Biochemical Pharmacology, Vol. 33, No. 2, pp. 187–190, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

AMINOPYRINE AND BIPHENYL METABOLISM IN CULTURED HEPATOCYTES

INDUCTION BY ALCOHOLS

JACQUELINE F. SINCLAIR,*†‡ PHILIP WIEBKIN,§ LINDA M. ZAITLIN,* E. LUCILE SMITH*† and PETER R. SINCLAIR*†

*VA Medical Center, White River Junction, VT 05001; and Departments of †Biochemistry and §Pathology, Dartmouth Medical School, Hanover, NH 03755, U.S.A.

(*Received* 13 *April* 1983; *accepted* 24 *June* 1983)

Abstract—Exposure of cultured chick embryo hepatocytes to ethanol, isobutanol, or isopentanol, the predominant alcohols present in commercial alcoholic beverages, resulted in increased metabolism of aminopyrine or biphenyl by the intact cells. The increases correlated with induction of cytochrome P-450. Hydroxylation of biphenyl at the 4-position was preferentially increased in cells pretreated with either the alcohols or propylisopropylacetamide, a barbiturate-like inducer of cytochrome P-450. In contrast, exposure of the cells to 3,4,3′,4′-tetrachlorobiphenyl, a planar polycyclic aromatic hydrocarbon inducer of P-450, resulted in preferential increased hydroxylation at the 2- and 3-positions of biphenyl.

Since the major site of drug metabolism in the body is the liver, many studies have focused on the effects of alcohol on hepatic functions involved in the metabolism of drugs. Enhanced drug metabolism is a major clinical finding in the alcoholic population [1–3]. This effect of alcohol may be related to induction of hepatic cytochrome P-450 since exposure of experimental animals to ethanol results in increased levels of hepatic cytochrome(s) P-450 and associated mixed-function oxidase activities [4–6]. We have shown that ethanol also induces P-450‖ in primary cultures of chick embryo hepatocytes [7].

Although ethanol is the major alcohol in commercial alcoholic beverages, higher chain alcohols are also present [8]. Isopentanol and isobutanol comprise 90–98% of these alcohols. In rats, a single oral dose of isobutanol has been found to increase hepatic microsomal mixed-function oxidase activity, but no increase in the amount of cytochrome P-450 was observed spectrophotometrically [9]. In contrast, in a system of cultured chick embryo hepatocytes which are inducible for various species of cytochrome(s) P-450, C_3- and C_4-chain alcohols, including isobutanol, induce both cytochrome P-450 and associated oxidase activities [10]. Isopentanol was not tested in those studies. In this paper, we report that isopentanol also induces cytochrome P-450 in these cultured hepatocytes. Exposure to ethanol, isobutanol, or isopentanol resulted in increased metabolism of aminopyrine and biphenyl by intact cells, the extent of metabolism correlating with the increase in cytochrome P-450.

METHODS

Preparation and treatment of cultured chick embryo hepatocytes. Primary cultures of chick embryo hepatocytes were prepared as described previously [10]. The size of the plates and amount of cell protein per plate are indicated in the figure and table legends. Twenty hours after plating the cells, the medium was changed to Williams E containing dexamethasone (0.3 μg/ml), 3,3′,5-triiodothyronine (1 μg/ml) and 20 mM HEPES, and the chemical inducers of cytochrome P-450 were added. The duration of exposure is indicated in the figure legends. For the 48-hr exposure, the medium was changed after the first 24 hr, and the chemicals were re-added. All plates containing alcohols were wrapped in Parafilm and Reynolds 912 film, a procedure found to minimize evaporation [7]. At the end of the treatment with inducers of P-450, the medium was removed, and fresh medium containing either biphenyl (70 μM, as described [11]) or aminopyrine (650 μM) was added. The duration of exposure to these substrates is indicated in the figure legends. Cells were routinely examined by phase contrast microscopy.

Assays. Cytochrome(s) P-450 was measured in the 8700 *g* supernatant fractions prepared from all homogenates, as described previously [12].

Protein concentrations were assayed by the procedure of Lowry *et al.* [13] using BSA as a standard.

The biphenyl hydroxylated products were determined fluorimetrically as described [14]. Cells and media were harvested together and incubated with β-glucuronidase (365 Fishman units/ml cell suspension) for 1 hr at 37°. The β-glucuronidase was dissolved in 0.2 M acetate buffer, pH 4.5. This fluorimetric analysis of the hydroxylated products of

‡ All correspondence should be sent to: Jacqueline F. Sinclair, Research Service (151A), Veterans Administration, White River Junction, VT 05001.

