Carbon tetrachloride-induced lipid peroxidation dependent on an ethanol-inducible form of rabbit liver microsomal cytochrome P-450

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Treatment of rats with ethanol or rabbits with either imidazole or pyrazole, agents known to induce the ethanol-inducible form of liver microsomal cytochrome P-450 (P-450 LMeb), caused, compared to controls, 3–25-fold enhanced rates of CCl_4-dependent lipid peroxidation or chloroform production in isolated liver microsomes. No significant differences were seen when the rate of CCl_4-dependent lipid peroxidation was expressed relative to the amount of P-450 LMeb in the various types of microsomal preparations. In reconstituted membranous systems, this type of P-450 was a 100-fold more effective catalyst of CCl_4 metabolism than either of the cytochromes P-450 LM_2 or P-450 LM_4. It is proposed that the induction of this isozyme provides the explanation on a molecular level for the synergism seen of ethanol on CCl_4-dependent hepatotoxicity.

Cytochrome P-450 *Hepatotoxicity* *Free radical* *Lipid peroxidation* *Ethanol* *Carbon tetrachloride*

1. INTRODUCTION

Ethanol, when administrated acutely or chronically, is known to increase the hepatotoxicity of carbon tetrachloride [1–7]. The potentiation is observed 16 h after the administration of the alcohol [8]. It has been proposed that the underlying mechanism involves the induction or activation of liver enzymes that accelerates the biotransformation of the solvent to highly toxic intermediates [5,6]. Also, administration of pyrazole, acetone or other aliphatic alcohols than ethanol, causes a 15–30-fold potentiation of the CCl_4-dependent hepatotoxicity [3,9,10].

Experiments performed in microsomes and in reconstituted cytochrome P-450-containing systems indicate that the enzyme responsible for the metabolic activation of CCl_4 is cytochrome P-450 rather than NADPH-cytochrome P-450 reductase [11,12]. We have previously reported the purification of an ethanol-inducible form of cytochrome P-450 from benzene- or imidazole-treated rabbits (cytochrome P-450 LMeb) which, based upon the amino acid composition, NH_2-terminal amino acid sequence, molecular mass and spectral properties [13,14], apparently is identical to cytochrome P-450 3a, purified in Professor M.J. Coon's laboratory [15]. This form of cytochrome P-450 is inducible by, besides ethanol, compounds like pyrazole [14] and acetone ([16], unpublished), i.e., by the same substances that also potentiate the toxicity of CCl_4. Accordingly, it was hypothesized that cytochrome P-450 LMeb would be an effective catalyst for the transformation of CCl_4 to toxic intermediates and might constitute the explanation on a molecular level for the synergistic effect of alcohols on the hepatotoxicity of CCl_4.

2. EXPERIMENTAL

2.1. *Materials*

NADPH, pyrazole and thiobarbituric acid (TBA) were purchased from Sigma. Carbon tetrachloride, imidazole and chloroform were

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from Merck. Cytochrome P-450 LM_2 (12–14 nmol/mg), cytochrome P-450 LMeb (17.5 nmol/mg), cytochrome P-450 LM_4 (14.5 nmol/mg) and NADPH-cytochrome P-450 reductase (22 nmol flavin/mg) were purified as described [17]. Microsomal phospholipids were extracted from liver microsomes of phenobarbital-treated rabbits according to the method of Bligh and Dyer [18].

2.2. *Methods*

Rabbits, obtained from a local farm, were treated with either imidazole (200 mg/kg) or pyrazole (200 mg/kg) by daily intraperitoneal injections for 3 consecutive days. Sprague-Dawley male rats (150 g) were fed liquid diets according to DeCarli and Lieber [19] for 20 days. The alcohol diet, which provides 36% of the calories from ethanol, and the control diet, were obtained from Bioserv Inc. (Frenchtown, NJ). The livers of the animals were homogenized with 2 vols of 1.14% (w/v) KCl containing 10 mM EDTA. The microsomes were isolated by centrifugation of the homogenate at 9000 × *g* for 40 min and a subsequent centrifugation at 100000 × *g* for 60 min of the 9000 × *g* supernatant. The microsomal pellets were washed once in the same medium and subsequently suspended in 50 mM potassium phosphate buffer, pH 7.4. The amount of cytochrome P-450 LMeb in the microsomal samples was determined using radial immunodiffusion as in [14]. Membranous vesicles were prepared by the cholate gel filtration technique [20] from microsomal phospholipids, NADPH-cytochrome P-450 reductase and one of the cytochromes P-450 LM_2, P-450 LM_4 or P-450 LMeb at a molar ratio of 1300:0.3:1, respectively. The Sephadex G-50 column was equilibrated in Chelex-100-treated 50 mM potassium phosphate buffer, pH 7.4, containing 50 mM KCl, and eluted in the same buffer.

