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Alcohol oxidizing enzymes and ethanol-induced cytotoxicity in rat pancreatic acinar AR42J cells

Kamlesh K. Bhopale,

Department of Pathology, The University of Texas Medical Branch, Galveston, TX 77555-0609, USA

Miriam Falzon,

Departments of Pharmacology, The University of Texas Medical Branch, Galveston, TX 77555, USA

G. A. S. Ansari, and

Department of Pathology, The University of Texas Medical Branch, Galveston, TX 77555-0609, USA

Bhupendra S. Kaphalia

Department of Pathology, The University of Texas Medical Branch, Galveston, TX 77555-0609, USA

Bhupendra S. Kaphalia: bkaphali@utmb.edu

Abstract

Alcoholic chronic pancreatitis (ACP) is a serious inflammatory disease causing significant morbidity and mortality. Due to lack of a suitable animal model, the underlying mechanism of ACP is poorly understood. Chronic alcohol abuse inhibits alcohol dehydrogenase (ADH) and facilitates nonoxidative metabolism of ethanol to fatty acid ethyl esters (FAEEs) in the pancreas frequently damaged during chronic ethanol abuse. Earlier, we reported a concentration-dependent formation of FAEEs and cytotoxicity in ethanol-treated rat pancreatic tumor (AR42J) cells, which express high FAEE synthase activity as compared to ADH and cytochrome P450 2E1. Therefore, the present study was undertaken to investigate the role of various ethanol oxidizing enzymes in ethanol-induced pancreatic acinar cell injury. Confluent AR42J cells were pre-treated with inhibitors of ADH class I and II [4-methylpyrazole (MP)] or class I, II, and III [1,10-phenanthroline (PT)], cytochrome P450 2E1 (trans-1,2-dichloroethylene) or catalase (sodium azide) followed by incubation with 800 mg% ethanol at 37°C for 6 h. Ethanol metabolism, cell viability, cytotoxicity (apoptosis and necrosis), cell proliferation status, and formation of FAEEs in AR42J cells were measured. The cell viability and cell proliferation rate were significantly reduced in cells pretreated with 1,10-PT + ethanol followed by those with 4-MP + ethanol. In situ formation of FAEEs was twofold greater in cells incubated with 1,10-PT + ethanol and ~1.5-fold in those treated with 4-MP + ethanol vs. respective controls. However, cells treated with inhibitors of cytochrome P450 2E1 or catalase in combination of ethanol showed no significant changes either for FAEE formation, cell death or proliferation rate. Therefore, an impaired ADH class I—III

catalyzed oxidation of ethanol appears to be a key contributing factor in ethanol-induced pancreatic injury via formation of nonoxidative metabolites of ethanol.

Keywords

Ethanol; Fatty acid ethyl esters; AR42J cells; Alcohol dehydrogenase; Cytochrome P450 2E1

Introduction

After the biliary duct diseases, chronic alcohol abuse is the second major cause of chronic pancreatitis and many alcoholic patients with pancreatitis die before reaching to the clinical stage of the disease (Pitchumoni et al. 1984; Singh and Simsek 1990; Lankisch and Banks 1998). Chronic pancreatitis is a serious inflammatory disease of exocrine pancreas resulting in fibrosis and can also cause pancreatic cancer in some cases (Lankisch and Banks 1998; Kaphalia 2011). About ~70% of the cases with chronic pancreatitis have been shown to be associated with chronic alcohol abuse (Brunner et al. 2004).

