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Rapid suppression of protein degradation in skeletal muscle after oral feeding of leucine in rats

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Abstract

A diet containing adequate amounts of protein rapidly suppresses myofibrillar protein degradation in rats and mice. This study determined whether dietary amino acids inhibit postprandial protein degradation in rat skeletal muscle. When rats fed on a 20% casein diet for 1 h after 18 h starvation, the rate of myofibrillar protein degradation measured by N^{τ}-methylhistidine release from the isolated extensor digitorum longus muscle was significantly ($p < 0.05$) decreased at 4 h after refeeding. A diet containing an amino acid mixture which is the same composition as casein also reduced myofibrillar protein degradation at 4 h after refeeding ($p < 0.05$). An essential amino acid mixture (15.1%, corresponding to casein composition) and a leucine (2.9%) diets reduced the rate of myofibrillar protein degradation after refeeding ($p < 0.05$), whereas a protein free diet did not. Administration of leucine alone (0.135 g/100 g body weight) by a feeding tube induced a decrease in the rate of myofibrillar protein degradation at 2 h after administration ($p < 0.05$), whereas the serum insulin concentration was constant after leucine administration. These results suggested that leucine is one of regulating factors of myofibrillar protein degradation after refeeding of a protein diet. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Postprandial protein degradation; Insulin; Amino acids; Myofibrillar protein; N^{τ}-methylhistidine

1. Introduction

Many catabolic diseases are accompanied by muscle wasting due to a decreased rate of synthesis and an accelerated rate of degradation of skeletal muscle protein [1]. It is therefore important to maintain muscle mass by regulating the synthesis and degradation of muscle protein resulting from disease and possibly aging. Numerous studies have investigated the factors regulating muscle protein turnover, which have included hormones and cytokines [2,3]. Muscle wasting is frequently associated with a decrease in food intake, which induces further severe metabolic disorders. The meal changes level of hormones and substrates which also affects protein synthesis and degradation.

Long-term starvation causes loss of muscle mass by both reducing protein synthesis and increasing protein degradation [4–9], and refeeding inhibits protein degradation [6,7] in rats. However, the changes in the protein degradation rate in short period after refeeding remain to be elucidated. Food intake is closely related insulin secretion. Therefore, it is conceivable that insulin modulates postprandial muscle protein turnover, because insulin stimulates muscle protein synthesis [10–12] and inhibits protein degradation [6,8,13–15], although the effect of insulin on protein turnover in vivo is as yet unclear [16,17]. We have previously shown that postprandial muscle protein synthesis is rapidly stimulated and degradation is subsequently decreased by refeeding in mice [18]. This is also shown in rats [19], although protein free diet does not reduce the rate of muscle protein degradation, suggesting that dietary protein and possibly amino acids rapidly alters postprandial muscle protein degradation.

Amino acids are a potent modulator of protein synthesis and degradation in muscle. Muscle protein synthesis is increased by amino acids that are infused [20–23], or orally administrated [24,25], whereas degradation is decreased by infused [20,22] or dietary amino acids [7]. Despite this knowledge, the early response of muscle protein degrada-

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Table 1
Composition of experimental diet

Composition (%)	20C[a]	AA	EAA	Leu	0C
Casein	22.7	0	0	0	0
Amino acid mixture[c]	0	22.5	0	0	0
Essential amino acid mixture[c]	0	0	15.1	0	0
Leucine[c]	0	0	0	2.9	0
D, L-Methionine	0.3	0	0	0	0
Sucrose	10.0	10.0	10.0	10.0	10.0
α-Cornstarch	50.3	50.8	58.2	70.4	73.3
Soy bean oil	7.0	7.0	7.0	7.0	7.0
AIN 93 Mineral mixture[b]	3.5	3.5	3.5	3.5	3.5
AIN 93 Vitamin mixture[b]	1.0	1.0	1.0	1.0	1.0
Choline bitartrate	0.2	0.2	0.2	0.2	0.2
Cellulose	5.0	5.0	5.0	5.0	5.0

[a] 20C, 20% casein diet; AA, amino acid mixture diet; EAA, essential amino acid mixture diet; Leu, leucine diet; 0C, protein free diet.
[b] AIN 93 composition [26].
[c] The content was based on amino acid composition of casein [26].

tion to orally administered amino acids has yet to be described. In this study we examined whether a diet containing amino acids or leucine induces suppression of myofibrillar protein degradation after refeeding.

