Dietary L-Lysine Suppresses Autophagic Proteolysis and Stimulates Akt/mTOR Signaling in the Skeletal Muscle of Rats Fed a Low-Protein Diet

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ABSTRACT: Amino acids, especially l-leucine, regulate protein turnover in skeletal muscle and have attracted attention as a means of increasing muscle mass in people suffering from malnutrition, aging (sarcopenia), or a bedridden state. We previously showed that oral administration of L-lysine (Lys) by gavage suppressed proteolysis in skeletal muscles of fasted rats. However, the intake of Lys in the absence of other dietary components is unlikely in a non-experimental setting, and other dietary components may interfere with the suppressive effect of Lys on proteolysis. We supplemented Lys to a 10% casein diet and investigated the effect of Lys on proteolysis and autophagy, a major proteolytic system, in the skeletal muscle of rats. The rate of proteolysis was evaluated from 3-methylhistidine (MeHis) released from isolated muscles, in plasma, and excreted in urine. Supplementing lysine with the 10% casein diet decreased the rate of proteolysis induced by intake of a low-protein diet. The upregulated autophagy activity [light chain 3 (LC3)-II/total LC3] caused by a low-protein diet was reduced, and the Akt/mTOR signaling pathway was activated by Lys. Importantly, continuous feeding of a Lys-rich 10% casein diet for 15 days increased the masses of the soleus and gastrocnemius muscles. Taken together, supplementation of Lys to a low-protein diet suppresses autophagic proteolysis through the Akt/mTOR signaling pathway, and continuous feeding of a Lys-rich diet may increase skeletal muscle mass.

KEYWORDS: L-lysine, sarcopenia, autophagy, Akt, mTOR, low-protein diet

INTRODUCTION

An energetic lifestyle requires adequate maintenance of muscle mass. Increased muscle mass is an effective strategy for preventing lifestyle-related diseases and metabolic syndrome as the skeletal muscle is the largest tissue in the human body that can regulate carbohydrate and lipid metabolism. Muscle mass is reduced by excess proteolysis in catabolic conditions caused by a low-protein diet, disuse of muscle, kidney disease, or aging (sarcopenia), and each requires an appropriate strategy for prevention of muscle atrophy.

L-Leucine (Leu), a branched chain amino acid, stimulates protein synthesis during the process of translation by targeting the mammalian target of rapamycin (mTOR) signaling and suppressing proteolysis in the skeletal muscle. Thus, Leu has attracted attention in recent years as a useful dietary means of increasing muscle mass in people suffering from malnutrition, aging (sarcopenia), or a bedridden state. We previously showed that oral administration of the essential amino acid l-lysine (Lys) by gavage (114 mg of Lys/100 g of body weight) suppressed myofibrillar proteolysis in fasted rats. Furthermore, Lys suppressed proteolysis by a major proteolytic system, autophagy, through protein kinase B (Akt), which is the upstream regulator of mTOR in the C2C12 myotube skeletal muscle model cell. On the other hand, the effects of Lys combined with other dietary components have not been investigated, even though Lys intake will always be in combination with other dietary components in daily life. Moreover, it is important to determine if the continuous intake of a Lys-rich diet can increase skeletal muscle mass by suppressing proteolysis in muscle.

We investigated the effect of a Lys-rich, 10% casein diet on the proteolysis of skeletal muscle in rats and confirmed that dietary Lys supplementation affects the activity of the Akt/mTOR signaling pathway, which regulates proteolysis. Furthermore, we investigated whether the continuous feeding of a Lys-rich diet can increase the mass of skeletal muscle.

