Development of an animal model of chronic alcohol-induced pancreatitis in the rat

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1Laboratory of Hepatology and Toxicology, Department of Pharmacology, 2Curriculum in Toxicology, and 3Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill 27599-7365; and 4Laboratory of Pharmacology and Chemistry, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709

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Kono, Hiroshi, Mikio Nakagami, Ivan Rusyn, Henry D. Connor, Branko Stefanovic, David A. Brenner, Ronald P. Mason, Gavin E. Arteel, and Ronald G. Thurman. Development of an animal model of chronic alcohol-induced pancreatitis in the rat. Am J Physiol Gastrointest Liver Physiol 280: G1178–G1186, 2001.—This study was designed to develop an animal model of alcoholic pancreatitis and to test the hypothesis that the dose of ethanol and the type of dietary fat affect free radical formation and pancreatic pathology. Female Wistar rats were fed liquid diets rich in corn oil (unsaturated fat), with or without a standard or high dose of ethanol, and medium-chain triglycerides (saturated fat) with a high dose of ethanol for 8 wk enterally. The dose of ethanol was increased as tolerance developed, which allowed approximately twice as much alcohol to be delivered in the high-dose group. Serum pancreatic enzymes and histology were normal after 4 wk of diets rich in unsaturated fat, with or without the standard dose of ethanol. In contrast, enzyme levels were elevated significantly by the high ethanol dose. Increases were blunted significantly by dietary saturated fat. Fibrosis and collagen α1(1) expression in the pancreas were not detectable after 4 wk of enteral ethanol feeding; however, they were enhanced significantly by the high dose after 8 wk. Furthermore, radical adducts detected by electron spin resonance were minimal with the standard dose; however, the high dose increased carbon-centered radical adducts as well as 4-hydroxynonenal, an index of lipid peroxidation, significantly. Radical adducts were also blunted by ~70% by dietary saturated fat. The animal model presented here is the first to demonstrate chronic alcohol-induced pancreatitis in a reproducible manner. The key factors responsible for pathology are the amount of ethanol administered and the type of dietary fat.

medium-chain triglycerides; fibrosis; free radical; enteral feeding

Epidemiological evidence indicates that chronic alcohol abuse, but not occasional alcohol intoxication, is a major cause of chronic pancreatitis in adult patients (26). Therapies are limited by an inadequate understanding of the mechanisms of pathophysiology, and progress has been slow because an appropriate animal model for alcohol-induced pancreatitis is lacking (6). The pathology of chronic alcoholic pancreatitis consists of both intralobular fibrosis involving pancreatic acini and interlobular fibrosis with fibrotic strictures of pancreatic ducts (8). Tsukamoto et al. (32) demonstrated that alcohol-induced pancreatitis can be studied in an intragastric enteral feeding model in the rat. In that study, enteral ethanol along with a high-fat diet caused atrophy and apoptosis in pancreatic acinar cells; however, focal necrosis and fibrosis were only present in ~30% of rats given ethanol chronically for 30–160 days. Thus modifications of this enteral feeding protocol could theoretically produce a useful model.

Free radicals are involved in alcohol-induced tissue injury (17). Indeed, α-hydroxyethyl free radical was detected in pancreatic secretions after alcohol exposure, but before increases in pancreatic enzymes and pathological changes occurred (15). The type of dietary fat is involved in free radical formation and pathogenesis in the liver after alcohol consumption. Indeed, medium-chain triglycerides (saturated fat) blunted increases in endotoxin levels in portal blood, free radical formation in the liver, and liver injury significantly in the Tsukamoto-French enteral model (18, 23). Moreover, a high-fat liquid diet (35% of total calories) rich in corn oil increased pancreatic injury in long-term enteral feeding (32). Thus dietary unsaturated fat has been associated with alcohol-induced pancreatic injury. Therefore, the present study was designed to develop an animal model and, using the intragastric alcohol feeding protocol, to test the hypothesis that the dose of ethanol and the type of dietary fat affect free radical formation and pathology in the pancreas. In the present study, rats received progressively increasing doses of ethanol through careful challenge as their tolerance developed over the 8 wk of the experimental period, because pancreatic injury was minimal in the

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Tsukamoto-French enteral protocol with the standard dose of ethanol (15).