Auto digestion of pancreatic tissue due to intra acinar activation of digestive enzymes (zymogens) in the exocrine pancreas is central to pancreatitis (Lankisch and Banks 1998). Exocrine pancreas is the major component of the pancreatic gland, composed of mainly acinar cells and a prime target of injury during chronic alcohol abuse (Kaphalia 2011). As compared to the livers, relatively small amounts of ethanol oxidizing enzymes [alcohol dehydrogenase (ADH), cytochrome P450 (CYP)2E1 and catalase] and very high activity of fatty acid ethyl ester (FAEE) synthase (catalyzes the formation of FAEEs, nonoxidative metabolites of ethanol) are reported in exocrine pancreatic tissue (Pfutzer et al. 2002; Kaphalia and Ansari 2003; Kaphalia 2011). Among ADH isozymes, ADH class 1, II, and III have also been reported in the human pancreas (Chrostek et al. 2003). However, major mechanism of ethanol disposition in extrahepatic organs such as pancreas frequently damaged by chronic alcohol abuse appears to be nonoxidative metabolism to FAEEs (Laposata and Lange 1986). Pancreatic toxicity of FAEEs has been reported in rats after their intra-arterial administration and in isolated acinar cells and rat pancreatic tumor (AR42J) cells in vitro (Werner et al. 1997; Haber et al. 1993; Gukovskaya et al. 2002; Criddle et al. 2004, 2006; Wu et al. 2008). Therefore, various ethanol metabolites formed in the pancreas during chronic alcohol abuse could be damaging to the acinar cells. However, a metabolic basis of alcoholic pancreatitis is not well understood due to a lack of suitable animal model. Owing to the short life span of freshly isolated pancreatic acinar cells, rat pancreatic tumor (AR42J) cells, which mimic most of the glandular functions of pancreatic acinar cells, have been used previously by us and others (Christophe 1994; Kaphalia et al. 1999; Wu et al. 2008).

The formation of FAEEs has been shown to be dose- and time-dependent in AR42J cells incubated with ethanol, and 3-benzyl-6-chloro-2-pyrone (FAEE synthase inhibitor) attenuates both FAEE synthesis as well as associated apoptotic cell death in AR42J cells (Wu et al. 2008). Therefore, the present study was undertaken to systemically investigate the

role of ADH isozymes, CYP2E1, and catalase in ethanol-induced pancreatic acinar cell toxicity using AR42J cell culture model.

Materials and Methods

Materials

HPLC grade solvents and SentiVerse^R (scintillation flour) from Fisher Scientific (Fairlawn, NJ) were used. [1-¹⁴C] Ethanol (specific activity 56 mCi/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Ethyl oleate (marker for FAEE formation assay), acetaldehyde, ethanol, target enzyme inhibitors; 1,10-phenanthroline (PT), 4-methylpyrazole (MP) and trans-1, 2-dichloroethylene (DCE) from Sigma-Aldrich (St. Louis, MO), sodium azide (SA) from Fluka, Biochemicals were used in our studies (Table 1).

Cell culture

Rat pancreatic tumor (AR42J) cells obtained from American Type Culture Collection (ATCC, Manassas, VA), were maintained in our lab in F12K medium supplemented with 2 mM L-glutamine, 20% fetal bovine serum, and 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified air atmosphere containing 5% CO₂ at 37°C and regularly monitored for the mycoplasma contamination (Wu et al. 2008). The cell cultures for inhibitor studies were conducted in T25 tissue culture flasks with 85% confluent cells in a total volume of 10-ml cell culture medium. For inhibitor studies, the confluent cell cultures were incubated with known predetermined concentrations of specific inhibitors of target enzymes in appropriate solvent(s) (Table 1; Haber et al. 1998; Mathews et al. 1998). The inhibitors were added to a set of flasks in culture medium 30 min prior to exposure to 800 mg% ethanol concentration, which we have used previously for cell culture studies (Wu et al. 2006, 2008). The cells were incubated at 37°C for 6 h. Control cells incubated with dimethyl sulfoxide (DMSO, 0.12% final concentration) with or without ethanol were used as controls for 1,10-PT and trans-1, 2-DCE, whereas untreated and ethanol-treated cells with or without ethanol for 4-MP and SA. In the present study, we used the reported optimal effective concentrations of inhibitors of ethanol oxidizing enzymes (Table 1).

Residual ethanol and acetaldehyde levels in culture medium

Concentration of ethanol and its oxidative metabolite, acetaldehyde, were determined in the culture medium of cells incubated with ethanol by head space gas chromatography analysis using a Hewlett Packard 5890 GC equipped with flame ionization detector as described previously (Wu et al. 2008). Recoveries for ethanol and acetaldehyde from the culture medium were >95% and final data corrected for the percentage recovery.

