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Lysine suppresses protein degradation through autophagic–lysosomal system in C2C12 myotubes

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Abstract Muscle mass is determined between protein synthesis and protein degradation. Reduction of muscle mass leads to bedridden condition and attenuation of resistance to diseases. Moreover, bedridden condition leads to additional muscle loss due to disuse muscle atrophy. In our previous study (Sato et al. 2013), we showed that administered lysine (Lys), one of essential amino acid, suppressed protein degradation in skeletal muscle. In this study, we investigated that the mechanism of the suppressive effects of Lys on skeletal muscle proteolysis in C2C12 cell line. C2C12 myotubes were incubated in the serum-free medium containing 10 mM Lys or 20 mM Lys, and myofibrillar protein degradation was determined by the rates of 3-methylhistidine (MeHis) release from the cells. The mammalian target of rapamycin (mTOR) activity from the phosphorylation levels of p70-ribosormal protein S6 kinase 1 and eIF4E-binding protein 1 and the autophagic–lysosomal system activity from the ratio of LC3-II/I in C2C12 myotubes stimulated by 10 mM Lys for 0–3 h were measured. The rates of MeHis release were markedly reduced by addition of Lys. The autophagic–lysosomal system activity was inhibited upon 30 min of Lys supplementation. The activity of mTOR was significantly increased upon 30 min of Lys supplementation. The suppressive effect of Lys on the proteolysis by the autophagic–lysosomal system was maintained partially when mTOR activity was inhibited by 100 nM rapamycin, suggesting that some regulator other than mTOR signaling, for example, Akt, might also suppress the autophagic–lysosomal system. From these results, we suggested that Lys suppressed the activity of the autophagic–lysosomal system in part through activation of mTOR and reduced myofibrillar protein degradation in C2C12 myotubes.

Keywords Lysine · Protein degradation · Autophagy · mTOR · C2C12 myotubes

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Introduction

A decreased rate of protein synthesis and an accelerated rate of protein degradation in skeletal muscle lead to muscle wasting [1]. In many catabolic states such as sepsis and cancer, and unloading, muscle wasting is induced, and these states accelerate a progressive reduction of skeletal muscle mass and muscle strength [2–4]. Furthermore, energy and/or protein deficiency in the food intake induce loss of muscle mass, which may cause accelerating catabolic diseases [5, 6]. Progression of skeletal muscle atrophy causes decreased physical activities which lead to bedridden state. Therefore, prevention of muscle atrophy is a really important in improvement of the quality of life.

Many studies have shown the factors regulating skeletal muscle protein turnover in catabolic conditions. Appropriate exercise regulates skeletal muscle protein turnover [7], and insulin-like growth factor-1 and insulin enhance protein synthesis, and suppress proteolysis in skeletal muscle [8]. Dietary components, particularly essential

amino acids (AA), are reported to regulate muscle protein turnover [9, 10]. Leucine (Leu), one of essential amino acid, is known to stimulate protein synthesis through activation of mammalian target of rapamycin (mTOR) [11, 12] and to suppress protein degradation [6, 13]. We demonstrated that myofibrillar protein degradation decreased in rats fed protein-free diet supplemented 1.5 % (w/w) Leu through inhibition of the autophagic–lysosomal system, one of the major proteolytic systems [14].

Other AA than Leu are also reported to regulate protein synthesis or protein degradation. For example, oral administration of arginine (Arg) suppresses muscle atrophy induced by hindlimb unloading [15]. The expression of atrogin-1 mRNA, one of an ubiquitin ligase E3, was suppressed in QT6 fibroblast cells treated with methionine [16]. Glutamine (Gln) enhances protein synthesis [17] and prevents protein hypercatabolism by inhibition of myostatin hyperexpression [18].

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 [19, 20]. 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 unclear. We showed that oral administration of Lys to rats suppressed myofibrillar protein degradation through suppression of the autophagic–lysosomal system, not the ubiquitin–proteasomal system [21]. However, the mechanism of the suppressive effects of Lys on the autophagic–lysosomal system is still unclear.

In this study, we evaluate the effects of supplemented Lys to C2C12 murine muscle cells on suppression of myofibrillar protein degradation determined by release of 3-methylhistidine (MeHis) from cells. Furthermore, we analyzed the changes in proteolytic systems particularly the autophagic–lysosomal system and its relating signaling molecules by supplemented Lys.

Methods

Materials

Fetal bovine serum (FBS) and horse serum (HS) were purchased from BioWest (Nuaillé, France) and Invitrogen (Carlsbad, CA, USA), respectively. Dulbecco's modified eagle's medium (DMEM, low glucose) and minimum essential medium (MEM) Vitamin mix were obtained from Sigma (St. Louis, MO, USA). HEPES was obtained from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). MG132 was obtained from peptide institute, Inc. (Osaka, Japan). LC3B antibody, Beclin-1 antibody, and phospho-p70 S6 kinase (Thr 389) (1A5) mouse mAb were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Rabbit anti-p70 S6K polyclonal antibody was 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, USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma. Rapamycin was obtained from Calbiochem (Darmstadt, Germany). Other chemicals were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Cell culture

C2C12 myoblasts (5.0×10^4 cells/cm^2) were seeded to DMEM-containing antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), 10 % (v/v) FBS and 44 mM sodium bicarbonate, and then cultured for 2 days. After that, fusion and differentiation of myoblasts into myotubes were induced by replacing the medium containing 10 % FBS with the medium containing 2 % (v/v) HS. Fusion and differentiation were kept for 6 days, and all medium were changed every day during differentiation period.