METHODS

Diets and animals. A liquid diet described first by Thompson and Reitz (27), supplemented with lipotropes as described by Morimoto et al. (20), was used in this study. It consisted of either corn oil (unsaturated fat) or medium-chain triglycerides (saturated fat) as a source of fat (37% of total calories), protein (23%), carbohydrate (5%), minerals, and vitamins plus either ethanol (35–40% of total calories) or maltose-dextrin (control diet), as described in detail elsewhere (29).

Female Wistar rats (200–225 g) were fed high-fat liquid diets (200 kcal·kg body wt⁻¹·day⁻¹) rich in unsaturated fat, without or with ethanol (standard dose, 8–12 g·kg⁻¹·day⁻¹; high dose, 10–16 g·kg⁻¹·day⁻¹) or a diet rich in saturated fat along with a high dose of ethanol continuously for up to 8 wk, using the enteral protocol developed by Tsukamoto and French (15, 30). In the standard dose protocol, ethanol was initially delivered at 8.5 g·kg⁻¹·day⁻¹ (35% of total calories) and was increased 0.6 g/kg every 2 days until the end of the first week; then it was increased 0.6 g/kg every 4 days up to 11.5 g·kg⁻¹·day⁻¹ of ethanol (37% of total calories). This dose was continued until the end of the experimental period. On the other hand, in the high-dose protocol, ethanol was delivered initially at 10.2 g·kg⁻¹·day⁻¹ (35% of total calories) and was increased 0.6 g/kg every 4 days up to the end of the first week and then it was increased 0.6 g/kg every 4 days up to the end of week 4. During the second 4-wk period, ethanol delivery was increased 0.6·0.6 g/kg each week up to 17–18 g·kg⁻¹·day⁻¹ (40% of total calories). All animals received humane care in compliance with institutional guidelines, and alcohol intoxication was assessed carefully to evaluate the development of tolerance to ethanol with the use of a 0–3 scoring system (0, normal; 1, sluggish movement; 2, loss of movement but still moving if stimulated; 3, loss of consciousness). The ethanol dose was increased progressively to challenge animals based on this assessment, allowing ~1-fold more ethanol to be delivered to adult Wistar rats than in previous studies by this and other laboratories (15, 22, 28).

Clinical chemistry. Ethanol concentrations in urine, which are representative of blood ethanol levels (3), were measured daily. Rats were housed in metabolic cages that separated urine from feces, and urine was collected over 24 h in bottles containing mineral oil to prevent evaporation. Each day at 9:00 AM, the urine collection bottles were changed and a 1-ml sample was stored at –80°C until analysis of free radical adducts by electron spin resonance (ESR) spectroscopy (17).

Samples were thawed and transferred to a quartz flat cell, and ESR spectra were obtained with a Varian E-109 spectrometer equipped with a TM110 cavity. Instrument conditions were as follows: 20-mW microwave power, 1.0-G modulation amplitude, 80-G scan width, 16-min scan, and 1-s time constant. Spectral data were stored on an IBM-compatible computer and were analyzed for ESR hyperfine coupling constants by computer simulation (10). ESR signal intensity was determined from the amplitude of the high-field member of the low-field doublet (second line from the left) of the ESR spectra and expressed in arbitrary units (1 unit = 1 cm chart paper).

Immunohistochemical detection of 4-hydroxyhexenal-modified proteins. Paraffin-embedded sections of pancreatic tissue were deparaffinized, rehydrated, and stained immunohistochemically for the presence of an in vivo marker of lipid peroxidation, 4-hydroxyhexenal protein adducts, by sequential incubation with a polyclonal antibody (Alpha Diagnostic International, San Antonio, TX) in PBS (pH 7.4) containing 1% Tween 20 and 1% bovine serum albumin. Peroxidase-linked secondary antibody and diaminobenzidine (Peroxidase Envision kit, DAKO, Carpinteria, CA) were used to detect specific binding. The slides were rinsed twice with PBS-0.1% Tween 20 between all incubations, and sections were counterstained with hematoxylin as described elsewhere (12). To control for nonspecific binding of the secondary antibody, sections from the same animals were processed without the primary antibody, followed by the procedure detailed above. No positive staining was observed in this control experiment (data not shown).

