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Effects of Oxidative Alcohol Metabolism on the Mitochondrial Permeability Transition Pore and Necrosis in a Mouse Model of Alcoholic Pancreatitis

NATALIA SHALBUEVA^{1,2}, OLGA A. MARENINOVA¹, ANDREAS GERLOFF¹, JINGZHEN YUAN¹, RICHARD T. WALDRON¹, STEPHEN J. PANDOL¹, and ANNA S. GUKOVSKAYA¹

¹Veterans Affairs Greater Los Angeles Healthcare System, David Geffen School of Medicine, University of California at Los Angeles, Southern California Research Center for Alcoholic Liver and Pancreatic Disease and Cirrhosis, Los Angeles, California

²Institute of General and Experimental Biology, Russian Academy of Sciences, Siberian Branch, Ulan-Ude, Russian Federation

Abstract

BACKGROUND & AIMS—Opening of the mitochondrial permeability transition pore (MPTP) causes loss of the mitochondrial membrane potential ($\Delta\psi$) and, ultimately, adenosine triphosphate depletion and necrosis. Cells deficient in cyclophilin D (CypD), a component of the MPTP, are resistant to MPTP opening, loss of $\Delta\psi$, and necrosis. Alcohol abuse is a major risk factor for pancreatitis and is believed to sensitize the pancreas to stressors, by poorly understood mechanisms. We investigated the effects of ethanol on the pancreatic MPTP, the mechanisms of these effects, and their role in pancreatitis.

METHODS—We measured $\Delta\psi$ in mouse pancreatic acinar cells incubated with ethanol alone and in combination with physiologic and pathologic concentrations of cholecystokinin-8 (CCK). To examine the role of MPTP, we used ex vivo and in vivo models of pancreatitis, induced in wild-type and *CypD*^{-/-} mice by a combination of ethanol and CCK.

RESULTS—Ethanol reduced basal $\Delta\psi$ and converted a transient depolarization, induced by physiologic concentrations of CCK, into a sustained decrease in $\Delta\psi$, resulting in reduced cellular adenosine triphosphate and increased necrosis. The effects of ethanol and CCK were mediated by MPTP because they were not observed in *CypD*^{-/-} acinar cells. Ethanol and CCK activated MPTP through different mechanisms— ethanol by reducing the ratio of oxidized nicotinamide adenine dinucleotide to reduced nicotinamide adenine dinucleotide, as a result of oxidative metabolism, and CCK by increasing cytosolic Ca^{2+} . *CypD*^{-/-} mice developed a less-severe form of pancreatitis after administration of ethanol and CCK.

CONCLUSIONS—Oxidative metabolism of ethanol sensitizes pancreatic mitochondria to activate MPTP, leading to mitochondrial failure; this makes the pancreas susceptible to necrotizing pancreatitis.

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Address requests for reprints to: Anna S. Gukovskaya, PhD, UCLA/VA Greater Los Angeles Healthcare System, West Los Angeles VA Healthcare Center, 11301 Wilshire Boulevard, Building 258, Room 340, Los Angeles, California 90073. agukovsk@ucla.edu; fax: (310) 268-4578.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at <http://dx.doi.org/10.1053/j.gastro.2012.10.037>.

Conflicts of interest

The authors disclose no conflicts.

Keywords

Tissue Damage; Ethanol Toxicity; Cell Death; Exocrine Pancreas

Pancreatitis is a disease of the exocrine pancreas, the main pathologic responses of which are intrapancreatic activation of digestive enzymes, such as trypsin, inflammatory infiltration, acinar cell vacuolization, and necrosis. Necrosis is a key response of pancreatitis and is associated with significant mortality.¹

Mitochondrial membrane permeabilization (MMP) is a universal trigger for both necrosis and apoptosis types of cell death.^{2,3} One important consequence of MMP is loss of the mitochondrial membrane potential, $\Delta\psi$, which ultimately leads to adenosine triphosphate (ATP) depletion and necrosis. A major participant in MMP is the mitochondrial permeability transition pore (MPTP). In response to stressors (such as Ca^{2+} overload), the MPTP assumes a high-conductance conformation, allowing unregulated entry of solutes with molecular mass less than 1500 daltons, into the mitochondrial matrix, which results in the loss of $\Delta\psi$. This is referred to as the *open* or *activated* state. MPTP is a multiprotein complex, the key component of which is cyclophilin D (CypD). Cells from mice with genetic CypD ablation are more resistant to MPTP opening, mitochondrial depolarization, and necrosis.^{4,5} The mechanisms whereby CypD deficiency prevents MPTP activation are under investigation and not established yet.⁶⁻⁸

Abnormal (global and sustained) increase in acinar cell cytosolic Ca^{2+} is a pathologic signal observed in several experimental models of acute pancreatitis, and is believed to be involved in disease initiation.^{9,10} An increase in cytosolic Ca^{2+} in cerulein (a cholecystokinin [CCK] analogue) and bile acid models of pancreatitis causes depolarization of pancreatic mitochondria through MPTP opening.¹¹⁻¹⁴ The results over the past several years have suggested that acinar cell necrosis in experimental models of pancreatitis is mediated, at least partially, through MPTP opening.^{11-13,15-17}

