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ETHANOL-MEDIATED INCREASE IN CYTOCHROME P-450 IN CULTURED HEPATOCYTES

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Abstract—Cellular levels of cytochrome P-450 were increased on exposure of cultured chick embryo hepatocytes to ethanol. This increase correlated with increases in the enzymatic activities of both benzphetamine demethylase [associated with the species of P-450 induced by propylisoproplyacetamide (PIA) or phenobarbital] and ethoxyresorufin deethylase [associated with the P-448 species induced by β-naphthoflavone (β-NF)]. The increased enzymatic activity relative to P-450 more closely resembled that caused by PIA than by β-NF. Glucuronidation of phenol red was also increased by ethanol; the dose response resembled that for increases in cytochrome P-450. PIA induced the glucuronidation of phenol red, whereas β-NF did not. Therefore, ethanol exposure was found to increase cytochrome P-450 and glucuronyltransferase in these cells in a pattern similar to increases observed with PIA.

In rats [1, 2] and hamsters [3, 4], chronic exposure to ethanol increases hepatic microsomal mixed function oxidases and cytochrome P-450, the terminal enzyme. These findings may explain why experimental animals and humans chronically exposed to ethanol exhibit increased clearance of several drugs [5, 6] and why ethanol enhances the toxicity of CCl_4 and acetaminophen [7, 8], agents believed to be toxic as a result of metabolic activation by cytochrome P-450 [9, 10]. Increases in P-450 by ethanol may also explain why alcoholics have an incidence of cancer higher than the non-alcoholic population since many chemicals that are not themselves carcinogenic must be metabolized to the active form by cytochrome P-450 [11].

Increases in hepatic drug-metabolizing activities following chronic exposure to ethanol are difficult to characterize in intact animals or humans where numerous secondary effects are possible, such as altered blood flow, variations in hormone concentrations, and changes in the nervous system. It would be advantageous to study these processes in cultured hepatocytes in which the chemical environment of the cells is totally defined. Primary cultures of chick embryo hepatocytes provide such a system. Cultured in a defined medium devoid of serum, they are responsive, as *in ovo* or in adult animals, to drug induction of the different species of cytochrome P-450 [12, 13]. In the experiments presented in this paper we have shown that these cells also respond to ethanol by increasing the levels of cytochrome P-450 and associated oxidative activities. The species of P-450 that was increased by ethanol in cultured chick embryo hepatocytes had properties similar to those induced by propylisoproplacetamide, a phenobarbital-like drug.

METHODS

Preparation and treatment of cultured chick embryo hepatocytes

Cultures of 16 to 17-day embryonic livers were prepared in serum-free medium, as described previously [13]. The medium contained 20 mM Hepes‡ buffer in addition to the other supplements. Twenty hours after plating the cells, the medium was changed and drugs were added. For the 48-hr exposure to the various chemicals, the medium was changed again 24 hr later and chemicals were added again. To minimize evaporation, all plates containing ethanol were wrapped in two layers of Parafilm and one layer of polyvinyl chloride film (Arthur H. Thomas Co., Philadelphia, PA). This wrapping procedure was not used routinely with control cells or cells exposed to either PIA (propylisopropylacetamide) or β-NF (β-naphthoflavone), since in preliminary studies levels of cytochrome P-450 under these conditions were identical with or without the wrapping.

Glucuronidation of phenol red

Conjugation of phenol red was measured as a decrease of absorbance at 560 nm after a 1 : 4 dilution of medium with 0.1% SDS in 0.1 N NaOH [14]. The extinction coefficient (E mM) was determined to be 56 in this solution. To assess whether the decrease in absorption was due to glucuronide formation, the medium was treated with bovine β-glucuronidase (1000 units/ml) in the presence or absence of D-saccharic acid, 1,4-lactone (20 mM), and alkaline absorbance at 560 nm was measured after a 60-min incubation at 37°C.

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‡ Abbreviations: Hepes, H-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; SDS, sodium dodecylsulfate; and BSA, bovine serum albumin.

Measurement of cytochrome P-450

Cells were harvested in buffer containing 0.1% Emulgen 913, and cytochrome P-450 was measured in the 8700 *g* supernatant fraction of the homogenate, as described previously [13].

Enzyme assays

Lactate dehydrogenase (LDH). LDH was measured using the assay of Wroblewski and Ladue [15] in which oxidation of NADH is followed during the reduction of pyruvate.

