VIRAL HEPATITIS

Branched-chain amino acids reduce hepatic iron accumulation and oxidative stress in hepatitis C virus polyprotein-expressing mice

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Keywords
Hepatitis C virus – hepatic mitochondrial dysfunction – hepcidin-25 – iron metabolic disorder – reactive oxygen species

Abbreviations
BAP, biological antioxidant potential; BCAA, branched-chain amino acids; BTR, ratio of BCAA relative to tyrosine; CHOP, CCAAT/enhancer-binding protein homology protein; CPT I, carnitine palmitoyl transferase I; dROM, derivatives of reactive oxygen metabolites; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HCVTgM, transgenic mice expressing hepatitis C virus polyprotein; ROS, reactive oxygen species; SOD2, superoxide dismutase 2; SREBP, sterol regulatory element-binding protein.

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Abstract
Background & Aims: Branched-chain amino acids (BCAA) reduce the incidence of hepatocellular carcinoma (HCC) in patients with cirrhosis. However, the mechanisms that underlie these effects remain unknown. Previously, we reported that oxidative stress in male transgenic mice that expressed hepatitis C virus polyprotein (HCVTgM) caused hepatic iron accumulation by reducing hepcidin transcription, thereby leading to HCC development. This study investigated whether long-term treatment with BCAA reduced hepatic iron accumulation and oxidative stress in iron-overloaded HCVTgM and in patients with HCV-related advanced fibrosis. Methods: Male HCVTgM were fed an excess-iron diet that comprised either casein or 3.0% BCAA, or a control diet, for 6 months. Results: For HCVTgM, BCAA supplementation increased the serum hepcidin-25 levels and antioxidant status [ratio of biological antioxidant potential (BAP) relative to derivatives of reactive oxygen metabolites (dROM)], decreased the hepatic iron contents, attenuated reactive oxygen species generation, and restored mitochondrial superoxide dismutase expression and mitochondrial complex I activity in the liver compared with mice fed the control diet. After 48 weeks of BCAA supplementation in patients with HCV-related advanced fibrosis, BAP/dROM and serum hepcidin-25 increased and serum ferritin decreased compared with the pretreatment levels. Conclusions: BCAA supplementation reduced oxidative stress by restoring mitochondrial function and improved iron metabolism by increasing hepcidin-25 in both iron-overloaded HCVTgM and patients with HCV-related advanced fibrosis. These activities of BCAA may partially account for their inhibitory effects on HCC development in cirrhosis patients.

Hepatitis C virus (HCV) causes acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) (1). New direct-acting antiviral treatments are expected to eliminate this virus in about 90% of patients (2), but therapies that could reduce disease progression in chronically infected individuals would be highly beneficial.
Valine, leucine and isoleucine are essential branched-chain amino acids (BCAA). A decreased ratio of serum BCAA relative to aromatic amino acids, a hallmark of cirrhosis, is caused by several factors, including reduced nutritional intake and ammonia detoxification in skeletal muscles (3). BCAA supplementation can improve the nutritional status and albumin synthesis by activating the mammalian target of rapamycin signalling cascade (4, 5) and glucose metabolism in skeletal muscles (6, 7). Long-term oral BCAA supplementation decreases the frequency of HCC in male obese patients with cirrhosis (8). BCAA also had an antihepatocarcinogenic activity in an animal model of insulin resistance (9, 10). In addition, glucose intolerance is closely linked to hepatic iron overload and oxidative stress via mitochondrial injury (15). The liver tissue was homogenized and the lipids were extracted (19), and the triglyceride levels were measured using a TG-E-test Wako kit (Wako Pure Chemicals, Tokyo, Japan), according to the manufacturer’s instructions. The protein concentrations were determined by the Lowry method (20) using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

In situ ROS detection

In situ liver ROS production was assessed by staining with dihydroethidium (Invitrogen Corp., Carlsbad, CA, USA), as described previously (14). Dihydroethidium is oxidized to ethidium bromide in the presence of ROS, which stains the nuclei bright red via DNA intercalation (21). The intensity of the fluorescence was quantified using the NIH Image analysis program in three randomly selected areas of the digital images for each mouse.

Derivatives of reactive oxygen metabolites and biological antioxidant potential levels

The derivatives of reactive oxygen metabolites (dROM) and biological antioxidant potential (BAP) levels were measured using a Free Radical Elective Evaluator (Wismerll Co. Ltd, Tokyo, Japan) (22, 23). The dROM measurements were determined based on the ability of transition metals to catalyse the formation of coloured free radicals (detection at 505 nm). The results were expressed in Cartelli units (U.CARR), where 1 U.CARR = 0.8 mg/L of H2O2. To obtain the BAP measurements, the blood samples were added to a solution containing FeCl3 bound to a chromogenic substrate (AT, a derivative of thiocyanate). Fe3+ reduction to Fe2+ caused a chromatic change that was directly proportional to the plasma ROS reduction, which was measured at 505 nm using a photometer. Blood aliquots (10 µL) were mixed with the FeCl3 solution and incubated for 5 min at 37°C before the photometric analysis.

Clinical chemistry tests

The serum concentrations of alanine aminotransferase, aspartate aminotransferase (AST), albumin, glucose, insulin, BCAA, tyrosine and hepcidin-25 were determined in blood samples collected from the inferior vena cava of sacrificed mice at 12 h after fasting. The blood glucose levels were periodically measured using a glucometer (OneTouch Ultra, Lifescan, Inc., Milpitas, CA, USA). The serum insulin levels were measured using an ultrasensitive mouse insulin ELISA kit (Morinaga Milk, Kanagawa, Japan). The serum hepcidin-25 levels were determined by LC/MS/MS (18).