MATERIALS AND METHODS

Materials. LC3B antibody, phospho-Akt (Thr308) antibody, and phospho-p70 S6 kinase (Thr389) mouse mAb were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Anti-p70 S6K1 antibody and anti-ubiquitin rabbit polyclonal antibody were obtained from Stressgen (Victoria, British Columbia, Canada). 4E-BP1 (R-113) antibody, Akt1 (B-1) antibody, and p-Akt 1/2/3 (Ser 473) antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). β-Actin antibody (AC-15) was obtained from Novus Biologicals (Littleton, CO). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) and HRP-conjugated goat anti-mouse IgG were obtained from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). Other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animals. Male Wistar rats (30–50 g) were purchased from Japan SLC, Inc. (Shizuoka, Japan). Rats were individually housed in stainless-steel wire cages and maintained at 22 °C and 55% relative humidity with a 12 h light–dark cycle (light period, 6:00–18:00; dark period, 18:00–6:00). Animal care protocols in this study were approved by

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the Iwate University Animal Research Committee and conformed to the Guidelines for Animal Experiments of Iwate University.

**Experimental Design and Experimental Diets. Experiment 1.** A total of 20 rats were allowed free access to tap water and were provided with a 20% (w/w) casein diet based on AIN-93G for 12 days. On day 13, the rats were fasted for 18 h and then fed a control diet (Table 1; 20C, 20% casein diet; 10C, 10% casein diet) or the control diet (Table 1; 20CK, Lys-rich 20% casein diet; 10CK, Lys-rich 10% casein diet) for 1 h (9:00–10:00). After 3 h of fasting (10:00–13:00), each rat was anesthetized with pentobarbital, then its abdomen was opened, and blood was collected from the inferior vena cava. Each rat was euthanized after the collection of blood. The blood was centrifuged at 3500 rpm for 15 min to obtain plasma, which was frozen in liquid nitrogen and stored at −80°C until analysis. The soleus muscle, extensor digitorum longus (EDL) muscle, and gastrocnemius muscle were removed from the hind leg and weighed.

**Table 1. Compositions of the Control and Experimental Diets**

<table>
<thead>
<tr>
<th></th>
<th>control and experimental diets</th>
<th>20C</th>
<th>20CK</th>
<th>10C</th>
<th>10CK</th>
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<tbody>
<tr>
<td>milk casein&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Ingredient (g/kg of diet)</td>
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<td>100</td>
<td>100</td>
</tr>
<tr>
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<td>0</td>
<td>6.5</td>
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</tr>
<tr>
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<td>3.0</td>
<td>3.0</td>
<td></td>
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<tr>
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<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>a-cornstarch&lt;sup&gt;e&lt;/sup&gt;</td>
<td>529.5</td>
<td>523.0</td>
<td>629.5</td>
<td>623.0</td>
<td></td>
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<td>70</td>
<td>70</td>
<td>70</td>
<td></td>
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<tr>
<td>mineral mixture&lt;sup&gt;f&lt;/sup&gt;</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>vitamin mixture&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>10</td>
<td>10</td>
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<td></td>
</tr>
<tr>
<td>choline bitartrate&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>2.5</td>
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<td>2.5</td>
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<tr>
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<td>50</td>
<td>50</td>
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<td>20</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>total Lys&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Composition (%)</td>
<td>1.30</td>
<td>1.95</td>
<td>0.95</td>
<td>1.30</td>
</tr>
</tbody>
</table>

<sup>a</sup>Obtained from the Oriental Yeast Co., Tokyo, Japan. <sup>b</sup>Obtained from the Ajinomoto Co., Tokyo, Japan. <sup>c</sup>Obtained from the Toyo Sugar Refining Co., Tokyo, Japan. <sup>d</sup>Total Lys was calculated from the sum of Lys contained in casein<sup>11</sup> and the amounts of supplemented Lys with diet.

**Measurement of Myofibrillar Proteolysis.** 3-Methylhistidine (MeHis) released from the isolated muscles was measured as described previously<sup>8</sup> by incubating the isolated EDL muscle or soleus muscle in Krebs-Ringer bicarbonate buffer containing 10 mM glucose under 95% O<sub>2</sub>/5% CO<sub>2</sub> at 37°C for 2 h after a 30 min pre-incubation at 37°C. The amount of MeHis in the incubation buffer solution was measured using a high-performance liquid chromatography (HPLC) method.<sup>12</sup>

The plasma concentration of MeHis provides an index of myofibrillar proteolysis<sup>13</sup> and was measured by HPLC after derivatization with o-phthalaldehyde using the method of Nagasawa et al.<sup>14</sup> with a slight modification.

