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INDUCTION MECHANISMS OF CYTOCHROME P450 2E1 IN LIVER: INTERPLAY BETWEEN ETHANOL TREATMENT AND STARVATION

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Abstract—Chronic ethanol exposure causes marked induction of the ethanol-inducible cytochrome P450 (CYP) 2E1 isozyme in the centrilobular liver region, where alcoholic damage commonly is initiated. In contrast to most other CYP forms, which are ligand-activated at the transcriptional level, ethanol induction of CYP2E1 has been found to be post-translational. However, transcriptional activation of the CYP2E1 gene was recently described in fed animals maintained at very high ethanol levels. To further evaluate mechanisms of ethanol-mediated CYP2E1 induction we compared the effect of short-term heavy-ethanol treatment and fasting on CYP2E1 mRNA, protein and catalytic activity. High blood-ethanol levels (20–70 mM) were maintained for 3 days by regular alcohol intubations to fed or fasted rats. During this period, the amount of liver CYP2E1 apoprotein increased a maximum of 20-fold and catalytic activity 16-fold, both in fed and fasted animals, whereas starvation alone caused only a 4- to 5-fold increase. By comparison, the amount of CYP2E1 mRNA, as assayed both by Northern blot and slot blot, was significantly increased (5- to 6-fold) by ethanol only in fasted rats; this increase was smaller than that observed after fasting alone (8- to 9-fold). Analysis of cell lysates isolated from the periportal and perivenous region revealed that the increase in CYP2E1 mRNA by fasting occurred in the perivenous region. Thus no evidence was obtained for an increased pretranslational *CYP2E1* gene expression as a consequence of the continuous presence of ethanol at intoxicating levels for 3 days. CYP2E1 mRNA elevation seems to be strongly associated with starvation while alcohol treatment increases the amount of enzyme, primarily by ligand-dependent stabilization of the synthesized protein. Our results indicate that transcriptional activation of CYP2E1 requires the long-term presence of highly intoxicating ethanol levels. It is conceivable that such activation occurs via indirect physiological responses related to those triggered by starvation.

Key words: ethanol-inducible cytochrome P450; ethanol intoxication; starvation; *CYP2E1* gene transcription; liver zonation

The ethanol-inducible CYP2E1§ exhibits an exceptionally broad substrate specificity comprising many organic solvents, hepatotoxins and carcinogens in addition to ethanol [1, 2]. The enzyme is constitutively expressed in the liver, where it is responsible for the microsomal oxidation of ethanol. CYP2E1 is thought to mediate cell toxicity by generating oxygen species and radical intermediates [3, 4]. The expression of CYP2E1 is observed in the centrilobular region, both constitutively and after induction [5]. This zonated expression, related to a regional difference in the transcription rate of the *CYP2E1* gene [6], may be toxicologically crucial. Hepatotoxins activated by CYP2E1, such as carbon tetrachloride and acetaminophen, primarily damage cells in the centrilobular region [7], where the concentration of maximally-induced CYP2E1 has been calculated to be as high as 100 μM [8].

The *CYP2E1* gene is transcriptionally activated at birth [9]. Starvation leads to an increase both in the amount of CYP2E1 protein and in the corresponding mRNA [10], an effect shown to be due to transcriptional activation [6]. In contrast to most other CYP genes, the induction of CYP2E1 by a variety of chemicals commonly occurs at the post-translational level (for a review, see Ref. 2). However, in a recent study where ethanol was administered for 7 weeks by total enteral nutrition (the TEN model), Badger and collaborators reported increased CYP2E1 mRNA and an increased rate of *CYP2E1* gene transcription in rats with blood ethanol levels exceeding 3‰ [11, 12].

This indicated that there might exist a previously unrecognized ethanol-responsible element in the *CYP2E1* gene. We argued that such an element should also increase CYP2E1 mRNA during short-term treatment of rats, provided that ethanol administration resulted in high and consistent intoxication, a requirement seldom achieved previously. Since ethanol intoxication is commonly associated with reduced food intake and fasting is known to cause an increase in CYP2E1 mRNA, it was important to compare the data to those obtained by fasting alone and fasting in combination with ethanol treatment.

