Alcoholic pancreatitis in rats: injury from nonoxidative metabolites of ethanol

JENS WERNER,1 MOURIS SAGHIR,2 ANDREW L. WARSHAW,1 KENT B. LEWANDROWSKI,2 MICHAEL Laposata,2 RENATO V. IOZZO,4 EDWARD A. CARTER,3 RICHARD J. SCHATZ,5 AND CARLOS FERNÁNDEZ-DEL CASTILLO1

Departments of 1Surgery, 2Pathology, and 3Pediatric Gastroenterology, Massachusetts General Hospital and Harvard Medical School, Boston 02114; 5Department of Pharmaceutical Science, Northeastern University, Boston, Massachusetts 02115; and 4Department of Pathology, Anatomy, and Cell Biology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

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Werner, Jens, Mouris Saghir, Andrew L. Warshaw, Kent B. Lewandrowski, Michael Laposata, and Renato V. Iozzo, Edward A. Carter, Richard J. Schatz, and Carlos Fernández-del Castillo. Alcoholic pancreatitis in rats: injury from nonoxidative metabolites of ethanol. Am J Physiol Gastrointest Liver Physiol 283: G65–G73, 2002; 10.1152/ajpgi.00419.2001.—The mechanism by which alcohol injures the pancreas remains unknown. Recent investigations suggest a role for fatty acid ethyl ester (FAEE), a nonoxidative metabolite of ethanol, in the pathogenesis of alcohol pancreatitis. In this study, we characterized ethanol-induced injury in rats and evaluated the contribution of oxidative and nonoxidative ethanol metabolites in this form of acute pancreatitis. Pancreatic injury in rats was assessed by edema, intrapancreatic trypsinogen activation, and microscopy after infusing ethanol with or without inhibitors of oxidative ethanol metabolism. Plasma and tissue levels of FAEE and ethanol were measured and correlated with pancreatic injury. Ethanol infusion generated plasma and tissue FAEE and, in a dose-dependent fashion, induced a pancreas-specific injury consisting of edema, trypsinogen activation, and formation of vacuoles in the pancreatic acini. Inhibition of the oxidation of ethanol significantly increased both FAEE concentration in plasma and pancreas and worsened the pancreatitis-like injury. This study provides direct evidence that ethanol, through its nonoxidative metabolic pathway, can produce pancreas-specific toxicity in vivo and suggests that FAEE are responsible for the development of early pancreatic cell damage in acute alcohol-induced pancreatitis.

Pathophysiology; ethanol metabolism; fatty acid ethyl ester

ALTHOUGH ALCOHOL ABUSE IS a major cause of pancreatitis, the mechanisms whereby ethanol exerts its toxic effects are still incompletely understood. Lack of an adequate experimental model of alcoholic pancreatitis has hampered progress in the study of its pathogenesis.

Intrapancreatic premature activation of trypsin, blockade of luminal enzyme secretion, and appearance of intracellular vacuoles have been observed in many experimental models of acute pancreatitis and are believed to trigger the development of this disease (18, 26). Recent studies have demonstrated that ethanol sensitizes the pancreatic acinar cell to the effects of cholecystokinin on zymogen conversion in vitro (22) and enhances cholecystokinin release into the circulation in vivo (36); but injury to the pancreas after ethanol exposure was not observed in either study.

Ethanol metabolism occurs predominantly in the liver (27, 34), and many reports suggest that alcohol-induced liver injury is, in part, mediated by its oxidative metabolite acetaldehyde (2, 21, 41). Ethanol intake also results in an accumulation of the nonoxidative metabolite fatty acid ethyl ester (FAEE) in blood (10, 11, 17) and several organs, with the highest concentration found in the pancreas (25). There is now compelling evidence that FAEE are toxic mediators in isolated organelles (16, 20, 24) and in intact cells (42). Moreover, a recent study (45) from our laboratory demonstrated that a pancreatitis-like injury can be induced by FAEE infusions in rats. Both oxidative and nonoxidative pathways are present in the pancreas (4, 8, 12, 15, 17), but the pathological significance of the different ethanol metabolites in the pathogenesis of alcoholic pancreatitis has not yet been determined.

