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Alleviation of alcoholic liver injury by betaine involves an enhancement of antioxidant defense via regulation of sulfur amino acid metabolism

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ABSTRACT

Previous studies suggested that the hepatoprotective activity of betaine is associated with its effects on sulfur amino acid metabolism. We examined the mechanism by which betaine prevents the progression of alcoholic liver injury and its therapeutic potential. Rats received a liquid ethanol diet for 6 wk. Ethanol consumption elevated serum triglyceride and TNFα levels, alanine aminotransferase and aspartate aminotransferase activities, and lipid accumulation in liver. The oxyradical scavenging capacity of liver was reduced, and expression of CD14, TNFα, COX-2, and *i*NOS mRNAs was induced markedly. These ethanol-induced changes were all inhibited effectively by betaine supplementation. Hepatic *S*-adenosylmethionine, cysteine, and glutathione levels, reduced in the ethanol-fed rats, were increased by betaine supplementation. Methionine adenosyltransferase and cystathionine γ-lyase were induced, but cysteine dioxygenase was down-regulated, which appeared to account for the increment in cysteine availability for glutathione synthesis in the rats supplemented with betaine. Betaine supplementation for the final 2 wk of ethanol intake resulted in a similar degree of hepatoprotection, revealing its potential therapeutic value in alcoholic liver. It is concluded that the protective effects of betaine against alcoholic liver injury may be attributed to the fortification of antioxidant defense via improvement of impaired sulfur amino acid metabolism.

1. Introduction

Alcoholic liver disease (ALD) encompasses a wide spectrum of progressive pathologic stages including fatty liver, steatohepatitis, fibrosis, and cirrhosis. Among these, steatosis or fatty liver is the most common histopathologic change found in drinkers. Previously steatosis was considered to be a rather benign condition, primarily due to its reversibility upon alcohol withdrawal (MacSween and Burt, 1986). However, an increasing number of research and clinical data support a link between the severity of fat accumulation and the development of liver injury. It is suggested that, regardless of the etiology, progression of simple fat accumulation into steatohepatitis requires double hits; the first producing steatosis and the second a source of oxidative stress capable of initiating lipid peroxidation (Day and James, 1998). Accordingly, both fat accumulation and oxidative stress appear to be critical factors in the pathogenesis of chronic liver injury.

Ethanol metabolism in liver results in formation of reactive oxygen species (ROS) and free radicals, which initiate the peroxidation of polyunsaturated fatty acid side chains of membrane phospholipids and lipoproteins. Potential sources of free radicals include the ethanol-inducible CYP2E1 that generates superoxide, hydroxyl and hydroxyethyl radicals, the mitochondrial respiratory chain, *i*NOS, and peroximal β-oxidation of free fatty acids capable of generating hydrogen peroxide (Arteel, 2003). A strong oxidizing and nitrating species, peroxynitrite, is formed from superoxide

Abbreviations: ALD, alcoholic liver disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BED, betaine-supplemented diet concomitant with ethanol intake; BHMT, betaine–homocysteine methyltransferase; CβS, cystathionine β-synthase; CD, control diet; CD14, cluster of differentiation 14; CDO, cysteine dioxygenase; CγL, cystathionine γ-lyase; COX-2, cyclooxygenase-2; CYP, cytochrome P450; EBD, betaine-supplemented diet for the final 2 wk of ethanol intake; ED, ethanol diet; GCL, γ-glutamylcysteine ligase; GSH, glutathione; GST, glutathione *S*-transferase; *i*NOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MAT, methionine adenosyltransferase; NF-κB, nuclear factor-kappaB; ROS, reactive oxygen species; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; TNFα, tumor necrosis factor α; TOSC, total oxyradical scavenging capacity.

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and nitric oxide via an enzyme-independent reaction. Meanwhile, chronic ethanol intake elevates the level of bacterial-derived endotoxin that stimulates Kupffer cell to produce free radicals and cytokines (Bode and Bode, 2005). NADPH oxidase plays a key role in the generation of oxidants in Kupffer cells after ethanol intake. The oxidants generated activate NF-κB, which leads to an increase in the production of TNFα, followed eventually by tissue damage (Arteel, 2003).