The amount of MeHis in urine, commonly used as an index of myofibrillar proteolysis,<sup>15</sup> was measured by HPLC as described by Nagasawa et al.<sup>15,16</sup> It has been proposed that skeletal muscle accounts for 40% of the entire body mass of rats and that 700 nmol of MeHis is present in 1 g of skeletal muscle.<sup>15</sup> Therefore, we expressed our results as a fractional degradation rate (K<sub>d</sub>) calculated using the following formula:

\[ K_d(\%) = 100E -(0.4W + W_0)/700/2 \]

where E (nmol) is the amount of MeHis excretion over 24 h, W<sub>0</sub> (g) is the whole body weight when urine collection started, and W (g) is the whole body weight when urine collection finished.

**Measurement of Plasma Amino Acid Concentrations.** Plasma was mixed with an equal volume of 3% sulfosalicylic acid during overnight at 4°C, and then the mixture was centrifuged at 18000g for 15 min. Plasma amino acid concentrations in the supernatant were measured using an amino acid autoanalyzer (JLC-500/V, JEOI, Tokyo, Japan).

**Measurement of the Plasma Insulin Concentration.** The plasma insulin concentration was measured using an enzyme-linked immunosorbent assay (ELISA) according to the protocol (Moringana Institute of Biological Science, Yokohama, Japan).

**Western Blot Analysis.** Western blot analysis was performed as reported previously.<sup>17</sup> Gastrocnemius muscle was homogenized in 10 volumes of buffer solution followed by centrifugation at 3800g. The supernatant was used as the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample and was separated on a 10% polyacrylamide gel, followed by transfer to a polyvinylidene fluoride (PVDF) membrane (Millipore Corporation, Billerica, MA). 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, and then the membrane was incubated with HRP-conjugated goat anti-rabbit IgG or HRP-conjugated goat anti-mouse IgG in TBS-T. After detection, the membrane was stained with Coomassie Brilliant Blue (CBB) to verify that equal quantities of each protein had been transferred.

**Gene Expression of E3 Ubiquitin Ligases.** mRNA expression of E3 ubiquitin ligases was assessed by northern blotting as described previously.<sup>18</sup> A total of 10 µg of total RNA extracted from the gastrocnemius muscle were separated on a 1.2% agarose-formaldehyde gel and transferred to a positively charged nylon membrane (Pall Corporation, Port Washington, NY). After ultraviolet (UV) cross-linking, the membranes were hybridized with a digoxigenin-labeled cDNA probe specific to MuRF1, atrogin-1, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for 16 h at 50°C in hybridization solution. Specific hybridization was detected with an anti-digoxigenin antibody conjugated with alkaline phosphatase, and blots were developed with CDP-star reagent (Tropix, Bedford, MA).

**Statistical Analysis.** Data are expressed as the mean with standard error of the mean (SEM). Data analysis was performed using GraphPad InStat software, version 3.0a (2001, GraphPad Software, Inc., San Diego, CA). Data were analyzed by analysis of variance (ANOVA) and Tukey’s post-test in multi-group (more than three groups) comparisons to determine whether there were significant differences (p < 0.05) among the groups. Comparisons of two groups were performed using unpaired t tests, and the two-tailed p value was calculated.

