Effects of Ethanol Metabolites on Exocytosis of Pancreatic Acinar Cells in Rats

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BACKGROUND & AIMS: During development of alcoholic pancreatitis, oxidative (acetaldehyde) and nonoxidative metabolites (ethyl palmitate, ethyl oleate), rather than ethanol itself, mediate toxic injury. Exposure of pancreatic acini to ethanol blocks cholecystokinin (CCK)-8–stimulated apical exocytosis and redirects exocytosis to the basolateral plasma membrane, causing interstitial pancreatitis. We examined how each ethanol metabolite contributes to these changes in exocytosis. **METHODS:** Rat pancreatic acini were incubated with concentrations of ethanol associated with alcoholic pancreatitis (20–50 mmol/L) or ethanol metabolites (1–3 mmol/L) and then stimulated with CCK-8. We performed single zymogen granule (ZG) exocytosis assays, Ca^{2+} imaging studies, ultrastructural analyses (with electron microscopy), and confocal microscopy to assess the actin cytoskeleton and track the movement of vesicle-associated membrane protein (VAMP)-8–containing ZGs. Coimmunoprecipitation assays were used to identify complexes that contain the distinct combinations of Munc18 and the soluble *N*-ethylmaleimide sensitive factor attachment protein receptor proteins, which mediate apical (ZG–apical plasma membrane) and basolateral exocytosis and fusion between ZGs (ZG-ZG). **RESULTS:** The ethanol metabolites acetaldehyde, ethyl palmitate, and ethyl oleate reduced CCK-8–stimulated apical exocytosis and formation of apical exocytotic complexes (between Munc18b and Syntaxin-2, synaptosomal-associated protein of 23 kilodaltons [SNAP23], and VAMP2) in rat pancreatic acini. Acetaldehyde and ethyl oleate redirected CCK-8–stimulated exocytosis to the basal and lateral plasma membranes and translocation of VAMP8-containing ZGs toward the basolateral plasma membrane. This process was mediated primarily via formation of basolateral exocytotic complexes (between Munc18c and Syntaxin-4, SNAP23, and VAMP8). Exposure of the acini to acetaldehyde and ethyl oleate followed by CCK-8 stimulation mildly perturbed the actin cytoskeleton and Ca^{2+} signaling; exposure to ethyl palmitate severely affected Ca^{2+} signaling. Acetaldehyde, like ethanol, promoted fusion between ZGs by the formation of ZG-ZG exocytotic complexes (between Munc18b and Syntaxin-3, SNAP23, and VAMP8), whereas ethyl palmitate and ethyl oleate reduced ZG-ZG fusion and formation of these complexes. **CONCLUSIONS: The ethanol metabolites acetaldehyde, ethyl palmitate, and ethyl oleate perturb exocytosis processes in cultured rat pancreatic acini (apical blockade, basolateral exocytosis, and fusion between ZGs). Acetaldehyde and, to a lesser degree, ethyl oleate produce many of the same pathologic effects of ethanol on CCK-8–stimulated exocytosis in pancreatic acini.**

Keywords: SNARE Proteins; Exocrine Pancreas; Vesicle Transport; Mechanisms of Acute Pancreatitis.

Alcohol abuse and gallstone disease account for 70%–80% of cases of acute pancreatitis.[1,2] Gallstone pancreatitis often resolves completely by supportive management followed by surgical or endoscopic removal of gallstones.[1] Ethanol abuse, on the other hand, causes not only a single episode of acute pancreatitis; because it is often an addiction, it causes repeated recurrence of acute pancreatitis and progression to chronic pancreatitis.[1,2] However, in rodent animal models, EtOH alone does not cause pancreatitis,[1,2] but rather requires triggering factors, including virus infection,[3] high-fat diet,[4] or physiological postprandial cholecystokinin (CCK)-8 or cholinergic stimulation,[5–9] for acute pancreatitis to manifest itself. In vitro, EtOH per se at relevant clinical levels (10 mmol/L, social drinking; 50 mmol/L, moderate intoxication; 100 mmol/L, severe intoxication) likewise has little effect on pancreatic acini.[6–8] The molecular basis by which EtOH predisposes exocrine pancreas to injury is thus largely unknown.

It is not EtOH per se, but rather its metabolites generated from oxidative (acetaldehyde, acetate) and nonoxidative pathways (fatty acid ethyl esters) that cause toxic effects.[10] Oxidative pathways are dominant in liver; hence, acetaldehyde is a primary culprit for alcoholic liver damage.[10] However, postmortem studies from alcohol intoxication showed that pancreases had higher levels of fatty acid ethyl esters compared with liver,[11] and this was confirmed in rodent models.[12] Major fatty acid ethyl esters generated in the pancreas include ethyl palmitate and ethyl oleate.[10–12] At clinically relevant EtOH concentrations, pancreatic concentrations of these metabolites were

Abbreviations used in this paper: CCh, carbachol; KRBH, Krebs–Ringer bicarbonate HEPES; PM, plasma membrane; SNAP23, synaptosomal-associated protein of 23 kilodaltons; SNARE, soluble N-ethyl maleimide sensitive factor attachment protein receptor; VAMP, vesicle-associated membrane protein; ZG, zymogen granule.

