Note

High Incidence of Lipid Deposition in the Liver of Rats Fed a Diet Supplemented with Branched-Chain Amino Acids under Vitamin B₆ Deficiency

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Summary Male Wistar rats were fed four diets composed of purified 20% vitamin-free casein diet with (+) or without (–) vitamin B₆ (7.0 mg of pyridoxine HCl/kg of diet) and with (+) or without (–) branched-chain amino acids (BCAAs) of valine, leucine, and isoleucine (4.75%): B₆(+)BCAA(–); B₆(+)BCAA(+); B₆(–)BCAA(–); and B₆(–)BCAA(+) for 21 d. Among rats fed the B₆(–)BCAA(+) diet, about a half showed lipid deposition in the liver. On the other hand, serum triacylglycerol levels in the B₆(–)BCAA(+) group tended to be decreased. Hepatic triacylglycerol and cholesterol levels tended to increase in the B₆(–)BCAA(+) group compared with the other three groups. Serum apolipoprotein B and apolipoprotein E (apo E) levels in the B₆(–)BCAA(+) group were the lowest among the three groups. In contrast, hepatic apo E levels in the B₆(–)BCAA(+) group were the highest among the three groups. High-performance liquid chromatography of pooled serum of rats with lipid deposits revealed that triacylglycerol and cholesterol levels in very low-density lipoprotein (VLDL) were decreased compared with other diet groups. These results strongly suggest that one of the mechanisms of lipid deposition in rats fed a B₆(–)BCAA(+) diet is due to impaired secretion of VLDL.

Key Words vitamin B₆ deficiency, branched-chain amino acid, lipid deposition, triacylglycerol, very low-density lipoprotein

Branched-chain amino acids (BCAAs) composed of valine, leucine, and isoleucine are essential amino acids and account for about 40% of dietary essential amino acids in body protein and about 15% of total amino acids in muscle proteins (1). BCAAs play a role in delayed onset of muscle soreness and fatigue, and suppression of fatigue in the central nervous system (2, 3). BCAAs have been also used for the prevention of hepatic encephalopathy in patients with liver cirrhosis (4, 5). Recently, sport enthusiasts have created an increased demand for dietary supplements with BCAAs and have increased their consumption of BCAAs-enriched soft drinks.

The first step in the catabolism of BCAAs involves branched-chain aminotransferase (BCAT; EC 2.6.1.42), a common enzyme. This enzyme requires vitamin B₆, which functions as a cofactor in many enzyme reactions of amino acid metabolism (6, 7). However, little information is available on the relationship between supplementation with BCAAs and the recommended daily allowance of vitamin B₆. In addition, it is known that a high-protein diet (e.g., 70% casein diet) under vitamin B₆ deficiency has a negative impact on several biochemical parameters and leads to fatty liver in rats (8–10).

Our previous study showed that providing supple-

mented BCAAs under vitamin B₆ deficiency and a 20% casein diet caused an accumulation of triacylglycerol in the liver, and individual differences in biological responses were observed (11). It is possible that a disorder of amino acid metabolism in the presence of vitamin B₆ deficiency might influence the assembly of lipids and apolipoproteins and/or the secretion of lipoproteins. Among the serum lipoproteins, very low-density lipoprotein (VLDL) is present during endogenous lipogenesis in the liver and its major apolipoproteins are apolipoprotein (apo) B, apo C, and apo E (12). It is interesting to compare serum apo B and apo E levels, and hepatic apoE levels to evaluate lipid deposition, because apo B and apo E are key determinants responsible for cellular recognition and internalization of chylomicron remnants, VLDL, and high-density lipoprotein (HDL) (13, 14).

In this study, we investigated the possible mechanism of lipid deposition in the liver of rats fed with the supplementation of BCAA under vitamin B₆ deficiency and suggested the presence of impaired secretion of VLDL into the bloodstream from the liver.

Materials and Methods

Diets and animals. Four diets composed of purified 20% vitamin-free casein diet with (+) or without (–) vitamin B₆ (7.0 mg of pyridoxine HCl/kg of diet) and with (+) or without (–) branched-chain amino acids

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Table 1. Composition of the experimental diets (g/kg).

Ingredient	B ₆ (+)BCAA(-)	B ₆ (+)BCAA(+)	B ₆ (-)BCAA(-)	B ₆ (-)BCAA(+)
α -Cornstarch	569.5	522	569.5	522
Casein	200	200	200	200
Corn oil	80	80	80	80
Sucrose	100	100	100	100
Mineral mixture ¹	35	35	35	35
Vitamin mixture ¹	10	10	0	0
Vitamin mixture (Vitamin B ₆ free) ¹	0	0	10	10
DL-Methionine	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5
Leucine	0	20	0	20
Valine	0	15	0	15
Isoleucine	0	12.5	0	12.5

¹ Mineral (AIN-93G) and vitamin (AIN-93) mixes were obtained from Oriental Yeast Co., Ltd., Tokyo, Japan.

