Supplementation with branched-chain amino acids attenuates hepatic apoptosis in rats with chronic liver disease

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ABSTRACT

Branched-chain amino acids (BCAA) can function as pharmacologic nutrients for patients with decompensated cirrhosis. However, the effects of BCAA at the early stage of chronic liver disease are not clear. We hypothesized that early BCAA supplementation would attenuate the progression of chronic liver disease. The present study examined the effects of BCAA supplementation on the progression of chronic liver disease in rats caused by injected carbon tetrachloride (CCl4). Sprague-Dawley rats were fed with a casein diet (control group) or the same diet supplemented with BCAA (BCAA group) for 11 weeks, and all rats were repeatedly injected with CCl4. Food intake did not significantly differ between control and BCAA groups during the experimental period. Plasma alanine aminotransferase activities gradually increased during the experimental period in both groups but peaked later in the BCAA group. Liver fibrosis was more evident in the control group. Levels of connective tissue growth factor messenger RNA were significantly lower in the livers of rats in the BCAA group than in the control group. Terminal deoxynucleotidyl transferase–mediated deoxyuridine 5'-triphosphate nick end labeling assays found considerably more hepatic apoptosis in the control group. Liver cytosolic cytochrome c levels and expression of the proapoptotic Bax protein in the mitochondrial fraction were significantly lower in the BCAA group than in the control group. These results suggest that supplementation with BCAA delays the progression of chronic liver disease caused by CCl4 in rats by attenuating hepatic apoptosis.

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1. Introduction

Chronic liver diseases progress through mild to more severe inflammation and finally to fibrosis. Hepatocytes that become damaged during the progression of chronic liver diseases undergo apoptosis, which is a form of cell death that is primarily brought about by activation of the caspase family of cysteine proteases [1]. Several organelles can initiate the intracellular pathway of the apoptotic machinery in hepatocytes [2]. The release of cytochrome c from mitochondria is a common event in apoptosis that triggers activation of the caspase cascade [3]. The release of cytochrome c from
mitochondria is prevented by the antiapoptotic protein Bcl-xL and promoted by the proapoptotic protein Bax [2,4]. Hepatocyte-specific Bcl-xL disruption leads to continuous hepatocyte apoptosis, elevated serum alanine aminotransferase (ALT) activities, and hepatic fibrosis [5], which is the final common pathway for most chronic liver diseases. Continuous hepatocyte apoptosis is closely related to inflammation and fibrosis; it results in the formation of apoptotic bodies, and engulfment of these bodies by stellate cells promotes stellate cell activation. Activated stellate cells secrete transforming growth factor β1 (TGF-β1), thus promoting the development of fibrosis [6]. The progression of various liver diseases in humans is associated with hepatocyte apoptosis [7–10], and thus, inhibiting hepatocyte apoptosis might become a therapeutic strategy with which to delay the progression of liver diseases [11,12].

Patients with chronic liver diseases frequently have an imbalance among plasma amino acids such as decreased and increased levels of branched-chain amino acids (BCAA) and aromatic amino acids, respectively [13,14]. Pharmacologic BCAA supplementation is widely applied in Japan as a strategy to improve nutritional status [15–17], event-free survival, and the health-related quality of life of patients with decompensated liver cirrhosis [16]. Furthermore, BCAA administration might be more effective when implemented at the compensatory stage or even earlier rather than at the decompensated stage [18]. Several studies have shown that early oral supplementation with BCAA can maintain serum albumin levels and inhibit hepatic carcinogenesis in patients with compensated cirrhosis [19-21]. However, the mechanisms related to these effects of BCAA supplementation at the early stage have not yet been established.

We hypothesized that BCAA supplementation from the early stage of chronic liver disease would attenuate disease progression through suppressing hepatic apoptosis. Carbon tetrachloride (CCl4) induces hepatocyte apoptosis and liver fibrosis in animal models [22–24]. The present study investigates effects of BCAA supplementation on apoptosis-related factors in the livers of rats with chronic liver disease caused by injected CCl4.

2. Methods and materials

2.1. Materials

Anticleaved lamin A (Asp230), anticaspase 3, anticleaved caspase 3 (Asp175), anti-Bcl-xL, and anti-Bax antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Antitubulin and antiactin antibodies were purchased from Sigma (St Louis, MO, USA) and Millipore (Billerica, MA, USA), respectively. Horseradish peroxidase-conjugated antimouse and antirabbit immunoglobulin G antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Quantikine rat/mouse cytochrome C was purchased from R&D Systems, Inc (Minneapolis, MN, USA).