In the present study, we describe an animal model of ethanol-induced pancreatic injury consisting of edema, intrapancreatic trypsinogen activation and formation of vacuoles within the pancreatic acinar cell. With this new model, we investigated the pathophysiological relevance of oxidative vs. nonoxidative ethanol metabolites in alcoholic pancreatitis.

MATERIAL AND METHODS

Animals

Male Sprague-Dawley rats (350–400 g) were housed individually in hanging wire-bottomed cages in rooms main-
Characterization of Ultrastructural Changes

Frozen sections of the pancreas of eight additional rats that were infused with the same amount of ethanol as described above were assessed by oil red stain for intracellular fat accumulation, the earliest histological change after long-term ethanol feeding.

Further characterization of the pancreatic cell injury at 3h was performed by electron microscopy in additional rats that received either saline (n = 2) or ethanol (n = 2).

Correlation Between Ethanol and FAEE Concentrations in Blood

To evaluate whether ethanol-induced pancreatic injury is related to the level of FAEE generated, animals were allocated to four groups (n = 2) that received different concentrations of ethanol (0.4, 0.8, 1.6, and 3.2 g/kg). Control animals received saline only. Blood ethanol and FAEE concentrations were evaluated after 2h as described in Ethanol concentrations in blood.

Inhibition of the Oxidative Pathway During Ethanol Infusion

In these experiments, rats were infused with ethanol alone (2ml bolus at 0.8 g/kg; and 6ml infusion over 2h at 1.2 g·kg⁻¹·h⁻¹) or in combination with an intravenous infusion of inhibitors of the oxidative pathway: 4-methylpyrazole (4-MP), an alcohol dehydrogenase inhibitor; 3-aminopropyl-2,4-triazole (AT), a catalase inhibitor; and diethylsulfide (DES), an inhibitor of the microsomal ethanol oxidizing system (MEOS). Animals were randomly allocated to the following subgroups: 1) ethanol only, 2) ethanol and 4-MP, 3) ethanol and a combination of all three inhibitors, 4) saline only as a volume control, 5) saline and 4-MP, or 6) saline and a combination of all three inhibitors as a control for direct toxicity of the inhibitors.

The inhibitors were administered as follows: A 1ml bolus of either 10 mg/kg of 4-MP alone or in combination with 10 mg/kg DES and 1 g/kg AT, followed by 30-min preinfusion of either 10 mg·kg⁻¹·h⁻¹ 4-MP alone or in combination with 10 mg·kg⁻¹·h⁻¹ DES and 0.25 g·kg⁻¹·h⁻¹ AT in 0.5 ml saline. This was followed by the ethanol infusion, which was given simultaneously with the inhibitors.

Animals were killed for the evaluation of nonoxidative ethanol metabolism in pancreatic and liver tissue 2h after the infusion. In addition, we assessed the activity of the oxidative pathway enzymes alcohol dehydrogenase (ADH), catalase, and the MEOS 2h after the infusion in animals that received saline, ethanol only, or ethanol in combination with the three inhibitors (n = 6/group). Assessment of edema formation, TAP in pancreatic tissue, and myeloperoxidase activity was performed at 6h (n = 8/group). Histological assessment was performed at 12h postinfusion (n = 4 per group).

