Food and Chemical Toxicology 74 (2014) 225–232

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Berberine protects liver from ethanol-induced oxidative stress and steatosis in mice

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ARTICLE INFO

Article history:
Received 30 May 2014
Accepted 8 October 2014
Available online 16 October 2014

Keywords:
Berberine
Alcoholic liver disease
Oxidative stress
Steatosis

ABSTRACT

Alcohol consumption is customary in many cultures and it is a common human behavior worldwide. Binge ethanol and chronic alcohol consumption, two usual drinking patterns of human beings, produce a state of oxidative stress in liver and disturb the liver function. However, a safe and effective therapy for alcoholic liver disease in humans is still elusive. This study identified the natural product berberine as a potential agent for treating or preventing ethanol-induced liver injury. We demonstrated that berberine attenuated oxidative stress resulted from binge drinking in liver by reducing hepatic lipid peroxidation, glutathione exhaust and mitochondrial oxidative damage. Furthermore, berberine also prevented the oxidative stress and macrosteatosis in response to chronic ethanol exposure in mice. Either the total cytochrome P450 2E1 or the mitochondria-located cytochrome P450 2E1, which is implicated in ethanol-mediated oxidative stress, was suppressed by berberine. On the other hand, berberine significantly blunted the lipid accumulation in liver due to chronic alcohol consumption, at least partially, through restoring peroxisome proliferator-activated receptor α/peroxisome proliferator-activated receptor-gamma Co-activator-1α and hepatocyte nuclear factor 4α/microsomal triglyceride transfer protein pathways. These findings suggested that berberine could serve as a potential agent for preventing or treating human alcoholic liver disease.

1. Introduction

Alcoholic liver disease (ALD), a major cause of morbidity and mortality worldwide, includes a broad spectrum of disorders, ranging from simple steatosis to severe steatohepatitis, cirrhosis and hepatocellular carcinoma (Beier et al., 2011). In 2004 the alcohol consumption-related chronic liver disease accounted for 3.8% of all deaths around the world (Rehm et al., 2009). The mechanisms underlying the development of ALD are complicated; however, accumulating evidence indicated that oxidative stress played a central role in alcohol-induced liver injury (Hoek and Pastorino, 2002). Either acute or chronic ethanol consumption produces excessive reactive oxygen species (ROS), mitochondrial dysfunction and endoplasmic reticulum stress in liver. The ethanol-dependent oxidative stress has been proven to be predominantly generated by the induction of cytochrome P450-2E1 (CYP2E1) which induce abundant ROS such as hydrogen peroxide and superoxide anion radical when metabolizing ethanol in the membrane of the endoplasmic reticulum (ER) (Lu and Cederbaum, 2008). Interestingly, current attention is paid to CYP2E1 that locates in mitochondrial membrane. Even though the specific role of mitochondrial CYP2E1 (mtCYP2E1) remains unclear, it is generally recognized that accumulation of CYP2E1 in the mitochondria enhances local and cellular oxidative stress and plays an important pathogenic role in ALD (Bansal et al., 2010; Knockaert et al., 2011; Laetitia et al., 2011). The localization of CYP2E1 within the mitochondria sufficiently resulted in mitochondrial ROS overproduction and GSH depletion under the condition of ethanol exposure (Knockaert et al., 2011).

The fatty liver was another important character of ALD which usually cause a disorder of lipid metabolism in the liver (Purohit et al., 2004). Recent studies demonstrated that ethanol feeding in

Abbreviations: ALD, alcoholic liver disease; BBR, berberine; ROS, reactive oxygen species; TBARS, TBA reactive substances; GSH, glutathione; CYP2E1, cytochrome P450 2E1; ALD, alcoholic liver disease; PPARα, peroxisome proliferator-activated receptor α; PGC-1α, peroxisome proliferator-activated receptor-gamma Co-activator-1α; HNF4α, hepatocyte nuclear factor 4α; MTTP, microsomal triglyceride transfer protein; VLDL, very low-density lipoprotein; ACO, acyl-CoA oxidase; MCAD, mitochondrial medium-chain acyl-CoA dehydrogenase; CALD, chronic alcoholic liver disease; TG, triglyceride; TC, total cholesterol; ALT, alanine aminotransferase; MEOS, microsomal ethanol oxidizing system; OG, orally gavage; IHC, immunohistochemistry; CMC, carboxymethyl cellulose.

