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# INCREASES IN CYTOCHROME P-450 IN CULTURED HEPATOCYTES MEDIATED BY 3- AND 4-CARBON ALCOHOLS

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**Abstract**—The amount of cytochrome P-450 was increased to different extents after treatment of cultured chick embryo hepatocytes with *n*-propanol, isopropanol, *n*-butanol, or isobutanol. These increases were associated with increases in benzphetamine demethylase activity, a cytochrome P-450-catalyzed oxidation, and glucuronidation of phenol red, catalyzed by UDP-glucuronyl transferase. The responses were similar to those obtained with ethanol or propylisopropylacetamide, which are phenobarbital-like inducers. Pretreatment of cells with cycloheximide prevented the increases in both cytochrome P-450 and glucuronidation of phenol red, indicating that protein synthesis was required for these responses.

Chronic consumption of alcoholic beverages by humans is associated with an increased clearance of several drugs [1]. Similar phenomena have been demonstrated in experimental animals and in humans exposed chronically to pure ethanol [2–4].

In rats [5, 6], hamsters [7] and cultured chick embryo hepatocytes [8], ethanol treatment results in increased levels of cytochrome P-450 and associated mixed-function oxidase activities, proteins involved in the oxidative metabolism of several drugs. The toxicities of $CCl_4$ [9, 10] and acetaminophen [11–13] are enhanced by either commercial alcoholic beverages or by pure ethanol. These chemicals ($CCl_4$ and acetaminophen) are rendered toxic as a result of their oxidation by cytochrome P-450.

Commercial alcoholic beverages contain, in addition to ethanol, higher chain alcohols. The proportion of higher chain alcohols present depends on the type of alcoholic beverage and the commercial preparation [14]. Several workers have shown that, like ethanol, higher chain alcohols increase the hepatotoxicity of $CCl_4$ in rats [9, 15, 16]. Increases in hepatic mixed-function oxidase activities have been found after 15–24 hr of exposure to some higher chain alcohols, although no increases in hepatic cytochrome P-450 have been found [17, 18].

In this paper, we show that cytochrome P-450 and associated enzymatic activities were increased in primary cultures of chick embryo hepatocytes treated with higher chain alcohols. The overall response of the cells to higher chain alcohols was similar to that seen after treatment of the cells with ethanol or propylisopropylacetamide. Propylisopropylacetamide is an effective inducer of cytochrome P-450, but not of P-448, in these hepatocytes [19].

## METHODS

*Preparation and treatment of cultured chick embryo hepatocytes.* Cultures of chick embryo hepatocytes were prepared in serum-free medium as described previously [19] with the following modifications. Livers were digested with 0.066% trypsin (Gibco, Grand Island, NY), and Varidase (Lederle Laboratories, Pearl River, NY) was added during the trypsinization [20]. The medium contained 20 mM HEPES‖ buffer in addition to the other supplements. Twenty hours after plating the cells, the medium was changed, and the chemicals were added. After 24 hr of exposure to the chemicals, the medium was changed again and the chemicals were re-added, unless stated otherwise. All plates containing alcohols were wrapped in Parafilm and Reynolds 912 film, a procedure found to minimize evaporation [8].

*Other assays.* Cytochrome P-450, glucuronidation of phenol red, 7-ethoxyresorufin-*O*-deethylase and benzphetamine-*N*-demethylase activities were all measured as described previously [8].

The protein concentrations in cell homogenates were measured by the procedure of Lowry *et al.* [21]. In cell preparations containing the detergent Emulgen 913, protein concentrations were assayed by either the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA) (Fig. 1 and Table 3) or the procedure of Lowry *et al.* [21] (Table 1), using BSA as the standard in both assays. In earlier experiments

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‖ Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; BSA, bovine serum albumin; SDS, sodium dodecylsulfate; ALA, δ-aminolevulinate; β-NF, β-naphthoflavone; Cx, cycloheximide; P-450, cytochrome P-450; PIA, propylisopropylacctamide; and TCA, trichloroacetic acid.