Ethanol Concentrations in Blood

Plasma ethanol levels were determined by gas chromatography (GC) (39). The plasma sample was mixed with an internal standard of 1-propanol, and a 1-µl sample was injected into a Hewlett-Packard 5890 GC containing a 5% carbowax 20 M 60/80 carpack B column (Supelco, Bellefonte, PA). The oven temperature was set isothermally at 100°C, and the ethanol peak was identified by comparison with the standard.
Determination of FAEE After Ethanol Infusion

FAEE isolation and quantitation. FAEE concentrations were determined by GC-mass spectroscopy (MS). An internal standard of 1 nmol of ethyl heptadecanoate (ethyl 17:0, Nu-Chek Prep, Elysian, MN) was added to each sample, and lipids were extracted by a modified Folch method (9). FAEE was isolated from the organic phase by solid phase as described by Bernhardt et al. (3) and concentrated by drying the sample under nitrogen. FAEE were then quantitated by GC-MS analysis on a Hewlett-Packard 5890 gas chromatograph coupled to Hewlett-Packard 5970 mass spectrometer with a WCOT Supelcowax capillary column (Supelco). The injector was maintained at 280°C, the MS detector at 280°C, and the oven was heated from 150 to 250°C, increasing at a rate of 10°C/min, and maintained at 250°C for 6 min. Total ion chromatograms were generated using an ionization energy of 70 eV.

FAEE in blood. To inactivate FAEE hydrolysis and to prevent any artifactual formation of FAEE, 1 ml of acetone was immediately added to 0.5 ml of blood prior to FAEE quantitation.

FAEE in pancreatic and liver tissue. Pancreatic and liver tissues were excised after the rats were killed and stored at −80°C until assayed. A homogenization buffer (phosphate-buffered saline containing 1 mM benzamidine, 20 mg/l n-phenylmethylsulfonyl fluoride, 100 mg/l soybean trypsin inhibitor, and 10 mg/l aprotinin) was added to a tube with pancreatic or liver tissue in a 1:2 ratio (tissue to buffer). The tissues were then homogenized (Power Gen 125 homogenizer, Fisher Scientific, Pittsburgh, PA), and 2 ml of acetone were added to 1 ml of pancreatic or liver homogenate before FAEE concentrations were determined.

Assessment of Enzyme Activity of Oxidative Ethanol Metabolism

Alcohol dehydrogenase activity. Alcohol dehydrogenase activity in liver tissues was determined spectrophotometrically as described by Bonnichsen and colleagues (5, 6). A sample of the tissues (1.0 g) was immediately shock frozen in liquid nitrogen after the animal was killed and stored until assayed at −80°C. Tissues were weighed and homogenized in 3 ml of 0.01 M sodium pyrophosphate (pH 9.6). Then, 0.1 ml of nicotinamide adenine diphosphate (10 mg/ml) was added, and the optical density was recorded with Beckman DU-65 spectrophotometer at 240 nm after the addition of ethanol. The change in optical density without the addition of ethanol was used as the blank and was subtracted from the value obtained with ethanol. The enzyme activity is expressed as change of optical density (OD) per gram wet tissue weight per hour.

Catalase activity. Catalase activity was determined spectrophotometrically as described in detail by Aebi (1). Liver homogenates were prepared as described in "FAEE in pancreatic and liver tissue" for the alcohol dehydrogenase assay and were then further diluted with 0.1 M sodium phosphate buffer (pH 7.0). The catalase activity was measured spectrophotometrically (Beckman DU-65) at 240 nm after the addition of H₂O₂ and is expressed as change of OD per gram wet tissue weight per hour.

MEOS. The increase in activity of the specific isozyme cytochrome P-450 2E1 (P-450) was measured by the N-nitrosoimidethyamine demethylase assay (NDMA) according to the method of Carlson (7) with minor modifications. Briefly, microsomes were prepared by homogenizing liver or pancreas in 0.1 M phosphate buffer (pH 7.4) and centrifuging the homogenate at 9,000 g for 20 min followed by ultracentrifugation of the resulting supernatant at 105,000 g. The incubation mixture contained ~1.0 mg of microsomal protein in 100 mM potassium phosphate buffer (pH 7.4) and 1.0 mM NADM in a total volume of 1.0 ml. The reaction was initiated by the addition of NADPH (final concentration of 1 mM). Incubations were performed at 37°C for 20 min and were terminated by the addition of 0.1 ml of 0.6 N perchloric acid. Formaldehyde was determined by incubating the sample for 40 min at 60°C with Nash reagent, recentrifuging, and measuring the absorbance at 412 nm. Total protein was measured using the Lowry method (28).

