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Suppression of myofibrillar proteolysis in chick skeletal muscles by α-ketoisocaproate

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Summary. We previously reported that L-leucine suppresses myofibrillar proteolysis in chick skeletal muscles. In the current study, we compared the effects of L- and D-enantiomers of leucine on myofibrillar proteolysis in skeletal muscle of chicks. We also assessed whether leucine itself or its metabolite, α-ketoisocaproate (α-KIC), mediates the effects of leucine. Food-deprived (24 h) chicks were orally administered 225 mg/100 g body weight L-leucine, D-leucine or α-KIC and were sacrificed after 2 h. L-Leucine administration had an obvious inhibitory effect on myofibrillar proteolysis (plasma N^{τ}-methylhistidine concentration) in chicks while D-leucine and α-KIC were much more effective. We also examined the expression of the proteolytic-related genes (ubiquitin, proteasome, m-calpain and cathepsin B) by real-time PCR of cDNA in chick skeletal muscles. Ubiquitin mRNA expression was decreased by D-leucine and α-KIC but not L-leucine. Proteasome and m-calpain mRNA expressions as well as cathepsin B mRNA expression were likewise decreased by L-leucine, D-leucine and α-KIC. These results indicate that D-leucine and α-KIC suppress proteolytic-related genes, resulting in an decrease in myofibrillar proteolysis while L-leucine is much less effective in skeletal muscle of chicks, may be explain by conversion of D-leucine to α-KIC.

Keywords: Myofibrillar proteolysis – Leucine – α-Ketoisocaproate – Skeletal muscle – Chick

Introduction

Branched chain amino acids (valine, leucine, and isoleucine, and BCAAs) are essential amino acids and the major nitrogen source in skeletal muscles. Among the BCAAs, particularly leucine stimulates protein synthesis (Anthony et al., 2000; Yoshizawa, 2004; Yoshizawa et al., 2002) but suppresses protein degradation (Buse and Reid, 1975; Fulks et al., 1975; Li and Jefferson, 1978) in skeletal muscles. Recently, it was found that leucine specifically stimulates phosphotidyl-inositol 3-kinase (PI3K) (Peyrollier et al., 2000; Tesseraud et al., 2003) and the kinase for the mammalian target of rapamycin (mTOR) (Anthony et al., 2000; Yoshizawa, 2004; Yoshizawa et al., 2002) resulting in stimulation of the protein synthesis machinery, including ribosomal S6 kinase and 4E binding protein in skeletal muscles. However, suppression of proteolysis by leucine does not involve an mTOR signaling pathway in mouse C2C12 myotubes (Mordier et al., 2000) and isolated rat hepatocytes (Kanazawa et al., 2004). We also showed that suppression of myofibrillar proteolysis by leucine does not involve an mTOR signaling pathway chick myotube cultures in vitro and oral administration of leucine suppresses myofibrillar proteolysis in chicks in vivo (Nakashima et al., 2005). Tischler et al. (1982) showed that leucine metabolite, α-ketoisocaproate (α-KIC), suppresses proteolysis in incubated skeletal muscles, indicating that transamination of L-leucine is essential for the inhibitory effect on proteolysis (Tischler et al., 1982). However, it is not known if this reflects the myofibrillar proteolysis caused by leucine or leucine metabolite, and the mechanism of the effects of each of the leucine is not understood in vivo.

Like other tissues, skeletal muscle contains multiple proteolytic systems, each of which could play an important role in muscle atrophy. Intracellular proteolysis is carried out by lysosomal and non-lysosomal pathways in which intracellular proteases are directly responsible for the degradation of proteins. Calpains, i.e., cysteine proteases in the cytosol, are thought to be the main agents of non-lysosomal Ca^{2+}-dependent proteolysis which occurs within the myofibril, and have been shown capable of carrying out the initial step in myofibrillar proteolysis (Goll et al., 1991, 1992). Proteasome, the multicatalytic proteinase complexes in the cytosol, is also thought to be responsible for non-lysosomal ATP-dependent proteolysis

(Coux et al., 1996). Although proteasome has been implicated in the regulation of myofibrillar protein degradation (Furuno et al., 1990; Solomon and Goldberg, 1996), its substrates and the control of its activity has not been fully studied in muscles. Proteasome may degrade muscle proteins released due to the action of the other proteases. Cathepsins, i.e., the main agents of lysosomal degradation, have been well established as contributing to muscle protein breakdown (Hall-Angeras et al., 1991). Lysosomal proteases degrade sarcoplasmic proteins and released myofibrillar proteins (Lowell et al., 1986). However, the precise roles of all these degradation systems in the breakdown of skeletal muscle proteins have yet to be determined.

