Pre-exercise casein peptide supplementation enhances endurance training-induced mitochondrial enzyme activity in slow twitch muscle, but not fast twitch muscle of high fat diet-fed mice

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Abstract To establish an efficient method of enhancing mitochondrial biogenesis, we investigated the effect of casein peptide supplementation. The aim of this study was to examine whether oral casein peptide ingestion enhances exercise-induced mitochondrial adaptation in high fat diet-induced obese diabetes mice. Mice received either casein peptide or water (0.2 mg/g body weight, 7 times/week) and were subjected to treadmill running (20–25 m/min × 60 min, 5 times/week for 6 weeks) 30 min later. In plantaris muscle (higher proportion of fast-twitch muscle fibers), casein peptide treatment did not impact mitochondrial adaptation. However, in soleus muscle (higher proportion of slow-twitch muscle fibers) and heart, casein peptide supplementation with exercise increased mitochondrial enzyme activity (citrate synthase and β-hydroxyacyl CoA dehydrogenase activity). To clarify the mechanisms underlying mitochondrial adaptation enhancement, we investigated the acute effects of pre-exercise casein peptide ingestion on the phosphorylation status of cellular signaling cascades associated with mitochondrial adaptations. We observed that casein peptide ingestion boosted exercise-induced AMPK phosphorylation in soleus, but not plantaris muscle. Thus, our present investigation suggested that casein peptide ingestion enhanced exercise-induced mitochondrial adaptation in slow twitch muscle, but not fast twitch muscle in high fat diet-induced obese-diabetes mice.

Keywords: casein peptide supplementation, mitochondrial adaptation, fiber type, endurance training

Introduction

Mitochondria play a central role in energy production. Previous studies have reported that mitochondrial content was closely related to endurance exercise capacity8, and that endurance training increased mitochondrial biogenesis5. Enhanced mitochondrial content and function contribute to improved exercise capacity; however, not all people can perform sufficient training. For example, obese individuals may have a low exercise capacity7 and/or poor physical fitness. Thus, it is necessary to establish an efficient method for enhancing exercise training-induced mitochondrial biogenesis.

To enhance mitochondrial biogenesis, we investigated nutrient supplements in this study. Previous studies have reported that branched-chain amino acid (BCAA: valine, leucine, isoleucine) supplementation enhanced mitochondrial biogenesis in middle-aged mice6. Furthermore, leucine supplementation prevented high fat diet-induced metabolic disorders by enhancing mitochondrial biogenesis5. Moreover, glutamine is involved in leucine incorporation into the cell7. Thus, it is possible that amino acid supplementation contributes to the enhancement of mitochondrial biogenesis. However, it is well known that ingestion of only one type of amino acid is not sufficient for muscular adaptation, because other amino acids are also required for muscle protein synthesis. Moreover, it takes more time to digest and absorb protein compared with that of an amino acid. Therefore, it is ideal that we ingest various types of amino acids and supply them to the body as quickly as possible. In this study, we focused on the effects of casein peptide (hydrolysate) supplementation. Casein, which accounts for 80% of the protein in bovine milk, is nutritionally dense, with an amino acid score of 1006, and is rich in BCAA. In addition, the peptide is absorbed more rapidly than protein or other amino acids6. Hence, casein peptide may be well suited as a sports supplement.

In the current study, we hypothesized that casein peptide supplementation enhances exercise-induced mitochon-
drial biogenesis. If casein peptide ingestion contributes to mitochondrial adaptation, it is considered likely to be an effective supplement for low physical fitness patients with obesity and/or diabetes. To test this hypothesis, we used a high fat diet in the experimental diet to induce an obese-diabetes condition and examined whether long-term casein peptide ingestion combined with exercise training enhanced mitochondrial adaptation, such as mitochondrial enzyme (citrate synthase [CS] and β-hydroxyacyl CoA dehydrogenase [β-HAD]) activities, biomarkers of mitochondrial oxidative capacity. Furthermore, to clarify the mechanisms underlying mitochondrial adaptation enhancement, we investigated the acute responses of two types of signaling cascades, AMP-activated protein kinase (AMPK) and mammalian target of rapamycin complex 1 (mTORC1), both of which are associated with mitochondrial biogenesis. These signaling cascades have been previously reported to be phosphorylated and activated by leucine.