MATERIALS AND METHODS

Reagents. Caesium-trifluoroacetate was purchased

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§ Abbrevations: CYP2E1, cytochrome P450 2E1; SDS, sodium dodecyl sulphate; IgG, immunoglobulin G; TE, Tris–HCl–EDTA.

from Pharmacia AB (Uppsala, Sweden). CYP2E1 cDNA was a gift from Dr Frank J. Gonzalez (National Cancer Institute, National Institute of Health, Bethesda, MD, U.S.A.). Mouse β-actin cDNA was a kind gift from the late Professor Håkan Persson (Karolinska Institutet, Stockholm, Sweden).

Animal treatment. Male Wistar rats, initially weighing 170–190 g, with free access to standard laboratory diet (R3 diet, Ewos AB, Södertälje, Sweden) were used. Six days prior to the forced ethanol administration regimen, tolerance to ethanol was initiated by addition of ethanol to the drinking water by stepwise increase from 3 to 7%. The mean daily ethanol intake of rats during the last day of this period was 6.5 g/kg. Controls were given water only. Thereafter, one group of animals was fasted while the others had continuous access to food.

Ethanol was then administered by gavage as a 20% (v/v) solution given in three doses at 8 a.m., 2 p.m. and 8 p.m. The total daily dose was increased from 8.5 to 12 g/kg body wt in fed animals and from 8.5 to 9.5 g/kg in fasted animals. Fasted rats were given approx. 20% less ethanol to compensate for their slower rate of ethanol elimination. In addition, all animals given ethanol were maintained on the 7% ethanol drinking fluid.

Methods. Blood ethanol was determined by head-space gas chromatography from 50 μL samples taken from the tail vein.

Cell lysates from the periportal and perivenous region were obtained after digitonin infusion during *in situ* liver perfusion by the dual pulsing principle [13] as modified in [14] and samples for analysis of protein and mRNA treated as recently described [15]. Briefly, periportal cells were lysed by infusing 6.7 mL/kg body wt of 3.5 mM digitonin (ICN Chemicals, Cleveland, OH, U.S.A.) via the portal vein and the lysate collected by immediate retrograde flushing. Perivenous cell lysates were obtained by infusing 10 ml/kg body wt digitonin solution via the upper vena cava followed by antegrade flushing. The zone-specificity of the lysates was verified by measuring the activity of alanine aminotransferase (EC 2.6.1.2), a periportal marker enzyme, as before [5].

Liver microsomes were prepared by homogenizing liver tissue in 10 mM sodium/potassium phosphate buffer, pH 7.4, containing 1.14% KCl. The microsomal pellet obtained after ultracentrifugation at 105 000 *g* was washed once and resuspended in 50 mM potassium phosphate buffer, pH 7.4. The catalytic activity of CYP2E1 was estimated by measuring the hydroxylation of *p*-nitrophenol using 0.2 mg microsomal protein [16].

Quantitation of CYP2E1 protein was done by Western blot analysis. Five or 10 μg microsomal protein was SDS–polyacrylamide gel electrophoresed [17] and electroblotted onto nitrocellulose (Bio-Rad) in buffer with SDS and methanol [18]. CYP2E1 was detected with anti-CYP2E1 IgG [19] using horseradish peroxidase and 4-chloro-1-naphthol. A preparation of CYP2E1 purified from rat liver as described in [19] was used as standard.

For isolation of total RNA, livers were homogenized in 4 M guanidine thiocyanate buffer. Total RNA was isolated by ultracentrifugation using a cesium-trifluoroacetate gradient method [20]. The RNA was dissolved in TE-buffer and stored at −70°. Total RNA of the cell lysates was isolated by acid guanidinum thiocyanate–phenol–chloroform extraction as described previously [15, 21].