2. Materials and methods

2.1. Animal preparation and experimental protocol

We performed the following three experiments. In the first experiment, thirty male 3-week-old Wistar rats obtained from CLEA Japan Inc. (Tokyo, Japan) were used in this experiment. The animals were individually housed in stainless steel wire cages and maintained at 22°C and 55% relative humidity on a 12 h light:dark cycle (6:00 a.m.-6:00 p.m.). They were allowed free access to water and a 20% casein diet (20C, Table 1) [26] for 3 days. To accustom them to feeding within a short period, the diet was given at 10:00 a.m. and was withdrawn at 10:00 p.m. for 3 days, then was given at 10:00 a.m. and withdrawn at 7:00 p.m. for 3 days, and finally given at 10:00 a.m. and withdrawn at 4:00 p.m. for 6 days. At the end of this feeding period, the rats were randomly assigned to 6 group (n = 5 each group) and were starved for 18 h (4:00 p.m.-10:00 a.m.). Rats of three groups were refed an amino acid mixture diet (AA, Table 1) for 1 h (10:00 a.m.-11:00 a.m.), and were sacrificed at 1, 4, or 7 h after refeeding. The amino acid mixture was the same amino acid composition as casein [26]. The rats of two groups fed on a 20C or a protein free diet (0C, Table 1) for 1 h were sacrificed at 4 h after refeeding. Five rats in remaining group were sacrificed just before refeeding (0 h after refeeding, 10:00 a.m.). All rats were anesthetized with diethylether. The abdomen was opened and blood was collected from the inferior vena cava to obtain serum. The extensor digitorum longus (EDL) muscle was removed from right leg. Serum was frozen in liquid nitrogen and stored at -80°C until analysis. The animal care protocols in this study were approved by the Iwate University Animal Research Committee and the Iwate University Guidelines for Animal Experimentation were followed.

In the second experiment, 25 male 3-week-old Wistar rats were used. The feeding protocol was the same as that for the first experiment. At the end of the feeding period, the rats were randomly divided into 5 groups (n = 5 each group). The rats in one group were killed before refeeding commenced (10:00 a.m.). Rats of two groups were refed an essential amino acid mixture diet (EAA, Table 1) corresponding to essential amino acids in the 20C [26], or a leucine diet (Leu, Table 1) corresponding to leucine in the 20C [26] for 1 h. The rats in remaining 2 groups were refed either 20C or 0C as a positive or a negative control. They were all sacrificed at 4 h after refeeding and the EDL muscle was removed.

In the third experiment, thirty male 4-week-old Wistar rats were used. They were fed on a 20C ad libitum for 5 days. On the fifth day of feeding, the rats were randomly assigned to 6 groups (n = 5 each group) and food-deprived at 4:00 p.m. One group of rats was killed at 10:00 a.m. (unfed for at least 18h). The rats in the other group were administered 2.5 ml of leucine in suspension in distilled water/100 g body wt (0.135g/100g body wt) by a feeding tube (FE86s, Terumo Co., Tokyo, Japan) at 10:00 a.m. The amount of administered leucine was calculated by the method based upon that of Anthony et al. [25]. The rats were killed at 1, 2, 3, 4, or 7 h after administration and the EDL muscle and serum samples were obtained.

2.2. Muscle protein degradation

To measure the rate of protein degradation, we incubated the isolated EDL muscles from rats fed on the experimental diet or leucine with stainless steel support in Krebs-Ringer bicarbonate buffer containing 10 mmol/L glucose under 95% O_2–5% CO_2 at 37°C for 2 h following a 30-min preincubation at 37°C [5,27]. In the third experiment, 0.5 mmol/L cycloheximide was contained in the Krebs-Ringer bicarbonate buffer. N^{τ}-methylhistidine (MeHis) and tyrosine released into the incubation buffer were measured by the HPLC method after derivatization of fluorescamin with a treatment of perchloric acid and heating [28] and fluorometry [29], respectively.

