Modulation of Ethanol-Mediated CYP2E1 Induction by Clofibrate and L-Carnitine in Rat Liver

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To understand the effects of lipid-lowering agents on the ethanol-induction of hepatic CYP2E1, clofibrate and l-carnitine were administered to adult male rats. The administration of ethanol in the diet (containing 21% calories as ethanol, given for 3 weeks) increased levels of hepatic CYP2E1 protein (1.9-fold that of untreated controls) and mRNA (2.1-fold). In contrast, the administration of clofibrate (0.1% v/v) in an ethanol-containing diet did not significantly increase either CYP2E1 protein (1.1-fold) or mRNA (0.8-fold), in spite of the significant increases in blood ketone bodies. Administration of l-carnitine alone had no clear effect on CYP2E1 and blood ketone body levels. Co-administration of l-carnitine, however, increased liver microsomal CYP2E1 protein (2.5-fold) in rats given an ethanol-containing diet. No difference was observed in the mRNA levels in rats receiving ethanol with and without l-carnitine. These results indicate that clofibrate and l-carnitine modulate ethanol-mediated induction of hepatic CYP2E1 independent of blood levels of ketone bodies. It is also suggested that these lipid-lowering agents affected hepatic CYP2E1 through particular mechanisms, suppression of the specific mRNA and post-translation stabilization.

Keywords CYP2E1; ethanol; induction; clofibrate; l-carnitine; mRNA

Chronic ethanol intake has been shown to promote disorders in a wide range of cells and organs in human and experimental animals.1,2) Ethanol is regarded as one of the hepatotoxic agents which cause lipid accumulation, fibrosis, cirrhosis and necrosis in the liver.3) Hepatic CYP2E1 (P450j) was first characterized as a central component of the microsomal ethanol-oxidizing system (MEOS).4) The contribution of CYP2E1 to hepatic pathogenesis has been studied, based on the hepatotoxicity of drugs such as acetaminophen,5) and the oxidative stress produced by the metabolic activities of this enzyme.5-9)

Nonetheless, the molecular mechanism of induction of CYP2E1 remains unclear, because of the complexity of the regulatory mechanism.10,11) Studies on ethanol-mediated induction of CYP2E1 are further hampered by the fact that ethanol metabolism interacts with both lipid and carbohydrate metabolism. Thus, the combined effects rather than the direct effect of ethanol appeared in vivo.2,12) Many efforts have been made to differentiate the direct effect due to ethanol from those of associated phenomena. Since the induction of hepatic CYP2E1 often involves increases in liver lipids and blood ketone body levels, the question has been raised whether induction represents a response to these changes. In this study, we performed experiments to assess the interaction between lipid metabolism and the ethanol-mediated induction of hepatic CYP2E1. We altered lipid metabolism by administering clofibrate and l-carnitine and investigated the interaction of these lipid-lowering agents on mRNA and protein levels of CYP2E1 under ethanol-induced conditions.

Materials and Methods

Antibodies and Chemicals Rabbit antibody raised against CYP2E1 was purchased from Oxygene (Dallas, U.S.A.), goat anti-rabbit IgG (biotinylated) and alkaline phosphatase-streptavidin conjugate were from Amersham International plc. (Buckinghamshire, UK). Clofibrate was obtained from Wako Pure Chemical (Osaka, Japan). Other chemicals were from Sigma Chemical Co. (St. Louis, U.S.A.).

Animals Thirty-six male Sprague-Dawley rats, weighing about 160 g (Nihon Charles River Inc. Kanagawa, Japan) were divided into 6 groups of 6 rats, housed individually and receiving equal volumes of liquid diets. Chronic ethanol administration was achieved using the liquid diet of Liver and DeCarb14 with slight modifications. Control diet contained 9.3% calories as protein, 13.9% as fat and 76.8% as carbohydrate. Ethanol-supplemented diet was added with 3% (v/v) ethanol (as 21.3% of total calories, substituting sucrose isorotically). Clofibrate- or l-carnitine-supplemented diets were added with 0.11% (w/v) clofibrate14 or 1% (w/v) l-carnitine,15 respectively. Co-treatment groups received diets containing clofibrate or l-carnitine in combination with ethanol. All groups were maintained on their respective diets for 3 weeks before sacrifice. The livers were then excised for preparation of microsomes and extraction of RNA. Blood samples were also collected.