‖ Abbreviations: P-450, cytochrome P-450; PIA, propylisopropylacetamide; TCB, 3,4,3′,4′-tetrachlorobiphenyl; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid; BSA, bovine serum albumin; and PAH planar polycyclic aromatic halogenated hydrocarbons.

biphenyl metabolism does not resolve 2- and 3-hydroxybiphenyls and, therefore, these two products can only be treated as a single metabolite.

4-Aminoantipyrine, a metabolite of aminopyrine, was measured in the medium as described [15], after treatment with Glusulase (37° 24 hr, units/ml: glucuronidase 572, sulfatase 104). We have confirmed the observation of Poland and Kappas [15] that all 4-aminoantipyrine formed is recovered in the media (results not shown).

Source of chemicals. Biphenyl was purchased from Pfaltz & Bauer, Inc. Stamford, CT; aminopyrine from Merck, Darmstadt, Germany; and Glusulase from Endo Laboratories, New York, NY. PIA was a gift from Hoffmann–LaRoche, Nutley, NJ; Emulgen 913 was a gift from the Kao Soap Co., Tokyo, Japan, HEPES buffer was purchased from the Calbiochem-Behring Corp., La Jolla, CA; BSA, insulin (bovine pancreas), T3 (sodium salt) and β-glucuronidase, Type H-1, were from the Sigma Chemical Co., St. Louis, MO; dexamethasone (sodium phosphate salt) was from Lypho-Med Inc., Chicago, IL; and 3,4,3′,4′-tetrachlorobiphenyl was from Ultrascience, Hope, RI, Ethanol (USP grade), purchased from the U.S. Industrial Chemical Co., Tuscola, IL and isobutanol (certified ACS) and isopentanol (certified), purchased from the Fisher Scientific Co., Fair Lawn, NJ, were all glass distilled before use.

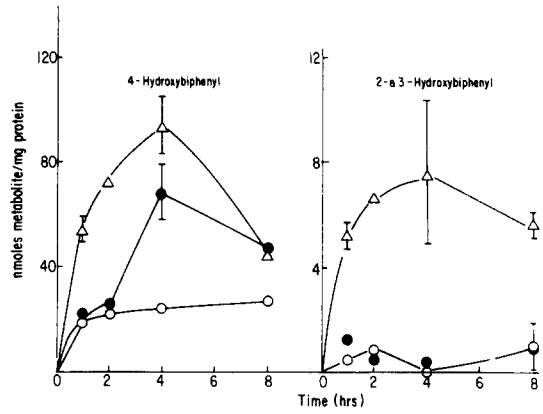

Fig. 2. Time course of biphenyl metabolism. Hepatocytes, plated on 10 cm dishes (1.5 to 2 mg protein/plate), were treated with inducers of P-450 for 23 hr. At the end of this time period, the medium was changed to remove the inducing chemical, and biphenyl was added to a concentration of 70 μM. At each time point, plates were chilled, and cells and medium were harvested together and stored frozen until analysis (see Methods). Symbols for the various inducers were as follows: (○) none; (●) PIA, 140 μM; (△) TCB, 70 nM. The vertical bars represent individual values that do not fall within the symbols. The results presented were obtained from a single time course experiment.

RESULTS

Time course of aminopyrine metabolism. Primary cultures of chick embryo hepatocytes were exposed to PIA, a phenobarbital-like inducer of P-450 in these cells [7, 10], for 21 hr. After removal of PIA by changing the medium, addition of 4-aminopyrine resulted in a nearly constant rate of 4-aminoantipyrine formation over 8 hr (Fig. 1) with no change in the level of cytochrome P-450 (161 ± 13 pmoles/mg of 8700 *g* supernatant protein). In cells not exposed to PIA, the rate of 4-aminoantipyrine formation was less and decreased rapidly with time (Fig. 1) even though P-450 levels remained fairly constant up to 4 hr (47 ± 10 pmoles/mg of 8700 *g* supernatant protein) and increased at the later time points (86 ± 10 pmoles/mg of 8700 *g* supernatant protein).

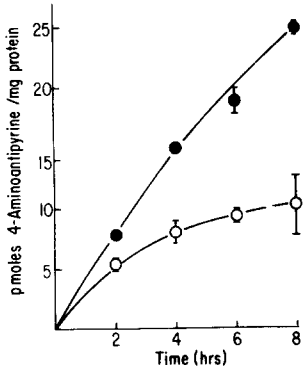

Fig. 1. Time course of aminopyrine metabolism. Hepatocytes, plated on 6 cm dishes (0.7 to 0.9 mg protein/plate) were either untreated or exposed to PIA (140 μM) for 21 hr. At the end of this time period, the medium was changed, and aminopyrine was added to a concentration of 650 μM. After 2, 4, 6 and 8 hr, the medium was removed and analyzed for 4-aminoantipyrine as described in Methods. Key: (○) untreated cells; and (●) PIA. Each measurement represents the mean value from duplicate plates. The vertical bars indicate individual values that do not fall within the symbols.

