The effects of branched-chain amino acid granules on the accumulation of tissue triglycerides and uncoupling proteins in diet-induced obese mice

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Abstract. It has been demonstrated the involvement of branched-chain amino acids (BCAA) on obesity and related metabolic disorder. We investigated the effects of branched-chain amino acids (BCAA) on obesity and on glucose/fat homeostasis in mice fed on a high-fat (45%) diet. BCAA was dissolved in 0.5% methylcellulose and added to the drinking water (BCAA-treated group). A high-fat diet was provided for 6 weeks and BCAA was given for 2 weeks. The BCAA-treated group gained almost 7% less body weight and had less epididymal adipose tissue (WAT) mass than the control group ($p<0.05$). BCAA supplementation also reduced the hepatic and skeletal muscle triglyceride (TG) concentrations ($p<0.05$). The hepatic levels of PPAR-alpha and uncoupling protein (UCP) 2, and the level of PPAR-alpha and UCP3 in the skeletal muscle were greater in the BCAA-treated group than in the control mice ($p<0.05$). These results demonstrate that the liver and muscle TG concentration are less in BCAA-treated group. BCAA affects PPAR-alpha and UCP expression in muscle and liver tissue.

Key words: BCAA, Tissue triglyceride, Obesity, Adiposity
carnitine palmitoyltransferase (CPT-1) all of which are regulators of lipid oxidation, in each target tissue. The goal of the present study therefore was to confirm the usefulness of BCAA as a therapeutic tool for visceral adiposity and related metabolic disorders.

Materials and Methods

Mice
Mature male mice (C57Bl/6J; KBT Oriental, Fukuoka, Japan; n=8 for each group) were housed in a light, temperature, and humidity-controlled room (12L:12D, 0700-1900 h; 21 ± 1°C; 55 ± 5% relative humidity). The mice were allowed free access to 45% or 60% HF food laboratory food (Research Diets jpn, Tokyo, Japan). All mice were treated in accordance with the Oita University Guidelines for the Care and Use of Laboratory mice.

BCAA preparation and treatment
The ratio of LIVACT BCAA (Ajinomoto Pharm, Tokyo, Japan) is 0.229g isoleucine/g, leucine 0.459g/g and 0.276g/g valine. BCAA was dissolved in 0.5% methylcellulose and added to the drinking water of the mice at a concentration of 0-2% (BCAA-treated group: 20mgBCAA/mL). A control group was also established that was administrated 0.5% methylcellulose only. Each solution was freshly prepared on the day of administration. The dose of BCAA was based on our preliminary results and a previous study. The 45% HF diets are commercially available (Research Diet jpn, Tokyo, Japan).

Mice were selected and divided into two treatment groups (n=8 for each). We assigned the mice by body weight. HF food was provided for six weeks (8-14 weeks of age). BCAA was given as a methylcellulose-BCAA solution at a concentration of 0-2% for 14 consecutive days (12-14 weeks of age), whereas the control mice received vehicle alone. During the BCAA treatment period, the mice were housed individually. The cumulative food and water intake were measured every 24 hours (once daily) during the 14 days of treatment. For the treatment of PPAR-alpha antagonist, MK-886 (Wako Pure Chemicals Inc., Tokyo, Japan) (50microg/kg s.c.) was injected during last 7 days of BCAA treatment. Body weight, tissue histology, and protein expression levels and energy homeostasis were measured in all mice at the end of this treatment period. The daily intake of food and fluid was monitored using a feeding/drinking autoanalyzer system (Sintechno, Fukuoka, Japan). The food and water intake were measured every 24 hours prior to the dark phase (1500). Body weight and serum and tissue variables were measured at the end of the 14-day treatment period.

