Branched-chain amino acid supplementation suppresses the detraining-induced reduction of mitochondrial content in mouse skeletal muscle

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Abstract
Exercise training enhances oxidative capacity whereas detraining reduces mitochondrial content in skeletal muscle. The strategy to suppress the detraining-induced reduction of mitochondrial content has not been fully elucidated. As previous studies reported that branched-chain amino acid (BCAA) ingestion increased mitochondrial content in skeletal muscle, we evaluated whether BCAA supplementation could suppress the detraining-induced reduction of mitochondrial content. Six-week-old male Institute of Cancer Research (ICR) mice were randomly divided into four groups as follows: control (Con), endurance training (Tr), detraining (DeTr), and detraining with BCAA supplementation (DeTr + BCAA). Mice in Tr, DeTr, and DeTr + BCAA performed treadmill running exercises [20–30 m/min, 60 min, 5 times/week, 4 weeks]. Then, mice in DeTr and DeTr + BCAA were administered with water or BCAA [0.6 mg/g of body weight, twice daily] for 2 weeks of detraining. In whole skeletal muscle, mitochondrial enzyme activities and protein content were decreased after 2 weeks of detraining, but the reduction was suppressed by BCAA supplementation. Peroxisome proliferator-activated receptor \( \gamma \) coactivator-1\( \alpha \) (PGC-1\( \alpha \)) protein content, a master regulator of mitochondrial biogenesis, was decreased by detraining irrespective of BCAA ingestion. Regarding mitochondrial degradation, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), a mitophagy-related protein, was significantly higher in the Tr group than in the DeTr + BCAA group, but not different from in the DeTr group. With respect to mitochondrial quality, BCAA ingestion...
1 INTRODUCTION

People are encouraged toward exercise training to promote health while they often have to interrupt training due to sports injuries or medical illnesses. Training cessation results in various unwanted outcomes in the body as detraining suppresses muscle strength, blood volume, and stroke volume. Therefore, it is necessary to establish a way to prevent the negative effects of detraining.

Mitochondria play a key role in energy production and are associated with endurance exercise capacity. Mitochondrial content in skeletal muscle is increased by endurance exercise training, while it is also affected by training cessation. Mitochondrial enzyme activities are decreased after detraining. Reduction in training volume decreases mass-specific mitochondrial respiration, and training cessation reduces mitochondrial ATP production. Consequently, detraining increases respiratory exchange ratio (RER) during submaximal intensity exercise and reduces endurance exercise capacity. Considering that mitochondrial dysfunction is related to disease development such as insulin resistance, maintaining mitochondrial quality and quantity is essential. However, the strategy for suppressing detraining-induced reductions in mitochondrial content and function is not yet fully elucidated.

In the present study, nutritional intake was assessed with a particular focus on branched-chain amino acids (BCAA) as a strategy to prevent the detraining-induced reduction of mitochondrial content. Mixed amino acid infusion, which increases the essential amino acid concentration in blood, enhances mitochondrial protein synthesis in human skeletal muscle. Leucine, one of the BCAAs, activates the mechanistic/mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK) signaling pathways involved in mitochondrial biogenesis. Leucine and its metabolite β-hydroxy-β-methylbutyrate (HMB) enhance mitochondrial content in C2C12 myotubes. BCAA ingestion after exercise stimulates mTOR signaling more effectively than leucine alone. BCAA consumption increases mitochondrial biogenesis and extends life span in middle-aged mice. Therefore, it is probable that BCAA supplementation enhances mitochondrial biogenesis and suppresses the detraining-induced reduction of mitochondrial content.

Furthermore, mitochondria have not only modified the contents but also coordinated the cycles of mitochondrial dynamics (fusion and fission) and clearance (mitophagy) that control mitochondrial quality and quantity. Mitochondria are divided by fission and then degraded by mitophagy. Coordination of mitochondrial biogenesis and degradation is essential to maintain mitochondrial content. However, the effects of detraining and BCAA supplementation on mitochondrial dynamics and clearance are not clear, although it is crucial for mitochondrial regulation. In addition, leucine and HMB enhance mitochondrial function in C2C12 myotubes. On the other hand, BCAA at supraphysiologically high levels (20 mM) decreases mitochondrial function in C2C12 myotubes. Therefore, the effect of BCAA on mitochondrial function has been controversial. Thus, the purpose of this study was to clarify the effects of BCAA supplementation on mitochondrial adaptations in skeletal muscle during detraining.

2 MATERIALS AND METHODS

2.1 Experimental animals

Six-week-old male Institute of Cancer Research (ICR) mice were obtained from Clea Japan Inc. (Tokyo, Japan). Mice were housed (one per cage) in an environment maintained at 23°C in a 12-h light-dark cycle (dark: 7:00–19:00, light: 19:00–7:00) provided with water and standard chow diet (3.59 kcal/g; 23.1% protein, 5.1% fat, 55.3% carbohydrates, 5.8% ash, 2.8% fiber, and 7.9% moisture; MF diet; Oriental Yeast Co., Ltd., Tokyo, Japan) ad libitum. Mice were acclimated in the environment for 1 week and were familiarized with the treadmill running exercise at a speed of 10–20 m/min for 10 min for 3 days before starting the experiment. All experimental protocols were approved by the animal experimental committee of The University of Tokyo (No.30–6).
2.2 | Materials

Leucine, isoleucine, and valine were obtained from Sigma-Aldrich Japan (L-Leucine: L8000, L-Isoleucine: I2752, L-Valine: V0500, Tokyo, Japan). These materials were mixed with leucine/isoleucine/valine in a 2:1:1 ratio and dissolved in water. A known amount of BCAA was administered as a solution by oral gavage using a sonde.