2.3. Analytical methods

Insulin was measured by the RIA method using a kit (Insulin RIA Eiken, Eiken Chemical Co., Tokyo, Japan). Serum amino acid concentrations were measured by an automatic amino acid analyzer (AminoTac, JLC-500/V, JEOL, Tokyo, Japan) following sulfosalicylic acid treatment (final 1.5%).

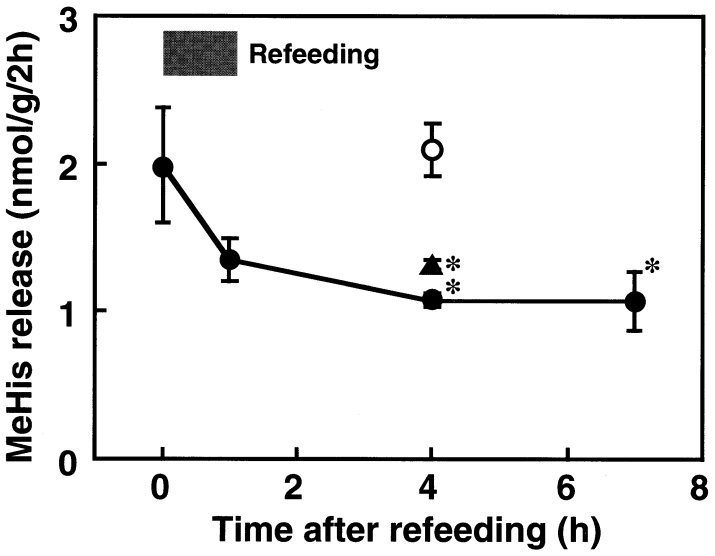

Fig. 1. Changes in N^{τ}-methylhistidine (MeHis) release from extensor digitorum longus muscle after refeeding of amino acid mixture diet. Closed circle, amino acid mixture diet; closed triangle, 20% casein diet; open circle, protein free diet. Values are means ± SE of 5 rats. *Significant difference from 0 h (18 h starvation) $p < 0.05$.

2.4. Statistical analysis

Data analysis (GraphPad InStat Software version 2.03, 1995, San Diego, CA) involved estimation of means and SEM for each of the groups. Analysis of variance was performed to determine whether there were significant ($p < 0.05$) differences among the groups. When an ANOVA indicated any significant difference among the means, the Newman-Keuls multiple comparison test was used to determine which means were significantly different.

3. Results

3.1. Diet containing amino acid mixture

After refeeding on the amino acid mixture diet (AA), the rate of MeHis release was gradually suppressed and at 4 h after refeeding the rate was 40% lower than that before refeeding (Fig. 1). Rats fed on a 20% casein diet (20C) also showed a suppression of MeHis the same extent as AA, whereas the protein free diet (0C) did not show this suppression (Fig. 1). Food intake of the rats fed on the 20C was 8.7 ± 0.8 g, whereas that on the AA was 3.7 ± 0.3 g. Therefore we prepared rats fed on the 20C which is the same amount as AA (3.7g, pair-fed). The pair-fed rats showed the same level of myofibrillar protein degradation (1.48 ± 0.12 nmol MeHis/g/2h) as rats fed on the 20C.

Fig. 2 shows the changes in serum amino acid concentrations in rats fed on the AA. All amino acid concentrations were immediately increased after refeeding ($p < 0.05$), and then most of the amino acids decreased in concentration to the pre-feeding level (at 18 h starvation). Rats fed on the 20C showed the same level of serum amino acid concentrations as those that consumed AA, although those fed on the 0C showed the same level as pre-feeding level.

3.2. Diet containing essential amino acid or leucine

When rats were fed on the essential amino acid mixture diet (EAA) in which amino acid content corresponded to the essential amino acids in 20% casein diet, MeHis release at 4 h after refeeding was suppressed by 50% ($p < 0.05$ vs Starvation, Fig. 3). Furthermore, rats fed on a leucine diet (Leu) showed the same suppression of myofibrillar protein degradation ($p < 0.05$ vs starvation), which was similar reduction to rats fed on the EAA or the 20C.

