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RESEARCH ARTICLE

Involvement of Caspase-Dependent and Independent Pathway in Apoptotic Action of Extrinsic β-Hydroxybutyrate in Liver Tissue of Mice

Teng Wei[§], Mingfang Bi[§], Fangning Liu[§], Tingting Lv[§], Wulin Tian[§] and Guanghong Xie*

College of Veterinary Medicine, Jilin University, Changchun 130062, China
*Corresponding author: xiegh@jlu.edu.cn

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ABSTRACT

β-hydroxybutyric acid (β-HB) is generated by degradation of fatty acids in the liver. β-HB level in serum increases significantly in a variety of metabolic disorder diseases, such as hyperketonemia and diabetic ketoacidosis, in which hepatocyte apoptosis have an important role. To investigate the effect of exogenous β-HB on the liver, mice were injected intraperitoneally with β-HB (7.5 mmol/kg) one time, three times and five times at 24 h intervals, respectively. Compared to the distilled water injection group, β-HB level in liver tissue increased significantly, while the triglycerides, high density lipoprotein cholesferol (HDL), low density lipoprotein cholestorol (LDL), alkaline phosphatase (ALP) in serum have not changed in the three β-HB injection groups. β-HB promoted the activation of caspase-12, caspase-9, caspase-3 and EndoG, up-regulated Bax and down-regulated of Bcl-2.

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INTRODUCTION

Ketone bodies are produced by the liver and used as an energy source by brain, skeletal muscle and heart when glucose is not readily available. Ketone bodies includes acetoacetate, acetone and β-hydroxybutyric acid (β-HB), while β-HB accounted for seventy percent of total. Generally β-HB level maintains at a low level in serum, for example, its postprandial level is approximately 0.05 mM in humans (Laeger *et al*., 2010; Zhang *et al*., 2013). Significant increase of circulating ketone bodies level is observed in some pathological physiological states such as diabetic ketoacidosis, alcoholic ketoacidosis, salicylate poisoning and other rare conditions (Jain *et al*., 1998; Laffel, 1999).

Most investigators agree that normal serum level of ketone bodies is less than 0.5 mM, more than 1.0 mM can be defined as hyperketonemia, and more than 3.0 mM can be defined as ketoacidosis (Fulop *et al*., 1999). In these diseases, liver function is impaired in different degrees. It suggests us whether increased β-HB level is associated with the development of these diseases.

Hepatocyte apoptosis occurs in many metabolic disorder diseases, such as toxic liver injury, chronic viral hepatitis, non-alcoholic and alcoholic liver disease (Alkhouri *et al*., 2011; Schattenberg *et al*., 2006). A known endogenous path trigger by endoplasmic reticulum stress, leading to caspase-12 activation, finally activates downstream caspase-3, a executor of apoptosis (Filipiak *et al*., 2012). Another exogenous pathway is associated with mitochondrial dysfunction, impaired mitochondrial releases cytochrome C, then activates caspase-9, and finally leads activation of caspase-3. Release of cytochrome C might accompany with AIF and EndoG into the nucleus, leading to DNA damage (Matias *et al*., 2013). Bax and Bcl-2 locate on mitochondrial membrane and endoplasmic reticulum membrane, their ratio can reflect the extent of membrane damage (Qi *et al*., 2012).

Several studies have found that parenteral administration of β-HB in pygmy goats suppress feed intake and subcutaneously injected β-HB (10 mmol/kg body weight) significantly reduced feeding in rats (Rossi *et al*., 2000). However, less well known is the role of β-HB in the pathogenesis of hepatocyte apoptosis. Hence, we study the pro-apoptotic effect of β-HB on hepatocyte in liver of mice. This research provides a novel understanding of β-HB, and contributes to explain those diseases accompanied by increased β-HB level.

MATERIALS AND METHODS

Materials: β-HB was obtained from Sigma Aldrich (USA). β-actin antibody was obtained from Sungene Biotech (China). Caspase-9, caspase-3, Bax, Bcl-2

[§]These authors contributed equally to this work

antibodies were obtained from Boster Biotechnology (China). Caspase-12 antibody was obtained from CST (USA). AIF and ENDOG antibodies were obtained from Santa Cruz (USA). All the other reagents, unless otherwise stated, were purchased from Sigma Aldrich (USA).