Thus, in the present study, we have compared the effects of leucine enantiomers on myofibrillar proteolysis and assessed whether leucine itself or the metabolite of leucine, α-ketoisocaproate (α-KIC), mediates the effects of leucine.

Materials and methods

Animal preparation and experimental protocol

One-day-old male layer chicks were supplied by a local commercial hatchery (Miyake-Furanjo, Chiba, Japan). They were housed in an electrically-heated battery brooder and were provided with water and a commercial starter diet (Toyohashi-shiryou, Aichi, Japan) *ad libitum* for 7 days. On day 7, twenty-four birds of similar body weight (about 80 g) were selected and housed in wire-bottomed aluminum cages, and six replications were made per treatment. The temperature of the room was 25 °C, and relative humidity was maintained at 50–70% throughout the experiment. Chicks were given free access to a semi-purified corn soybean meal diet (Crude protein 20%, metabolizable energy 2900 kcal/kg diet) and water for 7 d. At the start of the experiment, 14-day-old chicks weighing 140 ± 10 g were divided into groups and caged separately. Chicks were deprived of food for 24 h and then randomly assigned to continue as food-deprived (Control) or to receive one of three dietary treatments by oral gavage as follows: L-leucine (L-Leu), D-leucine (D-Leu) or α-ketoisocaproate (α-KIC). The amount of each amino acid administered was 225 mg/100 g body weight, prepared as 3.8 g/50 ml in distilled water. Food-deprived chicks (Control) received an equal volume of saline by oral gavage. After amino acid administration, chicks were returned to their cages where they were permitted free access to water only. The amount of each amino acid administered was equivalent to the amount of leucine consumed by chicks of this age and strain during 24 h of free access to a diet. The chicks were killed 2 h after administration, and gastrocnemius muscles and blood samples were obtained. The experimental procedures used in this study met the guidelines of the Animal Care and Use Committee of the National Institute of Livestock and Grassland Science.

RNA isolation and real-time PCR

Gastrocnemius muscles were rapidly excised, frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted from gastrocnemius muscles by the using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's protocols. cDNA was synthesized from 1 to 1000 ng of total RNA using random hexamer (TaKaRa, Tokyo, Japan) and ReverTra Ace (TOYOBO, Tokyo, Japan). Real-time PCR primers were designed (software Primer3, http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi) for ubiquitin, proteasome C2 subunit, m-calpain large subunit, cathepsin B and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Sequences of the forward primers and reverse primers are as follows: chicken ubiquitin, 5′-CGC ACC CTG TCT GAC TAC AA-3′–5′-GCC TTC ACG TTC TCA ATG GT-3, chicken proteasome C2 subunit, 5′-AAC ACA CGC TGT TCT GGT TG-3′–5′-CTG CGT TGG TAT CTG GGT TT-3, chicken m-calpain large subunit, 5′-ACA TCA TCG TGC CCT CTA CC-3′–5′-GAG ATC TCT GCA TCG CTT CC-3, chicken cathepsin B, 5′-CAA GCT CAA CAC CAC TGG AA-3′–5′-TCA AAG GTA TCC GGC AAA TC-3, and chicken GAPDH, 5′-CCT CTC TGG CAA AGT CCA AG-3′–5′-CAT CTG CCC ATT TGA TGT TG-3. Gene expression was measured by real-time PCR using a LightCycler (Roche Diagnostics, Mannheim, Germany) instrument with the QuantiTect SYBR Geen PCR system (Qiagen K. K., Tokyo, Japan) starting with 25 ng of reverse-transcribed total RNA. GAPDH expression was used as an internal control. For quantification of the levels of mRNA expression, PCR product roughly equivalent in size and equivalent primer lengths and GC contents of each primer (50–60%) were selected. PCR was performed under the following conditions: 95 °C × 15 min, 45 × (94 °C × 15 sec, 55 °C × 20 sec, 72 °C × 12 sec).

N^{τ}-methylhistidine analysis

Plasma N^{τ}-methylhistidine concentration was measured by the HPLC method after derivatization of fluorescamine with a treatment of perchloric acid and heating (Wassner et al., 1980).

Statistical analysis

Data were analyzed by Student's *t*-test. A p value <0.05 was considered statistically significant. Each result is expressed as the mean ± SD.