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http://dx.doi.org/10.1016/j.fct.2014.10.005
0278-6915/

mice induced fat accumulation in liver through suppression of PPARα and PGC-1α which account for the process of fatty acid oxidation (Chaung et al., 2008; Nanji et al., 2004). Expression of genes encoding enzymes involved in hepatic fatty acid β-oxidation is under dynamic transcriptional control by the nuclear receptor peroxisome proliferator-activated receptor α (PPARα) (Desvergne and Wahli, 1999). The activity of PPARα is regulated at multiple levels including fatty acid ligand availability and interaction with the highly-inducible transcriptional coactivator, PPARγ coactivator-1α (PGC-1α) (Vega et al., 2000). It is well documented that mice deficient for either PPARα or PGC-1α exhibit fasting-induced hepatic steatosis due to diminished capacity for fatty acid catabolism in the face of increased hepatic delivery of free fatty acids (Kersten et al., 1999; Leone et al., 2005). The findings illustrate the critical role of the hepatic PPARα/PGC-1α system in matching fatty acid oxidative capacity to substrate availability.

HNF4α is also an important transcription factor, regulating lipid utilization and export in liver, which has been determined to be suppressed in ethanol feeding mice via oxidative stress, zinc deficiency and adiponectin deficiency (Inoue et al., 2004; Martinez-Jimenez et al., 2010). Mice of liver-specific knockout of HNF4α exhibited severe fat accumulation in liver which was related to the decrease in gene expression of microsomal triglyceride transfer protein (MTTP) involved in very low-density lipoprotein (VLDL) secretion (Hayhurst et al., 2001). A recent study showed that berberine (BBR) increased the expression of HNF4α in mouse primary hepatocytes (Yan et al., 2008). It strongly suggests that berberine would protect liver from ethanol-induced steatosis partially through activating the HNF4α pathway.

Berberine is an alkaloid from the *Coptis chinensis* species which has a long history of use for treating diarrhea in traditional Chinese medicine. Growing studies revealed that a wide variety of biological activities were contributed to BBR, including anti-tumor and cardiovascular-protective actions (Singh and Mahajan, 2013). However, there is no investigation concerning the effect of BBR on ALD. This study was aimed to determine the role of BBR in preventing acute and chronic alcoholic liver injury and to explore the specific mechanisms.

2. Materials and methods

2.1. Materials

Berberine was purchased from Nanjing Zelang Medical Technology Co. Ltd. (Nanjing, China). The purity of BBR was more than 98%. Alanine aminotransferase (ALT), thiobarbituric acid reactive substances (TBARS) and glutathione (GSH) test kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Triglyceride (TG), total cholesterol (TC) assay kits were purchased from Siosino Sio-Technology and Science Inc (Beijing, China).

2.2. Animals

Adult male ICR mice (8 weeks, 24–26 g) from College of Veterinary Medicine, Yangzhou University were housed in temperature- and humidity-controlled rooms. The mice were maintained at a 12-hour light/dark cycle and had free access to rodent chow and tap water. All procedures were approved by the Institutional Animal Care and Use Committee at China Pharmaceutical University and adhered to the Jiangsu Provincial Guidelines for the use of experimental animals.

2.3. Experimental protocol

The acute alcohol exposure model was set up to mimic acute alcoholic liver injury in patients as well as to be used as a predictive/screening tool for evaluating the protective effect of BBR. Experimental mice were randomly divided into four groups – Control, Ethanol and two BBR-pretreated groups, each group contained eight animals. BBR-pretreated groups were orally administrated with BBR 200 mg/kg/day and 300 mg/kg/day for 10 consecutive days, while other groups received an equal volume of vehicle as control. The dosages of BBR were based on preliminary range-finding studies. After the last dose of BBR all animals were exposed to three doses of ethanol (6 g/kg, OG) at 12-h intervals, while mice in the control group received the same volume of saline as a vehicle control (Richard et al., 2014).