Serum and tissue measurements
The body fat mass was measured in each mice to assess changes in body fat accumulation. The tissues were removed, weighed, and frozen immediately in liquid nitrogen. Blood was withdrawn from the jugular vein, and the serum was separated and frozen immediately at –20°C until the assay. The mice were killed by decapitation and blood was collected after 8 hours of fasting and before the start of the dark phase (1500). Mice had free access to BCAA containing water during fasting. The levels of serum BCAA (SRL, Tokyo, JAPAN), glucose (Sanwa, Tokyo, Japan), insulin (Morinaga, Tokyo, Japan), TG (E-test kit, Wako, Osaka, Japan), and free fatty acids (FFA) (E-test kit, Wako, Osaka, Japan) were measured using commercial assay kits.

Histological analysis
Epididymal WAT and liver samples were fixed in 10% formalin and embedded in paraffin. The sections were then cut (5 µm) and stained with hematoxylin and eosin to examine the histology of the white adipocytes used in the analysis system (Olympus, Tokyo, Japan). Replicate sections were stained with hematoxylin and eosin for evaluation of WAT and liver. We examined the fat cell size in the BCAA and control groups using a fat cell analyzer (Olympus system, Tokyo, Japan). Multilocular adipocytes in the sections were not counted. The pathologist also evaluated all histological sections in a blinded fashion. In general, WAT and liver were estimated at low power (x100); questionable areas were evaluated at higher magnification (x200 or x400).

Tissue triglycerides
We have examined the tissue triglyceride according to previous studies [15, 16]. Briefly, skeletal muscle (combined soleus muscles) and liver samples (200 mg each) were homogenized for 1 min in 1 mL of buffer (150 mM NaCl, 10 mM Tris, and 0.1% Triton X-100) using a polytron homogenizer (NS-310E; Miero Tech Nichion, Chiba, Japan). The TG content of the samples (0.1mL) was then determined using a commercial kit.
**Western blotting**

Western blotting was performed as described previously [17]. The frozen-tissue preparations were homogenized in sodium dodecyl sulfate (SDS) sample buffer, centrifuged, and boiled. The total protein concentrations in the tissue samples were quantified using the Bradford method [18]. 10μg total protein per sample were separated by SDS-PAGE electrophoresis using a 8% SDS-polyacrylamide gels, and then electrophoretically transferred onto a PVDF membranes (Bio-Rad Laboratories, Richmond, CA). The PVDF membranes were developed with an enhanced chemiluminescence ECL plus western blot detection kit (GE healthcare jpn, Tokyo, JPN). The primary antibody solution consisted of 5 g/L of polyclonal antiserum with specificity for UCP2, UCP3, PPAR-alpha, CD36/FAT, CPT-1, alpha-tubulin (dilution 1/1000; Santa Cruz Biotechnology, Santa Cruz, CA). UCP2, UCP3, PPAR-alpha, and CPT-1 (Santa Cruz Biotechnology) and acyl-CoA oxidase 1 (ACOX1) (abcam) were detected by enhanced chemiluminescence (Amersham Life Sciences, Buckinghamshire, UK) and quantified using the National Institutes of Health imaging software (NIH, Bethesda, MD). The molecular weights of PPAR- alpha, UCP2, UCP3, and CD36 are 58, 33, 35 and 88 kDa, respectively.

**Statistical analysis**

Values in the text are given as the means ± SEM. We employed the student t-test to analyze differences between the two groups. All analyses were conducted with StatView 4.0 (SAS Institute, Cary, NC).

**Results**

**Concentration of BCAA and essential amino acids.**

The serum BCAA concentrations in 2% BCAA-treated mice in the HF group were greater than low-fat controls by 227%, and greater than HF controls (CONT) by almost 149%. Although the levels of other essential amino acids were not found to differ significantly, the trend was lower for all except arginine.

**Body weight and intakes of food, energy, fluid, and BCAA**

Intake of the 45% HF diet did not differ between the groups but weight gain was less in the HF diet group than in the Control group from 0 day through 14 day (Fig.1-A, B). Average daily energy intake did not differ and was 14.0± 1.8KJ/d in the Control group and 13.9± 1.6 KJ/d in the BCAA group (Fig. 1-A). Similar results were observed in body weight even in pair-feed mice. Daily fluid intake also did not differ and was 5.1 ± 0.3mL/d in the Control group and 5.3 ± 0.4mL/d in the BCAA group. Intake of the 60% HF diet did not differ between the groups from 0 day through 14 day (Fig.1-C).

