博士論文

Significance of amino acid intake during postexercise recovery phase

（運動後の回復過程におけるアミノ酸摂取の意義）

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Contents

Overall introduction

Introduction

Physical activity has well-known to be beneficial for the prevention and treatment of major chronic disease [1]. Resistance training increases muscle mass which is essential for strength, metabolic function, athletic performance and daily activities, while endurance training can reduce risk of cardiovascular disease [2]. Skeletal muscles are plastic tissues, which change their phenotype in response to stimuli such as exercise and nutritional availability [3]. Appropriate intake of nutrition is required for achieving health and athletic benefit from exercise. Postexercise nutritional strategies should cover 3 aspects: refuel, remodel and repair [4].

First, energy substrate stores are expended during exercise, which has to be refueled by the beginning of next exercise session. Refueling is paid much attention to especially after endurance exercise, because the type of exercise generally expend a lot of energy. The primary energy source during exercise are carbohydrate and fat [5]. However, amino acids are also utilized as energy source during exercise. Following overnight fasting, which might have increased the contribution of amino acids to energy production up to 10% because exercise under conditions of low muscle glycogen enhances amino acid oxidation during exercise [6, 7]. Therefore, amino acid intake should be considered as refueling aspect. Protein requirements in trained-subjects are suggested to be higher than those in sedentary individuals, to enhance the recovery from daily exercise as well as for training adaptation [8, 9]. However, the nitrogen balance technique, which has been widely employed to determine protein requirements in trained subjects [10, 11], requires an adaptation period—up to 7 days [12]; therefore, it remains unclear whether the training status or acute exercise could affect daily protein requirements. The indicator amino acid oxidation (IAAO) method was developed to determine the optimal amount of amino acid intake to maximize whole-body protein synthesis in a fed state, as measured by $^{13}CO_2$ during continuous ingestion of ^{13}C-labeled amino acid [13, 14] (**Figure 0.1 and 0.2**). Through the use of stable isotopes, the IAAO method requires no adaptation period [15]. Due to this advantages, the IAAO method is considered appropriate for the determination of individual amino acid requirements [16] and provides meaningful insights for protein requirements in various healthy and clinical populations [15, 17-19]. Recently, the IAAO method was utilized in trained subjects [20-22]. In those studies, the protein requirements were investigated at resting condition [22] or after exercise [20, 21]. However, there is no evidence that acute exercise can affect the protein requirement determined by IAAO method.

Second, exercise induces some adaptive responses (i.e., mitochondrial density and (or) cross-sectional area) which requires remodeling of several proteins in skeletal muscle. The remodeling can be achieved by synthesizing new protein and degrading old and/or damaged protein. Following exercise, muscle protein synthesis (MPS) and muscle protein breakdown (MPB) are increased for remodeling protein [23]. In aspect of muscle hypertrophy, net protein balance (= MPS-MPB) should

be positive. However, NB remains negative without increased amino acid availability [24]. Therefore, nutrient intake (especially, amino acid) is thought to be essential for training adaptation[25]. Although the interaction between training-induced adaptation and nutrient availability has been investigated [26], there are some issues to be clear as below. As I mentioned, positive NB at postexercise phase is thought to be essential for training adaptation. For that purpose, postexercise protein ingestion is widely used [27]. Furthermore, essential amino acids has primary role for muscle protein synthesis [28]. Among essential amino acids, leucine has potent role to activate master regulator of muscle protein synthesis, mammalian target of rapamycin (mTOR) [29]. However, ingestion of leucine alone decreases concentrations of other essential amino acids [30]. Therefore, leucine-enriched essential amino acids (LEAAs) is recently used for inducing positive NB through increasing muscle protein synthesis [31, 32].

Muscle protein synthesis is increased within 1-4 hour(s) and remains elevated up to 24-48 hours after resistance exercise [23]. Also, combination with protein or amino acid consumption with resistance exercise synergistically increase muscle protein synthesis within the immediate 1–4 hour(s) postexercise [25, 31, 33], and 24 hours after resistance exercise [34]. Therefore, given that resistance exercise enhances MPS up to 48h and dietary amino acids augments the process, it would be valuable to take amino acids over longer periods of post-exercise recovery (e.g. >24 hours). However, it remains to be clear that LEAA can synergistically augment the exercise-induced increment in MPS 24 hours after exercise. In addition, global muscle protein synthesis should be increased for inducing muscle hypertrophy through increasing MPS. However, for recovery from exercise-induced muscle damage, specific protein metabolism should be assessed. Collagen protein, the major protein of the extracellular matrix of skeletal muscle, has crucial roles in mechanical strength, and transmission of forces generated by muscle contractions [35]. Eccentric contractions result in muscle damage that involves muscle fibers and the extracellular matrix [36]. Failure in the re-organization of the extracellular matrix after exercise leads to accumulation of connective tissue which may interfere with tissue repair and functional recovery [37]. However, it remains to be clear whether LEAA could affect muscle collagen protein synthesis after exercise.

Glucose intake induces insulin excretion from pancreas, which induces to increase MPS and suppress MPB. Therefore, co-ingestion of carbohydrate with amino acid or protein might have the additive effect on increasing MPS [38] (**Figure 0.3**). However, the necessity of co-ingestion of glucose with protein or amino acids to augment postexercise MPS remains unclear [39]. Especially, insulin secretion is inhibited to below basal levels by adrenergic receptor activation, both via the sympathetic innervation of the islets and by circulating catecholamines [40]. As a result, insulin concentrations decrease to less than the basal level according to the intensity and duration of the exercise and the duration of fasting before exercise [41]. However, whether the co-ingestion of carbohydrate (CHO) with amino acids can affect the augmented protein synthesis in a hypoinsulinemic state warrants

clarification.

Third, exercise induces damage in some tissues (mainly muscle). Eccentric contractions occur when a force applied to muscle exceeds the momentary force generated by the muscle itself [42]. Training that incorporates eccentric exercise results in greater gains in muscle strength and size [43]. On the other hand, eccentric contractions are also well known to induce muscle damage and dysfunction lasting several days to several weeks, and thereby reduce the ability to perform athletic activities and potentially prevent regular exercise [44]. Therefore, strategies to repair the exercise-induced damages may promote general health and athletic performance. The repair process is regulated by intracellular signaling pathways that control protein turnover, maintaining a balance between muscle protein synthesis and muscle protein degradation. Among these pathways, mTOR pathway is an essential step for muscle regeneration [45]. Indeed, LEAA can stimulate muscle protein synthesis via mTOR [46]. Thus, I hypothesized LEAA could affect repair process from exercise-induced muscle damage (**Figure 0.4**).

To clarify the significance of amino acid intake after exercise, the following studies were conducted covering 3 fundamental concepts (i.e. refuel, remodel and repair) in postexercise period (**Figure 0.5**).
1) To evaluate the optimal amino acid intake that maximizes whole-body protein synthesis in endurance-trained rats at rest and immediately after endurance exercise by using the IAAO method (Chapter 1),
2) to investigate the effect of LEAA on muscle mixed protein and collagen protein synthesis immediately and 1 day after exercise and the additive effect of co-ingestion of carbohydrate with LEAA on muscle protein synthesis after exercise (Chapter 2)
3) To investigate the effect of LEAA on recovery from exercise-induced muscle damage after eccentric contractions (Chapter3).

Figures

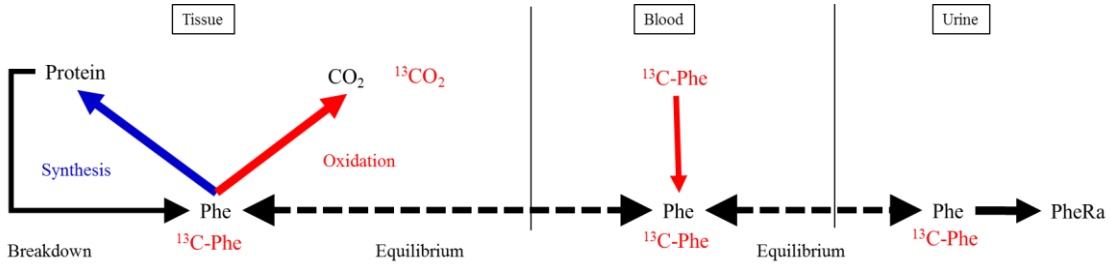


Figure 0.1 Assumption of indicator amino acid oxidation method (1) – Metabolic fate of phenylalanine.

The enrichment of ^{13}C-Phe is determined by amount of ingestion of ^{13}C-Phe and exogenous Phe (mainly from protein breakdown). Therefore, the enrichment of Phe tells whole body protein breakdown (PheRa). The 2 major metabolic fates of phenylalanine are protein synthesis and oxidation in tissue, which enables responses of oxidation to protein/amino acid ingestion to reflect responses of protein synthesis to protein/amino acid ingestion, inversely. Using ^{13}C-labeled amino acid as an indicator amino acid, the amino acid oxidation can be detected in breath $^{13}CO_2$ excretion.

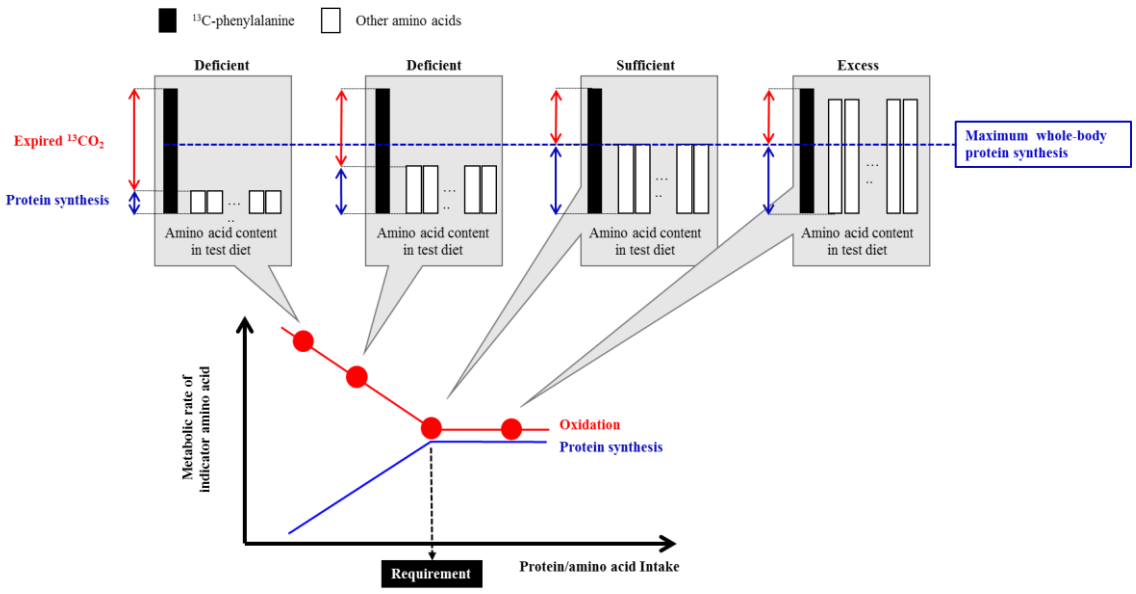


Figure 0.2 Assumption of indicator amino acid oxidation method (2) –Bi-phasic response of ^{13}C-Phe oxidation in response to protein/amino acid intake.

In the tissue, the amino acid is used for protein synthesis or oxidation. The portioning of ^{13}C-Phe is determined by the availability of other amino acid than phenylalanine. If the availability of other amino acid is sufficient for protein synthesis, phenylalanine is used for protein synthesis, but if the availability of the other amino acid is not enough for protein synthesis, the remaining phenylalanine acids will be oxidized. When the deficient protein/amino acid intake with sufficient amount of ^{13}C-Phe is consumed, the oxidation of ^{13}C-Phe is substantially high, which indicates low protein synthesis. Then, according to the increase of protein/amino acid intake, the oxidation of ^{13}C-Phe decreases. Once the protein/amino acid requirement is met, there will be no further increase in the oxidation of ^{13}C-Phe. The protein/amino acid requirement is determined as the point at which the slope of the regression line changes.

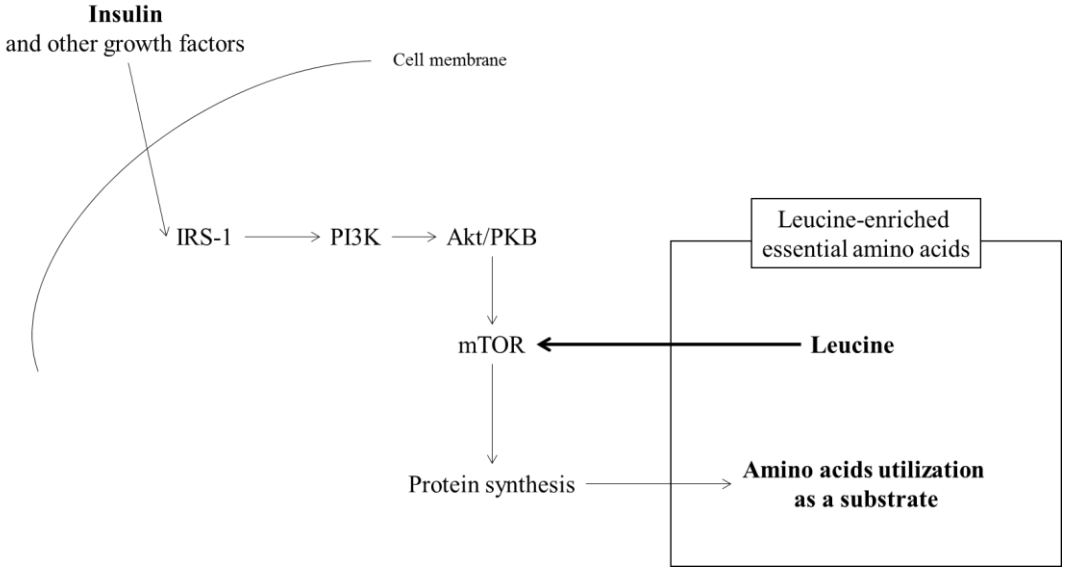


Figure 0.3 Schematic of mTOR pathway mediated by insulin and leucine

mTOR pathway is increased by insulin, also activated by leucine independent of insulin response.

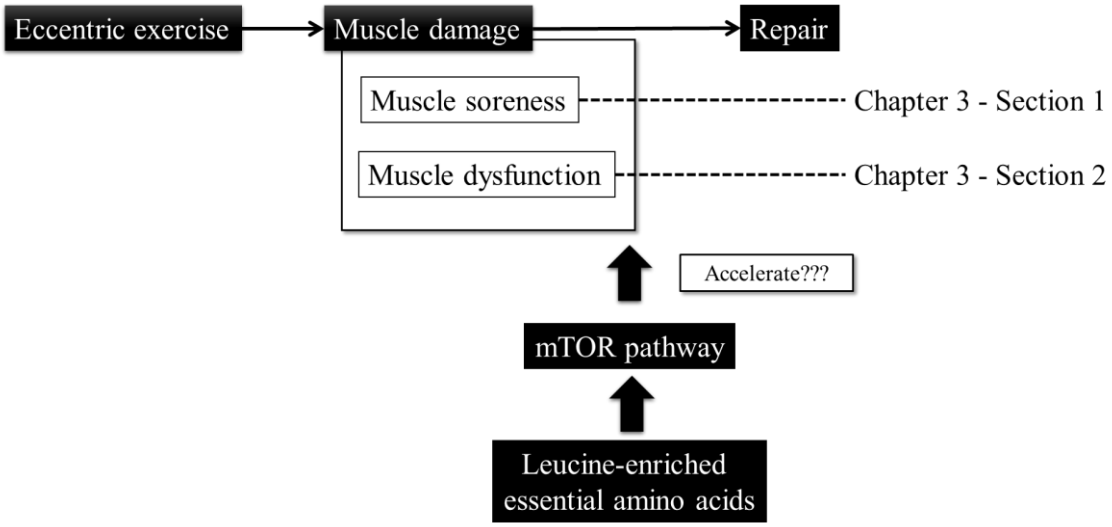


Figure 0.4 Schematic of hypothesis regarding leucine-enriched essential amino acids affects repair of exercise-induced muscle damage through mTOR pathway.

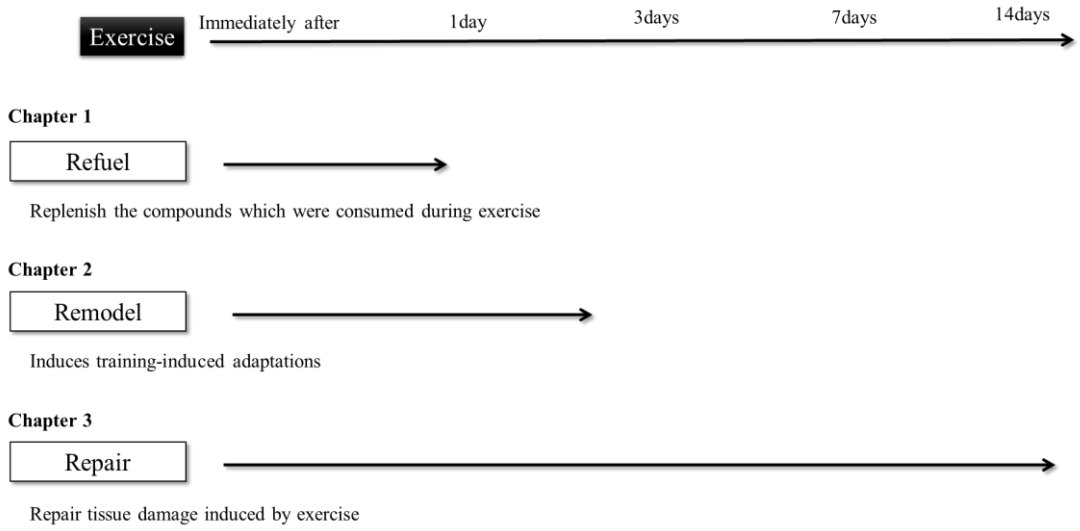


Figure 0.5 Summary of overall introduction and topics examined in each chapter.

Abbreviation

BW	body weight
CHO	carbohydrate
CI	confidential interval
DOMS	delayed onset muscle soreness
EAAs	essential amino acids
EC	eccentric contraction
EEE	energy expenditure during exercise
F_{CO2}	CO_2 production rate
FFM	fat-free mass
FM	fat mass
FSR	fractional synthesis rate
GAS	gastrocnemius muscle
GAAPDH	glyceraldehyde-3-phosphate dehydrogenase
IAAO	indicator amino acid oxidation
IGF-1	insulin-like growth factor-1
IL-1	interleukin-1
IL-6	interleukin-6
LEAA	leucine-enriched essential amino acid
Leu	leucine
MPB	muscle protein breakdown
MPE	mole per excess
MPS	muscle protein synthesis
mTOR	mammalian target of rapamycin
PheOx	phenylalanine oxidation rate
PheRa	phenylalanine flux
REE	resting energy expenditure
SED	sedentary rats
TA	tibialis anterior
TR	trained rats
TR-PostEx	trained rats during postexercise recovery
TR-Rest	trained rats at rest

Chapter 1: Greater amino acid intake is required to maximize whole-body protein synthesis immediately after endurance exercise than at rest in endurance-trained rats, as determined by an indicator amino acid oxidation method

1.1.1. Introduction

Protein requirements for endurance athletes are suggested to be higher than those for sedentary individuals, to enhance the recovery from daily endurance training as well as for training adaptation [8, 9] mainly because the utilization of amino acids is augmented during exercise [10]. Training that aims to enhance athletic performance involves a varying amount of purposely-oriented physical loads on a daily basis [47]. Therefore, because the metabolic demands for protein that increase with an acute bout of exercise should be replenished on the same day [48], the recommended protein intake for endurance athletes should vary depending on the daily physical load. However, the nitrogen balance technique, which has been widely employed to determine protein requirements in endurance-trained subjects [10, 11], requires an adaptation period—up to 7 days [12]; therefore, it is not appropriate for determining protein requirements on the day. As a result, the amount of protein that endurance-trained athletes should consume depending on daily physical training remains unclear.

The IAAO method was developed to determine the optimal amount of amino acid intake to maximize whole-body protein synthesis in a fed state, as measured by $^{13}CO_2$ during continuous ingestion of ^{13}C-labeled amino acid [13, 14]. Through the use of stable isotopes, the IAAO method requires no adaptation period and allows a more robust bi-phase modeling of the data than a linear model, which is typically used for the nitrogen balance technique [15]. In addition, the optimal amino acid intake determined using the IAAO method is comparable with the protein requirement that is estimated using a biphasic linear regression model for data from nitrogen balance studies [15]. Due to these advantages, the IAAO method is considered appropriate for the determination of individual amino acid requirements [16] and provides meaningful insights for protein requirements in various healthy and clinical populations [15, 17-19]. Recently, the IAAO method was utilized in trained subjects [20-22]. In those studies, the protein requirements were investigated at resting condition [22] or after exercise [20, 21]. However, there is no evidence that acute exercise can affect the protein requirement determined by IAAO method.

Over the past 50 years, many studies have evaluated the benefits of combined exercise and nutrition in animals. However, the importance of protein intake during endurance training in animal models remains controversial [49-52]. In addition, the effect of chronic training on amino acid oxidation at rest and during exercise is not well understood; some studies have shown that training increases amino acid oxidation [53, 54], while another study demonstrated a decrease in leucine oxidation [55]. Therefore, to clarify the additive effect of protein intake on the benefits of exercise, it is important to determine the optimal amino acid intake that maximizes whole-body protein synthesis separately for

trained animals at rest and immediately after exercise.

The purpose of this study was to evaluate the optimal amino acid intake that maximizes whole-body protein synthesis in endurance-trained rats at rest and immediately after endurance exercise by using the IAAO method.

1.1.2. Material and method

Animals

This study was approved by the Institutional Animal Care and Use Committee of Ajinomoto Co., Inc. Male Fischer (F344) rats (3 weeks old; Charles River Laboratories Japan, Inc., Yokohama, Japan) were used and housed under controlled conditions with 12:12-h cycles of dark (10:00–22:00) and light (22:00–10:00) at 23 ± 1°C and 55 ± 1% humidity. Water was provided *ad libitum* throughout the experiment.

Study design

After a 1-week period of adaptation to the laboratory environment, rats were divided into 2 groups: sedentary group (SED; n = 42) and trained group (TR; n = 41). The TR group underwent treadmill training 5 days a week for 6 weeks. In the fourth week of training, resting energy expenditure (REE) and energy expenditure during exercise (EEE) were measured 1 day per week; then, in the fifth and sixth weeks, the metabolic trial was conducted. Half of the rats in the TR group (TR-PostEx; n = 19) were used for the metabolic trial after the daily treadmill training. The other half of the TR group (TR-Rest; n = 22) and all of the rats in the SED group were used at rest. After the metabolic trials, body composition and muscle glycogen content were compared between the SED and TR groups. The schematic of the entire study design is shown in **Figure 1.1.1**.

Diets

The rats were provided a purified diet (AIN93G, Oriental Yeast Co., Ltd., Tokyo, Japan) until the end of the third week of training [56]; then, they were provided a purified diet based on AIN-93G, which was modified by substituting corn starch (6.2 g/kg diet) and crystalline amino acids (13.8 g/kg diet; Ajinomoto Co., Inc., Tokyo, Japan) for casein (20 g/kg diet; **Table 1.1.1**). The amino acid composition was based on the recommendations of the National Research Council [57] (**Table 1.1.2**).

Training protocol

TR rats were exercised in the morning (10:00–12:00) on a mechanical treadmill apparatus (model KN-73, Natsume Seisakusho, Tokyo, Japan). The duration and intensity of exercise were gradually increased over the first 2 weeks. Then, the final 4 weeks of training consisted of running for 60 min at 26 m/min and an 18% grade.

Energy expenditure

REE and EEE were calculated from VCO_2 and VO_2 based on the Weir's equation [58]. Nine rats from each group were moved into the metabolic chamber at 17:00. An additional nine rats from the TR group underwent the daily training on a sealed treadmill (Columbus Instruments, Columbus, OH), and

VCO_2 and VO_2 were measured for 24 hours by using an O_2/CO_2 metabolism measuring system (MK-5000RQ; Muromachi Kikai, Tokyo, Japan).

Metabolic trial

Each rat was used 1–3 times in the metabolic trial, and 74, 46, and 38 data points were collected for the SED, TR-Rest, and TR-PostEx groups, respectively. The rats in the SED and TR-Rest groups were moved into the metabolic chamber at 9:00, after 17 h of food deprivation (**Figure 1.1.2**). The TR-PostEx group underwent the daily training at 9:00; then, the rats were moved into the metabolic chamber. Subsequently, all of the rats randomly received 1 of 13 test diets containing different amounts of amino acids (Tables 1.1.1 and 1.1.2). the amino acid composition was modeled after the amino acid requirement [59]. The test diet was provided in 7 isocaloric and isonitrogenous meals every hour starting at 10:00, and each meal represented 1/24 of the total daily food intake. Each meal was weighed precisely before the metabolic trial; all rats started to eat each meal immediately after it was offered and consumed the entire meal. The amount of the test diet provided to the TR-PostEx and TR-Rest groups was increased to account for their REE and EEE (**Figure 1.1.2**). L-[1-^{13}C] phenylalanine (99.9 atom per excess [APE]: Cambridge Isotope Laboratories, Woburn, MA) was administered by replacing the entire dose of phenylalanine in the fourth to seventh meals. Oral priming doses of 3.3 mg/kg L-[1-^{13}C] phe and 29.7 mg/kg L-Phe were administered with the fourth meal.