For determination of CYP2E1 mRNA, total RNA of four different rats from each experimental group was size fractionated by electrophoresis in a 1.25% agarose–formaldehyde gel. The amount of mRNA was carefully quantitated by dot blot analysis. Variable amounts of RNA were loaded on the wells in order to ensure linearity with respect to CYP2E1 mRNA concentration. Quantitation was carried out by loading three different concentrations of RNA from each rat onto the wells. Slot blot analysis was carried out using the Schleicher and Schuell slot blot system (minifold II). Northern blot analysis was performed as described in the literature [22]. Hybridizations were conducted using ^{32}P-labelled full length cDNA of rat CYP2E1 gene. ^{32}P-labelled β-actin cDNA from mouse was used as a standard during slot blot analysis.

RESULTS

Nutritional state

During the 3 day treatment the changes in body weight were monitored to estimate indirect nutritional effects. The untreated fed animals gained 12% body wt while the starved animals lost 19% (Table 1). The fed rats receiving ethanol suffered a slight loss in weight (5%), much less than ethanol-treated fasted animals (16%). The absence of weight gain in the fed ethanol-treated animals is an expected consequence of heavy ethanol intoxication in a regimen with free access to food. The influence on weight gain was minimized by a gradual adaption to ethanol, first by its inclusion in the drinking water and then by the step-wise increase in the ethanol dosage given by gavage. In the fasted rats, the fact that animals given ethanol lost less weight than fasted controls was probably due to the caloric value of the ethanol administered.

Blood ethanol levels

Ethanol administration was designed to maintain a state of ethanol intoxication throughout the treatment period. In a separate pilot experiment, the rate of elimination of an orally administered dose of ethanol (4 g/kg), given to similarly ethanol-treated fed rats on day 3, was 560 ± 34 mg/kg body wt per hour (N = 3). Peak blood ethanol levels, as estimated from samples taken 90 min after intubation, were 63.2 ± 5.7 mmol/L (N = 3), corresponding to approx. 2.8‰.

The hourly rate of elimination corresponds to a total daily elimination of 13.4 g ethanol per kg body wt. The mean daily fluid intake of the 7% ethanol drinking fluid during the 3 day treatment was approx. 8 mL, corresponding to approx. 3 g ethanol/kg body wt. The total daily intake of ethanol can therefore be estimated to be 11.5–16 g/kg, exceeding even the calculated elimination capacity. Since fasting reduces the rate of ethanol elimination [23], starved rats were administered 20% less ethanol on days 2 and 3. To verify that the ethanol treatment procedure

Table 1. Effect of fasting and ethanol treatment on body weight development*

Treatment	group	N	Day 0	Day 1	Day 2	Day 3
Fed	1	(5)	241 ± 10.8			269 ± 13.6
Fasted	2	(5)	234 ± 20.1			189 ± 15.5
Fed + ethanol	3	(4)	234 ± 14.1	228 ± 15.4		
	4	(4)	223 ± 13.3		217 ± 17.1	
	5	(6)	215 ± 4.5			205 ± 11.8
Fasted + ethanol	6	(4)	223 ± 14.9	206 ± 11.7		
	7	(4)	234 ± 3.5		207 ± 6.8	
	8	(6)	225 ± 12.8			189 ± 17.7

* Ethanol was given by gavage in three daily doses as described in Methods. Means ± SD is given with the number of animals in parentheses.

