Analysis of the Stimulative Effect of Tryptophan on Hepatic Protein Synthesis in Rats

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(Received February 20, 2022)

Summary  Tryptophan is an essential amino acid important as a protein building block, but it also serves as substrate for the generation of several bioactive compounds with important physiological roles. Furthermore, tryptophan has been reported to have a unique role as a nutritional signaling molecule that regulates protein synthesis in mouse and rat liver. In the present study, the acute effects of tryptophan on protein synthesis were confirmed and compared with those of leucine in rats. Eighteen hours fasted rats were orally administered of tryptophan or leucine at a dose of 135 mg/100 g body weight by gavage and then sacrificed 1 h after administration. The effects of tryptophan and leucine on the rate of protein synthesis were evaluated by the surface sensing of translation (SUnSET) method. We also examined the ability of tryptophan to induce activation of the mTOR pathway by measuring phosphorylation of 4E-BP1 and S6K1. Oral administration of tryptophan led to a stimulation of the rate of protein synthesis concomitant with activation of mTOR pathway in the liver, but not in skeletal muscle. We also investigated the sensitivity of liver protein synthesis to tryptophan administration. The half-maximal effective doses (ED50) of tryptophan in stimulating 4E-BP1 and S6K1 phosphorylation were both about 60% of daily intake. The effect of tryptophan on hepatic protein synthesis was similar to that of leucine on muscle protein synthesis, and the sensitivity of liver protein synthesis to tryptophan administration appeared to be almost the same or slightly lower than that of muscle protein synthesis to leucine administration.

Key Words  tryptophan, protein synthesis, liver, mTOR, 4E-BP1, S6K1, rat

Amino acids are not only important precursors for the synthesis of proteins and other N-containing compounds, but also participate in the regulation of major metabolic pathways. Specific amino acids stimulate protein synthesis and inhibit protein degradation. Leucine has been shown to modulate the rate of protein synthesis, particularly by stimulating the activity of proteins involved in the translation process, which is critical for cells to control protein synthesis (1, 2). This modulation depends on the activation of specific intracellular pathways involved in protein synthesis, including activation of the mechanistic target of rapamycin (mTOR) pathway (3). Furthermore, recent studies using cells in culture showed that the Sestrins bind leucine and are required for leucine-dependent activation of mTOR (4–6). The mechanism by which leucine activates mTOR has been investigated for many years and is being better understood.

It has also been reported that tryptophan has a unique role as a nutritional signaling molecule that regulates protein synthesis in mouse and rat liver. Sidransky and co-workers demonstrated that a single tube-feeding of tryptophan to fasted animals (mice or rats) rapidly shifted hepatic polyribosomes toward heavier aggregation states and increased hepatic protein synthesis as measured in vivo and in vitro (7, 8). Subsequently, they reported a tryptophan receptor in the hepatic nuclear envelope, and that the binding of tryptophan to this receptor is saturable, stereospecific, and of high affinity (9, 10). They speculated that this specific binding of tryptophan to hepatic nuclei plays a vital role in the ability of tryptophan to rapidly stimulate hepatic protein synthesis (11, 12). However, to date there have been no further reports concerning the mechanism of protein synthesis stimulation by tryptophan. The mechanism underlying the stimulating actions of tryptophan on hepatic protein synthesis has not been clearly elucidated.

Tryptophan has been suggested to stimulate protein synthesis at the translational level because polysome aggregation was observed shortly after oral administration of tryptophan to rats (8). In this regard, it was reported that treatment with tryptophan increased hepatic mTOR phosphorylation after food deprivation (13). The phosphorylation of mTOR leads to enhanced...
transduction initiation and increased protein synthesis via phosphorylation of downstream targets such as eukaryotic initiation factor 4E binding protein 1 (4E-BP1) and ribosomal protein S6 kinase 1 (S6K1) (14). Recently, we reported that the phosphorylation of 4E-BP1 and S6K1 in the liver of food-deprived rats were phosphorylated 3 h after oral administration of tryptophan (15).

In vivo effect of oral administration of tryptophan on translation initiation was investigated in some studies as above, but its influence on the total protein synthesis is still poorly understood. The aim of this study was to evaluate the ability of tryptophan to stimulate not only translation initiation but also total protein synthesis in the liver and skeletal muscle of rats and to compare this with that of leucine. We also determined the minimal dose of tryptophan required to enhance signaling through mTOR in the liver.