In a recent discussion, the IAAO method was criticized; it was stated that the IAAO just reflects the relative excess or limitation of the indicator rather than the utilization of the amino acid [60, 61]. To avoid this, the phenylalanine content was maintained at 0.680 g/kg diet in all of the diets; however, the tyrosine content varied in proportion to the reference protein composition. This manipulation resulted in a breakpoint (7.7 $g \cdot kg^{-1} \cdot day^{-1}$) that was obtained from the relationship between amino acid intake and the relative content of phenylalanine in the test diets, which is clearly below the amino acid requirements for rats [57, 62]. Furthermore, the phenylalanine (0.680 g/kg) content was equal to the phenylalanine requirement for growing rats when the rats are provided with a tyrosine-free diet [63]. Thus, the amount of phenylalanine + tyrosine in the current diets was assumed to be sufficient for protein synthesis, which is required for this method. Although the tyrosine content in the test diet could affect phenylalanine hydroxylation in humans [64, 65], tyrosine concentration within the physiological range has little effect on phenylalanine hydroxylation in primary rat hepatocytes [66].

During each metabolic trial, enrichment of $^{13}CO_2$ in breath was analyzed using continuous-flow isotope ratio mass spectrometry (ABCA; Sercon Ltd., Cheshire, United Kingdom). At the end of the metabolic trial, blood was collected from the tail vein. The isotopic enrichment of plasma phenylalanine was determined using an LC–MS/MS system (Prominence HPLC system; Shimadzu, Kyoto, Japan and API 3200; AB SCIEX, Framingham, MA) and the external standard curve approach, as previously described [67, 68].

Tracer kinetics

Phenylalanine flux (PheRa; μmol·kg^{-1}·min^{-1}) and rate of phenylalanine oxidation (PheOx; μmol·kg^{-1}·min^{-1}) were calculated using standard equations as below [69, 70]:

$$\text{PheRa} = \frac{Intake}{plasma\ phenylalanine\ enrichment}$$

$$\text{PheOx} = \frac{FCO_2 \times\ ^{13}CO_2}{plasma\ phenylalanine\ enrichment \times Body\ weight(BW)} \times 44.6\ \div 0.50$$

in which, Intake is the rate of L-[1-^{13}C] phe ingestion (μmol·kg^{-1}·min^{-1}); FCO$_2$ (cm^3/min) is the CO$_2$ production rate. An isotopic steady state at rest for the enrichment of L-[1-^{13}C] phe in plasma was confirmed 3 h after providing the hourly meal in our laboratory. In former reports, leucine oxidation recovered to the basal level by 5 h after exercise [71], and MPS remained steady up to 6–8 h after exercise in rats [72, 73]. In addition, VCO$_2$ and ^{13}CO$_2$ excretion rate were confirmed as steady 5–7 h after exercise in the present study. Therefore, the rats that underwent exercise before the metabolic trial were assumed to be in a metabolic steady state 7 h after exercise. A correction factor of 0.50 was used based on our previous bicarbonate studies to account for the retention of ^{13}CO$_2$ in the bicarbonate pool and enrichment of L-[1-^{13}C] phe in plasma. ^{13}C bicarbonate recovery rate was increased during exercise, then recovered to the basal level within 2 hours after exercise completion [74]. Therefore, a single factor was used in all the groups in the current study.

Body composition and tissue sampling

Total body fat mass (FM) and total fat-free mass (FFM) were determined as measured by the ^{2}H$_2$O dilution method as previously described [75]. Briefly, after an intraperitoneal injection of ^{2}H$_2$O (15 mL/kg; 99.9 APE ^{2}H$_2$O), blood was collected under inhalation anesthesia with 1.5% isoflurane. The gastrocnemius muscle was then removed, frozen in liquid nitrogen, and stored at -80°C. The enrichment of ^{2}H$_2$O in plasma was determined using an Agilent 5973 equipped with an Agilent 6890 GC system (Agilent Technologies, Santa Clara, CA) and a DB17-MS capillary column (30 m × 0.25 mm × 0.25 μm; Agilent Technologies, Santa Clara, CA) [76]. FFM, and FM were calculated from the enrichment (E_{water}, MPE) using the following equations [77, 78]:

$$\text{FFM} = \frac{\text{volume of injected } {}^2H_2O}{E_{water}} \times 1.0657\ \div 0.732$$

$$\text{FM} = \text{BW} - \text{FFM}$$

Muscle glycogen

A 30-mg muscle sample was hydrolyzed in 2 N HCl, neutralized by adding 2 N NaOH, and assayed for glucose content using the Glucose CII Test Wako kit (Wako Pure Chemical Industries, Ltd., Osaka,

Japan).

Statistical analysis

Optimal amino acid intakes are shown as the mean and 95% confidence interval (CI), and the remaining data are shown as mean ± standard error of the mean. Differences were considered significant at $P < 0.05$. Student's *t* tests were used to analyze differences in characteristics between the SED and TR groups using GraphPad Prism5 (GraphPad Software Inc., San Diego, CA). A linear mixed-effect model with individual rats as a random variable was used to analyze the effect of amino acid intakes on PheOx and PheRa by using PROC MIXED (SAS University Edition; SAS Institute Japan, Tokyo, Japan). The optimal amino acid intake was determined using the breakpoint identified by applying a biphasic linear regression crossover model [79], and the 95% CI was calculated with use of Fieller's Theorem, as previously described [80] The differences in the optimal amino acid intakes between the groups were examined using one way ANOVA, followed by Bonferroni test.

1.1.3. Results

Characteristics of the rats

After the 6-week training period, BW (7.6%, $P < 0.01$), FFM (3.6%, $P < 0.05$), and FM (33.2%, $P < 0.01$) were lower in the TR group than in the SED group (**Table 1.1.3**). Food intake during the experimental period was not different between the SED and TR groups, despite the increased energy expenditure from daily training. The relative negative energy balance in the TR group, compared with the SED group, resulted in a higher percentage of FFM to BW and lower percentage of FM to BW ($P < 0.01$, Table 1.1.3). Muscle glycogen content in the gastrocnemius muscle of the TR group was 20% higher than that in the SED group ($P < 0.05$, Table 1.1.3).

Energy expenditure at rest and during exercise and corrected food intake during the metabolic trial

The REE in the TR group was 9.6% higher than that in the SED group ($P < 0.01$). The total daily energy expenditure of the TR group on the training day, which included 1 hour of running on the treadmill and 23 h of rest, was 17.0% higher than that of the SED group.

Phenylalanine flux

The slopes of the fitted regression lines for the relationship between PheRa and amino acid intake in each group were not different from zero in the mixed linear regression analyses (**Figure 1.1.3**). Therefore, PheRa was not affected by different amino acid intakes, as required by the IAAO method.

Phenylalanine oxidation

PheOx linearly decreased in response to the graded amino acid intake until the breakpoints of 15.1 $g \cdot kg^{-1} \cdot day^{-1}$ (95% CI: 11.1–19.1), 13.3 $g \cdot kg^{-1} \cdot day^{-1}$ (95% CI: 10.9–15.7), and 26.8 $g \cdot kg^{-1} \cdot day^{-1}$ (95% CI: 21.5–32.1) for the SED, TR-Rest, and TR-PostEx groups, respectively (**Figure 1.1.4**). Subsequently, the breakpoint in the TR-PostEx group was significantly higher than the breakpoints in the SED and TR-Rest groups ($P < 0.05$); the SED and TR-Rest groups were not significantly different.

1.1.4. Discussion

To the best of our knowledge, this is the first study to use the IAAO method to examine the effect of an acute bout of endurance exercise on the optimal amino acid intake to maximize whole-body protein synthesis in endurance-trained rats. The breakpoint of the minimal rate at which PheOx occurred in response to graded amino acid intake in the TR-Rest group was not significantly different from that in the SED group; however, the breakpoint in the TR-PostEx group was 77% and 100% higher than those in the SED and TR-Rest groups, respectively; this was significant. These findings indicate that an acute bout of exercise has a large impact on the optimal amino acid intake in trained rats, but chronic endurance training (6-week period) does not have a measurable effect on optimal amino acid intake at rest.

In the present study, the optimal amino acid intake immediately after exercise was 100% higher than that at rest in trained rats. A single bout of endurance exercise is associated with oxidation of up to 86% of the daily leucine requirement [81]. The rats underwent exercise following overnight food deprivation, which might have increased the contribution of amino acids to energy production up to 10% because exercise under conditions of low muscle glycogen enhances amino acid oxidation during exercise [6, 7]. Therefore, the exercise-induced increase in amino acid oxidation can explain a large part of this phenomenon, but does not fully explain this phenomenon. In a rodent study, exercise blunted MPS [82, 83] and increased MPB [82]. Furthermore, administration of amino acids alleviated, but did not increase, MPS after exercise, as compared with at rest [84]. Therefore, greater amounts of amino acids might be required to compensate for the exercise-induced decrease in protein synthesis and to maximize post-exercise protein synthesis in rats. Finally, endurance exercise induces intestinal damage through a potential ischemia-reperfusion mechanism [85], which impairs dietary protein digestion and absorption [86]. Therefore, a greater amount of amino acids might be required to provide a sufficient amount of amino acids to the circulation. Furthermore, enterocytes use glutamine as the main energy source, and glutamine supplementation prevents exercise-induced permeability [87]. Thus, the intestinal damage might increase the need for dietary amino acids for tissue repair.

In the present study, the optimal amino acid intake in the TR-Rest group was not significantly different from that in the SED group. In most former studies, protein requirements were studied during a chronic training period, which included the exercise, post-exercise, and resting periods [10, 11, 88]. Thus, the protein requirements of endurance-trained subjects in only the resting condition are not fully understood. To our best knowledge, only a single former study reported that a 1.2 $g \cdot kg^{-1} \cdot day^{-1}$ protein intake was required to achieve zero nitrogen balance in trained subjects at rest [89], which is higher than the protein requirements of healthy adults [90]. However, some authors have suggested that the efficiency of protein utilization increases after chronic exercise training [91-94]. Endurance training can decrease leucine and protein oxidation at rest [55, 95]. Accordingly, the training adaptations of amino acid metabolism might decrease protein requirements at rest in endurance-trained subjects,

compared to that in non-trained, sedentary subjects. Thus, the protein requirements at rest in endurance-trained subjects might not be higher than those in non-trained, sedentary subjects.

This study has certain limitations. First, as Millward [60] and Millward and Jackson [61] indicated, the IAAO might reflect the relative excess/deficiency of phenylalanine, compared with the other amino acids. However, when phenylalanine content is sufficient, phenylalanine oxidation could inversely reflect protein synthesis [61, 96]. In addition, in the present study, through the manipulation of the ratio of phenylalanine and tyrosine to the total amino acid content in the test diet, the breakpoint for this ratio was lower than the protein requirements in rats. However, the breakpoint in the SED group was similar to previous recommendations [57, 62]. Therefore, the IAAO did not reflect the relative excess/deficiency of phenylalanine to total amino acids, but did reflect whole-body protein synthesis. Second, although the IAAO method can address the optimal amino acid intake that maximizes whole-body protein synthesis, which is comparable with the protein requirements estimated using a biphasic linear regression model for data from nitrogen balance studies [15], the effects of increased amino acid intake on physiologic, morphologic, and other important outcomes remain to be determined.

In conclusion, the results of this study, which investigated the optimal amino acid intake to maximize whole-body protein synthesis in rats at rest and after a single bout of exercise in trained rats using the IAAO method, suggest that greater amino acid intake is required to maximize whole-body protein synthesis after a single bout of endurance exercise in trained rats, while chronic endurance training might not affect the optimal amino acid intake.

1.1.5. Tables and figures

Table 1.1.1 Composition of test diets

Rats randomly received 1 of 13 different amount of amino acids intake on metabolic trial.

[1] AIN-93G mineral mixture; Oriental Yeast Co., Ltd., Tokyo, Japan.

[2] AIN-93G vitamin mixture, Oriental Yeast Co., Ltd., Tokyo, Japan

	Amino acids content, g/kg diet												
Ingredients	3.3	7.2	8.5	11.1	13.8	17.7	19.0	21.6	24.2	26.8	29.5	33.4	37.3
Soy oil	70.0	70.0	70.0	70.0	70.0	70.0	70.0	70.0	70.0	70.0	70.0	70.0	70.0
Cellouse	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Sucrose	100	100	100	100	100	100	100	100	100	100	100	100	100
Mineral mix[1]	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0
Vitamin mix[2]	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Choline bitartrate	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50
t-butylyhydroxy quinone	0.0140	0.0140	0.0140	0.0140	0.0140	0.0140	0.0140	0.0140	0.0140	0.0140	0.0140	0.0140	0.0140
Corn starch	699	660	647	621	595	556	542	516	490	464	438	399	359
L-Phenylalanine	6.80	6.80	6.80	6.80	6.80	6.80	6.80	6.80	6.80	6.80	6.80	6.80	6.80
Other amino Acids	26.2	65.4	78.5	105	131	170	183	209	236	262	288	327	366

Table 1.1.2 Amino acid composition of reference protein and test diets

[1]: This pattern was demonstrated by Gahl et al. [62] to support weight gain.

[2]: Phenylalanine content was kept constant at 0.680 g/kg diet in all of the diets.

		Amino acids content, g/kg diet												
Amino Acid	Reference[1]	3.3	7.2	8.5	11.1	13.8	17.7	19.0	21.6	24.2	26.8	29.5	33.4	37.3
Arg	4.3	0.086	0.215	0.258	0.344	0.430	0.559	0.602	0.688	0.774	0.860	0.946	1.08	1.20
His	2.8	0.056	0.140	0.168	0.224	0.280	0.364	0.392	0.448	0.504	0.560	0.616	0.700	0.784
Lys HCl	11.5	0.230	0.575	0.689	0.919	1.15	1.49	1.61	1.84	2.07	2.30	2.53	2.87	3.22
Met	6.5	0.130	0.325	0.390	0.520	0.650	0.845	0.910	1.04	1.17	1.30	1.43	1.63	1.82
Cys-Cys	3.3	0.066	0.165	0.198	0.264	0.330	0.429	0.462	0.528	0.594	0.660	0.726	0.825	0.924
Phe[2]	6.8	0.680	0.680	0.680	0.680	0.680	0.680	0.680	0.680	0.680	0.680	0.680	0.680	0.680
Tyr	3.4	0.068	0.170	0.204	0.272	0.340	0.442	0.476	0.544	0.612	0.680	0.748	0.850	0.952
Thr	6.2	0.124	0.310	0.372	0.496	0.620	0.806	0.868	0.992	1.12	1.24	1.36	1.55	1.74
Trp	2.0	0.040	0.100	0.120	0.160	0.200	0.260	0.280	0.320	0.360	0.400	0.440	0.500	0.560
Ala	4.0	0.080	0.200	0.240	0.320	0.400	0.520	0.560	0.640	0.720	0.800	0.880	1.00	1.12
Asp	4.0	0.080	0.200	0.240	0.320	0.400	0.520	0.560	0.640	0.720	0.800	0.880	1.00	1.12
Glu	40.0	0.800	2.00	2.40	3.20	4.00	5.20	5.60	6.40	7.20	8.00	8.80	10.0	11.2
Gly	6.0	0.120	0.300	0.360	0.480	0.600	0.780	0.840	0.960	1.08	1.20	1.32	1.50	1.68
Pro	4.0	0.080	0.200	0.240	0.320	0.400	0.520	0.560	0.640	0.720	0.800	0.880	1.00	1.12
Ser	4.0	0.080	0.200	0.240	0.320	0.400	0.520	0.560	0.640	0.720	0.800	0.880	1.00	1.12
Asn H_2O	4.6	0.091	0.228	0.273	0.364	0.455	0.592	0.637	0.728	0.819	0.910	1.00	1.14	1.27
Leu	10.7	0.214	0.535	0.642	0.856	1.07	1.39	1.50	1.71	1.93	2.14	2.35	2.68	3.00
Ile	6.2	0.124	0.310	0.372	0.496	0.620	0.806	0.868	0.992	1.12	1.24	1.36	1.55	1.74
Val	7.4	0.148	0.370	0.444	0.592	0.740	0.962	1.04	1.18	1.33	1.48	1.63	1.85	2.07

Table 1.1.3 Characteristics of the rats after 6 weeks of endurance training

Values are reported as mean ± SEM, n = 42 (SED) and n = 41 (TR).

Body weight, fat free mass, fat mass, and muscle glycogen were measured after 6 weeks of training.

Total food intake was measured during the 6-week training period.

NS, not significant; SED, sedentary group; TR, Trained group

Characteristics	SED	TR	*P*
Body weight, *g*	220 ± 2	203 ± 2	< 0.01
Fat free mass, *g*	193 ± 2	186 ± 2	< 0.05
Fat free mass,%	87.7 ± 0.6	91.1 ± 0.4	< 0.01
Fat mass, *g*	27.3 ± 1.3	18.2 ± 0.8	< 0.01
Fat mass, %	12.3 ± 0.6	8.9 ± 0.4	< 0.01
Total food intake, g	498 ± 4	499 ± 4	NS
Muscle glycogen, mg/g tissue	2.79 ± 0.16	3.34 ± 0.16	< 0.05

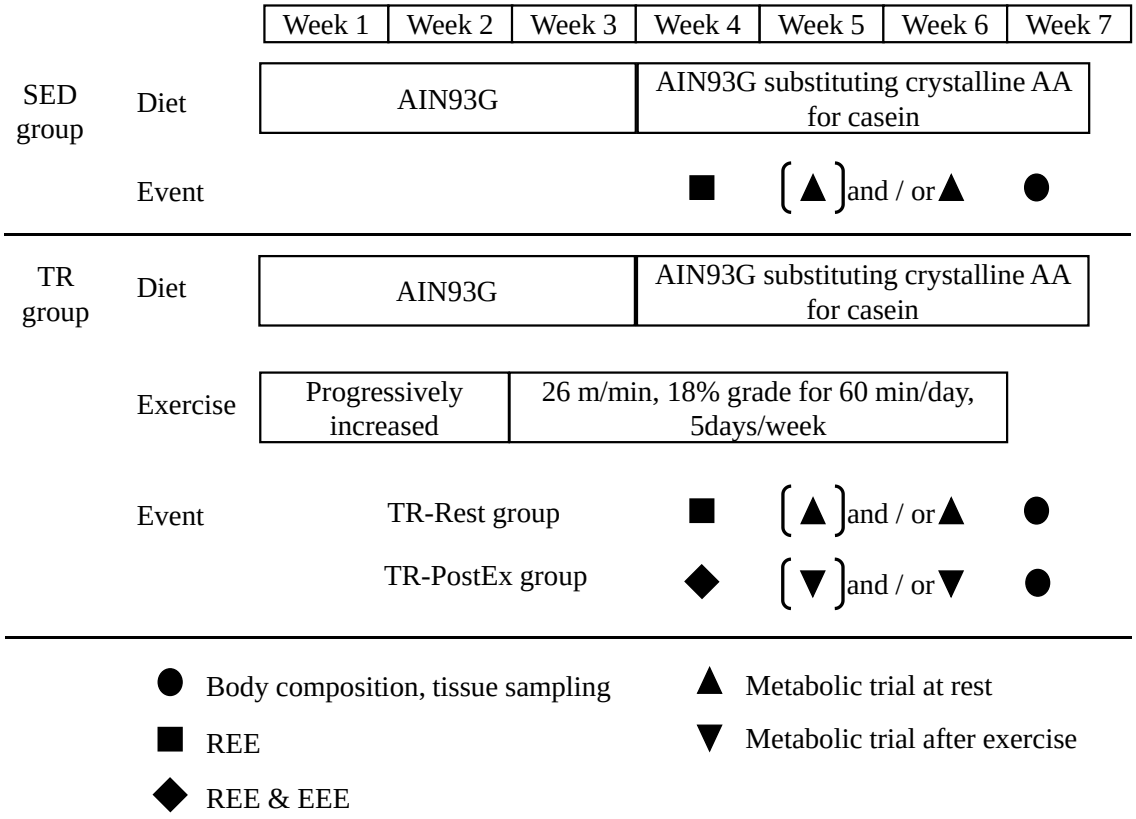


Figure 1.1.1 Study design to determine optimal amino acid intake to maximize whole-body protein synthesis in resting sedentary rats, resting and postexercise trained rats

After a 1-week adaptation period to the laboratory environment, rats were divided into 2 groups: sedentary group (SED; n = 42) and trained group (TR; n = 41). They were provided a purified diet (AIN93G; Oriental Yeast Co., Ltd., Tokyo, Japan) until the end of the third week of experimental period, then a purified diet based on AIN-93G containing crystalline amino acids as the protein source. TR group underwent treadmill running for 6 weeks. In the fourth week of experimental period, resting energy expenditure (REE) and/or energy expenditure during exercise (EEE) were measured; then, in the fifth and sixth weeks, the metabolic trial was conducted once or twice per rat. Half of rats in TR group underwent metabolic trial after exercise (TR-PostEx; n = 46 (22 rats, 2 or 3 measurements/rat), the other half (TR-Rest, n =38 (19 rats, 2 measurements/rat)) and the SED group (SED, n = 74 (42 rats, 1 or 2 measurements/rat) underwent metabolic trial at rest. Measurement of body composition and tissue sampling were conducted following metabolic trials.

n, number of rats in groups or total data points on metabolic trial; SED, sedentary group; TR-rest, trained rats at rest; TR-PostEx, trained rats after exercise

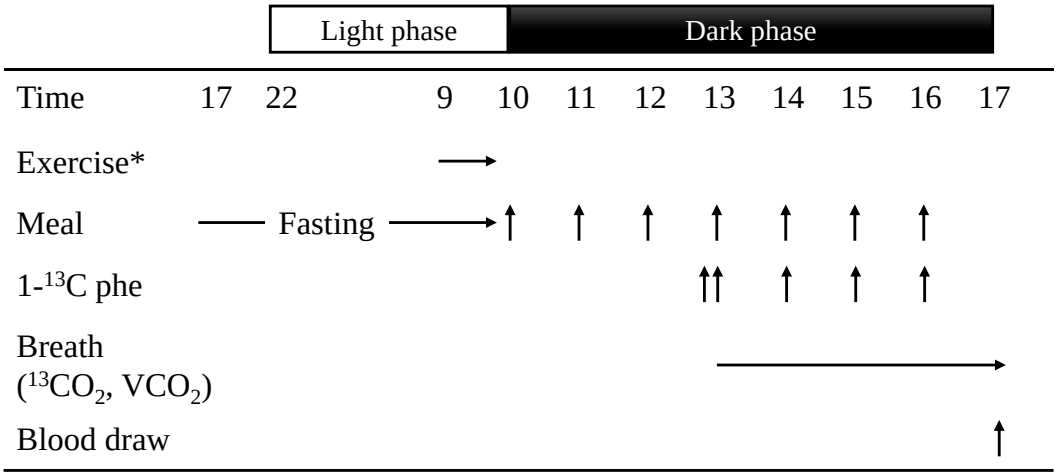


Figure 1.1.2 Protocol for measuring phenylalanine oxidation in resting sedentary rats, resting and postexercise trained rats on each metabolic trial day

The test diet was provided every hour for 7 h. Each meal was isocaloric and isonitrogenous, and represented 1/24 of each rats daily food intake adjusted with daily and exercise-induced energy expenditure as below:

- Hourly meal amount for the SED group (g/kg) =1/24 of mean daily food intake (mg · kg^{-1} · day^{-1}) in the SED group for the 3 days before the metabolic trial
- Hourly diet amount for the TR-Rest group (g/kg) = 1/24 of mean daily food intake of the SED group (g · kg^{-1} · day^{-1}) × [REE in the TR group (kcal · kg^{-1} · day^{-1})/REE in the SED group (kcal · kg^{-1} · day^{-1})].
- Hourly diet amount for the TR-PostEx group (g/kg) = 1/24 of mean daily food intake of the SED group (g · kg^{-1} · day^{-1}) × [REE in the TR group (kcal · kg^{-1} · day^{-1}) × 23/24] + (EEE in the TR group (kcal · kg^{-1} · day^{-1}) × 1/24)] / REE in the SED group (kcal · kg^{-1} · day^{-1})

Priming dose of L-[1-^{13}C] Phe was administrated at 4th meal, then a simulated continuous dose of L-[1-^{13}C] Phe was provided simultaneously and continued every hour throughout the remaining 4 hours of protocol. The enrichment of $^{13}CO_2$ in breath and CO_2 production rate (VCO_2) was determined by indirect calorimetry continuously for last 4 hours of protocol.

