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EFFECT OF XENOBIOTICS ON MONOOXYGENASE ACTIVITIES IN CULTURED HUMAN HEPATOCYTES

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Abstract—The activity of human cytochrome P450 monooxygenases, aryl hydrocarbon hydroxylase and 7-ethoxycoumarin *O*-deethylase can be increased by 3-methylcholanthrene, phenobarbital and ethanol in human hepatocytes maintained in primary culture. Total cytochrome P450 content increased two-fold after 48 hr of incubation with methylcholanthrene or phenobarbital and 1.5-fold after incubation with ethanol. The three chemicals elicited different effects on cytochrome P450 dependent activities. Addition of 3-methylcholanthrene caused a time- and concentration-dependent increase in both monooxygenase activities, aryl hydrocarbon hydroxylase and 7-ethoxycoumarin *O*-deethylase, while phenobarbital and ethanol increased 7-ethoxycoumarin *O*-deethylase activity but had no effect on aryl hydrocarbon hydroxylase. Dexamethasone *per se* had little or no effect on either monooxygenase activities, but potentiated the effect of the three chemicals on 7-ethoxycoumarin *O*-deethylase.

Xenobiotics, among them drugs administered to humans for therapeutical purposes, are biotransformed in the liver by two types of reactions: metabolization by the cytochrome P450 associated monooxygenases (Phase I reactions), and/or conjugation with glucuronic acid, sulphate or reduced glutathione (Phase II). This greatly facilitates their further excretion into bile and urine. The need for broad substrate specificity is fulfilled by the existence of multiple forms of cytochrome P450, each separately regulated and with limited substrate specificity [1]. Some monooxygenases are constitutively expressed in hepatocytes, while others can be induced by xenobiotics [2]. The substrate of a particular cytochrome P450 form can often induce the levels of the isozyme in question. Monooxygenases can be induced in experimental animals by polycyclic hydrocarbons (methylcholanthrene, MC†) and barbiturates (phenobarbital, Pb), which represent the two major types of enzyme inducers [3]. A third group is exemplified by hormonal steroids such as pregnenolone 16α-carbonitrile [4]. Chronic exposure to ethanol (Et-OH) also increases hepatic monooxygenases in experimental animals [5, 6]. These findings may explain why animals exposed to ethanol exhibit increased clearance of some drugs, and why ethanol enhances the toxicity of agents believed to be toxic as a result of metabolic activation of monooxygenases [7, 8].

The study of enzyme induction is of great relevance to an understanding of drug hepatotoxicity in man. Induction of drug-metabolizing enzymes may alter the rate and extent of singular biotransformation pathways of a certain drug and thereby contribute to interindividual metabolic differences and toxicity [2, 3]. Increases in hepatic drug-metabolizing enzyme activities after exposure to drug-inducers is very difficult to characterize in man, where experiments, for obvious ethical reasons, are very limited. Primary cultures of adult human hepatocytes offer an attractive model for characterizing human liver drug metabolism and its response to xenobiotics.

In the experiments presented in this paper we have studied dose and time-course activity of two representative monooxygenases (aryl hydrocarbon hydroxylase, AHH, and 7-ethoxycoumarin *O*-deethylase, ECOD) in presence of MC and Pb, and the role of glucocorticoids on drug effects. In addition, we present evidence showing that human hepatocytes also respond to Et-OH by increasing the levels of cytochrome P450 and ECOD activity.

MATERIALS AND METHODS

Material. Collagenase was obtained from Boehringer (Mannheim, F.R.G.) (Art. N 103 586); Fetal and adult calf sera were purchased from Gibco (Paisley, U.K.); Culture media were from Flow (Irvine, U.K.). Et-OH was obtained from Merck (Darmstadt, F.R.G.); Pb, MC, benzo(*a*)pyrene and 7-ethoxycoumarin were purchased from the Sigma Chemical Co. (St Louis, MO). All other reagents used in this study were of analytical grade.

Isolation and culture of hepatocytes. Surgical liver biopsies (1–3 g), were obtained from patients undergoing cholecistectomy after informed consent was obtained. Patients had no other liver pathology nor did they receive medication during the week prior to surgery. None of the patients were habitual consumers of alcohol or other drugs. The patient's ages ranged 45 to 65 years old. Five liver biopsies were obtained from males (A, B, G and K) and seven

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† Abbreviations: AHH, aryl hydrocarbon hydroxylase; Dex, dexamethasone; DMSO, dimethyl sulfoxide; ECOD, 7-ethoxycoumarin *O*-deethylase; EGTA, ethylenglycol-bis-(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid; Et-OH, ethanol; HEPES, *N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid; MC, 3-methylcholanthrene; Pb, phenobarbital.

