Expression of tubulin folding cofactor B in mouse hepatic ischemia-reperfusion injury

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Abstract. The aim of the present study was to investigate the association between tubulin folding cofactor B (TBCB) expression and ischemia-reperfusion injury (IRI) in mice. A total of 48 C57BL/6 mice were randomly divided into a control group (Sham, n=6) and an ischemia-reperfusion group (n=42). The ischemia-reperfusion group was further divided into 6 subgroups as per different times after reperfusion (2, 4, 6, 8, 12 and 24 h), with 7 mice per subgroup. A hepatic IRI model was established in mice by clamping the hepatic hilum. Morphology, serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α), and the expression level of TBCB were detected. Compared with the control group, the livers from the ischemia-reperfusion group were significantly changed, particularly at 12 h following ischemia-reperfusion, with obvious hepatic cell degeneration and necrosis. The ALT, AST, IL-6 and TNF-α levels in the sera of the mice in the hepatic ischemia-reperfusion group were increased at all time points following ischemia-reperfusion, and were the highest at 12 h, demonstrating statistically significant differences when compared with the control group (P<0.05). Furthermore, the expression levels of TBCB, TNF-α and IL-6 were significantly increased at all time-points following ischemia-reperfusion, and were the most significant at 12 h. At 24 h following ischemia-reperfusion, the expression levels had decreased. The present study indicated that TBCB expression is associated with TNF-α and IL-6 expression levels in mice with hepatic ischemia-reperfusion, and may be key in the development of liver injury during ischemia-reperfusion in mice.

Introduction

Ischemia-reperfusion injury (IRI) refers to the phenomenon in which the injury to tissues or organs is aggravated subsequent to restoration of the blood or oxygen supply based on tissue or organ ischemia. The degree of IRI experienced in liver surgery directly affects the function of the liver and its viability following surgery, affecting clinical prognosis; therefore, the pathophysiological changes of hepatic IRI have consistently been the focus of studies (1,2).

Tubulin folding cofactor B (TBCB) is an important member of the TBC family in cells. It is important for the proper folding of β-tubulin and the formation of α/β-tubulin heterodimers, which are critical for the normal growth of mammalian cells (3). Cell microtubules predominantly exist in the cytoplasm. As a component of the cellular spindle, eukaryotic cilium, centrosomes and other organelles, cell microtubules participate in the maintenance of cell morphology, cell polarity, cell motility, cell division, and intracellular transport, along with other cell biological functions, such as cytoskeleton formation (4).

Hypoxia-ischemia is a common clinical pathological process that causes extensive cell injury. The pathological process of this injury is extremely complex and is associated with a variety of factors. Changes in the cytoskeleton are significant in the occurrence and development of hypoxic-ischemic injury (5,6). However, although TBCs may be involved in tumorigenesis, there are few studies on the expression of TBCs in IRI. In the current study, a hepatic ischemia-reperfusion model was established by clamping of the hepatic hilum of the mice in order to restore blood perfusion (7). TBCB expression in the liver at certain time-points and the association between the changes in TBCB expression and liver function under such pathological conditions were observed to investigate the pathophysiological changes in hepatic IRI from a novel perspective, and to provide a theoretical basis for its prevention and treatment.
Materials and methods

Reagents and instruments. TRIZol reagent was purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) kits (SYBR® Premix Ex Taq™II (cat. no. DRR820A) and Prime Script RT Reagent kit (cat. no. RR047A)) were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Enzyme-linked immunosorbent assay (ELISA) kits (Mouse IL-6 Quantikine ELISA kit; cat. no. M6000B) for interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α) were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). A biochemical analyzer was purchased from Olympus and the ABI Prism 7300 Real-Time PCR System was purchased from Applied Biosystems (Thermo Fisher Scientific, Inc.).