*Only for TR-PostEx

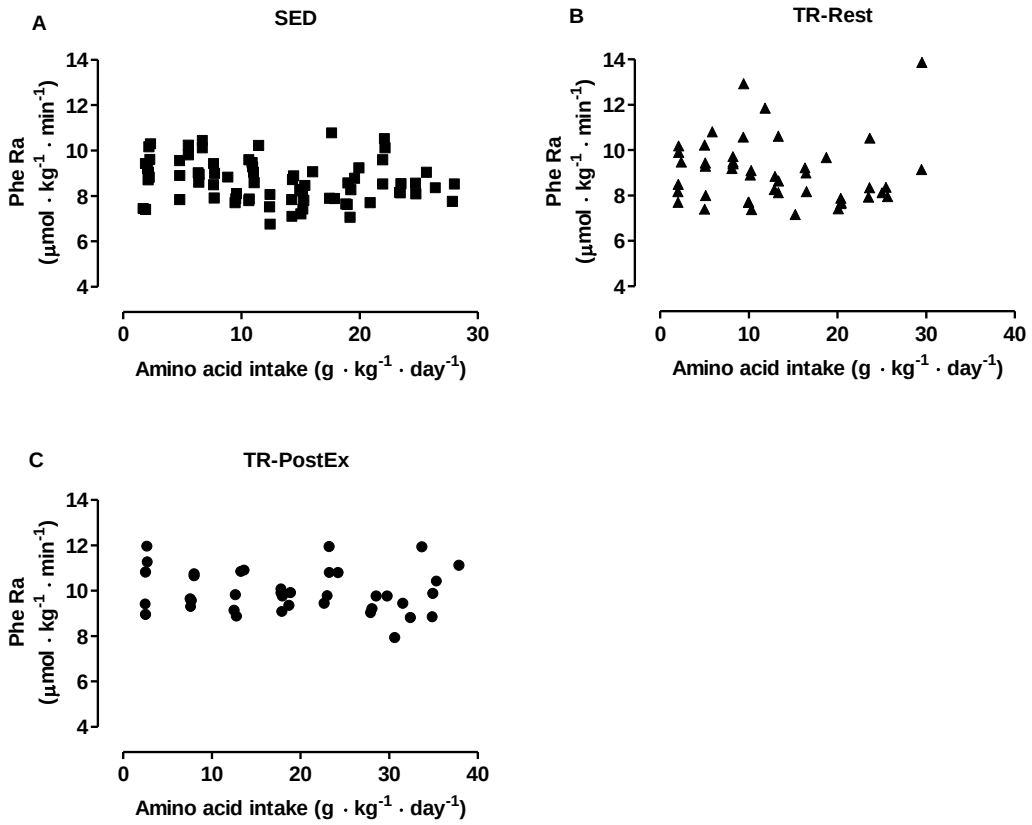


Figure 1.1.3 Phenylalanine flux (PheRa) in sedentary rats (SED; A, n = 74 (42 rats, 1 or 2 measurements/rat) at rest and in trained rats at rest (TR-rest; B, n = 46 (22 rats, 2 or 3 measurements/rat)) or postexercise (TR-PostEx; C, n = 38 (19 rats, 2 measurements/rat) in response to test amino acid intakes during the metabolic trial.

The data points represent the individual PheRa during the metabolic trial. The rats consumed a range of test amino acid intakes during the metabolic trial.

n, number of data points from each metabolic trial

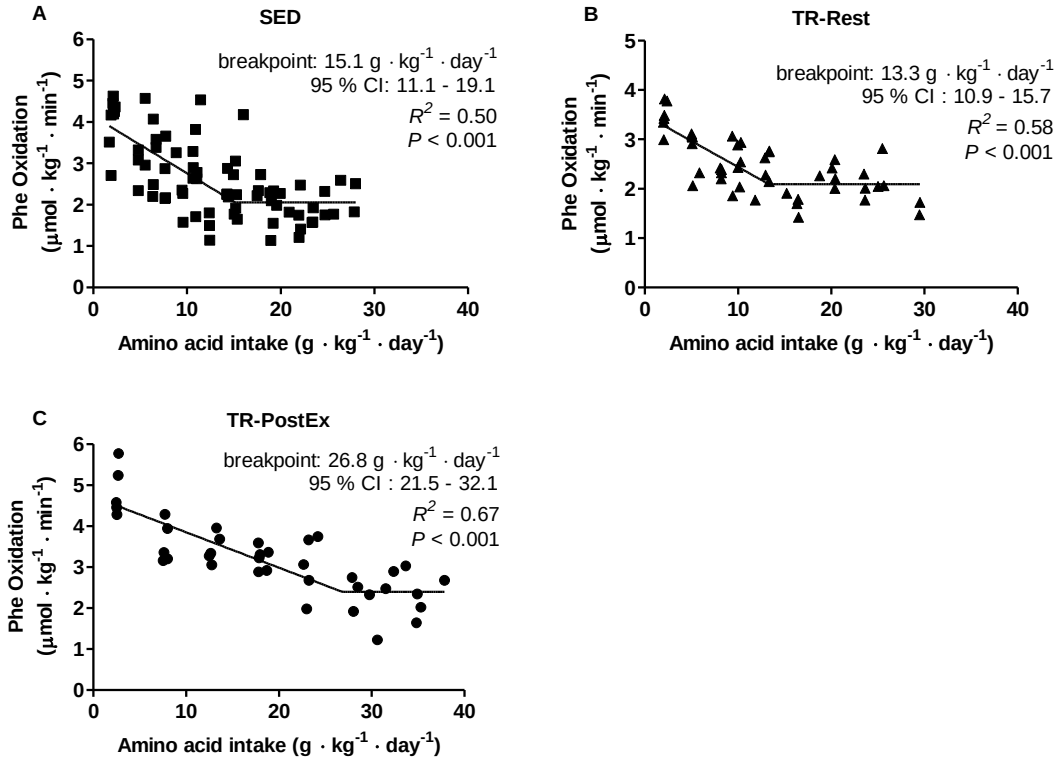


Figure 1.1.4 Optimal amino acid intakes of sedentary rats (SED; A, n = 74 (42 rats, 1 or 2 measurements/rat) at rest and in trained rats at rest (TR-rest; B, n = 46 (22 rats, 2 or 3 measurements/rat)) or postexercise (TR-PostEx; C, n = 38 (19 rats, 2 measurements/rat)

The data points represent individual phenylalanine oxidation during the metabolic trial. The rats consumed a range of test amino acid intakes. The breakpoints were determined using a biphasic linear regression model and represent the optimal amino acid intake to maximize whole-body protein synthesis. The breakpoint in the TR-PostEx group was significantly higher than those in the SED and TR-Rest groups ($P < 0.05$).

n, number of data points from each metabolic trial; CI, confidence interval

Chapter 2: Effect of leucine-enriched essential amino acids on muscle protein synthesis after exercise.

In this chapter, 2 studies were conducted to investigate the way of utilizing LEAAs to maximize MPS during postexercise recovery phase.

Section 1: LEAAs augment mixed protein synthesis, but not collagen protein synthesis, in rat skeletal muscle after downhill running

Section 2: Co-ingestion of carbohydrate with LEAAs does not augment acute postexercise MPS in a strenuous exercise-induced hypoinsulinemic state

2.1. Leucine-enriched essential amino acids augment mixed protein synthesis, but not collagen protein synthesis, in rat skeletal muscle after downhill running

2.1.1. Introduction

Skeletal muscles are plastic tissues, which change their phenotype in response to stimuli such as exercise and nutritional availability [3]. MPS is increased within 1-4 hour(s) and remains elevated up to 24-48 hours after resistance exercise [23]. Also, combination with protein or amino acid consumption with resistance exercise synergistically increase MPS within the immediate 1–4 hour(s) postexercise [25, 31, 33], and 24 hours after resistance exercise [34]. Therefore, given that resistance exercise enhances MPS up to 48 hours and dietary amino acids augments the process, it would be valuable to take amino acids over longer periods of post-exercise recovery (e.g. >24 hours).

It is well known that resistance exercise alone or that followed by the ingestion of essential amino acids (EAA), LEAA, or protein with or without carbohydrate (CHO) increases MPS in humans [25, 31, 97, 98]. Furthermore, protein or amino acid ingestion increases muscle mass during training periods [27]. Recently, the importance of protein or amino acid ingestion following endurance exercise has been attracting attention [4]. In particular, a mixture of LEAAs has been found to induce greater MPS than a standard EAA mixture [32]. Therefore, the importance of LEAA ingestion following both resistance exercise and endurance exercise is well-accepted. However, it remains to be clear that LEAA can synergistically augment the exercise-induced increment in MPS 24 hours after exercise.

In addition, global MPS should be increased for inducing muscle hypertrophy. However, for recovery from exercise-induced muscle damage, specific protein metabolism should be assessed. Collagen protein, the major protein of the extracellular matrix of skeletal muscle, has crucial roles in mechanical strength, and transmission of forces generated by muscle contractions [35]. The muscle collagen network provides a structural framework for skeletal muscle cells and should therefore grow with muscle hypertrophy. Collagen content can be increased by endurance training [99] or experimental compensatory hypertrophy [100]. Eccentric contractions result in muscle damage that involves muscle fibers and the extracellular matrix [36]. Failure in the re-organization of the extracellular matrix after exercise leads to accumulation of connective tissue which may interfere with tissue repair and functional recovery [37]. Thus, post-exercise re-organization of the skeletal muscle extracellular matrix is necessary for recovery from muscle damage.

Muscle collagen protein synthesis increases acutely following exercise [101, 102] and remains elevated for as long as 3 days [16]; the same is true for mixed MPS [23, 24, 103]. Whereas mixed protein synthesis is increased by enhanced amino acid availability both at rest and following exercise [25, 33], collagen protein synthesis in skeletal muscle is unaffected by enhanced availability of amino acids [104, 105]. In addition, post-exercise meal ingestion does not induce further increases in already elevated muscle collagen protein synthesis 0.5–5 hours following exercise [106]. However, muscle

collagen synthesis peaks between 6 hours and 1 day after exercise [16]. Therefore, questions remain as to whether the enhanced availability of amino acids might affect muscle collagen protein synthesis, particularly when post-exercise muscle collagen protein synthesis has peaked.

LEAA stimulates MPS at rest [107] and following exercise [31, 97] via activation of mammalian target of rapamycin (mTOR) [46]. Consumption of a leucine-enriched protein supplement induces gene expression related to extracellular matrix protein 30 min following exercise, and decreases the expression 4 hours after exercise [108]. Therefore, I hypothesized that LEAA may affect muscle collagen synthesis after exercise.

Thus, I investigated the effects of LEAA on muscle collagen protein synthesis after downhill running exercise, which can increase the expression of collagen in rodents [109]. First, I examined time-dependent changes of muscle collagen protein synthesis after exercise by measuring the enrichment of d_3-hydroxyproline in skeletal muscle after a flooding dose injection of d_3-proline. Next, I examined whether an amino acid mixture could affect collagen protein synthesis and mixed protein synthesis in skeletal muscle immediately after exercise, and 1 day after exercise at which the mixed protein and collagen protein synthesis peaked.

2.1.2. Material and method

Animals

This study was approved by the Institutional Animal Care and Use Committee of Ajinomoto Co., Inc. on March 30, 2011 (No. 20111210). All applicable international, national, and institutional guidelines for the care and use of animals were followed. Female 7-week-old Wistar rats (Charles River Laboratories Japan, Inc., Yokohama, Japan) were housed in a temperature-controlled room on a 12-h light-dark cycle (light 1000–2200 hours and dark 2200–1000 hours), and provided water and CR-F1 standard commercial chow (Charles River Laboratories Japan, Inc., Yokohama, Japan) ad libitum. After 1 week of acclimatization, rats were used for this experiment.

Experimental design

Data were obtained from multiple studies. A total of thirty overnight-fasted rats were used as a sedentary control group (SED), and a total of sixty-four rats underwent intermittent running on a motor-driven treadmill, at a speed of 17 m/min, for a total of 130 min on downhill (–13.5°) tracks [109]. During the exercise, 26 repetitions of five-minute running bouts were separated by 2-min intervals. The rats were adapted to the treadmill running with a lower speed for 3 days before the trial. Rats were orally administered distilled water (Con), immediately, 3 hours, 1 day, 2 days, 4 days, and 7 days after the completion of the exercise, or a LEAA mixture (1 g/kg; AminoL40) immediately and 1 day after the exercise. Mixed protein synthesis and collagen protein synthesis in skeletal muscle were determined as fractional synthesis rates (FSR, %/h), using the flooding dose method. Thirty minutes after the oral administration, rats were intravenously injected with flooding doses of proline (2.8 mmol/kg) containing L-2, 5, 5-d_3-proline (50 MPE, Sigma-Aldrich, St. Louis, MO) into their tail veins. This flooding dose of proline was selected because I had previously confirmed that it increases the intracellular enrichment of proline and remains steady for 30 min following injection. A blood sample was withdrawn from abdominal aorta of each rat under inhalation anesthesia (1.5% isoflurane). Subsequently, vastus lateralis muscle was removed, frozen in liquid nitrogen, and stored at −80 °C.

Leucine-enriched essential amino acids

The LEAAs mixture consisted of essential amino acids in the following proportions: histidine, 2%; isoleucine, 11%; leucine, 40%; lysine, 17%; methionine, 3%; phenylalanine, 7%; threonine, 9%; tryptophan, 1%; and valine, 11%. Except for the elevated proportion of leucine, this mixture contains the ratio of essential amino acids found in whey protein; all amino acids were manufactured by Ajinomoto Co., Inc., Tokyo, Japan. The AminoL40 mixture was deliberately developed to avoid decreasing the availability of the other EAAs while increasing the proportion of leucine.

Measurements of blood variables

Blood was separated from plasma by centrifugation at 10,000 × g for 10 min at 4 ℃, and the plasma was stored at −80 °C. Plasma amino acid concentrations in a part of sedentary rats and exercised groups of rats administered water (Control) or AminoL40 (1 g LEAA/kg) immediately or 1 day after exercise were measured with an automatic amino acid analyzer (JLC-500; JEOL, Tokyo, Japan).

Measurement of mixed and collagen protein synthesis

Contamination from non-collagen protein can lead to misinterpretations of collagen metabolism. This misinterpretation risk stems from protein synthesis rates in the protein fractions (i.e. myofibrillar proteins and sarcoplasmic proteins) which are substantially greater than those rates for collagen protein fractions [16, 104-106]; moreover, myofibrillar protein synthesis is increased by exercise and amino acid availability [110]. To avoid this potential problem, collagen protein synthesis was calculated by measuring labeled hydroxyproline, which had been generated by the hydroxylation of injected labeled proline in skeletal muscle [111-114]. The collagen molecule undergoes posttranslational hydroxylation of proline before being extruded from the endoplasmic reticulum [36]. Hydroxyproline cannot be recycled into protein, so it should be found in only collagen protein [115]. Approximately 30 mg of vastus lateralis muscle was homogenized in 15% sulfosalicylic acid, and the homogenate was centrifuged at 10000 × g for 10 min at 4°C. The supernatant was used for measurement of enrichment of intracellular free proline in vastus lateralis muscle. The precipitate, which was hydrolyzed in 2 ml of 6N hydrochloric acid at 90°C for 16 hours, was used for measurement of enrichment of protein-bound proline and hydroxyproline in vastus lateralis muscle. Amino acids in the supernatant and the hydrolysate were purified using cation exchange chromatography (Dowex 50 W 8X; Bio-Rad Laboratories, Hercules, CA), and dried in a rotary evaporator (Nakajima Corp., Tokyo Japan). D_3-proline enrichment ($E_{(Pro,\ muscle\ free)}$) in the supernatant was determined by its tert-butyl dimethyl silyl derivatization (N-Methyl-N-tert-Butyl dimethyl silyl trifluoroacetamide, Thermo Fisher Scientific, Waltham, MA) using gas chromatography–mass spectrometry (GC–MS; 6890 GC system and 5973 Network Mass Selective Detector, Agilent, Santa Clara, CA). I used the MS to monitor ions 286.2 and 289.2 in the electron impact mode. Muscle protein-bound d3-proline and d3-hydroxyproline enrichment were determined by measuring the butyl derivatization (HCl-n-butanol [10% v/v]: GL Science Inc., Japan) with liquid chromatography–mass spectrometry-enabled ion monitoring based on the former study with some modifications [116]. Seventy-five μL of 3N HCl-n-butanol was added to the sample residue and incubated for 15 min at 80 °C. Following butylation, the mixture was dried in a rotary evaporator, reconstituted in 400 μL of mobile phase (0.2% acetic acid). The butylated samples were separated on a 2.1 × 150 mm × 3 μm L-Column2 (Chemicals Evaluation and Research Institute, Tokyo, Japan) using a Prominence HPLC system (Shimadzu, Kyoto, Japan). Mobile phase A was 0.2% acetic acid, and mobile phase B was 0.2% acetic acid in acetonitrile. Gradient conditions were initial = 95% A and 5% B; 2.1 minutes = 85% A and 15% B; 6 minutes = 80% A and 20% B; 9 minutes =

65% A and 35% B; and 12.5 minutes = 98% A and 2% B, followed by 5 minutes equilibration with initial mobile phase (95% A and 5% B). MS/MS analysis was carried out using an API 3200 Triple quadrupole mass spectrometry system (SCIEX, Framingham, MA). Mobile phase was introduced into the mass spectrometer via the electrospray ionization source operating in the positive ion mode at 5500 V, curtain gas at 20 psi, collision gas at 6 psi, ion source gas1 at 60 psi and ion source gas2 at 70 psi. I monitored ions 174.2, 175.2 (for proline), 190.1, and 191.1 (for hydroxyproline) with the first mass spectrometer; I monitored ions 72.1, 72.3 (for proline), 88.2, and 89.2 (for hydroxyproline) with the second mass spectrometer; these analytical procedures used the external standard curve approach [67]. The FSR of muscle protein was calculated using the precursor–product model as described below,

$$\text{Mixed protein FSR (\%/h)} = E_{(\text{Pro, protein-bound})} / (E_{(\text{Pro, muscle free})} * t) * 100 \quad (1)$$

$$\text{Collagen protein FSR (\%/h)} = E_{(\text{HydroPro, protein-bound})} / (E_{(\text{Pro, muscle free})} * t) * 100 \quad (2)$$

In Equations (1) and (2), t represents the time interval between d_3-proline injection and tissue sampling; $E_{(\text{Pro, muscle free})}$ represents enrichment of precursor (intracellular proline); and $E_{(\text{HydroPro, protein-bound})}$ and $E_{(\text{Pro, protein-bound})}$ represent the enrichments of proline and hydroxyproline in product, respectively.

Statistical analysis

I report measurements as means ± SEM. One-way ANOVA followed by Bonferroni's multiple comparisons test was performed to test for significant differences between measurements. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). Tests with $P < 0.05$ were considered significant.

2.1.3. Results

Intracellular proline enrichment in vastus lateralis

There were no significant changes in the intracellular enrichment of proline in the vastus lateralis muscle between groups (**Table 2.1.1**). This result indicated that the enrichment of precursor was not affected by exercise or administration.

Mixed protein synthesis after downhill running exercise

Mixed protein synthesis in vastus lateralis muscle was elevated 1 day after the exercise (**Figure 2.1.1**, $P < 0.001$). LEAA administration led to heightened mixed protein synthesis in vastus lateralis muscle 1 hour and 1 day after the exercise, compared to distilled water administration, at each time point ($P < 0.05, 0.05$, respectively).

Collagen protein synthesis after downhill running exercise

Collagen protein synthesis in vastus lateralis muscle was elevated 1 and 2 days after the exercise (**Figure 2.1.2**, $P < 0.01, 0.01$, respectively), relative to the sedentary group. In contrast to the changes observed for mixed protein synthesis, LEAA administration did not induce a further increase in muscle collagen protein synthesis 1 h or 1 day after the exercise, relative to distilled water administration ($P > 0.05, 0.05$).

Amino acid concentrations after administration of LEAAs following downhill running exercise

Essential amino acid concentrations in plasma are shown in **Table 2.1.2.** Downhill running exercise did not affect essential amino acid concentrations in plasma 1 hour or 1 day after the exercise. At each time point, LEAA administration significantly increased the essential amino acid concentrations by 40–130%, with the exception of Trp concentrations.

2.1.4. Discussion

Through measuring enrichment of hydroxyproline in protein-bound fractions following flooding doses of intravenous d_3-proline, I found that muscle collagen protein synthesis is elevated and peaks at 1 day after downhill exercise, consistent with reported changes in collagen gene expression [109]. Subsequently, LEAAs do not affect muscle collagen protein synthesis 1 hour or 1 day after exercise, while the amino acids induce a further increase in mixed MPS 1 day after exercise compared with control. These results are consistent with a previous study on the effects of meal ingestion immediately after exercise [106]. While a peak in muscle collagen protein synthesis presents 1 day after exercise, this process does not respond to enhanced availability of amino acids. As collagen protein synthesis is not affected by nutritional availability at resting state [104, 105], muscle collagen protein synthesis might be regulated by mechanical stress rather than nutritional availability.

Nutritional and contractile regulation of global protein synthesis in skeletal muscle is well studied. Exercise increases MPS by activating the mTOR pathway along with the extracellular signal regulated kinase 1/2 pathway [117]. On the other hand, nutritional availability, particularly of leucine, also leads to MPS by stimulating the mTOR pathway [29]; such nutritional availability leads to further increases in MPS after exercise [31, 97]. Nutrient and contractile stimuli often converge at mTOR, suggesting that mTOR is an important modulator of protein synthesis. Although molecular signaling activation is not investigated in this study, essential amino acids such as leucine are sufficient to stimulate the mTOR pathway [29, 84], and mixed protein synthesis is increased by amino acid intake even in the absence of exercise. I initially hypothesized that mTOR activation would increase muscle collagen protein synthesis, but our observations contradicted this hypothesis. Focusing on the specific regulation of muscle collagen synthesis, the elevated collagen protein synthesis after exercise could be related to mechanical stress by itself and inflammation induced by muscle damage. Mechanical stress induces collagen gene expression in skeletal muscle via integrin [36]. Furthermore, interleukin-6, which is produced following muscle damage [118], induces fibroblast to produce collagen [119]. I have previously reported that a LEAA mixture suppresses the inflammatory response after eccentric exercise [120]. In addition, leucine administration alleviates the accumulation of collagen protein in muscle after cryolesion in rodents [121]. The absence of a difference in muscle collagen protein synthesis between LEAA-administered and non-administered groups may result from this amino acid supplement simultaneously activating the mTOR pathway and suppressed inflammation.

I found collagen protein synthesis was increased 1 and 2 days after the downhill running, while mixed protein synthesis was increased 1 day, but not 2 days after the exercise. Muscle collagen protein synthesis is increased by mechanical stress [36, 122] and inflammation [119]. In fact, 4.5-8.5 h after exercise, increases in collagen protein synthesis are identical for both shortening contractions and lengthening contractions [101]. On the other hand, downhill running in rats, which requires eccentric contraction of the quadriceps, results in more muscle damage than either uphill or level running [123].

For rats, muscle collagen accumulation occurs during the repair process of exercise-induced muscle injuries in rats [124]. Thus, muscle damage-related inflammation seems the most probable explanation for the presently reported increase in muscle collagen protein synthesis. Furthermore, muscle damage-induced inflammation is found starting at 1 day after the exercise, and not sooner [118]. In addition, gene expression of collagen protein was increased 6 hours to 4 days after the downhill running [109, 122]. Therefore, beginning 1 day after exercise, muscle damage-induced inflammation may increase muscle collagen protein synthesis. This result applies to the period following eccentric, muscle damage-inducing exercise.

Gene expression for muscle collagen protein is stimulated by mechanical stress [36, 122]. Next, procollagen is synthesized in the endoplasmic reticulum, and it undergoes posttranslational modification before being extruded from the endoplasmic reticulum to extracellular space. To the best of our knowledge, the present study is the first to assess muscle collagen protein synthesis measuring hydroxyproline enrichment in the protein bound fraction after stable isotope labeled-proline injection. This fraction is thought to contain pre-matured collagen (tropocollagen) along with matured collagen molecules. In contrast to previous studies in which the insoluble collagen protein (i.e. matured collagen) is assessed [104-106], the present measurements are sensitive to changes in muscle collagen protein. Due to using of stable isotope-labeled proline, the present study's method should be applicable for human study to assess collagen protein synthesis measuring enrichment of hydroxyproline.

In conclusion, through measuring the enrichment of hydroxyproline and proline in skeletal muscle, I find that downhill running exercise increases mixed protein synthesis for 1 day following exercise and collagen protein synthesis for 2 days following exercise. At 1 day post-exercise – the peak of post-exercise protein synthesis – administration of LEAA can lead to a further increase in mixed protein synthesis, but not collagen protein synthesis. These results suggest that, contrary to regulation of mixed protein synthesis, muscle collagen protein synthesis is not affected by nutrient availability.