RNase protection assays for detection of collagen I mRNA levels. Briefly, the template for the cRNA probe for rat collagen α1(I) was generated by subcloning the AvaI/PstI fragment of its cDNA into the in vitro transcription plasmid pGME3 (5). The antisense collagen α1(I) probe was generated by digestion of this plasmid with HindIII restriction endonuclease and transcribed with the T7 RNA polymerase in the presence of [α-³²P]UTP. The antisense rat L-32 probe was generated according to the manufacturer’s instructions (Ambion, Austin, TX). Both radiolabeled probes were simultaneously hybridized to total RNA and digested with RNase A2 and RNase T1. Protected fragments were resolved by electrophoresis on a 6% polyacrylamide sequencing gel and
quantified by Phospholmage analysis (Molecular Dynamics, Sunnyvale, CA).

Statistics. A multiple-comparison ANOVA with Bonferroni’s post hoc analysis was used for the determination of statistical significance, as appropriate. For comparison of pathology scores, the Mann-Whitney rank sum test was used. Before the study, a \( P \) value < 0.05 was selected as the level of significance.

RESULTS

Physiological parameters. The diets were initiated 1 wk after surgery to allow for full recovery, and steady growth rates were observed during enteral feeding. Weight gain in rats fed high-fat liquid diets with or without ethanol was similar to that in rats fed the chow diet [high-fat control (without ethanol), 2.0 ± 0.2 g/day; with standard dose of ethanol, 1.9 ± 0.1; with high dose of ethanol, 1.9 ± 0.2], indicating that the rats were adequately nourished. Including medium-chain triglycerides as the source of dietary fat did not affect weight gain. As previously reported (1, 22, 28), for unknown reasons, daily urine alcohol concentrations fluctuated between 0 and 600 mg/dl in all groups studied (Fig. 1). In rats fed the standard dose of ethanol, mean urine alcohol concentration was 185 ± 11 mg/dl (Fig. 1A); however, the high dose of ethanol increased these values significantly, by ~50% (274 ± 15 mg/dl; Fig. 1B). Furthermore, in the second 4-wk period, the mean urine alcohol concentrations were 319 mg/dl in the high-dose group and 246 mg/dl in the standard-dose group. There were no differences in mean urine alcohol concentrations between rats fed the high dose of ethanol with unsaturated fat and those fed the high dose with saturated fat, as expected (271 ± 17 mg/dl).

In the first 4 wk, all animals were healthy and survived during enteral feeding; however, ~30% mortality was observed in the high-dose ethanol group during the second 4 wk. Because analysis of these animals was postmortem, the cause of mortality was not clear in all cases, although it was observed that some animals died because of convulsions during alcohol withdrawal when urine alcohol levels were low. Serum creatine kinase values were not significantly elevated as would be expected if circulatory shock occurred. Furthermore, there was no effect of alcohol on average systemic blood pressure.

Pathophysiology. In rats fed a high-fat control diet for 4 wk, the values of the serum pancreatic enzymes \( \alpha \)-amylase and lipase were normal (Fig. 2). Increases in these values were minimal in rats given the standard dose of ethanol; however, values increased significantly, approximately threefold, in rats given higher doses of ethanol (Fig. 2). After 8 wk, the amylase and lipase values in the animals given higher doses of ethanol were also significantly elevated compared with those in animals that received the standard dose of ethanol (amylose, 1,877 ± 88 IU/l; lipase, 82 ± 38 IU/l) or the high-fat control animals (amylose, 1,477 ± 173 IU/l; lipase, 136 ± 26 IU/l). However, amylase levels at 8 wk in animals given the higher doses of ethanol were significantly lower compared with the values at 4 wk in the same group (Fig. 2A); this effect is similar to that observed in humans. Specifically, it is known that serum amylase levels decrease as pancreatic exocrine function declines during the progression of pancreatic damage (9).

