Table 3 The comparison of the rate of sustained virological response of patients with genotype 1 receiving a dose of 80% or more of pegylated interferon α-2b plus 60% or more of ribavirin and the reduced dosage group *n* (%)

	Male		Female		Total	
	n	SVR	*n*	SVR	*n*	SVR
Group A						
Minimum acceptable	168	116 (69.0)	110	69 (62.7)	278	185 (66.5)
Reduced	206	73 (35.4)	201	66 (32.8)	407	139 (34.2)
Total	374	189 (50.5)	311	135 (43.4)	685	324 (47.3)
Group B						
Minimum acceptable	31	15 (48.4)	31	13 (41.9)	62	28 (45.2)
Reduced	91	18 (19.8)	100	12 (12.0)	191	30 (15.7)
Total	122	33 (27.0)	131	25 (19.1)	253	58 (22.9)

Minimum acceptable: patients who received 80% or more of the target dose of pegylated interferon (IFN) α-2b and 60% or more of ribavirin (RBV). Reduced: Patients who received less than 80% of pegylated IFN α-2b and less than 60% of RBV. SVR: Sustained virological response.

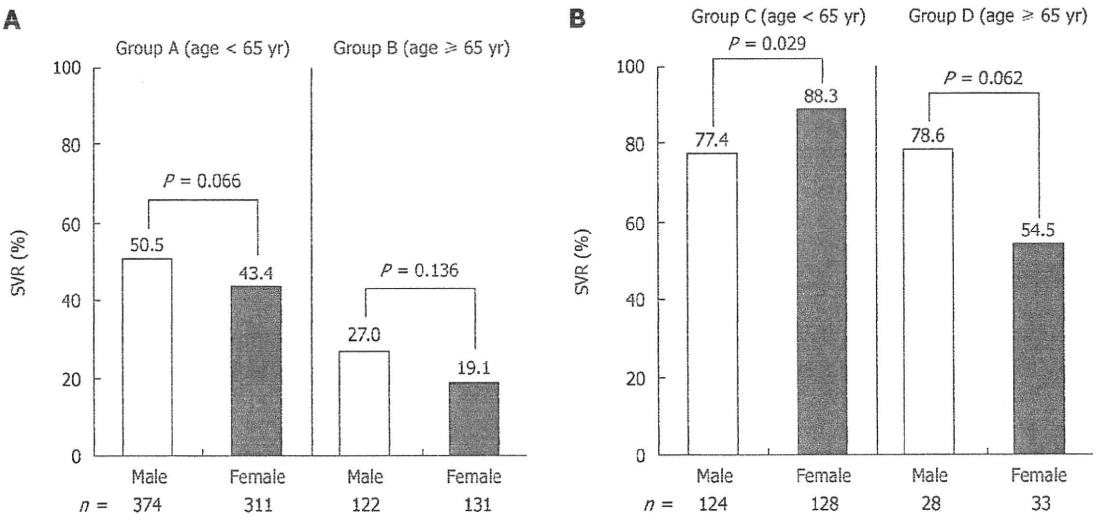

Figure 1 Virological response to the combination treatment by age and sex of patients with genotype 1 (A) and genotype 2 (B). SVR: Sustained virological response.

($P < 0.001$) in group B patients. No significant difference between groups C and D was observed. On comparing patients whose platelet count was under 10×10^{10}/L, the SVR rate for genotype 1 was significantly lower in group B (2 of 36, 5.6%) than in group A (16 of 56, 28.6%) ($P < 0.001$). Among the patients with genotype 2, SVR was not significantly different between group C (9 of 16, 56.3%) and group D (2 of 7, 28.6%).

In a comparison of the SVR rate in patients with or without one or more previous courses of IFN plus RBV, there was no significant difference between the genotypes (genotype 1: 118 of 310, 38.1% *vs* 264 of 628, 42.0%, genotype 2: 44 of 72, 61.1% *vs* 141 of 241, 58.5%). Furthermore, we compared the EOT response rate and SVR rate of cirrhosis patients whose liver fibrosis was F4, and found no significant difference between groups A (EOT: 16 of 30, 53.3%, SVR: 7 of 30, 23.3%) and B (EOT: 6 of 17, 35.3%, SVR: 2 of 17, 11.8%). In addition, no significant difference was found between groups C (EOT: 8 of 10, 80.0%, SVR: 6 of 10, 60.0%) and D (EOT: 9 of 12, 75.0%, SVR: 5 of 12, 41.7%).

Discontinuation of PEG-IFN α-2b plus RBV treatment and adverse effects

Of 1251 patients, 314 (25.1%) did not complete PEG-IFN α-2b plus RBV treatment due to adverse effects or other reasons. The discontinuation rate was significantly higher in patients with genotype 1 (273 of 938, 29.1%) than in those with genotype 2 (41 of 313, 13.1%) ($P < 0.001$) (Tables 4 and 5). Furthermore, the rate of discontinuation due to adverse effects was significantly higher in patients with genotype 1 (135 of 938, 14.4%) than in those with genotype 2 (23 of 313, 7.3%) ($P < 0.010$). The rates of discontinuation due to lack of treatment efficacy and for economic reasons (loss of job, inability to pay the medical costs) were also significantly higher in patients with genotype 1 (55 of 938, 5.9%, 15 of 938, 1.6%) than in those with genotype 2 (1 of 313, 0.3%, 0 of 938, 0%) ($P < 0.001$ and $P = 0.025$, respectively).

For genotype 1 patients, the discontinuation rate was significantly higher in group B (106 of 253, 42.9%) than in group A (167 of 685, 24.4%) ($P < 0.001$), and the rate of discontinuation due to adverse effects was also significantly

