Study of branched chain amino acids as physiologically active substances

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2016

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Chapter 1

General introduction

Amino acids constitute proteins and are primary components of the human body. A total of 20 amino acids are needed for the synthesis of proteins, of which 9 are not synthesized by humans and are called essential amino acids (31). Hence, intake of essential amino acids via consumption of various foods is necessary. Among these essential amino acids, leucine, isoleucine, and valine are collectively referred to as branched-chain amino acids (BCAA) owing to their branched structures. Primary sources of BCAA are animal and plant proteins. Animal protein contains leucine (7–9%), isoleucine (3–6%), and valine (4–6%) in the approximate ratio of 2:1:1 (18-20, 27). Recently, BCAA are obtained from supplements other than food items.

The balance of amino acids in diet is very important. It is well known that addition of one or some amino acids to a low protein diet could cause growth retardation in experimental animals (2, 13, 15, 21, 23-25). Additionally, administration of an excess amount of amino acid could decrease body weight, damage various organs and tissues, and in some serious cases, result in death (2, 23). In particular, the addition of an excess amount of leucine causes decreased food intake and growth retardation in experimental animals (13, 24). However, the underlying mechanism is not yet clear. In chapter 2 and 3, I examined this mechanism to gain further insights.

It was found that amino acids have various functions in addition to being the building blocks of proteins (3, 9, 12, 28, 30-32). Notably, leucine promotes protein translation in the following manner: leucine activates protein kinase mammalian target of rapamycin (mTOR） and increases the phosphorylation of eukaryotic initiation factor 4E-binding protein (4EBP) (3, 33). However, the mechanism by which cells recognize and respond to leucine is not clear (10, 16, 17). As mTOR is reportedly associated with peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (8), I examined the potential contribution of PGC-1α in leucine-activated mTOR (4EBP) signaling in chapter 4.

Another important role of amino acids is that they also act as important energy sources. Most amino acids are metabolized in the liver, but BCAA are metabolized primarily in skeletal muscles (11). BCAA contribute to energy production in skeletal muscles in human during exercise (1, 11). BCAA supplementation has been reported to improve endurance performance (4-7, 22). Mice that lacked the capacity to degrade BCAA showed decreased endurance performance (26). It was observed that transgenic mice overexpressing PGC-1α in skeletal muscles showed increased running capacity with concomitant upregulated expression of branched chain aminotransferase 2 and branched chain α-keto acid dehydrogenase and decreased blood and muscle BCAA concentrations (14, 29). However, the mechanisms by which BCAA improves exercise performance remain unclear. In chapter 5, I examined whether increased PGC-1α-mediated BCAA degradation is required for enhanced endurance performance

after BCAA supplementation.

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Chapter 2

Asparagine synthetase and 3-phosphoglycerate dehydrogenase downregulation in rat liver caused by excess leucine intake are not associated with leucine-caused growth retardation

Introduction

It is well known that leucine is not only a component amino acid of proteins, but also a signaling molecule, promoting protein synthesis and inhibiting protein degradation (2, 3, 19, 31). Because of these roles, leucine is used as a supplement to activate muscle protein synthesis. Although the supplemental intake of amino acids is considered to be safe, the intake of indispensable amino acids such as leucine or methionine causes anorexia, growth retardation and a fatty liver under low-protein dietary conditions in animal experiments (12, 13, 27). These phenomena are called amino acid “imbalance”, i.e., supplementation of a single indispensable amino acid decreases the nutritive value of a low-protein diet rather than increases it (15, 29). Although there have been many reports concerning amino acid imbalance, the mechanism causing it is not yet clear.

Recently, using an animal model, I assessed the safe level of leucine intake from the viewpoint of gene expression (18). The results demonstrated that the tolerable upper intake level of supplemental leucine is 2% of the diet of rats maintained on 6% casein as the sole protein source. Supplementation of leucine at over 2% of the diet reduced food intake and body weight gain. The pathway analysis after the cDNA microarray analysis of the

rat liver showed that leucine most significantly affected the metabolism of alanine and aspartate, in addition to the metabolism of glycine, serine and threonine.

It has been reported that asparagine synthetase (AS, EC 6.3.1.1) and 3-phosphoglycerate dehydrogenase (PHGDH, EC 1.1.1.95) are markedly induced in the liver of rodents that are fed a low-protein diet (14, 16).

The mammalian AS is the only enzyme that catalyzes asparagine synthesis and converts aspartate and glutamine to asparagine and glutamate, respectively, in an ATP-dependent manner (4, 5). The required amount of asparagine is usually sufficiently provided by biosynthesis; hence, asparagine is considered to be a dispensable amino acid. However, weaned rats that were fed an asparagine-deprived diet showed growth retardation. This growth retardation recovered within several days after asparagine deprivation, probably because of the induction of AS in the liver (26). It has also been reported that when cells lacking a functional AS are exposed to asparaginase (EC 3.5.1.1), they undergo cell cycle arrest in G1, and in some cases such as acute lymphatic leukemia (ALL), die by apoptosis (28). Thus, asparaginase has been used as a therapeutic drug against ALL (25). These studies indicate that *de novo* synthesis of asparagine by AS is essential for survival when the availability of asparagine is insufficient to fulfill cellular metabolic demands.

Although there are two ways to synthesize serine - the phosphorylation pathway and the combined action of the glycine cleavage system and serine hydroxymethyltransferase - it is

primarily synthesized by the phosphorylation pathway. In the first step of the phosphorylation pathway, 3-phosphoglycerate derived from glycolysis is metabolized into phosphohydroxypyruvate by PHGDH, a rate-limiting enzyme. Yoshida et al. have successfully generated PHGDH-knockout mice and demonstrated that systemic PHGDH-knockout embryos die 13.5 days post coitum (33), whereas brain-specific PHGDH-knockout mice are viable despite microcephaly (32). These results clearly showed that the serine provided from the mother did not satisfy their requirement and the fetus needs the *de novo* synthesis of most of the serine for tissue growth and development. Recently, significant upregulation of PHGDH expression was observed in proliferating, differentiating and neoplastic tissues (21, 24). Thus, these results suggest that the expression of PHGDH is critical for serine *de novo* synthesis to fulfill the cellular requirement of serine for growth and differentiation.

In contrast to a low-protein diet, a high-protein diet induces serine dehydratase (SDH, EC 4.2.1.13). SDH is expressed specifically in the liver and kidneys, and catalyzes serine and threonine to produce pyruvate and α-ketobutyrate, respectively. It has been reported that SDH in the liver plays an important role in serine catabolism and contributes to 90% of serine degradation (30). Comparing growing rats with mature rats, Iwami's group has demonstrated that SDH expression in the liver is induced by protein intake beyond their protein requirement (17, 20). It thus shows that the induction of SDH is closely related to protein nutrition. Zhong et al. have also

revealed that SDH is strongly induced by the intake of leucine, compared to that of other branched-chain amino acids (BCAA) (34), suggesting that leucine contributes to the regulation of SDH expression.

Marked induction of AS and PHGDH in the liver by a low-protein diet and of SDH by a high-protein diet is considered to be a very important adaptive mechanism to abnormal protein nutrition. If leucine disturbs the expression of these genes, it may affect the growth of animals. For this reason, I examined AS, PHGDH and SDH expression in relation to leucine-induced amino acid imbalance.

Materials and Methods

Animals

Male Sprague-Dawley rats (10 weeks old) were purchased from Japan SLC. (Shizuoka, Japan). They were housed individually in stainless-steel cages in an air-conditioned room at 23°C ± 1˚C with a 12-h light/dark cycle (lights on from 08:00 to 20:00). Rats were acclimated for 3 or 4 days and provided *ad libitum* access to water and a 20% casein diet based on AIN-93G (18). Then, the rats were assigned to the experimental groups described below. Food intake and body weight were measured daily. Growth rate was calculated by dividing initial body weight into final body weight of the experiment. The range of the initial body weight was 280-320 g. All experimental methods were approved by the Animal Experiment Committee of Kyoto Prefectural University and The University of Tokyo. All the rats were managed in line with the “Guidelines for Care and Use of

Laboratory Animals”.

Experimental design

In this study, I used 10-week-old rats in which the protein requirements are fulfilled by ingestion of a 10–12% casein diet (17, 20).

Experiment 1 Rats (n=72) were divided into 12 groups and fed a diet containing 6% (low), 12% (normal) or 40% (high) casein supplemented with 0, 2, 4 or 8% leucine (kindly provided by Ajinomoto, Kanagawa, Japan) for 1 week. The composition of the experimental diets is described elsewhere (18). The rats were allowed free access to food and water during the experimental period.

Experiment 2 Rats (n=72) were fed a 6, 12 or 40% casein diet for 1 week. Food was given *ad libitum* from 20:00 to 08:00 and water was available at all times. Each casein diet group was separated into four groups and orally administrated leucine at 0, 0.13, 0.25 or 0.50 g/mL/100 g body weight, at 11:00 daily. The amounts of administrated leucine corresponded to a 0, 2, 4, 8% leucine diet based on the assumption that the average intake of diet was 6 g/100 g body weight/day. The appropriate amount of leucine was suspended in 0.5% xanthane gum (San-Ei Gen F.F.I, Osaka, Japan) in phosphate-buffered saline (PBS).

Experiment 3 To examine the specificity of leucine, rats (n=20) were divided into four groups and fed a 6% casein diet, a 6% casein diet containing 8% leucine, or a 6% casein diet containing 8% isoleucine or 7.2% valine (equal to 8% leucine on a nitrogen basis). Food and water were available at all times.

Sample Preparation

On the final day, the diet was removed at 08:00. The rats were anesthetized by intraperitoneal administration of sodium pentobarbital (50 mg/kg body weight) and killed by exsanguination with transection of the inferior vena cava in a randomized order at 12:00. The livers were excised, rinsed with ice-cold PBS, frozen in liquid nitrogen and stored at –30˚C.

