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ETHANOL INCREASES CONTENT AND ACTIVITY OF HUMAN CYTOCHROME P4502E1 IN A TRANSDUCED HepG2 CELL LINE

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Using recombinant retroviral expression, a HepG2 cell line which stably and constitutively expresses the coding sequences of the human cytochrome P4502E1 was previously established. Addition of ethanol (2 to 100 mM) to the culture medium of this cell line for two days resulted in an increase in the content of P4502E1 as determined by immunoblotting and an increase in HepG2 microsomal oxidation of p-nitrophenol, aniline, and N,N-dimethylnitrosamine. The ethanol-induced increase in microsomal oxidation of these substrates was prevented by ligands and inhibitors of P4502E1 as well as anti-human P4502E1 IgG and corresponded to the increase in P4502E1 content. Several other agents including pyrazole, 4-methylpyrazole, isoniazid, pyridine, and DMSO also increased the content of P4502E1 in this cell line but not oxidation of substrates, presumably a reflection of remaining tightly bound to the active site of P4502E1. Slot blot analysis indicated that ethanol addition did not increase P4502E1 mRNA levels. These results indicate that ethanol can increase the content of P4502E1 as well as catalytic oxidation of substrates dependent on P4502E1 in this experimental model, perhaps by stabilization of the protein against degradation.

Cytochrome P4502E1 is induced by a variety of low molecular weight chemicals such as ethanol, acetone, isoniazid, pyridine, pyrazole, 4-methylpyrazole, and DMSO (1-11). The mechanism of induction of P4502E1 is complex and depending on the experimental conditions, the concentration of P4502E1 may be regulated by mechanisms involving transcription, stabilization of CYP2E1 mRNA, enhanced translation via polysome formation, and increased stabilization of P4502E1 protein against degradation (12-18). Induction of P4502E1 by ethanol and other alcohols was initially shown to be largely due to stabilization of the protein (9, 11, 14, 15); more recent studies have shown that induction of P4502E1 by ethanol can occur with associated increases in CYP2E1 mRNA levels (19-21). The above studies have been carried out largely with rats or rabbits; there are no apparent studies on regulation or stabilization of the human P4502E1 by ethanol. Our laboratory recently established a HepG2 cell line which stably and constitutively expresses the coding sequences of the human P4502E1 under the control of the LTR's of the retrovirus (22). Western blot analysis showed that the transduced clones

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produced a protein with molecular weight identical to purified human P4502E1, and microsomes isolated from these clones were catalytically active with a variety of P4502E1 substrates (22). Experiments were carried out using this model to determine whether addition of ethanol to the culture medium can increase the content and catalytic activity of the constitutively expressed human P4502E1.

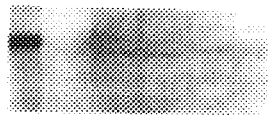
MATERIALS AND METHODS

The HepG2 cell line which stably and constitutively expresses the coding sequence of the human cytochrome P4502E1 was previously described (22). Clone MV2E1-9 was utilized for these experiments. Stable-transduced HepG2 cells were grown in MEM medium containing 0.2 mg/ml G418 in the absence of ethanol or presence of varying concentrations of ethanol for two days. Cells were grown in petrie dishes wrapped in Parafilm. The ethanol was present in the culture medium until the cells were washed and harvested by scrapping and subsequent sonication for 45s. Microsomes were prepared by differential centrifugation and resuspended in 0.125M KCl - 0.01M potassium phosphate (pH 7.4) buffer containing 20% glycerol and kept at -70°C.

