The Effects of Branched-Chain Amino Acids on the Akt/mTOR Pathway and Nebulin Protein in Joint Fixation-Induced Muscle Atrophy

Akira NISHIKAWA1,2,* Akiko NISHIKAWA1, Noboru KAMAJIRI2, Keisuke OKADA2 and Hidetaka IMAGITA2,**

1 Faculty of Business Information Sciences, Jobu University, 634–1 Toyazuka-machi, Iseaki, Gunma 372–0825, Japan
2 Graduate School of Health Sciences, Kio University, 4–2–2 Umaminaka, Koryo-cho, Kitakatsuragi-gun, Nara 635–0832, Japan

Summary   It is well known that branched-chain amino acids (BCAAs) promote protein synthesis in skeletal muscle and can cause muscle hypertrophy. However, it has also been reported that they may inhibit muscle atrophy induced by load-bearing and age-related changes. In this study, we investigated the effects of BCAA intake during joint fixation on the levels of protein kinase B (Akt), mammalian target of rapamycin (mTOR), and nebulin in a rat model of joint fixation. Akt and mTOR are signal factors of protein synthesis, whereas nebulin is a structural protein in the muscle. The effects of BCAAs on muscle atrophy were also investigated. The phosphorylation rate of mTOR was higher than that of Akt and increased with BCAA intake in the rat hind limb muscles (soleus) when the ankle joint was fixed. The relative level of nebulin and the phosphorylation rate of Neural Wiskott-Aldrich syndrome protein (N-WASP) also increased as a result of BCAA intake during fixation. This is important because nebulin and N-WASP are involved in the formation of the structure of sarcomere thin filaments. Furthermore, when the cross-sectional areas (CSAs) of different types of muscle fibers were measured during histological evaluation of muscle atrophy, it was found that the inhibitory effect of BCAA on muscle atrophy was higher in Type 1 fibers. Additionally, a positive correlation was found between nebulin level and the CSAs of the muscle fibers. It was found that there is a close relationship between the content of structural proteins and muscle atrophy.

Key Words   BCAAs, Akt/mTOR pathway, N-WASP, nebulin, muscle atrophy, joint fixation

The skeletal muscle is one of the organs that are particularly prone to thermoplastic changes in the body. Muscle hypertrophy promotes the production of proteins that make up the skeletal muscle, and conversely, muscle atrophy progresses when the former is suppressed. Furthermore, when skeletal muscle activity is reduced, muscle fibers undergo structural changes such as a decrease in cross-sectional area (CSA), shortening of muscle length due to a decrease in the number of sarcomeres, and disordered arrangement of myofibrils (1, 2). It has also been reported that changes in the type of myosin heavy chain (MHC) isoform and changes in sarcoplasmic reticulum function affect muscle contraction characteristics (3, 4). In our study (5), the CSAs of the fibers in the soleus muscle immobilized in the shortened position was reduced by the Type 1 fiber rather than by the Type 2 fiber. Furthermore, it is reported that the MHC isoform changes from slow to fast, and as a result, contraction time is shortened and contraction tension is decreased.

