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MIXED FUNCTION OXIDASE AND UDP-GLUCURONYLTRANSFERASE ACTIVITIES IN THE HUMAN HEP G2 HEPATOMA CELL LINE

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Abstract—In cultured human hepatoma cells phenolphthalein glucuronidation was increased 3-fold by 2 mM phenobarbitone (PB) in the culture medium but not by 25 μM benz(*a*)anthracene (BA), while 1-naphthol glucuronidation was not increased by either PB or BA. Ethoxyresorufin *O*-deethylation (EROD) was increased 15-fold by BA but not by PB, while the *O*-dealkylations of pentoxyresorufin (PROD) and benzyloxyresorufin (BROD) were increased by either PB or BA. The BROD activity increased by BA was sensitive to inhibition by α-naphthoflavone whereas that induced by PB was not. This suggests induction of different cytochrome P-450 isoenzymes. Control Hep G2 cells had similar glucuronide conjugation and cytochrome reductase activities to freshly isolated human adult hepatocytes, but had lower *O*-dealkylation and elevated microsomal epoxide hydrolase activities.

Human hepatoma cell lines may provide a useful model system in which to investigate human hepatic drug metabolism. Certain cell lines, including Hep G2, Hep 3B and Sk-Hep-1, can carry out cytochrome P-450-dependent mixed function oxidase (MFO)§ and conjugation reactions [1–4] and are capable of activating benzo(*a*)pyrene, aflatoxin B1 and cyclophosphamide to cytotoxic and mutagenic metabolites [2, 5–7]. Hep G2 cells form similar DNA-adducts with benzo(*a*)pyrene as are formed in explants from normal human tissue [2] and it may therefore be a suitable cell line for predicting drug-induced cytotoxicities in man.

Many drug-metabolising enzyme activities are increased by inducing agents. From experiments using laboratory animals *in vivo* inducers were originally classified into two main families. One family includes phenobarbitone (PB) and increases the activities of many MFO reactions, including the epoxidation of aldrin and the *O*-depentylation of pentoxyresorufin (PROD) [8], and the glucuronidation of substrates such as phenolphthalein and morphine [9, 10]. The other family of inducers are the polycyclic aromatic hydrocarbons, for example benz(*a*)anthracene (BA) and 3-methylcholanthrene, which increase the activities of a different set of reactions, including benzo(*a*)pyrene hydroxylation, ethoxyresorufin *O*-deethylation (EROD) [8] and the glucuronidation of 4-nitrophenol and 1-naphthol [9, 10]. Some MFO reactions, for example the *O*-debenzylation of benzyloxyresorufin (BROD), are induced by both PB and polycyclic aromatic hydrocarbon type inducers [8]. Several additional categories of inducing agent have been described in recent years.

The induction of drug metabolism is difficult to study in man. Most of the information available is from pharmacokinetic studies, although some studies describe the measurement of enzyme activities in biopsies or liver samples from surgical patients or kidney donors who have been treated previously with inducing drugs, such as barbiturates or rifampicin. These *in vivo* and *in vitro* studies show that the human drug-metabolising enzymes can be induced and that this induction results in altered drug pharmacokinetics. Anticonvulsants, including barbiturates, phenytoin and carbamazepine, increase the clearance of antipyrine and raise hepatic cytochrome P-450 levels [11–13], whilst rifampicin induces antipyrine and diazepam elimination and increases hepatic cytochrome P-450 content and the hydroxylation of steroids [14, 15]. Human hepatic microsomal EROD, PROD and BROD are induced differently by anticonvulsants and cigarette smoking respectively [16]. These effects can all be attributed to induction of MFO metabolism in man. Phenytoin and barbiturates also increase the clearance of oxazepam, a drug which is metabolised by a single step glucuronidation reaction [17] and *in vitro* studies on three livers have shown that the glucuronidation of 1-naphthol and 4-methylumbelliferone was increased by phenytoin and barbiturate treatment [18].

