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Detection of α-Hydroxyethyl Free Radical Adducts in the Pancreas after Chronic Exposure to Alcohol in the Rat

YUJI IIMURO, BLAIR U. BRADFORD, WENSHI GAO, MARIA KADIISKA, RONALD P. MASON, BRANKO STEFANOVIC, DAVID A. BRENNER, AND RONALD G. THURMAN

Laboratory of Hepatobiology and Toxicology, Departments of Pharmacology (Y.I., B.U.B., W.G., R.G.T.) and Medicine (B.S., D.A.B.), University of North Carolina at Chapel Hill, and National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina (M.K., R.P.M.)

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SUMMARY

Chronic pancreatitis is characterized by inflammation and fibrosis leading to tissue destruction; in industrialized nations, alcohol abuse is the cause of 70–80% of cases of pancreatitis in adults. The purpose of the current work was to determine whether free radical adducts are produced by the pancreas during the early phases of chronic exposure to ethanol. Accordingly, rats were chronically fed ethanol using the model of continuous enteral infusion developed by Tsukamoto *et al*. [*Am. J. Physiol*. **247:** R595–R599 (1984)]. Histological evaluation revealed only mild acinar steatosis and spotty necrosis after 4 weeks of alcohol treatment; the pancreatic enzymes lipase and amylase were not elevated. Furthermore, no fibrosis was detected, nor were there differences in pancreatic collagen α1(I) mRNA levels between the dietary control and ethanol-treated groups. After 4 weeks, rats were injected with the spin trap α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (1 g/kg intravenously), and pancreatic secretions were collected over a 4-hr period. A six-line free radical adduct spectrum indicative of a carbon-centered free radical was detected in pancreatic secretions and in Folch extracts of pancreatic tissue by electron spin resonance spectroscopy. Control experiments ruled out *ex vivo* radical formation. This study represents the first detection of radical adducts in pancreatic secretions. When [^{13}C]ethanol (3 g/kg intragastrically) was administered, a definitive 12-line spectrum was detected in pancreatic secretions, demonstrating that the α-hydroxyethyl radical adduct was formed in the pancreas from [^{13}C]ethanol. Interestingly, only a six-line signal was detected in tissue extracts under these conditions. Free radicals, therefore, are formed in the pancreas during the early phases of chronic alcohol intake in rats before the development of overt pathology.

Chronic pancreatitis is an inflammatory disease characterized by marked tissue destruction. This painful ailment results in the progressive deterioration of exocrine and endocrine function, leading to multiple associated diseases such as maldigestion and diabetes (1, 2). Epidemiological evidence indicates that alcohol abuse is the cause of chronic pancreatitis in 70–80% of adult patients in the United States and other industrialized countries (3, 4). The known pathology of chronic pancreatitis consists of both intralobular fibrosis involving pancreatic acini and interlobular fibrosis with fibrotic strictures of pancreatic ducts (5). The degree of fibrosis is greater in chronic alcoholic pancreatitis than in nonalcoholic idiopathic chronic pancreatitis or obstructive chronic pancreatitis (5). However, because an appropriate animal model for studying chronic pancreatitis is lacking, therapies are limited due to an inadequate understanding of the mechanisms of pathophysiology.

Acute animal models have been developed to examine pathophysiology and potentially therapeutic interventions in pancreatitis. These models are based in large part on enzymatic autolysis, bile salt toxicity, and cholecystokinin stimulation. The most frequently studied model of acute pancreatitis uses administration of the cholecystokinin analog cerulein (6). However, repetitive injection of cerulein does not cause chronic pancreatic fibrosis (6), making this model unsuitable for evaluation of the mechanisms of chronic alcoholic pancreatitis.

One study by Tsukamoto *et al*. (7) demonstrated that ethanol-induced pancreatic injury can be studied in an intragastric model of alcohol delivery to rats. They administered alcohol for 30–160 days and continuously maintained blood alcohol in the 250–300 mg/dl range. Control animals displayed normal histology except for occasional mild fat accumulation; however, alcohol caused hypogranulation and apoptosis of pancreatic acinar cells. Focal lesions characteristic of chronic pancreatitis (e.g., interlobular fibrosis, ductal dilatation, and mononuclear cell infiltration) were present in ~30% of rats exposed to ethanol; however, free radicals were not evaluated in that study.

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ABBREVIATIONS: POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide.

