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Metabolic basis of ethanol-induced cytotoxicity in recombinant HepG2 cells: Role of nonoxidative metabolism

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Abstract

Chronic alcohol abuse, a major health problem, causes liver and pancreatic diseases and is known to impair hepatic alcohol dehydrogenase (ADH). Hepatic ADH-catalyzed oxidation of ethanol is a major pathway for the ethanol disposition in the body. Hepatic microsomal cytochrome P450 (CYP2E1), induced in chronic alcohol abuse, is also reported to oxidize ethanol. However, impaired hepatic ADH activity in a rat model is known to facilitate a nonoxidative metabolism resulting in formation of nonoxidative metabolites of ethanol such as fatty acid ethyl esters (FAEEs) via a nonoxidative pathway catalyzed by FAEE synthase. Therefore, the metabolic basis of ethanol-induced cytotoxicity was determined in HepG2 cells and recombinant HepG2 cells transfected with ADH (VA-13), CYP2E1 (E47) or ADH + CYP2E1 (VL-17A). Western blot analysis shows ADH deficiency in HepG2 and E47 cells, compared to ADH-overexpressed VA-13 and VL-17A cells. Attached HepG2 cells and the recombinant cells were incubated with ethanol, and nonoxidative metabolism of ethanol was determined by measuring the formation of FAEEs. Significantly higher levels of FAEEs were synthesized in HepG2 and E47 cells than in VA-13 and VL-17A cells at all concentrations of ethanol (100–800 mg %) incubated for 6 h (optimal time for the synthesis of FAEEs) in cell culture. These results suggest that ADH-catalyzed oxidative metabolism of ethanol is the major mechanism of its disposition, regardless of CYP2E1 overexpression. On the other hand, diminished ADH activity facilitates nonoxidative metabolism of ethanol to FAEEs as found in E47 cells, regardless of CYP2E1 overexpression. Therefore, CYP2E1-mediated oxidation of ethanol could be a minor mechanism of ethanol disposition. Further studies conducted only in HepG2 and VA-13 cells showed lower ethanol disposition and ATP concentration and higher accumulation of neutral lipids and cytotoxicity (apoptosis) in HepG2 cells than in VA-13 cells. The apoptosis observed in HepG2 vs. VA-13 cells incubated with ethanol appears to be mediated by release of mitochondrial cytochrome *c* via activation of caspase-9 and caspase-3. These results strongly support our hypothesis that diminished hepatic ADH activity facilitates nonoxidative metabolism of ethanol and the products of ethanol nonoxidative metabolism cause apoptosis in HepG2 cells via intrinsic pathway.

Keywords: Ethanol; Fatty acid ethyl esters; HepG2 cells; VA-13 cells; Nonoxidative metabolism; Alcohol dehydrogenase; Cytochrome P450 2E1

Introduction

Chronic alcohol abuse and alcoholism, associated with high mortality and morbidity, are known to cause such several major health problems as alcoholic liver disease (ALD), myocardial infarction, pancreatitis and disorders of the immune, endocrine and reproductive systems (NIAAA, 2000). ALD is a major illness and the second leading cause of death among all the liver diseases (Grant et al., 1988; NIAAA, 2000; Purohit et al., 2004; Lieber, 2004). An early stage of ALD, known as fatty liver (steatosis), is reversible and can progress to hepatitis (inflammation and cell injury), fibrosis and finally to cirrhosis (in 5–15% individuals) after 10–20 years of alcohol abuse (Sorensen et al., 1984; Worner and Lieber, 1985). However, the mechanism(s) of ALD, including alcoholic steatosis, are not well understood. Oxidative as well as nonoxidative metabolism of ethanol has been implicated in the ethanol-induced target organ injury (Laposata and Lange, 1986; Lieber, 2004).

Abbreviations: ADH, alcohol dehydrogenase; ALD, alcoholic liver disease; ATP, adenosine triphosphate; FAEE, fatty acid ethyl esters; ELISA, enzyme-linked immunosorbent assay; CYP2E1, cytochrome P450 2E1.

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Therefore, understanding the metabolic basis of ethanol-induced toxicity could be an important step in delineating the mechanism of ALD and other alcohol-related target organ injuries.

Liver is the primary organ for the metabolism, disposition and toxicity of ingested ethanol (Lieber, 2004; Ramaiah et al., 2004). Alcohol dehydrogenase (ADH) oxidizes the majority of ingested alcohol to acetaldehyde in the liver as compared to that by microsomal cytochrome P450 2E1 (CYP2E1) and catalase (Lieber, 2004). Although hepatic microsomal CYP2E1 is significantly induced during chronic alcohol abuse, the K_m of CYP2E1 for ethanol is much higher than that of ADH (8–10 mM compared to 0.2–2.0 mM for hepatic ADH) (Lieber, 1997). Further oxidation of acetaldehyde to acetate is achieved in the mitochondria by aldehyde dehydrogenase (ALDH). Production of acetaldehyde via hepatic ADH and/or CYP2E1 is reported to generate reactive oxygen species and oxidative stress, which results in a deficiency in respiratory chain components and ATP production, decreased GSH levels and enhanced peroxidation of lipids, proteins and DNA (Cunningham et al., 1990; Kukielka et al., 1994; Wu and Cederbaum, 1999; Caro and Cederbaum, 2004; Lieber, 2004).