2.3 | Study design

Figure 1 presents a schematic overview of the experimental procedures. We had to harvest skeletal muscle for analysis in all four experiments; therefore, different mice were used in each of the four experiments.

2.3.1 | Single bout experiment

Experiment 1: Evaluation of blood BCAA concentration at rest

Initially, the effects of BCAA ingestion on blood substrate concentrations and signaling pathways at rest were evaluated. Following an acclimatization period of 1 week, mice were randomly divided into two groups as follows: control (Con, n = 7) and BCAA treatment (BCAA, n = 7). After 1 h of fasting, mice were orally administered with water or BCAA solution by oral gavage using a sonde [0.6 mg/g of body weight (BW)]. Blood samples were collected from the tail vein (before treatment and at 15, 30, 60, 90, and 120 min). Samples were centrifuged (10400g, 5 min) and plasma was rapidly frozen in nitrogen and stored at −80°C until analysis. After a one-week washout period, mice were administered BCAA using the same protocol. After 30 min, mice were anesthetized using isoflurane and euthanized by collecting blood from the postcaval vein. The plantaris muscle was then collected, directly frozen in nitrogen, and stored at −80°C until analysis.

2.3.2 | Detraining experiment

Experiment 2: Evaluation of mitochondrial enzyme activities and protein

Following an acclimatization period of 1 week, mice were randomly divided into four groups as follows: control (Con, n = 7), endurance training (Tr, n = 7), detraining (DeTr, n = 7), and detraining with BCAA supplementation (DeTr+BCAA, n = 7). Mice in the Tr, DeTr, and DeTr+BCAA were subjected to endurance training (20–30 m/min, 60 min, 5 times/week, 4 weeks) on a motor-driven treadmill. After a 4-week training period, mice in DeTr and DeTr+BCAA were housed under resting conditions for 2 weeks and were administered with water or BCAA solution during the detraining period by oral gavage using a sonde [0.6 mg/g, twice a day, a total 1.2 mg/g BW, every day, for 2 weeks]. We weighed the mice daily and adjusted the BCAA dosage according to their weight. To match the timing of tissue sampling, the Tr group rested for 2 weeks followed by 4 weeks of training. Twenty-four hours after the last intervention, mice were anesthetized using isoflurane and plantaris muscles were dissected, directly frozen in liquid nitrogen, and stored at −80°C until further analysis.

Experiment 3: Evaluation of mitochondrial dynamics, mitophagy, and respiration

Mice were divided into four groups as follows: Con (n = 7), Tr (n = 9), DeTr (n = 9), and DeTr+BCAA (n = 9), and were housed under the same conditions as in Experiment 2. Twenty-four hours after the last administration, mice were anesthetized using isoflurane, and plantaris and gastrocnemius muscles were dissected. Regarding plantaris muscle, samples were directly frozen in liquid nitrogen and stored at −80°C until further analysis. With respect to gastrocnemius muscle, samples were used to isolate mitochondria on ice after tissue collection.

Experiment 4: Exercise test

Mice were divided into four groups as follows: Con (n = 7), Tr (n = 7), DeTr (n = 7), and DeTr+BCAA (n = 7), and were housed under the same conditions as in Experiment 2. Twenty-four hours after the last treatment, mice in Tr, DeTr, and DeTr+BCAA were subjected to running on an airtight treadmill (25 m/min, for 60 min). During exercise, their exhalation was collected (1.5 L/min). Mice were anesthetized using isoflurane directly after exercise, and liver and skeletal muscle (soleus, plantaris, gastrocnemius, and tibialis anterior muscle) were dissected, directly frozen in liquid nitrogen and stored at −80°C until further analysis. In the Con group, tissues were removed under resting conditions for reference markers.

2.4 | Analysis

2.4.1 | Blood substrate concentration

Plasma BCAA concentration was measured using a branched-chain amino acid assay kit (K564-100, Bio Vision, CA, USA). Blood glucose concentration was measured using an auto-analyzer (GT-1840; GLUCOCARD Plus Care, Arkray Inc., Kyoto, Japan). Blood lactate concentration was measured using an auto-analyzer (Lactate Pro 2, Arkray Inc.).
2.4.2 Mitochondrial enzyme activity

Maximal Citrate synthase (CS) and 3-hydroxyacyl-CoA dehydrogenase (\(\beta\)-HAD) activities, biomarkers of mitochondrial content, were determined in whole plantaris muscle homogenates. The whole plantaris muscles were weighted and homogenized 100 times (v/w) in 100 mM potassium phosphate buffer. Maximal CS and
\( \beta \)-HAD activities were measured using a spectrophotometer based on previous studies.\(^{25,26}\) Protein concentration in each sample was measured using the Bradford method,\(^{27}\) and mitochondrial enzyme activity was compared after normalization to protein concentration.