Formation of FAEEs

Formation of total FAEEs was determined according to the method described earlier (Kaphalia et al. 1999; Wu et al. 2008). Confluent AR42J cells were incubated with specific inhibitors for 30 min followed by continuous exposure to 800 mg% [1-¹⁴C] ethanol (final specific activity 0.125 µCi/mM) at 37°C for 6 h. After incubation, cells were scrapped along with culture media and lipids extracted with chloroform/methanol (2:1, by volume) and

separated by thin layer chromatography (Kaphalia et al. 1999; Wu et al. 2006, 2008). The silica gel corresponding to the ester region was scrapped and radioactivity measured using liquid scintillation counter. The total radioactive disintegration per minute was adjusted to 25×10^6 cells/flasks and expressed as nmol FAEEs formed/ 25×10^6 cells.

Viability and cytotoxicity assays

Cell viability was assessed by MTT assay (R&D Systems, Minneapolis, MN) as per manufacturer's instructions. Effect of ethanol on cell proliferation was assessed by Cell Proliferation kit (XTT, Roche Diagnostic GmbH, Germany) as per manufacturer's instruction in cells pre-incubated with or without inhibitors. For determining apoptosis and necrosis, 5×10^5 cells/well were seeded in 24-well plates and incubated at 37°C overnight. Cells were pretreated with the inhibitors as described before followed by incubation with ethanol at 37°C for 6 h. Cells were harvested and apoptosis and necrosis were measured by TACS™ Annexin V-FITC Apoptosis Detection kit (R&D Systems) as per manufacturer's instructions as described previously (Wu et al. 2008). The apoptosis and necrosis were expressed as percent of total number of cells.

Ethanol-induced morphological changes in cells pre-treated with 1,10-PT or 4-MP

Cells pre-treated with 1,10-PT, or 4-MP were incubated with ethanol at 37°C for 6 h, examined under IX71 microscope equipped with digital camera DP71 and OLYMPUS MICRO DP71 using VER 03.01 software and images captured.

Statistical analysis

The data is presented as mean $\pm$ standard deviation (SD). The data were analyzed by employing unpaired Student's *t* test and Analysis of variance for multiple group comparisons followed by Student–Newman Kucl's post hoc test using the InStat programs. *P* values ≤ 0.05 were considered as statistically significant.

Results

Residual ethanol and acetaldehyde levels in culture medium

The residual levels of ethanol at 6 h of incubation are shown in Fig. 1. Mean values for ethanol were found to be 508 mg% as compared to 15 mg% in the culture medium of ethanol treated and control cells, respectively (Fig. 1). The mean values of ethanol concentrations in the culture media of treated cells indicate that ~70% of the total ethanol initially added was found at 6 h of incubation. The acetaldehyde concentration was found to be very low in the medium of ethanol-treated (0.02 mg%) and control cells (0.01 mg%) and the differences between the groups were not significant.

Formation of FAEEs

Cells exposed to ethanol or DMSO + ethanol are considered as control groups based on solubility of the inhibitors. For example, ethanol was used as control for 4-MP + ethanol and SA + ethanol or DMSO + ethanol as control for 1,10-PT + ethanol and DCE + ethanol-treated cells. Mean levels $\pm$ SD of total FAEEs in cells incubated with ethanol or DMSO +

ethanol were found to be 50 ± 11 and 47 ± 4 nmoles/ 25×10^6 cells, respectively. Synthesis of total FAEEs in cells incubated with 1,10-PT, 4-MP, DCE, or SA in combination with ethanol as summarized in Fig. 2 was found to be increased to 205, 150, 107, or 103% as compared to their respective ethanol control group with mean $\pm$ SD values being 106 ± 33 , 71 ± 16 , 53 ± 14 , and 55 ± 12 nmoles/ 25×10^6 cells, respectively. However, significantly high FAEEs levels were found only in the cells incubated with 1,10-PT + ethanol as compared to those with other inhibitors + ethanol. Therefore, inhibition of ADH class I—III combined appears to be a major contributor in ethanol metabolism to FAEEs via nonoxidative pathway.