Free radical formation has been demonstrated to be an important mediator of alcoholic liver disease (8), yet only one study has examined free radical formation in the pancreas. Nonaka *et al.* (9) produced acute experimental pancreatitis using endotoxin and ethionine. After 6 and 24 hr, the spin trap DMPO was added to pancreatic homogenates, and ESR spectra characteristic of DMPO-hydroxyl radical adduct were detected. Although this study supports the hypothesis that oxygen-derived free radicals are involved in acute experimental pancreatitis, the possibility that radicals were formed *ex vivo* in the homogenate from the release of redox active trace iron was not excluded, and information in chronic, clinically relevant models is absent. Thus, the purpose of the current study was to determine whether free radicals can be detected in pancreatic secretions during chronic ethanol intake under conditions carefully controlled to exclude *ex vivo* radical generation. Preliminary accounts of this work have appeared elsewhere (10).

Materials and Methods

Animals and sample collection. Male Wistar rats (300–320 g) were used in this study. An intragastric cannula was surgically implanted as described by Tsukamoto *et al.* (11), and rats were fed a high-fat liquid diet for 4 weeks via the cannula. The diet consisted of corn oil (37% of calories), protein (23% of calories), carbohydrate, minerals, vitamins, and either ethanol (35% of calories) or isocaloric dextrose (control diet). The ethanol concentration ranged between 8% and 9% (v/v) depending on the demonstrated degree of intoxication and the urine alcohol concentration. Each morning, 24-hr urine samples were collected, and ethanol concentrations were determined enzymatically (12). Blood was sampled weekly from the tail vein and centrifuged, and serum was stored frozen before determination of lipase, α-amylase, and aspartate aminotransferase using Sigma Chemical analytical kits (St. Louis, MO).

Collection of pancreatic samples. To reduce the likelihood of *ex vivo* radical formation (9), it was necessary to develop a new method to obtain free radical adducts without tissue disruption. To achieve this goal, the rat was anesthetized with pentobarbital (75 mg/kg), and the abdomen was opened. The spin trap POBN (1 g/kg) was administered intravenously, just before the bile duct was cut near the liver. As depicted in Fig. 1, the distal bile duct was connected to a small length of PE-20 tubing, and the bile duct was flushed with 0.2 ml of air to prevent bile contamination of pancreatic secretions. A ligature was then placed on the distal end of the pancreatic duct near the sphincter. Over a 4-hr period, pancreatic secretions were collected into 35 μl of 0.5 mM Desferal (deferoxamine mesylate) via retrograde flow from the ligated bile duct. Samples were stored at −80° before analysis for free radical adducts by ESR.

To identify whether the radical adducts arose from ethanol, [^{13}C]ethanol was used. Rats were removed from the alcohol-containing diet, so blood alcohol was negligible before [^{13}C]ethanol (3 g/kg) was administered intragastrically. Pancreatic secretions were sequentially collected into Desferal as described above. In control experiments, *ex vivo* radical formation was evaluated. Samples of pancreatic juice from ethanol-fed rats were collected into a tube containing 1 mg/ml POBN and 35 μl of 0.5 mM Desferal. After all of the experiments, samples of pancreatic tissue were frozen in 35 μl of 0.5 mM Desferal at −80°. Subsequently, tissue was homogenized in Folch medium, the organic extract was collected, and 0.5 ml of phosphate buffer, pH 7.4, was added before analysis for radicals with ESR. The remainder of the pancreatic tissue was fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin as well as with trichrome for histological evaluation.

ESR. The ESR spectra of radical adducts collected in pancreatic secretions or extracted from tissue were obtained using a Varian E-109 spectrometer operating with 1.0 G modulation amplitude, 40 mW microwave power, and 9.33 GHz with 100 kHz modulation frequency. Samples were placed into a quartz flat cell centered in an E-238 TM_{110} microwave cavity. Simulations of ESR spectra were performed on an IBM-compatible computer equipped with a math coprocessor.