3.3. Oral administration of leucine

MeHis release from the isolated EDL muscle before oral administration of leucine was 5.6 nmol/g muscle/2h (Fig. 4A), which was at a higher rate than that of rats fed on an amino acid mixture diet (2.0 nmol/g muscle/2h, Fig. 1). This difference was caused by the starvation period; the rats in the first experiment were unfed for 18 h but those in the third experiment were unfed for more than 18 h. This longer starvation period may increase myofibrillar protein degradation [9].

The MeHis release from the isolated EDL muscle of rats was significantly suppressed by the orally administered leucine (Fig. 4A). Suppression occurred slightly more rapidly than for rats fed on a casein diet [19] or the amino acid mixture diet (Fig. 1). Maximum suppression occurred at 2–4 h post administration ($p < 0.05$ vs 0 h). In contrast, the rate of tyrosine release showed a different pattern from that of MeHis release. Tyrosine release after leucine oral administration was immediately suppressed ($p < 0.05$) then in-

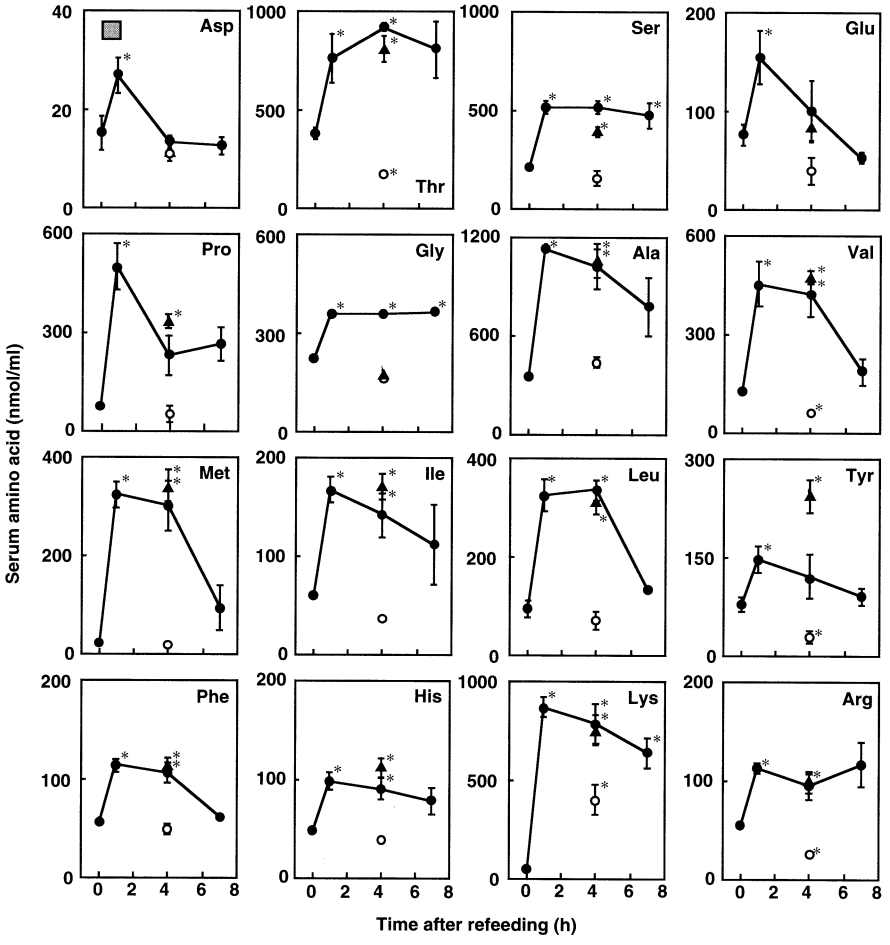

Fig. 2. Changes in serum amino acid concentration after refeeding of amino acid mixture diet. Closed circle, amino acid mixture diet; closed triangle, 20% casein diet; open circle, protein free diet. Values are means ± SE of 5 rats. *Significant difference from 0 h (18 h starvation) $p < 0.05$.

creased at 2 h after administration. At 3 h it was again decreased ($p < 0.05$) before increasing once more. Thus, tyrosine release shows a biphasic pattern.