Cell viability and proliferation assays

The cell viability was greater than 90% for control groups that were incubated with ethanol or DMSO + ethanol. We have used reported optimum effective concentration of inhibitors in our study. Ethanol itself decreased viability by ~7%, but 1,10-PT or 4-MP diminished viability by ~20 and ~17%, respectively. The cell viability rates were significantly decreased to ~62 and ~72% in cells treated with 1,10-PT + ethanol or 4-MP + ethanol, respectively, as compared to their respective controls (Fig. 3). Cells incubated with DCE or SA in combination of ethanol diminished cell viability by 12 and 8% only as compared to their respective DMSO + ethanol or ethanol controls.

The proliferation rates were also reduced significantly in the cells treated with 1,10-PT or 4-MP in combination with ethanol as compared to DMSO + ethanol or ethanol controls, respectively (Fig. 4). However, cell proliferation rates were not altered for the cells treated with 1,10-PT or 4-MP alone as compared to DMSO or untreated control cells, respectively. The cell death and cell proliferation data appears to be complementary to each other for the ADH inhibitors and collectively indicate that inhibition of pancreatic ADH isozyme(s) plays key role in ethanol-induced pancreatic injury. From present studies, we also conclude that role of CYP2E1 and catalase may not be significant in ethanol-induced pancreatic acinar cell toxicity. Therefore, we used cells treated with ADH inhibitors (1,10-PT or 4-MP) only for measuring ethanol-induced cell death by apoptosis and necrosis.

Apoptotic and necrotic cell deaths

Cells treated with inhibitors alone did not produce significant apoptosis and necrosis as compared to their respective controls. Ethanol caused significant apoptosis as compared to untreated or DMSO controls or those treated with inhibitors itself (Fig. 5A). Most importantly, cells treated with 1,10-PT + ethanol showed very significant increases in apoptosis and necrosis as compared to DMSO + ethanol control group (Fig. 5A,B). A representative histogram with quadrant of Annexin V stained subpopulation of AR42J cells is depicted in Fig. 5C for control group (DMSO + ethanol) and Fig. 5D for 1,10-PT + ethanol group showing increased in cell percentage for both apoptotic and necrotic cells.

Ethanol-induced morphological changes in cells treated with 1, 10-PT and 4-MP

Ethanol-induced morphological characteristics of cells were remarkably altered by ethanol in cells treated with 1,10-PT in combination with ethanol (cellular shrinkage and dissociation) (Fig. 6C). Only minor morphological alterations were found in the cells treated

with 4-MP. Significant morphological changes were also not observed in the cells treated with inhibitors alone, ethanol, or DMSO + ethanol controls.

Overall, inhibition of ADH isozymes appears to be critical for the facilitating FAEE synthase-catalyzed formation of FAEEs; a major pathway involved in ethanol-induced pancreatic injury during chronic alcohol abuse.

Discussion

Oxidation of ethanol catalyzed by hepatic ADH is a major mechanism of its metabolism and disposition in the body (Rognstead and Grunnet 1979). It is well known that chronic alcohol ingestion inhibits hepatic ADH and impairs oxidation of ethanol required for its further metabolism and excretion (Nuutinen et al. 1983; Panes et al. 1989, 1993; Kaphalia et al. 1996; Fernando et al. 2013). However, long-term ethanol ingestion also induces CYP2E1, which is known to be involved in ethanol oxidation during chronic alcohol abuse (Kaphalia et al. 1996). It is also well known that inhibition or deficiency of hepatic ADH facilitates nonoxidative metabolism of ethanol to FAEEs in extrahepatic organs such as pancreas, frequently damaged during chronic alcohol abuse (Laposata and Lange 1986; Bhopale et al. 2006). This FAEE formation takes place irrespective of CYP2E1 overexpression (Wu et al. 2006).