Assessment of Injury to the Pancreas and Other Organs

Pancreatic edema. Pancreatic edema was evaluated by measuring the wet/dry ratio. After the animals were killed, a portion of the tail of the pancreas was removed, trimmed of fat, and weighed immediately. Pancreatic water content was determined by calculating the ratio (wet/dry ratio) from the initial wet weight of the pancreas (wet wt) to its weight after incubation at 160°C for 24 h (dry wt). The wet/dry ratio was also determined for other organs, including liver, lung, kidney, spleen, skeletal muscle, subcutaneous fat, and mesentery.

TAP measurement. Blood samples (0.5 ml) were collected in 0.2 M EDTA to inactivate peptidases. Samples were stored at −20°C and assayed without knowledge of previous treatments. For measurement of TAP concentrations in pancreatic tissue, two portions (0.1–0.3 g) were excised from the pancreas and measured in duplicates. Specimens were then immersed in 0.2 M Tris–HCL buffer (pH 7.3) containing 20 mM EDTA, immediately boiled (100°C) for 10 min to denature remaining protease activity, and homogenized in a Brinkman Polytron for 30 s. After subsequent centrifugation (1,500 rpm, 10 min, 4°C), the resulting supernatant was assayed. TAP was measured by an enzyme-linked immunosorbent assay (37). Synthetic TAP (YD4K), a conjugate of rabbit serum albumin with YD4K, and rabbit anti-TAP antiserum containing calcium-independent anti-TAP antibodies were kindly provided by Professor J. Hermon-Taylor (St. George’s Hospital Medical School, London, England). Biotin-goat anti-rabbit IgG antibody and alkaline phosphatase-labeled Extravadin was purchased from Sigma (St. Louis, MO).

Amylase in plasma. Amylase levels were assessed in plasma using the Phadebas amylase test (Pharmacia, Columbus, OH).

Liver enzymes. Serum levels of alanine transaminase, aspartate transaminase, alkaline phosphatase, and bilirubin were measured on a Hitachi 911 analyzer (Boehringer-Mannheim, Indianapolis, IN).

Myeloperoxidase activity. Excised pancreatic tissues were rinsed with saline, blotted dry, shock frozen in liquid nitrogen, and stored frozen at −80°C until thawing for determination of myeloperoxidase activity using methods previously described (23, 44) with minor modifications. Briefly, pancreatic tissue was homogenized in 0.1 M sodium phosphate buffer containing 0.5% hexadecyltrimethyl ammonium bromide (Sigma) and 5% soybean trypsin inhibitor (Sigma) before sonication and was then frozen directly on dry ice. The specimens were freeze-thawed three times, and after each cycle, sonication was repeated. Suspensions were then centrifuged at 20,000 g for 15 min, and the resulting supernatant was assayed. Myeloperoxidase activity was measured with a spectrophotometer (Shimadzu, UV-160, Japan) at 470 nm by mixing an aliquot (25 μl) of the supernatant with 1.0
ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 1.6 µl of Guaiacol (Sigma) and 0.0005% hydrogen peroxide (Sigma) as substrates. Myeloperoxidase activity is expressed as units per mg wet weight.

**Histological assessment.** Evaluation of the histological appearance of the pancreas was performed by a pathologist (K. B. Lewandrowski) unaware of the treatment administered to the individual rats, as described previously (38). Briefly, the head of the pancreas was removed, fixed in 10% buffered formalin, and embedded in paraffin. One coronal section was made in the plane of the flattened pancreas and stained with hematoxylin-eosin. Morphometric documentation for inflammation and acinar necrosis was obtained by mapping the surface of the head of the pancreas into 10 geographical fields and evaluating each field independently. The mean histological score (scoring system: 0–3) was calculated for each variable in each animal. Other changes such as vacuolization of the cytoplasm were noted. In addition, the liver was also evaluated for possible pathological findings.

