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The effects of dimethylsulphoxide and 5-aminolaevulinic acid on the activities of cytochrome P450-dependent mixed function oxidase and UDP-glucuronosyl transferase activities in human Hep G2 hepatoma cells

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Hep G2 is a highly differentiated human hepatoma cell line which has retained many of the specialized functions normally lost by hepatocytes in culture [1]. There have been a number of reports suggesting that Hep G2 cells may provide a suitable *in vitro* system for studying drug metabolism and toxicity directly in man. These cells can carry out cytochrome P450 dependent mixed function oxidase (MFO*) and conjugation reactions and are capable of activating several xenobiotics to form cytotoxic and/or mutagenic metabolites [2–6]. The activity of these drug metabolizing enzymes in the Hep G2 cells can be manipulated by altering the composition of the growth medium [5] and several groups have shown that the activity of the MFO system can be increased by inducing agents such as benz(*a*)anthracene and phenobarbitone [2, 4, 7].

There is still some debate about the extent to which the metabolic profile of Hep G2 cells reflects that of normal human hepatocytes and the activities of several drug metabolizing enzymes have been compared in the two cell types [4, 8]. In general, the activity of the MFO system is lower in Hep G2 cells than in freshly isolated hepatocytes and the activities of epoxide hydrolase and DT-diaphorase (M. H. Grant and S. J. Duthie, unpublished results) are elevated in the hepatoma cells. These observations are typical of the changes which occur in the liver during de-differentiation of preneoplastic tissue [9] and, together with the absence of the cystathionine pathway in the Hep G2 cells [10], they suggest that these cells have undergone de-differentiation to some extent.

Primary cultures of hepatocytes are known to undergo rapid de-differentiation within 24–72 hr of culture and the activity of the MFO system is particularly unstable in cultured hepatocytes from animals and man [8, 11]. Several attempts have been made to modify media and substrata composition to resolve this problem. Dimethylsulphoxide (DMSO) has been shown to increase the secretion of albumin in cultured rat hepatocytes and hepatoma cells [12, 13], to elevate the level of cytochrome P450 in primary cultures of rat hepatocytes [14] and to promote cell differentiation in a variety of cell systems [15, 16]. In the present study we have investigated the effects of DMSO and 5-aminolaevulinic acid (ALA), a precursor required for haem synthesis, on the activities of the MFO system and UDP-glucuronosyltransferase (GT) in Hep G2 cells. The experiments were carried out in two different growth media—Dulbecco's modification of Eagle's medium, which is the medium usually used for growing Hep G2 cells, and Williams' E medium, which is extensively used for maintaining primary hepatocyte cultures.

Materials and Methods

Materials. Dulbecco's and Williams' E media were from ICN Flow Laboratories (Irvine, U.K.) and foetal calf serum was from Gibco (Paisley, U.K.). 1-Naphthol, 1-naphthol glucuronide, testosterone, bilirubin, UDP-glucuronic acid, 5-aminolaevulinic acid and NADPH were obtained from the Sigma Chemical Co. (Poole, U.K.). Methoxyresorufin, ethoxyresorufin, pentoxyresorufin and benzyloxyresorufin were synthesized as described by Burke and Mayer [17]. The Hep G2 Cell line was obtained from Prof. C. N. Hales, Department of Clinical Biochemistry, Addenbrooke's Hospital, Cambridge.

Cell culture. Hep G2 cells were grown as monolayer or multilayer cultures in 75 cm^2 flasks in either Dulbecco's modified Eagle's medium or Williams' E medium supplemented with 10% (v/v) foetal calf serum, penicillin (100 units/L) and streptomycin (100 mg/L). They were subcultured every 7 days at a 1:3 split ratio (seeding density 3.5×10^6 cells in a 75 cm^2 flask). The medium was renewed routinely at 3 and 6 days after passage and all enzyme measurements were carried out on confluent cells at 7 days after subculture. To investigate the effects of DMSO (2% v/v) and ALA (10^{-4} M), they were added either alone or in combination at the time of subculture and metabolism was measured 7 days later.