MATERIALS AND METHODS

Animal and experimental design. The animal care protocol was approved by the Utsunomiya University Animal Research Committee under the Guidelines for Animal Experiments of Utsunomiya University (approval number: A13-0004, approval date: 26 April 2013). Three-week-old male Wistar rats purchased from CLEA Japan, Inc. (Tokyo) were maintained at 22˚C under a 12 h light-dark cycle (6:00–18:00). They were allowed free access to water and a 20% casein diet for 18 h prior to experimentation.

**Experiment 1.** Food-deprived rats were assigned randomly to one of the following three dietary treatments: control (Con), or administered 135 mg/100 g body weight l-leucine (Leu) or l-tryptophan (Trp) by oral gavage (2, 3). The dose for all experimental meals was 2.5 mL/100 g body weight (prepared as 54.0 g/L in distilled water). Control rats were fed 2.5 mL/100 g body weight saline (0.155 mol/L of NaCl). The amount of each amino acid administered was equivalent to the amount of leucine consumed by rats of this age and strain during 24 h (17) of free access and food-deprived for 18 h prior to experimentation.

**Experiment 2.** A solution of l-tryptophan (3.68, 7.36, 14.72, and 29.44 g/L of water) was prepared, and the rats were administered volumes of solution corresponding to 9.2 (50%), 18.4 (100%), 36.8 (200%), and 73.6 mg of l-tryptophan/100 g of body weight (400%) by oral gavage. The highest concentration of l-tryptophan employed was four times the amount of tryptophan consumed by the rats during 24 h of free access to an AIN-93 powdered diet (15). Control rats were administered saline at a volume of 2.5 mL/100 g of body weight. This volume of saline was equivalent to the volume of tryptophan solution administered to the rats. The rats were anesthetized with isoflurane and sacrificed 1 h after administration of tryptophan or saline.

**Evaluation of the rate of protein synthesis using the SUnSET method.** The effects of tryptophan and leucine on the rate of protein synthesis were evaluated by the surface sensing of translation (SUnSET) method, which measures the incorporation of puromycin into nascent peptide chains (18, 19). Briefly, the amount of puromycin incorporated into the newly synthesized protein was determined using western blotting. A mouse monoclonal anti-puromycin antibody (MABE343, Merck Millipore) was used to detect puromycin incorporation, which was evaluated as the sum of the intensity of all protein bands in the western blot. The membrane was stained with Ponceau S (P7170, Sigma-Aldrich) to verify that equal quantities of each protein had been transferred, before the western blotting was performed.

**Western blotting analysis.** The tissues were excised and immediately weighed and homogenized in 7 volumes of buffer A (20 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid 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, 1 mM dithiothreitol, 50 mM NaF, 50 mM β-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 0.5 mM sodium vanadate) by the use of a Dounce homogenizer (for the liver) (Wheaton, Millville, NJ) or a Polytron homogenizer (for the muscle) (Kinematica, Littau, Switzerland). The homogenates were centrifuged at 10,000 × g for 10 min at 4˚C (20). The resulting supernatants were examined for the phosphorylation states of S6K1 and 4E-BP1.

Half of the supernatant was combined with an equal volume of 2XSDS sample buffer (2 mL of 0.5 M Tris pH 6.8, 2 mL of glycerol, 2 mL of 10% SDS, 0.2 mL of β-mercaptoethanol, 0.4 mL of a 4% solution of bromophenol blue, and 1.4 mL of water to a final volume of 8 mL), and the diluted sample was subjected to electrophoresis on a 7.5% polyacrylamide gel (21). The samples were then subjected to protein immunoblot analysis using antibodies specific for S6K1 (cat. no. 230, Santa Cruz Biotechnology, Santa Cruz, CA). The other half of the supernatant was heated for 10 min at 100˚C, cooled to room temperature, and centrifuged at 10,000 × g for 30 min at 4˚C. The resulting supernatant was mixed with an equal volume of 2XSDS sample buffer, and the diluted sample was subjected to electrophoresis on a 15% polyacrylamide gel. The samples were subjected to protein immunoblot analysis using rabbit anti-4E-BP1 polyclonal antibodies (cat. no. 6963, Santa Cruz Biotechnology), as described previously (20).
Serum amino acid analysis. Serum amino acid concentrations were measured means of an automatic amino acid analyzer (JLC-500/V2, JEOL, Tokyo) after sulfosalicylic acid treatment (final concentration 1.5%).

Determination of the half-maximal effective doses (ED50). For the calculation of ED50, the variable slope model was used with the following equation:

\[ Y = \frac{Y_{\text{min}} + X_{\text{HillSlope}}(Y_{\text{max}} - Y_{\text{min}})}{X_{\text{HillSlope}} + ED50_{\text{HillSlope}}} \]

Statistical analyses. Data are means±SE. All statistical analyses were performed with Graphpad Prism 7 software (GraphPad Software Inc., La Jolla, CA). 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 comparison test. The level of significance was set at \( p < 0.05 \) for all statistical tests.