Hepatic iron and triglyceride contents

The hepatic iron concentrations were measured by atomic absorption spectrometry, as described previously (15). The liver tissue was homogenized and the lipids were extracted (19), and the triglyceride levels were measured using a TG-E-test Wako kit (Wako Pure Chemicals, Tokyo, Japan), according to the manufacturer’s instructions. The protein concentrations were determined by the Lowry method (20) using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Materials and methods

Animals and experimental design

The pAlbSVP-HCV transgene contains the full-length HCV polyprotein-coding region under the control of the murine albumin promoter/enhancer (16, 17). HCV polyprotein is processed into individual proteins in the liver and expressed at biologically relevant levels in FL-N/35 transgenic mice (HCVTgM) (17). In the present study, male HCVTgM (8 weeks old) were fed a normal rodent diet, including carbonyl iron (45 mg/kg; control, n = 6), or an excess-iron diet (carbonyl iron, 225 mg/kg) that contained either 3.0% BCAA (BCAA/iron; n = 5) or casein (casein/iron; n = 7). Six months later, the mice were sacrificed by CO2 asphyxiation after a 12-h fast, according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals.

Clinical chemistry tests

The serum concentrations of alanine aminotransferase, aspartate aminotransferase (AST), albumin, glucose,
and iron concentration. The remaining liver tissue was fixed in 4% parformaldehyde in phosphate-buffered saline and embedded in paraffin for use in the histological analysis. The liver sections were stained with haematoxylin and eosin.

Real-time reverse transcriptase-PCR
One-step real-time reverse transcriptase-PCR (RT-PCR) was performed, as described previously (14), where the results were expressed as the hepcidin, interleukin 6 (IL6), BMP6 and superoxide dismutase 2 (SOD2) gene mRNA levels relative to β-actin mRNA.

Extraction of nuclear and histone deacetylase activity assay
For isolation of nuclear proteins from mice liver, Nuclear Extraction Kit 1 (Epigentek, Farmingdale, NY, USA) was used. Histone deacetylase (HDAC) activity was assessed using HDAC Activity/Inhibition Direct Assay Kit (Epigentek) according to the manufacturer’s instruction.

Isolation of mitochondria and complex I activity determination
Liver mitochondria were isolated and the activity of complex I was assayed (at 25°C) as described previously (3, 24).

Protein extraction and Western blotting
The liver lysate and mitochondrial lysate proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. These proteins were then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) and blocked overnight at 4°C with 1–3% skim milk and 0.1% Tween 20 in Tris-buffered saline, which was followed by incubation at room temperature for 1 h with a primary antibody. Anti-rabbit carnitine palmitoyl transferase I (CPT I), anti-rabbit CPT II (Alpha Diagnostic International, San Antonio, TX, USA), anti-rabbit SREBP1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), or anti-bacterially expressed mouse CCAAT/enhancer-binding protein homology protein (CHOP) fusion protein (Abcam, Cambridge, England) were used for the liver lysate proteins. Anti-SOD2 (Abcam), anti-Grp75 (mitochondrial heat shock protein70; Abcam), or anti-NDUFB8 (mitochondrial complex I) antibody (Abcam) were used for the mitochondrial lysates. The proteins were blocked for 1 h at room temperature and then incubated overnight at 4°C with a Phospho-stat3 (pSTAT3) antibody (Cell Signaling Technology Inc., Danvers, MA, USA) and a Phospho-Smad1/5/8 antibody (Cell Signaling Technology Inc.). The anti-acetyl-histoneH3K9 and anti-histoneH3 (Cell Signaling Technology Inc,) were used for the nuclear lysates.

Human BCAA supplementation study design
We screened 68 HCV RNA-positive patients who were aged >65 years (Fig. S1). We enrolled 25 patients with HCV-related advanced fibrosis who satisfied the following criteria: serum albumin = 3.5–4.2 g/dl; platelet counts <15 × 10⁹/µl; amino acid imbalance [based on the ratio of BCAA relative to tyrosine (BTR) <4.40, which was lower than the normal limits]; and no HCC or symptoms of chronic liver failure such as ascites, varices or hepatic encephalopathy. Advanced fibrosis defined liver specimens (METAVIR fibrosis staging: >F3,4) or Fib-4 index (>3.25). The patients were assigned randomly to receive BCAA supplementation (BCAA group; n = 12) or follow-up without treatment (non-BCAA group; n = 13). BCAA group were given a 4 g BCAA preparation (LIVACT Granules; Ajinomoto, Tokyo, Japan) administered orally three times daily after meals. We measured the plasma oxidized/reduced albumin and serum dROM and BAP as oxidative stress markers at 12, 24 and 48 weeks after starting the treatment. We also measured the levels of serum iron, ferritin, transferrin saturation (TSAT) and hepcidin-25 to evaluate the oxidative stress-associated iron metabolism. Moreover, type IV collagen 7s, type III procollagen peptide (PIIP) and Fib-4 index were measured to confirm the degree of hepatic fibrosis.

Written informed consent was obtained from each study participant. This study was conducted in accordance with the provisions of the 1975 Declaration of Helsinki and it was approved by the Institutional Ethics Committee of Kawasaki Medical School.