Mechanical stress on the skeletal muscle as a result of strength training promotes the production of insulin-like growth factor-1 (IGF-1), which is a factor of protein synthesis. This hormone induces the phosphorylation of phosphatidylinositol 3-kinase (PI3K) and its downstream molecule protein kinase B (Akt). Phosphorylated Akt then promotes the activation of the downstream mammalian target of rapamycin (mTOR) and inactivation of glycogen synthase kinase 3β (GSK3β), which result in the promotion of protein synthesis via the activities of factors involved in mRNA translation (6, 7). Previous studies have shown that reducing mechanical stress by immobilization results in suppressed activation of the Akt/mTOR pathway, which further suppresses protein synthesis and leads to muscle atrophy (6). Furthermore, inactivation of GSK3β promotes the binding of Neural Wiskott-Aldrich syndrome protein (N-WASP) to nebulin, a structural protein in the skeletal muscle (8). Nebulin is a large protein with a molecular weight of approximately 770 kDa in humans and is localized along the entire length of the thin filament in the sarcomere. This protein is thought to regulate the length of the thin filament to 1 μm (9). It has been clarified that when N-WASP binds to nebulin, actin polymerizes along the length of nebulin to form actin fibers. Additionally, N-WASP has been reported to play an essential role in IGF-1-induced muscle hypertrophy (8).
It is reported that branched-chain amino acids (BCAAs) promote protein synthesis in skeletal muscles during mechanical stress. BCAAs is a general term that refers to amino acids that have a branched structure in their side chains. Examples of BCAAs are leucine, isoleucine, and valine. Leucine has a particularly strong effect on protein synthesis. It has been clarified that, following oral intake, leucine activates mTOR, promotes protein synthesis in skeletal muscle, and causes muscle hypertrophy (10). It has also been reported that activated mTOR suppresses muscle atrophy by suppressing autophagosome formation (11).

Previous studies on the effects of BCAAs on muscle atrophy involved the use of experimental models based on hindlimb suspension, denervation, and dexamethasone-induced sarcopenia (12–14). However, muscle atrophy caused by immobilization due to joint fixation has not been investigated. In the model of disuse muscle atrophy, unloading is assumed with hindlimb suspension, and motor paralysis owing to peripheral nerve injury is assumed with denervation. These are not suitable models for investigating muscle atrophy that occurs during the treatment of traumatic disorders of the locomotorium (fractures, dislocations, sprains, etc.). Therefore, in the present study, we aimed to clarify the effects of BCAA intake on the factors involved in signaling in the protein synthesis system using the joint fixation model rat (5) that we have established. Additionally, we investigated the effects of BCAA intake on muscle atrophy. Furthermore, we evaluated nebulin level to investigate the relationship between muscle atrophy and structural proteins in the skeletal muscles.

**MATERIALS AND METHODS**

**Animals.** Male Wistar rats (10 wk old; weight, 321.4 ± 7.1 g, Japan SLC, Inc., Shizuoka, Japan) were used in this experiment. The animal breeding room was maintained at room temperature (23 ± 2°C) and at a humidity of 55 ± 5%. The animals were bred individually in a cage under a 12/12 h dark/light cycle. During the breeding period, rats were allowed free access to food (Rodent diet; CE-2, CLEA Japan, Inc., Tokyo, Japan) and drinking water. The study protocol was approved by the Animal Experiment Ethics Committee of Kio University (Koryo, Japan) and the Animal Experiment Committee of the Institute of Medical Physiology, Jobu University (Ishikawa, Japan). The study was carried out according to the respective regulations for animal experiments (No. H30-07, 19-AN01).

**Experimental design.** The animals were randomly assigned to one of the following four groups: Con (ingested water without joint fixation), Fix (ingested water and underwent joint fixation), BCAA (ingested BCAAs without joint fixation), and Fix + BCAA (ingested BCAAs and underwent joint fixation). Each group was comprised of six animals.

The method described by Okita et al. (15) with slight modifications was used to perform joint fixation. External fixation was maintained for 2 wk (Fig. 1). During the fixation period, follow-up examination was performed every day, and if the fixture was loosened or damaged, it was re-fixed as appropriate.

Whey protein, the protein with the highest BCAA content, contains valine, leucine, and isoleucine at a ratio of approximately 1:2:1. It is known that leucine, which comprises 50% of the content, has a strong effect on muscle protein synthesis. In addition, a study on the oral administration of leucine to rats after fasting revealed that the 50% effective dose of leucine for the soleus muscle was approximately 30% of the amount taken from the standard diet (about 40 mg/100 g body weight [BW]). After referring to these results, a mixture of BCAAs was prepared containing valine, leucine, and isoleucine at a ratio of 1:2:1, respectively, according to the method described by Jang et al. (16). The intake of the BCAAs was assessed based on the average daily intake of water (approximately 30 g) obtained by preliminary experiments. The dose of BCAAs was adjusted to 60 mg/100 g BW every day. The prepared solution was placed in a water bottle for intake by the rats during the experiment.