2.1.5. Tables and figures

Table 2.1.1 Intracellular proline enrichment in vastus lateralis muscle in sedentary (SED) and exercised groups of rats administered with water (Con) or LEAA (1 g/kg; AminoL40).

Data are shown as means ± SEM (n in parenthesis). No significant differences between groups.

	Intracellular proline enrichment (%MPE)
Sed (30)	40.5 ± 0.2
1h-Con (6)	42.2 ± 0.2
1h-AminoL40 (6)	42.2 ± 0.3
4h (6)	40.9 ± 0.6
Day1-Con (15)	40.9 ± 0.4
Day1-AminoL40 (7)	39.5 ± 1.0
Day2 (9)	39.4 ± 0.9
Day4 (7)	40.6 ± 0.2
Day7 (8)	39.5 ± 1.0

Table 2.1.2 Plasma essential amino acid concentrations in sedentary (SED) and exercised (Con) groups of rats administered water (Control) or AminoL40 (1 g LEAA/kg) immediately or 1 day after exercise.

Data are shown as means ± SEM (n in parenthesis) in μM. Different letters denote significance of difference, at least $P < 0.05$.

Amino Acid					1 Hour								Day1							
(μM)	SED (14)				Con (6)				LEAA (6)				Con (7)				LEAA (8)			
His	47	±	2	c,d	57	±	3	b,c	71	±	3	a	39	±	2	d	58	±	3	b
Ile	72	±	2	b	85	±	3	b	368	±	26	a	62	±	3	b	313	±	22	a
Leu	115	±	4	b	140	±	4	b	1,449	±	101	a	100	±	5	b	1,305	±	89	a
Lys	574	±	24	c	631	±	31	c	1,850	±	91	a	530	±	33	c	1,599	±	48	b
Met	59	±	2	c	53	±	2	c	84	±	5	b	50	±	2	c	99	±	5	a
Phe	60	±	2	b	65	±	2	b	118	±	7	a	54	±	2	b	101	±	6	a
Thr	233	±	10	b	208	±	6	b	633	±	71	a	223	±	12	b	747	±	46	a
Trp	99	±	4	b, c	116	±	13	a, b	146	±	10	a	83	±	7	c	111	±	4	b, c
Val	164	±	6	c	196	±	5	c	1,034	±	50	a	145	±	6	c	875	±	45	b

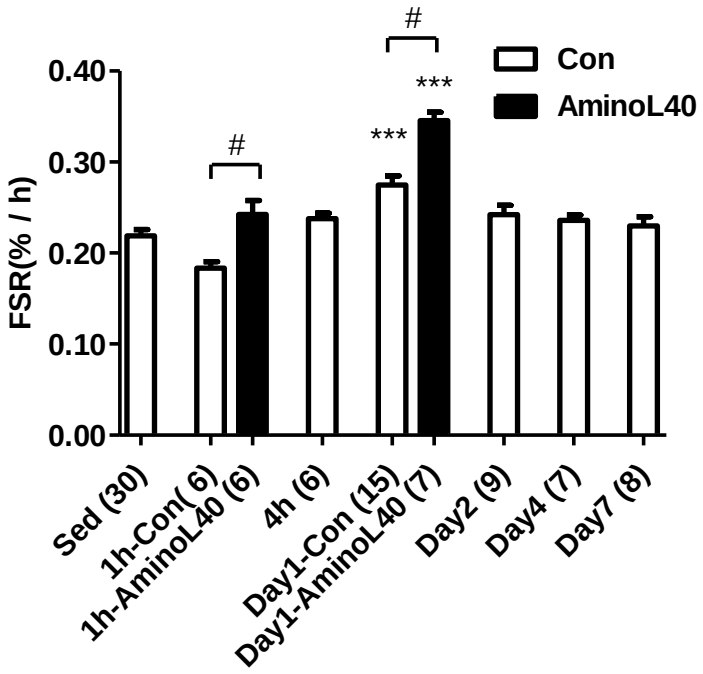


Figure 2.1.1 R**ates of mixed muscle protein synthesis for vastus lateralis muscle after downhill running exercise.**

Mixed MPS was measured in exercised groups with administration of water (open bars) before exercise, 1 h, 4 h, 1 day, 2 days, 4 days, and 7 days, and in exercised groups with administration of LEAA (filled bars) 1 hour and 1 day after downhill running exercise. Values are means ± SEM, with n below each bar. #, $p < 0.05$; ***, $p < 0.001$ vs. sedentary group

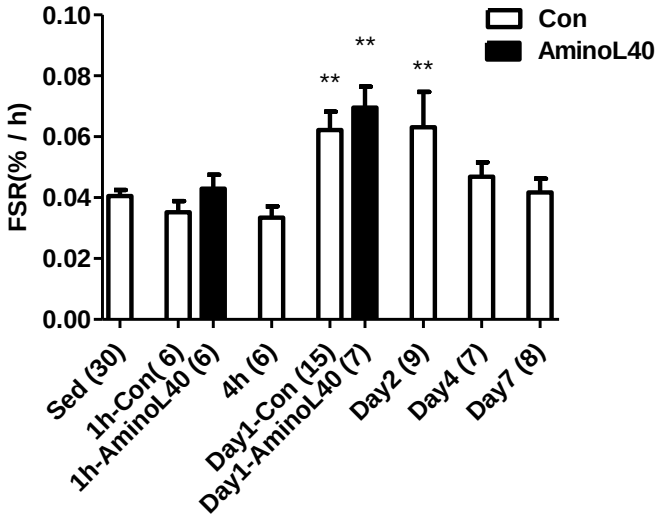


Figure 2.1.2 Rates of collagen protein synthesis for vastus lateralis muscle after downhill running exercise.

Collagen protein synthesis was measured in exercised groups with administration of water (open bars) before exercise, 1 h, 4 h, 1 day, 2 days, 4 days, and 7 days, and in exercised groups with administration of LEAA (filled bars) 1 hour and 1 day after downhill running exercise. Data are means ± SEM, with n below each bar. **, $p < 0.01$ vs. sedentary group

2.2. Co-ingestion of carbohydrate with leucine-enriched essential amino acids does not augment acute postexercise muscle protein synthesis in a strenuous exercise-induced hypoinsulinemic state

2.2.1. Introduction

Skeletal muscles are plastic tissues, which change their phenotype in response to stimuli such as exercise and nutritional availability [3]. Furthermore, the interaction between training-induced adaptation and nutrient availability has been investigated in detail [26]. Classically, high carbohydrate availability has been reported to ensure recovery from endurance exercise [125]. However, recent studies have reported that low carbohydrate availability can modify training adaptation [126]. Reduced carbohydrate availability because of low carbohydrate intake or overnight fasting increases fat oxidation during exercise and mitochondrial biogenesis [126]. Thus, endurance athletes should incorporate their training with low or high carbohydrate intake according to their training schedule [127]. However, there are some concerns regarding exercise with low glycogen availability. Exercise with low muscle glycogen can enhance amino acid oxidation during exercise [7]. Furthermore, a low-carbohydrate diet or overnight fasting before exercise has been associated with a decrease in plasma insulin, a well-known anabolic hormone [128, 129]. These changes can lead to muscle protein catabolism. Therefore, during the low-carbohydrate training period, close attention should be paid to maintain muscle mass.

Although a recent review suggested that CHO should be consumed with protein to maximize muscle hypertrophy by inducing an additive effect of insulin and leucine on protein synthesis [38], the necessity of CHO co-ingestion with protein or amino acids to augment postexercise MPS remains unclear [39]. In fact, hyperinsulinemia is reported to stimulate MPS rates [130, 131]. However, recent reports suggested that physiological hyperinsulinemia stimulated by the co-ingestion of CHO with protein or amino acid does not induce further increase in MPS [132-135]. Even basal levels of insulin after fasting are sufficient to enable amino acids to increase MPS under conditions where ample protein is ingested [136]. On the other hand, insulin secretion is inhibited to below basal levels by adrenergic receptor activation, both via the sympathetic innervation of the islets and by circulating catecholamines [40]. As a result, insulin concentrations decrease to less than the basal level according to the intensity and duration of the exercise and the duration of fasting before exercise [41]. However, whether the co-ingestion of CHO with amino acids can affect the augmented protein synthesis in a hypoinsulinemic state warrants clarification.

Thus, the purpose of this study was to investigate the effect of the co-ingestion of CHO with LEAAs on MPS in a hypoinsulinemic state induced by strenuous exercise following starvation. To this end, I assessed MPS by measuring the fractional synthesis rate (FSR) using the flooding dose method after the ingestion of LEAAs with or without glucose after jumping exercise in overnight fasted rats.

2.2.2. Material and method

Animals

This study was approved by the Institutional Animal Care and Use Committee of Ajinomoto Co., Inc. on December 24th, 2010 (No. 2010416). All applicable international, national, and institutional guidelines for the care and use of animals were followed. Eight-week-old male Sprague–Dawley rats (Charles River Laboratories Japan, Inc., Yokohama, Japan) were used in this study after 1 week of habituation. The rats were housed in a temperature-controlled room under a 12-hours light–dark cycle. They were also provided standard commercial chow (CR-F1; Charles River Laboratories Japan, Inc.), and water was provided ad libitum throughout the experiment.

Experimental design

The first step (Experiment 1) was to establish the exercise intensity of jumping exercise by measuring blood lactate concentration during the exercise. Six rats were made to perform the jumping exercise mentioned below after overnight fasting. Before the exercise, after 50, 100, and 200 jumps, blood samples were withdrawn from the tail vein. Immediately after blood sampling, blood glucose and lactate concentrations were measured using the Lactate Pro test meter (Arkray, Kyoto, Japan) and the Dia-sensor blood glucose tester (Arkray).

Having confirmed the exercise intensity, I proceeded to Experiment 2, in which the effect of the strenuous jumping exercise on plasma insulin and MPS was investigated. The study protocol is shown in **Fig. 2.2.1a**. Forty rats were divided into the following 6 groups: sedentary (SED, n = 6); immediately after exercise (PostEx, n = 7); and 1, 2, 4, or 6 h after exercise (n = 7 for 1, 2, and 4 h, n = 6 for 6 h). After overnight fasting, the rats underwent 200 repetitions of jumping exercise. MPS was determined as the FSR (%/h) using the flooding dose method as described by Garlick and McNurlan [137]. Briefly, rats were injected with flooding doses of phenylalanine (1.5 mmol/kg) containing L-[ring-$^{2}H_{5}$]-phenylalanine (50 MPE; Cambridge isotope, Cambridge, MA, USA) intravenously into the tail vein at rest (SED); before the exercise (PostEx); and 30 min (1 h), 100 min (2 h), 220 min (4 h), or 340 min (6 h) after the completion of the exercise. Twenty minutes after the tracer injection, blood samples were collected from the abdominal aorta under inhalation anesthesia with 1.5% isoflurane. The gastrocnemius (GAS) muscle was then removed, frozen in liquid nitrogen, and stored at −80°C.

Finally, having established the changes in plasma insulin concentrations and MPS after exercise, I proceeded to Experiment 3, in which I investigated the effect of LEAA administration and the addition of CHO to LEAA on MPS at a hypoinsulinemic state induced by strenuous jumping exercise. The study protocol is shown in **Fig. 2.2.1b**. Twenty-four rats were divided into the following 4 groups: sedentary (SED, n = 6) and rats administered distilled water as a negative control (Control, n = 5), LEAA mixture (AminoL40, n = 6), or LEAAs with glucose (AminoL40G, n = 7) following jumping exercise. After overnight fasting, rats in the Control, AminoL40, and AminoL40G groups performed

the jumping exercise. Immediately after the exercise, rats of the AminoL40 and AminoL40G groups were administered LEAAs (1 g/kg body weight) and LEAAs along with glucose (1 g/kg body weight) by oral gavage, respectively. As controls, rats of the SED and Control groups were administered distilled water. Thirty minutes after the oral administration, rats in all the groups were injected with tracer. Twenty minutes after the tracer injection, blood samples were collected from the abdominal aorta, and the GAS muscle was removed under anesthesia.

LEAAs and glucose

The LEAA mixture consisted of EAAs in the following proportion: histidine, 2%; isoleucine, 11%; leucine, 40%; lysine, 17%; methionine, 3%; phenylalanine, 7%; threonine, 9%; tryptophan, 1%; and valine, 11%. Except for the higher proportion of leucine, this mixture contains the ratio of EAAs found in whey protein. All amino acids were manufactured by Ajinomoto Co., Inc. The AminoL40 mixture was developed with the specific purpose of avoiding a substantial decrease in the availability of the other EAAs while increasing the proportion of leucine. For rescuing the decreased insulin concentration after exercise, 1 g glucose/kg was provided. This dose of glucose was selected to ensure the increase in glucose and insulin concentration after exercise in rats [138].

Jumping exercise

Rats were made to perform strenuous jumping exercise (height 35 cm, 200 jumps, 3-s intervals) as previously described in detail [139]. Such jumping training has been reported to induce an increase in the ratio of type II fiber to type I fiber [140] and bone mass [139]. This exercise model was selected to provide strenuous exercise with little acclimatization. Two days before the experiment, the rats were acclimatized to the jumping exercise as follows. The rats were placed in the jumping box, at the bottom of which an electrode plate was installed. Initially, the rats jumped upon electrical stimulation. Through acclimatization, the rats became accustomed to jump without electrical stimulation. On the experimental day, the rats were placed in the jumping box following overnight fasting. The rats then jumped and grasped the top of the box with their forelimbs, after which the rats climbed onto the wall of the box. Subsequently, the rats were caught by the investigators and returned to the bottom of the box for the next jump. This was repeated 200 times, and the total exercise time was roughly 14 min.

Measurements of blood variables

Blood was separated from plasma by centrifugation at 10,000 ×g for 10 min at 4°C, and the plasma was stored at −80°C. Plasma insulin concentrations were measured using a commercial ELISA kit (Morinaga Institute Biological Science, Yokohama, Japan). Plasma amino acid concentrations were measured with an automatic amino acid analyzer (JLC-500; JEOL, Tokyo, Japan). Plasma glucose

concentration was assayed for glucose content using the Glucose CII Test Wako kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) using glucose oxidase.

Measurement of the FSR

Muscle samples were ground, and intracellular free amino acids and muscle proteins were extracted as previously described [84]. Subsequently, phenylalanine enrichment (E(muscle free)) in the supernatant was determined by its *tert*-butyl dimethyl silyl derivatization (N-methyl-N-*tert*-butyl dimethyl silyl trifluoroacetamide; Thermo Fisher Scientific, Waltham, MA, USA) using gas chromatography–mass spectrometry (GC–MS; 6890 GC system and 5973 Network Mass Selective Detector, Agilent, Santa Clara, CA, USA) to monitor ions 336 and 341 in the electron impact mode. Muscle protein-bound phenylalanine enrichment (E(protein-bound)) was determined by measuring the butyl derivatization (HCl-*n*-butanol [10 v/v%]: GL Science Inc., Tokyo, Japan) using liquid chromatography–mass spectrometry to monitor ions 224 and 227 at the first mass spectrometry, and 122 and 125 at the second mass spectrometry (LC–MS/MS; Prominence HPLC system, Shimadzu, Kyoto, Japan and API 3200, SCIEX, Framingham, MA, USA) using the external standard curve approach [67]. The FSR of GAS muscle protein was calculated with the precursor-product model as previously described [84]. Briefly, MPS was calculated as follows: FSR (%/h) = E (protein-bound) / (E (muscle free) × t) × 100, where t represents the time interval between phenylalanine injection and tissue sampling.

Statistical analysis

Values are shown as means ± SEM. Repeated-measures ANOVA followed by Bonferroni's multiple comparison test was used to analyze the changes in blood glucose and lactate concentrations in Experiment 1. One-way ANOVA followed by Bonferroni's multiple comparison test was performed to test the changes in the other parameters. All the statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). Values of $P < 0.05$ were considered significant.

2.2.3. Results

Blood glucose and lactate concentrations during jumping exercise

Blood glucose was significantly lower after 200 jumps than that before the jumps (**Table 2.2.1**, $P < 0.01$). Blood lactate concentration increased significantly after 50 jumps and remained high until 200 jumps, compared with the pre value (Table 2.2.1, $P < 0.01$). The intensity of exercise was considered strenuous or high when lactate concentrations were >4 mM.

Changes in plasma glucose and insulin concentrations and MPS after jumping exercise

The plasma glucose concentration significantly decreased immediately after the jumping exercise (PostEx), gradually returning to the level of the SED groups until 6 h after exercise (**Fig. 2.2.2a**). Accordingly, the plasma insulin concentration decreased significantly immediately after the jumping exercise (PostEx), recovering 4 hours after the exercise (**Fig. 2.2.2b**). The FSR in GAS muscle protein was significantly lower in the PostEx group compared with all the other groups (**Figure 2.2.3,** $P < 0.05$).

Changes in MPS after the administration of LEAAs with or without glucose after jumping exercise

Although the jumping exercise alone did not increase MPS 1 hour after the exercise, the administration of LEAAs after the jumping exercise increased MPS compared with that in the SED and Control groups (**Figure 2.2.4**, $P < 0.05$). The co-ingestion of CHO with LEAAs following the jumping exercise increased MPS, while it did not induce any further increase in MPS compared with that in the AminoL40 group (Figure 2.2.4).

Blood variables after the administration of LEAAs with or without glucose following jumping exercise

Plasma glucose concentration was significantly lower in the Control group than that in the SED group (**Figure 2.2.5a**). Moreover, the administration of LEAAs induced a further decrease in plasma glucose concentration compared with those in the SED and Control groups. On the other hand, the administration of LEAAs with glucose recovered plasma glucose concentration to the level of the SED group (Figure 2.2.5a). The plasma insulin concentration after the administration of LEAAs with glucose was significantly greater than with the administration of LEAAs alone (**Figure 2.2.5b**, $P < 0.05$). Plasma amino acid concentrations are shown in **Table 2.2.2**. EAA concentrations, except for those of His and Trp, were significantly greater (2–9-fold greater) in the AminoL40 group than those in the SED and Control groups (Table 2.2.2, $P < 0.05$). Furthermore, the co-ingestion of glucose with LEAAs decreased the concentrations of Ile, Leu, Lys, and Val compared with those in the AminoL40

group, whereas the concentrations of EAAs except for His and Trp were significantly higher in the AminoL40G group compared with the SED and Control groups (**Table 2.2.2**, $P < 0.05$).

2.2.4. Discussion

The objective of this study was to investigate the effect of the co-ingestion of glucose with LEAAs on MPS under a hypoinsulinemic state induced by strenuous exercise following overnight fasting. First, by measuring insulin concentration after jumping exercise, I established the hypoinsulinemic state induced by jumping exercise. In addition, MPS was suppressed during exercise, reverting to the level of the sedentary rats 1 hour after the exercise. Second, LEAA administration with or without glucose following strenuous exercise augmented MPS. However, the co-ingestion of glucose with LEAAs did not induce any further increase in MPS compared with LEAAs alone, despite a recovery of the decrease in insulin concentration. Leucine-enriched protein feeding was recently reported to not impair exercise-induced fat oxidation during carbohydrate-restricted training [141]. Therefore, LEAA supplementation without carbohydrate intake is assumed to contribute to maintaining lean body mass without impairing training-induced adaptation during carbohydrate-restricted training.

Although amino acids, particularly leucine, are known to stimulate insulin secretion [29, 132, 142], LEAA administration alone did not alleviate the decrease in insulin concentration induced by strenuous exercise after overnight fasting. In previous studies, leucine or protein ingestion after exercise induced no or minimal increase in insulin concentration [134, 135, 138]. Therefore, the effect of amino acid or protein ingestion on insulin secretion is not sufficient to increase insulin concentration after exercise. In contrast to the ingestion of LEAAs alone, the co-ingestion of glucose with LEAAs reversed the insulin concentration to the basal level. However, the recovery of insulin concentration by adding glucose did not lead to any further increase in MPS, which was augmented by LEAA administration. Our results are consistent with former studies [134, 135], where hyperinsulinemia did not induce further increase in MPS compared with protein and/or amino acid ingestion. Therefore, based on our present findings and former studies, I surmise that the co-ingestion of CHO with protein or amino acids does not increase MPS, regardless of insulin concentrations. Leucine is also known to enhance protein synthesis by stimulating mTOR pathway [29]. Moreover, insulin affects mTOR activity by stimulating the insulin receptor substrate-1-Akt pathway [143]. Therefore, the lack of insulin mediated-augmentation of MPS reflected the fact that insulin shares the molecular pathway to stimulate MPS with leucine. In the current study, the dose of leucine administered was 0.4 g/kg, which is considered sufficient to maximize MPS [29]. However, in a different study, insulin co-ingestion with amino acid increased MPS when the administered dose of amino acid was not sufficient to augment MPS [144]. Therefore, when a smaller amount of LEAA is provided, the additive effect of co-ingested CHO might increase MPS. Similarly, insulin-like growth factor-1 (IGF-1) is well known to increase muscle protein synthesis in rodents [145] and in humans [144, 146]. However, there are some evidences arguing against IGF-1-mTOR pathway in the regulation of amino acid-induced MPS. At resting state, IGF-1 combined with amino acid infusion enhanced MPS more than amino acid infusion alone [144]. However, after resistance exercise under conditions of high endogenous hormone

(testosterone, IGF-1 and growth hormone) or low endogenous hormone concentrations, 25 g of whey protein, the amount of which is reported to maximize MPS after resistance exercise [33], induced similar responses in mTOR signaling or MPS [147]. The results indicated that, when amino acids are sufficiently ingested to maximize MPS, transient increases in endogenous anabolic hormones may not enhance fed-state anabolic signaling or MPS following resistance exercise. One possible mechanism is that amino acids and exercise itself fully activate mTOR pathway independent of insulin/IGF-1 signaling pathway [148, 149].

In addition to the effect of insulin on MPS, insulin has been reported to inhibit MPB without the ingestion of amino acids [131]. In addition, amino acids may enhance this effect [150]. Moreover, hyperinsulinemia has been reported to attenuate MPB following resistance exercise [151, 152]. Although MPB was not investigated in the current study, lower plasma concentrations of Leu, Ile, Val, and Lys were found after CHO co-ingestion compared to the plasma concentrations of these EAAs after the ingestion of LEAAs alone. This suggests that protein breakdown was reduced. Repeated, acute, net-positive protein balance induced by exercise results in chronic adaptation (i.e. muscle hypertrophy) [153]. However, MPB is likely to have a smaller impact on hypertrophy than MPS, because the magnitude of change in MPB is much lower than that in MPS [133].

In conclusion, our present results indicated that when a sufficient amount of LEAAs for maximizing MPS is provided, the co-ingestion of glucose with LEAA intake is not necessary to induce a maximal increase in MPS, even under a very low plasma insulin concentration induced by strenuous exercise following overnight fasting.

2.2.5. Tables and figures

Table 2.2.1 Blood glucose and lactate concentrations during dynamic exercise.

Data are shown as mean ± SEM (n = 6); **, P < 0.01 (significantly different from the pre value)

		Jump		
Variables	**Pre value**	**50**	**100**	**200**
Glucose, ng/mL	4.5 ± 0.2	4.6 ± 0.5	3.9 ± 0.5	3.5 ± 0.4**
Lactate, mM	1.8 ± 0.1	4.6 ± 0.4**	4.9 ± 0.6**	5.0 ± 0.5**

Table 2.2.2 Plasma essential amino acid concentrations in sedentary (SED) and exercised groups of rats administered water (Control), LEAA (AminoL40; 1 g LEAA/kg), or LEAA + glucose (AminoL40G; 1 g LEAA + 1 g glucose/kg) after exercise.

Data are shown as mean ± SEM (n = 6 for the SED and AminoL40 groups, n = 5 for the Control group, and n = 7 for the AminoL40G group). Different letters denote significant difference ($P < 0.05$).