Analytical methods. Enzyme activities were measured in cell homogenates prepared in 0.1 M sodium phosphate buffer, pH 7.6, as described previously [4]. MFO activities were measured by the O-dealkylation of ethoxyresorufin (EROD), methoxyresorufin (MROD), benzyloxyresorufin (BROD) and pentoxyresorufin (PROD), using 5 μM of each substrate as described previously [4]. Glucuronidation was assessed using 50 μM 1-naphthol, 0.4 mM bilirubin or 1 mM testosterone as substrate. 1-Naphthol GT activity was quantified in the presence of 0.5 mM UDP-glucuronic acid and 5 mM $MgCl_2$ by continuous fluorescence detection of the glucuronide formed [18]. Bilirubin and testosterone activities were measured in the presence of 4 mM UDP-glucuronic acid and 10 mM $MgCl_2$, the former using the diazotization procedure and the latter using ^{14}C-labelled substrate as described previously [19]. Cell protein was measured by the method of Lowry *et al.* [20] using bovine serum albumin as the standard.

Results

The activities of both the MFO system and GT in the Hep G2 cells were influenced by medium composition. Table 1 shows that there was a substrate-dependent increase in the enzyme activities of cells grown in Williams' E medium compared with those grown in Dulbecco's. The greatest increase was observed in the activities with EROD and MROD, substrates with selectivity for the P450IA family [21].

The effects of DMSO and ALA on the activities of the MFO and GT enzymes were investigated in both media. Figure 1A and B illustrates that not only is the basal enzyme activity affected by medium composition but the choice of medium is also crucial for observing the influence of other chemicals on the enzyme activity. There was a 20–25-fold increase in MFO activities towards the ethoxy-, methoxy- and benzyloxyresorufins by the separate addition of either DMSO or ALA to cells grown in Dulbecco's medium (Fig. 1A). This marked increase in MFO activity did not occur, however, with pentoxyresorufin as substrate;

* Abbreviations: MFO, mixed function oxidase; GT-UDP-glucuronosyl transferase; DMSO, dimethylsulphoxide; ALA, 5-aminolaevulinic acid; ALAD, 5-aminolaevulinic acid dehydratase; EROD, ethoxyresorufin O-dealkylation; MROD, methoxyresorufin O-dealkylation; PROD, pentoxyresorufin O-dealkylation and BROD, benzyloxyresorufin O-dealkylation.

Table 1. Mixed function oxidase and UDP-glucuronosyl transferase activities in control Hep G2 cells grown in Williams' E and Dulbecco's media

	Dulbecco's	Williams' E	
	(pmol/min/mg protein)		
EROD	2.30 ± 0.17 (3)	13.20 ± 1.90 (3)	[5.7]
MROD	0.97 ± 0.13 (3)	5.00 ± 0.75 (3)	[5.2]
BROD	0.66 ± 0.19 (3)	0.77 ± 0.07 (3)	[1.2]
PROD	0.41 ± 0.05 (3)	0.52 ± 0.19 (3)	[1.3]
NGT	573.0 ± 47.0 (5)	1269.0 ± 160.0 (10)	[2.2]
BGT	3.75 ± 0.11 (3)	13.75 ± 1.25 (8)	[3.7]
TGT	16.0 ± 0.10 (3)	13.0 ± 0.10 (5)	

EROD, ethoxyresorufin O-dealkylation; MROD, methoxyresorufin O-dealkylation; BROD, benzyloxyresorufin O-dealkylation; PROD, pentoxyresorufin O-dealkylation; NGT, 1-naphthol glucuronosyl transferase; BGT, bilirubin glucuronosyl transferase; TGT, testosterone glucuronosyl transferase activities. All enzyme activities were measured on confluent cultures 7 days after passage.

Results are means ± SEM with the number of experiments given in parentheses.

Figures in square brackets refer to the fold increase in activities measured in cells cultured in Williams' E medium compared with those cultured in Dulbecco's medium.