Animals and treatment: Twenty-four adult female Kunming mice weighing 25-28 g were used, provided by the Center of Experimental Animals of Baiqiuen Medical College, Jilin University. All animal experiments were approved by the Jilin University IACUC.

The mice were randomly divided into four groups (6 mice per group) as follows: Control group (CG) were injected intraperitoneally with vehicle five times at 24 h intervals then sacrificed 24 h after last injection. The first, second and third group were injected intraperitoneally with β-HB (7.5 mmol/kg), one time, three times and five times at 24 h intervals, respectively, then sacrificed 24 h after last injection. Liver tissues were rapidly excised, snap-frozen in liquid nitrogen and kept at -80°C.

Sample preparation and detection: Blood was gained from the eyeballs of mice and centrifuged for serum. Liver tissues were made 20% homogenate and centrifuged for supernate. Triglycerides and β-HB were measured by enzymic method, ALP was measured by rate method, HDL and LDL were measured by direct one-step method in the second hospital of Jilin University..

Western blot analysis: Liver tissues were lysed in RIPA lysis buffer containing complete protease inhibitor cocktail and phosphatase inhibitors mixture. Proteins (30 μg) were separated by SDS-PAGE and electro-transferred onto polyvinylidene fluoride membranes. The blots were incubated with specific antibodies. After rinsed in TBST, the blots were incubated with appropriate secondary antibodies conjugated to horseradish peroxidase. Immunoreactive band of proteins were detected by an enhanced chemiluminescence detection system, and the membrane was exposed to an X-ray film.

Data analysis: Statistical analyses were performed using the SPSS software. Significances between groups were determined by ANOVA. All values are expressed as the mean±S.E.M. The statistical significances were achieved when *P<0.05 or ** P<0.01.

RESULTS

Biochemical parameters: Compared to the Control group, concentrations of triglycerides, HDL, LDL, ALP in serum have no significantly difference, while the concentrations of β-HB in liver have significantly increased in the three β-HB injection groups (Table 1).

Effects of β-HB on Bax and Bcl-2 expression: To investigate the effect of β-HB on Bax and Bcl-2, two protein in caspase-dependent apoptotic pathway were detected by Western blotting in liver tissues.. Compared to the Control group, we found that β-HB induced the ratio of Bax/Bcl-2 increased by about 2.2, 11.5, 77.3 fold in the first, second and third group, respectively (Fig. 1A and B).

Effects of β-HB on caspase expression: To study whether β-HB induces stressed-ER in liver tissues, the level of caspase-12 was measured, total protein samples from liver were analyzed by Western blotting. Compared to the Control group, we found that β-HB increased caspase-12 activation by 2.3, 3.0, 2.2 fold in the first, second and third group, respectively (Fig. 2A and B).

The activation of caspase-3 and caspase-9 were detected by Western blot analyses. As can be seen in Fig. 3A and B. Compared to the Control group, we found that β-HB significantly elevated the expression of cleavaged caspase-9 by 1.3, 2.7, 3.0 fold, cleavaged caspase-3 to 1.7, 3.4, 6.0 fold in the first, second and third group, respectively.

Effects of β-HB on AIF and EndoG expression: AIF and EndoG were measured by Western blotting. Compared to the Control group, we found that β-HB increased the expression of EndoG by 1.5, 2.3, 3.5 fold in the first, second and third group, respectively, while the expression AIF have not changed significantly (Fig. 4A and B).

DISCUSSION

Apoptosis is a highly preserved and controlled mechanism to achieve tissue homeostasis through targeted elimination of single cells without disrupting the biological functionality of the tissue. Some common clinical metabolic disorder diseases are characterized by chronic course, accompanied by liver function dysfunction, liver atrophy, even development for liver cirrhosis or liver cancer (Guicciardi and Gores, 2010). Meanwhile, high β-HB level is observed in these diseases (Laffel, 1999). As the main metabolic place of β-HB between liver function dysfunction and increased β-HB level there may be inevitable connection. Therefore, we suspect high level of β-HB leads hepatocyte apoptosis, and further deterioration of liver diseases.