Measurement of myofibrillar protein degradation

MeHis is mainly located in myosin and actin in myofibrillar protein and is not reuse for protein synthesis [22]; therefore, MeHis release from muscle directly shows myofibrillar protein degradation. To measure the rates of MeHis release from C2C12, we prepared serum and AA-deprived medium [0.001 g/L ferric nitrate·$9H_2O$, 0.4 g/L KCl, 6.4 g/L NaCl, 0.142 g/L $NaH_2PO_4{\cdot}2H_2O$, 1.0 g/L glucose, 0.265 g/L $CaCl_2{\cdot}2H_2O$, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 0.1 % (w/v) bovine serum albumin (BSA), 1 % (v/v) MEM Vitamin mix, 20 mM HEPES and antibiotics], because serum and AA strongly interfere with the measurement of MeHis. The myotube cells were rinsed twice with phosphate buffer saline (PBS) after 6 days of differentiation. Then, the cells were cultured in serum and AA-deprived medium (C), serum and AA-deprived medium containing 20 mM Leu (L), AA-deprived medium containing 10 mM Lys (K), AA-deprived medium containing 20 mM Lys (HK), or AA-deprived medium containing 20 mM Glycine (Gly, G) for 0–4 h to measure the rate of myofibrillar protein degradation. To test whether the rates of MeHis release from C2C12 were appropriate marker for measurement of myofibrilar protein degradation, MG132 (10 μM, which was dissolved in DMSO), an inhibitor of proteasome and calpain [23], was added to the medium and measured MeHis release from C2C12 myotubes during 4 h incubation. The amounts of MeHis in the medium were measured by the HPLC method after derivatization by *o*-phthalaldehyde [5].

Western blot analysis

All medium were replaced with DMEM containing 0.1 % (w/v) BSA and incubated for 6 h at 6 days of differentiation. Then, the medium was replaced with new DMEM containing 0.1 % (w/v) BSA, DMEM containing 0.1 % (w/v) BSA, and 10 mM Leu or DMEM containing 0.1 % (w/v) BSA and 10 mM Lys. Myotubes cultured in DMEM containing 0.1 % (w/v) BSA were immediately treated with lysis buffer solution (1 % (v/v) Triton-X, 5 % (w/v) deoxycholic acid, 0.1 % (v/w) SDS, 20 mM Tris-HCl (pH 7.4), 150 mM sodium chloride, 0.5 mM sodium orthovanadate (V), and 5 mM EDTA) and collected (C). Myotubes cultured in DMEM containing 0.1 % (w/v) BSA and 10 mM Leu were kept culturing for 30 min and treated with lysis buffer solution and collected (L). Myotubes cultured in DMEM containing 0.1 % (w/v) BSA and 10 mM Lys were kept culturing for 30 min (0.5 K), 1 h (1 K), 2 h (2 K), or 3 h (3 K), and treated with lysis buffer solution and then collected. Collected myotubes were centrifuged at 4 °C, 17,900×*g* for 10 min, and the supernatant was used as the SDS-PAGE sample. The equal protein amount of the sample was separated on 10 % SDS-PAGE 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. After incubation with primary antibody, membrane was incubated with HRP-conjugated goat anti-rabbit IgG or HRP-conjugated goat anti-mouse IgG in TBS-T. Detection of secondary antibody was performed using an ECL western blot detection kit (Amersham Bioscience). The bands were scanned using luminescent image analyzer (ImageQuant LAS 4000, GE healthcare, Tokyo, Japan), and the relative intensity of each band was estimated using NIH Image.

Inhibitor experiment

Rapamycin (100 nM) dissolved in DMSO (final concentration of 0.05 % (v/v)) or DMSO (final concentration of 0.05 % (v/v)) alone was added to the medium 30 min before amino acid stimulation. Then, the samples were prepared for Western blotting, and the mTOR activity and the autophagic–lysosomal system activity were assessed. To measure the protein degradation, after 30 min of rapamycin treatment, cells were incubated with 10 mM Lys in serum and AA-deprived medium for 4 h, and then the rates of MeHis release from the myotubes were measured.

Gene expression of ubiquitin ligase

The myotube cells were rinsed twice with PBS after 6 days of differentiation. Then, the cells were cultured in serum and AA-deprived medium containing 20 mM Leu (L), 10 mM Lys (K), or 20 mM Gly (G) for 0–4 h to measure the mRNA expression of E3 ubiquitin ligase, MuRF1. Total RNA was extracted from treated cells at the indicated time. 10 μg of total RNA from C2C12 was separated on a 1.2 % agarose-formaldehyde gel and transferred to a positively charged nylon membrane (Amersham Biosciences). After UV cross-linking, membranes were hybridized with a digoxigenin-labeled cDNA probe specific to MuRF1 for 12–16 h at 50 °C in hybridization solution (5× 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× SSC-0.1 % SDS for 15 min at room temperature and twice with 0.1× 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, USA). To estimate the relative intensity of each band, X-ray films were scanned using NIH Image.