Serum leucine concentration was immediately increased after oral administration ($p < 0.05$, Fig. 5A) before decreasing to the pre-administration level at 4 h after treatment, while the rate of MeHis release was still suppressed at this time point. The maximum concentration was higher than that of the rats fed on an amino acid mixture diet (Fig. 2), which was probably due to a higher dose of leucine and no competition of absorption with other amino acids. The serum insulin concentration increased only slightly after leucine administration (Fig. 5B). The low level of insulin concentration continued until 7 h after treatment.

4. Discussion

This series of experiments clearly demonstrated that amino acids, particularly leucine suppress myofibrillar protein degradation at early time after oral administration. We

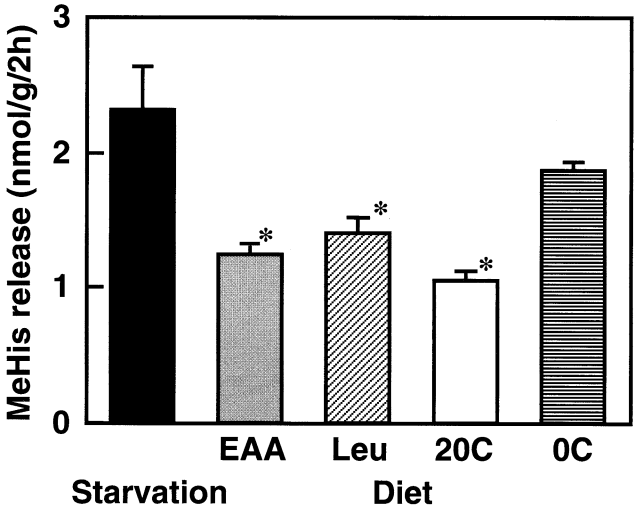

Fig. 3. N^{τ}-methylhistidine (MeHis) release from extensor digitorum longus muscle in rats fed on an essential amino acid mixture or leucine diet. The value is 0 h (Starvation, 18 h starvation) and 4 h after refeeding. Values are means ± SE of 5 rats. *Significant difference from 0 h (18 h starvation) $p < 0.05$.

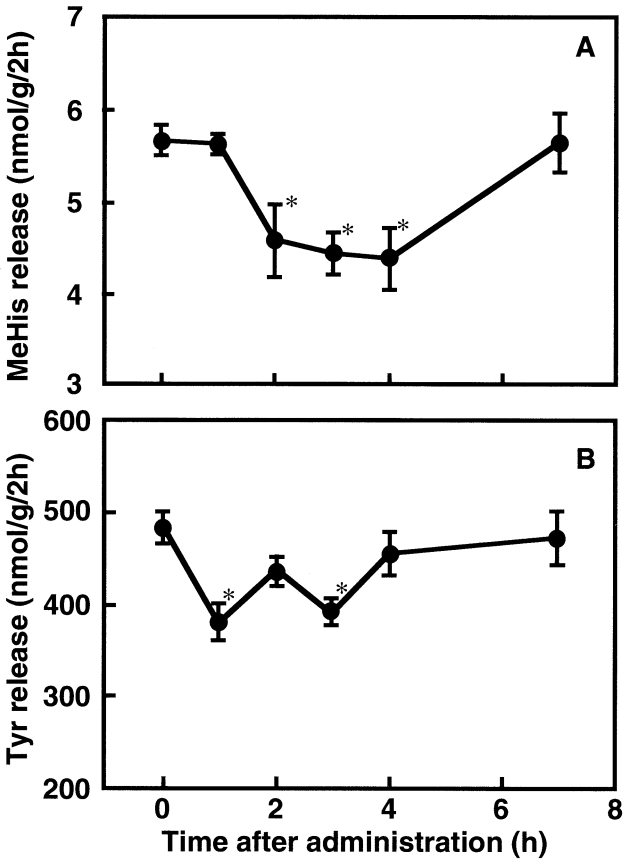

Fig. 4. Changes in N^{τ}-methylhistidine (MeHis) release (A) and tyrosine release (B) from extensor digitorum longus muscle after oral administration of leucine. Values are means ± SE of 5 rats. *Significant difference from 0 h (18 h starvation) $p < 0.05$.