	SED		Control		AminoL40		AminoL40G	
His	50.8 ± 3.8		48.3 ± 7.3		56.2 ± 4.9		51.8 ± 4.9	
Ile	98.1 ± 14.4	a	110.9 ± 14.7	a	443.3 ± 50.8	b	326.3 ± 46.2	c
Leu	149.0 ± 16.0	a	175.8 ± 32.7	a	1,594.0 ± 177.0	b	1,219.6 ± 163.6	c
Lys	558.3 ± 79.9	a	566.3 ± 142.7	a	1,499.2 ± 170.7	b	1,175.7 ± 165.8	c
Met	64.4 ± 13.1	a	83.0 ± 17.4	a	131.2 ± 13.2	b	125.1 ± 15.5	b
Thr	314.5 ± 39.5	a	311.5 ± 61.4	a	435.7 ± 59.5	b	515.0 ± 89.7	b
Trp	94.1 ± 20.1		95.4 ± 13.0		84.1 ± 9.4		98.9 ± 18.1	
Val	269.1 ± 30.4	a	328.7 ± 52.5	a	1,073.9 ± 125.1	b	869.9 ± 56.1	c

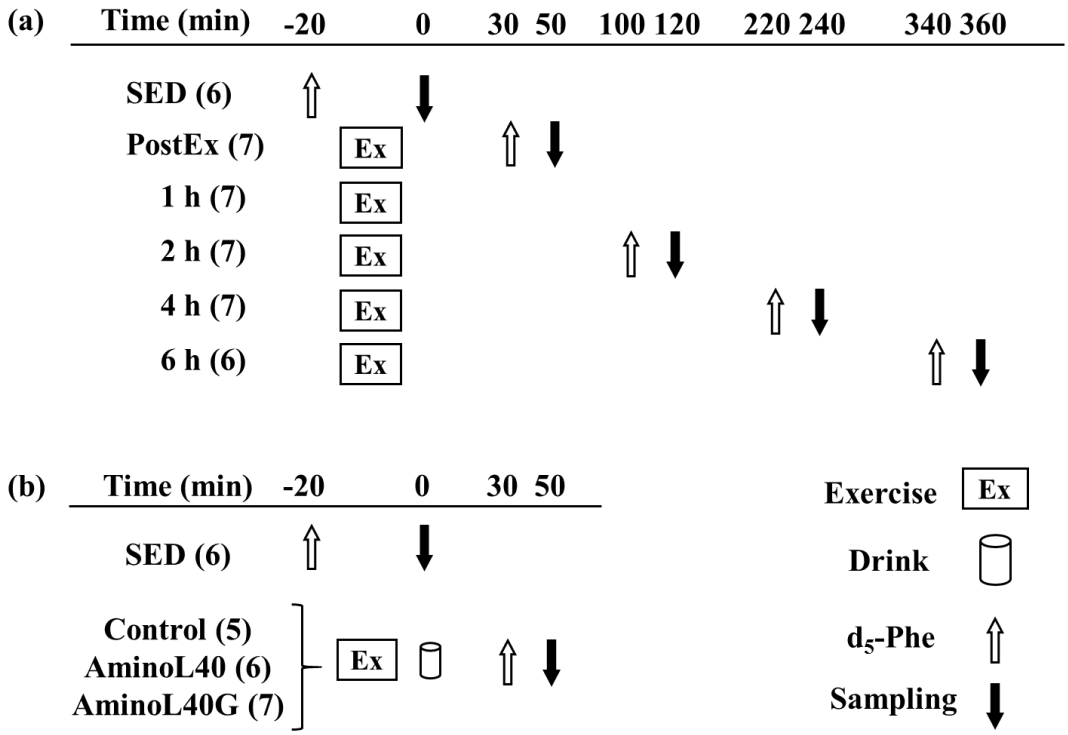


Figure 2.2.1 Schematics of the study protocols.

(a) Study design of Experiment 2 to measure MPS and plasma glucose and insulin concentrations after the jumping exercise following overnight fasting; (b) study design of Experiment 3 to measure MPS and plasma glucose and insulin concentrations after the administration of water (as a control), LEAAs, or LEAAs with glucose after the jumping exercise. The numbers of rats in each group are shown in parentheses.

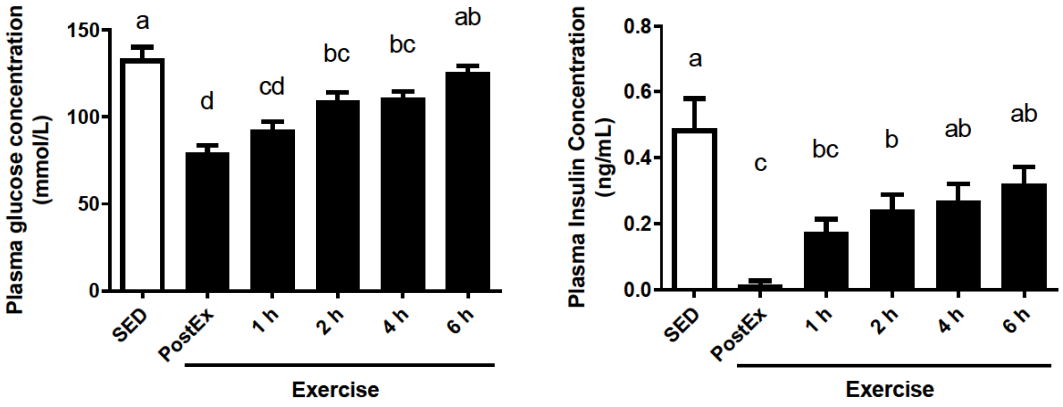


Figure 2.2.2 Plasma glucose (a) and insulin (b) concentrations in sedentary (SED; open bars) and exercised (ex; solid bars) groups of rats studied 1, 2, 4, and 6 h after dynamic exercise. Data are shown as mean ± SEM (n = 6 for the SED and 6 h groups, and n = 7 for the PostEx, 1 h, 2 h, and 4 h groups). Different letters denote significant difference ($P < 0.05$).

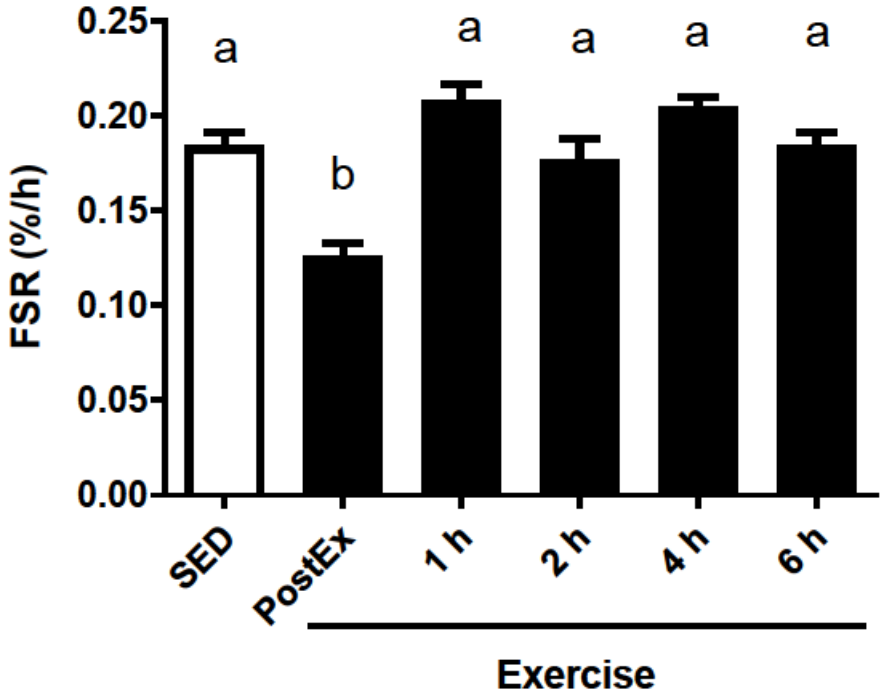


Figure 2.2.3 Rates of protein synthesis for mixed gastrocnemius muscle in sedentary (SED; open bars) and exercised (ex; solid bars) groups of rats studied 1, 2, 4, and 6 h after dynamic exercise. Data are shown as mean ± SEM (n = 6 for the SED and 6 h groups, and n = 7 for the PostEx, 1 h, 2 h, and 4 h groups). Different letters denote significant difference ($P < 0.05$).

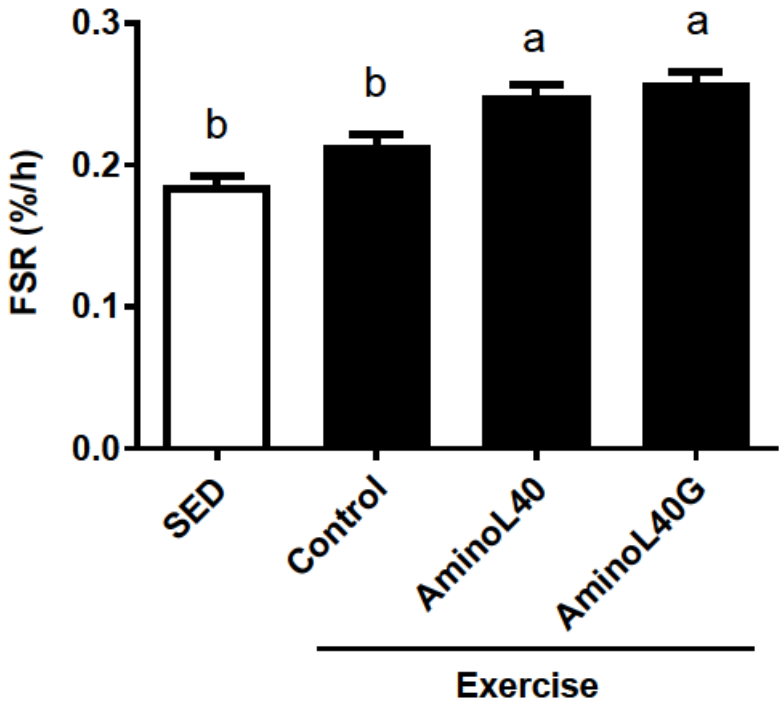


Figure 2.2.4 Rates of protein synthesis for mixed gastrocnemius muscle in sedentary (SED; open bars) and exercised (ex; solid bars) groups of rats administered water (Control), AminoL40 (1 g LEAA/kg), or AminoL40G (1 g LEAA + 1 g glucose/kg) after exercise. Data are shown as mean ± SEM (n = 6 for the SED and AminoL40 groups, n = 5 for the Control group, and n = 7 for the AminoL40G group). Different letters denote significant difference ($P < 0.05$).

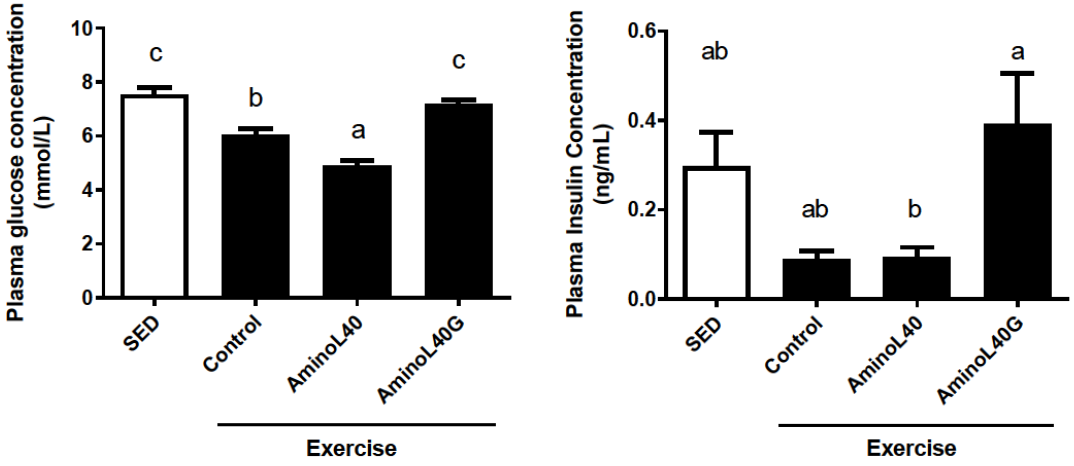


Figure 2.2.5 Plasma glucose (a) and insulin (b) concentrations in sedentary (SED; open bars) and exercised (ex; solid bars) groups of rats administered water (Control), AminoL40 (1 g LEAA/kg), or AminoL40G (1 g LEAA + 1 g glucose/kg) after exercise. Data are shown as mean ± SEM (n = 6 for the SED and AminoL40 groups, n = 5 for the Control group, and n = 7 for the AminoL40G group). Different letters denote significant difference ($P < 0.05$).

2.3. Summary of this chapter

I found that LEAA can synergistically increase MPS 24 hours after exercise, despite of no further increment in muscle collagen protein synthesis. Therefore, LEAA ingestion might be beneficial for inducing muscle protein anabolism not only immediately after exercise (i.e. within several hours after exercise), but also over longer time-frame (i.e. 1 day- several days after exercise). In addition, when a sufficient amount of LEAAs for maximizing MPS is provided, the co-ingestion of glucose with LEAA intake is not necessary to induce a maximal increase in MPS, even under a very low plasma insulin concentration induced by strenuous exercise following overnight fasting.

Chapter 3: Effect of leucine-enriched essential amino acids on muscle damage after eccentric contractions

In this chapter, 2 studies were conducted to investigate the effect of LEAA on recovery after eccentric exercise inducing muscle damage.

1) LEAA attenuate muscle soreness and improve MPS after eccentric contractions in rats
2) LEAA attenuate inflammation in rat muscle and enhance muscle repair after eccentric contraction

3.1. Leucine-enriched essential amino acids attenuate muscle soreness and improve muscle protein synthesis after eccentric contractions in rats

3.1.1. Introduction

The importance of muscle mass, strength, and metabolic function in athletic performance, daily activities, and general health is widely recognized. Resistance exercise increases muscle size and strength. The effect of resistance exercise on skeletal muscle growth depends on the mode of contraction and duration, intensity, and frequency of exercise [154]. Several studies have compared the effects of resistance exercise with concentric and/or eccentric contractions, showing that eccentric exercise results in greater gains in muscle strength and size than concentric exercise because of greater overload induced by eccentric contractions (ECs) [43]. Therefore, eccentric exercise is considered important for muscle growth.

However, eccentric exercise-biased training is not widely used, partly because eccentric exercise has some adverse effects. Eccentric exercise results in prolonged muscle weakness and muscle soreness [155], which are symptoms of muscle damage. This muscle soreness gradually develops and lasts for several days [156]. Muscle soreness and muscle weakness reduce the ability to perform athletic activities and potentially prevent regular exercise [44]. Therefore, to make eccentric exercise possible for various practical uses, (e.g., promoting general health and athletic performance), an effective intervention that decreases the adverse effects, and augments the positive effects of the eccentric exercise, needs to be identified.

The initial phase of the recovery process from muscle damage is characterized by inflammation and degeneration of damaged tissue. Satellite cells are then activated, and they proliferate, differentiate, and fuse to myofibrils to repair muscle tissue [157]. The recovery process is regulated by intracellular signaling pathways that control protein turnover, maintaining a balance between MPS and muscle protein degradation. Among these pathways, mTOR pathway is an essential step for muscle regeneration [45].

As described in previous chapter, LEAAs increase the rate of MPS after several types of exercise [31, 32]. However, there are no reports on the effect of LEAAs on recovery from muscle soreness after eccentric exercise. Some reports have shown that administration of amino acids [158], particularly leucine [159] or BCAAs [160], administration suppresses delayed-onset muscle soreness (DOMS) 1 day after exercise in humans. However, the mechanism by which amino acids act to decrease the degree of muscle soreness, and the optimal composition of amino acids to reduce the muscle soreness, remain unclear.

Wide individual variability of muscle soreness after eccentric exercise have been reported [161], and this creates a problem in assessing the effects of prophylactic or therapeutic interventions on muscle soreness. The wide individual variability isn’t attributed to genetic variability, and may be attributed

to the preconditioning induced by daily muscle contractile activity [161]. Thus, in order to investigate the optimal composition of amino acid to decrease muscle soreness or the mechanism by which amino acids acts to decrease muscle soreness, the animal model in which muscle contractile activity can be properly controlled, is required.

The purpose of this study was to investigate 1) the effect of LEAAs on muscle soreness, which is a typical symptom of exercise-induced muscle damage, and 2) the effect of LEAAs on the rate of MPS after ECs in rats. The tibialis anterior (TA) muscle of rats was forced to contract by electrical stimulation. The fractional synthesis rate (FSR; %/h) was determined by calculating the incorporation rate of L-[ring-$^{2}H_{5}$] phenylalanine into the skeletal mixed muscle protein pool. Muscle soreness was evaluated by measuring the mechanical paw withdrawal threshold according to the pressure stimulus.

3.1.2. Material and method

Animals

This study was approved by the Institutional Animal Care and Use Committee of Ajinomoto Co., Inc. Male Sprague–Dawley rats (300–350 g; Charles River Laboratories Japan, Inc., Yokohama, Japan) were used in this study. The rats were housed in a temperature-controlled room on a 12-h light–dark cycle. They were also provided a standard commercial chow (CR-F1; Charles River Laboratories Japan, Inc., Yokohama, Japan) and water was provided *ad libitum* throughout the experiment.

Experimental design

Experiment 1: The effect of LEAAs on the FSR in the TA muscle after ECs was investigated in rats after 3 h of food deprivation. The rats were divided into one of three groups: Sed, sedentary controls and administration of distilled water as a control (n= 9); EC-Con, ECs and administration of distilled water as a control (n= 10); EC-AminoL40, ECs and administration of LEAAs (n= 9). The TA muscle was stimulated electrically via needle electrodes that were inserted near the common peroneal nerve under anesthesia with sodium pentobarbital (50 mg/kg, i. p.). Electrical stimulation was applied for 1 s with current strength, which is three times as much as the twitch threshold (<100 μ A), and a frequency of 50 Hz with a pulse duration of 1 msec was used [162]. The TA muscle was simultaneously stretched with electrical stimulation from an ankle position of 45° to 135° over a 1-s period with the use of a linearized servomotor (CPL28T2B-06KD, OrientalMotor Co. Ltd, Tokyo, Japan), and then returned to the starting position over 3 s (ankle position was defined as the angle between the tibia and the plantar surface of the foot, with 180° representing a completely extended foot). The electrical stimulation was repeated every 4 s for a total of 500 repetitions. EC-AminoL40 group was administered LEAAs (1 g/kg body weight) by oral gavage 30 min before and 10 min after EC. The AminoL40 mixture consisted of essential amino acids in the following proportions: histidine, 2%; isoleucine, 11%; leucine, 40%; lysine, 17%; methionine, 3%; phenylalanine, 7%; threonine, 9%; tryptophan, 1%; and valine, 11%. Both the Sed and EC-Con groups were administrated distilled water as a control. The FSR was evaluated using the flooding dose method as described by Garlick and McNurlan [137]. Briefly, 30 min after second oral administration, rats in all groups were injected with flooding doses of phenylalanine (1.5 mmol/kg body weight) containing L-[ring-$^{2}H_{5}$]-phenylalanine (50 MPE, Cambridge isotope, Cambridge, MA) i. v. into the tail vein. 20 minutes after the phenylalanine injection, blood was collected from the abdominal aorta, the TA muscle was then removed under anesthesia, and frozen in liquid nitrogen and stored at −80°C. Blood was separated from plasma by centrifugation at 10,000 × g for 10 min at 4°C, and the plasma was stored at −80°C.

Experiment 2: EC and administration of LEAAs or distilled water were applied under the same conditions as experiment 1 (EC-Con and EC-AminoL40 groups, n=11 per group). Muscle soreness

was evaluated by mechanical paw withdrawal threshold as previously described [162]. Briefly, a Randall–Selitto analgesiometer (Ugo Basile, Italy) equipped with a probe with a tip diameter of 2.6 mm was used to measure mechanical paw withdrawal threshold. The TA muscle was pushed by the probe through shaved skin. The speed of force increment was set at 157 mN/s. The intensity of pressure causing an escape reaction was defined as the mechanical paw withdrawal threshold. The mechanical paw withdrawal threshold was evaluated before, and 1, 2, and 3 days after EC.

Measurement of the FSR

Approximately 30 mg of TA muscle was homogenized in 15% sulfosalicylic acid, and the homogenate was centrifuged at 10000 × g for 10 min at 4°C. The supernatant was used for measurement of enrichment of free phenylalanine in TA muscle. The precipitate, which was hydrolyzed in 2 ml of 6N hydrochloric acid at 90°C for 16 h, was used for measurement of enrichment of protein-bound phenylalanine in TA muscle. Amino acids in the supernatant and the hydrolysate were purified using cation exchange chromatography (Dowex 50 W 8X; Bio-Rad Laboratories, Hercules, CA), and dried in a rotary evaporator (Nakajima Corp., Tokyo Japan). Phenylalanine enrichment ($E_{(muscle\ free)}$) in the supernatant was determined by its tert-butyl dimethyl silyl derivatization (Thermo Fisher Scientific, Waltham, MA). Gas chromatography–mass spectrometry was used to monitor ions 336 and 341 in the electron impact mode (GC–MS; 6890 GC system and 5473 Network Mass Selective Detector, Agilent, Santa Clara, CA). Muscle protein-bound phenylalanine enrichment ($E_{(protein\text{-}bound)}$) was determined by measuring the butyl derivatization (HCl-n-butanol [10 v/v%]: GL Science Inc., Japan) using liquid chromatography–mass spectrometry to monitor ions 224 and 227 in the first mass spectrometry, and 122 and 125 in the second mass spectrometry (LC–MS/MS; Prominence HPLC system, Shimadzu, Kyoto, Japan and API 3200, Applied Biosystems, Carlsbad, CA) using the external standard curve approach [67].

Immunoblot analysis

Muscles were homogenized on ice in a five times volume of homogenization buffer (25 mM Tris-HCl [pH 7.6], 1% NP-40, 0.5% sodium deoxy cholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 1% protease inhibitor cocktail and 1% phosphatase inhibitor (Sigma Aldrich, St. Louis, MO)). The homogenates were separated by centrifugation at 19,800 × g for 30 min at 4°C. Protein concentrations were assessed in duplicate using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Muscle homogenates were solubilized in sample loading buffer (50 mM Tris-HCl [pH 6.8], 10% glycerol, 6% beta-mercaptoethanol, 2% SDS, and 0.1% bromophenol blue) at a concentration of 7.5 mg/ml and boiled for 3 minutes. Samples were loaded on SDS-polyacrylamide gels (TGX 4–15% gradient gel (Bio-Rad, Hercules, CA)). Protein was then separated by electrophoresis (200 V for 40 min at room temperature).

Separated proteins were transferred onto a polyvinylidene fluoride membrane (#162-0176; Bio-Rad, Hercules, CA) at 200 mA in transfer buffer (25 mM Tris base, 192 mM glycine, and 20% methanol). The membranes were blocked for 1 hour at room temperature in TBS-T (20 mM Tris-base, 150 mM NaCl, and 0.1% Tween-20) containing 5% bovine serum albumin (BSA), and were serially washed in TBS-T at room temperature. Membranes were then probed for specific signaling proteins using antibodies for detection of p70S6K1 and phospho-p70S6K1 (Thr 389). All antibodies were purchased from Cell Signaling Technology (Beverly, MA). Membranes were incubated overnight at 4°C in primary antibody buffer (5% BSA in TBS-T, pH 7.6, primary antibody diluted 1:1,000). The membranes were serially washed in TBS-T, incubated with horseradish peroxidase-conjugated secondary antibody (dilution, 1:10,000) in TBS-T for 1 hour, and serially washed in TBS-T. Horseradish peroxidase activity was detected using enhanced chemiluminescence reagent (Prime Western Blotting Detection System; Bio-Rad, Hercules, CA). Optical density measurements were obtained with densitometric scanning using the LAS3000 (Fuji Film, Tokyo, Japan). Membranes containing phospho-detected proteins were stripped of primary and secondary antibodies by use of stripping buffer (Nacalai Tesque, Kyoto, Japan). One hour after incubation in stripping buffer, membranes were reprobed for total protein with the specific antibody of interest. Immunoblot data are expressed as normalized phosphor-protein divided by total protein (phospho/total).

Measurements of blood variables

Insulin is a powerful stimulator of protein synthesis, and leucine is known to stimulate insulin secretion [29]. Therefore, to investigate the relationship between the stimulation of the insulin secretion by AminoL40 and MPS, plasma insulin concentrations were measured using a commercial ELISA kit (Morinaga Institute Biological Science, Yokohama, Japan). Plasma amino acid concentrations were measured with an automatic amino acid analyzer (JLC-500; JEOL, Tokyo, Japan).

Calculations

The FSR of TA muscle protein was calculated with the precursor-product model. The enrichment of precursor was represented by $E_{(muscle\ free)}$, and the enrichment of product was represented by $E_{(protein\text{-}bound)}$. MPS was calculated as FSR (%/h) = $E_{(protein\text{-}bound)}$ / ($E_{(muscle\ free)}$ *t)*100, where t represents the time interval between phenylalanine injection and tissue sampling.

Statistical analysis

Values are shown as means ± SE. A repeated measures two-way ANOVA followed by Bonferroni's multiple comparisons test was performed to test the mechanical withdrawal threshold with LEAA administration and various time points were the independent variables (GraphPad Prism; GraphPad Software Inc., San Diego, CA). Changes in the other measurements were examined with one-way

ANOVA followed by Tukey's multiple-comparisons test. Values of $p < 0.05$ were considered significant.

3.1.3. Results

Measurement of the FSR

The FSR in TA muscle protein was significantly lower in the EC-Con group compared with the Sed group (**Figure 3.1.1**, $p < 0.05$). The FSR was significantly higher in the EC-AminoL40 group compared with the EC-Con group (Figure 3.1.1, $p < 0.05$). There was no significant difference in the FSR between the Sed and EC-AminoL40 groups.

Immunoblot analysis

The phosphorylation of p70S6K1 at Thr389 was significantly higher in the EC-Con group compared with the Sed group (**Figure 3.1.2**, $p < 0.01$). There was a further increase in phosphorylation of p70S6K1 at Thr389 in the EC-AminoL40 group compared with the EC-Con group (Figure 3.1.2, $p < 0.05$).