Western blot analysis was carried out using 0.1 mg microsomal protein as previously described (22). The immunoblots were developed using an anti-human P4502E1 polyclonal antibody provided by Dr. J. Lasker (Biochemistry Department, Mount Sinai School of Medicine) and horseradish peroxidase conjugated goat anti-rabbit IgG as the second antibody. Microsomal oxidation of 0.2 mM p-nitrophenol, 4 mM N,N-dimethylnitrosamine, or 1 mM aniline was determined in a reaction system containing 0.1M potassium phosphate, pH 7.4, 1 mM NADPH and about 0.25 mg microsomal protein in a final reaction volume of 0.1 ml. Experiments were carried out for 60 min as previously described (22). Inhibition by antibodies was carried out by incubation of the microsomes with pre-immune IgG or anti-human P4502E1 IgG for 5 min at room temperature prior to the addition of NADPH to initiate the reaction. Reactions were carried out in duplicate and are from two experiments. Catalytic activity was corrected for values obtained in zero-time controls in which trichloroacetic acid was added before NADPH. Slot blot analysis was carried out using 20 μg total RNA as previously described (23). The CYP2E1 oligonucleotide probe was a 51-mer which corresponds to the DNA sequence encoding amino acids 254 to 270 of P4502E1. The probe for β-actin was a 27-mer oligonucleotide purchased from Clontech (Palo Alto, CA). The probes were labelled at the 5' end with [γ-^{32}P]ATP and T4 polynucleotide kinase. Densitometry of the slot blots and Western blots were carried out using a LKB ultraScan XL Laser Densitometer.

RESULTS AND DISCUSSION

Content of P4502E1 in HepG2 Cells Incubated with Ethanol - Western blot analysis was carried out to measure the expression level of P4502E1 in the microsomal fraction of HepG2 cells which had been infected with recombinant retrovirus containing the cDNA coding sequences of the human P4502E1. Lane 2 of Fig. 1 shows that the microsomes from the HepG2 cells incubated in the absence of ethanol produce a protein band of mol wt of 54 kDa when reacted with anti-human P4502E1 IgG, which migrates at the same position as purified human P4502E1 (lane 1). Microsomes isolated from HepG2 cells incubated with varying concentrations of ethanol for two days had an increased content of P4502E1 (Fig. 1, lanes 3 to 6). Densitometric analyses revealed the following values in arbitrary units (or pmoles per 100 μg microsomal protein); lane 2 - no ethanol, 0.026 (1.1); lane 3-5 mM ethanol, 0.057 (2.4); lane 4-20 mM ethanol, 0.061 (2.6); lane 5-100 mM ethanol, 0.056 (2.4), and lane 6-200 mM ethanol, 0.040 (1.7). Thus incubating the



1 2 3 4 5 6

Fig. 1 - Western blot analysis of the content of P4502E1 in microsomes isolated from HepG2 cells cultured in the absence and presence of ethanol. HepG2 cells (clone MV2E1-9) constitutively expressing human P4502E1 were cultured for two days in the presence of 0, 5, 20, 100, and 200 mM ethanol. Microsomes were prepared and 0.1 mg microsomal protein was analyzed using anti-human P4502E1 IgG as the first antibody and goat anti-rabbit IgG conjugated to horseradish peroxidase as the second antibody. Lanes correspond to the following: 1, 5 pmol human P4502E1; 2, HepG2 cells incubated in the absence of ethanol; 3 to 6, HepG2 cells incubated in the presence of 5, 20, 100, and 200 mM ethanol, respectively.

HepG2 cells in the presence of 5 to 100 mM ethanol increased the content of P4502E1 2- to 2½-fold.

Oxidation of Substrates - To validate that the P4502E1 in microsomes isolated from HepG2 cells incubated in the absence or presence of ethanol was catalytically active, the oxidation of substrates such as p-nitrophenol, aniline, and DMN was determined. As reported previously (22), all three substrates were oxidized by the control HepG2 cells constitutively expressing the P4502E1 (Table I. Exp A). There was an approximate two-fold increase in the rate of oxidation of the three substrates with microsomes isolated from HepG2 cells which were incubated with 5 mM ethanol for two days (Table I, Exp. A). A concentration curve for the stimulation of p-nitrophenol oxidation by ethanol is shown in Exp. B, Table I. Significant stimulation was observed at 2 mM ethanol, and concentrations of ethanol ranging from 2 mM to 20 mM produced a comparable two-fold increase in the oxidation of p-nitrophenol. The increase in substrate oxidation produced by ethanol is similar to the increase in P4502E1 content produced by ethanol.

