

研究ノート

Comparative analysis of the effect of leucine, arginine, and lysine on regulation of protein degradation pathways in mouse skeletal muscle and liver

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経口摂取したロイシン、アルギニン、あるいはリジンのマウス骨格筋、肝臓におけるタンパク質分解系に及ぼす影響

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要 旨

アミノ酸やタンパク質はタンパク質合成を促進し、分解を抑制する。以前、我々はロイシン、アルギニン、リジンの中でロイシン摂取が最もタンパク質合成促進作用を示すことを報告した。しかし、最もタンパク質分解抑制作用を示すものは明らかにできていない。そこで、本研究では、いずれかのアミノ酸を摂取させ、マウス腓腹筋と肝臓におけるオートファジー-リソソーム系とユビキチン-プロテアソーム系へ及ぼす効果を解析した。ロイシン摂取によりリン酸化ULK1は、両組織において最も増加し、オートファジー-リソソーム系の開始段階を最も抑制することが示唆された。一方、LC3B-II/I比とユビキチン化タンパク質量の減少はどの群においても認められず、LC3B-II/I比とユビキチン化タンパク質量の変化には、60分以上の時間を要する可能性があり、より詳細な経時的実験が必要であることが推察された。

キーワード

ロイシン、アルギニン、リジン、オートファジー-リソソーム系、ユビキチン-プロテアソーム系

Abstract

Amino acids and proteins promote protein synthesis and inhibit protein degradation. We previously reported that among leucine, arginine, and lysine, leucine intake promotes protein synthesis to the greatest extent. However, the amino acids that most effectively suppress protein degradation remained unclear. We therefore investigated the effects of oral administration of these amino acids on the autophagy-lysosome and ubiquitin-proteasome pathways in the gastrocnemius muscle and liver of C57BL/6J mice. We analyzed the same protein-extracted samples used in the previous study. The phosphorylation levels of ULK1, which regulates the initiation phase of the autophagy-lysosome pathway, were increased by leucine intake in both tissues. However, no significant reduction in the LC3B-II/I ratio or ubiquitinated protein levels was observed for any amino acid, and more time may be required to detect such changes. These findings suggest that leucine most effectively suppresses initiation of the autophagy-lysosome pathway. More detailed time-course studies were required to fully evaluate the effects of these amino acids on the overall process of the autophagy-lysosome and ubiquitin-proteasome pathways.

Key words

leucine, arginine, lysine, autophagy-lysosome pathway, ubiquitin-proteasome pathway

1. Introduction

Tissue protein content is determined by the balance between protein synthesis and degradation. Protein synthesis and degradation rates are continuously regulated to maintain body protein homeostasis (Inami et al., 2011). However, it is difficult to determine a priori whether a high or low rate of protein degradation is preferable for a particular physiological state. When protein degradation is an accelerated condition beyond a normal range, reducing it to normal levels can be beneficial; for example, the inhibition of muscle protein degradation in disuse muscle atrophy (Pang et al., 2023). In contrast, sustaining a higher rate of protein degradation with increasing age within the normal physiological range may be advantageous for health (Cavallini et al., 2001). Amino acids act as signaling molecules that regulate both protein synthesis and degradation. Therefore, understanding the regulatory role of amino acids in protein degradation is crucial for maintaining and improving health and for the prevention and treatment of diseases through nutritional management.

Protein degradation is regulated by autophagy-lysosome and ubiquitin-proteasome pathways, and amino acids are reported to regulate protein degradation via these pathways (Béchet et al., 2005; Chotechuang et al., 2011). At high nutrient concentrations, unc-51 like kinase 1 (ULK1) is phosphorylated and inactivated by the mammalian target of rapamycin complex 1 (mTORC1), which negatively regulates autophagy (Dikic & Elazar, 2018; Lee et al., 2019). During starvation-induced autophagy, mTORC1 dissociates from the ULK1