Quantitative real-time RT-PCR analysis

Total RNA from the liver was isolated by the acid guanidinium isothiocyanate-phenol-chloroform method as described elsewhere (7). Total RNA (10 ng) was used for the reverse transcription reaction using the PrimeScript™ RT reagent Kit (Takara Bio, Siga, Japan), according to the manufacturer's protocol. Quantitative PCR was performed using SYBR Premix Taq™ II (Takara Bio, Siga, Japan) with Rotor-Gene Q (Qiagen, Hilden, Germany). The expression levels of AS, PHGDH, SDH and β-actin were measured individually. To normalize, the relative expression level of AS, PHGDH and SDH was obtained by dividing their expression levels by the expression level of β-actin, which is a housekeeping gene. Primers used for cDNA amplification:

AS Fw, 5′- ACTGCTGTTTTGGCTTC -3′;

AS Rv, 5′- TCTCACCGTCCACATTG -3′;

PHGDH Fw, 5′- TCTGAAGAATGCTGGGACCT -3′;

PHGDH Rv, 5′- GCTTAGCGTTCACCAAGTTCA -3′;

SDH Fw, 5′- TCACCAGTGTTGCCAAGG -3′;

SDH Rv, 5′- TCGTCTACGAACTTCTCG -3′ and

β-actin Fw, 5′- CTACAATGAGCTGCGTGTGG -3′;

β-actin Rv, 5′- ATGGCTACGTACATGGCTGG -3′.

Pre-incubation was performed at 94°C (PHGDH) or at 95°C (AS, SDH and β-actin). The thermal cycling conditions for AS, PHGDH, SDH and β-actin are summarized in Table 1.

Table 1. Thermal cycling parameters for primer optimization. The thermal cycling conditions for AS, PHGDH, SDH and β-actin.

Gene	Temperature (°C)	Time (s)	# of cycles
AS	95	10	50
	58	30	
	72	45	
PHGDH	95	4	45
	60	20	
	72	30	
SDH	95	5	45
	53	30	
	72	30	
β-actin	94	4	35
	62	20	
	72	30	

Western blot

Liver (300 mg) was homogenized in a buffer comprising 0.1 M potassium hydrogen phosphate, 0.1 M potassium dihydrogenphosphate, pH 8.0, 1 mM EDTA, 0.1 mM [(4-formyl-5-hydroxy-6-methylpyridin-3-yl) methoxy] phosphonic acid, 1 mM (2S,3S)-1,4-bis (sulfanyl) butane-2,3-diol and 0.5 mM phenylmethanesulfonyl fluoride using a Polytron homogenizer. Insoluble material was removed

by centrifugation for 30 min at 4°C and 10,000 g, and the supernatant was centrifuged for 60 min at 4°C and 100,000 g. The protein concentration was measured using the Lowry method. Equal amounts of protein were separated by SDS-PAGE and transferred to PVDF membranes (Merck Millipore, Darmstadt, Germany). The membranes were blocked with 5% skim milk in PBS containing 0.1% Tween 20 (blocking buffer) for 1 h at room temperature. The membranes were reacted with primary antibodies (AS: Epitomics, California, USA; PHGDH: supplied by Furuya S.; SDH: Sigma-Aldrich, Missouri, USA; β-actin: Imgenex, California, USA) diluted 1:1,000 in blocking buffer overnight at 4°C or for 1 h at room temperature. The blots were washed 3×10 min with PBS containing 0.1% Tween 20, and then incubated with secondary antibodies (anti-rabbit horseradish peroxidase: Vector Laboratories, California, USA) diluted 1:1,000 in blocking buffer for 1 h at room temperature. The blot was washed 3×10 min with PBS containing 0.1% Tween 20, and then chemiluminescence was detected with an image analyzer (LAS-1000, Fuji Film, Tokyo, Japan).

Statistical analysis

Data were expressed as mean ± SD. Data were tested by two-way ANOVA, to analyze the casein and leucine effect (8). Tukey-Kramer tests for multiple comparisons were performed to determine the significance of differences (8). A difference was considered significant at $P < 0.05$. The analysis was performed by JMP 5.1.2 for Macintosh computers (SAS Institute, North Carolina, USA).

Results

Effects of leucine administered with diet or by oral supplementation between meals on food intake and growth rate of rats (Experiments 1 and 2)

I examined the effects of varying amounts of leucine given with the diet or orally on the food intake and growth rate of rats maintained on a 6, 12 or 40% casein diet. Two-way ANOVA revealed a significant effect of leucine on the food intake and growth rate of rats when it was included in the diet (Table 2), but not when given orally (Table 3). However, there was a significant interaction between casein and leucine in both the administration methods; thus, I performed multiple comparisons to determine the significance of the differences (Table 2, 3). The results showed that leucine supplementation in the diet significantly decreased the food intake and growth rate in a dose-dependent manner in the 6% casein groups, but not in the 12 or 40% casein groups (Table 2). Namely, the food intake of the rats fed a diet containing 8% leucine decreased to 46% compared with that of the rats fed a 6% casein diet without leucine addition. Similarly, the growth rate decreased to 0.89 ± 0.04 when given a diet containing 8% leucine (Table 2). However, in contrast to the diet containing leucine, there were no significant differences within the 6, 12 and 40% casein diet groups when leucine was supplemented orally (Table 3)

Table 2. Food intake and growth rate of rats fed a 6, 12 or 40% casein diet containing 0, 2, 4 or 8% leucine

Caseine	Leucine	Food intake	Body weight[1]		Growth rate
			Beginning	End	
(%)	(%)	(g/100 g B.W./day)	(g)		(Fold)
6	0	6.3±0.4^{ab}	326.6±8.1	352.1±12.6	1.08±0.02^{a}
	2	6.3±0.8^{a}	318.4±13.0	343.1±25.2	1.08±0.04^{a}
	4	5.1±0.5^{c}	325.7±10.5	326.7±10.2	1.00±0.04^{b}
	8	2.9±1.0^{d}	327.0±15.6	290.9±18.5	0.89±0.04^{c}
12	0	5.2±0.6^{bc}	326.3±7.8	345.2±8.2	1.06±0.03^{ab}
	2	5.7±0.2^{abc}	325.5±15.1	355.0±16.3	1.09±0.02^{a}
	4	5.6±0.5^{abc}	326.0±7.5	360.2±10.7	1.11±0.04^{a}
	8	5.3±0.8^{abc}	321.7±6.1	347.8±7.3	1.08±0.03^{a}
40	0	5.2±0.4^{abc}	327.5±6.7	358.4±3.4	1.09±0.01^{a}
	2	5.2±0.5^{bc}	319.2±10.9	345.7±13.7	1.08±0.03^{a}
	4	5.3±0.3^{abc}	322.5±10.1	345.4±11.8	1.07±0.02^{a}
	8	5.1±0.2^{c}	326.5±13.7	352.0±20.7	1.08±0.02^{a}
Statistical significance					
Casein		NS			$P < 0.001$
Leucine		$P < 0.001$			$P < 0.001$
Casein × Leucine		$P < 0.001$			$P < 0.001$

Values are mean ± SD. n = 6. Data were tested by two-way ANOVA, to analyze the casein and leucine effect. When a significant interaction was detected, Tukey-Kramer tests for multiple comparisons were performed to determine significance of differences among individual groups. Labeled values in the same column without a common letter are statistically different, $P < 0.05$.

[1]Original data used were the same as Figure 1 cited in reference (Imamura et al. 2013), and were recalculated to evaluate the effect of casein and leucine on growth of rats by two-way ANOVA.

Table 3. Food intake and growth rate of rats fed a 6, 12 or 40% casein diet and subjected to oral administration of leucine

Casein	Leucine	Food intake	Body weight		Growth rate
			Beginning	End	
(%)	(g/100 g B.W.)	(g/100 g B.W./day)	(g)		(Fold)
6	0.00	5.1±0.3^{ab}	322.6±11.3	338.1±18.1	1.03±0.02^{ab}
	0.13	5.9±0.3^{a}	322.8±6.9	341.3±15.0	1.05±0.03^{ab}
	0.25	5.7±0.3^{ab}	319.4±4.0	329.7±10.8	1.03±0.02^{ab}
	0.50	5.0±0.6^{ab}	322.1±9.3	321.6±12.3	0.99±0.03^{b}
12	0.00	4.8±0.3^{b}	318.1±6.9	333.2±5.9	1.04±0.03^{ab}
	0.13	5.0±0.8^{ab}	320.5±8.1	330.7±24.0	1.03±0.06^{ab}
	0.25	5.1±0.6^{ab}	315.0±14.8	322.8±23.6	1.02±0.03^{ab}
	0.50	5.6±0.4^{ab}	319.5±6.3	342.4±12.0	1.07±0.03^{a}
40	0.00	5.0±0.3^{ab}	324.9±4.7	346.6±5.8	1.06±0.02^{a}
	0.13	4.9±0.4^{b}	322.5±10.6	338.5±12.3	1.04±0.01^{ab}
	0.25	5.2±0.4^{ab}	320.4±5.3	347.8±7.0	1.08±0.01^{a}
	0.50	4.9±0.4^{ab}	321.3±5.5	337.5±1.9	1.04±0.02^{ab}
Statistical significance					
Casein		$P = 0.027$			$P = 0.041$
Leucine		NS			NS
Casein × Leucine		$P = 0.008$			$P = 0.006$

Values are mean ± SD. n = 3–6. Data were tested by two-way ANOVA, to analyze the casein and leucine effect. When a significant interaction was detected, Tukey-Kramer tests for multiple comparisons were performed to determine significant differences among individual groups. Labeled values in the same column without a common letter are statistically different, $P < 0.05$.

