Original Article

HIBADH Plays an Important Role in the Course of Liver Cell Necrosis

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Objective To observe the biological function of human 3-hydroxyisobutyrate dehydrogenase (HIBADH).

Methods Human 3-hydroxyisobutyrate dehydrogenase (HIBADH, 3-hydroxy-2-methyl propanoate: NAD⁺ oxidoreductase) recombinant protein was expressed in *E. coli* BL21, and purified by Ni²⁺ column. The special antisera was obtained from rabbits immunized by this purified antigen. On the distribution of HIBADH, it was found that HIBADH over-expressed in the injured liver cells when serious hepatitis occurred. The phenomenon was confirmed in the animal models of SD rats with acute liver cell injury induced by CCl₄, but this phenomenon did not exist in the models induced by endotoxin combined with galactosamine. Further more, HIBADH's overexpression in liver cells will induce cell necrosis through the pathway of oxidative stress.

Results When the liver cells injured by drug or other chemical materials, HIBADH will be compensationally over-expressed for the deficiency of energy, so liver cells can make enough ATP through brand-chain amino acid catabolism. However, the overexpression of HIBADH will be harmful for liver cells through the product of much more active oxygens which will induce the cell necrosis.

Conclusions HIBADH over-expression is a signal of the liver cell metabolism injury, and it can aggravate the liver cell injury through oxidative stress.

Key words: 3-hydroxyisobutyrate dehydrogenase; Brand-chain amino acid; Liver cell; Necrosis; Oxidative stress

The 3-hydroxyisobutyrate dehydrogenase (HIBADH) is an enzyme in the catabolic pathway of valine. It was named NS5ATP1 in our Lab. The catabolic pathway of valine shares some similarities with that of the other brand-chain amino acids (BCAAs) and can be divided into three stages. During the first stage, valine is reversibly transaminated to 2-oxoisovalerate. In the second stage, 2-oxoisovalerate enters the irreversible part of valine catabolism and be converted to 3-hydroxyisobutyrate (HIB) by mitochondrial enzymes. In the third stage, HIB is oxidized by 3-hydroxyisobutyrate dehydrogenase (HIBADH) into methylmalonic-semialdehyde, and then enzymatically converted to a member of the citrate cycle, succinyl-CoA. Thus, the catabolism of valine can serve an anaplerotic function for the citrate cycle.

Whether HIBADH/NS5ATP1 is important in liver injury remains unclear. The results in this paper indicated that HIBADH/NS5ATP1 was forced to overexpressed and overexpression of HIBADH/NS5ATP1 aggravated liver injury through oxidative stress.

MATERIALS AND METHODS

Materials

There are some researches focused on purified 3-hydroxyisobutyrate dehydrogenase from rabbit liver, its construction and enzymology feature.¹⁻³ But there is no document on its medical value. Therefore, in this study, HIBADH was prepared through the method of prokaryotic expression and then antisera was obtained from immunized rabbits. Then immunohistochemistry of liver tissue under physiological and pathological conditions was carried out with the antisera. In order to get more information about HIBADH, the plasmid

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of pEGFP-HIBADH was constructed and expressed in liver cells to observe its biological functions.

Restriction enzyme *EcoR* I and *BamH* I and DNA marker was purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. pEGFP-C1 vector was the product of BD Biosciences Clontech. pET32a(+) vector was from EMD Chemicals Inc. A mixture of dNTPs and Taq DNA polymerase were from Bio-lab Co., Ltd. Sequence specific primers were purchased from Invitrogen. Skimmed milk powder was from Clontech Inc. Rabbit polyclonal antibody to GFP were purchased from Proteintech Group Inc. Goat anti-rabbit IgG conjugated peroxidases were from Santa Cruz Biotechnology Inc. BALB/c mice and SD rats were purchased from Vitalriver Inc. (Beijing, China).

Cloning and expression of the HIBADH, purification of HIBADH and generation of antisera

HIBADH coding sequence was subcloned into the pET-32a(+) prokaryotic expression vector (his-tag) and sequenced for confirmation. Then the pET-32a-HIBADH recombinant expression plasmid was transformed into competent BL21 cells. HIBADH was harvested as inclusion body when the *E. coli* BL21 was induced by 1 mM IPTG at 37°C. Inclusion was dissolved in Buffer A (8 M Urea, 0.5 M NaCl, 20 mM PB, 20 mM imidazole, pH 7.4). HIBADH recombinant protein was bound to Ni²⁺ column in Buffer A. After the column was washed by large volume of Buffer B (20 mM imidazole, 0.5 M NaCl, 20 mM PB, pH 7.4), the purified protein was eluted from the column by Buffer C (500 mM imidazole, 0.5 M NaCl, 20 mM PB, pH 7.4). For immunization, two rabbits were initially (day 0) injected subcutaneously with 2 ml of emulsion containing 1 mg of purified HIBADH in complete Freund's adjuvant. Boosting was carried out on days 14, 28 and 42 using 0.5 mg of HIBADH in Freund's incomplete adjuvant. Antibody titers were determined by ELISA, the two rabbits showing titers > 1 : 100 000. A week after the last booster injection, rabbits were decapitated on day 71 and sera were prepared.

