Chronic ethanol administration causes oxidative stress in the rat pancreas

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There is increasing evidence implicating oxidative stress in the pathogenesis of both acute and chronic pancreatitis. Because ethanol is a major cause of pancreatitis in Western society, the aim of this study was to determine whether chronic ethanol administration results in oxidative stress in the pancreas. Twelve pairs of rats were fed a diet containing ethanol as 36% of calories or an isocaloric control diet for 4 weeks. Ethanol feeding resulted in a 46% increase in pancreatic malondialdehyde (p = 0.006). In addition, total pancreatic glutathione was increased by 22% (p = 0.005). These biochemical changes occurred in the absence of histologic evidence of inflammation or necrosis, implying that the observed oxidative stress is a primary phenomenon rather than part of an inflammatory response.

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Abbreviations: BHT = Butylated hydroxytoluene; EDTA = ethylenediamine tetra-acetic acid; GSH = glutathione; HPLC = high-performance liquid chromatography; MDA = malondialdehyde; TBA = thiobarbituric acid

Pancreatitis is an important complication of alcoholism that often results in chronic pain, exocrine and endocrine pancreatic failure, and reduced life expectancy.1,2 The pathogenesis of this disease remains unknown. However, recent reports have implicated oxidative stress in both human3,4 and experimental pancreatitis.5-8

Oxidative stress may be important in the pathogenesis of ethanol-induced pancreatic injury. Ethanol is the most common cause of chronic pancreatitis in Western society.9 However, to date the effect of ethanol on pancreatic oxidative stress has received relatively little attention. Braganza and colleagues demonstrated a beneficial effect of antioxidant therapy (with respect to pain) in patients with chronic pancreatitis.10 However, it is unclear whether such oxidative stress was a primary event or a secondary effect possibly caused by the pancreatic inflammatory reaction or pancreatic insufficiency.11 Altimare et al.12 reported that a single acute dose of ethanol results in oxidative stress in rat pancreas; however, alcoholic pancreatitis in humans rarely develops after a single dose of ethanol (binge)13 but rather after 5 to 15 years of heavy drinking. The aim of this study was to determine the effect of chronic ethanol consumption per se on pancreatic oxidative stress, using a well-established model of chronic ethanol administration known not to result in morphologic evidence of pancreatic inflammation.14

METHODS

Animals. Twenty-four male Sprague-Dawley rat littersmates weighing 90 to 100 gm were housed in individual cages and pair-fed, for a period of 4 weeks, liquid diets with or without ethanol as 36% of calories. Control rats received an isocaloric diet with carbohydrate substituting for ethanol. The diets had an energy density of 1000 kcal (4200 kcal) per liter and were prepared according to the general formulation of Lieber et al.15 The two diets contained equal amounts of antioxidants and micronutrients. At the end of the feeding period the rats were killed by decapitation in the fed state. On the day before killing, diets were fed in divided portions to standardize the rate of food intake. After decapitation, the pancreas was quickly removed and trimmed of excess fat. All procedures were performed with ice cooling. The pancreas was weighed,
Table I. General characteristics of control and ethanol-fed animal

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Ethanol-fed</th>
<th>Probability value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat weight (gm)</td>
<td>190 ± 2.7</td>
<td>170 ± 3.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Pancreas weight (mg)</td>
<td>654 ± 0.2</td>
<td>652 ± 2.4</td>
<td>NS</td>
</tr>
<tr>
<td>Caloric intake (kcal/day)</td>
<td>32 ± 0.5</td>
<td>32 ± 0.8</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS: Not significant.

and portions were removed for histologic analysis and for measurement of GSH and MDA.

**Histology.** Samples of pancreatic tissue removed at the time the rat was killed were placed immediately in 10% buffered formalin and subsequently embedded in paraffin. Four-micrometer sections were stained with hematoxylin and eosin using standard techniques. Sections were viewed without knowledge of the treatment group to which each animal had belonged.