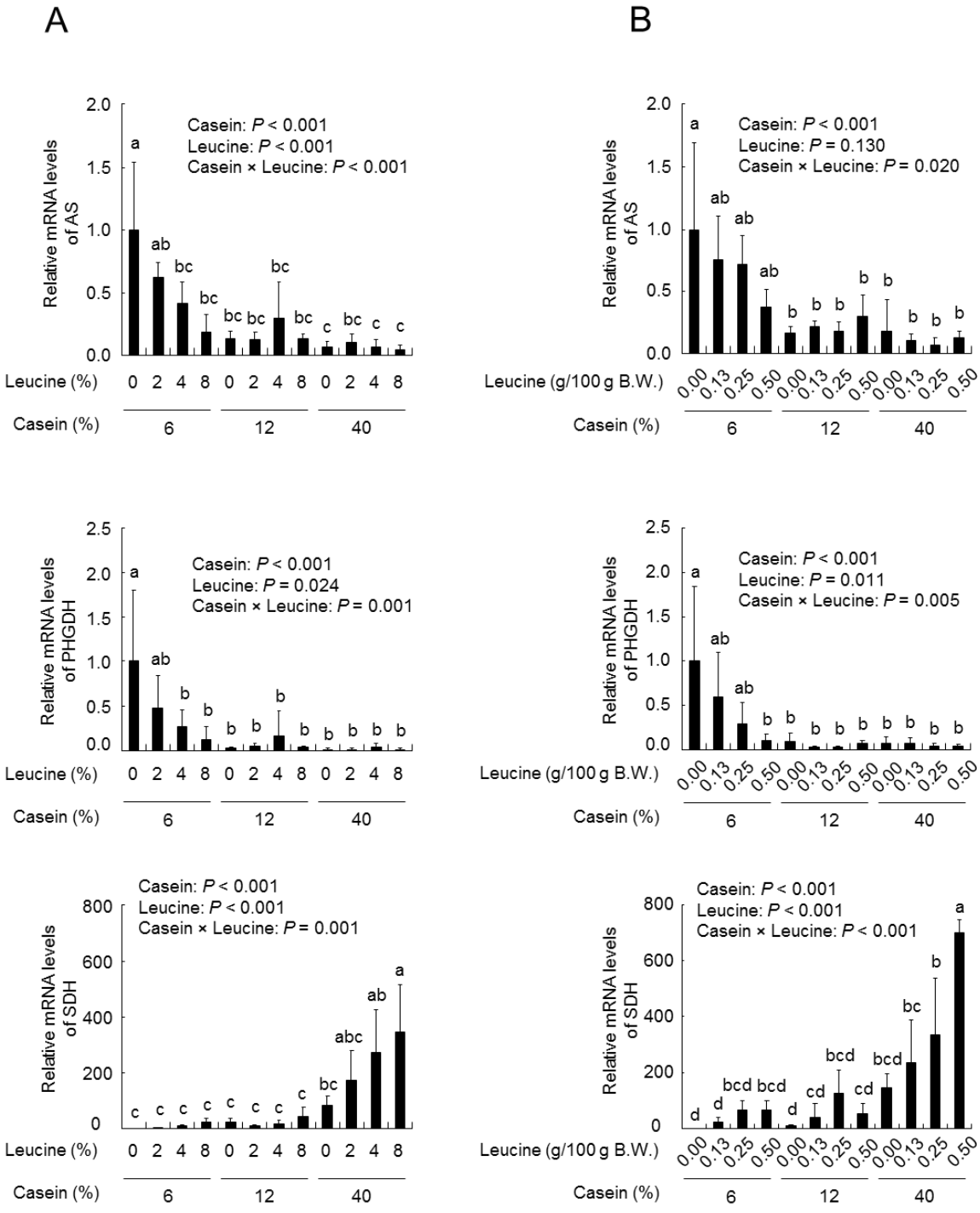

Fig. 1. Effects of diet and oral administration of leucine on AS, PHGDH and SDH mRNA expression in the rat liver. (A) Rats were fed a 6, 12 or 40% casein diet containing 0, 2, 4 or 8% leucine for 1 week. (B) Rats fed a 6, 12 or 40% casein diet were subjected to oral administration of leucine at 0, 0.13, 0.25 or 0.50 g/100 g body weight for 1 week. Data were tested by two-way ANOVA, to analyze the casein and leucine effect. Results are shown in figures, respectively. When a significant interaction was detected, Tukey-Kramer tests for multiple comparisons were performed to determine the significance of the differences among individual groups. Values are mean ± SD. n = 3–6. Labeled values without a common letter are statistically different, $P < 0.05$

Leucine either administered with diet or orally between meals similarly affected the expression of AS, PHGDH and SDH (Experiments 1 and 2)

Gene expression measured by quantitative real-time PCR was analyzed by the two-way ANOVA. The result indicated that both casein and leucine affected the expression of AS, PHGDH and SDH mRNA. Leucine administered either with the diet or orally between meals decreased the high expression of AS and PHGDH that was induced by the 6% casein diet in a dose-dependent manner. On the other hand, the SDH expression was induced by the 40% casein diet, and leucine increased the SDH mRNA expression in a dose-dependent manner (Fig. 1A, B).

Specific effects of leucine among the BCAA on AS, PHGDH and SDH gene expression (Experiment 3)

I fed rats with a leucine, isoleucine or valine diet, containing the same amount of nitrogen. The effect of isoleucine and valine was marginal compared with that of leucine on the food intake and growth rate of rats (Table 4). Leucine suppressed the food intake to 50% compared with that in the control, and decreased the growth rate to 0.89 ± 0.05. However, isoleucine and valine suppressed the food intake to 80% compared with that in the control, and the growth rate was 1.06 ± 0.02. The data indicated that among the BCAA, excess leucine intake strongly suppressed food intake and weight gain. Moreover, compared with leucine, isoleucine and valine had weaker effects on the expression of AS and PHGDH mRNA, and they did not induce SDH mRNA expression (Fig. 2A). These changes in the mRNA

levels were accompanied by similar changes in protein levels (Fig. 2B).

Table 4. Food intake and growth rate of rats fed with branched-chain amino acids

Group	Food intake	Body weight		Growth rate
		Beginning	End	
	(g/100 g B.W./day)	(g)		(Fold)
Control	6.4±0.4[a]	355.2±11.5	406.1±21.3	1.13±0.03[a]
Leucine	3.3±0.7[c]	351.5±5.8	315.3±17.5	0.89±0.05[c]
Isoleucine	5.2±0.2[b]	358.0±7.7	379.3±5.4	1.06±0.02[b]
Valine	5.2±0.2[b]	350.0±8.7	372.3±10.9	1.06±0.02[b]

Values are mean ± SD. n = 5. Data were tested using Tukey-kramer tests. Labeled values in the same column without a common letter are statistically different, $P < 0.05$.

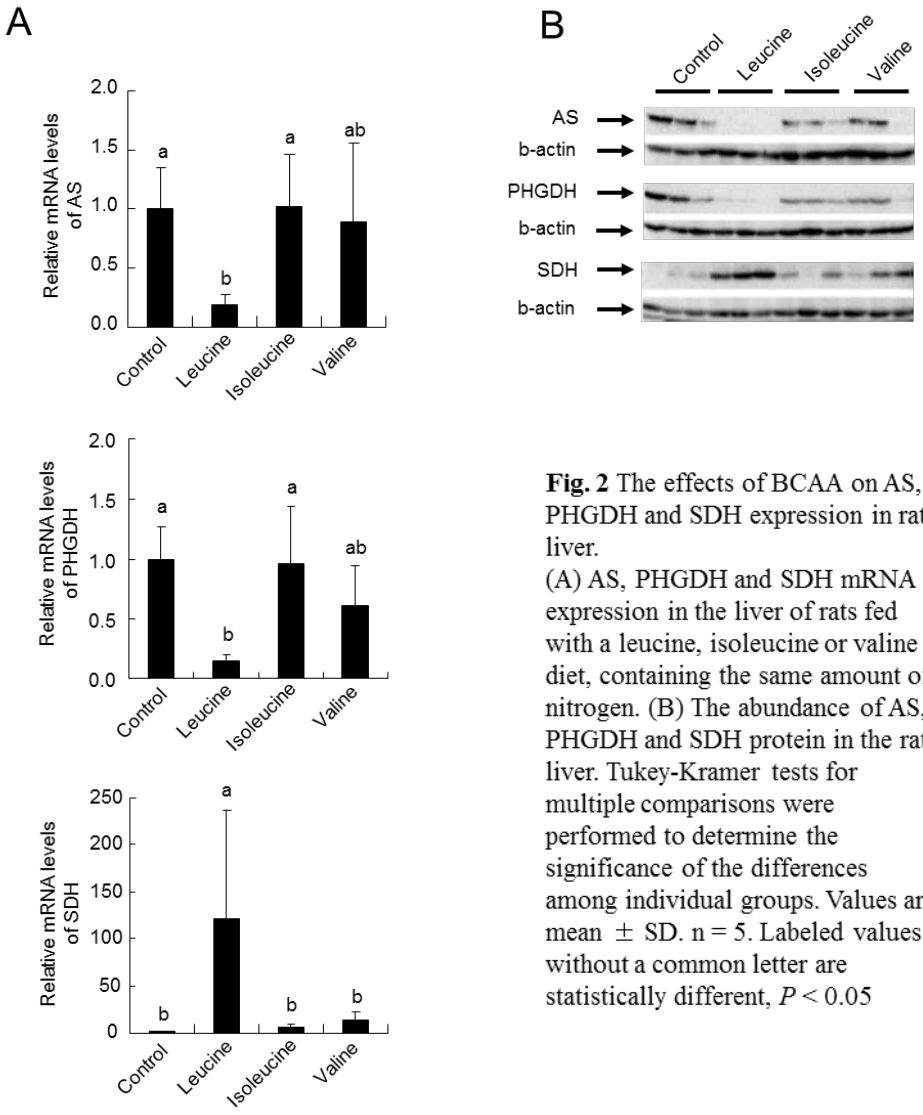

Fig. 2 The effects of BCAA on AS, PHGDH and SDH expression in rat liver.
(A) AS, PHGDH and SDH mRNA expression in the liver of rats fed with a leucine, isoleucine or valine diet, containing the same amount of nitrogen. (B) The abundance of AS, PHGDH and SDH protein in the rat liver. Tukey-Kramer tests for multiple comparisons were performed to determine the significance of the differences among individual groups. Values are mean ± SD. n = 5. Labeled values without a common letter are statistically different, $P < 0.05$

Discussion

The food intake and growth rate decreased with excess leucine intake in the 6% casein diet group, but not in the 12 or 40% casein diet groups (Table 2). In contrast, oral administration of leucine did not significantly change the food intake or growth rate (Table 3), indicating that the effects of leucine depend on the timing of administration. On the other hand, both dietary intake and oral administration of leucine decreased AS and PHGDH mRNA expression in the 6% casein diet and increased SDH mRNA expression in the 40% casein diet in a dose-dependent manner (Fig. 1A, B). The effect of leucine on gene expression appears to be specific, because isoleucine and valine did not change the expression of AS, PHGDH or SDH (Fig. 2). Although the expression of AS and PHGDH in rat liver is known to be induced by a low-protein diet (14, 16), I revealed here for the first time that leucine downregulates the expression of AS and PHGDH *in vivo*.

I hypothesized that changes in AS, PHGDH and SDH expression contribute to growth retardation in rats during excess leucine intake. Decreased expression of AS and PHGDH, amino acid synthesis enzymes, and increased expression of SDH, an amino acid catabolic enzyme, may cause an amino acid imbalance. Interestingly, although dietary leucine intake caused growth retardation while oral administration did not, the gene expression patterns of AS, PHGDH and SDH were similar in both cases. This suggests that there are no causal relationships between AS, PHGDH and SDH expression and growth retardation.