**Joint range of motion (ROM).** Joint ROM was measured by following the procedure described by Ono et al. (17) with some modifications. The states in which the
ankle joint was maximally flexed and dorsiflexed by pressing (approximately 490 hPa) the center of the sole using a manual tender meter (015-07461, Matsumiya medical equipment factory, Ltd., Tokyo, Japan) were directly photographed. The dorsiflexion ROM of the ankle joint of each rat was calculated using ImageJ software (version 1.8.0; National Institutes of Health, Bethesda, MD, USA).

Excision of skeletal muscle. After the 2-wk fixation period, three mixed anesthetics were intraperitoneally administered to all groups, and deep anesthesia was applied to remove the soleus muscle (SOL) of the right hind limb. After that, animals were euthanized via blood removal. After measuring the muscle wet weight, the SOL was divided into two parts, one for histochemical specimens and the other for biochemical specimens, and stored in an ultra-low temperature freezer at −80°C.

Enzyme staining. A 10-µm-thick continuous cross section of tissue was prepared from the histochemical specimen and subjected to myosin adenosine triphosphatase (ATPase) staining. Alkaline pretreatment (pH 10.4) was performed according to the method described by Brooke and Kaiser (18), followed by incubation in a solution containing ATP for 15 min. The success of this staining method is based on the sensitivity of myosin ATPase to pH. Additionally, myosin ATPase in Type 1 fiber loses its activity when pretreated with alkali. Therefore, the Type 1 fiber is lightly dyed, whereas the Type 2 fiber is deeply dyed. Images of the stained sections were captured using an optical microscope and classified into Type 1 and 2 fibers. The CSA of each muscle fiber type was measured using ImageJ software.

Western blotting. Biochemical specimens were homogenized in a 1:1 mixture of 0.1 M dithiothreitol and Tris-SDS solution (8% sodium dodecyl sulfate [SDS], 10 mM ethylenediaminetetraacetic acid, and 0.2 M Tris-HCl). The mixtures were then boiled in water for 3 min, and centrifuged at 13,600 × g at 4°C for 10 min, after which the supernatant was collected and used as a sample for electrophoresis. Protein concentration was determined using the Bradford assay (19). An amount of sample containing 5 µg of protein was analyzed in each instance.

Next, western blotting was performed according to the method described by Towbin et al. (20) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 10% linear gel and 2–15% gradient gel according to a previously reported method (21). The 10% linear gel was used to react with Act (Cell Signaling Technology, Inc., Danvers, MA, USA, #4685), phospho-Akt (Ser473) (Cell Signaling Technology, #4060), N-WASP (ECM Biosciences, Versailles, KY, USA, #WP2401), and phospho-N-WASP (Ser485/Ser485) (ECM Biosciences, #WP2201), whereas the 2–15% gradient gel was used for reaction with mTOR (Cell Signaling Technology, #2983), phospho-mTOR (Ser2448) (Cell Signaling Technology, #2971), and nebulin (Proteintech, Rosemont, IL, USA, #19706-1-AP). After SDS-PAGE, proteins were electrophoretically transferred from the gel onto a nitrocellulose membrane for immunoblotting. After blocking with 5% bovine serum albumin (BSA) in a mixture of Tris-buffered saline and Tween 20 (TBS-T; 0.5 M Tris, 1.38 M NaCl, 27 mM KCl, and 0.05% Tween 20) for 60 min, the primary antibody was diluted 1,000-fold with 1% BSA in TBS-T and incubated at 4°C overnight. After washing with TBS-T, horseradish peroxidase–labeled anti-rabbit IgG was reacted with a secondary antibody diluted 10,000-fold with 1% BSA in TBS-T for 60 min. The membranes were exposed to an X-ray film, and bands of interest were quantified with ImageJ software. Relative quantification of the target protein was performed using β-actin (Proteintech, #20536-1-AP) as the control protein.

