Expression of a high-affinity form of UDP-glucuronosyltransferase in human foetal liver cells in culture on exposure to mercuric chloride

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The activity of UDP-glucuronosyltransferase (UDPGT, EC 2.4.1.17) in human foetal liver cells in culture was measured with two acceptor substrates, namely harmol and 1-naphthol. There was a dose-dependent increase of about 10–400 % in UDPGT activity when the cells were exposed to 1–30 μM - HgCl_2 . Above a critical concentration of 30 μM - HgCl_2 , the heavy metal ion was toxic to the cells. Kinetic studies of the glucuronidation reaction with harmol and 1-naphthol showed that Hg^{2+} ions seemed to induce the expression of a high-affinity form of UDPGT, which was absent from the normal controls. The dramatic increase in specific activity in UDPGT was accompanied by a parallel increase in V_{max} measured with harmol and UDP-glucuronic acid. The significance of a possible induction of UDPGT in human foetal liver cells by HgCl_2 is discussed.

INTRODUCTION

Mercury is found in the environment in different chemical and physical forms. Although its toxicity is well known (Bidstrup, 1964), this heavy metal continues to be used in many commercial products, including thermometers, fungicides (Clarkson, 1988) as well as in Chinese medicine (Koh *et al.*, 1977; Sin *et al.*, 1983). Pregnant women and the developing foetuses may acquire mercury through occupational exposure or through the ingestion of contaminated fish or grain by the mother (Koos & Longo, 1976). Mercury compounds can pass through the placenta into the foetal circulation, and cases of intrauterine mercury poisoning have been reported (Matsumoto *et al.*, 1965; Amin-Zaki *et al.*, 1974).

UDP-glucuronosyltransferase (UDPGT) is an enzyme involved in Phase II detoxification process. It catalyses the transfer of glucuronic acid from UDP-glucuronic acid (UDPGA) to various xenobiotics and endobiotics. In this study, the effects of HgCl_2 on UDPGT of human foetal liver cells in culture were examined following from our previous observation of increase in UDPGT activity after the oral administration of mercury to mice (Tan *et al.*, 1990a). The bivalent (Hg^{2+}) ion was employed, as it is believed to be the proximate species in mediating mercury toxicity (Clarkson, 1988). The toxic effects of all forms of inorganic mercury are ascribed to the action of ionic mercury (Nordberg & Skefving, 1972). Organic mercury compounds such as methylmercury and mercury vapour can be transformed into the inorganic form by the processes of demethylation and oxidation, respectively (Norseth & Clarkson, 1970a,b; Clarkson, 1972; Rodier *et al.*, 1988; Hursh *et al.*, 1988).

MATERIALS AND METHODS

Chemicals

UDP-[U- ^{14}C]glucuronic acid (sp. radioactivity 303 mCi/mmol) was purchased from New England Nuclear Corp., Boston, MA, U.S.A. 1-[1- ^{14}C]Naphthol (sp. radioactivity 57 mCi/mmol) was from Amersham International, Amersham, Bucks., U.K. HgCl_2 and harmol hydrochloride were from Aldrich Chemical

Co., Milwaukee, WI, U.S.A. UDPGA (ammonium salt), cycloheximide and octyl sulphate (sodium salt) were from Sigma Chemical Co., St. Louis, MO, U.S.A. 1-Naphthol was obtained from E. Merck, Darmstadt, Germany. Methanol of h.p.l.c. grade was from J. T. Baker, Phillipsburg, NJ, U.S.A. Dulbecco's modified Eagle medium and foetal-bovine serum were obtained from ICN Flow, Irvine, CA, U.S.A.

Cell culture

Livers from second-trimester prostaglandin-induced abortuses were used. The preparation of liver cells from the tissues and the conditions of culture have been described (Tan *et al.*, 1988). Routinely, $(5\text{--}10) \times 10^6$ cells were plated in 25 cm² Lux culture flasks in Dulbecco's modified Eagle medium supplemented with 10 % (v/v) foetal-calf serum.