PROD increased only 3–4-fold in the presence of DMSO or ALA. The effects of DMSO and ALA on the MFO activities were not additive. In cells grown in Williams' E medium the effects of DMSO and ALA were very much less, and did not show the selective effect of MROD and EROD activities. BROD activity was increased selectively compared with MROD, EROD and PROD activities by ALA, but not by DMSO.

Figure 2A and B shows the effect of DMSO and ALA on GT activities in the Hep G2 cells cultured in Dulbecco's and Williams' E media, respectively. The glucuronidation of 1-naphthol and testosterone were unaffected by either DMSO or ALA, either alone or in combination. In contrast, bilirubin GT activity was increased 8–9-fold by either DMSO or ALA added separately to cells cultured in Dulbecco's medium (Fig. 2A). In contrast to the effect on MFO activities, the effect on bilirubin GT was additive, and when both DMSO and ALA were added together, bilirubin GT activity was increased 19-fold in cells grown in Dulbecco's medium. The effects of DMSO and ALA on bilirubin GT activity were also observed in Williams' E medium but to a lesser extent. The maximum effect when both agents were present was a 5-fold increase in GT activity (Fig. 2B).

Discussion

The results of this study illustrate the importance of defining and standardizing the culture conditions used for investigating factors affecting drug metabolism and cytotoxicity in cultured cells. Although the effects of DMSO and ALA were marked in the Hep G2 cells cultured in Dulbecco's medium, they would not have been detected if the experiments had been carried out entirely in Williams' E medium.

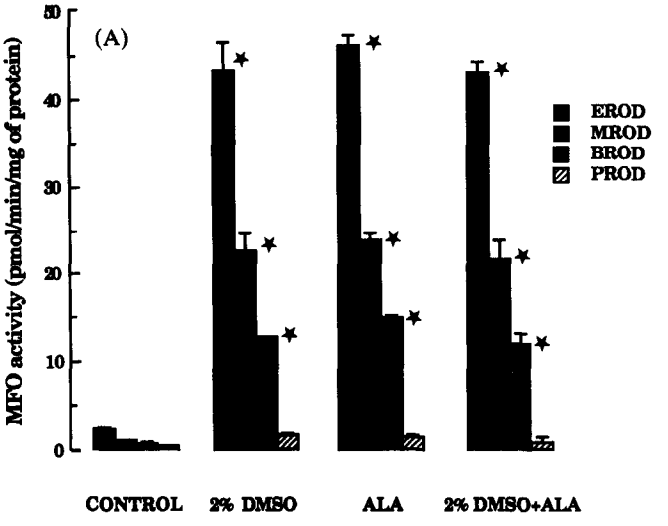
DMSO is known to induce cellular differentiation and it is possible that the effects on enzyme activities could be due to different growth characteristics of cells cultured in DMSO. However, this is unlikely because there is no evidence that ALA can induce cellular differentiation, it produced effects on enzyme activities similar to those of DMSO and the effect of DMSO and ALA together was not additive, suggesting that they are acting through a common mechanism. Furthermore, the protein content of control cell homogenates and cells treated with DMSO and/or ALA were not significantly different (results not shown), suggesting that the growth rate of the cultures had not been altered by the treatment.

In rodent liver, BROD and MROD activities are catalysed preferentially by the polycyclic, aromatic, hydrocarbon-induced P450IA family of isoenzymes, whereas PROD activity is catalysed mainly by the phenobarbitone-induced P450IIB1 isoenzyme; BROD activity reflects both the induced families, P450IA and P450IIB [21]. It should be noted, however, that the substrate specificities of the P450 isoenzymes of human liver tissue for resorufin ethers have not been fully investigated. DMSO and ALA caused a 20–25-fold increase in EROD, MROD and BROD activities in cells grown in Dulbecco's medium, compared with only a 2–4-fold increase in PROD activity. The effect of these agents on P450-dependent activities appeared, therefore, to be isoenzyme selective and apparently occurred with the P450IA family of isoenzymes but not with P450IIB.