Effect of Inhibitors - Oxidation of p-nitrophenol by microsomes isolated from control HepG2 cells expressing P4502E1 was sensitive to the addition of 4-methylpyrazole, a ligand for P4502E1, to ethanol, a competitive substrate for P4502E1, and to diethyldithiocarbamate, an effective inhibitor of P4502E1-catalyzed substrate oxidation (23-25) (Table II). The elevated oxidation of p-nitrophenol found after incubation of the cells with 5 mM ethanol for two days was also very sensitive to these additions. Anti-human P4502E1 IgG was a strong inhibitor of the oxidation of p-nitrophenol by microsomes isolated from HepG2 cells incubated in the absence or presence of 5 mM ethanol; pre-immune IgG had little or no effect (Table II). Thus the elevated oxidation of p-nitrophenol produced by ethanol treatment was sensitive to various inhibitors of P4502E1-dependent catalytic activity, validating its dependence on P4502E1.

P4502E1 mRNA Levels - Northern blot analysis previously showed that the HepG2 cells expressing the coding sequences of the P4502E1 hybridized with CYP2E1 cDNA and produced

TABLE I

Increased Oxidation of Substrates After Growth of HepG2 Cells in the Presence of Ethanol

A. Substrate	Rate of oxidation (nmol/min/mg microsomal protein)		Effect of Ethanol (%)
	Control	Ethanol	
p-Nitrophenol	0.065	0.154	+137
Aniline	0.013	0.031	+138
DMN	0.010	0.024	+140

B. Substrate	Concentration of Ethanol (mM)	Rate of Oxidation (nmol/min/mg)	Effect of Ethanol (%)
p-Nitrophenol	0	0.061	-
"	2	0.115	+89
"	5	0.127	+108
"	20	0.111	+82
"	100	0.094	+54

HepG2 cells constitutively expressing the human P4502E1 (clone MV2E1-9) were grown for two days in the absence or presence of ethanol and microsomal oxidation of 0.2 mM p-nitrophenol, 1 mM aniline, or 4 mM DMN was determined. In Exp. A, the concentration of ethanol was 5 mM, while in Exp. B, the cells were grown in the presence of the indicated concentrations of ethanol.

an RNA species consistent with the 1.7 Kb CYP2E1 cDNA insert (22). Slot blots using an oligonucleotide probe specific for P4502E1 were carried out with total RNA isolated from HepG2 clone E9 cells incubated in the absence or presence of either 5 mM or 100 mM ethanol. Results shown in Fig. 2 indicate that incubation with ethanol did not increase the P4502E1 mRNA levels; in fact a decrease in intensity was observed. Arbitrary units were: lane 1, no ethanol, 100 units; lane 2, 5 mM ethanol, 87 units; lane 3, 100 mM ethanol, 67 units. Fig. 2B shows the β-actin mRNA levels, where no effect by ethanol is noted. Ratios of P4502E1/β-actin RNA levels were 1.0, 0.9, and 0.7 for lanes 1, 2 and 3, respectively.

Effect ofOther P4502E1 Inducing Agents - Besides ethanol, the content of P4502E1 can be increased by a diverse group of low molecular weight chemicals, including pyrazole, 4-methylpyrazole, isoniazid, and DMSO (1-18). Western blot analysis showed that similar to the ethanol treatment, the content of P4502E1 was elevated two-to-three-fold when the HepG2 cells were incubated for two days with these agents (data not shown). However, oxidation of p-nitrophenol was inhibited when microsomes isolated from cells incubated with these additions were utilized (Table III). In this experimental protocol, the various inducers were present in the

TABLE II

Effect of Inhibitors of P4502E1 on Ethanol Stimulation of the Oxidation of p-Nitrophenol