Liver samples are only available opportunistically from patients receiving likely inducing agents. Moreover, interpretation of results from such samples is complicated by the tendency for most patients to be receiving more than one drug, plus the unreliability,

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§ Abbreviations used: EROD, ethoxyresorufin *O*-deethylation; PROD, pentoxyresorufin *O*-depentylation; BROD, benzyloxyresorufin *O*-debenzylation; BA, benz(*a*)anthracene; PB; phenobarbitone; MFO, mixed function oxidase; GT, UDP-glucuronyltransferase; EH, epoxide hydrolase; ANF, α-naphthoflavone.

with smokers, of information about the number of cigarettes smoked. Consequently, the study of the induction of human drug metabolism would be aided by a suitable human cell culture model. As a first step towards this, we have compared between cultured human Hep G2 hepatoma cells and freshly isolated human hepatocytes several reactions that are considered in animals to be *in vitro* "markers" of PB or BA type induction *in vivo*, and we have measured the response of the activities to inducers in the cell culture medium.

MATERIALS

Phenolphthalein glucuronide, 1-naphthol sulphate, 1-naphthol glucuronide, NADPH, α-naphthoflavone and benz(*a*)anthracene were obtained from Sigma (St Louis, MO). Dulbecco's medium was from Flow Laboratories (Herts, England) and foetal calf serum from Gibco (Paisley, Scotland). Resorufin, ethoxyresorufin, pentoxyresorufin and benzyloxyresorufin were synthesised as described by Burke and Mayer [19]. Phenanthrene-9,10-oxide and phenanthrene-9,10-dihydrodiol were a generous gift from Dr P. Dansette, Institute of Biochemistry, Universite Paris-Sud, France. The Hep G2 cell line was obtained by Dr W. T. Melvin, Department of Biochemistry, Aberdeen University from Professor C. N. Hales, Department of Clinical Biochemistry, Addenbrooke's Hospital, Cambridge.

METHODS

1. *Cell culture.* Hep G2 cells were grown as monolayer or multilayer cultures in 75 cm^2 flasks in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal calf serum, penicillin 100 Units/litre and streptomycin 100 mg/litre. They were grown in a humidified atmosphere of 5% CO_2 in air and subcultured every 7–9 days at a 1:3 split ratio (seeding density 3.5×10^6 cells in a 75 cm^2 flask).

2. *Analytical methods.* MFO activities (EROD, PROD and BROD) were measured on confluent cultures at day 9–10 following subculture and the measurements were carried out on cell homogenates prepared as described previously for rat hepatocyte cultures [20]. Briefly, cultures were washed twice with phosphate buffered saline, pH 7.6, scraped off the flasks into 0.1 M sodium phosphate buffer, pH 7.6, using a "rubber policeman" and homogenised using a Teflon–glass Potter–Elvehjem type homogeniser. Homogenates were stored at −80° until analysed. MFO activities were measured using 0.1 ml cell homogenate with 0.3 mM NADPH and 5 μM substrate in a final volume of 0.5 ml 0.1 M sodium phosphate buffer, pH 7.6, at 37° in a shaking water bath. Reactions were stopped by the addition of 0.75 ml ice-cold acetone and the samples were centrifuged at 12,000 *g* for 5 min. The fluorescence of the supernatant was read at an emission wavelength of 600 nm and an excitation wavelength of 580 nm. The use of these higher wavelengths (compared with the assay as previously described [19]) facilitated the measurement of very low activities by increasing the ratio of resorufin to substrate fluorescence. These experiments were carried out under yellow light. The activities were measured every 5 min for 30 min and the reaction rates calculated over the linear part of the time curve.

Microsomal epoxide hydrolase (EH) activities were measured in 0.4 ml cell homogenate with 20 μM phenanthrene-9,10-oxide [21] using the method described by Grant *et al.* [22] for human hepatocytes. NADH-cytochrome b_5 reductase and NADPH-cytochrome *c* reductase were measured in cell homogenates (0.1 ml) as described by Falzon and coworkers [23].

The conjugation reactions were measured in intact cells. This avoided the problem of artefactual activation of UDP-glucuronyltransferases (GT) in cell homogenates and enabled measurement of both the glucuronidation and sulphation of 1-naphthol. Phenolphthalein or 1-naphthol (each 100 μM) was added to the confluent culture flasks (day 9–10) containing a final volume of 20 ml Dulbecco's medium without foetal calf serum. Incubations in the culture flasks were carried out for 8 hr and 1 ml samples removed every hour. Samples were stored at −20° until analysis by high pressure liquid chromatography as described previously [24].