**Serum glucose, insulin, FFA, and TG**

The serum insulin concentration in the BCAA treatment group was lower than in the controls ($p < 0.05$) (Table 1). However, whereas the serum glucose, TG, and FFA concentrations did not differ between the groups ($p > 0.1$ in all cases; Table 1).

**WAT weight and histology**

The epididymal WAT weight was less in BCAA-treated group compared to the non-treated group ($p < 0.05$; Fig. 2-A). The adipocyte size in the BCAA-treated mice was also less than that in the control group mice (Fig. 2-B). The average fat cell diameter in the BCAA-treated group was also less than the control group (BCAA-treated group vs control group: 58±12 vs 79±14 μm, $p<0.01$ vs controls). The expression of PPAR-alpha, but not that of UCP2 or CD36/FAT, was greater in the BCAA-treated group compared with the control group (Fig. 2-C-E).

**Fat accumulation and molecular markers related to lipid mobilization in the liver**

BCAA treatment was found to lower the TG content in the liver ($p<0.05$; Fig. 3-B). The treatment of PPAR-alpha MK-886 partially attenuated the effects of BCAA-induced the reduction of TG accumulation of liver in DIO mice (MK-886-treated group vs control group: 0.52±0.02 vs 0.44±0.03mmol/L, $p<0.05$). Histological examinations revealed reduced levels of fat deposition in the liver after BCAA treatment (Fig. 3-C). In the liver also, the PPAR-alpha, and UCP2 expression levels were greater in the BCAA-treated group compared with the control group ($p<0.05$, Fig. 4). In the liver, PPAR-α target ACOX1 was also greater in the BCAA treatment group than in the controls ($p < 0.05$).
Tissue fat accumulation is less in BCAA-treated group compared with control group. The TG content in the liver and skeletal muscle was also less in BCAA-treated group, and the lowering of fat deposition in these two tissues was confirmed by histological examination. The adiposity of WAT was less in BCAA-treated group, as assessed by changes in tissue weight and the adipocyte morphology. Hence, BCAA may play a protective role. BCAA treatment was observed to significantly lower the TG content in skeletal muscle ($p<0.05$; Fig. 5). The dose dependent effects of BCAA on TG concentrations in muscle were observed in 0%, 0.5%, 1%, 2% (0.21±0.01, 0.20±0.03, 0.18±0.02, 0.11±0.01 mmol/L; Fishers $r$ test: $r=-0.86$, $p<0.01$). In skeletal muscle, the PPAR-alpha, and UCP3 levels were higher in the BCAA-treated group than in the controls ($p<0.05$ in each case; Fig. 5). In addition, PPAR-alpha target ACOX1 was also greater in the BCAA treatment group than in the controls ($p<0.05$). The treatment of PPAR-alpha MK-886 partially attenuated the effects of BCAA-induced the reduction of TG accumulation of muscle in DIO mice (MK-886-treated group vs control group: 0.15±0.01 vs 0.10±0.01 mmol/L, $p<0.05$). BCAA treatment was further found to prevent the accumulation of tissue TGs and to activate tissue-specific differences in lipid oxidation in DIO.

**Discussion**

The most striking finding of our present study is that tissue fat accumulation is less in BCAA-treated group compared with control group. The TG content in the liver and skeletal muscle was also less in BCAA-treated group, and the lowering of fat deposition in these two tissues was confirmed by histological examination. The adiposity of WAT was less in BCAA-treated group, as assessed by changes in tissue weight and the adipocyte morphology. Hence, BCAA may play a protective role.
Fig. 2 The changes of weight, histology, PPAR- alpha, UCP2 and CD36 expression of WAT in control and BCAA-treated DIO mice. (A) WAT weight; (B) histology of WAT; (C) PPAR- alpha expression in WAT; (D) UCP2 expression in WAT; (E) CD36 expression in WAT. CONT; 0.5% methylcellulose alone, BCAA; BCAA treatment with 0.5% methylcellulose. Values are the means ± SEM (n=8) *p<0.05 vs CONT.