complex, which is composed of ULK1, autophagy-related (ATG) 13, ATG101, and focal adhesion kinase family interacting protein of 200-kDa. Subsequently, the ULK1 complex initiates autophagosome formation and cytosolic microtubule-associated protein light chain 3 (LC3-I) is converted to membrane-associated phosphatidylethanolamine-conjugated LC3 (LC3-II), which is necessary for autophagosome production. A protein-free diet supplemented with 1.5% leucine for one week suppresses the reduction in gastrocnemius weight and decreases LC3-II levels (Sugawara et al., 2009). However, Maki *et al.* did not detect an effect of branched-chain amino acids on the LC3-I/II ratio in hindlimb suspension rats (Maki et al., 2012). Although arginine depletion induces autophagy in cultured cell lines (García-Navas et al., 2012; Xia et al., 2016), few studies have focused on the direct effect of arginine intake on autophagy suppression. Additionally, lysine decreased the LC3B-II/I ratio and levels of Beclin-1, a marker of autophagic-lysosomal system activity, and suppressed protein degradation in C2C12 cells (Sato et al., 2014). In contrast, lysine did not alter the LC3B-II/I ratio in the skeletal muscle of rats after administration for 1–6 h (Sato et al., 2013).

In the ubiquitin-proteasome pathway, proteins destined for degradation are ubiquitinated by conjugation with E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase) (Nandi et al., 2006). Ubiquitinated proteins are then degraded by proteasomes, followed by proteases and peptidases. The expression of E3, atrophy gene-1 (atrogin-1),

and muscle ring-finger protein 1 (MuRF1) is upregulated under conditions of muscle atrophy, such as immobilization, denervation, hindlimb suspension, dexamethasone treatment (Bodine et al., 2001), and fasting (Gomes et al., 2001). Moreover, the expression of the ubiquitin ligase neural precursor cells expressed developmentally downregulated-4-like (NEDD4L) is induced in the mouse liver during fasting (Fu et al., 2013). Ingestion of a leucine or branched-chain amino acid mixture suppresses atrogen-1 and MuRF1 mRNA levels in vivo and in vitro (Herningtyas et al., 2008; Maki et al., 2012; Yamamoto et al., 2010). Notably, the suppression of mTORC1 activity by rapamycin revealed that the effect of leucine on the downregulation of atrogen-1 expression was mediated by mTORC1 (Herningtyas et al., 2008). A previous study showed that arginine suppresses atrogen-1 and MuRF1 mRNA expression in C2C12 cells (Herningtyas et al., 2008). However, lysine does not affect atrogen-1 mRNA expression in rat skeletal muscle or MuRF1 expression in C2C12 cells (Sato et al., 2013; Sato et al., 2014).

As noted above, several studies describe the effect of leucine, arginine, and lysine on protein degradation. However, regarding the oral intake of leucine, arginine, and lysine, it is unclear which shows the greatest inhibition of the autophagy-lysosome and ubiquitin-proteasome pathways in various tissues. Previously, we showed that among the above amino acids, leucine most effectively activates the mTORC1 pathway, a protein synthesis pathway, in both skeletal muscle and liver, as well as protein synthesis in skeletal muscle. Thus, in this

study, we investigated the effectiveness of the oral intake of each of the amino acids leucine, arginine, and lysine in suppressing the autophagy-lysosome and ubiquitin-proteasome pathways in mouse skeletal muscle and liver used in our previous study.

2. Materials and methods

2.1. Animals and experimental design

Male C57BL/6J mice (8 weeks old) were purchased from Nihon S.L.C. (Hamamatsu, Japan) were randomly assigned to control (Cont, $n = 4$), leucine (Leu, $n = 4$), arginine (Arg, $n = 4$), and lysine (Lys, $n = 3$) groups (Quy et al., 2013). They were housed individually in plastic cages at $22 \pm 3^\circ\text{C}$ and $55 \pm 7\%$ humidity with a 12-h light/dark cycle (lights on from 07:00 to 19:00). The mice were acclimated for three days and given ad libitum access to water and an AIN-93G rodent composition diet (Reeves et al., 1993). On days 4 and 5, the mice were administered saline or 0.2% xanthan gum/saline solution. No medications were administered on days 6 and 7. After 24 h fasting, the mice were administered 0.2% xanthan gum solution, leucine, arginine, or lysine at 1.35 mg/10 μL 0.2% xanthan gum solution/g body weight by gavage. To mitigate sampling order effects, one mouse per group was dosed sequentially. The average body weights of mice among the groups were similar (control group, 19.7 [SD 1.1] g; leucine group, 19.9 [SD 0.5] g; arginine group, 19.6 [SD 0.7] g; and lysine group, 19.4 [SD 0.7] g) after 24 h fasting. The fasting duration was determined based on previous reports (Anthony et al., 2002; Sato et al., 2013; Yoshimura et al., 2016; Yoshimura & Nomura, 2022). The amount of leucine