Exposure of cells to HgCl_2

At 4 days after seeding, 0.1 ml of an aqueous solution of HgCl_2 (final concn. of Hg^{2+} of 1–400 μM) per 10 ml of culture medium was added daily to the experimental flasks. Each concentration of HgCl_2 was added in duplicates. The culture medium was changed daily. Control cultures were maintained similarly without addition of HgCl_2 . The cells were harvested on day 7 or 9 after addition of HgCl_2 .

Short-term exposure of cells to HgCl_2 was also examined. At 7 days after seeding, 30 μM - HgCl_2 was added to the experimental flasks, this being the concentration of HgCl_2 observed in the preceding series of experiments which elicited maximal effects on the UDPGT activity. Cells from control and experimental flasks were harvested at time intervals of 30 up to 120 min after addition of HgCl_2 .

Preparation of extracts from human foetal liver cells

The medium was discarded, and the cells were scraped with a rubber policeman and resuspended in 25 ml of cold 0.15 M-KCl, followed by centrifugation at 15000 *g* for 15 min. This step was repeated, and a final suspension of 50 % (w/v) was prepared in 0.15 M-KCl/3 mM-dithiothreitol. This was stored in small batches

Abbreviations used: UDPGT, UDP-glucuronosyltransferase; UDPGA, UDP-glucuronic acid.

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at -80°C and used as the enzyme source for the assay of UDPGT activity.

Measurement of UDPGT activity

(a) With harmol as acceptor substrate. A typical assay mixture contained the following, with final concentrations in parentheses: $5\ \mu\text{l}$ of 100 mM-UDPGA (5 mM), $5\ \mu\text{l}$ of 5 mM-harmol (250 μM), $5\ \mu\text{l}$ of 100 mM- Mg^{2+} (5 mM), $70\ \mu\text{l}$ of 50 mM-glycine/NaOH buffer, pH 8.6, and $15\ \mu\text{l}$ of enzyme extract containing 100–200 μg of protein. The protein content was measured by the procedure of Lowry *et al.* (1951). After 20 min of incubation at 37°C , the reaction was terminated with 0.3 M- $\text{Ba}(\text{OH})_2$ and 5% (w/v) ZnSO_4 . The incubation mixture was then adjusted to 250 μl with water, and $150\ \mu\text{l}$ of 0.2 M- Na_2HPO_4 buffer, pH 9.5, was added. It was then extracted with $2 \times 0.5\ \text{ml}$ of ethyl acetate by the procedure of Pang *et al.* (1981). After centrifugation, the aqueous layer was filtered and $20\ \mu\text{l}$ was injected for the quantification of harmol glucuronide by h.p.l.c./fluorimetry by the method described previously (Tan *et al.*, 1990b).

Kinetic studies were carried out at pH 8.6 in 50 mM-glycine/NaOH buffer and in the presence of 5 mM- Mg^{2+} . UDPGA concentration was varied from 20 μM to 5 mM in the presence of 250 μM -harmol. Another set of data was obtained by varying harmol concentration from 1.25 to 250 μM in the presence of 5 mM-UDPGA. Extracts from human foetal liver cells treated with 30 μM - HgCl_2 daily for 7 days were used.

(b) With 1-naphthol as acceptor substrate. The incubation mixture contained the following: 6.547 μM -UDP- ^{14}C GA, 100 μM -1-naphthol, 5 mM- Mg^{2+} and $10\ \mu\text{l}$ of enzyme extract containing 50–100 μg of protein made up to a final volume of $50\ \mu\text{l}$ with 50 mM-glycine/NaOH buffer, pH 8.6. At time intervals up to 30 min, $10\ \mu\text{l}$ of each incubation mixture was spotted on a strip (60 cm $\times$ 0.75 cm) of Whatman no. 1 paper. The chromatograms were developed in the organic phase of the solvent system consisting of butan-1-ol/acetic acid/water (4:1:5, by vol.). The sections of the chromatograms corresponding to 1-naphthol ^{14}C glucuronide, with R_f 0.88, were cut and counted for radioactivity by liquid scintillation as described previously for glucuronides (Wong, 1976). Standards of UDP- ^{14}C GA were similarly counted, and the amount of 1-naphthol ^{14}C glucuronide formed was extrapolated from the standard curve.