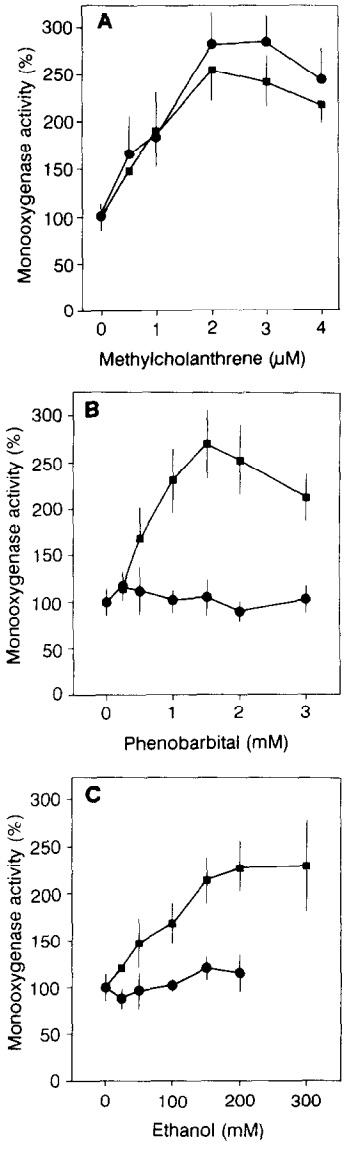

Fig. 1. Dose-dependent effect of 3-methylcholanthrene, phenobarbital and ethanol on monooxygenase activities. Cultures were exposed 6 hr after cell-attachment to increasing concentrations of 3-methylcholanthrene (A), phenobarbital (B) or ethanol (C), and AHH (●) and ECOD (■) activities were measured 42 hr later. AHH and ECOD activities in control cultures were 7.94 ± 1.32 and 2.10 ± 0.28 pmol/min/mg, respectively. Values represent mean ± SD of five to six independently treated plates from two different hepatocyte preparations (cultures F and G) and the data are expressed as percent of controls.

from females (C–F, H–J and L). Care was taken to ensure that the liver pieces had only one cut surface.

Hepatocytes were isolated by a two-step technique involving perfusion of the liver biopsy [9]. During the first step, the tissue was perfused with 250 mL of a calcium-free buffer (137 mM NaCl, 2.68 mM KCl, 0.7 mM $Na_2PO_4H.12H_2O$, 10 mM HEPES, 10 mM glucose and 0.5 mM EGTA, pH 7.5). The flow rate was 10 mL/min/catheter. The washing perfusate was allowed to drain and was not recirculated. The second step was a recirculating perfusion with the same buffer without EGTA but with 5 mM $CaCl_2$ and 0.5 mg/mL collagenase (enzymatic activity 0.37 units/mg), at the same flow rate for about 30 min, until sufficient softening of the tissue was obtained. The perfusion buffers were continuously oxygenated, with carbogen and delivered to the liver at 37°. The cellular suspension was washed in ice-cold buffer and centrifuged for 2 min at 50 *g*. The washing was repeated once more with ice-cold Ham F-12 medium complemented with 0.2% albumin and 10^{-8} M insulin. The cellular viability was estimated by the dye exclusion test with 0.4% Trypan blue in saline.

Hepatocytes were resuspended in culture medium and seeded on fibronectin coated plastic dishes, at a density of 80×10^3 viable cells/cm^2 [9]. Culture medium was Ham F-12 supplemented with 2% newborn calf serum, 50 units/mL penicillin, 50 μg/mL streptomycin, 0.2% bovine serum albumin and 10^{-8} M insulin. One hour after cell plating the medium was renewed and after 24 hr cells were shifted to serum-free hormone-supplemented medium (10^{-8} M insulin and dexamethasone, Dex) and the medium was renewed daily. Dex was suppressed from medium only when indicated.

Biochemical assays. Culture plates were washed in 0.1 M potassium phosphate buffer, 1.15% KCl (pH 7.4), and detached with a rubber policeman. The S-9 microsomal fraction was prepared as described [10]. The cytochrome P450 content was measured by the method of Omura and Sato [11]. AHH [12] and ECOD [13] were measured as described. Pb and Et-OH were prepared as an aqueous solution and added directly to cultures. MC was dissolved in dimethyl sulfoxide (DMSO) and added to cultures (0.5% final concentration of DMSO in culture medium). In the latter case, control cultures were also treated with the same concentration of the DMSO. Proteins were measured according to the Lowry [14] method. Tetrazolium MTT test was assayed and described [15].