Incubations with carbon tetrachloride were carried out with phospholipid vesicles corresponding to 0.1 nmol P-450 or with liver microsomes corresponding to 0.5–1 mg protein in a total volume of 1 ml 50 mM potassium phosphate buffer, pH 7.4, containing 2.15 mM carbon tetrachloride (added in 5–10 μl acetone) and 0.25 mM NADPH. Control incubations were performed in the absence of NADPH or CCl_4.

Products from lipid peroxidation were detected using the TBA assay [21]. The incubations were terminated by the addition of 0.25 ml of 40% (w/v) trichloroacetic acid and 0.125 ml of 5 M HCl. Subsequently, 0.25 ml of 2% (w/v) TBA was added. The samples were heated in boiling water for 10 min, centrifuged and the amount of TBA reactive material was quantitated spectrophotometrically in the supernatant at 532 nm using 156 $mM^{-1} \cdot cm^{-1}$ as absorption coefficient.

Chloroform was determined using the head space technique. The incubations were performed under anaerobic conditions in 5-ml tubes equipped with rubber membrane caps. The incubation mixtures were flushed with nitrogen for 45 min on ice prior to the addition of carbon tetrachloride dissolved in 5 μl acetone. After a 5 min preincubation period at 37°C, the incubations were started by the addition of NADPH. One ml aliquots of the head space volume were analyzed on a GLC (at 100°C) equipped with a Carbopack C 0.1% SP-1000 column and a flame ionization detector (120°C). Residual carbon tetrachloride was used as internal standard for quantitation of the chloroform formed, since the total conversion was usually less than 0.05%.

3. RESULTS

The rate of carbon tetrachloride-dependent lipid peroxidation was determined in liver microsomes isolated from variously treated rabbits and rats (table 1). Both imidazole and pyrazole were potent inducers of the microsomal CCl_4-induced lipid peroxidation; 4–5-fold higher rates were reached here, compared to controls, when the conversion was expressed per nmol P-450. However, when expressed per nmol cytochrome P-450 LMeb in the microsomal membranes, no significant differences were seen (table 1). Ethanol treatment of rats caused a 25-fold induction of the microsomal CCl_4- dependent lipid peroxidation, compared to controls, when expressed per mg microsomal protein (table 1). The reaction in liver microsomes of ethanol-treated rats was linear only for 2 min, whereas the linear phase using rabbit liver microsomes was 15 min (not shown).

To evaluate further to what extent the effect of the inducers on the CCl_4-induced lipid peroxidation in rabbit liver microsomes was attributed to the induction of the ethanol-inducible P-450 form, various forms of purified microsomal cytochromes

Table 1

Carbon tetrachloride-dependent lipid peroxidation in liver microsomes

Type of microsomes	TBA-reactive products		
	nmol/mg protein per min	nmol/nmol P-450 per min	nmol/nmol LMeb per min
Rat			
Control	0.077 ± 0.012 (5)	0.15 ± 0.21 (5)	
Ethanol	1.98 ± 0.30 (5)[a]	1.71 ± 0.17 (5)[a]	
Rabbit			
Control	0.16 ± 0.04 (4)	0.12 ± 0.01 (4)	1.07 ± 0.34 (4)
Pyrazole	0.42 ± 0.18 (6)[c]	0.44 ± 0.21 (6)[c]	1.16 ± 0.30 (6)
Imidazole	0.77 ± 0.21 (8)[a]	0.44 ± 0.14 (8)[b]	1.47 ± 0.47 (4)

