Glucuronidation of the environmental oestrogen bisphenol A by an isoform of UDP-glucuronosyltransferase, UGT2B1, in the rat liver

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Bisphenol A, an environmental oestrogenic chemical, was found to conjugate highly with glucuronic acid in male rat liver microsomes studied *in vitro*. In the various isoforms tested (1A1, 1A3, 1A5, 1A6, 1A7 and 2B1), glucuronidation of bisphenol A and of diethylstilboestrol, a synthetic crystalline compound possessing oestrogenic activity and known to be glucuronidated by liver microsomes, was catalysed by an isoform of UDP-glucuronosyltransferase (UGT), namely UGT2B1, which glucuronidates some endogenous androgens. UGT activity towards bisphenol A in liver microsomes and in UGT2B1 expressed in yeast AH22 cells (22.9 and 0.58 nmol/min per mg of microsomal proteins respectively) was higher than that towards diethylstilboestrol (75.0 and 4.66 pmol/min per mg of micro-

somal proteins respectively). UGT activities towards both bisphenol A and diethylstilboestrol were distributed mainly in the liver but were also observed at substantial levels in the kidney and testis. Northern blot analysis disclosed the presence of UGT2B1 solely in the liver, and about 65 % of the male rat liver microsomal UGT activities towards bisphenol A were absorbed by the anti-UGT2B1 antibody. These results indicate that bisphenol A, in male rat liver, is glucuronidated by UGT2B1, an isoform of UGT.

Key words: diethylstilboestrol, environmental chemicals, oestrogens.

INTRODUCTION

A number of environmental chemicals have been implicated in the abnormal sexual development of reptiles [1,2] and birds [3], in feminized responses of male fish [4,5] and in an increased incidence of human breast cancer [6,7] as well as the attenuation of sperm count and fertility [8,9]. Various substances are currently considered to be environmental oestrogens, including pesticides, pollutants and certain chemicals [10]. One such compound, bisphenol A (BPA), is widely used by the chemical industry in the manufacture of epoxy, polycarbonate and polyester-styrene resins [11], and trace levels of BPA leak out from polycarbonate plastic ware and from resins used in food-packaging materials for industrial processing and consumer use [12,13].

BPA has been shown to act as an oestrogen on MCF-7 human breast cancer cells, stimulating cell proliferation and inducing progesterone receptors [12]. BPA was shown to bind to the oestrogen receptors; the oestrogenic effects elicited by BPA were blocked by the oestrogen antagonist tamoxifen, thus lending credibility to the notion that the oestrogenic activities of BPA are mediated via the oestrogen receptor [12]. Although so far the information available is insufficient to support a causal relationship between BPA and breast cancer or any general adverse effects, this potential hazard of BPA could conceivably occur on a global scale, affecting the entire population. Because BPA is a weak oestrogen, approx. 1/1000 to 1/2000 as potent as oestradiol [12], the potential public health hazards of BPA cannot be estimated without a further study of its metabolism in cells and in the whole body.

BPA has been reported to be hydroxylated *in vivo* [13] and to bind to DNA [14]; 28 % of the ¹⁴C-labelled BPA administered to rats was excreted in the urine, primarily as glucuronide [13]. Glucuronidation is a major pathway for the metabolism and excretion of both endogenous and exogenous compounds in vertebrates [15]. In the present study, BPA and diethylstilboestrol (DES), another compound widely known to have severe oestrogenic toxicity [16] and to undergo glucuronidation [17], were shown to be glucuronidated in liver from male rats by an isoform of UDP-glucuronosyltransferase (UGT) (EC 2.4.1.17), namely UGT2B1.

EXPERIMENTAL

Materials

Cholic acid, purchased from Nissui Yakuhin Co., was further purified and converted to its sodium salt [18]. UDP-glucuronic acid was obtained from Nakarai Yakuhin Co. BPA, DES, DES glucuronide, testosterone, oestradiol and oestradiol glucuronide were obtained from Sigma Chemicals. Nylon membrane (Hybond N⁺) was purchased from Amersham Co., and TRIzol[®] reagent was from Gibco BRL. Other reagents used were of the highest grade available.