Time course of biphenyl metabolism. The effects of PIA and TCB on biphenyl metabolism are shown in Fig. 2. Treatment of cells with either PIA or TCB resulted in a marked increase in 4-hydroxybiphenyl production compared to untreated cells. This increased formation of 4-hydroxybiphenyl compared to untreated cells was observed over the entire 8 hr incubation with TCB, but only at the later time points (4 hr and 8 hr) with PIA.

Maximal formation of 4-hydroxybiphenyl was detected at 4 hr following prior exposure of the hepatocytes to either inducer. A subsequent decrease in recoverable metabolite was noted at the 8 hr time point (TCB, 53%; PIA, 30%). These decreases were not due to apparent toxicity since the cells appeared normal on examination by phase contrast microscopy, and there was no significant difference in the amount of cell protein adhering to the plates (results not shown). In addition, there was no decrease in cytochrome P-450 in either untreated or TCB-treated cells (results not shown).

In contrast to the hydroxylation of biphenyl at the 4-position, there was little to no formation of 2- and 3-hydroxybiphenyl in either control or PIA-treated cells over 8 hr. Treatment of cells with TCB, how-

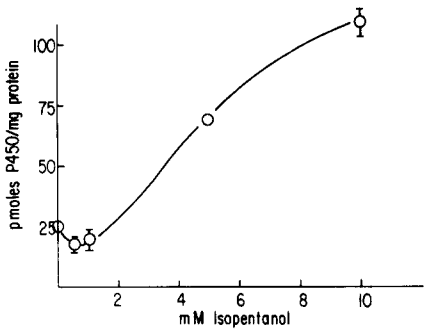

Fig. 3. Dose response of isopentanol on induction of cytochrome P-450. Hepatocytes were plated on 10 cm dishes (2 mg protein/plate) and exposed to increasing concentrations of isopentanol for 24 hr. Cytochrome P-450 was measured in the 8700 *g* supernatant fraction [12]. Each measurement represents the mean value from duplicate plates. The vertical bars represent individual values that do not fall within the symbols.

ever, caused an increase in 2- and 3-hydroxybiphenyl formation up to 4 hr. On longer incubation, there was a small decrease in 2- and 3-hydroxybiphenyl recovered.

Effect of isopentanol on levels of cytochrome P-450. In a previous paper, we reported that 2 to 4 carbon chain alcohols induce cytochrome P-450 [10]. Isopentanol, a prominent alcohol in commercial alcoholic beverages [8], was not tested in that study. Here we show induction of cytochrome P-450 in cultured hepatocytes exposed to increasing concentrations of isopentanol (Fig. 3). A 10 mM concentration of isopentanol caused a 4-fold increase over control levels in this experiment. At concentrations greater than 10 mM, increased numbers of floating cells and rounding-up of cells still attached to the plate were observed, and levels of cytochrome P-450 were below those detected in untreated cells (results not shown).

Effects of ethanol, isobutanol, and isopentanol on metabolism of aminopyrine and biphenyl. Cells were exposed to either aminopyrine or biphenyl for only 4 hr due to the decrease in rate of biphenyl metabolism (Fig. 2) and possible induction of cytochrome P-450 at the later time point. As shown in Table 1, prior exposure of hepatocytes to concentrations of ethanol, isobutanol or isopentanol found maximal for induction of P-450 ([7, 103], Fig. 1) resulted in increases in the metabolism of both aminopyrine and biphenyl.

Only formation of 4-hydroxybiphenyl by the intact cells was increased by the alcohol pretreatment. Hydroxylation at the 2- and 3-positions was either identical to the control, or lower.

Both isobutanol and isopentanol caused greater increases in 4-hydroxybiphenyl and 4-aminoantipyrine formation than ethanol, with the actual amounts of metabolites formed being similar to those detected after treatment of the cells with PIA, the most potent inducer of P-450 in this system.

DISCUSSION

We have reported previously that treatment of cultured chick embryo hepatocytes with ethanol increases both cytochrome P-450 and UDP glucuronyl transferase [7]. Isobutanol and isopentanol, the predominant higher chain alcohols in commercial alcoholic beverages [8], also induced cytochrome P-450 in these cells (Fig. 3, Table 1) to amounts 1.5 times the ethanol-induced levels. Since P-450 is involved in the metabolism of several drugs, induction of the cytochrome could lead to an increase in drug metabolism. In this paper, we demonstrate a direct correlation between increased metabolism of P-450 substrates by intact hepatocytes and induction of cytochrome P-450 in these cells. The inducers of P-450 tested caused increased metabolism of 4-aminopyrine and biphenyl by intact cells.