(BCAAs) of valine, leucine, and isoleucine (4.75%) were used (15): B₆(+)BCAA(-); B₆(+)BCAA(+); B₆(-)BCAA(-); and B₆(-)BCAA(+) for 21 d (Table 1). Male Wistar rats, 4 wk of age, weighing 75–80 g, were fed the B₆(+)BCAA(-) diet ad libitum for 7 d as they adjusted to their new environment; they were then assigned to one of the four groups and given free access to diet for 21 d. Each group used six rats except for the B₆(-)BCAA(+) group, which used 12 rats. Animals were individually housed in a controlled room temperature (24°C ± 2) and light cycle (lighting 8:00 to 20:00). Rats were deprived of food for 4 h before sacrifice and were bled from the abdominal aorta under anesthesia by ethyl ether. Livers were immediately removed, frozen in dry ice, and stored at -70°C pending analyses. This study was conducted in accordance with the guidelines established by the Japanese Society of Nutrition and Food Science, and the experimental protocol was approved by the Institutional Animal Care and Use Committee of Shikoku University.

Isolation of lipoproteins and purification of apo E. Isolation and purification of VLDL ($d < 1.006$ g/mL) using preparative ultracentrifugation from pooled serum of male Wistar rats and delipidation of VLDL were performed as described previously (16–18). Delipidated VLDL (40–45 mg) was solubilized in 6 M guanidine HCl and 0.05 M Tris-HCl (pH 8.4) containing 0.1% NaN₃ and was applied on Sepharose CL-6B. Apo E fractions corresponding to a molecular weight of 34,000–36,000 were exhaustively dialyzed against distilled water, lyophilized, and used for calibration of apo E.

Enzyme-linked immunoassay (ELISA) of serum and hepatic apolipoproteins. Serum apo B levels were determined using an ELISA kit (Usen, Life Science, Inc., Wuhan, China). Serum and hepatic apo E levels were determined as follows. Polyvinylchloride microtiter plates were coated with the diluted rat serum in carbonate buffer (pH 9.6) containing 0.02% NaN₃ at 4°C overnight. For the preparation of liver samples, liver was homogenized with PBS buffer (pH 7.4) and an aliquot of solution was freeze-dried. Delipidation of freeze-dried liver was performed as described previously (18). Delipi-

dated liver was solubilized with carbonate buffer (pH 9.6) containing 0.02% NaN₃, and the supernatant was used for coating plates. Apo E levels were measured by ELISA using two antibodies against goat anti-rat apo E as a first antibody (Santa Cruz Biotechnology, Inc., CA, USA) and horseradish peroxidase conjugated rabbit anti-goat IgG as a second antibody (R & G Systems, MN, USA). The procedure was previously described in detail except for 230 μ M 3,3',5,5'-tetramethylbenzidine and 0.02% H₂O₂ in 200 mM citrate buffer (pH 5.3) (19). The developed color was determined at 450 nm for apo B and apo E with a microplate reader (model 680, Bio-Rad Laboratories, Richmond, CA, USA).

Lipoprotein analysis. Serum lipoproteins were analyzed by an on-line dual detection high performance lipid chromatography (HPLC) system (Skylight Biotech, Inc., Akita, Japan) according to the procedure described by Usui et al. (20, 21).

Lipid analysis and other measurements. Hepatic lipids were extracted and purified according to the method of Folch et al. (22). Hepatic triacylglycerol, phospholipid, and cholesterol levels were determined colorimetrically as previously described (23). Serum triacylglycerol, phospholipids, and HDL cholesterol were determined using an enzymatic assay kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Serum total cholesterol, albumin, and aspartate aminotransferase levels were determined by Fuji DRI-CHEM 3030 (Fuji Film Medical Co., Inc., Tokyo, Japan). Protein concentrations of the liver protein and purified apo E protein were determined using with bovine serum albumin as the standard (24).

Statistical analysis was performed using the Tukey-Kramer multiple range test. All statistical tests were done with SPSS 14.0J for Windows (SPSS Inc., IL, USA). A *p* value < 0.05 was considered statistically significant.

Results and Discussion

Growth parameters and tissue weight of rats fed different diets are shown in Table 2. After 21 d, fat infiltration of the liver was observed via gross examination, liver weight per body weight, and hepatic triacylglycerol

Table 2. Growth parameters and tissue weight of rats fed experimental diets.