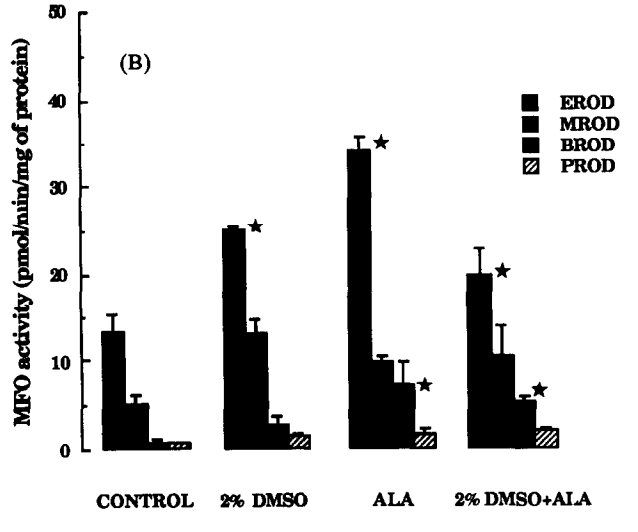
Galbraith *et al.* [22] have shown that the addition of 2% (v/v) DMSO to Hep G2 cells grown in a minimal essential medium results in a 2-fold increase in the concentration and activity of 5-aminolaevulinic acid dehydratase (ALAD), the second enzyme in the haem biosynthetic pathway. The increase in ALAD was due to *de novo* induction of enzyme protein in the Hep G2 cells and as a result of the induction the concentration of haem in the cells increased [23]. Haem is known to regulate a variety of cellular processes, such as differentiation, transcription and post-translational processing in different systems [24, 25]. In the rat, *in vivo*, haem has been shown to be a positive regulator of cytochrome P450 gene transcription and it also stabilizes freshly synthesized P450 apoprotein [26]. These published studies showed that haem optimized the transcription rates of cytochrome P450IA1 and P450IA2 [27], and P450IIB1 and P450IIB2 [26] at concentrations very much lower than those required to exert feedback regulation on its own biosynthesis. This may explain why the effects of DMSO and ALA on MFO activities were not additive in our experiments.

In the present study DMSO treatment did not increase the P450IIB1-associated PROD activity to the same extent as it increased the MROD and EROD activities associated with the P450IA family. Transcription of P450IIB1 may not be modulated by haem in Hep G2 cells. On the other hand, the expression, activity and induction of P450IIB1 has been difficult to demonstrate both in hepatocyte cultures and in cell lines [28, 29]. There may be some defect in the transcription process for this P450 isoenzyme in partially de-differentiated tissue which prevents a response both to inducers and to modulators like haem. Recent studies have demonstrated *in vitro* induction of P450IIB1 and P450IIB2 in rat hepatocytes cultured in serum-free medium with up to a 90-fold increase in PROD activity [30]. These studies showed that the presence of 10% foetal calf serum inhibited the induction process by more than 90%. The media used to culture HepG2 cells in the present study also contained 10% foetal calf serum and this may have influenced the effect of DMSO on the P450IIB1-associated PROD activity. The effect of serum on the response of Hep G2 cells to inducing agents is currently being investigated.

DMSO and ALA increased GT activity towards bilirubin



		CONTROL	2% DMSO	ALA	2% DMSO+ALA
Fold increase	EROD	1	19	20	19
	MROD	1	23	24	22
	BROD	1	18	22	19
	PROD	1	4	3.5	2



		CONTROL	2% DMSO	ALA	2% DMSO+ALA
Fold increase	EROD	1	2	2.5	1.5
	MROD	1	2.5	2	2
	BROD	1	3.5	9.5	7
	PROD	1	2	3	4

Fig. 1. Mixed function oxidase (MFO) activities of Hep G2 cells cultured in Dulbecco's medium (A) and Williams' E medium (B), and supplemented with 2% (v/v) dimethylsulphoxide (DMSO) and/or 10^{-4} M 5-aminolaevulinic acid (ALA). MFO activity is measured as the O-dealkylation of methoxyresorufin (MROD), ethoxyresorufin (EROD), pentoxyresorufin (PROD) and benzyloxyresorufin (BROD) as described in Materials and Methods. Results are means of three experiments and error bars represent SEM. *$P < 0.01$, using ANOVA followed by Dunnett's test. Significance values refer to differences between enzyme activities in control and treated cells. Fold increase values represent the increase in activities of treated cells compared with controls.