Analytical Procedures Protein concentrations were determined by the method of Lowry et al.16) with bovine serum albumin as a standard. A portion of whole blood was quickly deproteinized with ice-cold perchloric acid reagent for determination of acetone by headspace gaschromatography.17) The headspace gas (1 ml) was analyzed using a gaschromatograph equipped with a capillary column (polyethylene glycol (PEG) 20M, 0.53 mm i.d. and 90 m length, Supelco, U.S.A.) and an flame-ionization detector (FID). Blood 3-hydroxybutyrylactone concentrations were determined by a colorimetric method18) using 3-hydroxybutyrate dehydrogenase and iodonitrotetrazolium as a chromogenic substrate for diaphorase (Boehringer Mannheim GMBH, Germany). Total liver lipids were extracted according to Folch et al.19) Cholesterolacetate was added as an internal standard. An aliquot was fractionated on silica-gel rods (Iatron, Tokyo, Japan) with hexane-dimethylther-formic acid (85:15:1), followed by quantification using scanning FID (Iatron, Tokyo, Japan).

Microsomal protein samples (3.75 ug), separated on 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel,20) were electrophoretically transferred to nitrocellulose membranes (Bio-Rad, U.S.A.) and probed with rabbit antibodies raised against CYP2E1. Alkaline phosphatase activities on the immunoblots were quantitated essentially by the method of Blake et al.,21) using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as chromogenic substrates. Resulting bands were quantitated with a scanning densitometer (Biomed, U.S.A.) with purified CYP2E1 as a standard. Microsomal hydroxylation of p-nitrophenol was measured by means of the procedure used by Reinke and Moyer.22) The reaction mixture contained 0.05 mg microsomal protein in a final volume of 0.5 ml K-phosphate buffer (0.15M, pH 6.8), EDTA (0.15 mm), ascorbate (1 mm), magnesium chloride (0.4 mm), p-nitrophenol (0.4 mm) and an NADPH-generating system. Incubation was performed at 37°C for 10 min. Resulting p-nitrocatechol was analyzed by HPLC using an electro-chemical detector. The specific mRNA of CYP2E1 was analyzed by means of Northern blots.
Total RNA was prepared from livers by a one-step precipitation method. A CYP2E1 cDNA probe was prepared by a polymerase chain reaction from the cDNA library of normal rat liver (Clontech, U.S.A.) and biotin-labelled by a random-primed polymerase reaction with Klenow fragment. Northern hybridization was performed at 42°C overnight in a solution containing 5 x sodium chloride/sodium phosphate/EDTA buffer, 5 x Denhardt's solution, 0.1% SDS, 5% PEG 6000 and 0.1 mg/ml denatured salmon sperm DNA. Hybridized membranes were washed twice at room temperature in 2 x sodium chloride/sodium citrate buffer (SSC) and 0.1% SDS, followed by repeated washes (15 min twice) at 42°C in 0.1 x SSC and 0.1% SDS. Quantitative determination of the specific mRNA level was performed with slot blots. Total RNA samples (1-10 μg) were transferred to nylon membranes (Millipore, U.S.A.) using a filtration manifold having 0.5 by 4 mm slots. Hybridization was performed under the same condition as the Northern blots. After washing, signals from the biotinylated probe were detected as described. The intensities of the signals from each slot were quantitated by scanning densitometry.