RNase protection assays for quantification 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 pGEM3 (13). The antisense collagen α1(I) probe was generated by digestion of this plasmid with the *Hin*dIII restriction endonuclease and transcribed with the T7 RNA polymerase in the presence of α^{32}[P]UTP. The antisense rat glyceraldehyde-3-phosphate dehydrogenase probe was generated according to the manufacturer's recommendation (Ambion, Austin, TX). Both radiolabeled probes were simultaneously

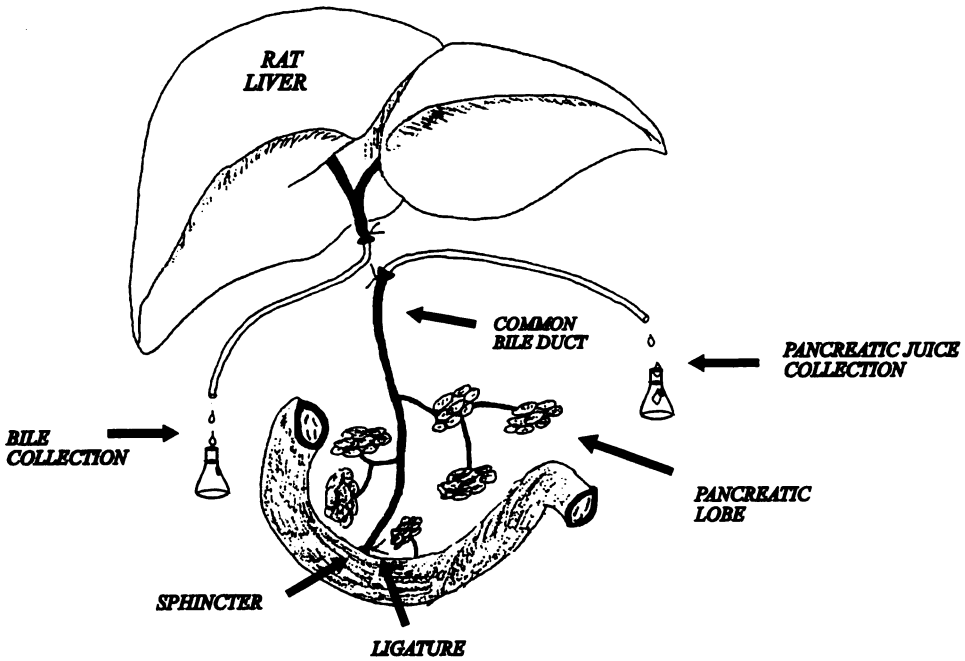

Fig. 1. Simultaneous collection of pancreatic secretions and bile from alcohol-treated rats. The bile duct was cut near the liver just after POBN injection intravenously, and a small piece of PE-20 tubing was inserted into the distal bile duct. After the remaining bile in the distal duct was flushed into the duodenum, a ligature was placed on the distal end of the pancreatic duct near the sphincter. The bile duct was also cannulated. Samples of pancreatic secretions were collected over 4 hr via retrograde flow from the ligated bile duct while bile was collected from the second cannula.

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 PhosphoImage analysis (Molecular Dynamics, Sunnyvale, CA).

Results

Physiological parameters. Both dietary control and ethanol-treated rats maintained on the Tsukamoto-French diet regimen lost a little weight (~10%) in the first week after surgery. The body weight then stabilized and was not different between the groups.

Urine alcohol concentration was determined daily. On average, the urine alcohol concentration was 216 ± 8 mg/dl throughout the course of the study. As reported previously, the daily urine alcohol concentration cycled even though the alcohol concentration in the diet remained constant (14) (Fig. 2). Daily urine alcohol concentrations fluctuated between 0 and 375 mg/dl alcohol every ~5-6 days, similar to previous reports (15, 16). The cause of this pattern is not presently understood (14).

Histological studies. Histological evaluation of pancreatic tissue after 4 weeks of ethanol treatment revealed mild fat deposition but no evidence of fibrosis from trichrome staining. Interstitial edema or necrosis was very mild (data not shown). Minimal changes in pancreatic histology after 1 month of alcohol exposure were confirmed by pancreatic enzyme activity (lipase and α-amylase) in serum collected at death. Lipase and α-amylase activity after ethanol treatment did not differ from controls (lipase: control, 45 ± 9 units/liter; ethanol, 34 ± 10 units/liter; α-amylase: control, 1416 ± 54 units/liter; ethanol, 1297 ± 56 units/liter). Pancreatic samples were also assayed for fibrillar collagen α1(I) mRNA using an RNase protection assay, whereas the mRNA level of the housekeeping gene *GAPDH* was measured as an internal control. The ratio of pancreatic collagen mRNA to glyceraldehyde-3-phosphate dehydrogenase was 1.54 ± 0.07 from control organs and 1.73 ± 0.05 from ethanol-treated tissue, values that were not significantly different. Despite the lack of pathological changes in the pancreas, liver injury occurred with this treatment as described previously (14). Aspartate aminotransferase levels were increased significantly in ethanol-treated rats over controls in 4 weeks (72 ± 8 versus 113 ± 15 IU/liter; $p < 0.05$).