Statistical analysis. The study was carried out in cultures from 12 (A–L) different human liver biopsies. Each experiment was performed in at least two preparations from different liver biopsies. For each culture the results are expressed as the mean ± SD of three different plates per point.

RESULTS

Dose–response curve of monooxygenase activities to 3-methylcholanthrene, phenobarbital and ethanol

The dose-dependent effect of Pb, MC and Et-OH on AHH and ECOD activities were measured after 48 hr of continuous exposure of hepatocytes to increasing concentrations of the chemicals. Compounds were added 6 hr after cell plating and thereafter with the medium renewal. Both AHH and ECOD activities increased after exposure to MC. Half maximal effect was achieved at about 1 μM, and reached plateau at 2 μM (2.5–2.8-fold increase) (Fig. 1A). DMSO (0.5%, v/v) did not produce any effects on both monooxygenase activities (data not shown). ECOD activity increased after exposure of human

Table 1. Time-course of aryl hydrocarbon hydroxylase activity in presence of 3-methylcholanthrene, phenobarbital and ethanol

Biopsy		Days of culture				
		0	1	2	3	4
H	Control	3.44 ± 0.21	3.01 ± 0.18	1.62 ± 0.59	1.38 ± 0.11	0.98 ± 0.08
	MC		3.66 ± 0.71	4.37 ± 1.23	3.99 ± 0.51	3.91 ± 0.73
	Pb		3.13 ± 0.01	1.77 ± 0.33	1.35 ± 0.05	0.80 ± 0.21
	Et-OH		2.78 ± 0.28	1.84 ± 0.15	1.31 ± 0.16	
I	Control	3.57 ± 0.43		3.17 ± 0.20	2.51	1.77 ± 0.28
	MC		4.05 ± 0.69	6.96 ± 1.36	6.24 ± 0.47	6.45 ± 0.12
	Pb		3.41 ± 0.38	3.15 ± 0.95	2.49 ± 0.41	1.69 ± 0.13
	Et-OH		3.37 ± 0.51	2.96	2.73 ± 0.21	1.80 ± 0.12
J	Control	2.08 ± 0.30	1.83 ± 0.18	1.24 ± 0.10	0.85 ± 0.09	0.74 ± 0.08
	MC		2.42 ± 0.30	4.11 ± 0.36	4.42 ± 0.65	4.35 ± 0.30
	Pb			1.18 ± 0.12	0.86 ± 0.22	0.78 ± 0.12
	Et-OH			1.24 ± 0.34	0.71 ± 0.22	

3-Methylcholanthrene (MC) 2 μM, phenobarbital (Pb) 1.5 mM and ethanol (Et-OH) 200 mM were added 6 hr after initiating the culture. AHH activity was estimated daily in the S-9 fraction of hepatocytes and expressed as pmol of 3-hydroxybenzo(*a*)pyrene formed per min and per mg cellular protein. Each point represents the mean ± SD of three different plates.

hepatocytes either to Pb or Et-OH. Maximal increase of ECOD activity (2.3–3-fold increase) was achieved at 1.5 mM Pb and 200 mM ethanol (Fig. 1B and C). Neither Pb nor Et-OH had any appreciable effect on AHH activity.

Cellular protein attached to plates and tetrazolium salt MTT test were used to assess cell damage after exposure of the hepatocytes to increasing concentrations of MC, Pb and Et-OH. MC and Pb did not produce any cytotoxic effect in the range of concentrations used in this study, while Et-OH at concentrations above 200 mM produced a 15% inhibitry effect on the MTT test, but at this same concentration did not affect cellular protein.

Time-course of AHH and ECOD activities after exposure to 3-methylcholanthrene, phenobarbital and ethanol

The time-course of AHH and ECOD activities was examined in three different culture preparations exposed to the optimal concentration of the chemicals. Compounds (2 μM MC, 1.5 mM Pb and 200 mM Et-OH) were added 6 hr after cell plating and thereafter with each medium renewal. AHH and ECOD activities were measured daily and a decrease was observed in control cells with time of culture. AHH activity was increased only by MC exposure and reached the maximum (2.2–2.8-fold of the control at the same day) after 48 hr (Table 1). ECOD activity was increased by MC, Pb and Et-OH to about the same extent and reached the maximal activity (2.2–3.8-fold of the control at the same day) after 72 hr of exposure to the three chemicals (Table 2). Inter-individual variations in the extent of the response to xenobiotics were observed (Tables 1 and 2).