Addition to Microsomes	Rate of oxidation (nmol/min/mg protein)		Effect of addition (%)	
	Control	Ethanol	Control	Ethanol
None	0.061	0.133	-	-
1 mM 4-Methylpyrazole	0.021	0.015	-66	-89
0.1 mM Diethyldithiocarbamate	0.022	0.005	-64	-96
100 mM Ethanol	0.019	0.013	-69	-90
4 mg/mg Pre-immune IgG	0.059	0.110	-3	-17
4 mg/mg Anti-2E1 IgG	0.016	0.012	-74	-91

HepG2 cells constitutively expressing the human P4502E1 (clone MV2E1-9) were grown for two days in the absence or presence of 5 mM ethanol. Microsomes were prepared and the oxidation of 0.2 mM p-nitrophenol was determined in the presence of the indicated additions.

culture medium until the time of harvesting the cells. In a second series of experiments, the medium with the inducers was replaced by fresh medium lacking the inducers for two hours prior to harvesting the cells in an attempt to "wash-out" the inducers. However, results were essentially similar to the no wash-out experiments shown in Table III, i.e., oxidation of p-nitrophenol by microsomes isolated from HepG2 cells treated with the inducers was less than control activity. Longer wash-out periods were not evaluated. It would appear that, in contrast to ethanol, inducers such as pyrazole, 4-methylpyrazole, isoniazid, pyridine, and DMSO, bind more tightly to P4502E1 and remain bound at the catalytic site even after preparation of the microsomes such that substrate oxidation is impaired.

In summary, the addition of ethanol to HepG2 cells which express the human P4502E1 results in an increase in the content of this P450 as well as an increase in the oxidation of substrates which are relatively specific for this isoform. The increased catalytic activity is

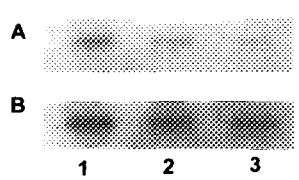


Fig. 2 - Slot blot analysis of the content of P4502E1 mRNA (A) and β-actin mRNA (B) from HepG2 clone 9 cells incubated in the absence (lane 1) or presence of either 5 mM (lane 2) or 100 mM (lane 3) ethanol. Total RNA was isolated by an acid guanidinium thiocyanate-phenol-chloroform extraction procedure and hybridization was carried out using 20 μg total RNA and either a 51-mer CYP2E1 oligonucleotide probe (A) or a 27-mer β-actin oligonucleotide probe (B).

TABLE III

Effect of P4502E1 Inducing Agents on HepG2 Microsomal Oxidation of p-Nitrophenol

Addition	Concentration (mM)	Rate of Oxidation (nmol/min/mg protein)	Effect of Addition (%)
None	-	0.078	-
Pyrazole	1	0.064	-18
	5	0.050	-36
4-Methylpyrazole	0.2	0.039	-50
	1	0.028	-64
Isoniazid	0.2	0.016	-79
	1	0.018	-77
Pyridine	5	0.027	-65
	10	0.015	-81
DMSO	10	0.020	-74

HepG2 cells constitutively expressing the human P4502E1 (clone MV2E1-9) were incubated for two days in the presence of the indicated additions: microsomes were isolated from each sample and oxidation of p-nitrophenol was assayed.

prevented by various inhibitors of P4502E1. Although several other P4502E1 inducers can increase the content of the enzyme, increased catalytic activity with substrates is observed only with ethanol as the inducer, most likely a reflection of the weaker binding and ease of removal of ethanol from the P4502E1 active site. In rodent and rabbit models, ethanol has been shown to increase the content of P4502E1 by a variety of mechanisms, including stabilization of the P4502E1 protein against degradation (12, 13). As expected, ethanol did not increase P4502E1 mRNA levels since expression of the P4502E1 is under the control of the LTR's of the viral promotor. In view of the constitutive expression of the P4502E1 in this model, further studies are underway to evaluate whether the mechanism responsible for this increase is due to stabilization of the P4502E1 against degradation.

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