Protective effects of branched-chain amino acids on hepatic ischemia-reperfusion-induced liver injury in rats: a direct attenuation of Kupffer cell activation

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Kitagawa T, Yokoyama Y, Kokuryo T, Nagino M. Protective effects of branched-chain amino acids on hepatic ischemia-reperfusion-induced liver injury in rats: a direct attenuation of Kupffer cell activation. Am J Physiol Gastrointest Liver Physiol 304: G346–G355, 2013. First published December 28, 2012; doi:10.1152/ajpgi.00391.2012.—We determined whether there is a protective effect of branched-chain amino acid (BCAA) on hepatic ischemia-reperfusion (I/R)-induced acute liver injury. Wister rats were divided into the following four groups: simple laparotomy with vehicle; simple laparotomy with BCAA (1 g/kg body wt orally); I/R (30 min clamp) with vehicle; and I/R with BCAA. Serum liver function tests and the gene expression of adhesion molecules (intercellular adhesion molecule and vascular cell adhesion molecule) and vasoconstrictor-related genes (endothelin-1) in the liver were examined. In the in vivo study, portal venous pressure, leukocyte adhesion, and hepatic microcirculation were evaluated. Furthermore, Kupffer cells were isolated and cultured with various concentrations of BCAA in the presence or absence of lipopolysaccharide (LPS). Increased levels of liver function tests following I/R were significantly attenuated by BCAA treatment. The increased expression of adhesion molecules and endothelin-1 was also significantly attenuated by BCAA treatment. Moreover, increased portal venous pressure, enhanced leukocyte adhesion, and deteriorated hepatic microcirculation following I/R were all improved by BCAA treatment. In the experiment using isolated Kupffer cells, the expression of interleukin-6, interleukin-1β, and endothelin-1 in response to LPS stimulation was attenuated by BCAA in a dose-dependent fashion. These results indicate that perioperative oral administration of BCAA has excellent therapeutic potential to reduce I/R-induced liver injury. These beneficial effects may result from the direct attenuation of Kupffer cell activation under stressful conditions.

Inflammatory responses; microcirculatory failure

During liver resection, ischemia-reperfusion (I/R) is a mandatory procedure to minimize intraoperative blood loss. However, this procedure unfailingly induces liver injury and may sometimes lead to major postoperative complications such as liver failure (9). I/R-induced liver injury is also a major problem in the process of liver transplantation (3, 6). Therefore, minimizing I/R-induced liver injury is crucial to safely performing liver surgery.

The major mechanism of hepatic I/R-induced liver injury includes an enhanced inflammatory response (12) and microcirculatory failure (7). The upregulation of inflammatory cytokines in combination with an upregulation of adhesion molecules in the sinusoidal endothelial cells leads to the rolling, adhesion, and migration of leukocytes (14). The increased production of vasoconstrictors such as endothelin-1 (ET-1) leads to presinusoidal and sinusoidal vasoconstriction in the liver through an autocrine or paracrine mechanism (7). Moreover, the sensitivity of the hepatic microcirculatory system to vasoconstrictors is also enhanced after I/R (36). These changes activate inflammatory processes and microcirculatory disturbances in the liver, which finally results in severe hepatic injury.

Branched-chain amino acid (BCAA) is a group of essential amino acids comprised of valine, leucine, and isoleucine. In Japan, oral BCAA preparations containing valine, leucine, and isoleucine in a composition ratio of 1.2:2:1 have been used to correct protein and amino acid abnormalities in patients with chronic liver disease and hepatic encephalopathy (26). In addition, recent studies indicate that long-term administration of BCAA is effective in reducing the risk of cancer development in viral hepatitis (10, 39). However, the effect of BCAA administration on hepatic I/R-induced liver injury has never been investigated. In this study, we hypothesized that oral administration of BCAA can attenuate excessive inflammatory responses, leukocyte adhesion, upregulation of vasoconstrictors, and microcirculatory liver failure and may also prevent liver damage following I/R.

Materials and Methods

Animals

Male Wistar rats (Charles River Labs, Wilmington, MA) weighing 250–300 grams were purchased from Japan SLC (Nagoya, Japan) and housed in a temperature- and humidity-controlled room under a constant 12:12-h light-dark cycle. Animals were allowed free access to water and food at all times. All experiments were performed in compliance with the guidelines of and approved by the Institute for Laboratory Animal Research, Nagoya University Graduate School of Medicine.