Materials and Methods

Ethical approval
All experiments were approved by the animal experimental committee of The University of Tokyo.

Experimental animals and supplements
Male 6-week-old ICR mice were purchased from CLEA Japan Inc. (CLEA Japan, Tokyo, Japan). Mice were housed on a 12 h/12 h light-dark cycle (dark: 7:00–19:00) in an air-conditioned room (temperature: 23°C) and were adapted to these conditions for 1 week prior to experimentation. In order to cause a diet-induced obesity condition, all mice were provided with a high fat diet (High Fat Diet 32, CLEA Japan Inc, Tokyo, Japan) and water ad libitum throughout the experimental period. Mice were orally administered with either casein peptide (0.2 mg/g body weight [BW], CU2500A; Morinaga Milk industry Co. Ltd, Tokyo, Japan) or water. Nutrient composition of the high-fat diet and amino acid composition of casein peptide are listed (Table 1 A-B).

Table 1. General component composition in high fat diet (g/100g) (A), amino acids composition in casein peptide (mg/g) (B)

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
</tr>
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<tbody>
<tr>
<td>Protein</td>
<td>25.5</td>
</tr>
<tr>
<td>Fat</td>
<td>32.0</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>32.3</td>
</tr>
<tr>
<td>(Fiber)</td>
<td>(2.9)</td>
</tr>
<tr>
<td>Ash</td>
<td>4.0</td>
</tr>
<tr>
<td>Moisture</td>
<td>6.2</td>
</tr>
<tr>
<td>Energy (kcal/100g)</td>
<td>507.6</td>
</tr>
<tr>
<td>Fat (% Energy)</td>
<td>56.7</td>
</tr>
<tr>
<td>Lys</td>
<td>68</td>
</tr>
<tr>
<td>Thr</td>
<td>44</td>
</tr>
<tr>
<td>Val</td>
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<tr>
<td>Met</td>
<td>24</td>
</tr>
<tr>
<td>Cys</td>
<td>3</td>
</tr>
<tr>
<td>Ile</td>
<td>46</td>
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<tr>
<td>Leu</td>
<td>72</td>
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<tr>
<td>Phe</td>
<td>31</td>
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<tr>
<td>Tyr</td>
<td>33</td>
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<tr>
<td>Trp</td>
<td>2</td>
</tr>
<tr>
<td>His</td>
<td>22</td>
</tr>
<tr>
<td>Asp • Asn</td>
<td>75</td>
</tr>
<tr>
<td>Ser</td>
<td>56</td>
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<tr>
<td>Glu • Gln</td>
<td>250</td>
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<td>Ala</td>
<td>30</td>
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<tr>
<td>Arg</td>
<td>22</td>
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</tbody>
</table>

Experimental validation of the effects of casein peptide ingestion on plasma BCAA concentration
Following a 1-week acclimation period, mice were randomly assigned to the casein peptide ingestion group (CP: 0.2 mg/g BW; n = 7) or the water ingestion group (CON; equal volume to the CP group; n = 7). After casein peptide or water ingestion, circulating blood samples were collected from the tail vein (0, 15, 30, 45, and 60 min). To measure the plasma BCAA concentration, plasma samples were collected from the tail vein, rapidly frozen in liquid nitrogen, and stored at −80°C until further analysis.

Long-term administration experiment with endurance training
Following a 1-week acclimation period, mice were randomly divided into three treatment groups: the control group (CON; n = 6), the endurance training group (Tr; n = 6), and the endurance training with casein peptide supplementation group (CP+Tr; n = 7). Mice were treated with either casein peptide or water (0.2 mg/g BW, 7 times/week) and subjected to endurance training (20–25 m/min × 60 min, 5 times/week for 6 weeks) using a motor-driven treadmill 30 min after supplementation. All mice were sacrificed 48 hours after the final training session by
cervical dislocation. Soleus and plantaris muscles were dissected out, rapidly frozen in liquid nitrogen, and stored at −80°C until further analysis.