Alcohol abuse is a major etiologic factor for necrotizing pancreatitis. Because clinically relevant disease develops in fewer than 10% of heavy drinkers and because animals do not develop pancreatitis with alcohol treatment alone, it is believed that alcohol sensitizes the pancreas to the action of other stressors for the development of the disease.¹ For example, we showed that ethanol feeding sensitizes rats to acute cerulein-induced pancreatitis and thus decreases the threshold for the initiation of pancreatic injury.¹⁸

Sensitization to pathologic effects of CCK or cerulein also occurs in pancreatic acinar cells incubated with ethanol. Combinations of ethanol and low concentrations of CCK (but not low concentrations of CCK itself) were shown to trigger pathologic responses of pancreatitis in acinar cells, such as activation of nuclear factor- κ B, pathologic protease activation, necrosis, and basolateral exocytosis.¹⁹⁻²² This experimental system enables us to study the mechanisms by which ethanol predisposes to pancreatitis in an ex vivo model.

The mechanisms of the sensitizing effect of alcohol only partly are known. For example, very little is known about the effect of alcohol on pancreatic mitochondria or the role of mitochondrial MPTP on the effects of ethanol.

Ethanol's toxicity is mediated through the action of ethanol itself or through oxidative and nonoxidative ethanol metabolism.²³⁻²⁶ In oxidative metabolism alcohol dehydrogenase (ADH) oxidizes ethanol to acetaldehyde followed by chemical transformation of acetaldehyde to acetate by isoform 2 of aldehyde dehydrogenase (ALDH2) located specifically to mitochondria. This metabolism also depletes oxidized nicotinamide adenine

dinucleotide (NAD⁺) levels while increasing reduced nicotinamide adenine dinucleotide (NADH) because ADH and ALDH are both NAD⁺-consuming enzymes producing NADH.²⁶ In nonoxidative metabolism, fatty acid ethyl ester (FAEE) synthase converts ethanol to FAEE.²³⁻²⁵ Thus, ethanol metabolism results in accumulation of acetaldehyde, acetate, NADH, and FAEE, as well as changes in cellular and mitochondrial NAD⁺ and NADH levels.

Here, we show that ethanol causes a MPTP-dependent loss of Ca^{2+} in pancreatic acinar cells. Further, ethanol converts a transient Ca^{2+} response induced by physiologic concentrations of CCK into a sustained decrease in Ca^{2+} , resulting in decreased cellular ATP and necrosis. Ethanol-induced MPTP activation is mediated by a decrease in NAD⁺ resulting from oxidative ethanol metabolism whereas the effect of CCK is caused by a CCK-induced increase in free cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$). Genetic CypD ablation prevents these effects of ethanol and CCK and improves the severity of alcoholic pancreatitis induced by the combination of EtOH+CCK.

Materials and Methods

Measurements of Ca^{2+} in pancreatic acinar cells using Ca^{2+} -sensitive fluorescence probes, tetramethylrhodamine (TMRM) and JC-1,¹² digitonin permeabilization of acinar cells,²⁷ isolation of pancreatic mitochondria¹² and pancreatic acinar cells, and measurements of $[\text{Ca}^{2+}]_i$ ²⁸ are described in the Supplementary Materials and Methods section.

Animals

Cyclophilin D– deficient mice (of the same C57BL/6 background as wild-type controls)⁶ were kindly provided by Dr Michael A. Forte (Oregon Health and Sciences University).

Models of Pancreatitis

In experiments on cells we used an ex vivo model in which mouse pancreatic acinar cells were preincubated for 1–3 hours without and with ethanol, and then were stimulated for the indicated time with physiologic 10 pmol/L CCK (or vehicle added for control).¹⁹⁻²² We also measured the effects of ethanol on the ex vivo model of fully blown pancreatitis that is in cells preincubated with and without ethanol as described earlier and stimulated with supraphysiologic 0.1 $\mu\text{mol/L}$ CCK. In animal experiments mice were pair-fed for 6 weeks the Lieber–DeCarli ethanol or control diet,¹⁸ after which they received cerulein (a stable CCK analog) at a dose of 3 $\mu\text{g/kg}$ or saline as 7-hourly intraperitoneal injections, and mice were killed 1 hour after the last injection. Animals in the control group received a control diet and saline injections.

Quantification of Necrosis in Pancreatic Tissue

Quantification of necrosis was performed on H&E-stained sections.¹⁸ Necrosis in pancreatic acinar cells was determined using the Vybrant Cytotoxicity Assay Kit (Life Technologies, Grand Island, NY).