Statistical analysis. All data have been expressed as

![Fig. 2. ROM of the right ankle dorsiflexion (after the fixation period). As a result of two-way ANOVA, there were significant differences in the two factors (joint fixation, branched-chain amino acid (BCAA) intake), and interactions were also observed (all p<0.001).](image-url)

**Table 1. Body weight and index of muscle atrophy.**

|          | BW (g)    | MW (mg)    | MW: BW ratio |
|----------|-----------|------------|--------------|
| Con      | 352.0±5.7 | 171.6±13.2 | 0.49±0.03    |
| Fix      | 345.1±11.6| 102.5±22.8*| 0.30±0.08*   |
| BCAA     | 373.1±5.9*| 180.4±19.1†| 0.48±0.06†   |
| Fix+BCAA | 343.3±10.3§| 132.7±25.6*‡| 0.39±0.07*‡  |

As a result of two-way ANOVA, for body weight there were significant differences in the two factors (joint fixation, branched-chain amino acid (BCAA) intake), and interactions were also observed (p<0.001, p=0.020, p=0.007, respectively). For muscle weight there were significant differences in the two factors (p<0.001, p=0.041 respectively), and the ratio of muscle weight to body weight also showed the same association (p<0.001, p=0.013, respectively). *p<0.05 (compared with the Con group).
†p<0.05 (compared with the Fix group).
§p<0.05 (compared with the BCAA group).
The Effects of Branched-Chain Amino Acids in Muscle Atrophy

mean standard deviation. SPSS Statistics 27 (IBM Corp., Armonk, NY, USA) was used for statistical processing of data. Tukey’s multiple comparison test was performed after a two-way analysis of variance for comparison between groups. In addition, Pearson’s correlation coefficient was used to test correlation. In each case, the significance level was set at <5%.

RESULTS

Water and food consumption

Drinking water was measured at 10 am every day. No statistically significant differences were observed in the average daily water consumption (Con: 29.6±1.1 g, Fix: 30.2±1.3 g, BCAA: 29.8±0.8 g, Fix+BCAA: 30.7±2.7 g) and total water consumption during the experiment (Con: 384.6±14.8 g, Fix: 392.9±16.7 g, BCAA: 387.8±10.9 g, Fix+BCAA: 399.6±35.6 g) between the groups. In addition, there was no statistically significant difference between the groups in the total food consumption (Con: 288.9±25.8 g, Fix: 285.4±20.5 g, BCAA: 296.8±29.0 g, Fix+BCAA: 290.6±22.7 g).

Effect of BCAAs on ROM during joint fixation

Figure 2 shows the ROM of the right ankle dorsiflexion after the fixation period. The ROM value for the Fix group (14.8±2.2˚) was only 9% of the value obtained for the Con group (162.1±0.9˚). Additionally, the ROM value for the Fix+BCAA group (22.0±1.0˚) was higher than that for the Fix group but only 14% of the value obtained for the Con group. Overall, ROM limitation following BCAA intake during fixation was suppressed by only 5%.

BCAAs suppressed decrease in muscle weight (MW) during joint fixation

Table 1 shows the BW, MW, and MW : BW ratio in the groups after the fixed period. The data show that BW increased only in the BCAA group. Additionally, the MW : BW ratio for the Fix group was 60% lower than that for the Con group. The MW : BW ratio for the Fix+BCAA group was higher than that for the Fix group but 80% of the value for the Con group. The results showed that the BCAAs suppressed the decrease in MW during fixation.

Effects of BCAAs on the CSAs of Type 1 and 2 fibers during joint fixation

Figure 3 shows the CSAs of the different muscle fibers. The CSA of Type 1 fiber in the Fix group (2076.0±167.7 μm²) was 63% of the value obtained...
Nishikawa A et al.