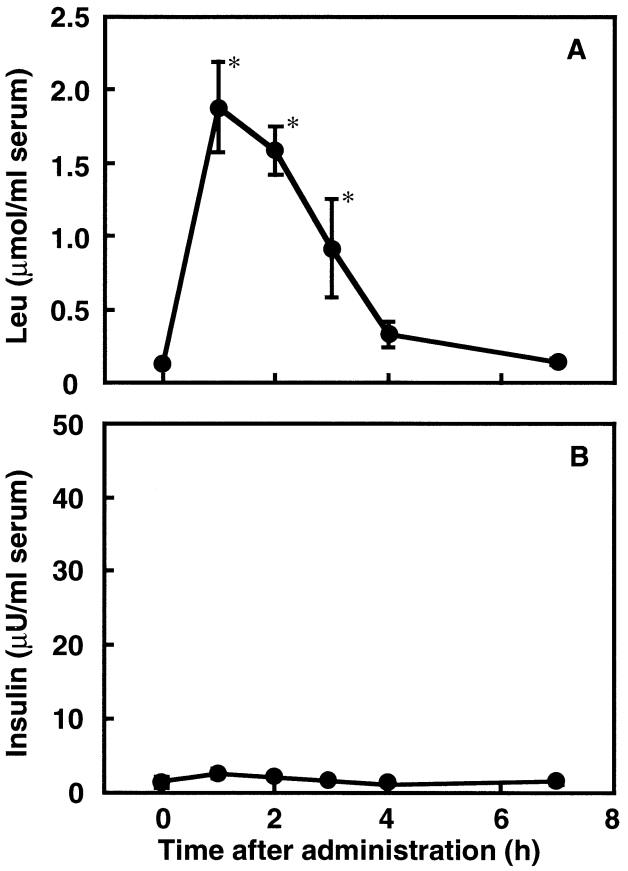

Fig. 5. Changes in serum leucine concentration (A) and insulin concentration (B) after oral administration of leucine. Values are means ± SE of 5 rats. *Significant difference from 0 h (18 h starvation) $p < 0.05$.

report here for the first time by direct measurements that orally administrated leucine regulates muscle protein degradation.

We measured the rate of MeHis release from isolated EDL muscles that show a greater response to physiological changes than does red, soleus muscle [27]. MeHis is contained only in myofibrillar protein (myosin and actin) and is not metabolized and used for protein synthesis in muscle cells [30], therefore the MeHis release from the isolated muscle reflects its myofibrillar protein degradation. On the other hand, many studies have evaluated the degradation rate of total muscle protein by measuring the rate of tyrosine release from the isolated muscle after inhibiting protein synthesis by cycloheximide [5], because tyrosine does not metabolize in muscle cells**.** The rate of released MeHis or tyrosine from the isolated muscle is used in many studies, because it reflects in vivo rate of myofibrillar or muscle protein degradation in many circumstances such as starvation [9], feeding on a protein free diet [19], sepsis [27], burn injury [31], and tumor-bearing [32]. Furthermore, it is easy to evaluate an early response of muscle protein degradation.

In our previous study [19], a 20% casein diet reduced myofibrillar protein degradation at 4–7 h after refeeding, although a protein free diet did not suppress it. We suggested that dietary protein is an important factor for the suppression of myofibrillar protein degradation after refeeding. In the present study, MeHis release from the EDL muscle was significantly reduced at 4h after feeding on an amino acid mixture diet, as was also found with a 20% casein diet (Fig. 1), indicating that amino acids are necessary to inhibit myofibrillar protein degradation after refeeding. The essential amino acid and leucine diets suppressed myofibrillar protein degradation like the amino acid mixture or casein diets (Fig. 3). Moreover, serum leucine concentration was increased after refeeding (Fig. 2). Therefore, leucine is one potential candidate amino acid for the inhibition of myofibrillar protein degradation.