Preparation of microsomes from rat tissues

Male and female adult Wistar rats (250 g) were killed by cervical dislocation, and the liver, kidneys, lungs, intestines, testes and brain were minced and homogenized with 4 vol. of 0.15 M KCl solution containing 1 mM EDTA. The homogenate was centri-

Abbreviations used: BPA, bisphenol A; DES, diethylstilboestrol; RT-PCR, reverse transcriptase-mediated PCR; UGT, UDP-glucuronosyltransferase.

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fused for 15 min at 9000 *g* and the supernatant fraction was centrifuged at 105 000 *g* for 60 min to obtain the microsomes. Protein concentration was determined by the method of Lowry et al. [19], with BSA as the standard.

Preparation of anti-UGT2B1 antibody

UGT2B1 specific anti-peptide (the C-terminal region 517–529, CRKTANMGKKKE) antibody was prepared and the specificity was confirmed as described by Ikushiro et al. [20].

Northern blot analysis

Total RNA (20 mg), isolated from 0.2 g of each tissue preparation by using TRIzol reagent, was subjected to electrophoresis with denaturation by formamide; the total RNA species then were transferred to a nylon membrane. A digoxigenin-labelled UGT2B1 cRNA probe was used to detect mRNA-encoded UGT2B1, as described by Kohri et al. [21]. A 1.6 kb full-length cDNA of UGT2B1 was subcloned into Bluescript pKS(–). A digoxigenin-UTP-labelled anti-sense cRNA probe was prepared with a DIG RNA labelling kit (Boehringer Mannheim) in accordance with the manufacturer's instructions.

cDNA cloning and expression of UGTs

From the livers of phenobarbital-treated rats, rat UGT isoforms UGT1A1, UGT1A3, UGT1A5 and UGT2B1 were obtained by reverse transcriptase-mediated PCR (RT-PCR). A single-strand cDNA was synthesized from the total RNA by Super Script II reverse transcriptase. The coding regions of the respective cDNA species were amplified by PCR with oligonucleotide primers, which were designed by reference to the sequences of rat UGT cDNA species of UGT1A1, UGT1A3, UGT1A5, UGT1A6 and UGT1A7 as reported by Emi et al. [22] and of UGT2B1 as reported by Mackenzie [23]. The forward primers (containing *SpeI* sites) were 5'-ATACTAGTATGTCCGTGGTG-TGCCGGAGCTC-3' for UGT1A1, 5'-ATACTAGTATGG-GAATCCAAGGGTTCCTGC-3' for UGT1A3, and 5'-ATAA-CTAGTATGGGACTCCATGTGACC-3' for UGT1A5; the reverse primers (containing *BspEI* sites) were 5-GTTCTCCGG-AGGCGTTGACATAG-3' for UGT1A1, UGT1A3 and UGT1A5. PCR mixture (100 μ l), containing each dNTP at 0.2 mM, each primer at 0.2 mM and 25 units/ml *Pfu* DNA polymerase (Stratagene), was subjected to 30 cycles of amplification: 1 min at 94 °C, 2 min at 45 °C and 4 min at 72 °C. The amplified cDNA species (exon 1) were subcloned into the pUC119 vector and sequenced; the individual isoenzymes were identified by an Applied Biosystems Model 377 sequencer. The cDNA species obtained were joined to the exon 2–5 fragment from the previously cloned UGT1A6; the full-length cDNA was then constructed for each isoform. A full-length UGT2B1 cDNA was obtained by using the forward primer (containing a *SpeI* site), 5'-CGCACTAGTATGTCTATGAAACAGACTTCA-3', and the reverse primer (containing a *SpeI* site), 5'-CGCACTAGTCTA-CTCTTTCTTCTTCTT-3'. From the liver of β -naphthoflavone-treated rats, a full-length UGT1A7 cDNA was cloned by RT-PCR by using a forward primer (containing a *SpeI* site), 5'-TTATTACTAGACCTCGAGATGGCTCCTGCAGACGTTCC-3' and reverse primer (containing a *SpeI* site), 5'-GGGA-CTAGTTCAGTGGGTCTTGGATTTG-3' [24]. A full-length UGT1A6 cDNA was prepared from the liver of 3-methylcholanthrene-treated rats as described previously [25]; these cDNA species were subcloned into the yeast expression vector pAAH5 and expressed in yeast strain AH22 cells [25]. Yeast transformants were cultivated in a synthetic minimal medium

[0.67% yeast nitrogen base without amino acids/2% (w/v) dextrose] supplemented with 20 mg/l histidine, and finally in YPD [1% (w/v) yeast extract/2% (w/v) peptone/2% (w/v) dextrose]. Yeast microsomes were prepared by the method of Oeda et al. [26].