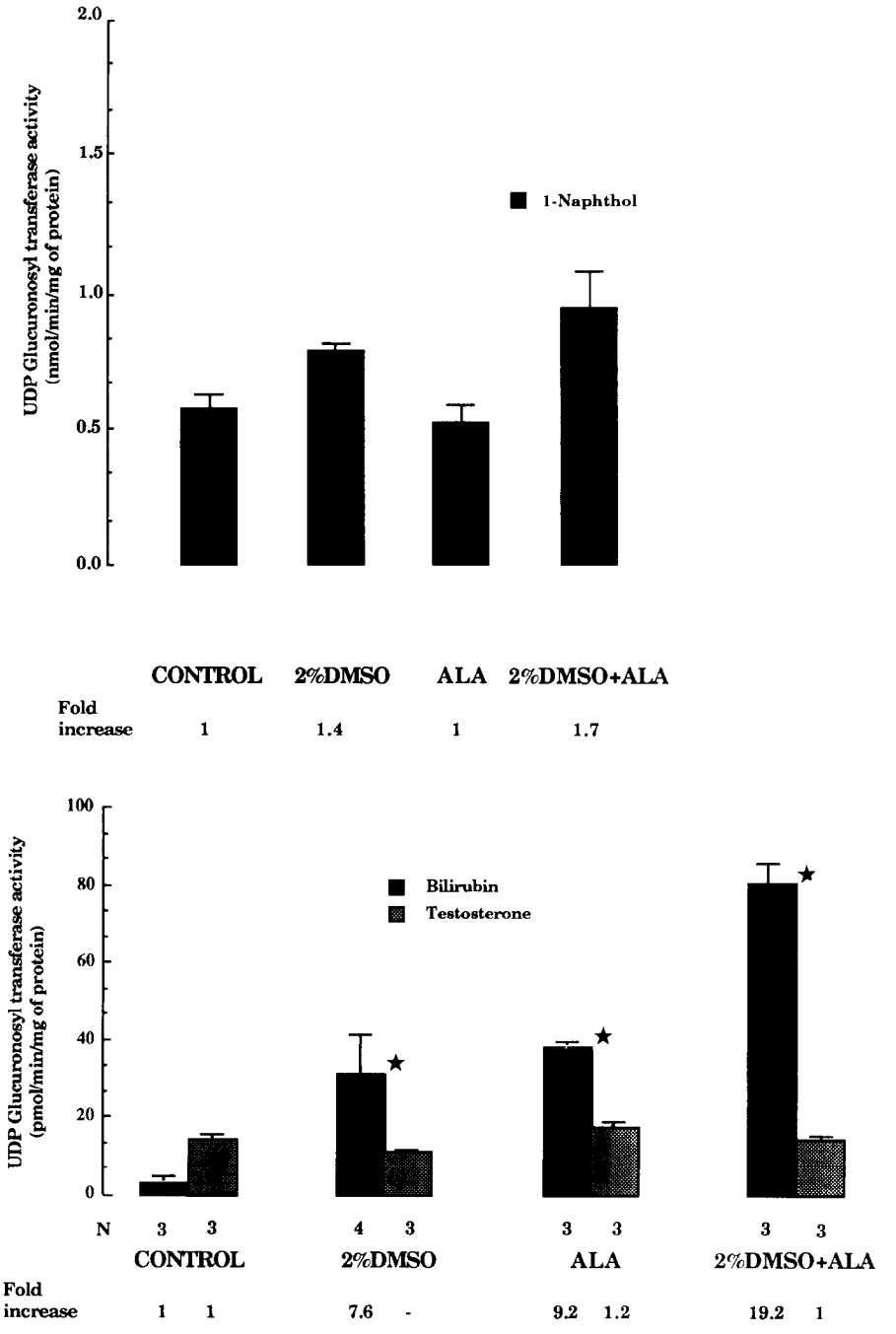

UDP Glucuronosyl transferase activity (nmol/min/mg of protein)
2.0
1.5
1.0
0.5
0.0
1-Naphthol
CONTROL
2%DMSO
ALA
2%DMSO+ALA
Fold increase
1
1.4
1
1.7
UDP Glucuronosyl transferase activity (pmol/min/mg of protein)
100
80
60
40
20
0
Bilirubin
Testosterone
N
3 3
4 3
3 3
3 3
CONTROL
2%DMSO
ALA
2%DMSO+ALA
Fold increase
1 1
7.6 -
9.2 1.2
19.2 1

Fig. 2A.

