the mean of six mice and each vertical bar indicates one SE.
*Significantly different from control ($P < 0.05$).

the liver was blocked by indomethacin, whereas indomethacin treatment caused no significant change in the FFR value in the muscle *(Figure 3)*.

Insulin injection caused a significant rise of FFR in muscles but not in the liver of diabetic animals *(Figure 4)*. Indomethacin exerted no influence on the insulin-induced rise of FFR in muscles of diabetic mice *(Figure 4)*.

The hepatic ribosome aggregation was slightly decreased in diabetic mice by a single injection of leucine, while no significant decrease in aggregation was observed in non-diabetic mice *(Table 1)*. Leucine injection had no influence on ribosome aggregation of skeletal muscle in either diabetic or non-diabetic mice *(Table 1)*.

When diabetic mice were injected with insulin, ribosome aggregation of muscles increased significantly from 20.2 ± 0.9% ($n = 6$, *Table 1*) to 27.2 ± 2.3% ($n = 6$, data not shown), while no change in hepatic ribosome aggregation was found in diabetic mice after insulin injection.

The concentration of glucose (mg/100 mL) and insulin (μ units/mL) was >400 and 6, respectively, in diabetic mice, while in non-diabetic mice those values were 105 and 18, respectively. There was a marked decrease in serum glucose (205 mg/100 mL) and an increase in serum insulin (240μ units/100 mL) in diabetic mice by subcutaneous injection of insulin (5 units/kg body weight).

Table 1 Effect of L-leucine administration on ribosomal aggregation in diabetic or non-diabetic mice[a]

Treatment		Polysomal fraction	
Animal	Administered compound	Liver	Muscle
Diabetic	Saline (control)	52.8 ± 2.4 (5)	20.2 ± 0.9 (6)
	Leucine	43.7 ± 4.2 (6)[b]	17.0 ± 1.8 (4)
Non-diabetic	Saline	52.6 ± 1.1 (7)	37.6 ± 1.0 (5)
	Leucine	49.1 ± 2.8 (7)	40.0 ± 1.5 (7)

[a]Values are means ± SEM of the number of mice indicated in parenthesis and are expressed as percentage of polysomal fraction per total ribosome.
[b]Significantly different from control group, $P < 0.05$.

Discussion

The amino acid massive dose technique[16,17], i.e., the injection of a large amount of amino acid together with a tracer dose of the same radiolabeled amino acid, is commonly used to measure the rate of protein synthesis in tissues of animals under certain physiological and nutritional conditions in place of the constant intra-

venous injection of labeled amino acids. Although problems in determining the specific activity of the precursor of protein synthesis were overcome by injection of a large amount of amino acid, it is still uncertain whether a large administration affects the measurement of tissue protein synthesis. Nakano and Hara[18] described the technique for estimating the number of active ribosomes in tissue in vivo by injection of a radioactive puromycin combined with a large quantity of unlabeled puromycin. The rate of translation in a tissue is not solely determined by the number of active ribosomes but is related to both this number and the rate of peptide-chain elongation. We developed a new technique that we called the "labeled puromycin constant pool method" with which it is possible to measure FFR value without the problem of determination of precursor radioactivity. The constant supply of newly formed ribosomes through ceaseless operation of the ribosome cycle with the administration of a tracer dose of (^{3}H)puromycin guarantees the linear increase of peptidyl-puromycin formation for up to 20 minutes *(Figure 1)*.

The data presented here *(Figure 2)* are compatible with the results obtained by McNurlan et al.,[19] who failed to detect any effect of 100 μ mole leucine administration on the rate of protein synthesis, measured by a phenylalanine large-dose method, in the liver and muscle of either fed, starved (2 days), or protein-deprived (9 days) rats. The stimulatory action of leucine on liver protein synthesis without cooperation with insulin was clearly demonstrated in diabetic mice *(Figure 3)*. But the increment of FFR in the liver with leucine injection, granted that it was so, may be hidden under the high basal level of FFR in non-diabetic animals *(Figure 2)*. Leucine exerted no influence on muscle protein synthesis judging from FFR values in both non-diabetic and diabetic mice *(Figures 2 and 3)*. The lack of stimulating effect of leucine injection on muscle protein synthesis in diabetic mice *(Figure 3)* does not conflict with the results showing that the response of muscle protein synthesis to food intake is mediated by both insulin and amino acid in concert, particularly the branched-chain ones.[8,20] Although serum insulin concentration was not determined after leucine injection in diabetic mice, it seems probable that the insulin concentration was too low to affect the stimulation of muscle protein synthesis.

The blocking of a leucine-induced rise in the rate of peptidyl puromycin formation by the cyclooxygenase inhibitor, indomethacin, suggests an involvement of prostaglandins (PGs), thromboxanes, or both produced via the cyclooxygenase pathway in leucine-induced stimulation of liver protein synthesis in diabetic mice.

