Regulation of Skeletal Muscle Protein Degradation and Synthesis by Oral Administration of Lysine in Rats

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Summary Several catabolic diseases and unloading induce muscle mass wasting, which causes severe pathological progression in various diseases and aging. Leucine is known to attenuate muscle loss via stimulation of protein synthesis and suppression of protein degradation in skeletal muscle. The aim of this study was to investigate the effects of lysine intake on protein degradation and synthesis in skeletal muscle. Fasted rats were administered 22.8–570 mg Lys/100 g body weight and the rates of myofibrillar protein degradation were assessed for 0–6 h after Lys administration. The rates of myofibrillar protein degradation evaluated by MeHis release from the isolated muscles were markedly suppressed after administration of 114 mg Lys/100 g body weight and of 570 mg Lys/100 g body weight. LC3-II, a marker of the autophagic-lysosomal pathway, tended to decrease ($p=0.05$, 0.08) after Lys intake (114 mg/100 g body weight). However, expression of ubiquitin ligase E3 atrogin-1 mRNA and levels of ubiquitinated proteins were not suppressed by Lys intake. Phosphorylation levels of mTOR, S6K1 and 4E-BP1 in the gastrocnemius muscle were not altered after Lys intake. These results suggest that Lys is able to suppress myofibrillar protein degradation at least partially through the autophagic-lysosomal pathway, not the ubiquitin-proteasomal pathway, whereas Lys might be unable to stimulate protein synthesis within this time frame.

Key Words lysine, muscle wasting, protein degradation, protein synthesis, rat

Muscle mass is regulated by the balance between the synthesis and degradation of muscle protein. Hence, a decreased rate of protein synthesis and an accelerated rate of protein degradation in skeletal muscle lead to muscle wasting (1). Muscle wasting is induced in many catabolic conditions such as sepsis and cancer, and unloading. These states induce a progressive reduction of skeletal muscle mass and muscle strength (2, 3). Progression of muscle atrophy leads to a decrease in physical activity. Energy and/or protein deficiency in the diet also induces loss of muscle mass, which may be an important factor in muscle atrophy.

In several studies, the factors regulating skeletal muscle protein turnover under catabolic conditions have been investigated. IGF-1 and insulin enhance protein synthesis, and suppress protein degradation in skeletal muscle (4). Appropriate exercise also regulates skeletal muscle protein turnover (5, 6). In addition, dietary components, particularly essential amino acids, are reported to affect muscle protein turnover (7, 8).

Many studies have suggested that leucine (Leu) activates the mTOR signaling and stimulates protein synthesis (9, 10). We have reported that supplementation with Leu under a protein-deficient diet suppresses myofibrillar protein degradation in rats (11, 12). We reported that the suppression effect of Leu occurred by inhibition of the autophagic-lysosomal pathway, a proteolytic system (13). Thus, Leu also regulates muscle protein degradation.

Yao et al. (14) showed that dietary arginine (Arg) supplementation increased mTOR signaling activity in the skeletal muscle of neonatal pigs. Oral intake of glutamine (Gln) stimulates protein synthesis (15) and prevents protein hypercatabolism by inhibition of myostatin hyperexpression (16). However, the effects of other amino acids on muscle protein synthesis and degradation are unclear.

Lysine (Lys) is a major limiting essential amino acid in some plant proteins. Animals fed low Lys diets grow more slowly than those fed standard diets (17, 18). Therefore, addition of Lys to low-Lys diets is considered to be essential for maturity in growing animals. Nevertheless, the effects of Lys on skeletal muscle protein turnover are unknown. Thus, it is important to clarify to the regulatory role of Lys on muscle degradation and protein synthesis.

In this study, we administered Lys to rats, and investigated the changes in skeletal muscle turnover. Our results indicate that dietary Lys suppresses myofibrillar protein degradation, whereas Lys intake does not affect

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Abbreviations: AMPK, AMP-activated protein kinase; EDL, extensor digitorum longus; IGF-1, insulin-like growth factor-1; LC3, microtubule-associated protein 1 light chain 3; MeHis, 3-methylhistidine; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3'-kinase; S6K1, ribosomal protein S6 kinase 1; 4E-BP1, eukaryotic initiation factor (eIF) 4E-binding protein 1.

protein synthesis.

