Gadolinium Chloride Blocks Alcohol-Dependent Liver Toxicity in Rats Treated Chronically with Intragastric Alcohol Despite the Induction of CYP2E1

DENNIS R. KOOP, BETHANY KLOPFENSTEIN, YUJI IIMURO, and RONALD G. THURMAN

Department of Physiology and Pharmacology, Oregon Health Sciences University, Portland, Oregon 97201 (D.R.K., B.K.), and Department of Pharmacology, University of North Carolina, Chapel Hill, North Carolina 27599 (Y.I., R.G.T.)

Received October 9, 1996; Accepted February 14, 1997

SUMMARY

Hepatic CYP2E1 is induced in several models of alcohol administration, but clinically relevant pathology is only observed in rats in a model involving the continuous intragastric administration of an ethanol-containing, corn oil-based, high-fat diet. The level of CYP2E1 correlates with the degree of liver pathology in the intragastric feeding model, which leads to the hypothesis that radical production by CYP2E1 is responsible for the pathology. Destruction of the Kupffer cells with gadolinium chloride (GdCl₃) prevented the development of ethanol-dependent pathology and decreased the production of radicals that appeared in the bile of intragastrically alcohol-fed rats. If the induction of CYP2E1 and subsequent formation of oxidant species by the enzyme is causative in the ethanol-dependent hepatic pathology, then protection by GdCl₃ could be due an inhibition of CYP2E1 induction. In the current study, ethanol-administration for 4 wk produced marked steatosis, necrosis, and inflammation not seen in control rats. Immunochemically, CYP2E1 was induced 5- to 6-fold in microsomes from the ethanol-treated animals. Rates of p-nitrophenol and chlorzoxazone hydroxylation were elevated approximately 3-fold, consistent with CYP2E1 induction. When GdCl₃ was administered with ethanol, there was a decrease of approximately 80% in Kupffer cell receptor expression, and there was a significant decrease in hepatic pathology, which confirms previous studies. However, in the ethanol and GdCl₃-treated animals, there was no significant decrease in the induction of CYP2E1. CYP2E1 was elevated approximately 5-fold, as estimated by immunoblot analysis, and rates of p-nitrophenol and chlorzoxazone hydroxylation were elevated 3- to 4-fold in ethanol + GdCl₃-treated rats. Thus, these results clearly dissociate the induction of CYP2E1 by intragastric infusion of ethanol from the generation of early alcohol-induced liver disease. It is concluded that Kupffer cells rather than CYP2E1 play the major role in the initiation of hepatocyte damage caused by alcohol.

Alcohol is a well known hepatotoxin, and alcoholic hepatitis is a serious health problem (1). The mechanisms responsible for ethanol-induced hepatotoxicity have been the subject of investigation for many years. Unfortunately, answers remain elusive. One approach to this problem is to use a clinically relevant model in which an ethanol-containing, corn oil-based, high-fat diet is given continuously to rats, as described originally by Tsukamoto et al. (2). Unlike other models that only produce steatosis, this model also exhibits inflammation and necrosis that is similar to that observed in humans.

Many studies have shown that ethanol administration in vivo is associated with the formation of free radicals and oxidative stress (1, 3–5). Lipid peroxidation increases (6) and hepatic antioxidants decrease (7) after ethanol administra-

This research was supported, in part, by Grants AA03624 (R.G.T.) and AA08608 (D.R.K.) from the National Institute on Alcohol Abuse and Alcoholism.

ABBREVIATIONS: RT, reverse-transcriptase/transcription; KCR, Kupffer cell receptor; PNP, p-nitrophenol; PCR, polymerase chain reaction; P450, cytochrome P450.
ethyl radical adduct produced in vitro and in vivo (4). In addition, inhibitors of CYP2E1 (diallyl sulfide and phenethyl isothiocyanate) decreased ethanol-induced pathology (12). Because CYP2E1 is a rich source of oxygen radicals in vitro, these data suggested that when CYP2E1 is induced, significant hepatic pathology should be observed.

Reactive oxygen species are also produced by various white blood cells as part of the response to pathogens. Of particular interest is the production of radical species by Kupffer cells, the resident macrophages of the liver. Kupffer cells have been shown to be activated by ethanol treatment (13). Destruction of large Kupffer cells with GdCl3 prevented the development of ethanol-dependent pathology (14). Further, GdCl3 blocked α-hydroxyethyl radical formation from ethanol in vivo in the Tsukamoto-French model (15). GdCl3 administration also reduced the hypoxia observed in this animal model, possibly reducing free radicals produced via hypoxia-reoxygenation mechanisms in parenchymal cells (15).