Statistical analysis
The results were expressed as mean ± SD. The group results were compared using Levene’s or Welch’s tests. The changes in the levels of the iron metabolism and oxidative stress markers between the BCAA and the non-BCAA groups were analysed using Wilcoxon rank-sum tests. Pearson’s product moment correlation coefficient was used to assess associations between the dihydroethioporum and hepcidin-25, BAP and dROM ratios. Differences were considered statistically significant at P < 0.05. The statistical analyses were performed using SPSS software (IBM SPSS Statistics 20.0 for Windows).

Results
AST, fasting blood sugar, plasma BCAA and tyrosine levels in HCVTgM
The dietary intake and body weight did not differ significantly between the three groups of mice. BCAA administration for 6 months significantly reduced the serum
AST (P < 0.05) and fasting blood sugar (FBS) levels (P < 0.05) compared with HCV TgM fed the excess-iron diet with casein (casein/iron group) (Table 1). However, the FBS levels remained higher in the HCV TgM fed the excess-iron diet with BCAA (BCAA/iron group) compared with the HCV TgM fed a normal rodent diet (control group) (P < 0.05). The casein/iron group had significantly lower plasma BCAA and the ratio of BCAA relative to tyrosine (BTR) levels (P < 0.05) compared with the BCAA/iron and control groups (Table 1). The tyrosine levels were significantly higher in the casein/iron group than the control group (P < 0.05).

Hepatic iron contents and hepcidin-25 levels in HCVTgM

The hepatic iron contents of HCVTgM fed the excess-iron diet with casein were significantly higher than those of HCVTgM fed an excess-iron diet with BCAA or a control diet at 6 months after the treatment commenced (Fig. 1A). The hepatic iron contents and hepcidin-25 levels in HCVTgM fed the excess-iron diet with casein or the control diet (Fig. 1A). The serum hepcidin to ferritin ratio was lower in patients with HCV (25). The serum hepcidin-25 to hepatic iron ratio was significantly higher in HCVTgM fed the excess-iron diet with BCAA compared with those fed the excess-iron diet with casein or the control diet.

Table 1. Effects of casein/iron and branched-chain amino acids (BCAA) diets on the liver to body weight ratios and blood chemistry results in hepatitis C virus transgenic mice

|                | Control | Casein/iron | BCAA/iron |
|----------------|---------|-------------|-----------|
| Mice (N)       | 6       | 7           | 5         |
| Liver weight/  | 3.32 ± 0.30 | 3.50 ± 0.60 | 2.97 ± 0.28 |
| Body weight (%)| 100     | 100         | 100       |
| AST (IU/L)     | 61 ± 23 | 92 ± 47     | 39 ± 7‡   |
| ALT (IU/L)     | 14 ± 4  | 61 ± 60     | 16 ± 4    |
| FBS (mg/dl)    | 115 ± 11| 299 ± 49†   | 184 ± 47‡ |
| Insulin (ng/ml)| 0.89 ± 0.36| 1.19 ± 0.20| 0.93 ± 0.39|
| Albumin (g/dl)| 2.82 ± 0.04| 2.77 ± 0.15| 2.96 ± 0.15|
| BCAA (nmol/ml)| 313 ± 22| 275 ± 31†   | 318 ± 35‡ |
| Tyrosine (nmol/ml)| 63 ± 5| 82 ± 11†   | 69 ± 11   |
| BTR            | 5.01 ± 0.20| 3.41 ± 0.40‡| 4.67 ± 0.40‡|

*Results are mean ± SD.
†P < 0.05 vs. transgenic mice expressing hepatitis C virus polyprotein (HCVTgM) on control diet for 6 months.
‡P < 0.05 vs. transgenic mice expressing hepatitis C virus polyprotein (HCVTgM) on control diet for 6 months.
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Factors that affected hepcidin upregulation

HCV-induced ROS production downregulates hepcidin transcription by inhibiting the C/EBPα DNA-binding activity of CHOP (14). Thus, we examined CHOP expression and the hepcidin mRNA levels. Hepatic CHOP expression was significantly lower and hepatic hepcidin expression was significantly higher in HCVTgM fed the excess-iron diet with BCAA compared with the levels in HCVTgM fed the excess-iron diet plus casein (Fig. 2A,B). The IL-6-gp130/signal transducer and activator of transcription are involved in the regulation of hepcidin transcription (26). Another pathway that regulates hepcidin expression involves the TGF-β/bone morphogenetic protein superfamily (27, 28). Thus, we examined the STAT-IL6 and SMAD-BMP signalling pathways. There were no differences in the phosphate STAT3, IL6, phosphor- SMAD1/5/8 and BMP6 expression levels between the BCAA and casein groups (Fig. 2C). In addition, HCV-induced oxidative stress inhibited hepcidin expression through increased histone deacetylase (HDAC) activity in cell culture system (29). HDAC activity of HCV TgM fed the excess-iron diet with BCAA was significantly lower than those of HCVTgM fed the excess-iron diet with casein or the control diet (Fig. S2). These results suggested that BCAA induced the upregulation of hepatic hepcidin by enhancing the antioxidant potential.