The kinetic constants were also determined with 1-naphthol as the acceptor by using extracts of control human foetal liver cells and those cultures treated with 30 μM - HgCl_2 for 7 days. 1-Naphthol was varied from 0.1 to 375 μM in the presence of 6.547 μM -UDP- ^{14}C GA.

Analysis of kinetic data

A computer program has been reported to give a good approximation of kinetic parameters of K_m and $V_{\max}$ values for two enzymes or two forms of an enzyme acting on a common substrate (Spears *et al.*, 1971). A modified version of this program, entitled 'KINSTWO', was kindly supplied to us by Dr. E. G. Loten (University of Otago), one of the authors. It uses the method of Cornish-Bowden (Eisenthal & Cornish-Bowden, 1974; Cornish-Bowden & Eisenthal, 1974) to calculate these values. The values which exhibited deviant Michaelis-Menten kinetics were drawn as Eadie-Hofstee plots (Figs. 3a and 3b) and fitted by the isoenzyme model in the EZ-FIT program, developed by Dr. Frank W. Perrella (E.I. DuPont de Nemours & Co.). The equation employed was:

$$v = \frac{V_{\max,1}[\text{S}]}{K_{m1} + [\text{S}]} + \frac{V_{\max,2}[\text{S}]}{K_{m2} + [\text{S}]}$$

Effects of Hg^{2+} on UDPGT activity *in vitro*

Extracts from control foetal liver cells were used in these assays. Hg^{2+} (in the form of an aqueous solution of HgCl_2) was added to the standard assay mixture in final concentrations ranging from 1 nM to 1 μM . Harmol glucuronide formed was quantified as described above.

Effects of cycloheximide on HgCl_2 -treated cultures

Cultures of human foetal liver cells were treated with cycloheximide and with cycloheximide plus HgCl_2 for 7 days. The culture medium was changed daily. Cycloheximide was added at final concentrations of 0.1, 0.5 and 1 μM to normal cultures and also to those treated simultaneously with 30 μM - HgCl_2 . The choice of these concentrations was based on a preliminary set of experiments which showed that cycloheximide at concentrations greater than 2.5 μM was cytotoxic. Another batch of culture was treated with only 30 μM - HgCl_2 , whereas control cultures were untreated.

RESULTS

Effects of HgCl_2 on human foetal liver cells

HgCl_2 at concentrations higher than 30 μM was found to be cytotoxic; the lethal effect was apparent 1 day after exposure to concentrations from 50 to 400 μM . On the other hand, UDPGT activity was increased in cultures treated with 1–30 μM - HgCl_2 .

Fig. 1. Dose-dependent increase of UDPGT activity by HgCl_2 treatment of human foetal cells in culture for 7 days, measured with harmol (●) and 1-naphthol (○)

Control values were designated as 100%.

Fig. 2. Percentage increase in UDPGT activity after treatment of human foetal liver cells in culture with 30 μM - HgCl_2 for 7 days (●) and 9 days (○)

Control values were designated as 100%.

Table 1. Specific activities of UDPGT of human foetal liver cells after short-term exposure to 30 μM - HgCl_2

Values are expressed in pmol of harmol glucuronide/min per mg of protein. Each value was an average of the UDPGT activities of two cultures, except for the value for 0 h, which was the average of four cultures.

Length of exposure (h)	Specific activity
0	25.46
0.5	27.82
1.0	33.90
1.5	23.85
2.0	26.69

Fig. 3. Eadie-Hofstee plots for the glucuronidation of harmol with cells treated with 30 μM - HgCl_2 for 7 days

The velocity (v) was expressed in pmol of harmol glucuronide/min per mg of protein, and the respective harmol and UDPGA concentrations were expressed in μM : (a) harmol from 1.25 μM to 10 μM (●) and 10 μM to 250 μM (○); (b) UDPGA from 20 μM to 100 μM (●) and 200 μM to 5 mm (○). The data were fitted by the isoenzyme model in the EZ-FIT program by using an unweighted least-squares procedure.