In rats showing growth retardation, the food intake was markedly reduced. Cota et al. have reported that intraventricular administration of leucine suppressed food intake through the hypothalamic mammalian target of rapamycin (mTOR) activation (9). Thus, leucine may suppress food intake by activation of the hypothalamic mTOR pathway. From this point of view, growth retardation is primarily mediated through the suppression of food intake. In contrast to dietary leucine, the orally administered leucine did not cause suppression of food intake, which could be because of the time of leucine intake. When rats were given leucine orally between meals, the effect of leucine was attenuated, and by the next feeding the rats ate a normal amount. Indeed, plasma leucine transiently increased at 30 min and returned to the basal level 2 h after oral administration of BCAA enrichment (10). On the other hand, I did not observe the decreased food intake caused by an excess amount of leucine when the rats were fed a 12% or 40% casein diet. Niijima et al. have reported that the sensitivity of lysine sensors in the hepato-portal region was 100-fold higher in lysine-deficient rats than in normal rats (22). Thus, sensitivity of the amino acids sensors may therefore have increased in the rats maintained on a 6% casein diet.

It has been demonstrated that *de novo* synthesis of asparagine and serine was critical for cellular growth and function (21, 24, 28, 32, 33). AS and PHGDH are known to be expressed in several tissues at different levels. This suggests that the expression of these enzymes depends on the metabolic demand of the tissues for asparagine and serine. If leucine

changes the expression of AS and PHGDH in respective tissues, it may affect their physiological function in the tissues. For example, an adverse effect of leucine on the immune system has been reported (11). Thus, to further understand the effects of the leucine-induced amino acid imbalance, it should be determined whether leucine affects the expression of AS and PHGDH in tissues other than the liver in relation to the tissue function.

Because the liver is a dominant organ for amino acid homeostasis, induction of AS and PHGDH seems to be an adaptation to amino acid deficiency, to provide adequate asparagine and serine to peripheral tissues to fulfill their metabolic demand for maintaining cellular functions under low-protein nutrition. The amino acid response (AAR) pathway, a signal transduction pathway activated to sense amino acid deficiency, was found during the study of the induction mechanism of AS in a cultured cell line. The AAR pathway is activated by a deficiency in amino acids, particularly indispensable amino acids, and the translation of the downstream activating transcription factor 4 (ATF4) is stimulated (6, 23). ATF4 binds to a specific element called nutrient-sensing response element-1 (NSRE1), which exists within the AS promoter, and activates AS transcription. The AAR pathway is suppressed by addition of a single indispensable amino acid including leucine to the culture medium. I demonstrated here that the expression of AS was increased by feeding rats a low-protein diet, and the induction was suppressed by administration of leucine, suggesting that the AAR pathway is involved in the adaptive change in AS in response to protein nutrition *in vivo*. It

is conceivable that the concomitant expression of PHGDH and AS is regulated through the AAR pathway. However, Kanamoto et al. could not find a putative NSRE1 in the promoter region of PHGDH with a data base search (data not published). On the other hand, it has been reported that PHGDH expression in rat liver is induced by insulin and suppressed by glucocorticoid (1, 14), though there were no reports of hormonal regulation of AS. Recently, three signal transduction pathways, mTOR, 5' AMP-activated protein kinase and general control nonderepressible 2 have been proposed to be involved in amino acid sensing in the liver, and to coordinately regulate the hepatic energy metabolic pathway in response to protein intake (8). Although I did not examine the regulatory mechanisms of the expression of AS, PHGDH and SDH in current study, it seemed that these enzymes were coordinately expressed in response to protein nutrition. Clarifying the regulatory mechanism of these enzymes' expression may provide useful information for understanding the amino acid-sensing mechanism in the liver and the adaptive changes in the hepatic amino acid metabolism in response to protein nutrition.

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Chapter 3

The Vagotomy Alleviates the Anorectic Effect of an Excess Amount of Dietary Leucine on Rats Fed a Low-protein Diet

Introduction

Leucine has been reported to have a role as a signal factor to stimulate protein synthesis and inhibit protein degradation in animal muscles. Leucine is therefore taken by athletes in a relatively high amount with the expectation of such effects. However, it is also well known that leucine has an adverse effect on experimental animals. An excess amount of leucine fed with a low-protein diet has reduced the food intake and body weight of rats (5, 11). To evaluate the adverse effects of an excessive leucine intake, I have previously conducted a study to identify the gene expression markers reflecting such an excessive intake of leucine by using a microarray analysis. Six genes known to be regulators of growth or of the cell cycle were identified as biomarkers of the adverse effects of excessive leucine. The cut-off value for the biomarker panel indicated that a leucine level of no more than 2% with 6% dietary protein had no adverse effects, but a level higher than 3% was a potential hazard. A leucine level higher than 3% also showed growth retardation and a reduced food intake (6). However, the mechanism for the anorectic effect caused by an excess amount of dietary leucine fed with a low protein diet had not been elucidated.

The vagal sensory mechanism plays a crucial role in the neural mechanism for satiation. Afferent fibers of the vagal nerve are an integral part of the brain-gut axis which take part in feedback loop controlling food intake induced by presence of food (13). Ohinata et al.

have demonstrated by using a vagotomy that the orexigenic activity of zinc was mediated by the afferent vagal nerve in a zinc-deficient rat (10). I performed a vagotomy in the present study to examine whether the anorectic effect of an excess amount of leucine was mediated by the afferent vagal nerve.

Material and Methods

Animals

Nine-week-old male Sprague-Dawley rats (SLC, Shizuoka, Japan) were individually housed under regulated conditions (23 ± 1˚C with a 12-h-light/12-h-dark cycle, lights on 08.00-20.00). The rats were acclimatized for 3 d and provided *ad libitum* access to a 20% casein (20C) diet, based on the AIN93G diet described elsewhere (6), (Table 1) and water. The experiment was approved by the Kyoto University Ethics Committee for Animal Research Use.

Table 1. Composition of the Diets (%)

Nutrient	20C	6C	6C+8L
Casein	20.0	6.0	6.0
Leucine	-	-	8.0
Corn starch	60.50	77.41	69.41
Cysteine	3.0	0.09	0.09
Soy bean oil	7.0	7.0	7.0
Cellulose	5.0	5.0	5.0
Mineral mix (AIN-93G)	3.5	3.5	3.5
Vitamin mix (AIN-93)	1.0	1.0	1.0
Total	100.0	100.0	100.0

Experimental procedures

A truncal vagotomy was performed as previously described (10), the vagal nerves being cut above the hepatic and celiac branch. The rats

(two normal rats without the surgical operation and three vagotomized rats) were fed the 20C diet until the vagotomized rats had ingested an equal amount of the diet to that of the normal rats. The experiment was started to feed all the rats a 6% casein (6C) diet at 08.00, the food intake and body weight being measured on the following 2 d at 08.00 every morning. The rats were next fed the 20C diet for 4 d. The diet was then changed to the 6% casein diet containing 8% leucine (6C + 8L) at 08.00, and the food intake and body weight were measured on the following 2 d as already described. This feeding regimen was conducted twice (Fig. 1A). The vagotomy was assessed by performing a food intake analysis to treat the rat intraperitoneally with cholecystokinin-octapeptide (CCK8; Peptide Institute, Osaka, Japan) at a dose of 16 μg/kg of body weight, since the satiety induced by CCK8 is mediated by the afferent vagus nerve (2, 8). I confirmed that the vagotomized rats did not show any significant decrease in their food intake by injecting CCK8 (data not shown).

Statistical analysis

Results are presented as the mean ± SD. The Tukey-Kramer method was used for multiple comparisons to determine significant differences, differences being considered significant at $p < 0.05$. The analysis was performed by using JMP 5.1.2 for the Macintosh computer (SAS Institute, North Carolina, USA).

Results and Discussion

Figure 1B shows that the food intake by the normal and vagotomized rats did not differ by feeding either the 20C or 6C diet. Two days feeding of the 6C + 8L diet reduced the food intake of the normal

rats to 40%, whereas only a slight decrease of food intake was apparent in the vagotomized rats.

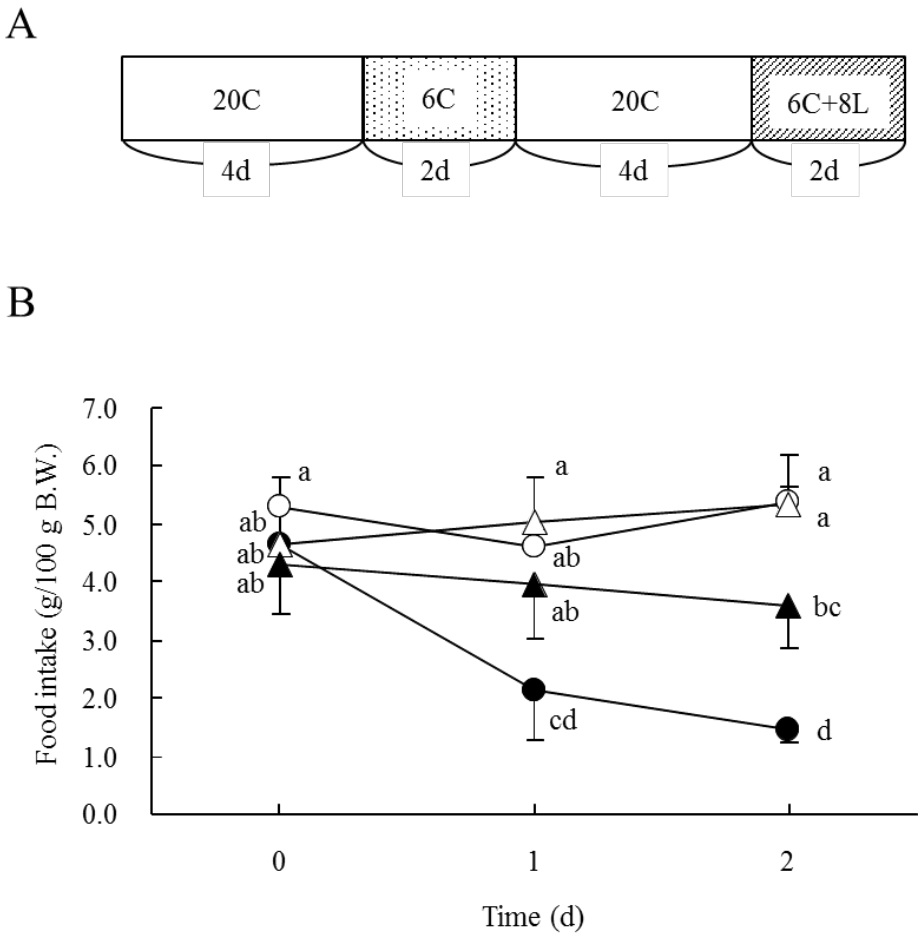

Fig. 1. The Vagotomy Alleviated the Anorectic Effect of an Excess Amount of Dietary Leucine.
A, Outline of the experimental protocol for measuring the food intake. 20C, the 20% casein diet; 6C, the 6% casein diet; 6C + 8L, the 6% casein diet containing 8% leucine. This feeding regimen was conducted twice. B, Two normal (○, ●) and three vagotomized rats (△, ▲) were fed the 6C (○, △) or 6C+8L (●, ▲) diet. The results of repeated experiments were combined and are shown as the mean ± SD (normal n=4, vagotomized n=6). Symbols not sharing common letters were significantly different at $p < 0.05$.