RESULTS

Relative to control, leucine-treated rats had highly elevated leucine concentrations in serum as expected (Table 1). The serum concentration of leucine was 13-fold greater in rats administered leucine compared with control rats. In contrast, serum concentration of isoleucine and valine were significantly reduced by oral administration of leucine (Table 1), while almost all of the other amino acids were not significantly affected (data not shown). The changes in serum concentrations of isoleucine and valine were essentially the reverse of those of leucine. The mechanism of this effect is
unknown, but may involve specific stimulation of branched-chain amino acid oxidation in order to consume excess leucine (22), an increase in uptake of those amino acids to support enhanced rates of protein synthesis (3) or a combination of these factors. On the other hand, oral administration of tryptophan increased the serum concentration of tryptophan by 12-fold compared to control (Table 1). The concentrations of 19 of the 20 serum amino acids that are common in proteins were not significantly different between the control and tryptophan-treated rats (Table 1).

In this investigation, the ability of the tryptophan to stimulate translation initiation and protein synthesis was examined with that of leucine. Oral administration of leucine stimulated the rate of protein synthesis in both the liver and skeletal muscle. In contrast, provision of tryptophan increased protein synthesis only in the liver, not in skeletal muscle (Fig. 1).

Increases in skeletal muscle protein synthesis following leucine ingestion have been known to be associated with enhanced translation initiation via activation of mTOR and downstream targets S6K1 and 4E-BP1. Activation of mTOR results in phosphorylation of 4E-BP1 and S6K1, the best studied downstream targets of mTOR. The phosphorylation state of 4E-BP1 can conveniently be examined by resolution of the phosphory-
lated forms of the protein during SDS-polyacrylamide gel electrophoresis. During this, 4E-BP1 is resolved into multiple electrophoretic forms, α, β, and γ, representing differentially phosphorylated forms of the protein. The γ form is the most highly phosphorylated form and exhibits the lowest electrophoretic mobility, and it is the only one of the three that does not bind to eIF4E. In this study, therefore, the phosphorylation of 4E-BP1 was expressed as a percentage of the protein in the γ form. Oral administration of leucine to food-deprived rats increased the phosphorylation of 4E-BP1 in the liver by 4-fold (Fig. 2A) and in skeletal muscle by 2-fold compared with food-deprived controls (Fig. 2B). This result is consistent with previous reports showing that the oral administration of leucine resulted in an enhanced phosphorylation of 4E-BP1 in both the liver and skeletal muscle (20). In contrast to oral administration of leucine, oral administration of tryptophan induced a 4-fold hyperphosphorylation of 4E-BP1 in the liver (Fig. 2A) but did not induce observable changes in the phosphorylation state of 4E-BP1 in skeletal muscle (Fig. 2B).

Upon activation, S6K1 is typically resolved into multiple electrophoretic forms after separation of it by electrophoresis on SDS-polyacrylamide gel, increased phosphorylation being associated with decreased electrophoretic mobility (23). In the present study, therefore, the effect of leucine or tryptophan administration on the phosphorylation of S6K1 was investigated in the liver and skeletal muscle by protein immunoblot analysis. For S6K1, we quantified the ratio of the more heavily phosphorylated (more slowly migrating) forms to total immune reactivity, because it is the phosphorylated forms that have kinase activity. The phosphorylation of S6K1 in both the liver and skeletal muscle was increased 2-fold by oral administration of leucine (Fig. 3). In contrast, oral administration of tryptophan had

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**Fig. 4.** Changes in serum tryptophan concentrations following oral tryptophan administration in rats. Serum tryptophan concentrations were measured 1 h after administration of saline or tryptophan at doses ranging from 9.2 g (50%) to 73.6 g (400%) of l-tryptophan/100 g of body weight. Results represent mean ± SE for 4 animals. Values not sharing the same superscript letter are significantly different (p<0.05) by the Tukey-Kramer multiple comparison Test.