Hepatic steatosis and CPT1 expression

HCVTgM fed the excess-iron diet developed severe steatosis, including the centrilobular microvesicular type (15, 17). Previous studies showed that the antioxidant drugs N-acetylcysteine (NAC) and Stronger Neomorphagen C (SNMC) reduce the hepatic triglyceride levels in a dose-dependent manner (30, 31). In the present study, BCAA administration tended to reduce the hepatic triglyceride levels (P = 0.055; Fig. 3A).

Thus, we examined the effects of BCAA on CPT1 and CPT2, which are proteins that regulate long-chain fatty acid oxidation in mitochondria, and SREBP1 expression, which is a transcription factor that activates genes required for lipogenesis. Our previous study indicated that decreased CPT1 and increased SREBP1 expression contribute to the development of hepatic steatosis in HCVTgM fed an excess-iron diet (30). In the present study, CPT1 expression increased significantly in HCVTgM fed the excess-iron diet with BCAA after 6 months (P < 0.05, Fig. 3C), whereas CPT2 expression
Fig. 1. (A) Hepatic iron contents, hepcidin-25 levels, and hepcidin-25 to iron content ratios (hepcidin/iron). (Left) Hepatic iron contents in mice at 6 months after starting treatment for the control (n = 6), casein/iron (n = 7) and BCAA/iron groups (n = 5). (Centre) Serum hepcidin-25 levels. (Right) The hepcidin/iron ratios were used as an index of the sensitivity of hepcidin upregulation against iron overload. (B) Oxidative stress markers in serum. (Left) dROM and (centre) BAP were measured at 6 months after starting treatment. (Right) The antioxidant status was determined as the BAP to dROM ratio. (C) Dihydroethidium fluorescence intensity was quantified for three randomly selected areas in digital images for the control (n = 3), casein/iron (n = 7), and BCAA/iron groups (n = 5) at 6 months after starting treatment. (D) Correlations between the BAP/dROM ratios and fluorescence-positive areas in liver. *P < 0.05 vs control group; #P < 0.05 vs casein/iron group.
did not increase significantly. However, SREBP1 expression did not decrease in HCVTgM fed the excess-iron diet with BCAA ($P = 0.082$; Fig. 3B). These results suggest that the administration of BCAA was insufficient to prevent iron-induced steatosis in HCVTgM because BCAA failed to reduce SREBP1 expression.

**SOD2 expression and mitochondrial complex I activity**

CPT1 is localized to the mitochondrial outer membrane. Decreased CPT1 expression may be related to the HCV core protein’s association with the mitochondrial outer membrane. The HCV core protein interacts with mitochondria complex I, which generates ROS (13). Alterations in the mitochondrial ultrastructure were observed in HCVTgM fed the excess-iron diet after 6 months, as described previously (15, 30). We examined whether BCAA supplementation reduced iron- and HCV-induced mitochondrial injury.

The mitochondrial SOD2 mRNA levels were significantly higher in HCVTgM fed the excess-iron diet with BCAA compared with those fed the excess-iron diet with casein or the control diet. The SOD2 expression levels in mice fed the excess-iron diet with casein were significantly lower than those fed the control diet. However, the SOD2 expression levels were restored by BCAA supplementation (Fig. 4A). After 6 months, the mitochondrial complex I expression levels were significantly lower in mice fed the excess-iron diet with casein compared with those fed the control diet. Similar to SOD2, the mitochondrial complex I expression levels were restored by BCAA supplementation (Fig. 4B).

The enzymatic activity of mitochondrial complex I was significantly lower in mice fed the excess-iron diet...
The activity was restored by BCAA supplementation (Fig. 4C). Thus, these improvements in the mitochondrial complex I activity and CPT1, SOD2, and mitochondrial complex I expression indicate that BCAA may protect against the mitochondrial injury induced by HCV proteins and iron overload.

Antioxidant effects of BCAA supplementation in patients with HCV-related severe fibrosis

Next, we determined whether oral BCAA supplementation reduced oxidative stress and affected iron metabolism in patients with HCV-related advanced liver fibrosis. We assigned 25 patients to receive either BCAA supplementation (BCAA group; n = 12) or follow-up without treatment (non-BCAA group; n = 13). There were no differences in the clinical characteristics, oxidative stress markers, or iron metabolic markers at baseline between these groups (Table 2). Serum albumin and AST levels in BCAA group tended to be lower than those in non-BCAA group, although these differences were not statistically significant (P = 0.071 and P = 0.074 respectively).

The dROM levels increased significantly at weeks 24 and 48 in the non-BCAA group, whereas they did not in the BCAA group. The BAP levels also increased at weeks 12 and 24 in the non-BCAA group, and at weeks 12, 24 and 48 in the BCAA group (Table 3). The BAP/dROM ratio, an indicator of antioxidant potential, decreased significantly at week 48 in the non-BCAA group, but increased at weeks 24 and 48 in the BCAA group. This suggests that the BAP levels of the non-BCAA group increased in response to oxidative stress, while the increased BAP levels in the BCAA group indicated enhanced antioxidant potential.
In agreement with the antioxidant status, the serum ferritin levels were significantly lower after week 48 of BCAA supplementation (137 ± 109 mg/dl; \( P < 0.05 \)) compared with those before treatment (Table 3). BCAA supplementation significantly increased the serum hepcidin-25 levels at week 48 (20.2 ± 14.5 mg/dl; \( P < 0.05 \)). In addition, we determined the level of albumin synthesis after BCAA supplementation, because the oxidized albumin to total albumin ratio increases with cirrhosis progression and it is related to oxidative stress (32,33). In the present study, there were no differences in the total albumin changes in the non-BCAA or BCAA groups (Table 4). However, the amount of albumin present in the reduced form increased significantly in the BCAA group at week 48 compared with that before the study. By contrast, the level of reduced albumin decreased significantly at week 48 in the non-BCAA group. This suggests that long-term BCAA supplementation reduced iron overload by upregulating antioxidant potential and this improved the albumin status in patients without hypoalbuminaemia and chronic liver failure.