(Fig. 1), the Bio-Rad Assay was used for a rapid determination of the protein concentration in multiple samples. However, we later found that, although the Bio-Rad assay was linear with protein concentration, higher values of protein concentrations were obtained using the procedure of Lowry *et al.* In addition, the Bio-Rad Assay could not be used on cell homogenates not containing detergent. Because of these complications, we now measure all protein concentrations by the procedure of Lowry *et al.*

To measure the effect of cycloheximide on protein synthesis, hepatocytes plated in 3.5 cm plates were exposed to cycloheximide (0.5 μg/ml) for 24 hr and pulsed with [$^{14}$C]leucine (0.5 μCi/ml, 1 μCi/μmole) 2 hr before harvesting. At the end of the pulse period, cells were chilled, washed 3 times with 5% (w/v) TCA and rinsed with saline. The cells were then harvested in 0.8 ml of 1 N NaOH, transferred to a scintillation vial, and counted after the addition of 10 ml of scintillation fluid (ACS, Amersham, Arlington Heights, IL).

## RESULTS

*Effects of different alcohols on levels of cytochrome P-450 and glucuronidation of phenol red.* Figure 1 shows dose-dependent increases in cytochrome P-450 after exposure of cultured hepatocytes to different alcohols for 48 hr. Up to 50 mM propanol increased cytochrome P-450. Above 50 mM, the concentration of P-450 began to decrease. This decrease may reflect cytotoxicity, since cells came off the plate at 100 mM propanol (results not shown). In contrast, isopropanol at 100 mM was not toxic, as shown by continued increase of P-450. Both butanol and isobutanol were more potent and more efficacious inducers of cytochrome P-450 than *n*-propanol or isopropanol. In the experiments presented in Fig. 1, a 2-fold increase in cytochrome P-450 was obtained with the following concentrations of the $C_3$–$C_4$ alcohols: propanol and isopropanol, 40 mM; butanol, 5.5 mM; and isobutanol, 8 mM. In contrast, 200 mM ethanol was required to yield a 2-fold increase in P-450 in these experiments.

With all the alcohols except isobutanol, the dose-dependent increases in P-450 paralleled increases in the glucuronidation of phenol red, measured as decreases in the phenol red concentration in the medium (Fig. 1). The decrease in phenol red was due to glucuronidation, since treatment of the medium with β-glucuronidase restored phenol red to the initial concentration [8].

*Effect of cycloheximide on cytochrome P-450 and phenol red.* Table 1 shows that exposure of cells to cycloheximide, an inhibitor of protein synthesis, prevented the increases in both P-450 and glucuronidation of phenol red. Only a small decrease in P-450 was observed in control cells treated with cycloheximide. The species of P-450 present in control cells may have a longer half-life than the chemically induced forms [22]. In these experiments, cells were exposed to cycloheximide and the various chemical inducers for only 24 hr instead of the 48-hr exposure in the experiments of Fig. 1 and Table 2, to avoid any possible non-specific cytotoxicity that may have developed on a longer-term exposure to both drugs. At 24 hr the exposure of cycloheximide was not lethal to the cells since drug-mediated induction of δ-aminolevulinate (ALA) synthase was obtained after cycloheximide had been removed by changing the medium (results not shown).

Table 1. Effect of cycloheximide on cytochrome P-450 and phenol red*

| Additions | | Cytochrome P-450 (pmoles/mg protein) | Residual phenol red (μM) |
|---|---|---|---|
| None | | 49 | 18 |
| | | (48, 51) | (16.7, 19.3) |
| | + Cx | 34 | 18.6 |
| | | (32, 36) | (19.0, 18.2) |
| PIA | | 227 | 10.7 |
| | | (216, 239) | (11.5, 10.0) |
| | + Cx | 35 | 17.4 |
| | | (41, 30) | (17.6, 17.3) |
| Ethanol | | 72 | 15.7 |
| | | (72, 73) | (16.0, 15.4) |
| | + Cx | 32 | 18.4 |
| | | (31, 34) | (18.6, 18.2) |
| Butanol | | 147 | 15.6 |
| | | (141, 153) | (15.0, 16.2) |
| | + Cx | 36 | 18.3 |
| | | (37, 35) | (18.2, 18.5) |

* Cells were prepared as described in Methods and harvested 24 hr after the addition of the various chemicals. Cycloheximide (0.5 μg/ml) was added 1 hr prior to addition of the other chemicals. The concentrations of the chemicals were as follows: PIA, 140 μM; ethanol, 200 mM; and butanol, 20 mM. All plates contained ALA (25 μM) (see text). P-450 was measured in the 8700 *g* supernatant fraction of cells harvested in buffer containing Emulgen [19]. The concentration of phenol red in the media was measured as described in the legend to Fig. 1. The protein concentration was measured by the procedure of Lowry *et al.* [21] using BSA as a standard. The numbers in parentheses represent the individual data for duplicate plates.