**Investigation of acute cellular signaling cascade responses associated with mitochondrial adaptations under exercise conditions**

Following a 1-week acclimation period, mice were randomly assigned to the casein peptide ingestion with exercise group (CP+Ex: 0.2 mg/g BW; n = 7) or the water ingestion with exercise group (Ex; equal volume with CP+Ex group; n = 7). Mice were subjected to endurance exercise (20 min/min, 60 min) 30 min after supplementation. Blood samples were collected from the tail vein (0, 15, 30, 90, 105, and 120 min). Mice were sacrificed by cervical dislocation at 120 min after oral administration. Plasma and soleus and plantaris muscles were isolated and rapidly frozen in liquid nitrogen. Samples were stored at −80°C until further analysis.

**Analysis**

**Blood BCAA concentrations**

4-Fluoro-7-nitrobenzofurazan (NBD-F, Dojindo Laboratories, Kumamoto, Japan) in ethanol was used as a derivatization reagent. Plasma (20 μl) was mixed with 4% sulfosalicylic acid (20 μl) and incubated at 0°C for 60 min. The mixture was then centrifuged at 3,500 × g for 5 min, and the supernatant was obtained. The supernatant (4 μl) was mixed with 0.1 M borate buffer (16 μl, pH 8.5) and was added to 25 mM NBD-F (10 μl) to derivatize the amino acids at 60°C for 5 min. The reaction was terminated by adding 300 μl of eluent A (10 mM citrate buffer, pH 6.2, containing 75 mM sodium perchlorate), and the samples were transferred to autosampler vials. The sample (4 μl) was then injected onto the HPLC column. The chromatographic system was a NANOSPACE SI-2 series HPLC instrument (Shiseido, Tokyo, Japan) consisting of two single pumps (type 3001), an autosampler (type 3023), and a fluorescence detector (type 3013) operating the EZChrom Elite for Shiseido (Scientific Software Group, Salt Lake, USA). Separations were achieved using a reversed-phase ODS column (Capcell Pak C18 AQ S-5 μm, 1.5 mm ID×150 mm, Shiseido, Tokyo, Japan). For NBD-F derivatives, the eluent system consisted of two components: eluent (A) was 10 mM citrate buffer, pH 6.2, containing 75 mM sodium perchlorate, while eluent (B) was 80% acetonitrile (v/v). The eluent flow rate was 100 μl/min. The fluorescence intensity was detected at 530 nm with excitation at 480 nm.

**Mitochondrial enzyme activity analysis**

The mitochondrial CS and β-HAD enzyme activities in whole muscle homogenates were determined. Plantaris, soleus, and heart muscle were homogenized in 100% (vol/wt) 100 mM potassium phosphate buffer. CS and β-HAD activities were measured spectrophotometrically in these homogenates using the methods of Srere and Bass et al.

**Western blot analysis**

Soleus and plantaris muscle samples were homogenized as previously described using lysis buffer (1% Triton X-100, 50 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 10 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, and 2 mM DTT; pH 7.5) containing 10 μg/ml of pepstatin A, aprotinin, and leupeptin, 1 mM sodium orthovanadate, and 0.177 mg/ml phenylmethylsulfonyl fluoride (PMSF). Sample protein concentrations were measured by the Bradford method. We loaded an equal amount (20–30 μg) of protein for each antibody. Proteins were separated using standard SDS-PAGE procedures (7.5–12% polyacrylamide gels) and transferred to a polyvinylidene difluoride (PVDF) membrane (Hvond-P, GE Healthcare Japan, Tokyo, Japan). Membranes were blocked with 3–7.5% BSA in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 hour and incubated overnight with the following primary antibodies: phosphorylated (p-)AMPKα (Thr172, no. 2532, CST Japan), AMPKα (no. 2513, Cell Signaling Technology [CST] Japan, Tokyo, Japan), p70S6K (Thr389, no. 9205, CST Japan), and p-p70S6K (index of mTORC1 activation; no. 9202, CST Japan). After incubation, membranes were washed in TBST, incubated for 1 hour at room temperature with secondary antibodies (A102PT, American Qualex), and washed again in TBST. Chemiluminescent reagents (SuperSignal West Pico Chemiluminescent Substrate, Thermo Fisher Scientific) were used to facilitate blot detection. Blots were scanned and quantified using ChemiDoc XRS (170-8071, Bio-Rad) and Quantity One (170-9600, version 4.5.2, Windows, Bio-Rad).