### 2.4.3 Mitochondrial isolation

Gastrocnemius muscle was used to isolate mitochondria as this procedure requires a large muscle volume. Mitochondrial fractions were isolated using differential centrifugation as previously described.\(^{28,29}\) Briefly, the gastrocnemius muscles were homogenized in mitochondrial isolation buffer (67 mM sucrose, 50 mM Tris, 50 mM KCl, 10 mM EDTA and 0.2 \(^{\text{w/v}}\) of fatty acid-free bovine serum albumin, pH 7.4). The homogenate was centrifuged (700 \(g\), 15 min, 4°C), and the supernatant was further centrifuged (12000 \(g\), 20 min, 4°C). The pellet was washed and resuspended in a mitochondrial isolation buffer. The protein concentration of each sample was determined using a protein assay bicinchoninic acid (BCA) kit (297-73101, Fujifilm Wako Chemical Corporation, Osaka, Japan).

### 2.4.4 Mitochondrial respiration and reactive oxygen species (ROS) production

Mitochondrial respiration was measured as previously described.\(^{30-32}\) Freshly isolated mitochondria (30 \(\mu\)g) were incubated in a reaction buffer (10 mM Tris, 30 mM KCl, 10 mM \( \text{KH}_2\text{PO}_4\), 5 mM \(\text{MgCl}_2\), 1 mM EGTA, 2.5 g/L BSA, pH 7.2). Mitochondrial oxygen consumption was measured using the Tecan Spark multimode plate reader (Spark 20 M, Tecan, Männedorf, Switzerland) with a 96-well oxygen sensor plate (OP96C; PreScan Precision Sensing GmbH, Regensburg, Germany; Ex: 540 nm/ Em: 650 nm). The homogenate was centrifuged (700 \(g\), 15 min, 4°C), and the supernatant was further centrifuged (12000 \(g\), 20 min, 4°C). The pellet was washed and resuspended in a mitochondrial isolation buffer. The protein concentration of each sample was determined using a protein assay bicinchoninic acid (BCA) kit (297-73101, Fujifilm Wako Chemical Corporation, Osaka, Japan).

### 2.4.5 RNA isolation and real-time quantitative PCR

Gastrocnemius muscle was homogenized on ice in ISOGEN II (311-07361, NIPPON GENE CO., LTD., Tokyo, Japan). According to the manufacturer’s instructions, total RNA was isolated using the RNeasy Mini kit (74104, Qiagen, Venlo, Netherlands). After RNA concentration was measured using spectrophotometry (Nanodrop ND1000, Thermo Scientific, Waltham, MA), first-strand cDNA synthesis was performed using a high-capacity cDNA reverse transcription kit (4368814, Applied Biosystems, Foster City, CA). The gene expression was quantified using the Thermal Cycler Dice Real-Time System and PowerUp™ SYBR™ Green Master Mix (A25742, Applied Biosystems). All the samples were run in duplicate. GAPDH was used as the control housekeeping gene, the expression of which did not alter between groups. Primers used for reverse transcription/PCR (RT-PCR) analysis were: FOXO1-F: GCTGGGTGTCAGGCTAAGAG, FOXO1-R: AGGGCATCTTTGGACTGCT, FOXO3-F: GGAAATGGGCAAAGCAGA, FOXO3-R: AAACGATCATGTCACCACTCCAGTTG, GAPDH-F: ATGTGAGCAATGCTATCTG, GAPDH-R: ATGGAACGTGATCTAGGACC, MAFbx-F: AGTGAGGACGGCTACTGTT, MAFbx-R: GATCAAACGCTTGGCAATCT, MuRF1-F: TCCTCAGAGTGGACAAAGG, and MuRF1-R: GGGTGAGGGGTTGCAAC, as previously described.\(^{33}\)

### 2.4.6 Western blot analysis

Samples were homogenized in a lysis buffer (1% Triton X-100, 50 mM Tris–HCl, 1 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 10 mM sodium \( \beta \)-glycerol phosphate, 5 mM sodium pyrophosphate, and 2 mM DTT; pH 7.5) containing protease and phosphatase inhibitors (1183617001, Complete Mini EDTA-free, and 0490637001, PhosSTOP phosphatase inhibitor cocktail, Roche Life Science, IN, USA). The protein concentration of each sample was determined using the BCA protein assay. An equal amount of protein (10 \(\mu\)g) was loaded onto 7.5%–15% polyacrylamide gels and separated by SDS polyacrylamide gel electrophoresis before being transferred to a polyvinylidene difluoride (PVDF) membrane (Hybond-P, GE Healthcare Japan, Tokyo, Japan). Membranes were blocked using 5% BSA in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h and incubated overnight (4°C) with primary antibodies. After overnight incubation, membranes were washed in TBS-T (10 min \( \times \) 3), incubated for 1 h at room temperature with secondary antibodies (A102PT and A106PU, American Qualex, CA, USA), and washed in TBS-T. Chemiluminescent reagents (Pierce Western Blotting Substrate, Thermo Fisher Scientific, MA,
USA) were used to detect the protein blots. Blots were then scanned and quantified using ChemiDoc XRS (170-8071, Bio-Rad Laboratories, Hercules, CA) and Quantity One (170–9600, version 4.5.2, Bio-Rad).