2.2. Animals

The animal facilities and protocol were reviewed and approved by the institutional animal care and use committee at Kyoto Prefectural University. Male Sprague-Dawley rats weighing approximately 200 g were housed under a 12-hour light/dark cycle, with access to a commercial diet and water ad libitum. Chronic liver disease was induced by injecting the rats with CCl4 (Wako Pure Chemical, Osaka, Japan), as follows. After an acclimatization period of 1 week, CCl4 mixed with an equal volume of olive oil was injected subcutaneously twice a week at a dose of 1.0 mL/kg of body weight for 11 weeks. The rats were fed with either a casein diet (control group, n = 13) or the same diet supplemented with BCAA (BCAA group, n = 13) (Table 1) as well as drinking water ad libitum. Dietary intake was measured, and the rats were weighed daily during the study. Blood was withdrawn from the tail vein every week, and plasma was separated by centrifugation. After 11 weeks, the rats were euthanized with diethyl ether. Blood was drawn and centrifuged to separate plasma. Livers were rapidly removed and weighed. Pieces of liver were fixed in 10% buffered formaldehyde for histologic analysis. The remaining liver and plasma samples were stored at −70°C until analysis.

2.3. Measurement of plasma aminotransferase, total protein, and albumin

Activities of aspartate aminotransferase (AST) and ALT in plasma were determined using ultraviolet methods with malate and lactate dehydrogenases, respectively [25]. Plasma concentrations of total protein and albumin were, respectively, determined using the Biuret [26] and bromocresol green methods [27]. Each of AST, ALT, total protein, and albumin was measured using GOT-L, GPT-L, TP-L, and BCG-L kits (Cerotec, Sapporo, Japan), respectively, and a CL-8000 auto-analyzer (Shimadzu, Kyoto, Japan).

2.4. Histologic analysis

Pieces of liver fixed in 10% buffered formaldehyde were embedded in paraffin, cut into 3-μm-thick sections, and stained with Azan to examine liver fibrosis by microscopy (Olympus Co, Tokyo, Japan).

| Table 1 – Nutrient and ingredient composition of the experimental diets |
|------------------|-----------------|-----------------|
| Component        | Control diet    | BCAA diet       |
|                  | g/kg diet       |                 |
| Casein           | 200             | 175             |
| Valine           | 0               | 7               |
| Leucine          | 0               | 12              |
| Isoleucine       | 0               | 6               |
| Cornstarch       | 457             | 457             |
| Sucrose          | 228             | 228             |
| Rapeseed oil     | 35              | 35              |
| Soybean oil      | 15              | 15              |
| Cellulose        | 20              | 20              |
| Vitamin mixture  | 10              | 10              |
| Mineral mixture  | 35              | 35              |

* AIN-76 vitamin mixture.

b AIN-76 mineral mixture.
2.5. Preparation of total RNA and quantitative real-time polymerase chain reaction

Total RNA was extracted using the ISOGEN system (Nippon Gene, Tokyo, Japan) according to the manufacturer’s protocol. First-strand complementary DNA was synthesized using M-MLV reverse transcriptase (Invitrogen Corp, Carlsbad, CA, USA). Real-time polymerase chain reactions (PCRs) (DNA Engine Opticon; Bio-Rad Laboratories, Hercules, CA, USA) was proceeded using SYBR Premix Ex Taq (perfect real time) (TaKaRa Bio Inc, Shiga, Japan) and the following specific primers: α-smooth muscle actin (α-SMA): forward, 5′-ACAACGTGCTATCTCTGAGGGCTT-3′; reverse, 5′-AGCGACATAGCACAGCTTCTCCT-3′; TGF-β1: forward, 5′-TGGCCTGCAGAGATTCAAG-3′; reverse, 5′-AGGTAACGCCAGGAATTGTTGCTA-3′; connective tissue growth factor (CTGF): forward, 5′-AGACGTTTGTGCCTATTGTTTGGA-3′; reverse, 5′-TGTCCTATCGATGGTGTTTGGA-3′; and 18s ribosomal RNA (rRNA): forward, 5′-GTAACCGTGTGAACCCCATTT-3′; reverse, 5′-CGCTACTACGGATTTGGGAG-3′. Levels of messenger RNA (mRNA) were normalized to that of 18s rRNA.