Discussion

Hepatic iron overload and ROS production are both pathophysiological features of HCV-associated chronic liver disease (34) and risk factors for HCC development (35). The reduced hepatic oxidative stress observed after oral BCAA supplementation may be related to changes in the albumin redox state (32, 36). However, previous studies did not determine how BCAA affects iron metabolism and ROS generation.

The mouse model used in the present study shared similarities with the patients who had HCV-associated chronic liver disease in terms of hepatic ROS production and steatosis (14) at 6 months after treatment, followed by hepatocarcinogenesis (15). Furthermore, the hepatic iron concentrations in HCVTgM fed the excess-iron diet were comparable to those of a large number of patients with chronic hepatitis C (30, 37, 38). Thus, HCVTgM fed the excess-iron diet is a suitable model for assessing the effects of long-term supplementation with BCAA on disordered iron metabolism and ROS production in HCV infection.
Table 2. Patient baseline characteristics

|                        | non-BCAA | BCAA   | P-value |
|------------------------|----------|--------|---------|
| Patients (N)           | 13       | 12     | N.S.    |
| Age (years)            | 73.5 (65–87) | 74.9 (65–83) | N.S.    |
| Sex (male/female)      | 6/7      | 6/6    | N.S.    |
| White blood cell count (×10^3/μl) | 46.2 (30.1–63.4) | 45.1 (27.1–84.5) | N.S.    |
| Haemoglobin concentration (g/dl) | 13.3 (10.6–16.3) | 13.2 (11.4–15.7) | N.S.    |
| Platelet counts (×10^3/μl) | 12.2 (4.9–15) | 10.5 (3.7–15) | N.S.    |
| Ferritin (ng/ml)       | 0.7 (0.5–1.1) | 1.0 (0.3–1.7) | N.S.    |
| Albumin (g/dl)         | 4.0 (3.5–4.2) | 3.9 (3.5–4.2) | N.S.    |
| ALT (IU/L)             | 33 (23–47) | 41 (21–55) | N.S.    |
| AST (IU/L)             | 43 (32–54) | 48 (32–54) | N.S.    |
| ALP (IU/L)             | 286 (158–435) | 302 (145–491) | N.S.    |
| GTP (IU/L)             | 46 (15–177) | 53 (16–137) | N.S.    |
| FBS (mg/dl)            | 94 (70–130) | 107 (72–158) | N.S.    |
| Insulin (μU/ml)        | 14 (5.3–29) | 14 (6.3–28) | N.S.    |
| Tyrosine (nmol/ml)     | 104 (76–123) | 103 (63–149) | N.S.    |
| BCAA (nmol/ml)         | 424 (319–606) | 401 (269–617) | N.S.    |
| BTR                    | 4.1 (2.6–4.9) | 4.0 (2.7–4.4) | N.S.    |
| AFP (ng/dl)            | 11 (2–61) | 18 (2–95) | N.S.    |
| Serum iron (μg/ml)     | 134 (50–255) | 136 (37–256) | N.S.    |
| TSAT (%)               | 38 (11–70) | 44 (12–88) | N.S.    |
| Ferritin (ng/ml)       | 120 (30–429) | 190 (30–346) | N.S.    |

Results are mean (range). Comparisons between branched-chain amino acids (BCAA) and non-BCAA groups were made using Levene’s or Welch’s tests. AFP, α-fetoprotein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyltransferase; BTR, the ratio of BCAA relative to tyrosine; FBS, fasting blood sugar; N.S., Not significant; TSAT, transferrin saturation.

BCAA supplementation improves the nutritional status, prognosis and quality of life for patients with cirrhosis (39, 40). A randomized, controlled trial demonstrated that BCAA supplementation reduced the frequency of HCC in obese male patients with cirrhosis and HCV infection (18). BCAA treatment also reduced the hepatocarcinogenic activity in obese diabetic animals with insulin resistance (9, 10). Insulin resistance promotes hepatocarcinogenesis by activating the mitogen-activated protein kinase (MAPK) pathway and insulin-like growth factor 1 (IGF-1) receptors, which further activates the Raf/MAPK kinase/MAPK cascade (41, 42). BCAA suppress the IGF/IGF-1R axis by down-regulating IGF-1, IGF-2 and IGF-1R mRNA expression, thereby leading to the inhibition of mitosis and cell growth (9). BCAA reduce HCC development by inhibiting insulin resistance (43).

In the present study, the FBS levels of HCV TgM fed the excess-iron diet with casein increased after 6 months. BCAA supplementation reduced the iron overload-induced elevation of the FBS. There was no intrahepatic inflammation or fibrosis in the HCV TgM fed the excess-iron diet, but those fed the excess-iron diet with casein had significantly lower plasma BCAA levels and a lower BTR compared with those fed excess-iron with BCAA and the control diet. An amino acid imbalance, which is indicated by a lower BTR, has been observed in patients with compensated cirrhosis or chronic hepatitis (44, 45). This suggests that BCAA might potentially reduce hepatic iron accumulation and ROS in patients with HCV-related advanced fibrosis.