Radical adducts from pancreas. Rats were injected with POBN, and radical adducts were measured in pancreatic secretions and tissue using ESR spectroscopy. In rats fed an ethanol-free control diet, only an ascorbate semiquinone signal was detected in pancreatic secretions (Fig. 3A), whereas a robust six-line signal indicative of a carbon-centered radical adduct was observed in pancreatic juice from ethanol-treated rats (Fig. 3B). In a control experiment, pancreatic juice was collected from an ethanol-treated rat into a tube containing POBN and Desferal. Radical adducts were undetectable even at high gain (2×), demonstrating that radicals were not formed *ex vivo* under these conditions (Fig. 3C). When [^{13}C]ethanol (3.0 g/kg) was given acutely to rats fed an ethanol-containing diet for 1 month, the resulting spectrum exhibited 12 lines (Fig. 4A). Computer simulation identified this species as the POBN/α-hydroxyethyl radical adduct (Fig. 4B). The hyperfine coupling constants (a^N = 15.8 G, a_β^H = 2.3 G, and $a_\beta^{13}C$ = 4.5 G) match literature values for this species (17).

Pancreatic tissue was also extracted with Folch reagent, and radical adducts were measured in tissue from both control and ethanol-treated rats (Fig. 5). No evidence for radicals was observed in pancreatic tissue from rats fed an ethanol-free control diet (Fig. 5A); however, a six-line radical adduct spectrum of unknown origin was detected in pancreatic tissue from ethanol-treated rats (Fig. 5B). Interestingly, the

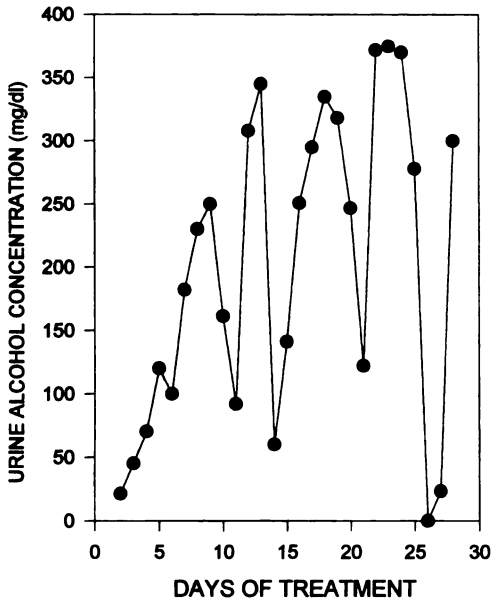

Fig. 2. Urine alcohol concentrations were determined daily in rats administered an ethanol-containing diet. Alcohol measurements were performed enzymatically as described in Materials and Methods. A typical cyclic pattern is shown.

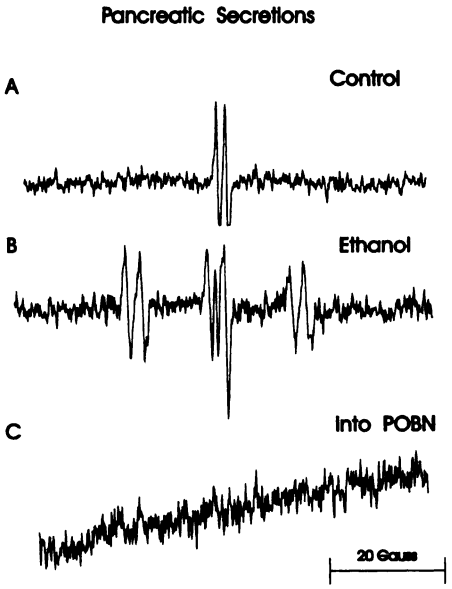

Fig. 3. Radical adducts in pancreatic secretions. Rats were maintained on a continuous enteral infusion of dietary control (A) or ethanol-containing (B) diets for 4 weeks. Pancreatic secretions were collected as described in Materials and Methods. To rule out *ex vivo* radical formation, pancreatic secretions from an ethanol-treated rat (C) were collected into a tube containing 1 mg/ml POBN and 0.5 mM Desferal and were analyzed by ESR as described in Materials and Methods. Gain was twice as high in C as in A and B. Representative experiments were repeated three times.