This stimulatory effect was consistently observed in the UDPGT activity measured with harmol or 1-naphthol as the acceptor substrate. It is noteworthy that similar bimodal phenomena at high and low concentrations of heavy metals were observed in oligodendroglial cells (Grundt & Neskovic, 1989). The increase in UDPGT activity was dependent on the concentration of HgCl_2 added to the medium (Fig. 1). The maximal UDPGT activity, of about 5-fold over the controls, was found in cells treated for 7 days with 30 μM - HgCl_2 . A greater increase in UDPGT activity was observed at 9 days than at 7 days of exposure (Fig. 2). Morphologically, there was no difference between mercury-treated and control cells when viewed under the light microscope. At this same concentration of 30 μM , HgCl_2 had no effect on UDPGT activity on short-term exposure up to 2 h (Table 1). Likewise, HgCl_2 between 1 nM and 1 mM added to

Table 2. Kinetic data for the glucuronidation of harmol and 1-naphthol by extracts of control and/or mercury-treated human foetal liver cells

Values of V_{max} are expressed in pmol of harmol or 1-naphthol glucuronide/min per mg of protein. K_m and V_{max} values were obtained by the 'KINSTWO' program.

Substrate (μM)	Treatment	K_{m1} (μM)	$V_{\text{max},1}$	K_{m2} (μM)	$V_{\text{max},2}$
Harmol					
10–375	Control	—	—	51.4*	19.8*
1.25–10	30 μM - HgCl_2	0.52	15.9		
10–250	30 μM - HgCl_2			140.7	143.7
UDPGA					
200–500	Control	—	—	396*	15.4*
20–100	30 μM - HgCl_2	23.0	15.9		
200–5000	30 μM - HgCl_2			800	80
1-Naphthol					
0.1–0.5	20 μM - HgCl_2	0.19	7.1		
0.5–31.25	20 μM - HgCl_2			8.1	9.5

* Reproduced from data published previously (Tan *et al.*, 1990b).

the assay incubation mixture had no effect on the UDPGT activity. At concentrations of 0.5 mM- HgCl_2 and above, quenching of the fluorescence of both harmol and harmol glucuronide occurred. Addition of a boiled extract of cells which had been treated with 30 μM - HgCl_2 also had no effect on the UDPGT activity, suggesting that the effect of Hg^{2+} was not mediated by metabolite(s) present in the extracts of mercury-treated cells.

Kinetic data

The formation of harmol glucuronide by extracts of mercury-treated cells exhibited deviant Michaelis-Menten kinetics. This anomalous kinetic behaviour was evident from the non-linear Eadie-Hofstee plots for both harmol and UDPGA (Figs. 3a and 3b). Two apparent K_m and V_{max} values were obtained for both harmol and 1-naphthol, the acceptor substrates, and for UDPGA, the conjugating agent (Table 2). These values were established by the 'KINSTWO' program, described in the Materials and methods section.

Kinetic studies were also carried out for 1-naphthol glucuronidation with control cultures and cultures treated with 30 μM - HgCl_2 . Normal Michaelis-Menten kinetics were observed for 1-naphthol glucuronidation in extracts of untreated cells. The apparent K_m was 4.34 μM for 1-naphthol. In contrast, deviant kinetic behaviour was observed in extracts of cultures treated with 30 μM - HgCl_2 (Table 2). The K_{m1} value of 0.19 μM was about 43 times less than the K_{m2} value, which was 8.1 μM . Unfortunately, a similar set of experiments could not be conducted with UDPGA, the limitation being imposed by the low concentration of UDP- ^{14}C GA employed in the radiometric assay. Attempts to overcome this problem by employing labelled 1- ^{14}C naphthol in conjunction with unlabelled UDPGA were unsuccessful; controls in which UDPGA was omitted invariably produced unacceptably high background values when 1- ^{14}C naphthyl glucuronide was analysed by paper chromatography as detailed in the Materials and methods section or by h.p.l.c./radiometry using several different mobile solvent systems.