Table 3. Changes in oxidative stress and iron metabolism markers during branched-chain amino acids (BCAA) administration

|                        | Week 0     | Week 12    | Week 24    | Week 48    |
|------------------------|------------|------------|------------|------------|
| Hepcidin (ng/ml)       | 11.6 ± 7.9 | 10.4 ± 9.8 | 11.8 ± 9.5 | 10.5 ± 8.8 |
| BCAA                   | 9.5 ± 8.7  | 9.2 ± 9.5  | 11.0 ± 9.1 | 20.2 ± 14.5†|
| Ferritin (ng/ml)       | 120 ± 121  | 112 ± 105  | 100 ± 108  | 118 ± 120  |
| non-BCAA               | 190 ± 135  | 164 ± 129  | 163 ± 130  | 137 ± 109† |
| BCAA                   | 136 ± 64   | 131 ± 63   | 134 ± 68   | 117 ± 57   |
| Serum iron (μg/ml)     | 134 ± 52   | 143 ± 60   | 133 ± 55   | 142 ± 38   |
| non-BCAA               | 136 ± 64   | 131 ± 63   | 134 ± 68   | 117 ± 57   |
| TSAT (%)               | 38 ± 14    | 42 ± 19    | 39 ± 15    | 42 ± 19    |
| BCAA                   | 45 ± 29    | 42 ± 24    | 35 ± 19†   | 33 ± 16†   |
| non-BCAA               | 342 ± 64   | 405 ± 84   | 431 ± 76†  | 455 ± 96†  |
| BCAA                   | 360 ± 113  | 372 ± 80   | 361 ± 118  | 359 ± 65   |
| BAP (μM)               | 2369 ± 386 | 2772 ± 487†| 2798 ± 337†| 2630 ± 64  |
| non-BCAA               | 2139 ± 587 | 2516 ± 678‡| 2601 ± 647‡| 2758 ± 413‡|
| BCAA                   | 6.1 ± 1.3  | 6.8 ± 1.5  | 7.5 ± 1.6† | 7.8 ± 1.5† |
| BAP/dROM               | 7.0 ± 1.0  | 7.1 ± 1.7  | 6.6 ± 0.7  | 6.0 ± 1.0† |
| non-BCAA               | 6.1 ± 1.3  | 6.8 ± 1.5  | 7.5 ± 1.6† | 7.8 ± 1.5† |
| BCAA                   |              |            |            |            |

Results are mean ± SD.

†P < 0.05 vs. before BCAA treatment, Wilcoxon rank-sum test; U.CARR, Cartelli Units (1 U.CARR = 0.8 mg/L of H₂O₂), TSAT, transferrin saturation.
and tyrosine levels of HCVTgM and non-transgenic differences in the liver enzyme, glucose, insulin, BCAA without the excess-iron diet. However, there were no transgenic mice (14), but we did not test whether BCAA HCVTgM fed the control diet compared with non-

The hepatic ROS production increased more in the induced ROS production and mitochondrial injury? antioxidant potential and mitochondrial complex I mediated product of fatty acid synthesis (47). Decreased transcriptional level by malonyl-CoA, which is an inter-

Our previous study indicated that the antioxidant N-acetylcysteine (NAC) almost completely blocked ROS production and abrogated the hepatic steatosis induced by HCV proteins and iron (30). In the present study, the hepatic triglyceride levels tended to be lower in mice fed the excess-iron diet with BCAA compared with those fed the excess-iron diet with casein, although these differences were not statistically significant (P = 0.055). This may have been because BCAA reduce ROS production to a lesser degree than NAC, or because BCAA supplementation did not completely inhibit the ROS-associated unfolded protein response or improve glucose intolerance compared with the control diet. SREBP1 expression is positively regulated by insulin signalling pathways (46). Therefore, further studies are needed to determine whether BCAA reduce hepatic iron accumulation without affecting hepatic steatosis.

CPT1, a transmembrane enzyme in the mitochondrial outer membrane, is negatively regulated at the transcriptional level by malonyl-CoA, which is an intermediate product of fatty acid synthesis (47). Decreased CPT1 expression may be related to the HCV core protein, which is also located in the mitochondrial outer membrane and it generates mitochondrial ROS production indirectly (13). BCAA enhanced protection against mitochondrial injury by restoring the mitochondrial antioxidant potential and mitochondrial complex I activity. Thus, how does BCAA protect from HCV-induced ROS production and mitochondrial injury? The hepatic ROS production increased more in the HCVTgM fed the control diet compared with non-transgenic mice (14), but we did not test whether BCAA supplements reduced ROS production in HCVTgM without the excess-iron diet. However, there were no differences in the liver enzyme, glucose, insulin, BCAA and tyrosine levels of HCVTgM and non-transgenic mice. Furthermore, HCVTgM without the excess-iron diet did not develop severe steatosis and HCC. This indicates that HCVTgM without the excess-iron diet are not a suitable model for long-term treatments with BCAA.