It is now well established that different species of cytochrome P-450 are inducible depending on the chemical inducers and the animals being tested. In

Table 1. Metabolism of aminopyrine or biphenyl after treatment with various inducers of cytochrome P-450*

Inducer	Concn	Cytochrome P-450†	Metabolite formation‡ 4-Aminoantipyrine	4-OH Biphenyl	2- and 3-OH Biphenyl
None		56	1.6	15.9	2.7
		(65, 46)	(1.8, 1.4)	(14.3, 17.6)	(2.6, 2.9)
PIA	140 μM	141	7.1	56.2	4.8
		(148, 133)	(7.3, 6.9)	(55.8, 56.7)	(4.9, 4.8)
TCB	70 nM	140	3.5	57.9	15.6
		(140, 140)	(3.4, 3.6)	(67.3, 48.6)	(14.4, 16.9)
Ethanol	200 mM	94	2.8	29.4	3.7
		(89, 99)	(3.3, 2.3)	(32.3, 26.5)	(3.1, 4.4)
Isobutanol	30 mM	136	6.7	57.6	0.8
		(132, 140)	(6.8, 6.6)	(59.0, 56.2)	(1.1, 0.5)
Isopentanol	10 mM	154	5.3	67.6	0.9
		(152, 156)	(5.7, 4.9)	(63.4, 71.8)	(1.8, 0)

* Hepatocytes were plated on 10 cm dishes (1 to 1.5 mg protein/plate) and treated with the chemicals indicated for 48 hr. At the end of this time period, the medium was changed to remove the inducing chemical. Separate sets of duplicate plates were exposed to either biphenyl (70 μM) or aminopyrine (650 μM) for 4 hr. Cultures were analyzed for cytochrome P-450 and/or metabolites as described in Methods. Each value represents the mean of duplicate plates with individual values in parentheses. Abbreviations: PIA, propylisopropylacetamide; and TCB, 3,4,3′,4′-tetrachlorobiphenyl.

† Expressed in pmoles/mg of 8700 *g* supernatant protein.

‡ Expressed in nmoles/mg cellular protein.

addition, certain substrates are metabolized to different products depending on the species of P-450 induced. In rats and hamsters, PAH induce hepatic hydroxylation of biphenyl at both the 2- and 4-positions while barbiturates induce hydroxylation at the 4-position only. These metabolic profiles have been detected in hepatic microsomes, homogenates [14, 16, 17], and suspensions of hepatocytes [18] isolated from rats treated with either phenobarbital or 3-methylcholanthrene.

Similar profiles of biphenyl metabolism occurred in cultured chick embryo hepatocytes induced for cytochrome(s) P-450 with either PIA or TCB (Fig. 2, Table 1). Exposure to either PIA or TCB increased hydroxylation of biphenyl at the 4-position by intact hepatocytes (Fig. 2). Only TCB, however, caused a modest increase in the formation of 2- and 3-hydroxybiphenyls. These results contrast with the findings in the intact chick embryo, in which β-naphthoflavone, though a PAH, did not induce hepatic biphenyl 2-hydroxylation [19].

When 4-hydroxybiphenyl formation was monitored for 8 hr after exposure of the cells to biphenyl, a decrease in metabolites was observed at the later time points. This is possibly due to further metabolism of the 4-hydroxybiphenyl to 4,4′-dihydroxybiphenyl, which exhibits a weaker fluorescence at the wavelength maxima used to measure 4-hydroxybiphenyl [11]. The decrease of total metabolites detected cannot be attributed to cell toxicity since P-450 levels were maintained (results not shown), no protein was lost from the plate, and direct microscopic examination revealed normal morphology.

Treatment of the cultured hepatocytes with ethanol, isobutanol, or isopentanol resulted in increased metabolism of biphenyl to the 4-hydroxy product and no detectable increase in 2- and 3-hydroxybiphenyl formation. With isobutanol and isopentanol pretreatment, the amount of biphenyl metabolites formed was similar to that detected after prior exposure of the cells to PIA (Table 1).