Cell protein was measured by the method of Lowry and coworkers using bovine serum albumin as the standard [25].

Enzyme activities were compared using one-way analysis of variance followed by Dunnett's test and considered significantly different if $P \leq 0.05$.

3. *Induction and inhibition.* Inducing agents (25 μM BA or 2 mM PB) were added at confluency (day 7) and the cells were exposed to the inducers for 3 days before metabolism was measured on day 10. The medium was changed every day in both control and inducer-treated flasks. α-Naphthoflavone (ANF) was used as a selective inhibitor of MFO activities induced by BA [26] and was added to the EROD and BROD reactions at the concentrations indicated.

RESULTS

In Table 1 the MFO, EH, GT, NADH-cytochrome b_5 reductase and NADPH-cytochrome *c* reductase activities of Hep G2 cells measured here are compared with those measured in freshly isolated human hepatocytes (obtained from renal transplant donors with no evidence of hepatic disease), which we have published previously [22].

The toxicity of inducing agents to the Hep G2 cells was investigated to find the maximum tolerated concentration of each. 3-Methylcholanthrene was too toxic to the cells to be used as an inducer. The 3-methylcholanthrene concentration of 5 μM, used by others for *in vitro* induction in Hep G2 cells [3], caused loss of approximately 60% of the cultured cells over 3 days (data not shown). PB and BA were comparatively non-toxic and at the concentrations used routinely for induction experiments with animal hepatocyte cultures (2 mM PB and 25 μM BA) they caused no significant detachment of cultured Hep G2 cells.

Table 2 shows the responses of 1-naphthol GT, phenolphthalein GT and EH activities to PB and BA in the culture medium. 1-Naphthol formed both glucuronic acid and sulphate conjugates, with glu-

Table 1. Enzyme activities in isolated human adult hepatocytes and control Hep G2 hepatoma cells

	Human adult hepatocytes	Control Hep G2 cells
	(pmol/min/mg/protein)	
EROD	23.3 ± 2.8 (4)	2.3 ± 0.1 (4)
BROD	9.3 ± 0.7 (4)	0.4 ± 0.1 (4)
PROD	1.2 ± 0.8 (4)	0.2 ± 0.03 (6)
EH	73.4 ± 22.0 (4)	491.0 ± 77.6 (5)
N-GT	772.2 ± 135.8 (4)	496.7 ± 23.7 (10)
P-GT	216.7 ± 61.2 (3)	106.8 ± 13.3 (4)
	(nmol/min/mg protein)	
NADH-cyt. b_5 reductase	322.5 ± 94.2 (4)	560.5 ± 42.3 (14)
NADPH-cyt. *c*-reductase	21.5 ± 2.2 (4)	27.3 ± 1.5 (16)
EROD/BROD ratio	2.5	6.6
EROD/PROD ratio	19.3	12.2
N-GT/P-GT ratio	3.5	4.5

The data are expressed as mean ± SEM with the number of experiments in parentheses. The Hep G2 cells were cultured in the absence of any inducing agent. The enzyme activities in freshly isolated human adult hepatocytes are taken from Grant *et al.* [22]. Conjugation reactions in hepatocytes were measured in suspensions of 10^6 cells/ml using the conditions and methods described by Grant *et al.* [22], while mixed function oxidase and epoxide hydrolase activities were measured in cell homogenates (10^6 cells/ml) prepared in 0.1 M sodium phosphate buffer, pH 7.6, using the conditions described in the present manuscript. The ratios of enzyme activities were calculated from the mean enzyme activity data. EROD, ethoxyresorufin *O*-deethylation; BROD, benzyloxyresorufin *O*-debenzylation; PROD, pentoxyresorufin *O*-depentylation; N-GT, 1-naphthol glucuronyltransferase; P-GT, phenolphthalein glucuronyltransferase; EH, epoxide hydrolase.

curonide formation being 5-fold that of sulphation and neither BA nor PB caused any significant increase in 1-naphthol conjugation. Phenolphthalein was not sulphated to any great extent in the Hep G2 cells, and whilst BA caused a non-significant increase in phenolphthalein glucuronidation, the reaction was increased 3-fold by PB. EH activity was decreased significantly by PB and to a smaller non-significant extent by BA.