The results indicate that the increases observed in both P-450 and glucuronidation of phenol red required protein synthesis. In this experiment, all media contained ALA (25 μM) since cycloheximide prevents induction of ALA synthase, the rate-limiting enzyme in heme biosynthesis. Preventing induction of ALA synthase of itself can cause a decrease in chemically induced cytochrome P-450 [23].

In separate plates, in which cell proteins were labeled with [$^{14}$C]leucine, the concentration of cycloheximide used was found to inhibit protein synthesis by 80–85%.

*Effects of various alcohols on mixed-function oxidase activities.* The results presented in Table 2 demonstrate alcohol-mediated increases in benzphetamine demethylase activity, but no increase in ethoxyresorufin deethylase activity. This pattern of increase in mixed-function oxidase activities is similar to that following treatment with propylisopropylacetamide, a drug which causes a phenobarbital-type response in the induction of cytochrome P-450 in these cells [24]. In contrast, the poly-

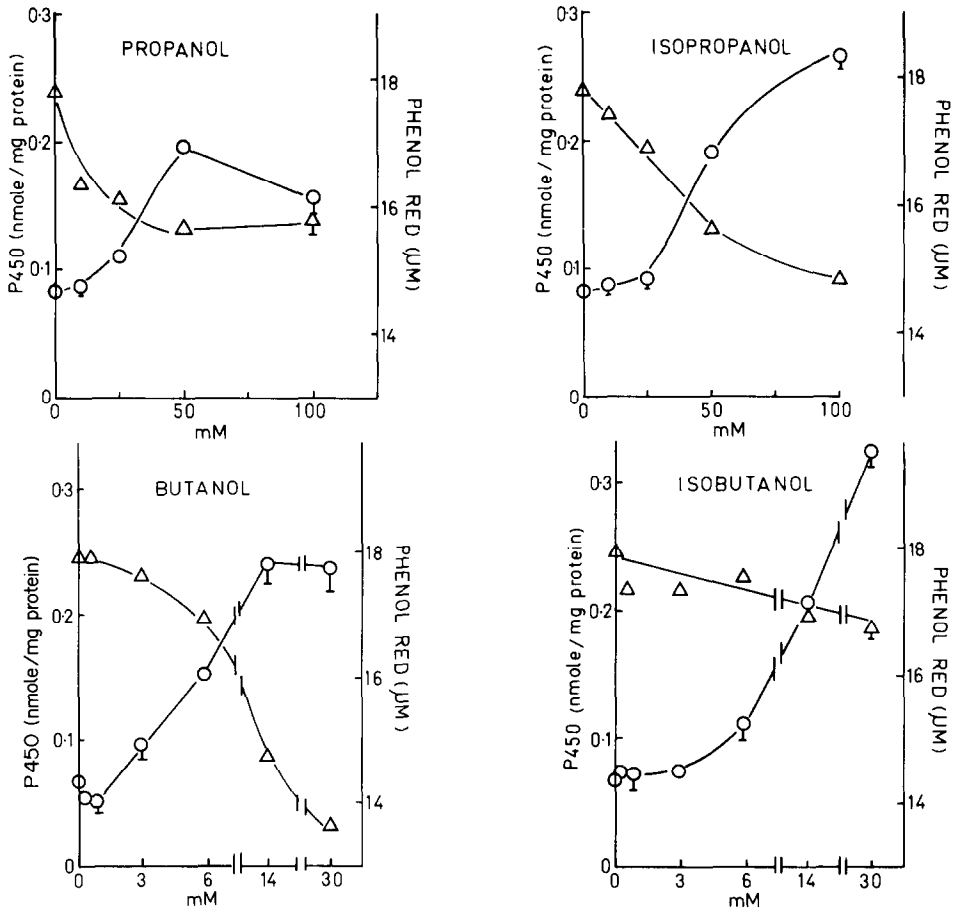


Fig. 1. Effects of $C_3$–$C_4$ alcohols on cytochrome P-450 and the glucuronidation of phenol red. Cells were prepared and treated with the various alcohols for 48 hr as described in Methods. Cytochrome P-450 (○) was measured in the 8700 *g* supernatant fraction of cells harvested in buffer containing the detergent Emulgen [19]. Protein concentrations were measured by the Bio-Rad Assay. Phenol red (△) was assayed from the O.D. at 560 nm after a 1:3 dilution of the media with 0.1% SDS, 0.1 N NaOH. Each point is the mean of three plates. Standard deviations fall within the symbols, unless shown.

Table 2. Effects of different chemicals on mixed-function oxidase activities*

| Additions | Dose | P-450† | Benzphetamine demethylase‡ | Ethoxyresorufin deethylase§ |
|---|---|---|---|---|
| None | | 50 (45, 55) | 3 (3, 3) | 0.48 (0.57, 0.40) |
| PIA | 140 μM | 192 (195, 190) | 52 (46, 58) | 0.63 (0.63, 0.64) |
| β-NF | 15 μM | 118 (114, 92) | 11 (13, 10) | 26.40 (29.0, 23.9) |
| Ethanol | 200 mM | 138 (152, 124) | 15 (13, 17) | 0.43 (0.44, 0.42) |
| *n*-Propanol | 25 mM | 76 (71, 81) | 13 (13, 13) | 0.40 (0.30, 0.50) |
| Isopropanol | 100 mM | 114 (120, 109) | 14 (13, 16) | 0.28 (0.30, 0.27) |
| *n*-Butanol | 14 mM | 187 (186, 189) | 42 (40, 44) | 0.50 (0.50, 0.50) |
| Isobutanol | 30 mM | 185 (170, 201) | 28 (27, 29) | 0.57 (0.60, 0.55) |