Results and discussion

In the present study, we compared the effects of L- and D-enantiomers of leucine on myofibrillar proteolysis in skeletal muscle of chicks. We also assessed whether leucine itself or its metabolite, α-KIC, mediates the effects of leucine. The BCAAs together or simply leucine alone stimulates protein synthesis and inhibits protein degradation in isolated muscles (Buse and Reid, 1975; Fulks et al., 1975). The mechanisms by which the BCAAs exert these effects on protein balance remain unidentified. The acceleration of protein synthesis by leucine seems to occur at the level of peptide chain initiation (Morgan et al., 1971). Even less information is available concerning the mechanism by which leucine inhibits proteolysis.

The result of plasma N^{τ}-methylhistidine concentration, as an index of myofibrillar proteolysis, is shown in Fig. 1. Plasma N^{τ}-methylhistidine concentration was decreased by L-leucine, D-leucine and α-KIC. However, D-leucine and α-KIC were much more effective than L-leucine. This result indicates that L-leucine administration had an obvious inhibitory effect on myofibrillar proteolysis in chicks while D-leucine and α-KIC were much more

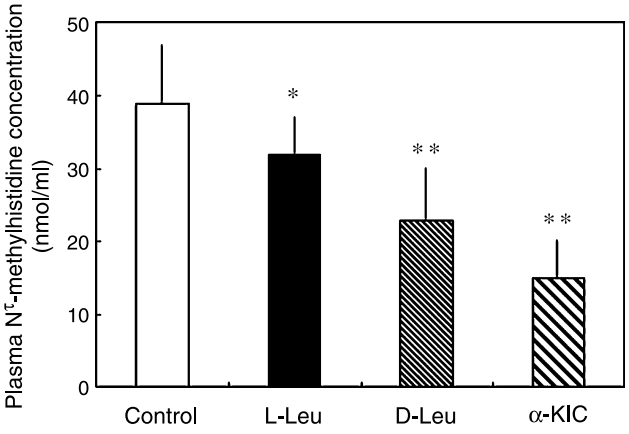

Fig. 1. Effects of oral administration of L-leucine (L-Leu), D-leucine (D-Leu) or α-ketoisocaproate (α-KIC) on plasma N$^{\tau}$-methylhistidine concentrations in chicks. Food-deprived (24 h) chicks were orally administered 225 mg/100 g body weight L-Leu, D-Leu and α-KIC and were sacrificed after 2 h. Data are expressed as means ± SD, $n = 6$. A Student's t-test was performed. $^{**}p < 0.01$, $^{*}p < 0.05$

effective. N$^{\tau}$-methylhistidine is an amino acid formed by the post-translational methylation of specific histidine residues on two myofibrillar proteins, actin and myosin. N$^{\tau}$-methylhistidine cannot charge tRNA, and therefore cannot be reutilized for protein synthesis. Because of this, and also because it does not undergo catabolism, the output of N$^{\tau}$-methylhistidine has been used as an index of myofibrillar proteolysis (Thompson et al., 1996; Young et al., 1972). The degradation rate of skeletal muscle protein can be measured by the urinary excretion of N$^{\tau}$-methylhistidine, which is localized in the myofibrillar proteins, myosin and actin, but is not reused for protein synthesis (Young et al., 1972). Measuring the urinary excretion of N$^{\tau}$-methylhistidine will not detect acute changes in myofibrillar protein degradation, since the collection period for urine is usually 1 d. In contrast, the plasma N$^{\tau}$-methylhistidine concentration can reflect an acute change in the degradation of myofibrillar protein (Nagasawa et al., 1996, 1998). In the previous study, we showed that L-leucine reduces N$^{\tau}$-methylhistidine release into medium in chick myotubes, indicating that L-leucine suppresses myofibrillar proteolysis in vitro (Nakashima et al., 2005). We also showed that L-leucine reduces the plasma N$^{\tau}$-methylhistidine concentration in orally administrated chicks. This result also provides clear evidence that L-leucine suppresses myofibrillar proteolysis in chicks (Nakashima et al., 2005). The recent findings that protein feeding (Nagasawa et al., 1998) and even feeding L-leucine alone (Nagasawa et al., 2002) rapidly reduced the plasma level of N$^{\tau}$-methylhistidine, as a specific marker of myofibrillar proteolysis in vivo, together with its release from muscle incubation in vitro, strongly indicate that amino acids control myofibrillar proteins. These results are consistent with our result. However, in the present study, D-leucine and α-KIC as well as L-leucine have an inhibitory effect of myofibillar proteolysis in chicks, and were much more effective. Tischler et al. (1982) showed that α-KIC suppresses proteolysis in skeletal muscles, indicating that transamination of L-leucine is essential for the inhibitory effect on proteolysis (Tischler et al., 1982). D-Leucine is thought to be inverted to the L-enantiomer by two steps. First, it is oxidatively deaminated to α-KIC by D-amino oxidase. Next, the α-keto acid is asymmetrically reaminated by transaminase to form L-leucine (Yoshizawa et al., 2004). A resent study demonstrated that about 30% of an administrated dose of the D-leucine was inverted to L-leucine (Hasegawa et al., 2000). In the present study, we showed that α-KIC suppresses myofibrillar proteolysis in skeletal muscles, indicating that transamination of L-leucine might be essential for the inhibitory effect on myofibrillar proteolysis.