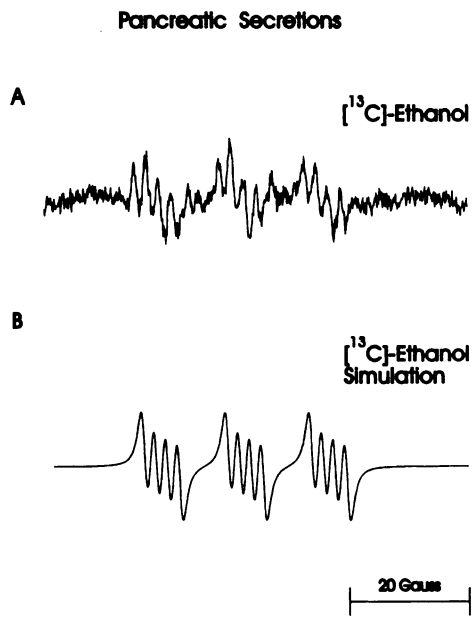

Fig. 4. Identification of radical adducts in pancreatic secretions with [^{13}C]ethanol. Conditions were the same as for Fig. 3. [^{13}C]Ethanol (3.0 g/kg intragastrically) was administered to ethanol-treated rats before the administration of POBN as described in Materials and Methods (A). Computer simulation (B) identified the species as the α-hydroxyethyl radical adduct. Representative spectra from the experiment were repeated three times.

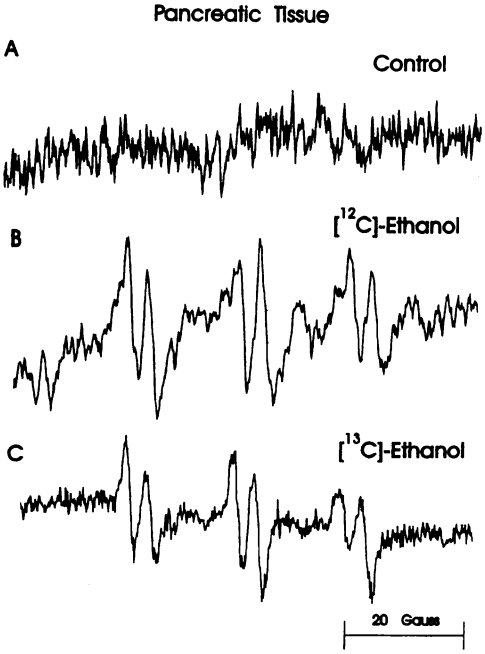

Fig. 5. Radical adducts in pancreatic tissue. Conditions were the same as for Fig. 3. Pancreatic tissue was collected from rats after sampling of pancreatic secretions from control (A), [^{12}C]ethanol-treated (B), or [^{13}C]ethanol-treated (C) rats. The tissue was flash-frozen and homogenized in Folch (chloroform/methanol 2:1) solution. Radical adducts were measured as described in Materials and Methods (n = 3).

administration of [^{13}C]ethanol also produced a six-line radical adduct spectrum in pancreatic tissue, rather than the expected 12-line spectrum, ruling out the possibility that this species is the α-hydroxyl ethanol adduct (Fig. 5C). For this experiment, pancreatic juice and tissue were harvested from the same rat, which yielded a 12-line POBN/α-hydroxyethyl spectrum in pancreatic secretions (Fig. 4A). It is not clear why α-hydroxyethyl free radical could not be detected in pancreatic tissue; however, one possibility is that it is secreted into pancreatic juice so fast that it does not accumulate in tissue.

Discussion

This study represents the first detection of α-hydroxyethyl free radical adduct in pancreatic secretions in the early phases of chronic exposure to ethanol before the development of overt pathology. Previously, information on free radicals in the pancreas was scant, although free radicals have been detected in other tissues treated with ethanol, including the liver. For example, in the 1960s DiLuzio (18) characterized lipid peroxidation in the liver after chronic exposure to alcohol, a finding later confirmed by others (19). After the introduction of the spin trapping technique, Tomasi *et al.* (20), Reinke *et al.* (21), and Rashba-Step *et al.* (22, 23) presented *in vitro* evidence for a free radical derived from ethanol, the α-hydroxyethyl free radical. Using a deer mouse model, we were the first to detect this radical *in vivo* (17). [^{13}C]Ethanol produced a 12-line ESR signal, proving unequivocally that the free radical was derived from ethanol (17). In early studies, detection of the α-hydroxyethyl radical from ethanol was only possible in deer mice fed a high-fat, ethanol-containing diet. Later, however, we detected α-hydroxyethyl radicals in the bile of Wistar rats continuously exposed to ethanol via the Tsukamoto-French protocol (8), and it was recently detected in bile after acute administration of ethanol in rats (24). In the deer mouse study, however, ESR evidence for a lipid free radical was also obtained (17).