Enzymatic Assays

Trypsin activity was measured in homogenized cells or pancreatic tissue by a fluorogenic assay as described¹¹ using Boc-Gln-Ala-Arg-AMC as a substrate. ATP levels in pancreatic tissue samples were measured as described by Sung et al¹⁵ using an ATP determination kit (Molecular Probes, Eugene, OR). NAD⁺/NADH levels were measured in cell lysates using a fluorescent detection kit (Cell Technology, Mountain View, CA).

Statistical Analysis

Data are presented as the mean $\pm$ standard error of the mean. Statistical evaluation was performed using a 2-tailed Student *t* test.

Results

Ethanol Causes MPTP-Dependent Loss of $\Delta\Psi_m$ in Pancreatic Acinar Cells

To determine the effect of ethanol treatment on mitochondrial membrane potential in acinar cells we used the ψ_m -sensitive fluorescence probe TMRM. Incubation of pancreatic acinar cells with ethanol resulted in increases in TMRM fluorescence, indicating mitochondrial depolarization (Figure 1). Mitochondrial uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, which completely cancels ψ_m , increased TMRM fluorescence maximally (Figure 1A). Ethanol at 25–100 mmol/L caused a time- and dose-dependent decrease of ψ_m in acinar cells. The decrease was significant after a 3-hour incubation with 50 mmol/L or a 1-hour incubation with 100 mmol/L ethanol (Figure 1E). Of note, acute addition of EtOH had no effect on ψ_m (not shown).

In acinar cells isolated from mice deficient in CypD, a key MPTP component, depolarization induced by 50 and 100 mmol/L ethanol was largely prevented, indicating that it is mediated through MPTP (Figure 1D and E).

The results with TMRM (Figure 1) were confirmed with another ψ_m -sensitive fluorescence probe, JC-1 (Supplementary Figure 1).

Ethanol Potentiates CCK-Induced Depolarization by Sensitizing Pancreatic Mitochondria to Ca^{2+} -Induced MPTP Activation

Physiologic (10 pmol/L) and pathologic (0.1 μ mol/L) doses of C-terminal octapeptide of CCK both induced mitochondrial depolarization (Figure 2A), which agrees with findings by Odínokova et al,¹² Booth et al,¹³ Voronina et al,¹⁴ and Sung et al.¹⁵ A dose of 10 pmol/L CCK caused a rapid (within 1 minute) decrease in ψ_m followed by a plateau sustained for up to 16 minutes, and then ψ_m recovered back to the basal level (Figure 2A). Of note, basal ψ_m slowly decreases with time (Figure 1A), so that by 25 minutes there was no difference between ψ_m in untreated cells and those treated with 10 pmol/L CCK (Figure 2A and C). A dose of 0.1 μ mol/L CCK also caused a rapid decrease in ψ_m , but the decrease here was sustained (Figure 2A and C). Thus, although physiologic doses of CCK cause transient depolarization, pathologic concentrations of CCK cause a sustained depolarization.

Preincubation with 50 or 100 mmol/L EtOH potentiated the depolarizing effects of both physiologic and pathologic doses of CCK (Figure 2A–D). Importantly, without ethanol, depolarization induced by 10 pmol/L CCK was transient, whereas in cells preincubated with 50 or 100 mmol/L EtOH the same dose of CCK caused progressive depolarization (Figure 2B and Supplementary Figure 2).

MPTP inactivation largely prevented the depolarizing effects of both EtOH and CCK (Figure 2D–F). In particular, potentiation by ethanol of the depolarization induced by 10 pmol/L CCK was completely prevented in CypD^{−/−} cells (*n* = 5) and the response to 0.1 μ mol/L CCK was inhibited by 87% $\pm$ 4% (in cells preincubated with 50 mmol/L EtOH; Figure 2E) and by 90% $\pm$ 3% (in cells preincubated with 100 mmol/L EtOH; Figure 2F; *n* = 4). Thus, the results in Figures 1 and 2 indicate that ethanol, CCK, and the combination of ethanol+CCK treatments decrease ψ_m through a CypD/MPTP-dependent mechanism.

As discussed earlier, CCK-induced depolarization is mediated through an increase in cytosolic Ca^{2+} , leading to the mitochondrial Ca^{2+} overload.¹²⁻¹⁴ Previous results^{29,30} have indicated that ethanol had only minor effects on Ca^{2+} homeostasis and at concentrations up to 850 mmol/L produced only small increases in $[\text{Ca}^{2+}]_i$. In accordance with these results,^{29,30} we found that preincubation with 50 or 100 mmol/L ethanol had no effect on $[\text{Ca}^{2+}]_i$ in approximately 95% of cells and caused oscillations, which were small and irregular in both amplitude and frequency, in approximately 5% of cells (Supplementary Figure 3A–D). Both in control and ethanol-treated cells, 10 pmol/L CCK stimulated similar $[\text{Ca}^{2+}]_i$ oscillations (Supplementary Figure 3C and D). $[\text{Ca}^{2+}]_i$ measurements in cell suspension (Supplementary Figure 3) showed no effect of pretreatment with ethanol on the magnitude of CCK-induced Ca^{2+} responses.