Animals. A total of 48 healthy, male C57BL/6 mice (body weight, 18-20 g) were purchased from and reared at the Experimental Animal Center of Guilin Medical College (Guilin, China). All animals were used in accordance with institutional guidelines and the current experiments were approved by the Animal Ethics Committee of Guilin Medical College. The mice were randomly divided into a control group (Sham, n=6) and an ischemia-reperfusion group (n=42). The mice in the ischemia-reperfusion group were further divided into 6 subgroups according to the different time durations following reperfusion (2, 4, 6, 8, 12 and 24 h), with 7 mice per subgroup (ischemia and reperfusion group at six time points, n=7 per group, and n=6 in the corresponding control group). Prior to the experiment, the mice were fasted for 12 h, with free access to drinking water. According to Pringle's method (8) (hepatic portal occlusion), a model of total hepatic IRI (hepatic portal occlusion for 30 min) was established. The mice were sacrificed at 2, 4, 6, 8, 12 and 24 h after reperfusion, and the specimens were subsequently collected.

Western blot analysis. To prepare the protein lysate, 1 ml radioimmunoprecipitation assay buffer and 10 µl phenylmethylsulfonyl fluoride were added to 100-µg tissue samples, followed by homogenization on ice. After the protein concentration was measured using the bicinchoninic acid method, the protein samples (sham, 2.77 µl; 2 h, 3.23 µl; 4 h, 3.15 µl; 6 h, 3.4 µl; 8 h, 3.31 µl; 12 h, 3.4 µl; 24 h, 3.72 µl) were subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (at 80 V for 60 min) and blocked with 5% skimmed milk. The membrane was then incubated with the primary antibodies (TBCB, IL-6 and TNF-α; dilution, 1:1,000 overnight and then with the secondary antibodies, peroxidase-conjugated rabbit anti goat IgG (H+L) [dilution, 1:5,000 (cat. no. ZB-2306); ZSGB-BIO] and peroxidase-conjugated mouse anti rabbit IHC kit; Maixin, Fujian, China) to detect the absorbance in each field for statistical analysis.

Biochemical test. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured using an Olympus AU2000 automatic biochemical analyzer. Serum IL-6 and TNF-α levels were determined by competitive inhibition ELISA. The procedures were performed according to the ELISA kit manufacturer instructions.

Immunohistochemistry (IHC) assay. The liver tissue samples were fixed in 10% formaldehyde solution, embedded in paraffin, and sliced into sections with a thickness of 5 µm. Following dehydration in an ethanol gradient that was replaced with xylene, hematoxylin and eosin (H&E) staining was performed to observe the morphological changes under a light microscope. Pathological scores were determined according to the degree of tissue injury. The TBCB (cat. no. ab96101), IL-6 (cat. no. ab7737) and TNF-α (cat. no. ab6671) antibodies were all purchased from Abcam (Cambridge, MA, USA). Subsequent to dewaxing with xylene and dehydration through an ethanol gradient, the sections were incubated with 0.3% hydrogen peroxide for 10 min to remove the endogenous peroxidase, followed by a phosphate-buffered saline rinse, antigen repair in ethylenediaminetetraacetic acid solution at high pressure, and blocking with horse serum (cat. no. ZLI-9023; ZSGB-BIO, Beijing, China). The sections were incubated with primary antibody (TBCB, 1:300; IL-6, 1:200; TNF-α, 1:50) for 1 h and then with biotin-labeled secondary antibody (cat. no. KIT-5920; MaxVisionTM2 kit goat anti-mouse/rabbit IHC kit; Maixin, Fujian, China) for 1 h at 37°C. After adding 100 µl freshly prepared diaminobenzidine solution, color development was terminated in a timely manner (10-20 min) and observations were made under an Olympus CX41 microscope. This was followed by rinsing with distilled water, counterstaining with hematoxylin and rinsing with tap water for blue color recovery. The sections were then dried through an ethanol gradient, cleared by xylene and mounted with neutral balsam. Five non-overlapping visual fields were randomly selected. The positive-expression regions in the fields were observed at a magnification of x400, with density scanning performed using a quantitative digital pathology image analysis system (cat. no. H9-HMIAS-2000; Xuzhou City Technology Co., Ltd., Xuzhou, China) to detect the absorbance in each field for statistical analysis.