Results

Microsomal CYP2E1 Protein Content. The effects of ethanol, clofibrate and L-carnitine on the CYP2E1 content of rat livers were investigated by Western blots. As shown in Fig. 1A, the microsomal content of CYP2E1 in the liver was increased (45.3 ± 4.6 pmol/mg) in rats received ethanol alone, compared with control (23.8 ± 3.2 pmol/mg). Clofibrate treatment had no clear effect on the microsomal content of CYP2E1 (20.4 ± 0.9 pmol/mg). Administration of clofibrate in an ethanol-containing diet had no effect on CYP2E1 content (27.3 ± 5.1 pmol/mg). Feeding L-carnitine in an ethanol-containing diet increased the microsomal CYP2E1 content (60.2 ± 4.9 pmol/mg) to a level significantly higher than that produced by ethanol alone. No effect was produced by L-carnitine alone.

Microsomal p-Nitrophenol Hydroxylation. As a selective marker of CYP2E1 induction, p-nitrophenol hydroxylation was determined in the same liver samples used for the immunochemical quantification of CYP2E1. In the present study, changes in microsomal hydroxylation of p-nitrophenol (Fig. 1B) were largely consistent with those in Western blots in all the groups examined. The p-nitrocatechol formation in microsomes from ethanol-treated rats (1.4 ± 0.1 nmol min⁻¹ mg⁻¹) and ethanol plus L-carnitine co-treated rats (2.3 ± 0.6 nmol min⁻¹ mg⁻¹) was increased 1.7- and 2.9-fold compared with untreated controls (0.8 ± 0.2 nmol min⁻¹ mg⁻¹), respectively. These

Fig. 2. Hepatic Levels of CYP2E1 mRNA

Fig. 3. Blood Concentrations of Acetone (A) and 3-Hydroxybutyrate (B)

a) and b) refer to p < 0.05 and p < 0.01 by Student's t-test, respectively.
results provide further support for the enhancing effect of L-carnitine on CYP2E1 protein.

**CYP2E1 mRNA Levels** To assess the relationship between mRNA and protein in CYP2E1, hepatic levels of CYP2E1 mRNA were measured. A 2.1-fold increase in mRNA level was observed in rats treated with ethanol (Fig. 2). The mRNA level was suppressed in accordance with the reduced level of CYP2E1 protein in the group treated with clofibrate together with ethanol. On the other hand, in the L-carnitine co-treated group, no further increase in mRNA was observed compared with the ethanol-treated group (1.9-fold higher than untreated control).

**Blood Ketone Bodies** The effects of ethanol, clofibrate, and L-carnitine treatments on blood ketone bodies were examined. As described in Fig. 3, blood acetone and 3-hydroxybutyrate levels were increased in animals treated with ethanol and/or clofibrate. The blood acetone level in rats treated with ethanol alone was increased (60.2 ± 12.4 μM) to a level 1.5-fold higher than in untreated controls, although this value was considerably lower than reported previously in fasting or diabetic rats. In clofibrate-treated rats, the blood acetone level was 3.3-fold higher than in untreated controls. Co-administration of clofibrate with ethanol resulted in a marked increase in blood acetone (8.5-fold higher than in untreated controls).

**Liver Triglycerides** The liver triglyceride content was increased significantly in the ethanol-fed group (Fig. 4). However, co-feeding clofibrate or L-carnitine with ethanol markedly reduced the ethanol-mediated accumulation of liver triglycerides. Although the lipid-lowering phenomena in these co-treated groups could be ascribed to both the pharmacodynamic actions of these drugs and lowered food intake, the weight gain and dietary intake of ethanol-fed rats were not significantly different from those of the clofibrate or L-carnitine co-treated groups (Table 1).

**Discussion** Induction of CYP2E1 occurs under pathological conditions such as diabetes, fasting, and obesity, where changes in endogenous components including blood ketone bodies, endocrine factors and liver fat have been observed. HEPATIC CYP2E1 levels have been shown to be under multistage control involving protein synthesis and degradation. A post-translational stabilization of the enzyme protein, without elevation of the specific mRNA level, has been proposed as the mechanism of ethanol-mediated induction of CYP2E1, while pre-translational (transcriptional) activation is commonly accepted as the mechanism for other P450 species.