Fig. 3 The changes of weight, triglyceride and histology of liver in control and BCAA-treated DIO mice. (A) liver weight; (B) the levels of triglyceride of liver; (C) histology of liver. Values are the means ± SEM (n=8). *p<0.05 vs CONT.
Fig. 4  The levels of CD36, UCP2, PPAR- alpha and CPT-1 expression of liver in control and BCAA-treated DIO mice. (A) CD36 expression in liver; (B) UCP2 expression in liver; (C) PPAR- alpha expression in liver; (D) CPT-1 expression in liver. Representative western blot showing CD36, UCP2, PPAR- alpha and CPT-1 expression (upper panel) in the WAT of CONT and BCAA-treated group. Values are the means ± SEM (n=8). *p<0.05 vs CONT.

Fig. 5  The levels of triglyceride, PPAR- alpha, UCP3, CD36 and CPT-1 expression of skeletal muscle in control and BCAA-treated DIO mice. (A) The levels of triglyceride of skeletal muscle in control and BCAA-treated DIO mice. Values are the means ± SEM (n=8). (A) PPAR- alpha expression in skeletal muscle; (B) UCP3 expression in skeletal muscle; (C) CD36 expression in skeletal muscle; (D) CPT-1 expression in skeletal muscle. Values are the means ± SEM (n=8). *p<0.05 vs CONT.
role against fat accumulation in tissues. The present data also show that the fasting insulin level is less in BCAA-treated groups without producing a change in the glucose level. An increased tissue TG content has been reported to interfere with insulin-stimulated phosphatidylinositol 3-kinase activation and/or subsequent glucose transporter 4 translocation and glucose uptake, leading to insulin resistance [19].

Thus, it is highly probable that BCAA-induced reduction of the TG content in muscle and/or liver may contribute to the improvement of impaired insulin signal transduction in DIO mice.

It is interesting to note from our present results that body weight, the tissue TG content, and also adiposity are less in BCAA-treated group. In a previous study, the activation of PPAR- alpha was found to prevent a HF diet-induced increase in body weight and adipose tissue mass without influencing food intake, and also that insulin resistance was concomitantly improved by the same treatment [20]. In contrast, PPAR- alpha knockout mice have been shown in another report to become obese when fed a HF diet [21]. These findings highlight the importance of our present data on the effects of BCAA on PPAR- alpha and the related molecular parameters for lipid mobilization, including fatty acid oxidation, in skeletal muscle, liver, and WAT [20-23]. Our results further show that the level of PPAR-alpha expression in the BCAA treatment group was greater than the control mice in all of the tissues examined. PPAR-alpha is mainly expressed in muscle and liver, where it regulates various target genes, including those involved in fatty acid oxidation and lipid metabolism and activation of PPAR-alpha accelerates fatty acid oxidation in skeletal muscle and liver tissues [24-26]. The treatment of PPAR-alpha MK-886 partially attenuated the effects of BCAA-induced the reduction of TG accumulation of those tissues in DIO mice. Thus, the results indicated that the activation of PPAR-alpha may have contributed to the reduction of TG accumulation in those tissues.

In our present study also, PPAR- alpha expression in WAT in the BCAA-treated group was higher compared with the controls. The exact function of PPAR-alpha in WAT has not received much attention because its expression level is lower compared with liver and muscle tissues. Nevertheless, our current data and the results of previous studies have identified the expression of PPAR-alpha in WAT and 3T3-L1 adipocytes [27]. The role of WAT PPAR- alpha in fatty acid oxidation is still unclear. One earlier study has found that the activation of PPAR- alpha prevents adipocyte hypertrophy in obese mice [28], and another report has shown that a PPAR-alpha agonist directly accelerated lipolysis in isolated adipocytes [29]. Thus, the BCAA-induced activation of PPAR-alpha in WAT may potentially reduce the adiposity of this tissue, but the underlying mechanism remains unknown.