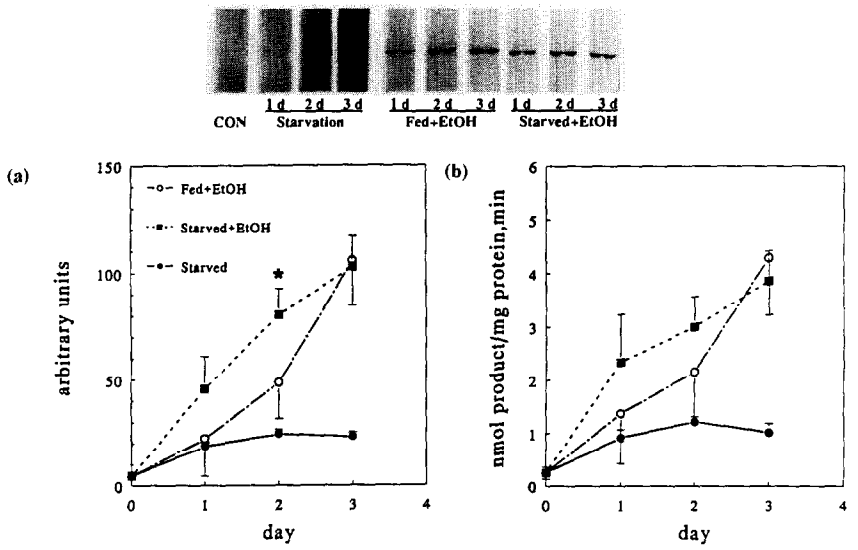

Fig. 1. Effects of short-term ethanol exposure and starvation on CYP2E1 apoprotein levels and *p*-nitrophenol hydroxylase activity. Western blots of samples representative of respective treatment are shown at the top of the figure. The graphs show means ± SD (N = 5) of CYP2E1 apoprotein levels (a) and microsomal *p*-nitrophenol hydroxylase activities (b). Values at day 0 represent untreated controls. *= $P < 0.05$ compared to corresponding fed rats.

resulted in continuous presence of ethanol, tail blood samples were taken from both fed and fasted animals during the last day of treatment at 1 p.m., 10 p.m. and 8 a.m. Sampling was performed 1–2 hr before the next dosage, i.e. during periods when blood ethanol levels approached their lowest values. In the fed animals blood ethanol levels on these occasions were 43 ± 8 mM, 18 ± 14 mM and 38 ± 22 mM, respectively (mean ± SD, N = 6). The corresponding concentrations for fasted animals were 39 ± 16 mM, 20 ± 17 mM and 55 ± 23 mM. Fed and fasted animals thus had approximately similar ethanol levels, while during the course of this study blood ethanol levels fluctuated between 20 and 70 mM (1–3‰).

CYP2E1 enzyme induction

During the 3 day ethanol treatment period both the amount of CYP2E1 protein and catalytic activity, as estimated from microsomal *p*-nitrophenol hydroxylase activity, increased continuously both in fed and starved animals (Fig. 1). The induction process was more rapid in the fasted animals, but at day 3 the amount of CYP2E1 protein had increased approx. 20-fold and microsomal *p*-nitrophenol hydroxylase activity approx. 16-fold, both in fed and fasted rats. Fasting alone also caused an induction both in the amount of protein and in catalytic activity, but only a 4- to 5-fold increase was observed and this effect was maximal after 2 days of starvation.

CYP2E1 mRNA

The relative effects of ethanol and fasting on CYP2E1 were quite different when analysed at the level of mRNA. Starvation alone caused an over 9-fold accumulation of CYP2E1 mRNA as compared to fed controls (Fig. 2). This effect was larger than that observed in similarly fasted animals