We further showed that the intracellular Ca^{2+} chelator 2-bis (2-aminophenoxy)-ethane-*N,N,N,N*-tetraacetic acid, which fully abolished whatever increases ethanol might have induced in $[\text{Ca}^{2+}]_i$, did not prevent ethanol-induced depolarization (Supplementary Figure 3). These data indicate that an increase in $[\text{Ca}^{2+}]_i$ is not the key mechanism of ethanol-induced depolarization.

Considering the earlier-described results, we hypothesized that ethanol enhances the sensitivity of pancreatic mitochondria to Ca^{2+} -mediated MPTP opening. To test this possibility we compared the depolarizing effects of different concentrations of free Ca^{2+} on $\Delta\psi_m$ of pancreatic mitochondria from control cells and from cells pre-incubated with ethanol (Figure 3). The experiments were performed on digitonin-permeabilized cells. At the concentration used, digitonin binds to plasma membrane cholesterol, making plasma membrane permeable to solutes and molecules without affecting the structure or permeability of mitochondrial membranes.²⁷ Pancreatic acinar cells were preincubated with or without EtOH, permeabilized with digitonin, and changes in $\Delta\psi_m$ were measured at the indicated concentrations of free Ca^{2+} clamped with Ca^{2+} /ethylene glycol tetraacetic acid buffers in the presence of glutamate/malate as the respiratory substrate (Figure 3).

Plasma membrane permeabilization with digitonin led to an increase in $\Delta\psi_m$ as a result of delivery of respiratory substrates to the mitochondria (Figure 3A). Depending on the concentration of free Ca^{2+} , the mitochondria either maintain $\Delta\psi_m$ or show time-dependent depolarization (Figure 3A). Of note, we showed that Ca^{2+} at micromolar concentrations caused a marked loss of $\Delta\psi_m$ of isolated pancreatic mitochondria.¹²

Preincubation of cells with ethanol enhanced the sensitivity of mitochondria to Ca^{2+} -induced depolarization. The effect of ethanol was evident at 300 nmol/L Ca^{2+} and more pronounced at greater Ca^{2+} concentrations (Figure 3A). For example, mitochondria from cells preincubated with both 50 mmol/L and 100 mmol/L EtOH and exposed to 1 $\mu\text{mol/L}$ Ca^{2+} lose potential faster than mitochondria from control cells (Supplementary Figure 4, Figure 3A and B). Thus, Ca^{2+} and EtOH act in concert to potentiate the depolarizing effect of each other on $\Delta\psi_m$.

We previously showed that Ca^{2+} -induced activation of pancreatic MPTP is partially inhibited by bongkreikic acid, and fully by a combination of bongkreikic acid and cyclosporin A.¹² The same pharmacologic PTP inhibitors prevented depolarization induced by ethanol (Figure 3A and Supplementary Figure 4). CypD deficiency also greatly attenuated the effects of ethanol and Ca^{2+} on depolarization (Figure 3B and C and Supplementary Figure 4). Thus, pharmacologic and genetic approaches both indicate that ethanol-induced mitochondrial depolarization is mediated through MPTP opening.

Of note, we measured the effects of the inhibitors on m in permeabilized cells because bongkreikic acid is toxic for acinar cells, and cyclosporin A inhibits not only CypD but also cytosolic calcineurin.^{2,3}

Ethanol Activates MPTP Through a Decreasing NAD^+/NADH Ratio

The NAD^+/NADH ratio is known to regulate MPTP opening.^{31,32} Further, it is well established that ethanol oxidation results in a decrease of NAD^+/NADH ratio in the liver, which may contribute to the formation of fatty liver.³³ This is because ethanol oxidation is mediated by 2 enzymes, both of which consume NAD^+ (ie, ADH, which converts alcohol into acetaldehyde, and ALDH, which oxidizes acetaldehyde into acetate).³³ We found that incubation with EtOH significantly decreases the NAD^+/NADH ratio in pancreatic acinar cells (Figure 4A). Furthermore, this decrease was prevented by daidzin, a specific inhibitor of ALDH2, which resides in mitochondria (Figure 4, inset), and converts acetaldehyde generated by alcohol metabolism into acetate.^{33,34} Restoring NAD^+ with either daidzin or by adding exogenous NAD^+ both fully prevented depolarizing effects of ethanol (Figure 4B–D).

To determine whether an ethanol-unrelated decrease in NAD^+ can cause depolarization, we applied FK866, a specific inhibitor of nicotinamide phosphoribosyltransferase, the key enzyme in the NAD^+ synthesis pathway.³⁵ FK866 significantly decreased both the cellular NAD^+ level and m (Figure 4E and F) and potentiated depolarization induced by 10 pmol/L or 0.1 $\mu\text{mol/L}$ CCK (Figure 4E) similar to the effects of ethanol. Furthermore, exogenous NAD^+ prevented depolarization induced by FK866 (Figure 4E).