Pancreatic histology data from adult female Wistar rats on the chow diet are shown in Fig. 3A. In rats fed the high-fat diet with or without the standard dose of ethanol, pathological changes were minimal after 4 wk (Fig. 3, B and C). In contrast, the high dose of enteral ethanol given with unsaturated fat for 4 wk caused acinar cell atrophy, fatty infiltration in acinar and islet cells (Fig. 3D), inflammatory cell infiltration (Fig. 3E), and focal necrosis (Fig. 3F) in the pancreas, resulting in the total pathology score of 4.4 ± 0.5 (Table 1). The
infiltrating cells were predominantly lymphocytes. Dietary medium-chain triglycerides prevented these pathological changes nearly completely (total pathology score, 1.0 ± 0.4). Furthermore, after 8 wk of the high dose of ethanol, pathological changes were greater than after 4 wk (total pathology score, 5.0 ± 0.4; Fig. 3E).

Fibrogenesis in the pancreas after chronic enteral ethanol feeding. Trichrome staining of the pancreas from adult female Wistar rats on the chow diet is shown in Fig. 4A. No significant fibrotic changes were detected after 4 wk of enteral feeding as reflected by the fibrosis score (Table 2), confirming a previous study from this laboratory (15). However, 8 wk of enteral feeding of the high dose of ethanol significantly increased the fibrotic score compared with the fibrotic score of animals receiving the standard dose of alcohol or the high-fat control diet (Fig. 4E, Table 2). Under conditions in which fibrosis was detected, these changes tended to be focal. In addition to the scoring of

Table 1. Pathological scores in the pancreas

<table>
<thead>
<tr>
<th>No. of Weeks</th>
<th>Steatosis</th>
<th>Inflammation</th>
<th>Necrosis</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Standard dose</td>
<td>4</td>
<td>1.0 ± 0.4</td>
<td>0.3 ± 0.2</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>High dose</td>
<td>4</td>
<td>2.8 ± 0.5*</td>
<td>1.0 ± 0.3*</td>
<td>1.0 ± 0.3*</td>
</tr>
<tr>
<td>High dose</td>
<td>8</td>
<td>2.2 ± 0.5*</td>
<td>1.3 ± 0.3*</td>
<td>1.8 ± 0.3*</td>
</tr>
<tr>
<td>High dose + MCT</td>
<td>4</td>
<td>0.5 ± 0.2†</td>
<td>0.5 ± 0.2†</td>
<td>0.4 ± 0.1†</td>
</tr>
</tbody>
</table>

Pathological changes in the pancreas (means ± SE) were scored as described in METHODS. Control, high-fat control diet; standard dose, high-fat diet + standard ethanol dose; high dose, high-fat diet + high ethanol dose; high dose + MCT, high-fat (medium-chain triglycerides [MCT]) diet + high ethanol dose. *P < 0.05 compared with standard-dose group; †P < 0.05 compared with high-dose group by Mann-Whitney rank sum test.
pancreata for fibrotic changes, levels of collagen α1(I) mRNA expression in the pancreas were also determined (Fig. 5). There were no detectable signals in animals fed the chow or high-fat diets, regardless of the length of treatment (Fig. 5). Enteral feeding with ethanol for 4 wk also did not affect collagen α1(I) mRNA expression (Fig. 5), as observed previously (15). However, high-dose ethanol given for 8 wk caused extensive increases in collagen α1(I) mRNA expression.