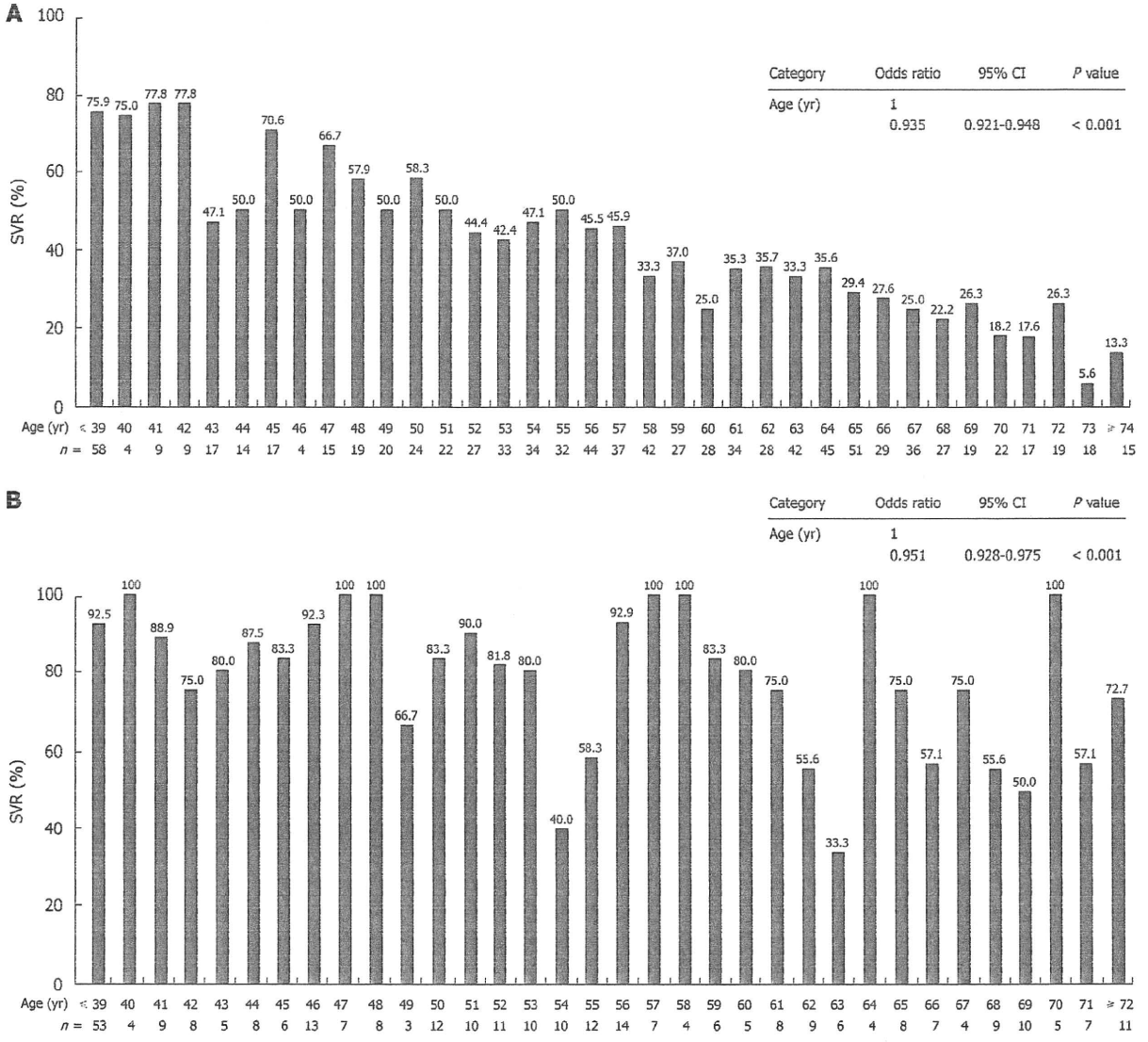

Figure 2 Virological response to the combination treatment by age of patients with genotype 1 (A) and genotype 2 (B). SVR: Sustained virological response; CI: Confidence interval.

higher in group B (61 of 253, 24.1%) than in group A (74 of 685, 10.8%) ($P < 0.001$). General fatigue was the most frequent adverse effect, and was significantly more frequent in group B than in group A ($P < 0.001$). However, in these group 1 patients, RBV was reduced due to anemia in 12.5% (3 of 24) of group A and in 30.4% (7 of 23) of group B. Furthermore, rash and thrombocytopenia were significantly more frequent in group B than in group A ($P = 0.014$ and $P = 0.007$, respectively). In group A, depression was significantly more frequent in females than in males ($P = 0.012$). In genotype 2 patients, treatment discontinuation did not differ between group C (33 of 252, 13.1%) and group D (8 of 61, 13.1%), and the rate of discontinuation due to adverse effects did not differ between these groups (17 of 252, 6.7%, 6 of 61, 9.8%, respectively).

The mean time to discontinuation in group A (21.6 ± 11.9 wk) was not significantly different from group B (21.5 ± 12.6 wk), and the mean time in group C (11.0 ± 6.8 wk) was also not significantly different from group D (11.6 ± 6.0 wk). There was no significant difference between male and female patients in each group (male: 21.0 ± 12.4 *vs* female: 22.1 ± 11.8 in group 1, male: 11.3 ± 7.1 *vs* female: 10.9 ± 6.1 in group 2).

HCC was not seen in genotype 2 patients; only in patients with genotype 1 (29.5 ± 9.9 wk) and was more frequent in group B (5 of 253, 2.0%) than in group A (2 of 685, 0.3%) ($P = 0.008$).

DISCUSSION

In a large, national, multicenter Greek study involving 993 treated and 734 untreated patients with chronic hepatitis C, patients with cirrhosis, showed a protective effect of treatment even among those without SVR. For patients without cirrhosis, the beneficial effect of IFN α treatment was particularly evident in older patients; patients with the worst prognosis if left untreated. Therefore, IFN α-based treatment should be offered to older persons, as these are

Table 4 Reasons for discontinuation of pegylated interferon plus ribavirin treatment by hepatitis C virus genotype 1 patients

	Group A (age < 65 yr)		Group B (age ≥ 65 yr)		Total
	Male (n = 374)	Female (n = 311)	Male (n = 122)	Female (n = 131)	
Discontinued number	101	66	52	54	273
Adverse effects	43	31	33	28	135
General fatigue	17	7	12	11	47
Depression	3	11	4	5	23
Appetite loss	1	0	1	0	2
Rash	3	2	3	4	12
Encephalopathy	1	0	0	0	1
Neutropenia	2	0	0	0	2
Anemia	3	2	4	1	10
Thrombocytopenia	1	0	3	1	5
Elevation of ALT	1	0	0	0	1
Hyperthyroidism	3	2	0	1	6
Hypothyroidism	0	1	0	0	1
Retinopathy	1	0	1	0	2
Interstitial pneumonia	2	0	1	1	4
Pulmonary disease (others)[1]	0	1	1	1	3
Psychoneurotic disorder[2]	2	0	2	0	4
Nervous disease[3]	1	1	0	1	3
Autoimmune disease[4]	0	2	0	1	3
Metabolic disease[5]	0	2	0	0	2
Digestive disorder[6]	2	0	1	1	4
Hepatocellular carcinoma	2	0	4	1	7
Malignancy (extra-liver)	0	1	1	0	2
No effect of treatment	22	18	7	8	55
Economic problem	9	3	0	3	15
Others[7]	25	13	7	14	59

[1]Includes pulmonary tuberculosis (n = 1), pneumonia (n = 1), tuberculous pleuritis (n = 1); [2]Includes psychiatric disorder (n = 2), disquiet (n = 1), insomnia (n = 1); [3]Includes nerve paralysis (n = 1), cerebral infarction (n = 1); [4]Includes rheumatoid arthritis (n = 2), myasthenia gravis (n = 1); [5]Includes diabetes mellitus (n = 1), hypertriglycemia (n = 1); [6]Includes cholecystitis (n = 3), pancreatitis (n = 1); [7]Includes 25, 13, 6 and 13 drop-outs from groups A, B, C and D, respectively: One for excessive alcohol consumption in group C and one was nursing in group D. ALT: Alanine aminotransferase.

the patients with the greatest potential benefit and may achieve SVR[16]. In Japan, the prevalence of chronic HCV infection increases with age, however, the optimal management of older patients has not yet been accurately defined. Whether or not to treat patients older than 65 years with antiviral treatment is highly debated, especially in terms of cost/benefit ratio. In addition, the natural history of chronic hepatitis C in elderly patients is not accurately known, as the presence of comorbidity can affect illness progression and life expectancy. HCV became more prevalent in Japan decades before the United States[17]. Japanese patients with chronic hepatitis C treated with IFN are currently 10 to 15 years older than corresponding patients in the United States and European countries, where patients treated with antiviral treatment tend to average 45 years of age[18-20]. Therefore, our results can serve as a world-wide model for the treatment of older chronic hepatitis C patients.