If the induction of CYP2E1 and the subsequent production of oxygen radicals is causative of hepatic pathology as a result of ethanol, then GdCl3 could prevent ethanol-dependent CYP2E1 induction and thus prevent toxicity. Alternatively, if CYP2E1 is induced but pathology is reduced by GdCl3, arguments against the involvement of this cytochrome in the initial stages of alcohol-induced liver injury would have to be raised. The purpose of this study was therefore to compare the levels of CYP2E1 and alcohol-induced liver injury in animals given intragastric ethanol for 4 wk in the presence and absence of GdCl3.

**Experimental Procedures**

**Materials.** Rabbit anti-sheep, goat anti-rabbit, and sheep peroxidase-anti-peroxidase IgG were from Organon Teknika-Cappel (Durham, NC). Prestained high-range molecular weight markers were from Gibco BRL (Gaithersburg, MD). The enhanced chemiluminescence Western blotting detection system was from Amersham (Arlington Heights, IL). Moloney murine Leukemia virus RT and polyT oligonucleotides were from Perkin-Elmer/Applied Biosystems (Norwalk, CT). All other chemicals were of the highest grade commercially available. 5-Fluoro-benzoxazole and 6-hydroxychloroxazone were generously provided by Dr. Raimund Peter from the University of Washington.

**Animals.** Male Wistar rats weighing 300–340 g each were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-approved facility with lights on between 6 a.m. and 6 p.m. Intragastric cannulas were inserted as described by Tsukamoto et al. (12). Cannulas were tunneled subcutaneously to the dorsal aspect of the neck and attached to infusion pumps by means of a spring-tether device and swivel, allowing complete mobility of rats in metabolic cages. Animals were infused continuously with a high-fat liquid diet containing ethanol for 4 wk. All animals received humane care in compliance with institutional guidelines.

**Diets.** A liquid diet described previously by Thompson and Reitz (16) was used. It contained corn oil as fat (37% of total calories), protein (23%), carbohydrate (5%), minerals and vitamins, plus ethanol or isocaloric maltose-dextrin (35%). GdCl3 (10 mg/kg in acidic saline) was injected intravenous twice weekly to destroy Kupffer cells as described previously (14).

**Urine collection and assay of ethanol.** Ethanol concentration in urine, which is representative of blood alcohol levels, was measured daily. Rats were housed in metabolic cages that separated urine from feces, and urine was collected over 24 hr in bottles containing mineral oil to prevent evaporation. Each day at 9 a.m., urine collection bottles were changed, and a 1-ml sample was stored at –20°C in a microtube for later ethanol analysis. Ethanol concentration was determined by measuring absorbance at 360 nm resulting from the reduction of NAD+ to NADH by alcohol dehydrogenase (17).

**Pathological evaluation.** After 4 wk of treatment, portions of the livers were fixed with formalin, embedded in paraffin, and stained with hematoxylin and eosin to assess steatosis, inflammation, and necrosis. Liver pathology was scored as described by Nanji et al. (18) as follows: steatosis (the percentage of liver cells containing fat): <25% = 1+, <50% = 2+, <75% = 3+, >75% = 4+, inflammation and necrosis: 1 focus per low-power field = 1+, 2 or more foci = 2+. Pathology was scored in a blinded manner by one of the authors.

**Preparation of microsomes.** Rat livers were immediately excised after death, placed into liquid nitrogen, and stored at –70°C. Microsomes were isolated from livers by differential centrifugation. All procedures were conducted at 4°C. Tissue was homogenized in four volumes of Tris/chloride buffer, pH 7.4, containing 150 mM potassium chloride and 1 mM EDTA, with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) and centrifuged at 10,000 × g for 20 min. The supernatant was collected and centrifuged at 211,000 × g for 40 min. The microsomal pellet was resuspended and washed in sodium pyrophosphate buffer, pH 7.4, containing 1 mM EDTA and centrifuged again at 211,000 × g for 40 min at 4°C. The washed microsomal pellet was resuspended in a Tris-chloride buffer, pH 7.4, containing 20% glycerol, with a ground glass tissue grinder and stored at –70°C. Protein was determined as described by Lowry et al. (19).