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measured to be at submillimolar to low millimolar ranges.[12] Of these, ethyl palmitate was postulated to be the major culprit inducing pathologic events in acini, including evoking global and sustained high Ca^{2+} elevation[13] (instead of physiologic apical oscillations) that blocks apical exocytosis and initiates premature zymogen protease activation,[14] biogenesis of large vacuole formation,[15] and mitochondrial injury. In contrast, acetaldehyde and ethyl oleate have little effect on Ca^{2+} signaling in acinar cells.[13] Acetaldehyde at high concentrations blocked CCK-8–stimulated secretion by unclear mechanisms[16] and activated proinflammatory transcription factors nuclear factor κB and cytokines.[12] Little is known about the toxic effects of ethyl oleate and thus seems to have been ignored as a cause of acinar injury.

Pancreatic acinar cells are highly polarized cells wherein regulated exocytosis occurs at the apical pole, with initial fusion of zymogen granules (ZGs) to apical plasma membrane (PM) followed by sequential fusion of ZGs behind apically fused ZGs.[17,18] Once exposed to supramaximal CCK-8 or carbachol (CCh), apical exocytosis is reduced and instead redirected to basolateral PM,[19] resulting in interstitial pancreatitis as observed clinically.[9,20] Pretreatment of acini with 10–50 mmol/L EtOH followed by postprandial CCK-8 or CCh similarly induced basolateral exocytosis in vitro that may lead to in vivo pancreatitis in rodents.[6–9] The molecular mechanism mediating this process was found to be consistent with the soluble *N*-ethyl maleimide sensitive factor attachment protein receptor (SNARE) hypothesis for membrane fusion[21]; that is, basolateral PM Munc18c binds basolateral PM Syntaxin-4 in closed conformation, but when threonine phosphorylated by protein kinase C α is stimulated by supramaximal CCK-8/CCh or EtOH/submaximal CCK-8 or CCh, Munc18c is activated and becomes displaced from Syntaxin-4, inducing Syntaxin-4 into open conformation capable of binding synaptosomal-associated protein of 23 kilodaltons (SNAP23) and ZG-bound vesicle-associated membrane protein (VAMP)-8 to form a fusion complex that mediates ZG fusion with basolateral PM.[6–9,22] Whereas Munc18c/(Syntaxin-4, SNAP23, VAMP8) complex mediates basolateral exocytosis, apical exocytosis, composed of ZG-apical PM fusion and sequential ZG-ZG fusion, is mediated by 2 other fusion complexes, including Munc18b/(Syntaxin-2, SNAP23, VAMP2) and Munc18b/(Syntaxin-3, SNAP23, VAMP8),[9,18,23] respectively.

We here dissected the actions of each EtOH metabolite (acetaldehyde, ethyl palmitate, and ethyl oleate) on pancreatic acinar exocytosis. We found that pretreatment with ethyl palmitate followed by maximal CCK-8 stimulation caused mild reduction of apical exocytosis consistent with its actions on disrupting Ca^{2+} signaling and did not affect basolateral exocytosis. Remarkably, pretreatment of acini with acetaldehyde and ethyl oleate severely abrogated CCK-8–stimulated apical exocytosis and redirected exocytosis to the basal and lateral PM due to their actions on apical and basolateral exocytotic fusion complexes.

Materials and Methods

Antibodies and Reagents

Sources of antibodies are detailed in Supplementary Materials and Methods.

Acinar Isolation and Culture, Amylase Secretion and Cytosolic Ca^{2+} Recording, and Confocal and Electron Microscopy

Dispersion of pancreatic acini from male Sprague–Dawley rats (125–150 g) and the previously mentioned assays have all been reported[6–9] and are described in Supplementary Materials and Methods.

Exocytosis Imaging

FM1-43 epifluorescence imaging[6–9] and imaging of pancreatic acini infected with syncollin-pHluorin by spinning disk confocal microscopy[24] were performed as previously reported and described in Supplementary Materials and Methods.

Immunoprecipitation

Immunoprecipitation was performed similarly as previously described[9,23] using Syntaxin-2, Syntaxin-3, or Syntaxin-4 antibodies as detailed in Supplementary Materials and Methods.

Statistical Analysis

Data are presented as means ± SEM analyzed by one-way analysis of variance followed by Tukey post hoc test, with $P < .05$ considered statistically significant.

Results

Effects of Alcohol Metabolites on CCK-8–Stimulated Amylase Secretion

Previous work has implicated ethyl palmitate as the putative culprit that perturbs acinar Ca^{2+} signaling and induces necrosis,[13,14,25] whereas the role of other major metabolites, acetaldehyde and ethyl oleate, has not been elucidated. Rat pancreatic acini preincubated with clinically relevant and minimal effective concentrations[12] (Supplementary Figure 1) of acetaldehyde (1 mmol/L) and either fatty acid ethyl ester ethyl palmitate (3 mmol/L) or ethyl oleate (3 mmol/L), followed by maximal CCK-8 (100 pmol/L) stimulation, caused an inhibition of maximal CCK-8–stimulated amylase secretion (Figure 1*A*), very similar to the low EtOH (10–50 mmol/L) preincubation we previously reported.[6–9] When respective basal levels (EtOH or EtOH metabolites alone) were subtracted from stimulated release (with 100 pmol/L CCK-8), the reduction of secretion caused by EtOH and its metabolites was approximately one-third of maximal secretion. In the succeeding studies described in the following text, these were the minimally effective concentrations of the EtOH metabolites used.

The effects of ethyl palmitate on CCK-8–stimulated secretion could be due to disruption of Ca^{2+} signaling, but the effects of acetaldehyde and ethyl oleate were postulated not to affect acinar Ca^{2+} signaling.[13,14] We performed Ca^{2+} microfluorimetry and found that Ca^{2+} oscillatory responses evoked by 200 pmol/L CCK-8 (Figure 1*Bi*) were largely preserved after pretreatments with