Effect of dexamethasone on monooxygenase activity

In order to examine the combined effect of glucocorticoids and MC, Pb and Et-OH on monooxygenase activities two sets of plates (with or without Dex) were used. Chemicals (2 μM MC, 1.5 mM Pb and 200 mM Et-OH) were added to cultures 6 hr after cell plating and daily at the time of medium renewal. Dex (10^{-8} M), when used, was added at 24 hr of culture and daily with the culture medium. The enzyme activities were measured after 72 hr of culture. Dex only produced a moderate increase of the basal ECOD activity levels in one out of five biopsies. However, when the hormone was added in combination with MC and Pb, a significant increase in their effect on ECOD activity was found in four (biopsies A, B, C and D) of the five cultures examined (Table 3). Dex did not produce any effects on AHH activity either in control or MC treated cultures (data not shown).

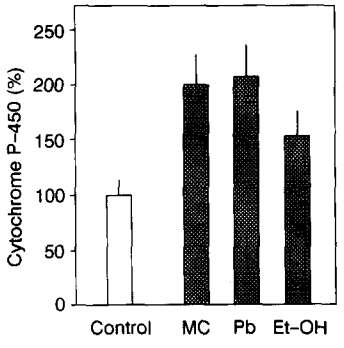

Fig. 2. Increased cytochrome P450 content by exposure to 3-methylcholanthrene, phenobarbital and ethanol. Hepatocytes were exposed 6 hr after attachment to 2 μM 3-methylcholanthrene, 1.5 mM phenobarbital or 200 mM ethanol and cytochrome P450 content were evaluated after 48 hr. Values represent mean ± SD of six independently treated plates from two different hepatocyte preparations (cultures K and L) and the data are expressed as percent of controls (38.5 ± 5.6 pmol/mg). Cytochrome P450 content in freshly isolated hepatocytes was 71.2 ± 13.3 pmol/mg.

Table 2. Time-course of 7-ethoxycoumarin *O*-deethylase activity in presence of 3-methylcholanthrene, phenobarbital and ethanol

Biopsy		Days of culture				
		0	1	2	3	4
H	Control	10.51 ± 1.32	8.10 ± 1.26	6.85 ± 2.09	4.53 ± 1.13	2.87 ± 0.62
	MC		12.40 ± 1.08	16.71 ± 1.82	17.06 ± 1.69	16.39 ± 2.16
	Pb		10.32 ± 1.51	12.61 ± 0.99	16.39 ± 1.96	13.00 ± 1.81
	Et-OH		9.61 ± 1.04	14.50 ± 1.61	17.03 ± 1.61	14.18 ± 1.96
I	Control	20.21 ± 1.70		13.20 ± 2.06	12.87	8.49 ± 0.04
	MC		24.51 ± 3.06	34.57 ± 1.82	32.28 ± 0.37	29.69 ± 1.08
	Pb		20.96 ± 2.13	24.13 ± 1.82	28.39 ± 1.69	22.71 ± 3.06
	Et-OH		18.83 ± 1.29	26.13 ± 3.11	29.11 ± 3.13	24.68 ± 2.73
J	Control	10.63 ± 1.29	8.93 ± 1.95	7.56 ± 1.35	5.01 ± 0.96	3.88 ± 0.74
	MC		17.10 ± 2.43	30.76 ± 0.61	24.28 ± 1.69	16.89 + 1.32
	Pb		13.17 ± 2.60	15.78 ± 0.74	18.40 ± 1.53	15.10 ± 2.33
	Et-OH			19.82 ± 2.99	13.63 ± 3.66	

3-Methylcholanthrene (MC) 2 μM, phenobarbital (Pb) 1.5 mM and ethanol (Et-OH) 200 mM were added 6 hr after hepatocyte seeding. ECOD activity was estimated daily in the S-9 fraction of hepatocytes and expressed as pmol of 7-hydroxycoumarin formed per min and per mg cellular protein. Each point represents the mean ± SD of three different plates.