Importantly, the effect of exogenous NAD^+ was prevented in $\text{CypD}^{-/-}$ cells, indicating that a NAD^+ decrease caused depolarization through activating MPTP (Supplementary Figure 5). NAD^+ restoration prevented ethanol but not CCK-induced depolarization, indicating that these agents activate MPTP through different mechanisms. (Figure 4B and D).

We next used digitonin-permeabilized cells to test whether NAD^+ affects Ca^{2+} -induced MPTP opening (Figure 5). Cells preincubated in the presence and absence of EtOH and exogenous NAD^+ were permeabilized with digitonin, and changes in m were measured with $[\text{Ca}^{2+}]_i$ clamped at 1 $\mu\text{mol/L}$. In the absence of ethanol, the addition of NAD^+ only slightly alleviated depolarization induced by 1 $\mu\text{mol/L}$ Ca^{2+} in permeabilized cells. However, NAD^+ largely prevented the potentiating effect of ethanol on Ca^{2+} -induced depolarization (Figure 5B and C). Importantly, this effect of NAD^+ was absent in $\text{CypD}^{-/-}$ cells (Figure 5A and B), suggesting that NAD^+ acts by inhibiting MPTP.

The results in Figures 4 and 5 show a critical role of NAD^+/NADH in ethanol-induced MPTP activation.

Major products of ethanol metabolism are acetaldehyde and FAEs.^{23–26} Acetaldehyde at concentrations up to 1 mmol/L had no effect on m in both intact and permeabilized acinar cells (Supplementary Figure 6). As shown by Criddle et al,^{29,36} FAEs stimulate Ca^{2+} release from intracellular stores, producing an increase in $[\text{Ca}^{2+}]_i$ and Ca^{2+} -induced depolarization. Our measurements indicated that in permeabilized cells, FAEs, at a total concentration of up to 1.5 mmol/L, did not change m (Supplementary Figure 6), indicating no direct Ca^{2+} -independent effect of FAEs on mitochondria. Of note, in these experiments $[\text{Ca}^{2+}]_i$ was clamped at 100 nmol/L.

MPTP Activation Mediates Pathologic Responses of Alcoholic Pancreatitis

We assessed the role of MPTP in acinar cell necrosis, in an ex vivo model of pancreatitis, in which mouse pancreatic acinar cells were treated with both ethanol and physiologic 10

pmol/L CCK. Combinations of ethanol and low-dose CCK (but not low-dose CCK itself) were shown previously to trigger essential pathologic responses of pancreatitis in acinar cells.¹⁹⁻²² This experimental system enables one to study *ex vivo* the mechanisms by which ethanol predisposes to pancreatitis. Incubation with ethanol by itself slightly increased the basal acinar cell necrosis rate; whereas 10 pmol/L CCK alone had no effect. However, the combination of EtOH and 10 pmol/L CCK synergistically increased necrosis 2-fold compared with control (Figure 6). For comparison, the supramaximal (0.1 μ mol/L) CCK alone in the same conditions increased acinar cell necrosis to 23% $\pm$ 2% (n = 4).

The increase in necrosis induced by EtOH + 10 pmol/L CCK completely was prevented in CypD-null acinar cells (Figure 6), indicating a key mediatory role for MPTP in acinar cell necrosis. Also, the increase in necrosis fully was prevented by daidzin, indicating that ALDH2 and loss of NAD⁺ mediate necrosis in ethanol-treated acinar cells.

We further examined the role of mitochondrial depolarization in the mouse model of alcoholic pancreatitis induced by a combination of alcohol feeding and low-dose cerulein.¹⁸ We first measured the effect of Ca²⁺ on $\Delta\psi_m$ in isolated mitochondria, showing that pancreatic mitochondria isolated from CypD^{-/-} mice with alcoholic pancreatitis were more resistant to Ca²⁺-induced depolarization compared with mitochondria from wild-type mice with alcoholic pancreatitis (Figure 7A). Further, CypD deficiency improved parameters of alcoholic pancreatitis. For example, CypD deficiency prevented the decrease in pancreatic ATP induced by alcoholic pancreatitis (Figure 7B). Correspondingly, there was less necrosis in alcoholic pancreatitis in CypD^{-/-} mice (Figure 7C). We also found that CypD^{-/-} deficiency decreased serum hyperamylasemia and pancreatic trypsinogen activation (Figure 7D and E).

Discussion

The combined results of our study indicate that ethanol causes a time- and dose-dependent opening of the pancreatic MPTP, resulting in mitochondria depolarization. The mediatory role of MPTP in the mitochondrial failure is supported by the findings that both genetic ablation of CypD and pharmacologic MPTP blockers prevented the depolarizing effects of ethanol on pancreatic mitochondria.

Depolarization was observed in cells incubated with 50 or 100 mmol/L ethanol for 1–3 hours. These ethanol concentrations are within the range of blood alcohol levels (BAL) occurring in rodent models of ethanol-mediated pancreatitis.^{18,37} They also are within the range of BALs reported in drinkers cited for driving under the influence (the mean and the highest BAL levels being 0.17% and 0.52%, respectively,³⁸ which are equivalent to 40 and 112 mmol/L ethanol).