administered in this study has been frequently used in previous studies on the effects of leucine on protein synthesis (Anthony et al., 2002; Yoshimura et al., 2016; Yoshimura & Nomura, 2022). This amount was equivalent to that consumed in a 24-h period when male Sprague-Dawley rats were provided free access to the AIN-93 powdered diet (Anthony et al., 1999). Evaluation of protein synthesis levels was performed as described in a previous study (Yoshimura et al., 2024). Thirty minutes after administration, the mice were administered puromycin (Sigma-Aldrich) at 0.04 $\mu\text{mol}/10 \mu\text{L}$ saline/g body weight by intraperitoneal injection (Goodman et al., 2011; Hayasaka et al., 2014; Hulmi et al., 2013). Thirty minutes after the puromycin injection, the mice were sacrificed by cervical dislocation and then bled by decapitation. Gastrocnemius muscle and liver were harvested and snap-frozen in liquid nitrogen. Tissues were stored at -80°C until analysis. All experimental protocols were approved by the Research Ethics Committee and Animal Experiment Ethics Committee of the Nagasaki International University (No. 17A05). All mice were handled according to the institutional guidelines for the care and use of laboratory animals.

2.2. Western blotting

The gastrocnemius muscle and liver tissues were homogenized in 7 volumes of a buffer consisting of 20 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid pH 7.4, 100 mM KCl, 0.2 mM ethylenediaminetetraacetic acid, 2 mM ethylene glycol-bis (β -aminoethyl ether)-

N,N,N',N'-tetraacetic acid, 1 mM dithiothreitol, 50 mM sodium fluoride, 50 mM β -glycerophosphate, 0.1 mM phenylmethylsulphonyl fluoride, 1 mM benzamidine, and 0.5 mM sodium vanadate using a bead-homogenizer (TAITEC, Koshigaya, Japan). Insoluble materials were removed using centrifugation of the gastrocnemius muscle at $10,000 \times g$ for 10 min at 4°C and of the liver at $18,000 \times g$ for 20 min at 4°C . The protein concentration in the supernatant was measured using a Bradford Protein Assay Kit (Takara Bio, Shiga, Japan). The supernatant was mixed with $6\times$ sample buffer (0.35 M Tris-HCl pH 6.8, 10% sodium dodecyl sulfate [SDS], 10% glycerol, 9.3% dithiothreitol, 0.012% bromophenol blue). The samples were heated for 5 min at 100°C and cooled on ice. Equal amounts of protein were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany). The membranes were blocked with blocking buffer (5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST)) for 1 h at approximately $25 \pm 2^{\circ}\text{C}$. The membranes were washed three times for 5 min each with TBST and then incubated with primary antibodies against phospho-ULK1 at Ser757 (Cell Signaling Technology, #14202), LC3B (Cell Signaling Technology, #2775), ubiquitin (Cell Signaling Technology, #3936), and GAPDH (Cell Signaling Technology, #3683) diluted in 5% bovine serum albumin in TBST. The membranes were then washed three times for 5 min each with TBST, and incubated with anti-rabbit IgG horseradish peroxidase secondary

antibody (Cell Signaling Technology, #7074) or anti-mouse IgG horseradish peroxidase secondary antibody (Cell Signaling Technology, #7076) diluted in blocking buffer at $25 \pm 2^\circ\text{C}$. Membranes were washed three times for 5 min each with TBST, and band densities were detected with enhanced chemiluminescence reagent (Chemi-Lumi One Super, Nacalai Tesque, Kyoto, Japan) and quantified using a ChemiDoc XRC Plus system and Image Lab 5.2.1 (Bio-Rad, Hercules, CA, USA). After the first detection, membranes were washed twice for 5 min each with TBST and incubated twice for 5 min each with stripping buffer (6 M guanidine hydrochloride, 0.2% Nonidet P-40, 10 mM dithiothreitol, 20 mM Tris-HCl pH 7.5) (Yeung & Stanley, 2009). The membranes were washed four times (3 min each) with TBST. Membranes were blocked and incubated to detect the total target against the phosphorylated form.

2.3. Statistical analysis

Experiments were conducted using three or four mice in each group ($n = 3\text{--}4$) (Quy et al., 2013). Data were expressed as means \pm SD. One-way analysis of variance and Tukey-Kramer tests for multiple comparisons were performed to determine the significance of the differences. Differences were considered statistically significant at $P < 0.05$. Analysis was performed using the statistical software Statcel4.