**Oil red O stain.** To identify possible fat accumulation within the pancreas, pancreatic tissue of ethanol-infused rats was embedded in optimum cutting temperature compound (Tissue-Tek OCT compound 4583, Sakura Finetek, Torrance, CA) and immediately frozen at −80°C. Frozen sections of 5-µm thickness (cryostat 2800 Frigocut-E; Reichert-Jung, Nussloch, Germany) were stained with Oil red O staining solution (0.75 g Oil red O, Fisher Biotech, Fair Lawn, NJ; 300 ml isopropanol, 99%, Fisher Scientific; and 200 ml distilled water) for 5 min and then counterstained with hematoxylin (Fisher Scientific) for 30 s.

**Ultrastructural changes.** Pancreatic cell injury was characterized further by electron microscopy in a blinded fashion for ultrastructural changes. Pancreatic tissue was fixed in Karnovsky solution, postfixed in osmium tetroxide, stained en bloc with uranyl acetate, dehydrated in ethanol, infiltrated with propylene oxide/epon, and embedded in epon-812 (Polyscience, Warrington, PA). Representative areas were chosen and evaluated with Jeol 100 B electron microscope (Jeol, Tokyo, Japan).

### Statistical Analysis

Data with normal distribution are presented as means ± SE, and differences between groups were evaluated by ANOVA followed by the Student's t-test. Data without a normal distribution (TAP values) are presented as median, 25th, and 75th percentile values, and statistical analysis was performed by using the nonparametric ANOVA (Kruskal-Wallis test) to show an overall difference between the groups, and the Mann-Whitney rank-sum test was used to make subsequent pairwise comparisons indicating the exact differences. A P value of 0.05 was considered statistically significant.

### RESULTS

#### Ethanol-Induced Pancreatic Injury

The following blood ethanol concentrations (mg ethanol/100 ml blood) were achieved by using the infusion regimen (2 ml bolus and a subsequent 6-ml infusion over 2 h) with different concentrations of ethanol (2.5–20%): 0.31 ± 0.02 (2.5%), 0.67 ± 0.04 (5%), 1.16 ± 0.08 (10%), and 2.08 ± 0.07 (20%).

Pancreatic injury 3 h after onset of ethanol infusion was characterized by edema formation and intrapancreatic trypsinogen activation (TAP). A 2-ml bolus and 6-ml infusion of 2.5% (0.4 g/kg) ethanol did not induce pancreatic edema or TAP in pancreatic tissue. However, both parameters increased with doses of 5% ethanol (0.8 g/kg) or greater (Figs. 1 and 2).

The time course of the pancreatic injury induced by the ethanol infusion regimen demonstrated significant pancreatic edema formation at 3 h, which subsequently decreased (Fig. 3). At 24 h, there was no detectable edema. TAP in pancreatic tissue increased over the first hours after the ethanol bolus was administered, with a peak at 6 h (Fig. 4).

Amylase and TAP were not increased in plasma (data not shown). Increased myeloperoxidase activity in pancreatic tissue was not observed in any group at any time (data not shown).
Histologically, vacuoles in the cytoplasm of pancreatic acinar cells were observed in all groups that received ethanol, including those infused with a 2.5% solution and killed at 3 h. There was no apparent relationship between the number of vacuoles and either dose or time within the study subgroups. Vacuoles were still detectable in ethanol-infused rats at 24 h but were not found in animals killed 1 wk after the administration of the ethanol bolus. Characterization of these vacuoles by Oil red O stain revealed the absence of fat.

Electron microscopy of animals infused with ethanol revealed extensive morphological changes characterized by accumulation of secondary lysosomes and deposition of lipids (Fig. 5B). In addition, the pancreatic acinar cells...
often showed dilation of the rough endoplasmic reticulum (Fig. 5C). In contrast, the control animals did not show any such pathological changes (Fig. 5A).