2.4.7 | Primary antibodies for western blot analysis

The following primary antibodies were used: phospho-mTOR<sup>Ser2448</sup> (2971, Cell Signaling Technology [CST] Japan, Tokyo, Japan), mTOR (2972, CST), phospho-p70S6K<sup>Thr389</sup> (9205, CST), p70S6K (9202, CST), phospho-ULK1<sup>Ser757</sup> (6888, CST), ULK1 (8054; CST), phospho-AMPK<sup>α</sup><sup>Thr172</sup> (9475, CST), AMPK (5832, CST), phospho-acyetyl-CoA carboxylase (ACC)<sup>Ser79</sup> (3661, CST), ACC (3662, CST), COX IV (ab14744, Abcam, Cambridge, UK), peroxisome proliferator-activated receptor <i>γ</i> coactivator 1-α (PGC-<i>γ</i>-<i>α</i>) (516557, Millipore, CA, USA), sirtuin 1 (SIRT1) (9475, CST), GAPDH (2118, CST), Optic Atrophy 1 (Opa1) (612606, BD Transduction Laboratories, Tokyo, Japan), mitofusion 2 (Mfn2) (ab124773, abcam), dynamin-related protein 1 (Drp1) (ab56788, abcam), mitochondrial fission protein 1 (Fis1) (ab96764, abcam), parkin (M230-3, Medical & Biological Laboratories [MBL], Tokyo, Japan), p62/seques-tosome 1 (p62/SQSTM1) (PM045, MBL), BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) (3769, CST), BNIP3-like/NIX (BNIP3/NIX) (12396, CST), LC3 (M186-3, MBL), and CS (129095, abcam).

2.4.8 | Tissue glycogen concentration

Glycogen concentrations in the liver and skeletal muscle (soleus, plantaris, gastrocnemius, and tibialis anterior muscle) were measured using the phenol–sulfuric acid assay, as previously described.<sup>34</sup> Liver fragments (approximately 10 mg) and whole muscles were weighed, added in 300 μl of 30% (w/v) KOH saturated with Na<sub>2</sub>SO<sub>4</sub>, and heated at 98°C until completely dissolved. The homogenates were then mixed in 360 μl of ethanol and placed on ice for 30 min, followed by centrifugation (5000g, 15 min, 4°C). The glyco-gen-containing pellets were dissolved in distilled water. Then, 5% (v/v) phenol and sulfuric acid were added to the solutions, and the mixture was allowed to react for 15 min before measuring their absorbance at 490 nm.

2.4.9 | Expired gas analysis

The expired gas during the exercise test was evaluated using the O<sub>2</sub>/CO<sub>2</sub> metabolism measuring system for small animals (MK-5000RQ, Muromachi kikai, Tokyo, Japan). Respiratory exchange ratio (RER) was calculated as expired CO<sub>2</sub>/inspired O<sub>2</sub> (VCO<sub>2</sub>/VO<sub>2</sub>). The carbohydrate oxidation was calculated using the following formula based on a previous study.<sup>35</sup> Carbohydrate oxidation (mg/min) = 4.55 VCO<sub>2</sub> (ml/min) – 3.21 VO<sub>2</sub> (ml/min) and normalized to metabolic body size (kg<sup>0.75</sup>).

2.5 | Statistical analysis

All data herein are expressed as mean ± standard error of the mean (SEM). Regarding BCAA concentrations and RER, a two-way repeated measures analysis of variance (ANOVA) was performed to assess the effects of time and supplementation. For other analysis in experiment 1, statistical analysis was conducted using the Student’s t-test, while for additional analyses in experiments 2–4, one-way ANOVA and Tukey–Kramer post hoc tests were performed. Significant differences and trends were defined as <i>p</i> < .05 and <i>p</i> < .10, respectively.

3 | RESULTS

3.1 | Experiment 1

3.1.1 | Plasma BCAA concentration

Initially, it was assessed whether a single period of BCAA supplementation increases blood BCAA levels in sedentary mice. Plasma BCAA levels at pre-ingestion were not significantly different among the two groups (Figure 2A). In the BCAA group, plasma BCAA levels were significantly higher up to 120 min compared to pre-ingestion (<i>p</i> < .01). Plasma BCAA levels at 15, 30, 60, and 90 min in the BCAA group were significantly higher than those in the Con group (Figure 2A, at 15–60 min: <i>p</i> < .01, at 90 min: <i>p</i> < .05). Plasma BCAA incremental area under the curve (iAUC) in the BCAA group was significantly higher than that of the Con group (Figure 2B, <i>p</i> < .01).