It has been well documented that the combination therapy of PEG-IFN α-2b plus RBV is more effective than previous IFN monotherapy in chronic hepatitis C patients[7,8]. There have been four studies on the efficacy of PEG-IFN plus RBV therapy in patients 65 years or older with genotype 1, which revealed low rates of SVR (31.1%-51.9%)[21-24]. However, these studies were too small (11-93 patients) for conclusive recommendations to be made. Because the present study was a large multicenter design, it is useful for clarifying the efficacy and safety of PEG-IFN plus RBV combination therapy in older patients. The present study confirmed the results of our previous study which showed that the SVR rate was significantly higher for genotype 2 than for genotype 1 patients[8]. Another important result was that the ability to take at least a minimum acceptable dosage during treatment increased the SVR rate by about three times in older patients with genotype 1. This result also confirmed previous studies which indicated the importance of giving at least the minimum acceptable treatment dosage in patients infected with HCV genotype 1, especially older patients[23,24].

Secondly, we compared discontinuation of treatment by genotype and sex. In genotype 1 patients, adverse effects were seen more often in older than in younger patients. This was the most important reason why the rate of treatment discontinuation was higher in older than in younger patients, and affected the outcome of PEG-IFN α-2b plus RBV combination therapy. General fatigue was the most common adverse effect in older patients. Because older patients often have impaired renal function, they have increased blood levels of RBV[25,26]. They are also inclined to be anemic and to have general fatigue. However, only a small number of older patients in the present study had reduced RBV due to anemia. Therefore, general fatigue is probably a direct adverse effect of PEG-IFN α-2b. We previously reported that herbal medicine

Table 5 Reasons for discontinuation of pegylated interferon plus ribavirin treatment by hepatitis C virus genotype 2 patients

	Group C (age < 65 yr)		Group D (age ≥ 65 yr)		Total
	Male (n = 124)	Female (n = 128)	Male (n = 28)	Female (n = 33)	
Discontinued number	18	15	4	4	41
Adverse effects	6	11	3	3	23
General fatigue	1	3	1	0	5
Depression	0	2	0	0	2
Appetite loss	0	0	0	0	0
Rash	2	1	0	2	5
Encephalopathy	0	0	0	1	1
Neutropenia	0	2	0	0	2
Anemia	0	0	2	0	2
Thrombocytopenia	2	0	0	0	2
Elevation of ALT	0	0	0	0	0
Hyperthyroidism	0	1	0	0	1
Hypothyroidism	0	1	0	0	1
Retinopathy	0	0	0	0	0
Interstitial pneumonia	0	0	0	0	0
Pulmonary disease(others)	0	0	0	0	0
Psychoneurotic disorder	0	0	0	0	0
Nervous disease[1]	1	1	0	0	2
Autoimmune disease	0	0	0	0	0
Metabolic disease	0	0	0	0	0
Digestive disorder	0	0	0	0	0
Hepatocellular carcinoma	0	0	0	0	0
Malignancy (extra-liver)	1	0	0	0	1
No effect of treatment	1	0	0	0	1
Economic problem	0	0	0	0	0
Others[2]	10	4	1	1	16

[1]Includes nerve paralysis (n = 1), tetany (n = 1); [2]All patients were drop out. ALT: Alanine aminotransferase.

relieved the adverse effects of IFN, including general fatigue[27]. Herbal medicine may be useful for mitigating general fatigue during PEG-IFN α-2b plus RBV combination treatment, especially in older patients.

The rate of discontinuation was lower in patients with genotype 2 than in patients with genotype 1, and there was no difference between the older and the younger patients with genotype 2. These results are possibly a consequence of the shorter term of treatment in genotype 2 and the many genotype 1 patients who discontinued due to lack of efficacy.

Two of the characteristics of older patients in the present study were that both hemoglobin and platelet count were significantly lower than in younger patients. The SVR rate was significantly lower when the platelet count was less than 10×10^{10}/L. Furthermore, the older genotype 1 patients were often forced to discontinue treatment due to thrombocytopenia and the occurrence of HCC. These findings appear to result from advanced liver fibrosis in older chronic hepatitis C patients. Therefore, the possibility of HCC during long-term IFN treatment in older patients must be considered.

We previously reported that older female patients had a low response to IFN-α monotherapy[9], and other investigators have reported that older female patients have a poor response to PEG-IFN α-2b plus RBV[22,28]. Although our data showed that sex was not related to SVR, the reason for this finding was not fully elucidated. In any case, studies have conclusively shown that it is important to begin treatment with PEG-IFN α-2b plus RBV combination therapy as soon as possible. Our data suggest that age may be a more important factor than sex for increasing the rate of SVR. Resistance to treatment in older patients may be due to IFN-immunomodulation, advanced liver fibrosis, or reduced dosage.

To maximize adherence to the optimal treatment regimen, the treatment schedule can be modified or other therapeutic modalities added, such as hematopoietic growth factors[29] or the new thrombopoietin-receptor agonist, eltrombopag, for the antiviral treatment of older patients with chronic hepatitis C[30]. A further individualized treatment protocol based on viral kinetics might be more practical[31].

In conclusion, PEG-IFN α-2b plus RBV treatment was effective in the treatment of older chronic hepatitis C patients when they received at least the minimum acceptable treatment dosage. However, there were frequent adverse effects and treatment discontinuation. It is necessary to control for adverse effects that might interrupt treatment and to begin this combination therapy as soon as possible, especially in older patients.

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COMMENTS

Background

Whether or not to treat patients older than 65 years with antiviral treatment is highly debated, especially in terms of cost/benefit ratio. However, there is little data concerning the response and safety of combination treatment for a large number of older patients with chronic hepatitis C virus infection. Therefore, in an attempt to ameliorate these problems, the authors decided to treat older patients with pegylated interferon (PEG-IFN) α-2b plus ribavirin (RBV) combination therapy.

Research frontiers

The combination treatment of PEG-IFN α-2b plus RBV improved the sustained virological response rate in chronic hepatitis C patients. However, the current issue is whether or not to treat older patients because of low response and high dropout rate.

Innovations and breakthroughs

There have been four studies on the efficacy of PEG-IFN plus RBV therapy in patients 65 years or older with genotype 1. However, these studies were too small (11-93 patients) for conclusive recommendations to be made. This study is very useful for clarifying the efficacy and safety of PEG-IFN plus RBV combination therapy in older patients, because of its large scale, multicenter design.

Applications

The study demonstrated that PEG-IFN α-2b plus RBV treatment was effective in chronic hepatitis C patients 65 years or older who completed treatment with at least the minimum required treatment dosage. Furthermore, this study suggested that the combination treatment and beginning this therapy as soon as possible are important, especially in older patients.

Peer review

The study has been well conducted and includes a large number of patients. Results have been described in a lucid and informative manner and are of clinical relevance.