Other organs, including the liver and lung, as evaluated by wet/dry ratio, did not show any changes compared with saline control groups at any ethanol concentration and time point (data not shown). Additionally, histological examination of the liver did not show any pathological findings (data not shown).

**Correlation Between Ethanol and FAEE Concentrations in Blood**

There was a positive linear correlation ($r = 0.94$) between blood concentrations of ethanol and endogenously formed FAEE (Fig. 6).

**Ethanol Metabolism In Vivo After Inhibition of the Oxidative Ethanol Pathway**

Evaluation of FAEE production in the pancreas and liver after ethanol infusion revealed significantly higher FAEE synthesis by the pancreas than by the liver (Fig. 7). Inhibition of the oxidative ethanol pathway by administration of three inhibitors (4-MP, DAS, and AT) reduced the activity of alcohol dehydrogenase in the liver by 65%, the MEOS activity by 50%, and catalase activity by 60% (Table 1). The production of FAEE in both the pancreas and liver increased significantly after the administration of 4-MP (Fig. 7), with a much greater increase in pancreas. The same was true with the subsequent addition of DAS and AT, which resulted in a more than threefold increase of FAEE in pancreatic tissue (Fig. 7).

**Pancreatic Injury In Vivo After Inhibition of the Oxidative Ethanol Pathway**

Pancreatic injury 6 h after the administration of the initial ethanol bolus was assessed in animals in which the oxidative ethanol metabolism was inhibited with 4-MP alone or in combination with DAS and AT, and the results were compared with those from animals that received no inhibitors. Control animals received either saline only or inhibitors in the saline infusion.

There was no difference in pancreatic water content or intrapancreatic trypsinogen activation between the saline-infused control animals that received inhibitors and those that received no inhibitors. This is consistent with the absence of pancreatic toxicity of these compounds as used. The addition of inhibitors to the ethanol infusion increased edema and TAP in pancreatic tissue over that produced by ethanol alone (Figs. 8 and 9). TAP in plasma, serum amylase, liver enzymes (alanine transaminase, aspartate transaminase, alkaline phosphatase), and bilirubin were normal in all groups (data not shown). The number of vacuoles in pancreatic cells did not increase with inhibition of the oxidative ethanol pathway.

**Fig. 6.** Correlation between ethanol and fatty acid ethyl ester (FAEE) concentrations in blood. Different concentrations of ethanol (0.4, 0.8, 1.6, and 3.2 g/kg) were infused in animals over 2 h, and plasma ethanol levels were determined by gas chromatography and FAEE concentrations in blood by gas chromatography-mass spectroscopy ($n = 2$group). Control animals received saline only. There was a positive linear correlation ($r = 0.94$) between ethanol concentrations and endogenously formed FAEE concentrations in blood.**

**Fig. 7.** Ethanol metabolism in vivo after inhibition of the oxidative ethanol pathway. FAEE concentration in liver and pancreas were evaluated by gas chromatography-mass spectroscopy 2 h after the start of ethanol infusion. Measurements were made after ethanol infusion alone (none), ethanol with 4-methyl pyrazole (4-MP), or ethanol with 4-MP and diallyl sulfide and aminotriazole (TRIPLE). Data are presented as means ± SE ($n = 6$group).