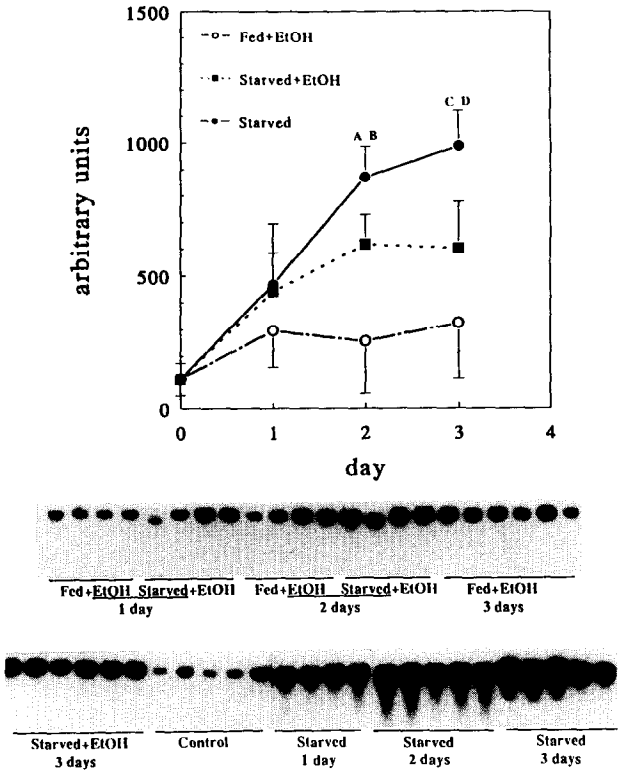

Fig. 2. Effects of short-term ethanol exposure and starvation of CYP2E1 mRNA levels. The graph shows means ± SD (N = 5) of each group. Values at day 0 represent untreated controls. Results of Northern blot analysis performed with 10 μg of total RNA are shown at the lower part of the figure. A and C: $P < 0.001$ as compared to corresponding fed + EtOH rats. B = $P < 0.05$ and D = $P < 0.005$ as compared to corresponding fasted + EtOH rats.

which also received ethanol. The amount of CYP2E1 mRNA in non-starved rats given ethanol was approx. twice as high as in fed controls, but this increase was not statistically significant.

Regional distribution of CYP2E1 induction

We have previously demonstrated that CYP2E1, both at the protein level and the mRNA level, is expressed mainly in the perivenous liver region and that ethanol induction of CYP2E1 protein occurs in the same cells [5]. In the present study we studied the localization of CYP2E1 protein and mRNA after 3 days of starvation by comparing the amount of CYP2E1 protein and mRNA in periportal and perivenous cell lysates. The amount of CYP2E1 protein and mRNA was eight to nine times higher in perivenous (centrilobular) than in periportal cell samples, in agreement with our previous data. Compared to fed controls, fasting seemed to increase both protein and mRNA mainly in the perivenous (centrilobular) cells (Fig. 3). After starvation, the amount of CYP2E1 protein was 18-fold higher than that of mRNA 5.6 times higher in perivenous than in corresponding periportal samples. No significant effect of fasting on periportal CYP2E1 protein and mRNA was discerned. Thus CYP2E1 in hepatocytes of the same perivenous region, where the constitutive CYP2E1 protein and mRNA expression dominates, seems to be pretranslationally activated by fasting.

DISCUSSION

Among the numerous members of the microsomal cytochrome P450 isozymes responsible for metabolic activation of xenobiotics in the liver, the ethanol-inducible CYP2E1 form appears to be of particular interest due to its inducibility by ethanol and to its capacity to activate important hepatotoxins such as carbon tetrachloride and acetaminophen [2]. In contrast to most other P450 forms [24], the substrate induction of CYP2E1 does not generally involve transcriptional activation. This has been demonstrated repeatedly in intact rodents, where ethanol, pyrazole, acetone and a number of other CYP2E1 substrates seem to act primarily by means of protein stabilization [9, 25]. Although pyridine and isoniazid have been shown to increase CYP2E1 mRNA translation efficiency, mRNA levels are not affected [26, 27]. It is therefore generally held that induction of CYP2E1 by exogenous ligands does not involve transcriptional activation. Recent reports of elevated CYP2E1 mRNA levels and activation of CYP2E1 transcription in animals maintained on very high blood ethanol levels ($\leq$ 3‰) [11, 12] and of increased CYP2E1 mRNA levels in livers of alcoholics [28]