Statistical analysis

Data are expressed as mean with SEM. Data analysis was performed using GraphPad Instat Software version 2.03 (1995, GraphPad Software, Inc., San Diego, CA, USA). 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.

Results

Lys suppresses myofibrillar protein degradation in C2C12 myotubes

At first, we evaluated whether MeHis release from myotubes into the medium was available as an index of myofibrillar protein degradation. We measured MeHis release during starvation of cells. The changes in the rates of MeHis release from C2C12 myotube are shown in Fig. 1a. The rates of MeHis release from C2C12 myotubes significantly increased in starvation time-dependent manner, which indicated that the released MeHis could be an index of myofibrillar protein degradation.

To test whether C2C12 myotubes during starvation response to a known inhibitor of protein degradation, we

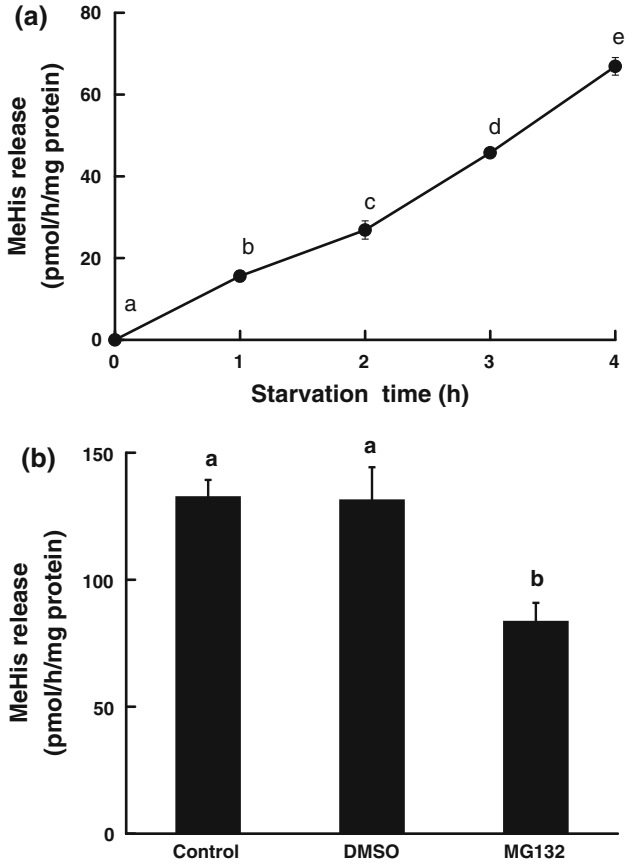

Fig. 1 Time course of the rates of 3-methylhistidine (MeHis) release from C2C12 myotube incubated in AA-deprived medium (**a**) and DMSO or MG132 (10 μM) containing medium (**b**). Values are mean ± SE ($n = 4$). C2C12 myotubes were incubated in AA-deprived medium for 0–4 h. *Different letters* indicate significant differences among the groups ($p < 0.05$)

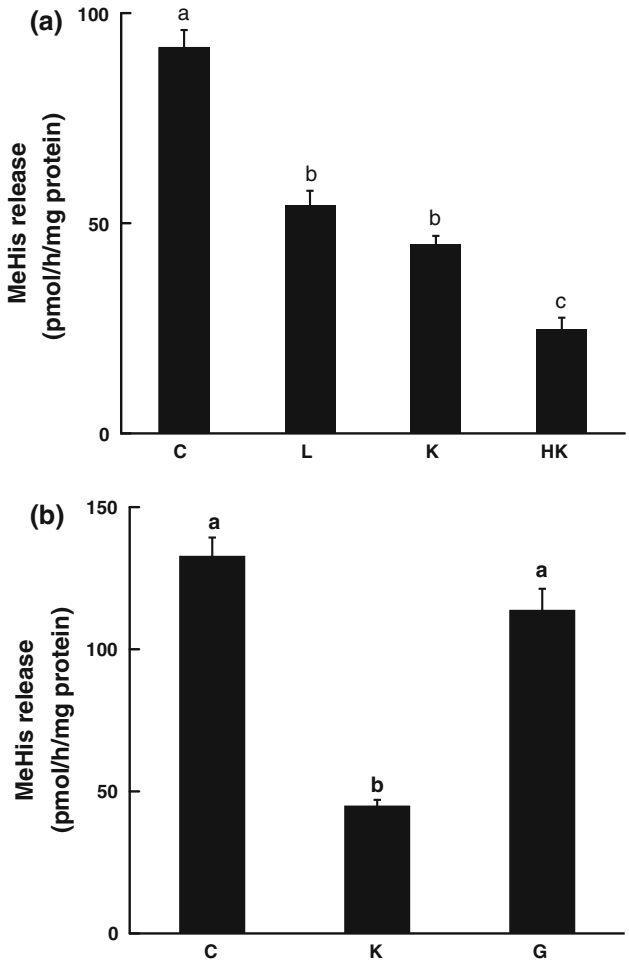

Fig. 2 The rates of 3-methylhistidine (MeHis) release from C2C12 myotubes incubated in the medium containing Leu or Lys (**a**), and in the medium containing Lys or Gly (**b**). C2C12 myotubes were incubated in serum and AA-deprived medium (C), serum and AA-deprived medium containing 20 mM Leu (L), serum and AA-deprived medium containing 10 mM Lys (K), serum and AA-deprived medium containing 20 mM Lys (HK), or serum and AA-deprived medium containing 20 mM Gly (G) for 4 h. Values are mean ± SE ($n = 4$–5). *Different letters* indicate significant differences among the groups ($p < 0.05$)

measured MeHis release from the cells treated with MG132, an inhibitor of proteasome and calpain. MG132 significantly suppressed the rates of MeHis release from C2C12 (Fig. 1b).

