Supplementation with Dietary Leucine to a Protein-Deficient Diet Suppresses Myofibrillar Protein Degradation in Rats

Takayuki Sugawara1, Yoshiaki Ito2, Naoyuki Nishizawa2 and Takashi Nagasawa2,*

1United Graduate School of Agricultural Sciences, Iwate University, Morioka, Iwate 020–8550, Japan
2Biological Chemistry and Food Sciences, Faculty of Agriculture, Iwate University, Morioka, Iwate 020–8550, Japan
(Received November 24, 2006)

Summary Muscle mass is regulated by the synthesis and degradation of muscle protein, which in turn are affected by aging, several catabolic diseases, and malnutrition. Amino acids, particularly leucine, are known to stimulate muscle protein synthesis and suppress muscle protein degradation, although their long-term effects are unclear. The objective of our research was to elucidate whether long-term feeding of a protein-free or low-protein diet supplemented with leucine suppresses myofibrillar protein degradation. The rate of myofibrillar protein degradation was measured by the rate of release of 3-methylhistidine (MeHIs) from isolated extensor digitorum longus (EDL) muscle. The weight of gastrocnemius muscle decreased in rats fed a protein-free diet for 7 d; however, a leucine-supplemented (1.5%) diet tended to suppress this decrease. The release of MeHIs from EDL muscle was increased by the protein-free diet and decreased by the feeding of a diet supplemented with leucine to the level of a 20% casein diet. When rats were fed a 5% casein diet, the gastrocnemius muscle weight decreased and MeHIs release from EDL muscle increased compared to those fed a 20% casein diet. However, feeding of a 5% casein diet supplemented with leucine (1.15%) reduced muscle weight loss and MeHIs release. These results suggest that long-term feeding of leucine suppresses the rate of myofibrillar protein degradation and muscle weight loss in rats fed a protein-deficient diet.

Key Words myofibrillar protein degradation, leucine, sarcopenia, muscle protein metabolism

Many catabolic diseases and states such as burns, sepsis, and cancer induce muscle wasting due to a decreased rate of protein synthesis and an accelerated rate of protein degradation in skeletal muscle. These diseases induce progressive reduction in the muscle mass and muscle strength (1, 2). Malnutrition also causes loss of muscle mass, which may be an important factor in the sarcopenia of aging. A protein-deficient diet reduces muscle mass (3) because dietary protein is an important factor regulating muscle protein turnover.

Several studies have investigated factors regulating muscle protein turnover under catabolic conditions. Hormones such as insulin, insulin-like growth factor-1, growth hormones, and glucocorticoid are known to be factors that regulate muscle protein turnover (4). Dietary components also affect muscle protein turnover. It is well known that high-quality dietary protein maintains a higher rate of muscle growth due to higher protein synthesis than low-quality protein does (5). Essential amino acids stimulate muscle protein synthesis, whereas nonessential amino acids are ineffective (6). These increases in protein synthesis are partially explained by the effects of leucine.

On the other hand, the effect of dietary protein on muscle protein degradation is not clear. We have previously shown that myofibrillar protein degradation was suppressed by refeeding of a casein diet after starvation in mice and rats, while it was not suppressed by a protein-deficient diet (7, 8). These results suggested that dietary protein regulates muscle protein degradation. In our recent observations, administration of leucine alone suppressed myofibrillar muscle protein degradation (9).

Thus, dietary protein and leucine may have beneficial effects on muscle atrophy by increasing the rate of protein synthesis and decreasing the rate of protein degradation. The aim of the present study was to elucidate the effect of long-term intake of leucine on muscle protein degradation when supplemented to a low-protein diet. Our results indicate that long-term feeding of leucine suppresses the increased rates of muscle protein degradation and muscle mass decline due to feeding a low-protein diet.

Materials And Methods

Animals and diet. Experiment 1. Wistar rats, aged 3 wk, were purchased from SLC (Shizuoka, Japan). They were individually housed in stainless steel wire cages and maintained at 22°C and 55% relative humidity.
with a 12-h light-dark cycle (6:00–18:00). They were allowed free access to water and a 20% casein diet according to AIN-93G (10) for a week. The rats were then randomly assigned to the following three dietary groups; OC (protein-free), OC+L (protein-free supplemented with 1.5% leucine, equal to the amount of leucine in 20C), and 20C (20% casein) (Table 1). The rats were fed the experimental diets ad libitum for a week. During this period, the food intake of rats fed OC+L was less than that of those fed 20C ad libitum in a preliminary experiment. Therefore, we used the pair-feeding method because the reduced amount of food intake may affect body weight, muscle weight, and protein metabolism. On the final day, the rats were anesthetized with diethyl ether, the abdomen was 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 \(-80^\circ C\) until analysis. The extensor digitorum longus (EDL) muscle and gastrocnemius muscle were removed from the leg to measure the rate of protein degradation and muscle weight. The animal care protocol in this study was approved by the Iwate University Animal Research Committee under Guidelines for Animal Experiments in Iwate University.