MATERIALS AND METHODS

Animals and experimental design.

Experiment 1: Male Wistar rats (4 wk of age) were purchased from Japan SLC, Inc. (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 tap water and a 20% casein diet based on AIN-93G (19). The animal care protocols in this study were approved by the Iwate University Animal Research Committee under Guidelines for Animal Experiments in Iwate University. When the weight of the rats reached approximately 80 g (5 wk of age), they were randomly assigned to the following five groups ($n=5$, in each group) and subjected to fasting for 18 h. MeHis release from the isolated skeletal muscle is unable to be measured in rats with a body weight of more than 80 g, because the isolated muscle must be thin (20). Therefore, we used growing rats. After fasting, the rats in each group were administered Lys (Ajinomoto Co., Inc., Tokyo, Japan) solution at 22.8 mg/100 g body weight (0.2K), 114 mg/100 g body weight (K), or 570 mg/100 g body weight (5K), or were administered glycine (Gly) (Ajinomoto Co., Inc.) solution at 94 mg/100 g body weight (G). The amounts of 114 mg Lys was equivalent to the amounts of Lys contained in 8.8 g of 20% casein diet which was eaten by rats per day. The nitrogen level of 94 mg Gly is equivalent to that of 114 mg Lys which contained a 20% casein diet consumed by the rats each day. Lys and Gly were dissolved in saline. The control group consisted of rats administered saline (2 mL/100 g body weight) after 18 h starvation and killed 3 h after saline administration (C). Lys, Gly and saline were intragastrically administered to rats with a feeding tube (Atom Indwelling Feeding Tube T 5Fr, Atom Medical, Tokyo, Japan). At 3 h after administration of amino acid solution, rats were anesthetized with diethylether, and their abdomens were opened and blood was collected from the inferior vena cava. The blood was centrifuged at $3,500 \times g$ for 15 min to obtain plasma, which was frozen in liquid nitrogen and stored at -80°C until analysis. EDL muscle, soleus muscle and gastrocnemius muscle were removed from the hind leg to measure the rate of protein degradation and muscle weight.

Experiment 2: Rats (~ 80 g) were randomly assigned to the following four groups ($n=5$, in each group) and subjected to fasting for 18 h. After fasting, the first group was administered saline, and soon after administration, blood and muscle samples were collected by the same method as described in Experiment 1. The other three groups were administered Lys (114 mg/100 g body weight) and at 1 h, 3 h or 6 h after administration, their blood and muscle samples were collected.

Measurement of myofibrillar protein degradation. We incubated the isolated EDL muscle and soleus muscle in a Krebs-Ringer bicarbonate buffer containing 10 mM glucose under 95% O_2 –5% CO_2 at 37°C for 2 h after a

30 min pre-incubation at 37°C in order to directly measure the rate of myofibrillar protein degradation (21). The amount of MeHis in the incubation buffer solution, which was used as a marker of myofibrillar proteolysis (11, 21, 22), was measured by the HPLC method (23).

Plasma Lys, Leu and MeHis concentration. Plasma Lys, Leu, Arg and histidine (His) concentrations were measured using an Amino Acids Autoanalyzer (JLC-500/V; JEOL, Tokyo, Japan). Plasma MeHis concentration was determined by HPLC method using fluorescence derivatization (24).

Plasma insulin concentration. Plasma insulin concentration was measured by enzyme-linked immunosorbent assay (ELISA) (Morinaga Institute of Biological Science, Yokohama, Japan).