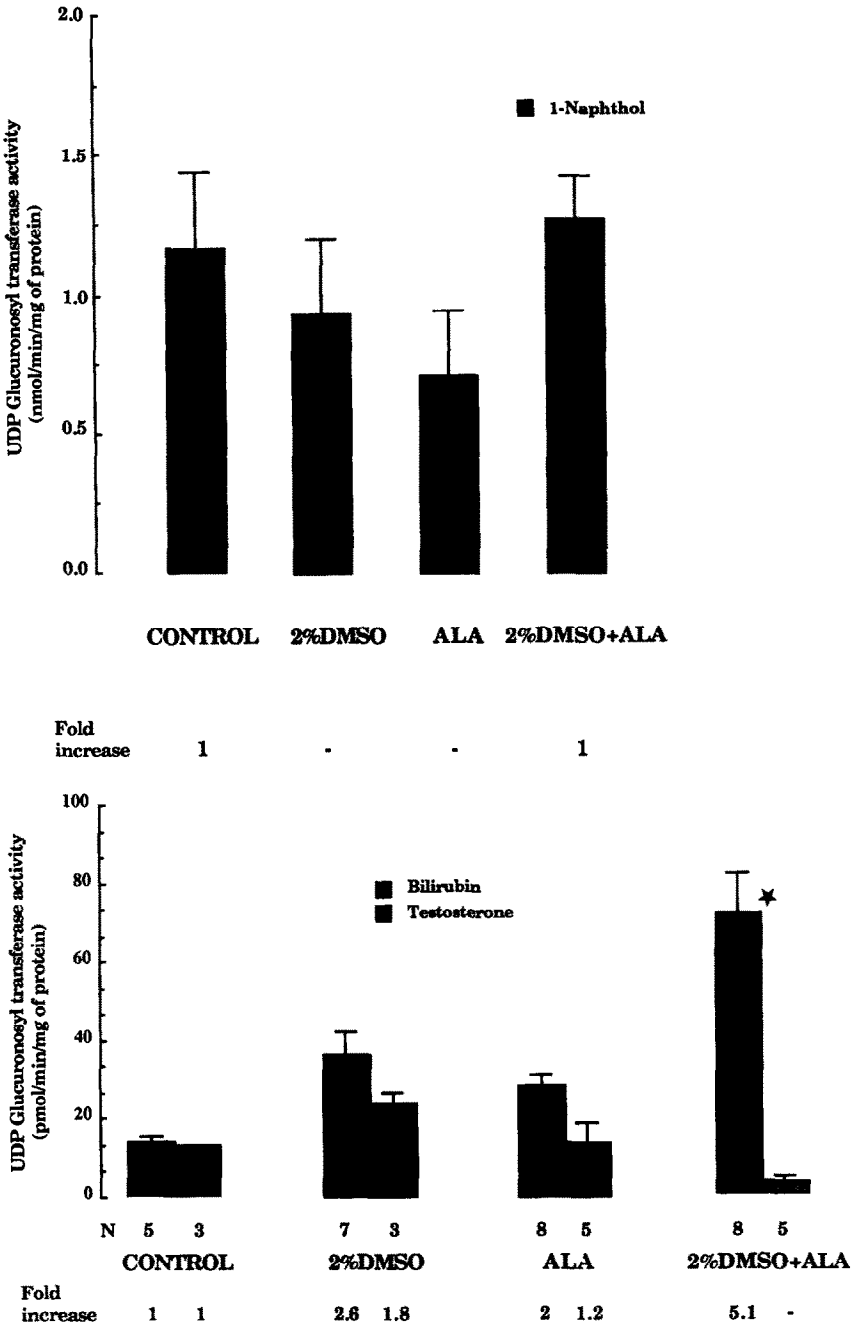


Fig. 2B.

Fig. 2. UDP-glucuronosyl transferase activities of Hep G2 cells cultured in Dulbecco's medium (A) and Williams' E medium (B), and supplemented with 2% (v/v) dimethylsulphoxide (DMSO) and/or 10^{-4} M 5-aminolaevulinic acid (ALA). For 1-naphthol the results are means of five experiments in Dulbecco's medium and 10 experiments in Williams' E medium. The number of experiments (N) for bilirubin and testosterone are shown. Error bars represent SEM. *$P < 0.01$, using ANOVA followed by Dunnett's test. Significance values refer to differences between enzyme activities in control and treated cells. Fold increase values represent the increase in activities of treated cells compared with controls.

but not towards 1-naphthol or testosterone. Bilirubin is the breakdown product of haem metabolism and it is further metabolized exclusively by conjugation with glucuronic acid prior to biliary excretion. A specific GT isoenzyme responsible for conjugating bilirubin has been identified in rat liver [31, 32]. Pretreatment of Hep G2 cells with either DMSO or ALA resulted in a 2-fold increase in the concentration of total bilirubin (conjugated and free) excreted into the medium and treatment with both agents together caused a 3-fold increase (H. Doostdar, unpublished results). The elevated concentrations of intracellular bilirubin appear to result in an increase in the specific GT isoenzyme activity required for detoxification. Whether or not this increase reflects *de novo* synthesis of the GT protein is not known at present. GT is a membrane-bound latent enzyme [32] and its activity may, therefore, be modulated not only by synthesis of new protein but also by modification of its relationship with the endoplasmic reticulum membrane.