Increases in [Ca²⁺]_i mediate MPTP opening induced by CCK and bile acids.^{12,14} However, published data^{29,30} as well as our results (Supplementary Figure 3) indicate that ethanol alone has only a minor effect on [Ca²⁺]_i in intact acinar cells. Moreover, ethanol caused depolarization even in 2-bis (2-aminophenoxy)-ethane-*N,N,N,N*-tetraacetic acid-loaded intact cells or in permeabilized cells with clamped [Ca²⁺]_i. These data indicate that ethanol stimulates MPTP opening through mechanisms other than [Ca²⁺]_i increase.

The probability of MPTP opening can be enhanced by factors that increase the pore sensitivity to Ca²⁺.^{2,3} Our results indicate that ethanol depolarizes pancreatic mitochondria through this mechanism, and further indicate that the effect of ethanol on $\Delta\psi_m$ critically depends on the NAD⁺/NADH ratio. Ethanol significantly decreased the NAD⁺/NADH ratio in acinar cells. Our results indicate that ALDH2, the mitochondrial enzyme that converts acetaldehyde to acetate,³⁹ mediates an alcohol-induced decrease in NAD⁺ in acinar cells.

Restoring the NAD^+ level prevented ethanol-induced mitochondrial depolarization. Conversely, decreasing NAD^+ through an ethanol-unrelated mechanism, by inhibiting its synthesis with the inhibitor FK866, significantly decreased $\Delta\psi_m$, thus mimicking the effect of ethanol.

The effects of NAD^+ on $\Delta\psi_m$ were not observed in $\text{CypD}^{-/-}$ mitochondria, further supporting the likelihood that NAD^+ acts through MPTP. Decreases in NAD^+ long have been associated with MPTP opening, although the underlying mechanisms still are poorly understood.^{31,32} However, recent data on the liver suggest that a decrease in NAD^+ may lead to the inhibition of deacetylase SIRT3, resulting in hyperacetylation of CypD, which causes MPTP opening.^{40,41}

In contrast to NAD^+ , acetaldehyde, the main product of ethanol oxidation, did not affect $\Delta\psi_m$ (Supplementary Figure 6), and acetaldehyde did not affect $[\text{Ca}^{2+}]_i$ in acinar cells.²⁹ In contrast, FAEEs, nonoxidative ethanol metabolites, stimulate Ca^{2+} release from intracellular stores, resulting in Ca^{2+} -induced depolarization.^{29,36} FAEE did not have any direct Ca^{2+} -independent effect on $\Delta\psi_m$ (Supplementary Figure 6). The fact that ethanol depolarizes pancreatic mitochondria without increasing $[\text{Ca}^{2+}]_i$ argues against a significant contribution of FAEEs to ethanol's effects on $\Delta\psi_m$ under the conditions applied; CCK is a principal inducer of physiologic pancreatic secretion, but at high (supramaximal) doses it stimulates signaling mechanisms leading to pancreatitis. It is believed that abnormal Ca^{2+} signaling mediates pancreatitis induced by supraphysiologic doses of CCK.^{9,10} Indeed, although physiologic doses of the hormone induce Ca^{2+} oscillations, supraphysiologic CCK concentrations cause sustained and global increases in $[\text{Ca}^{2+}]_i$. We found that the patterns of $\Delta\psi_m$ responses to CCK are similar to those of Ca^{2+} . That is, physiologic doses of CCK cause small and transient mitochondrial depolarization, which is not harmful for acinar cells. In contrast, high doses of CCK cause a sustained loss of $\Delta\psi_m$, leading to necrosis in pancreatitis.^{13,15}

Alcohol not only decreases basal $\Delta\psi_m$ in acinar cells but also increases the magnitude of depolarization induced by both low and high CCK concentrations. Importantly, ethanol converts a transient depolarization induced by physiologic doses of CCK into sustained losses of $\Delta\psi_m$. Our data further indicate that the depolarizing effect of EtOH+CCK is mediated through MPTP activation by the synergistic action of 2 factors, a decrease in NAD^+/NADH ratio induced by ethanol and an increase in $[\text{Ca}^{2+}]_i$ by CCK.