Experiment 2. Wistar rats, aged 4 wk, were purchased from SLC. They were allowed free access to water and a 20% casein diet according to AIN-93G for 2 d. Then the rats were randomly assigned to the following four groups; OC (protein-free), 5C (5% casein), 5C+L (5% casein supplemented with 1.15% leucine, equal to the amount of leucine in 20C), and 20C (20% casein) (Table 1). The rats were fed the experimental diets for a week. The rats fed 5C, 5C+L, and 20C consumed almost the same amount of diet as those in the OC group did. Blood and muscle samples were then collected by the same methods as described in Experiment 1.

Table 1. Composition of the experimental diets in Experiments 1 and 2.

<table>
<thead>
<tr>
<th>Composition (g/kg)</th>
<th>OC</th>
<th>OC+L</th>
<th>5C</th>
<th>5C+L</th>
<th>20C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein*</td>
<td>0</td>
<td>0</td>
<td>50</td>
<td>50</td>
<td>200</td>
</tr>
<tr>
<td>Leucine*</td>
<td>0</td>
<td>0</td>
<td>50</td>
<td>50</td>
<td>200</td>
</tr>
<tr>
<td>Cystine*</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Sucrose*</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>α-Cornstarch*</td>
<td>732.5</td>
<td>717.5</td>
<td>682.5</td>
<td>671</td>
<td>529.5</td>
</tr>
<tr>
<td>Soybean oil*</td>
<td>70</td>
<td>70</td>
<td>70</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Mineral mixture*</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin mixture*</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Choline bitartrate*</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Cellulose*</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

OC, 0% casein diet (protein-free diet); OC+L, OC supplemented with 1.5% leucine; 5C, 5% casein diet; 5C+L, 5C supplemented with 1.15% leucine; 20C, 20% casein diet. * Oriental Yeast Co., Ltd., Tokyo, Japan. † Ajinomoto Co., Inc., Tokyo, Japan. © Toyo Sugar Refining Co., Tokyo, Japan. + Wako Pure Chemical Industries, Ltd., Osaka, Japan. ^ AIN 93 composition (10).

Myofibrillar protein degradation. We incubated the isolated EDL muscle in a Krebs-Ringer bicarbonate buffer containing 10 mM glucose under 95% O₂–5% CO₂ at 37°C for 2 h after a 30-min pre-incubation at 37°C to measure the rate of myofibrillar protein degradation directly (8). The amount of 3-methylhistidine (MeHis) in the incubation buffer, which has been used as an index of myofibrillar proteolysis (8, 9), was measured by the HPLC method after derivatization by fluorescamine and treatment with perchloric acid and heating (11).

Concentration of plasma leucine. Plasma leucine concentration was measured by HPLC after derivatization of α-phthalaldehyde by our previous method with a small modification (12).

Statistical analyses. Data are expressed as means and SE. Comparisons between the OC groups and other groups were carried out using the two-tailed unpaired t-test. In Experiment 2, comparisons of body weight and muscle weight between the 20C groups and other groups were also carried out using the two-tailed unpaired t-test. Differences were considered significant at \( p<0.05 \).

Results

Experiment 1

The final body weights of rats fed OC and OC+L did not differ, but the rats fed 20C were significantly heavier than those fed OC (Table 2). Food intake in all three groups was 32.2 g. The weight of the gastrocnemius muscle tended to increase with feeding of OC+L \((p=0.16)\) and significantly increased with feeding of 20C compared to OC. Although statistical significance was not achieved, there was a trend toward increasing weight of the gastrocnemius muscle per body weight in rats fed OC+L compared with those fed OC \((p=0.13)\) (Table 2).

The rates of MeHis release from the isolated EDL muscle in rats fed OC+L and 20C were significantly suppressed by 25% and 20%, respectively, compared with that in rats fed OC (Fig. 1). These results suggest that feeding OC+L or 20C suppressed the increased degradation of myofibrillar protein caused by a protein-free diet.

The plasma leucine concentrations of rats fed OC+L (115.3±10.3 nmol/mL) and 20C (111.0±6.0 nmol/...
ml) were slightly higher than that of rats fed 0C (86.2±2.4 nmol/mL).

**Experiment 2**

The rats fed 5C or 5C+L were significantly heavier than those fed 0C, and those fed 20C were significantly heavier than those in the other three groups. The total food intake of rats fed 0C was 48.0 g and of those fed 5C, 5C+L, and 20C was 50.0 g. The weight of the gastrocnemius muscle of rats fed 5C+L or 20C was greater than that of those fed 0C (Table 3).

The rates of MeHis release from the isolated EDL muscle in rats fed 5C+L and 20C were significantly suppressed by 13% and 25% respectively, compared with those in rats fed 0C, although the rate of MeHis release in rats fed 5C was the same as that in rats fed 0C (Fig. 2). These results suggest that feeding of a low-protein diet as well as a protein-free diet induces myofibrillar protein degradation, and leucine supplementation could minimize the effect of a low-protein diet on myofibrillar protein degradation.