**RESULTS**

**Experiment 1. Lys-Rich 10% Casein Diet Suppressed the Rate of Proteolysis and Autophagy Activity.** The average food intake for 1 h after 18 h of fasting on the final day did not differ between the groups [20C, 4.4 ± 0.6 g (average ± SE); 20CK, 4.5 ± 0.1 g; 10C, 4.4 ± 0.1 g; and 10CK, 4.3 ± 0.2 g]. The rates of MeHis released from isolated muscles (EDL and soleus muscles) and the plasma MeHis concentrations significantly increased in rats fed the standard 10C diet compared to rats fed the 20C diet (panels A and B of Figure 1). However, the values of MeHis in rats fed the 10CK diet were lower than those in the 10C group (panels A and B of Figure 1), whereas...
Supplementation of Lys to the 20% casein diet did not affect the rate of proteolysis (panels A and B of Figure 1). We evaluated autophagy activity from the light chain 3 (LC3)-II/total LC3 ratio,\textsuperscript{17} and ubiquitin–proteasomal system activity was assessed from the levels of ubiquitinated proteins and the mRNA expression of the E3 ubiquitin ligases, muscle RING-finger protein 1 (MuRF1) and atrogin-1. Consistent with the rate of proteolysis (panels A and B of Figure 1), autophagy activity was suppressed by 45% in rats fed the 10CK diet compared to those fed the standard 10C diet (Figure 1C). The levels of ubiquitinated proteins significantly increased in rats fed the low-protein diets (Figure 1D), despite the same amount of protein being transferred to the membrane for all groups (evaluated by CBB staining; data not shown). Lys supplementation did not suppress ubiquitin–proteasomal activity (panels D and E of Figure 1). These results indicated that supplementing Lys with the 10% casein diet suppresses autophagic proteolysis.

**Lys-Rich 10% Casein Diet Stimulated Akt Phosphorylation and Recovered mTOR Signaling Activity.** The phosphorylation level of Akt Thr308 increased by 32% in rats fed the 10CK diet compared to rats fed the 10C diet (Figure 2B). A similar increase was observed for Ser473 phosphorylation in Akt (Figure 2B). The mTOR signaling activity was evaluated from the phosphorylation levels of eIF4E-binding protein 1 (4E-BP1) and p70 ribosomal protein S6 kinase 1 (S6K1), which are downstream targets of mTOR. mTOR signaling activity (both of 4E-BP1 and S6K1) in rats fed the 10C diet decreased by 30% compared to rats fed the 20C diet, but supplementation of the 10C diet with Lys restored mTOR signaling activity (panels C and D of Figure 2). This activation effect of Lys on Akt/mTOR signaling was not observed in rats fed the 20CK diet (panels B–D of Figure 2). The phosphorylation level of eukaryotic initiation factor 2 α (eIF2α), which increases in amino-acid-limited conditions and regulates translation and autophagy,\textsuperscript{18,19} did not differ among the groups (Figure 2E). These results indicated that supplementing Lys with 10% casein suppressed autophagy through Akt/mTOR signaling activation. On the other hand, the phosphorylation level of eIF2α tended to decrease in 20CK (Figure 2E). Therefore, supplementing Lys with a high-casein diet may stimulate protein synthesis through a mTOR-independent pathway.

**Lys-Rich 10% Casein Diet Did Not Affect the Plasma Concentrations of Insulin and Basic Amino Acids Other than Lys.** The plasma Lys concentration was significantly higher in rats fed Lys-rich diets (10CK and 20CK) compared to those fed the standard diets (10C and 20C) (Figure 3A). However, the plasma concentrations of two basic amino acids, histidine (His) and arginine (Arg), were unaffected by Lys supplementation of the diet (panels B and C of Figure 3) nor was the plasma insulin concentration (Figure 3D). The plasma concentration of amino acids other than Lys did not differ between the standard diet group and the Lys-rich diet group (20C versus 20CK and 10C versus 10CK), whereas the plasma threonine, asparagine, valine, isoleucine, leucine, phenylalanine, and tryptophan concentrations in rats fed the 10C diet were significantly lower than those fed the 20C diet.