It has been realized that chronic liver injury is closely associated with an impairment of hepatic sulfur-containing amino acid metabolism, which is attributed to abnormality in the critical enzymes involved in the metabolic reactions including methionine adenosyltransferase (MAT), methionine synthase, betaine-homocysteine methyltransferase (BHMT), and cystathionine β-synthase (CβS) (Duce et al., 1988; García-Tevijano et al., 2001). Previous studies have indicated that alcoholic liver injury entails an imbalance in the metabolomics of sulfur-containing substances such as methionine, *S*-adenosylmethionine (SAM), homocysteine, *S*-adenosylhomocysteine (SAH), and glutathione (GSH) (Lee et al., 2004; Kim et al., 2009a). Among these metabolites, SAM is the principal biological methyl donor, the precursor of aminopropyl group used in polyamine synthesis, and a provider of cysteine for synthesis of GSH. In addition, SAM was shown to have a direct antioxidant activity via ROS scavenging and chelating iron ions to inhibit hydroxyl radical generation (Caro and Cederbaum, 2004). Meanwhile, elevation of homocysteine and/or SAH is suggested to be a major component in the induction of endoplasmic reticulum stress in alcoholic livers (Ji and Kaplowitz, 2004). Other studies showed that inadequate transfer of cytosolic GSH, a final product in the transsulfuration reactions, to its predominant mitochondrial site of antioxidant function may play a critical role in the development of ALD (Fernández-Checa et al., 2002). Reduction of hepatic GSH by ethanol consumption results in increased susceptibility of hepatocytes to oxidative stress and TNFα (Colell et al., 1998).

Betaine, an oxidative metabolite of choline, is involved in the synthesis of methionine from homocysteine in liver. It has been shown that betaine administration increases hepatic concentrations of methionine and SAM while reducing homocysteine and cystathionine in rats and mice (Kim et al., 2003; Kim and Kim, 2005). A rise in plasma homocysteine concentrations after methionine intake was also inhibited by betaine (Steenge et al., 2003). Previous studies have shown that betaine administration protects the liver from hepatotoxicants such as ethanol, lipopolysaccharide (LPS), α-naphthylisothiocyanate, and dimethylnitrosamine (Barak et al., 2003; Kim and Kim, 2002; Kim et al., 2005, 2009b). Since oxidative stress is implicated in the liver injury provoked by exposure to these toxic substances, the hepatoprotective activity of betaine appears to be due to the retrieval of antioxidant defense via normalization of the metabolomics of sulfur amino acids in liver. Therefore, it was of interest to examine the effects of betaine on oxidative liver injury in association with improvement of impaired sulfur amino acid metabolism in animals fed ethanol chronically. We also determined the therapeutic potential of betaine in alcoholic liver by administrating this substance to the animals exposed to ethanol beforehand.

2. Methods

2.1. Animals and treatments

Male Wistar rats were purchased from Dae-Han (Seoul, Korea). The use of animals was in compliance with the guidelines established by the Animal Care Committee in this institute. Animals were acclimated to temperature (22 ± 2 °C) and humidity (55 ± 5%) controlled rooms with a 12-h light/dark cycle for 1 wk prior to use. Twenty-four rats, weighing 100–120 g, were randomly divided into 4 groups and fed a Lieber–DeCarli liquid diet with or without ethanol for 6 wk. Control rats were provided with a control Lieber–DeCarli liquid diet (CD); ethanol-treated rats with a liquid diet containing 5% (w/v) ethanol (ED); betaine-supplemented rats with a liquid ethanol diet containing 1% (w/v) betaine (BED). An additional group of rats fed a liquid ethanol diet for 6 wk was supplemented with betaine for the final 2 wk of ethanol administration (EBD). For the control liquid diet, 35% of energy was derived from fat, 18% from protein, and 47% from carbohydrates; the liquid ethanol diet contained 35% of energy from fat, 18% from protein, 11% from carbohydrates, and 36% from ethanol. The overall dietary compositions of the control diet and the ethanol diet, including vitamins and minerals, were identical to the ones suggested by Lieber et al. (1989). The diets were purchased from Dyets Inc. (Bethlehem, PA).

