Cytochrome P4502E1 is present in rat pancreas and is induced by chronic ethanol administration

I D Norton, M V Apte, P S Haber, G W McCaughan, R C Pirola, J S Wilson

Abstract

Background—The mechanisms responsible for the initiation of alcoholic pancreatitis remain elusive. However, there is an increasing body of evidence that reactive oxygen species play a role in both acute and chronic pancreatitis. In the liver, cytochrome P4502E1 (CYP2E1, the inducible ethanol metabolising enzyme) is one of the proposed pathways by which ethanol induces oxidative stress.

Aims—To determine whether CYP2E1 is present in the pancreas and, if so, whether it is inducible by chronic ethanol feeding.

Methods—Eighteen male Sprague-Dawley rats were pair fed liquid diets with or without ethanol as 36% of energy for four weeks. CYP2E1 levels were determined by western blotting of microsomal protein from both pancreas and liver. Messenger RNA (mRNA) levels for CYP2E1 were quantified using dot blots of total pancreatic RNA.

Results—CYP2E1 was found in the pancreas. Furthermore, the amount of CYP2E1 was greater in the pancreas of rats fed ethanol compared with controls (mean increase over controls 5.1-fold, 95% confidence intervals 2.4 to 7.7, p<0.02). In the liver, induction by ethanol of CYP2E1 was similar (mean increase over controls 7.9-fold, 95% confidence intervals 5.2 to 10.6, p<0.005). Pancreatic mRNA levels for CYP2E1 were similar in ethanol fed and control rats.

Conclusions—CYP2E1 is present in the rat pancreas and is inducible by chronic ethanol administration. Induction of pancreatic CYP2E1 is not regulated at the mRNA level. The metabolism of ethanol via CYP2E1 may contribute to oxidative stress in the pancreas during chronic ethanol consumption.

Keywords: cytochrome P4502E1; rat pancreas; chronic ethanol administration

Pancreatitis is a major cause of morbidity and mortality among alcoholics. The pathogenesis of alcoholic pancreatitis is poorly understood. A possible role for oxidative stress has been suggested by studies in both human and experimental pancreatitis. Reactive oxygen species have the potential to damage lipid membranes, intracellular proteins, and DNA. A recent study of acute ethanol administration in rats has provided evidence of oxidative stress in the pancreas as indicated by increased malondialdehyde levels as well as an increased ratio of oxidised to reduced glutathione in the pancreas of rats gavaged with ethanol compared with controls. Furthermore, hydroxethyl radical (a free radical form of ethanol) has been found in the pancreas of rats chronically fed ethanol.

Cytochrome P4502E1 (CYP2E1) is the ethanol inducible form of cytochrome P450. In heavy drinkers, this enzyme is a major pathway of ethanol metabolism. Chronic ethanol exposure leads to a five to 10-fold increase in this protein within the hepatocyte. One consequence of the metabolism of ethanol via CYP2E1 is the formation of reactive oxygen species. To date, CYP2E1 has not been shown in pancreatic tissue. Therefore, the aims of this study were: (1) to determine whether CYP2E1 occurs in rat pancreas; (2) to determine whether this protein is inducible by chronic ethanol administration; and (3) to determine whether this induction is accompanied by an increase in the level of messenger RNA (mRNA) for this enzyme.

Materials and methods

ANIMALS

Eighteen male weanling Sprague-Dawley rats weighing between 80 and 100 g were pair fed, for four weeks, either a control diet or a diet containing ethanol as 36% of energy. Control rats received an isonenergy diet with carbohydrate substituting for ethanol. The diet was prepared according to the formulation of Lieber and DeCarli. It had an energy density of 4.18 MJ/litre and was supplemented with adequate amounts of vitamins and minerals. Both alcohol and control diets contained equal amounts of lipid and polyunsaturated fatty acids.