Western blot analysis. We used gastrocnemius muscle for Western blot analysis. Because the weight of the soleus and EDL muscles are quite low, it is difficult to prepare the sample for Western blot analysis. Gastrocnemius muscle (200 mg) was homogenized with 10 volumes buffer solution A (0.25 M sucrose/1 mM EDTA, pH 7.4) or 7 volumes buffer solution B (20 μM phenylmethylsulfonyl fluoride, 2 μM HEPES, 2 μM EGTA, 50 μM sodium fluoride, 1 μM potassium chloride, 0.2 μM EDTA, 50 μM glycerol 2-phosphate disodium salt *n*-hydrate, 1 mM benzamidine hydrochloride *n*-hydrate, 0.5 mM sodium orthovanadate (V) and 1 mM ($\pm$)-dithiothreitol) using a Polytron homogenizer (Kinematica, Lucerne, Switzerland) followed by centrifugation at $3,800 \times g$. Homogenates in buffer solution A were used for quantitative determination of LC3, and homogenates in buffer solution B were used for quantitative determination of other proteins.

The supernatant was used as the SDS-PAGE sample. The sample was separated on 10% polyacrylamide gel and transferred to a PVDF membrane (Amersham Bioscience, Little Chalfont, UK). The membrane was blocked for 1 h with 5% skim milk in Tris buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) at room temperature. The membrane was incubated overnight at 4°C with primary antibodies.

LC3B antibody, phospho-S6K1 (Thr 389) (1A5) mouse mAb, mTOR antibody and phospho-mTOR (Ser 2448) antibody were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Rabbit anti-S6K1 polyclonal antibody and anti-ubiquitin rabbit-polyclonal antibody were obtained from Stressgen (Victoria, Canada). 4E-BP1 (R-113), Akt1 (B-1) and p-Akt 1/2/3 (Ser 473) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). After incubation with the primary antibody, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG (Stressgen) or HRP-conjugated goat anti-mouse IgG (Stressgen) in TBS-T. Detection of the secondary antibody was performed using an ECL western blot detection kit (Amersham Bioscience). To estimate the relative intensity of each band, X-ray films were scanned using NIH Image.

Gene expression of ubiquitin-ligase. Ten micrograms of total RNA from gastrocnemius muscle were separated on a 1.2% agarose-formaldehyde gel and transferred

Fig. 1. Rates of MeHis release from isolated EDL and soleus muscle (A), plasma Lys concentrations (B) and plasma MeHis concentrations (C) after different amount of Lys administration. Values are means $\pm$ SE ($n=5$). C: rats administered saline; 0.2K: rats administered 22.8 mg Lys/100 g body weight; K: rats administered 114 mg Lys/100 g body weight; 5K: rats administered 570 mg Lys/100 g body weight; G: rats administered 94 mg Gly/100 g body weight. Different letters indicate significant ($p<0.05$) differences among the groups.

to a positively charged nylon membrane (Amersham Biosciences). After UV cross-linking, membranes were hybridized with a digoxigenin-labeled cDNA probe specific to atrogin-1 for 12 to 16 h at 50°C in hybridization solution (5 $\times$ SSC, 50% formamide, 50 mM sodium phosphate buffer, pH 7.0, 7% sodium dodecyl sulfate (SDS), 2% blocking reagent (Roche Diagnostics, Mannheim, Germany), and 0.1% N-lauroylsarcosine). Membranes were washed twice with 2 $\times$ SSC-0.1% SDS for 15 min at room temperature and twice with 0.1 $\times$ SSC-0.1% SDS for 15 min at 68°C. Specific hybridization was detected with an anti-digoxigenin antibody conjugated with alkaline phosphatase, and blots were developed with CDP-star reagent (Tropix; Bedford, MA). To estimate the relative intensity of each band, X-ray films were scanned using NIH Image.

Statistical analyses. Data are expressed as mean with SE. Data analysis was performed using GraphPad Instat Software version 2.03 (1995, GraphPad Software, Inc., San Diego, CA). Data were analyzed by analysis of variance (ANOVA) and Tukey's post-test in multigroup comparisons to determine whether there were significant differences ($p<0.05$) among the groups when ANOVA indicated a difference among the means.

RESULTS

Experiment 1

The rates of MeHis release from the isolated EDL muscle and soleus muscle are shown in Fig. 1A. The rates of MeHis release from the isolated EDL muscle in the K group and 5K group were significantly suppressed by 40% and 55%, respectively, as compared with the

Fig. 2. Time course of rates of MeHis release from EDL and soleus muscle. Values are means $\pm$ SE ($n=5$). Rats were administered Lys (114 mg/100 g body weight). Different letters indicate significant ($p<0.05$) differences among the groups.