Blood variables

Plasma insulin levels were significantly higher in the EC-AminoL40 group than in the EC-Con and Sed groups (**Figure 3.1.3**, $p < 0.05$). Furthermore, the data points from the EC-Con and EC-AminoL40 groups have been pooled in **Figure 3.1.4**; the data of EC-Con and EC-AminoL40 groups were pooled to examine the relationship between plasma insulin and FSR. ($R = 0.24$, $p = 0.33$, Figure 3.1.4). Plasma amino acid concentrations are shown in Table 3.1.1. Essential amino acid concentrations, except for histidine and tryptophan, were significantly greater (2 to 10-fold greater) in the EC-AminoL40 group compared with the Sed and EC-Con groups (**Table 3.1.1**, $p < 0.05$). However, histidine and tryptophan concentrations were lower in the EC-AminoL40 group compared with the Sed and EC-Con groups, despite LEAA administration (Table 3.1.1, $p < 0.05$).

Muscle soreness

The time course of the change in the withdrawal threshold after EC is shown in **Figure 3.1.5**. The decrease in mechanical paw withdrawal threshold to a pressure stimulus was significantly suppressed in the EC-AminoL40 group at 1 and 2 days after ECs compared with the EC-Con group ($p < 0.05$, Figure 3.1.5).

3.1.4. Discussion

The objective of this study was to investigate the effect of LEAAs on the rate of MPS and muscle soreness after eccentric exercise in rats. First, through the comparison of the EC-Con the Sed groups (which both received distilled water) I observed that the MPS was blunted 60 minutes after EC. Second, through the comparison between EC-AminoL40 and EC-Con groups, I found that administration of LEAAs significantly alleviated the EC-induced impairment of MPS, and induced greater phosphorylation of p70S6K1 than EC alone. Furthermore, LEAA reduced the muscle soreness evidenced by the decrease in the mechanical paw withdrawal threshold 1 and 2 days after EC. The current results suggested that LEAA administration could alleviate the impaired MPS and muscle soreness induced by eccentric exercise.

I found that EC decreased MPS. MPS is blunted 1 hour after prolonged exercise in rodents [138]. However, exercise is associated with maintenance or hypertrophy of skeletal muscle and not atrophy. Therefore, the rate of MPS must increase during recovery from exercise. In humans, a previous study showed that MPS is blunted during resistance exercise. The same study also reported that an increase in AMP-activated protein kinase (AMPK) activity and a reduced phosphorylation of 4E-BP1 may contribute to the suppression of MPS. [163]. It has been generally accepted that mTOR controls both 4E-BP1 and p70S6K1 directly. However, mTOR phosphorylation was unchanged immediately after exercise during the time when 4E-BP1 phosphorylation was reduced in Dreyer's study [163]. Furthermore, during post-exercise recovery, when MPS was stimulated, the increase in mTOR phosphorylation was associated with an increase in p70S6K1 phosphorylation. It suggested that the signaling mechanisms of mTOR to 4E-BP1 and p70S6K1 are regulated differentially and may be associated with the other mechanisms such as upstream regulation by TCS2[164]. In the current study, 60 minutes after EC the phosphorylation of p70S6K1 was five times higher in the EC-Con than in the Sed group. However, MPS remained blunted. It indicated that AMPK activation or the other mechanisms which suppress the MPS still remained to be clear.

Notably, LEAA administration further increased the phosphorylation of p70S6K1 relatively to that induced by EC alone. Although I did not measure any other signaling molecules related to a mTOR pathway, the dose of leucine administered in our study (0.4 g/kg body weight twice, with a total dose of 0.8 g/kg body weight) has been previously reported to be sufficient to increase mTOR pathway activity [29]. Resistance exercise and amino acid administration affects MPS additively and BCAA administration increases the augmented phosphorylation of p70S6K1 by resistance exercise [165]. Therefore, different mechanisms between exercise and amino acids are proposed for stimulating the mTOR pathway. However, in Crozier's study (2005), administration of 0.135 or 0.337 g/kg BW leucine increased the phosphorylation of p70S6K1 by four or six times its level in the control group, respectively; furthermore, MPS rates were not different between the groups. This suggests that, in the present study, the difference that I observed in the phosphorylation of p706SK1 between the EC-Con

and EC-AminoL40 groups might not be large enough to be a physiologically important. MPS was higher in the EC-AminoL40 group than in the EC-Con group, but was not different from the Sed group. This indicates that the LEAA administration could partly improve the negative effect of EC on MPS, but the factors which suppress the MPS still remained.

In the current study, MPS and p70S6K1 phosphorylation were measured 50 minutes after administration of LEAAs or 60 minutes after ECs. Amino acid concentrations were elevated immediately, reached a peak at 30 to 60 minutes, and returned to basal levels approximately 240 minutes after LEAAs administration. Furthermore, Katta et al. reported that the phosphorylation of p70S6K1 was increased 0 and 1 hour after ECs in rats [166]. Thus, measurement at 50 minutes post-administration of LEAAs, 60 minutes post-ECs allowed us to detect the peak change in MPS and p70S6K1 phosphorylation induced by the combination of LEAA administration and ECs.

I found that LEAA administration increased plasma insulin concentrations in plasma (Figure 3.1.3). Insulin is a powerful stimulator of protein synthesis, and leucine is known to stimulate insulin secretion. However, previous studies have reported that the stimulatory effect of leucine on MPS occurs without a concomitant increase in serum insulin concentrations [167]. Furthermore, a previous study demonstrated that contractile activity is a potent stimulus for blunting the net protein synthesis rate in rat skeletal muscle *ex vivo* and can even override the anabolic effect of insulin [168]. In our study, there was no correlation between insulin concentrations and MPS (Figure 3.1.4). Therefore, an increase in insulin concentration might not have contributed to the alleviation of reduction in MPS seen in our study.

I found that LEAA suppressed muscle soreness after EC (Figure 3.1.5). This is the first study to investigate the effect of amino acids on muscle soreness after EC in rats. Although muscle soreness is a typical consequence of eccentric exercise-induced muscle damage, the underlying mechanisms of muscle soreness are not clearly understood, but are probably related to the inflammatory response to muscle damage [169]. Ge et al. reported that the muscle regeneration process is regulated by the mTOR pathway [45]. Therefore, I investigated the effect of LEAAs, as a potent mTOR stimulator, on muscle soreness. EC increased the phosphorylation of p70S6K1, and the combination of EC and LEAA administration increased the phosphorylation of p70S6K1 more than EC alone. The precise mechanisms by which LEAAs act to cause such effects remain to be determined, but LEAAs may alleviate muscle soreness, an index of muscle damage, partly via activation of the mTOR pathway. BCAAs can be transaminated to glutamate to synthesize glutamine, which is highly consumed by inflammatory cells under inflammatory conditions [170]. Furthermore, BCAAs play a role as a precursor of glutamine and are metabolized to glutamine in skeletal muscle [171]. Therefore, BCAAs may affect the inflammatory status of damaged muscle by increased availability of amino acids as substrates for immune cells.

Pereira et al. reported that leucine administration improves the recovery of myofibril size and strength

after cryolesion-induced muscle damage [172]. The dose of administered leucine in our study (0.4 g/kg body weight twice, for a total dose of 0.8 g/kg body weight) was less than the dose of 1.35 g/kg body weight/d used in the study conducted by Pereira et al. (2014). However, in the current study, plasma BCAA concentrations in the EC-AminoL40 group were increased by 4–10 times compared with the EC-con group (Table 3.1.1). The increase in BCAA concentrations in plasma was sufficient to activate the mTOR pathway [29]. In the current study, LEAAs stimulated the phosphorylation of p70S6K1 augmented by eccentric contractions. However, in a study conducted by Pereira et al. (2014), these beneficial effects of leucine were not associated with activation of the mTOR pathway in regenerating muscle. This difference between studies in intracellular signaling pathways remains unknown, but it could be because of the difference in the degree of muscle damage. Muscle damage was not recovered until post-cryolesion day 10 in the study conducted by Pereira et al. (2014). However, in our study, DOMS was recovered at 3 days after EC. Therefore, the degree of muscle damage might be more moderate in this exercise-induced muscle damage model than in the cryolesion model. In moderately damaged muscle, protein synthesis may be more essential for regeneration than in the greatly damaged muscle. In the current study, muscle damage and muscle function were not specifically investigated because the blood sampling and tests required to assess the muscle function could have altered the results of the pain test.

In our study, I examined LEAAs, not leucine alone or BCAAs. Administration of leucine alone or BCAAs leads to a decrease in plasma concentrations of other essential amino acids in neonatal pigs [30] and in humans [165]. In normal swine, reduced availability of amino acids leads to blunted MPS and amino acid supplementation recovers this reduced MPS [173]. Furthermore, insulin-mediated hypoaminoacidemia reduces protein synthesis, and increased availability of all amino acids, but not leucine alone, recovers this reduction in protein synthesis. Therefore, other essential amino acids, except for BCAAs and leucine, may be essential for sustaining augmented MPS. Accordingly, LEAAs have been investigated to stimulate MPS after several types of exercise [31, 32]). Additionally, in our study, I found that LEAAs alleviated muscle damage after eccentric exercise. Therefore, LEAAs are effective for recovery after exercise via alleviation of muscle damage and enhancement of muscle adaptation.

In conclusion, LEAAs suppress the decrease in mechanical paw withdrawal threshold induced by EC in rats. Additionally, LEAAs alleviate impaired MPS by EC and further increases phosphorylation of p70S6K1 by EC. However, the precise mechanisms by which LEAAs act to confer such effects still need to be clarified. These findings suggest that LEAA administration before and after eccentric exercise hastens the recovery from muscle soreness.

3.1.5. Tables and figures

Table 3.1.1 Plasma amino acid concentrations. Amino acid concentrations (μM) in plasma were measured 30 min following the second administration of distilled water or LEAA. Values are means ± SEM, n = 9-10. Different characters indicate results significantly different ($p < 0.05$).

	Sedentary	EC-Con	EC-amino L40
Histidine	105.1 ± 5.0 a	110.0 ± 5.4 a	97.0 ± 6.5 b
Isoleucine	106.8 ± 6.7 a	99.1 ± 6.0 a	409.3 ± 42.7 b
Leucine	177.1 ± 10.5 a	160.9 ± 10.0 a	1,652.8 ± 157.2 b
Lysine	522.3 ± 33.2 a	474.6 ± 21.6 a	1,240.7 ± 102.9 b
Methionine	83.2 ± 5.4 a	82.4 ± 5.6 a	161.9 ± 12.0 b
Phenylalanine	1,337.0 ± 67.4	1,327.5 ± 92.4	1,368.3 ± 95.9
Threonine	487.5 ± 32.1 a	488.7 ± 35.1 a	1,051.5 ± 101.7 b
Tryptophan	138.4 ± 11.6 a	107.8 ± 6.6 ab	101.6 ± 8.4 b
Valine	268.4 ± 16.9 a	250.8 ± 14.8 a	891.7 ± 69.8 b

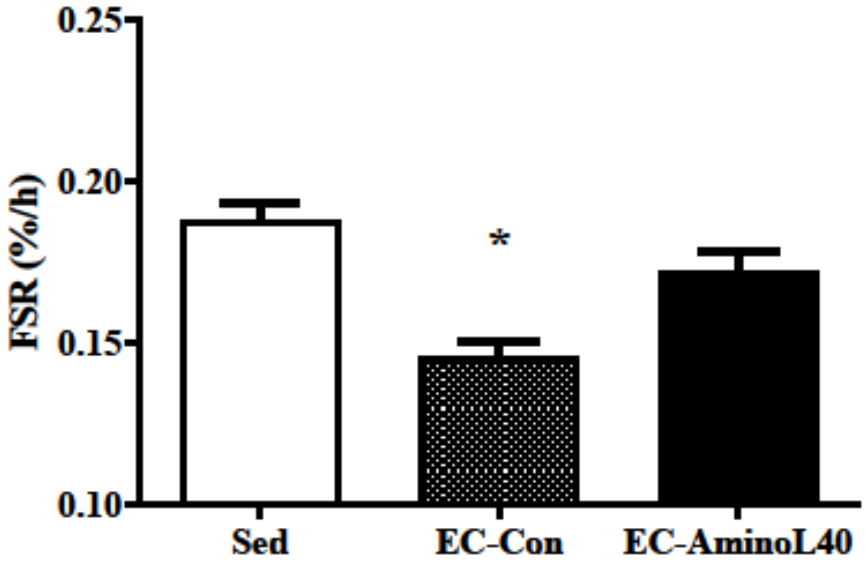


Figure 3.1.1 Effect of EC and administration of LEAA on the FSR (%/h) of TA muscle protein. The FSR in TA muscle protein was significantly lower in the EC-Con group compared with the Sed group. The FSR was significantly higher in the EC-AminoL40 group compared with the EC-Con group. There was no significant difference in the FSR between the Sed and EC-AminoL40 groups. Data are shown as mean ± SE (n = 9 for the Sed and EC-AminoL40 groups, and n = 10 for the EC-Con group). *p < 0.05 significantly different from other groups

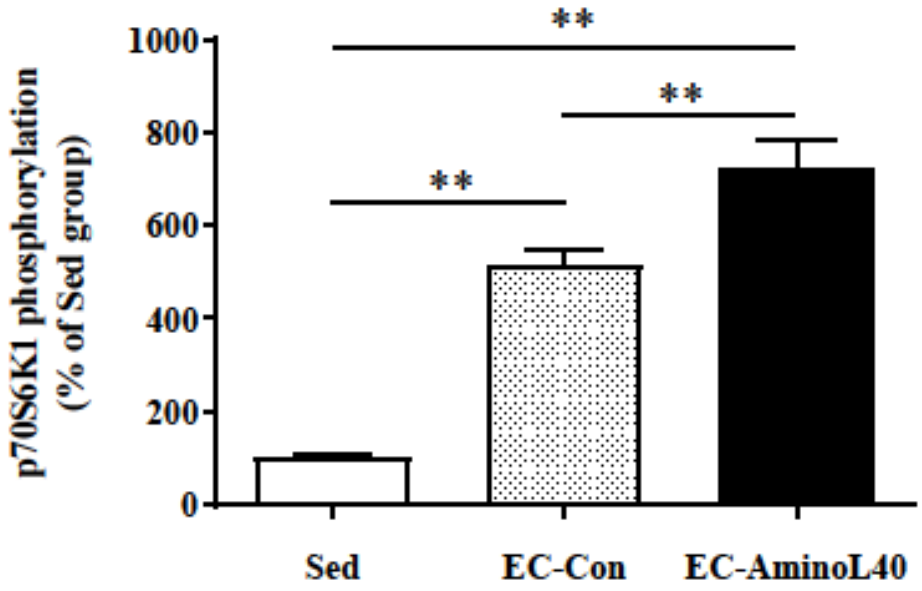


Figure 3.1.2 Effect of EC and administration of LEAA on the relative phosphorylation state of ribosomal S6 protein kinase 1 (phospho-S6K1 [Thr389]/total S6K1) in TA muscle. Phosphorylation of p70S6K1 was higher in the EC-Con group than the Sed group. Administration of AminoL40 further increased phosphorylation. Values are arbitrary units and presented as mean ± SE (n = 9 for the Sed and EC-AminoL40 groups, and n = 10 for the EC-Con group). **p < 0.01 between groups

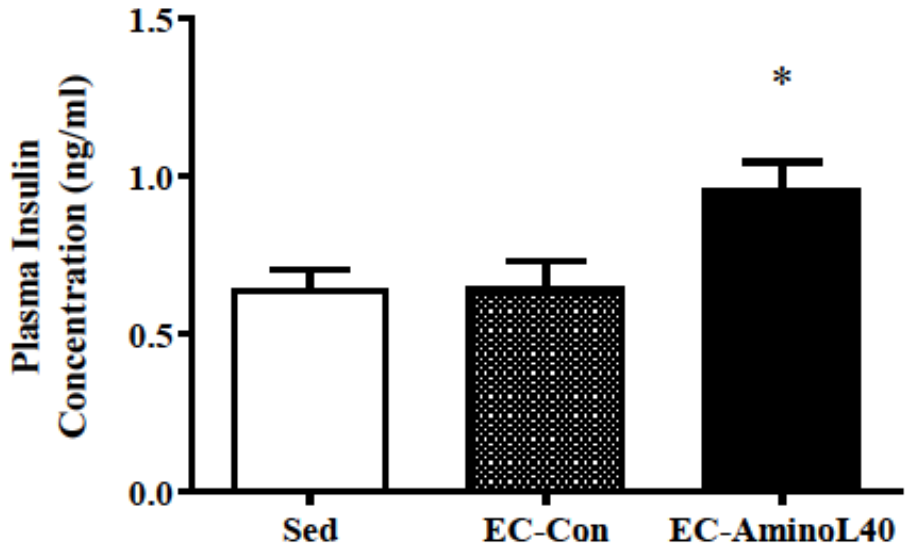


Figure 3.1.3 Effect of EC and administration of LEAA on plasma insulin concentrations. Insulin concentrations did not differ between the Sed and EC-Con groups, but were significantly higher in the EC-AminoL40 group compared with both the Sed and EC-Con groups. Data are shown as mean ± SE (n = 9 for the Sed and EC-AminoL40 groups, and n = 10 for the EC-Con group) *p < 0.05 significantly different from other groups

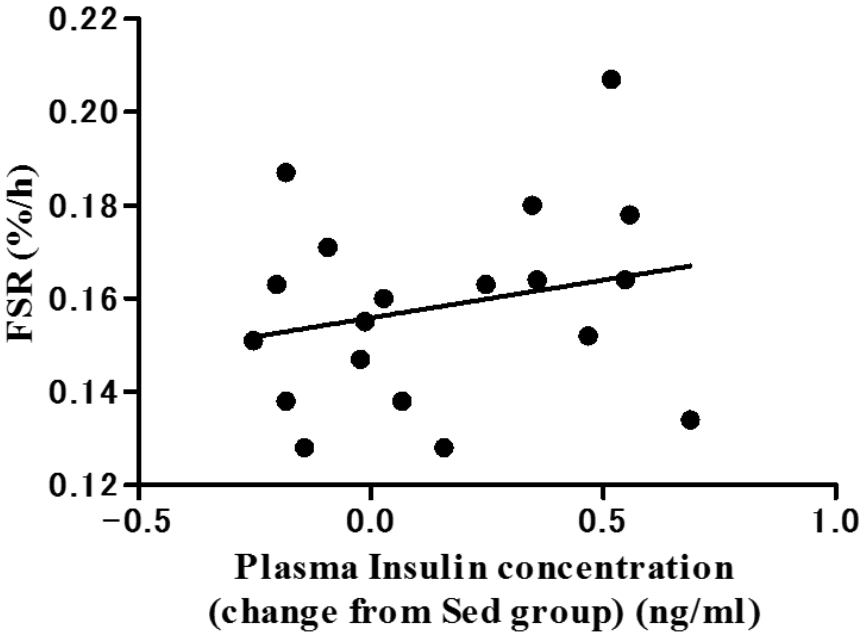


Figure 3.1.4 Correlation between the FSR in TA muscle and change in plasma insulin concentrations from Sed group. The 19 data points from EC-Con and EC-AminoL40 have been pooled in this figure. There was no significant correlation between the FSR in TA muscle and change in plasma insulin concentrations (R = 0.24, p = 0.33)

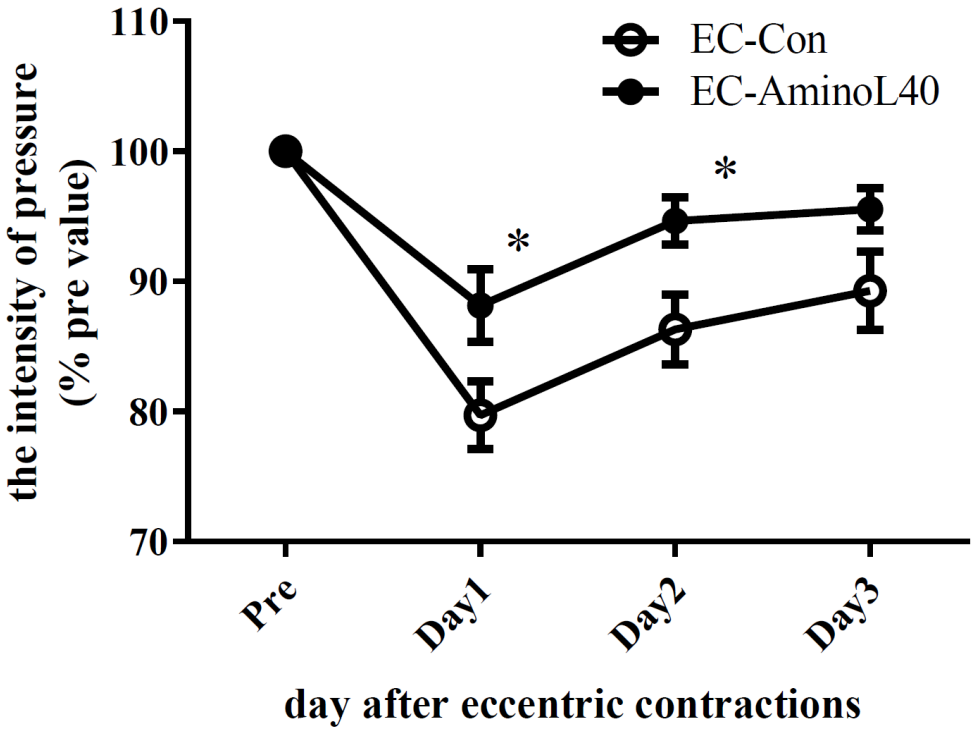


Figure 3.1.5 Effect of LEAA administration on mechanical paw withdrawal threshold after EC. The mechanical withdrawal threshold in the EC-AminoL40 group was significantly higher than in the EC-Con group on days 1 and 2. Values are shown as mean ± SE (EC-Con and EC-AminoL40 groups, n=11 for each group).*: $p < 0.05$ compared with the EC-Con group

3.2. Leucine-enriched essential amino acids attenuate inflammation in rat muscle and enhance muscle repair after eccentric contraction

3.2.1. Introduction

Eccentric contractions occur when a force applied to muscle exceeds the momentary force generated by the muscle itself [42]. Such contractions impact the human body in both adverse and favourable ways, as higher maximum force can be generated than during concentric or isometric contractions. Consequently, training that incorporates eccentric exercise results in greater gains in muscle strength and size [43]. On the other hand, eccentric contractions are also well known to induce muscle damage and dysfunction lasting several days to several weeks, and thereby reduce the ability to perform athletic activities and potentially prevent regular exercise [44]. Therefore, strategies to mitigate adverse effects may promote general health and athletic performance.

Initial muscle damage induced by mechanical stress triggers prominent local inflammation to remove cellular debris [174, 175]. In particular, increased Ca^{2+} influx due to disruption of the cell membrane in mechanically stressed skeletal muscle induces expression of the pro-inflammatory cytokine interleukin-6 (IL-6) [176]. In turn, IL-6 recruits and activates inflammatory cells to produce a host of cytotoxic substances, including superoxide anions and hydrogen peroxide [174, 177-179]. By producing additional pro-inflammatory cytokines [180], infiltrating inflammatory cells may induce secondary muscle damage. On the other hand, IL-6 is also essential for regeneration, a process controlled by the myogenic regulatory factors MyoD, Myf5, myogenin, and MRF4. For instance, IL-6 regulates myoblast proliferation and differentiation via MyoD and myogenin [181] , and controls macrophage migration during muscle regeneration [182]. Therefore, to improve muscle repair, it is important to suppress the pro-inflammatory effects of IL-6 without inhibiting regenerative activity.

Leucine has well known to stimulate mTOR, also known as the potent stimulator of protein synthesis [29]. Indeed, recent reports demonstrated that mTOR is a key pathway in inflammation-dependent muscle regeneration, and that mTOR overexpression attenuates inflammation in cardiomyocytes, and prevents cardiac dysfunction [183]. In addition, the potent mTOR inhibitor rapamycin impairs muscle regeneration after injury [45]. Accordingly, some reports have shown that administration of amino acids [158], particularly leucine [159] or branched chain amino acids (BCAAs) [160], suppresses delayed-onset muscle soreness and blood creatine kinase activity, which are typical symptoms of muscle damage. However, due to some limitations of the parameters measured [184], the impact of amino acids on recovery from muscle damage was not definitively established. Indeed, the most reliable markers of muscle damage are histology and muscle function [185]. However, repeated biopsies may also elicit increased inflammation in muscle [186]. In addition, a small muscle biopsy might not be sufficiently representative of the whole muscle [187]. Thus, it is necessary to analyse the whole muscle in order to understand the recovery process.