ISOLATION OF MICROSOMAL PROTEIN

Animals were killed by decapitation in the fed state. The pancreas was quickly removed and trimmed of excess fat. All procedures were performed on ice. The pancreas was minced with scissors in a homogenising solution (1/50 wt/vol) comprising 0.3 M sucrose containing a combination of protease inhibitors: phenylmethylsulphonyl fluoride (PMSF, 20 µg/ml), soybean trypsin inhibitor (100 µg/ml), leupeptin (10 µg/ml), and benzamidine (20 µg/ml). The tissue was then homogenised, firstly using an Ultra Turrax homogeniser (Janke and Kunkel, Germany) for 30 seconds and then with four strokes of a Potter S homogeniser (B Braun, Australia) at 1300 rpm.

The liver was perfused with 50 ml iced saline via the portal vein to wash out excess blood and then removed. After weighing, a piece of liver was cut and homogenised with a combination of protease inhibitors as above.
was homogenised using the same protocol as for the pancreas. Liver and pancreatic microsomes were prepared using the method of Meldolesi et al. Using a Sorvall RC-5B centrifuge with an SS-34 rotor (DuPont, Wilmington, Delaware), crude homogenate was centrifuged at 1000 g for 10 minutes to remove unbroken cells and nuclear debris. The supernatant was then centrifuged at 12 500 g for 10 minutes and the pellet discarded. Using a Beckman L8–70M ultracentrifuge with an SW-41 rotor (Beckman Instruments, Palo Alto, California), the resulting supernatant was centrifuged at 27 000 g for 10 minutes to sediment the rough endoplasmic reticulum. The subsequent supernatant was centrifuged for one hour at 109 000 g to yield a fraction rich in smooth endoplasmic reticulum (microsomes). All procedures were performed at 4°C. The 109 000 g pellet was resuspended in homogenising solution and stored at −70°C for further analysis. Microsomal protein was determined by the method of Lowry et al.

Identification of CYP2E1

Microsomal proteins from pancreas and liver (50 and 1.25 µg, respectively) were separated by electrophoresis (in 12.5% polyacrylamide gel) using Biorad Minigel apparatus (Biorad Laboratories, Richmond, California). Protein migration was assessed using protein standards (Kaleidoscope, Biorad). Protein bands were transferred to a nitrocellulose membrane (Hofffer Scientific, San Francisco, California) using an IMM-1 semi-dry blotter (WEP Instruments, Seattle, Washington) at 80 mA for 90 minutes. The membrane was blocked with 5% skimmed milk in Tris buffered saline (TBS, pH 7.6) for one hour and incubated overnight with a polyclonal rabbit antirat CYP2E1 antibody (1/1000, in 5% skimmed milk in TBS). Following incubation with primary antibody, membranes were washed in TBS and incubated for one hour with secondary antibody (donkey antirabbit antibody, Amersham, UK; 1/1000 in TBS). CYP2E1 bands were detected on radiographic film (Eastman Kodak, Rochester, New York) using enhanced chemiluminescence (ECL, Amersham). Bands were quantified via videodensitometry (Tracktel, Vision Systems, Australia) and CYP2E1 levels were expressed in arbitrary densitometer units.

Analysis of RNA

Pancreatic RNA was isolated by a modification of the method of Chomczynski and Sacchi as previously described using a frozen portion of the gland.

Analysis of messenger RNA

Analysis of CYP2E1 messenger RNA was performed qualitatively via northern blotting and quantitatively via dot blotting according to previously published methods. CYP2E1 mRNA was detected using a 1.2 kilobase cDNA probe. This probe was radiolabelled with 32P deoxyctosine triphosphate using a random priming kit (Megaprime labelling systems, Amersham, UK). Unincorporated nucleotides were removed using nucleic acid purification columns (NENsorb 20, DuPont). Labelling typically resulted in 5–7 × 106 cpm/µg DNA.

An oligonucleotide probe for β actin (5' CT CGTCATACTCCTGCTTGCTGATC 3') was used as an internal control to ensure equal loading for both northern and dot blots. This probe was labelled with a 5' end labelling kit (NEN oligonucleotide end labelling kit, DuPont).