**Statistical analysis**

All data are presented as mean ± standard error of the mean. A two-way ANOVA was performed to determine the effects of time and casein peptide supplementation on BCAA concentrations. For analysis of the long-term endurance training experiment, a one-way ANOVA was performed. When differences were found to be significant, comparisons were made using the Tukey-Kramer post-hoc test. For all other experiments, statistical analysis was performed using the Student’s t-test. Statistical significance was defined as p < 0.05.

**Results**

**Casein peptide supplementation increased plasma BCAA concentration**

The main positive effect of casein peptide, but not time, on plasma BCAA concentration was observed in a sedentary condition (Fig. 1A). Similarly, positive main effect of casein peptide, but not time, on plasma BCAA concen-
Pre-exercise concentrations were observed in pre and post-exercise conditions (Fig. 1B). These results demonstrated that casein peptide supplementation increased plasma BCAA levels in sedentary and exercise condition.

**Casein peptide supplementation did not affect energy intake or body weight gain**

We investigated the effects of long-term casein peptide supplementation. Total energy intake during the 6-week experimental period was similar in all groups (Table 2). However, the final body weights and body weight gain were significantly lower in the Tr and CP+Tr groups than those in the CON group (Table 2). These results suggest that endurance training suppressed body weight gain compared with that of sedentary activity independent of casein peptide supplementation.

**Long-term casein peptide supplementation with endurance training increased mitochondrial enzyme activity in slow twitch muscle, but not fast twitch muscle**

To clarify the effects of casein peptide on mitochondrial enzyme activity, we measured CS and β-HAD activities. In plantaris muscle (higher proportion of fast-twitch muscle fibers), CS and β-HAD activities were significantly higher in the Tr and CP+Tr groups than those in the CON group (Fig. 2A and 3A, respectively). In contrast, in soleus muscle (higher proportion of slow-twitch fibers), CS and β-HAD activities were significantly higher in the CP+Tr group than those in the CON and Tr groups (Fig. 2B and 3B, respectively). Similarly, in heart muscle, CS and β-HAD activities were significantly higher in the CP+Tr group than those in other groups (Fig. 2C and 3C, respectively).

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**Fig. 1**  Plasma branched-chain amino acid (BCAA) concentration after oral administration of casein peptide (0.2 mg/g BW) or water in sedentary condition (A), in exercise condition (B). Values are presented as mean ± SEM.
Table 2. Body weight and total energy intake

<table>
<thead>
<tr>
<th></th>
<th>Con</th>
<th>Tr</th>
<th>CP+Tr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>34.9±0.4</td>
<td>34.9±0.4</td>
<td>35.0±0.4</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>49.2±0.7</td>
<td>44.1±0.4**</td>
<td>45.4±1.1*</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
<td>15.2±0.5</td>
<td>9.9±0.9**</td>
<td>10.4±1.0**</td>
</tr>
<tr>
<td>Total energy intake (kcal)</td>
<td>724.6±17.3</td>
<td>688.9±13.0</td>
<td>718.0±12.5</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SEM
*p<0.05 and **p<0.01 vs. Con

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**Fig. 2** Long-term adaptations to casein peptide supplementation with endurance training on CS activities in plantaris muscle (A), soleus muscle (B) and heart (C). Values are presented as mean ± SEM. *p < 0.05 and **p < 0.01 vs. the control group. †p < 0.05 and ††p < 0.01 vs. the Tr group.

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**Fig. 3** Long-term adaptations to casein peptide supplementation with endurance training on β-HAD activities in plantaris muscle (A), soleus muscle (B) and heart (C). Values are presented as mean ± SEM. *p < 0.05 and **p < 0.01 vs. the control group. †p < 0.05 and ††p < 0.01 vs. the Tr group.
Casein peptide ingestion combined with exercise acutely activated AMPK phosphorylation in soleus muscle

To clarify the mechanism underlying mitochondrial enzyme activity enhancement following casein peptide treatment with exercise, we investigated the acute responses of the signaling cascades associated with mitochondrial biogenesis. Thirty minutes after exercise, p70S6K phosphorylation status was not significantly different in soleus and plantaris muscles with or without casein peptide treatment (Fig. 4B and 4D). Similarly, no significant difference between the two groups was observed in AMPK phosphorylation status in plantaris muscle (Fig. 4A). However, in soleus muscle, AMPK phosphorylation status in the CP+Ex group was significantly higher than that in the Ex group (Fig. 4C).