Ketogenesis takes place in the mitochondria of hepatocytes. The process includes the following steps: β-oxidation of fatty acids to acetyl CoA, formation of acetoacetyl CoA, conversion of acetoacetyl CoA to 3-hydroxy-3-methylglutaryl CoA (HMG CoA) and then to AcAc; and finally reduction of AcAc to β-HB (Crawford *et al*., 2009). As store form of fatty acid in the body,

Table 1: Triglycerides, HDL, LDL, ALP in serum and β-HB in liver of mice

	Control group	1st group	2nd group	3rd group
TG (mmol/l)	1.03±0.42	0.63±0.13	0.83±0.34	0.73±0.07
HDL (mmol/l)	1.11±0.08	1.21±0.26	1.15±0.78	1.02±0.19
LDL (mmol/l)	0.34±0.02	0.47±0.14	0.49±0.88	0.33±0.03
ALP (U/L)	269.67±86.75	230.33±22.23	269.33±75.75	244.00±20.88
β-HB (mmol/kg)	0.17±0.07	0.50±0.05**	0.36±0.05*	0.47±0.16**

Mice were injected intraperitoneally with distilled water and β-HB one time, three times and five times at 24 h intervals, respectively, to assess the contents of triglycerides, HDL, LDL, ALP in serum and β-HB in liver tissues. The data were presented as means±S.E.M (n=6). *P<0.05, **P<0.01 vs distilled water group

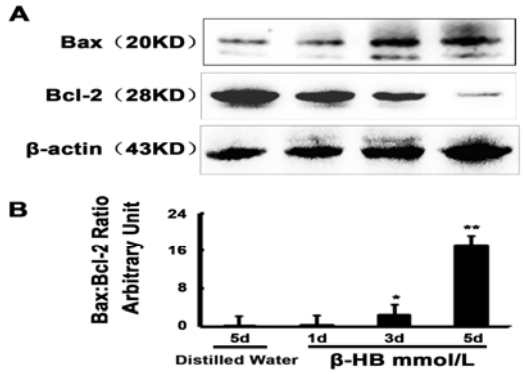

Fig. 1: Western blot analysis to measure the effects of exogenous β-HB on Bax, Bcl-2 in the liver tissue of mice. A) Bax and Bcl-2 response to β-HB(one time, three times, and five times) in liver tissue of mice (One representative western blot is shown, n=3); B) Bax to Bcl-2 ratio was measured and their ratio was calculated. *P<0.05, **P<0.01 vs distilled water group.

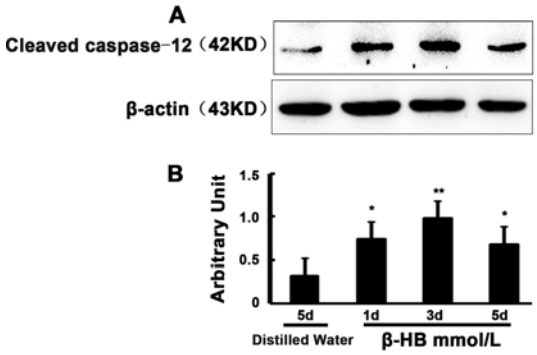

Fig. 2: Western blot analysis to measure the effects of exogenous β-HB on Caspase-12 in the liver tissue of mice. A) Caspase-12 response to β-HB (one time, three times, and five times) in liver tissue of mice (One representative western blot is shown, n=3); B) The densities of Caspase-12 was measured and their ratio was calculated. *P<0.05, **P<0.01 vs distilled water group.

serum triglycerides level can reflect situation of fatty acid metabolism in body (Kawano and Cohen, 2013). HDL helps transport free cholesterol and lipoprotein from peripheral tissue and blood circulation to the liver. LDL reflects outward transportation ability of fat in liver (Dubrovsky *et al*., 2012). ALP reflects whether liver function is damaged. However, these indexs were not significantly changed, which means short-term β-HB injection has no acute effect on liver lipid metabolism and liver function.