Such gastrointestinal hormones as CCK, glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) have been shown to exert an anorectic effect through the vagus nerve (7, 12, 14), and the excretion of these hormones is known to be stimulated by protein and amino acids (1, 3).

On the other hand, it has been reported that the satiety signal of amino acids was mediated by vagal chemical sensors in the hepato-portal region and duodeno-intestinal canal. An intraduodenal infusion of a large amount of leucine also evoked an excitatory response in the vagal celiac afferents (9). My current observations suggest that an excess amount of dietary leucine exerted an anorectic effect through the afferent vagus nerve, at least in part. It can be speculated that leucine stimulated the excretion of such gastrointestinal hormones as CCK, GLP-1 and PYY, and/or directly acted on the vagal chemical sensors.

The direct effect of leucine on the central nervous system has recently been reported. The central administration of leucine has thus increased hypothalamic mTOR signaling and decreased the food intake and body weight (4). Figure 1B also shows that the reduced food intake by an excess amount of dietary leucine was not completely recovered in the vagotomized rat. It is therefore possible that part of the anorectic effect of dietary leucine would be to increase the leucine concentration in the brain that activates the mTOR pathway to reduce the food intake.

However, I did not observe the anorectic effect of an excess amount of leucine when the rats were maintained on a normal or high-protein diet (6). Niijima et al. have reported that the sensitivity of lysine sensors in the hepato-portal region was 100-fold higher in lysine-deficient rats than in normal rats (9). The sensitivity of the amino acids sensors may therefore have increased in the rats maintained on a low-protein diet.

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Chapter 4

Phosphorylation of 4EBP by oral leucine administration was suppressed in the skeletal muscle of PGC-1α knockout mice

Introduction

Branched chain amino acids (BCAA) such as valine, leucine, and isoleucine are essential amino acids and components of proteins which are known to stimulate protein biosynthesis (15). Among the BCAA, leucine is known to be a signaling molecule that stimulates protein biosynthesis in tissues such as the skeletal muscle and liver (16). The primary regulator of protein biosynthesis is mammalian target of rapamycin (mTOR), which is an evolutionally conserved serine/threonine kinase (13). Eukaryotic initiation factor 4E-binding protein (4EBP) is a suppressor of protein translation and a substrate for mTOR. The phosphorylation of 4EBP by mTOR prevents the suppressor activity of 4EBP and, thus, increases protein biosynthesis (15). The phosphorylation status of 4EBP determines its binding to eukaryotic translation initiation factor 4E (eIF4E), a rate-limiting component of the eukaryotic translation apparatus, and suppressing protein translation; γ phosphorylation form, but not α, β phosphorylation forms, of 4EBP does not bind to eIF4E, and does not inhibit translation (14). Further, it has been reported that the oral administration of leucine in rodents increases the phosphorylation of 4EBP (γ form) and stimulates protein synthesis (1, 2).

Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) is a co-activator of transcription factors, including nuclear receptors and is known to increase mitochondrial biogenesis and mitochondria-rich type-I fiber formation in skeletal muscle (8, 11). D'Antona et al. reported

that the administration of BCAA in aged mice caused an increase in PGC-1α levels in their skeletal muscle (6). PGC-1α is reported to associate with mTOR (5) and may play a role in mTOR-mediated protein biosynthesis. However, the relationship between BCAA (particularly leucine), mTOR, and PGC-1α has not yet been investigated. Thus, in this study, I examined the role of PGC-1α in leucine-activated mTOR (4EBP) signaling using PGC-1α knockout (PGC-1α KO) mice in skeletal muscle.

Material and Methods

Genetically modified animals

To control the ablation of PGC-1α, I generated a conditional KO version of the PGC-1α gene using the Cre-loxP recombination system. Exons 3 to 5 of the PGC-1α gene were flanked by loxP sites in the target construct (10). Mice with the conditional allele of PGC-1α were crossed with transgenic mice expressing the Cre recombinase in skeletal muscle driven by the human α-actin promoter (3). Homozygous PGC-1α lox allele mice were crossed with heterozygous Cre transgenic mice, and the offsprings were used for experiments. The genotypes of offspring were PGC-1α flox/flox with Cre (PGC-1α KO) and PGC-1α flox/flox without Cre (wildtype, WT). Mice were maintained in a 12-h light/dark cycle at 24°C and were fed a normal chow diet ad libitum (CRF-1; Oriental Yeast, Tokyo, Japan). Mice were cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and my institutional guidelines. All animal experiments were conducted with the approval of the Institutional Animal Care and Use Committee of Kyoto Prefectural University (No. KPU260407).

Quantitative real-time RT-PCR analysis

Total RNA was prepared using TRIzol (Life Technologies, Carlsbad, CA, USA). cDNA was synthesized from 500 ng of total RNA using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). Gene expression levels were measured with ABI PRISM 7000 using Thunderberd SYBR qPCR Mix (Toyobo, Osaka, Japan) designed to detect cDNAs. The following primers were used:

PGC-1α Fw, 5′- CGGAAATCATATCCAACCAG -3′;

PGC-1α Rv, 5′- TGAGGACCGCTAGCAAGTTTG -3′ and

36B4 Fw, 5′- GGCCCTGCACTCTCGCTTTC -3′;

36B4 Rv, 5′- TGCCAGGACGCGCTTGT -3′.

Measurement of citrate synthase activity

The enzyme activity of citrate synthase was measured by spectrophotometric analysis. The citrate synthase assay was performed at 412 nm following the reduction of 5, 5'-dithiobis (2-nitrobenzoic acid) as previously described (12).

Histological analysis

The samples of the tibialis anterior muscle from WT and PGC-1α KO mice at 12 weeks of age were frozen in liquid nitrogen-cooled isopentane, and transverse sections were analyzed by enzyme histochemistry to evaluate succinate dehydrogenase activities (9).

Western blot

After fasting for 24 h, leucine (1.35 mg/g body weight) or vehicle were administered to the experimental mice. Thirty minutes later, the samples of skeletal muscle and liver were obtained. Western blot analysis

was performed as previously described (7). The following primary antibodies were used: anti-phospho 4EBP (#2855; Cell Signaling Technology Japan, Tokyo, Japan) and anti-GAPDH (C14C10; Cell Signaling Technology Japan, Tokyo, Japan). Western blot signals were calculated using densitometry (LAS 1000; Fuji Film, Tokyo, Japan).

Statistical analysis

Data were evaluated by Student's t-test or one-way analysis of variance followed by Tukey's honestly post-hoc test. P values below 0.05 were considered statistically significant.

Results and Discussion

Fig. 1A shows that PGC-1α mRNA level was markedly decreased in the skeletal muscle of PGC-1α KO mice compared with that of WT mice, but not in the liver of WT and PGC-1α KO mice. To assess functionality of the decreased PGC-1α mRNA in PGC-1α KO mice, I examined mitochondrial marker levels in the skeletal muscle of the PGC-1α KO mice, as PGC-1α is a regulator of mitochondrial biogenesis. Fig. 1B shows that a decrease in the activity of citrate synthase, a mitochondrial enzyme of the TCA cycle (4), was observed. The histological staining of succinate dehydrogenase, another mitochondrial enzyme of the TCA cycle, also showed decreased signal in the transverse sections of the skeletal muscle of PGC-1α KO mice (Fig. 1C). Thus, in the PGC-1α KO mice, mitochondrial activity is decreased, suggesting that PGC-1α is functionally knocked out in the skeletal muscle.