**GSH assay.** Approximately 200 mg of pancreatic tissue was homogenized by hand for 1 minute in cold potassium phosphate buffer containing 0.1 mol/L EDTA, 10 mmol/L HCl, and 5% trichloroacetic acid, pH 7.0. The dilution of tissue in homogenizing buffer was 1:25 (weight:volume). The homogenate was then centrifuged at 10,000 g at 4°C for 5 minutes, and the supernatant was assayed for total GSH. The DTNB-GSSG reductase recycling assay described by Tietze was used, and measurements were made with a DU 640 spectrophotometer (Beckman Instruments, Palo Alto, CA).

**MDA assay.** Levels of MDA, a lipid peroxidation product, were measured by formation of an adduct comprising MDA and TBA, using a modification of the method of Buege and Aust. Approximately 100 mg of tissue was homogenized by hand for 1 minute in 1.5% KCl. The dilution of tissue in the homogenizing solution was 1:7.5 (weight:volume). Two hundred microliters of homogenate was then added to a reaction mixture containing 300 μl glacial acetic acid, 200 μl of 8.1% sodium dodecyl sulfate, 1.5 ml of 0.8% TBA, and 2.8 ml water. BHT in 50 μl of ethanol was added to prevent artefactual formation of MDA during the assay. This mixture was heated at 95°C for 1 hour in glass tubes and then cooled to room temperature. Pilot experiments determined that 2 mmol/L BHT was required to maximally inhibit spurious MDA formation during the heating step. Three milliliters of butanol was added to the mixture, and MDA-TBA complexes were extracted into the butanol phase by vigorous shaking. This mixture was centrifuged for 5 minutes at 2000 g, after which 200-μl aliquots of the butanol phase were transferred into HPLC vials.

MDA-TBA adducts were assayed by reverse-phase HPLC using a modification of the method of Young and Trimble. Briefly, sample was injected with the use of a BAS-200 pump (Bioanalytical Systems) in an isocratic mobile phase composed of 25% acetonitrile and 75% 25 mmol/L phosphate buffer (pH 7.0) at a flow rate of 1.2 ml/min. A 250 × 4.6 mm ALLTIMA™ C18 SU column (Alltech) was used. Detection was accomplished with a Waters 470 scanning fluorescence detector (Millipore, Sydney, Australia) with an excitation wavelength of 532 nm and an emission wavelength of 553 nm.

**Materials.** All chemicals were of analytic grade and were purchased from the Sigma Chemical Company (St Louis, MO).

**Statistical analysis.** Data were expressed as mean ± SEM. Statistical significance of difference between groups was determined by the Student t-test.

**Ethics committee approval.** This project was approved by the Animal Care and Ethics Committee of the University of New South Wales.

**RESULTS**

General characteristics of the test animals are shown in Table I. All animals remained well for the duration of feeding. The rate of weight gain was slightly less in ethanol-fed animals, in accord with the previously demonstrated relative inefficiency of ethanol-derived calories. Pancreatic weights were similar in both groups.

At the light microscopic level, pancreas from both control and ethanol-fed animals appeared normal. Specifically, there was no evidence of necrosis or inflammatory infiltrate in the pancreas of rats fed ethanol. This is in accord with previous observations by our group.

There was a 46% increase in MDA content in the pancreas of ethanol-fed rats compared with controls (70 ± 10 versus 48 ± 4.6 nmol/gm, p = 0.006, Fig. 1).

There was a 22% increase in total GSH content in the pancreas of ethanol-fed rats compared with controls (0.60 ± 0.04 versus 0.49 ± 0.02 mg/gm, p = 0.005, Fig. 2).

**DISCUSSION**

With this study we demonstrate for the first time that chronic ethanol administration leads to oxidative stress within the pancreas. These changes occur in the absence of histologic evidence of pancreatic damage or inflammation, suggesting that this oxidative stress is primary, rather than secondary to an inflammatory cell infiltrate.