We treated the mice with exogenous β-HB, increasing the β-HB level in liver, accordingly, pro-apoptosis protein caspase-3, caspase-9, caspase-12 and Bax activated and down-regulation of anti-apoptosis protein Bcl-2. Therefore, we consider that, β-HB induces mitochondrial stress through up-regulation of pro-apoptotic Bax and down-regulation of anti-apoptotic Bcl-2, their ratio has been reported to be correlated to apoptosis (Desagher *et al*., 1999). Mitochondria convert pore permeability changes and then release caspase-9. Activated caspase-9 can activate the downstream executor caspase-3 (Brentnall *et al*., 2013). Meanwhile, β-HB activates caspase-12, a known ER-stress marker, which is located on surface of endoplasmic reticulum. Activated caspase-12 further activated caspase-9 and ultimately activate caspase-3

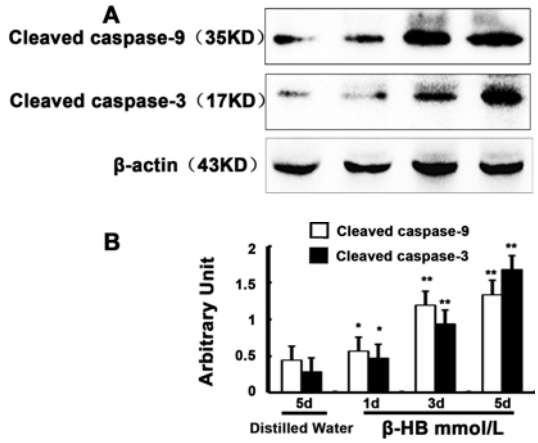

Fig. 3: Western blot analysis to measure the effects of exogenous β-HB on Caspase-9 and Caspase-3 in the liver tissue of mice. A) Caspase-9 and Caspase-3 response to β-HB (one time, three times, and five times) in liver tissue of mice (One representative western blot is shown, n= 3); B) Caspase-9 and Caspase-3 bands was measured and their ratio was calculated. *P<0.05, **P<0.01 vs distilled water group.

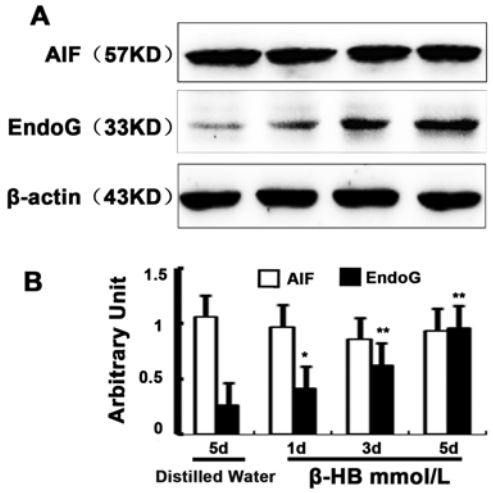

Fig. 4: Western blot analysis to measure the effects of exogenous β-HB on AIF and Endo G in the liver tissue of mice. A) AIF and Endo G response to β-HB (one time, three times, and five times) in liver tissue of mice (One representative western blot is shown, n=3); B) AIF and Endo G bands was measured and their ratio was calculated. *P<0.05, **P<0.01 vs distilled water group.

(Morishima *et al*., 2002). AIF and EndoG are two caspase-independent apoptosis proteins. Their translocation from mitochondria to the nucleus causes chromatin condensation and large-scale DNA fragmentation (Yu *et al*., 2012). Moreover, we observed expression of EndoG increased while AIF not increased, meaning that DNA may be damaged through caspase-independent pathway.

Ketogenic diet has been used in treatment of refractory epilepsy for more than 80 years, in addition, it has been reported that extrinsic β-HB has a therapeutic effect on hemorrhagic shock (Hiraide *et al*., 1991; Katayama *et al*., 1994), large area burns (Mizobata *et al*., 1996), lack of oxygen to the brain, hypoxia and ischemia (Suzuki *et al*., 2001). As a metabolic intermediate, physiological function of β-HB is complex and less known. This research focused on the apoptosis effect of β-

HB in liver. However, long-term, high concentration of exogenous β-HB exposure, whether affecting the liver lipid metabolism and liver function, still needs further discussion.

Conclusion: The present investigation provides strong evidences supporting the apoptotic action of extrinsic β-HB in liver of mice *in vivo* through activation of caspase-dependent pathway and caspase-independent pathway. β-HB increased the ratio of Bax/Bcl-2 and activated caspase-12, -9, -3. β-HB increased EndoG expression. However, there is no effect of β-HB on the concentrations of triglycerides, HDL, LDL, ALP in serum, which means the liver lipid metabolism and liver function have no change during the experiment.

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