Prehybridisation and hybridisation

When using the cDNA probe for CYP2E1, blots were prehybridised in a solution containing: 5 × sodium chloride sodium citrate (SSC), 0.025 mol/l sodium phosphate buffer (pH 7.0), 0.1% sodium dodecyl sulphate (SDS), 0.5 mg/ml salmon sperm DNA, and 5 × Denhardt’s solution. Prehybridisation was carried out at 65°C for one hour. The cDNA probe for CYP2E1 was then added at a final concentration of 1 × 106 cpm/ml of prehybridisation mix. Membranes were hybridised overnight at 65°C and membranes were then washed with 1 × SSC, 0.1% SDS for 30 minutes at room temperature, followed by four washes (2 × 30 minutes, 1 × 60 minutes, and 1 × 30 minutes) in 0.2% SSC, 0.1% SDS at 65°C. Prehybridisation, hybridisation, and washing conditions for the β actin probe were as previously described.

Autoradiography and densitometry

Northern blots were exposed to autoradiography film at −70°C for 48 hours. Quantitation of dot blots for CYP2E1 was determined using a microchannel array detector (InstantImager, Packard Instruments, Downers Grove, Illinois) for 12 hours. Autoradiographs of dot blots for β actin were quantified by videodensitometry (vide supra).

Materials

All chemicals were of analytical grade and purchased from the Sigma Chemical Company (St Louis, Missouri). 32P dCTP was purchased from Amersham. Molecular biology reagents were purchased from Biorad Laboratories. Anti-CYP2E1 antibody was kindly donated by Professor Magnus Ingelman-Sundberg, Karolinska Institute, Stockholm, Sweden, the 1.2 kilobase CYP2E1 cDNA probe was kindly donated by Dr Maurice Veronese, Flinders University, Adelaide, Australia, and the β actin probe was kindly donated by Dr Alex Bishop, Royal Prince Alfred Hospital, Sydney, Australia.

Statistical analysis

Western blot data were expressed as a relative increase compared with control with 95% confidence intervals. RNA data were expressed as means (SEM). Student’s t test for paired samples was used to determine statistical significance between groups. Analyses were performed using the Statview II statistical program.
ETHICS APPROVAL
This project was approved by the Animal Care and Ethics Committee of the University of New South Wales.

Results
GENERAL PARAMETERS
All animals remained well for the duration of feeding. The rate of weight gain was slightly less in ethanol fed animals (table 1), in accord with the relative inefficiency of ethanol derived calories.\(^{20}\) Pancreatic weights were similar in both groups.

PANCREATIC CYP2E1
Western blots of pancreatic microsomal protein from control rats showed a single band (56 000 daltons), corresponding to the known molecular weight of CYP2E1 (fig 1).

INDUCTION OF CYP2E1 IN RESPONSE TO CHRONIC ETHANOL ADMINISTRATION
The administration of ethanol led to induction of CYP2E1 in the pancreas (fig 1) with a mean increase of 5.1-fold over controls (95% confidence intervals 2.4 to 7.7, \(p<0.02\); fig 3). The induction of CYP2E1 in the liver (fig 2) was similar to that of the pancreas, 7.9-fold over controls (95% confidence intervals 5.2 to 10.6, \(p<0.005\); fig 3). Based on the amounts of protein loaded per well, and densitometry results, there was approximately 1/150 the amount of CYP2E1 in the pancreas compared with the liver per µg of microsomal protein (fig 4).

MSSANGER RNA LEVELS FOR PANCREATIC CYP2E1
Northern hybridisation (fig 5) showed that the mRNA for CYP2E1 was of the expected size. Figure 6 shows representative dot blots of RNA probed for CYP2E1 from control and ethanol fed animals. Quantitation of the dots showed that CYP2E1 mRNA levels in ethanol fed rats were similar to controls (894 (95) versus 829 (65) counts per 12 hours, \(p=0.5\); figs 6 and 7). Reprobing of dot blots for β actin mRNA showed that there was equal loading of RNA from both control and ethanol fed animals.

Discussion
This study has shown for the first time that CYP2E1 is present in the pancreas of rats and is induced in response to chronic ethanol administration. The degree of this induction is similar to that seen in the liver in this model.