2.6. Preparation of protein extracts for Western blotting

Whole protein fractions were prepared as described by Schaefer et al [28]. Livers were homogenized in 3 volumes of buffer containing 10 mmol/L Tris-HCl (pH 7.5), 10 mmol/L NaCl, 0.1 mmol/L EDTA, 0.2 mmol/L phenylmethylsulfonyl fluoride (PMSF), 0.5% Triton X-100, and a protease inhibitor cocktail tablet (Complete Mini; Roche Diagnostics, Mannheim, Germany). The homogenate was incubated for 30 minutes on ice and then separated by centrifugation at 16000 g for 30 minutes at 4°C. The supernatant was Western blotted against anticaspase 3 and anticleaved caspase 3 antibodies.

Nuclear fractions were prepared as described by Blobel and Potter [29]. Livers were homogenized in 2 volumes of 0.25 mol/L sucrose in buffer A containing 50 mmol/L Tris-HCl (pH 6.5), 25 mmol/L KCl, and 10 mmol/L MgCl2, and filtered through sterile gauze. The filtrate was then thoroughly mixed with 2 volumes of 2.2 mol/L sucrose in buffer A. The mixture layered on the top of 2.2 mol/L sucrose in buffer A was separated by centrifugation at 100000g for 1 hour at 4°C. The pellet was lysed in buffer containing 20 mmol/L Tris–HCl (pH 7.4), 10 mmol/L KCl, 1.5 mmol/L MgCl2, 1 mmol/L EDTA, 1 mmol/L ethyleneglycoltetraacetic acid (EGTA), 1 mmol/L dithiothreitol, 1 mmol/L PMSF, 1 mmol/L sodium vanadate, and 1 mmol/L β-glycerophosphate and separated by centrifugation at 800g for 10 minutes at 4°C. The supernatant (postnuclear supernatant) was separated by centrifugation at 110000g for 25 minutes at 4°C. The pellet was lysed in buffer containing 10 mmol/L Tris–Cl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L PMSF, 1 mmol/L sodium vanadate, 1 mmol/L β-glycerophosphate, and 1% Triton X-100, and the supernatant after centrifugation at 20000g for 15 minutes at 4°C was taken as the mitochondrial fraction. Both the mitochondrial fraction and the postnuclear supernatant were Western blotted against anti-Bcl-xL, anti-Bax, and antiactin antibodies.

The protein concentration was determined using the BCA protein assay regent (Pierce, Rockford, IL, USA).

2.7. Western blotting

Proteins separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under standard reducing conditions were electrophoretically transferred to Immun-Blot PVDF membranes (Bio-Rad Laboratories) and incubated with antibodies to caspase 3, cleaved caspase 3, cleaved lamin A, tubulin, Bcl-xL, Bax, or actin. Horse radish peroxidase–conjugated antimouse or antirabbit immunoglobulin G antibodies served as secondary antibodies, and

| Table 2 – Body weight, liver weight, and blood measurements of rats injected with CCl4 and fed the control of BCAA-supplemented diets |
|-----------------|------------------|------------------|
|                  | Control          | BCAA             |
| Body weight (g)  | 397 ± 14         | 387 ± 16         |
| Liver weight (g/100g body weight) | 4.7 ± 0.3 | 4.8 ± 0.2 |
| Blood biochemistry |                  |                  |
| AST (IU/L)       | 801 ± 79         | 624 ± 83         |
| ALT (IU/L)       | 480 ± 63         | 475 ± 111        |
| Total protein (g/dL) | 5.4 ± 0.1 | 5.6± 0.1        |
| Albumin (g/dL)   | 2.6 ± 0.1        | 2.8 ± 0.1        |

Values are shown as means ± SEM (n = 13).
signals were detected using the Immobilon Western system (Millipore).

2.8. Terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick end labeling assay

The terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick end labeling (TUNEL) assay proceeded using the In Situ Cell Death Detection kit Fluorescein (Roche Diagnostics), according to the manufacturer’s protocol. Nuclei were counterstained with propidium iodide. Cells labeled by TUNEL were analyzed using fluorescence microscopy (Olympus).