Fig. 4. Phosphorylation of Akt. As a result of two-way ANOVA, there were significant differences in the two factors (joint fixation, branched-chain amino acid (BCAA) intake), and interactions were also observed (all \( p < 0.001 \)).

Fig. 5. Phosphorylation of mTOR. As a result of two-way ANOVA, there were significant differences in the two factors (joint fixation, branched-chain amino acid (BCAA) intake), and interactions were also observed (all \( p < 0.001 \)).

Fig. 6. Phosphorylation of N-WASP. As a result of two-way ANOVA, there were significant differences in the two factors (joint fixation, branched-chain amino acid (BCAA) intake), and interactions were also observed (all \( p < 0.001 \)).

Fig. 7. Phosphorylation of N-WASP. As a result of two-way ANOVA, there were significant differences in the two factors (joint fixation, branched-chain amino acid (BCAA) intake), and interactions were also observed (all \( p < 0.001 \)).

Fig. 8. Phosphorylation of N-WASP. As a result of two-way ANOVA, there were significant differences in the two factors (joint fixation, branched-chain amino acid (BCAA) intake), and interactions were also observed (all \( p < 0.001 \)).

Fig. 9. Phosphorylation of N-WASP. As a result of two-way ANOVA, there were significant differences in the two factors (joint fixation, branched-chain amino acid (BCAA) intake), and interactions were also observed (all \( p < 0.001 \)).

for the Con group (3282.3 ± 192.0 \( \mu m^2 \)). Additionally, the CSA of Type 1 fiber in the Fix+BCAA group (2611.4 ± 143.9 \( \mu m^2 \)) was higher than that in the Fix group, and was 80% of the value for the Con group. Furthermore, the CSA of the Type 2 fiber in the Fix group (1357.9 ± 70.7 \( \mu m^2 \)) was approximately 65% of the value obtained for the Con group (2087.1 ± 101.5 \( \mu m^2 \)). However, the CSA of the Type 2 fiber in the Fix+BCAA group (1561.1 ± 169.5 \( \mu m^2 \)) was almost the same as that in the Fix group. We also found that the rate of decrease in CSA due to joint fixation and the rate of increase in CSA due to BCAA intake during fixation were greater for the Type 1 fiber.

Effects of BCAAs on Akt phosphorylation during joint fixation

Figure 4 shows the ratio of phospho-Akt (p-Akt) to total Akt (t-Akt). The Fix group (0.38 ± 0.12) and Fix + BCAA group (0.28 ± 0.13) had lower ratios than the Con group (0.46 ± 0.16). BCAA intake during fixation continued to suppress Akt phosphorylation.

BCAAs increase mTOR phosphorylation during joint fixation

Figure 5 shows the ratio of phospho-mTOR (p-mTOR) to total mTOR (t-mTOR). The Fix group (0.36 ± 0.09) had almost the same ratio as the Con group (0.40 ± 0.16), but the Fix + BCAA group (0.84 ± 0.12) had a significantly higher ratio than the Fix group. BCAA intake during fixation thus promoted mTOR phosphorylation.

BCAAs increase N-WASP phosphorylation during joint fixation

Figure 6 shows the ratio of phospho-N-WASP (p-N-WASP) to total-N-WASP (t-N-WASP). The Fix group (0.61 ± 0.09) had almost the same ratio as the Con group (0.62 ± 0.20), but in the Fix + BCAA group (1.49 ± 0.41), this was significantly higher than that in the Fix group. Accordingly, BCAA intake during fixation promoted N-WASP phosphorylation.

BCAAs increased the production of nebulin

Figure 7 shows the relative nebulin levels. That in the Fix group (0.25 ± 0.04) was lower than that in the Con group (0.62 ± 0.12). However, the Fix + BCAA group (0.46 ± 0.09) showed higher levels than those in the Fix group but lower levels than those in the Con group. BCAA intake during fixation therefore increased the nebulin content.