Surgical Procedures

Rats were anesthetized with diethyl ether. After an upper abdominal midline laparotomy, the hepatoduodenal ligament was clamped for 30 min with a vascular clip (BEAR Medic, Chiba, Japan) for the I/R groups. Thereafter, the vascular clip was removed, and the liver was reperfused for 24 h. Although severe mesenteric congestion was observed during the clamping of the hepatoduodenal ligament, no animal died within 24 h. In the sham group, only laparotomy and mobilization of the hepatoduodenal ligament were performed. Twenty-four hours after the I/R or sham operation, blood samples were collected, and liver tissue samples were removed for analysis (n = 6 in each group).

Administration of BCAA

Oral BCAA preparations (containing valine, leucine, and isoleucine in a composition ratio of 1.2:2:1) were provided by Ajinomoto Pharmaceutical (Tokyo, Japan). The protocol of BCAA administration was determined based on previously published studies with minor...
modification (20, 23). Briefly, BCAA was dissolved in 0.5% methylcellulose solution and administered orally (1 g/kg body wt). The same volume of 0.5% methylcellulose solution was used as a vehicle. In the I/R group, BCAA or vehicle was administered 0.5 h before ischemia and 7 h after reperfusion and 0.5 h before sampling. This administration protocol was established through multiple preliminary studies. We performed a protocol with only one time administration before I/R, only one time administration after I/R, or multiple administrations before and after I/R. Although these protocols showed a similar trend, multiple administrations showed best results. BCAA or vehicle was also administered on the same time scale in the sham operation group.

Analysis of Blood Samples

Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), hyaluronic acid (HA), and total bilirubin (T-Bil) were measured by standard laboratory methods (SRL, Tokyo, Japan). Serum concentrations of aromatic amino acids (AAA), BCAA, and methionine were also measured by standard laboratory methods (SRL). Fischer’s ratio was calculated by the ratio of serum BCAA to AAA concentrations.

Histologic Evaluation

The liver tissue samples were immersed immediately in 10% buffered formalin and stored overnight. The samples were then dehydrated in a graded ethanol series and embedded in paraffin. Six-micrometer-thick sections were mounted on glass slides and stained with hematoxylin and eosin. The number of necrotic focus was counted in paraffin-embedded liver samples by a third researcher who has no association with this study. The data were expressed as the number of necrotic foci in high-power fields (HPF). Ten fields from one specimen were evaluated for three animals in each group. Other sections were subjected to immunohistochemistry to detect the expression of vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM). The automated slide preparation system Discovery XT (Ventana Medical Systems, Tucson, AZ) was used. Before staining, paraffin sections were heated at 37°C for 30 min in a paraffin oven and were blocked with 5% nonfat milk. The staining procedure was carried out according to the manufacturer’s protocol (Ventana Medical Systems). Anti-VCAM antibody (Abcam, Cambridge, UK) and anti-ICAM antibody (Abcam) were diluted in Discovery Ab diluent (Ventana Medical Systems).

Real-Time PCR for Inflammatory Cytokines and Vasoconstrictor Genes

To validate the gene expression changes in the liver, quantitative real-time PCR analysis was performed with a Prism 7300 sequence detection system (Applied Biosystems, Foster City, CA). Total RNA was isolated from whole liver or Kupffer cells using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. cDNA was generated from total RNA samples by use of the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Each reaction was performed in a 20-μl mixture with TaqMan universal PCR master mix according to the manufacturer’s instructions (Applied Biosystems). The expression of the genes encoding adhesion molecules [VCAM (assay identification no. Rn00563627_m1) and ICAM (assay identification no. Rn00564227_m1)], inflammatory cytokines [interleukin-1β (IL-1β; assay identification no. Rn00580432_m1) and interleukin-6 (IL-6; assay identification no. Rn99999011_m1)], and vasoconstrictor-related proteins (ET-1; assay identification no. Rn00561129_m1) in the liver homogenate or the isolated Kupffer cells was determined by comparative quantitative real-time PCR using the Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA). 18S rRNA (assay identification no. Hs99999901_s1) was used as an endogenous control. The reaction mixture was denatured for 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, 50 cycles at 95°C for 15 s, and 1 cycle at 60°C for 1 min. All
samples were tested in duplicate, and the average values were used for quantification. The analysis was performed using MxPro TM Software version 2.00 (Stratagene) according to the manufacturer’s instructions. The comparative cycle threshold ($C_T$) method ($\Delta \Delta C_T$) was used for quantification of gene expression. The average of the sham group with vehicle treatment was set as onefold induction, and the data were adjusted to that baseline.