Nonaka *et al.* (9) reported the production of free radicals in homogenates of mouse pancreatic tissue after acute pancreatitis produced by endotoxin and ethionine. In this study, mice were killed, and pancreatic tissue was frozen rapidly and subsequently homogenized in DMPO and saline. Radicals were measured in homogenate samples incubated at room temperature with subsequent ESR analysis 0, 6, and 24 hr later. Although spectral evidence for a DMPO-hydroxyl radical adduct was presented (9), these results must be viewed cautiously because radicals can be formed *ex vivo* from metals released from tissue homogenization or present in buffers. Our data with pancreatic tissue are similarly ambiguous.

To avoid this possible confounding variable, we developed new methods to collect pancreatic juice and detect free radicals. Using these methods, a carbon-centered six-line species was detected in pancreatic secretions (Fig. 4). Furthermore, the identity of this species was confirmed by the use of [^{13}C]ethanol. The 12-line species was identified as the α-hydroxyethyl free radical (Fig. 3). An important advantage of this new methodology is that possible artifactual production of free radicals due to tissue destruction does not occur (e.g., Fig 3C). Thus, free radicals are indeed produced by the pancreas using new methods that do not require tissue disruption.

What is the mechanism for the formation of α-hydroxyethyl free radicals in the pancreas? Cytochrome P-450 can form α-hydroxyethyl free radicals in the liver (25–27) and also exists in pancreatic acinar cells in rodents (28) and humans (29), even though its levels in pancreas are much

lower than in hepatocytes. Furthermore, it has been reported that cytochrome P-450 is induced in pancreatic tissues from patients with chronic pancreatitis (30). Thus, it is possible that cytochrome P-450 is induced in pancreatic acinar cells after chronic exposure to ethanol and is the source of free radicals in the current study. On the other hand, hypoxia/reoxygenation could also account for α-hydroxyethyl radical formation. In support of this idea, it has been reported that pancreatic capillary blood flow is decreased with intravenous ethanol administration in rats (31). In the current study, urine alcohol concentrations in ethanol-treated rats, which are representative of blood alcohol levels (15), fluctuated (Fig. 2). Therefore, cycles of hypoxia/reoxygenation could occur in the pancreas during chronic exposure to ethanol, leading to the formation of oxygen radicals, which attack ethanol to produce the α-hydroxyethyl species. At present, it is not possible to choose between these two putative mechanisms. However, DMSO (3 g/kg), which reacts with hydroxyl radicals, abolished the α-hydroxyethyl free radical signal in pancreatic juice in the presence of ethanol (3 g/kg) (data not shown). These observation support the hypothesis that α-hydroxyethyl free radical formation results from the reaction of ethanol with hydroxyl radical.

Lipid peroxidation and free radical formation are associated with ethanol-induced fibrosis in the liver (8, 32, 33). Acetaldehyde, the first metabolite of ethanol, and malondialdehyde, a lipid peroxidation product, stimulate collagen synthesis in cultured cells (34, 35). The pathogenic sequelae of the generation of free radicals by ethanol in the pancreas are unknown, even though a direct cytotoxic effect of oxygen-derived free radicals on pancreatic acinar cells has been reported *in vitro* (36). Perhaps the role free radicals play in the pancreas is similar to their role in the liver, with prolonged ethanol intake stimulating fibrinogenesis, resulting in chronic alcoholic pancreatitis. In conclusion, the results from this study are consistent with the hypothesis that oxidative stress occurs in the pancreas during the early phases of chronic alcohol intake before the development of overt pathology. Understanding the source of the radical species could lead to the development of better chronic pancreatic injury models and, ultimately, to an understanding of the mechanisms underlying clinical pathology in patients.

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Send reprint requests to: Ronald G. Thurman, Ph.D., Laboratory of Hepatobiology and Toxicology, Department of Pharmacology, CB 7365, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7365.