Correlation between nebulin levels and the CSAs of the muscle fibers

Figure 8 shows the correlation between nebulin lev-
The Effects of Branched-Chain Amino Acids in Muscle Atrophy

DISCUSSION

An average of 147˚ limited the ROM of ankle joint dorsiflexion due to the joint fixation for 2 wk. The rate at which ROM limitation could be suppressed by BCAAs during fixation was as low as 5% (approximately 7˚). Various factors are involved in the limitation of ROM, and these include relative increase in fascia thickness (atrophy of muscle fiber and thickening of fascia), a decrease in the number of sarcomeres (shortening of muscle length), cross-linking between collagen fibers, disordered arrangement (decreased extensibility), decreased glucosaminoglycan and water content (decreased viscosity), among others (2–4, 22). The results of this study confirmed that BCAA intake during fixation suppresses muscle fiber atrophy. However, the inhibitory effect on ROM limitation was slight, suggesting that the effects of other factors related to ROM limitation were considerable.

There was no difference in BW between the Con, Fix, and Fix+BCAA groups. On the other hand, MW was reduced by 40% due to fixation and by 20% due to BCAA intake. This was similar to the previous studies (12, 16) in which it was reported that BCAA intake during hindlimb suspension could suppress the decrease in muscle weight. In the present study, the rate of decrease in MW during fixation and the rate of increase in MW due to BCAA intake during fixation were the same as the respective rates of decrease and increase in the CSA of the Type 1 fiber, which occupies most of the soleus muscle. This indicates that the changes in MW reflect changes in the Type 1 fiber, especially in the soleus muscle. Additionally, BCAA intake during fixation resulted in increased myofibril production and suppression of muscle atrophy, which is considered to be caused by the effect on Type 1 fibers in particular.

The results showed that the CSAs of both Type 1 and 2 fibers decreased significantly owing to joint fixation. However, the rate of decrease was more remarkable for the Type 1 fibers. These results are similar to the data we obtained in our previous studies in a rat model of fracture treatment (5). According to Fujino et al. (23), capillary/muscle fiber ratio and capillary diameter decrease during muscle atrophy caused by non-loading. As a result, blood flow to the muscle fibers also decreases. They also reported that the aerobic ATP synthase system has a more significant effect on immobilization than the anaerobic ATP synthase system has. This indicates that muscle atrophy progressed selectively in Type 1 fibers containing a large amount of mitochondria, which are the site for aerobic ATP syn-
thesis, due to decreased blood flow.

We found that there were increases in the CSAs of both Type 1 and 2 fibers in the group that ingested BCAAs during fixation. However, the rate of CSA increase was more significant for the Type 1 fiber. Wang et al. (24) reported that vascular endothelial growth factor is activated via the Akt/mTOR pathway, promoting the formation of capillaries. Thus, we believe that mTOR activated as a result of BCAA intake during fixation may promote the formation of capillaries in the skeletal muscles of the rats and promote the formation of capillaries. Consequently, the Type 1 fibers, which have a high capillary density, were affected by the BCAAs more than the Type 2 fibers were, thus resulting in a more significant inhibitory effect on muscle atrophy.

It is reported that BCAAs promote protein synthesis by directly phosphorylating mTOR and activating the translation factor mTOR complex 2, a component of mTOR, exerts its effect on Akt phosphorylation (27). However, in this study, BCAA intake during fixation did not promote Akt phosphorylation. This suggests that the pathway associated with mTOR-to-Akt phosphorylation might be suppressed by joint fixation.

The rate of mTOR phosphorylation during joint fixation was almost the same as that of Akt. This indicated that the Akt/mTOR pathway was involved and suppressed in the atrophied muscle. However, BCAA intake during fixation promoted mTOR phosphorylation approximately twice as effectively as that in the control group. BCAAs promote protein synthesis by directly activating mTOR without activating upstream signaling factors such as IGF-1 and Akt; therefore, we believe that the same effect was observed in the rats that were subjected to joint fixation in this study.