Tissue samples, cells and animal model of liver cell injuries induced by CCl₄, lipopolysaccharide and galactosamine

The human severe hepatitis specimens and normal liver tissues were collected from the Beijing Ditan

Hospital, Capital Medical University. The Bel-7402 cell lines were provided by our lab and cultured in standard conditions (10% fetal bovine serum, 5% CO₂). The SD rats models of acute liver injury were induced by intraperitoneal inject with 0.03 ml/kg of 50% CCl₄ diluted with olive oil or 10 µg/kg of lipopolysaccharide with 900 µg/kg galactosamine. The liver tissues of SD rats were obtained 8 hours later.

Immunohistochemistry analysis on the distribution of HIBADH in liver tissues

The tissue array plate was blocked with 3% BSA at 37°C for 1 hour, then probed with HIBADH antibody (1 : 10 000) at 4°C in a humid chamber for 12 hours. After that, they were rinsed three times by PBS and incubated with secondary rabbit antibody for 1 hour at 37°C (1 : 1 000). This antibody complex was visualized by 3, 3'-diaminobenzidine (Zymed Laboratories Inc.). Sections were counterstained with hematoxylin for 5 min and mounted with Eukit (Calibrated Instrument). The results were observed under a light microscope.

HIBADH expression in vitro

HIBADH coding sequence was subcloned into the pEGFP-C1 expression vector (GFP-tag) and sequenced for confirmation. The sense primer for pEGFP-C1 was 5'-CGGAATTC-TATGGCAGCCTCCTTG-3' and the antisense was 5'-CGGGATCCCGGAAGGTCTCCTC-3'. The sequence underlined is restriction site for subcloning. Then the pEGFP-C1-HIBADH recombinant expression plasmid was transformed into competent *DH5α* cells. The purified plasmid was extracted, and the A₂₆₀/A₂₈₀ was 1.9. Human hepatocellular carcinoma Bel-7402 cells conserved by our laboratory were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 µg/ml of streptomycin in a humidified chamber at 37°C in 5% CO₂. Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Triplicates were carried out for each transfections.

HIBADH expression in vivo

For high expression of HIBADH in liver cells, mice were injected with 25 µg pEGFP-C1-HIBADH

Figure 1. 12% SDS-PAGE analysis on the purified His-HIBADH recombinant protein.

Notes: The purified His-HIBADH recombinant protein was analysed by 12% SDS-PAGE. A purified His-HIBADH recombinant protein of 56 kD was observed and the purity was in line with the requirements of experiment.

plasmid DNA in 2.5 ml saline via tail veins.⁴ The course of DNA injection was completed in less than 5 s. After 8 h, animals were killed and the liver was dissected and immediately frozen by dry ice. Ten-micron sections were made using Cryostat (CMT 950 Series, Research Manufacturing Company, Tucson, AZ, USA). Tissue samples were observed under fluorescence microscope.

Detection of the oxidative stress reaction in liver cells

Bel-7402 cells transfected with pEGFP-C1-HIBADH was cultured as description. Dihydroethidium was added into medium (10 μ M). After incubated for 30 min away from light at 37°C, the cells were observed. The nuclear will be stained into red under the fluorescence microscope.

Immunoblot analysis

Bel-7402 cell culture homogenates were analyzed by SDS-polyacrylamide gel electrophoresis by 5% stacking gel and 12% separating gel. Proteins were transferred onto a nitrocellulose membrane (45 V constant voltage in pre-cooled transfer buffer for 1.5 h) and residual antibody binding sites on the membrane were saturated by incubation in Tris-buffered saline (TBS), pH 7.5, supplemented with 0.05% (w/v) Tween 20/5% (w/v) skimmed milk powder for 4 h. The membranes were washed three times with washing buffer [TBS/0.05% (w/v) Tween 20] and subsequently incubated with anti-HIBADH serum (diluted

Figure 2. Detection of GFP-HIBADH recombinant protein expressed in the Bel-7402 cells by Western blot.