The rates of MeHis release were significantly suppressed by 20 mM Leu by 40 % (Fig. 2a). On the other hand, the rates of MeHis release from C2C12 myotubes were markedly suppressed by 10 mM Lys and 20 mM Lys (by 45 and 70 %, respectively), suggesting myofibrillar protein degradation in C2C12 cells could be suppressed by Lys. However, Gly supplementation was quite ineffective in suppression of MeHis release from C2C12 myotubes (Fig. 2b).

Lys suppresses activity of autophagy

LC3 is a mammalian homolog of Apg8p that is essential for the autophagic–lysosomal system in yeast, and has two forms, known as LC3-I and LC3-II. The ratio of LC3-II to LC3-I is reported to correspond to the extent of the autophagic–lysosomal system activity [24]. To determine whether the activity of autophagic–lysosomal system was suppressed by Leu or Lys, we measured the ratio of LC3-II to LC3-I by the Western blotting upon 0–3 h of Leu or Lys supplementation. The ratio of LC3-II to LC3-I was significantly decreased by 30 % in the cells treated with Leu for 30 min (Fig. 3a). Similarly, treatment with Lys for 30 min (0.5 K) also significantly reduced the ratio of LC3-II to LC3-I by 30 % (Fig. 3a). We also assessed the autophagic–lysosomal system activity from the amounts of Beclin-1. The mammalian ortholog of yeast Atg6, Beclin-1 has a central role in autophagy [25, 26] and is as a marker of the activity of the autophagic–lysosomal system [27].

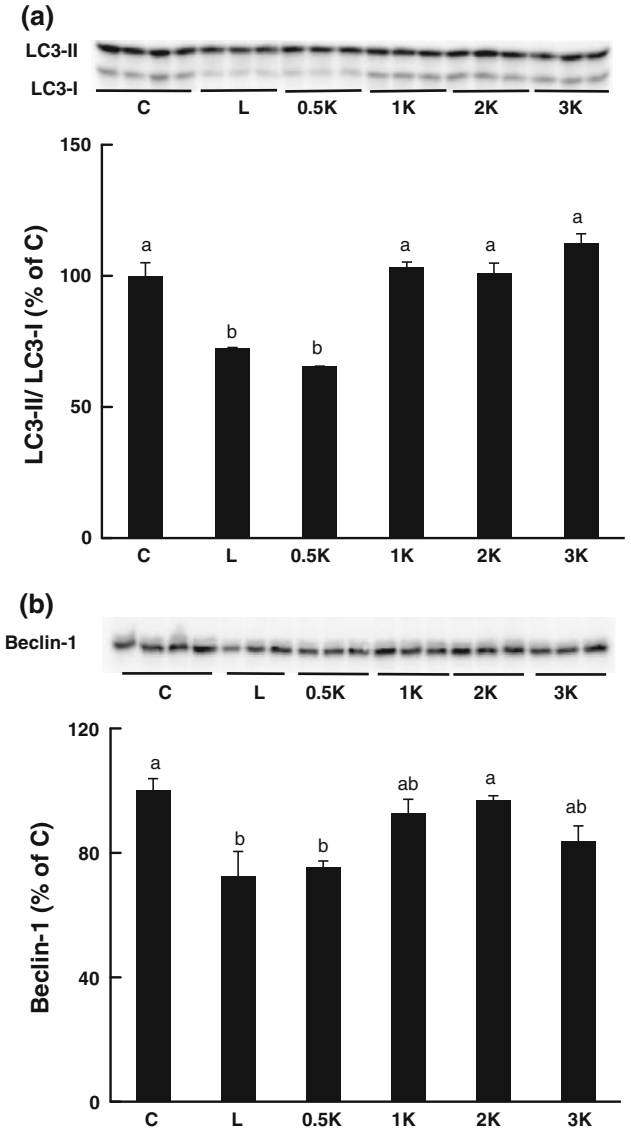

Fig. 3 The ratio of LC3-II to LC3-I (**a**) and the amounts of Beclin-1 (**b**) treated with Leu or Lys. C2C12 myotubes were untreated (C) or treated with 10 mM Leu (L) or 10 mM Lys for 0.5–3 h (0.5, 1, 2, 3 K). Values are mean ± SE ($n = 3$–4). *Different letters* indicate significant differences among the groups ($p < 0.05$)

The amounts of Beclin-1 protein in C2C12 myotubes were significantly decreased by 25 % in the cells treated with 10 mM Leu (L) or 10 mM Lys (0.5 K) for 30 min (Fig. 3b).