Table 3. Effect of dexamethasone in combination with 3-methylcholanthrene, phenobarbital and ethanol on 7-ethoxycoumarin *O*-deethylase activity

	Biopsy				
	A	B	C	D	E
Control	3.16 ± 1.01	5.84 ± 0.40	3.04 ± 0.43	2.70 ± 0.40	8.66 ± 1.72
Dex	2.86 ± 0.31	8.03 ± 0.92	3.17 ± 0.74		9.87 ± 1.20
MC	5.96 ± 1.75	15.55 ± 3.64	9.03 ± 1.78	14.37 + 2.21	19.78 + 4.36
MC + Dex	8.56 ± 0.20	21.46 ± 3.10	13.75 ± 1.08	15.43 ± 0.11	21.96 ± 0.24
Pb	5.05 ± 1.15		8.39 ± 0.36	7.38 ± 0.28	16.83 ± 0.04
Pb + Dex	7.44 ± 0.83		11.43 ± 1.21	7.10 ± 0.98	21.96 ± 0.24
Et-OH			10.96 ± 0.88	8.63 ± 0.14	18.02 ± 3.52
Et-OH + Dex			16.99 ± 5.31	8.78 ± 0.14	14.08 ± 4.76

3-Methylcholanthrene (MC) 2 μM, phenobarbital (Pb) 1.5 mM and ethanol (Et-OH) 200 mM were added 6 hr after hepatocyte seeding. After 72 hr of continuous incubation with the chemical, ECOD activity was estimated in the S-9 fraction and expressed as pmol of 7-hydroxycoumarin formed per min and per mg cellular protein. Dexamethasone (Dex) at 10^{-8} M was added after 24 hr of culture. ECOD activity in freshly isolated hepatocytes was 7.89 ± 1.01, 16.70 ± 2.15, 10.99 ± 1.18, 10.52 ± 0.93 and 18.04 ± 0.21 pmol/min/mg respectively for biopsies A–E. Each point represents the mean ± SD of three different plates.

Effect of methylcholanthrene, phenobarbital and ethanol on cytochrome P450 levels

The levels of total cytochrome P450 were evaluated after 48 hr of treatment with 2 μM MC, 1.5 mM Pb and 200 mM Et-OH. As seen in Fig. 2, all chemicals increased the basal hemoprotein content and MC and Pb produced higher effect (2-fold) than Et-OH (1.5-fold). Addition of 0.5% DMSO did not produce variations on cytochrome P450 content.

DISCUSSION

Our work presents direct evidence that Pb, MC and Et-OH produce important increases in total cytochrome P450 as well as in the activities of two representative monooxygenases in human hepatocytes. The dose and time-course activity of AHH and ECOD by MC and of ECOD by Pb in human hepatocytes is similar to what is known to occur in rat liver *in vivo* [16, 17] and in cultured rat hepatocytes [18–20]. In addition, our findings show that both chemicals produce different and selective effects on cytochrome P450 dependent monooxygenases in human hepatocytes *in vitro*, as they do in rat [21, 22]. Interindividual variability of the basal levels of monooxygenases and of the extent of the effects produced by MC, Pb and Et-OH was observed in the biopsies studied. Recent reports have shown an increase in cytochrome P450 [23], ethoxyresorufin O-deethylation and pentoxyresorufin O-dealkylation activities [24] in cultured human hepatocytes exposed to Pb. Our study confirms these results, but in addition shows the effect of MC on AHH and ECOD activities in human cultured hepatocytes that has not been reported previously.

Enzyme induction alters the rate of drug metabolism and is a factor that increases the incidence of toxic disease of liver [3]. Studies in experimental animals have shown that intake of Et-OH increases the activity of hepatic monooxygenase and conjugating enzymes [5, 6, 25]. Increases in drug-metabolizing activities following exposure to Et-OH are difficult to characterize in humans but are important since in humans drug intake is frequently associated with chronic Et-OH consumption. By using cultured human hepatocytes we have shown for the first time that Et-OH produces a dose- and time-dependent increase in ECOD activity in human hepatocytes but has no effect on AHH activity.

The present study reveals that the effects known to be produced by glucocorticoids on monooxygenase activities in cultured rat hepatocytes [18, 26] are also produced in human hepatocytes. Our results showed that although Dex had no appreciable effect *per se*, when added simultaneously with MC, Pb and Et-OH to human hepatocytes, it potentiated their effect on monooxygenase activities. Glucocorticoids are very frequently administered over long periods of time for the treatment of important diseases, i.e. hepatic diseases, inflammatory and allergic processes, autoimmune diseases and for immunosuppression after organ transplantations. The results here presented suggest that although treatment with Dex is not likely to increase drug-metabolizing enzyme activities by itself, it may potentiate the action of other drugs administered concomitantly to these patients.

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