The main purpose of the present experiments was to determine the proteolytic mechanism(s) involved in the

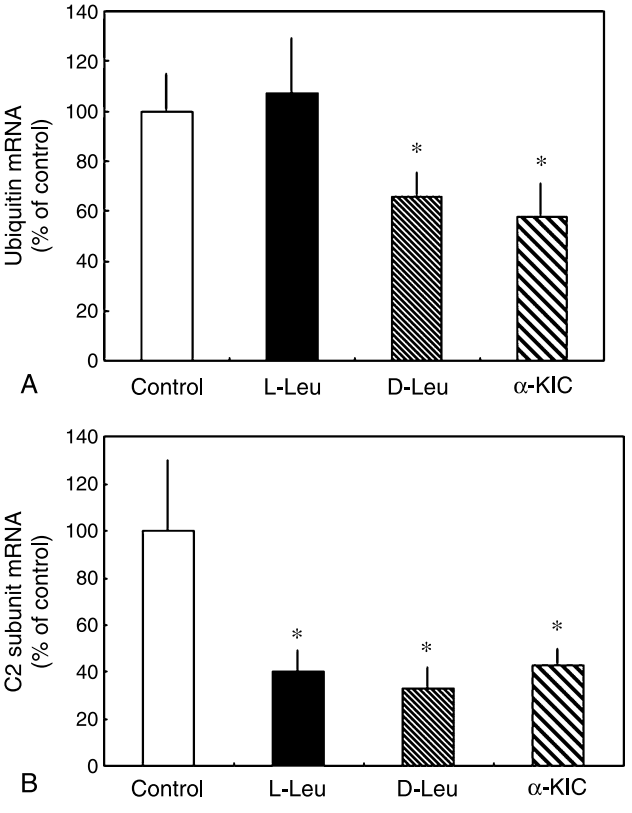

Fig. 2. Effects of oral administration of L-leucine (L-Leu), D-leucine (D-Leu) or α-ketoisocaproate (α-KIC) on expressions of ubiquitin (**A**) and C2 subunit mRNAs (**B**) in skeletal muscle of chicks. Same procedure as described for Fig. 1. C2 subunit, 20S proteasome C2 subunit

L-leucine, D-leucine or α-KIC-dependent suppress in myofibrillar proteolysis. The biochemical pathways for the degradation of muscle protein and its mode of activation under leucine and leucine metabolite are remain unclear. We examined the expression of the proteolytic-related genes (ubiquitin, proteasome, m-calpain and cathepsin B) by real-time PCR of cDNA in skeletal muscle of orally leucine or leucine metabolite administrated chicks. We measured the mRNA levels of components of the ubiquitin-proteasome system (non-lysosomal ATP-dependent proteolysis). The results of the mRNA expression of ubiquitin and proteasome subunit C2 are shown in Figs. 2A and B. Ubiquitin mRNA expression (A) was significantly decreased by D-leucine and α-KIC but not L-leucine. The proteasome C2 subunit mRNA expression (B) was significantly decreased by L-leucine, D-leucine and α-KIC. Busquets et al. (2000) have reported that high concentration of leucine (10 mM) inhibits proteolysis and ubiquitin and proteasome C8 subunit in incubated rat skeletal muscles (Busquets et al., 2000). We previously showed that L-leucine suppresses expressions of ubiquitin and proteasome C2 subunit in chick myotube cultures (Nakashima et al., 2005). On the other hand, orally administrated L-leucine suppressed proteasome C2 subunit but not ubiquitin expression chick skeletal muscles (Nakashima et al., 2005). These results are consistent with ours. In the present study, orally administrated D-leucine and α-KIC suppressed ubiquitin and proteasome C2 subunit expressions in skeletal muscle of chicks.