**Fig. 5.** Changes in the phosphorylation states of 4E-BP1 and S6K1 in the livers of rats following oral tryptophan administration. A, Phosphorylation of 4E-BP1 was expressed as the amount of protein present in the γ-form as a percentage of total 4E-BP1. Results represent mean ± SE for 4 animals. Values not sharing the same superscript letter are significantly different (p<0.05) by the Tukey-Kramer multiple comparison Test. Insets: results of representative immunoblots, positions of the α-, β-, and γ-forms of 4E-BP1 noted on the left. Lanes represent samples from 0% dose (0%), 50% dose (50%), 100% dose (100%), 200% dose (200%), and 400% dose (400%) animals. ED50 = 55.5% of daily intake. B, Phosphorylation of S6K1 was expressed as the amount of S6K1 in the β and γ forms as percentages of total S6K1. Results represent mean ± SE for 4 animals. The inset shows a representative immunoblot, and the positions of the α-, β-, and γ-forms of S6K1 are noted on the left. ED50 = 59.1% of daily intake.
an obvious stimulatory effect on the phosphorylation of S6K1 in the liver (Fig. 3A), but it had no effect on the phosphorylation of S6K1 in skeletal muscle (Fig. 3B).

The sensitivity of mTOR signaling to tryptophan in the liver was determined as the half-maximal effective doses (ED50) of tryptophan in stimulating 4E-BP1 and S6K1 phosphorylation. The serum tryptophan concentrations rose in proportion to the amount of amino acid administered, and by 1 h they were significantly greater than the control at all compared doses (Fig. 4). The phosphorylation of 4E-BP1 in the liver increased beyond the control by 631%, 544% and 542% after the administration of the 400%, 200% and 100% doses of tryptophan respectively (Fig. 5A). At the 50% dose, there was a 304% increase in the phosphorylation of 4E-BP1 beyond the control (Fig. 5A). Similarly, S6K1 phosphorylation in the liver increased in a dose-dependent manner in response to increasing amounts of tryptophan (Fig. 5B). With respect to typical dose responses (Fig. 5), the ED50 of tryptophan in stimulating 4E-BP1 and S6K1 phosphorylation were 55.5% and 59.1% of daily intake respectively.

**DISCUSSION**

In this study, the rate of protein synthesis in the liver and skeletal muscle was evaluated by SUnSET method to confirm the effects of tryptophan and leucine on protein synthesis in these tissues. As previously reported (3), oral administration of leucine to fasted rats stimulated protein synthesis in skeletal muscle in the present study (Fig. 1). Leucine also stimulated protein synthesis in the liver as well as muscle (Fig. 1). This is not consistent with the earlier finding that oral administration of leucine did not stimulate fractional rates of protein synthesis in the liver of food-deprived rats (24). The basis for this discrepancy is unclear, but it may be due to differences in the methods for measuring the rate of protein synthesis used in each study. For example, while SUnSET has been shown to be valid for the measurement of relative rates, or relative changes in protein synthesis, it remains to be determined whether SUnSET could be used to calculate absolute or fractional synthesis rates similar to those measured with isotope techniques.

In contrast to administration of leucine, administered tryptophan increased protein synthesis only in the liver, not in skeletal muscle (Fig. 1). The results in the liver were similar to those found in previous studies that have reported that administration of tryptophan rapidly stimulates hepatic protein synthesis (7, 25). We can find few studies which have focused on the acute effects of tryptophan on protein synthesis in skeletal muscle. Taken together, tryptophan appears to play an important regulatory role in the stimulation of protein synthesis in the liver, but not in skeletal muscle. However, it is unknown why the effect of tryptophan on protein synthesis is tissue specific.

Insulin is known to stimulate protein synthesis by translational effects in many tissues. Therefore, serum insulin concentrations were measured in all groups. Oral administration of tryptophan or leucine to food-deprived rats did not alter circulating insulin concentrations compared with food-deprived controls (data not shown). It has already been demonstrated that oral administration of leucine stimulates synthesis of skeletal muscle proteins in food-deprived rats independently of changes in the circulating concentration of insulin (3). The results of present study suggest that the stimulating effect of tryptophan on hepatic protein synthesis, as well as that of leucine on muscle protein synthesis, does not require an elevation in circulating insulin concentrations. Therefore, it is unlikely that the stimulatory effect of tryptophan on hepatic protein synthesis is an indirect effect mediated through increased insulin secretion.

Sidransky and co-workers demonstrated that a single tube-feeding of tryptophan to fasted animals (mice or rats) rapidly shifted hepatic polyribosomes toward heavier aggregation states and increased hepatic protein synthesis as measured in vivo and in vitro (7, 8). These results show that tryptophan has stimulative effects on protein synthesis at the translation initiation level. However, surprisingly, there are little reports about the effect of tryptophan on translation initiation.