As reported for the livers, mammalian pancreata also possess similar ethanol oxidizing enzymes including ADH isozymes, CYP2E1, and catalase, but in smaller amounts (Foster et al. 1993; Kessova et al. 1998; Norton et al. 1998; Chrostek et al. 2003; Wu et al. 2008; Kaphalia 2011). The observation that a direct inhibition of pancreatic ADH isozyme(s) shifts ethanol metabolism to FAEEs via nonoxidative pathway catalyzed by FAEE synthase resulting in increased cell death in culture as reported herein could be an important underlying mechanism of alcoholic pancreatitis. Among all organs, pancreas possesses the highest activity of FAEE synthase, which can be up-regulated by ethanol exposure (Laposata and Lange 1986; Bhat and Ansari 1990; Pfutzer et al. 2002; Kaphalia and Ansari 2003). However, a significant FAEE synthase catalyzed formation of FAEEs takes place in the pancreas only at high alcohol levels or when oxidative metabolism is impaired during chronic alcohol abuse or after ethanol exposure in experimental animals pre-treated with 4-MP (Laposata and Lange 1986; Manautou and Carlson 1991; Kaphalia and Ansari 2001; Werner et al. 2002).

FAEEs display several fold greater cytotoxicity in freshly isolated rat pancreatic acinar cells than ethanol itself or its oxidative metabolite, acetaldehyde, (Gukovskaya et al. 2002; Criddle et al. 2004, 2006). Due to the short life span of dispersed freshly isolated pancreatic acinar cells, AR42J cell line, which display most of the biochemical characteristics of pancreatic acinar cells, have been used as a cell culture model to investigate the biology and mechanism of pancreatic injury (Christophe 1994). Remarkably increased levels of FAEEs in cells treated with inhibitors of ADH class I—III (1,10-PT) or ADH I—II (4-MP) in combination with ethanol as compared to DMSO + ethanol or ethanol controls, respectively, suggest that ADH are housekeeping enzymes integral for oxidation and removal of xenobiotic alcohols including ethanol from the cells. It has been reported that ADH class III

form is the most active form; about sevenfold greater than ADH class I in human pancreata (Chrostek et al. 2003). However, estimated levels of FAEEs found in the present study are far below than those reported in the pancreata of alcohol abusers and deer mice (Laposata and Lange 1986; Kaphalia et al. 2004; Bhopale et al. 2006). Being lipophilic, FAEEs accumulate in the lipid-rich membranes and cause such mitochondrial toxicity as uncoupling of oxidative phosphorylation and membrane toxicity (Lange and Sobel 1983; Criddle et al. 2004, 2006). However, the formation of FAEEs and their toxicity can be attenuated by 3-benzyl-6-chloro-2-pyrone (specific inhibitor of pancreatic FAEE synthase) in AR42J cell culture model (Wu et al. 2008).

A significant cell death by apoptosis and necrosis as found in the present study in cells treated with ADH inhibitors in combination of ethanol could be related to impaired ATP synthesis caused by the uncoupling effect of FAEEs and their mitochondrial toxicity in general (Lange and Sobel 1983; Wu et al. 2006). Ethanol-induced cytotoxicity as observed in the cells treated with 1,10-PT or 4-MP in combination with ethanol could be related to the increased formation of FAEEs as supported by the reports on FAEE-induced pancreatic acinar cell toxicity (Wilson et al. 1990; Gukovskaya et al. 2002; Criddle et al. 2004, 2006; Wu et al. 2008). Like as livers, pancreatic ADH isozymes can also differentially metabolize and dispose ethanol from pancreata as defense mechanism against the ethanol-induced toxicity. However, pancreas appear to be a major organ for ethanol metabolism via nonoxidative metabolism to FAEEs and a target of toxicity during chronic alcohol abuse because of very high FAEE synthase activity and very low levels of ADH and CYP2E1 (Laposata and Lange 1986; Kaphalia 2011). Therefore, increased formation and accumulation of FAEEs can be a risk factor for alcohol-induced pancreatic injury during its chronic abuse. To the best of our knowledge, so far, no reports are available regarding the inhibition of pancreatic ADH. Therefore, we hypothesize that the continuous formation and accumulation of FAEEs to a toxic concentration in the pancreas during chronic alcohol abuse could be a potential underlying mechanism of alcoholic pancreatitis. Further studies regarding the distribution and differential inhibition of pancreatic ADH isozymes after chronic ethanol consumption along with ethanol metabolism should be undertaken to test the hypothesis mentioned above.