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Isoleucine Prevents the Accumulation of Tissue Triglycerides and Upregulates the Expression of PPARα and Uncoupling Protein in Diet-Induced Obese Mice[1–3]

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Abstract

In this study, we investigated the effects of the branched-chain amino acid L-isoleucine (Ile) on both obesity and glucose/fat homeostasis in mice that were fed a high-fat (45% energy) diet. The mice were divided into different treatment groups and given a high-fat diet for 6 wk. During the last 4 wk, Ile was dissolved and added to the drinking water to a final concentration of 2.5%. The control mice received vehicle alone. The mice in the Ile group had an almost 6% lower body weight gain and 49% less epididymal white adipose tissue (WAT) mass with the control group ($P < 0.05$). The hepatic and skeletal muscle triglyceride (TG) concentrations and degree of hyperinsulinemia in the Ile group mice were also lower than the control group by 38, 47, and 39%, respectively ($P < 0.05$). The WAT leptin concentration was also lower, whereas that of adiponectin was higher, in the Ile group compared with the control group ($P < 0.05$). The hepatic levels of protein CD36/fatty acid translocase, PPARα, and uncoupling protein (UCP) 2 and the levels of UCP3 in skeletal muscle were all greater in the Ile group than in the control mice ($P < 0.05$). These results demonstrate that the liver and muscle TG concentrations are both lowered by Ile treatment. In addition, the PPARα and UCP expression levels in the mouse tissues were greater in the Ile group compared with the controls. Our current data thus suggest that supplementation with Ile might be useful in the treatment of metabolic syndrome. J. Nutr. 140: 496–500, 2010.

Introduction

Obesity and its related metabolic disorders such as diabetes are now major global health problems. The importance of low-carbohydrate, low-fat, and relatively high-protein diets, which include branched chain amino acids (BCAA),[4] has been recognized as an effective strategy to reduce the incidence of these diseases (1–8). BCAA, most notably leucine, are thought to play a key role in regulating glucose and fat homeostasis (8–11). It has been shown also that leucine stimulates the glucose uptake in isolated soleus muscles under insulin-free conditions (12,13), whereas glycogen synthesis in myoblasts is activated by the mammalian target of rapamycin (mTOR) (14,15). In addition, a further report has demonstrated that during energy restriction, leucine supplementation results in enhanced fat loss and improved hepatic and muscle protein synthesis (16).

Isoleucine (Ile), a positional isomer of leucine, is an essential BCAA that suppresses the plasma glucose concentration in rats in a more potent manner than leucine (17,18). In addition, Ile is a potent activator of the glucose transporter (17,18). These findings suggest that Ile functions as a regulator of glucose homeostasis, but it remains unclear how this amino acid affects the development of diet-induced obesity (DIO) and obesity-related metabolic disorders. In this study, we employed a DIO mouse model to investigate the effects of Ile on food intake; body weight changes; adiposity in white adipose tissue (WAT); serum glucose, insulin, and triglyceride (TG) levels; liver and skeletal muscle TG content; WAT adiponectin and leptin levels; liver and skeletal muscle PPARα and CD36 fatty acid translocase (CD36/FAT); and uncoupling protein (UCP) in the liver and skeletal muscle. We performed these analyses to evaluate the usefulness of Ile as a novel therapeutic tool for visceral obesity and related metabolic disorders.

Materials and Methods

Mice. Mature male mice (C57Bl/6J; KBT Oriental; $n = 8$ for each group) were housed in a light-, temperature-, and humidity-controlled room (12 h light:12 h dark, 0700–1900 h; 21 ± 1°C; 55 ± 5% relative

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[3] Supplemental Figure 1 is available with the online posting of this paper at jn.nutrition.org.
[4] Abbreviations used: BAT, brown adipose tissue; BCAA, branched-chain amino acid; CD36/FAT, CD36 fatty acid translocase; DIO, diet-induced obesity; ITT, insulin tolerance test; mTOR, mammalian target of rapamycin; TG, triglyceride; UCP, uncoupling protein; WAT, white adipose tissue.
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humidity). Mice were allowed free access to 45% high-fat food (Research Diets). The ingredients and the nutrient characteristics of this diet are shown in **Table 1.** All mice were treated in accordance with the Oita University Guidelines for the Care and Use of Laboratory Mice.

Isoleucine preparation. L-Ile (Wako) was dissolved in 0.5% methylcellulose and added to the drinking water of the mice at a final concentration of 2.5% Ile (wt:v) in 0.5% (v:v) methylcellulose. The control mice received the vehicle alone (0.5% methylcellulose). All solutions were prepared freshly each day.

Chronic Ile treatment. Mice were selected and divided into different treatment groups and provided with a high-fat diet during a 6-wk period. For the first 2 wk, the mice received the high-fat diet and vehicle. L-Ile or vehicle control was administrated for the subsequent 4 wk as detailed above. The dose of Ile used was based on our preliminary results and a previous study (11).

During the Ile treatment period, the mice were housed individually and the daily intake of food and fluid was monitored using a feeding/drinking autoanalyzer system (Sintechno) every 24 h prior to the dark phase (at 1500 each day). Body weight and serum and tissue variables were measured at the end of the 28-d treatment period. For the insulin tolerance test (ITT), the mice were administered 0.5 mU regular insulin/g body weight by intraperitoneal injection (Nobolin R; Novo Nordisk). Blood was sampled from the tail vein prior to and at 15, 30, 60, and 90 min after these injections and the serum glucose concentrations were then determined.

Serum and tissue measurements. The body fat mass was measured in each mouse to assess changes in body fat accumulation. Briefly, 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 use in the assay. The mice were killed by decapitation and the blood collected before the dark phase (at 1500). The levels of serum glucose (Sanwa), insulin (Morinaga), TG (E-test kit, Wako), and FFA (E-test kit, Wako) were measured using commercial assay kits. The leptin (MBL) and adiponectin concentrations in the WAT were measured using a sandwich enzyme immunoassay kit according to the manufacturer's instructions (Otsuka Pharm).

Histological analysis. Epididymal WAT samples were fixed in 10% formalin and embedded in paraffin. The sections were cut (5 μm) and stained with hematoxylin and eosin to examine the histology of the white adipocytes used in the analysis (Olympus).

Tissue TG. Skeletal muscle and liver samples (100 mg each) were homogenized for 1 min in 2 mL of a buffer containing 150 mmol/L NaCl, 10 mmol/L Tris, and 0.1% Triton ×100 using a Polytron homogenizer (NS-310E; Micro Tech Nichion). The TG level of the samples (0.1 mL) was determined using a commercial kit (Triglyceride E-test kit, Wako).

Western blotting. Western blotting was performed as described previously (19). Briefly, the frozen-tissue preparations were homogenized with SDS sample buffer, centrifuged, and then boiled for 3 min. The total protein concentrations of the tissue samples were quantified using the Bradford method (20). Equal amounts of total protein were electrophoresed on 8% SDS-polyacrylamide gels and then transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories).

The membranes were blocked with 5% nonfat milk for 1 h, incubated overnight with primary antibodies at 4°C, and then incubated with the secondary antibody for 1 h at room temperature. The primary antibody solution consisted of 5 g/L of polyclonal antiserum with specificity for UCP2, UCP3, PPARα, and CD36/FAT (Santa Cruz Biotechnology). UCP2, UCP3, PPARα, and CD36/FAT were detected by enhanced chemiluminescence (Amersham Life Sciences) and quantified using the NIH imaging software. The molecular weights of Acrp30, Ob, PPARα, UCP2, UCP3, and CD36 were 30, 16, 58, 33, and 88 kDa, respectively.

Statistical analysis. Values in the text are given as the means ± SEM. We employed the Mann-Whitney U-test to analyze differences between the 2 groups. All analyses were conducted with StatView 4.0 (SAS Institute).