In neonatal pigs [30] and humans [165], administration of leucine or a mixture of BCAAs elicits a decrease in plasma concentrations of other essential amino acids. However, essential amino acids other than leucine are also required to sustain leucine-induced synthesis of muscle protein [188]. Indeed, LEAA can stimulate MPS via mTOR [46]. Therefore, I hypothesized that leucine-enriched amino acids may enhance muscle repair by modulating inflammation. Thus, I investigated the effects of LEAA on muscle damage, inflammatory response, and expression of myogenic factors after eccentric contractions in rats.

3.2.2. Material and method

Animals

This study was approved by the Institutional Animal Care and Use Committee of Ajinomoto Co., Inc. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Male Sprague–Dawley rats 8-9 weeks old (Charles River Laboratories Japan, Inc., Yokohama, Japan) were housed in a temperature-controlled room on a 12-h light-dark cycle, and provided water and CR-F1 standard commercial chow (Charles River Laboratories Japan, Inc., Yokohama, Japan) *ad libitum*.

Experimental design

The study design is illustrated in **Figure 3.2.1**. Sedentary rats received distilled water by oral gavage throughout the experiment (Sed, n = 24). Rats similarly treated with distilled water underwent electrical stimulation to induce eccentric contraction (EC-Con, n = 24). Finally, a group of rats that also underwent electrical stimulation to induce eccentric contraction received oral doses of LEAA instead (EC-AminoL40, n = 25). It was previously confirmed that daily administration of amino acids did not affect food intake. Under inhalation anaesthesia with 1.5 % isoflurane, rats were sacrificed immediately (n = 4), 1, 3, 7 days (n = 5), and 14 days (n = 5-6) after eccentric contraction. The tibialis anterior muscle was collected at various time points. The mid-belly of the muscle was fixed by neutralized 10 % formalin for histochemistry. The remaining tissue was frozen in liquid nitrogen, and stored at -80°C until gene expression analysis. The muscle samples collected 14 days after eccentric contraction were not used for these analyses because muscle damage and gene expression recovered within 7 days after eccentric contraction.

Eccentric contraction

Eccentric contraction was induced as previously described [189]. Briefly, animals were fasted for 3 h, and electrically stimulated to induce a total of five sets of 10 eccentric contractions, each set separated by 1 min. The tibialis anterior muscle was stimulated percutaneously under inhalation anaesthesia with 1.5 % isoflurane, using a pair of surface electrodes. Electrical stimuli were applied for 1100 msec with constant current 4-5 mA, frequency 100 Hz, and pulse duration 1 msec via a SEN-3301 electrical stimulator (Nihon Kohden Corp., Tokyo, Japan) fitted with an SS-202J isolator (Nihon Kohden Corp., Tokyo, Japan). The muscle was simultaneously stretched over 900 msec from an ankle position of 45 ° to 135 ° using a customized NDH-1 device (Bio Research Center Co., Ltd., Nagoya, Japan), beginning at 200 msec after the start of electrical stimulation.

Leucine-enriched essential amino acids

LEAAs (1 g/kg body weight) were administered by oral gavage once a day beginning at 2 days prior

to eccentric contraction until sacrifice. On the day of eccentric contraction, amino acids were administered immediately after the rats came out of anaesthesia, a process that took 30 minutes. The mixture consisted of 2 % histidine, 11 % isoleucine, 40 % leucine, 17 % lysine, 3 % methionine, 7 % phenylalanine, 9 % threonine, 1 % tryptophan, and 11 % valine (Ajinomoto Co., Inc., Tokyo, Japan). All control animals received distilled water by oral gavage once a day over the same period.

Muscle function

Under inhalation anaesthesia with 1.5% isoflurane, maximum isometric dorsiflexion torque was measured prior to (Pre-EC; n = 24-25) and immediately after eccentric contraction (Post-EC; n = 24-25), as well as 1, 3, 7 (n = 10-11), and 14 days after stimulation (n = 5-6). Maximal dorsiflexion was elicited using supramaximal tetanic current with train duration 650 msec, pulse 1 msec at 100 Hz, constant current 4-5 mA, and both knee and ankle joints set at 90 °.

Histochemical analysis

The tibialis anterior muscle was aligned in cross-section, and immersed in neutralized 10 % formalin for at least 3 days, and embedded in paraffin. Subsequently, cross-sections (5 µm thick) from paraffin-embedded tissue were stained with haematoxylin and eosin. Muscle fibres with nuclei stained by haematoxylin and infiltrated with inflammatory cells were considered damaged, and damage was assessed by point counting [190]. Briefly, four regions were randomly selected in each cross-section, and covered with a 792-point grid at 20× magnification to mark points over damaged muscle fibres. Damage is reported as percentage of damaged grid points. Histochemistry was assessed by investigators who were blinded to experimental conditions.

Semi-quantitative real-time PCR

Gene expression of cytokines and myogenic regulator factors was determined using semi-quantitative real-time PCR. Total RNA was extracted using RNeasy Fibrous Tissue Mini Kit (QIAGEN, Valencia, CA). Yield was measured using Nanodrop 1000 (Thermo Scientific, Waltham, MA), and quality was assessed by the ratio of absorbance at 260 nm to 280 nm. Total RNA (1 µg) was then reverse transcribed using PrimeScript RT Master Mix (Takara, Ohtsu, Japan). RNA and cDNA samples were then stored at -80 °C until further analysis. Relative mRNA expression was determined by real-time PCR using TP80 Thermal Cycler Dice Real-Time System (Takara Bio, Ohtsu, Japan) and SYBR Premix Ex Taq Tli RNaseH Plus (Takara Bio, Ohtsu, Japan). The details concerning these primers are shown in **Table 3.2.1**. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used control, and relative fold change was determined from cycle threshold (C_T) values using the $2^{-\Delta\Delta CT}$ method [191].

Measurement of amino acid concentrations in blood and muscle

Plasma samples were deproteinized by precipitation with 5% sulfosalicylic acid (1:1) and centrifugation for 10 min at 10,000 rpm and 4 °C. On the other hand, TA muscle was pulverized using Multi beads shocker (Yasui Kikai Corporation, Osaka, Japan), extracted with 17 volumes of 5% sulfosalicylic acid, and centrifuged for 10 min at 10,000 rpm and 4 °C. The supernatant was filtered at 10,000 rpm and 4 °C for 30 min using Amicon Ultra 0.5 mL (Merck Millipore, Billerica, MA). Amino acid concentrations in plasma and muscle were measured on an automatic amino acid analyser (JLC-500; JEOL, Tokyo, Japan).

Statistical analysis

Values are reported as mean ± SEM. All variables were examined by two-way ANOVA, with treatment and time as factors. When a significant main effect or interaction was observed, Bonferroni's multiple comparisons test was used to compare groups. Data were analysed in GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA), with $p < 0.05$ considered significant.

3.2.3. Results

Body weight and muscle weight

There was no significant difference in body weight and the weight of the tibialis anterior muscle among groups at all time points (**Figure 3.2.2**).

Muscle function

Maximum isometric dorsiflexion torque gradually increased over 14 days in Sed rats (**Figure 3.3.3**) as a result of muscle growth. Maximum isometric dorsiflexion torque significantly decreased immediately after eccentric contraction (Post-EC) relative to sedentary rats ($p < 0.001$), but gradually increased over the next 14 days. Indeed, there were significant differences throughout the experiment between sedentary rats and rats that underwent eccentric contraction. Notably, rats that received LEAA (EC-AminoL40) gained within 14 days the same level of isometric dorsiflexion torque as in Sed rats, while rats dosed with distilled water (EC-con) presented lower isometric dorsiflexion torque than Sed rats, indicating incomplete recovery. In addition, rats treated with amino acids regained stronger muscle function within 3 days relative to rats treated with distilled water ($p < 0.05$).

Histochemical features

Inspection of sections stained with haematoxylin and eosin did not show morphological changes in muscle fibres or presence of inflammatory cells in sedentary rats (**Figure 3.2.4a**). In contrast, damaged muscle fibres were observed 1, 3, and 7 days after eccentric contraction (**Figure 3.2.4b-g**). In rats treated with distilled water, damage due to eccentric contraction peaked at 41% of the total area of the tibialis anterior muscle within 3 days (**Figure 3.2.4h**). Notably, the number of damaged muscle fibres was significantly lower in rats treated with leucine-enriched amino acids than in animals treated with distilled water ($p < 0.001$, Figure 3.2.4h).

Gene expression

Expression of all genes tested did not change immediately after eccentric contraction (**Figure 3.2.5**). However, IL-6 expression significantly increased 1 day after eccentric contraction in rats treated with distilled water ($p < 0.001$ vs. sedentary rats). Subsequently, IL-6 levels returned to baseline within 3-7 days (Figure 3.2.5a). Leucine-rich essential amino acids significantly suppressed IL-6 expression 1 day after eccentric contraction ($p < 0.001$). Expression of IL-1β, myogenin, and MyoD increased within 3 days after eccentric contraction, but to levels comparable between rats treated with distilled water and those treated with leucine-enriched amino acids (Figure 3.2.5b-d).

Amino acid concentrations

The concentrations of essential amino acids in plasma and muscle tissue are listed in **Tables 3.2.2** and

3.2.3. Plasma Met decreased 1 day after eccentric contraction. Following LEAA administration, plasma His decreased in comparison with Sed rats, while plasma Leu and Met decreased relative to EC-Con rats. On the other hand, muscle His and Thr increased the day following eccentric contraction, and Ile, Leu, Met, Phe, and Val rose 2 days thereafter. However, LEAA administration significantly decreased His concentration 3 days after eccentric contraction.

3.2.4. Discussion

I found that LEAAs attenuated IL-6 expression in mechanically stressed muscles, in line with data obtained during recovery from endurance exercise [108]. One possible mechanism of this phenomena is that the amino acids modulate inflammation and IL-6 expression via activation of mTOR pathway. Indeed, mTOR overexpression suppresses IL-6 secretion from cardiomyocytes exposed to lipopolysaccharides [183], as well as inflammation in the heart after ischemia-reperfusion injury [192]. In addition, mTOR activation reduces activation and expression of intercellular adhesion molecule 1 in endothelial cells, and thereby inhibits neutrophil invasion [193]. Collectively, these observations indicate that mTOR may alleviate inflammation in some tissues and organs.

Alleviation of excessive inflammation is traditionally believed to enhance muscle repair [194]. Thus, the apparent ability of leucine-enriched amino acids to mitigate muscle damage likely depends on its ability to attenuate excessive inflammation due to IL-6, which is expressed in inflammatory and skeletal muscle cells [185], and believed to amplify the inflammatory response by inducing monocyte differentiation into macrophages and to increase the oxidative burst [195]. I note that IL-6 may also be involved in degrading muscle proteins during pathologic muscle-wasting [196, 197]. However, IL-6 is also essential for muscle regeneration [182], because it regulates myoblast proliferation and differentiation [181]. Thus, whether anti-inflammatory agents should be taken to enhance recovery from exercise remains an open question [194].

Accordingly, I found that LEAA also alleviated muscle dysfunction, which is correlated with the extent of muscle damage [189, 198]. Notably, these amino acids restored full muscle function 14 days after eccentric contraction without an observable increase in muscle mass, in line with previous results demonstrating that leucine accelerated connective tissue repair and functional recovery after cryolesion muscle damage [121], and also reduced macrophage infiltration into muscle tissue [172]. One possibility is that LEAAs help prevent inflammation-induced fibrosis [199], which may interfere with tissue repair and functional recovery [37].

Due to lack of isonitrogenous control, I cannot exclude the possibility that the beneficial effects of LEAA may be due to leucine alone, especially in light of reports indicating that branched-chain amino acids like leucine decrease markers of muscle damage in rats [121, 172] and humans [158-160, 200], including creatine phosphokinase and muscle soreness. In addition, branched-chain amino acids also accelerated recovery of maximum voluntary contraction after squat exercises [200]. However, even if this were the case, the identity of such amino acid may no longer be needed in practical terms, especially since other essential amino acids rather than leucine or a branched-chain amino acids are needed to maintain the increase in MPS induced by leucine [188]. In addition, LEAAs have been investigated to stimulate MPS after several types of exercise [31, 32]. Thus, the mixture of leucine-enriched amino acids, rather than branched-chain amino acids or leucine alone, may have sufficient biological activity in the end to promote post-exercise recovery by alleviating muscle damage,

enhancing muscle adaptation, and increasing protein synthesis.

Following oral administration, amino acid concentrations transiently increase, and return to basal levels within 9 hours [201]. Thus, plasma amino acids were comparable between EC-Con and EC-AminoL40 rats, because amino acids were not administered on the morning of the sampling day. Notably, most plasma amino acids were not affected by eccentric contraction, but higher intracellular concentrations of Ile, Leu, Met, Phe, and Val were observed 3 days later, which may indicate increased protein breakdown. On the other hand, LEAA administration did not alter the concentration of essential amino acids except Lys and His, implying that exogenous LEAAs probably do not affect the degradation of muscle protein.

In summary, I found that daily administration of leucine-enriched amino acids restored full muscle function 14 days after eccentric contraction, an effect not achieved using a similar regimen of distilled water. Indeed, muscle function was stronger within 3 days in rats treated with amino acids than in those treated with distilled water. I also found that leucine-enriched amino acids modulated IL-6 expression 1 day after contraction, as well as the ensuing muscle damage 3 days after contraction. However, the amino acids did not impact expression of the myogenic regulatory factors MyoD and myogenin. Taken together, the results suggest that the amino acids enhance muscle repair by suppressing excessive inflammation without impeding regeneration.

3.2.5. Tables and figures

Table 3.2.1 Primers for real-time quantitative PCR. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-6, interleukin-6; IL-1β, Interleukin-1β

Gene	Accession No.	Forward primer	Reverse Primer
GAPDH	NM_017008	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA
Myogenin	NM_017115	CCAGTGAATGCAACTCCCACA	GTAGGGTCAGCTGCGAGCAA
MyoD	NM_176079	CACACCTCTGACAGGACAGGACA	TTCTGACGGTTGGAATGCACA
IL-6	NM_001008725	ATTGTATGAACAGCGATGATGCAC	CCAGGTAGAAACGGAACTCCAGA
IL-1β	NM_031512	CTACCTATGTCTTGCCCGTGGAG	GGGAACATCACACACTAGCAGGTC

Table 3.2.2 Plasma amino acid concentrations. Plasma amino acid concentrations (μM) were measured 2 hours after measurement of muscle function. Values are means ± SE (n = 4-5). *, **: $P < 0.05$, 0.01 significantly different from the Sed group. +: $P < 0.05$ significantly different from the EC-Con group.

	Group	PostEC	1 day		3 days		7 days
His	Sed	69±4	66±1		67±1		67±1
	EC-Con	69±2	61±1		60±1		60±2
	EC-AminoL40	78±1	56±2	*	68±0		65±3
Ile	Sed	93±7	100±8		91±1		92±2
	EC-Con	93±6	88±3		83±2		84±3
	EC-AminoL40	108±6	88±4		101±3		100±2
Leu	Sed	163±10	177±14		163±4		165±4
	EC-Con	168±14	158±6		149±4		148±4
	EC-AminoL40	194±11	156±6		183±6	+	177±4
Lys	Sed	355±15	337±37		402±17		389±11
	EC-Con	336±10	324±17		374±13		362±12
	EC-AminoL40	348±14	322±22		406±10		380±14
Met	Sed	85±0	83±3		79±4		67±2
	EC-Con	78±4	63±4	**	67±3		73±1
	EC-AminoL40	89±3	76±3		83±5	+	77±4
Phe	Sed	71±4	75±2		76±1		75±4
	EC-Con	64±2	70±4		70±1		68±1
	EC-AminoL40	70±1	67±3		76±1		75±1
Thr	Sed	290±19	292±17		317±13		313±10
	EC-Con	344±24	292±18		297±11		298±5
	EC-AminoL40	383±26	305±40		326±25		293±9
Trp	Sed	97±7	102±4		83±6		109±9
	EC-Con	79±5	90±4		84±8		93±3
	EC-AminoL40	105±6	88±5		105±5		94±5
Val	Sed	206±16	225±17		211±4		208±6
	EC-Con	221±17	196±7		193±6		190±6
	EC-AminoL40	254±12	201±12		234±9		223±5

Table 3.2.3 Intramuscular amino acid concentrations. Intramuscular amino acid concentrations (μM) were measured 2 hours after measurement of muscle function. Values are means ± SE (n = 4-5). **: $P < 0.01$ significantly different from the Sed group. +: $P < 0.05$ significantly different from the EC-Con group. N.D = not detected.

	Group	PostEC	1 day		3 days		7 days
His	Sed	266±19	270±14		328±5		306±19
	EC-Con	283±16	396±44	**	267±16		251±22
	EC-AminoL40	247±20	328±26		167±15	**, +	187±30
Ile	Sed	131±9	125±9		129±5		121±5
	EC-Con	143±6	152±12		205±21	**	115±6
	EC-AminoL40	134±13	147±7		199±14	**	110±5
Leu	Sed	176±12	179±12		177±9		164±7
	EC-Con	198±8	230±17		338±35	**	171±8
	EC-AminoL40	194±14	226±11		318±23	**	176±5
Lys	Sed	1260±177	1191±140		1565±65		1429±69
	EC-Con	1158±120	1164±87		1204±100		1875±150
	EC-AminoL40	965±99	1129±129		1076±48	**	1766±148
Met	Sed	85±7	80±2		63±8		69±6
	EC-Con	104±9	114±7		146±22	**	73±11
	EC-AminoL40	99±6	109±8		148±11	**	77±5
Phe	Sed	96±3	91±3		98±7		89±4
	EC-Con	93±3	116±11		154±10	**	87±4
	EC-AminoL40	89±6	112±5		137±5	**	89±6
Thr	Sed	855±49	818±30		989±35		859±33
	EC-Con	908±82	1332±96	**	1246±41		1163±50
	EC-AminoL40	876±105	1360±206	**	1144±85		913±38
Trp	Sed	N. D.	N. D.		N. D.		N. D.
	EC-Con	N. D.	N. D.		N. D.		N. D.
	EC-AminoL40	N. D.	N. D.		N. D.		N. D.
Val	Sed	259±21	255±13		256±11		236±8
	EC-Con	287±18	318±23		376±29	**	232±10
	EC-AminoL40	270±18	304±18		363±18	**	232±3

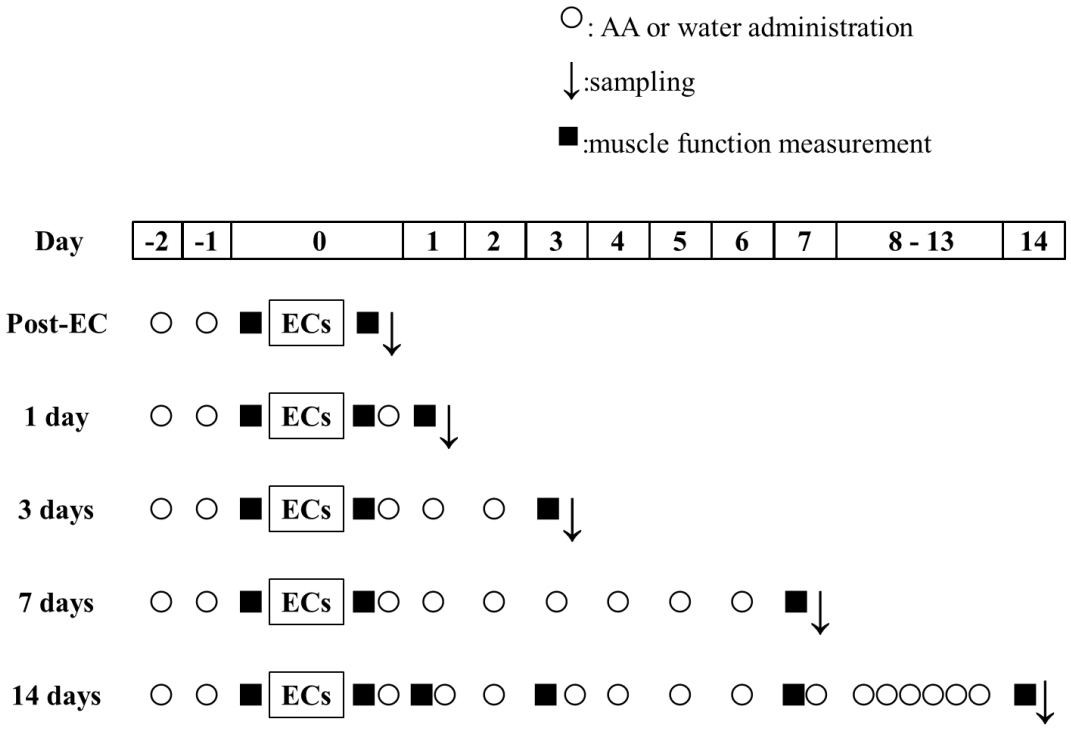


Figure 3.2.1 Overall experimental design. Rats treated with distilled water (EC-Con, n = 24) and leucine-rich essential amino acids (EC-AminoL40, n = 25) underwent electrical stimulation under anaesthesia to induce 5 sets of 10 eccentric contractions. Sedentary rats (n = 24) treated with distilled water were used as control. Muscle function was evaluated by measuring maximum isometric dorsiflexion under electrical stimulation. The tibialis anterior muscle was excised immediately after eccentric contraction (Post-EC, n = 4), and 1 (n = 5), 3 (n = 5), 7 (n = 5), and 14 days (n = 5-6) thereafter. ↓, tissue sampling; ■, measurement of muscle function; ○, daily dosing with LEAA or water

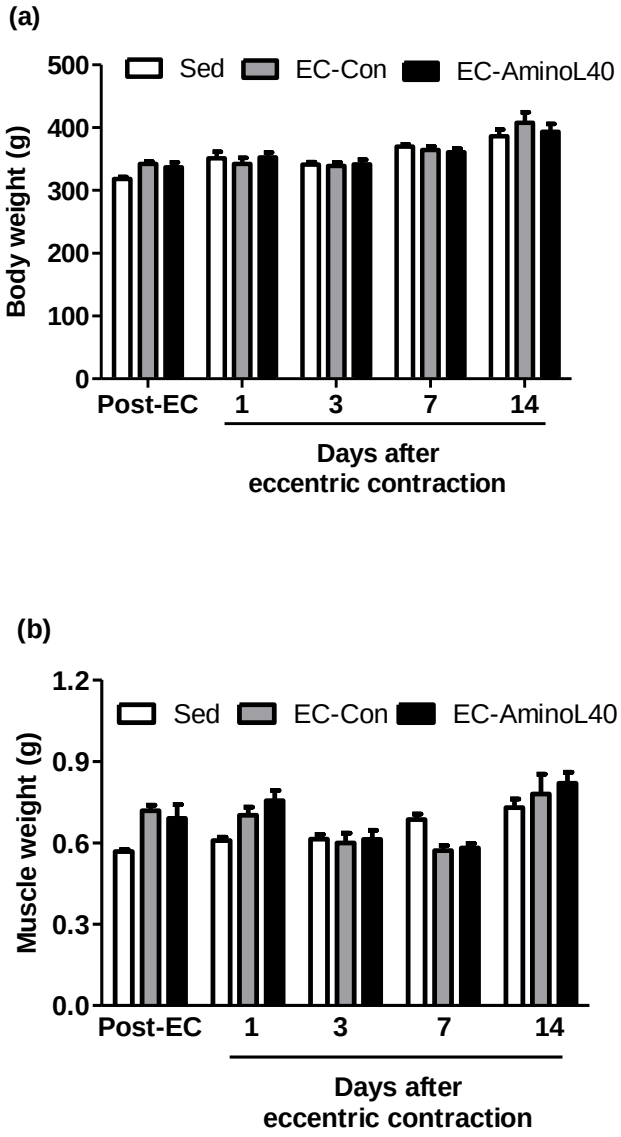


Figure 3.2.2 Body weight (a) and weight of the tibialis anterior muscle (b) immediately after (Post-EC), and 1, 3, 7, and 14 days after eccentric contraction. Data are mean ± SEM ((a) n = 5-25, (b) n = 4-6). There was no significant difference at all time points among sedentary rats treated orally with water (SED), and rats that underwent eccentric contractions and were treated orally with water (EC-Con) or LEAA (EC-AminoL40)