However, the above diets contained carbohydrate (starch and sucrose, Table 1) which affects insulin secretion. Insulin is reported to be a potent modulator of protein synthesis in skeletal muscle [10,11], being associated with the activation of translational factors [12]. Moreover, insulin is a factor that inhibits proteolysis in skeletal muscle [6,8,13–15], although to date the mechanisms remain unclear. Insulin is known to inhibit autophagic proteolysis in the liver [33]. Recently, Bennett et al. [34] suggested that insulin mediates proteolysis by the 26S proteasome in vitro, and Solomon et al. [35] have shown that insulin suppresses the

activities of lysosomal cathepsins and ubiquitination in the muscle of burn-injured rats. Thus, insulin has anabolic effects on skeletal muscle through both synthesis and degradation. Consequently, the postprandial inhibition of myofibrillar protein degradation by feeding may be regulated by insulin. To account for this possibility, we avoided this effect by examining the effect of oral administration of leucine alone.

Myofibrillar protein degradation was suppressed by oral administration of leucine alone (Fig. 4A), although the response was seen at 2–4 h after administration. This result is consistent with those of Anthony et al. [36] who found that the serum MeHis concentration was not suppressed at 1 h after the oral administration of leucine. The serum insulin concentration was nearly constant after leucine administration, although leucine is one of the insulin secretagogue amino acids [37]. These findings strongly suggest that leucine can regulate myofibrillar protein degradation without insulin action. Branched chain amino acids infusion cause induction of muscle protein synthesis [20,23] and suppression of degradation [20,22] in humans and animals. In addition, muscle protein synthesis was increased by the oral administration of amino acids [38,39] or leucine [24, 25,36] in association with an increased eIF4E complex [36]. This postprandial activation of the eIF4E complex is reported to be independent of changes in insulin [40]. Thus leucine may regulate muscle protein metabolism independent of insulin. However, oral leucine administration suppressed myofibrillar protein degradation by 20% (Fig. 4A), whereas leucine diet containing carbohydrate did by 40% (Fig. 3), suggesting that both leucine and insulin may suppress myofibrillar protein degradation after refeeding.

Despite our knowledge of the induction of protein synthesis by the oral administration of amino acids or leucine, there are few studies on the suppression of protein degradation. Goodman and del Pilar Gomez [7] have reported that myofibrillar protein degradation was decreased by refeeding with a diet including protein or amino acids using perfused rat muscle. On the other hand, Rasmussen et al. [38] have shown that the rate of muscle protein degradation measured by phenylalanine kinetics did not alter in response to drinking essential amino acids and carbohydrate after exercise in humans. The difference between their results and ours may be due to the less response of muscle protein degradation to administered amino acids in humans than in rats. Venerando et al. [41] demonstrated that leucine suppresses autophagically mediated proteolysis in isolated rat hepatocytes. Recently, Mordier et al. [42] showed that leucine limitation induces protein degradation in C2C12 myotube cells via the activation of autophagy without mRNA levels of cathepsins. Thus, the mechanisms of inhibition by leucine appear to be related to lysosomal proteolysis.

It is noteworthy that tyrosine release indicating total muscle protein degradation showed a biphasic reduction pattern (Fig. 4B), when rats were administered leucine. The first suppression was rapid and the second was slow. The second reduction corresponded to myofibrillar protein degradation, therefore, the first reduction might be due to the suppression of degradation of sarcoplasmic protein. The different responses between sarcoplasmic and myofibrillar protein degradation have been reported by several authors [8,15,27]. This possibility also means that the mechanisms of degradation including signal transduction are different between them.

In conclusion, postprandial suppression of myofibrillar protein degradation is mainly due to amino acids, particularly leucine. Administration of leucine alone suppressed protein degradation without insulin action. However, it is necessary to examine the effects of other amino acids as well as insulin on muscle protein degradation, and to evaluate the effect in humans.

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