Conclusions

ADH class I, II and/or especially class III in combination appear to be major contributor of ethanol oxidative metabolism and their inhibition facilitates formation of FAEEs (nonoxidative metabolites of ethanol) contributing to ethanol-induced cytotoxicity in pancreatic acinar cells. We also conclude that CYP2E1 and catalase-mediated oxidation of ethanol might not be significant mechanisms of ethanol oxidative metabolism and related pancreatic injury.

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Figure 1. Residual ethanol levels in the culture media of AR42J cells incubated with ethanol at 37°C for 6 h. * P 0.001 as compared to controls. Values are mean $\pm$ SD (n = 4 in each group).

Figure 2.

Formation of FAEs in AR42J cells pre-treated with specific inhibitors of ethanol oxidizing enzymes followed by incubation with [1-¹⁴C]ethanol at 37°C for 6 h. 1,10-PT + ethanol treatment enhanced the formation of FAEs as compared to DMSO + ethanol control (*b*, **P* < 0.01). Values are mean ± SD (*n* = 4 in each group).

Figure 3.

Effects of inhibitors in combination with ethanol on the viability of AR42J cells. The cell viability decreased significantly both in cell incubated with 1,10-PT + ethanol (* P 0.001) as compared to DMSO + ethanol control (d), and 4-MP + ethanol (* P 0.001 as compared to ethanol control (b). The viability of the cells treated with 1,10-PT or 4-MP only as compared to DMSO (c) or untreated (a) controls, respectively, was also diminished significantly (* P 0.01 and P 0.05, respectively). Values are mean $\pm$ SD (n = 6 in each group).

Figure 4.

Effects of inhibitors or combination of inhibitors and ethanol on cell proliferation of AR42J cells. Only ADH inhibitors 1,10-PT or 4-MP in combination of ethanol showed decreased cell proliferation, * $P < 0.001$ as compared to DMSO + ethanol (d) or ethanol (b) controls, respectively. Values are mean $\pm$ SD ($n = 5$ in each group).

Figure 5.

Effects of inhibitors in combination of ethanol on apoptosis (A) and necrosis (B) of AR42J cells. **P* value = 0.01 against untreated control (a) and ethanol (b), DMSO (c) or DMSO + ethanol (d) treated controls. 1,10-PT + ethanol-treated cells showed significant apoptosis (**P* value = 0.01) and necrosis (**P* value = 0.05) as compared to DMSO + ethanol control (d). Two color quadrant of Annexin V stained showing three subpopulation (blue live cells, green apoptotic cells, and red necrotic cells) in DMSO + ethanol control (C) and 1,10-PT + ethanol group (D). Values are mean $\pm$ SD (*n* = 4 in each group).

Figure 6.

Effect of ethanol on morphology of AR42J cells treated with specific inhibitors of ethanol oxidizing enzymes in combination of ethanol; untreated control cells (**A**), ethanol-treated control cells (**B**), cells treated with 1,10-PT + ethanol (**C**), 4-MP + ethanol (**D**) or DMSO (solvent) + ethanol control (**E**). Cell shrinking and dissociation was observed in cells treated with 1, 10-PT in combination of ethanol (**C**, magnification $\times 20$).

Table 1

Inhibitors of ethanol oxidizing enzymes and their optimal inhibitory concentrations

Inhibitors	Final concentration	Inhibited isozyme
1,10-phenanthroline [*] (1,10-PT, Sigma) (soluble in DMSO)	1 mmol/L	Alcohol dehydrogenase (ADH) class I, II, III
4-methylpyrazole [*] (4-MP, Sigma) (soluble in aqueous medium)	5 mmol/L	ADH class I, II
Trans – 1,2– dichloroethylene ^{**} (DCE, Aldrich) (soluble in DMSO)	5 mmol/L	Cytochrome P450 2E1 (CYP2E1)
Sodium azide [*] (SA, Fluka Biochemicals) (soluble in aqueous medium)	1 mmol/L	Catalase

^{*} Haber et al. 1998

^{**} Mathews et al. 1998