Enzyme analysis and HPLC

UGT activities towards the various substrates were assayed in 200 μ l of 50 mM Tris/HCl buffer (pH 7.4)/0.5 mM MgCl₂ containing 0.25 mM BPA and 0.25 mM DES maintained at 37 °C. The resulting enzyme reaction products were filtered by a disposable disc filter (HPLC-DISK₃; Kanto Co., Tokyo, Japan) and analysed by an HPLC system consisting of a Tosoh TSKgel 80TM reverse-phase column (7.8 mm $\times$ 30 cm). The filtered samples were injected and eluted with acetonitrile/water/acetic acid (35:65:0.1, by vol.).

Absorption of UGT activities

Liver microsomes (50 μ g of protein) solubilized with 0.6% sodium cholate were incubated with various amounts of anti-UGT2B1 antibody serum and with unimmunized serum (15 mg/ml total protein) in 0.05 M potassium phosphate buffer, pH 7.4, for 2 h at room temperature. An excess of Protein A–Sepharose powder was added to the solution and incubated at 4 °C for 2 h. The mixture was then centrifuged at 35 *g* for 5 min to sediment only the Protein A–Sepharose, leaving microsomal protein bound to the UGT2B1 antibody. The supernatant fluid was subsequently assayed for UGT activities.

RESULTS

The results of the HPLC analysis of the reaction products of BPA obtained *in vitro* from rat liver microsomes in the presence of UDP-glucuronic acid are shown in Figure 1. Unconjugated BPA in the absence of UDP-glucuronic acid was eluted at 27 min, which was the same as the elution time of standard BPA (results not shown). The reaction products of BPA in rat liver microsomes in the presence of UDP-glucuronic acid contained a new peak that, by the disappearance of the peak after β -glucuronidase treatment, was confirmed as BPA-glucuronide. We cloned various full-length cDNA species encoding UGT isoforms (UGT1A1, 1A3, 1A5, 1A6, 1A7 and 2B1) by RT-PCR and expressed them functionally in yeast AH22 cells, as described previously for UGT1A6 [25] and UGT2B1 [27]. UGT1A1 and UGT1A5 could glucuronidate bilirubin and UGT1A7 could glucuronidate 1-naphthol slowly (results not shown). The only isoform that did not show glucuronidation activity towards any of the substrates tested was UGT1A3. However, all the isoforms cited above were highly expressed in yeast microsomes, as shown on a Western blot with polyclonal antibodies prepared previously [28]. The chromatogram in Figure 1 shows that BPA was conjugated with glucuronic acid by UGT2B1, an isoform of UGT. None of the other isoforms could glucuronidate BPA (results not shown).

The chemical structure of BPA is similar to that of DES, which is known as a potent non-steroidal oestrogenic compound and is glucuronidated by liver microsomes [17]. The reaction products of DES in rat liver microsomes (Figure 1) were analysed by HPLC (Figure 2). An authentic standard of DES glucuronide was detected at 9 min (Figure 2A), and the same peak was eluted from the reaction products in rat liver microsomes (Figure 2B). Minor peaks were observed at 16–17 min (Figure 2B). After treatment of the products with β -glucuronidase, DES glucuronide and the minor unknown peaks disappeared. The kidney micro-

Figure 1 HPLC analysis of BPA glucuronidation in rat liver microsomes and in cloned UGT2B1 expressed in microsomes of yeast AH22 cells

Chromatograms were generated from HPLC of the reaction products of BPA in rat liver microsomes without (A) or with UDP-glucuronic acid (B) as described in the Experimental section. (C) Chromatogram of β -glucuronidase treatment of the same products as those shown in (B). (D) Chromatogram of the reaction products of BPA in the expressed UGT2B1 in the presence of UDP-glucuronic acid. The peak eluted at 27 min in (A) was confirmed as unreacted BPA.

somes could produce DES glucuronide but not the unknown minor peaks at 16–17 min. Although various UGT isoforms were tested (see Figure 1), only the isoform UGT2B1 was found to glucuronidate DES and also to produce unknown minor peaks at 16–17 min.