Notes: To investigate the specificity of rabbit antisera, Bel-7402 cells were transfected with the vector pEGFP-C1-HIBADH (Lane 2) or pEGFP-C1 (Lane 1). After incubated for 24 h, total protein was extracted. After electrophoresis by 12% SDS-PAGE, Western blot was performed. The first antibody was polyclonal antibody made by ourselves and the titer was 1 : 10 000. The second antibody was goat anti-rabbit IgG conjugated with peroxidase diluted by 1 : 2 000. The immuno-signal was visualized by an enhanced chemiluminescent detection system. Lane 1, total protein from the cells transfected with pEGFP-C1. Lane 2, total protein from the cells transfected with pEGFP-C1-HIBADH.

as 1 : 10 000 in washing buffer) at 22°C for 4 h. After three times of washing with washing buffer, the membrane was incubated with goat anti-rabbit IgG-HRP conjugates [diluted 1 : 2 000 in TBS/0.05% (w/v) Tween 20] at 22°C for 1 h. The membrane was washed three times with washing buffer and once with PBS. Subsequently, a signal was developed by an enhanced chemiluminescent detection kit, according to the manufacturer's instructions.

RESULTS

Preparation of His-HIBADH fusion protein and its polyclonal antibody

As the antibody for HIBADH was not commercially available, full-length His-HIBADH fusion protein (Figure 1) and the polyclonal rabbit antibody against it were prepared. The pEGFP-HIBADH recombinant plasmid was constructed and transfected into Bel-7402 cells. The whole proteins of the transfected cells were extracted by Western blot using the HIBADH rabbit antibody to detect the expression of HIBADH. As illustrated in Figure 2, a band of pEGFP-HIBADH fused protein could be detected by anti-HIBADH antibodies. On the contrary, no band was observed for pEGFP vector-transfected cell protein by anti-HIBADH

antibody. Therefore, the prepared rabbit polyclonal antibody for HIBADH was specific, and it could be used for further experiments. Furthermore, it was confirmed that the pEGFP-HIBADH fusion protein was expressed exactly in liver cells.

Up-regulation of HIBADH in chemical damaged liver cells and tissues

To demonstrate HIBADH expression at the protein level in human liver tissues, liver tissues from patients with serious hepatitis and normal liver were analyzed by immunohistochemistry. It was illustrated in Figure 3, the different intensity between live tissues with serious hepatitis and normal liver tissues. The image showed that HIBADH was not expressed in normal liver tissues, but there were many positive stained cells in tissues with serious hepatitis. Even more, the stained cells in tissues with serious hepatitis lost the normal shape and occurred necrosis.

In order to clarify HIBADH over-expression in which kinds of liver cells, two kinds of SD rats liver injury models were created. One was acute liver injury induced by CCl_4 , the other was induced by LPS associated with galactosamine and the control was normal SD rats. The liver tissues were sliced and then analyzed by HE staining and immunohistochemistry.

It could be concluded that HIBADH had no expression in normal liver tissues and SD rats model induced by LPS associated with galactosamine, but there were many positive stained cells in the models induced by CCl_4 (Figure 4).

Over-expression of HIBADH induces liver cells necrosis

In order to clarify what happened on earth in the cells when they express HIBADH, a plasmid expressing GFP-HIBADH (pEGFP-C1-HIBADH) was constructed. After transfected with the plasmid, HCC Bel-7402 cells which expressed GFP-HIBADH can be found under fluorescence microscope. Through in vitro experiment, the course of cell necrosis can be observed and finally the green fluorescence expressed by cells disappeared in no longer than 30 hours (Figure 5).

In order to confirm this phenomenon, an animal model was established, pEGFP-C1-HIBADH plasmid was transfected into mouse liver by tail vein hyperpnea injection. Eight hours later, the liver was frozen and then sliced immediately. Frozen sections were observed under fluorescence microscope and the images were obtained. There were many cells expressing GFP-HIBADH in the liver of mice, but they had lost normal shape. In order to get a clearer

Figure 3. Immunohistochemical analysis of HIBADH in human liver tissues.

Notes: B, C and D were specimens from patients with severe hepatitis observed by immunohistochemical methods. As control, A was from normal liver tissue. The first antibody was polyclonal antibody and the titer was 1 : 10 000. The second antibody was goat anti-rabbit IgG conjugated with peroxidase diluted by 1 : 2 000. The immuno-signal was visualized by an enhanced chemiluminescent detection system. In the figure, there were many positive stained cells in tissues with serious hepatitis, HIBADH was over-expressed and cells shape were severely damaged with cracked or tumid nuclear.

Figure 4. HIBADH over-expression is different in two kinds of SD rats liver injury models (200×).

Notes: A and B were normal SD rats as control. C and D were from acute liver injury models induced by CCl_4 . E and F were from models induced by LPS associated with galactosamine. The different liver tissues from SD rats were sliced and then analyzed by HE staining (left) and immunohistochemical analysis (right). The liver pathological changes mainly around central veins were induced by CCl_4 and the immunohistochemistry test was also positive; the liver pathological changes induced by LPS associated with galactosamine were also diffused, but immunohistochemistry test was negative.