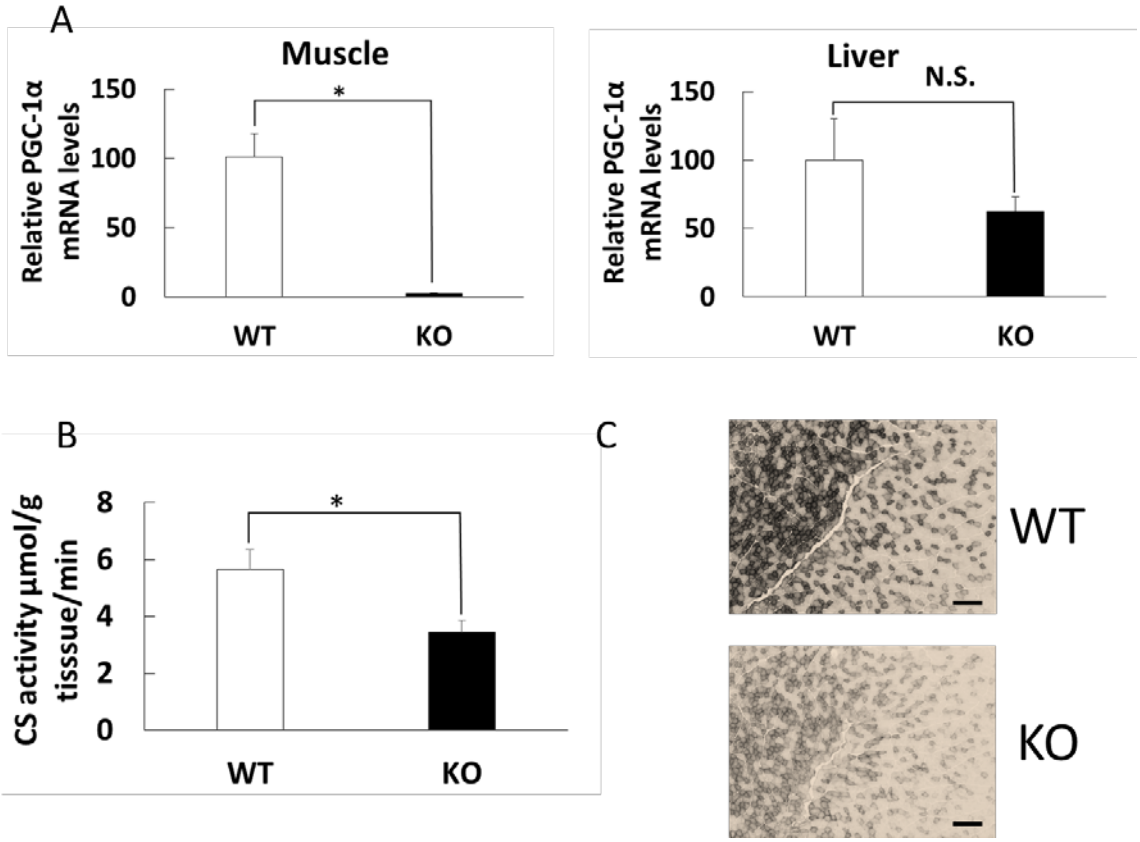

Fig. 1 PGC-1α mRNA level in skeletal muscle of WT and PGC-1α KO mice and mitochondrial enzyme activities in skeletal muscle of PGC-1α KO mice
(A) mRNA levels of PGC-1α in the skeletal muscle and liver of WT and PGC-1α KO mice. Relative mRNA levels are shown. White bar is WT and black bar is PGC-1α KO. Values are expressed as mean ± SE (n=6 for WT, n=7 for KO). *P < 0.05. (B) Citrate synthase activity. White bar is WT and black bar is PGC-1α KO. Values are expressed as mean ± SE (n=7 for WT, n=9 for KO). *P < 0.05. (C) Succinate dehydrogenase staining in WT and PGC-1α KO. Scale bar is 200 mm.

I orally administered leucine to WT and PGC-1α KO mice, after which the phosphorylation of 4EBP in the skeletal muscle and liver was examined. In WT mice, consistent with the previous reports (1, 2, 16), the phosphorylation of 4EBP, including the γ form, was increased in the skeletal muscle (Fig. 2A, B). In contrast, phospho-4EBP (γ form) level was markedly reduced in the skeletal muscle of PGC-1α KO mice (Fig. 2A, B). In the liver, leucine administration increased phospho-4EBP level (γ form) both in both WT and PGC-1α KO mice (Fig. 2C, D). Thus, PGC-1α is involved in leucine-mediated mTOR activation and possibly in

protein biosynthesis.

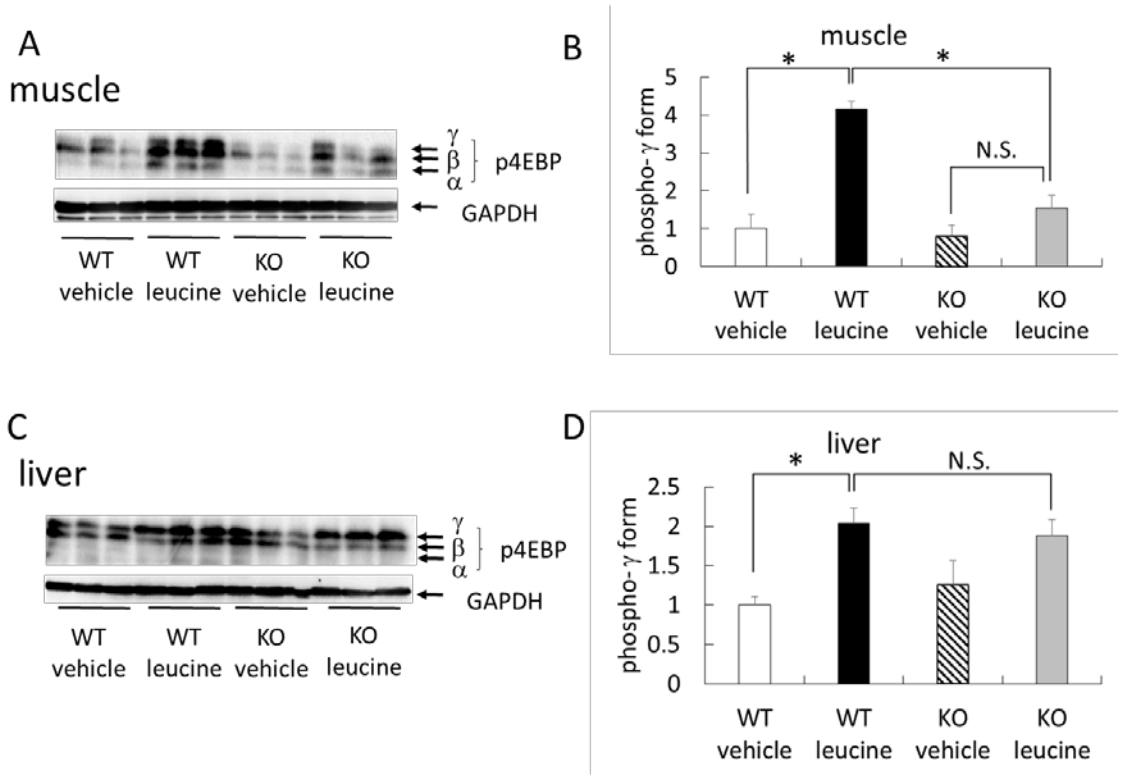

Fig. 2 Phospho-4EBP levels in the skeletal muscle and liver of mice following leucine administration (A) Representative Western blot analysis of skeletal muscle. Phospho-4EBP levels are shown. Positions of the α, β, and γ forms of 4EBP are indicated by arrows. Oral leucine or vehicle was administered to WT and PGC-1α KO mice. Representative blots are shown. (B) Densitometric analysis of the western blot shown in A. White bar: WT with vehicle, black bar: WT with leucine, white bar with slash: PGC-1α KO with vehicle, gray bar: PGC-1α KO with leucine. Values are expressed as mean ± SE (n = 3). *P < 0.05. (C) Western blot analysis of the liver. (D) Densitometric analysis of the western blot shown in C. Values are expressed as mean ± SE (n = 3). *P < 0.05.

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Chapter 5

Enhanced exercise performance through the intake of branched chain amino acids requires PGC-1α in murine skeletal muscles

Introduction

During endurance exercise, the body consumes energy from many sources, including carbohydrates, fats, and proteins (7). Proteins are degraded to amino acids, which are used as energy sources during exercise. Leucine, isoleucine, and valine are collectively referred to as branched-chain amino acids (BCAA). BCAA are oxidized in skeletal muscles and are important energy sources during exercise (1, 7). The first and second steps of the muscle BCAA degradation pathway are catalyzed by the branched-chain aminotransferase 2 (BCAT2) and branched-chain α-keto acid dehydrogenase (BCKDH), respectively (18). BCAT2 catalyzes the reversible transamination of BCAA into branched-chain α-keto acids (BCKA). BCKDH irreversibly catabolizes BCKAs into CoA compounds. These catabolites then enter the TCA cycle for energy production.

Peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) is a transcriptional coactivator, whose expression is induced by exercise in the skeletal muscle (11). PGC-1α plays critical roles in the regulation of mitochondrial content and function. PGC-1α also induces the upregulation of fatty acid oxidation and oxidative phosphorylation (11, 19). Tadaishi et al. have reported that transgenic mice overexpressing skeletal muscle PGC-1α (PGC-1α Tg) displayed increased running capacity during a treadmill experiment with a concomitant increase in mitochondria and fatty acid oxidation (19). Additionally,

Hatazawa et al. found that PGC-1α Tg mice demonstrated upregulated BCAT2 and BCKDH expressions in the skeletal muscle (10). BCAA levels in the skeletal muscle and plasma were decreased in these mice, suggesting increased BCAA degradation in PGC-1α Tg (9, 10). A recent study showed that rats with high exercise capacity showed increased BCAA metabolism compared to those with low exercise capacity (15). Moreover, in humans and rats, BCAA administration appears to improve endurance exercise capacity (3-6, 13). However, the mechanisms by which BCAA improves exercise performance remain unclear.

In this study, I examined whether increased PGC-1α-mediated BCAA degradation is required for enhanced endurance exercise capacity after BCAA supplementation.

Material and Methods

Animals

Skeletal muscle-specific PGC-1α knockout mice (PGC-1α KO) were generated as previously described (16, 17, 21). PGC-1α KO mice (males) and age- and sex-matched wild-type (WT) littermate mice were maintained in a 12-h light/dark cycle at 24°C and fed a normal chow diet *ad libitum* (AIN93G; Research Diet, New Jersey, USA). Food intake, body weight, and voluntary wheel running were measured daily. Mice were cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and my institutional guidelines. All animal experiments were conducted with the approval of the Institutional Animal Care and Use Committee of Kyoto Prefectural University (No. KPU260407).

Exercise protocol

WT and PGC-1 KO mice were housed individually in cages equipped with a voluntary running wheel (diameter 200 mm, width 20 mm) for 3 weeks of voluntary training. The mice were administered either saline or BCAA [0.15-mg/g body weight; LIVACT (46% leucine, 28% valine, and 23% isoleucine), Ajinomoto, Tokyo, Japan] 30 min before the exercise tolerance test. The exercise capacity was determined based on a previously described exercise tolerance test with slight modifications (8). Mice were subjected to a running test on a treadmill. The mice were then challenged with a 10% uphill run starting at 10 m/min for 5 min. The speed was increased by 2 m/min in 2-min increments, up to a maximum speed of 30 m/min. The exhaustion was indicated by a mouse remaining on the shocker plate for over 20 sec. Then, all mice were again housed individually in cages equipped with a voluntary running wheel for 6 days. Mice were sacrificed after saline or BCAA administration 30 min prior to the running session at 20 m/min for 25 min.