* Cells were treated with each drug for 41 hr and then harvested in 0.1 M phosphate buffer. Each value is the mean from two pools of three 10 cm plates each. The values for each pool are given in parentheses. Protein was measured by the procedure of Lowry *et al.* [21].

† Expressed in pmoles P-450/mg 8700 *g* supernatant protein.

‡ Expressed in nmoles HCHO per mg homogenate protein per hr.

§ Expressed in pmoles resorufin per mg homogenate protein per min.

cyclic hydrocarbon, β-naphthoflavone, an inducer of cytochrome P-448 [25], caused a greater increase in the deethylation of ethoxyresorufin than in the demethylation of benzphetamine. Isopropanol appears to have decreased ethoxyresorufin deethylation compared to control levels. However, the absolute fluorescent units in all treatments except for β-naphthoflavone were not much higher than the background fluorescence obtained from the appropriate unincubated sample. Therefore, the apparent decrease may not have been significant.

## DISCUSSION

We previously reported that treatment of cultured chick embryo hepatocytes with ethanol resulted in increases in cytochrome P-450 and associated oxidase activities, as well as in the glucuronidation of phenol red, the pH indicator in the medium [8]. Parallel inductions of P-450 and glucuronidation of phenol red were also obtained after treatment of the cells with propylisopropylacetamide (PIA), a chemical that induces P-450 (as opposed to P-448). The response to PIA was greater than that caused by phenobarbital (results not shown). Here we show that the higher chain alcohols, *n*-propanol, isopropanol, *n*-butanol and isobutanol, also caused increases in cytochrome P-450 in cultured chick embryo hepatocytes (Fig. 1). These alcohol-mediated increases in P-450 were associated with increases in the activity of benzphetamine demethylase (Table 2), a P-450-catalyzed reaction. There was no increase, however, in the activity of ethoxyresorufin deethylase, a reaction preferentially catalyzed by cytochrome P-448 ([26]; Table 2).

The $C_2$ to $C_4$ alcohols constitute the simplest molecules reported to elicit a phenobarbital-like induction of cytochrome P-450 in hepatocytes. Like most P-450 inducers [27], the potency of the alcohols increased with increasing lipophilicity (Fig. 1). Other than lipophilicity, no specific structure–function relationship has been established for chemicals that induce cytochrome P-450 [27].