Figure 5. The necrosis course of Bel-7402 cells expressing GFP-HIBADH (100×).

Notes: Bel-7402 cells expressing GFP-HIBADH at 15 (A, B and C), 17 (D, E and F) and 19 (G, H and I) hours after transfection were observed, respectively. The cells expressing GFP only (J, K and L) were taken as the control. Every three horizontal pictures formed a group, the left pictures were observed with a microscope under white light, the middle ones with blue light to stimulate GFP for observation, and the right pictures were images that overlapped the left and middle images. All magnification of these pictures was 100 times. The course of necrosis was observed and finally the cells expressing green fluorescence disappeared in no longer than 30 hours. However, the control cells which only expressed GFP remained the normal shape.

Figure 6. The liver cells expressing GFP-HIBADH were damaged in SD rats (400 $\times$).

Notes: A and B were from the mouse liver transfected with pEGFP-C1 plasmid by tail vein hyperpiesia injection. C and D were transfected with pEGFP-C1-HIBADH. From the control (A and B), the cells expressing GFP kept normal shape. On the contrary (C and D), necrosis was observed in the cells expressing GFP-HIBADH. A and C were observed with a microscope under blue light. B and D were stained with immunohistochemistry method.

Figure 7. The cells over-expressing GFP-HIBADH were damaged by oxidative stress (200 $\times$).

Notes: Cellular nuclei were stained with dihydroethidium, figures A and D were observed with green fluorescence under fluorescence microscope, B and E were observed with blue fluorescence, and C and F were overlaps of A and B, D and E, respectively. In the left 3 images (A, B and C), the cells expressing GFP-HIBADH was in the course of necrosis. The nuclear was highlighted with red fluorescent staining on behalf of the cells under oxidative stress (A). The green fluorescence representing GFP-HIBADH was successfully expressed (B). Many cells lost normal shape and their nuclear was stained into red for oxygenic stress. However, in the right 3 images as control, the cells expressed GFP kept normal shape and their nuclear were not stained.

conclusion, the left lobe of liver tissue was paraffin embedded and sliced. Immunohistochemical detection was performed with the antibody of anti-GFP. As the same to the images under fluorescence, these cells lost normal shape and were dyed brown. On the control, the liver cells only expressing GFP kept normal (Figure 6).

The cells over-expressing HIBADH lysed for oxidative stress

In order to explore the reasons of necrosis, the pEGFP-C1-HIBADH plasmid was transfected into HCC Bel-7402 cells and then dihydroethidium was added into medium (10 μ M) at the peak time of green fluorescence. The cellular nuclear under oxidative stress was stained into red under the fluorescence microscope. Necrosis and red nuclear were observed in HCC Bel-7402 cells expressing GFP-HIBADH, which were not observed in cells only expressing GFP (Figure 7).

DISCUSSION

HIBADH is an enzyme of valine catabolism.⁵ When cells can not get enough energy under pathologic condition, they will metabolize branched-chain amino acid for more energy. The HIBADH reflects the level of valine catabolism. The pathway responsible for valine catabolism has been partially characterized in mammals.

HIBADH has been previously purified from rabbit liver,¹ while not from human. In this article, sequence of HIBADH from human was obtained and expressed by prokaryotes and eukaryotes. Because it was completely from human, its advantages were more than those obtained from animals.

HIBADH expression in normal liver cells was hardly found before. On the contrary, when hepatitis happened, the expression of HIBADH changed significantly. Furthermore, the results in different SD rat liver injury models induced by CCl₄ or galactosamine with endotoxin were different. HIBADH was over-expressed in the injured liver induced by medicines, but this phenomenon was not observed in livers injured by infectious diseases. The result was in accordance with the function of HIBADH, so it can be concluded that HIBADH overexpression is relevant to liver medical injury. When injured by medicine, liver cells will over-

express HIBADH in human liver. The possible reason may be that cells decompose branched-chain amino acid for compensatory energy.⁵

When artificially overexpress HIBADH in liver cells both in vivo and in vitro, cells necrosis would happen for oxidative stress. Thus HIBADH overexpression is not beneficial for the inner condition of cells, oxide over-production would induce necrosis. Necrosis must be associated with the overconsumption of NADH or NADPH by HIBADH in the course of decomposing branched-chain amino acid for compensatory energy.^{3,6}

These results demonstrate that HIBADH is relevant to the necrosis of liver cells. When liver was injured by medicine, the level of HIBADH in liver cells will rise, because HIBADH can decompose branch chain amino acid and supply more energy. However, HIBADH over-expression will make liver cells under oxidative stress and then damage. Thus, the supply of enough energy or reduced concentration of chemical substances in the blood is helpful for liver cells injured by medicine. HIBADH plays an important role in the course of liver cell necrosis.

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