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Figure 1. Bolus intake of a Lys-rich 10% casein diet suppresses proteolysis and autophagy. Rats were fed a standard 20% casein diet (20C), Lys-rich 20% casein diet (20CK), standard 10% casein diet (10C), or Lys-rich 10% casein diet (10CK) for 1 h and then fasted for 3 h. (A) Rate of myofibrillar proteolysis was evaluated from the amount of MeHis released from isolated EDL muscle (black bars) or soleus muscle (white bars). (B) Plasma concentration of MeHis was also used as an index of proteolysis. (C) Autophagy activity was evaluated from LC3-II/total LC3 by western blotting. Activity of the ubiquitin–proteasomal system was evaluated from (D) levels of ubiquitinated proteins determined by western blotting and (E) mRNA levels of the E3 ubiquitin ligases, MuRF1 (black bars) and atrogin-1 (white bars), determined by northern blotting. Values are means with SE (n = 5). Different letters indicate significant differences among the groups (p < 0.05).
Suppression of Lys on autophagy did not depend on the increase of insulin or other amino acids than Lys.


We investigated whether the continuous intake of a Lys-rich diet results in increased skeletal muscle mass in experiment 2, given that bolus intake of a Lys-rich diet suppressed proteolysis (panels A–C of Figure 1) and restored Akt/mTOR signaling activity (panels A–D of Figure 2) in experiment 1. The average food intake per day by rats fed the 10C diet was 9.6 ± 0.6 g (SE) (62 mg of total Lys/day), and those fed the 10CK diet was 9.9 ± 0.6 g (SE) (129 mg of total Lys/day). As expected, 15 days of Lys-rich diet intake significantly increased the masses of the soleus and gastrocnemius muscles, whereas the final body weight and mass of the EDL muscle did not increase (panels A–C of Figure 4). The weights of the soleus and gastrocnemius muscles adjusted for body weight in rats fed the Lys-rich diets also tended to be higher than those of rats fed the corresponding control diet (p = 0.07 and 0.09, respectively). The rate of proteolysis was calculated from the amount of MeHis excreted in the urine and was suppressed by 33% in rats fed Lys-rich diets (Figure 4D). Similarly, autophagy was reduced by 30% in rats fed Lys-rich diets (Figure 4E). These results indicated that continuous intake of the Lys-rich 10% casein diet increased skeletal muscle mass through inhibition of autophagy.

Discussion

A low-protein diet causes loss of skeletal muscle, and Leu has attracted attention as a dietary supplement, which preserves muscle mass in mammals fed a low-protein diet. Yin et al. showed that supplementation of Leu to a low-protein diet (10% whey protein-based diet, 1.88% total Leu) improved protein synthesis in pigs. Similar results have been reported in rats and humans. In addition, it was reported that intake of a 10% whey diet supplemented with Leu (6% total Leu) resulted in increased skeletal muscle mass, although supplementation of Leu to a 20% whey protein diet did not result in increased muscle mass in mice. To our knowledge, there has been no previous study investigating supplementation of a single amino acid other than Leu that diminishes muscle mass loss caused by a low-protein diet.

We previously showed that oral administration of Lys by gavage (114 mg/100 g of body weight) markedly suppressed the rate of proteolysis in fasted rats, and therefore, we
anticipated that Lys may be a useful dietary supplement for decreasing muscle mass loss. In this study, we supplemented Lys with a low-protein diet and investigated the suppressive effect of dietary Lys on proteolysis, autophagy, and the loss of muscle mass induced by intake of a low-protein diet in rats.

The effect of dietary Lys on proteolysis was evaluated from the rate of proteolysis by measuring MeHis released from isolated muscles (EDL and soleus muscles), the plasma concentration of MeHis, and the amount of MeHis excreted in the urine. In addition, the activity of autophagy, a major proteolytic system, was evaluated from LC3-II/total LC3 (because LC3-II localizes at the autophagosome membrane). First, we investigated the suppressive effect of a bolus intake of a Lys-rich diet on proteolysis and on proteolytic systems (autophagy and the ubiquitin–proteasomal system) after fasting. Supplementation of Lys with the 10% casein diet clearly suppressed the rate of proteolysis and autophagy activity stimulated by the intake of a low-protein diet (Figure 1), whereas the activity of the ubiquitin–proteasomal system was not suppressed by a Lys-rich diet (panels D and E of Figure 1). Therefore, the present results indicate that supplementing Lys with the 10% casein diet would suppress autophagy but not the ubiquitin–proteasomal system, consistent with our previous studies.