2.2. Measurement of hepatic injury

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in serum were determined to estimate the ethanol-induced hepatotoxicity. Total serum triglyceride was measured using a commercially available kit (Catalog # TR0100; Sigma, St. Louis, MO). A commercial ELISA kit (Endogen, Woburn, MA) was employed for measurement of TNFα level in serum. For histopathologic evaluation, sections of frozen liver were sliced at 10 μm, immersed in propylene glycol for 5 min, and stained with Oil red O for 7 min. After rinsing with 85% propylene glycol and distilled water, the sections were counterstained with hematoxylin for 2 min before microscopic examination.

2.3. Measurement of sulfur-containing amino acids and related enzyme activities

Livers were homogenized in a 4-fold volume of cold 1 M perchloric acid. Denatured protein was removed by centrifugation. Total GSH concentrations were determined using an enzymatic recycling method (Griffith, 1980). Cysteine concentrations were estimated by the acid-ninhydrin method (Gaitonde, 1967). The method of She et al. (1994) was employed to determine SAM and SAH concentrations. The supernatant was directly injected into HPLC equipped with a UV detector and TSK-GEL ODS-80TM column (4.6 × 250 mm) (Tosoh, Tokyo, Japan).

Free amino acids, hypotaurine and taurine were derivatized with *O*-phthalaldehyde/2-mercaptoethanol prior to quantification using HPLC with a fluorescence detector and 3.5 μm Kromasil C18 column (4.6 × 100 mm) (Eka, Bohus, Sweden). Free amino acids were separated by using the method of Rajendra (1987). The method of Ide (1997) was used to quantify hypotaurine and taurine.

Livers were homogenized in a 3-fold volume of ice-cold buffer containing 1 mM EDTA in 0.154 M KCl and 50 mM Tris–HCl (pH 7.4). The 10,000*g* supernatant was centrifuged at 104,000*g* for 60 min. The 104,000*g* supernatant fraction (cytosol) was used to determine the enzyme activities. MAT activity was estimated by quantifying SAM and SAH production using the method described above. CβS activity was determined by cystathionine formation (Kashiwamata and Greenberg, 1970). Cystathionine γ-lyase (CγL) activity was estimated by α-ketobutyrate production (Matsuo and Greenberg, 1957). γ-Glutamylcysteine ligase (GCL) and cysteine dioxygenase (CDO) activities were measured by generation of γ-glutamylcysteine (Yan and Huxtable, 1995) and cysteinesulfinate (Bagley et al., 1995), respectively. Glutathione *S*-transferase (GST) activity was measured using 1-chloro-2,4-dinitrobenzene as a substrate (Habig et al., 1974).

2.4. Measurement of total oxyradical scavenging capacity (TOSC)

The antioxidant capacity of liver cytosol was determined with the TOSC assay (Regoli and Winston, 1999), which is based on the ethylene-yielding reaction of α-keto-γ-methiolbutyric acid with hydroxyl, peroxyl radicals, and peroxynitrite. The ethylene production was measured using GC equipped with a flame ionization detector and Porapack N column (Supelco, Bellefonte, PA). TOSC values were quantified from the equation TOSC = 100 − ($\int$SA/$\int$CA × 100), where $\int$SA and $\int$CA were the integrated ethylene peak areas obtained from the sample and control reactions, respectively. Because TOSC is calculated from the inhibition of ethylene generation relative to control reaction, this value is unitless.

2.5. Western blotting analysis

Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes by electroblotting. The membranes were blocked in 5% nonfat dry milk in 0.05% Tween 20 in phosphate buffered saline. The blots were incubated overnight with antibodies diluted in 5% bovine serum albumin at 4 °C followed by incubation with secondary antibodies conjugated to horseradish peroxidase. Polyclonal antibodies against rat GCL with a catalytic subunit (GCLC; NeoMarkers, Fremont, CA), GSTα (Detroit R&D, Detroit, MI), MAT, and CDO were used as probes. Antibodies against MAT and CDO were kind gifts from Dr. María A. Pajares (Instituto de Investigaciones Biomédicas "Alberto Sols", Madrid, Spain) and Dr. Yu Hosokawa (Faculty of Human Sciences, Jissen Women's University, Tokyo, Japan), respectively. Proteins were detected by enhanced chemiluminescence.

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