Effects of cycloheximide on mercury-treated cells

The effects of cycloheximide were studied by adding HgCl_2 and cycloheximide both independently and simultaneously to the

Table 3. Specific activities of UDPGT of cultures treated with cycloheximide alone or plus HgCl₂ (30 µM)

Concn. of cycloheximide (µM)	UDPGT (expressed in pmol of harmol glucuronide/min per mg of protein)		Ratio (a)/(b)
	(a) Mercury-treated cultures	(b) Control cultures	
0	134.8	39.2	3.44
0.1	107.1	29.1	3.68
0.5	70.7	27.1	2.61
1.0	63.8	22.9	2.79

culture. Cycloheximide added at a final concentration of 2.5 µM or more was found to be toxic to untreated and mercury-treated cultures. As observed in a previous experiment (Fig. 1), the UDPGT activity of cultures treated with 30 µM-HgCl₂ was higher than the control (Table 3). A similar ratio was obtained in cultures co-treated with 0.1 µM-cycloheximide. However, when the concentrations of cycloheximide were increased to 0.5 µM and 1 µM, the ratio of mercury-treated:control values decreased to 2.61 and 2.79 respectively (Table 3), showing that the stimulatory effect of Hg²⁺ was somewhat negated by the inhibitor of protein synthesis.

DISCUSSION

The inducibility of UDPGT by phenobarbital and 3-methylcholanthrene in experimental animals, particularly in the rat, has been well documented (Bock *et al.*, 1973; Wishart, 1978; Lilienblum *et al.*, 1982). This has led to the classification of rat liver UDPGT activity into two groups on the basis of their inducibility by these two prototype inducers (Bock *et al.*, 1983). Little is known about the inducibility of human UDPGTs. In view of the species differences which exist in differential inducibility of UDPGT (Bock & Bock-Hennig, 1987), it would appear that the use of human tissues would be obligatory for such investigations. In contrast with the conventional method of using whole experimental animals for such studies, which cannot be extended or extrapolated to humans, the present study was performed on human liver cells in culture, which seem to offer a useful model system for study of the induction phenomenon in man. Elevated UDPGT activities were observed in cells treated with HgCl₂; the degree of increase was dependent on the dose of HgCl₂ as well as the length of treatment. This phenomenon resembles the induction of UDPGT by the prototype inducers mentioned above in terms of the several-fold increase in enzyme activity. In this context, it is noteworthy that induction of UDPGT had been demonstrated previously in the HepG2 cell line of human origin (Dawson *et al.*, 1985) by 3-methylcholanthrene at micromolar concentration, similar to that of mercury used in the present study. The cytotoxic effect was also observed at slightly higher concentration (15 µM) of 3-methylcholanthrene. However, Hg²⁺ added to the assay mixture had no effect on the UDPGT activity. This was in contrast with the stimulatory effects observed with other bivalent ions such as Mn²⁺ and Mg²⁺ (Zakim *et al.*, 1973; Tan *et al.*, 1990b).

From our study, there was suggestive evidence that mercury treatment causes induction of UDPGT, as the stimulatory effect of this heavy metal was diminished, but not completely abolished, by inclusion of cycloheximide in the culture medium. Although this did not show conclusively that induction did occur, our

results were indicative of such a mechanism. Furthermore, in contrast with control cells, where data for UDPGT activity conformed to the normal Michaelis-Menten kinetics, with one apparent *K_m* value for harmol (Table 2) and for 1-naphthol, mercury-treated cells showed the presence of two affinity components in the glucuronidation of harmol (Table 2 and Fig. 3a) and 1-naphthol (Table 2). This was similar to the kinetic profiles of UDPGT of adult human liver, where two *K_m* values were obtained for both of these acceptors (Miners *et al.*, 1988a; Tan *et al.*, 1990b). If these two different components indeed represent two different isoenzyme forms of adult human liver UDPGT, as suggested by Miners *et al.* (1988b), it would seem possible that Hg²⁺ ions could have induced the expression of the high-affinity form in the human foetal liver cells. The significant increase in *V_{max}* of 5–9-fold in mercury-treated cells (see Table 2) appeared to add credence to this proposition. This magnitude of increase was not observed with 1-naphthol because of the limitation imposed by the sub-optimal concentration of labelled UDPGA employed in the present experiments.

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