3. Results And Discussion

Dietary amino acids and proteins are known to suppress protein degradation

(Kamei et al., 2020). However, the amino acid that is most effective at suppressing protein degradation has not yet been elucidated. In this study, we analyzed the phosphorylation of ULK1, LC3B-II/I ratio, and levels of ubiquitinated proteins. As shown in Fig. 1(a), leucine caused the greatest increase in phosphorylated ULK1 levels in skeletal muscle ($F = 11.5$, $P < 0.05$). However, none of the amino acids altered the LC3B-II/I ratio ($F = 2.8$, $P > 0.05$), which is another marker of autophagy-lysosome pathway activity (Fig. 1(b)). Although a significant difference was observed in the liver between the Leu and Lys groups, Leu also showed the greatest increase in phosphorylated ULK1, as observed in skeletal muscle ($F = 4.5$, $P > 0.05$; Fig. 1[c]). Similar to the skeletal muscle results, the LC3B-II/I ratio was not altered by any amino acid in the liver ($F = 1.7$, $P > 0.05$; Fig. 1[d]). As phosphorylated ULK1 cannot promote autophagy initiation, the results suggest that among the amino acids studied, leucine most effectively suppressed protein degradation by autophagy. However, another autophagy marker, the LC3B-II/I ratio, did not change in any group. The effects of leucine and arginine on mTORC1 activation were observed at least one hour after administration or addition to medium (Ham et al., 2014; Yoshizawa et al., 2001). The LC3B-II/I ratio is decreased by amino acid stimulation after 120 min in rat liver H4-II-E cells (Karim et al., 2014). Therefore, the conversion of LC3B-I to LC3B-II, which results in the formation of autophagosomes, may require a longer time than that required for the phosphorylation of ULK1 by mTORC1 during the initiation of