The kinetic parameters of UGT towards BPA, DES and 4-hydroxybiphenyl, which is known to be highly glucuronidated by UGT2B1, are compared in Table 1. The $V_{\max}$ of UGT in rat liver microsomes towards BPA was more than 3-fold that towards DES (Table 1). The K_m towards BPA was approx. one-third less than that towards DES. The $V_{\max}$ towards BPA of UGT2B1 was 125-fold that towards DES. Parameters of BPA UGT activity in both liver microsomes and the UGT2B1 microsomes expressed in yeast were of the same order as those of 4-hydroxybiphenyl

Figure 2 HPLC analysis of DES glucuronidation in rat liver and kidney microsomes and in the expressed UGT2B1

(A) HPLC of the authentic standard of DES glucuronide. (B, D, E) HPLC of the products of incubation reactions of DES with the following samples as described in the Experimental section: rat liver microsomes (B), rat kidney microsomes (D) and yeast microsomes expressing UGT2B1 (E). (C) Chromatogram of β -glucuronidase treatment of the same products as those shown in (B). Elution of unconjugated DES was confirmed as a gentle peak at 45 min. Unknown peaks (arrows) were eluted at 16–17 min from the incubation reactions of DES by rat liver microsomes (B) and in the expressed 2B1 (E).

glucuronidation. These results show that BPA was a much better substrate than DES in the rat liver microsomes.

The distribution of UGT activities towards both BPA and DES in the various rat tissues is shown in Figure 3. BPA glucuronidation activity was evident in the rat liver, kidney and testis (Figure 3A), and a comparable distribution was found for DES glucuronidation activity (Figure 3B). Minimal glucuronidation of BPA was noted in the lung and brain microsomes (Figure 3A).

Table 1 Kinetic parameters of UGTs in rat liver microsomes and UGT2B1 expressed in yeast microsomes

Enzymic activities were assayed as described in the Experimental section. $V_{\max}$ values were determined with 8–16 adult male rats and three experiments of the assay of UGT2B1 activities. $V_{\max}$ values are means $\pm$ S.E.M.

	BPA		DES		4-Hydroxybiphenyl	
	$V_{\max}$ (nmol/min per mg)	K_m (μ M)	$V_{\max}$ (pmol/min per mg)	K_m (μ M)	$V_{\max}$ (nmol/min per mg)	K_m (μ M)
Liver microsomes	22.9 $\pm$ 2.4	25	75.0 $\pm$ 5.8	77	31.2 $\pm$ 5.1	67
UGT2B1	0.58 $\pm$ 0.17	42	4.66 $\pm$ 0.8	146	0.91 $\pm$ 0.23	124

Figure 3 Tissue distributions of UGT activities towards BPA and DES

Cholate-activated microsomes from the various organs of the rat (Li, liver; Ki, kidney; Lu, lung; In, small intestine; Te, testis; Br, brain) were assayed for UGT activities towards BPA (A) and DES (B), as described in the Experimental section. Results are means $\pm$ S.D. (error bar).

The results of Northern blot analysis with UGT2B1 cDNA as a probe are shown in Figure 4 for the various tissues. UGT2B1 was highly expressed only in the liver (Figure 4). Figure 5 shows the remaining UGT activities assayed in the supernatant fluid separated from the protein that was binding to the anti-UGT2B1 antibody that recognized only the UGT2B1 isoform [20]. UGT activity towards 1-naphthol, a lipophilic xenobiotic glucuronidated by isoforms other than UGT2B1, was not absorbed by anti-UGT2B1 antibody in the liver microsomes of either the male or female rats. Approx. 65% of UGT activity towards BPA was found to bind to the antibody in male rat liver, indicating that at least 65% of BPA glucuronidation activity was determined by the UGT2B1 isoform. Conversely, in female rat liver 65% of the activity remained in the supernatant (Figure 5), suggesting the presence of other isoform(s) responsible for glucuronidating BPA in the liver of female rats.

Figure 4 Northern blotting analysis of the rat tissues, with UGT2B1 cDNA as a probe

Total RNA species were prepared from the liver (Li), kidney (Kd), lung (Lu), intestine (In), testis (Te) and brain (Br) of Wistar rats. Each lane contained 20 μ g of total RNA, as judged by staining with ethidium bromide. Hybridization was performed with a UGT2B1 probe as described in the Experimental section. The relative mobilities of the 18 S and 28 S ribosomal RNA species are shown as size markers.