2.9. Enzyme-linked immunosorbent assay

Livers were homogenized in 10 volumes of buffer containing 250 mmol/L mannitol, 0.5 mmol/L EGTA, 5 mmol/L N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 0.1% bovine serum albumin (BSA), and a protease inhibitor cocktail tablet (Complete Mini) (pH 7.2) and separated by centrifugation at 600g for 5 minutes at 4°C. The supernatant was further processed by centrifugation at 10000g for 10 minutes at 4°C, and then cytochrome c was analyzed in this supernatant using Quantikine rat/mouse cytochrome c enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer’s protocol. The protein concentration was determined using the BCA protein assay regent.

2.10. Statistical analyses

Data are expressed as means ± SEM of 3 independent experiments (experiments 1 and 2, n = 4; experiment 3, n = 5). The uniformity of SD within groups was evaluated using the F test. The significance of differences was assessed using Student t test. Data were analyzed using Statcel2 software.

**Fig. 2** - Effect of BCAA supplementation on liver fibrosis in rats injected with CCl4. Representative Azan-stained sections from livers of rats in control (A) and BCAA (B) groups (original magnification ×40). Bar, 200 μm. Levels of mRNAs encoding α-SMA (C), TGF-β1 (D), and CTGF (E) in the livers were measured using real-time PCR and normalized to that of 18s rRNA. All values are shown as means ± SEM (n = 13). Data were analyzed by Student t test. *P < .05 vs corresponding control group.
3. Results

3.1. Physical and biochemical parameters in CCl₄-injected rats

Plasma AST and ALT activities that gradually increased after repeated CCl₄ injections in both the control and BCAA groups (Fig. 1) did not significantly differ between the control and BCAA groups throughout the study. The activities of ALT peaked after CCl₄ injections for 8 and 9 weeks in the control and BCAA groups, respectively, and then decreased thereafter (Fig. 1B). Food intake throughout the study did not significantly differ between the control and BCAA groups ($19.6 \pm 0.6$ vs $19.2 \pm 0.8$ g/d). Body and liver weights as well as aminotransferase activities, total protein, and albumin concentrations did not significantly differ between the 2 groups after 11 weeks of CCl₄ administration (Table 2).

Fig. 3 – Effect of BCAA supplementation on hepatic apoptosis in rats injected with CCl₄. A, Whole protein fraction prepared from livers analyzed by Western blotting against anticaspase 3 and anticleaved caspase 3 antibodies. Corresponding densitometric findings are shown graphically. B, Nuclear fraction prepared from livers analyzed by Western blotting against anticleaved lamin A and antitubulin antibodies. Corresponding densitometric findings are shown graphically. All values are shown as means ± SEM (n = 13). Data were analyzed by Student t test. *P < .05 vs corresponding control group. C, Fluorescence microscopy images of TUNEL-positive cells (green) and nuclei (red) in sections from livers of control (upper panel) and BCAA (lower panel) groups (original magnification ×20). Arrows indicate positively stained cells.
3.2. Liver fibrosis in rats caused by CCl4

Liver fibrosis visualized by Azan staining was more evident in the control group than in the BCAA group (Fig. 2A and B). Levels of α-SMA mRNA (Fig. 2C) and of TGF-β1 mRNA (Fig. 2D) did not significantly differ between the 2 groups, whereas those of CTGF mRNA were significantly lower in livers from the BCAA group than in those from the control group (Fig. 2E).

3.3. Hepatic apoptosis in rats injected with CCl4

Levels of cleaved (activated) caspase 3 (Fig. 3A) and of cleaved nuclear membrane protein lamin A (Fig. 3B) were significantly lower in livers from the BCAA group than the control group, and TUNEL assays revealed fewer stained hepatocytes in the BCAA group (Fig. 3C).

3.4. Cytosolic cytochrome c levels and expression of antiapoptotic protein Bcl-xL and of proapoptotic protein Bax in livers of rats injected with CCl4

Levels of cytosolic cytochrome c were significantly lower in the livers of rats in the BCAA group than in the control group (Fig. 4A). Both pro- and antiapoptotic Bcl-2 proteins regulate cytochrome c release from mitochondria. Levels of the antiapoptotic protein Bcl-xL did not significantly differ between the 2 groups in the mitochondrial fraction (Fig. 4B and C), whereas those of the proapoptotic protein Bax were significantly decreased in livers from the BCAA group (Fig. 4B and D). Levels of Bax in postnuclear supernatant fractions did not significantly differ between the 2 groups (Fig. 4E).