TABLE 1 Ingredient and nutrient values of the high-fat diet used in this study

Ingredient	Concentration, %
Casein, 80 mesh	23.31
L-Cystine	0.350
Corn starch	8.48
Maltodextrin 10	11.65
Sucrose	20.14
Cellulose, BW200	5.83
Soybean oil	2.91
Lard	20.68
Mineral mix S10026[1]	1.17
Dicalcium phosphate	1.51
Calcium carbonate	0.641
Potassium citrate, 1 H_2O	1.92
Vitamin mix V10001[2]	1.17
Choline bitartrate	0.233

[1] Containing the following (g/kg mineral mix): magnesium oxide, 41.9; magnesium sulfate·7H_2O, 257.6; sodium chloride, 259; chromium KSO_4·12H_2O, 1.925; cupric carbonate, 1.05; potassium iodate, 0.035; ferric citrate, 21; manganous carbonate, 12.25; sodium selenite, 0.035; zinc carbonate, 5.6; sodium fluoride, 0.20; ammonium molybdate·4H_2O, 0.30; sucrose, 399.105. Sucrose in the mineral mix provided 63 kJ energy/kg diet.

[2] Containing the following (g/kg vitamin mix): retinyl palmitate, 0.80; cholecalciferol, 1.0; all-rac-α-tocopheryl acetate, 10; menadione sodium bisulfite, 0.08; biotin (1.0%), 2.0; cyancocobalamin (0.1%), 1.0; folic acid, 0.20; nicotinic acid, 3.0; calcium pantothenate, 1.6; pyridoxine-HCl, 0.70; riboflavin, 0.60; thiamin-HCl, 0.60; and sucrose, 978.42. Sucrose in the vitamin mix provided 159 kJ energy/kg diet.

Results

Measurement of body weight and intake of food, energy, fluid, and Ile. The intake of the high-fat diet did not differ between the 2 study groups, but the weight gain was less in the Ile group than in the control group from d 17 over the 28-d treatment period (**Fig. 1**). The daily energy intake did not

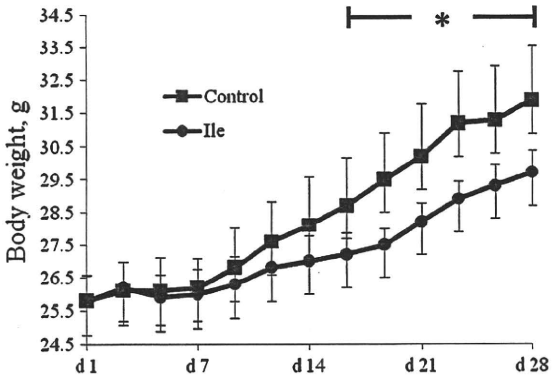

FIGURE 1 Body weight changes in control and Ile-treated DIO mice. Values are means ± SEM, n = 8.*Groups differed at these times, $P < 0.05$.

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TABLE 2 Serum glucose, insulin, TG, and FFA concentrations and the ITT area under the curve of control and Ile-treated DIO mice[1]

	Glucose	Insulin	TG	FFA	ITT area under the curve
	mmol/L	*pmol/L*	*mmol/L*	*mmol/L*	*mmol/L·h*
Control	17.3 ± 0.6	44.8 ± 5.2	0.85 ± 0.05	0.75 ± 0.05	17.25 ± 0.7
Ile	16.3 ± 0.9	27.6 ± 6.9*	0.81 ± 0.07	0.71 ± 0.03	16.09 ± 0.5*

[1] Values are means ± SEM, $n = 8$. *Different from control, $P < 0.05$.

significantly differ between the groups (56.8 ± 5.8 kJ/d in the control group and 55.2 ± 7.1 kJ/d in the Ile group) nor did the daily fluid intake (5.1 ± 0.1mL/d in the control group and 5.3 ± 0.2mL /d in the Ile group).

Serum glucose, insulin, FFA, TG, and ITT levels. The serum insulin concentration in the Ile treatment group was less than in the controls ($P < 0.05$) (**Table 2**), whereas the serum glucose, TG, and FFA concentrations did not differ between the groups ($P > 0.1$ in all cases; Table 2). The ITT values further showed that the hypoglycemic response was exaggerated in the Ile-treated mice compared with the controls (Table 2; $P < 0.05$).

WAT weights. The epididymal WAT weights were lower in the Ile-treated group ($P < 0.05$; **Table 3**) and the adipocyte sizes in the Ile-treated mice were also less than those in the controls (**Supplemental Fig. 1**).

Leptin, adiponectin, PPARα, CD36/FAT, and UCP 2 levels in the WAT. The WAT leptin levels were lower, whereas the WAT adiponectin levels were higher, in the Ile group compared with the control group ($P < 0.05$; Table 3). However, the WAT CD36/FAT, PPARα, and UCP2 levels did not differ between the groups (Table 3).

Fat accumulation and molecular markers related to lipid mobilization in the liver. Ile treatment lowered the TG level in the liver ($P < 0.05$; Table 3). The hepatic fat deposition in the Ile group was also lower than the control group (Supplemental Fig. 1). In the liver also, the CD36/FAT, PPARα, and UCP2 levels were greater in the Ile-treated mice ($P < 0.05$ in each assay; Table 3).

Fat accumulation and molecular markers related to lipid mobilization in the skeletal muscle. The Ile-treated group had a lower TG level than the control group in the skeletal muscle ($P < 0.05$; Table 3), whereas CD36/FAT, PPARα, and UCP3 levels were higher ($P < 0.05$; Table 3). Ile dietary supplements prevented the accumulation of tissue TG and activated tissue-specific lipid oxidation in DIO mice.

Discussion

Leucine, Ile, and valine are essential amino acids for normal growth and development. Leucine appears to modulate insulin signaling and glucose utilization in skeletal muscle and plays an important role in mTOR signal transduction (14,15). In addition, the regulation of these fuel-sensing mTOR mechanisms in the brain may contribute to the metabolic dysregulation that underlies diseases such as type 2 diabetes (21).

In support of this hypothesis, a previous report has demonstrated that increases in dietary leucine substantially alleviate the onset of obesity, hyperglycemia, and hyperinsulinemia in high-fat DIO mice (22). Hence, leucine may function to promote body fat loss while decreasing insulin resistance.

The results of our current study in mice demonstrate a possible role for Ile in the regulation of adiposity and glucose homeostasis. Our data reveal that the weight of the WAT in our Ile group is less than in the control group and that this is accompanied by an improvement in the onset of hyperinsulinemia in the DIO mice. In addition, the ITT levels showed that the hypoglycemic response was exaggerated in Ile-treated mice compared with the controls. Previous studies using a glucose tolerance test in rats found that Ile induces a more beneficial effect upon glucose metabolism than either leucine or valine and also has positive effects on insulin-stimulated glucose uptake in C2C12 myotubes (17,18).

These previous findings indicate that PI3K or PKC signaling is involved in the enhancement of glucose uptake by Ile. Another recent study has further demonstrated the beneficial effects of Ile on glucose homeostasis in DIO mice (23). In our present analysis, we further examined the effects of Ile on glucose and lipid metabolism in mice, including the tissue levels of leptin, adiponectin, and PPARα.