RT-qPCR. Total RNA was extracted from the liver tissue samples using TRizol, and 2 µg total RNA was used to synthesize cDNA with the RT kit (Prime Script RT Reagent kit). The primer sequences were as follows: Forward, 5'-AGT AGGCTTTCCTCCATTCAC-3' and reverse, 5'-ACTTAC AGATTTCAAGCCA for TBCB; forward, 5'-GCATGGAGCTCTGGGCAAT-3', and reverse, 5'-CTAGAGAAGTCTTG CGTGG3'-3' for β-actin. The concentrations of each of the primers were 100 nmol/l. PCR amplification was performed
using the fluorescence qPCR kit (SYBR® Premix Ex Taq™ II). The cycle threshold values were obtained from the PCR curve, and the relative expression levels of the target genes were calculated, with β-actin serving as the internal reference (9).

**Statistical analysis.** The experimental data were analyzed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). The data were in line with the normal distribution in the normal test and are represented as means ± standard deviations. One-way analysis of variance (ANOVA) was used for inter-group comparisons, and ANOVA with repeated measurements was used for intra-group comparisons. P<0.05 was considered to indicate a statistically significant difference and pairwise t-tests were used to compare quantitative data.

**Results**

**Hepatic IRI model established using Pringle’s method.** Not including the the group in which mice were sacrificed at 2 h after perfusion, the mice were awake and had free access to food and drinking water within 4 h of surgery. No abdominal infection was detected and all of the mice survived. In the control group (Fig. 1A), the morphology of the hepatic cells was observed under a light microscope and appeared normal, with no obvious oedema. At 2 h after ischemia-reperfusion (Fig. 1B) the changes were mild and at 4 h after reperfusion (Fig. 1C), obvious oedema was apparent in the hepatic cells. Six hours after reperfusion (Fig. 1D), the oedema in the hepatic cells was further aggravated and at 8 h after perfusion (Fig. 1E), point or flaky necrosis, or large zones of necrosis were observed in the liver samples. At 12 h after reperfusion (Fig. 1F), the necrosis was most severe, showing sheet necrosis, hepatic sinusoidal pressure and no obvious liver cord structure, with a large quantity of infiltrated lymphocytes in the portal area, demonstrating the most obvious changes in cell morphology. At 24 h after reperfusion (Fig. 1G), compensatory recovery of hepatic cells was observed, with mild oedema in the hepatic cells.

**Expression levels of ALT, AST, IL-6 and TNF-α were upregulated in hepatic IRI.** ALT (Fig. 2A) and AST (Fig. 2B) levels in the serum at 2, 4, 6, 8, 12 and 24 h following ischemia-reperfusion were significantly higher than those in the control group. At 12 h post-surgery, the expression levels were the highest, but decreased by 24 h post-surgery and the differences were statistically significant (P<0.05).

IL-6 and TNF-α serum levels of the ischemia-reperfusion group were significantly higher than those of the control group (P<0.05). The IL-6 (Fig. 2C) and TNF-α (Fig. 2D) expression levels at 2, 4, 6, 8, 12 and 24 h after hepatic ischemia-reperfusion were significantly higher than those of the control group at the corresponding time-points after ischemia-reperfusion. At 12 h post-surgery, the expression levels were the highest, but decreased by 24 h post-surgery; the differences were statistically significant (P<0.05).