Figure 5 Absorption of UGT by anti-UGT2B1 antibody

Solubilized microsomes from the liver of adult male rats (●, ■) and adult female rats (○, △, □) were incubated with the indicated amount of anti-UGT2B1 antibody serum and unimmunized serum (total 15 mg/ml protein) at room temperature for 2 h. Protein A–Sepharose was added and the mixture was incubated at 4 °C for 2 h, then centrifuged at 35 g for 5 min. The resultant supernatant was assayed for UGT activities towards BPA (○, ●) and naphth-1-ol (■, □).

DISCUSSION

The results of the present study show that BPA and DES, two environmental oestrogenic chemicals, are glucuronidated in rat liver microsomes and, furthermore, that the isoform UGT2B1 is responsible for their glucuronidation (Figure 5). UGT isoforms can be divided into two families on the basis of their amino acid sequences [29]. Previously we cloned UGT1 family isoforms UGT1A1, UGT1A3, UGT1A5, UGT1A6 and UGT1A7, all of which can glucuronidate bilirubin and various xenobiotics [29,30], and the UGT2 family isoform UGT2B1, which has been

shown to glucuronidate some endogenous androgens [31,32] and some xenobiotics such as 4-hydroxybiphenyl and opioid compounds [33]. These cDNA clones are also expressed functionally in yeast AH22 cells. To clarify which isoforms can glucuronidate BPA and DES, we developed a method for assaying the two chemicals *in vitro*, verifying the glucuronidation by reverse-phase HPLC.

The toxicity and metabolism of DES in mammals have attracted serious research during the last quarter of the twentieth century [17,34,35]. Knaak and Sullivan [13] reported that 28% of BPA was excreted in urine, primarily as the glucuronide. In the rat tissues used in the present investigation, however, UGT activities towards both BPA and DES were found mainly in the liver (Figure 3), and UGT2B1 was expressed solely in the liver (Figure 4). These distributions are consistent with reports showing that, in DES metabolism, the liver is the major site of accumulation of DES and its glucuronide [17] and that after conjugation of DES in the liver the main route of excretion is via the bile [34].

During organogenesis in mice, BPA treatment at maternally toxic doses was reported to produce foetal toxicosis, manifested adversely as a decrease in both foetal body weight and gravid uterine weight [36]. Shah et al. [17] observed, in foetal rat liver, low but significant levels of UGT activity towards DES and the accumulated DES conjugates [17]. In addition, UGT activity towards testosterone [37] as well as towards DES [38] was reported to be low in neonatal rats. The deficiency in testosterone glucuronidation in the foetus was shown by an immunoblot analysis to be due to the absence of the UGT isoenzyme protein responsible for this conjugation [39]. Those results suggest that, in the mammalian foetus and in the newborn, the toxicity of BPA and DES is attributable to the low expression of UGT2B1 in the developmental stages of the animal. Curiously, the size of the testis and the diameter of the seminiferous tubules were markedly decreased in newborns exposed to DES but not in those exposed to BPA [40]. In the light of our present results, this phenomenon can be ascribed to the UGT activity, which is much higher towards BPA than that towards DES, as shown by UGT2B1 in the rat liver.

Glucuronidation is a major detoxification pathway in all vertebrates, whereas it is rare in invertebrates [15]. In both fish and birds, glucuronidation is common; however, the rates of glucuronidation of steroid hormones in plaice liver microsomes [41] and of chloramphenicol in chicken liver microsomes [15], in which substrates are glucuronidated by a family 2 isoform corresponding to UGT2B1 [30–32], are lower than those observed in rats. The diverse effects of oestrogenic toxicity in animals might depend on the presence or absence of the UGT isoform corresponding to UGT2B1 in the liver.

In the present study, considerable UGT activities towards BPA and towards DES were also observed in the kidney and testis (Figure 3), neither of which contains UGT2B1 (Figure 4) [30]. This indicates that in the kidney and the testis, which is the target organ of oestrogenic toxins, the glucuronidation of BPA and of DES is catalysed by one or more isoforms other than UGT2B1.

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