Takano et al. (8) have reported that the complex formation between N-WASP and nebulin is regulated by the IGF-1/Pi3K/Akt pathway. Signals transmitted from IGF-1 result in Akt phosphorylation. Additionally, GSK3β that is further downstream is phosphorylated and inactivated. As a result, GSK3β suppresses the phosphorylation of the C-terminal of nebulin, which enables binding to N-WASP. In collaboration with the nebulin module with the actin monomeric binding motif, the bound N-WASP induces the formation of actin fibrils from the Z band. This mechanism is thought to be involved in muscle maturation and hypertrophy; however, it has also been suggested that disruption of this mechanism may cause muscle atrophy. In addition, it has been reported that N-WASP is activated by Src tyrosine kinase during muscle regeneration, and that phosphorylated N-WASP is transferred from around the nucleus into myofibrils. Therefore, N-WASP phosphorylation leads to an increase in actin polymerization (28). In this study, N-WASP phosphorylation during joint fixation was approximately the same as that in the control group, but it was significantly increased when BCAAs were ingested during fixation. This result was closer to the result of mTOR phosphorylation than to that of Akt. This suggests that mTOR is directly activated by the action of BCAAs, which might also be involved in the activity of N-WASP. In this study, it was impossible to clarify the effect of BCAA intake on the binding between N-WASP and nebulin during joint fixation. However, we hypothesized that the increased phosphorylation rate of N-WASP and the increase in nebulin level promoted the regeneration of myofibrils.

The IGF-1/Pi3K/Akt pathway also acts on the control of Forkhead box O (FOXO) downstream of this axis. Activated Akt phosphorylates FOXO, which suppresses the expression of muscle proteolytic genes by mediating the export of FOXO out of the nucleus. It is known that during muscle atrophy, the inhibitory phosphorylation of FOXO by Akt is suppressed, and as a result, the expression of ubiquitin ligases, such as muscle atrophy F-box (MAFbx/Atrogin-1) and muscle ring finger 1 (MuRF1), is increased, with subsequent muscle protein degradation (29). Previous studies using rats reported that BCAA intake during hindlimb suspension suppressed the increase in Atrogin-1 and MuRF1 and also suppresses the decrease in CSAs (12). It is considered that the inhibitory effect of BCAA intake during fixation in this study might have acted not only on the muscle protein synthesis system but also on the degradation system.

The structural proteins in skeletal muscles include nebulin, titin (connectin), myosin, and actin. Among these proteins, nebulin, which was the subject of this study, is considered the most sensitive to muscle atrophy (30). The results of the present study showed a positive correlation between the CSAs of muscle fibers and nebulin level. From this, it was clarified that the increase/decrease in the content of structural proteins in skeletal muscle was accompanied by plasticity such as muscle hypertrophy and muscle atrophy.

In conclusion, we used a rat model of joint fixation to investigate the effect of BCAA intake during fixation on the signal factors of the protein synthesis system in this study. We found that muscle atrophy was suppressed as a result of BCAA intake via increased phosphorylation of mTOR; however, the effect on Akt phosphorylation was weak. Furthermore, it was shown that BCAA intake during fixation promoted the phosphorylation of N-WASP and might be involved in the regeneration of myofibrils containing nebulin. Additionally, it was also clarified that the nebulin content has a strong correlation with the CSAs of muscle fibers and is closely related to muscle atrophy.

Authorship
Research conception and design: Akira N and HI; experiments: Akira N, NK, KO and HI; statistical analysis of the data: Akira N and HI; interpretation of the data: Akira N and HI; writing of the manuscript: Akira N, Akiko N and HI.

Disclosure of state of COI
The authors declare no conflicts of interest.
Acknowledgments
This study was partly supported by the 2020 Jobu University Mitsumata Memorial Fund. The authors are grateful for the support received.