Table 1 General characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control</th>
<th>Ethanol fed</th>
<th>(p) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight gain/day (g/day)</td>
<td>2.8 (0.1)</td>
<td>2.3 (0.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pancreatic weight (g)</td>
<td>0.80 (0.06)</td>
<td>0.77 (0.04)</td>
<td>NS</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>7.3 (0.2)</td>
<td>7.3 (0.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Microsomal protein</td>
<td>6.2 (0.7)</td>
<td>6.2 (1.5)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Figure 1 Representative western blot of pancreatic microsomal protein showing the presence of cytochrome P4502E1 in control pancreas and induction of this protein due to chronic ethanol administration.

Figure 2 Representative western blot of hepatic microsomal protein showing the presence of cytochrome P4502E1 in control liver and induction of this protein due to chronic ethanol administration.

Figure 3 Induction of CYP2E1 in pancreas and liver of rats fed ethanol. Results expressed as x fold increase over control, calculated from arbitrary densitometer units per µg of microsomal protein. \(n=9\) pairs. \(\ast p<0.02, \dagger p<0.005\) compared with control.

Figure 4 Representative western blot comparing the induction of CYP2E1 in the pancreas and liver during chronic ethanol exposure.

Figure 5 Northern hybridisation of pancreateic CYP2E1 mRNA from control and ethanol fed animals.
CytochromeP4502E1isanisoformofcyto-
chromeP450inducedduringchronicethanol
consumption. In the liver, the metabolism of
ethanol by this enzyme system leads to the
generation of reactive oxygen species. Indices
of oxidative stress (increased oxidised to
reduced glutathione ratio and increased lipid
peroxidation products) have been shown in the
livers of animals chronically fed ethanol.
Moreover, studies of ethanol metabolism by
isolated hepatic microsomes have shown the
generation of reactive oxygen species—a proc-

ess which is inhibited by prior addition of anti-
CYP2E1 antibodies. 

It should be emphasised that CYP2E1 is not
the only possible mechanism for ethanol
induced oxidative stress in the pancreas. Altomare et al have hypothesised a role for
acetaldehyde mediated reduction in glutath-
ione in the pancreas of rats fed ethanol.
Acetaldehyde can be generated by a number of
enzyme systems in diverse cellular compart-
ments: alcohol dehydrogenase in the cytosol,
catalase in peroxisomes, and CYP2E1 in the
endoplasmic reticulum. 

In the pancreas, reactive oxygen species have
been implicated both in experimental and
clinical pancreatitis. Animal models of acute
pancreatitis have shown evidence of oxidative
stress (increased oxidised to reduced glutath-
ione ratio and increased lipoperoxides). These studies suggest that the balance between oxidative stress and antioxidant defence mechanisms may be important in the pathogenesis of pancreatitis.

Only a minority of alcoholics develop clinical
pancreatitis, raising the possibility that some
factor predisposes a subgroup of heavy drink-
ers to the disease. To date, the reason for this
individual susceptibility remains obscure, de-
spite investigations of genetic factors such as α,
antitrypsin phenotype, blood group antigens,
HLA antigens, and environmental factors such
as type and pattern of alcohol consumption,
dietary and nutritional factors, and smoking. With regard to CYP2E1, a polymorphism in
the CYP2E1 gene has been described which
results in increased transcription of the
enzyme. This could lead to increased metabo-


stabilisation” have both been found to be modes of induction of CYP2E1.

It is clear that the pancreatic level of CYP2E1 is substantially lower than that of the liver, suggesting that pancreatic CYP2E1 contributes little to total body ethanol metabolism. Nonetheless, within an individual pancreatic cell, CYP2E1 could generate free radicals during chronic ethanol exposure, leading to accumulation of damage to organelle membranes and intracellular proteins.

In conclusion, this study has shown for the first time that CYP2E1 is present in the rat pancreas and is induced in response to chronic ethanol exposure. These observations may be relevant to the pathogenesis of alcoholic pancreatitis.

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