7-Ethoxyresorufin-O-deethylase (7-EROD). The deethylation of ethoxyresorufin was measured in cell homogenates prepared in 0.1 M phosphate buffer, pH 7.8. The reaction mixture contained, as final concentrations in a 150 μl volume: 1.67 mM NADPH, 13 mM nicotinamide, 12.5 mM $MgCl_2$, 20 mM DL-isocitrate, 0.2 units/ml isocitrate dehydrogenase, 1.33 mg/ml BSA, and 5 mg/ml cell homogenate protein. The mixture was incubated at 37° for 15 min, and the reaction was terminated by the addition of 1 ml of cold acetone [16]. Resorufin was measured in the 1000 *g* supernatant fraction by determining the fluorescence at 590 nm emission following excitation at 530 nm on a Perkin–Elmer 512 spectrofluorometer. The amount of resorufin was calibrated with a known standard.

Benzphetamine demethylase (β-phet DME). This activity was measured according to the procedure of Poland and Nebert [17] with the following modifications. Enzyme activity was measured in cell homogenates. The reaction mixture consisted of 140 μl total volume and contained 0.5 mg cell homogenate protein. Isocitrate (20 mM) and isocitrate dehydrogenase (0.21 units/ml) were used to generate NADPH. The product of the reaction, HCHO, is converted to dihydrolutinide, which is measured by its fluorescence (420 nm excitation/514 nm emission) [18].

Ethanol concentration

The concentration of ethanol in the medium was measured by the absorbance at 340 nm due to the appearance of NADH after incubation with alcohol dehydrogenase in pyrophosphate buffer (75 mM, pH 9.2) and NAD^+ (0.56 mM) (Sigma standard kit). Ethanol standards were included in each reaction set. The other components of the culture medium had no effect on the reaction (results not shown).

Proteins

The Bio-Rad Protein Assay (Bio-Rad Laboratories, Rockville Center, NY) was used to measure proteins in cell preparations containing Emulgen. All other proteins were determined by the method of Lowry *et al.* [19].

Sources of chemicals

PIA was a gift from Hoffman–LaRoche, Nutley, NJ; β-NF was purchased from the Aldrich Chemical Co., Milwaukee, WI; ethanol (USP grade) was supplied by the U.S. Industrial Chemical Co., Tuscola, IL; Hepes buffer was obtained from ICN Pharmaceuticals, Inc., Plainview, NY; Emulgen 913 was a gift from the Kao Soap Co., Tokyo, Japan; ethoxyresorufin was purchased from Pierce Chemicals, Rockford, IL; resorufin was obtained from Phaltz & Bauer, Stamford, CT; NADPH, nicotinamide, DL-isocitrate, isocitrate dehydrogenase, bovine β-glucuronidase, D-saccharic acid, 1,4-lactone, and bovine serum albumin were all purchased from the Sigma Chemical Co., St. Louis, MO.

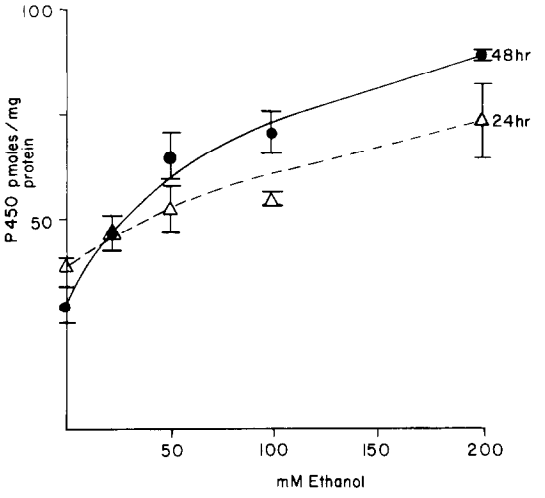

Fig. 1. Changes in cytochrome P-450 as a function of ethanol concentration. Cultures of chick embryo hepatocytes were prepared and treated with ethanol as described in Methods. Cytochrome P-450 was measured in the 8700 *g* supernatant fraction of cell homogenates [13]. Each value is the mean of measurements from duplicate plates.

RESULTS

Effect of ethanol on cytochrome P-450 and glucuronidation of phenol red

Figure 1 shows a dose-dependent increase in cytochrome P-450 after exposure of cultured hepatocytes to ethanol for either 24 or 48 hr. After 48 hr of exposure to ethanol at concentrations of more than 25 mM, there was an increase in P-450 compared to the 24-hr exposure. Therefore, cells were exposed to ethanol for 48 hr in all subsequent studies.

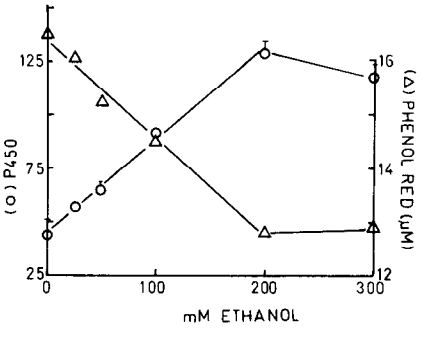

Fig. 2. Effect of ethanol on P-450 and glucuronidation of phenol red. Cultures of chick embryo hepatocytes were treated with increasing concentrations of ethanol for 48 hr. P-450 (pmole/mg protein) was measured in the 8700 *g* supernatant fraction of cell homogenates (see Methods). Glucuronidation of phenol red was measured as a decrease of absorption in the medium at 560 nm (see Methods). Each value is the mean and standard deviation from three individual 10 cm plates. Except where indicated, the standard deviation fell within the range of the symbols.