C group. The rates of MeHis release from the isolated soleus muscle in the K group and the 5K group were significantly suppressed by 25% and 35%, respectively, as compared with the C group. However, Gly and smaller amounts of Lys (0.2K) did not suppress the rate of MeHis release from isolated muscles. These results demonstrate that oral administration of Lys reduced myofibrillar protein degradation. Plasma Lys concentrations in the K group and 5K group were significantly increased by 4.5-fold and 8.8-fold, respectively, when compared with that in the C group (Fig. 1B). Plasma MeHis concentration showing the protein degradation of whole muscle was also suppressed by administration of Lys (Fig. 1C).

Fig. 3. Time course of plasma Lys concentrations (A), plasma Leu concentrations (B) and plasma insulin concentrations (C). Values are means $\pm$ SE ($n=5$). Rats were administered Lys (114 mg/100 g body weight). Different letters indicate significant ($p < 0.05$) differences among the groups.

Experiment 2

Figure 2 shows the changes in rates of MeHis release from the isolated EDL muscle and soleus muscle. The rate of MeHis release from the isolated EDL muscle decreased gradually after Lys administration and that at 3 h after administration of Lys was significantly suppressed by 40% when compared with that in rats before administration (0 h). Subsequently, the rate of MeHis release was maintained at lower levels. The rate of MeHis release from the isolated soleus muscle at 3 h and 6 h after administration of Lys was significantly suppressed by 35% when compared with that in rats given saline. Plasma Lys concentrations at 1 h, 3 h and 6 h after administration of Lys increased significantly (Fig. 3A). On the other hand, plasma Leu concentrations were not increased after Lys administration (Fig. 3B). Plasma insulin concentrations showed no changes at any time points after Lys intake (Fig. 3C).

LC3 is a mammalian homologue of Apg8p that is essential for the autophagic-lysosomal pathway in yeast, and has two forms, known as LC3-I and LC3-II. The ratio of sarcoplasmic LC3-II to LC3-I is reported to correspond to the extent of autophagic-lysosomal activity (25). To determine whether the autophagic-lysosomal pathway is suppressed by administration of Lys, we measured the ratio of LC3-II to LC3-I by Western blotting. Although the ratio of sarcoplasmic LC3-II to LC3-I in the gastrocnemius muscle in rats given Lys was not significantly reduced (Fig. 4), the ratio of LC3-II to LC3-I tended to decrease at 1 h and 6 h after administration of Lys ($p=0.05$, 0.08).

The activity of the ubiquitin-proteasomal system upon administration of Lys was determined, as the ubiquitin-proteasomal system is the other major pathway of protein degradation. No significant differences in the

Fig. 4. Time course of ratio of LC3-II/LC3-I after Lys administration. Values are means $\pm$ SE ($n=5$). Different letters indicate significant ($p < 0.05$) differences among the groups.

expression of atrogin-1 mRNA were observed between the rats given saline and those given Lys (Fig. 5A). Furthermore, the levels of the ubiquitinated proteins did not differ between the rats given saline and those given Lys (Fig. 5B).

It is known that Leu activates the mTOR pathway (26). We analyzed the phosphorylation levels of mTOR, S6K1 and 4E-BP1, which are signaling targets downstream of mTOR in the gastrocnemius muscle. The phosphorylation levels of mTOR, p70 S6K1 and 4E-BP1 in rats given Lys were unchanged (Fig. 6A, B, C). Both the autophagic-lysosomal and the ubiquitin-protea-

Fig. 5. Time course of atrogin-1 mRNA expression (A) and ubiquitinated protein content after Lys administration (B). Values are means $\pm$ SE ($n=5$). Different letters indicate significant ($p<0.05$) differences among the groups.