It is believed that modulation of fatty acid metabolism is one of the factors in ethanol-mediated pathogenesis in the liver. Liver lipids accumulate in the early stages of alcoholic liver injuries, probably due to the decreased rate of β-oxidation, which competes with ethanol oxidation for a cofactor, NAD+. Clofibrate and L-carnitine are known to reduce lipid accumulation in fatty liver. In the present study, liver lipid levels were markedly lowered in clofibrate-treated animals, accompanied by significant increases in blood ketone bodies. These facts suggest that clofibrate and L-carnitine modulate lipid metabolism through separate mechanisms. As shown in Fig. 1, clofibrate and L-carnitine have distinct effects on the hepatic levels of CYP2E1 protein. Hepatic CYP2E1 levels were raised independently of liver triglycerides, which were lowered by treatment with clofibrate and L-carnitine (Fig. 4). In addition, no direct relationship between blood ketone bodies and CYP2E1 content was observed (Fig. 3).

Two-fold increases in CYP2E1 mRNA were observed in groups receiving diets containing ethanol alone or together with L-carnitine. Association between the levels of specific mRNA and protein was also observed in the clofibrate co-treated group; the suppression of ethanol-induction of CYP2E1 protein was associated with a reduction in the specific mRNA level. These results suggest that the change in mRNA level is one of the factors involved in the suppression of hepatic CYP2E1. Yun et al. have reported a positive correlation between CYP2E1 mRNA and blood ketone bodies (acetocetate and 3-hydroxybutyrate) in rats fed high fat diets, suggesting a pre-translational activation by ketone bodies. In contrast to their results, no positive correlation was observed between mRNA and ketone bodies in the clofibrate treated rats, where blood acetone levels were higher than those in the ethanol-treated animals. The enhancement of CYP2E1 protein in the L-carnitine co-treated group was not accompanied by an increase in mRNA. The reason for this discrepancy between mRNA and protein levels remains unclear, but might reflect the complexity of the mechanism by which CYP2E1 levels are modulated. In other words, the elevation of CYP2E1 protein might reflect post-translational stabilization. A post-translational activation, without elevation of CYP2E1

![Fig. 4. Hepatic Triglyceride Content](image)

*Fig. 4. Hepatic Triglyceride Content*

*a* and *b* refer to *p* < 0.05 and *p* < 0.01 by Student's *t*-test, respectively.

**Table 1. Total Calorie Intake and Body Weight of Animals after the Feeding Period (Three Weeks)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Calorie intake (g)</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1797 ± 106</td>
<td>267 ± 10</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1498 ± 110</td>
<td>244 ± 8</td>
</tr>
<tr>
<td>Clofibrate</td>
<td>1439 ± 119</td>
<td>234 ± 17</td>
</tr>
<tr>
<td>L-Carnitine</td>
<td>1540 ± 97</td>
<td>227 ± 12</td>
</tr>
<tr>
<td>Ethanol + clofibrate</td>
<td>1367 ± 65</td>
<td>239 ± 11</td>
</tr>
<tr>
<td>Ethanol + L-carnitine</td>
<td>1356 ± 106</td>
<td>234 ± 9</td>
</tr>
</tbody>
</table>

Diet consumption and body weight were recorded every morning. Values represent the mean ± S.D. (*n* = 6).

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mRNA, by acetone and pyridine has been reported. On the other hand, pre-translational regulation of CYP2E1 has been found in fasted or hypophysectomized rats. Consistent with these results previously reported, we found multistage modulation of hepatic CYP2E1 under ethanol-induced conditions; clofibrate suppressed both CYP2E1 protein and mRNA, while t-carnitine increased only the protein level.

The mechanism by which clofibrate and t-carnitine modulate the ethanol-mediated induction of CYP2E1 is not known. Changing the levels of the inducer, ethanol, might be one of the factors. Clofibrate increases the oxidation of ethanol through catalase, whereas t-carnitine lowers ethanol oxidation through a competition for cofactor (NAD\(^+\)). Further studies are required to clarify the interaction of lipid-modulating agents on CYP2E1 induction.

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