The chronic ethanol feeding model was set up to mimic chronic alcoholic liver injury in human beings. Experimental mice were pair-fed isocaloric Lieber–DeCarli liquid diets (LD Diet) containing 0% or 36% ethanol by caloric content for 5 weeks (Lieber and DeCarli, 1982). Mice were divided into three groups, (1) Control, (2) LD Diet, (3) LD Diet + BBR (120 mg/kg/day, OG), n = 12. Control mice were fed with liquid diet containing 0% ethanol, while the other two groups were fed with isocaloric liquid diet containing 36% ethanol. The dosage of BBR was based on the preliminary range-finding studies.

At sacrifice, animals were anesthetized with sodium pento-barbital (80 mg/kg, IP) 6 hours after ethanol administration. Blood was collected with cardiac puncture just prior to sacrifice. Liver tissues were harvested at the time of sacrifice.

2.4. Blood biochemical assay

Blood was placed into a centrifuge tube and allowed to obtain the serum that was separated by centrifugation 800 *g*, 4 °C for 10 min. Serum alanine aminotransferase activity was measured using commercial diagnostic kits.

2.5. Determination of lipid accumulation

Quantitative assay of lipids was conducted by measuring the concentrations of triglycerides, cholesterol in the liver tissue using commercial diagnostic kits. Oil red O staining of neutral lipid was performed on cryostat liver sections.

2.6. Measurement of hepatic oxidative stress

The liver was removed and 100 mg of samples were homogenized in 1 ml of cold 0.1 M phosphate buffer. Tissue homogenates were centrifuged at 1000 *g*, 4 °C for 10 min. The supernatant was analyzed for total protein by bicinchoninic acid (BCA) method. Hepatic lipid peroxidative product (TBARS) and antioxidant substance (GSH) were measured by the commercial detection kits according to the manufacturer's instruction.

2.7. Histopathological analysis

After sacrificing the mice, small pieces of liver from same location were harvested and washed with ice-cold saline. Tissue fragments were then fixed in 4% neutral buffered formalin solution, embedded in paraffin and used for histopathological examination. Sections of 5 μm thickness were cut, deparaffinized, hydrated and stained with hematoxylin and eosin. Liver sections were examined by the same individual (N.N.) in a blind fashion. Steatosis was scored as described by Nanji et al: steatosis <5%, 1; 25%–50%, 2; 50%–75%, 3; > 75%, 4 (Nanji et al., 1989).

2.8. Apoptosis assay

Samples of 4 μm thick were cut and stained with the TUNEL assay. This assay is performed by labeling the free 3′-OH ends of DNA strand breaks that were generated after DNA fragmentation during apoptosis with specific nucleotides. In this study a commercial kit Situ TUNEL Apoptosis Detection Kit obtained from Nanjing Jiancheng Bioengineering Institute was used for the TUNEL assay, according to the manufacturer' instructions. They were analyzed by light microscopy. The cell was considered TUNEL-positive when the nuclear staining was dark brown and homogenous. Apoptotic index was calculated by dividing the number of TUNEL-positive cells in a randomly focused field to the total number of cells in that field, and the result was multiplied by 100. The apoptotic indexes of 10 random fields for each slide were evaluated and the mean apoptotic index of each case was calculated. Three slides per animal were analyzed in this study.

2.9. Measurement of mitochondrial oxidative damage

Mitochondria were isolated by differential centrifugation of the liver homogenate (Frezza et al., 2007). The total protein of isolated mitochondria was measured by BCA method. Mitochondrial lipid peroxidative product (TBARS) and antioxidant substance (GSH) were determined by the commercial kits according to the manufacturer's instruction.

2.10. Mitochondrial swelling test

The assays were performed in 1 ml of the reaction media (250 mM sucrose, 3 mM sodium succinate, 5 mM KH_2PO_4, 0.3 mM $CaCl_2$) (Galindo et al., 2003). Changes of absorbance at 520 nm in 10 minutes were recorded after 0.5 mg of protein was added to the solution. Mitochondrial osmotic volume changes were presented by the decrease of absorbance at 520 nm. Damaged mitochondria reflected much less sensitivity to the Ca^{2+} loading, which was demonstrated by blunted decrease of 520 nm absorbance.