Table 3 shows the effects of PB and BA on the

Table 2. Conjugation reactions and epoxide hydrolase activity in Hep G2 cells

Inducer	1-Naphthol glucuronide formation	1-Naphthol sulphate formation	Phenolphthalein glucuronide formation	Epoxide hydrolase activity
	(pmol/min/mg protein)			
Control	496.7 ± 23.7 (10)	97.3 ± 4.5 (10)	106.8 ± 13.3 (4)	491.0 ± 77.6 (5)
BA (25 μM)	478.2 ± 33.7 (6)	99.5 ± 6.5 (6)	134.7 ± 20.0 (4)	308.0 ± 65.3 (7)
PB (2 mM)	476.3 ± 8.8 (3)	102.7 ± 11.0 (3)	267.2 ± 49.3* (5)	200.7 ± 48.7* (6)

Results are expressed as mean ± SEM with the number of experiments in parentheses. Cells were treated with benz(*a*)anthracene (BA) or phenobarbitone (PB) for 3 days before enzyme activities were measured.

* $P < 0.05$. Significance values refer to differences in activity between control and inducer-treated cells.

Table 3. Mixed function oxidase activities in Hep G2 cells

Inducer	EROD	PROD (pmol/min/mg protein)	BROD
Control	2.3 ± 0.1 (4)	0.2 ± 0.03 (6)	0.4 ± 0.1 (4)
BA (25 μM)	35.6 ± 3.1** (5)	0.7 ± 0.2* (7)	1.0 ± 0.1* (5)
PB (2 mM)	2.3 ± 0.5 (5)	0.5 ± 0.1 (7)	1.0 ± 0.2* (5)

Results are expressed as mean ± SEM with the number of experiments in parentheses. Cells were treated with benz(*a*)anthracene (BA) or phenobarbitone (PB) for 3 days before enzyme activities were measured. EROD, ethoxyresorufin *O*-deethylation; PROD, pentoxyresorufin *O*-dependentylation; BROD, benzyloxyresorufin *O*-debenzylation.

** $P < 0.01$, * $P < 0.05$. Significance values refer to differences in activity between control and inducer-treated cells.

MFO activities, EROD, BROD and PROD. BA increased all three activities but the increase was greatest for EROD (15-fold). PB did not significantly increase EROD but increased BROD and PROD to similar extents (2–3-fold). In order to differentiate between the apparent inducing effect of PB and BA, the MFO inhibitor ANF was used which in animal experiments is a selective inhibitor of activities induced by BA type agents [26]. Figure 1 shows the inhibition of the EROD and BROD reactions by ANF. (BROD was measured rather than PROD because the former reaction was 2-fold faster and so inhibition could be detected more easily.) EROD was inhibited by ANF in both BA and PB treated cells. In contrast, while BROD in BA-treated cells was inhibited by ANF, BROD in PB-treated cells was resistant to ANF inhibition.

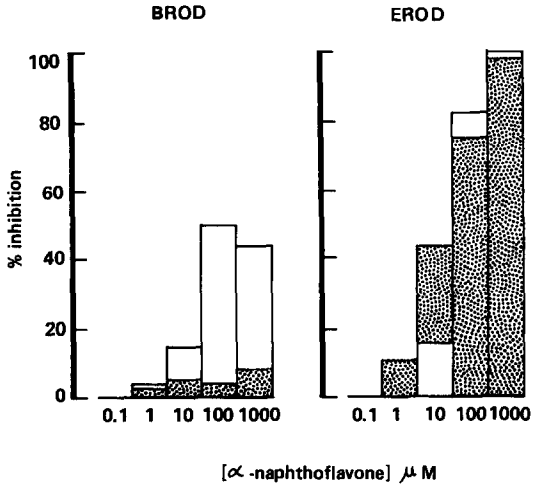

Fig. 1. Inhibition of benzyloxyresorufin (BROD) and ethoxyresorufin (EROD) *O*-dealkylations by α-naphthoflavone. Different concentrations of α-naphthoflavone were added directly to reactions containing cells pretreated with either phenobarbitone (▩) or benz(*a*)anthracene (□) for 3 days. Results are the means of either 3 or 4 experiments. Histogram bars for phenobarbitone and benz(*a*) anthracene are overlaid from zero.