Table 1. Effect of ethanol and other chemicals on P-450 and associated oxidases*

Treatment	P-450†	Soret peak (nm)	Benzphetamine demethylation‡		Ethoxyresorufin deethylation§		Concentration of phenol red‖ (μM)
			Activity	Activity vs P-450 × 10^{-2}	Activity	Activity vs P-450 × 10^{-2}	
None	43 ± 9	452	3.1 ± 0.1	7.4 ± 1.6	0.25 ± 0.08	0.59 ± 0.09	16.5 ± 0.1
Ethanol (50 mM)	64 ± 5	452	6.2 ± 0.7	9.8 ± 1.3	0.84 ± 0.03	1.3 ± 1.0	15.7 ± 0.2
Ethanol (200 mM)	128.6 ± 6	452	14.2 ± 3.3	11.1 ± 2.3	0.90 ± 0.14	0.7 ± 0.09	12.8 ± 0.3
PIA (20 μg/ml)	354 ± 13	451.6	38.9 ± 2.8	11.0 ± 0.44	1.59 ± 0.02	0.45 ± 0.02	6.7 ± 0.11
β-NF (4 μg/ml)	171 ± 19	449	11.1 ± 0.8	6.5 ± 0.26	37.7 ± 2.1	22.3 ± 2.5	15.6 ± 0.1

* Cells were treated with each drug for 48 hr, as described in Methods. All cells were harvested in 0.1 M phosphate buffer (pH 7.4). Each value for P-450 and the associated enzyme activities is the mean ±S.D. from three pools of three plates each.
† Expressed as pmoles/mg protein.
‡ Expressed as nmoles HCHO produced per mg homogenate protein per hr.
§ Expressed as pmoles resorufin per min per mg homogenate protein.
‖ Each value is the mean ±S.D. from nine plates, measured separately.

Table 2. Ethanol concentrations in culture medium*

Initial concn. (mM)	+Cells	−Cells
25	25 (25, 25)	ND†
50	49 (49, 50)	49 (54, 45)
100	87 (84, 91)	ND
200	167 (163, 172)	160 (156, 164)

* The concentrations of ethanol in the medium were measured according to the procedure given in Methods. Each value is the mean of duplicate treatments (individual values are given in parentheses).
† Not determined.

Figure 2 compares the dose-response on P-450 with glucuronidation of phenol red. The dose-dependent increase in P-450 by ethanol paralleled the increase in glucuronidation of phenol red. Treatment of the medium from ethanol-treated cells with β-glucuronidaase resulted in complete recovery of the original absorbance to control levels. Saccharolactone, a competitive inhibitor of β-glucuronidase, prevented this restoration [alkaline absorbance at 560 nm: control, 0.124 ± 0.001 (S.D.); 200 mM ethanol, 0.093 ± 0.001; 200 mM ethanol and β-glucuronidase, 0.12 ± 0.001; 200 mM ethanol, β-glucuronidase and D-saccharic acid, 1,4-lactone, 0.091 ± 0.001].

PIA, a phenobarbital-like drug [14], also increased P-450 and the glucuronidation of phenol red in parallel, whereas β-NF, a P-448 inducer, did not (Table 1; Ref. 14).

Concentration of ethanol in the medium

Ethanol concentrations in the medium were measured after 48 hr of exposure to hepatocytes. As a control, ethanol was also measured in plates that contained medium, but no cells, and had been incubated for 48 hr. The results are presented in Table 2. There was no measurable difference between the concentrations of ethanol with and without cells. If

Table 3. Ethanol toxicity*

Treatment	Relative LDH activity in medium 24 hr	48 hr
None	1.0	1.0
Ethanol, 50 mM	1.32 (1.20, 1.45)	1.04 (0.98, 1.11)
100 mM	1.30 (1.37, 1.23)	1.01 (1.17, 0.85)
200 mM	1.35 (1.50, 1.19)	1.10 (0.99, 1.22)
300 mM	1.32 (1.17, 1.46)	2.03 (2.03, 2.03)
Triton X-100, 1%		6.80 (6.70, 6.90)

* Activities of LDH were measured in culture medium according to the procedure given in Methods, and are presented in relation to control activity. Each value is the mean of duplicate samples, with the individual ratios given in parentheses. Control activity for 24 hr = 6.07 units/6 cm plate (6.25, 5.90); 48 hr = 2.52 units/6 cm plate (2.43, 2.61). One unit is the amount of enzyme which causes oxidation of 1 μmole of NADH in 1 min.

chick cells metabolize ethanol at the same rate as rat hepatocytes [20], under our culture conditions no measurable change in ethanol concentration would be expected.