3.1.2 | Signaling pathways related to mitochondrial adaptations

After 30 min of supplementation, mTOR phosphorylation in the BCAA group was significantly higher compared to that of the Con group (Figure 2C,D). Accordingly, the phosphorylation state of p70S6K, one of the downstream effectors of mTOR, tended to be higher in the BCAA group compared to the Con group (Figure 2C,D, <i>p</i> = .096). The phosphorylation state of ULK1, one of the downstream effectors of mTOR, showed a similar phosphorylation pattern...
Figure 2: Effects of single BCAA ingestion on blood substrate and signaling pathways. BCAA supplementation increased plasma BCAA levels and induced the mTOR signaling pathway in plantaris muscle at rest. (A) Plasma BCAA concentration. (B) Plasma BCAA iAUC. (C) Phosphorylation states of mTOR, p70S6K, ULK1, AMPK, and ACC. (D) Representative immunoblot images of phosphorylation states of mTOR, p70S6K, ULK1, AMPK, and ACC. Data are expressed as mean ± SEM. n = 6–7 per group. *p < 0.05, **p < 0.01, vs. Con group. ††p < 0.01, vs. 0 min at BCAA group.

as mTOR and p70S6K, but no statistically significant difference was observed between the groups (Figure 2C,D). AMPK and its downstream target ACC did not differentiate between the two groups (Figure 2C,D).

3.2 | Experiment 2

3.2.1 | Animal characteristics

Animal characteristics are presented in Table 1. Initial body weights, final body weights, and body weight gain did not differ among all groups. Total energy intake was similar among the assessed groups. Plantaris muscle weight did not differ among the examined groups.

3.2.2 | Mitochondrial content and biogenesis

According to the previous study, CS activity is the most prevalent indicator of mitochondrial content and is strongly associated with it. Therefore, we evaluated CS activity in this study. CS activity in the Tr group was significantly higher than that in the DeTr group (Figure 3A,
3.3.1 | Markers of mitochondrial dynamics

Mitochondria are dynamic organelles that undergo fusion and fission dependent on cellular conditions. Mitochondrial dynamics regulate mitochondrial homeostasis and quality. 37 Mitochondrial fusion-related protein (OPA1 and Mfn2) and fission-related protein (DRP1 and Fis1) levels were then measured as markers involved in the regulation of mitochondrial morphology. Opa1 protein levels in DeTr and DeTr+BCAA groups were found to be significantly lower than those of the Tr group (Figure 4A,B, p < .01). Mfn2 protein levels demonstrated a similar pattern as Opa1, but the statistical difference was not detected among the groups (Figure 4A,C). Drp1 protein levels in the Tr group were significantly higher than those in the DeTr + BCAA group (p < .05), but they were similar to those of the DeTr group (Figure 4B,C). Fis1 protein levels demonstrated a similar pattern to DRP1, although no statistical difference was detected among the groups (Figure 4B,C).

3.3.2 | Markers of protein degradation and mitophagy

As mitochondria are divided by fission and then degraded by mitophagy, 20,21 protein degradation- and mitophagy-related genes and proteins were then measured in whole muscle and isolated mitochondria. Regarding protein degradation, FoxO1, FoxO3, and MAFbx/atorogin-1 mRNA expression did not differentiate among the groups in whole skeletal muscle (Figure 5A). MuRF-1 mRNA expression in the Tr group tended to be lower than that of the DeTr group (p = .059), while it was not different from that of the DeTr+BCAA group (Figure 5A). In terms of mitophagy, Parkin protein content in the Tr group was significantly lower than that of the DeTr group (p < .01) and tended to be lower than that of the DeTr+BCAA group (Figure 5B,C, p = .057). p62/SQSTM1 protein content was similar among the evaluated groups (Figure 5B,C), while BNIP3 protein content in the Tr group was significantly higher than that of the DeTr+BCAA group (p < .05) but not different from that of the DeTr group (Figure 5B,C). BNIP3L/NIX and LC3B-II protein levels were similar among the groups (Figure 5B,C).

In isolated mitochondria from gastrocnemius muscle that were then analyzed, Parkin and p62/SQSTM1 protein levels did not differentiate among the evaluated groups (Figure 5D,E). BNIP3 protein levels in the Tr group were significantly higher compared to those of the DeTr+BCAA group (p < .01), but not different from those in the DeTr group (Figure 5D,E). Accordingly, BNIP3L/NIX protein content in the DeTr+BCAA group tended to be lower than that of the Tr group (Figure 5D,E, p = .060). Furthermore, LC3B-II in the DeTr group tended to be higher compared to that of the Tr group (p = .100), while it was significantly higher than that of the DeTr+BCAA group (Figure 5D,E, p < .05).

3.3.3 | Mitochondrial respiration and ROS production

We further measured the respiratory capacity and oxidative stress of the isolated mitochondria to assess
mitochondrial function. Mitochondrial oxygen consumption rate (OCR) in both states 2 (4) and 3 were similar among the evaluated groups (Figure 6A,B). ROS production under state 2 (4) and 3 respiratory conditions (Figure 6C,D) as well as 4-HNE-conjugated protein levels, which is a biomarker of oxidative stress (Figure 6E,F), were similar among the evaluated groups.

3.4 | Experiment 4

3.4.1 | Exercise test

BCAA supplementation was demonstrated to suppress the detraining-induced reduction of mitochondrial content without altering the mitochondrial quality. One of the characteristic symptoms of increased mitochondrial

FIGURE 3  Effects of BCAA ingestion on mitochondrial enzyme activities and protein at detraining. BCAA supplementation suppressed the detraining-induced reduction of mitochondrial content. (A) CS and β-HAD activities. (B) COXIV protein content. (C) PGC-1α and SIRT1 protein content. (D) Representative immunoblot images of COXIV, PGC-1α, and SIRT1. Protein expression comparison was performed after normalization to GAPDH. Data are expressed as mean ± SEM. n = 6–7 per group. *p < .05, **p < .01, vs. Tr group.
content is the reduction of carbohydrate utilization during exercise.38 Substrate utilization during exercise and glycogen content were subsequently assessed.