We found that Ile dietary supplementation induced differences in the levels of adiponectin and leptin in the WAT, possibly due to a reduction in WAT adiposity. In addition, our results indicate that the adiposity of liver and skeletal muscle in the Ile group was less than in the control group, indicating that the TG levels in both tissues are lowered by Ile treatment. Given that adiponectin acts as an insulin-sensitizing hormone (24), the

TABLE 3 Leptin, adiponectin, PPARα, UCP, and CD36/FAT levels in WAT, liver, and muscle in control and Ile-treated DIO mice[1]

	Control	Ile
WAT		
Weight, *g*	1.2 ± 0.2	0.6 ± 0.1*
Leptin,[2] *rau*	23.2 ± 0.7	10.2 ± 1.0*
Adiponectin, *rau*	13.9 ± 1.0	19.4 ± 2.6*
PPARα, *rau*	18.5 ± 2.6	14.9 ± 0.6
UCP2, *rau*	15.7 ± 0.8	17.7 ± 1.1
CD36, *rau*	16.0 ± 1.8	17.4 ± 1.7
Liver		
TG, *mmol/g protein*	0.48 ± 0.05	0.29 ± 0.03*
PPARα, *rau*	6.5 ± 0.7	26.8 ± 4.5*
UCP2, *rau*	10.1 ± 0.8	23.2 ± 4.0*
CD36, *rau*	8.7 ± 0.6	24.6 ± 4.2*
Muscle		
TG, *mmol/g protein*	0.25 ± 0.01	0.13 ± 0.01*
PPARα, *rau*	12.9 ± 1.1	20.4 ± 1.0*
UCP3, *rau*	6.0 ± 2.3	27.3 ± 2.4*
CD36, *rau*	12.7 ± 0.7	20.7 ± 1.3*

[1] Values are means ± SEM, $n = 8$. *Different from control, $P < 0.05$.
[2] rau, Relative arbitrary unit.

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removal of TG from the liver and skeletal muscle may also contribute to the improvement in the impaired insulin sensitivity induced by lipotoxicity (25). Taken together, our data suggest that these factors may restore hyperinsulinemia by improving insulin resistance.

Ile treatment was further found to reduce the levels of adiposity in each analyzed tissue without affecting food intake of the DIO mice, which suggests that this amino acid is involved in energy metabolism or lipid mobilization. Because the brown adipose tissue (BAT) UCP1 gene plays an important role in energy expenditure (26–28), it is a very likely target for Ile. However, our unpublished results additionally show that Ile treatment does not affect BAT UCP1 expression. This implies that a mechanism other than the regulation of food intake and BAT energy expenditure contributes to the reduction in tissue adiposity we observed.

The levels of PPARα expression in the liver and skeletal muscle of the Ile-treated mice were higher than in the controls. Given that the activation of PPARα in these tissues accelerates FFA oxidation (29), we speculate that Ile reduces tissue TG accumulation by affecting fatty acid oxidation. In this regard, a previous study has demonstrated that PPARα activation prevents the development of DIO and insulin resistance without influencing food intake (30). In contrast, PPARα-knockout mice become obese when fed a high-fat diet (31).

The levels of liver UCP2 and muscle UCP3 were greater after Ile treatment than in the controls. UCP is mitochondrial membrane transporter and is regulated by PPAR subtypes, i.e. the UCP2 and UCP3 genes are regulated by PPARα in liver and muscle, respectively (32,33). Our results thus suggest that the Ile-induced UCP expression in both tissues is mediated by PPARα.

Recently, UCP2 and UCP3 were reported to play a physiological role in the control of reactive oxygen production or FFA oxidation rather than in the regulation of energy expenditure through thermogenesis (34,35). If this is correct, we hypothesize that the upregulation of muscle UCP3 and hepatic UCP2 may also contribute to the activation of FFA oxidation in the tissues.

The levels of FAT/CD36, a key protein regulator of FFA uptake in skeletal muscle and liver, were greater in the Ile treatment group than in the control mice. Although FFA transporters other than FAT/CD36 have been shown to function in the liver, the Ile-induced differences in skeletal muscle and liver FAT/CD36 levels that we observed indicate that FFA uptake may be accelerated in muscle and liver but not in WAT (36). Taken together, our present findings suggest that Ile simultaneously activates liver and skeletal muscle FFA uptake and oxidation.

It must be noted that various, and as yet unidentified, mediators may play roles in regulating fat and energy metabolism and further research is needed to address the detailed signaling mechanisms of Ile in these processes.

Our current data indicate, however, that Ile may prevent the development of visceral obesity and hyperinsulinemia by affecting WAT, liver, and muscle adiposity in DIO mice. Our results thus provide new insights into possible future therapeutic approaches to the treatment of metabolic syndrome.

Acknowledgments

J.N., M.S., and T.M. designed research; J.N. conducted research; M.A. analyzed data; J.N., T.M., and H.Y. wrote the paper; and H.Y. had primary responsibility for final content. All authors read and approved the final manuscript.

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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

ACCUMULATING evidence now indicates that a substantial role of amino acid metabolism in obesity and development of insulin resistance [1, 2]. Protein sparing during weight loss was previously shown in a study of high-protein diets to induce rapid weight loss [3, 4]. These same studies also found that high-protein diets reduce urinary nitrogen loss, and concluded that a high protein intake is beneficial in minimizing of the loss lean body mass during weight loss treatments for obesity [3, 4]. Similar studies have also indicated that diets with reduced ratios of carbohydrate to protein accelerate the reduction of body weight as well as fat tissue [5, 6]. In addition, the beneficial effects of a high-protein diet have been observed not only in terms of the prevention of muscle protein loss but also for glycemic control [5-9].

In general, branched-chain amino acid was developed for the purpose of improving hypo-albuminemia in patients with uncompensated liver cirrhosis [10]. In addition, previous study has also demonstrated however that this BCAA preparation produces improvements in glucose metabolic disorders [11]. Although several studies have also now shown that BCAA improves glucose metabolism through its actions on skeletal muscle [12-14], the effects of BCAA on metabolic disorders have not yet been fully elucidated. To further explore these mechanisms in our current study, we employed a diet-induced obese (DIO) diabetic mouse model and investigated the effects of BCAA on the following: food intake; body weight changes; serum metabolic parameters such as glucose, insulin, and triglyceride (TG); tissue TG content in the liver and skeletal muscle; adiposity in white adipose tissue (WAT); and the expression of PPAR-alpha, uncoupling proteins (UCPs), and

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Abbreviations: ACOX1 acyl-CoA oxidase 1 (ACOX1): BCAA, branched-chain amino acids, BCAA-treated group: 0.5% methylcelluose with BCAA treated 45% high-fat diet group, CD36/FAT, CD36 fatty-acid translocase; Control group: 0.5% methylcelluose treated 45% high-fat diet group; DIO, diet-induced obesity; FFA; free fatty acid, HF, high-fat; %E, percent of energy; PPAR, peroxisome proliferator-activated receptor; rau: relative arbitrary unit, TG, triglyceride; UCP, uncoupling protein: WAT, white adipose tissue

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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:*ie* 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; Micro Tech Nichion, Chiba, Japan). The TG content of the samples (0.1mL) was then determined using a commercial kit

(Triglyceride E-test kit, Wako, Osaka, Japan).

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). Interestingly, the results of BCAA on body weight were not observed in 60% high-fat diet (Fig. 1-D).

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 ($p < 0.05$).