Measurement of Portal Venous Pressure

A total of 24 h after the I/R or sham operation, the animals were anesthetized with pentobarbital sodium (50 mg/kg body wt, ip). Portal venous pressure was measured by Power Lab AD Instruments (Castle Hill, Bella Vista, Australia) through a catheter (22-gauge) inserted in the portal vein ($n = 6–8$ in each group).

In Situ Intravital Microscopy

**Evaluation of leukocyte adhesion.** A total of 24 h after the I/R or sham operation, the animals were anesthetized with pentobarbital sodium (50 mg/kg body wt, ip). Leukocytes were labeled by intravenous administration of rhodamine 6G (0.1, 437 mg/kg; Sigma-Aldrich, St. Louis, MO). As the liver preparation was stabilized on a fluorescence microscope, the liver surface was epi-illuminated with a LED excitation system (Cool LED pE excitation system) using 532–554 nm excitation and 573–613 nm emission band-pass filters to visualize fluorescent rhodamine 6G-labeled leukocytes in the sinusoids. In all experiments, four fields consisting of four to eight acini per field were recorded using a x20 objective ($n = 6$ in each group). The number of adherent leukocytes was determined during playback of videotaped images.

**Evaluation of liver microcirculation.** Red blood cells (RBCs) were isolated and labeled using the PKH 67 Green Fluorescent Cell Linker Mini Kit for general cell membrane labeling (Sigma-Aldrich) according to the manufacturer’s protocol. The liver surface was epi-illuminated with an LED excitation system (Cool LED pE excitation system) using 464.5–499.5 nm excitation and 516–556 nm emission band-pass filters. The images were recorded by a Digital CameraC10600 ORCA-R2 and processed using Aquacosmos software (Hamamatsu Photonics, Hamamatsu, Japan). Three acini were randomly recorded in each rat ($n = 4$ in each group). Measurements of perfused sinusoidal diameters ($D_s$) were made directly from video playback (5). For measurement of the RBC velocity ($V_{RBC}$) in the sinusoids, 0.2 ml of fluorescent-labeled RBCs were injected in the carotid artery. The volumetric flow (VF) was calculated from the $D_s$ and $V_{RBC}$ by the following equation as previously described: $VF = V_{RBC} \times \pi \times (D_s/2)^2$ (38).

**Kupffer Cell Isolation**

The Kupffer cells were obtained by an in situ collagenase digestion method. The liver was perfused by oxygenized Hanks’ balanced salt solution (GIBCO-BRL, Gaithersburg, MD) for 10 min to wash out the blood and was perfused with 0.05% collagenase (Sigma-Aldrich) for 5 min. After the digestion, the cell suspension was filtered through a sieve and centrifuged two times at 50 g for 2 min to separate the parenchymal and nonparenchymal cells. Nonparenchymal cells were collected and centrifuged at 2,000 g for 15 min (4 °C). Supernatants were discarded, and pellets were suspended with RPMI 1640 (Invitrogen, Carlsbad, CA). Cells were centrifuged at 800 g for 15 min through Percoll (GE Healthcare, Little Chalfont, UK) to remove contaminating nonparenchymal cells. Collected Kupffer cells were centrifuged at 300 g for 15 min. The pellets were collected, and the cells were resuspended in RPMI 1640. The concentration of cells was adjusted to $1 \times 10^5$ cells/ml in RPMI, and 1 ml of cell suspension was added per well to a 12-well culture dish. After incubation for 3 h, the plates were washed with warm RPMI to remove nonadherent cells, and 1 ml of RPMI was added. After 24 h, the adherent Kupffer cells were treated with vehicle or various concentrations of BCAA. After 30 min of vehicle or BCAA treatment, the Kupffer cells were stimulated with LPS (5 µg/ml) in the medium to induce inflammatory cytokine and ET-1 gene expression. Purity (>90%) was checked by the uptake of latex beads (Sigma-Aldrich) in the Kupffer cells.

**Assessment of Proinflammatory Cytokine Release**

IL-6 levels in the supernatant from the Kupffer cell culture were determined by sandwich enzyme-linked immunosorbent assay (ELISA).
methods. The assay was performed according to the manufacturer’s protocol (R&D, Minneapolis, MN).