Previously, the measurement of MDA-TBA complexes by spectrophotometry has lacked adequate specificity, necessitating the use of other corroborative measures of oxidative stress. The use of reverse-phase HPLC to measure MDA-TBA complexes provides a high degree
of specificity because the technique adds the dimension of column transit time to the assay; other compounds with the same absorbance and fluorescence can be excluded from measurement because their migration characteristics differ from those of the MDA-TBA complexes.\textsuperscript{19}

Reactive oxygen species are capable of damaging the cell in many ways. They have been shown to react with all major classes of cellular constituents: lipids, proteins, and nucleic acids.\textsuperscript{23} Peroxidation of lipid membranes is likely to be an important mechanism of cellular dysfunction caused by oxidative stress. For example, lipid peroxidation products have been shown to alter mitochondrial membrane fluidity,\textsuperscript{24} and oxidative damage to cultured hepatocytes has been shown to be associated with fragmentation of lysosomal membranes.\textsuperscript{25} Both lysosomal and zymogen granule fragility have been demonstrated in this model of chronic ethanol consumption.\textsuperscript{26,27} Such changes may facilitate contact between digestive and lysosomal enzymes with subsequent initiation of autodigestion.

Relatively little is known concerning oxidative stress in chronic pancreatitis. Braganza and coworkers studied various populations with chronic pancreatitis in Madras,\textsuperscript{3} Manchester,\textsuperscript{3} and Soweto.\textsuperscript{28} Blood antioxidant levels were reduced in patients with chronic pancreatitis, suggesting either that a dietary deficiency predisposed to pancreatitis or that oxidative stress caused by the pancreatitis process depleted antioxidants. This indirect evidence that antioxidant status is an important factor in chronic pancreatitis led to a study of antioxidant supplementation in patients with chronic pancreatitis. Although improvement in patient pain was documented,\textsuperscript{10} these encouraging results require confirmation in view of the relapsing and remitting nature of chronic pancreatitis.

It is possible that metabolism of ethanol within the pancreas could have led to the oxidative stress observed in our study. Pancreatic acinar cells in culture have been shown to metabolize ethanol.\textsuperscript{29} It is well established that chronic ethanol administration leads to oxidative stress in the liver.\textsuperscript{30} A number of mechanisms are potentially responsible for this effect, including acetaldehyde-mediated depletion of GSH,\textsuperscript{31} metabolism of acetaldehyde by aldehyde oxidase,\textsuperscript{32} and metabolism of ethanol via cytochrome P4502E1 (CYP2E1).\textsuperscript{33} We recently demonstrated that CYP2E1 is expressed in the rat pancreas and is induced during chronic ethanol administration.\textsuperscript{34} Therefore, metabolism of ethanol by CYP2E1 is a potential source of the oxidative stress observed in the present study. However, there are other potential mechanisms whereby ethanol consumption may result in pancreatic oxidative stress. Aitomare et al.\textsuperscript{12} recently demonstrated the generation of pancreat-
ic oxidative stress after an acute single dose of ethanol in naive rats. This was associated with a reduction in total pancreatic GSH, and the authors suggested acetaldehyde-mediated reduction in GSH as a possible mechanism for the oxidative stress demonstrated. It is unlikely that such a mechanism was operative in the present study, because pancreatic GSH levels were increased in the ethanol-fed rats.

With respect to the observed ethanol-induced increase in pancreatic GSH, a parallel situation has been described in the livers of rats chronically fed ethanol. There is evidence that synthesis of GSH is up-regulated in the presence of oxidative stress as a protective measure. It is therefore possible that the increase in GSH observed in our study was an adaptive response to chronic oxidative stress within the pancreas.

In conclusion, we have demonstrated for the first time that chronic ethanol administration results in oxidative stress in the pancreas. As in the liver, these changes may be of importance in the pathogenesis of ethanol-induced pancreatic injury.

REFERENCES