Discussion

A novel perspective of casein peptide ingestion on sports nutrition

In the present investigation, we investigated the effect of casein peptide ingestion on mitochondrial adaptation in diet-induced obese-diabetes mice. We demonstrated that casein peptide ingestion with exercise training enhanced mitochondrial enzyme activity in soleus muscle and the heart in high fat diet-induced obese-diabetes mice. Total energy intake for the 6-week experimental period was similar in all groups (Table 2). Thus, the results obtained in this experiment were not caused by differences in energy intake. In addition, mitochondrial enzyme activities were not increased by training (Fig. 2B, 3B) or casein peptide supplementation alone (control, 665.4 ± 29.1 μmol/min/mg protein vs. casein peptide, 699.5 ± 41.5 μmol/min/mg protein).

Fig. 4  Acute responses of casein peptide supplementation with endurance exercise on the AMPK and p70S6K. The phosphorylation status of AMPK and p70S6K were measured in plantaris (A, B) and soleus (C, D) muscle. Values are presented as mean ± SEM. *p < 0.05 vs. the Ex group.
Effects of casein peptide supplementation on mitochondrial adaptation were muscle fiber type-dependent

In this study, endurance training alone enhanced mitochondrial enzyme activity in plantaris, but not soleus muscle. Initial post-exercise blood lactate concentrations in the control mice running at 20–25 m/min for 60 min were 3.1 ± 0.2 mmol/l. This suggests that the exercise intensity used in this experiment was at approximately the lactate threshold level. A previous study reported that mitochondrial adaptations occurred more readily in low-oxidative muscles, such as plantaris muscle, compared with that of high-oxidative muscles, such as soleus muscle. Therefore, it is possible that the exercise stimulation used in this study was sufficient to cause mitochondrial adaptation in the plantaris but not in the soleus muscle.

Casein peptide supplementation with endurance training influenced soleus muscle and the heart, but not plantaris muscle. We hypothesize that this may be due to the diabetest-like condition induced by high fat diet consumption. To cause a diet-induced obese-diabetes condition, mice were provided with a high-fat diet throughout all experiments. High-fat diet consumption increased body weight and fat accumulation, and induced glucose tolerance deterioration in mice. Indeed, we confirmed that resting body weight and fat accumulation, and induced glucose tolerance deterioration in mice. In this report, mice were supplemented with 1.5 mg/g BW BCAA for 3 months. In the current study, mice were supplemented with a lower amount of casein peptide (0.2 mg/g BW) for 6 weeks. These results suggest that casein peptide supplementation is effective even in small quantities combined with exercise. Additionally, a previous study demonstrated that post-exercise whey protein hydrolysate supplementation increased muscle protein synthesis compared with ingestion of its constituent amino acids in a rat model. Therefore, it was suggested that the peptide (protein hydrolysate) has bioactive functions, and that peptide ingestion might be more effective than amino acid supplementation.

Conclusion

The present investigation demonstrated that pre-exercise casein peptide supplementation increased mitochondrial enzyme activity in slow twitch muscle, but not fast twitch muscle after endurance training at low intensity. Furthermore, pre-exercise casein peptide supplementation acutely activated AMPK phosphorylation in soleus muscle, but not in plantaris muscle. mTORC1 was not activated in either muscle (Fig. 4A-D). Therefore, casein peptide ingestion with exercise affected AMPK activation rather than mTORC1 activation. Furthermore, it can be concluded that AMPK activation by pre-exercise casein peptide ingestion at least partly contributes to the enhancement of mitochondrial enzyme activity in the soleus muscle.

Conflict of Interests

The co-authors of this study, Noriko Saito, Hirohiko Nakamura and Yasuhiro Takeda are employees of Morinaga Milk Industry Co., Ltd.
References