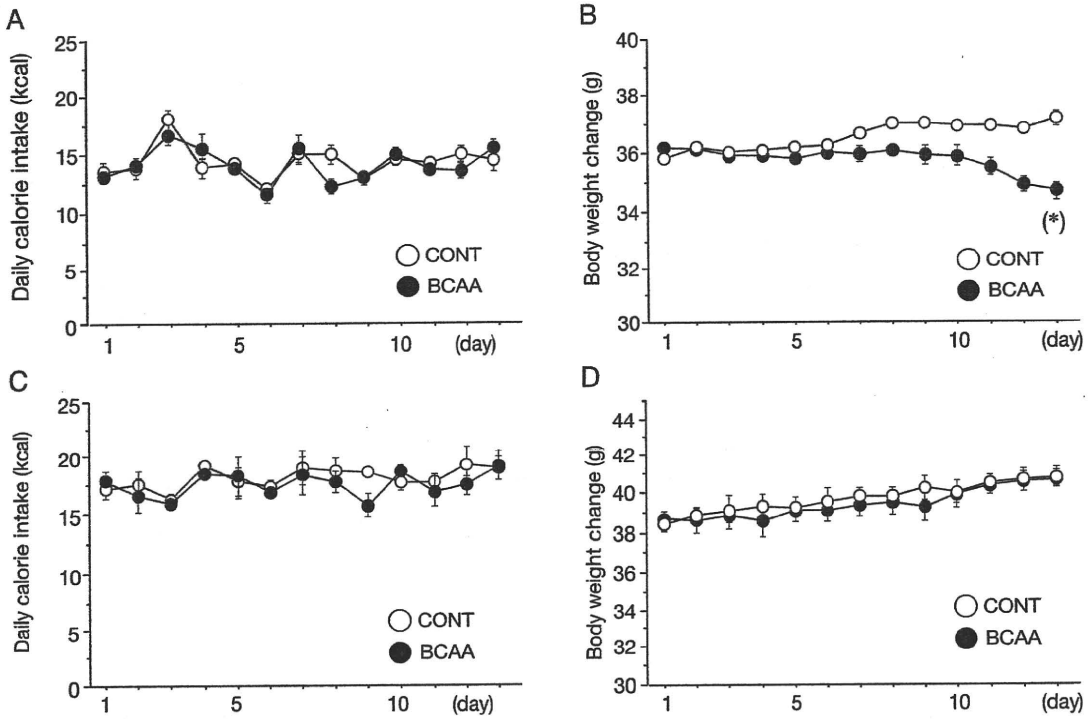

Fig. 1 The change of energy intake and body weight in control and BCAA-treated DIO mice. (A) Energy intake in the 45% HF diet (B) Body weight in the 45% HF diet (C) Energy intake in the 60% HF diet (D) Body weight in the 60% HF diet Values are the means ± SEM (n=8) *$p<0.05$ *vs* CONT.

Table 1 The levels of serum glucose, serum insulin, triglyceride (TG), free fatty acid (FFA) by BCAA treatment.

	Glucose (mmol/L)	Insulin (pmol/L)	TG (mmol/L)	FFA (mmol/L)
CONT	13.7±1.2	28.8±6.9	0.43±0.02	0.44±0.01
BCAA	13.6±1.8	20.2±2.6*	0.40±0.02	0.46±0.01

*Significant difference at $p<0.05$ *versus* CONT; TG: triglyceride. FFA: free fatty acid

TG content and molecular markers related to lipid mobilization in the skeletal muscle

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

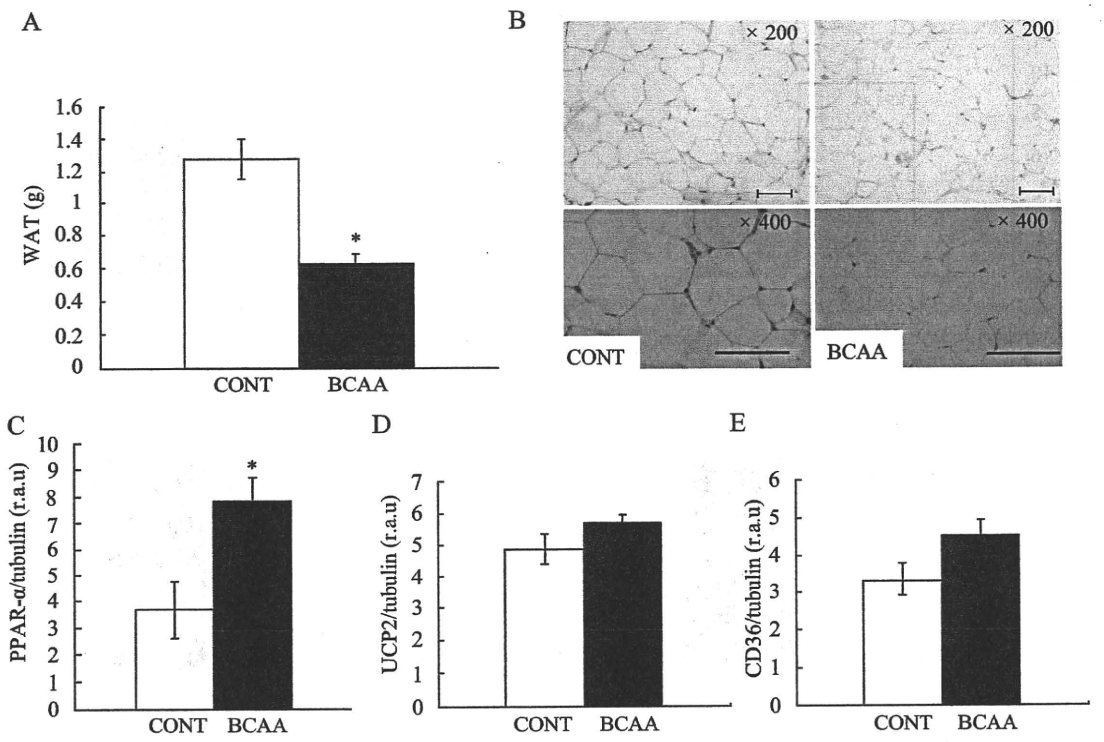

Fig. 2 The changes of weight, histology, PPAR- alpha, UCP2 and CD36 expression of WAT in control and BCAA-treated DIO mice. (A) WAT weight; (B) histology of WAT; (C) PPAR- alpha expression in WAT; (D) UCP2 expression in WAT; (E) CD36 expression in WAT. CONT; 0.5% methylcellulose alone, BCAA; BCAA treatment with 0.5% methylcellulose. Values are the means ± SEM (n=8) *$p<0.05$ *vs* CONT.