Suppression of autophagic–lysosomal activity by Lys is modulated through mTOR pathway

We analyzed the phosphorylation levels of p70 S6K1 and 4E-BP1 which are downstream target of mTOR in C2C12 myotubes treated with Leu or Lys. Similarly to Leu, treatment of Lys for 30 min (0.5 K) significantly enhanced

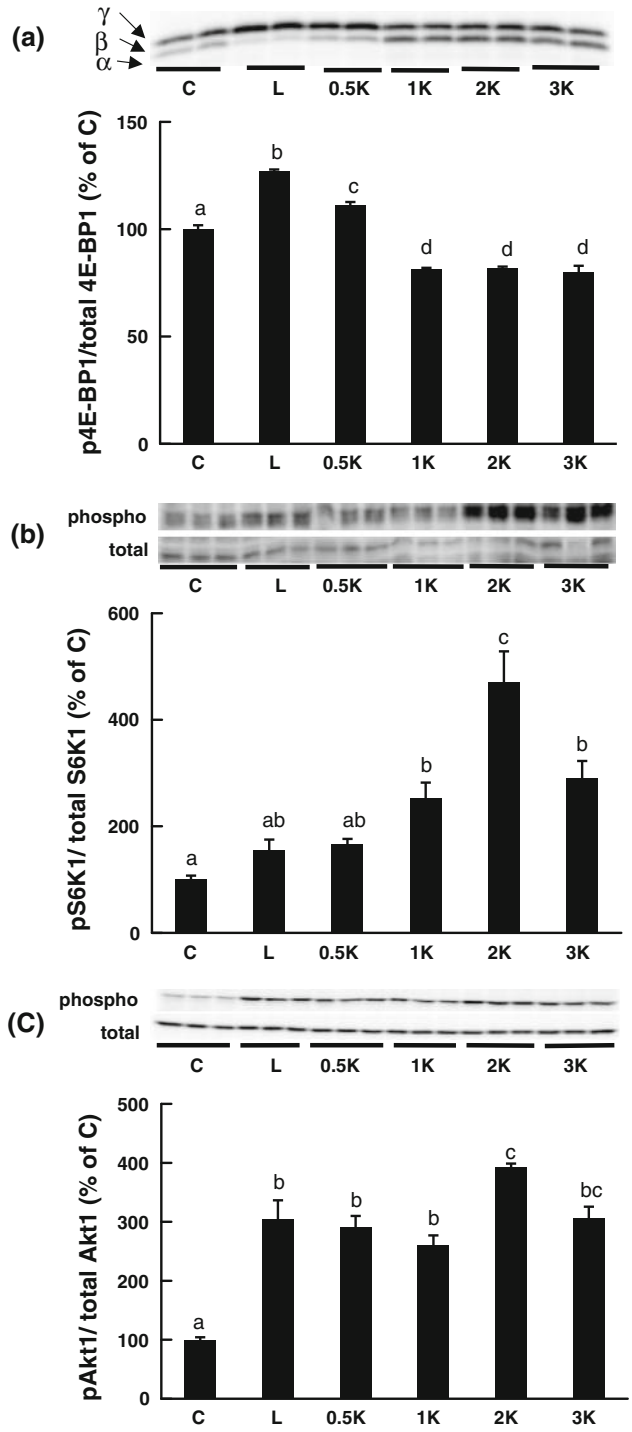

Fig. 4 Phosphorylation levels of 4E-BP1 (**a**), phosphorylation levels of p70 S6K1 (S6K1) (**b**), and phosphorylation levels of Akt (**c**) treated with Leu or Lys. C2C12 myotubes were untreated (C) or treated with 10 mM Leu (L) or 10 mM Lys for 0.5–3 h (0.5, 1, 2, 3 K). Values are mean ± SE ($n = 3$–4). *Different letters* indicate significant differences among the groups ($p < 0.05$)

the phosphorylation levels of 4E-BP1 by 10 % (Fig. 4a) and increased that of p70 S6K1 by 60 % ($p = 0.06$; Fig. 4b). Interestingly, Lys significantly enhanced the