The most important outcome of decreased $\Delta\psi_m$ is acinar cell necrosis.^{13,15-17} Ethanol and low-dose CCK each have little effect on necrosis; however, necrosis is induced synergistically by their combination. The effect was prevented by a blockade of ALDH2 as well as of MPTP. MPTP blockade also prevented ATP decrease, decreased necrosis, and improved other pathologic responses in the mouse model of alcoholic pancreatitis induced by EtOH and low-dose cerulein. The combined effects of ethanol and CCK on MPTP can explain the finding that ethanol feeding sensitizes animals to pancreatitis caused by low-dose CCK¹⁸ and, in particular, promotes necrosis in pancreatitis.⁴²

In conclusion, our results suggest that alcohol acts to worsen pancreatitis by its effects on pancreatic mitochondria to promote necrosis. Ethanol oxidative metabolism leads to a decreased NAD^+/NADH ratio, resulting in MPTP activation, mitochondrial depolarization, ATP depletion, and necrosis. These effects of ethanol on mitochondrial depolarization, ATP depletion, and necrosis can be prevented by MPTP inhibition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
ATP	adenosine triphosphate
BAL	blood alcohol level
[Ca²⁺]_i	free cytosolic Ca ²⁺
CCK	cholecystokinin-8
CypD	cyclophilin D
m	mitochondrial membrane potential
FAEE	fatty acid ethyl ester
MMP	mitochondrial membrane permeabilization
MPTP	mitochondrial permeability transition pore
NAD⁺	oxidized nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
TMRM	tetramethylrhodamine

Figure 1.

Ethanol time- and dose-dependently causes mitochondrial depolarization, which is prevented by CypD genetic ablation. (A–D) Pancreatic acinar cells isolated from wild-type (Wt) and CypD^{-/-} mice for 1 hour (c, d, and f) and 3 hours (a, b, e, and g) without (a and c) and with 25 mmol/L (b), 50 mmol/L (d and e), or 100 mmol/L (f and g) EtOH. Measurements of the mitochondrial membrane potential Ψ_m were performed in cells loaded with the fluorescence probe TMRM (1 μ mol/L) in the cuvette of the spectrofluorometer. TMRM is redistributed between cytosol and mitochondrial matrix along the membrane potential. An increase in Ψ_m causes the formation of low-fluorescence TMRM aggregates within mitochondria, thereby quenching TMRM fluorescence. The mitochondrial uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP; 15 μ mol/L) was added at the end of the experiments to completely dissipate Ψ_m . The results are expressed as changes in fluorescence intensity in arbitrary units (a.u.). (E) Quantification of the changes in Ψ_m in wild-type and CypD^{-/-} acinar cells incubated for 1 hour and 3 hours with and without ethanol. The difference between the levels of TMRM fluorescence in control cells treated without and with FCCP (zero Ψ_m) was taken as 100%. Wild-type acinar cells incubated without ethanol for 1 hour were considered as control. Values are means $\pm$ standard error (n = 5). * P < .05 and ^ P < .05 compared with corresponding control cells; # P < .05 compared with CypD^{-/-} cells in the same conditions.

Figure 2.

Ethanol potentiates CCK-induced mitochondrial depolarization. CypD genetic ablation prevents the loss of $\Delta\Psi^m$ in cells treated with EtOH+CCK. Changes in $\Delta\Psi^m$ were measured as in Figure 1 in pancreatic acinar cells isolated from (A–D) wild-type and (E and F) CypD^{-/-} mice. Cells were incubated for (A, B, D, F) 1 hour or (C and E) 3 hours without and with indicated doses of EtOH, and treated without (a and d) and with 10 pmol/L (b) and 0.1 μmol/L (c) or indicated doses (e, f, and g) of CCK. CCK additions to the cell suspension are shown by arrows. The data are representative of at least 4 experiments with similar results. (C) Quantification of the CCK-induced changes in $\Delta\Psi^m$ in acinar cells incubated with and without 100 mmol/L EtOH. The differences between $\Delta\Psi^m$ in control cells (not treated with CCK) and in cells treated with CCK for 2 and 25 minutes were quantified. The difference between levels of TMRM fluorescence in control cells treated without and with carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (zero $\Delta\Psi^m$) was taken as 100%. Values are means ± standard error (n = 5). **P* < .05 compared with control cells not treated with CCK. #*P* < .05 compared with the same conditions without EtOH.

Figure 3.

Ethanol increases the sensitivity of pancreatic mitochondria to Ca^{2+} -induced depolarization through a MPTP-dependent mechanism. (A–C) Pancreatic acinar cells isolated from (A and B) Wt and (C) $\text{CypD}^{-/-}$ mice were preincubated for 1 hour without (black) and with (red) 100 mmol/L EtOH. Then acinar cells were transferred to the cytosol-like medium B (see the Materials and Methods section), which contained 10 mmol/L glutamate/2 mmol/L malate as the respiratory substrate and, where indicated, 100 mmol/L EtOH and 4 $\mu\text{mol/L}$ bongkrekic acid (BKA). The concentration of free Ca^{2+} in the medium B ($[\text{Ca}^{2+}]_i$) was maintained with Ca^{2+} /ethylene glycol tetraacetic acid buffers at 100 and 300 nmol/L, and 1 $\mu\text{mol/L}$ (as indicated). TMRM was added to the cell suspension followed by digitonin (arrows) 30 seconds later. Plasma membrane permeabilization by digitonin results in mitochondria exposure to the respiratory substrate, leading to a decrease in TMRM fluorescence indicating an increase in Ψ_m . Depending on the concentration of free Ca^{2+} , the mitochondria either maintain Ψ_m or show a time-dependent increase in TMRM fluorescence, indicating depolarization. Protonophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) was added at the end of each experiment to cancel Ψ_m as shown (arrows). The data are representative of at least 3 experiments with similar results. (B) Quantification of the changes in Ψ_m after 20 minutes of exposure of permeabilized cells to 1 $\mu\text{mol/L}$ Ca^{2+} . Maximal Ψ_m in permeabilized cells in the presence of 100 nmol/L Ca^{2+} was considered 100% as shown in panel A. Values are means $\pm$

standard error ($n = 5$). $*P < .05$ compared with cells incubated without EtOH. $\#P < .05$ compared with CypD^{-/-} cells in the same conditions.