PPAR-alpha regulates the UCPs, which are mitochondrial membrane transporters involved in the control of energy conversion [30-32]. PPAR-alpha regulates the UCP3 gene in muscle and controls the UCP2 gene in liver, and both of these genes in other tissues [33-35]. Our present data also highlight a tissue-specific effect of BCAA on CD36/FAT expression as well as on the UCPs. Our findings suggest that the oxidation of fatty acids may be activated by BCAA treatment in liver and skeletal muscle. CD36/FAT is not controlled by PPAR-alpha in the liver. The other factors than PPAR-alpha might influence the levels of CD36 in liver. BCAA may regulate triglycerides in muscle, liver, and WAT tissues by affecting PPAR-alpha, UCPs, and CD36/FAT in a tissue-specific manner. It was discovered that several factors are the PPAR-alpha ligands [36-38]. PPAR-alpha activity was shown to be induced by several cytokines and hormones [38, 39]. Thus, it cannot be exclude the possibility that BCAA can indirectly activate PPAR-alpha especially in liver through these factors.

In the present study, ACOX1 both in liver and skeletal muscle in BCAA treatment group were greater than controls. ACOX1 is a peroxisomal enzyme of the fatty acid beta-oxidation pathway, which catalyzes the desaturation of acyl-CoAs to 2-trans-enoyl-CoAs [40]. It donates electrons directly to molecular oxygen, thereby producing hydrogen peroxide. The findings suggested that the oxidation of fatty acids may be activated by BCAA treatment in liver and skeletal muscle. BCAA may regulate the tissue triglyceride of muscle, liver, and WAT tissues by affecting ACOX1, PPAR-alpha, and UCPs, in a tissue-specific manner.

Recently, it was reported that an increased leucine intake decreases the body adiposity in HF diet induced obese mice [41]. This reduction of adiposity was found to be the result of an increased resting energy expenditure since food intake was not decreased [41]. Increasing the leucine intake was further found to prevent HF diet-induced hyperglycemia, which was associated with improved insulin sensitivity. These previous results
indicate that increases in dietary leucine intake substantially decrease diet-induced obesity and hyperglycemia [41]. In addition, elevated BCAA and/or loss of BCAA catabolism in peripheral tissues has been shown to play an important role in regulating energy expenditure [42]. In this regard, BCATm (-/-) mice were found to exhibit elevated plasma BCAA and a decreased body size along with increased energy expenditure and protection from diet-induced obesity [42].

Recent study demonstrated that BCAA supplementation did not regulate the energy expenditure and diet-induced obesity induced with a 60-KJ% fat diet [43]. Consistently, we found in our current experiments that BCAA did not ameliorate diet-induced obesity induced with a 60-KJ% fat diet. Given that BCAA does ameliorate diet-induced obesity associated with a 45-KJ% fat diet, the lipolytic effects of BCAA appear to be sensitive to fat levels in the diet.

Our study has some noteworthy limitations that must be highlighted. First, we are not able to explain the energy expenditure and weight loss in BCAA-treated mice by the changes of UCP2 and UCP3. The marker of energy expenditure may influence on the energy expenditure and weight loss in the present study. In the present study, we cannot determine the levels as physiological or pharmacological levels. In addition, it is possibility that high volume of nitrogen itself or nitrogen unbalance might be able to exert preferable effects.

In summary, BCAA treatment may prevent fat accumulation in muscle, liver, and WAT, accompanied by improved insulin resistance, in DIO mice. Associated changes in PPAR-alpha may also contribute to changes in adiposity in these tissues by affecting fatty acid oxidation and uptake. The results of our present study thus provide new insights into possible therapeutic approaches for obesity-related metabolic disorders.

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References

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