Fig. 6. Time course of phosphorylation levels of mTOR (A), S6K1 (B) and 4E-BP1 after Lys administration (C). We measured γ and β forms of 4E-BP1 as phosphorylated 4E-BP1, and measured total of γ , β and α forms of 4E-BP1 as total 4E-BP1. Values are means $\pm$ SE ($n=5$). Different letters indicate significant ($p<0.05$) differences among the groups.

somal pathways are regulated through the mTOR and insulin/IGF-1 signaling pathways. Therefore, we analyzed the phosphorylation levels of Akt (Ser 473) in the rat gastrocnemius by Western blotting. No significant differences were seen in phosphorylation levels of Akt (Ser 473) between the rats given saline and those given Lys (Fig. 7).

DISCUSSION

Dietary Leu suppresses skeletal muscle protein deg-

radation (11, 12, 27), and enhances protein synthesis through activation of mTOR signaling (9, 10, 26). However, the effects of other amino acids on protein degradation and protein synthesis in skeletal muscle have scarcely been investigated. Therefore, we determined whether administration of Lys, a major limiting essential amino acid, is able to regulate protein synthesis and protein degradation in the skeletal muscles of rats administered Lys.

First, we administered Lys to the fasted rats at three

Fig. 7. Time course of phosphorylation levels of Akt after Lys administration. Values are means $\pm$ SE ($n=5$). Different letters indicate significant ($p<0.05$) differences among the groups.

concentration levels, and measured the rates of myofibrillar protein degradation of the isolated muscles. Myofibrillar protein degradation of EDL and soleus muscle was suppressed in rats administered 114 mg or 570 mg of Lys/100 g body weight, but suppression of myofibrillar protein degradation was not observed in rats administered 22.8 mg of Lys (Fig. 1A). These results strongly indicate that Lys intake suppresses myofibrillar protein degradation in a concentration-dependent manner. We thought that ingested nitrogen, not Lys itself, might decrease the myofibrillar protein degradation. Thus, we administered Gly, which has an equivalent nitrogen level to 114 mg of Lys, to rats. The rates of myofibrillar protein degradation of EDL and soleus muscle were not suppressed in the rats administered Gly (Fig. 1A), which suggests that the inhibitory effects of Lys on the degradation of myofibrillar protein are not due to nitrogen intake. Furthermore, the plasma concentration of MeHis, which indicates whole body muscle protein degradation, decreased with the administration of Lys. The suppressive effects on myofibrillar proteolysis were therefore Lys-specific.

Next, we investigated the time-course of skeletal muscle protein turnover after Lys administration (114 mg/100 g body weight). Nagasawa et al. (11) showed that oral administration of Leu suppressed myofibrillar protein degradation for 2–4 h after administration. The rate of myofibrillar protein degradation of EDL was notably suppressed after 3 h of Lys administration (Fig. 2). On the other hand, myofibrillar protein degradation of the isolated soleus muscle was markedly suppressed at 3 h and 6 h after Lys administration (Fig. 2). From these results, we believe that the suppressive effects of Lys were most notable at 3 h after administration, similar to the results observed for Leu (11), although the responses differed slightly between the EDL muscle and soleus muscles. Nakashima et al. (28) showed that oral administration of Lys suppressed the proteolysis of

skeletal muscle in chicks, measured by plasma MeHis concentrations. Here, we directly measured the rate of myofibrillar protein degradation in skeletal muscle and found that Lys suppressed myofibrillar proteolysis at 3 h after Lys administration. This study is therefore the first to evaluate the suppressive effects of Lys on mammalian skeletal muscle proteolysis based on the rate of myofibrillar protein degradation.

There are three proteolytic systems in skeletal muscle: the autophagic-lysosomal system, the ubiquitin-proteasomal system and the calpain system (29). Many studies have shown that various catabolic conditions activate the ubiquitin-proteasomal pathway (30, 31). We have previously shown that dietary Leu intake suppresses myofibrillar proteolysis by inhibiting the autophagic-lysosomal pathway (13). Ogata et al. (32) demonstrated that the ratio of LC3-II/LC3-I, a marker of the autophagic-lysosomal pathway activity, increased in a fasting duration-dependent manner. In this study, the ratio of LC3-II/LC3-I tended to decrease after Lys administration, although statistical significance was not achieved (Fig. 4). In the cultured muscle cells, we found that Lys significantly suppressed the autophagic-lysosomal activity assessed from the ratio of LC3-II/LC3-I (data not shown).