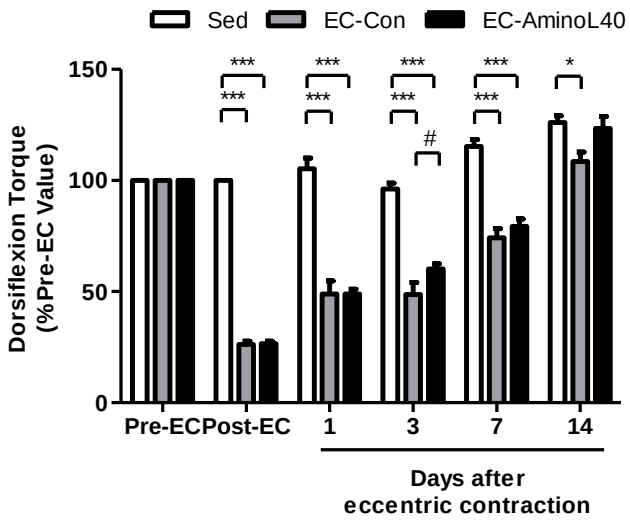
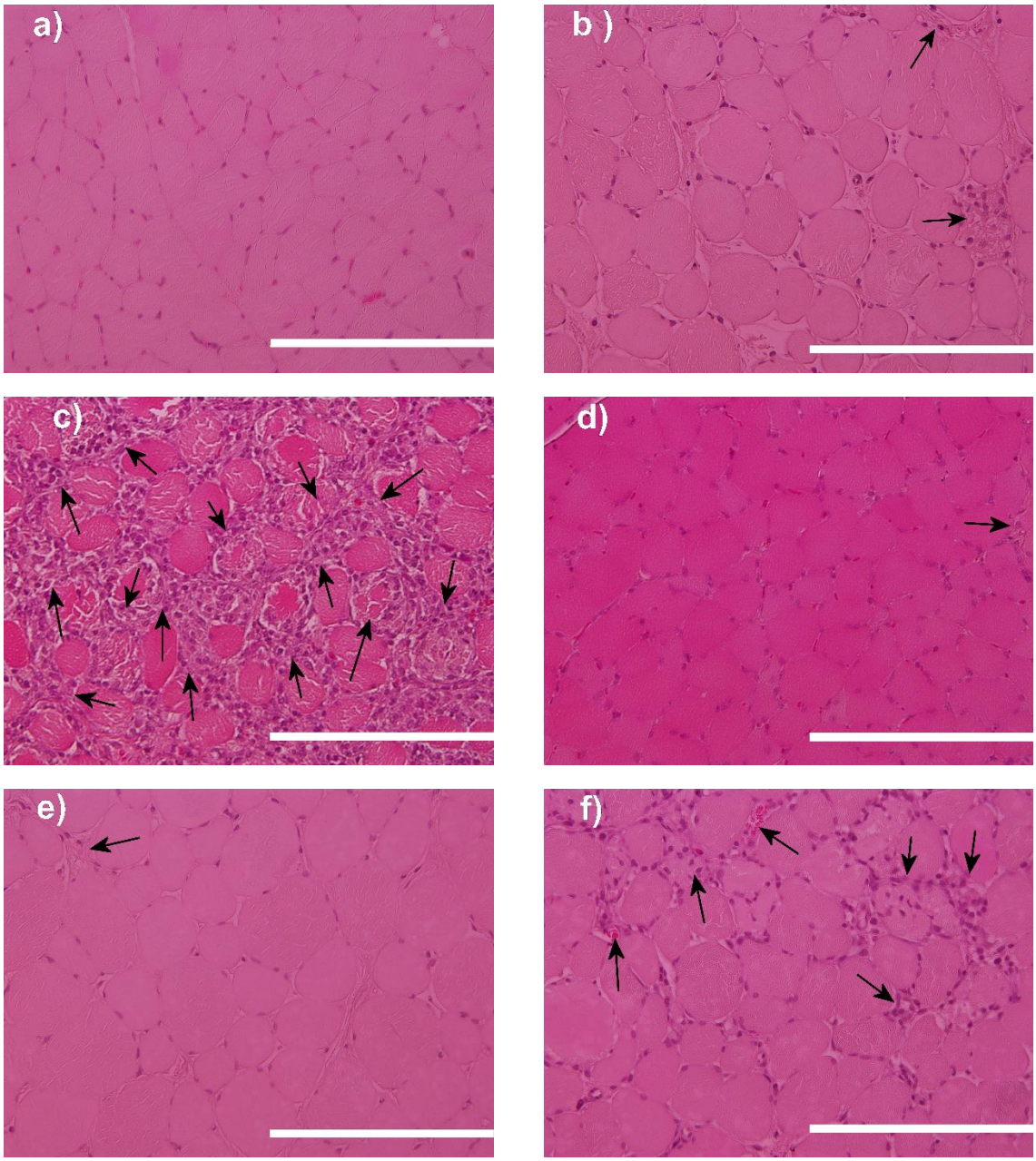


Figure 3.2.3 Time course of maximum isometric dorsiflexion torque. Maximum isometric dorsiflexion torque was measured before (Pre-EC, n = 24-25), immediately after (Post-EC, n = 24-25), as well as 1, 3, 7 (n = 10-11), and 14 days (n = 5-6) after eccentric contraction. Data are mean ± SEM. * and #, $p < 0.05$; ***, $p < 0.001$


a)
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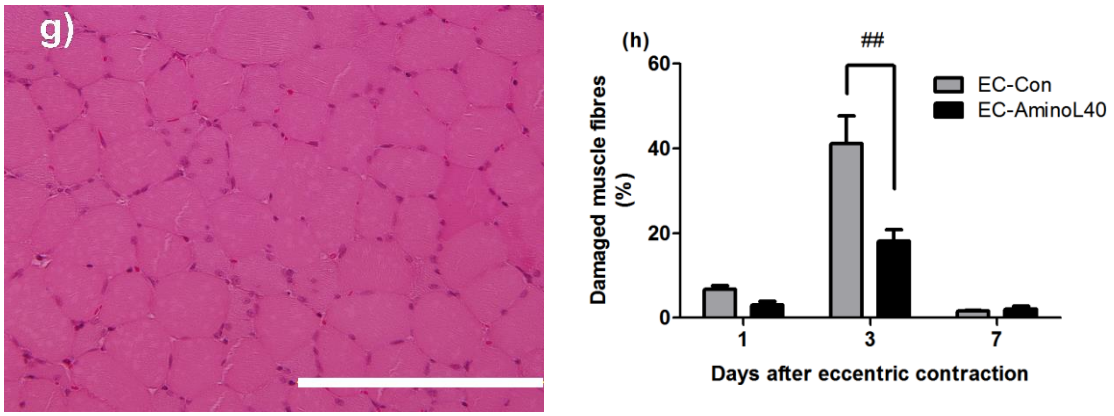

Figure 3.2.4 Histochemical analysis of the tibialis anterior muscle before and after eccentric contraction. Haematoxylin and eosin staining of transverse sections of the muscle in (a) sedentary animals (SED), and from (b-d) rats that underwent eccentric contraction and received water (EC-con) or (e-g) LEAA (EC-AminoL40). Tissues were collected (b, e) 1 day, (c, f) 3 days, and (d, g) 7 days after eccentric contraction. Images are representative sections from 4-5 rats sacrificed at a given time point, with magnification 40×. Scale bars, 100 μm. (h) Muscle damage 1, 3, and 7 days after eccentric contraction, as assessed by point counting, and expressed as percentage of muscle fibres infiltrated with inflammatory cells (arrows) in each sampling grid. Data are mean ± SEM (n = 4-6). ##, $p < 0.001$

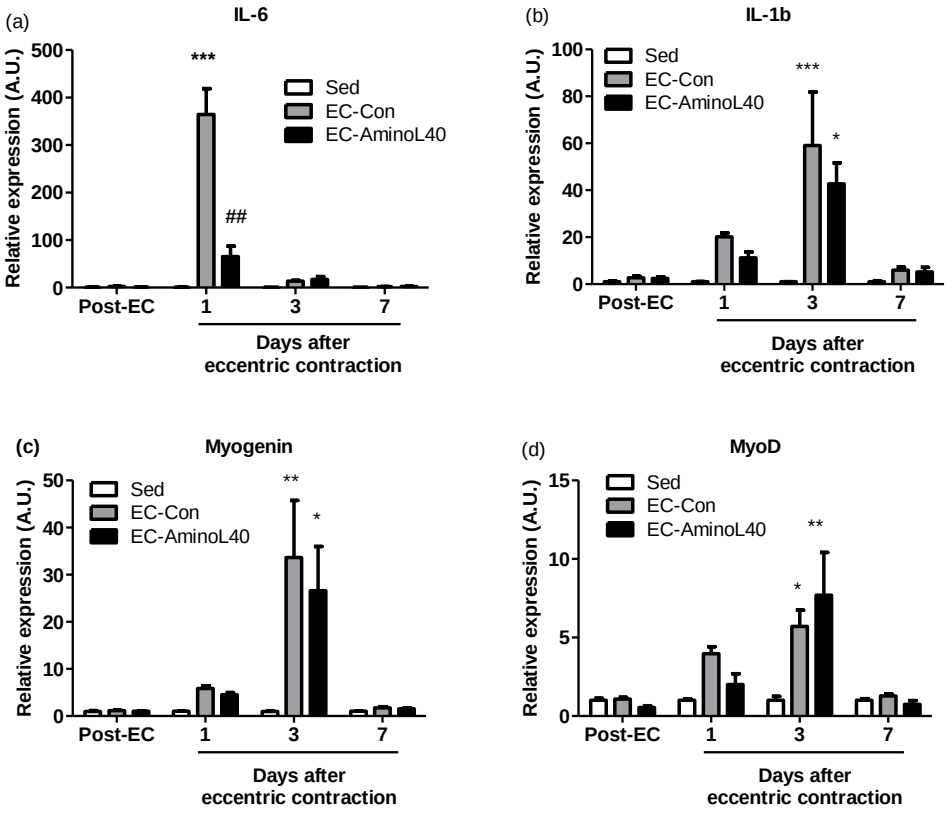

Figure 3.2.5 Time course of gene expression in muscle tissue. The tibialis anterior muscle was obtained at various time points from sedentary rats (SED), and from rats that underwent eccentric contraction and treated with distilled water (EC-Con) or LEAAs (EC-AminoL40). Samples were analysed by real time PCR to quantify mRNA transcripts of IL-6 (a), IL-1β (b), myogenin (c), and MyoD (d). Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase, and is reported as fold-increase relative to sedentary rats at each time point. Data are mean ± SEM (n = 4-6). *, **, and ***, $p < 0.05$, 0.01, 0.001 vs. sedentary rats; ##, $p < 0.01$ between EC-Con and EC-AminoL40 rats

3.3. Summary of chapter 3

I found that LEAAs can alleviate muscle soreness, and can enhance muscle recovery from exercise-induced muscle damage at least due to suppressed over-inflammation.

Overall discussion

Discussion

Postexercise nutritional strategies for competitive athletes should consider 3 approaches: refuel, remodel and repair [4]. First, substrate stores consumed during exercise, has to be refueled by the beginning of next exercise session. Refueling is paid much attention to especially after endurance exercise, because the type of exercise generally expend a lot of energy. To evaluate the amount of amino acid to be refueled, endurance exercise was applied in the first chapter in this thesis, because the amino acid requirements are increased after endurance exercise. Second, exercise induces some adaptive responses (i.e., mitochondrial density and (or) cross-sectional area) which requires remodeling of several proteins in skeletal muscle. In the process, enhanced MPS requires amino acids as substrate. Furthermore, increase in MPS derived from amino acid/protein ingestion induced better outcome in training adaptation [27]. In addition, MPS is also related with the third aspect, repair. Exercise induces damage in some tissues (mainly muscle). Mainly, the exercise which contains eccentric contraction, leads to muscle damage which last for several days to 2 weeks. The muscle damage reduce the ability to perform athletic activities and potentially prevent regular exercise [44]. Therefore, strategies to mitigate adverse effects may promote general health and athletic performance. To evaluate the significance of amino acid intake in the repair and remodeling process, eccentric-biased exercises were applied in second and third chapter. Downhill running and electrical stimulation model were widely utilized to investigate the muscle damage in rodents [189, 202-204]. After downhill running, cytoskeletal proteins were diminished in quadriceps muscle, which works for absorbing the impact of landing [202]. In addition, the model was recruited to investigate the muscle collagen turnover [109]. Similarly, loss of cytoskeletal proteins were found after eccentric contractions [189, 203, 204]. Furthermore, impaired muscle exercise performance (i.e. power output or muscle soreness) was found dramatically after eccentric exercise [162, 189, 204, 205]. The jumping model was applied to investigate bone metabolism in former studies [139, 206, 207], but not muscle damage. The chronic training utilizing the jumping exercise induced bone stiffness and bone mass [139, 207]. Mechanical loading is known to play a key role in the change of bone mass and strength[208]. Taking these facts into account, the jumping exercise induced mechanical stress into skeletal muscle too. In addition, this model can easily induce hypoinsulinemia combined with overnight fasting [209]. Although different exercise model were recruited in this thesis in order to investigate specific purposes in each chapter, all the exercise model are supposed to induce muscle damage. Therefore, the significance of amino acid intake in the repair and/or remodeling process can be addressed in chapter 2 and 3.

For refueling, carbohydrate intake is the primary issue because carbohydrate is the primary substrate at high intensity exercise [5]. Indeed, as aspect of energy source, amino acid contributes at most 10% [10]. Therefore, little attention has been paid to refuel of amino acids after endurance exercise. However, without adequate replacement of amino acid, substrate for repair and remodel after

endurance exercise is limited after endurance exercise due to expending amino acid as energy during exercise. Therefore, amino acid should be consumed after endurance exercise. In first chapter, I found the amino acid requirement after endurance exercise was doubled compared with sedentary rats or at resting state. The previous study showed that the protein requirements after 20 km treadmill running was reported 1.7 g/kg/day [20], which is nearly doubled compared with current recommended dietary allowance of protein [3]. However, since the amount of oxidative loss of amino acid during endurance exercise depends on the duration and intensity, protein requirements should be varied according to the exercise duration on the day. The previous reports indicated that the duration of exercise might affect amino acid requirements after exercise. The protein requirements after 20 km treadmill running was reported 1.7 g/kg/day [20], while 1.2 g/kg/d was determined as the protein requirement after various intensity game sports simulated running exercise which covers approximately 10 km over the exercise protocol [210]. Although the exercise intensity were not same between the studies, the duration of exercise is supposed to affect the protein requirement. The current recommendation for athlete and active population is 1.2-2.0 g/kg/d [211]. However, after prolonged exercise (i.e. marathon or triathlon), protein intake might be adjusted to the exercise duration on the day.

This is the first study to apply IAAO method to determine optimal amino acid intake to maximize whole body protein synthesis after an acute bout of exercise in rats. IAAO method determines a minimum amount of amino acid to induce maximum whole body protein synthesis using ^{13}C-labeled indicator amino acid [13, 14]. As previously reported, the classical method to determine protein requirements, nitrogen balance technique has been argued that nitrogen balance technique leads to underestimation of the protein requirements due to overestimation of nitrogen intake and an underestimation of nitrogen excretion [14]. Furthermore, although protein requirement is generally defined as the protein intake to maintain body weight and composition [212], for athlete, optimal protein/amino acid intake should be able to maximize to induce training adaptation and recovery from exercise-induced fatigue. The minimum amount of amino acid intake determined by IAAO to maximize whole body protein synthesis, is consistent with the that estimated by the application of a bi-phase linear regression model to the data from nitrogen balance studies [15]. However, the NBAL requires adaptation period that the body urea pool changes in response to test amino acid intake, which usually need up to 7 days [12]. Therefore, the IAAO method can evaluate the optimal amino acid/protein intake more conveniently and sensitive to amino acid intake compared with classical nitrogen balance technique [13, 14]. In practical, since athletes adjusts their exercise to their own purpose on daily basis, the recommended protein intake should be changed daily according their exercise on the day. However, nitrogen balance technique is not able to recommend the protein intake in response to their daily training, because of the adaptation periods. However, since the IAAO method can determine the protein requirements more sensitively and non-invasively, it has been utilized to determine the protein/amino acid requirements in vulnerable populations [17, 18, 80]. Taking

advantage of the virtue of the IAAO method, the IAAO method can manifest the effect of exercise intensity, exercise duration or subject characteristics (gender, training history or so on) on protein/amino acid requirements. Furthermore, the protein/amino acid requirements are supposed to be higher in the period of reducing body weight in weight class sports and aesthetic sports, which is difficult to assess by NBAL, can be addressed by the IAAO method. Indeed, some studies reported that postexercise IAAO method defined the recommended protein intake after prolonged exercise (20-km running) [20] or various intensity exercise simulated soccer match [21]. Applied to various conditions, the IAAO method can answer the practical questions, such as how much protein/amimo acid should be consumed after exercise. The current result is referred to support the future studies which assess the effect of an acute bout of exercise on the metabolic trial day on protein/amino acid requirements.

Greater amino acid/protein intake is needed in several tissues (skeletal muscle, liver and so on) after an acute bout of exercise. However, there is concern regarding consuming increased amounts of protein, particularly for renal and hepatic health. There is no indication of harm or damage in liver or kidney in competitive athletes and active individuals consuming increased protein intake [213, 214]. Chronic kidney disease has a high global prevalence with a global prevalence of 11-13% [215]. Moreover, exercise induces some changes in pathological renal or hepatic function tests [216-218]. Especially, since top athlete or heavily trained body builder train extremely hard which might cause to decrease kidney or liver function, their kidney or liver may be exposed to damage. The tissues have an important role on metabolism of nitrogen and urea production and excretion. To lower the burden of the tissues, it might be helpful to decrease nitrogen intake consuming essential amino acids or some rate-limiting amino acids. In addition, exercise causes another problem in tissue rather than muscle. Exercise induces gastrointestinal damage and gut barrier dysfunction, which impairs protein digestion and absorption [86]. The impairments in protein digestion and absorption may reduce amino acid availability from diet, for tissue repair and adaptation. Therefore, amino acid ingestion might be beneficial for bypassing some issues induced by exercise in the postexercise period. To decrease total amount of nitrogen in the diet, the rate-limiting amino acids should be ingested. The IAAO method can clarify the rate-limiting amino acids in the diet. Generally, egg protein is utilized for determining protein/ amino acid requirement because egg protein, which presumably has an ideal balance of essential amino acids as the major protein component of the diet [219]. A previous study utilizing the IAAO method indicated that valine may be the primary rate-limiting amino acid in egg protein for healthy non-exercised adults [220]. Along with the rate-limiting amino acid in egg protein, branched-chain amino acid catabolism is enhanced during exercise [221, 222]. The IAAO method can contribute on decreasing nitrogen intake clarifying rate-limiting amino acids, presumably essential amino acids or branched-chain amino acids, in specific conditions.

LEAA was utilized in the chapter 2 and 3, to meet increased amino acid requirements after exercise.

Among branched-chain amino acids, leucine is the primary stimulator of skeletal and liver protein synthesis through activating mTOR pathway [29, 223]. However, ingestion of leucine alone decreases some essential amino acids concentrations in neonatal pigs [30] and in humans [165]. In normal swine, reduced availability of amino acids leads to blunted MPS and amino acid supplementation recovers this reduced MPS [173]. In addition, recent report suggested that the ingestion of branched-chain amino acids without the other EAAs increases the stimulation of mTORC1 activity and MPS following exercise, but does not maximally stimulate MPS following exercise [224]. In addition, it is known that exercise increases the oxidation of branched chain amino acids [221, 222], and the oxidation of other amino acids according to increased total energy expenditure. Thus, I added sufficient essential amino acids to leucine to ensure that the amino acid mixture stimulates maximal MPS after exercise. LEAA contains 40% leucine of total amino acids in my studies, the ratio is fourth times higher compared with general dietary protein source (milk-based protein, whey, casein, or egg protein generally contains about 10 % leucine in total amino acids) [225]. Because leucine content of dietary proteins is a primary determinant of MPS in rodents [226], the total amount of amino acid can be reduced to one-fourth through utilizing LEAA substituted with whole protein. In addition, whole amino acid of the ingested protein cannot be utilized due to the digestibility of protein. According to the difference of digestibility of each protein, bioavailability of each amino acids will changes. Therefore, protein quality (reflected in essential amino acids, especially leucine content and protein digestibility) has an impact on changes in MPS. As mentioned above, exercise induces gastrointestinal damage which impairs protein digestion [86]. Since amino acids are absorbed without digestion, especially after exercise, significance of the form of amino acid can be emphasized compared with whole protein.

IAAO method revealed that amino acid requirements on the day is increased by an acute bout of exercise, but not at resting state. The trained rats executed same duration and intensity of endurance exercise the day the before metabolic trial. Thus, the result indicated that the endurance exercise did not affect amino acid requirement 1 day after the exercise. On the other hands, 2 doses of LEAAs ingestion on the day of exercise, decreased muscle soreness 1 day after eccentric contractions, also single dose of LEAA ingestion on the day of exercise, suppressed IL-6 gene expression 1 day after the exercise. The facts indicated that the amino acid ingestion on the day of exercise affected the recovery process. Taking these results into account, sufficient amount of amino acid is important for recovery process despite of exercise type (endurance and eccentric-biased exercise). On the other hands, LEAA enhanced MPS 24 hours after downhill running. In addition, repeated LEAA administration over 3 days alleviated muscle damage and muscle dysfunction after eccentric contractions. Therefore, chronic amino acid intake after exercise beneficial for recovery from muscle damage (summarized in **Figure 4.1**). The muscle damage lasts for several days to 2 weeks, the initial phase of which is characterized by inflammation and degeneration of damaged tissue. Satellite cells are then activated, and proliferate, differentiate, and fuse to myofibrils to repair muscle tissue. Therefore, amino acids are supposed to be

required as substrate on the process. However, LEAAs suppressed excessive inflammation 1 day after the exercise, which may be due to mTOR activation. Thus, LEAAs has a role not only as a substrate but also as an activator of recovery process. The contribution of the two roles of LEAAs depends on the exercise content (intensity, duration and so on.).

Female rats were used in downhill running study because collagen mRNA elevation was reported in the former study in which female rats were used [109]. Sex hormones are well known to affect protein metabolism. Generally speaking, testosterone has an anabolic effect in muscle [227], estrogen, which elevates in the late follicular phase and during the luteal phase, has an anabolic effect [228], while progesterone (which elevates during the luteal phase) has a catabolic effect [229]. The changes of protein metabolism is assumed to affect protein/amino acid requirements. Partly due to progesterone-mediated catabolism, lysine requirement [230], as well as leucine oxidation [231] are elevated during the luteal phase. However, Fujita et al. reported that the MPS was identical during the follicular and luteal phase despite clear differences in plasma estradiol and progesterone concentrations [232]. Similarly, the basal MPS in female was same as the basal MPS in men [233, 234]. Furthermore, no differences in amino acid-induced MPS between males and females [235]. These evidences indicated the responsibility of MPS to amino acid intake is found similarly in male and female. Thus, the current finding from female rats, that LEAA increased MPS 1 hour or 1 day after exercise, is thought to be applicable for male rats.

Figure

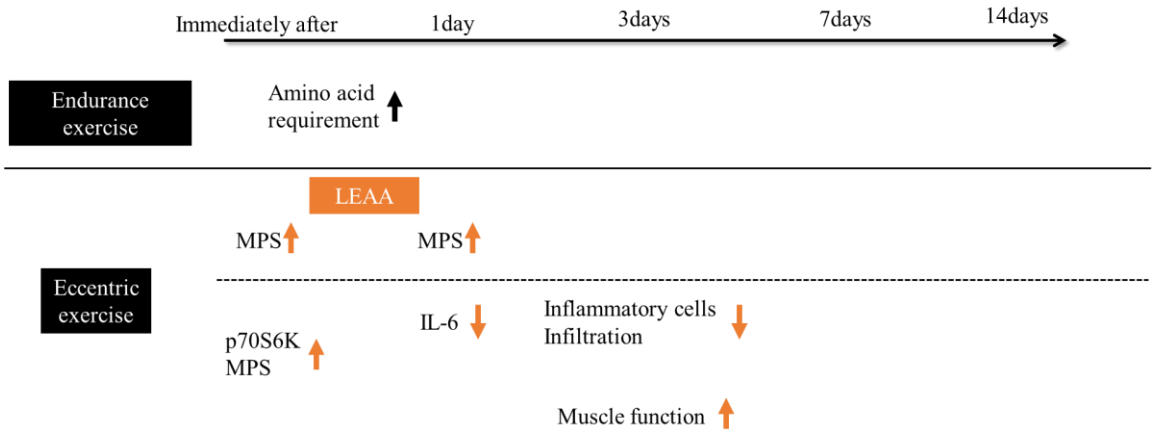

Figure 4.1 Schematic of significance of amino acid intake during postexercise phase according to exercise types.

Conclusion

Importance of amino acid intake for refuel, repair and remodel, were examined in the recovery period following exercise in the current thesis. I found

1) Amino acid requirements were doubled after acute exercise, but not changed at resting state after 6 weeks training, compared with sedentary rats at resting state.

2-1) LEAAs increased MPS immediately and 1 day after exercise, but not affect muscle collagen protein synthesis.

2-2) Co-ingestion of carbohydrate with LEAA could reverse hypoinsulinemic state, but not induced further increase in MPS augmented by LEAAs.

3-1) LEAA alleviated delayed-onset muscle soreness, which is typical symptom of exercise-induced muscle damage.

3-2) LEAA attenuated muscle dysfunction, and histochemical muscle damage through suppressing excessive inflammation after eccentric exercise.

The current study indicated that amino acid requirements is enhanced after endurance exercise, and, postexercise amino acid ingestion could affect the recovery from exercise-induced muscle damage, and has a potential to enhance training adaptation through increasing MPS. Therefore, I conclude that sufficient amino acid ingestion has significance during recovery period following exercise.

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