[a] $p < 0.001$; [b] $p < 0.005$; [c] $p < 0.05$, compared to control

The incubations were carried out as detailed in section 2 with microsomes corresponding to 0.5 mg protein. Rat liver microsomes were incubated with 2.15 mM CCl_4 for either 2 min, when microsomes from ethanol-treated animals were used, or for 10 min, in the case of control microsomes. Incubations with rabbit liver microsomes were carried out for 10 min with 8.6 mM substrate. The number of animals is indicated within parentheses

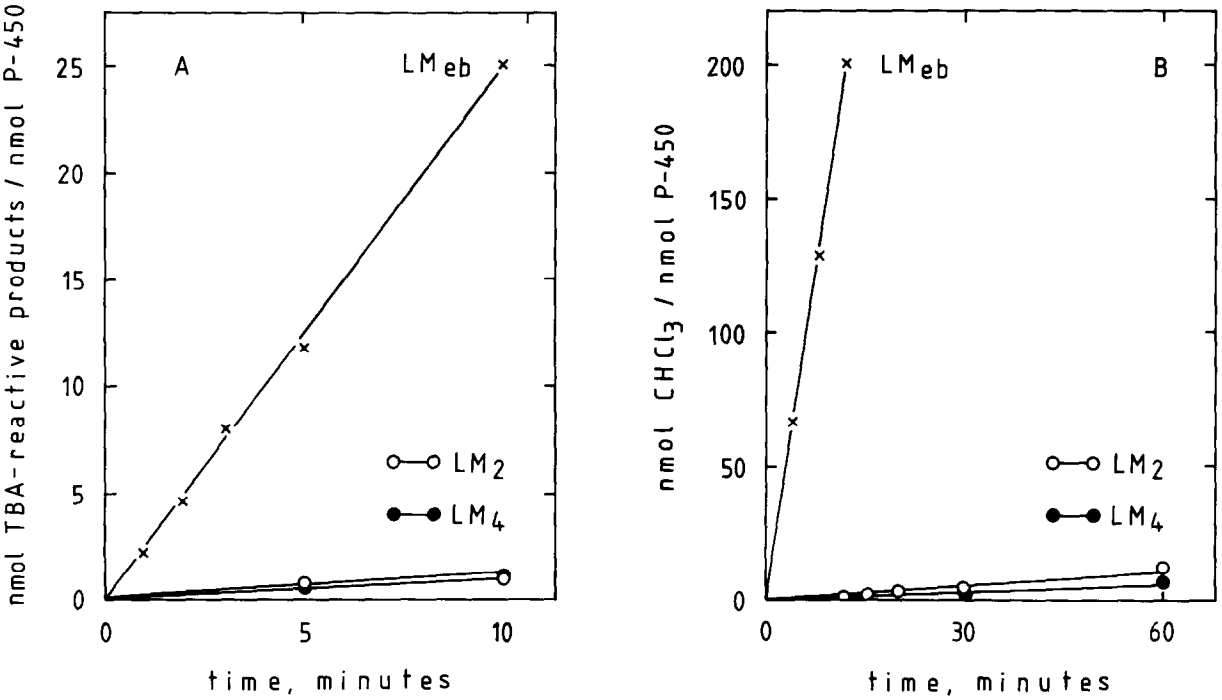

Fig.1. (A) CCl_4-dependent lipid peroxidation in reconstituted membrane vesicles. Liposomes corresponding to 0.1 nmol cytochrome P-450, containing NADPH-cytochrome P-450 reductase and the hemoprotein in a molar ratio of 0.3:1, were incubated in the presence of 10 μM EDTA with 2.15 mM CCl_4 at 12°C for the indicated time intervals. Other conditions as described in section 2. The apparent rates of product formation were for P-450 LMeb, 2.6; P-450 LM_2, 0.12; P-450 LM_4, 0.10 nmol TBA reactive products/nmol cytochrome P-450 and per min. (B) Cytochrome P-450-dependent conversion of carbon tetrachloride to chloroform in reconstituted membrane vesicles. Membrane vesicles corresponding to 0.15–0.6 nmol cytochrome P-450 were incubated for the indicated time intervals at 37°C. Other conditions as described in section 2. The apparent turnover numbers were for P-450 LMeb, 16.7; P-450 LM_2, 0.18; P-450 LM_4, 0.09 nmol/nmol P-450 per min.