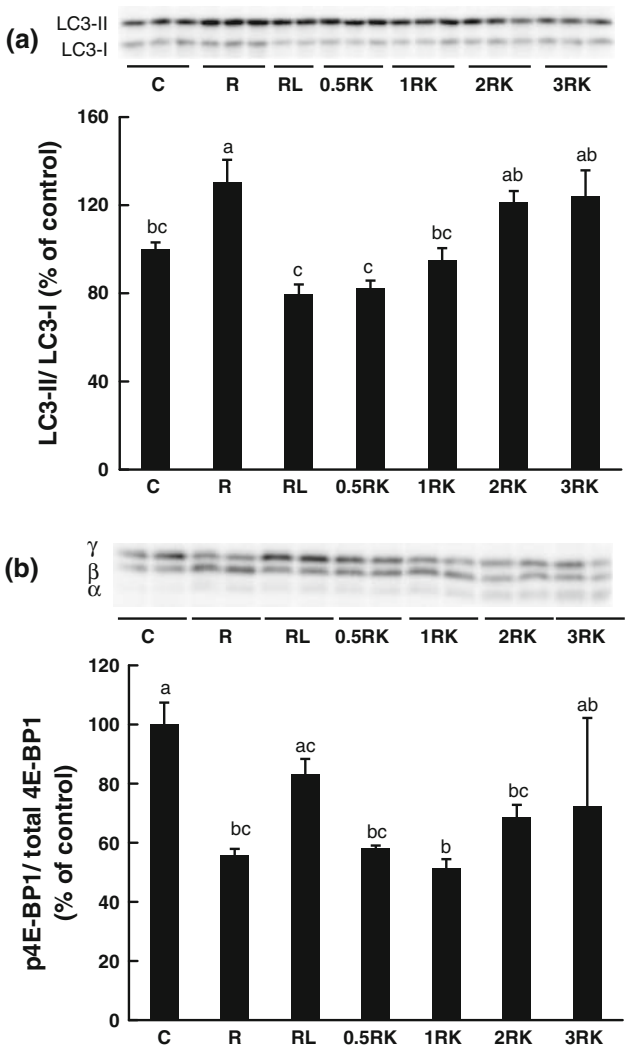

Fig. 5 The ratio of LC3-II to LC3-I (**a**) and phosphorylation levels of 4E-BP1 (**b**) treated with Leu or Lys for 0.5–2 h under the presence of mTOR inhibitor. C2C12 myotubes were treated with DMSO (C), 100 nM rapamycin (R), 10 mM Leu plus 100 nM rapamycin (RL), or 10 mM Lys for 0.5–3 h plus 100 nM rapamycin (0.5RK, 1RK, 2RK, 3RK). Values are mean ± SE ($n = 3$–4). *Different letters* indicate significant differences among the groups ($p < 0.05$)

phosphorylation of p70 S6K1 in the cells treated for 1 h (1 K), 2 h (2 K), and 3 h (3 K) by 150, 370, and 190 %, respectively (Fig. 4b). Akt is downstream target of phosphoinositide 3-kinase (PI3K) and is upstream target of mTOR. The phosphorylation levels of Akt were significantly increased by treated with Leu for 30 min (L) and that with Lys for 30 min (0.5 K), 1 h (1 K), 2 h (2 K), and 3 h (3 K) (by 200, 200, 160, 290, and 200 %, respectively) (Fig. 4c).

To confirm that the inhibitory effect of Lys on the autophagic–lysosomal system was mediated by activation of mTOR, we inhibited mTOR activity with 100 nM rapamycin, known as an mTOR inhibitor, and measured the ratio of LC3-II/I upon Lys treatment. The ratio of LC3-II/I was slightly decreased but not significant by treatment of 10 mM Lys for 30 min (0.5RK) and 1 h (1RK) as well as that of Leu for 30 min (RL) (Fig. 5a). The ratio of phosphorylation levels of 4E-BP1 was decreased by treatment of rapamycin compared to the control, but Lys did not increase the phosphorylation of 4E-BP1 which was decreased by rapamycin (Fig. 5b). On the other hand, the phosphorylation levels of 4E-BP1 when treated by rapamycin and Leu showed a tendency to increase compared to the control.

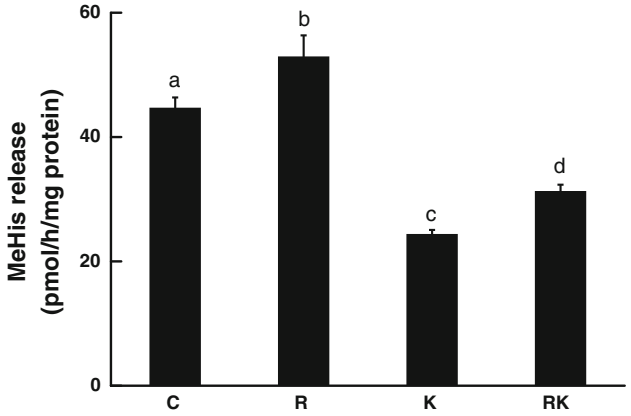

Fig. 6 The rates of MeHis release from C2C12 myotube incubated in the medium containing Lys and 100 nM rapamycin. Values are mean ± SE ($n = 4$–5). C2C12 myotubes were incubated in the medium containing DMSO (C), 100 nM rapamycin (R), 10 mM Lys (K), or 10 mM Lys with 100 nM rapamycin (RK) for 4 h. *Different letters* indicate significant ($p < 0.05$) differences among the groups

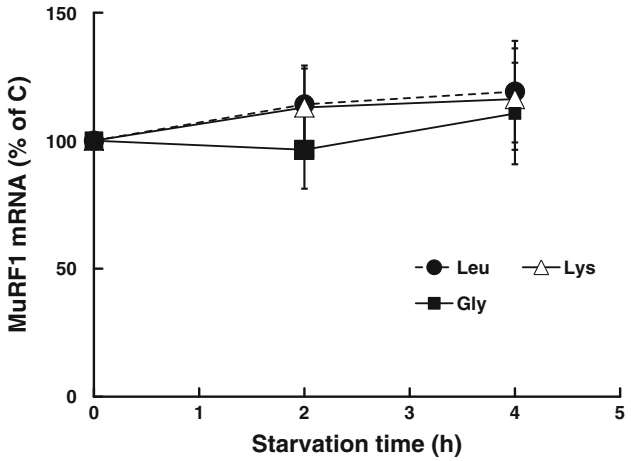

Fig. 7 The expression of MuRF1 mRNA in C2C12 myotubes incubated in the medium containing Leu, Lys, or Gly. C2C12 myotubes were incubated in the medium containing 20 mM Leu (L), 10 mM Lys (K), or 20 mM Gly (G) for 4 h. Values are mean ± SE ($n = 3$). *Different letters* indicate significant ($p < 0.05$) differences among the groups

The suppressive effects of Lys on the myofibrillar protein degradation were partially inhibited by rapamycin (Fig. 6) and suggested Lys suppressed myofibrillar protein degradation through partially mTOR.