**Gel electrophoresis and immunoblot analysis.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a 9% polyacrylamide separating gel and electrophoretic transfer of protein to nitrocellulose sheets were performed as previously described (20). Nitrocellulose sheets were blocked overnight in 3% (w/v) nonfat dry milk in Tris-buffered saline (10 mM Tris/chloride, pH 7.4, with 150 mM NaCl) at 4°C. CYP2E1 was detected using sheep anti-rabbit CYP2E1 antiserum, rabbit anti-sheep IgG, and sheep peroxidase-antiperoxidase. All antibodies were diluted 1:10,000 in 3% nonfat dry milk in Tris-buffered saline (10 mM Tris/chloride, pH 7.4, with 150 mM NaCl). Immune complexes were visualized using enhanced chemiluminescence reagent from Amersham. Staining intensity of the complex was determined with a BioRad GS-363 molecular imaging system (BioRad, Richmond, CA) with a CH-imaging screen and BioRad Molecular Analyst software. The response was linear when protein concentrations were varied over 2 orders of magnitude. Values were normalized to the level of CYP2E1 in microsomes from control animals.

**Catalytic activity of microsomal fractions.** All reactions were conducted under conditions that were linear with respect to time and protein concentration at 37°C. The hydroxylation of PNP was determined spectrally as described previously (21). Reaction mixtures (final volume of 1 ml) contained 100 μM PNP, 100 mM potassium phosphate buffer, pH 6.8, 0.2 mg of microsomal protein and were initiated by the addition of 1 mM NADPH. Reactions were run for 10 min and stopped with 0.2 ml of 1.5 M perchloric acid.

Reactions identical to those for PNP hydroxylation were used for chlorozoxazone metabolism except that the substrate concentration was 200 μM. A stock solution of 10 mM chlorozoxazone was prepared fresh daily in 60 mM KOH. Reactions were terminated with 50 μl of 43% phosphoric acid, and 2.5 mM of 5-fluorobenzoazole was added as an internal standard (22) and extracted as described by Maples et al. (23). Metabolites were separated by reverse-phase high-performance liquid chromatography using a Supelcosil LC-18 column (150 mm × 4.6 mm, 5 μm) (Supelco, Bellefonte, PA). The mobile phase, which consisted of 10% acetonitrile/90% water with 0.5% glacial acetic acid, was run isocratically for 16 min and then stepped to 25% acetonitrile and held for 4 min. The flow rate was 2.0 ml/min. The effluent was monitored at 287 nm, and the amount of product was determined from the peak area ratio of the metabolite and internal standard.
standard compared with standard curves generated with known amounts of product and internal standard.

Lipid peroxidation was determined by the formation of thiobarbituric acid reactive metabolites in the presence of NADPH and ferric-ADP as described previously (24).

RNA preparation and KCR RT-PCR. Total RNA was prepared from frozen liver tissues as described elsewhere (25). One microgram total liver RNA was reverse transcribed using polyT oligonucleotide and Moloney murine leukemia virus RT in a 25-μl volume. Rat β-actin was amplified using primers previously described (26) in a 50-μl PCR reaction containing 1 mM MgCl₂, 50 mM KCl, and 10 mM Tris, pH 8.3, using 1 μl of cDNA template. Rat KCR (27) was amplified using primers KCR5' [5'-ATG AAG GAG GCG GAA CTG AAC-3'] and KCR3' [5'-CAG CTC TGG TCC GTT CTG GC-3'] in the 50-μl reaction mixture described above for β-actin. The sizes of amplified PCR products were 1652 bp for KCR and 281 bp for β-actin. PCR samples were analyzed by agarose gel electrophoresis and ethidium bromide staining. The relative amount of mRNA transcript was determined by densitometric analysis with National Institutes of Health Image 1.54, and the area under the curve was normalized using β-actin. PCR cycle number was optimized to ensure that the conditions were not within the plateau phase of the amplification. Samples in which the RNA was not subjected to RT did not yield PCR products.

Results

Ethanol elimination and hepatic pathology. Dietary ethanol was increased gradually to approximately 10 g/kg/day during the first week and held constant (11–12 g/kg/day) for 3 subsequent weeks. There were no significant differences in ethanol delivery in the ethanol and ethanol-plus-GdCl₃ groups. As was reported previously (28) and observed in the present study (Fig. 1), alcohol levels fluctuate in a cyclic pattern for reasons that remain unclear. Mean urine alcohol concentrations were 240 ± 30 mg/dl in the ethanol groups (n = 6) and 229 ± 19 mg/dl in the ethanol-plus-GdCl₃-treated groups (n = 7). Photomicrographs of representative histology are depicted in Fig. 2 and summarized in Fig. 3. As observed previously, massive steatosis was observed with this model (Figs. 2B and 3A). Necrosis and inflammation were also observed in the alcohol-exposed groups (Figs. 2B and 3, B and C). The total pathology score reached values of 6 in the ethanol-treated group (Fig. 3D). All parameters of...
pathology studied were diminished by treatment with GdCl₃ (Figs. 2C and 3).