Before training cessation, an exercise test was conducted in the DeTr and DeTr+BCAA groups. The purpose of this measurement was to confirm that there were no differences between the groups prior to the intervention. The main effects of time were observed for blood glucose and lactate concentration (p < .01); however, there were no differences among the supplement groups (Figure S1A,B). Similarly, RER during the exercise test decreased with time (p < .01), but there were no differences among the treatment groups (Figure S1C). Carbohydrate oxidation was similar among the groups (Figure S1D).

After 2 weeks of detraining and BCAA supplementation, a similar exercise test was performed. Pre- and post-exercise glucose concentrations did not differ among the evaluated groups (Figure 7A). Blood lactate concentration was lower in the post-exercise compared to those in the pre-exercise group (p < .01), although there were no differences among the treatment groups (Figure 7B). RER during the exercise test decreased with time (p < .01); however, no differences were reported among the treatment groups (Figure 7C). Additionally, carbohydrate oxidation during the exercise test did not differ among the treatment groups (Figure 7D).

Post-exercise liver glycogen concentration was similar among the evaluated groups (Figure 7E). Post-exercise soleus and plantaris muscle glycogen concentrations in the Tr group were significantly higher than those in the DeTr and DeTr+BCAA groups (Figure 7F,G, p < .01). Post-exercise gastrocnemius and tibialis anterior muscle glycogen concentrations in the Tr group were significantly higher compared to those in the DeTr group (p < .01) but not significantly different from those of the DeTr+BCAA group (Figure 7H,I). Considering the results of post-exercise muscle glycogen levels, it should be confirmed whether BCAA supplementation alters basal muscle glycogen levels. There was no disparity in basal muscle glycogen concentration with BCAA administration using the tibialis anterior muscle in experiment 2 (Figure S2A,B).

4 | DISCUSSION

Mitochondria are organelles that undergo reversible changes with training and detraining in skeletal muscle. Previous studies reported that detraining reduced mitochondrial content,7,8 which was consistent with our observations that 2 weeks of detraining reduced mitochondrial enzyme activities and protein content. In this study, we found that BCAA supplementation suppressed the reduction of mitochondrial enzyme activities and protein content. To the best of our knowledge, this is the first study to determine the effect of BCAA ingestion during detraining on mitochondrial content.

Coordination of mitochondrial biogenesis and degradation is essential to maintain mitochondrial content.22 We first focused on mitochondrial biogenesis as the reason for the suppression of the detraining-induced reduction of mitochondrial content by BCAA ingestion. It is well known that mitochondrial biogenesis is regulated by PGC-1α.39 PGC-1α in skeletal muscle is increased by exercise40 and electrical stimulation-induced contractile activity.41 A previous study has also shown a robust positive correlation between the PGC-1α protein expression and mitochondrial enzyme activity.42 In this study, detraining decreased PGC-1α protein content, suggesting that

FIGURE 4 Effects of BCAA ingestion on mitochondrial dynamics markers. BCAA supplementation partly reduced mitochondrial fusion-related protein content. (A) Opa1 and Mfn2 protein content. (B) Drp1 and Fis1 protein content. (C) Representative immunoblot images of Opa1, Mfn2, Drp1, and Fis1. Protein expression comparison was performed after normalization to GAPDH. Data are expressed as mean ± SEM. n = 6–7 per group. *p < .05, **p < .01, vs. Tr group.
Effects of BCAA ingestion on protein degradation and mitophagy. BCAA supplementation partly reduced mitophagy-related protein content. (A) FoxO1, FoxO3, MAFbx/atrogin-1, and MuRF-1 gene expression in whole muscle. (A) Parkin, p62, BNIP3, BNIP3/NIX, and LC3B-II protein content in whole muscle. (B) Representative immunoblot images of Parkin, p62, BNIP3, BNIP3/NIX, and LC3B-II in whole muscle. In whole muscle, comparisons of gene expression and protein content were performed after normalization to GAPDH. (C) Parkin, p62, BNIP3, BNIP3/NIX, and LC3B-II protein content in isolated mitochondria. (D) Representative immunoblot images of Parkin, p62, BNIP3, BNIP3/NIX, and LC3B-II in isolated mitochondria. In isolated mitochondria, comparison of protein content was performed after normalization to CS. Data are expressed as mean ± SEM. n = 7–9 per group. *p < .05, **p < .01, vs. Tr group. †p < .05, vs. DeTr group.
training cessation suppresses mitochondrial biogenesis. Regarding BCAA ingestion and adaptation of PGC-1α, a previous study reported that the intake of leucine, one of the BCAAs, activated PGC-1α via deacetylation and enhanced mitochondrial enzyme activities, resulting in improvement in metabolic disorders in mice fed a high-fat diet.\(^4^3\) BCAA consumption increased PGC-1α mRNA levels and mitochondrial content, resulting in an extended life span of middle-aged mice.\(^{19}\) However, in contrast to earlier studies, the protein content of PGC-1α in this study was unaffected by BCAA ingestion (Figure 3C,D). On this point, D’Antona et al.\(^{19}\) also reported effects of BCAA consumption on mitochondrial adaptation were absent in young animals. Therefore, it is possible that BCAA ingestion enhances mitochondrial biogenesis in metabolic disease and aging conditions, but not in young healthy conditions. Collectively, we could not determine a clear effect of BCAA ingestion on PGC-1α protein levels and promote mitochondrial biogenesis in skeletal muscle. Our results suggest that other pathways were associated with the suppression of the detraining-induced reduction of mitochondrial content.