Lys is ineffective in the ubiquitin–proteasome system activity in C2C12

We measured the mRNA expression of E3 ubiquitin ligase, MuRF1, used as a marker of the ubiquitin–proteasomal system activity. The expression of MuRF1 mRNA was not affected by Lys in C2C12 (Fig. 7).

Discussion

Protein synthesis and degradation in skeletal muscle are known to be regulated by Leu intake [6, 12, 28, 29]. However, the effects of other AA than Leu on protein turnover in skeletal muscle have been scarcely investigated. Arg and Gln were reported to modulate muscle protein synthesis [15, 17]. In our previous study [21], we have shown that oral administration of Lys to rats suppressed the rate of muscle (myofibrillar) protein degradation, while the mechanism of the suppressive effect of Lys on muscle protein degradation was still unclear. The present study was undertaken with the aim of the suppressive effect of Lys on the autophagic–lysosomal system using C2C12 myotubes, and we clearly showed that Lys suppressed myofibrillar protein degradation through the autophagic–lysosomal system.

We measured time course of the rates of MeHis release from C2C12 myotubes in order to confirm that MeHis release from C2C12 cells could reflect myofibrillar protein (main protein in muscle, actin, and myosin) degradation. Nakashima et al. [30] measured the rates of MeHis release from the cells isolated from muscle in chick embryos. The rate of myofibrillar protein degradation can be measured by MeHis in urine [5, 19] and plasma [5]. Furthermore, we measured the rate of myofibrillar protein degradation with released MeHis from the isolated muscle [6] and that of arterio-venous differences (A–V differences) [31]. However, there is no report to measure the rates of MeHis release from the cell line. The rates of MeHis release from C2C12 myotubes linearly increased in a starvation time-dependent manner (Fig. 1a). The plasma MeHis concentration and urinary excretion of MeHis were increased in a starvation with time-dependent manner [5], which was consisted with the rates of MeHis release from C2C12 myotubes measured in this study. To further confirm whether measurement of MeHis release was appropriate to assess the myofibrillar protein degradation in C2C12, we tested the effects of protease inhibitor. The inhibition of the proteasome and calpain by MG132 significantly suppressed the rates of MeHis release (Fig. 1b). Therefore, MeHis release from C2C12 myotubes is a good index of myofibrillar protein degradation and reflects at least proteolysis by the ubiquitin–proteasome and/or calpain system. In addition, it should be noted that this index may reflect another proteolysis system, for instance, the autophagic–lysosomal system, because the inhibitory effect of MG132 on the MeHis release from C2C12 was not complete. Taken together, the method in this study may reflect the myofibrillar protein degradation by three proteolysis systems, the ubiquitin–proteasome system, the calpain system, and the autophagic–lysosomal system. Thus, we considered that the present method using MeHis was appropriate to assess the myofibrillar protein degradation by various proteolysis systems in cell line, C2C12.

To assess the suppressive effect of Lys on myofibrillar protein degradation in vitro, we cultured C2C12 myotubes in serum-free medium containing Leu, Lys, or Gly and measured the rates of MeHis release from myotubes. The rates of MeHis release from myotubes were significantly decreased by 20 mM Leu (Fig. 2a). Similarly to Leu, the rates of MeHis release from myotubes were markedly decreased by 10 mM Lys (by 50 %, Fig. 2a) and 20 mM Lys (by 70 %, Fig. 2a). It was interesting to note that the rates of MeHis release from myotubes cultured in the 20 mM Lys medium were lower than that incubated in 20 mM Leu, which implicated that the suppressive effect of Lys on myofibrillar protein degradation was more effective than that of Leu. However, the precise mechanisms that underlie the difference between Leu and Lys on myofibrillar protein degradation are unknown. On the other hands, Gly could not suppress the rates of MeHis release (Fig. 2b), and this result suggested that the suppressive effects on myofibrillar protein degradation in C2C12 were unique to Lys and Leu, not merely by supplement of nitrogen sources.

It is known that there are three proteolytic systems to degrade muscle proteins; the autophagic–lysosomal system, the ubiquitin–proteasomal system, and the calpain system [32]. Many studies have shown that various catabolic conditions induce the ubiquitin–proteasomal system by upregulating the expression of ubiquitin ligase [33, 34]. We have previously shown that dietary Leu intake suppressed myofibrillar proteolysis by inhibiting the autophagic–lysosomal system in rats fed protein deficient diet [14]. Thus, the autophagic–lysosomal system is also considered to be important in proteolysis. We showed that oral Lys administration to rats suppressed myofibrillar protein degradation at least partially through inhibition of the autophagic–lysosomal system, whereas Lys did not affect on the activity of the ubiquitin–proteasomal system [21]. To determine whether Lys suppressed the ubiquitin–proteasomal system

in C2C12, we assessed the mRNA expression of MuRF1, E3 ubiquitin ligase. However, MuRF1 mRNA expression was not changed by Lys (Fig. 7). Therefore, we particular focused on the regulation of the autophagic–lysosomal system by Lys in C2C12 myotubes in this study. The ratio of LC3-II to LC3-I, a marker of the autophagic–lysosomal system activity, was significantly suppressed by 35 % in treatment of Lys for 30 min (0.5 K) (Fig. 3a), while it was significantly suppressed by 30 % treated with 10 mM Leu for 30 min (Fig. 3a). The amount of Beclin-1 protein used as a marker of the activity of the autophagic–lysosomal system in C2C12 myotubes was also significantly decreased by 25 % treated with Leu or Lys for 30 min (Fig. 3b). Mordier et al. [35] have shown that Leu starvation induced increase in protein degradation in C2C12 myotubes through the autophagic–lysosomal activity. Our present results strongly suggested that Lys also suppressed myofibrillar protein degradation by the reduction of autophagic–lysosomal activity.