**KCR mRNA.** A carbohydrate binding receptor unique to Kupffer cells, KCR has been reported in the rat (27). In the present study, we examined the expression of KCR mRNA by RT-PCR in liver tissue from rats using the Tsukamoto-French protocol in the presence and absence of GdCl₃. An agarose gel with representative samples from individual livers is shown in Fig. 4A. A high-fat diet and 4 wk of ethanol treatment did not change KCR mRNA levels significantly compared with chow-fed animals (Fig. 4, lanes 2–8). In rats exposed to ethanol on the high-fat diet, GdCl₃ decreased KCR mRNA expression to approximately 20% of the level observed in animals receiving the control high-fat diet. Thus, chronic treatment with GdCl₃ eliminated most but not all Kupffer cells (Fig. 4, lanes 5–8 versus lanes 9–12). There was a relationship between the level of KCR mRNA and hepatic pathology. Liver samples with very low levels of KCR mRNA (Fig. 4, lanes 11 and 12) exhibited very little pathology. In contrast, livers with higher levels of KCR mRNA exhibited greater pathology. This suggests that the residual pathology seen in the ethanol-plus-GdCl₃ group (Fig. 3D) could be due to the activity of the remaining Kupffer cells.

**Hepatic levels of CYP2E1.** Hepatic microsomes were isolated from livers of each treatment group, and the level of CYP2E1 was determined by immunoblot analysis. As shown in Fig. 5 (compare controls in lanes 1–3 with the ethanol-treated rats in lanes 4–6) and as reported by others (6, 7, 29), intragastric feeding of ethanol resulted in a 5- to 6-fold increase in immunoreactive CYP2E1. The extent of induction was not significantly different in the ethanol and the ethanol-plus-GdCl₃ groups in this study (Fig. 5, lanes 4–6 versus lanes 7–9). The lower band observed in microsomes from control rats (Fig. 5, lanes 1–3) is an unidentified protein that cross-reacts with the sheep anti-CYP2E1 antibody used in these studies. Thus, although the liver pathology was significantly decreased by GdCl₃ (Figs. 2 and 3), the levels of CYP2E1 were not affected.

The results of immunoblots do not distinguish active from inactive enzymes, which raises the possibility that although the apoprotein level was not affected by GdCl₃, catalytic activity was affected. To assess catalytic activity of CYP2E1, the hydroxylation of PNP and chlorzoxazone was monitored with the same microsomal preparations evaluated for immunoreactive CYP2E1. These two hydroxylations are selective...
Kramer multiple comparisons test. Comparisons among groups with one-way analysis of variance and Tukey-Kramer multiple comparisons test. $E$, ethanol; Gd, GdCl$_3$.

for CYP2E1 (8). As shown in Fig. 6, ethanol treatment increased both activities 3- to 4-fold, as expected. Consistent with the immunoblot analysis, treatment with GdCl$_3$ had no effect on the induction of CYP2E1 activity by ethanol, as determined with either substrate. The extent of CYP2E1 induction was greater when measured immunologically than via catalytic activity. This has been observed previously (30, 31) and could be the result of limiting amounts of NADPH P450 reductase or cytochrome $b_5$. In vitro NADPH-dependent lipid peroxidation in the presence of ferric-ADP mirrored the increases observed in both chlorzoxazone and PNP hydroxylation and was also not affected by GdCl$_3$ treatment (results not shown).

Discussion

Microsomes from animals treated with inducers of CYP2E1 produce high levels of reduced oxygen species (i.e., superoxide and hydrogen peroxide) in an uncoupled reaction and exhibit high levels of lipid peroxidation in vitro in the absence of substrate (1, 8–10, 24). These in vitro observations and the correlation of CYP2E1 induction with the onset of pathophysiology in alcoholic liver disease in the Tsukamoto-French model (1, 6, 7, 11, 12) led to the suggestion that CYP2E1-dependent radical production was a causative factor in alcoholic liver disease. Previous studies with the Tsukamoto-French model demonstrated that liver pathology associated with alcohol intake could be significantly reduced by the inhibition of Kupffer cell function by GdCl$_3$ (14). The results of the current study confirm this earlier work and clearly demonstrate that in a model in which ethanol-induced pathology is decreased significantly by GdCl$_3$, induction of functional CYP2E1 in liver parenchymal cells was unaffected. This led us to the conclusion that the induction of CYP2E1 is not the primary cause of the pathology as a result of ethanol (i.e., induction of this enzyme alone is not sufficient to account for early alcohol-induced liver injury). Other studies support this conclusion. Steatosis but not hepatitis was observed when the Lieber-DeCarli diet was used, despite significantly elevated levels of CYP2E1. Further, pathology was reduced but CYP2E1 was induced in the Tsukamoto-French model when saturated fat was used in place of corn oil (18, 32, 33).