This study has shown that the choice of growth medium is of crucial importance when investigating control of drug metabolism in cultured cells. Nevertheless Hep G2 cells provide a useful *in vitro* model system in which to study the regulation of enzyme activities in human liver. We have shown that both DMSO and ALA can increase the activities of the MFO system and GT and they are believed to act by increasing intracellular haem concentrations.

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Departments of
**Medicine and*
Therapeutics,
†Biochemistry and
‡Pharmacology
Aberdeen University
Polwarth Building
Foresterhill
Aberdeen AB9 2ZD, U.K.

HAMED DOOSTDAR*†‡
M. DANNY BURKE‡
WILLIAM T. MELVIN†
M. HELEN GRANT*§

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§ Address correspondence to: Dr M. Helent Grant, Bioengineering Unit, Wolfson Centre, University of Strathclyde, 106 Rottenrow, Glasgow G4 0NW, U.K.

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Influence of halothane on the interactions of serotonin$_{1A}$ and adenosine A_1 receptors with G proteins in rat brain membranes

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Certain volatile general anesthetics depress synaptic transmission by interfering with receptor–G protein interactions [1]. Halothane and other volatile anesthetics disrupt signal transduction by two neurotransmitter receptors, the muscarinic acetylcholine and α_2-adrenergic receptors, whose synaptic actions are mediated by pertussis toxin-sensitive G proteins [1–5]. This disruption is seen as a depression of the guanine nucleotide sensitivity of agonist binding, as well as an interference with the inhibitory control of these receptors over adenylate cyclase activity. In contrast, halothane does not affect the stimulation of adenylate cyclase activity by β-adrenergic receptors, an action which is mediated by the stimulatory, pertussis toxin-insensitive G_s protein [6].