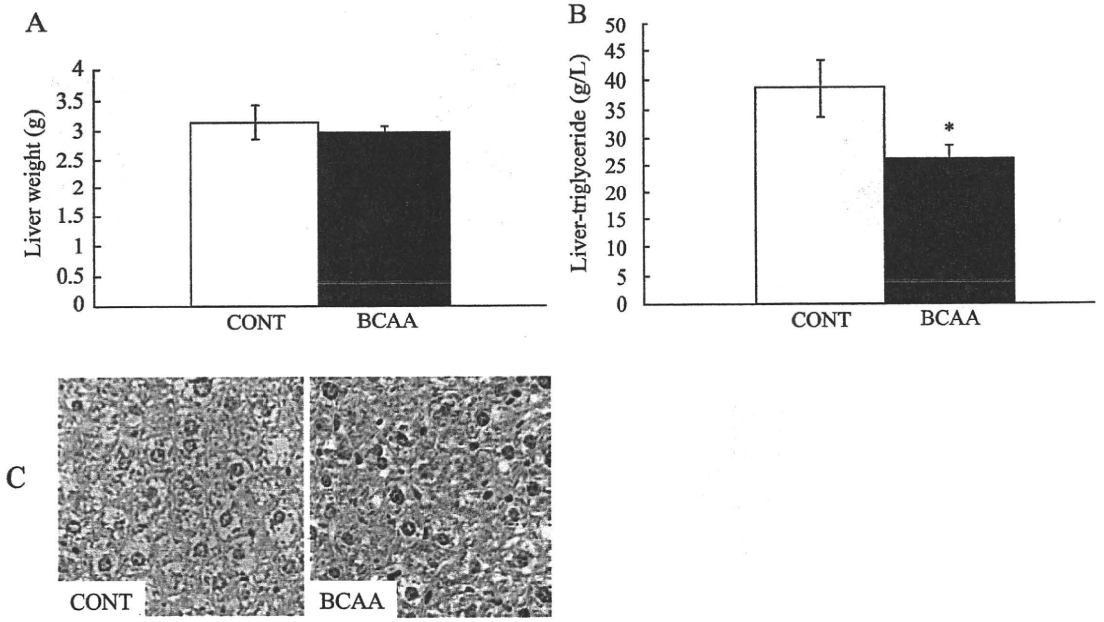

Fig. 3 The changes of weight, triglyceride and histology of liver in control and BCAA-treated DIO mice. (A) liver weight; (B) the levels of triglyceride of liver; (C) histology of liver. Values are the means ± SEM (n=8). *$p<0.05$ *vs* CONT.

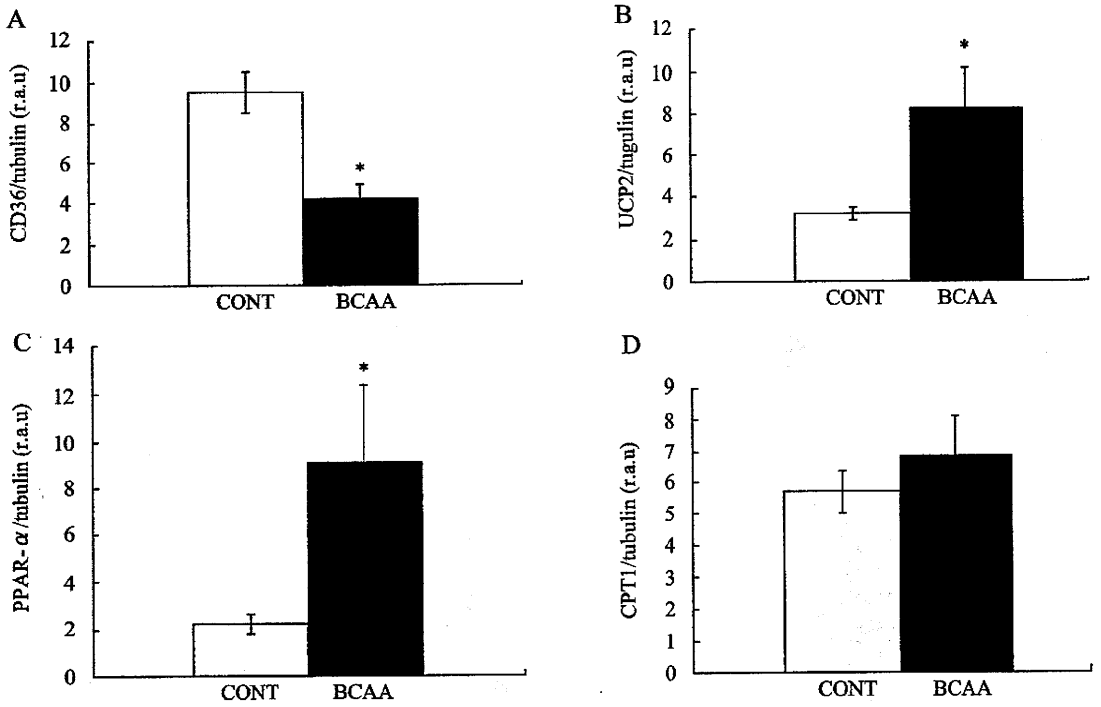

Fig. 4 The levels of CD36, UCP2, PPAR- alpha and CPT-1 expression of liver in control and BCAA-treated DIO mice. (A) CD36 expression in liver; (B) UCP2 expression in liver; (C) PPAR- alpha expression in liver; (D) CPT-1 expression in liver. Representative western blot showing CD36, UCP2, PPAR- alpha 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.

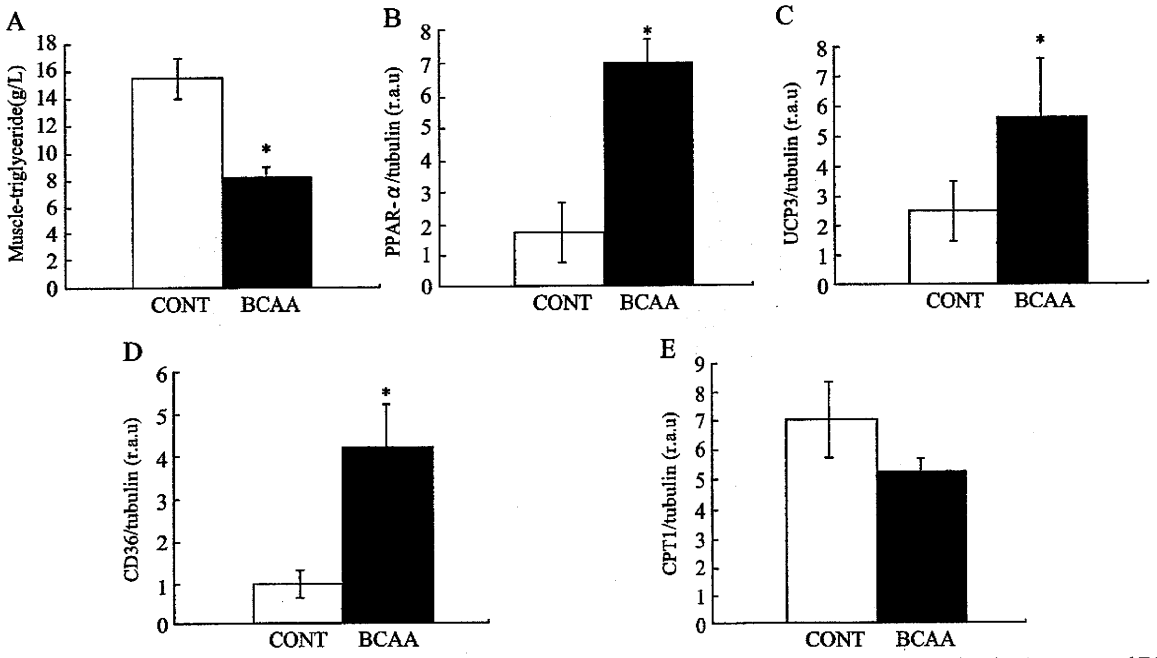

Fig. 5 The levels of triglyceridePPAR- alpha, UCP3, CD36 and CPT-1 expression of skeletal muscle in control and BCAA-treated DIO mice. (A) The levels of triglyceride of skeletal muscle in control and BCAA-treated DIO mice. Values are the means ± SEM (n=8). (A) PPAR- alpha expression in skeletal muscle; (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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