**Table 1.** Enzyme activity of the oxidative ethanol pathways

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ethanol</th>
<th>Ethanol + Inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol dehydrogenase</td>
<td>$1.30 \pm 0.11$</td>
<td>$1.33 \pm 0.28$</td>
<td>$0.56 \pm 0.05^*$</td>
</tr>
<tr>
<td>CYP 2E1</td>
<td>$179.5 \pm 26.0$</td>
<td>$185.7 \pm 23.0$</td>
<td>$96.7 \pm 24.0^*$</td>
</tr>
<tr>
<td>Catalase</td>
<td>$31.00 \pm 2.01$</td>
<td>$29.50 \pm 3.24$</td>
<td>$13.43 \pm 2.27^*$</td>
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Values are means ± SE ($n = 6/group$). Alcohol dehydrogenase and catalase activity are expressed as change in optical density (OD) per hour per gram of tissue weight. OD/hr/g tissue weight; CYP 2E1 is reported as nmol·min·mg protein$^{-1}$. $^*P < 0.05$ compared with control or ethanol infusion without inhibitors.
This study shows that ethanol infusion alone can induce pancreas-specific injury consisting of edema, intrapancreatic trypsinogen activation, and formation of vacuoles in the pancreatic acini of rats in a dose-dependent fashion. It also demonstrates in this model of alcohol-induced pancreatic injury that there is a very close correlation between plasma ethanol and FAEE concentrations, that FAEEs are increased in liver and pancreas after ethanol administration, and that both tissue levels of FAEE and parameters of pancreatic injury increase in parallel with inhibition of the oxidative pathway of ethanol metabolism.

The protocol of ethanol administration used in our experiments was designed to produce blood ethanol levels that are commonly observed in acutely intoxicated individuals. Because pilot studies revealed that intra-arterial infusion of concentrated ethanol induced hemodynamic instability, we developed a well-tolerated regimen of an initial bolus and a subsequent lower concentration infusion over 2 h. With this, we demonstrated a dose-dependent injury in the pancreas that was absent in other organs. It is characterized by edema, which was maximal at 3 h, intrapancreatic trypsinogen activation, which peaked at 6 h, and formation of vacuoles, which further accumulated at 12 h. Edema and premature activation of trypsinogen within the pancreas are common denominators in a variety of experimental models of pancreatitis (13, 14, 26, 29, 31, 32, 35, 37, 40). The presence of intracellular vacuoles similar to those produced by ethanol in our experiments is also found in several models of pancreatitis (13, 35, 43). In secretagogue-induced pancreatitis, these vacuoles have been found to harbor colocalized lysosomal hydrolases and digestive enzymes (32, 35, 43). Fat vacuoles within the pancreas were observed in experimental studies after long-term ethanol administration. Although our electron microscopy results show vacuoles resembling either lipid accumulation or degranulated zymogen granules, our Oil red O staining experiments after acute ethanol administration prove that they do not contain fat and thus they seem to represent degranulated zymogen granules. We did not investigate whether protease and hydrolyase colocalization occurred in the intra-acinar vacuoles observed in our study, but the increased ectopic trypsinogen activation found in pancreatic tissue after acute ethanol administration further supports this hypothesis.

The mechanism whereby alcohol injures the pancreas remains unknown, but this study provides further evidence that FAEEs play an important role. In a previous study, we described that infusion of FAEE reconstituted in low-density lipoprotein-produced morphological and biochemical changes in the pancreas and in other organs (45). This observation raised the possibility that this nonoxidative metabolite of ethanol was responsible, at least in part, for alcohol-induced pancreatitis. The present study reproduces that same injury by administering ethanol instead of FAEE and shows that ethanol blood concentrations in rats have a linear correlation with FAEE levels. This is strikingly similar to what is observed in acutely intoxicated humans (10). Furthermore, the FAEE levels found in pancreatic tissue are almost identical to those observed in an autopsy study of individuals who died with acute alcohol intoxication (25). The origin of FAEE in serum after alcohol ingestion has not been determined, but endogenous FAEE synthesis from ethanol is much
greater in the pancreas than in any other organ (25, 46), and this may explain our finding that the amount of FAEE detected in the pancreas after ethanol infusion was significantly higher than that of the liver.