Statistical Analysis

Student’s t-test was used to compare the significant differences between two groups. Significant differences among multiple groups were analyzed by a one-way ANOVA followed by the Dunnet test. When criteria for parametric testing were violated, the appropriate nonparametric (Mann-Whitney U-test) was used. A P value of <0.05 was considered significant. All results are presented as means ± SE.

RESULTS

Blood Tests

The serum levels of AST, ALT, HA, and T-Bil significantly increased following I/R with vehicle treatment (Fig. 1, A–D). However, all of these levels were significantly lower in the BCAA treatment group compared with the group with vehicle treatment. The serum levels of AAA were significantly higher following I/R compared with those levels following the sham operation (Fig. 2A). However, in the I/R with BCAA treatment group, the serum levels of AAA were significantly lower than in the I/R with vehicle treatment group (Fig. 2A). Fischer’s ratios (BCAA/AAA) were significantly lower following I/R compared with those following the sham operation in the group with vehicle treatment. However, with BCAA treatment, these ratios were substantially increased (Fig. 2B). The serum levels of methionine, another amino acid that is related to liver injury (25), were significantly higher following I/R compared with those following the sham operation in the group with vehicle treatment. However, these levels were restored to the levels of the sham group by BCAA treatment (Fig. 2C).

Histologic Evaluation

Livers in the sham group showed normal histology irrespective of BCAA administration, indicating that a simple laparotomy did not damage the liver parenchyma [sham + vehicle (Fig. 3A); sham + BCAA (Fig. 3B)]. In contrast, some necrotic foci were observed in the I/R + vehicle group (Fig. 3C).

![Fig. 3. Histology of the livers in the sham + vehicle (A), sham + BCAA (B), I/R + vehicle (C), and I/R + BCAA (D) groups. The micrographs depict representative hematoxylin and eosin staining of paraffin-embedded liver slides, recorded using a ×4 objective. Several necrotic foci were observed in the I/R + vehicle group (arrows). The average no. of necrotic foci in high-power fields (HPF) (E). Ten fields from one specimen were evaluated for three animals in each group. *P < 0.05 vs. I/R + vehicle.](http://ajpgi.physiology.org/doi/figure-pdf/10.1152/ajpgi.00391.2012/3)
However, signs of necrosis were rarely observed in the I/R + BCAA group (Fig. 3D). The average number of necrotic foci in randomly selected HPF was significantly higher in the I/R + vehicle group than that in the I/R + BCAA group (Fig. 3E).

**Leukocyte Adhesion**

The level of leukocyte adhesion observed by intravital microscopy was significantly greater in the I/R group with vehicle treatment compared with that in the sham group. However, the level was significantly lower in the I/R group with BCAA treatment (Fig. 4).

**Hepatic Hemodynamics**

As previously reported (16, 22, 38), the levels of portal venous pressure in the I/R group were significantly higher compared with the sham group (Fig. 5A). However, these levels in the I/R group with BCAA treatment were significantly lower compared with those in the I/R group with vehicle treatment. The average $D_s$ were significantly greater in the I/R with BCAA treatment group compared with the I/R with vehicle treatment group (Fig. 5B). Although the $V_{RBC}$ was almost identical between the I/R with vehicle treatment and BCAA treatment groups, the average $V_F$ of the sinusoids was significantly higher in the I/R with BCAA treatment group compared with the vehicle treatment group (Fig. 5, C and D). The representative images of intravital microscopy in the I/R with vehicle and BCAA treatment groups are shown in Fig. 5, E and F, respectively.

**Gene Expression of Adhesion Molecules and Vasoconstrictor Genes**

The gene expression levels of VCAM and ICAM in the liver were significantly higher in the I/R with vehicle treatment group with BCAA treatment were significantly lower compared with those in the I/R group with vehicle treatment. The average $D_s$ were significantly greater in the I/R with BCAA treatment group compared with the I/R with vehicle treatment group (Fig. 3E).