On the other hand, expression of atrogin-1 mRNA, muscle-specific ubiquitin ligase (E3), was unchanged by Lys administration (Fig. 5A). Similarly, the levels of ubiquitinated proteins were not affected by Lys administration (Fig. 5B). Therefore, it is possible that Lys suppresses the autophagic-lysosomal pathway, but does not suppress the ubiquitin-proteasomal pathway. The autophagic-lysosomal pathway may be a particularly important proteolytic pathway with regard to the inhibitory effects on amino acids, although Hamel et al. (33) reported that Lys directly inhibited the activity of proteasome prepared from rat muscle in vitro. Ishida et al. (18) suggested that dietary Lys decreased the fractional protein degradation rate in skeletal muscle in growing rats using urinary MeHis excretion, and they confirmed the suppression of atrogin-1 mRNA expression in the skeletal muscle of rats fed sufficient Lys. Thus, dietary Lys may suppress both the autophagic-lysosomal pathway and the ubiquitin-proteasomal pathway. Further study is required for clarification of this possibility.

The phosphorylation levels of mTOR, S6K1 and 4E-BP1 in gastrocnemius muscles were not affected by administration of Lys (Fig. 6A–C). These results suggest that a single oral administration of Lys suppresses degradation of skeletal muscle protein, but may not enhance protein synthesis through the mTOR signaling although we did not measure the fractional synthesis rate. Dietary Leu stimulates protein synthesis by activating mTOR signaling (10, 26, 34–36). Therefore, the regulation of protein turnover by Lys may be different from that of Leu. On the other hand, it has been reported that the rates of protein synthesis increased with feeding of a gluten diet supplemented with Lys (37). Thus, Lys might increase protein synthesis when it is added to a low-Lys diet like a gluten diet. Akt is a down-stream target of

mTOR complex 2 (mTORC2), and is an upstream target of mTOR complex 1 (mTORC1) (38). mTORC1 comprises mTOR, GβL, PRAS40, raptor, and DEPTOR, and phosphorylates S6K1 and 4E-BP1 in a rapamycin-sensitive manner (39, 40). mTORC2 comprises mTOR, Ric-1, mSIN1 and mLST8, and phosphorylates Akt (38). In this study, Lys did not affect the phosphorylation levels of Akt. Therefore, Lys was not considered to activate mTORC1 or mTORC2. On the other hand, AMPK is known to regulate the autophagic-lysosomal pathway (41) and PI3K, an upstream target of Akt, involved in protein turnover through the mTOR signaling pathway (42), although we did not measure the activity of AMPK or PI3K. Furthermore, release of growth hormone and plasma IGF-1 levels were increased by Lys intake (43), which may lead to elevated protein synthesis with long-term feeding of Lys.

It is known that insulin regulates protein turnover in skeletal muscle (44, 45). However, plasma insulin concentration was unchanged after Lys administration (Fig. 3). Thus, insulin was not involved in the suppressive effects of Lys. We measured plasma concentration of Leu, but Leu concentration was not increased by Lys administration. This result suggested that the suppressive effects of Lys were not associated with plasma Leu concentration.

The expression of atrogin-1 mRNA was suppressed in QT6 fibroblast cells treated with methionine, an essential amino acid (46). Wu and Thompson (15) showed that Gln stimulates protein synthesis. Tan et al. (47) reported that dietary arginine supplementation increased muscle gain and reduced body fat mass in pigs. However, the relationship between other amino acids and protein metabolism is unknown. In this study, we found that intragastric administration of Lys suppressed myofibrillar proteolysis, at least partially through the autophagic-lysosomal pathway in rats. Lys is nutritionally important, as it is a major limiting essential amino acid in some plant proteins. Therefore, the effects of Lys on muscle protein degradation appear to increase its nutritional value.

In conclusion, our results showed that administered Lys is able to suppress myofibrillar protein degradation and the effects of Lys may be expressed through the autophagic-lysosomal pathway in rats.

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