Numerous previous studies show that amino acids positively regulate mTOR signaling. Activated mTOR promotes the translation initiation process by phosphorylating two major targets: S6K1 and 4E-BP1 (26). Recently, we reported that oral administration of tryptophan stimulated phosphorylation of 4E-BP1 and S6K1 in the liver of food-deprived rats within 3 h (15). In this study, we demonstrated that oral administration of tryptophan as well as leucine promoted phosphorylation of S6K1 and 4E-BP1 in the liver of fasted rats within shorter period of time (1 h) (Figs. 2 and 3). These results demonstrate that tryptophan-dependent stimulation of translation initiation in the liver involves mTOR signaling. Taken together, these data support the idea that tryptophan acts as a signaling molecule that activates the hepatic protein synthetic machinery.

The amount of leucine or tryptophan administered was equivalent to the amount of leucine consumed by the rats during 24 h of free access to an AIN-93 powdered diet (17). This amount of tryptophan is equivalent to almost 7 times the amount of tryptophan consumed in a 24-h period by age- and strain-matched rats allowed free access to the AIN-93G standard diet and is very high (1.5). Therefore, a much lower and more physiological dose of tryptophan was used in dose-response studies which was conducted to find the minimal tryptophan dose activating phosphorylation of S6K1 or 4E-BP1 in the liver. With respect to typical dose responses (Fig. 5), the ED50 of tryptophan in stimulating 4E-BP1 and S6K1 phosphorylation were 55.5% and 59.1% of daily intake respectively. The sensitivity of hepatic protein synthesis to tryptophan administration appeared to be almost the same or slightly lower than that of muscle protein synthesis to leucine administration, because the ED50 of tryptophan in stimulating 4E-BP1 and S6K1 phosphorylation in the liver (~60%
dose) was close to that of leucine in skeletal muscle (~20% dose) reported in our previous study (20). The finding that phosphorylation of 4E-BP1 and S6K1 in the liver reached a plateau at the 100% dose, which increased the serum tryptophan concentrations 10.9-fold as compared to the fasted control, suggests that the effects were mediated through a receptor-based mechanism that can be expected to exhibit maximal activation at tryptophan concentrations near or slightly above physiological levels.

Previous studies established that leucine stimulates protein synthesis in skeletal muscle and the effect occurs through activation of mTOR (2, 3, 20). Recent studies using cells in culture showed that the Sestrins bind leucine and are required for leucine-dependent activation of mTOR (4–6). Leucine has been found to activate mTOR via Sestrin 2, i.e., binding of leucine to Sestrin 2 disrupts its interaction with GTPase-activating protein toward rags 2 (GATOR2), thereby activating the mTOR signaling pathway (27). Meanwhile, Xu et al. reported that Sestrin 1, but not Sestrin 2, is highly expressed in the skeletal muscle and that leucine activates mTOR via Sestrin 1 (28). Thus, Sestrin seems to be a leucine sensor in the mTOR pathway. Tryptophan stimulates the mTOR signaling pathway and protein synthesis in the liver, just as leucine stimulates those in skeletal muscle. But, it has not been clear how the cell senses extracellular tryptophan to promote mTOR activation. Sidransky and co-workers reported that hepatic nuclei, particularly their nuclear envelopes, had a specific receptor for tryptophan (9, 10). They have speculated that the nuclear envelope receptor binding of tryptophan could be important in the tryptophan-induced enhancement of nucleocytoplasmic transport of mRNA within liver cells, a process that has been speculated to be intimately involved in the enhanced protein synthesis (29–32). The relationship between the nuclear envelope receptor for tryptophan and mTOR signaling pathway is still not clear. But, it is possible that there is some linkage between the receptor and mTOR signaling, because the mTOR signaling was reported to regulate the nucleocytoplasmic shuttling in yeast (33). Nevertheless, further studies are required to resolve how the hepatocyte senses tryptophan and transforms the signal into a change in protein synthesis. If the tryptophan-sensing mechanism were elucidated, this might provide an answer to the question about the tissue-specificity of the effect of tryptophan on protein synthesis.

In summary, in this investigation, the ability of the tryptophan to stimulate translation initiation and protein synthesis was compared with that of leucine. Our study results show that tryptophan can stimulate protein synthesis in the liver but not in skeletal muscle of fasted rats. Our results also strongly support the notion that tryptophan stimulates hepatic protein synthesis by enhancing mTOR-dependent translation initiation signaling. The effect of tryptophan on hepatic protein synthesis was similar to that of leucine on muscle protein synthesis, and the sensitivity of liver protein synthesis to tryptophan administration appeared to be almost the same or slightly lower than that of muscle protein synthesis to leucine administration.

Acknowledgments
This research was funded by JSPS KAKENHI, grant number 22380071 and 22K05472.

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