Figure 4.

Ethanol-induced depolarization of pancreatic mitochondria is mediated by a NAD⁺ decrease. Pancreatic acinar cells were isolated from wild-type mice, preincubated for (A–D) 1 hour or (E and F) 3 hours in the presence or absence of 100 mmol/L EtOH, 10 μ mol/L daidzin, 1 mmol/L NAD⁺, 100 μ mol/L FK866 (FK), a combination of FK866 and NAD⁺, and treated (A and F) without and (B–E) with the indicated doses of CCK. *Inset*: immunoblot of ALDH2 analyzed in isolated mitochondria from cells treated with and without EtOH. The mitochondrial marker cyclooxygenase IV serves as loading control. Changes in cellular (A and F) NAD⁺/NADH ratios and in (B–E) TMRM fluorescence in cells incubated without and with indicated treatments. (B–E) The data are representative of 5 experiments with similar results. (B) Reproduced for convenience from Figure 2C. (A and F) Values are means $\pm$ standard error (n = 4). **P* < .05 compared with control cells not treated with EtOH, or inhibitors. #*P* < .05 compared with cells incubated with EtOH but without daidzin. The level of NAD⁺/NADH in control cells is taken as 100%.

Figure 5.

Restoration of NAD⁺ prevents ethanol-induced sensitization of pancreatic mitochondria to Ca²⁺-induced depolarization. (A–C) Pancreatic acinar cells isolated from Wt and CypD^{-/-} mice as indicated were preincubated for 1 hour without any treatments (*black*) with 100 mmol/L EtOH (*red*), without (a) and with 1 mmol/L NAD⁺ (b). Then, pancreatic acinar cells were transferred to a cytosol-like medium, containing 1 μ mol/L [Ca²⁺]_i maintained with Ca²⁺/ethylene glycol tetraacetic acid and changes in Ψ were measured as in Figure 3. The data are representative of at least 3 experiments with similar results. (C) Quantification of the changes in Ψ after 20 minutes of exposure of permeabilized cells to 1 μ mol/L Ca²⁺. Maximal Ψ in permeabilized cells in the presence of 100 nmol/L Ca²⁺ was considered 100% as in Figure 3A. Values are means $\pm$ standard error (n = 5). **P* < .05 compared with cells incubated without NAD⁺.

Figure 6.

CypD deficiency protects from necrosis in acinar cells treated with alcohol and CCK. Pancreatic acinar cells isolated from wild-type (Wt) and CypD^{-/-} mice were incubated for 3 hours with and without 100 mmol/L EtOH, 10 pmol/L CCK, and 10 μ mol/L daidzin. Necrosis was quantified as the percentage of G6PD release into extracellular medium. Values are means $\pm$ standard error (n = 4). **P* < .05 compared with control Wt cells treated without EtOH and CCK. #*P* < .05 compared with Wt cells treated with EtOH and CCK but without daidzin.

Figure 7.

CypD deficiency improves pathologic responses of alcoholic pancreatitis induced by EtOH + cerulein. Pancreatitis in wild-type (Wt) and CypD^{-/-} mice was induced by a combination of the alcohol-containing Lieber–deCarli diet and injections of cerulein (pancreatitis). Control mice received regular diet and saline injections. (A) Changes in $\Delta\Psi_m$ were measured with the tetraphenyl phosphonium (TPP)⁺ electrode in mitochondria isolated from the pancreas in buffer B in the presence of 10 mmol/L glutamate/2 mmol/L malate and at 1 μmol/L $[Ca^{2+}]_i$ maintained with Ca^{2+} /ethylene glycol tetraacetic acid (EGTA) buffers. (B) Changes in ATP were measured with an ATP determination kit and normalized to those in control tissue. (C) Necrosis was quantified on H&E-stained pancreatic tissue sections. (D) Amylase was measured in serum. (E) Trypsin activity was measured in pancreatic tissue

homogenates. (F) The proposed scheme of MPTP involvement in alcoholic pancreatitis induced by EtOH and low CCK. (A) The data are representative of experiments on 2 Wt and 2 CypD^{-/-} mice with similar results. (B–E) Values are means $\pm$ standard error, with at least 4 mice in each group. * $P < .05$ compared with control Wt group. # $P < .05$ compared with pancreatitis on Wt mice.