We continuously provided a Lys-rich low-protein diet to rats for 15 days and investigated the effect of Lys supplementation into the diet on muscle mass. The continuous feeding of a Lys-rich diet significantly increased the masses of the soleus and gastrocnemius muscles (panels B and C of Figure 4); furthermore, the rate of proteolysis, evaluated from MeHis excreted in the urine, was significantly suppressed by intake of a Lys-rich diet (Figure 4D), as was autophagy (Figure 4E). These results indicate that continuous feeding of a Lys-rich low-protein diet can preserve the mass of skeletal muscle.

Akt is an important regulator of autophagy. Zhao et al. showed that Akt and not mTOR is the central regulator of autophagic proteolysis in skeletal muscle cells. It has been reported that Leu stimulates Akt and that Leu supplementation increased muscle mass and Akt/mTOR signaling activity in rats suffering from malnutrition. We previously demonstrated that Lys suppresses autophagy and activates mTOR signaling, which regulates autophagy through Akt in C2C12 myotubes. In this study, we observed significant phosphorylation of Akt Thr308, which regulates Akt activity, in rats fed a Lys-rich diet (Figure 2B). In addition, the activity of mTOR signaling was recovered in rats fed a Lys-rich 10% casein diet, although the activity of mTOR signaling activity decreased in rats fed a standard 10% casein diet (panels C and D of Figure 2). Therefore, a Lys-supplemented diet likely regulates autophagy through the Akt/mTOR signaling pathway in rats fed a low-protein diet.

Akt is phosphorylated through the insulin signaling pathway, and Lys activates the secretion of insulin. However, the plasma insulin concentrations of rats fed a Lys-rich diet did not increase (Figure 3D). We previously showed that Lys markedly phosphorylated Akt in the absence of insulin. Hence, Akt would be stimulated by an insulin-independent pathway in rats fed a Lys-rich diet.

We previously showed that oral administration of glycine, which is the same nitrogen level of Lys, did not suppress the rate of protein degradation in fasted rats. In addition, we
protein diet. Tesseraud et al. indicated that a Lys-depleted protein diet suppresses muscle mass loss caused by the intake of a low-protein diet. Moreover, the present results may help overcome the increase of muscle in rats fed a Lys-rich low-protein diet. Supplementing Lys with a low-protein diet might be a beneficial strategy for preventing skeletal muscle loss in chronic kidney disease patients advised to adhere to a low-protein diet. Moreover, the present results may help overcome the increase of muscle in rats fed a Lys-rich low-protein diet.

The authors declare no competing financial interest.

ABBREVIATIONS USED
Akt, protein kinase B; Arg, arginine; CBB, Coomassie Brilliant Blue; eIF2α, eukaryotic initiation factor 2 α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; His, histidine; LC3, light chain 3; Leu, leucine; Lys, lysine; MeHis, 3-methylhistidine; MuRF1, muscle RING-finger protein 1; mTOR, mammalian target of rapamycin; S6K1, p70 ribosomal protein S6 kinase 1; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1

REFERENCES


Figure 4. Continuous feeding of a Lys-rich 10% casein diet results in increased muscle mass and suppresses proteolysis and autophagy. Rats were fed a standard 10% casein diet or a Lys-rich 10% casein diet for 15 days. Urine was collected for 24 h, from the morning of the 13th day to the morning of the 14th day. (A) Initial body weight was measured at the start of feeding the control (black bars) or experimental (white bars) diets, and final body weight was measured prior to dissection. The weights of the (B) soleus and (C) gastrocnemius muscles were measured following dissection. (D) Amount of MeHis excreted in urine was measured by HPLC. (E) Autophagy activity evaluated from LC3-II/total LC3 was analyzed by western blotting. Values are means with SE (n = 5). Different letters indicate significant differences among the groups (p < 0.05).