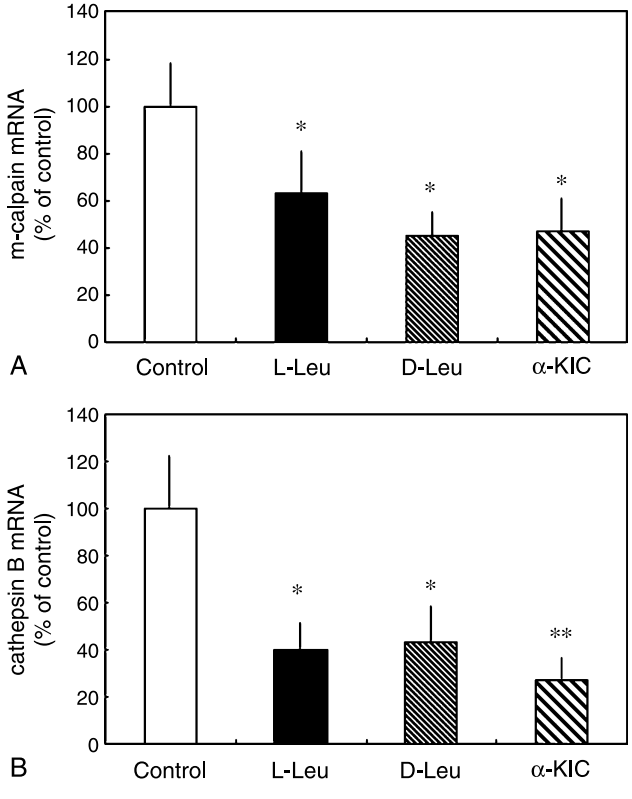

Fig. 3. Effects of oral administration of L-leucine (L-Leu), D-leucine (D-Leu) or α-ketoisocaproate (α-KIC) on expressions of m-calpain (**A**) and cathepsin B (**B**) in skeletal muscle of chicks. Same procedure as described in Fig. 1. M-calpain, m-calpain large subunit

We next measured the mRNA levels for calpain of the non-lysosomal Ca^{2+}-dependent proteolytic system. The result of mRNA expression of m-calpain large subunit is also shown in Fig. 3A. The m-calpain large subunit mRNA expression was significantly decreased by L-leucine, D-leucine and α-KIC. We previously showed that L-leucine suppresses m-calpain large subunit expression in chick myotube cultures (Nakashima et al., 2005). This result is consistent with ours. We also showed that D-leucine and α-KIC suppress expression of calpain in skeletal muscles in vivo. However, the effect of leucine itself or leucine metabolite on calpain expression in skeletal muscles in vivo had not been reported. The present study provides the first evidence that orally administrated leucine and leucine metabolite suppress expression of calpain in skeletal muscles in vivo.

We next measured the mRNA level for cathepsin B of the lysosomal-dependent proteolytic system. The result of the mRNA expression of cathepsin B is also shown in Fig. 3B. Cathepsin B mRNA expression was also significantly decreased by L-leucine, D-leucine and α-KIC. α-KIC was much more effective ($p < 0.01$). However, we previously showed that L-leucine did not suppress cathepsin B expression in chick myotube cultures (Nakashima et al., 2005). In the present study, we showed that L-leucine, D-leucine and α-KIC suppress expression of cathepsin B in skeletal muscles in vivo. However, the effect of leucine itself or leucine metabolite on cathepsin B expression in skeletal muscles in vivo had not been reported. The present study provides the first evidence that orally administrated leucine and leucine metabolite suppress expression of cathepsin B in skeletal muscles in vivo.

Like other tissues, skeletal muscle contains multiple proteolytic systems, each of which might play an important role in muscle atrophy. Calpains (non-lysosomal Ca^{2+}-dependent proteolysis), proteasome (non-lysosomal ATP-dependent proteolysis), and cathepsins (the main agents of lysosomal degradation) have been well established as contributing to muscle protein breakdown. In the present experiment, we measured calpain, proteasome, cathepsin expressions, and showed that leucine and leucine metabolite suppress all those expressions in skeletal muscle of chicks.

In conclusion, the present study shows that D-leucine and α-KIC stimulates proteolytic-related genes, resulting in an decrease in myofibrillar proteolysis while L-leucine is much less effective in skeletal muscle of chicks, may be explain by conversion of D-leucine to α-KIC.

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