Quantitative real-time RT-PCR analysis

Total RNA was prepared using Sepasol-RNA I Super G (Nakalai Tesque, Kyoto, Japan). cDNA was synthesized from 500 ng of total RNA, using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). Gene expression levels were measured as described previously (12). mRNA levels were normalized to those of a housekeeping gene 36B4 mRNA. The following primers were used:

PGC-1α Fw, 5′-CGGAAATCATATCCAACCAG-3′;

PGC-1α Rv, 5′-TGAGGACCGCTAGCAAGTTTG-3′;

BCAT2 Fw, 5′-CGGACCCTTCATTCGTCAGA-3′;

BCAT2 Rv, 5′-CCATAGTTCCCCCCCAACTT-3′;

BCKDH Fw, 5′-CGGCAACGATGTGTTTGCTG-3′;
BCKDH Rv, 5′-ATTGACCTCGTCCACCGAAC-3′; and
36B4 Fw, 5′-GGCCCTGCACTCTCGCTTTC-3′;
36B4 Rv, 5′-TGCCAGGACGCGCTTGT-3′.

Measurement of amino acid levels

Samples of muscle tissue were homogenized in five volumes of ice-cold 5% sulfosalicylic acid. After centrifugation at 20,400 × *g* for 10 min at 4°C, the levels of free amino acids in the supernatant were measured by high performance liquid chromatography assays (SRL, Tokyo, Japan). Blood free amino acids analyses were performed with liquid chromatography-mass spectrometry by SRL.

Statistical analysis

Tukey-Kramer tests for multiple comparisons were performed to determine the significance of differences. Data were expressed as the mean ± standard error (SE). P value of <0.05 was considered statistically significant.

Results and Discussion

The ability of mice (WT and PGC-1α KO) to tolerate a bout of exercise might be altered by BCAA administration. To examine this possibility, mice started to run on a treadmill at 10 m/min and then the speed was increased by 2 m/min in 2-min increments up to a maximum speed of 30 m/min. The mice ran until exhaustion, which is defined as remaining on the shocker plate for more than 20 sec. The exercise tolerance test showed that the running time in the WT saline-administered group (WT-Saline) was 21.4 ± 2.7 min, while that in the WT BCAA-

administered group (WT-BCAA) was 35.4 ± 5.1 min, indicating that the BCAA supplement significantly increased the exercise capacity (Figure 1A). To my knowledge, this is the first report of a BCAA-induced increase in the exercise capacity in mice. In contrast, the exercise tolerance test showed that the running times were not increased by BCAA in PGC-1α KO mice [PGC-1α KO saline-administered group (KO-Saline); 23.6 ± 3.9 min or the PGC-1α KO BCAA-administered group (KO-BCAA); 18.2 ± 1.6 min] (Figure 1A). The trends observed with running distances were similar to those observed with running time (Figure 1B).

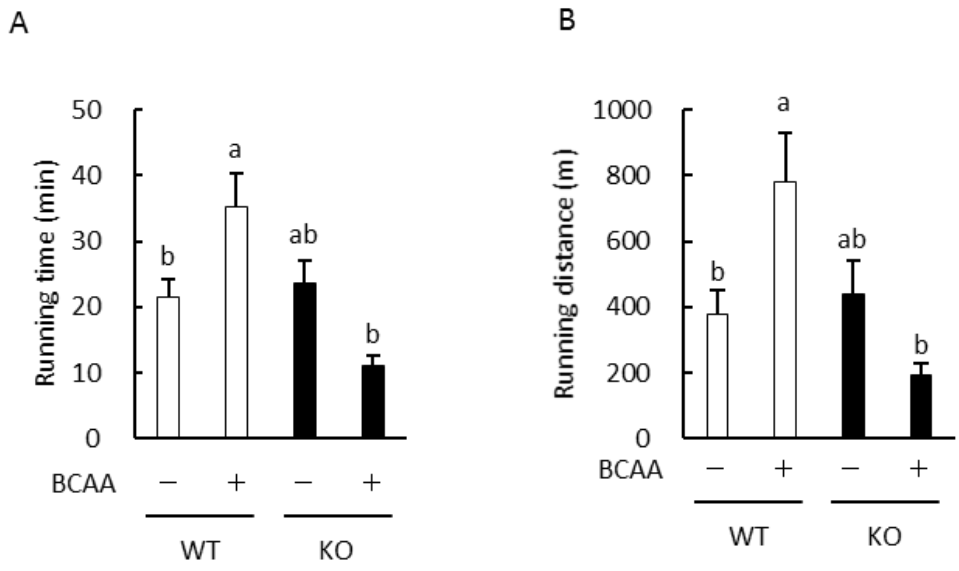


Fig. 1 Running times and distances for WT and PGC-1α KO mice.
Mice were administered saline or BCAA, and 30 min after, were made to run on a treadmill until exhausted. (A) running time and (B) running distance. Values are means ± SE; n = 6. Labeled values without a common letter are statistically different, $P < 0.05$.

Differences in food intake, voluntary wheel running, and skeletal muscles mass were not significant between any group (Figure 2A, B and Table 1). These results indicated that these parameters, among

experimental groups, do not contribute to the increase in exercise capacity.

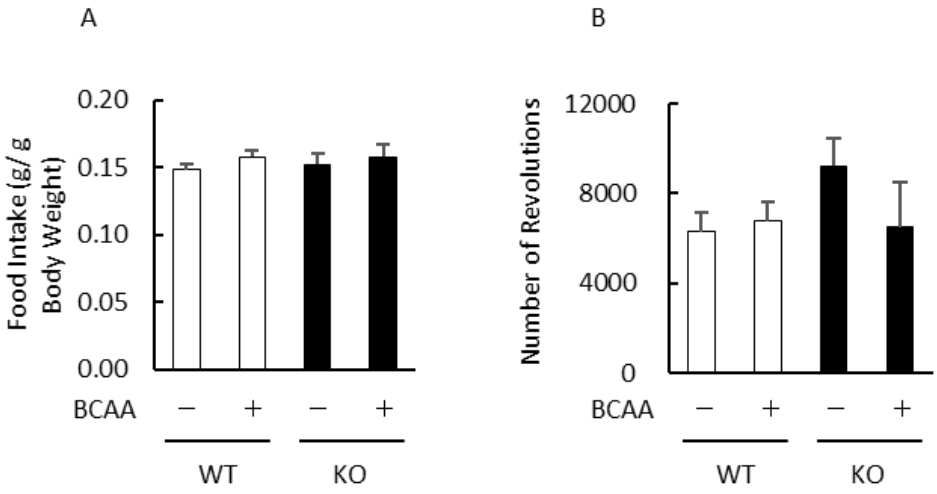


Fig. 2 Food intake and number of voluntary running wheel revolutions in WT and PGC-1α KO mice. (A) Food intake was measured daily and normalized by body weight. Values show mean food intake in 1 day.(B) Number of revolutions was measured daily. Values show the mean number of revolutions in 1 day. Values are means ± SE; n = 6.

Table 1. Skeletal muscle mass of WT and PGC-1α KO mice.

	WT		KO	
BCAA	−	+	−	+
Gastrocnemius (g)	0.326 ± 0.021	0.301 ± 0.006	0.300 ± 0.017	0.285 ± 0.014
Soleus (g)	0.034 ± 0.004	0.032 ± 0.006	0.029 ± 0.002	0.029 ± 0.003
Quadriceps (g)	0.360 ± 0.009	0.355 ± 0.012	0.372 ± 0.005	0.352 ± 0.016
Tibialis anterior (g)	0.092 ± 0.003	0.090 ± 0.003	0.092 ± 0.003	0.091 ± 0.001

Values are means ± SE; n = 6.

Further, I performed real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis. Muscle PGC-1α mRNA levels in PGC-1α KO mice were less compared with those in WT mice (Figure 3A). Muscle mRNA levels of the BCAA degradation enzymes, BCAT2 and

BCKDH, significantly decreased in PGC-1α KO mice compared with those in WT mice (Figure 3B and C).

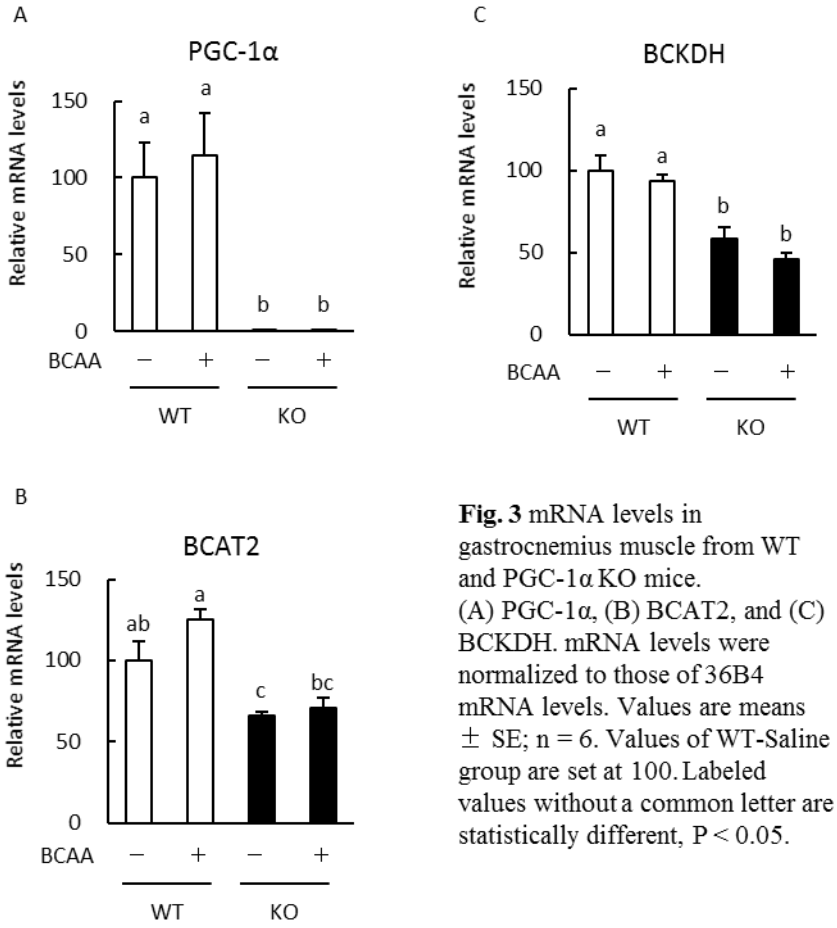
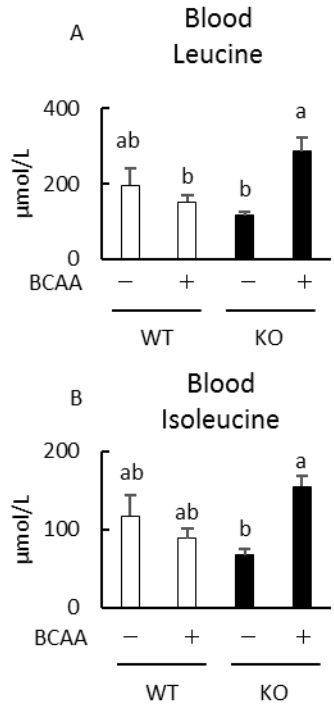


Fig. 3 mRNA levels in gastrocnemius muscle from WT and PGC-1α KO mice. (A) PGC-1α, (B) BCAT2, and (C) BCKDH. mRNA levels were normalized to those of 36B4 mRNA levels. Values are means ± SE; n = 6. Values of WT-Saline group are set at 100. Labeled values without a common letter are statistically different, $P < 0.05$.