The plasma leucine concentration was not changed when rats were fed 5C (118.4±3.4 nmol/mL) and 5C+L (118.7±4.7 nmol/mL) compared with 0C (111.1±3.7 nmol/mL), although rats fed 20C (134.6±11.6 nmol/mL) showed higher plasma leucine concentrations than those fed 0C (p=0.09).

**Table 3.** Body weight (BW) and gastrocnemius muscle weight of rats fed 0C, 5C, 5C+L, and 20C.

<table>
<thead>
<tr>
<th></th>
<th>Initial BW (g)</th>
<th>Final BW (g)</th>
<th>Gastrocnemius muscle (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0C</td>
<td>76.0±2.3</td>
<td>61.8±1.5*</td>
<td>0.55±0.02†</td>
</tr>
<tr>
<td>5C</td>
<td>75.5±1.9</td>
<td>66.9±1.5*†</td>
<td>(8.88±0.19)</td>
</tr>
<tr>
<td>5C+L</td>
<td>75.6±1.8</td>
<td>67.4±1.2*†</td>
<td>(8.83±0.17)†</td>
</tr>
<tr>
<td>20C</td>
<td>75.8±2.0</td>
<td>81.0±1.1*†</td>
<td>(9.15±0.10)</td>
</tr>
</tbody>
</table>

Value is the mean±SE (n=5). *p<0.05 vs. 0C, †p<0.05 vs. 20C.

**Discussion**

This study clearly demonstrated that the increased rate of myofibrillar protein degradation due to feeding a low-protein diet was suppressed to the level observed in rats fed 20C by feeding of supplemental leucine for 7 d. It is noteworthy that leucine supplementation tends to alleviate the decrease in muscle mass caused by feeding a protein-deficient diet. We have shown that oral administration of leucine alone to rats after 18 h starvation suppresses myofibrillar protein degradation (9). In our previous study (9), the rats were administered leucine at once with an equal amount to a daily intake (135 mg leucine per 100 g body weight). However, this level might be still unphysiologically, because it was taken as a bolus and made the plasma concentration of leucine about 3,000 nmol/mL (13). The effect of long-term feeding of leucine on myofibrillar protein degradation has not been established. Feeding of a protein-deficient diet induces muscle mass reduction (3). Therefore, we observed in this study the long-term effect of ad libitum feeding of leucine as a supplement, leading to a moderate increase in plasma concentration, and we showed that the long-term feeding of leucine had an anabolic effect on skeletal muscle.

Leucine is known to stimulate muscle protein synthesis, through activation of translation (13). Yoshizawa et al. (13) and Anthony et al. (14) showed that oral administration of leucine activates muscle protein synthesis. Rieu et al. (15) showed that feeding of a diet supplemented with leucine for 10 d stimulated muscle protein synthesis in old (21 mo) rats. In the present study, we did not measure the rate of muscle protein synthesis; however, it is possible that translation of protein synthesis was also stimulated by long-term feeding of leucine.

Several studies indicate that leucine suppresses tissue protein degradation. Combaret et al. (16) showed that dietary leucine suppresses muscle protein degradation by inhibition of ubiquitin-proteasome dependent proteolysis in rats. Mordier et al. (17) showed that leucine limitation induces formation of autophagy and activation of lysosomal-dependent proteolysis in C2C12 myotube through an mTOR-independent signaling path-
way. Thus leucine administration may regulate the ubiquitin-proteasome system or autophagy-lysosome system. In the present study, the chymotrypsin-like activity of the proteasome was not changed by feeding of leucine (data not shown). The involvement of leucine in the proteolytic system should be further investigated.

We suggested in previous reports (9, 18) that the plasma leucine concentration may be a key factor for inhibition of myofibrillar protein degradation. In this study, however, plasma leucine concentrations of rats fed OC+L and 5C+L were about 100 nmol/mL, which were slightly higher than the post-absorption level. Yoshizawa et al. (13) showed that administration of leucine (135 mg/100 g body weight) elevated the plasma concentration of leucine within 1 h after administration; the leucine concentration then decreased to the post-absorption level. The rapid disappearance of leucine from plasma implies that leucine is rapidly oxidized (19). Therefore, the plasma leucine concentration was thought to be elevated just after feeding of a leucine-supplemented diet, which was a signal of inhibition of myofibrillar protein degradation.

In conclusion, we suggest that long-term feeding of a diet supplemented with leucine could suppress myofibrillar protein degradation, which would alleviate loss of muscle mass by protein malnutrition. Based on the results of this study, we propose that feeding of leucine may have a beneficial effect in sarcopenia in humans who cannot eat sufficient dietary protein.

Acknowledgments
This work was supported in part by the 21st century COE in Iwate University program from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and Ajinomoto Co. Ltd.

REFERENCES