	B ₆ (+)BCAA(-)	B ₆ (+)BCAA(+)	B ₆ (-)BCAA(-)	B ₆ (-)BCAA(+)
Final body weight (g)	300±28 ^a	294±13 ^a	239±10 ^b	228±15 ^b
Food intake (g/d)	17.9±2.7 ^a	17.3±2.1 ^a	14.1±1.9 ^b	13.3±1.9 ^b
Liver weight (g)	12.6±1.4	12.2±0.3	10.6±1.2	11.6±1.6
Liver (g/100 g BW)	4.22±0.09 ^b	4.15±0.17 ^b	4.44±0.37 ^b	5.08±0.58 ^a
Kidney (g/100 g BW)	0.75±0.04 ^b	0.76±0.07 ^b	0.97±0.05 ^a	0.97±0.07 ^a
Epididymal adipose tissue (g/100 g BW)	1.51±0.40 ^a	1.33±0.12 ^{ab}	1.00±0.17 ^{bc}	0.95±0.17 ^c
Perirenal adipose tissue (g/100 g BW)	0.41±0.12 ^{ab}	0.50±0.11 ^a	0.34±0.10 ^b	0.29±0.06 ^b
Thymus (g/100 g BW)	0.26±0.03 ^a	0.32±0.04 ^a	0.14±0.01 ^b	0.10±0.05 ^b

The preparation of experimental diets and its abbreviation are described in Table 1.

Values are means±SD of six rats except for 12 rats of the B₆(-)BCAA(+) group. Different superscript letters indicate significant differences at $p<0.05$.

Table 3. Serum and hepatic lipid concentrations and growth indices of rats fed experimental diets.

	B ₆ (+)BCAA(-)	B ₆ (+)BCAA(+)	B ₆ (-)BCAA(-)	B ₆ (-)BCAA(+)
Serum				
Triacylglycerol (mg/dL)	236±71	191±48	160±80	154±55
Phospholipids (mg/dL)	175±24	166±22	159±19	143±26
Total cholesterol (mg/dL)	81.8±7.9	80.8±11.5	81.8±5.7	81.8±14.6
HDL-cholesterol (mg/dL)	48.9±3.7	47.7±6.8	46.2±4.5	46.2±5.8
Aspartate aminotransferase (U/L)	60.8±5.5 ^b	75.7±11.7 ^a	18.8±6.7 ^c	24.0±4.7 ^c
Albumin (g/dL)	3.70±0.17 ^{ab}	3.58±0.17 ^b	3.95±0.21 ^a	3.89±0.22 ^a
Hepatic lipids (mg/g liver)				
Triacylglycerol	16.9±6.1	12.8±3.2	20.5±3.3	44.6±40.9
Phospholipids	27.8±2.1	29.9±0.9	28.0±2.2	27.7±2.2
Total cholesterol	2.34±0.54 ^{ab}	2.03±0.18 ^b	2.60±0.30 ^{ab}	3.61±1.54 ^a

Values are means±SD of six rats except for 12 rats of the B₆(-)BCAA(+) group. Different superscript letters indicate significant differences at $p<0.05$.

Table 4. Serum and hepatic apolipoprotein levels of rats fed experimental diets.

	B ₆ (+)BCAA(-)	B ₆ (+)BCAA(+)	B ₆ (-)BCAA(-)	B ₆ (-)BCAA(+)
Serum				
Apolipoprotein B (mg/dL)	28.6±2.8 ^{ab}	24.0±6.7 ^b	33.7±4.4 ^a	16.9±5.0 ^c
Apolipoprotein E (mg/dL)	22.9±9.2 ^a	13.9±7.3 ^{ab}	10.6±5.4 ^{ab}	8.0±7.8 ^b
Hepatic				
Apolipoprotein E (ng/mg protein)	72.9±18.6	57.4±23.7	69.9±10.4	128±101

Values are means±SD of six rats except for 12 rats of the B₆(-)BCAA(+) group. Different superscript letters indicate significant differences at $p<0.05$.

levels in six of 12 rats fed the B₆(-)BCAA(+) diet. Final body weight and food intake were significantly lower in the two B₆(-) groups compared with the two B₆(+) groups. The relative liver weight in the B₆(-)BCAA(+) group was significantly increased compared with the other three groups. The relative kidney weight of the two B₆(-) groups was significantly increased compared with that of the two B₆(+) groups, and the relative epididymal adipose tissue weight of the B₆(-)BCAA(+) group was significantly lower than that of the two B₆(+) groups. In addition, the relative thymus weight of the

two B₆(-) groups was significantly decreased compared with that of the two B₆(+) groups.