BCAA supplementation increases the reduced form of albumin, which is a predictor of the cirrhosis prognosis (32, 33), while it also improves oxidative stress and iron metabolism in patients with decompensated cirrhosis (36), and in rats exposed to a fibrogenic agent (48). This suggests that the antioxidant effects of BCAA may be related to qualitative changes in serum albumin or the upregulation of albumin synthesis (4, 5, 9, 49). BCAA itself activates the mammalian target of rapamy-

Table 4. Changes in the serum albumin characteristics during branched-chain amino acid (BCAA) administration*

| Albumin (g/dl) | Week 0 | Week 12 | Week 24 | Week 48 |
|---------------|--------|---------|---------|---------|
| non-BCAA (13) | 4.0 ± 0.2 | 4.0 ± 0.2 | 4.0 ± 0.2 | 4.0 ± 0.2 |
| BCAA (12)     | 3.9 ± 0.3 | 3.8 ± 0.4 | 3.9 ± 0.3 | 4.0 ± 0.3 |
| Reduced albumin (%) | 66 ± 4.5 | 66 ± 5.3 | 66 ± 3.9 | 63 ± 4.9† |
| non-BCAA (10) | 66 ± 3.9 | 68 ± 2.8 | 68 ± 5.2 | 70 ± 3.2† |
| BCAA (10)     |        |         |         |         |
| Type IV collagen 7s (U/ml) | 5.8 ± 1.7 | 6.1 ± 2.5 | 6.0 ± 2.2 | 6.1 ± 2.1 |
| non-BCAA (13) | 6.8 ± 2.1 | 7.1 ± 1.8 | 7.0 ± 2.1 | 6.7 ± 2.0 |
| BCAA (12)     |        |         |         |         |
| P-III-P (U/ml) | 0.89 ± 0.19 | 0.83 ± 0.19 | 0.91 ± 0.24 | 0.83 ± 0.15 |
| non-BCAA (13) | 0.88 ± 0.23 | 0.90 ± 0.16 | 0.88 ± 0.18 | 0.86 ± 0.22 |
| BCAA (12)     |        |         |         |         |
| Fib4-index    | 5.0 ± 2.7 | 5.0 ± 2.5 | 5.4 ± 2.9 | 5.5 ± 3.7 |
| non-BCAA (13) | 6.8 ± 4.2 | 7.2 ± 3.7 | 6.4 ± 4.0 | 6.3 ± 4.0 |
| BCAA (12)     |        |         |         |         |

*Results are mean ± SD.
†P < 0.05 vs. before treatment, Wilcoxon rank-sum test, P-III-P: Type III procollagen peptide.

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which BCAA supplementation prevented fibrotic progression (Table 4). However, Fib-4 index in BCAA group at 48 weeks tended to be decreased compared with those at initial point, although these differences were not statistically significant ($P = 0.061$). Long-term BCAAs treatment might inhibit hepatic fibrosis in HCV patients with advanced fibrosis.

Our clinical study had some limitations, including a higher number of older patients who had higher serum albumin and ferritin levels than those in the cohorts reported in other studies, although they used small sample sizes and were not randomized. Further studies should use large cohorts to clarify these effects.

In conclusion, we demonstrated that BCAA administration reduced the hepatic iron contents and ROS levels, which were induced by HCV proteins and iron overloading in mice, probably by protecting the function of mitochondrial complex I. Furthermore, we confirmed that BCAA supplementation improved disordered iron metabolism and the antioxidant status in patients with HCV-related advanced fibrosis. These effects of BCAA may partially account for their inhibitory effects on HCC development in patients with cirrhosis.