There is little doubt that in isolated microsomes or in a reconstituted system, CYP2E1 produces oxygen radicals (1, 8–10, 24). However, whether uncoupled reactions of CYP2E1 are major contributors of oxygen radicals in vivo remains unclear. There is little experimental data in vivo to directly support the hypothesis that superoxide and peroxide produced in the cytosol are generated from the reduction of oxygen to superoxide and/or peroxide by P450. The reduction in ethanol-induced toxicity in the presence of GdCl$_3$ despite the induction of CYP2E1 is consistent with the hypothesis that CYP2E1 does not produce levels of reduced oxygen species that cannot be adequately scavenged in the intragastric fed model in vivo when Kupffer cell function is diminished. In the current study, RT-PCR analysis of KCR mRNA indicated that Kupffer cell function was not completely abolished by GdCl$_3$-treatment. The failure to completely block the pathology by GdCl$_3$ treatment could thus result from the activity of the residual Kupffer cell population. Whether Kupffer cells
produce a substrate or uncoupler of CYP2E1 still remains to be determined.

Morigoto et al. (12) demonstrated that when the P450 inhibitors diallyl sulfide and phenethyl isothiocyanate were administered to ethanol-fed animals in the intragastric model, a reduction in pathology because of ethanol correlated with levels of CYP2E1. This is at odds with the conclusions drawn above. However, these compounds are not selective for inactivation of CYP2E1. Diallyl sulfide can act as an antioxidant, and a possible effect of both compounds on Kupffer cell function was not assessed and remains a potential mechanism for protection. Further, a correlation between the level of CYP2E1 and other parameters does not a priori demonstrate a causal relationship. For example, the level of CYP2E1 correlated with the episodic blood ethanol levels observed in the intragastric feeding model (29, 34). Chlorothiazole, a compound that inhibits the transcription of CYP2E1 (35), had no effect on the episodic blood ethanol concentrations, which demonstrates that CYP2E1 was not responsible for the fluctuating levels of ethanol despite the correlation (36).

It was recently reported that CYP2E1 was induced in Kupffer cells from rats fed the Lieber/DeCarli diet (37). Although not assessed in the current study, CYP2E1 may participate in the formation of reduced oxygen species in Kupffer cells. Whether this would represent a significant portion of the total superoxide produced in activated Kupffer cells compared with NADPH oxidase remains to be determined. Further, it was reported that ethanol treatment produced a-hydroxyethyl adducts and that antibody to these adducts was present in the serum of alcoholic patients (4, 38). CYP2E1 can catalyze the formation of the a-hydroxyethyl radical and is recognized by antibody to the adducts (38). Thus, although the current study indicates that the induction of CYP2E1 alone cannot be responsible for alcohol-induced pathology, the activity of CYP2E1 may be important in subsequent immune responses as injury progresses.

A free radical was detected in bile from rats exposed to ethanol in the Tsukamoto-French model (14, 15). This free radical signal, which was largely a-hydroxyethyl (15), was reduced dramatically when Kupffer cells were eliminated with GdCl3. The exact pathways responsible for the formation of free radicals in alcohol-treated rats remains unclear. However, because the ESR signal was reduced with GdCl3 treatment, a likely candidate is oxygen radical production by the NADPH oxidase system in Kupffer cells. On the other hand, a repertusion injury involving hypoxia and free radical formation via the xanthine-xanthine oxidase system cannot be ruled out. Radicals in bile would be expected to arise from parenchymal cells, and Kupffer cells produce vasoactive mediators that could influence oxygen delivery to parenchymal cells.

In summary, the results of the current investigation clearly dissociate the induction of CYP2E1 by intragastric infusion of ethanol from the generation of early alcohol-induced liver injury. It seems that Kupffer cells play a major role in the initiation of hepatocyte damage.

References