**TBCB expression level was upregulated in hepatic IRI.** TBCB expression levels (mRNA and protein) at 2, 4, 6, 8, 12 and 24 h following ischemia-reperfusion were significantly higher than those of the control group (P<0.05). There were no significant differences in TBCB mRNA (Fig. 3A) and protein (Fig. 3B and C) expression levels among the different subgroups in the ischemia reperfusion group (P>0.05). The TBCB expression level was highest at 12 h post-surgery, but had decreased by 24 h post-surgery (P<0.05).

**Upregulated expression levels of IL-6, TNF-α and TBCB in hepatic IRI were correlated.** Expression levels of IL-6, TNF-α and TBCB at different time-points following hepatic IRI were detected by IHC. The positive expression of IL-6 in hepatic cells at 6, 8, 12 and 24 h following ischemia-reperfusion was observed. IL-6 expression gradually increased from 6 h to its highest level at 12 h, but decreased by 24 h (Fig. 4A). Compared with the control group, positive TNF-α expression in the hepatic cells at 4, 6, 8, 12 and 24 h following ischemia-reperfusion was observed. TNF-α expression gradually increased to its highest level at 12 h, but decreased by 24 h (Fig. 4B). TBCB expression levels at 6, 8, 12 and 24 h following ischemia-reperfusion
in the ischemia-reperfusion group were significantly higher compared with the control group, the expression gradually increased from 6 h to its highest level at 12 h, but decreased by 24 h (Fig. 4C).

To determine whether any correlation existed between TBCB and IL-6 and TNF-α expression during hepatic IRI of mice, the correlation between the level of TBCB protein expression in hepatic tissue, and serum IL-6 and TNF-α expression levels was evaluated. TBCB expression was identified to be positively correlated with IL-6 (Fig. 4D) and TFN-α (Fig. 4E).

**Discussion**

IRI is a common pathophysiological process in liver surgery and is inevitable in surgical procedures, including shock resuscitation, liver transplantation and liver lobectomy. IRI often markedly affects the clinical prognosis. Furthermore, the pathophysiology of hepatic IRI is very complex and has not been fully elucidated. Therefore, IRI prevention or mitigation is a current research focus.

The cytoskeleton is a complex three-dimensional network structure composed of protein filaments in the eukaryotic cytoplasm that consists of cell microtubules, microfilaments and intermediate fibers. The main role of the cytoskeleton is to stabilize and maintain the cell morphology, and to support the mutual association between the cellular and nuclear membranes. In addition, the cytoskeleton is involved in cell movement, cell polarity, cell division, and cytoplasmic transport, with an important significance in signal transduction (4).
Cell microtubules are one of the main components of the cytoskeleton. Microtubules primarily exist in the cytoplasm as a component of the cellular spindle, eukaryotic cilium, centrosomes and other organelles, and are composed of α- and β-tubulin (each with molecular weights of ~55 kDa). Microtubule formation is a complex process involving multiple molecules, such as TBCs (TBCA, TBCB, TBCC, TBCD and TBCE) and multiple microtubule-associated proteins. A heterodimer is formed with α- and β-tubulin, and 13 heterodimers arrange in a round coil to form a microtubule with a relatively stable tubular structure and a diameter of ~25 nm (10). The majority of the microtubule fibers are in a dynamic state of assembly and disassembly, which is a necessary process for performing their functions. Microtubules constitute the intracellular network scaffold to maintain cell morphology, which is associated with cell motility. In addition, microtubules are involved in the regulation of the L-calcium current on the cell membrane and are closely associated with cellular electrophysiological activities. Ischemia-reperfusion causes injuries to the microtubules and damages the network structure of the cytoplasm, leading to the loss of support in the cell membrane and increased cell fragility (11-13).