Cytotoxicity of ethanol

The amount of LDH released into the medium after exposure of the cells to increasing concentrations of ethanol was used to assess cell damage [15]. As shown in Table 3, concentrations of ethanol that caused increases in cytochrome P-450 caused very little release of LDH. Ethanol itself had no effect on the assay (results not shown). For comparison, we have also given the levels of LDH after treatment of the cells with 1% Triton X-100, which produces 100% cell lysis.

Increase in mixed-function oxidase activities associated with the increases in P-450

The results presented in Table 1 demonstrate an ethanol-mediated increase in both ethoxyresorufin deethylation and benzphetamine demethylation associated with the increase in cytochrome P-450. The demethylation of benzphetamine is a reaction preferentially catalyzed by P-450 rather than by P-448 (Ref. 17; Table 1), whereas the reverse is true for the deethylation of ethoxyresorufin (Ref. 21; Table 1). When the enzymatic activities are expressed relative to the amount of P-450, the effect of ethanol is seen to resemble that of PIA more closely than that of β-NF.

DISCUSSION

The results presented in this paper demonstrate that cellular levels of cytochrome P-450 are increased after exposure of cultured chick embryo hepatocytes to ethanol. The major advantages of this system, relative to the use of intact animals for study of both the mechanism of the increase and the overall effects on drug metabolism are: (1) the cells are maintained in a chemically defined environment, and (2) increases occur after only 24–48 hr of exposure to ethanol. In contrast, in intact rats a 19 per cent increase in cytochrome P-450 has been found 48 hr after administration of ethanol [22]. Larger increases (1.5 to 2-fold) require 7–30 days of ethanol treatment [1–3, 6, 22]. Treatment of the cells with 50 mM ethanol yielded a 2-fold increase in cytochrome P-450 (Figs. 1 and 2; Table 1). This concentration of ethanol is similar to plasma levels in man after an acute dose of alcohol [23]. The data help to explain the results of Traiger and Plaa [7] in which ethanol in rats increased the hepatotoxicity of CCl_4, a chemical that requires activation by P-450 in order to be hepatotoxic. The data may also explain why human alcoholics and mice given ethanol are more susceptible to hepatotoxic effects of acetaminophen, a compound that is rendered hepatotoxic through metabolism by P-450 [8].

The dose-response curve in Fig. 2 indicates that the maximum increase in P-450 occurred at ethanol concentrations that were two to three times the plasma levels after acute alcohol consumption in man. Cell toxicity, however, as measured by the release of LDH into the medium (Table 2), was negligible after a 48-hr exposure to ethanol in concentrations ranging from 25 to 200 mM. During the first 24 hr of exposure to ethanol, there was some release of LDH; the extent, however, was no greater at 200 mM ethanol than at 50 mM, a concentration which is similar to plasma levels in man after an acute dose of alcohol. The high concentration of ethanol needed for a maximum increase in P-450 (200 mM) may reflect a general property of water-soluble inducers of cytochrome P-450. In cultured chick embryo hepatocytes, phenobarbital and valproate, two inducers of cytochrome P-450 that are highly water-soluble, induce P-450 maximally at 1.57 mM and 10 mM respectively [14, 24]. PIA and AIA (allylisoproplacetamide), on the other hand, are more lipid-soluble, and both induce P-450 maximally at 0.08 mM in the culture (Ref. 13, results not shown).

One of the major detoxification pathways in the liver is conjugation of toxic metabolites with glucuronic acid. UDP-glucuronyltransferase, the enzyme that catalyzes this conjugation, is increased by inducers of cytochrome P-450. The transferase activity, however, differs depending on whether glucuronidation is stimulated by inducers of P-450 or P-448 [25]. Figure 2 indicates that the glucuronidation of phenol red was increased in a dose-dependent response to ethanol, in parallel with the increase in cytochrome P-450. The same response was obtained with PIA, a P-450 inducer, but not with β-NF, a P-448 inducer, indicating that phenol red is a substrate of a glucuronyltransferase that is increased by inducers of P-450. In intact rats, administration of ethanol for 15 days has also been found to increase hepatic glucuronyltransferase activity [26]. In contrast, short-term treatment with ethanol decreases glucuronyltransferase activity in isolated rat hepatocytes in suspension [27]. In conclusion, ethanol was found to increase cytochrome P-450 and glucuronyltransferase in cultured chick embryo hepatocytes in a manner similar to PIA, a phenobarbital-like inducer. It is anticipated that the chemically defined environment of the culture will facilitate further characterization of the effects of ethanol and aid in delineating the role of ethanol in affecting the metabolism of drugs, hepatotoxins, and carcinogens.

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