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References

1. Seeff LB. Natural history of chronic hepatitis C. Hepatology 2002; 36: S35–46.
2. Lawitz E, Mangia A, Wyles D, et al. Sofosbuvir for previously untreated chronic hepatitis C infection. N Engl J Med 2013b; 368(20): 1878–87.
3. Yamato M, Muto Y, Yoshida T, Kato M, Moriwaki H. Clearance rate of plasma branched-chain amino acids correlates significantly with blood ammonia level in patients with liver cirrhosis. Int Hepatol Commun 1995; 3: 91–6.
4. Nishitani S, Ijichi C, Takehana K, Fujitani S, Sonaka I. Pharmacological activities of branched-chain amino acids: specificity of tissue and signal transduction. Biochem Biophys Res Commun 2004; 313: 387–9.
5. Kuwahata M, Yoshimura T, Sawai Y, et al. Localization of polypyrimidine-tract-binding protein is involved in the regulation of albumin synthesis by branched-chain amino acids in HepG2 cells. J Nutr Biochem 2008; 19: 438–47.
6. Nishitani S, Matsumura T, Fujitani S, et al. Leucine promotes glucose uptake in skeletal muscles of rats. Biochem Biophys Res Commun 2002; 299: 693–6.
7. She P, Reid TM, Bronson SK, et al. Disruption of BCAATm in mice leads to increased energy expenditure associated with the activation of a futile protein turnover cycle. Cell Metab 2007; 6: 181–94.
8. Muto Y, Sato S, Watanabe A, et al. Overweight and obesity increase the risk for liver cancer in patients with liver cirrhosis and long-term oral supplementation with branched chain amino acid granules inhibits liver carcinogenesis in heavier patients with liver cirrhosis. Hepatol Res 2006; 35: 204–14.
9. Iwasa J, Shimizu M, Shiraki M, et al. Dietary supplementation with branched-chain amino acids suppresses diethylnitrosamine-induced liver tumorigenesis in obese and diabetic C57BL/KsJ-db/db mice. Cancer Sci 2010; 101: 460–7.
10. Yoshiji H, Noeguchi R, Kaji K, et al. Attenuation of insulin-resistance-based hepatocarcinogenesis and angiogenesis by combined treatment with branched-chain amino acids and angiotensin-converting enzyme inhibitor in obese diabetic rats. J Gastroenterol 2010; 45: 443–50.
11. Farinati F, Cardin R, De Maria N, et al. Iron storage, lipid peroxidation and glutathione turnover in chronic anti-HCV positive hepatitis. J Hepatol 1995; 22: 449–56.
12. Kato J, Kobune M, Nakamura T, et al. Normalization of elevated hepatic 8-hydroxy-2′-deoxyguanosine levels in chronic hepatitis C patients by phlebotomy and low iron diet. Cancer Res 2001; 61: 8697–702.
13. Korenaga M, Wang T, Li Y, et al. Hepatitis C virus core protein inhibits mitochondrial electron transport and increases reactive oxygen species (ROS) production. J Biol Chem 2005; 280: 37481–8.
14. Nishina S, Hino K, Korenaga M, et al. Hepatitis C virus-induced reactive oxygen species raise hepatic iron level in mice by reducing hepcidin transcription. Gastroenterology 2008; 134: 226–38.
15. Furutani T, Hino K, Okuda M, et al. Hepatic iron overload induces hepatocellular carcinoma in transgenic mice expressing the hepatitis C virus polyprotein. Gastroenterology 2006; 130: 2087–98.
16. Beard MR, Abell G, Honda M, et al. An infectious molecular clone of a Japanese genotype 1b hepatitis C virus. Hepatology 1999; 30: 316–24.
17. Lerat H, Honda M, Beard MR, et al. Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. Gastroenterology 2002; 122: 352–65.
18. Murao N, Ishigai M, Yasuno H, Shimonaka Y, Aso Y. Simple and sensitive quantification of bioactive peptides in biological matrices using liquid chromatography/selected reaction monitoring mass spectrometry coupled with trichloroacetic acid clean-up. Rapid Commun Mass Spectrom 2007; 21: 4033–8.
19. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959; 37: 911–7.
20. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265–75.
21. Harrison-Findik DD, Schafer D, Klein E, et al. Alcohol metabolism-mediated oxidative stress down-regulates hep-
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hepatitis C patients treated with interferon and ribavirin. Am J Gastroenterol 2005; 100: 332–7.

39. Kawaguchi T, Izumi N, Charton MR, Sata M. Branched-chain amino acids as pharmacological nutrients in chronic liver disease. Hepatology 2011; 54: 1063–70.

40. Kawaguchi T, Shiraiishi K, Ito T, et al. Branched-chain amino acids prevent hepatocarcinogenesis and prolong survival of patients with cirrhosis. Clin Gastroenterol Hepatol 2014; 12: 1012-8.e1.

41. Formisano P, Oriente F, Fiore F, et al. Insulin-activated protein kinase C beta bypasses Ras and stimulates mitogen-activated protein kinase activity and cell proliferation in muscle cells. Mol Cell Biol 2000; 20: 6323–33.

42. Sandhu MS, Dunger DB, Giovannucci EL. Insulin, insulin-like growth factor-I (IGF-I), IGF binding proteins, their biologic interactions, and colorectal cancer. J Natl Cancer Inst 2002; 94: 972–80.

43. Kawaguchi T, Nagao Y, Matsuoka H, Ide T, Sata M. Branched-chain amino acid-enriched supplementation improves insulin resistance in patients with chronic liver disease. Int J Mol Med 2006; 22: 105–12.

44. Suzuki K, Suzuki K, Koizumi K, et al. Measurement of serum branched-chain amino acids to tyrosine ratio level is useful in a prediction of a change of serum albumin level in chronic liver disease. Hepatol Res 2008; 38: 267–72.

45. Michitaka K, Hiraoka A, Kume M, et al. Amino acid imbalance in patients with chronic liver diseases. Hepatol Res 2010; 40: 393–9.

46. Kohijima M, Higuchi N, Kato M, et al. SREBP-1c, regulated by the insulin and AMPK signaling pathways, plays a role in nonalcoholic fatty liver disease. Int J Mol Med 2008; 21: 507–11.

47. Kerner J, Hoppel C. Fatty acid import into mitochondria. Biochim Biophys Acta 2000; 1486: 1–17.

48. Iwasa M, Kobayashi Y, Mifuji-Moroka R, et al. Branched-chain amino acid supplementation reduces oxidative stress and prolongs survival in rats with advanced liver cirrhosis. PLoS ONE 2013; 25(8); e70309.

49. Muto Y, Sato S, Watanabe A, et al. Effects of oral branched-chain amino acid granules on event-free survival in patients with liver cirrhosis. Clin Gastroenterol Hepatol 2005; 3: 705–13.

50. Ijichi C, Matsumura T, Tsuji T, Eto Y. Branched-chain amino acids promote albumin synthesis in rat primary hepatocytes through the mTOR signal transduction system. Biochem Biophys Res Commun 2003; 303: 59–64.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Twenty five patients with HCV-related advanced fibrosis enrolled Human BCAA supplementation study. Advanced fibrosis defined liver specimens (METAVIR fibrosis staging: >F3,4) or Fib-4 index (>3.25).

Fig. S2. HDAC activity of HCV TgM fed the excess-iron diet with BCAA was significantly lower than those of HCVTgM fed the excess-iron diet with casein or the control diet. All samples were nuclear which was extracted from liver tissue.