![Fig. 4. The level of leukocyte adhesion in the liver evaluated by intravital microscopy in the sham + vehicle group (A), sham + BCAA group (B), I/R + vehicle group (C), and I/R + BCAA group (D). The level of leukocyte adhesion shown in a HPF (E). Four fields consisting of 4–8 acini/field were recorded using a ×20 objective. The no. of adherent leukocytes was determined during playback of videotaped images. $\dagger P < 0.05$ vs. sham + vehicle; $\ast P < 0.05$ vs. I/R + vehicle.](http://ajpgi.physiology.org/)
compared with the sham group (Fig. 6, A and B). However, these changes were significantly attenuated by BCAA treatment. The gene expression levels of ET-1, a potent vasoconstrictor in the liver, showed a similar trend (Fig. 6 C).

Immunohistochemistry for VCAM and ICAM

To confirm the expression of VCAM and ICAM in the liver tissue, immunohistochemistry was performed. After I/R with vehicle treatment group, an increased expression of VCAM and ICAM in the sinusoidal endothelial cells was observed (Fig. 7, A and C). However, the expression was substantially suppressed in the I/R with BCAA treatment group (Fig. 7, B and D).

Study of Isolated Kupffer Cells

The mechanisms of liver injury in response to various hepatic stresses, including I/R (34), endotoxemia [exposure to the lipopolysaccharide (LPS)] (33), and alcohol consumption (2, 21), are similar. Under these stressful conditions, the Kupffer cells are activated and produce excessive levels of inflammatory cytokines (i.e., IL-6 and IL-1β) (1) and vasoconstrictors (i.e., ET-1) (29). These changes lead to an enhanced inflammatory response and microcirculatory failure, thereby resulting in liver damage (37). Therefore, we next determined whether the BCAA directly modulated the excessive activation of Kupffer cells. In isolated Kupffer cells stimulated with LPS, the gene expression of inflammatory cytokines (such as IL-6 and IL-1β) and ET-1 was significantly upregulated (Fig. 8, A–C). However, the expression levels were attenuated by BCAA administration in the medium in a dose-dependent manner. We also determined the protein levels of IL-6 after LPS treatment were substantially suppressed with 40 mM.
BCAA in the medium compared with the levels without BCAA administration (1,135 ± 612 pg/ml without BCAA vs. 78 ± 39 mg/ml with BCAA).

**DISCUSSION**

In this study, we found that the preoperative administration of BCAA significantly attenuated post-I/R liver injury. Moreover, we demonstrated that the beneficial effects of BCAA on I/R-induced liver injury are at least partly explained by the direct attenuation of Kupffer cell activation. These are novel findings that have never been reported before and may have a substantial clinical impact on liver surgery. The results indicate that the perioperative oral administration of BCAA has excellent therapeutic potential for use in attenuating I/R-induced liver injury.

Fischer’s ratio is important for assessing hepatic functional reserve in patients with liver cirrhosis (28). A low level of Fischer’s ratio indicates impaired physiological liver function, including protein metabolism and synthesis. The rationale of BCAA supplementation in these patients is to normalize amino acid profiles and nutritional status. In this study, we found significantly lower Fischer’s ratios following the I/R group compared with those following the sham group. Moreover, the levels of AAA and methionine were also significantly higher in the I/R group than the sham group. These results indicate a functional alteration in amino acid metabolism in the liver in response to I/R stress. Although the precise mechanism was not elucidated in this study, supplementation of BCAA at least alleviated these alterations. It should be noted that the dose of BCAA used in this study seems pretty high (1 g/kg body wt) compared with that used in humans. Metabolic pathway of BCAA in the liver may be different between rat and human (27). Therefore, it is difficult to directly extrapolate the results of animal studies to humans. Nevertheless, there are numerous reports showing a similar beneficial effect of BCAA administration both in the animal (11, 23) and human (15, 19) studies. To determine the optimal dose of BCAA in protecting I/R-induced liver damage in humans, a clinical study including patients who undergo hepatectomy while clamping hepatoduodenal ligament should be performed.

One of the major mechanisms of hepatic injury following I/R stress includes an excessive inflammatory response (18). Under the stress of I/R, the adhesion, rolling, and migration of leukocytes is induced through the upregulation of adhesion molecules in both leukocytes and sinusoidal endothelial cells (30). The ICAM and VCAM molecules are representative adhesion molecules that are upregulated on endothelial cells following I/R stress (8). As demonstrated in this study, BCAA treatment significantly attenuated the upregulation of ICAM and VCAM following I/R. Decreased leukocyte adhesion on the sinusoidal endothelial cells was also observed by intravital microscopy. These effects may reduce excessive inflammatory responses and ameliorate liver injury following I/R.