These findings raise the possibility that volatile anesthetic disruption of receptor–G protein coupling is restricted to the family of neurotransmitter receptors which preferentially couple to G proteins which serve as substrates for ADP-ribosylation catalyzed by pertussis toxin, particularly G_i and G_o. To examine the validity of this suggestion, we examined the effects of halothane on adenosine A_1 and serotonin$_{1A}$ (5-HT$_{1A}$) receptors. A_1 and 5-HT$_{1A}$ receptor agonists inhibit adenylate cyclase activity through G_i protein activation [7, 8]. Moreover, the involvement of A_1 and 5-HT$_{1A}$ receptors in the regulation of sleep and analgesia makes them reasonable potential targets of anesthetic action [9–11].

Materials and Methods

Rats were decapitated and brain tissue was removed and homogenized in 15 mL of 50 mM Tris–HCl buffer, pH 7.4, containing 2 mM $MgCl_2$ and 1 mM dithiothreitol. The homogenate was centrifuged at 15,000 *g* for 20 min. The supernatant was discarded and the pellet washed twice by resuspension and centrifugation under the same conditions. The final pellet was suspended in buffer at a final protein concentration of 1 mg/mL. The G protein interactions of muscarinic and α_2-adrenergic receptors in neural membranes prepared in 50 mM Tris–HCl/2 mM $MgCl_2$ buffer were disrupted by anesthetics (e.g. [4]). Moreover, inclusion of 1 mM dithiothreitol in the buffer (as in the present experiments) did not alter these actions of halothane (e.g. [2]). Hippocampal membranes were used for 5-HT$_{1A}$ binding assays, while forebrain membranes were used for A_1 binding assays. Membranes used in [^{3}H]-cyclohexyladenosine ([^{3}H]CHA) binding assays were incubated for 30 min at room temperature with adenosine deaminase (0.2 unit/mL) to remove endogenous adenosine.

Assays were initiated by addition of the membranes to a binding medium containing either 1 nM 8-hydroxy-dipropylaminotetralin ([^{3}H]8-OH-DPAT; DuPont-NEN; 142.9 Ci/mmol) or 1 nM cyclohexyladenosine ([^{3}H]CHA; DuPont-NEN; 34.4 Ci/mmol). Incubations were carried out at 37° until binding equilibrium was reached (10 min, [^{3}H]8-OH-DPAT; 60 min, [^{3}H]CHA). Guanyl-5'-imidodiphosphate (Gpp(NH)p; Sigma), a stable analogue of GTP, was included in some assays. Non-specific binding was determined in the presence of 100 μM 5-HT or 1 mM theophylline in the 5-HT$_{1A}$ and A_1 receptor binding assays, respectively. Specific binding represented 80–90% of the total binding of either probe at 1 nM. Membranes were collected by vacuum filtration on glass fiber filters, and the radioactivity content of the filters was determined by liquid scintillation counting.

Under these conditions, the density of [^{3}H]8-OH-DPAT binding sites was 258 ± 35 fmol/mg membrane protein, while the dissociation constant was 1.1 ± 0.2 nM (means ± SD, N = 4). [^{3}H]CHA labeled 222 ± 19 fmol binding sites/mg membrane protein with a dissociation constant of 39 ± 0.5 nM (means ± SD, N = 4).

Anesthetics were added to assay tubes as aliquots from stock buffers. Anesthetic concentrations were verified by gas–liquid chromatographic analysis of 1-μL aliquots obtained from sham reaction tubes.

Results

Halothane inhibited the binding of [^{3}H]8-OH-DPAT to 5-HT$_{1A}$ receptors in hippocampal membranes by up to 23% at 1.8 mM (Fig. 1A). Inclusion of the guanine nucleotide, Gpp(NH)p, during the incubation had little effect on the inhibition by halothane.

In agreement with previous reports [12, 13], Gpp(NH)p