Results in growth parameters and tissue weight in B₆ deficiency were similar to a previous study (11).

Serum and hepatic lipids levels and growth indices are shown in Table 3. Aspartate aminotransferase activity of the two B₆(-) groups was significantly decreased to one third of the two B₆(+) groups. Serum triacylglycerol, phospholipids, cholesterol, and HDL-cholesterol levels were not remarkably different among the four groups.

Fig. 1. HPLC profiles of serum lipid concentrations of lipoproteins of rats fed four different experimental diets. Pooled serum from each group was subjected to HPLC analysis, and triacylglycerol (solid lines) and cholesterol levels (dashed lines) in effluents were monitored. The retention times of very low-density lipoprotein, low-density lipoprotein, and high-density lipoprotein were 20.5 min, 22.6 min, and 25.5 min, respectively. The composition of the experimental diets is described in Table 1. The B₆(-)/BCAA(+) group was subdivided into two groups: normal liver or liver with lipid deposits, based on hepatic triacylglycerol levels.

Hepatic triacylglycerol and phospholipids levels were not significantly different among groups. However, hepatic triacylglycerol levels of the B₆(-)/BCAA(+) group were two to three-fold higher than those in the other three groups, although differences were not significant. The hepatic triacylglycerol levels of the 12 rats in the B₆(-)/BCAA(+) group ranged from 11.4 to 145 mg per g liver, which may explain the differences compared with the other groups. Hepatic cholesterol levels in the B₆(-)/BCAA(+) group were significantly increased compared with the B₆(+)/BCAA(+) group.

These results that about half of the rats fed the B₆(-)/BCAA(+) diet showed lipid deposition in the liver were not experimental errors. Similar results were also observed in our previous report (11) and another three experimental results (unpublished data).

Serum and hepatic apolipoprotein levels determined by ELISA are shown in Table 4. Serum apo B and apo E levels in the B₆(-)/BCAA(+) group were the lowest among all groups, and apo B levels in the B₆(-)/BCAA(+) group were significantly decreased compared with the other three groups. In addition, serum apo E levels in the B₆(-)/BCAA(+) group were also significantly decreased compared with that of the B₆(+)/BCAA(-) group. On the other hand, hepatic apo E levels were not significantly different among the four groups, although apo E levels in the B₆(-)/BCAA(+) group were the highest among the groups. In this study, hepatic apo B levels could not be determined because of insolubility of the characteristic of this apoprotein with the assay system (25). It is interesting to note that both hepatic triacylglycerol and apo E levels in the B₆(-)/BCAA(+) group

tended to increase, but serum triacylglycerol, apo B, and apo E levels tended to decrease. It is known that endogenously synthesized triacylglycerol in the liver is secreted as VLDL into the blood after being assembled with apolipoproteins such as apo B, apo C, and apo E as well as lipids (26). Therefore, it can be inferred from our data that the secretion of synthesized lipoproteins from the liver to the bloodstream might be impaired. As described in this study, hepatic triacylglycerol levels with the B₆(-)/BCAA(+) group of 12 rats ranged from 11.4 to 145 mg per g liver. We then subdivided the B₆(-)/BCAA(+) group into two groups on the basis of hepatic triacylglycerol levels: 11.4 to 17.1 mg per g liver was considered a normal liver, and 37.7 to 145 mg per g liver was considered to represent lipid deposits. Then, the profile of serum lipoproteins was analyzed and compared among the three other groups and the two B₆(-)/BCAA(+) subgroups.

HPLC analyses of serum lipoprotein profiles are shown in Fig. 1. The retention time of VLDL, LDL, and HDL were 20.5 min, 22.6 min, and 25.5 min, respectively. The pooled serum of lipid-deposited livers in the B₆(-)/BCAA(+) group showed decreased triacylglycerol levels in VLDL compared with the B₆(-)/BCAA(+) group with no fatty liver deposits and with the other three groups. However, no differences in triacylglycerol or cholesterol levels of LDL or HDL were observed among these five groups. These lipoprotein profiles also support the mechanism by which the B₆(-)/BCAA(+) diet induced the lipid deposition in the liver due to the impaired secretion of VLDL from the liver into the bloodstream.

In this study, it is not clear why only half the rats fed

the same diet developed lipids deposits in the liver and showed a divergent response under the same nutritional conditions. Further studies are underway to elucidate which factor(s) is more important in regulating hepatic triglycerol levels under BCAA supplementation and vitamin B₆ deficiency.

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