Another important mechanism of hepatic injury following I/R is microcirculatory failure (4, 31). Under I/R stress, ET-1, one of the most potent vasoconstrictors, is upregulated in the liver and acts on hepatic microvasculature through a paracrine and/or autocrine mechanism (17, 24). Moreover, the constricive response of the portal vasculature system to ET-1 is also enhanced through the upregulation and remodeling of endo-

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**Fig. 6.** The expression levels of the genes encoding adhesion molecules (vascular cell adhesion molecule [VCAM] (A) and intercellular adhesion molecule [ICAM] (B)) and vasoconstrictor-related proteins [endothelin-1 (ET-1)] (C) in the liver, detected by real-time RT-PCR. Total RNA was extracted from the frozen liver tissue and was subjected to comparative quantitative real-time RT-PCR using specific primers. 18S rRNA was used as an internal control for each sample. The average expression level of the sham + vehicle group’s data was set to 1.0, and other data were adjusted to this baseline. †P < 0.05 vs. sham + vehicle; *P < 0.05 vs. I/R + vehicle.
thelin receptors (35, 36). Although the mRNA expression of ET-1 is robustly upregulated following I/R, BCAA treatment significantly attenuated this change. In concordance with the upregulation of ET-1, hepatic microcirculatory failure is commonly observed following I/R stress. Although the sinusoidal $D_s$ become narrower compared with normal levels following I/R, they were significantly greater in the group with the BCAA treatment compared with the vehicle treatment. We speculate that these differences are partly explained by the attenuated expression of ET-1 in the liver due to BCAA treatment.

Under various hepatic stresses such as endotoxemia, I/R, alcoholic consumption, and biliary obstruction, enhanced inflammatory response (13) and microcirculatory failure (32) commonly occur, and these changes finally lead to liver damage. Kupffer cells, which are hepatic resident macrophages, play a crucial role in these pathophysiological changes (13). Therefore, in the final experiment, we hypothesized that BCAA treatment directly modulates Kupffer cell activation. Kupffer cells were isolated from normal liver, and these cells were stimulated with LPS with/without BCAA preadministration in the medium. As was expected, the upregulated mRNA expression of inflammatory cytokines and ET-1 by LPS stimulation were significantly attenuated by adding BCAA (30 min before LPS stimulation) to the medium in a dose-dependent fashion. Although the precise metabolic mechanisms on the molecular level remain unknown, we believe that the protective effect of BCAA on I/R-induced liver injury is at least partly mediated by the direct modulation of the Kupffer cell function by BCAA. Further study is needed to elucidate this novel pharmacological effect by BCAA.

During major hepatectomy, repeating I/R is a necessary procedure to minimize intraoperative bleeding. However, this procedure paradoxically induces I/R-related injury to the liver and may sometimes lead to severe postoperative complications. Based on our findings in this study, a preoperative administration of BCAA may have excellent therapeutic potential to reduce postoperative liver injury. This hypothesis should be investigated through a randomized controlled study.

In summary, this study is the first to demonstrate a protective effect of BCAA treatment on hepatic I/R-induced liver injury. The administration of BCAA before and following ischemia significantly attenuated I/R-induced inflammatory responses and microcirculatory failure. The major mechanisms of these beneficial effects may include the direct attenuation of Kupffer cell activation in response to I/R stress. These results strongly indicate the therapeutic potential of BCAA in preventing the hepatic I/R-induced liver injury that inevitably occurs during liver surgery.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

Author contributions: T. Kitagawa, Y.Y., and T. Kokuryo conception and design of research; T. Kitagawa and Y.Y. performed experiments; T. Kitagawa, Y.Y., and T. Kokuryo analyzed data; T. Kitagawa, Y.Y., and T. Kokuryo interpreted results of experiments; T. Kitagawa and Y.Y. prepared...
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**IL-6**

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**ET-1**

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Fig. 8. Kupffer cells were isolated and incubated for 20 h. The adherent Kupffer cells were treated with vehicle or various concentrations of BCAA. After 30 min of vehicle or BCAA treatment, the Kupffer cells were stimulated with LPS (5 μg/ml) in the medium. After 4 h, the expression levels of the genes encoding interleukin-6 (IL-6, A), interleukin-1β (IL-1β, B), and ET-1 (C) were detected by real-time RT-PCR. LPS, lipopolysaccharide. *P < 0.05 vs. 0 mM BCAA.

**REFERENCES**


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