Since higher chain alcohols are present in commercial alcoholic beverages, the amounts depending on the particular beverage and the commercial preparation [8], it is important to know what effect such combinations of ethanol with the higher chain alcohols have on drug metabolism. Wine contains 0.5–3 mM isobutanol and 1–4 mM isopentanol. Cognac contains larger amounts of these alcohols (1–5 mM isobutanol and 3–20 mM isopentanol) [8]. The concentrations of all the alcohols in the culture medium are higher than those detected in the plasma of experimental animals and humans after acute exposure to these alcohols [20]. The concentrations of isopentanol and isobutanol that occur in peripheral blood and livers of humans who consume commercial alcoholic beverages are not known. Hepatic levels may be considerably higher than those in peripheral blood since portal ethanol levels after an acute exposure are approximately 3 times the plasma levels [21]. In addition, the high concentrations of the alcohols required to induce P-450 in the cultured hepatocytes may reflect the aqueous environment of the culture, since we have found that higher concentrations of water-soluble, compared to lipid-soluble, chemicals are required to induce P-450 in these cultured hepatocytes [7]. In any case, in this study the inducing concentrations of the alcohols were not toxic to the cells since there was no increased release of lactate dehydrogenase into the media compared to untreated cells (results not shown). We have found recently that combinations of ethanol with isopentanol, but not with isobutanol, at the proportions present in commercial alcoholic beverages, caused synergistic inductions of cytochrome P-450 (results not shown).

Our results suggest that the longer chain alcohols, isobutanol and isopentanol, which comprise 90–98% of the higher chain alcohol constituents of commercial beverages, may contribute significantly to increased drug metabolism observed in chronic alcoholics.

Acknowledgements—We thank S. Shedlofsky for his assistance and fruitful discussions, J. Pomeroy for excellent technical assistance, and D. Burke for her patience and expertise in typing the manuscript. This work was supported by grants from the VA and NIH (CA 25012 and CA 30650).

REFERENCES

1. P. Sandor, E. Sellers, M. Dumbrell and V. Khouw, *Clin. Pharmac. Ther.* **30**, 390 (1981).
2. R. Kater, G. Roggin, F. Toban, P. Zieve and F. Iber, *Am. J. med. Sci.* **258**, 35 (1969).
3. E. Rubin, P. Bacchin, H. Gang and C. Lieber, *Lab. Invest.* **22**, 569 (1970).
4. J. Joly, J. Villeneuve and P. Mavier, *Alcoholism* **1**, 17 (1977).
5. G. McCoy, C. Chen, S. Hecht and E. McCoy, *Cancer Res.* **39**, 793 (1979).
6. R. Sharma, R. Cameron, E. Farber, M. Griffin, J. Joly and R. Murray, *Biochem. J.* **182**, 317 (1979).
7. J. F. Sinclair, P. R. Sinclair, E. L. Smith, W. J. Bement, J. Pomeroy and H. Bonkowsky, *Biochem. Pharmac.* **30**, 2805 (1981).
8. J. Ribereau-Gayon and E. Peynaud, in *International Encyclopedia of Pharmacology and Therapeutics* (Eds. C. Radouco-Thomas and J. Trémoliéres), Vol. I, p. 67. Pergamon Press, New York (1970).
9. G. Powis, *Biochem. J.* **148**, 269 (1975).
10. J. F. Sinclair, L. Smith, W. J. Bement, P. R. Sinclair and H. L. Bonkowsky, *Biochem. Pharmac.* **31**, 2811 (1982).
11. P. Wiebkin, J. Fry, C. Jones, R. Lowing and J. Bridges, *Xenobiotica* **6**, 725 (1976).
12. J. F. Sinclair, P. R. Sinclair and H. L. Bonkowsky, *Biochem. biophys. Res. Commun.* **86**, 710 (1979).
13. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
14. M. Burke and R. Prough, in *Methods in Enzymology* (Eds. S. Fleischer and L. Packer), Vol. 52, p. 399. Academic Press, New York (1978).
15. A. Poland and A. Kappas, *Molec. Pharmac.* **7**, 697 (1971).
16. M. Burke and J. Bridges, *Xenobiotica* **5**, 357 (1975).
17. P. Creaven and D. Parke, *Biochem. Pharmac.* **15**, 7 (1966).
18. P. Wiebkin, J. Fry, C. Jones, R. Lowing and J. Bridges, *Biochem. Pharmac.* **27**, 1899 (1978).
19. A. Rifkind, M. Troeger and H. Muschick, *J. biol. Chem.* **257**, 11717 (1982).
20. R. Derache, in *International Encyclopedia of Pharmacology and Therapeutics* (Eds. C. Radouco-Thomas and J. Trémoliéres), Vol. 2, p. 507. Pergamon Press, New York (1970).
21. I. Beck, G. Paloschi, P. Dinda and M. Beck, *Gastroenterology* **67**, 484 (1974).