Further, I analyzed blood and muscle amino acid levels. Blood leucine, isoleucine, and valine levels in the KO-BCAA group significantly increased compared with those in the KO-Saline group (Figure 4A, B, and C). In contrast, although BCAA administration possibly enhanced BCAA concentration in the WT-BCAA group, leucine, isoleucine, and valine levels did not increase compared with those in the WT-Saline group (Figure 4A, B, and C), suggesting that administered BCAA was degraded in WT mice but not in PGC-1α KO mice. The trends observed with muscle BCAA levels were similar to those observed with

blood BCAA levels (Figure 5A, B, and C). Other amino acid levels showed no marked differences between groups (data not shown).



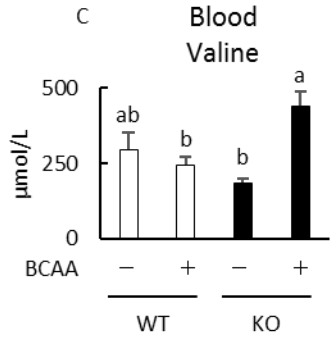
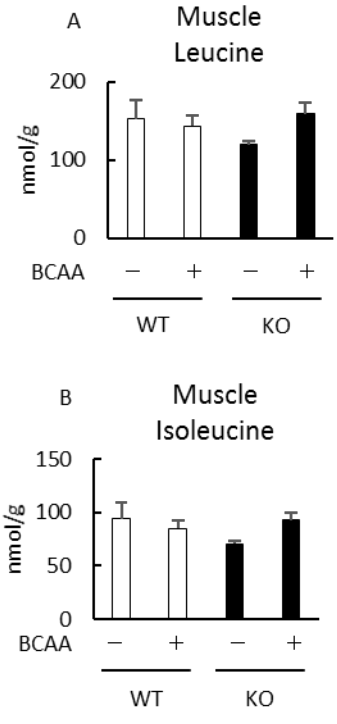


Fig. 4 BCAA concentrations in blood from WT and PGC-1α KO mice.
(A) leucine, (B) isoleucine, and (C) valine. Values are means ± SE; n = 5-6. Labeled values without a common letter are statistically different, $P < 0.05$.



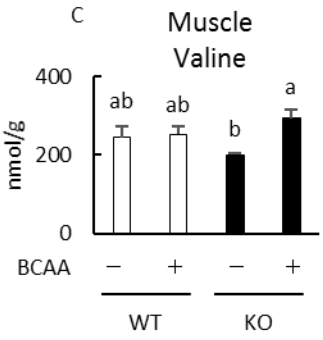


Fig. 5 BCAA concentrations in gastrocnemius muscle from WT and PGC-1α KO mice.
(A) leucine, (B) isoleucine, and (C) valine. Values are means ± SE; n = 6. Labeled values without a common letter are statistically different, $P < 0.05$.

In WT-BCAA group, I observed an increase in running time compared with that in WT-Saline group (Figure 1A and B). There are several reports examining the effects of BCAA on exercise performance in rats; however, there are both reports that BCAA increased (4, 5) and not increased (20) exercise capacity. Calders et al., using pre-trained rats that received BCAA, demonstrated an increase in the running time on a treadmill (4, 5). In this study, I used pre-trained mice, which were put in a wheel cage and trained voluntarily for 3 weeks; an increase in the running time was observed (Figure 1A and B). On the other hand, Verger et al. reported that rats without pre-training did not demonstrate an increase in the exercise capacity after BCAA administration (20). In my results with non-pre-trained mice, I did not observe an increase in the running time after BCAA administration (data not shown). Thus, BCAA-induced endurance exercise capacity may require pre-training of rodents. PGC-1α expression is known to be increased in the skeletal muscle by continuous exercise training (2, 7, 14). Because increased PGC-1α enhances BCAA degradation enzyme levels (10), it would be expected that an increase in PGC-1α levels induced by pre-training may be important for BCAA-induced exercise capacity.

In conclusion, my data suggests that BCAA-induced enhanced endurance performance requires PGC-1α in murine skeletal muscles, and PGC-1α-mediated BCAA degradation may contribute this process.

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Chapter 6

General discussion and Conclusion

Liver is an important tissue for amino acid metabolism. Asparagine synthetase (AS) and 3-phosphoglycerate dehydrogenase (PHGDH) are required for the synthesis of asparagine and serine, respectively, which are essential for growth. Low-protein diet has been reported to increase the expression of AS and PHGDH in rat liver. This increased expression of AS and PHGDH may be an adaptive response to amino acid deficiency. Furthermore, it has been reported that leucine supplementation lowered the amino acid deprivation-induced increase in AS expression in vitro. Therefore, I examined whether excess amount of leucine decreased the expression of AS and PHGDH in the liver of rats maintained on low-protein diet. Leucine supplementation in the diet suppressed AS and PHGDH expression in the liver. I also observed decreased food intake and growth retardation. On the other hand, oral administration of leucine by gavage after meal, but not dietary intake of leucine, decreased the expression of AS and PHGDH without growth retardation and reduction in food intake. These results suggest that there is no correlation between growth retardation caused by leucine and the decreased expression of AS and PHGDH and that growth retardation is, in fact, attributable to decreased food intake.

The vagal nerve mediates anorexigenic signals from the gastrointestinal tract to the brain. Therefore, I performed a vagotomy to examine whether the anorectic effect of excess leucine was mediated by the vagal nerve. Food intake of normal rats maintained on a diet supplemented with leucine was reduced, whereas only a slight decrease

was observed in the food intake of vagotomized rats. This shows that the anorectic effect of excess leucine is mediated by the vagal nerve and that that leucine directly or indirectly affects the vagal nerve to suppress food intake.

Leucine activates mammalian target of rapamycin (mTOR), serine/threonine protein kinase, and increases the phosphorylation of the eukaryotic initiation factor 4E-binding protein (4EBP), which acts downstream of mTOR. These actions promote protein translation. mTOR is known to form a complex with peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α). Therefore, I examined the role of PGC-1α in leucine-activated mTOR (4EBP) signaling using mice with skeletal muscle-specific knockout of PGC-1α (PGC-1α KO). Leucine administration resulted in marked increase in the phospho-4EBP level in the skeletal muscles of wild type mice (WT). In contrast, 4EBP phosphorylation did not increase in the skeletal muscles of PGC-1α KO mice following leucine administration. This result suggests that PGC-1α is involved in leucine-mediated mTOR activation.

Branched-chain amino acids (BCAA), such as leucine, contribute to energy production in skeletal muscles during exercise. BCAA supplementation has been reported to improve endurance performance. It was observed that transgenic mice overexpressing PGC-1α in the skeletal muscle showed increased running capacity with concomitant upregulation in the expression of branched-chain aminotransferase 2 (BCAT2) and branched-chain α-keto acid dehydrogenase (BCKDH) and decrease in BCAA concentrations in the blood and muscle. The mechanism underlying the enhancement of endurance performance by BCAA supplementation is not understood. Therefore, I investigated whether increased PGC-1α-mediated BCAA degradation is required for enhanced

endurance exercise capacity after BCAA supplementation. BCAA significantly increased running time in WT mice. However, BCAA supplementation did not enhance endurance capacity in PGC-1α KO. mRNA levels of BCAT2 and BCKDH in the PGC-1α KO mice were significantly lower than those in WT mice. Blood BCAA concentrations were higher in PGC-1α KO mice than in WT mice. These data suggest that PGC-1α is required for the BCAA-induced enhancement of endurance performance of murine skeletal muscles and that PGC-1α mediated BCAA degradation may contribute to this process.

The above results indicate that BCAA, including leucine, are physiologically active substances that regulate biological functions such as gene expression, food intake, protein translation, and endurance performance through various mechanisms.

List of publications

Chapter 2: Yoshimura R, Takai M, Namaki H, Minami K, Imamura W, Kato H, Kamei Y, Kanamoto R
Down regulation of asparagine synthetase and 3-phosphoglycerate dehydrogenase, and the up-regulation of serine dehydratase in rat liver from intake of excess amount of leucine are not related to leucine-caused amino acid imbalance.
J Nutr Sci Vitaminol (Tokyo) 61: 441-448 (2015)

Chapter 3: Yoshimura R, Ho Y Y, Mizushige T, Ohinata K, Kanamoto R
The vagotomy alleviates the anorectic effect of an excess amount of dietary leucine on rats fed a low-protein diet.
Biosci Biotechnol Biochem 77: 1593-1594 (2013)

Chapter 4: Yoshimura R, Minami K, Matsuda J, Sawada N, Miura S, Kamei Y
Phosphorylation of 4EBP by oral leucine administration was suppressed in the skeletal muscle of PGC-1α knockout mice.
Biosci Biotechnol Biochem 80: 288-290 (2016)

Chapter 5: Yoshimura R, Minami K, Fujita A, Hatazawa Y, Yamashita A, Hirose Y, Senoo N, Sawada N, Matsuda J, Miura S, Kamei Y

Enhanced exercise performance through the intake of branched-chain amino acids requiring PGC-1α in murine skeletal muscles.
submitted

Acknowledgements

The author wishes to express his sincerest gratitude to Dr. Yasutomi Kamei, professor of Kyoto Prefectural University, for his useful guidance, valuable discussions, and cordial encouragement throughout the course of this study.

The author wishes to express his grateful acknowledgement to Dr. Tohru Saeki, Kyoto Prefectural University, for his valuable suggestions and technical advice.

The author gratefully thanks to Dr. Ryuhei Kanamoto, professor of Kyoto University, for his valuable suggestion and discussion.

The author also thank Dr. Shinji Miura, professor of University of Shizuoka, for his meaningful discussion and helpful advices.

Finally, the author is grateful to the members of Laboratory of Molecular Nutrition, Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, for their encouragement and helpful suggestions.