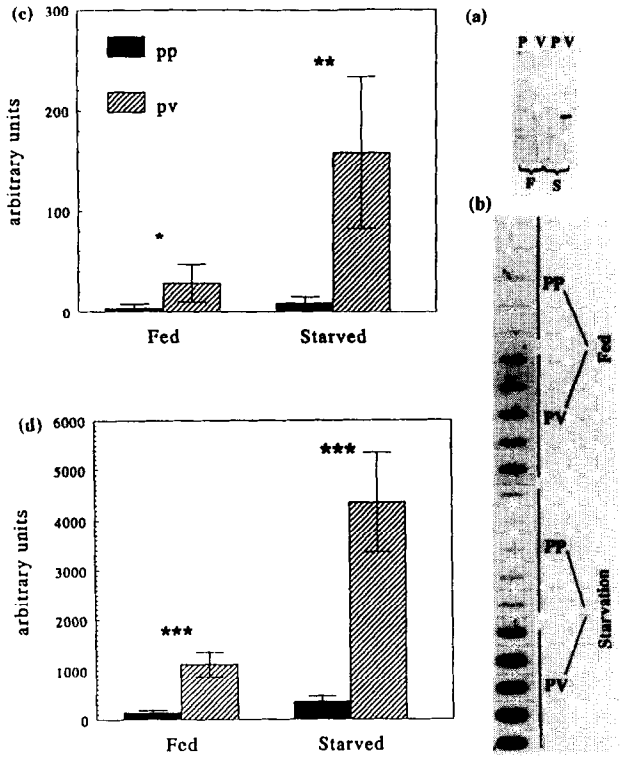

Fig. 3. Zonation of induction of CYP2E1 by starvation. Cell lysates were obtained from the perivenous (pv) and periportal (pp) region as described in the Methods section. On the right side are shown (a) Western blots of representative periportal (P) and perivenous (V) samples (10 μg protein) from fed (F) and starved (S) rats and (b) corresponding slot blots with 1 μg eluate RNA. The graphs show means ± SD of CYP2E1 apoprotein (c) and mRNA (d) (N = 5). *P < 0.05, **P < 0.01, ***P < 0.001 for pp–pv difference.

were therefore intriguing. This observation could be related to a recent report wherein exposure of primary cultured rabbit hepatocytes to high concentrations (17 mM) of acetone caused an increase in CYP2E1 mRNA [29], provided this occurred *in vivo* as well. Elevated levels of acetone are characteristic of starvation, a state known to lead to a marked increase in CYP2E1 mRNA [9] and transcriptional activation [6]. Any mode of chronic alcohol intake is likely to influence the recipient's nutritional status. This complicates evaluation of a true ethanol effect. Clearly, the nutritional status of alcoholics is commonly compromised and the induction of CYP2E1, with potentially toxicological consequences, probably results from a combination of ethanol exposure and poor dietary intake. The present animal study illuminates this situation. In the TEN animal model undernutrition seems excluded, since the animals continuously received a nutritionally adequate liquid diet containing ethanol and grew well.

In the present study we attempted to discriminate between the effect of ethanol as a solvent and intoxicant *per se* and its associated, secondary nutritional effects. We observed that in animals subject to prolonged heavy ethanol intoxication, there was a stepwise induction of CYP2E1 protein and catalytic activity both in fed and starved animals, but that at the level of CYP2E1 mRNA a significant induction by ethanol only was observed in fasted animals. It was of particular interest that the increase in CYP2E1 mRNA was smaller in fasted rats receiving ethanol than in similarly fasted animals receiving no ethanol. This indicates that the calories from ethanol relieve the fasting state and the associated increase in CYP2E1 mRNA. Administration of acetone, a solvent and a caloric source like ethanol, has also been shown to reduce the increase in CYP2E1 mRNA caused by starvation [19].

Thus, administration of ethanol for 3 days resulting in intoxicating levels (blood ethanol levels varying between 20 and 70 mM or 1 and 3‰) does not appear to activate transcription of the CYP2E1 gene. The absence of CYP2E1 transcriptional activation has been repeatedly observed beforehand, but at lower ethanol levels than those reported in the present study. Neither was any increase in CYP2E1 mRNA discerned in a recent study with hepatoma cell lines expressing CYP2E1 [30], even after exposure to high ethanol levels. Consequently, evidence for the existence of a specific ethanol-responsive element associated with the CYP2E1 gene has only been obtained after long-term heavy-ethanol exposure achieved by the TEN model [11]. Since a nutritionally adequate diet is provided with

this model, nutritional deficiency or semistarvation seems excluded. It cannot, however, be excluded that both starvation and prolonged and severe ethanol intoxication triggers similar hormonal responses, that ultimately lead to transcriptional activation of the CYP2E1 gene. Stress hormones such as glucocorticoids and thyroxine, both known to be affected by starvation and ethanol intoxication, could be common mediators of the observed effects.

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