Table 2. Fibrosis in the pancreas

<table>
<thead>
<tr>
<th>Enteral Feeding</th>
<th>4 Wk</th>
<th>8 Wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-fat control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Standard dose</td>
<td>0.2 ± 0.1</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>High dose</td>
<td>0.4 ± 0.2</td>
<td>1.3 ± 0.3*</td>
</tr>
<tr>
<td>High dose + MCT</td>
<td>0.2 ± 0.1</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Pancreatic fibrosis (means ± SE) was scored as described in METHODS. * P < 0.05 compared with rats fed the standard dose of ethanol by Mann-Whitney rank sum test. N.D., not determined.

Fig. 5. Effect of chronic ethanol and lipid type on collagen α1(I) mRNA expression in pancreatic tissue. Pancreatic tissues were assayed for fibrillar collagen α1(I) mRNA using an RNase protection assay with L32 as the housekeeping gene. Representative gels are shown. EtOH, ethanol.
Radical adducts in pancreatic secretions. Radical adducts were barely detectable in pancreatic secretions from rats fed either the chow or the high-fat control diet without ethanol (data not shown). Moreover, POBN radical adducts in pancreatic secretions from rats given the standard dose of ethanol were minimal after 4 wk (Fig. 6). In contrast, the high dose of ethanol increased radical adducts approximately twofold. Furthermore, dietary saturated fat in the form of medium-chain triglycerides blunted this increase significantly. Computer simulation of these spectra identified the POBN-α-hydroxyethyl radical adduct (17). The average radical intensity was approximately fourfold greater in pancreatic secretions from rats fed the high dose than those fed the standard dose of ethanol (Fig. 7). Furthermore, this increase was blunted significantly (~70%) by dietary saturated fat.

Immunohistochemical detection of 4-hydroxynonenal modified proteins. To test whether ethanol administration causes lipid peroxidation in the pancreas, 4-hydroxynonenal-modified proteins were detected by immunohistochemistry (24). Despite increases in free radical formation (Fig. 7), no detectable increases in 4-hydroxynonenal protein adducts were detected after 4 wk of enteral feeding in any group (data not shown). After 8 wk of enteral feeding, significant accumulation of 4-hydroxynonenal (brown staining) was observed in the pancreas of rats fed high-dose ethanol but not in rats fed the chow or high-fat control diets (Fig. 8) or in rats given the standard dose of ethanol (data not shown).

DISCUSSION

Development of a new animal model to study alcohol-induced pancreatic injury. The mechanism of chronic alcohol-induced pancreatitis remains unclear, and a major problem has been the lack of an appropriate animal model for study. Tsukamoto et al. (32) previously reported that feeding a high-fat diet with the standard dose of ethanol caused atrophy and apoptosis in pancreatic acinar cells; however, focal necrosis and fibrosis were only present in ~30% of the rats fed enteral ethanol for 30–160 days. Because the severity of clinical alcohol-induced pancreatitis and fibrosis is related to dose- and time-dependent alcohol consumption in humans (11), here, the chronic intragastric enteral feeding protocol was modified to increase ethanol delivery. This goal was achieved by increasing the alcohol levels in the diet based on behavioral assessment of the development of tolerance to ethanol with the use of the 0–3 score system. In this manner, alcohol delivery in female Wistar rats in the 200–225-g weight range could be increased to 17–18 g kg\(^{-1}\) day\(^{-1}\). Alcohol delivery was increased ~1.4-fold over previous studies from this and other laboratories with rats in the same weight range (Refs. 13, 15, 31; Fig. 2). As a result, pancreatic enzymes (amylase and lipase) and pathology scores were increased after 8 wk of the high dose of ethanol (Table 1, Figs. 2 and 3). Furthermore, fibrosis and collagen \(\alpha(1)\) mRNA expression increased significantly after 8 wk of the high dose of ethanol in nearly all animals (Table 2, Figs. 4 and 5).
Thus it is concluded that the total amount of alcohol consumed is a critical factor in producing chronic alcohol-induced pancreatic injury. Furthermore, the animal model presented here is the first reproducible demonstration of chronic alcohol-induced pancreatitis in the rat.