P-450 were incorporated into vesicles of microsomal phospholipids and the rate of lipid peroxidation was determined. The cytochrome P-450-dependent, but CCl_4-independent lipid peroxidation mechanism, was suppressed by the presence of EDTA during these incubations (cf. [22]). As shown in fig.1A, the ethanol-inducible P-450 isozyme was at least a 25-fold more potent catalyst than the other types of cytochrome P-450 in the transformation of carbon tetrachloride to products being able to initiate lipid peroxidation in the reconstituted vesicles. At maximum, 4.4 nmol TBA-reactive products were produced/nmol P-450 LMeb per min under the conditions used.

Under anaerobic conditions, chloroform is a product of the cytochrome P-450-dependent reductive metabolism of carbon tetrachloride [23–25]. As shown in table 2, the rate of chloroform production was about 4-fold higher in liver microsomes isolated from ethanol-treated rats or imidazole-treated rabbits, compared to controls. Also, the rate of chloroform production was severely elevated in the reconstituted rabbit liver microsomal system containing the ethanol-inducible cytochrome P-450 form compared to membranous systems containing either of the cytochromes P-450 LM_2 or P-450 LM_4 (fig.1B). Cytochrome P-450 LMeb reduced CCl_4 at a rate 100–200-fold higher compared to the other enzymes; at maximum, 17 nmol $CHCl_3$ was produced/nmol P-450 per min. It should be pointed out that by increasing the level of NADPH-cytochrome P-450 reductase in the membranes, thereby approaching the concentration of cytochrome P-450 LMeb, a turnover for CCl_4 of approx. 60 would be reached.

Table 2

Chloroform production in anaerobic incubation systems containing liver microsomes and carbon tetrachloride

Type of microsomes	Chloroform produced	
	nmol/mg protein per min	nmol/nmol P-450 per min
Rat		
Control	0.24 ± 0.07 (4)	0.48 ± 0.10 (4)
Ethanol	1.45 ± 0.21 (4)[a]	1.17 ± 0.10 (4)[a]
Rabbit		
Control	0.42 ± 0.09 (6)	0.35 ± 0.11 (6)
Imidazole	1.40 ± 0.39 (6)[a]	0.77 ± 0.22 (6)[b]

[a] $p < 0.001$; [b] $p < 0.002$, compared to control

The incubations were carried out as described in table 1 and in section 2 with liver microsomes corresponding to 0.5 mg (rat) or 1.0 mg (rabbit) protein. The number of animals used is indicated within parentheses

4. DISCUSSION

Our results indicate that the synergism seen on carbon tetrachloride hepatotoxicity by treatment of animals with ethanol prior to the administration of the halogenated solvent is attributed to the induction of the ethanol-inducible form(s) of cytochrome P-450 effective in the metabolism of CCl_4 to products acting as initiators of lipid peroxidation in membranous systems. The similar values of the rate of CCl_4-dependent lipid peroxidation in the various types of rabbit liver microsomes, when expressed relative to the amount of cytochrome P-450 LMeb in these membranes, indicate that this form of cytochrome P-450 to a major extent is responsible for the metabolic activation of the solvent. Previously, it has been proposed that CCl_4 preferentially interacts with phenobarbital inducible cytochrome P-450 [26]. Cytochrome P-450 LM_2 was reported to reduce the solvent at a rate of 1 nmol/nmol per min [11], a value which is comparable to our result, 0.6 nmol/nmol per min in a system optimized with respect to the concentration of NADPH-cytochrome P-450 reductase. However, in the reconstituted membranous system, cytochrome P-450 LMeb was at least a 100-fold more effective catalyst in the reduction of CCl_4, as based upon the determination of the rate of chloroform production, and a 25–50-fold more potent catalyst in the transformation of the compound to metabolites acting as initiators of lipid peroxidation, than either of the cytochromes P-450 LM_2 or P-450 LM_4.

The nature of the toxic intermediate responsible for initiation of CCl_4-dependent lipid peroxidation appears not yet unambiguously determined. The most likely candidate, according to the literature, is the trichloromethyl radical [23,27]. This compound is formed by a one-electron reduction of carbon tetrachloride. We have previously presented results indicating that cytochrome P-450

LMeb effectively reduces EDTA-chelated iron [17]. Therefore, it seems plausible that P-450 LMeb in a similar manner also reduces the halogenated solvent.

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