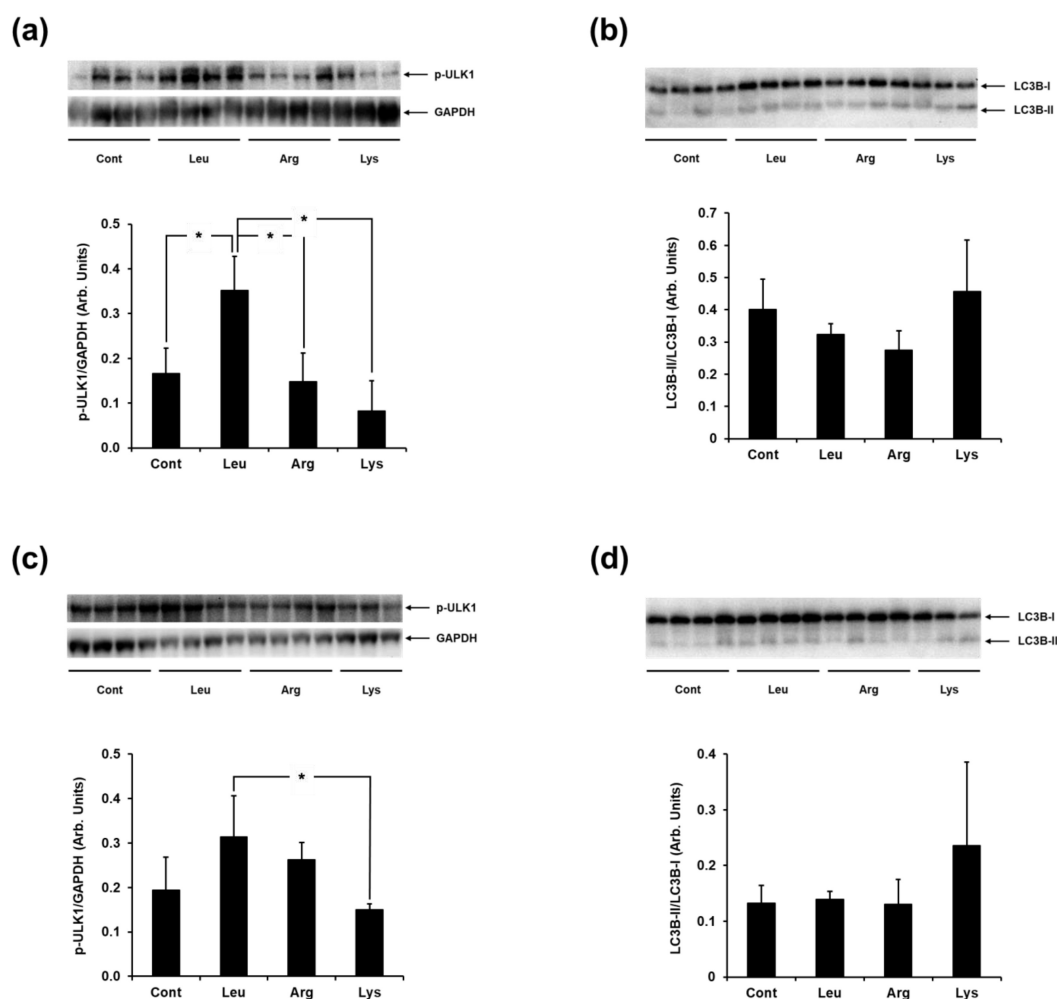


Figure 1 Changes in the protein level of phospho-ULK1 at Ser757 and LC3B in the gastrocnemius muscle (a, b) and liver (c, d) of mice administered 0.2% xanthan gum solution (Cont), leucine (Leu), arginine (Arg), or lysine (Lys) at 1.35 mg/g body weight after 24 h fasting.

Protein levels of phospho-ULK1 at Ser757, LC3B, and GAPDH were determined by western blot analysis. GAPDH was used as an internal standard for normalization. The densitometry of the bands is expressed as the mean \pm SD for $n = 3-4$. As the puromycin-labeled peptides were not detected at all in one mouse in the lysine group, we eliminated the results of that mouse from all evaluations to avoid drawing a false conclusion; it is possible that we may have missed injecting puromycin into that particular mouse. * Mean values with asterisks on different letters indicate significant differences between groups ($P < 0.05$).

autophagy. Since we performed sampling 60 min after amino acid administration, we detected no change in the LC3B-II/I ratio.

Next, we evaluated the amount of ubiquitinated protein as an index of ubiquitin-proteasome pathway activity (Fig.

2[a] and [b]) (Sato et al., 2013). Except for lysine, which induced a significant increase in the amount of ubiquitinated protein in skeletal muscle ($F = 10.1$, $P < 0.05$), none of the amino acids decreased the levels of ubiquitinated protein in either tissue (liver F

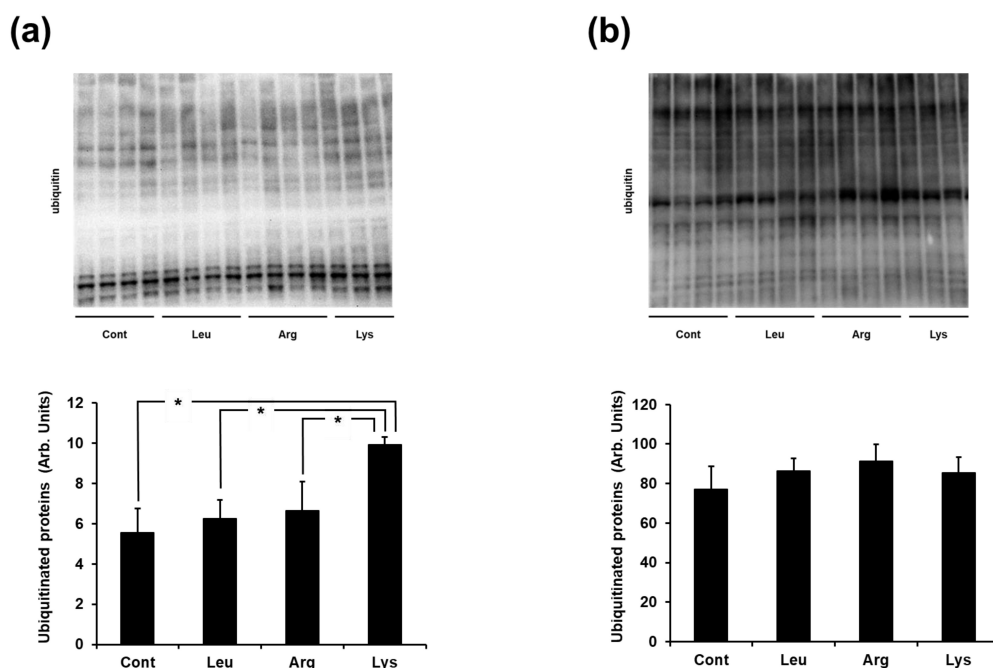


Figure 2 Ubiquitinated protein levels in the gastrocnemius muscle (a) and liver (b) of mice administered 0.2% xanthan gum solution (Cont), leucine (Leu), arginine (Arg), or lysine (Lys) at 1.35 mg/g body weight after 24 h fasting.