Mitochondrial content is regulated not only by mitochondrial biogenesis but also by mitochondrial degradation.\(^{20}\) In the mitochondrial degradation process, mitochondria are fragmented by fission and then eliminated by mitophagy. Therefore, we subsequently focused on markers of mitochondrial dynamics. Exercise training is reported to increase protein content related to mitochondrial fusion and fission in human skeletal muscle.\(^{4^4}\) In this study, we observed that training-induced enhancement of Opa1, but not DRP1, returned to baseline levels.

**FIGURE 6** Effects of BCAA ingestion on mitochondrial respiration and ROS production in isolated mitochondria. BCAA supplementation did not affect mitochondrial quality. (A) Oxygen consumption rate under state 2 (4). (B) Oxygen consumption rate under state 3. (C) ROS production under state 2 (4). (D) ROS production under state 3. (E) 4-HNE protein content as an index of oxidative stress. (F) Representative immunoblot images of 4-HNE. Protein expression comparisons were performed after normalization to CS. Data are expressed as mean ± SEM. \(n = 7–9\) per group.
following detraining (Figure 4A–C). These results indicate that detraining suppresses training-induced enhancement of the mitochondrial fusion marker, but not the mitochondrial fission marker, which results in reduced mitochondrial content. In this study, Drp1 protein levels in the DeTr + BCAA group were significantly lower than those in the Tr group (Figure 4B,C). Although the effects of BCAA ingestion on mitochondrial dynamics have not been known in skeletal muscle, in other tissue, it is reported that BCAA and L-carnitine administration reduces mitochondrial-localized DRP1 protein content in rat cirrhotic liver. Therefore, BCAA supplementation during detraining may suppress the mitochondrial fission process in skeletal muscle.

Fragmented mitochondria are eliminated by mitophagy. Several mechanisms of selective mitophagy have
been identified, such as PINK1/Parkin-mediated mitophagy and mitochondrial receptor-mediated mitophagy represented by BNIP3 and BNIP3L/NIX.46 PINK1/Parkin-mediated mitophagy eliminates damaged mitochondria. According to a prior study, PINK1/Parkin-mediated mitophagy is as follows:28 Mitochondria are tagged to clearance when Parkin is recruited from the cytosol to the membrane of dysfunctional mitochondria, which then ubiquitinates mitochondrial outer-membrane proteins. Ubiquitin-conjugated mitochondria are recognized by P62/SQSTM1. Mitochondria adapted with poly-ubiquitin and P62/SQSTM1 are engulfed by autophagosomes consisting of the biomarker protein LC3B-II and are finally degraded by lysosomes. Whereas, BNIP3 and BNIP3L/NIX-mediated mitophagy is PINK1/Parkin-independent mitophagy. BNIP3 and BNIP3L/NIX are well-known activators of cell death and act as receptors for targeting mitophagy. BNIP3 and BNIP3L/NIX bind directly to LC3 and degrade mitochondria without Parkin-mediated ubiquitination. A study has reported that 14 days of hindlimb immobilization enhanced BNIP3 and BNIP3L/NIX gene expressions and reduced PGC-1α gene expression and CS activity.46 In the current study, we demonstrated that BCAA supplementation during detraining decreased BNIP3, BNIP3L/NIX, and LC3B-II protein levels in isolated mitochondria (Figure 5D,E). Our results indicate that BCAA ingestion during detraining suppresses BNIP3 and BNIP3L/NIX-mediated mitophagy. Regarding BCAA ingestion and adaptation of mitophagy, leucine ingestion activates mTOR signaling.14 BCAA ingestion after exercise stimulates mTOR signaling more effectively than leucine alone.18 mTOR responsiveness to exercise was higher during the detraining period than repetitive training period.49 Therefore, it is possible that mTOR activation by BCAA ingestion was more likely to occur during the detraining period. In cell culture experiments, activation of mTOR suppresses mitophagy in cells lacking tuberous sclerosis complex 2 (TSC2), a critical negative regulator of mTOR.50 Conversely, the inhibition of mTOR increases BNIP3 in ventricular myocytes51 and causes mitophagy in human cytoplasmic cybrid cell lines.52 Thus, mTOR activation by BCAA intake may have also contributed to the suppression of BNIP3 and BNIP3L/NIX-mediated mitophagy in skeletal muscle. Further studies are warranted to investigate the mechanism by which BCAA ingestion suppresses mitophagy.