Gender is also an important factor in alcohol-induced tissue injury. Indeed, alcohol-induced liver injury is greater in females than in males in the enteral alcohol model (16). The present study differs from previous work (32) in that female rats were used, and gender may therefore play a role in the observed pancreatic damage; however, the damage resulting from the use of the alcohol dosing regimen described in the Methods section also occurs in males (Kono et al., unpublished observations), consistent with the idea that gender cannot completely explain the greater pathology observed in this study.

Dietary fat and alcohol-induced pancreatic injury. Dietary fat is also an important factor in alcohol-induced tissue injury (21). Indeed, dietary medium-chain triglycerides reduced lipid peroxidation in the enteral model (23). Furthermore, dietary medium-chain triglycerides prevented free radical formation and early alcohol-induced liver injury in the Tsukamoto-French enteral model (18). In the present study, dietary medium-chain triglycerides also blunted free radical formation and tissue injury in the pancreas (Figs. 6 and 7). Dietary medium-chain triglycerides significantly blunted the increase in plasma endotoxin levels after enteral ethanol, the response of Kupffer cells to endotoxin, and liver injury (18). Therefore, medium-chain triglycerides could affect activation of tissue macrophages or other critical cell types in the pancreas. Thus dietary medium-chain triglycerides prevented pancreatic injury, most likely by inhibition of free radical formation. Together, these data indicate that dietary fat is also an important factor in the pathogenesis of chronic alcohol-induced pancreatic injury.

Fig. 9. Working hypothesis. Ethanol changes gut permeability and microflora during enteral feeding, and the high dose of ethanol could increase endotoxin levels. Increased endotoxin most likely increases infiltration of leukocytes such as macrophages and neutrophils, which produce free radicals in the pancreas. One key factor in causing pathology seems to be the total amount of ethanol consumed chronically.
What is the mechanism of damage caused by alcohol in the pancreas? Figure 9 is a schematic depiction of our working hypothesis on the mechanism by which alcohol damages the pancreas. In the present study, enteral feeding with a higher delivery of ethanol increased formation of both carbon-centered free radicals and 4-hydroxynonenal-modified proteins in the pancreas (Figs. 6–8). These data are consistent with the hypothesis that oxidative stress is involved in the pathogenesis of early alcohol-induced pancreatic injury (see Fig. 9).

It is also proposed that the infiltration of inflammatory cells plays a key role in the progression of pancreatic damage (Fig. 9). Infiltrating leukocytes such as lymphocytes and macrophages can be activated by gut-derived endotoxin and/or other stimuli to produce oxidants after alcohol consumption (Fig. 9). Indeed, 4 wk of high-dose ethanol increased endotoxin levels in portal blood (e.g., 149 ± 17 pg/ml), and free radical formation (Figs. 6 and 7) was ~2.5-fold higher than in a previous study (16) performed with the standard ethanol protocol. Furthermore, inflammatory foci increased significantly in the high-dose group compared with those in the standard group in this study (Table 1). There are already links in the literature between acute pancreatitis, endotoxins, and inflammatory cells. For example, endotoxemia predicts outcome in acute pancreatitis in humans (34), and gut sterilization with antibiotics protects against acute pancreatitis in rats (19). Inflammatory cell infiltration is also known to well with the prognosis of pancreatitis in humans and in animal models (2, 7, 25, 33). The question remains whether these mechanisms are also important during the chronic pancreatitis caused by alcohol, and this question will be the focus of future research.

**Clinical implications.** The Tsukamoto-French enteral alcohol model has a strong nutritional component, with liver injury dependent on unsaturated fat in the diet (21). In this study, pancreatic injury caused by the high dose of ethanol was prevented by medium-chain triglycerides (Table 1). Importantly, in this study, increases in fibrosis were observed only after 8 wk (Figs. 4 and 5), leading to the conclusion that chronic alcohol-induced pancreatic injury is dependent on the total amount of alcohol consumed and the type of dietary fat. Furthermore, this is the first demonstration of reproducible chronic alcohol-induced pancreatic fibrosis in a relatively short experimental period. Thus this animal model may be useful for the study of mechanisms of chronic alcohol-induced pancreatitis and for developing useful therapeutic strategies.

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