REFERENCES
1) Fujita N, Fujimoto T, Tasuki H, Arakawa T, Matsubara T, Miki A. 2009. Influence of muscle length on muscle atrophy in the mouse tibialis anterior and soleus muscles. *Biomed Res* **30**: 39–45.
2) Gomes ARS, Coutinho EL, Franca CN, Polonio J, Salvini TF. 2004. Effect of one stretch a week applied to the immobilized soleus muscle on rat muscle fiber morphology. *Br J Med Biol Res* **37**: 1473–1480.
3) Zhong H, Roy RR, Siengthai B, Edgerton VR. 2005. Effects of inactivity on fiber size and myonuclear number in rat soleus muscle. *J Appl Physiol* **99**: 1494–1499.
4) Thom JM, Thompson MW, Ruell PA, Bryant GJ, Fonda JS, Harmer AR, De Jonge XA, Hunter SK. 2001. Effect of 10-day cast immobilization on sarcoplasmic reticulum calcium regulation in humans. *Acta Physiol Scand* **172**: 141–147.
5) Nishikawa A, Nishio S, Nishikawa A, Imagita H. 2012. The effects of fracture fixation on joint contracture and disuse atrophy in immobilized rat soleus muscle. *Taiyogakukaiga* **61**: 95–101 (in Japanese).
6) Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E, Scrimgeour A, Lawrence JC, Glass DJ, Yancopoulos GD. 2001. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat Cell Biol* **3**: 1014–1019.
7) Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, Yancopoulos GD, Glass DJ. 2001. Mediation of IGF-I-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. *Nat Cell Biol* **3**: 1009–1013.
8) Takanu K, Watanebe-Takanu H, Suet sugu S, Kurita S, Tsujita K, Kimura S, Karatsu T, Takenawa T, Endo T. 2010. Nebulin and N-WASP cooperate to cause IGF-1-induced sarcomeric actin filament formation. *Science* **330**: 1536–1540.
9) Labeit S, Kolmerer B. 1995. The complete primary structure of human nebulin and its correlation to muscle structure. *J Mol Biol* **248**: 308–315.
10) Proud CG. 2007. Signalling to translation: how signal transduction pathways control the protein synthetic machinery. *Biochem J* **403**: 217–234.
11) Kadowaki M, Karim MR, Carpi A, Miotto G. 2006. Adaptive changes in muscle fiber morphology and cell structure. *J Appl Physiol* **98**: 1407–1413.
12) Yu Q, Wu J, Yang H, Chen Y, Liu K. 2016. The mTOR/AP-1/VEGF signaling pathway regulates vascular endothelial cell growth. *Pharmacol Res* **68**: 680–685.
13) Anthony JC, Anthony TG, Layman DK. 1999. Leucine supplementation enhances skeletal muscle recovery in rats following exercise. *J Nutr* **129**: 1102–1106.
14) Lynch CJ. 2001. Role of leucine in the regulation of mTOR by amino acids: revelations from structure-activity studies. *J Nutr* **131**: 861S–868S.
15) Suet sugu S, Takenawa T. 2003. Translocation of N-WASP by nuclear localization and export signals into the nucleus modulates expression of HSP90. *Oncotarget* **7**: 53269–53276.
16) Case N, Thomas J, Sen B, Stynen M, Xie Z, Galior K, Rubin J. 2011. Mechanical regulation of glycosyn thase kinase 3β (GSK3β) in mesenchymal stem cells is dependent on Akt protein serine 473 phosphorylation via mTORC2 protein. *J Biol Chem* **286**: 39450–39456.
17) Sandri M, Sandri C, Gilbert A, Skuruk C, Calabria E, Picard A, Walsh K, Schiaffino S, Lecker SH, Goldberg AL. 2004. Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. *Cell* **117**: 399–412.
18) Weij HH, Chang NC, Chen SP, Geraldine P, Jayakumar T, Fong TH. 2015. Comparative decline of the protein profiles of nebulin in response to denervation in skeletal muscle. *Biochem Biophys Res Commun* **466**: 95–102.