The autophagic–lysosomal system is regulated by activity of mTOR [35, 36]. We evaluated mTOR activity from the phosphorylation levels of p70 S6K1 and of 4E-BP1, downstream targets of mTOR. It is reported that Leu enhances mTOR activity [12, 28]. The phosphorylation levels of 4E-BP1 were significantly increased by treatment with Leu within 30 min (Fig. 4a). Lys also increased the phosphorylation levels of 4E-BP1 and S6K1 within a short time, which suggested that Lys may enhance mTOR activity. The great increase of the phosphorylation levels of p70 S6K1 upon 1–3 h of 10 mM Lys treatment (1, 2, and 3 K) (Fig. 4b), although the phosphorylation levels of 4E-BP1 were unchanged upon 1–3 h of 10 mM Lys treatment (Fig. 4a). The reason for this discrepancy is unclear; however, there may present other pathway to enhance activity of p70 S6K1. 3-Phosphoinositide-dependent protein kinase 1 (PDK1) is downstream of PI3K and induces phosphorylation of Akt. Furthermore, PDK1 is reported to directly activate p70 S6K1 [37, 38]. The Akt activity was immediately increased following either Leu or Lys treatment (Fig. 4c), which suggested Lys and Leu activate Akt following mTOR. However, its activity remained at 2 and 3 h after treatment with Lys unlike the changes in phosphorylation of p70 S6K1. Therefore, it is suggested that activity of p70 S6K1 may be regulated by other mechanism Akt/mTOR pathway such as PDK1 which may be enhanced by Lys. Tato et al. [39] had reported that AA phosphorylated both of Thr308 site and Ser473 site through Class I PI3K and mTORC2 in starvation condition. From these data, Lys and/or Leu may phosphorylate Akt through Class I PI3K and mTORC2 in C2C12. However, the direct effects of Lys or Leu on PI3K and mTORC2 have not been proved in this study. The detailed regulatory mechanism of Lys on Akt activity needs to be clarified in the future study.

When mTOR was inhibited by rapamycin, the autophagic–lysosomal activity evaluated the ratio of LC3-II to LC3-I was suppressed nearly to the level of without inhibitor and amino acid in C2C12 treated with Lys and Leu (Figs. 3a, 5a); however, the activity was not suppressed significantly compared to vehicle control (C) (Fig. 5a). These results confirmed that Lys regulated the autophagic–lysosomal activity through mTOR-dependent pathway partially in C2C12 myotubes. In addition, the rates of myofibirillar protein degradation when C2C12 treated with Lys and rapamycin (RK) were lower than that of only rapamycin treated cells (R) (Fig. 6). These data support the hypothesis that Lys suppresses proteolysis through other regulator pathway. It was reported that regulatory amino acids (RegAA) suppressed proteolysis by the autophagic–lysosomal system through mTOR-independent pathway [40]. In addition, Akt suppresses the autophagy through FoxO3 [41]. Actually, Lys enhanced Akt phosphorylation in this study (Fig. 4c). The suppressive effect of Akt on the autophagic–lysosomal system may not be inhibited by rapamycin, because Akt locates upstream of mTOR, the target of rapamycin. Therefore, Lys might suppress the autophagic–lysosomal system through Akt/FoxO3 pathway or the pathway similar to RegAA. The phosphorylation levels of 4E-BP1 increased with treatment of Leu and the inhibitor, although mTOR was inhibited by rapamycin (0.5RK, Fig. 5b). It was reported that the phosphorylation of 4E-BP1 increased with Leu and rapamycin treatment while the activity of mTOR and S6K1 was suppressed at the same level of control [42]. Therefore, Leu may be able to stimulate 4E-BP1 phosphorylation through mTOR-independent pathway, because the phosphorylation levels of 4E-BP1 were not suppressed perfectly in this study.

It is well known that muscle protein synthesis is activated by Akt/mTOR pathway [12, 28]. The present results in which phosphorylation levels of 4E-BP1 and Akt were stimulated by treatment of Lys suggested that protein synthesis may be increased by Lys. In contrast to the present finding, we previously suggested that oral administration of Lys to rat did not activate mTOR and/or Akt [21]. The discrepancy of these results was not clear, possibly due to the difference between in vivo and in vitro, such as the effects of metabolites. We measured the activity of mTOR and Akt only at 0, 1, 3, and 6 h upon Lys administration to rats [21]. Therefore, Lys may activate mTOR and Akt in other time points and enhance protein synthesis in vivo. Further studies would be required to reveal these issues.

In conclusion, this study demonstrated for the first time that treatment with Lys suppresses myofibrillar protein degradation by MeHis release in C2C12 myotube cells. Furthermore, we can indicate that Lys suppresses the

activity of autophagic–lysosomal system in part through the activation of mTOR and Akt in C2C12 myotubes.

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