The results shown in *Figure 4* refer to the findings obtained in post-absorptive rats:[10] intravenous infusion of insulin resulted in a dose-dependent rise in the rate of muscle protein synthesis but not in the liver. A stimulatory effect of insulin on protein synthesis was exerted at the level of no less than 50 μ units/mL.[10] In the present experiment, 0.5 units/100 g body weight of insulin was injected into mice and the concentration of serum insulin after 30 minutes was >240 μ units/mL. The lack of effect of indomethacin on insulin-stimulated muscle protein synthesis obtained in diabetic mice *(Figure 4)* is identical to the effect of this inhibitor on the muscle of non-diabetic young rats.[10] In this study, indomethacin was injected into mice at a dose of 2000 μg/100 g body weight, compared with 250 μg/100 g body weight used in the rat,[10] because sensitivity to the drug is lower in mice.[21] Its administration to normal rats did not alter protein synthesis in either muscles or the liver.[22]

Insulin increased ribosome aggregation in the muscle of diabetic animals, as stated in Results. A novel role of insulin that could affect the rate of peptide-chain elongation was reported using NIH 3T3 cells.[23] However, in muscle, insulin has been reported to increase protein synthesis exclusively by enhancing initiation.[24] The increased ribosome aggregation in muscle of diabetic mice by insulin is thought to be due to the enhancement of peptide-chain initiation relative to that of elongation, because the insulin-induced rise of the FFR in muscle of diabetic mice compared with the control *(Figure 4)* could be interpreted as the result of increase in the rate of peptide-chain initiation relative to the elongation, along with ribosome aggregation. A slight decrease of polysome size in the liver of diabetic mice after leucine injection could be caused by the increase of peptide-chain elongation relative to that of initiation *(Table 1)*. On a parallel with increase in elongation rate, initiation should also be promoted to a lesser degree than elongation in the liver of diabetic mice by leucine injection. The value of FFR increased in spite of a slight decrease of ribosome aggregation by virtue of an increase in elongation rate *(Figure 3, Table 1)*. Refeeding a complete diet to 18 hour-fasted mice stimulated elongation activity in the liver to a large extent (unpublished data). Rapid aggregation of hepatic ribosome occurred in diabetic mice previously injected with cycloheximide (100 mg/100 g body weight, 10 minutes before sacrifice); this blocks elongation but allows initiation to proceed (results not shown). Experimental evidence that polysome size was increased with the cycloheximide indicates that ribosome aggregation does not always reflect the rate of protein synthesis in vivo, but it merely provides information on the relative rates of initiation and elongation.

Along with arginine and methionine, leucine has been shown to be one of the insulin secretagogue amino acids.[25] Insulin concentration in plasma was significantly elevated 2 minutes after leucine (100 μmol) and phenylalanine (150 μmol) injection in fed, starved (2 days), and protein-deprived (9 days) rats.[19] To examine the possible role of leucine alone on protein synthesis without concomitant increase in plasma insulin concentration, diabetic animals were used in this experiment. Plasma insulin concentration 1 hour after leucine injection in diabetic mice was not determined. However, such concentrations would be too low to be effective if leucine was injected into streptozotocin-induced diabetic mice. Leucine has been shown to stim-

ulate the secretion of glucagon and glucocorticoides in addition to insulin.[26,27] Plasma glucagon and glucocorticoid concentrations have not been measured in diabetic mice after leucine injection, so the possibility that glucagon, glucocorticoid, or both might promote liver protein synthesis by some unidentified mechanism cannot be ruled out entirely.

The abrupt increase of leucine concentration in serum (a six-fold increase: 1640 from 285 μmol/L in diabetic mice, data not shown) resulting from leucine injection may lead to a series of signal transductions. The hypothetical signaling pathway from leucine to ribosomal factor proteins mediated by arachidonic acid metabolism presented in this study are the same as that proposed by Reeds and Palmer[28] who depicted that the stimulus, insulin or other, increases $PGF_2\alpha$ release and may affect the rate of protein synthesis via changes in the phosphorylation of specific ribosomal or initiation-factor proteins. In this study, the following points differ from the original proposal:[28] the liver protein synthesis is stimulated by leucine instead of muscle being stimulated by insulin and the regulation may be caused at the step of elongation rather than initiation. In the case of insulin action, the signal transduction via cyclooxygenase pathway may be accepted because of the insulin receptor being ascertained on the muscle cell membrane, but to search for a possible signaling pathway from leucine to the stage of protein synthesis becomes a problem because no evidence has appeared on the binding of leucine to a specific receptor on the cell surface in the liver, hence the hypothetical pathway offered here is just a speculation. A missing link between the abrupt increase of leucine in extracellular environment and the change in ribosomal function remains to be found.

In this in vivo experiment it is difficult to evaluate whether the effect of indomethacin is direct or indirect on blocking the stimulation of liver protein synthesis by leucine injection. Besides its action as a first messenger, a hormone secretagogue, or both, the possibility that leucine exerts action as an allosteric effector on some step(s) involved in the series of protein synthesis, probably in some protein kinase(s), cannot be excluded.[29]

In conclusion, the present results support the view that leucine has a potential ability to stimulate the rate of liver protein synthesis in vivo, at least in the step of peptide elongation at the onset of the prandial state.

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