TBCs were initially demonstrated to be folding proteins. The current study demonstrated that TBCs are predominantly involved in the folding and degradation of the tubulin complex, playing a significant role in the functional diversity and dynamic equilibrium of microtubules (14). TBCB, one of the important members of the TBC family, is very important in the proper folding of β-tubulin and the formation of α/β-tubulin (3). Studies have found that abnormal expression levels of TBCB and TBCE may directly cause microtubule abnormalities (15). Furthermore, it has been confirmed that TBCB is closely associated with tumorigenesis and tumor metastasis. For example, the level of TBCB expression in breast cancer tissues was significantly upregulated, and TBCB overexpression may increase the degree of malignancy in breast cancer cells (16).

Currently, the majority of studies have suggested that IRI is associated with the excessive production of oxygen free radicals, calcium overload, inflammatory response, energy metabolism disorders and apoptosis (17-19). Apoptosis is an important cause of severe liver damage and organ dysfunction during ischemia-reperfusion (20,21). Apoptosis is a multifactorial, multi-step, and multi-path complex process, while oxygen free radicals, energy metabolism disorders, intracellular calcium overload, cytokines, caspases, and the B-cell lymphoma 2 family all induce apoptosis. In the implementation of cell apoptosis, the significance of the role of caspase proteins is often above that of cytoskeleton proteins. By activating and blocking certain specific substrates (i.e.,

Figure 4. TBCB expression level positively correlates with the expression levels of IL-6 and TNF-α. (A-C) Expression levels of IL-6, TNF-α and TBCB in hepatic cells at 6, 8, 12 and 24 h following ischemia-reperfusion were detected by immunohistochemistry. Correlation analysis was performed between (D) TBCB and IL-6 expression levels and (E) TBCB and TNF-α expression levels. TBCB, tubulin folding cofactor B; IL-6, interleukin 6; TNF-α, tumour necrosis factor-α.
proteins involved in cytoskeletal regulation), caspase proteins indirectly reconstitute the structure of cells, resulting in the morphological changes observed in apoptosis. Furthermore, after hypoxic-ischemic injury, the intracellular Ca\(^{2+}\) concentration may alter. The binding of Ca\(^{2+}\) and calmodulin activates a series of protein kinases to react on cytoskeleton proteins, thus leading to cytoskeletal disruption or reformation (22).

The alterations in the AST and ALT expression levels in the model group were significantly higher than those in the sham surgery group. ALT and AST are important indicators of liver injury. In the modeling process of hepatic IRI, these enzymes are effective indicators that demonstrate the success of modeling and the degree of liver injury. In IRI, numerous inflammatory mediators are released to activate the complement system. Kupffer cells, neutrophils, monocytes and eosinophils in the liver exhibit signs of infiltration and chemotaxis towards the ischemic region, thus activating the NADPH/NADH oxidase system and generating a large quantity of oxygen free radicals, also known as respiratory burst, resulting in hepatic cell injury (23). Numerous studies have confirmed that TNF-\(\alpha\) participates in the pathophysiology of hepatic IRI (24,25). TNF-\(\alpha\) causes the release of cytokines, such as IL-1\(\beta\), IL-6, and IL-8 (26). In the present study, during the process of hepatic ischemia-reperfusion, the IL-6 and TNF-\(\alpha\) expression levels were significantly higher than those of the sham surgery group, which was consistent with the findings in the pathological H&E staining, thus indicating that TNF-\(\alpha\) and IL-6 participate in the process of hepatic IRI, and are important in the IRI-induced inflammatory response. In the current study, the pathological changes of hepatic IRI were simulated by establishing a hepatic IRI model in mice. TBCB expression levels in injured liver tissue samples, at different time-points subsequent to reperfusion, were investigated. The TBCB expression level was identified to be significantly higher in injured liver tissue samples when compared with tissue samples from the sham surgery group, indicating that TBCB may be involved in hepatic IRI, indicating that early detection, diagnosis and prevention of liver diseases in clinical treatment are possible. However, the underlying mechanism of TBCB in hepatic IRI remains unclear. Therefore, further studies regarding TBCB expression levels and the signal transduction pathways involving TBCB have important clinical significance for improving IRI, organ transplantation and gene therapy.

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