Amino acids have long been known to be prime regulators of autophagy,53 and BCAA supplementation suppresses muscle protein breakdown.54,55 In this study, MuRF-1 mRNA expression in the Tr group tended to be lower than that of the DeTr group (Figure 5A) but not different from that of the DeTr+BCAA group. These findings suggest that BCAA ingestion may slightly inhibit muscle degradation. However, there were no differences in ULK1 signaling (Figure 2C,D) and plantaris muscle weight (Table 1). Whereas, LC3B-II in the DeTr group was significantly higher than that of the DeTr+BCAA group in isolated mitochondria (Figure 5D,E). Given these observations, BCAA supplementation during detraining may affect the inhibition of mitochondrial degradation rather than the inhibition of overall skeletal muscle degradation.

Previously, it has been shown that exercise training increases BNIP3 and LC3B-II levels.56 Based on results from a previous study, it is considered that exercise training promotes mitochondrial turnover, resulting in improved mitochondrial quality.57 Our data showed that BCAA supplementation during detraining decreased the levels of mitochondrial fission and mitophagy-related proteins. Inhibition of mitophagy causes the accumulation of dysfunctional mitochondria.58 Mitochondrial dysfunction leads to undesired events such as increased ROS production.59 For instance, hepatic mTOR activation by BCAA intake inhibits lipid-induced hepatic autophagy, increases hepatic apoptosis, and increases hepatocyte susceptibility to FFA-mediated lipotoxicity.60 BCAA at supraphysiologically high levels (20 mM) reduced mitochondrial function in C2C12 myotubes.24 Therefore, there was a concern that inhibition of the degradation process by BCAA suppressed mitochondrial clearance and caused the accumulation of low-quality mitochondria. In this study, we assessed Complexes II-driven state II (IV) and III respiration and found no difference. Further, as no difference was seen in ROS production, we concluded that BCAA ingestion did not affect mitochondrial function.

Enhancement of the mitochondrial content affects substrate oxidation during exercise. Exercise training enhances mitochondrial content,6 resulting in decreased carbohydrate utilization during exercise.61 In this study, RER and glucose oxidation during the exercise test did not differ among the assessed groups, and there was no difference in liver glycogen content. This indicates that BCAA supplementation throughout the 2-week detraining phase was insufficient to impact whole-body substrate utilization. However, post-exercise glycogen levels in the gastrocnemius and tibialis anterior muscles were lower after detraining, but BCAA ingestion prevented these changes. These results indicate that BCAA ingestion suppresses the detraining-induced reduction of mitochondrial content and muscle glycogen breakdown during exercise. Muscle glycogen content is related to endurance exercise performance,62 and muscle glycogen depletion decreases intermittent sprint exercise performance.63 It is therefore possible that BCAA ingestion during detraining suppresses the reduction of exercise performance.

In this study, we found that 0.6 g of BCAA supplementation activated mTOR, and continuous ingestion twice daily prevented detraining-induced reduction of mitochondrial content. Consuming such high doses would
be challenging in humans; nevertheless, Karlsson et al.\textsuperscript{64} reported that ingesting BCAA (100 mg/g BW)-activated mTOR signaling in humans. Moreover, 5.6 g of BCAA was reported to activate mTOR signaling in humans.\textsuperscript{65} Therefore, lower doses of BCAA may be effective in humans and should be investigated in future studies.

5 | LIMITATION

There are several limitations of this study. The first is to examine mitochondrial function in more detail. Due to limited sample volume, we could only measure Complexes II-driven state II (IV) and III respiration, and we found no difference. Further, as no difference was seen in ROS production, we concluded that there are no functional changes in the mitochondria. Complex I-driven respiration, however, was not measured in this study and should be examined in future studies. The second is to examine mitophagy flux. We could not measure autophagy and mitophagy flux. Autophagy and mitophagy flux assessment will also provide important insights concerning mitochondrial turnover.\textsuperscript{66} The last is to examine the mitochondrial ultrastructure. We did not analyze the mitochondrial ultrastructure because it was beyond the scope of this study. However, a structural analysis would provide more detailed findings on the effects of BCAA ingestion on mitochondrial adaptations. Therefore, this should be studied in the future.

6 | CONCLUSION

In the current study, the effect of BCAA supplementation during the detraining period was assessed on mitochondrial adaptations of the skeletal muscle (Figure 8). We demonstrated that BCAA supplementation in detraining: (i) suppressed loss of mitochondrial enzyme activities and protein content, (ii) partially suppressed mitochondrial fission- and mitophagy-related protein levels, (iii) did not affect mitochondrial respiration and ROS production, and (iv) partially enhanced post-exercise muscle glycogen concentrations. The presented findings provide a nutritional strategy for suppressing detraining-induced reductions in mitochondrial content and shed light on the relationship between BCAA ingestion and mitochondrial adaptations.

AUTHOR CONTRIBUTIONS

Yutaka Matsunaga, Yuki Tamura, Kenya Takahashi, Yu Kitaoka, Daisuke Hoshino, and Hideo Hatta conceived and designed the research; Yutaka Matsunaga, Yuki Tamura, Kenya Takahashi, and Yumiko Takahashi performed the research and acquired the data; and Yutaka Matsunaga, Yuki Tamura, Kenya Takahashi, Yu Kitaoka, and Tomoyasu Kadoguchi analyzed and interpreted the data. All authors were involved in drafting and revising the manuscript.

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DISCLOSURES

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article.

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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