DISCUSSION

The purpose of this study was to asses the potential of the human Hep G2 hepatoma cell line as a system in which to study the induction of human MFO, GT and EH activities, therefore substrates were used whose hepatic microsomal metabolism was known to be induced in rats *in vivo*. In rats phenolphthalein glucuronidation and PROD are selective markers for PB induction, whereas 1-naphthol glucuronidation and EROD are selective probes for induction by polycyclic aromatic hydrocarbons such as BA [8–10].

GT activities were similar in the Hep G2 cells and in normal human hepatocytes. MFO activities were markedly lower in the Hep G2 cells, but this was not accompanied by a decrease in the activities of the associated NADH-cytochrome b_5- and NADPH-cytochrome *c*-reductase. EH activities were 6-fold higher in the Hep G2 cells compared with normal hepatocytes. These changes in the activities of the MFO and EH enzymes are similar to those reported as occurring in various pre-neoplastic and neoplastic hepatic tumours [27] and are consistent with some degree of de-differentiation of the Hep G2 cells. Sulphate conjugation was low in Hep G2 cells but for 1-naphthol the ratio of glucuronidation to sulphation in Hep G2 cells (5:1) was similar to that in normal hepatocytes (4:1), whilst the virtually total lack of sulphation of phenolphthalein in Hep G2 cells is also seen in normal human hepatocytes [22]. The ratios between the three MFO activities and between the two GT activities in Hep G2 cells are similar to the respective ratios in normal human hepatocytes. This may indicate that the pattern of MFO and GT isoenzymes expressed are similar in Hep G2 and normal human hepatocytes, but the resolution of this question will require the direct quantification of each of the isoenzymes involved.

The increase in phenolphthalein glucuronidation by PB but not by BA and the increase in EROD by BA but not PB suggests that PB and BA exerted different and characteristic inducing effects on the cytochrome P-450 and GTs of Hep G2 cells in culture. The failure of 1-naphthol glucuronidation to be increased by either agent may have been due to a possible rate-limiting effect of the cofactor, UDP-

glucuronic acid, since in control cells this reaction was 5-fold faster than phenolphthalein glucuronidation. However, the phenanthrene-9,10-epoxide EH reaction, which is inducible in rat liver microsomes by either PB or BA type inducers *in vivo* (unpublished observations), also was not increased in Hep G2 cells. The reason for the marked decrease in EH activity by PB and BA in Hep G2 cells is not known at present. The increase of BROD in Hep G2 cells by either PB or BA was in accordance with its induction by either type of inducer in rats *in vivo* [8]. However, a truly characteristic effect in Hep G2 cells of PB and BA respectively (i.e. as seen in rats) demanded that PROD be induced by PB but not BA, whereas it was increased by either agent equally. It has been suggested that in cultured cells PB acts aberrently, like a BA-type inducer of cytochrome P-450 [28, 29]. Moreover, although Dawson *et al.* [3] reported the presence in Hep G2 cells of a protein related immunologically to a PB-induced isoenzyme of rat liver cytochrome P-450, Sassa *et al.* [4] were unable to detect such a protein. PB-induced BROD and other PB-induced MFO reactions are much less sensitive to inhibition by ANF than are the reactions when induced by BA-type agents [26, 30]. Thus, the inhibition by ANF of BROD in BA-treated but not in PB-treated Hep G2 cells suggests that PB was an inducer acting in true character in these cultured cells. Immunoinhibition experiments using monoclonal antibodies against rat and human cytochrome P-450 isoenzymes are currently underway in our laboratories to answer this question definitively.

In conclusion, we believe that Hep G2 cells, which we have shown to contain MFO, EH and GT activities and to respond to inducing agents, may provide a valuable model system in which to study human hepatic drug metabolism.

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