Ubiquitinated protein levels were determined by western blot analysis. The densitometry of the bands is expressed as the mean \pm SD for $n = 3-4$. As the puromycin-labeled peptides were not detected at all in one mouse in the lysine group, we eliminated the results of that mouse from all evaluations to avoid drawing a false conclusion; it is possible that we may have missed injecting puromycin into that particular mouse. * Mean values with asterisks on different letters indicate significant differences between groups ($P < 0.05$).

= 1.7, $P > 0.05$). Thus, oral intake of leucine, arginine, or lysine may not alter the amount of ubiquitinated protein. Although previous reports have described the effects of leucine, arginine, and lysine on the ubiquitin-proteasome pathway, they primarily evaluated their effects on E3 ubiquitin ligase expression. Moreover, the half-life of atrogin-1 mRNA evaluated after incubation with actinomycin D was approximately 100 min (Dehoux et al., 2004). Therefore, we could not reliably detect changes in ubiquitinated proteins because sampling was performed 60 min after intake.

In the present research, we demonstrated that in mouse skeletal muscle and liver tissues, leucine administered at 1.35 mg/g body weight was the more effective than arginine or lysine in increasing phosphorylated ULK1 levels, which regulates the initiation step in the autophagy-lysosome pathway, after 60 minutes of oral intake (Fig. 3). In contrast, we observed no significant suppressive effects on the LC3B-II/I ratio or ubiquitinated protein levels. Changes in the LC3B-II/I ratio and ubiquitinated proteins may occur > 60 min after intake. Therefore,

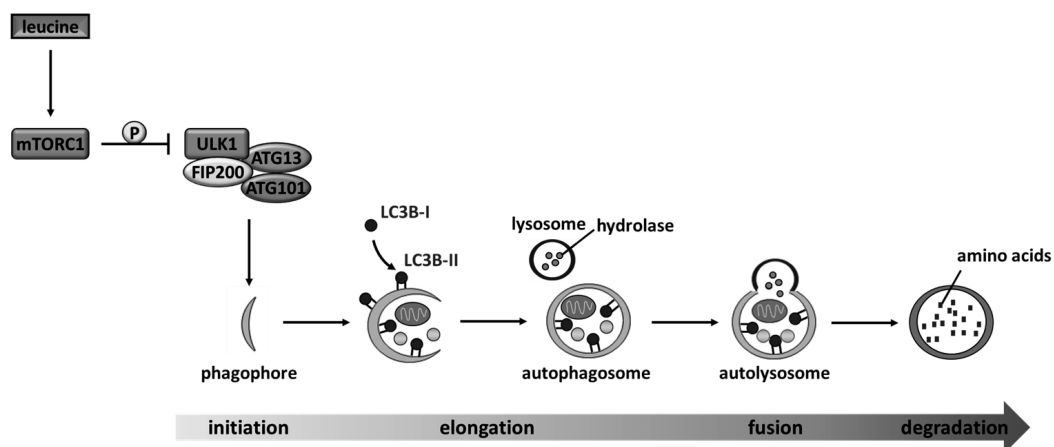


Figure 3 Suggested model for the regulation of autophagy by leucine.

a time-course study is required to evaluate the effects of these amino acids on the autophagy-lysosome and ubiquitin-proteasome pathways. Moreover, the autophagy-lysosome and ubiquitin-proteasome pathways are complex processes involving many factors (Liu et al., 2021; Park et al., 2020). In this study, we sought to evaluate the changes in specific markers in these pathways. Therefore, to evaluate the effects of these amino acids on the autophagy-lysosomal and ubiquitin-proteasome systems in detail, it is necessary to evaluate their effects on the regulators of each pathway under various conditions. Furthermore, because protein degradation is also regulated by calpains and endocytosis (Metwally et al., 2023; Rusilowicz-Jones et al., 2022), it is crucial to assess the effect of these amino acids on these molecules and/or pathways to understand the regulatory function of amino acids in the homeostasis of body proteins.

4. Disclosure statement

The authors declare that there are no

conflicts of interest.

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