The correlation of both FAEE levels in blood and pancreas and pancreatic injury with ethanol dosage is further substantiation of FAEE as a mediator of ethanol-induced pancreatic injury. To exclude the possible role of toxic products of oxidative metabolism, we administered ethanol in combination with inhibitors of the oxidative pathway. Our results show that inhibition of oxidative metabolism results in increase of FAEE levels in pancreas and liver, with consequent increase of pancreatic injury, and demonstrate that the oxidative and nonoxidative pathways are metabolically linked as alternatives in both organs. Although only a minor role for the oxidative pathway in the pancreas was suggested by direct assay of enzymes in the oxidative pathway in other studies (4, 12), we found that 50% inhibition of the oxidative pathway led to a threefold increase of nonoxidative metabolism and that the relative increases were similar in pancreas and liver. This observation is consistent with a significant capacity for oxidative metabolism of ethanol by the pancreas as was shown by Haber et al. (15), who demonstrated that isolated pancreatic acinar cells have a capacity for ethanol oxidation comparable with that of hepatocytes, although it is of note that the experiments of Haber et al. suggest that pancreatic ethanol oxidation is mediated by class 3 ADH, which is not inhibited by 4-MP. As the acinar cells in the experiments of Haber et al. were cultured and not used immediately after the animals were killed, their ethanol-oxidizing capacity and enzyme pattern may be different than the acinar cells used in our experiments and those of others (4, 12). Moreover, other types of pancreatic cells, e.g., duct cells or connective tissue, which were not included in the studies by Haber and colleagues, may also play a role in ethanol oxidation of the pancreas.

Nonetheless, the potential role of the oxidative metabolites of ethanol in pancreatic injury appears to be small, because injury increases rather than decreases when the oxidative pathway is inhibited, and there is a shift to production of nonoxidative metabolites. Because injury of the liver by acetaldehyde has been well documented, it has been proposed that this same oxidative metabolite might play a role in the development of pancreatitis (12, 40). Despite searches for decades, pancreatic injury by oxidative metabolites, including acetaldehyde, has never been shown in vivo or on isolated perfused pancreas (14, 19, 33). Our results cast further doubt on the relevance of acetaldehyde or other oxidative metabolites in the pathogenesis of alcoholic pancreatitis and give support to the hypothesis that the nonoxidative metabolites, namely FAEE, mediate the early pancreatic acinar cell injury caused by alcohol. Furthermore, although acetaldehyde and the other oxidative metabolites are rapidly metabolized, FAEEs accumulate in the pancreas after acute and long-term ethanol ingestion (11) and, thereby, can exert their toxicity over prolonged periods of exposure.

Pancreatic injury by FAEE may be a result of several intracellular effects of FAEE. FAEE, but not ethanol or acetaldehyde, have been shown to increase lysosomal fragility in vitro (16), and lysosomal disruption may facilitate contact between lysosomal and digestive enzymes, leading to intracellular autodigestion of proteases and cell injury as shown in other models of pancreatitis (35, 43). Others, such as Hungund et al. (20), have proposed that FAEE bind to intracellular membranes and directly alter their function and permeability. Yet another hypothesis states that FAEE induce mitochondrial dysfunction by uncoupling of oxidative phosphorylation, possibly via free fatty acids liberated from hydrolysis of FAEE within the cell (24).

In this study, which investigated only the effects of a very brief exposure of the pancreas to ethanol, we found both biochemical and morphological consequences that may be consistent with early alcohol-induced pancreatitis. We do not yet have evidence that repeated or long-term exposure would produce fully developed acute or chronic pancreatitis with inflammation and necrosis or chronic fibrosis and acinar loss. Because chronic alcohol consumption increases the pancreatic content of digestive and lysosomal enzymes and decreases the trypsin-inhibiting capacity (40), our finding that high FAEE levels induce trypsinogen activation may explain how chronic FAEE exposure could promote more severe and injurious inflammatory reactions. Cofactors such as diet or inherited predisposition may help to determine individual susceptibility, because alcohol abuse does not uniformly cause pancreatitis.

In summary, this study shows that ethanol infusion causes a dose-dependent injury to the pancreas, and that inhibition of the oxidative metabolic pathway of ethanol results in a shift to nonoxidative metabolism with consequent increase of FAEE and pancreatic injury. This, together with prior evidence of direct toxicity of FAEE on the pancreas, suggests that FAEE is directly involved in the pathogenesis of alcoholic pancreatitis.

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