Hepatic ADH and/or ALDH are generally inhibited in chronic alcohol abuse (Nuutinen et al., 1983; Palmer and Jenkins, 1985; Panes et al., 1989, 1993; Kharchenko, 1997). Similar inhibition can also be achieved after a large acute or subchronic ingestion of ethanol in vivo in experimental animals or after in vitro incubation with ethanol (Shore and Theorell, 1966; Zahlten et al., 1980; Sharkawi, 1984; Flora and Tandon, 1987; Kaphalia et al., 1996). However, very high levels of fatty acid ethyl esters (FAEEs) and phosphatidylethanol (PEt) reported in alcoholic patients indicate the predominance of nonoxidative metabolism during chronic alcohol abuse (Laposata and Lange, 1986; Kaphalia and Ansari, 2001a; Musshoff, 2002; Soderberg et al., 2003; Kaphalia et al., 2004). Both FAEEs and PEt are nonoxidative metabolites of ethanol. Several-fold higher levels of FAEEs, reported after ethanol exposure in the pancreas of rats pretreated with 4-methylpyrazole (4-MP, an inhibitor of hepatic ADH), are associated with pancreatic injury (Manautou and Carlson, 1991; Werner et al., 2002). Besides the pancreas, FAEEs are also detected in other tissues, including the livers of individuals intoxicated with alcohol at the time of their death (Laposata and Lange, 1986). Some of the FAEEs, when tested in cell culture and in vivo, are reported to cause hepatocellular and pancreatic toxicity (Kaphalia and Ansari, 2001b; Werner et al., 1997; Criddle et al., 2004; Aydin et al., 2005). Although toxicity of PEt has not been studied so far, nonoxidative metabolism of ethanol and its metabolites, in general, may play an important role in pathogenesis of ALD in chronic alcohol abusers.

Enzymes that synthesize FAEEs are collectively called FAEE synthases and have been purified and characterized from rat hepatic and pancreatic microsomes and human pancreas (Kaphalia and Ansari, 2001b, 2003; Kaphalia et al., 1997; Riley et al., 1990). These enzymes are shown to be up-regulated after ethanol exposure in rats (Pfutzer et al., 2002). However, contribution of such induction to overall levels of FAEEs in the liver and pancreas is not known.

The histopathological appearance of apoptotic and Mallary bodies, commonly detected in clinical and experimental ethanol-induced liver injury, increases in patients with alcoholic hepatitis (Natori et al., 2001; Ishii et al., 2003). A relationship between lipid accumulation and ethanol-induced cytotoxicity including the development of ALD has not been established yet. FAEEs detected in the plasma correlate better with the history of alcohol abuse than does the blood alcohol levels and are known to cause hepatocellular and pancreatic toxicity (Kaphalia and Ansari, 2001a; Kaphalia et al., 2004; Soderberg et al., 2003; Werner et al., 1997, 2002; Criddle et al., 2004; Aydin et al., 2005). However, very little is known about the role of nonoxidative metabolism of ethanol in the pathogenesis of ALD or other target organ diseases.

Human hepatocellular carcinoma (HepG2) cell line has been used extensively to understand the mechanism(s) of ethanol-induced hepatic injury (Caro and Cederbaum, 2004; Neuman et al., 1995, 1998, 1999). Since HepG2 cells are inherently ADH-deficient (a metabolic condition observed in chronic alcohol abusers) and synthesize FAEEs after ethanol exposure (Wolfla et al., 1988; Kabakibi et al., 1998; Kaphalia et al., 1999), we used HepG2 cells transfected with ADH and/or CYP2E1 in the present study to understand the metabolic basis of ethanol-induced hepatocellular injury.

Experimental procedures

Materials

[1-^{14}C] Ethanol and [1-^{14}C] oleic acid were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO) and Perkin Elmer Life Sciences (Boston, MA), respectively. Fine biochemicals, *p*-nitrophenyl acetate (PNPA), FAEE standards and ethyl heptadecanoate (internal standard) from Sigma (St. Louis, MO) and reagents and molecular weight markers for gel electrophoresis from Bio-Rad Laboratories (Hercules, CA) were used. Anti-alcohol dehydrogenase (ADH, yeast) and cytochrome P450 (CYP2E1, human) polyclonal antibodies were obtained from Rockland (Gilbertsville, PA) and Oxford Biomedical Research (Oxford, MI), respectively. BCA protein assay kit, electrophoresis reagents and goat anti-rabbit peroxidase IgG were purchased from Pierce Biotechnology Inc. (Rockford, IL). HPLC grade solvents and SentiVerse were from Fisher Scientific (Fairlawn, NJ).

Cell cultures

HepG2 cells, obtained from ATCC (Rockville, MD), were grown in Dulbecco's modified Eagle medium (DMEM) as described earlier (Kaphalia et al., 1999). HepG2 cells transfected with mouse ADH1 (VA-13 cells) and mouse ADH1 + human CYP2E1 (VL-17A cells) were established and maintained according to Clemens et al. (2002). E47 cells (HepG2 cells transfected with human CYP2E1) were generously provided by Dr. Cederbaum (Dai et al., 1993). The cells were subcultured at appropriate intervals and maintained at subconfluent densities. Overexpression of ADH and CYP2E1 in recombinant HepG2 cell lines was confirmed by Western blot analysis as described previously (Kaphalia et al., 1997, 1999), using anti-ADH and CYP2E1 antibodies (Fig. 1). The cells were counted in a hemocytometer after incubation with 0.05% trypsin and 0.02% EDTA. The viability of cells was measured by flow cytometry using TACS Annexin V FITC and propidium iodide assay (R&D Systems Inc., Minneapolis, MN). The protein was determined using a BCA protein assay kit using bovine serum albumin as standard. All the experiments were done in triplicate.