4. Discussion

Supplementation with BCAA confers a benefit upon patients with decompensated liver cirrhosis [15-17] who have very few functioning hepatocytes. The mechanism might be that BCAA activates the functions of remaining hepatocytes as overall nutritional status improves. Furthermore, the present findings suggest that early BCAA supplementation delays the progression of chronic liver disease.

Plasma AST and ALT activities are useful markers of liver injury. Especially, the rate of fibrosis progression or the occurrence of hepatocellular carcinoma is closely associated with ALT activity in patients with liver disease [31,32]. Levels of ALT activity decrease with more extreme liver damage because defective hepatocytes lose the ability to produce ALT [33]. Throughout the experimental period, ALT activities did not significantly differ between the control and BCAA groups. However, the delayed peak of ALT activity indicates that disease progression was suppressed in the BCAA group.

Hepatic fibrosis is a common pathway for most chronic liver diseases. The present study found that BCAA supplementation attenuated fibrosis and inhibited CTGF mRNA expression in the livers of rats injected with CCl4. The inhibition of CTGF by small interfering RNA (siRNA) confers a beneficial effect on experimental liver fibrosis [34,35]. On the other hand, expression levels of mRNAs encoding α-SMA and TGF-β1 that are related to the activation of stellate cells did not significantly differ between the control and BCAA groups. The expression of these proteins and liver fibrosis should be quantitatively analyzed. Furthermore, if the target of BCAA
supplementation consists of hepatocytes and not stellate cells, then the effects of BCAA on the progression of fibrosis should be investigated in both cell types.

Cytochrome c release is induced by Bax that is translocated from the cytosol to the mitochondria when the apoptotic pathway is activated[2]. The present study found that Bax translocation to mitochondria in the livers of CCl₄-injected rats was attenuated in the BCAA group. Apoptotic cells should be quantitatively analyzed. However, the present study suggests that BCAA supplementation decreases cytochrome c release via regulation of Bax translocation in the livers of rats with chronic liver disease induced by CCl₄. Antiapoptotic protein Bax inhibitor-1 suppresses Bax translocation to mitochondria [36]. The expression of the Bax inhibitor-1 decreases as chronic liver diseases progresses [37]. The mechanism through which BCAA regulated Bax translocation in the livers of rats with chronic liver disease induced by CCl₄ remains unclear.

We previously indicated that continuous BCAA supplementation activates mammalian target of rapamycin signaling in the livers of rats with chronic liver diseases [38]. Others have indicated that mammalian target of rapamycin signaling controls the apoptotic pathway through phosphorylation of the proapoptotic protein Bad [39] and expression of the antiapoptotic protein Mcl-1 [40]. The mitochondrial pathway of apoptosis is regulated by both pro- and antiapoptotic Bcl-2 proteins that interact with each other and/or with mitochondria [2,6]. Apoptosis in the livers of rats injected with CCl₄ and supplemented with BCAA seemed to be suppressed through not only Bax localization but also the regulation of other Bcl-2 proteins including Bad and Mcl-1.

Plasma ALT activity must be suppressed to prevent the aggravation of liver disease in the clinical setting. Here, we showed that BCAA supplementation delayed the peak of plasma ALT activity in CCl₄-injected rats. Although details of the mechanisms remain unknown, BCAA supplementation at least attenuated hepatocyte apoptosis in rats with liver damage induced by CCl₄ via controlling Bax localization. These findings are consistent with our hypothesis that BCAA supplementation from the early stage of chronic liver disease would help to protect hepatocytes. The findings of a recent study using asialo-scintigraphy suggest that a BCAA-enriched snack consumed late in the evening is more therapeutically useful for improving hepatic parenchymal cell mass during the early stages of liver cirrhosis [41].

The present study has some limitations. We did not determine the effects of BCAA supplementation in groups of rats without induced liver damage. Furthermore, CCl₄ induces not only apoptosis but also necrosis of the rat liver [22], and thus, further studies should evaluate the effects of BCAA supplementation on the progression of disease in other models of chronic liver disease.

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