Several phenobarbital-like inducers of P-450 also induce ALA synthase, the rate-limiting enzyme in heme biosynthesis [28]. As with P-450, there is no defined structure–function relationship for inducers of ALA synthase. However, potency has been found to correlate with lipophilicity and metabolic stability [28, 29]. Perhaps this is also true for inducers of P-450 [27]. In contrast, chemicals that induce cytochrome P-448 were found to have specific structural requirements [30].

The induction of P-450 by the alcohols studied in this paper may help explain the potentiation of $CCl_4$ hepatotoxicity by these higher chain alcohols since $CCl_4$ and acetaminophen must be metabolized by cytochrome P-450 to produce hepatotoxic effects [16]. In addition, numerous potential carcinogens in the environment are converted to their active forms via oxidation by cytochrome P-450. Heavy drinkers, particularly those who also smoke cigarettes, have a higher incidence of cancer than the non-alcoholic population, especially cancers of the upper respiratory and upper alimentary tracts [31]. Our results suggest that, in addition to ethanol, higher chain alcohols, present in commercial alcoholic beverages, may contribute to the increase in cancer incidence via their induction of cytochrome P-450. Isobutanol and isopentanol are the most abundant higher chain alcohols present in alcoholic beverages [14]. Red wine contains 1–3 mM isobutanol and cognac contains 1–5 mM isobutanol depending on the commercial preparation. The concentrations of higher chain alcohols that occur in peripheral blood and livers of humans who chronically ingest red wine or cognac are unknown. They may be lower than the concentrations required to induce cytochrome P-450 in our culture system. Higher concentrations of water-soluble, compared to lipid-soluble, chemicals are required to induce P-450 in the cultured hepatocytes (Fig. 1; [8]), a property which may be an artifact of the aqueous environment of the culture. In any case, the inducing concentrations of all the alcohols tested were not lethal to the cells, as evidenced by microscopic examination and by the increases in both P-450 and glucuronidation of phenol red (Fig. 1). In addition, the combination of the various alcohols at lower concentrations may have additive or even synergistic effects on the induction of P-450, a possibility that we are planning to investigate. All the exposures, in the studies presented in this paper, were relatively short-term (48 hr). Under these conditions, 5 mM butanol, a concentration present in certain commercial alcoholic beverages, yielded a 1.5-fold increase in P-450. Long-term exposure to these or lower concentrations, as occurs in heavy drinkers, may have more pronounced effects.

For all the alcohols, except isobutanol, the increases in cytochrome P-450 in cultured chick embryo hepatocytes were associated with increases in the glucuronidation of phenol red, the pH indicator in the media (Fig. 1). UDP-glucuronyl transferase is increased by chemicals that induce cytochrome P-450; however, the substrate specificity of the transferase depends on the type of inducer [32]. We have found that phenol red is a substrate of a transferase that is increased by inducers of P-450, but not by inducers of P-448 [23]. It is possible that isobutanol also increased glucuronyl transferase but acted as a competitive inhibitor of phenol red conjugation. In isolated microsomes, $C_1$–$C_4$ alcohols have been shown to competitively inhibit glucuronyl transferase activity when *p*-nitrophenol is the substrate [33]. Short-term treatment of rabbits with $C_1$–$C_2$ alcohols also inhibits glucuronyl transferase activity *in vivo* [34]. In contrast, chronic exposure of rats to ethanol results in an increase in this activity [35]. All the $C_2$–$C_4$ alcohols tested here were competitive inhibitors of the glucuronidation of phenol red, the extent of inhibition having increased with lipophilicity (results not shown). Therefore, the effect on phenol red conjugation that we observed after treatment with the $C_2$–$C_4$ alcohols may reflect the net effect of increasing the amounts of UDP-glucuronyl transferase and competitive inhibition by the alcohols of the glucuronidation of phenol red.

In summary, we have found that the higher chain alcohols, *n*-propanol, isopropanol, *n*-butanol and isobutanol, increased cytochrome P-450 and associated enzyme activities in cultured chick embryo

hepatocytes. All alcohols tested, except isobutanol, also caused an associated increase in the glucuronidation of phenol red.

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